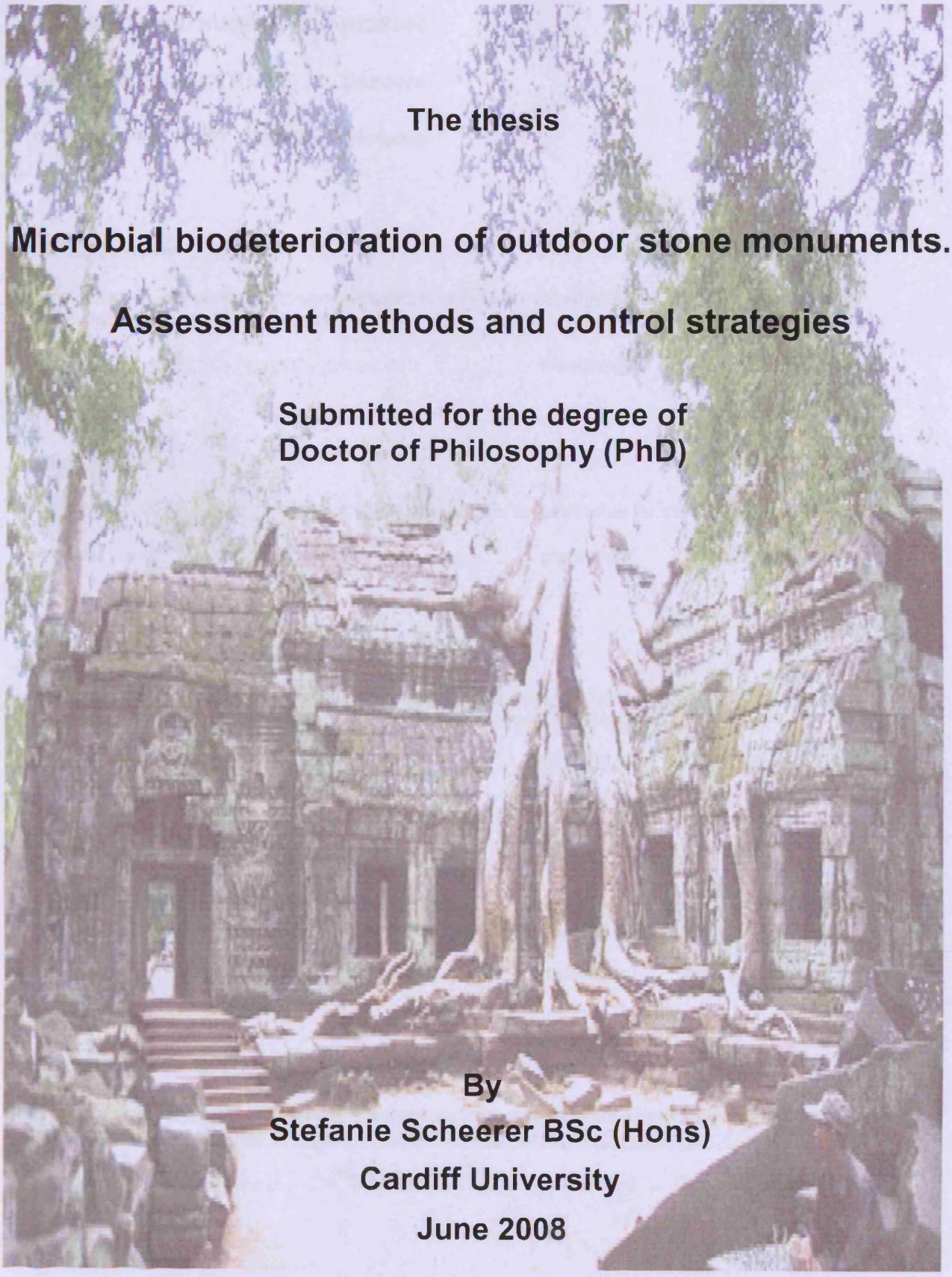

The thesis

Microbial biodeterioration of outdoor stone monuments.

Assessment methods and control strategies

Submitted for the degree of
Doctor of Philosophy (PhD)

By
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Chapter 2

Detection and assessment of microbial biodeterioration

Chapter 3

Development of a system to test sub-lethal and lethal effects on bacteria

Chapter 4

Antimicrobial treatments and their assessment

Chapter 5

Development of minimally-invasive antimicrobial treatments

Abstract

Biodeterioration is the least understood decay mechanisms of outdoor stone monuments. Microbial colonisation is largely determined by the properties of the stone and environmental conditions. The literature on microorganisms on outdoor stone monuments and their decay mechanisms was reviewed. For the assessment and quantification of microbial deterioration, methods that can be carried out by cultural heritage conservators with limited microbiological skills were selected and adjusted for the application on outdoor stone monuments. To this end, the total biomass was quantified by a protein assay (Folin-Lowry method), its phototrophic contribution through chlorophyll *a* absorbance and the amount of extracellular substances (EPS) were assessed by carbohydrate quantification (phenol method). Microbial activity was measured through two different enzyme assays: fluorescein diacetate cleavage and dehydrogenase activity (INT reduction). In order to develop a long-term monitoring strategy, these parameters were tested in the morning (8 am) and in the afternoon (4 pm) on biofilms from a sunny and a shady sampling site on a limestone wall in the south of Mexico. The experiments were performed in the dry season and the rainy season. Changes in biofilm composition and activity during the day were very small, while seasonal changes were more pronounced. The largest differences could be seen in samples from the different sampling sites (sun and shade), where the microbial population had established over years of distinct environmental conditions. Variations in biofilm composition and activity exceeding such natural variation may indicate the necessity for an antimicrobial treatment. The choice of an antimicrobial agent is difficult and the ideal treatment does not exist. Of the various chemical antimicrobial agents tested (Mergal K14, Parmetol DF12, Troysan S97, Preventol R50 hydrogen peroxide and ethanol) on microbial biofilms on stone, ethanol (70%) was the most effective, as revealed by ATP measurements. A flexible, non-invasive *in vivo* system, employing the bioluminescent bacterium *Vibrio fischeri*, was developed to assess sub-lethal effects of antimicrobial treatments and to test combined treatments for synergy. Various biocides and ultrasound (267 kHz, 20 kHz), alone and in combination, were tested for their effect on *V. fischeri* (Mergal K14, Parmetol DF12, Troysan S97, Preventol R50 hydrogen peroxide and ethanol) and a microbial biofilm on stone (Troysan S97, Preventol R50 and ethanol). The tests did not reveal synergistic effects; however, a systematic, comprehensive study on chemical and/or physical methods might reveal an innovative approach towards a more environmentally friendly microbial eradication method for outdoor stone monuments.

Long-term monitoring of the composition and activity of a microbial biofilm may provide data to determine if an antimicrobial treatment is necessary. If an antimicrobial intervention cannot be avoided, low-toxic substances, such as ethanol, should be considered first. For the evaluation of the success of an antimicrobial treatment, ATP measurement has proven to be a reliable and simple method that does not require specialised skills.

List of figures

List of tables

List of abbreviations

AAS	atomic absorption spectroscopy
AFM	atomic force microscopy
am	ante meridiem (at morning)
AO	acridine orange
App.	appendix
Ashade	samples taken from the shade-site in the afternoon (4pm)
Asun	samples taken from the sun-site in the afternoon (4pm)
ATP	adenosine triphosphate
A.U.	arbitrary units
BC	benzalkonium chloride
BES	N-Bis (hydroxyethyl)-2-aminoethanesulfonic acid
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
cm	centimetre
cm^2	square centimetre
cm^3	cubic centimetre
CO_2	carbon dioxide
CT	X-ray computed tomography
DAPI	4',6-diamidino-2-phenylindole
DGFA	diglyceride fatty acid
DGGE	denaturing gradient gel electrophoresis
DHA	dehydrogenase activity
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
DTG	derivative thermogravimetry
DW	dry weight
ECOS	environmental control of salts
EDTA	ethylenediaminetetraacetic acid
EDX/EDS	energy dispersive x-ray spectroscopy
e.g.	for example
EPS	extracellular polymeric substances
ESEM	environmental or low-vacuum SEM
etc.	et cetera
eV	electron volts
FAME	fatty acid methyl ester
FDA	fluorescein diacetate
fig.	figure
FISH	fluorescent *in situ* hybridization
FTIR	Fourier transform infra-red spectroscopy
FW	fresh weight
g	gram
GC	gas chromatography
h	hour
HCl	hydrochloric acid
H_2O	water
HPLC	high performance liquid chromatography
ICP-AES	inductively coupled plasma atomic emission spectroscopy

ICP-MS	inductively coupled plasma mass emission spectroscopy
i.e.	id est (that is)
INF	Iodonitrotetrazolium formazan
INT	Iodonitrotetrazolium chloride
IR	infra-red
kHz	kilohertz
km/h	kilometres per hour
LED	light emitting diode
LIBS	laser induced breakdown spectroscopy
m	metre
M	Mol
mbar	millibar
mg	milligram
min	minute
ml	millilitre
mM	millimol
mm	millimetre
MPN	most probable number
MR	Michael Rayner broth
MR new	Michael Rayner Broth with Oxoid nutrient broth No. 2
MS	mass spectroscopy
Mshade	samples taken from the shade-site in the morning (8am)
Msun	samples taken from the sun-site in the morning (8am)
MUF	4-methylumbelliferone
N	normal
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide – hydrogen (reduced)
NaOH	sodium hydroxide
nm	nanometre
NMR	nuclear magnetic resonance
OD	optical density
PAR	photosynthetically active radiation
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PD	photodiode
PEI	polyethyleneimine
PIXE	particle induced x-ray emission
PLFA	phospholipid fatty acid
PI	propidium iodide
pm	post meridiem (past morning)
PMT	photomultiplier tube
PYGV	peptone yeast extract glucose vitamin medium
quats	quaternary ammonium compounds
RH	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse-transcription polymerase chain reaction
s	second
SEM	scanning electron microscope
SEM-EDX	scanning electron microscopy/ energy dispersive x-ray analysis

SM	seawater medium
SSCP	single strand conformational polymorphism
StErrMean	standard error of mean
TEM	transmission electron microscope
TG	thermogravimetry
TOC	tin organic compound
TPF	triphenylformazan
TSB	tryptone soya broth
TTC	triphenytetrazolium chloride
UK	United Kingdom
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
XRF	X-ray fluorescence
°C	degree Celsius
µg	microgram
µm	micrometre

Chapter 1

Introduction to microbial biodeterioration of cultural heritage made of stone[1]

1.1 Microbial biodeterioration of cultural heritage made of stone

A large percentage of the world's tangible cultural heritage is made from stone, and it is slowly but irreversibly disappearing (fig. 1). It has been calculated that, for limestone, an average of 1.5–3 mm of rock will erode away in 100 years in temperate climates, leading to the disappearance of inscriptions on tombstones in the UK within 300 years (D. Allsopp, personal communication). The transformation of stone into sand and soil is a natural recycling process, essential to sustain life on earth. However, the deterioration of stone monuments represents a permanent loss of our cultural heritage.

Many different types of stone have been used by artists over the years. The most common are marble and limestone, of the calcareous type, sandstone (which is mainly quartz, feldspar and iron oxide) and granite (mainly quartz and feldspar) of siliceous type. These differ in hardness, porosity and alkalinity, properties that affect their susceptibility to biodeterioration. These stone types are not discrete; there is an overlap between calcareous and siliceous rocks, with types such as calcareous sandstone, or siliceous limestone, existing. Materials often used to stabilise the building blocks (mortar) and to coat the surface prior to painting (plaster or stucco) are man-made and very variable in composition, sometimes even containing high levels of organic materials. They are generally extremely susceptible to biodeterioration, as is the modern stone substitute, concrete.

Damage to stone caused by microorganisms is often referred to as bio-weathering but better called biodeterioration (Gorbushina & Krumbein, 2004); it is the least understood of degradation mechanisms of stone heritage. It was reviewed most recently by Warscheid and Braams (2000) and Gorbushina and Krumbein (2004) in general overviews on biodeterioration of stone, Kumar and Kumar (1999), who reported on biodeterioration of stone in the tropics and Urzi (2004), who examined these processes in the Mediterranean. Only within the last two decades has it

[1] parts of the following chapter have already been published (Scheerer et al, 2008), and are quoted here verbatim.

received serious attention from conservators and conservation scientists (Price, 1996; Schnabel, 1991). A thorough understanding of the factors and mechanisms involved in microbial biodeterioration is essential to develop appropriate methods for its control.

Figure 1 Biodeterioration of an angel statue
The influence of biodeterioration on an angel statue of the cathedral of Cologne (Germany) photographed 1880 by Anselm Schmitz, Cologne (a) and 1993 by Dombaumeister Prof. Dr. A. Wolff, Cologne (b) (Warscheid & Braams, 2000).

1.2 Microbial ecology of outdoor stone surfaces

The microflora of external stone surfaces represents a complex ecosystem, which includes not only algae, bacteria, fungi and lichens, but also protozoa. In addition, small animals, such as mites, may be present and lower and higher plants may develop, once the earlier colonisers have conditioned the surface.

Stone inhabiting microorganisms may grow on the surface (epilithic), in more protected habitats such as crevices and fissures (chasmolithic), or may penetrate some millimetres or even centimetres into the rock pore system (endolithic) (Tiano, 2002; Garcia-Vallès et al., 1997; Wolf & Krumbein, 1996; Saiz-Jimenez et al., 1990; Golubic et al., 1975). They can be found in environments as far apart as the Antarctic (Hirsch et al., 2004) and the tropics (Chacón et al., 2006; Golubic et al., 2005; Althukair & Golubic, 1991). Endoliths have been classified in more detail by Golubic et al. (1981), according to their presence in cracks (chasmoendoliths), pores (cryptoendoliths), or as euendoliths if they show a true boring ability into the stone matrix. The endolithic communities of limestone monuments have been shown, both

by culture (Ortega-Morales et al., 2005) and by molecular biology techniques (McNamara et al., 2006), to be different from those on the surface. These differences may be explained, at least in part, by the protective role of epilithic growth and varying availability of nutrients (Cockell et al., 2002). It is likely that these microbial communities are also different at the functional level, since increased exposure to ultraviolet (UV) radiation induces the synthesis of protective pigments (Ehling-Schultz et al., 1997). Warscheid et al. (1996) considered that, whereas microorganisms in moderate climates tend to colonise the surface of stones, their tropical and sub-tropical counterparts prefer to penetrate deeper into the rock profile in order to protect themselves from sunlight and desiccation. Matthes-Sears et al. (1997), however, suggested that organisms are driven to become endolithic not for protection, but in the search for increased nutrients and space (lack of competition). As endoliths in natural carbonate rocks are rarely associated with catastrophic failure, their slow growth rates within the rock would lead them to have a relatively stable life for considerable periods (Hoppert et al., 2004).

Microbial colonisation on outdoor stone surfaces generally initiates with a wide variety of phototrophic microorganisms (mainly cyanobacteria and algae). These accumulate biomass, usually embedded in a biofilm enriched with organic and inorganic substances and growth factors (Tiano, 2002; Tomaselli et al., 2000b). Lichens probably follow these on the stone surface (Hoppert et al., 2004). The accumulation of photosynthetic biomass provides an excellent organic nutrient base for the subsequent heterotrophic microflora. However, the establishment of heterotrophic communities on rocks is possible even without the pioneering participation of phototropic organisms and may in fact facilitate the subsequent growth of photosynthetic populations (Roeseler et al., 2007). In this case, organic substrates from various sources are used, including airborne particles and organic vapours, organic matter naturally present in sedimentary rock (usually between 0.2–2%), excreted organic metabolic products and biomass from other organisms, together with synthetic or natural organic substances from previous restoration treatments (Warscheid & Braams, 2000, Gorbushina et al., 1996). Highly degraded stone surfaces, with subsequent alteration of the physical condition of the rock, provide appropriate conditions (a "proto-soil") for the germination of reproductive structures from higher organisms such as cryptogams (mosses and ferns) and higher plants (Tiano, 2002).

Restoration treatments can, indeed, increase microbial colonisation when carried out by workers with no microbiological knowledge. Caneva and Nugari (2005) showed that a consolidant made from local plant mucilaginous (carbohydrate-like) extracts (Escobilla), used at the Mayan site of Joya de Ceren, El Salvador, supported the growth of fungi and, particularly, actinomycetes; its use should be critically evaluated. Other treatments, such as simple cleaning with water, have also been shown to exacerbate microbial growth (Young, 1997).

Biodeterioration processes are rarely caused by one distinct group of microorganisms, but are rather an interaction of co-existing groups (fig. 2). Table App.1 shows a list of those that have been detected on stone monuments; the functional microbial groups are discussed later in more detail.

Figure 2 Microbial community on sandstone
a. Mixed microbial community on a sandstone tomb in Cardiff, Wales
b. Scanning Electron Micrograph of a microbial community on sandstone
A= algal cells, B= bacterial aggregation, C= fungal hyphae and algal filaments (Cameron et al., 1997).

1.3 Molecular biology in the study of epi- and endolithic microorganisms

Our knowledge of the extent of the diversity of the microbial microflora is far from complete, since traditional culture techniques isolate less than 1% of the microbial community (Ward et al., 1990). In recent years, molecular methods have been developed that allow the identification and, to some extent, enumeration of microorganisms in environmental samples (Amann et al., 1995). Techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Single Strand Conformational Polymorphism (SSCP) and Fluorescent In Situ Hybridisation (FISH), point to the possibility that halophilic or alkanophilic eubacteria and archaea are also involved in stone decay (Ortega-Morales et al., 2004; McNamara et al., 2003; Saiz-Jimenez & Laiz, 2000; Roelleke at al., 1998). These extremophiles had not previously been isolated from stone monuments and thus never considered to play a role in their biodeterioration.

Further approaches employing molecular identification techniques have resulted in the identification of previously unknown species of bacteria, including some actinobacteria, and of organisms such as the Acidobacteria, a practically unknown division of bacteria that is widely distributed in a large variety of ecosystems (McNamara et al, 2006; Salazar et al., 2006; Zimmermann et al., 2005; Saarela et al., 2004; Heyrman, 2003). In addition to the Acidobacter group, other rare microorganisms have been detected on historic buildings. Ortega-Morales et al. (2004), using SSCP, showed that a pink-stained area of an external wall at the Mayan site of Uxmal contained predominantly bacteria related to the Actinobacteria genus, *Rubrobacter*. These authors also showed, for the first time, putative halophiles of the genera *Halothece* and *Salinibacter,* along with photosynthetic bacteria related to the *Ectothiorhodospiraceae*. The occurrence of this latter group expands our knowledge of the microorganisms that may contribute through their autotrophic metabolism to the fixation of carbon in these terrestrial ecosystems.

Most of the bacteria identified by molecular biology have not been cultured and their role in the ecology of stone surfaces is not understood (Schabereiter-Gurtner et al., 2003). Even less is known about the role of archaea in biodeterioration, but recent research sheds light on this microbial group. The archaeal species, *Halobacillus trueperi*, however, has been shown to participate in the mineralisation of

carbonates *in vitro* (Rivadeneyra et al., 2004) and this may be the first indication of the importance of previously uncultured microorganisms in stone deterioration.

There are many practical problems with community analysis using molecular biology methods involving DNA. These include, for example, selective extraction of DNA from different microorganisms, selective amplification in the PCR, lack of amplification of low levels of DNA in a mixture, and interference in the reaction by environmental materials such as polysaccharides or stone constituents. Nevertheless, it has been clearly demonstrated that organisms found by culture and those detected by sequencing methods are not the same. Roelleke et al. (1996) identified relatives of the genera *Halomonas, Clostridium* and *Frankia* in an ancient mural painting; these were not detected by culture, which showed the presence of bacteria such as *Bacillus, Micrococcus* and *Arthrobacter*, not detected by the molecular techniques. New strains of the actinomycete genus *Arthrobacter* were detected by a polyphasic study, including molecular analyses, in the internal biofilms on Servilia's tomb, Carmona, Spain, and St. Catherine's chapel, in the Castle of Herberstein, Austria (Heyrman et al., 2005).

Laiz et al. (2003) showed that the majority of bacteria detected by culture from building materials, artificially inoculated with a known consortium of bacteria and archaea, were spore-formers. A much greater diversity was apparent using the culture-independent technique of DGGE and sequencing. McNamara et al. (2006) found a very wide range of bacteria in and on limestone from the Mayan archaeological site of Ek' Balam, Mexico, using total DNA extraction from samples, PCR with 16S rDNA primers and cloning. Although they did not attempt to culture the organisms, comparison with other publications on similar sites indicated that many more, and different, bacterial groups were detected by this method. Using a combined approach of phospholipid fatty acid markers and SSCP profiling, Ortega-Morales et al. (2004) determined that the main colonisers in most biofilms at another Mexican Mayan site, Uxmal, were cyanobacteria of the *Pleurocapsales* group, although *Bacillus carboniphilus* was particularly abundant in internal sites (more dense biofilms) and *Rubrobacter*-related bacteria on external surfaces (higher UV radiation). The dangers of relying on only DNA analysis for evaluation of the cyanobacterial microflora were pointed out by Gaylarde et al. (2004, 2005) and Chacón (2006). Without any doubt, polyphasic detection methods are essential to determine the true nature of epilithic and endolithic communities.

1.4 Effect of climate and substrate on microflora

Apart from the microorganisms present in the immediate environment, many factors influence the deterioration of stone. Physical, chemical and biological agents act in associations ranging from synergistic to antagonistic. The physical properties of the stone influence the extent of degradation. For microbial growth, for example, rough surfaces and high porosity favour adhesion and colonisation (May et al., 2003; Warscheid & Braams, 2000; Caneva et al., 1991). Environmental pollution, which has increased rapidly within the last century (Wright, 2002), may influence stone degradation directly (e.g., acid rain) or indirectly, by supplying nutrients for microbial growth. It has been shown to enhance detrimental microbial activity on the stone substrate (Herrera & Videla, 2004; Sand et al., 2002; Mitchell & Gu, 2000). Monuments that have survived thousands of years as relicts of extinct cultures have experienced accelerated aging in recent years (Gaylarde & Morton, 2002).

The total properties of a substrate that determine its ability to be colonised by microorganisms have been termed its bioreceptivity (Guillitte, 1995). Although this concept is more used in the engineering field, it could be of interest for heritage conservation, to allow an understanding and assessment of materials to be used in restoration. Prieto and Silva (2005) published a set of simple and well-established methods for assessing bioreceptivity; abrasion pH, bulk density, open porosity and capillary water. The group is now working on a quantitative method to compare visual observations of surface biogenic colour changes on stone (Prieto et al., 2006).

Qualitative and quantitative attributes of the colonising microflora are strongly influenced by the properties of the stone substrate (Warscheid et al., 1996) and it is well known that different kinds of lichens prefer either calcareous or siliceous rocks (Allsopp et al., 2003). Some empirical evidence for the effect of substrate on microbial colonisation comes from studies on natural biofilms on various types of building surfaces (Gaylarde & Gaylarde, 2005). It has also been shown that pollutants deposited on the stone surface from the atmosphere can affect microbial colonisation and degradation (Zanardini et al., 2000).

The influence of the chemical composition of stone on general microbial colonisation remains unclear; however, the condition of the stone has been said to influence the microflora. May et al. (2000) reported that filamentous bacteria were almost never isolated from sound stone in temperate climates, whereas

actinomycetes of the genera *Streptomyces*, *Micromonospora* and *Microphylospora* were the dominant organisms on decayed stone. There is no empirical information on whether these organisms are the cause or the result of the damage, although Mansch and Bock (1998) suggested, with indirect evidence, that colonisation of sandstone by nitrifying bacteria is accelerated by chemical weathering.

Published data on the distribution of different taxa of photosynthetic microorganisms do not indicate a clear relationship between the organisms present and stone composition (Tomaselli et al., 2000b) and the major influence is considered to be climate, rather than substrate (Gaylarde & Gaylarde, 2005; Tiano et al., 1995). Tropical and sub-tropical climates enhance the destructive activity of microorganisms, while in moderate climates air-pollution significantly supports microbial biodeterioration.

According to Warscheid (2003), moderate climates with regular rainfall give rise to a mixed consortium of microorganisms on exposed stone surfaces, whereas semiarid climates, with less rain and higher temperatures, support the growth of more specialised microorganisms such as cyanobacteria, black yeasts and lichens, which tend to dominate the microflora. In arid zones the detrimental influence of microorganisms is low. Instead a "rock-varnish" is formed, mainly by cyanobacteria and mineral-oxidising fungi (Gorbushina & Krumbein, 2000; Krumbein & Jens 1981, Krumbein & Giele, 1979). The highest degree of biodeterioration occurs in the tropics, because of high humidity and temperatures. The stone microflora here is considered to be very aggressive, with a high capacity for "biocorrosion" (more properly called "bioerosion") and biofouling (Warscheid, 2003). These two terms are defined by Warscheid (2003) as 1) "microbially induced or influenced corrosion of materials", altering the structure and stability of the substrate, and 2) "the presence of colloidal microbial biofilms on or inside materials", leading to visual impairment and potentially altering the physiochemical characteristics of the substrate. The production of pigments, thick walls or capsules protect microorganisms from adverse climates; however, their aesthetic damage is severe. Deeply-coloured coccoid and filamentous cyanobacteria, which predominate in biofilms on buildings in the hot and humid climates of Latin America (Gaylarde & Gaylarde, 2005), are more frequently present on surfaces of buildings at high altitude in the tropics and sub-tropics than at lower altitudes (Gaylarde & Englert, 2006; Gaylarde & Gaylarde, 2005; Gaylarde et al., 2004 and fig. 3).

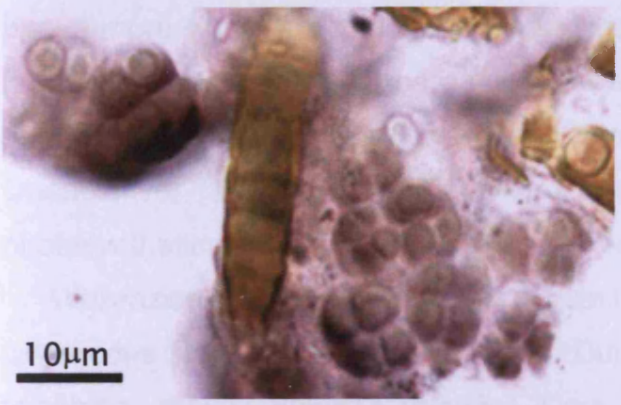


Figure 3 Mixed coccoid and filamentous cyanobacteria
Mixed coccoid and filamentous cyanobacteria on the external surface of a church in Minas Gerais, Brazil, showing intense pigmentation. This photo is from a rehydrated biofilm. Picture by C. Gaylarde.

Particularly sheltered areas on historic buildings in the UK have been shown to give rise to rich and homogenous biofilms consisting mainly of bacterial rods (May et al., 2003). Biofilms exposed to salt from marine aerosols were of heterogeneous structure with coagulated cells entangling stone particles; whether salting or microbial activity was the main cause of decay is not clear. Exposure to high levels of solar radiation in these temperate climates, with subsequent drying of the substrate, leads to preferential growth of spore forming bacteria, such as *Bacillus* and heat tolerant actinomycetes, over gram negative bacteria (May et al., 2000). Actinomycetes are frequently found in the more temperate climates of Europe (Palla et al., 2002; Warscheid et al., 1995). Gaylarde & Gaylarde (2005) suggested that they are more common on external surfaces in these milder conditions, whereas in the hot and humid tropics and semi-tropics, they seem to prefer the interiors of buildings, or to grow as endoliths. However, actinomycetes have also been reported on surfaces in areas of hot climate (Ortega-Morales et al., 2004), the genus *Geodermatophilus* apparently being common in calcareous stone (Eppard et al., 1996).

Tayler and May (1991) reported seasonal changes in the microbial community of sandstone from ancient monuments in the UK, with higher bacterial numbers in winter and early spring than in summer and early autumn. Seasonal climate changes tend to result in higher numbers and greater diversity of gram positive bacteria during summer months in temperate climates. In the warmer Mediterranean climate of Crete, no seasonal changes were observed in heterotrophic bacteria. In this geographical area, the location of the exposed surface (sheltered or not) seemed to

play a more significant role than climate change (May et al., 2000). Tomaselli et al. (2000a) also reported that there were few seasonal changes in the composition of photosynthetic populations on marble statues from different locations in Italy. Quantitative, rather than qualitative, differences were found, higher numbers of photosynthetic microorganisms being detected during summer months.

Wollenzien et al. (1995) showed qualitative differences in fungi occurring on calcareous stone in the Mediterranean. During periods of higher humidity and less sunshine, rapidly growing mycelial fungi, particularly of the genera *Alternaria, Aspergillus, Cladosporium, Phoma* and *Ulocladium*, were dominant, but they were rarely found during the dry season. Nitrifying bacteria, which are known to be highly dependent on the water regime, have been found to be more abundant in indoor environments and during the rainy season at the archaeological site of Uxmal (Ortega-Morales, 1999).

1.5 Mechanisms of microbial biodeterioration

The detrimental effects of microorganisms may be aesthetic, biogeochemical and/or biogeophysical. Microbial cells may contribute directly to the deterioration of stone by using it as a substrate or indirectly by imposing physical stress, serving as nutrients for other organisms, or providing compounds for secondary chemical reactions (Sand, 1996). May (2003) stated that the intimate association of microorganisms with the mineral substrate may reach more than 3 cm deep into the stone, while Wolf and Krumbein (1996) reported microbial contamination in highly degraded, fine grained marble to a depth of 20 cm. These may not have been active boring microorganisms, but such organisms do exist, although their mechanisms of penetration are not fully understood (Salvadori, 2000).

Microbial deterioration of stone is currently considered to be due to one or more of the below mentioned mechanisms. However, it must be pointed out that much of it is speculative. There is only little experimental evidence, for example, for the production of acids *in situ* and acidic polysaccharides on the stone surface; most investigators that have demonstrated the ability of stone-colonisers to produce acids have tested this in artificial media in the laboratory.

1.5.1 Biofilms

1.5.1.1 Biofilm structure and function

Biofilms are sessile microbial communities embedded in extracellular polymeric substances (EPS), which attach the cells to a surface (Nikolaev & Plakunov, 2007; Hall-Stoodley et al., 2004; Dunne, 2002). The surrounding matrix, which provides anchoring and protection for the microbial aggregates, contains predominantly a range of anionic sugar molecules (Dunne, 2002) but also pigments, proteins, nucleic acids, lipids, dead cell debris, water and airborne particulates (Kemmling et al, 2004; Donlan & Costerton, 2002; Dunne, 20002; Flemming & Wingender, 2002). This matrix, rather than the microorganisms themselves, is in close contact with the substrate and all microbial substances (e.g. extracellular enzymes or acids) are transferred through this matrix to the substrate (Kemmling et al, 2004). Biofilms are located at phase boundaries, most frequently at a solid-liquid interface but not uncommonly found at solid-gas interfaces (also called subaeral biofilms), such as air-exposed stone surfaces. The biofilm structure has been compared to that of multicellular organisms performing intracellular communication (Nikolaev & Plakunov, 2007; Parsek & Greenberg, 2005; Stewart & Costerton, 2001; Wimpenny, 2000). However, the ability of biofilms to react rapidly to the challenges of an ecological niche by means of genetic regulation (active response) and/or selection (passive response) is more flexible than that of multicellular eukaryotes (Hall-Stoodley et al., 2004). The spatial and metabolic biofilm structure depends largely on environmental conditions (e.g. physical and chemical stress, nutrients), the composition and character of the EPS and the character of the organisms present (Wimpenny, 2000). The architecture can be very complex; channels, pores and cavities may interfuse the matrix surrounding the microbial cells to provide the exchange of water, nutrients, metabolites and oxygen (Nikolaev & Plakunov, 2007; Hall-Stoodley et al., 2004). In aqueous niches that pose high shear forces (e.g. rivers), biofilms tend to be composed of filamentous microcolonies and ripple structures. In calm water, however, circular biofilm structures (often mushroom-shaped) are predominant. Biofilms on air-exposed rock surfaces, in addition to biofilms in pores and fissures, tend to have mineral grains and cementing material

incorporated (Gorbushina, 2007; Kemmling et al., 2004). Subaerial biofilms are less homogenous than subaqueous ones and have been described as "patchy" or network-like" (Gobushina, 2007).

For billions of years, the formation of biofilms has been a successful strategy for microorganisms to occupy a habitat by means of adhesion. The ability of biofilms to accumulate nutrients allows microorganisms to occupy oligotrophic habitats (Hall-Stoodley et al., 2004; Flemming & Wingender, 2002). Symbiotic associations of different species within a biofilm may degrade substrates through a combination of metabolic processes, which may not be possible by a single species (Nikolaev & Plakunov, 2007; Flemming & Wingender, 2002; Nielsen et al., 2000). Possibly the most important factor for the success of biofilms is its protective nature; biofilm formation increases the possibilities for microorganisms to survive adverse conditions such as desiccation, extreme temperatures, osmotic stress, UV radiation and toxic chemicals (Nikolaev & Plakunov, 2007; Hall-Stoodley et al., 2004; Kemmling et al, 2004).

1.5.1.2 Biofilm resistance

The protection against toxic substances (antibiotics being the best studied) can be attributed to several mechanisms. Most likely it is a complex phenomenon and can be explained by a combination of several of the following mechanisms: The EPS provides a physical barrier allowing only slow or incomplete penetration of an antimicrobial substance and makes its contact with the microbial cells more difficult and. Toxic substances are effectively diluted by the EPS before they reach the cells surface and some charged or reactive chemicals may be neutralised (Nikolaev & Plakunov, 2007; Hall-Stoodley, et al., 2004; Fux et al., 2003; Dunne, 2002; Fah & O`Toole, 2001; Stewart & Costerton, 2001). This physical barrier further protects the microbial cells from UV radiation and dehydration (Hall-Stoodley, et al., 2004); and a stress response system has been suggested to protect microbial cells from challenges like temperature shock, pH changes or chemical stress (Fah & O`Toole, 2001). Furthermore, cells within a biofilm tend to be in stationary phase and hence their metabolism is not highly active. Antibiotics tend to interrupt microbial processes and therefore need some metabolic activity to be effective. Stationary phase organisms are therefore less susceptible to antibiotics (Hall-Stoodley, et al., 2004; Fux et al., 2003; Fah & O`Toole, 2001). Conditions (e.g. oxygen concentration, pH,

nutrient concentration, metabolic waste products, signalling compounds) vary within a biofilm. As some of these conditions have an effect on the efficiency of an antimicrobial agent, the susceptibility of the microbial population is not the same in all parts of the biofilm (Fah & O`Toole, 2001; Stewart & Costerton, 2001). Another hypothesis is that of the existence of a resistant sub-population, so-called "persisters" and other resting structures (e.g. spores). Such a sub-population with a unique phenotype may consist of a very small fraction of the total biomass, however, surviving adverse conditions, they may further proliferate (Nikolaev & Plakunov, 2007; Hall-Stoodley, et al., 2004; Fux et al., 2003; Stewart & Costerton, 2001). Finally, phenotypic changes that occur during the transition from a planktonic to a sessile life form within a biofilm, may render microbial cells less susceptible to antibiotics by decreasing the number of targets while increasing resistance mechanisms such as efflux pumps (Pasmore & Costerton, 2003). As bacteria dispersed from biofilms tend to become susceptible to antibiotics again, it is unlikely that mutation or horizontal gene transfer might be the underlying factor (Fux et al., 2003; Stewart & Costerton, 2001).

1.5.1.3 Biofilms on cultural heritage monuments

The simple presence of a biofilm on a cultural heritage monument has aesthetic, chemical and/or physical effects on the stone. Biofilms are areas of stabilised metabolic activity, where digestive enzymes excreted by microorganisms are concentrated and protected. Kemmling et al. (2004) found that the EPS in a biofilm from the Market Gate of Miletus (Pergamon Museum) protected cell enzymes against repeated desiccation and rehydration cycles, thus offering the organisms within the biofilm a distinct advantage over non-embedded cells on external surfaces.

Microbial EPS, predominantly consisting of anionic sugar molecules, contains several types of functional groups, some of which are capable of binding cations in solution. Calcium can be leached from limestone surfaces, or chelated, once solubilised from the matrix, by hexuronic acids, carbonyl and hydroxyl groups (Ortega-Morales et al., 2001; Perry et al., 2004). Ortega-Morales et al. (2001) found higher levels of hexuronic acids in EPS directly extracted from degraded limestone surfaces at Uxmal than on sound stone blocks that were heavily colonised by

cyanobacterial biofilms. These molecules may have mediated the deposition of carbonate minerals around coccoid cells, previously demonstrated by scanning electron microscopy (SEM) (Ortega-Morales et al., 2000).

The formation of biofilms intensifies microbial attack by weakening the mineral lattice through repeated wetting and drying cycles and subsequent expansion and contraction (Warscheid, 1996a). They may change the pore size, dry density, water content, surface hardness and weight of the stone (May, 2003; Papida et al., 2000). Water is readily absorbed by the biofilm and does not evaporate as rapidly as it would from rock surfaces without a biofilm. This water may facilitate chemical reactions leading to stone degradation process, which under dry conditions would not be possible (Gorbushina, 2007). Biofilms have a lower thermal conductivity than stone, which may lead to uneven heat transfer within the artefact (Warscheid & Braams, 2000).

However, EPS from biofilms have also been reported to have a certain protective nature, owing to a consolidation effect (Kurtz, 2002). Microbial polysaccharides, and other naturally occurring biopolymers of various chemical compositions, have been shown inhibit dissolution under certain conditions (Papida et al., 2000; Welch & Vandevivere, 1994).

1.5.2 Discolouration

Discolouration is mainly an aesthetic problem (fig. 4). It may be caused by pigments released from, or contained within, the microorganisms (melanins, carotenes and photosynthetic pigments). Figure 5 shows carotene-packed cells of the alga *Trentepohlia umbrina* on a pink-stained limestone surface at the Mayan site of Edzna, Mexico. Crushed samples of stone showed that the calcite crystals had taken up the orange stain (Gaylarde et al., 2006).

Figure 4 Ambar Fort
Black biofilms on the surface of the Ambar Fort in Jaipur, India.
Picture by C. Gaylarde.

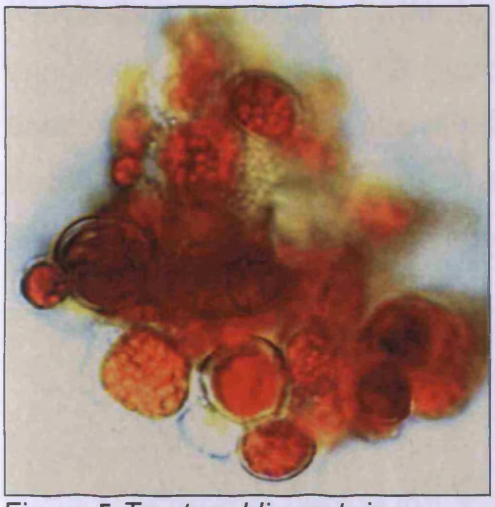


Figure 5 *Trentepohlia umbrina*
Bright orange (carotene-packed) cells of *Trentepohlia umbrina* on a limestone surface in Edzna, Mexico (Gaylarde et al., 2006).

Sulphur cycle bacteria can convert limestone into gypsum, common especially in sulphur-polluted environments. This can lead to the formation of dark surface colourations, even when the normally responsible fungi and cyanobacteria are not present. Dark discolourations may also be due to airborne particles trapped in EPS.

Discolouration is not, however, purely aesthetic. Discoloured areas may absorb more sunlight, which increases physical stress by expansion and contraction caused by temperature changes (Sand et al., 2002; Warscheid, 2000). Temperatures on darkly stained areas of stone have been shown by Garty (1990) to differ by as much as 8°C from lighter-coloured areas. This effect has been shown experimentally by Carter and Viles (2004), using limestone blocks with and without a lichen covering. Surface temperature change and thermal gradients were greater below the lichens.

The darkening of the stone surface decreases its albedo, so that it experiences increased heating/cooling and wetting/drying cycles, causing stresses within the stone (Warke et al., 1996).

A special example of this is the so-called "black crust" (fig. 6). Thick (several mm) crusts, such as those found on buildings in Aberdeen, Scotland (Urquart et al., 1996), or on Seville cathedral, Spain (Saiz-Jimenez, 1995), are rich in Ca, Si and C (Wright, 2002). The carbon component may be composed of hydrocarbons, deposited from vehicle exhausts, and microorganisms within the biofilm may be able to utilise these molecules as nutrients (Saiz-Jimenez, 1995). On the other hand, the more commonly seen thin black crusts (Figure 7) seem to be predominantly cyanobacterial (sub-tropics/tropics) or fungal (moderate climate) in composition (Gaylarde et al., 2007; Gaylarde et al., 2006; Pattanaik & Adhikary, 2002) and are enriched in Si and Fe (Gaylarde et al., 2006; Wright, 2002). Apart from their aesthetic effects, these crusts also block pores within the stone. This can result in water retention and subsequent spalling of the surface, although it is also possible that they protect the stone by reducing water infiltration (Garcia-Vallès et al., 1997).

Figure 6 Black crust on Seville cathedral
a. Thick black crust, with detail (b), on Seville cathedral, Spain with hydrocarbon-degrading microorganisms (Saiz-Jimenez, 1995).

Figure 7 Black crust on Campeche cathedral
a. A thin black crust on Campeche cathedral, Mexico, with detail (b). Dark brown branching filamentous cyanobacteria were the main component (Gaylarde & Ortega-Morales, personal communication 2006).

1.5.3 Salting

Salting, the production of efflorescence, involves secondary minerals produced through reaction of anions from excreted acids with cations from the stone (fig. 8); this mechanism is related to that discussed above. The damage caused by such salts is mainly of a physical nature, leading to blistering, flaking, scaling and granular disintegration, and this may often be the main mechanism of stone decay (Wright, 2002). Hydration and subsequent swelling of a salt molecule within a small stone pore may cause cracking. During desiccation, the salts crystallise, leading to an increase in volume. (Ortega-Morales et al., 2005). At low temperatures, hydration of salts increases the water content and may lead to mechanical damage through ice crystal formation. Salts of biotic or abiotic origin may also increase the production of EPS and biofilm density (May, 2003; Sand et al., 2002; Papida et al., 2000; Sand, 1996). Areas of efflorescence present a niche for halophilic/-tolerant microorganisms, e.g. several Archaea. These specialised microorganisms may have synergistic action with the salts and therefore severely enhance the physical and chemical deterioration processes (May, 2003; Papida et al., 2000).

Figure 8 Salting on tomb of Servilia
Salting on the internal walls of the tomb of Servilia, Carmona, Spain. Picture by C. Gaylarde.

1.5.4 Physical damage

Physical damage may be caused by penetration of filamentous microorganisms (particularly fungal hyphae) into the stone (Hirsch et al., 1995a). Many cyanobacteria, not necessarily filamentous, have also been shown to have this ability. Weakened areas of the stone will be affected first. Danin and Caneva (1990) proposed that calcareous stone is decayed by cyanobacteria by attachment of cyanobacterial cells in small fissures and growth within these fissures. This is followed by water uptake and expansion of cell mass, exerting pressure within the structure, precipitation of carbonates and oxalates around the cells, opening of the fissure with subsequent entry of dust, pollen, grains, etc. and death of some cyanobacterial cells, allowing the establishment of heterotrophic bacteria, fungi and small animals such as mites. The final increased internal pressure on the superficial layer of the structure leads to its detachment (spalling).

1.5.5 Inorganic acids

These are mainly nitric acid (HNO_3) and sulphuric acid (H_2SO_4), but also carbonic acid (H_2CO_3), sulphurous acid (H_2SO_3) and nitrous acid (HNO_2). The acids may dissolve acid-susceptible materials, with the production of substances more soluble in water (calcium sulphate, nitrates and calcium hydrogen carbonate). This leads to

weakening of the stone matrix. This dissolution action proceeds through the depth of the stone (Sand et al. 2002; Sand, 1996). Sulphuric and sulphurous acids are predominantly produced by *Thiobacillus,* as well as by *Thiothrix*, *Beggiatoa* and some fungi. The involvement of sulphur-oxidising bacteria in the degradation of sandstone was first proposed in 1904, but later workers did not find these bacteria on natural stone buildings (Bock & Krumbein, 1989), leading to doubts about their role in biodegradation.

Nitric and nitrous acids are produced by ammonia and nitrite oxidisers, heterotrophic nitrifiers and some fungi. Endolithic nitrifying bacteria were the first microorganisms to be proposed as the cause of stone decay, in 1890 (Muntz, 1890). However, since most natural stone is alkaline in reaction, nitrification will result in the production of nitrate and not nitric acid; thus acid attack is unlikely (Gaylarde & Gaylarde, 2004) and, indeed, Mansch and Bock (1998) suggested that acidic breakdown of stone is required prior to colonisation by these bacteria.

Carbonic acid is produced by all living organisms as an end product of energy metabolism after the reaction of CO_2 with water (Sand, 1996). It is a weak acid, however, and unlikely to contribute greatly to stone degradation, especially in calcareous stone, where it will react to form calcium bicarbonate, a weak alkali buffer.

1.5.6 Organic acids

These include oxalic, citric, acetic, gluconic, malic, succinic acid, but also amino acids, nucleic acids, uronic acids, etc. They may react with the stone, solubilising it via salt formation and complexation (Sand et al., 2002; de la Torre et al., 1993). The "biomineralisation" caused by this metabolic activity has been documented using transmission and scanning electron microscopy in association with electron dispersion spectroscopy (de los Rios & Ascaso, 2005; de los Rios et al., 2004; Ascaso et al., 2002). Complexation, or chelation, is not, of course, "acid degradation" and pH values may not be reduced. Almost all microorganisms can excrete organic acids, especially when growth is unbalanced (Sand, 1996); perhaps the most frequently mentioned is oxalic acid, produced by fungi and lichens, mainly as the monohydrate form (whewellite), but also the dihydrate (weddelite). Polyfunctional organic acids, such as oxalic, have been shown to enhance the dissolution of siliceous rocks (Bennett et al., 1988), but they may have a protective role in

calcareous rock through the formation of calcium oxalate (Di Bonaventura et al., 1999) or malonate (Salinas-Nolasco et al., 2004) films. The brown/yellow oxalate films on stone surfaces are known as "time patinas" and are regarded by many as an attractive feature, enhancing the aged appearance of a monument. They may, or may not, be biogenically produced.

1.5.7 Osmolytes

These are a diverse group of polyol substances (which includes glycerol, other sugars and polysaccharides) produced in response to changes in water activity; they are protectants against freezing, excessive heat and drying, salts, acids, alkalis and factors such as ethanol. They have been reported in all life forms except protozoa, myxomycetes and some simple animals. Under alkaline conditions (the majority of stone types), polyols degrade siliceous rock by binding to the crystalline layers, causing expansion, and by enhancing the solubility of organosilicon compounds (Gaylarde & Gaylarde, 2004).

1.6 Microorganisms detected on historic monuments

A list of microorganisms present on and in the stone of historic monuments, found in the literature, is given in table App.1.

1.6.1 Phototrophic microorganisms

Cyanobacteria and algae, as phototrophs, do not require organic material for their growth. They can form biofilms and crusts on stone surfaces, which, depending on the environmental conditions and the predominant strains, can be black, grey, brown, green or red. Under wet conditions, such biofilms tend to be green, while when dry they are grey or black (Ortega-Morales et al., 2004). This does not mean that the organisms within dry biofilms are dead; indeed, cyanobacterial biofilms have been stored for years in dry and dark conditions and remained viable (Gaylarde et al., 2006). It has been shown that certain cyanobacteria, such as *Chroococcidiopsis*, the most desiccation-resistant cyanobacterium known (Potts & Friedmann, 1981), regain photosynthetic activity within minutes when rehydrated (Hawes et al., 1992).

Green algae are found mainly in damper areas. Their contribution to biodegradation has not been researched thoroughly and is considered to be mainly to promote the growth of other organisms. This is not so for cyanobacteria, whose

role in the deterioration of surfaces of historic buildings has been the subject of several recent studies and reviews (Crispim & Gaylarde, 2004; Ortega-Morales et al., 2001; Tomaselli et al., 2000b). These organisms are generally adapted to resist adverse conditions because of their thick outer envelopes and the presence of protective pigments (Chazal & Smith, 1994; Garcia-Pichel et al., 1992). Cyanobacteria are probably the most resistant of the microflora on monument surfaces (with lichens in second place), if we relate this environment to desert crusts (West, 1990). The Atacama and Namib deserts, the most extreme on earth, have crusts in which cyanobacteria are the only phototrophs (Evenari et al., 1985). The ability of cyanobacteria to survive repeated cycles of dehydration and high levels of UV radiation (Chazal & Smith, 1994; Potts, 1994; Garcia-Pichel et al., 1992) makes them particularly important organisms on outdoor stone surfaces. In spite of their resistance to UV, superficial growth of cyanobacteria and algae is stronger on sheltered indoor surfaces of historic limestone buildings, which have reduced illumination, higher humidity and more organic nutrients (Gaylarde et al., 2001; Ortega-Morales et al., 2000).

Cyanobacteria have been suggested to be of higher ecological importance as pioneer organisms on exposed stone surfaces of buildings than any other organism (Grant, 1982) and may have the most important influence on weathering of exposed stone (Gaylarde & Morton, 2002). Cyanobacteria have been shown to constitute the major biomass on external surfaces of ancient stone structures in Latin America (Gaylarde et al., 2001; Ortega-Morales et al., 2000) Greece (Anagnostidis et al., 1983) and India (Tripathy et al., 1999). In fact, cyanobacteria and eukaryotic algae had been found, until the recent molecular biology study of McNamara et al. (2006), to be the most widespread microorganisms in the endolithic habitat (Sigler et al., 2003). The ability to fix carbon dioxide, and in some species atmospheric dinitrogen (N_2), gives the cyanobacteria an obvious advantage over heterotrophic bacteria. Light quality and intensity are the main factors that control the minimum and maximum depth at which endolithic phototrophic communities grow (Nienow et al., 1988). Figure 9 shows the cyanobacterial genus, *Nostoc*, growing endolithically in the granite pillar of a church in Parati, Brazil.

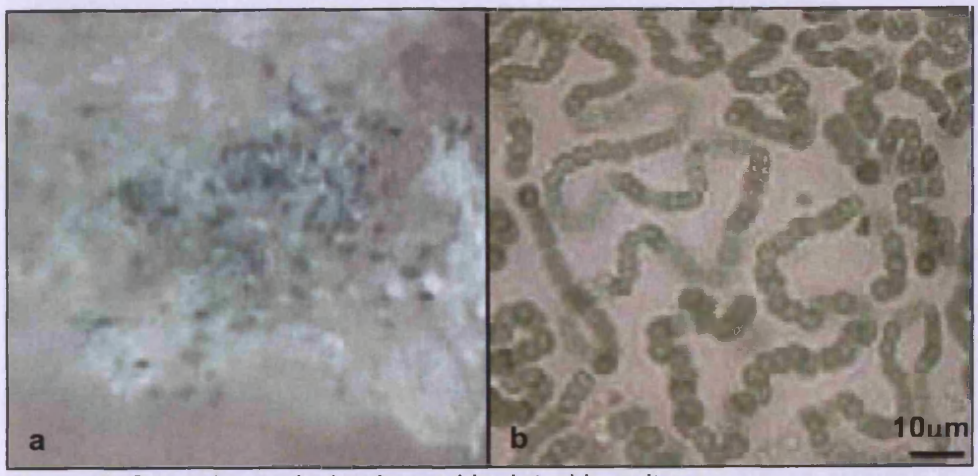


Figure 9 Cyanobacteria *in situ* and isolated in culture
a. Microscopic colonies of *Nostoc* on the inner surface of a rehydrated flake from the granite pillar of a church in Parati, Brazil
b. Isolated cyanobacteria in culture. Pictures by C. Gaylarde.

Apart from their evident aesthetic deterioration of the stone monument, these phototrophs may cause chemical and physical deterioration by the excretion of chelating agents and stone-dissolving acids (Albertano 2003; May 2003; Urzi & Krumbein, 1994), as well as by yet undefined boring activity (Garcia-Pichel, 2006), documented for the Pleurocapsa-group (Mao-Che et al., 1996), *Synechocystis, Gloeocapsa*, *Stigonema, Schizothrix* (Hoffman, 1989), *Scytonema* (Golubic et al., 2000) and *Mastigocladus* (*Fischerella*) (Boone et al., 2001). Ortega-Morales et al. (2000) and Gaylarde and Englert (2006) showed scanning electron micrographs that demonstrate coccoid cyanobacteria sitting in cell-sized depressions in the stone surface, while Gaylarde et al. (2006) report the presence of pure colonies of the alga *Trentepohlia umbrina* within colony-sized pits on limestone. All these images indicate that the cells themselves are causing the degradation.

Photosynthetic organisms deposit calcium carbonate ($CaCO_3$) in the presence of light and solubilise it at night. The precipitation of calcium salts on cyanobacterial cells growing on limestone suggests the migration of calcium from neighbouring sites (de los Rios, 2005; Ortega-Morales et al., 2000; Ascaso et al., 1998; Schultze-Lam & Beveridge, 1994). The external S-layer of *Synechococcus* GL24 binds calcium ions (Schultze-Lam & Beveridge, 1994), which complex with carbonate ions at the pH values (>8.3) produced around the cells (Miller et al., 1990). *Synechococcus* cells can become encrusted with calcite within 8 h in an aqueous environment and must continually shed patches of the mineralised S-layer to remain viable (Douglas & Beveridge, 1998). This mobilisation of calcium ions and the trapping of released

particles of calcite in the gelatinous sheaths or capsules of cyanobacterial cells (Pentecost, 1987, 1988) are important mechanisms of degradation of calcareous stone. Nitrogen and phosphorus may also be mobilised from the stone and metabolised or stored within the organisms (Albertano, 2003). Warscheid and Braams (2000) also mentioned the possibility that phototrophic organisms take up and accumulate sulphur and calcium into their cells.

Lichens are symbiotic associations between fungi and one or two photobionts, which can be algae or cyanobacteria. They are frequent colonisers of stone monuments and have been mistaken for the remains of ancient rendering when they cover substantial areas of the surface (Seaward, 2003). Lichens are particularly sensitive to air pollution and, indeed, are used as bioindicators of such. It has been suggested that improved air quality has already, or may in the future, lead to an increase in colonisation of stone by lichens (Ardron, 2002; Young, 1997). Although many people find lichen growth on stone pleasing, it can be a problem in obscuring fine details of carvings and it is certainly inherently damaging to the structure. Nimis and Monte (1988) reported an interesting effect of lichen growth on the Orvieto duomo (Italy). The alternating dark basalt and light limestone bands have been colonised, respectively, by light and dark, or orange, lichens, completely eliminating the effect desired by the artist on the northern facade.

Lichens cause mechanical damage due to penetration of their rhizines, composed of fungal filaments, and the expansion/contraction of the thallus on wetting/drying, which can lift grains of stone off the surface (De los Rios et al., 2004; Gaylarde & Morton, 2002, fig. 10).

Figure 10 Lichens on sandstone
A combination of lichen acids, physical action and possibly salt accumulation caused this extreme case of stone decay (Cameron et al., 1997).

Accumulation of small stone fragments (as small as 5 μm) within the lower thallus has been reported (Gadd, 2007). The depth of penetration depends on the stone substrate and the type of lichen; lichen structures can sometimes be found at least 3 cm below the stone surface (Lee et al., 2003). They also cause direct chemical attack by the production of significant amounts of acids. "Lichen acids" have been shown to cause damage at the stone/lichen interface (Seaward, 2003; Cameron et al., 1997). The principal acid produced is oxalic, which leads mainly to the formation of calcium oxalate and its different hydrate forms whewellite and weddellite (Gaylarde & Morton, 2002; Tiano, 2002). The lichen thallus has been shown to accumulate from 1-50% calcium oxalate, depending on the substrate. Even on siliceous stone, some lichens can accumulate this compound, using calcium from the air or leachates (Seaward, 2003). Lichens on historic stone buildings have been reviewed recently by Lisci et al. (2003).

The CO_2 produced by lichens is transformed within the thallus to carbonic acid (Tiano, 2002), which, although a weak acid, seems to be able to solubilise some calcium and magnesium carbonates in calcareous stone. Lichens have been demonstrated to biomobilise certain elements from the stone matrix (de los Rios et al., 2004; Tiano, 2002). The former workers demonstrated magnesium-depleted areas of the stone substrate around the lichen thallus. Saxicolous lichens mobilise magnesium and silicon in rock, causing biochemical weathering (Aghamiri & Schwartzman, 2002). Gordon and Dorn (2005) calculated that a saxicolous lichen increased the weathering rate of basalt by a factor of at least 1.7. The weathering was greatest directly under the lichen colony. 0.5 mm below the colony, weathering rates fell to those of uncolonised surfaces. Banfield et al. (1999) proposed a model, based on high resolution transmission electron microscopy, of the weathering of silicate rocks by lichen activity. Clear boundaries are shown in the vertical profile, with a direct "biochemical" effect first produced, followed by predominantly biophysical action in the deeper layer of material.

Certain lichens can grow endolithically (Gaylarde & Gaylarde, 2005; Gerrath et al., 1995). They are slow-growing, stress-tolerant organisms, which have been stated to have a similar physiology to epilithic crustose lichens (Tretiach & Pecchiari, 1995), and lead to similar destructive effects.

Under conditions of high abiotic weathering, lichens have been suggested to provide protection for the stone surface from wind and rain through the insoluble oxalate layer (Bungartz et al., 2004; Warscheid & Braams, 2000; Di Bonaventura et al., 1999), or to limit erosion by reducing the level of water within the rock (Garcia-Vallès et al., 2003); their retention of moisture within the thallus reduces thermal stress on a limestone surface (Carter & Viles, 2003). However, they are generally defacing and intrinsically damaging. Even when a protective effect can be shown, subsequent decay of the lichen thallus (which occurs in the centre of the colony of some species) can open this area to further weathering, resulting in cratered mounds on the rock surface (Mottershead & Lucas, 2000). The mechanical removal of crustose lichens is particularly difficult because the thallus forms an intimate association with the substrate. Hence, its removal leads to severe structural damage (Allsopp & Gaylarde, 2004; Gaylarde & Morton, 2002).

Recent work based on molecular approaches has shown that, in addition to algae, lichens and cyanobacteria, other previously unrecognised phototrophic microorganisms may occur in stone monuments. Ortega-Morales et al. (2004) found bacteria related to the *Ectothiorhodospiraceae* in certain samples at the Mayan site of Uxmal, while McNamara et al. (2006) detected *Chloroflexi* organisms. These new data, added to the already known complex nature of lithic biofilms on historic monuments, indicated that these organisms may contribute to the carbon pool in autotrophic biofilms. It is likely that their role in stone deterioration, as for algae, is supporting the growth of associated heterotrophs, although the production of osmolytes cannot be ruled out. Interestingly, the halophily of these organisms is congruent with the measured levels of salts in some monuments, where significant amounts of sulphate, chloride and nitrate have been found (Ortega-Morales, 1999; Ortega-Morales et al., 2004, 2005).

1.6.2 Chemoorganotrophic microorganisms

The contribution of heterotrophic/chemoorganotrophic microorganisms to stone deterioration, particularly as pioneering colonisers, had long been neglected; however, their degradative role by acid/alkali production and by chelation is now well accepted (Gaylarde & Morton, 2002).

1.6.2.1 Fungi

The effects of fungi are due to physical and chemical actions, which are often synergistic in the degradation of stone. They were recently reviewed by Gadd (2007). The fungal stone flora consists of filamentous fungi (ubiquitous hyphomycetes and coleomycetes) and microcolonial fungi (black yeasts and yeast-like meristematic fungi) (Gorbushina et al., 2003; 2002; Sterflinger, 2000; May, 2003; Urzi et al., 2000). Meristematic fungi produce swollen, isodiameteric cells with thick, melanin containing cell walls. They remain metabolically active even in low nutrient conditions and have high resistance to desiccation, UV radiation and osmotic stress (Urzi et al., 2000), thus being well adapted to growth on external walls. Wollenzien et al. (1995) suggested that these are the resident fungi in Mediterranean climates; the fast growing, filamentous hyphomycetes being present only in the colder and more humid winter months and therefore considered contamination in this climatic area. Hyphomycetes tend to be the major fungal population in more northerly parts of Europe (Sterflinger, 2002). However, the ubiquitous hyphomycetes can also be found in tropical and sub-tropical climates. Resende et al. (1996) identified a wide range of filamentous fungi in soapstone and quartzite in churches in the Brazilian state of Minas Gerais. The most common genera were Cladosporium and Penicillium. However, it must be emphasised that the detection technique affects the results of such investigations.

Gorbushina et al. (2002) detected mainly deuteromycetes, such as Alternaria, Cladosporium and Trichoderma, on historic marble monuments in St. Petersburg and Moscow. Many of the organisms were obviously derived from the surrounding plants. They applied Koch's postulates to two of the isolates and showed that they could grow on and discolour sterile marble blocks.

Sterflinger (2000) indicated *Aspergillus niger*, *Penicillium simplissimum* and *Scopulariopsis brevicaulis* as important fungi that attack siliceous stone. These dark pigmented mitosporic fungi ("black fungi") can actively penetrate limestone and marble and produce pits of up to 2 cm diameter on rock surfaces (Sterflinger & Krumbein, 1997). They are especially important in arid and semi-arid environments (hot and cold deserts) because of their ability to resist high temperatures, desiccation and osmotic stress (Sterflinger, 1998).

In fact, several cryptoendolithic fungi may actively bore into the stone and hence physically disrupt its integrity (Gadd, 2007; Hoffland et al., 2004). Fungi, unlike the phototrophs, do not require light for growth, and so their boring activity can penetrate to greater depths. Golubic et al. (2005) discussed such a tunnelling activity in carbonate substrates (particularly mollusk shells) in marine environments. Hyphal penetration of materials involves swelling/deflation effects and channelling of water into the substrate. It can form cracks, fissures and crevices, extend existing ones and lead to the detachment of crystals (Sterflinger, 2000; Urzi et al., 2000). Weaker areas of the stone will be preferably penetrated by thigmotropism (contact guidance on solid surfaces to explore new substrates) (Gadd, 2007; Watts et al., 1998).

Biochemical actions of fungi can lead to microtopological alterations through pitting and etching, mineral dislocation and dissolution (Gadd, 2007). They are associated with extracellular mucilaginous substances, which contain, amongst many other metabolites, acidic and metal-chelating compounds (Burford et al., 2003). Acidic metabolites (oxalic, acetic, citric and other carbonic acids) deteriorate the stone minerals by a solubilising and chelating effect (Sterflinger, 2000; Urzi & Krumbein, 1994). Ortega-Morales et al. (unpublished results) showed that fungi isolated from deteriorated limestone at the Mayan site of Uxmal, Mexico, produced oxalic acid, which reacted with solubilised calcium from the stone to produce crystals of whewellite and weddelite. Fungal oxalic acid had previously been reported to solubilise metals (e.g. iron, aluminium, lithium, manganese) from various other substrates to form oxalates (Devevre et al. 1996; Strasser et al. 1994). Acidolysis and complexolysis, which have been reported to be the primary deteriorative mechanisms of fungi (Gadd, 2007), act on the stone mineral by proton efflux (plasma membrane H^+-ATPase, maintenance of charge balance during nutrient uptake) and siderophores, which mobilise iron (III), or CO_2 production (Gadd, 2007).

Oxidation and reduction of mineral cations are also triggered by fungal activity (Gadd, 2007). Iron and manganese particularly are removed from the stone lattice by redox processes (Warscheid & Braams, 2000) and may be reoxidised at the stone surface, forming "patinas" or "crusts". This biotransfer of metal ions and subsequent formation of patina (called "rock varnish" by Krumbein and Jens [1981] and Krumbein and Giele [1979]), can lead to hardening of the surface layer and

exfoliation (Tiano, 2002). However, Urzi et al. (2000) emphasised that there is no evidence that meristematic fungi produce acids, oxidise manganese or are directly responsible for the formation of "rock" or "desert varnish".

Various metabolic substances excreted by fungi are coloured, leading to staining of the substrate (Tiano, 2002). The production of melanins by dematiaceous (dark pigmented mitosporic) fungi darkens the stone surface, leading to significant aesthetic alterations and physical stress.

The literature suggests that fungi are present in low numbers on the surfaces of historic stone buildings. Populations of 10^2-10^5 cfu·g^{-1} are common (Gaylarde et al., 2001; Ortega-Morales et al., 2000; Hirsch et al., 1995b; Urzi, 1993; Resende et al., 1992). However, this does not mean that they are unimportant; their activity may be high and erosive. In addition, fungi may be the most important endoliths in built stone, according to de los Rios and Ascaso (2005). They have higher tolerance of low water activity than algae and bacteria and require low nutrient concentrations, as well as having no need for light.

1.6.2.2 Actinomycetes

These filamentous bacteria penetrate their substrate by mechanisms similar to those employed by fungi; they also excrete a wide range of enzymes. They can form a whitish veil on stone or produce various water-soluble dark pigments. Laboratory experiments have demonstrated their ability to utilise nitrites and nitrates and to reduce sulphates (Caneva et al., 1991), and, of course, they are well recognised as degraders of a wide range of different carbon and nitrogen sources. Probably for these reasons, the gram-positive actinomycetes tend to predominate over gram-negative bacteria on exposed stone surfaces (Saarela et al., 2004; Dornieden et al., 2000a; Warscheid & Braams, 2000). Their acidic metabolic products can attack calcareous stone, hydrolyse some silicate minerals and chelate metal ions (Kumar & Kumar, 1999). However, they have been reported to rarely, if ever, produce noteworthy amounts of organic acids and chelates in a rock decay environment (Urzi & Krumbein, 1994). In spite of this, they may cause structural damage by their extensive biofilm formation and penetration of their filaments into the stone substrate.

Actinomycetes have been found as important endoliths in various types of built stone (McNamara et al., 2006; Ortega-Morales et al., 2005), emphasising their degradative ability in this situation. Ortega-Morales et al. (2004), for example, found almost exclusively *Rubrobacter xylanophilus*-related bacteria on external biofilms in Uxmal. Although the genus Geodermatophilus has been suggested to be common on and in limestone (Eppard et al., 1996), Urzi et al. (2001), using amplified 16S rDNA analysis (ARDRA) and partial sequencing, found that many of the Geodermatophilaceae family on stone in the Mediterranean belonged to other genera (closest to *Modestobacter multiseptatus*).

There have been a number of publications on the presence of actinomycetes in caves (Schabereiter-Gurtner et al., 2004; Laiz et al., 2000), but, in spite of their obvious importance, there is little in the built cultural heritage literature on this group of microorganisms, apart from mainly superficial comments about their presence. This is an area that demands further attention.

1.6.2.3 Non-filamentous bacteria

The contribution of heterotrophic bacteria to stone deterioration had long been neglected, as insufficient organic nutrients were assumed to be present on stone surfaces. However, these organisms have been isolated frequently from such surfaces; and it has been found that organic contaminants, such as soil, dust and dirt, are sufficient to support heterotrophic growth. Furthermore, several of these heterotrophic bacteria are oligotrophic (May, 2003). Chemoorganotrophic bacteria utilise a wide range of nutrients and may serve other microorganisms by the breakdown of poorly degradable compounds (e.g. from atmospheric pollution), which could otherwise not be utilised.

Organisms of the genus *Bacillus* have been very frequently identified on stone buildings (Kiel & Gaylarde, 2006; Laiz et al., 2003; Gaylarde et al., 2001; Heyrman & Swings, 2001; Blazquez, 2000; Roelleke et al., 1996). This is not unexpected due to their ease of culture; also they are very common in soil and able to withstand extreme environments because of their spore-forming ability. Laiz et al. (2003), comparing culture and molecular biology techniques, suggested that their proportion of the biofilm on external surfaces of historic buildings is overestimated. However,

McNamara et al. (2006), using only molecular biology, found that many of the clones were closely related to the Low GC Fermicutes and considered that culture techniques may not, in fact, be entirely misleading.

Rather more important than the simple presence of these bacteria in the biofilm, is their potential degradative activity. Kiel and Gaylarde (2006) found that some of their *Bacillus* isolates produced acids and surfactants with auto-emulsifying activity in the laboratory, indicating that they had the capacity to accelerate stone degradation. Once again, however, beware extrapolation from laboratory experiments to the real world!

One surprising component of the stone microflora is the group of bacteria producing or utilising methane. These were isolated from 44 of 225 stone samples from 19 historic buildings in Germany and Italy (Kussmaul et al., 1998). All were Type II methanotrophs, i.e. those found at oligotrophic sites under nitrogen limiting conditions. It was suggested that the methane necessary for methanotrophic growth could originate from anthropogenic sources and from endolithic methanogens, which were detected in four of the samples, presumably in anaerobic niches. "Mini-methane producers", such as *Clostridium*, were found in almost half of the 47 samples tested for this activity.

1.6.3 Chemolithotrophic microorganisms

The presence of chemolithoautotrophic microorganisms, such as sulphur-oxidisers, nitrifying bacteria and iron- and manganese oxidisers, depends on the availability of the specific nutrients supporting their growth (Warscheid & Braams, 2000). Although they were the first group of microorganisms to be implicated in stone decay, their assumed importance has been superseded by later research that suggested the greater role of phototrophs and chemoorganotrophs. Gaylarde and Morton (2002) emphasised that there is little doubt that chemolithotrophic microorganisms have the potential to cause damage to stone; however, their significance to biodeterioration of outdoor stone monuments is still in question.

It appears that sulphur-oxidisers and nitrifying bacteria play a more significant role in biodeterioration in humid areas, because of their sensitivity to desiccation (Warscheid & Braams, 2000). In fact, nitrifying bacteria have been suggested to be the most important microbial factors in the decay of sandstone in northern Europe (Bock et al., 1988; Meincke et al., 1988).

1.6.3.1 Sulphur compound oxidisers and reducers

Sulphur-oxidising bacteria obtain energy by the oxidation of reduced or elemental sulphur to sulphuric acid. Sulphuric acid may react with calcium carbonate to form calcium sulphate (gypsum), which is more soluble in water than the calcium carbonate of the parental rock (Warscheid & Braams, 2000; Urzi & Krumbein, 1994), and thus more readily leached. However, sulphuric acid and calcium sulphate are not always of biogenic origin; they may also derive from atmospheric pollution and acid rain (May, 2003). In fact, Tiano (2002) emphasised that there is as yet no experimental evidence that confirms the direct action of sulphur-oxidising bacteria in the development of gypsum layers on stone surfaces.

Sulphate-reducing bacteria (which are not chemolithotrophic, but chemoorganotrophs) have been detected in biofilms on limestone (Gaylarde et al., 2001; Ortega-Morales & Hernández-Duque, 1998), but this is apparently rare and no role has been suggested for them in stone decay.

1.6.2.2 Nitrifying bacteria

Ammonia and nitrites on the stone surface are oxidised by chemolithotrophic and, partly, by heterotrophic ammonia- and nitrite- oxidisers to nitrous and nitric acid, respectively. Ammonia tends to derive from airborne ammonium salts, whereas nitrites may originate from automobiles, industry and soil (May, 2003). The acids that are produced attack calcium carbonate and other minerals (Warscheid & Braams, 2000; Urzi & Krumbein, 1994). The CO_2 produced can be utilised by the cells to form organic compounds, while calcium cations from the stone matrix form nitrates and nitrites, which are more soluble again than the original mineral phases and thus are leached out of the stone by rain. The characteristic symptom of the activity of nitrifying bacteria is a change in stone properties with no obvious biofilm. It becomes more porous, exfoliation occurs and fine powder may fall off (Urzi & Krumbein, 1994).

1.6.2.3 Iron- and manganese oxidising microorganisms

Iron oxidation is usually rapid and sensitive to pH and oxygen concentrations. Iron-oxidising bacteria obtain energy by oxidising ferrous iron in iron-containing minerals to ferric iron, which reacts with oxygen to form iron oxide. The latter process determines the characteristic discolouration and patina formation on stones. Many

bacteria and fungi, even algae, are capable of these oxidation steps, causing damaging lesions (Barrionuevo & Gaylarde, 2005; Urzi & Krumbein, 1994; Caneva et al., 1991). It is difficult to distinguish such bio-oxidation from abiotic chemical processes, although evidence for the involvement of living organisms in the formation of a red patina on a dolomite cathedral in Spain has been presented by Valls del Barrio et al. (2002).

1.7 Conclusion

Although it is well established that microorganisms can cause serious damage to stone monuments, knowledge of the precise mechanisms of decay is still fragmentary. This is a field that demands more attention. The development of new identification methods provides us with a broader understanding of the diversity of organisms present on outdoor monuments, and may expand our knowledge of new types of microbial metabolism occurring in these habitats. Most likely the list of organisms will expand dramatically as further analytical methods for detection and taxonomy are developed. However, very little work has been carried out in studying the general physiology and potential deteriorative activity of the newly identified organisms, using, for example, proteomics. A proteomic and genomic approach would not only shed light on the potential activity of microorganisms, but would also help to design new strategies for isolating and successfully culturing new organisms. Even microorganisms that have long been known to occupy the surface of stone monuments have only rarely been appropriately examined for their actual contribution to stone decay *in vivo*. In order to compare results of different research groups, a standardisation of methods for the detection, assessment and quantification of biodeterioration is necessary.

The possibility of biologically induced stabilisation of stone needs to be more thoroughly investigated. Understanding the interactions between microorganisms and with their environment is crucial to determine whether the organism is damaging or protective to the art object. A description of criteria for determining that the decay of a monument is due to microbial action is rare in the literature. Similarly, very few studies aim to quantify biodeterioration processes. In fact, there have been no attempts to define the degree of biodeterioration of an artefact and at what stage

antimicrobial actions should be initiated. In order to assess the contribution of microorganisms to the deterioration of cultural heritage objects, as well as the possibilities for their control, interdisciplinary research projects between conservators and scientists, such as microbiologists, geologists and chemists, are needed.

1.8 Aims

The overall aim of this thesis was to investigate protocols that give conservators tools to perform simple investigation towards the degree of biodeterioration. Identification of the microbial community was not attempted, as this would require specialised microbiological skills. Instead the activity of the entire microbial community was assessed. To be able to interpret the activity, it was attempted to put microbial activity in context with biomass, extra-polymeric substances and moisture present in the sample.

There are no data at what level of contamination and microbial activity antimicrobial action has to be taken. Therefore, the possibilities of long-term monitoring of the above parameters were assessed. If the condition of the artefact changes as well as activity and composition of the biofilm, this may indicate that antimicrobial measures need to be performed.

The second part of the experimental section focused on potential antimicrobial action, which could be performed if biodeterioration had reached an unacceptable level. Antimicrobial treatments for cultural heritage monuments are problematic, because methods of moderate toxicity are not effective enough and such that are effective tend to be hazardous to operating staff, visitors and non-target organisms. The aim was to test an approach how more environmentally friendly methods could be developed. To this end a combination of a chemical and a physical antimicrobial treatment method was tested, which could potentially have synergistic effects to make the combined treatment more efficient than each of the methods alone.

Chapter 2

Detection and assessment of microbial biodeterioration

2.1 Introduction to the detection and assessment of microbial biodeterioration

The contribution of algae, bacteria, fungi and lichens to the decay of stone in cultural heritage monuments has long been recognised (Müntz, 1890) but great challenges for investigation of deterioration processes remain. Determination of whether the degraded features of the substratum are the direct result of microbial activity or if abiotic chemical deterioration made the substratum susceptible for biological colonisation and further degradation is difficult. Assessment of the influence of microorganisms on building materials (Welton et al., 2005) provide methods that cannot always be used with cultural heritage material due to restrictions in sampling size and sample representativeness. The lack of standardised procedures for the detection, assessment and quantification of microbial deterioration makes the comparison of results difficult.

2.1.1 Assessment of stone degradation

Stone decay may be caused in a variety of ways and is not restricted to biological deterioration processes; hence it is important that the assessment of degradation be accompanied by microbiological studies if biodeterioration is suspected. Although water is recognised as the main factor affecting microbial growth (fig. 11), chemical and physical properties of the stone (e.g. rough surfaces and high porosity) influence its extent and may favour colonisation of more susceptible stone (fig. 12) (May et al., 2003; Warscheid & Braams, 2000; Caneva et al., 1991).

Figure 11 Wet facade in Bridport
A facade in Bridport, Dorset, UK, shows that those parts that remain wet longer after the rain develop more biofilm than surfaces that dry more rapidly. Picture by C. Gaylarde.

Figure 12 Mosaic with selective algal growth
The green colour in this mosaic from Aquileia, Italy, derived from algal growth, which selectively colonised only one type of stone. Picture by C. Gaylarde.

Decayed stone adopts a wide variety of macroscopic appearances and assessment of the attack will begin with visual inspection (Siegesmund et al., 2004). Erosion (fig. 13) often extends over a large area and the surface of the material is slowly worn down. Pitting (fig. 13) consists of small holes that can extend to deeper layers of the stone. Blistering and exfoliation (fig. 14) are the result of processes that usually occur within the stone and lift whole flakes from the surface. Microorganisms are often involved in all these processes.

Figure 13 (left) Limestone statue showing erosion and pitting
Limestone statue in Tikal, Guatemala showing severe cases of erosion and pitting (arrows).
Figure 14 (right) Limestone wall showing exfoliation
Limestone wall in Tikal, Guatemala with large areas or material loss in the surface due to exfoliation.

Determination of the intrinsic properties of the material is fundamental to the prognosis of stone bioreceptivity (Prieto & Silva, 2005) and a comparison between sound and deteriorated areas can give an idea of the degree of decay. Techniques for the characterisation of stone decay include chemical analysis of major, minor and trace elements; mineralogical and petrological descriptions. A description of non-destructive methods that can be used on cultural heritage objects is to be found in Janssens and Van Grieken (2005), although stone is not included in their examples. Currently available microscope techniques were reviewed by de los Ríos and Ascaso (2005). The more important methods are shown in table 1.

Table 1
Methods for the assessment of stone monument degradation

Method	Parameter determined	Comments/use	References
Visual inspection	Pattern of stone loss, basic characterisation of deterioration mechanisms	Non-destructive. First and simplest investigation. Amount of information depends on experience.	Siegesmund et al., 2004
Inspection of physical properties	Surface hardness, surface roughness, porosity, pore size distribution, water absorption, capillarity, compressive strength, sound velocity, resistance to salt crystallisation	These properties give an indication of the bioreceptivity of the stone	Barrionuevo & Gaylarde, 2005; Vladimirov, 2005; Moropoulou et al., 2003; Topal & Sözmen, 2003; Galán et al., 1999; Price, 1996; Ribas Silva, 1995
Scanning electron microscopy (SEM)	Topography, surface porosity, surface deposits, material loss	Small fragment needed. Useful to assess conservation treatments.	De Graef et al., 2005; Benavente et al., 2004; Moropoulou et al., 2003; McNamara et al., 2003; Esbert et al., 2001; Maravelaki-Kalaitzaki et al., 2001; Bartosch et al., 2000; Garcia-Vallès et al., 2000
X-ray computed tomography (CT)	Physical changes, pore size, density, material loss	Small objects: non-destructive, large objects: fragment needed. Provides 3-dimensional information.	De Graef et al., 2005; Jacobs & Cnudde; 2005; Cnudde et al., 2004; McNamara et al., 2003
Nuclear magnetic resonance (NMR)	Porosity, water saturation, elements	Normally destructive, unless portable instrument used.	Casieri et al., 2005; McNamara et al., 2003
Acoustic wave velocity	Physical properties (e.g. density, fissures and other discontinuities)	Non-destructive (however, a conductive medium may have to be applied), useful to assess conservation treatments.	Chukwunweike & Rutter, 2006; Vladimirov 2005; McNamara et al., 2003; Galán et al. 1999; Siegesmund et al., 2004

Energy dispersive x-ray spectroscopy (EDX/EDS)	Elements present	Small fragment needed. One of the most common methods. Useful for patina analysis.	Barrionuevo & Gaylarde, 2005; De Graef et al., 2005; Maravelaki-Kalaitzaki, 2005; Ghedini et al., 2003; Moropoulou et al., 2003; Esbert et al., 2001
X-ray diffraction (XRD)	Mineralogical composition	Generally a very small (µgs), powdered sample needed, but objects can be examined non-destructively *in situ* with hand-held instruments.	Maravelaki-Kalaitzaki, 2005; Sawdy & Price, 2005; Benavente et al., 2004; Moropoulou et al., 2003; Topal & Sözmen, 2003
X-ray photoelectron spectroscopy (XPS)	Elements present	Surface analysis of small fragments.	Maravelaki-Kalaitzaki et al., 2002
Atomic absorption spectroscopy (AAS)	Elements present	Small samples needed (µg to mg).	Topal & Sözmen, 2003; Galán et al., 1999
X-ray fluorescence (XRF)	Elements present	Non-destructive if object or instrument is mobile. Elemental mapping of several cm^2 possible. Stone matrix can affect results.	Powers et al., 2005; Cesareo et al., 2004;1999; Moropoulou et al., 2003; Bronk et al., 2001
Inductively coupled plasma mass/atomic emission spectroscopy (ICP-MS, ICP-AES)	Elements present	Small samples needed (mgs). Very sensitive.	Sawdy & Price, 2005; Galán et al., 1999
Particle induced x-ray emission (PIXE)	Metals present	Non-destructive if object or instrument is mobile. Limited to near-surface analysis. Very sensitive and good for analysis of trace elements (from Na to the end of the periodic table).	Del Monte et al., 2001
Laser induced breakdown spectroscopy (LIBS)	Elements present	Minimal destruction (size of the laser beam). Stratigraphic mapping possible.	Maravelaki-Kalaitzaki, 2005; Maravelaki-Kalaitzaki et al., 2001; Klein et al., 2000
Thermogravimetry, derivative thermogravimetry, differential scanning calorimetry	Heat-labile materials	Small samples needed (mgs). Can differentiate organic and inorganic carbon compounds.	Ghedini et al., 2003; Barcina et al., 1997

Weathering of rocks causes changes in chemical composition of the stone by enrichment, as well as leaching. The chemical analysis of rainwater run-off (both as dry and wet deposition) provides estimates of the adsorption of pollutants onto the stone, migrating salts and stone dissolution, once the ionic balance is calculated

(Bravo et al., 2006; Baedecker et al. 1992; Lipfert 1989). The assessment of stone dissolution by run-off analysis is particularly useful for rocks with high weathering rates (e.g. limestone), while for more durable materials, such as granite, material losses may be below the detection limit (Sweevers et al., 1995). Leached cations may also be analysed in laboratory simulations of weathering. Ion-selective electrodes and the Ca^{2+} binding fluorochrome Rhod-5N have been used to measure Ca^{2+} release from limestone (McNamara et al., 2005).

The approximate depth of weathering may be determined by either optical or chemical methods; both provide similar results (Topal & Sözmen, 2003). Analysis of the mineral structure is frequently performed by X-ray diffraction (XRD), provided the material possess a crystalline structure (Sawdy & Price, 2005; Benavente et al., 2004; Moropoulou et al., 2003; Topal & Sözmen, 2003). This can provide information on the structure of crystalline materials by powder diffraction, where a small powdered sample is analysed, or by surface analysis. If the object is small, or the equipment portable, the latter can be performed *in situ*.

X-ray fluorescence (XRF) allows rapid characterisation of surface chemicals and their quantification if appropriate standards are available. However, elements of low atomic number (generally below 9, fluorine) cannot be detected. Mapping of selected elements in centimetre-sized areas is possible. Castellano and Cesareo (1997) developed a portable energy-dispersive XRF system, which was used to detect sulphur and chlorine traces before and after the restoration of various frescos in Italy (Cesareo et al., 2004). A portable micro-XRF equipment has also been produced (Bronk et al., 2001).

Maravelaki-Kalaitzaki et al. (2002) demonstrated the higher sensitivity of X-ray photoelectron spectroscopy (XPS, also called ESCA, Electron Spectroscopy for Chemical Analysis) over Fourier transform infra-red spectroscopy (FTIR) and scanning electron microscopy / energy dispersive using x-ray (SEM-EDX) in the detection and chemical characterisation of early stages of stone weathering. The alteration products initially remain within nanometres of the exposed surface; hence, a minimally destructive surface-sensitive technique, such as XPS, is most appropriate.

In-depth profiling of the elemental composition of deterioration layers is possible with laser-induced breakdown spectroscopy (LIBS) (Maravelaki-Kalaitzaki et al., 2001). This is a quick *in situ* technique of minimal destructivity that can provide

elemental profiles of even highly inhomogeneous crusts. Sampling is performed by successive laser pulses and the emission spectrum is recorded for the samples removed by each laser pulse, allowing stratigraphic mapping of the elements present (from Li to U).

Generally, a variety of methods are used for compositional analyses of stone and their degradation products. For instance, Maravelaki-Kalaitzaki (2005) showed S, Fe, Si and Al in black crusts on Parthenon marble using SEM-EDX and LIBS, while XRD and FTIR allowed elemental stone analysis and demonstrated the presence of calcium oxalate in patinas. These techniques did not, however, allow the discrimination of biogenic from chemically-formed oxalate. The biogenic origin of black crusts could, however, be demonstrated when EDX was used together with spectrophotometric techniques to detect and quantify blackening microbial pigments (Gaylarde et al., 2007).

SEM and related methods (SEM-EDX, ESEM [environmental, or low-vacuum SEM]) can be used to investigate topography, material composition and mineral structure of small stone samples (De Graef et al., 2005; Benavente et al., 2004; Moropoulou et al., 2003; Esbert et al., 2001; Bartosch et al., 2000). Surface changes are detected, but for deeper studies sections are required (McNamara et al., 2003; Maravelaki-Kalaitzaki et al., 2001). Patinas are readily characterised (Garcia-Vallès et al., 2000). An interesting addition to SEM-EDX is the software program "Featurescan", which allows the individual detection, measurement and elemental analysis of features such as surface particles or pore sizes (Esbert et al., 2001).

X-ray computed tomography (CT) allows the visualisation and characterisation of artefact materials. The major advantage over conventional medical scanners is its increased spatial resolution (De Graef et al., 2005; McNamara et al., 2003). Although often described as non-destructive, a sample must generally be taken; for stone monuments, a core of several millimetres has to be drilled or a stone fragment has to be removed (De Graef et al., 2005). McNamara et al. (2003) adapted high-resolution three-dimensional micro-CT (μCT) for qualitative and quantitative analysis of the deterioration of carbonate-based stone. The authors produced a three-dimensional composite image of the citric acid-etched calcareous stone coupon prepared from a limestone of a Mayan building. Cnudde et al. (2004) used μCT to visualise porosity and pore size distribution of natural building stones and to determine the penetration depth of water repellents and consolidants. Their instrument allowed the 3-

dimensional visualisation of pores as small as 10 μm. μCT instruments that will allow investigations of even higher resolution have already been developed. De Graef et al. (2005) used μCT to investigate the depth of material loss, areas of leaching and changes in porosity and density during bacterial weathering of stones and Jacobs and Cnudde (2005) discussed the use of the method for cultural heritage materials.

Thermogravimetry (TG), derivative thermogravimetry (DTG) and differential scanning calorimetry (DSC) are rapid methods that require relatively small amounts of sample (approximately 25 mg). TG and DTG measure the weight-loss of a heated sample, which is characteristic for its nature. They give information on chemical (compositional analysis of solid and volatile components) and physical (e.g. thermal stability, water content, oxidation) parameters of the sample. Coupled TG/DSC analysers exist, which can be interfaced with other analytical units (e.g. mass spectroscopy [MS]; FTIR, gas chromatography [GC]). Using a combination of TG and DTG, Ghedini et al. (2003) distinguished between the carbon components in black crusts derived from the stone matrix (carbonate carbon), atmospheric pollution (elemental carbon), biological weathering and organic conservation materials (organic carbon) and quantified the amounts present. Barcina et al. (1997) used DTG to differentiate between calcite and dolomite.

The physical properties give an indication of the bioreceptivity of the stone. Profiling the properties of a stone at different levels allows an indication of the depth of decay. Topal and Sözmen (2003) found that effective porosity, water absorption and sonic velocity were the most useful parameters for the quantification of stone damage. The effective porosity determines the interconnected pores of a stone. It is related to fluid flow. Pore size distribution is an important factor for decay resistance as it contributes to penetration depth and distribution of water. It is a critical factor for microbial transport and growth within stone, a threshold value of 1 μm having been estimated for nitrifying bacteria (Mansch & Bock, 1998). Pore size also significantly influences the damage caused by salt crystallisation, through its effect on crystallisation pressure (higher in stone with small pores), and mineral penetration (deeper in stone with small pores) (Benavente et al., 2004). Porosity and water saturation may be measured by nuclear magnetic resonance (NMR), which, however, cannot discriminate between biological and abiotic changes in pore size distribution (McNamara et al., 2003). Portable NMR devices recently became

available to perform non-destructive *in situ* measurements (Casieri et al., 2005). For cultural heritage materials, these non-destructive methods should be preferred over others, such as those employing Hg-injection, absorption of nitrogen, or treatment with a microscopically detectable resin that fills the pores (Moropoulou et al., 2003; Moropoulou & Kefalonitou 2001; Galán et al., 1999). The ability to take up water is related to stone porosity. It can be directly measured using the non-destructive Karsten-Tube-Test and other capillary methods (Moropoulou et al., 2003; Moropoulou & Kefalonitou, 2001; Wendler & Snethlage, 1989). Capillary water uptake may be influenced by biofilms on the stone substrate (Warscheid, 2000; Wendler & Snethlage, 1989). Vladimirov (2005) used the free-water saturation of a marble votive plaque to determine the effective porosity (percentage of total stone volume), the conditional momentary saturation (water saturation within the first 20 min, which determines liquid permeability), the fraction of pores of different sizes, the saturation constant (a parameter different from total porosity that determines how fast water saturation is reached) and the weight in water, which determines the stone density. The effect of complete water saturation on an artefact, however, needs to be carefully considered.

Acoustic wave velocity gives information on discontinuities in the stone and whether changes in stone density correlates with its decay (Siegesmund et al., 2004). It has been used to characterise stones for provenance purposes (Galán et al. 1999) and to investigate the success of a consolidation treatment (Vladimirov, 2005). The higher the porosity or larger the micro-cracks, the lower the acoustic wave velocity (Chukwunweike & Rutter, 2006, Siegesmund et al., 2004). The level of moisture within the stone significantly influences the velocity, which has to be taken into account for the interpretation of the results (Siegesmund et al., 2004). Correlation of the results with other techniques is generally necessary (McNamara et al., 2003).

The phase transition of salts present on building stones can be predicted using the computer programme, Environmental Control of Salts (ECOS; Sawdy & Price, 2005). The thermodynamic behaviour of the salts was correlated to relative humidity (RH) and temperature at the site. The researchers demonstrated that there was a high correlation between the phenomenological observations and the calculated thermodynamic behaviour of the salts present, predicting which of the salts would crystallise first.

2.1.2 Evaluation of the microbiological flora on stone monuments

Microorganisms on or from stone may be enumerated and identified by a wide range of culture-dependent and culture-independent techniques (table 2).

Table 2
Methods for the assessment of stone-colonising microorganisms

Method	**Microbial cells/activity assessed**	**Comments/use**	**References**
Visual inspection	Presence of biofilm. Basic categorisation of organisms involved	Limited in distinguishing biofilm from non-living film. Information increases with experience	Prieto et al., 2004; Urzi & Realini, 1998
SEM, environmental SEM (ESEM), transmission EM (TEM), atomic force microscopy (AFM)	Presence of cells and EPS	Microbial type and activity not identified, but may offer evidence of direct involvement of cells with degradation	de los Ríos & Ascaso, 2005 and references therein
Epifluorescence microscopy	Cell component/activity depends on fluorescent stain used	May be used directly on sample surface. Presence of autofluorescent organisms can be assessed without stain	McNamara et al., 2006
confocal laser scanning microscopy (CLSM)	As above	Three-dimensional visualisation within stone possible at depths up to a few mm	de los Ríos & Ascaso, 2005; Bartosch et al., 2003; Blazquez et al., 2000; Barker et al., 1998
Activity dyes, fluorescent or non-fluorescent	Activity, viability, identification of enzymes	May be possible to visualise colour change on stone surface with the naked eye and measure fluorescence *in situ* with fibre optic probes	Prieto et al., 2004; Hirsch et al., 1995a; Tayler & May, 1995; Warscheid et al., 1990
Culture on microbiological media	Cell numbers, viability, some types of activity, substrate utilisation	Biofilm is generally disrupted. Many microorganisms are "non-culturable". Organisms in culture may exhibit different activities than in the environment.	Gaylarde et al., 2004; Dornieden et al., 2000a, b; Saiz-Jimenez & Laiz, 2000; Tomaselli et al., 2000a; Ortega Morales et el, 1999; Urzi et al., 1999; Urzi & Realini, 1998; Hirsch et al., 1995a

Adenosine triphosphate (ATP)	Active (energy-storing) cells	Type of cells not identified. Must be performed rapidly after sampling as ATP degrades quickly, but equipment available for *in situ* measurement	Cappitelli et al., 2006; Ranalli et al., 2003; Allsopp et al., 2003; Gorbushina et al., 2002; Praderio et al., 1993; Tiano et al., 1989
phospholipid fatty acids, ergosterol analysis	Cell membrane lipids	Signature PLFAs allow some level of identification. Ergosterol specific for fungi. Samples must be taken to the laboratory	Ortega-Morales et al., 2004, 2000, 1999; Saad et al., 2003; Gutarowska & Zakowska, 2002; Palmer, 1994; Hirsch et al., 1995a;
Chlorophyll analysis	Photosynthetic cells	Related to viability and activity of cells. May be performed directly on stone surface	Gaylarde & Englert, 2006; Schumann et al., 2005; Tomaselli et al., 2002
Non-photosynthetic pigments e.g., scytonemin, melanin, carotene	Pigmented microorganisms	Detects disfiguring activity	
Analysis of protein, lipid, carbohydrate, nucleic acids	All cells, viable or not, and extracellular compounds	Simple methods can be used. Not specific to microbial genera	Bellezza et al., 2006; Warscheid et al., 1990
Respirometry, CO_2 production	Cell respiration	All active organisms measured. Photosynthetic cells can interfere as they utilise CO_2	Koestler & Salvadori, 1996; Albertano, 2003
Molecular methods based on DNA amplification (PCR)	Specific genes	Very small samples required. Conditions of PCR may be chosen to detect specific organisms or activities. Isolation and culture not necessary	McNamara et al., 2006; Salazar et al., 2006; Gaylarde et al., 2004; Ortega-Morales et al., 2004; Saarela et al., 2004; Abbruscato et al., 2003; Heyrman, 2003; Laiz et al., 2003; Saad & Gaylarde, 2003; Heyrman et al., 1999; Roelleke et al., 1998
Metagenomics	Community genes	Isolation and culture not required. Much work still needed before this can be used in the cultural heritage field	

2.1.2.1 Sampling techniques

The method used for sampling environmental microorganisms is an underestimated source of errors in determining microbial diversity. Assessment of microorganisms associated with cultural heritage objects is particularly difficult; the damage caused in removing a representative sample must be balanced against the information gained. Non-destructive sampling and micro-sampling methods are preferred. However, these samples may not produce results that are representative of the entire extant microflora; particularly endolithic microorganisms are often overlooked by such techniques.

The only published comparison of different sampling techniques for the isolation of microorganisms from cultural heritage materials was carried out by Kyi (2001). She found that velvet and 3M "Post-it Index" tape were the most successful for detecting bacteria that caused pink and yellow staining on murals. Very low numbers of target bacteria were found with the inoculation loop, needle, fragment and swab method. Brushing resulted in the isolation of fungi only. It must be remembered that the aim of this research was to isolate those bacteria that caused staining. The target organisms were surface colonisers and these methods would not necessarily be suitable for more detailed studies.

The limits of minimal-destructive methods, such as washing off microorganisms from rock surfaces (Hirsch et al., 1995a), damp cotton swaps (Mitchell & Gu, 2000; Taylor-George et al., 1983) adhesive tape (Gaylarde & Gaylarde, 2005; Shirakawa et al., 2002; Urzi & De Leo, 2001; Gaylarde & Gaylarde, 1998) and direct contact agar (Bridson, 1969), are that only organisms on the upper surface layers may be sampled, whereas chasmolithic organisms and those that are intimately attached to the substrate may not be removed. The brushing equipment attached to a water spray, devised by Peters et al. (2005), improves removal, but may damage the surface. As with washing and swabbing, the microbial biofilm is disrupted by this method and this may lead to the non-detection of those species that cannot grow alone on laboratory media. Whatever the method chosen, the airborne microflora should also be analysed to evaluate recent contaminants that may not proliferate on the artefact or participate in deterioration.

Photographic monitoring is an inexpensive and simple non-destructive method that gives information on changes in microbial colonisation of a surface without the

necessity for sample removal. Changes in colour and area of discolouration over time may provide information on microbial activity and biomass (Hirsch et al., 1995a) or the water availability (Ortega-Morales et al., 2004). The results can be quantified by image analysis. Murphy et al. (2006) used an adaptation of this technique to determine phototroph colonisation on aquatic rocks. Digital colour infra-red (IR) imagery showed that the ratio of near IR to red absorption was linearly related to chlorophyll content.

Adhesive tape sampling allows stratigraphic examination of biofilms by consecutive sampling of the same area. Community composition may vary throughout the depth of the biofilm, although major biomass has been suggested to remain constant in successive layers in mature biofilms (Gaylarde & Gaylarde, 2005). Normally, microbial numbers decrease with rock depth (Bartosch et al., 2003), apart from in rocks exposed to high irradiance (Urzi, 1993; Palmer & Hirsch, 1991). Adhesive tape sampling can also be used for fluorescent *in situ* hybridisation (see 2.1.3) (La Cono & Urzi, 2003; Urzi & de Leo, 2001). The major advantage, in addition to its minimal to non-destructive nature, is that tape sampling is readily performed by non-microbiologists (Urzi & De Leo, 2001).

Destructive sampling methods include surface scraping and the removal of fragments with sterile tools (Cappitelli et al., 2006; De los Ríos et al., 2004; Herrera & Videla, 2004; Tayler & May, 2000; Ortega-Morales et al., 2000). These methods allow the assessment of more intimately attached microorganisms and those that dwell deeper in the stone. The analysis of fragments can provide information on the spatial distribution of the organisms.

The selection of sampling sites is critical. Microenvironmental conditions must be carefully documented to allow appropriate interpretation of the results. There are significant differences between total microbial biomass and microbial community composition in exposed and protected stone environments (Herrera & Videla, 2004; Ortega-Morales et al., 1999, 2000).

2.1.2.2 Detection, identification and quantification of the microbial flora on stone

Total microorganisms may be quantified by culture methods, such as the most probable number (MPN) technique (estimation of the microbial population size through culturing known dilutions of a sample), by microscope counting methods, often in combination with fluorescent dyes (Hirsch et al., 1995a; McNamara et al.,

2006), by nephelometry (a counting technique that is based on the light scattering caused by cells in a liquid suspension) and laser particle counting (measures light absorption by particles in a liquid suspension). For the study of the physiology of microorganisms cultivation-based methods are still the most common approach. However, molecular methods are becoming more and more widespread in the detection, identification and, to some extent, quantification of microorganisms on cultural heritage objects (table 2).

2.1.2.2.1 *In situ* microscopy techniques

The term "*in situ*" is used here in the microbiologist's, rather than the conservator's sense. It implies that microorganisms are detected in their original positions on or in the stone, and not that the stone sample itself has remained an integral part of the artefact. Indeed, small samples of stone must generally be removed to the laboratory for visualisation. Although destructive, these methods are culture-independent.

The methods and instruments used to examine microorganisms within the biofilm on a stone surface include epifluorescence microscopy, with fluorescent dye staining for non-autofluorescent organisms, atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM), various modifications of SEM and ESEM (for example, SEM in the back-scattered mode) and transmission electron microscopy. Some of these have been reviewed recently by de los Ríos and Ascaso (2005). CLSM, a high-resolution microscopic method that allows optical sectioning of the sample, is particularly appropriate for visualising cells within the stone. The use of fluorescent dyes specific for certain kinds of microorganisms or physiological or metabolic characteristics allows the categorisation of the microbial community with spatial reference. It has been used to show that many more active bacteria were present in north, east and west as compared to south facing natural stone exposed for 9 years in Germany, correlating with the higher sun exposure of south facing stone (Bartosch et al., 2003). The use of pH-sensitive fluorescent probes allows pH measurement around microbial cells within the stone, giving fundamental information on breakdown mechanisms linked to metabolic activity (Barker et al., 1998).

2.1.2.2.2 Cultivation of microorganisms

The cultivation of microorganisms on laboratory media has produced a wide range of pure cultures whose physiology and potential deteriogenic activities can be studied. However, culture-based techniques detect only a small fraction of the total population of an environmental sample, leading to a serious under-estimation of microbial abundance and diversity, the so-called "Great Plate Count Anomaly" (Amann et al., 1995; Staley & Konopka, 1985). Most of the bacteria in culture collections were isolated on conventional, rich media, which do not mimic the oligotrophic status of natural habitats (Fry, 2004). The development of molecular methods (see following section) has circumvented this problem to a certain extent; "total" genomic DNA from a community can be extracted and analysed by various techniques. This has increased our understanding of the diversity of microorganisms dwelling on outdoor stone monuments. However, in order to assess their physiology, cultivation is still essential. Most of the latest improvements in culture techniques (e.g. inoculation with highly diluted samples [extinction culture], using growth medium that mimics the natural substrate, specific sampling by micromanipulation employing "optical tweezers", colony excision, etc.) have not been used on cultural heritage samples.

Conditions on outdoor stone monuments are extremely variable, with changes in nutrients ("feast and famine"), as well as seasonal temperature and humidity. Direct exposure to sunlight on exposed surfaces results in higher thermal stress, desiccation and UV exposure, and is a major factor determining the composition and functional ecology of microbial colonizers (Gaylarde et al., 2006). Nutrient-rich media tend to preferentially isolate fast-growing organisms, which may not be the dominant microflora. A large fraction of the microbial population on outdoor stone monuments is oligo- or poikilotrophic. They grow slowly but are resistant to adverse conditions, enabling them to out-grow less resistant microorganisms on stone surfaces. Poikilotrophic microorganisms are said to have high deteriogenic potential (Dornieden et al., 2000a, b).

Hirsch et al. (1995a) suggested the inoculation of several particles of rock into a small volume of oligotrophic peptone yeast extract glucose vitamin medium (PYGV) and into Hutner's basal salts, incubated in dim light for long periods. For the isolation of the endolithic microbial community, they suggested a solid medium containing

2% agar, with a thin liquid film on its surface. This film covered the small rock samples (1–3 mm), providing a surface layer enriched in rock nutrients. They observed that microorganisms growing on solid medium were different from those developing in liquid culture; the percentage of exopolymer-forming microorganisms was higher on solid medium.

Interactions between organisms in the microbial community are also important. Antimicrobial substances may hinder the growth of some organisms, whereas other substances (e.g. exo-enzymes) may support growth. Gaylarde et al. (2004) noted that the cyanobacterial diversity seen microscopically in rehydrated biofilms on surfaces is much larger than suggested by standard isolation techniques and that many cyanobacteria are lost in culture if the initial spatial relationships present in the biofilm are not maintained.

2.1.3 Molecular methods

Molecular methods are becoming more common in the detection, identification and, to some extent, quantification of microorganisms on cultural heritage objects. Their great advantages are that they require very small sample sizes and are independent of the biases inherent in cultivation. Often, they provide a better picture of the microbial diversity, once the procedures are optimised. Laiz et al. (2003) found that a larger variety of microorganisms were identified with molecular techniques and that cultivation based methods led to an overestimation of *Bacillus* species; these findings corroborated previous observations (Heyrman et al., 1999). However, under certain conditions, *Bacillus* spp. may indeed be dominant, as demonstrated by both culture (Kiel & Gaylarde, 2006; May et al., 2000) and molecular (McNamara et al., 2006) approaches.

The use of molecular identification techniques has led to the discovery of novel microbial species (Salazar et al., 2006; Saarela et al., 2004; Heyrman, 2003), identified organisms phylogenetically related to microbes of a certain physiology (Ortega-Morales et al., 2004) and detected community differences between epilithic and endolithic habitats (McNamara et al., 2006). Denaturing Gradient Gel Electrophoresis (DGGE) profiling methods have dominated the literature (Ortega-Morales et al., 2004; Laiz et al., 2003; Saad & Gaylarde, 2003; Roelleke et al., 1998). The DGGE mediated separation of small DNA fragments from a microbial community into microbial species provide profiles that are useful to monitor changes

in microflora diversity (Petersen & Toepfer, 2001) and show differences of the microbial community between deteriorated and undeteriorated surfaces (Abbruscato et al., 2003). They could be applied to determine the microbial diversity before and after conservation or biocide treatment. DGGE targeting specific genes may allow the detection of phenotypic potential and, together with the analysis of community function, is possible by reverse-transcription-PCR (RT-PCR). Despite the relevance of these methods, they have not, to date, been applied to biofilms on stone, although they have been used on complex environmental samples to detect active fungi (Bleve et al., 2003) and bacteria (Marchant et al., 2006; Otawa et al., 2006; Bürgmann et al., 2003).

Emerging techniques for assessing the stone microbial community include single strand conformation polymorphism (SSCP) (Ortega-Morales et al., 2004). SSCP assesses single stranded DNA fragments, which underwent a 3-dimensional folding after denaturation. This characteristic folding allows the fragments to travel faster or slower in an electrophoresis gel and thus mediates the differentiation of DNA molecules of the same length but with different nucleotide (base) sequences. As with all PCR-based methods, DNA extraction and amplification biases may lead to erroneous conclusions about the main organisms present (Fry, 2004; Gaylarde et al., 2004). Further interfering factors, such as presence of PCR inhibitors, certain ions, metal pigments, cellulose and cellulose derivatives, or some synthetic polymers, may render results unreliable (Fry, 2004).

Fluorescent *in situ* hybridisation (FISH) is an extremely useful molecular method for the study of environmental microorganisms. In its normal application, it is a DNA amplification–independent method, making it less biased than PCR-based protocols. It applies commercially available fluorescently-tagged DNA sequences which can bind to those parts of the target RNA to which they have sequence identity. The availability of a large quantity of different fluorescent probes makes its application extremely versatile. Depending on the specificity of the fluorescent probe it permits the visualisation of specific organisms or genes within complex microbial communities and allows the correlation of identity, quantity, physiology, metabolic activity, morphology and spatial distribution (Urzi et al., 2004). This highly sensitive technique seems to be most successful for bacteria; however, fungi (where problems may derive from thick cell walls), the autofluorescent cyanobacteria and the archaea can be investigated with modified protocols (Gonzalez, 2003; Urzi et al., 2003). The

method is not always suitable for cultural heritage artefacts; in oligotrophic environments like monument surfaces, low nutrient availability may result in microorganisms possessing too few ribosomes for sufficient oligonucleotide probe binding.

Many bacteria identified by molecular biology have not yet been cultured and thus their metabolic activities that might influence stone are unknown (Schabereiter-Gurtner et al., 2003). Metagenomics, the study of the total microbial genetic material in an environment, raises the possibility of detecting genes that could be involved in deterioration processes without the identification or culture of microbial species. A review of the potential of metagenomics for microbial ecology is given in Cowan et al. (2005). Gene-specific PCR has been used for environmental samples from habitats other than cultural heritage objects to selectively amplify genes involved in certain metabolic processes or biodegradative capabilities (Henckel et al., 2000; Sheu et al., 2000; Watanabe et al., 1998). It has not been approached on samples from cultural heritage objects, however, it would be of great interest to identify the presence of genes coding for the production of pigments, extracellular polymers, osmolytes or substrate-degrading enzymes. Research in this direction is desperately needed, as it would greatly enhance our knowledge of the physiology and metabolic capacities of a stone inhabiting community without having to culture them.

Although important, molecular biology techniques must be teamed with other microbiological studies and the dangers of relying on only DNA analysis for evaluation of cyanobacterial populations on stone have been pointed out (Chacón et al., 2006; Gaylarde et al. 2005; 2004).

2.1.4 Biomolecules

Biomolecules, other than nucleic acids, can be readily extracted from microbial communities and used to quantify microbial biomass and to provide an estimate of community composition, at least to the microbial group level. Microbial biomass determination on the surface of a cultural heritage building may be required when monitoring conservation or biocide treatment. However, sequential and not single absolute measurements are needed. Comparison of microbial biomass on different sites may give an insight into environmental conditions favouring colonisation but has limited importance in terms of the damaging potential of the community, for which identification of microbial species or activities is more relevant.

Indirect biomass assessment by measuring proteins, phospholipids or biogenic pigments (e.g. chlorophyll *a*, scytonemin, carotenoids) by spectrophotometry, UV-Vis spectroscopy, high performance liquid chromatography (HPLC), GC or FTIR (Gaylarde et al., 2006; Prieto et al., 2004; Tomaselli 2002; Ortega-Morales et al., 2000, 2004; Warscheid, 1996b; Hirsch et al., 1995a; Gerhard et al., 1994; Warscheid et al., 1990; Whitlatch & Johnson, 1974) must be carefully interpreted, as it does not necessarily indicate microbial origin.

Carbohydrates are also used for assessment of microbial contamination, but do not allow quantification of microbial biomass; however, they give an idea of the amount of biofilm present, of which they are the predominant constituent. Polysaccharides may be microscopically visualised with periodic acid-Schiff reagent (PAS) (Whitlatch & Johnson, 1974). The periodic acid of the PAS stain oxidises the carbon to carbon bond forming aldehydes. These aldehydes react with the fuchsin-sulphurous acid resulting in the red colour indicating the presence of glycogen. Dubois et al. (1956) suggested that the phenol-sulphuric acid method for colorimetric assay of sugars was preferred over others for its simplicity, sensitivity, speed and reliability. This determination has been used on directly extracted microbial EPS from stone samples and correlated well with the amount of biofilm present in the sample (Ortega-Morales et al., 2001). For determining monosaccharide composition, EPS directly extracted from pulverised stone can be analysed by HPLC (Bellezza et al., 2006) or as the methyl glycosides by GC (Ortega-Morales et al., 2001).

The phototrophic community in a sample can be semi-quantified by assessing the chlorophyll *a* content. After the extraction of the pigment (e.g. in acetone, methanol or dimethyl formamide) from the ground stone sample, it is quantified by absorption measurement in a spectrophotometer (Schumann et al., 2005; Prieto et al., 2004; Tomaselli et al., 2002; Ortega-Morales et al., 2000). Measurements can also be made directly on the stone surface, using a spectrofluorimeter with a fibre-optic attachment (Miller et al., 2006). For small artefacts this method provides a non-destructive, *in situ* assessment of phototrophs. However, the stone sample has to be examined in a light-tight containment, rendering it unfeasible for large monuments.

Biomarkers (also called biosignatures) are constitutive molecules in organisms that can indicate the presence and quantify the biomass of (micro-)organisms at varying levels of taxonomic resolution, ranging from microbial groups to species. The analysis of cell components has been shown to give essentially the same cell

number estimates as more traditional microbiological counting techniques (Balkwill et al., 1988). Signature biomarkers such as phospholipid fatty acids (PLFA) can be analysed by GC and MS (Ortega-Morales et al., 2004). Phospholipids can provide an indirect quantification of the viable microflora, since cellular phosphatases degrade phospholipids rapidly after cell death, releasing the phosphate group and diglyceride with its fatty acids (DGFA). The ratio DGFA/PLFA provides an estimate of the proportion of non-viable to viable microbes (White & Ringelberg, 1997). This approach is commonly used for determining community composition and viable microbial biomass in environmental matrices and for monitoring changes in the microbial community composition (Pinkart et al., 2000 and references therein). However, it has been only rarely used on historic monuments (Ortega-Morales et al., 2004, 2000, 1999; Palmer, 1994). PLFA analysis also provides an estimate of physiological status, as the ratio of trans/cis monoenoic PLFA can be related to environmental stress (Villanueva et al., 2004; White & Ringelberg, 1997). Despite the power of the lipid biomarker method for microbial community analysis, clear limitations exist in identifying community components at a fine taxonomic level; most bacteria contain simple PLFA profiles of straight chain fatty acids, which do not provide sufficient variety to distinguish between species (White & Ringelberg, 1997). The identification of species using fatty acid methyl ester (FAME) profiles requires cultivation of the organism for comparison with a database. The major drawback is that it is prone to the bias of cultivation procedures evoked above and may require the generation of a library of environmental bacterial profiles, since the commercial database contains mostly medically-relevant microorganisms. It has been successfully applied, however, to identify heterotrophic bacteria on three mural paintings showing severe biodeterioration (Heyrman et al., 1999).

Ergosterol, the major sterol in fungal membranes is absent from other microorganisms, plants or animals (Weete, 1989). It may be used as an indicator of metabolically active cells because of its ready oxidative degradation at the double bonds and hence its lability in dead cells (Montgomery et al., 2000). Ergosterol has been quantified in biofilms on building materials by HPLC to assess fungal biomass (Saad et al., 2003; Gutarowska & Zakowska, 2002). This is a rapid method that requires removal of the biofilm, but is not necessarily destructive of the underlying material. It does not allow species identification, but gives a fast laboratory measure of the viable fungal biomass.

Such indirect quantification methods do not yield microbial numbers; the individual cell content of each component is rarely constant, often depending on the physiological state of the cell (Schumann et al., 2005; Pasanen et al., 1999) and conversion factors may lead to error. However, as pointed out at the beginning of this section, exact numbers are rarely required.

2.1.5 Microbial activity measurement

Inactive or dormant cells usually do not contribute to the deterioration of a substrate. It is assumed that the more active the metabolism of a microbial cell the more likely it is to contribute to material degradation through its metabolism. Therefore, determination of metabolic activity is an appropriate way to assess the degradative potential of a microbial community. However, it is important to remember that microbial activity may display oscillations and rhythms throughout the year and even the day (Lloyd & Hayes, 1995).

Active microorganisms may be assessed by epifluorescence microscopy. Specific fluorochromes can indicate cell viability by differences in cytoplasmic redox potential, electron transport activity, enzymatic activity, cell membrane potential or membrane integrity (McFeters et al., 1995; Kepner & Pratt, 1994). For example, acridine orange binds to both DNA and RNA. Single stranded nucleic acid (RNA) emits an orange-red and double stranded (DNA) a green fluorescence, under the appropriate conditions. DAPI (4',6-diamidino-2-phenylindole) fluoresces blue or bluish-white when bound to DNA and yellow when bound to non-DNA material. However, only dead cells can be differentiated by these two dyes; living active and inactive cells will stain similarly. Other fluorochromes are available to differentiate highly active, frail and injured cells (Lloyd & Hayes, 1995).

Adenosine triphosphate (ATP), measured in a bioluminescence assay with firefly luciferin/luciferase, offers a rapid and highly sensitive tool for the determination of microbial activity (Ranalli et al., 2003; Rakotonirainy et al., 2001; Fung, 1995; Tiano et al., 1989). In the presence of ATP, luciferase catalyses the oxidation of luciferin producing visible light. If excess luciferin is available the amount of ATP is directly proportional to the amount of light emitted. All active organisms produce ATP and will be measured in the assay, therefore, care must be taken not to include the microscopic insects and arthropods commonly found on external stone surfaces. Biofilms may be removed from an artefact to assess surface contamination and

ground stone samples may be used if endolithic activity is also required. The assay has been used to monitor contamination, estimate bacterial cell numbers growing on stone monuments (Gorbushina et al., 2002; Praderio et al., 1993; Tiano et al., 1989), assess the decrease of microbial activity after a biocide treatment (Ranalli et al, 2003) and to monitor microbial activity during and after bioremediation of historic stone artefacts (Cappitelli et al., 2006). Kits are commercially available, allowing the method to be employed by non-microbiologists. ATP data for the estimation of biomass have to be interpreted carefully, as the ATP content varies significantly with species and the metabolic state of the individual.

Microbial activity may also be assessed by quantification of enzymes within a sample. Dehydrogenase activity (DHA) is a measurement of the oxidative activity of a microbial cell under aerobic conditions. This is linked to the membrane-bound electron transfer chain where oxygen serves as a final electron acceptor. Dehydrogenases, a group of oxido-reductases, oxidise substrates by the liberation of electrons, which are funnelled into the respiratory chain (von Mersi & Schinner, 1990). The activity of enzymes of the respiratory chain has long been used as an index of the overall microbial activity in cells of soil organisms. Chemicals such as 2,3,5-triphenytetrazolium chloride (TTC) or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) can act as artificial electron acceptors, being reduced by the action of DHA to coloured triphenylformazan (TPF) or INT-formazan (INF) respectively, which can be assessed colourimetrically (Alef & Nannipieri, 1995; Hirsch et al., 1995a; Tayler & May, 1995; Warscheid et al., 1990). For soil samples it was concluded that the sensitivity of each method depended largely on the experimental conditions (Trevors, 1984; Benefield et al., 1997; Gong, 1997). Areas of microbial activity and their spatial distribution within the rock may be visualised by microscopy *in situ* (i.e. on a rock fragment) as red reduced TTC (Warscheid et al., 1990). A potential limitation is that some organisms may not absorb the dye due to the nature of their cell walls and plasma membranes. This is a major concern in biofilms containing phototrophs, where thick-sheathed cyanobacteria are often the major biomass (Crispim et al., 2006; Ortega-Morales et al., 2004). Conversely, other reducing substances within a microbial cell may trigger the colour change, and hence a DHA-negative cell may be rendered red (Petersen & Toepfer, 2001).

Esterases, lipases and, partially, proteases, present in all microbial (and other) cells, can be assessed by fluorescein diacetate (FDA) hydrolysis. The molecule enters the cell by passive diffusion through the cell membrane, where it is enzymatically hydrolysed into two molecules of acetate and one molecule of yellow-fluorescing fluorescein (Wanandy et al., 2005). Fluorescein can be quantified spectrophotometrically or microscopically visualised in single cells. Both intracellular and extra-cellular enzymes can cleave FDA (Tayler & May, 1995), but this is unlikely to be a problem on stone surfaces, as released enzymes are rapidly inactivated in dry conditions. The pH of the sample is critical, as pH >8.5, not uncommon on stone surfaces, supports non-enzymatic breakdown (Petersen & Toepfer, 2001; Tayler & May, 1995).

The action of hydrolases may be visualised with 4-methylumbelliferone (MUF)-labelled substrate analogues. Hydrolytic cleavage produces free MUF, which shows a strong fluorescence at 450nm. While this method cannot prove that the substrate is utilised by the organisms, it demonstrates their potential to perform hydrolytic cleavage (Colombo et al., 2004; Hirsch et al., 1995a). An interesting non-destructive *in situ* modification of this method was reported by Hirsch et al. (1995a). Chromatography paper impregnated with MUF-labelled substrate analogues was pressed against the rock surface with weights and covered to avoid desiccation. After 1 min to 2.5 h, the chromatography paper was treated with sodium bicarbonate ($NaHCO_3$) and examined under UV light (365 nm) to assess cleavage of the substrate analogues by hydrolytic enzymes. Colourless phenolphthalein phosphate-impregnated filter paper can be used in a similar fashion, being treated after exposure with ammonia (NH_3) vapour to reveal red phenolphthalein, released by phosphatases (Hirsch et al., 1995a). It may be inadvisable to use the above methods directly on monuments, as substrate analogues are nutrients for microorganisms and may increase their growth and metabolic activity.

An *in situ* method that does not require the application of substances onto the artefact has been tested by Koestler and Salvadori (1996). The authors measured the respiration product CO_2 from living organisms (microorganisms or insects) growing on art objects by FTIR. As long as either the artefact or the FTIR instrument is portable this method can be used *in situ* without taking a sample.

Phototrophic microorganisms can be investigated by infrared photography, using the characteristic reflection of chlorophyll *a* in the near infrared (Van der Molen et al., 1980). Chlorophyll also fluoresces after activation with UV light and hence phototrophs can be assessed with a UV microscope (Gaylarde & Englert, 2006). Non-fluorescing cells can be considered as photosynthetically inactive. Tomaselli et al. (2002) reported the successful use of chlorophyll *a* fluorescence analysis to assess the efficiency of biocides.

2.1.6 Simulation experiments

Simulation experiments aim to demonstrate the effects of microorganisms placed in contact with a substrate and the mechanisms involved. Their advantage over *in situ* studies is the possibility of comparing the substrate before and after exposure. Limitations are that they are performed under controlled and optimised laboratory conditions in order to accelerate natural processes and provide reproducibility (Sand et al., 2002). The sample materials are generally inoculated with selected organisms of a chosen functional group (Prieto et al., 2004; Tomaselli et al., 2002) or a mixed microbial model community previously isolated from monuments (Papida et al., 2000; May et al., 2003). Hence, only culturable microorganisms can be investigated. A more acceptable inoculum might be composed of an entire biofilm recently removed from the surface of a similar type of stone; however, this would not be reproducible. Incubation is generally performed under what are considered optimised conditions, which do not mimic those to which the organisms are exposed in their natural habitat. Experiments may include cycling of temperature and RH (Papida et al., 2000; May et al., 2003) or of illumination (Prieto et al., 2004; Tomaselli et al., 2002). The incubation times vary from 2 days (Prieto et al., 2004) to over 100 days (Papida et al., 2000; May et al., 2003), depending on the aim of the study. After incubation, stone properties and microorganisms must be assessed and attempts made to correlate the latter with changes in the former. Microbial activity (Prieto et al., 2004), direct or indirect biomass concentration (Prieto et al., 2004; May et al., 2003; Tomaselli et al., 2002), leaching of cations (McNamara et al., 2005) and weight, volume, porosity, surface hardness and sound velocity in the substrate (Papida et al., 2000; May et al., 2003) have been assessed in simulation experiments.

2.1.7 Aims of detection and assessment of microbial biodeterioration

In this chapter available techniques are presented and their relevance and applications are discussed. New methods were developed and existing ones improved especially those that can be carried out by cultural heritage conservators with limited microbiological skills and such that do not employ sophisticated technical equipment. Identification of the microbial community was not attempted, as this requires special microbiological skills. Instead, the total biomass, its phototrophic contribution, biofilm content and most importantly the activity of the microbial population were assessed. As no data was available on a "critical level" of microbial contamination, an approach was made to determine the feasibility of long-term monitoring. Changes in activity or total biomass present may indicate the necessity for an antimicrobial treatment. To this end a representative "baseline" can be developed. Natural changes in the composition and activity of a microbial biofilm due to the micro-conditions at a given sampling site as well as daily and seasonal changes of the climatic conditions were monitored and guidelines for the sampling strategy were developed.

2.2 Materials and methods

All glassware and laboratory equipment used was rinsed with acetone and autoclaved where possible. Solutions were stored under refrigeration (7°C). Sterile deionised water was used throughout. All experiments were performed in triplicate unless otherwise noted.

2.2.1 Sampling site and time

Samples for the detection and assessment of microbial biodeterioration were taken from an approximately 30-year old, unpainted limestone wall outside of the "Centro Cultural Universitario" of the Universidad Autónoma de Campeche, Mexico. The wall was selected because it was located within less than 5 min walking distance from the laboratory, where the experiments were performed. An antique site was not selected, as for the optimisation of the protocols and the measurement of circadian and seasonal differences in microbial activity, continuous sampling of large amounts of biofilm with stone substrate was necessary. The wall was strongly colonised with microbial biofilms (mainly phototrophs, dominated by coccoid

cyanobacteria but also filamentous cyanobacteria, coccoid and filamentous algae existed, personal communication O. Ortega-Morales and C. Gaylarde, 2006. The heterotrophic microbial community was not analysed but assumed to be closely related to that found in related archaeological sites nearby [Proteobacteria and Actinobacteria, McNamara et al., 2006). Two sampling sites were selected within the same wall, approximately 4 m away from each other. The site that will be referred to as "sun" site faced south and the site that will be referred to as "shady" site faced east. This eastern side of the wall was rectangular to the right of the "sun-site" (fig. 15a, b). In the morning (the morning samples were taken at 8 am) none of the two sites were exposed to direct sunlight. The sun-site was directly irradiated by sunlight from approximately 11 am until sunset (approximately 5 pm during the dry season and approximately 7 pm during the rainy season). The shade-site did not receive any direct sunlight, as it was protected by the shadow of surrounding trees. Samples taken in the morning (8 am) will be referred to as "Msun" (from the sun-site) and "Mshade" (from the shade-site), whereas those taken in the afternoon (4 pm) as "Asun" and "Ashade". Samples from the dry season were taken between March and May and those from the rainy season between July and October.

Figure 15 Sampling sites with partially removed biofilm
Partially removed biofilm on a: the sun-site at the south-facing part of the wall and b: the shade-site at the east-facing part of the wall.

2.2.2 Preparation of biofilm samples

The surface layer of the stone wall with thick black biofilm was scraped off the stone with an ethanol sterilised scalpel. The approximate surface area and the weight of the sample was documented. The sample was finely ground in a mortar. The powdered sample was suspended in either 0.9 (w/v) NaCl or 10 mM N or N-Bis (hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (Sigma, pH 7.0).

2.2.3 Absorbance reading

The absorption of the samples was measured in a Jenway 6300 spectrophotometer.

2.2.4 Biofilm weight

The biofilm weight was documented as:

$$\text{biofilm weight } (mg/cm^2) = \frac{\text{total weight of the sample (mg)}}{\text{area of biofilm removed } (cm^2)}$$

2.2.5 Moisture content of the biofilm

For the analysis of the moisture content, immediately after sampling, a known amount of the ground sample (approximately 100 mg) was placed in an aluminium foil covered glass beaker and dried for 24 h at 90°C. The weight-loss was measured and expressed as the total amount of moisture (mg H_2O / mg sample) and as % of fresh weight (FW). As large amounts of sample were needed to receive reliable moisture content measurements, the moisture content was performed on one sample per experiment and not in triplicate.

2.2.6 Irradiation measurement at the sampling site

The intensity of the light incident on the biofilm at the time of sampling was measured with a LI-1400 Data Logger (Li-COR, Lincoln, Nebraska) equipped with a silicon photodiode sensor (LI-190SA Quantum Sensor). It measured photosynthetically active radiation (PAR) in the 400–700 nm waveband. The unit of measurement was micromoles per second per square metre ($\mu mol \cdot s^{-1} \cdot m^{-2}$). The irradiation intensity was measured directly before and after sampling and the mean was documented.

2.2.7 Quantification of proteins

For the quantification of proteins a modified protocol of that described by Warscheid et al., (1990) was employed. Finely ground biofilm suspensions (0.5 ml in 10 mM BES buffer, pH 7.0) at concentrations between 1.25 mg/ml and 10 mg/ml were mixed with 0.5 ml 1 M NaOH and heated for 5 min in a heating block (Multi Block heater, Lab Line) at 100°C. After cooling it was mixed with 2.5 ml of solution 3 (see below) and allowed to react for 10 min. Subsequently, 0.5 ml Folin & Ciocalteu's

phenol reagent (Sigma) (1:2 dilution in water) was added, shaken immediately and kept for 30 min in the dark. The absorption was read at A_{750} against a blank. The blank (0.5 ml 10 mM BES buffer, pH 7.0) and protein standards for the calibration curve (bovine serum albumin, Sigma; 1, 10, 50, 100, 250, 500, 750 and 1000 µg/ml, in 10 mM BES buffer, pH 7.0) were treated in the same manner as the biofilm samples.

Solution 1: 5% (w/v) $NaCO_3$ in water

Solution 2: 0.5% (w/v) $CuSO_4 \cdot 5H_2O$ in 1% K-Na-tartrate

Solution 3: mixture of 50 ml of solution 1 and 2 ml of solution 2

2.2.8 Quantification of carbohydrates

2.2.8.1 Anthrone method

A modified protocol by Gerhard et al. (1994) was applied. The biofilm solutions (0.6 ml of 5 mg/ml ground sample in 0.9% (w/v) NaCl) and a blank (0.6 ml 0.9% (w/v) NaCl) were refrigerated in thick walled Pyrex tubes. When cold, 3 ml cooled anthrone reagent (100 µg anthrone mixed with 2.5 ml absolute ethanol added to 47.5 ml 75% sulphuric acid) was added and mixed for 5 min in an ice-water bath. The tubes were then transferred for precisely 10 min into a in a heating block (Multi Block heater, Lab Line) at 100°C and subsequently returned to the ice-water bath. Care had to be taken during this procedure not to contaminate the samples with water, as this caused turbidity. A calibration curve was prepared of glucose (Difco) standards (1, 10, 25, 50, 75, 100, 250 and 300 µg/ml in 0.9% (w/v) NaCl). The absorbance was measured at 625 nm.

2.2.8.2 Phenol method

A modified protocol by Gerhard et al. (1994) was applied. Finely ground biofilm suspensions (0.5 ml of 1 mg/ml in 10 mM BES buffer, pH 7.0) and a blank (0.5 ml 10 mM BES buffer, pH 7.0) were mixed thoroughly in Pyrex tubes and with 0.5 ml of phenol reagent (5% w/v in H_2O). Concentrated sulphuric acid (2.5 ml) was added, thoroughly mixed and allowed to react for 10 min at room temperature. Subsequently, the samples were incubated for 15 min in a water bath at 25°C. A calibration curve was prepared of glucose (Difco) standards (1, 10, 25, 50, 75, 100, 250 and 300 µg/ml in 10mM BES buffer, pH 7.0). The absorbance was read at 488 nm.

2.2.9 Quantification of phospholipids

Extraction of phospholipids

A modified protocol after Findlay et al. (1989) was employed for the extraction of lipids from biofilm samples. A ground biofilm sample (100 mg) was added to 50 ml extraction mixture (dichloromethane : methanol : water in the ratio 1:2:0.8) and stirred for 2 h (400 rpm). The sample was then filtered (Whatman filter paper 4, pore size: 20–25 µm) into a partitioning funnel. After doubling the initial amount of dichloromethane and water, the sample was shaken thoroughly and allowed to stand for approximately 24 h to divide the lipid fraction (within the dichloromethane phase) from other cell components (within the water phase). The dichloromethane phase was transferred into a distillation apparatus (Büchi Rotavapor R-200) and the dichloromethane was evaporated at 40°C and 700 mbar at low rotation speed until approximately 1 ml sample was left. This sample was pipetted into a small Pyrex tube and combined with washings from the distillation receiver (1 ml dichloromethane). This sample (approximately 2 ml) was dried under a stream of nitrogen.

Assessment of phospholipid phosphorous

A modified protocol by Gerhard et al. (1994) was applied. All glassware was washed for 1 h in hot 1 N nitric acid and rinsed with water. The extracted dry phospholipid fraction of the biofilm samples was mixed with 70% perchloric acid (0.4 ml) and heated at 100°C for 2 h with a lid to avoid evaporation. Subsequently, 4.2 ml water, 0.2 ml ammonium molybdate solution (5% w/v) and 0.2 ml amidol reagent (0.5 g 2,4-diaminophenol dihydrochloride and 50 ml of 20% (w/v) sodium bisulphite solution) were added. The sample was mixed and heated for 7 min at 100°C in a heating block (Multi Block heater, Lab Line). After rapid cooling in an ice-water bath the absorbance was read at 830 nm against a blank. A phosphorus standard (20 µg/ml) consisted of 1.097 g KH_2PO_4 (monobasic potassium phosphate) in 250 ml water diluted (1:5) with methanol. Phosphorus standards of between 0.1 and 20 µg/ml were prepared for the calibration curve.

2.2.10 Chlorophyll content

Chlorophyll was sampled from finely ground biofilm (approximately 30 mg) with acetone (3 ml). After the extraction in the dark (24 h at 7°C), the sample was centrifuged (5 min, 3000 rpm) and the absorbance was read at 663 nm. The chlorophyll content was expressed as µg chlorophyll / g biofilm (adapted from Ortega-Morales et al. 1999).

2.2.11 Assessment of microbial activity

2.2.11.1 Unspecific enzyme assay with fluorescein diacetate

FDA stock solution (10 mg/ml) was prepared in acetone and stored at -40°C. Dilutions of the stock solution were made with 10 mM BES buffer (pH 7.0). Suspensions of finely ground biofilm (0.5 ml of 10 mg/ml in 10 mM BES buffer, pH 7.0), negative control samples (autoclaved biofilm suspensions of the same concentration) and a blank (0.5 ml 10 mM BES buffer, pH 7.0) in ethanol-sterilised spectrophotometry cuvettes (1 ml capacity). After mixing with 0.5 ml FDA solution (100 µg/ml in 10 mM BES buffer, pH 7.0) and sealing with Nesco film® (Fisons Scientific Apparatus, Loughborough, UK), incubation was for 2 h at 33°C. During the incubation the samples were shaken approximately every 30 min. The absorbance was read against the blank at A_{490}. A calibration curve was prepared from fluorescein, free acid (Fluka), standards (between 0.1 and 25 µg/ml in 10 mM BES buffer, pH 7.0).

The enzyme activity was expressed as µg fluorescein · g FW^{-1}, which was calculated as follows:

$$\text{fluorescein (µg)} \cdot \text{g } FW^{-1} \text{ after 2 h at 33°C} = \frac{S_1 - S_0}{FW}$$

S_1: fluorescein concentration (µg) of the live sample; S_0: fluorescein concentration (µg) abiotically produced by the negative control. The enzyme activity was subsequently expressed per protein, chlorophyll and carbohydrate within the sample dry weight (DW).

2.2.11.2 Dehydrogenase activity (DHA) assay using iodonitrotetrazolium (INT) reduction

Suspensions of finely ground biofilm (0.5 ml of 10 mg/ml in 10 mM BES buffer, pH 7.0) and negative control samples (autoclaved biofilm suspensions of the same concentration) in 2 ml screw top vials were mixed with 0.5 ml INT solution (200 µg/ml 10 mM BES buffer, pH 7.0). During the incubation time (5 h at 33°C in the dark) the samples were shaken regularly (approximately every 30 min). Subsequently, they were centrifuged at 6000 rpm for 4 min, the supernatant was discarded and the pellet was resuspended in 1 ml methanol. Extraction of the INT formazan (INF) was carried out in the dark for 30 min with shaking at approximately 10 min intervals. The absorbance was read against a blank of methanol at A_{490}. A calibration curve was prepared from INF (Sigma, 0.5 ml of dilutions between 1 and 100 µg/ml). All standards were prepared fresh before the assay, as freezing resulted in precipitation of the INF. DHA was expressed as µg INF · g FW^{-1}, which was calculated as follows:

$$\text{INF (µg)} \cdot \text{g FW}^{-1} \text{ after 5 h at 33°C} = \frac{S_1 - S_0}{FW}$$

S_1: INF concentration (µg) of the live sample; S_0: INF concentration (µg) of the negative control. The DHA was subsequently expressed per protein, chlorophyll and carbohydrate within the sample DW.

2.3 Results and discussion

2.3.1 Optimisation of the assessment methods

To date, it has been impossible to isolate all the different microbial species present on a stone monument. Most molecular methods, used for the identification of microorganisms, do not give much information on the organisms' physiology or viability; furthermore, high professional skills are needed for their performance. An approach towards assessing total biomass, EPS, viable biomass and activity of the microbial population might therefore be more useful within a conservation context to assess if an antimicrobial treatment is necessary or not.

Most of the protocols employed were developed for the assessment of microorganisms from different environments, such as soil or aquatic organisms. They had to be optimised for the application for biofilms on outdoor stone monuments.

2.3.1.1 Quantification of the total microbial biomass by protein assay

For the quantification of proteins within stone samples, a modified protocol by Warscheid et al. (1990) was followed. The authors recommended stone samples of 50 mg powdered stone in 0.45 ml 0.9% NaCl. However, the assay proved to be sensitive enough to use 0.5 ml of a 10 mg/ml stone concentration, which is preferable for the application on cultural heritage objects, as less sample is needed. It was essential to maintain the pH at around neutral for other assays performed at the same time, therefore the powdered sample was diluted in 10 mM BES buffer (pH 7.0) rather than in 0.9% NaCl; this did not affect the results of the protein assay. The protocol described by Warscheid et al. (1990) did not specify the method of cooling the samples after the initial treatment with NaOH. The intensity of the colour developed during the assay proved to vary significantly depending on the cooling time. Rapid cooling in a water bath resulted in less colour development than slow cooling at room temperature. For improving the sensitivity of the method, all samples were allowed to cool at room temperature, which was constantly maintained at 25°C. The standard curve for protein (fig. 16) showed a straight line until approximately 125 µg/ml protein. At higher concentrations the increase in absorbance described a flatter straight line. However, when the protein content was read from the line of best fit, the method demonstrated reliability and repeatability for biofilm suspensions extracted from limestone surfaces (fig. 17).

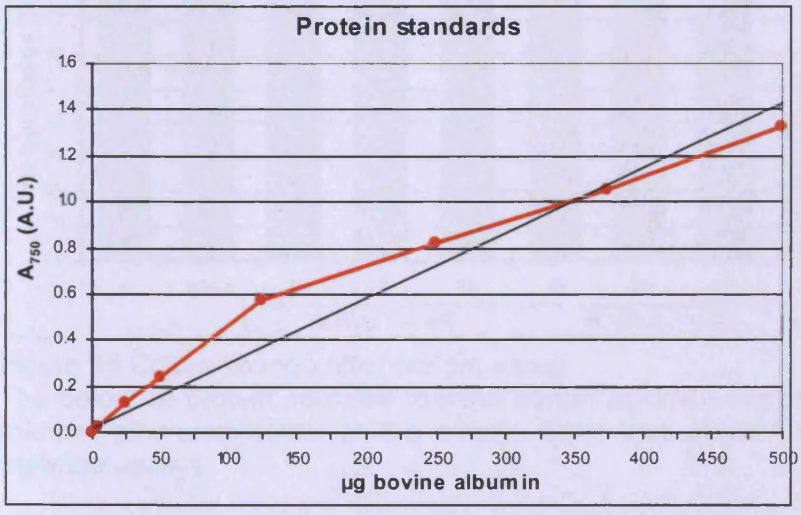


Figure 16 Protein standard curve
The standard curve for protein assay employing bovine serum albumin (Sigma) showed a straight line until 125 µg/ml protein. At higher concentrations the increase in absorbance described a flatter straight line. Error bars show the standard error of mean for triplicate assays.

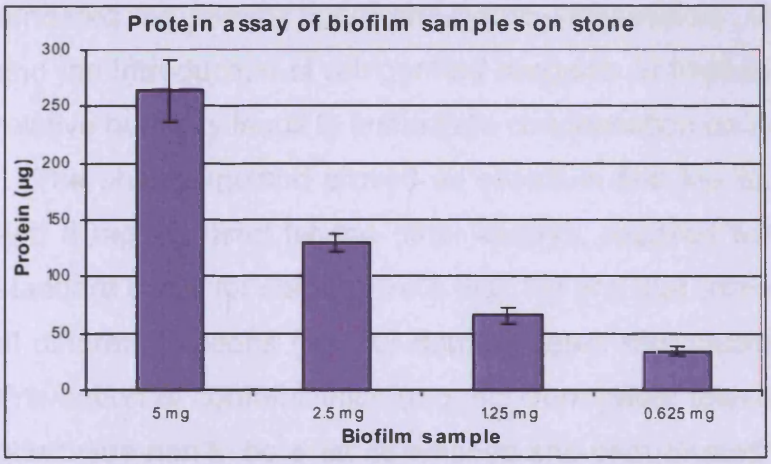


Figure 17 Protein assay of biofilm suspensions
The decrease in protein content of biofilm suspensions from a limestone wall from Campeche, Mexico corresponded with the decrease in biofilm concentration. Error bars show the standard error of mean for triplicate assays.

The detection limit was approximately 5 µg protein. The intensity of the developed colour decreased approximately 3–4% h^{-1}, however, the colour increase was not uniform for different replicates (fig. 18). Therefore care had to be taken that the absorption measurement was always performed immediately after finishing the assay. The time needed for the assay was approximately 60 min (not including sampling time).

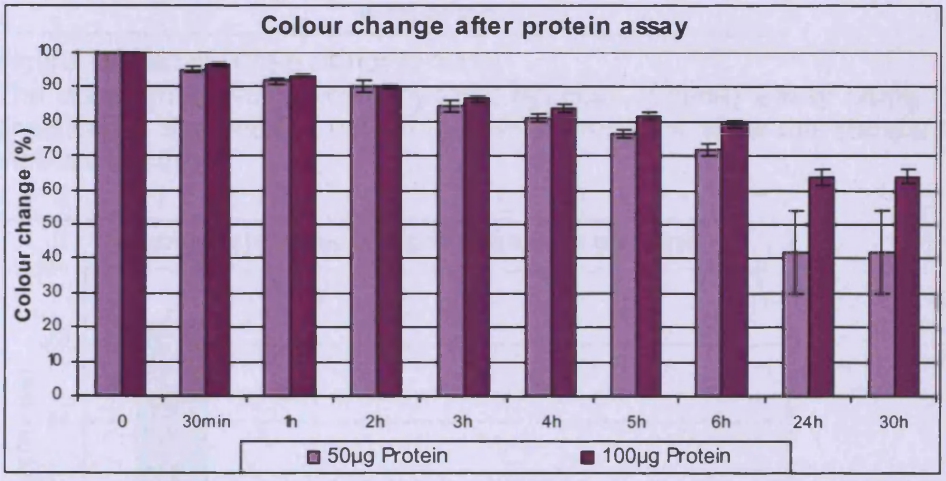


Figure 18 Colour change after protein assay
The colour of protein samples (bovine serum albumin, Sigma) decreased in a non-uniform manner after completion of the assay. Error bars show the standard error of mean for triplicate assays.

2.3.1.2 Quantification of the EPS by carbohydrate assay

For the assessment of the EPS concentration, two different methods after Gerhard et al. (1994) were tested. The anthrone method was less reliable than the phenol method, as the slightest water contamination (e.g. from condensation)

rendered the sample turbid and hence inassessible. The refrigeration of the sample and the introduction of refrigerated reagents in tropical climates with extremely high relative humidity leads to immediate condensation on any cold surfaces.

The phenol method proved so sensitive that the stone suspensions of 10 mg/ml and 5 mg/ml, used for the other assays, required further dilution to 1 mg/ml. The standard curve for carbohydrate (fig. 19) and that extracted from biofilm suspensions at different dilutions (fig. 20) demonstrated reproducible sensitivity as low as 1 µg. Prevention of contamination (e.g. lint from paper towels) was essential, therefore, all glassware had to be acetone-washed and heat-treated for 2 h at 230°C.

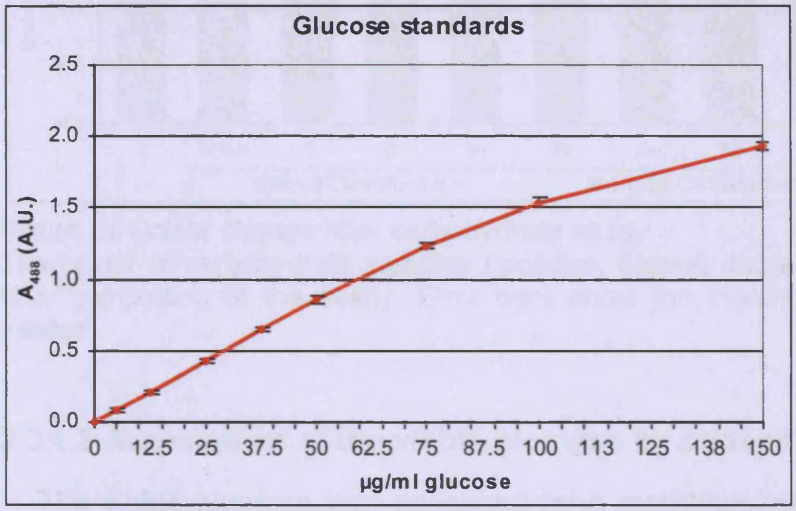


Figure 19 Carbohydrate standard curve
The standard curve for carbohydrate (glucose, Sigma) assay using the phenol method described a straight line until 100 µg/ml. Error bars show the standard error of mean for triplicate assays.

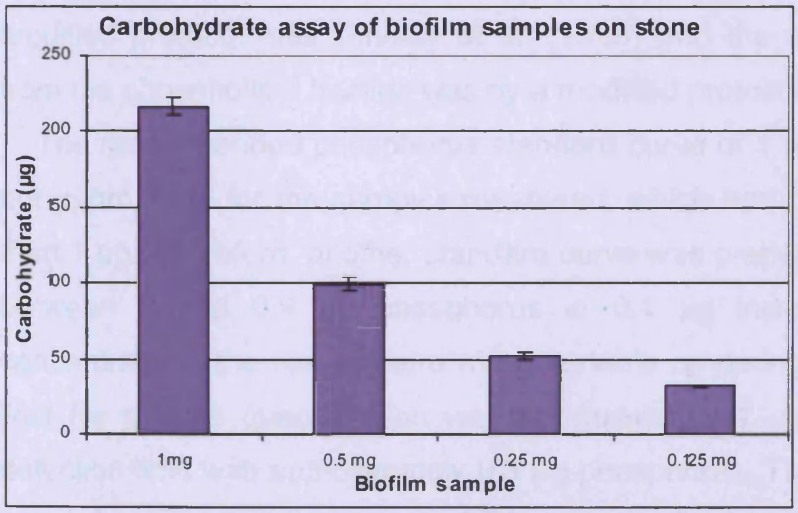


Figure 20 Carbohydrate assay of biofilm suspensions
The decrease in carbohydrate content (assessed employing the phenol method) of biofilm suspensions from a limestone wall from Campeche, Mexico corresponded with the decrease in biofilm concentration. Error bars show the standard error of mean for triplicate assays.

After the recommended reaction time for colour development the absorption of the samples at A_{488} was very stable over a period of 30 h with colour intensities between 99.2 and 100.2% of the value immediately after completion of the assay (fig. 21). The total time needed for the assay was approximately 50 min.

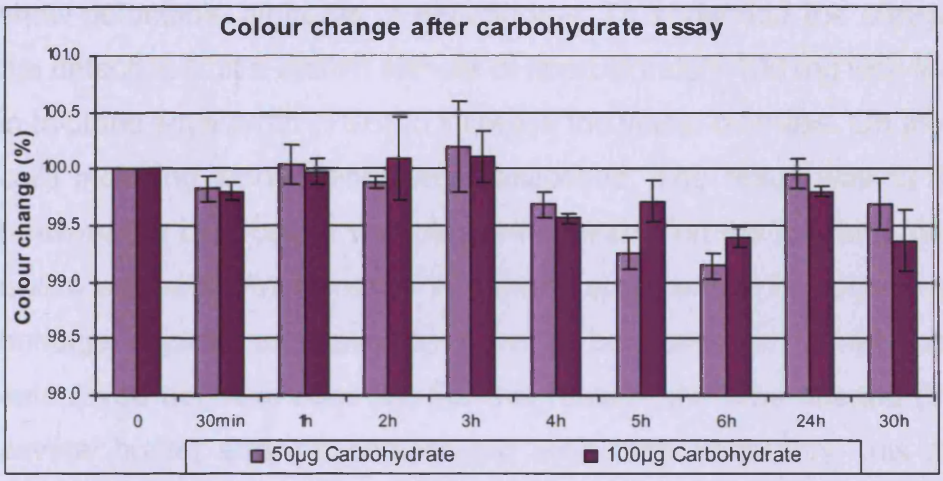


Figure 21 Colour change after carbohydrate assay
The colour of carbohydrate samples (glucose, Sigma) decreased in a non-uniform manner after completion of the assay. Error bars show the standard error of mean for triplicate assays.

2.3.1.3 Assessment of the viable biomass by phospholipid assay

The viable biomass was assessed by a quantification of the phosphorus content from the phospholipids of the sample. Phospholipases and phosphatases readily degrade phospholipids after cell death, therefore only the viable portion of the total biomass was assessed by this assay. Lipid extraction was performed following a modified protocol after Findlay et al. (1989) and the quantification of phosphates from the phospholipid fraction was by a modified protocol after Gerhard et al. (1994).

The recommended phosphorus standard curve of 1 µg to 10 µg phosphorus was not appropriate for the samples measured, which had phosphorus contents of less than 1 µg. Therefore, another standard curve was prepared for phosphorus contents between 0 and 0.9 µg phosphorus in 0.1 µg increments (fig. 22). At these concentrations the results were more variable rendering the results unreliable. The limit for reliable quantification was approximately 1 µg phosphorus, whereas the detection limit was approximately 0.3 µg phosphorus. The intensity of the developed colour increased approximately 3-7% h^{-1} after finishing the assay in a non-uniform manner (fig. 23). Hence, care had to be taken that the measurement of the samples was always performed immediately after completion of the assay. The colour of the

pigments within the biofilm may interfere with the absorption measurement leading to an overestimation of the phosphorus content. Therefore the value of an autoclaved negative control was subtracted from that of the biofilm sample under investigation.

Biofilm samples of 100 mg, assessed for phosphorus with this method, did not show detectable amounts of phosphorus. To show that the phosphorus was below the detection limit a biofilm sample of approximately 100 mg was incubated overnight in tryptone soya broth (TSB) to increase the viable biomass. On the following day the cells including stone debris were assessed. The result was at the detection limit however the blue colour was clearly noticeable on the following day, but was not an useful estimate. An increase in sample size cannot be recommended for cultural heritage objects, as triplicates have to be assessed for all samples as well as autoclaved negative controls. For this reason, the time needed (2 days rather than several hours) and the complicated procedure necessary, this method cannot be recommended for the assessment of phospholipids in the biomass of cultural heritage samples performed by conservators in a laboratory not equipped for biological and biochemical operations. Other protocols for the assessment of the viable fraction of a microbial community (e.g. visual assessment with an epifluorescent microscope employing a viability stain such as acridine orange) were not tested, as the equipment needed cannot be expected to be available to conservation staff. The estimation of the viable biomass by its ATP content, as suggested by Gorbushina et al. (2002), was considered too unreliable due to significant daily and seasonal variations in microbial activity due to environmental conditions.

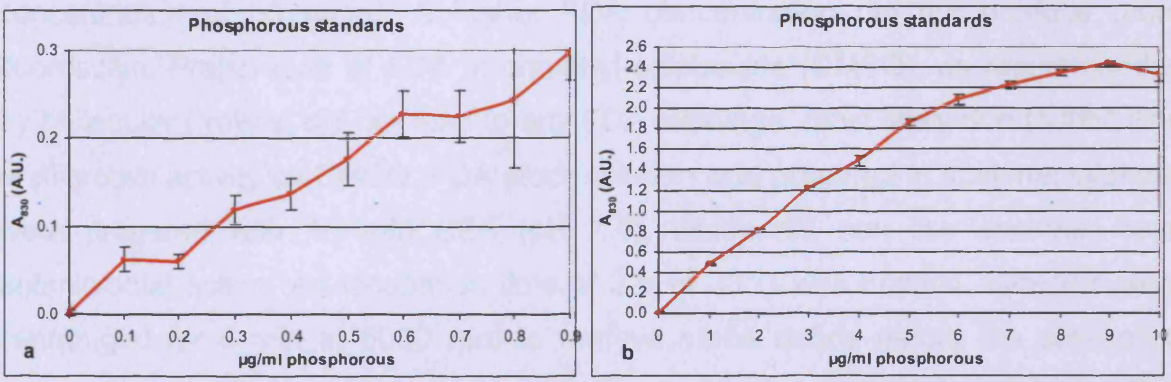


Figure 22 Phosphorous standard curve
The standard curve for phospholipid assay employing monobasic potassium phosphate (KH_2PO_4) did not describe a straight line at concentrations found in biofilm samples (a: 0.1–0.9 µg). Also at higher concentrations the standard curve did not describe a straight line (b) Error bars show the standard error of mean for triplicate assays.

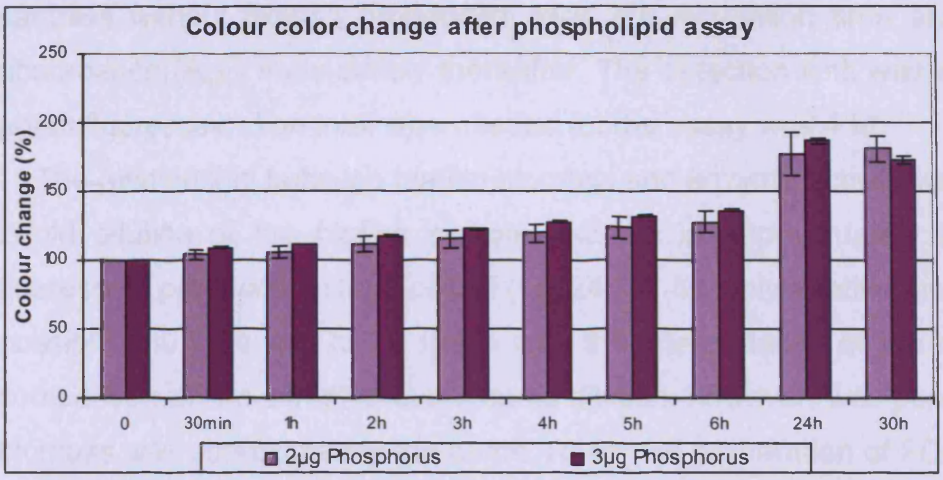


Figure 23 Colour change after phospholipid assay
The colour of phosphorous samples increased in a non-uniform manner after completion of the assay. Error bars show the standard error of mean for triplicate assays.

2.3.1.4 Assessment of microbial activity

2.3.1.4.1 Unspecific enzyme assay with fluorescein diacetate

The assessment of microbial activity by FDA, which can be cleaved by non-specific enzymes such as lipases, esterases and by some proteases, has been frequently employed for cultural heritage objects. Colourless FDA is hydrolysed both within intact cells and by extracellular enzymes (Wanandy et al., 2005; Tayler & May, 1995) to produce fluorescein (yellow). FDA cleavage is highly pH dependent, so that whereas most protocols found in the conservation literature reported on reactions performed in either water or in 0.9% NaCl, results were more reliable when the biofilm sample was suspended in 10 mM BES buffer (pH 7.0). Optimum conditions were with biofilm suspensions of 10 mg/ml treated with excess FDA at a final concentration of 50 µg/ml. A higher FDA concentration did not produce more fluorescein. Preparation of FDA in dimethyl sulphoxide (DMSO), as recommended by Molecular Probes, did not lead to any FDA cleavage, most likely due to inhibition of microbial activity by DMSO. FDA stock solution was prepared in acetone; dilutions were prepared with 10 mM BES (pH 7.0), which did not, like acetone, have antimicrobial action. An incubation time of 2 h at 33°C was optimal. Samples were centrifuged for 4 min at 6000 rpm to remove stone debris before the absorption measurement. Fixation of the samples after incubation with glutaraldehyde (final concentration 10%), as sometimes recommended, is to be avoided as it promoted FDA cleavage to give bright yellow solutions. Therefore, centrifugation of the

samples without fixation proceeded after 2 h incubation time and assessment of absorbance (A_{490}) immediately thereafter. The detection limit was approximately 0.5 µg/ml fluorescein. The total time needed for the assay was 4 h.

The relationship between biofilm biomass and enzyme activity was non-linear as a 2-fold dilution of the biofilm sample resulted in approximately two thirds of the fluorescein produced instead of half (fig. 24). Thus, only relative measurements were possible and care had to be taken with the interpretation of the results. A relative comparison of two samples at the same dilution, however, was possible, if the viable biomass was approximately the same. As for the preparation of FDA solutions, stock solutions for the fluorescein standards were prepared in acetone and diluted with 10 mM BES (pH 7.0).

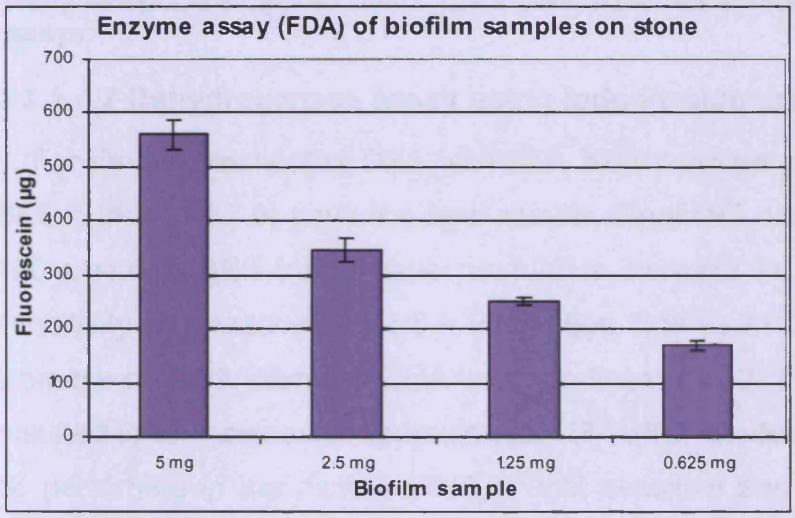


Figure 24 Fluorescein diacetate hydrolysis of biofilm suspensions
The decrease in fluorescein diacetate hydrolysis of biofilm suspensions from a limestone wall from Campeche, Mexico corresponded with the decrease in biofilm concentration. Error bars show the standard error of mean for triplicate assays.

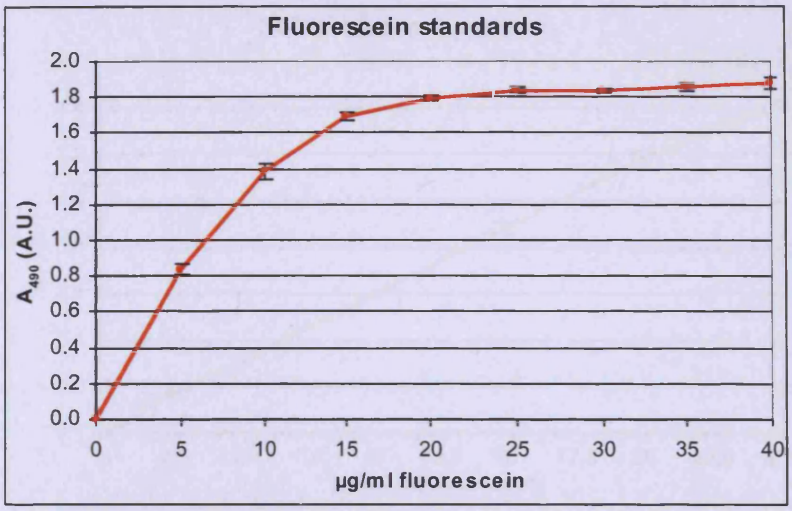


Figure 25 Fluorescein standard curve
The standard curve for unspecific enzyme assay employing fluorescein (Fluka) described a nearly straight line until 10 µg/ml. Error bars show the standard error of mean for triplicate assays.

2.3.1.4.2 Dehydrogenase assay using iodonitrotetrazolium (INT) reduction

For the assessment of DHA with INT, biofilm suspensions of 10 mg/ml in 10 mM BES buffer (pH 7.0) gave the best results. Final INT concentrations of greater than 100 µg/ml in BES buffer gave no further increase in colour intensity. Significant sensitivity was reached after 5 h incubation time at 33°C. The relationship between biomass concentration and DHA was non-linear (fig. 27); doubling the sample weight resulted in an increase of approximately 50% INF production. All experiments had to be performed in the dark, as INT is light sensitive and its photodegradation might lead to an overestimation of DHA activity. The detection limit was approximately at 3 µg INF (fig. 26). The total time needed for the assay was 7 h.

The assessment of very wet biofilm samples was problematic. Even well-ground wet biofilm produced clumps rather than powder: poor substrate accessibility then made the INT test unreliable.

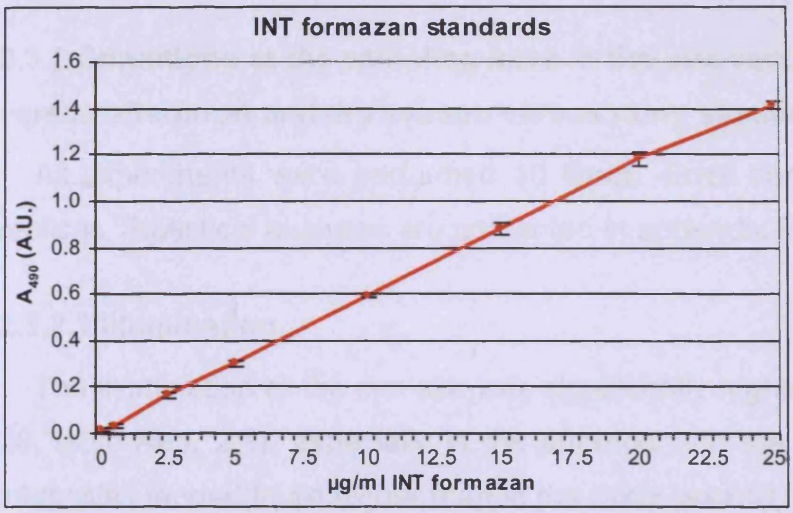


Figure 26 INT formazan standard curve
The standard curve for dehydrogenase activity assay employing iodonitrotetrazolium violet-formazan (Sigma) described a straight line. Error bars show the standard error of mean for triplicate assays.

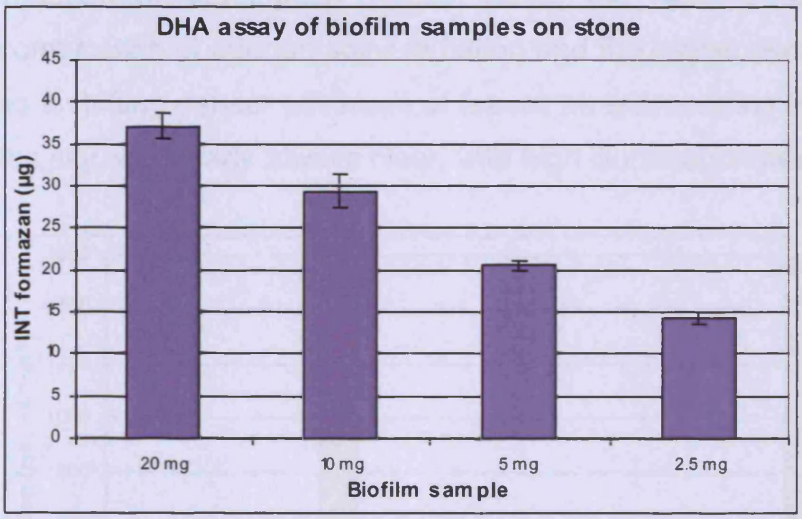


Figure 27 Iodonitrotetrazolium reduction of biofilm suspensions
The decrease in dehydrogenase activity of biofilm suspensions from a limestone wall from Campeche, Mexico corresponded with the decrease in biofilm concentration. Error bars show the standard error of mean for triplicate assays.

2.3.2 Conditions at the sampling sites in the sun versus shade, morning versus afternoon and dry season versus rainy season

All experiments were performed 10 times. Error bars are the StErrMean of 10 replicas. Statistical analyses are presented in appendix 2.

2.3.2.1 Illumination

The illumination at the sun-site was significantly higher than at the shade site (fig. 28, table App. 2.1), especially in the afternoon. In the dry season the illumination intensities tended to be higher than in the rainy season, except for the Asun sample, where the difference was statistically insignificant. The divergence in light condition between the morning and the afternoon, as well as at the same time on different days was more variable in the rainy season than in the dry season. The higher variation of illumination values during the rainy season were a result of the combination of intense solar radiation and the higher likelihood of cloudy sky as well as a shifting denser coverage of leaves on surrounding trees. During the dry season the sky was nearly always clear, with high illumination intensities.

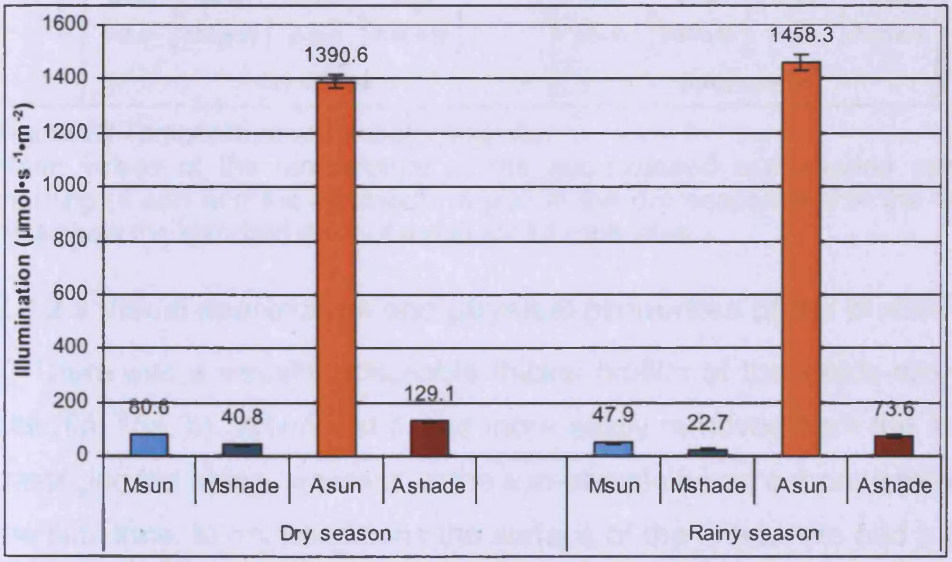


Figure 28 Illumination at the sampling site
Mean values of the illumination at the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season. Error bars show the standard error of mean for 10 replicates.

2.3.2.2 Temperature

In the morning, the temperature was similar in the sunny and the shady location, whereas in the afternoon the more intense solar radiation was reflected by a significantly higher temperature in the sun than in the shade (fig. 29). In the afternoon, the temperature increased significantly more in the sun site than in the shade. The seasonal tendencies in temperature were similar to those of the illumination. The temperature was generally higher in the dry season than in the rainy season with the exception of the Asun site, where the difference was statistically insignificant (table App. 2.2).

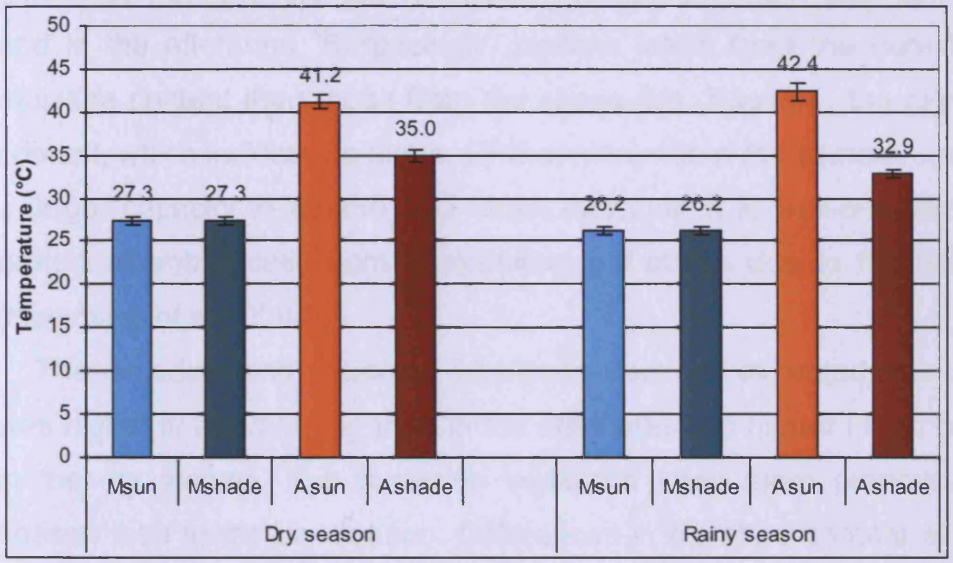


Figure 29 Temperature at the sampling site
Mean values of the temperature at the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season. Error bars show the standard error of mean for 10 replicates.

2.3.2.3 Visual appearance and physical properties of the biofilm

There was a visually noticeable thicker biofilm at the shade-site than at the sun-site (fig. 15a, b). When wet it was more easily removed from the shade-site without damaging the stone, whereas at the sun-sample it had a more intimate attachment to the substrate. In dry conditions the surface of the shade-site had a tendency to form loosely attached flakes. However, in some areas the stone itself formed poorly connected layers, which detached easily when biofilm samples were taken.

2.3.3 Composition of the sample in the sun versus shade, morning versus afternoon and dry season versus rainy season

2.3.3.1 Moisture content

The average moisture content (mg/g FW) of the biofilm depended largely on the climate conditions before and during sampling (fig. 30, table App. 2.3). In highly humid conditions with several consecutive days of heavy rainfall, the moisture content was the highest and reached in some cases up to 50%. The percentage difference between the two locations was approximately the same in the morning and in the afternoon. Surprisingly, biofilms taken from the sun-site had a higher moisture content than those from the shade-site. Possibly, the higher carbohydrate content, which indicated a higher EPS content within the sample, was responsible for a larger capacity to absorb and retain moisture. It is well-established that biofilms protect microbial cells from desiccation and stress due to fluctuations in humidity (Kemmling et al., 2004).

The circadian and seasonal variations were as expected: the moisture content was higher in the morning than in the afternoon and higher in the rainy season than in the dry season. The circadian variations were more pronounced in the rainy season than in the dry season. Differences in moisture content of the biofilm were visually noticeable. While a humid biofilm tended to be of a darker colour and soft consistency, often accompanied by strong odour, a dry biofilm was lighter in colour and formed a hard crust over the stone, which formed a craquelé in areas of thick biofilm growth (fig. 31a). Furthermore, a wet biofilm had more capacity to absorb water, while a dry biofilm was water repelling (fig. 31b). This demonstrated that the variations in moisture content were very high, which will inevitably result in high variation in volume of the biofilm, leading to physical stress on the substrate.

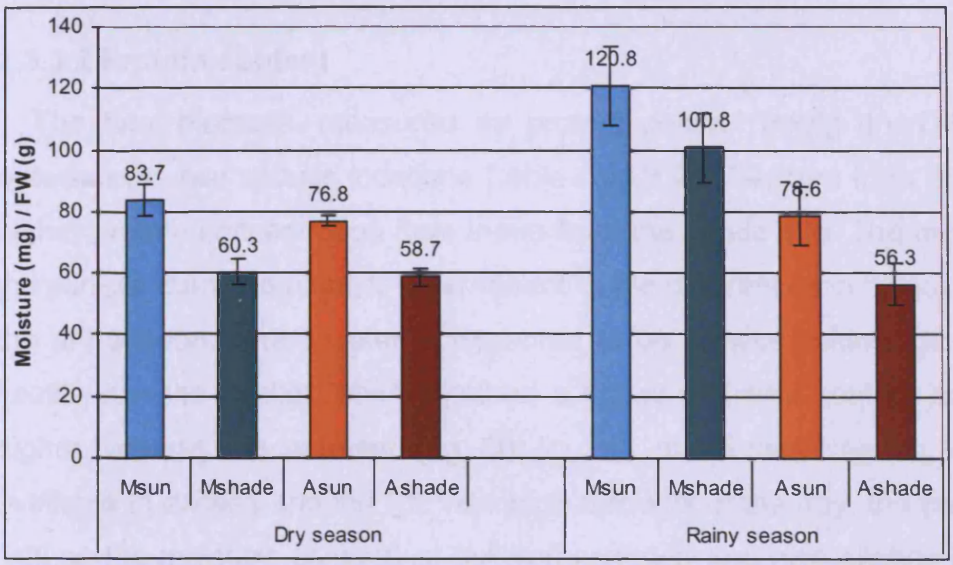


Figure 30 Moisture content of biofilm samples

Mean values of the moisture content at the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season. Error bars show the standard error of mean for 10 replicates.

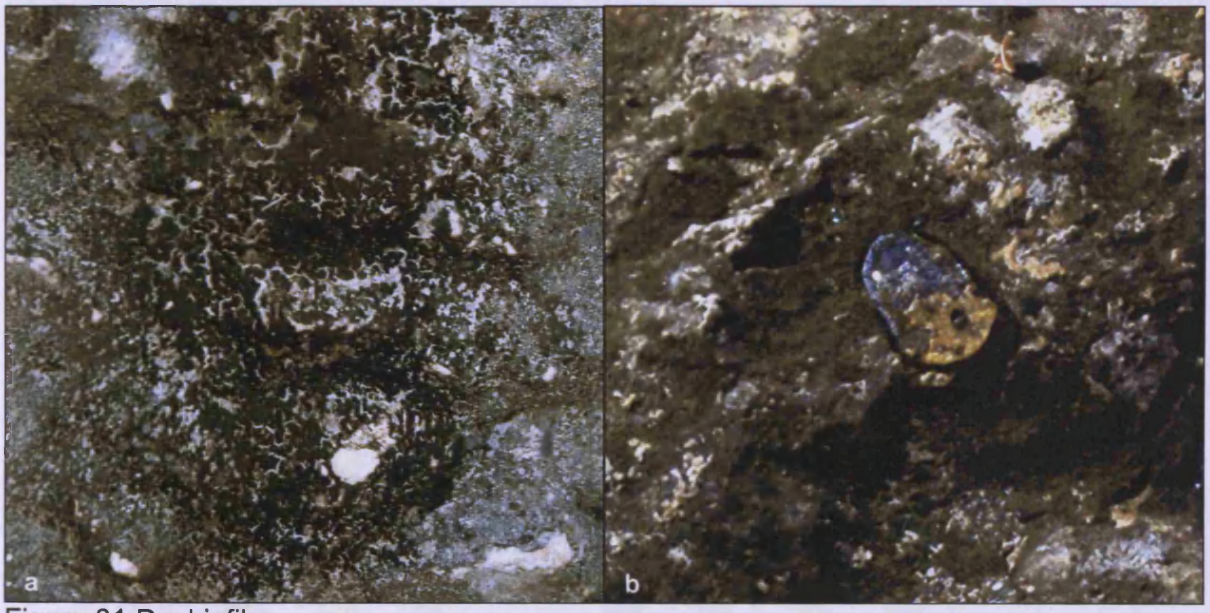


Figure 31 Dry biofilm

a. A dry biofilm from a limestone wall from Campeche, Mexico after several days without rain.

b. A drop of water on a dry biofilm from a limestone wall from Campeche, Mexico, which does not wet the surface.

2.3.3.2 Protein content

The total biomass, measured as protein content (mg/g dry DW) was different between the two sample locations (table App.2.4). Samples from the sun-site had a higher protein concentration than those from the shade-site. The moisture content of the sample could be a major determinant of the differences in biomass, particularly in the dry season, where water is expected to be growth limiting. Biofilm of the more hostile sun-site habitat, which retained a higher moisture content had a significantly higher biomass concentration (fig. 30, fig. 32). In the rainy season, where water was available in excess and the RH was high throughout the day, the protein content (as well as the moisture content) of the sun-samples was also slightly higher, however, the differences were not statistically different. Furthermore, the microbial biomass was dominated by phototrophs, as shown in 2.3.3.3 (most likely cyanobacteria, personal communication C. Gaylarde, 2006).

Circadian differences at both sampling sites were statistically insignificant and neither did a comparison of the mean values reveal a trend. The protein content of the afternoon was between 98 and 113% (table App.2.4) of that of the morning; these were within the usual sampling errors indicating that the total biomass remained approximately the same during the day.

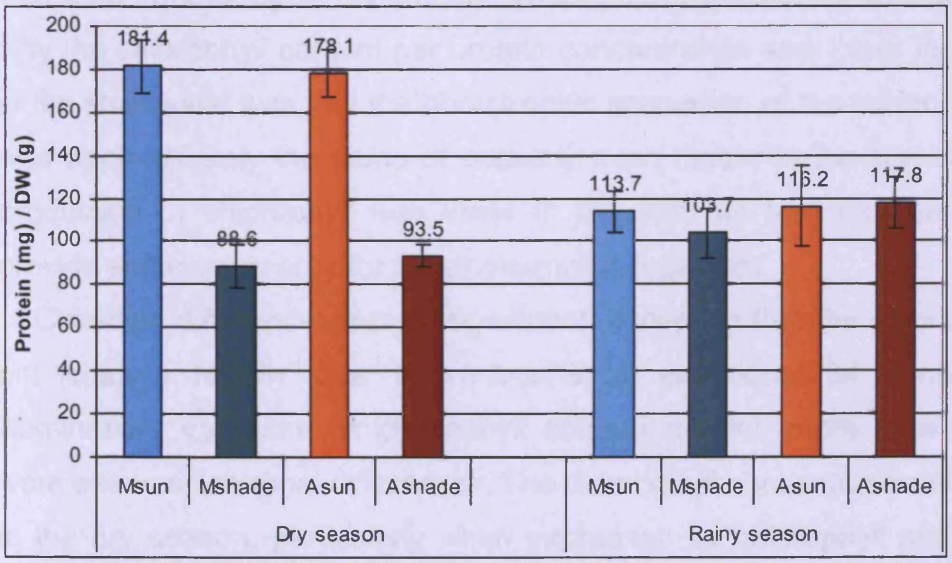


Figure 32 Protein content of biofilm samples
Mean values of the protein content from the sun-exposed and shaded sample in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season. Error bars show the standard error of mean for 10 replicates.

2.3.3.3 Chlorophyll content

Altered chlorophyll content corresponded well with the protein content between samples from different sites and under different conditions, indicating that much of the microbial biomass was composed of phototrophs. It was previously observed that the microbial biomass on buildings in the tropics and sub-tropics of Latin America is dominated by cyanobacteria (Gaylarde & Englert, 2006; Gaylarde & Gaylarde, 2005; Gaylarde et al., 2004). Significantly more chlorophyll per DW was detected in the sun-sample, particularly during the dry season (fig. 33a), where the solar illumination was slightly higher (fig. 28). However, when the chlorophyll concentration per protein concentration was determined, it was demonstrated that, per unit biomass, the differences of chlorophyll in the sun versus the shade-site was insignificant (table App. 2.5) and in some cases the sun-sample had actually a lower chlorophyll content than the shade-sample (fig. 33b).

Possibly the biofilm in the sun-site, even though being dominated by phototrophs, contained a higher concentration of heterotrophic microorganisms than the shade-site. This theory may be supported by a comparison of the carbohydrate content per protein and per chlorophyll (see chapter 2.3.3.4, fig. 34b, c). It showed that the variation of the carbohydrate content between the sun-sample and the shade-sample was greater when related to the chlorophyll content. This might suggest that there was a non-phototrophic community present that produced EPS. Another explanation why the chlorophyll content per protein concentration was lower in the sun-site than in the shade-site was that the phototrophic proportion of the community of both sites was approximately the same or possibly even higher in the sun-site, however, the production of chlorophyll was lower in the sun, as less pigment was needed to provide sufficient energy for the phototrophic organism.

Circadian differences were insignificant, indicating that the chlorophyll content did not change rapidly due to variations in environmental conditions, such as illumination. Variations in chlorophyll content needed more time to establish and were seen as seasonal differences. The chlorophyll concentration tended to be lower in the dry season, particularly when expressed as chlorophyll per biomass. Again, this result is difficult to interpret but the lower chlorophyll concentration during the season of higher solar radiation could again be explained by a reduced requirement of the photosynthetic pigment to obtain sufficient energy for the phototroph.

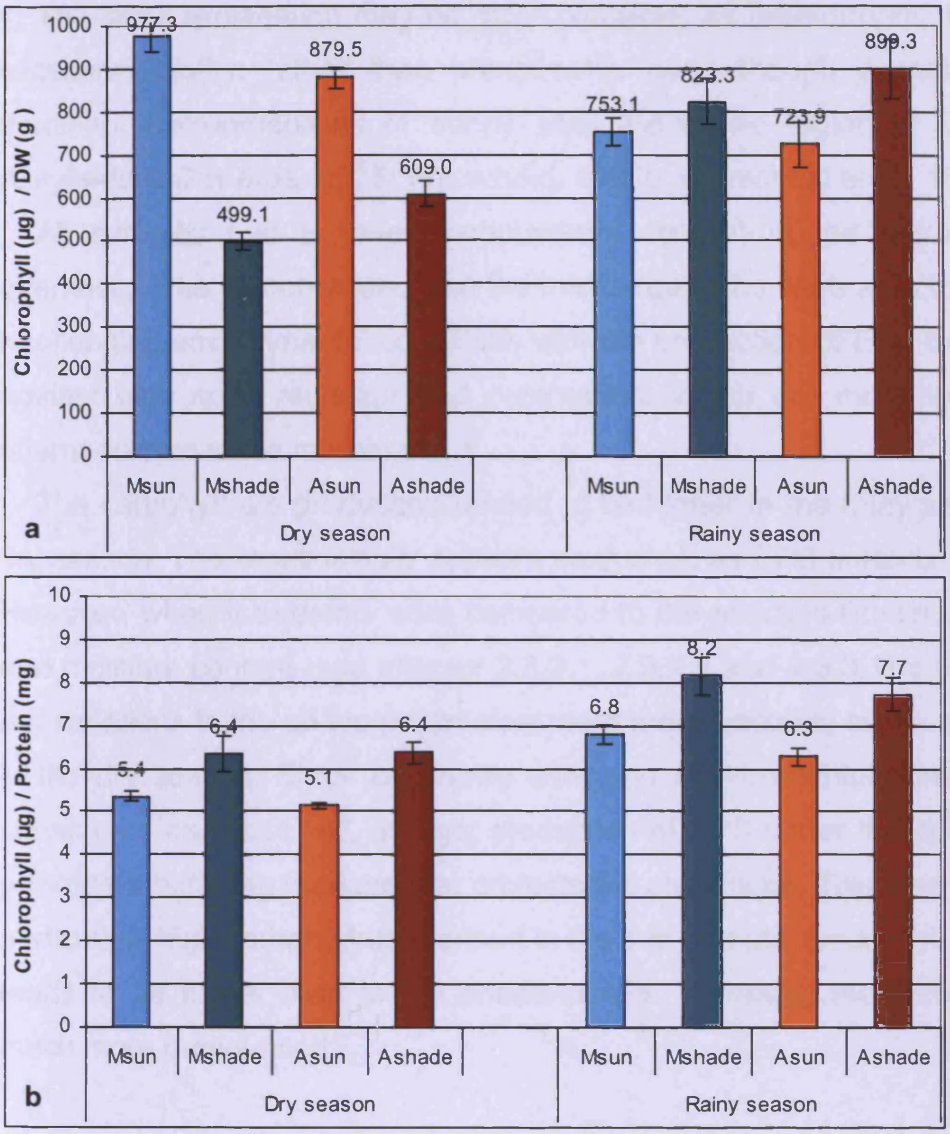


Figure 33 Chlorophyll content of biofilm samples
Mean values of the chlorophyll content from the sun-exposed and shaded sample in the morning (8 am) and the afternoon (4 pm), related to the sample dry weight (a) and protein content of the sample (b) in the dry season (March - May) and in the rainy season (July - October). Error bars show the standard error of mean for 10 replicates.

2.3.3.4 Carbohydrate content

The carbohydrate content was in all cases significantly higher in the sun-sample (fig. 34a-c, table App. 2.6). The EPS production per chlorophyll concentration (fig. 34c) had to be analysed with caution for the problematic interpretation for the low chlorophyll content discussed in 2.3.3.3. On the one hand, a potentially high proportion of phototrophs with few chloroplasts in the sun was the origin for high EPS production. On the other hand, the potentially dominating heterotrophic community in the sun-site was responsible for the high concentration of EPS.

The latter explanation may be more plausible, as heterotrophic bacteria produce excessive biofilm rather than phototrophs, even though cyanobacteria are the dominant microorganisms in sunny sites within the region of Southern Mexico (Gaylarde & Gaylarde, 2005; Warscheid, 1996b; Warscheid et al., 1991).

All samples had a lower carbohydrate content in the morning than in the afternoon. This demonstrated that the microorganisms were able to respond quickly to changing environmental conditions with the production of EPS to protect the cells against high solar radiation and desiccation, which are more threatening in the afternoon than in the morning.

The carbohydrate production tended to be higher in the rainy season than in the dry season. This result initially appears confusing, as EPS protects from desiccation. However, when the results were compared to the average illumination, temperature and moisture content (see chapter 2.3.2.1, 2.3.2.2 and 2.3.3.1) it became clear that the variations in the above parameters were more unstable in the rainy season than in the dry season. Since constantly changing environmental conditions pose high stress on a microbial cell, a larger production of EPS under those hostile conditions provides a buffering medium that protects the organisms. This was supported by the particularly high carbohydrate content in the sun-sample, where the moisture content tends to be higher than in the shade-sample, however, the daily variations were much more pronounced.

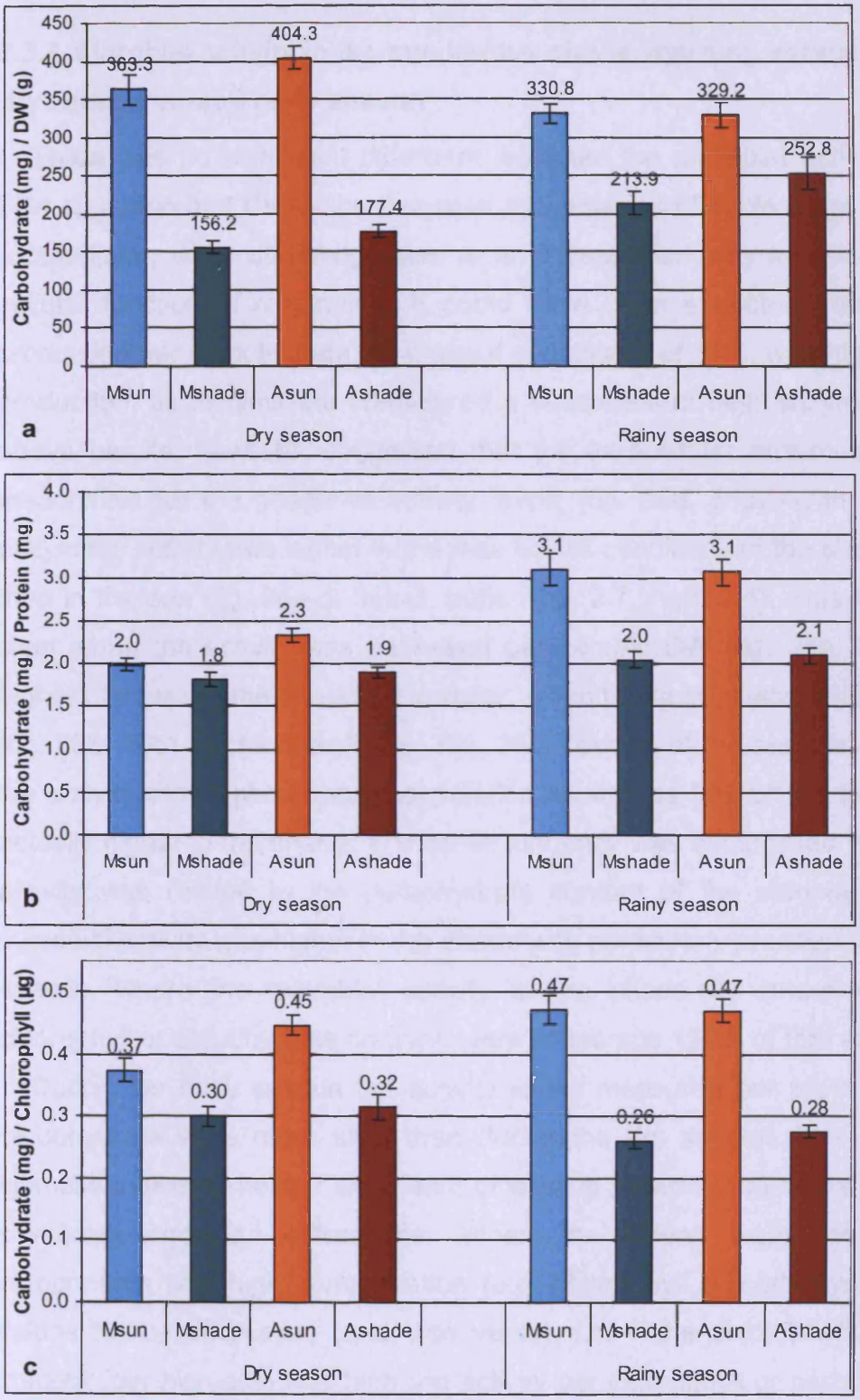


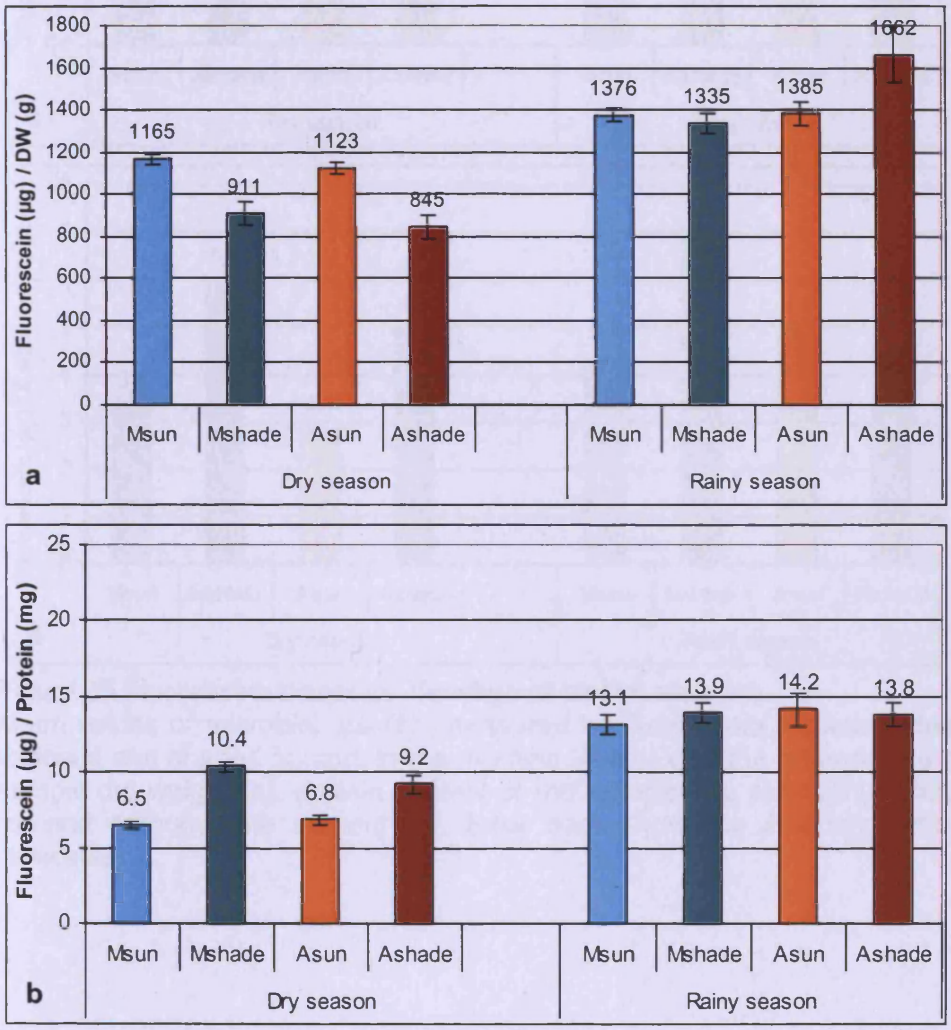
Figure 34 Carbohydrate content of biofilm samples
Mean values of the carbohydrate content from the sun-exposed and shaded sample in the morning (8 am) and the afternoon (4 pm), related to the sample dry weight (a), protein content of the sample (b) and chlorophyll content of the sample (c). Error bars show the standard error of mean for 10 replicates.

2.3.4 Microbial activity in the sun versus shade, morning versus afternoon and dry season versus rainy season

There was no significant difference between the microbial activity measured by FDA cleavage and DHA. The enzymes assessed by FDA cleavage may be intra- or extracellular, while dehydrogenase is an intracellular enzyme related to the basic cellular function of respiration. It could have been expected that the fluorescein production per carbohydrate, as a major constituent of EPS, was higher than the INF production, as biofilms are considered a substance of high enzymatic activity. The above results, however, suggested that the intracellular enzymes were the ones responsible for the assessed activity levels (fig. 35d, 36d). With both assays the enzymatic activity was higher in the less hostile conditions of the shady sampling site than in the sun (fig. 35a-d, 36a-d, table App. 2.7, App. 2.8). This trend was not as clear when the activity was assessed per sample DW (fig. 35a, 36a), due to the higher biomass at the sun site. However, when the activity was related to the protein (fig. 35b, 36b) or chlorophyll (fig. 35c, 36c) content of the sample, it was clear that the activity of the phototrophic population as well as that of the total biomass was actually higher in the shade. The same tendency was established when the enzyme activity was related to the carbohydrate content of the sample. This trend that microbial activity was higher in the shade was particularly pronounced during the dry season, where the microbial activity at the shade-site (measured per protein, chlorophyll or carbohydrate content) were in average 130% of that at the sun-site.

During the rainy season the activity levels measured per protein, chlorophyll or carbohydrate were more alike than during the dry season. The activity per total biomass indicated neither significant difference between the two sampling sites nor significant circadian differences. When the values were related to sample components with high concentration (e.g. chlorophyll or carbohydrate) the activity values hence decreased (and vice versa), i.e. if the chlorophyll or carbohydrate content (per biomass) was high the activity per chlorophyll or carbohydrate was low and vice versa. These results indicated that during the rainy season the enzymatic activity was reasonably similar for all four samples (morning, afternoon, sun, shade) as shown in fig. 35.

The circadian variations of the enzymatic activity was surprisingly small. This was the case at both sampling sites in the dry and rainy season. It could be expected to see more rapid variations in enzymatic activity during the day, as enzymes are known to react very quickly upon environmental stimuli. The results may indicate that the environmental parameters such as temperature and UV radiation within the biofilm are by far not as variable as in the surrounding environment.



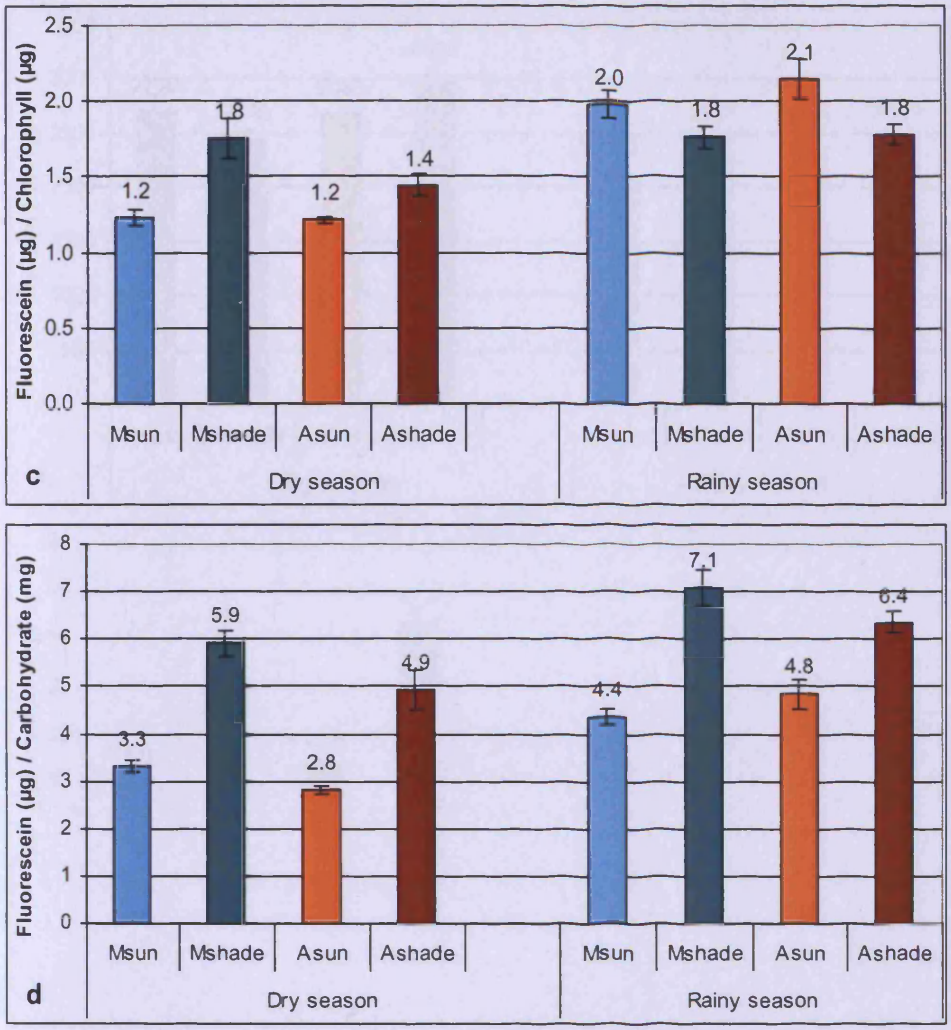
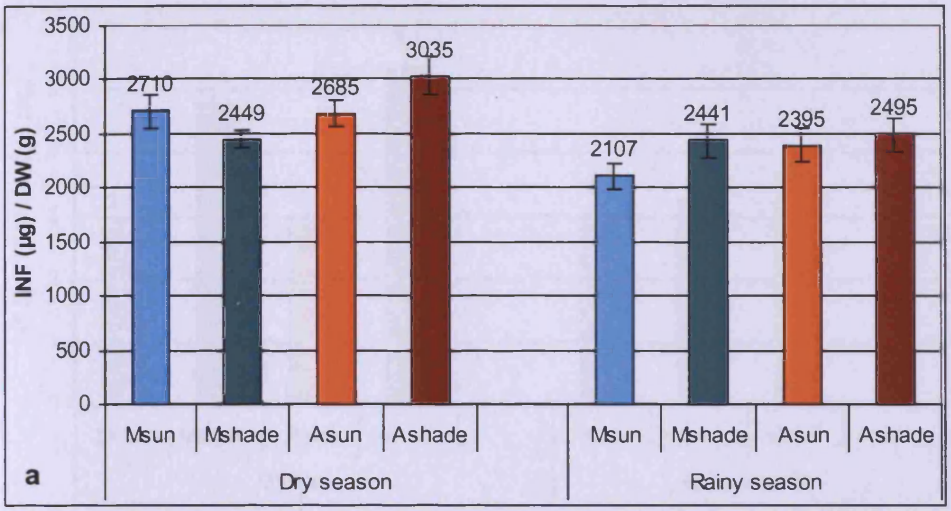


Figure 35 Fluorescein diacetate cleavage of biofilm samples
Mean values of microbial activity (measured by fluorescein diacetate cleavage) of the sun-exposed and shaded sample in the morning (8 am) and the afternoon (4 pm), related to the sample dry weight (a), protein content of the sample (b), chlorophyll content of the sample (c) and carbohydrate content (d). Error bars show the standard error of mean for 10 replicates.


3500
3000
2500
2000
1500
1000
500
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INF (µg) / DW (g)
2710
2449
2685
3035
2107
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Mshade
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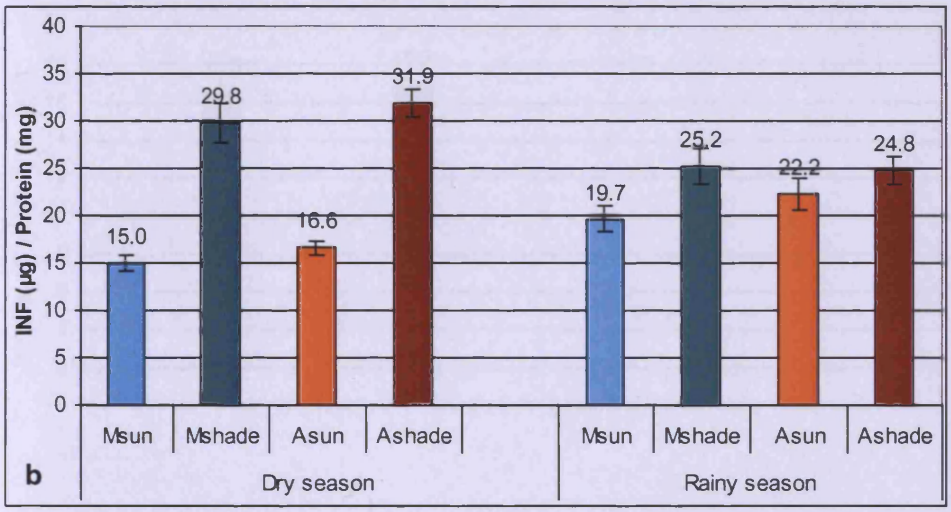
Ashade
a
Dry season
Rainy season


40
35
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INF (µg) / Protein (mg)
15.0
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Dry season
Rainy season

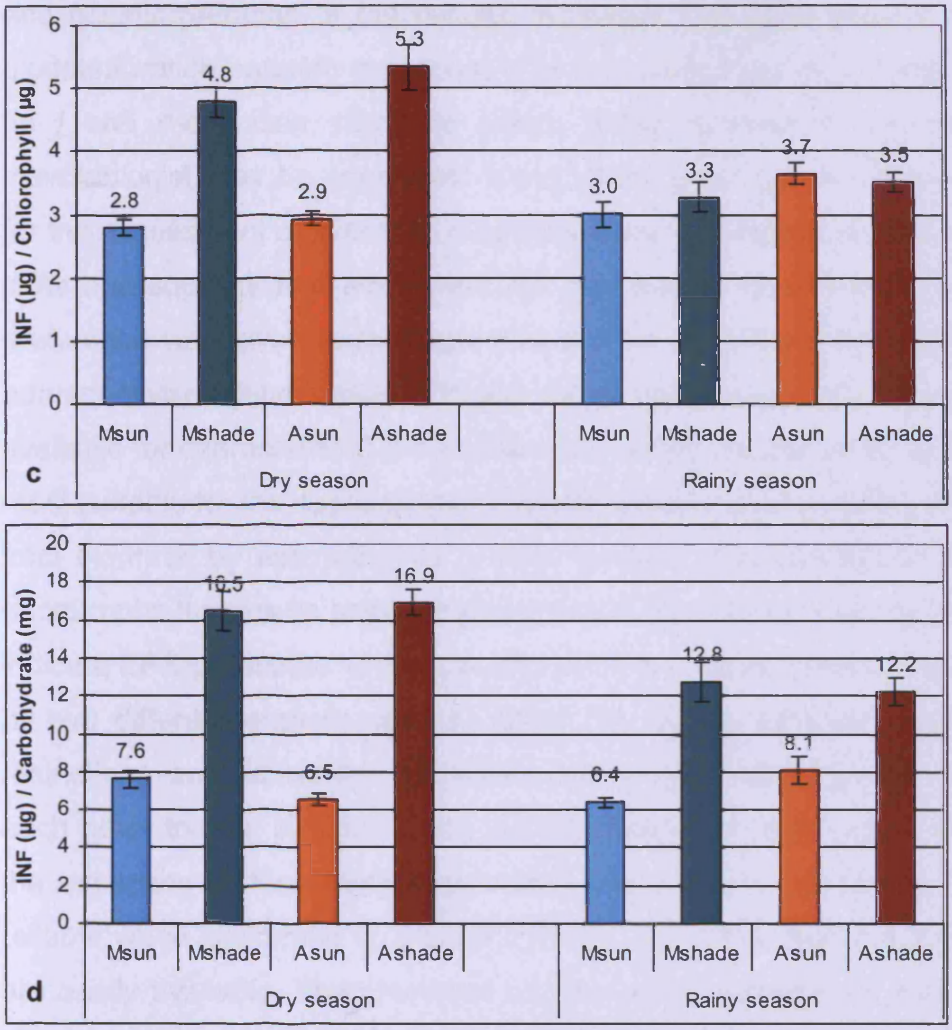


Figure 36 Iodonitrotetrazolium reduction of biofilm samples
Mean values of dehydrogenase activity (measured by iodonitrotetrazolium reduction) of the sun-exposed and shaded sample in the morning (8 am) and the afternoon (4 pm), related to the sample dry weight (a), protein content of the sample (b), chlorophyll content of the sample (c) and carbohydrate content (d). Error bars show the standard error of mean for 10 replicates.

2.3.5 Conclusion

The mere presence of microorganisms on cultural heritage objects does not prove that biodeterioration is occurring, but it is indicative of a possible danger (Urzi, 2004; Sand et al., 2002; Hirsch et al., 1995a). Monitoring the condition of the artefact, together with the quantity, composition and activity of the microbial population, may give an indication when microbial colonisation might be reaching a critical level and an intervention is necessary.

Many microbiologists find the idea that conservators carry out microbiological investigations dangerous due to potential errors in the performance of the protocols

and misinterpretation of the results. However, the initial step of identifying that a biodeterioration problem is present is usually performed by a conservator and only if he / she recognises microbial action being involved in the decay process, a microbiologist may be consulted. Many of the most commonly performed methods for the assessment of microbial biodeterioration of outdoor stone monuments require skills and sophisticated equipment not available to conservation staff. To this end, preference was given to methods that can be carried out by non-microbiologists in non-specialised laboratories. Of the many parameters and assessment methods available for characterising a microbial community, the following were considered the most suitable for the application in cultural heritage conservation: investigation of the total biomass by assessing its protein content, characterisation of the amount of phototrophs through its chlorophyll *a* content, quantification of the carbohydrates that indicate EPS production and most important the assessment of the microbial activity by two different enzyme assays. All of the above discussed methods have their restrictions and limitations. However, when performed together they complement each other to give an idea of the microbial activity, composition and quantity. With the exception of the phospholipid assay, they are all simple methods, which are reliable when performed in a constant way. All of the equipment and consumables are easily available. The protocols do not require specialised microbiological skills, however, some time is needed to get familiarised with the procedure and to optimise the methods for each field of application. Therefore, all of the above discussed methods, except for the phospholipid assay, are recommended to be used by conservators for the assessment of microbial activity and biomass on cultural heritage objects.

It is recommended to assess the above parameters together with a thorough condition report of the artefact immediately when biodeterioration is assumed. If the object's condition allows monitoring rather than immediate action, a "baseline" of the above biodeterioration parameters should be created. Care has to be taken to document the exact condition under which the experiment was performed (sampling time and location, climate condition before and during sampling and any changes made to the protocols). A monitoring plan should be developed indicating the frequency of sampling/assessments. The sampling site has to be selected with care: it should be either representative of the overall contamination and environmental condition of the site, a place with a contamination level above the average or a

location in particularly poor condition. The advantages of taking samples from various sampling sites have to be evaluated against. When choosing the site it has to be considered that enough sample material is present to perform long-term monitoring with sequential sampling. The environmental conditions at a sunny sampling site are much more hostile and variable than in the shade. The total microbial biomass and microbial activity are usually much higher in protected areas, while EPS production tends to be higher at a sunny site to protect the cells from the adverse conditions. The sampling time is also a critical factor that may influence the results, as the temperature and illumination at various sampling sites are much more similar in the morning than in the afternoon. However, not many parameters assessed responded rapidly to changes over the day. Only the carbohydrate content showed the tendency to be slightly higher in the afternoon than in the morning. Seasonal differences, again, were more pronounced, as the differences between various sites were much more significant during the dry season than during the rainy season.

The results demonstrated that differences in microbial activity and biofilm composition were more obvious the longer the specific environmental conditions acted on the sample. Hence, changing conditions during the day were statistically insignificant, while seasonal changes were more pronounced. The largest divergence could be seen in samples from different sampling sites, where the microbial population could establish over years of distinct environmental conditions. This shows that it is very important to always sample at the same site for long-term monitoring, while the time of the day is less critical. Seasonal changes have to be considered when interpreting the results to avoid wrong conclusions on the level of biodeterioration.

Chapter 3

Development of a system to test sub-lethal and lethal effects on bacteria[2]

3.1 Assessment of sub-lethal perturbation of bacteria

The evaluation of the efficiency of antimicrobial treatments entails serious problems. Effective doses of an antimicrobial agent cause either bacteriostatic (growth inhibiting) or bactericidal effects (causing cell death). The assessment method to identify the efficiency of an antimicrobial agent is a great source of error. One of the most commonly used methods, counting the number of colony forming units (CFU) underlies general cultivation errors and may not distinguish between bacteriostatic or bactericidal effects. Another common method, which has also been used in the evaluation of biocides against biodeterioration of outdoor stone monuments (Blazquez, 2000), is the assessment of the minimal growth-inhibiting concentrations (MIC). This semi-quantitative method depends on the ability of the biocide to diffuse through a solid growth medium. Furthermore, it depends on the incubation time and the specific growth rate of the test organism (Rodin et al., 2005).

Antimicrobial treatments that readily perturb microorganisms, however, that do not act bactericidal or –static at the dose tested, are even more difficult to assess. Decreased growth rates and extended lag-phases of microorganisms may serve to evaluate the effectiveness of a biocide (Rodin et al., 2005), however, the slightest difference in cultivation conditions can lead to significant variations in growth dynamics, rendering this method unreliable. Visual inspection (under the microscope with or without the help of activity stains) may give an indication of physiological condition of microorganisms. However, the results are often unreliable as discussed in chapter 2.1.5 and chapter 4.

[2] parts of the following chapter have already been published (Scheerer et al, 2006), and are quoted here verbatim.

3.1.1 Bioindicators

Bioindicators are organisms used to monitor environmental changes. They may be employed to indicate sub-lethal biological effects. The use of bioluminescent bacteria as bioindicators dates back to the 1950s (Rayner-Brandes, 1996). Examples of the variety of applications range from the monitoring, detection and/or quantification of particular chemicals, genetically engineered organisms released into the environment; assessment and monitoring of the toxicity of compounds and investigations on remedial operations in environmental samples (Nunes-Halldorson & Duran, 2003; Steinberg et al., 1995). Sub-lethal treatment procedures may be studied employing bacterial bioluminescence. They are based on the activity of electron transport systems, which is a requirement for the production of reduced substrates used for bioluminescence. Processes like electron transport are part of the cell's basic metabolism and anything affecting it has an effect on bioluminescence. Consequently, bioluminescence has been suggested to provide a direct assessment of the effect of a toxin on the microbial metabolism by changes in bioluminescence (Nunes-Halldorson & Duran, 2003).

The response of luminous bacteria to drugs has been demonstrated to vary during their growth (Middleton, 1973 cited by Wardley-Smith & White, 1975). Hence, to be reproducible, tests need to be performed on bacteria at the same stage of their growth. This is not easily possible with organisms grown in batch culture. Cultivation of bacteria in a fermenter provides a permanent supply of cells in exponential growth phase. However, light intensity has been reported to be very difficult to control (Pooley, 2003). All the published data on "continuous culture" of bioluminescent bacteria were actually on quasi-continuously grown bacteria. These devices were either controlled by turbidity or by luminosity; hence they were batch-fed systems (Pooley, 2003; Zavoruev & Mezhevikin, 1982; Wardley-Smith & White, 1975). The disadvantage of the batch-fed mode is that the cells experience frequent changes in environmental conditions and hence exhibit discontinuous metabolic activity, with episodes of exponential growth interspersed by starvation. To be able to use bioluminescent bacteria as a biosensor for sub-lethal effects, it is essential to keep the environmental conditions constant. This ensures that the only variable in the system is imposed by the source that caused the sub-lethal effect.

3.1.1.1 *Vibrio fischeri* bioluminescence for the indication of bacterial stress

V. fischeri is the best-described bioluminescent bacterium used as an indicator for toxicity tests. The marine bacterium, which has a world-wide distribution, can be found preferentially in temperate and sub-tropical waters at depths of about 1000 m. *V. fischeri* occupies a variety of niches. It may grow in a free-living planktonic state or in a symbiotic relationship with certain fish and squid (Hastings, 2004; Ruby & Nealson, 1976). It may also grow as a member of the enteric microbiota of a variety of marine animals or as a pathogen of certain invertebrates (Herring, 2002). Different from various other members of the genus *Vibrio*, *V. fischeri* is non-pathogenic to humans. It is a motile, chemoorganotrophic, gram-negative bacterium belonging to the subgroup Vibrionaceae of the gamma-proteobacteria. It has a typical vibroid shape as straight or curved rods of 0.5–0.8 µm in width and 1.4–2.6 µm in length (fig. 37). It is facultatively anaerobic with the option of a respiratory or a fermentative metabolism. However, bioluminescence can only be performed in the presence of oxygen.

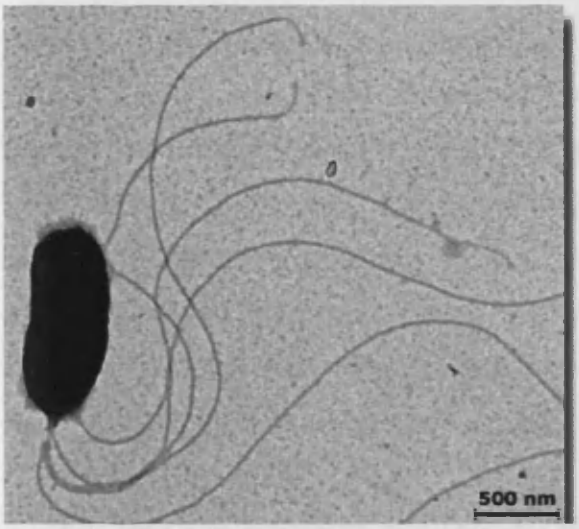


Figure 37 *Vibrio fischeri* cell
Scanning electron micrograph of a flagellated *V. fischeri* cell.
Source: Integrated Genomics Inc, 2002; photo by D.S. Millikan.

3.1.1.2 Ecology of bioluminescence

Bioluminescence is a rare event in nature that is performed by few, remarkably diverse groups of organisms comprising 13 Phyla mainly in marine habitats, but some may also be found in freshwater and terrestrial environments. Luminous organisms include bacteria, dinoflagellates, fungi, unicellular algae, jellyfish, annelids, molluscs, shrimp, fireflies, echinoderms and fish. Bioluminescence has not

been found to exist in higher plants or vertebrates above fish (Hastings, 1995; Meighen, 1991). In contrast to bioluminescent dinoflagellates, where light production occurs in organelles called scintillons, bacteria do not have specialised compartments for the production of light (Hastings, 1995).

The biochemical and physiological mechanisms that underlie bioluminescence in various groups of organisms are very different, suggesting that it evolved independently in different Phyla. It has been estimated that about 30 distinct evolutionary events led to today's luminous organisms (Hastings, 1983). Further, it may have been lost in different evolutionary lines where it has not proven to be of great advantage to the organism (Hastings, 1995).

Most luminous organisms produce their own light, but some fish and squid live in a symbiotic relationship with bioluminescent bacteria such as *V. fischeri*, which grow within light organs consisting of specialised tissue. These highly vascularised compartments provide nutrients and oxygen for the bacteria. Ducts to the exterior allow growing bacteria to exit (Tebo et al., 1979). In fact, about 90% of the bacteria are expelled every day from a symbiotic luminescent squid whose light organ contains a dense culture of generally over 10^{10} cells ml^{-1} (Graf, 2007; Herring, 2002; Wilson & Hastings, 1998). Fish are reported to release 10^{7}–10^{8} cells h^{-1} from light organs that have a symbiont density of 10^{8}–$5x10^{9}$ ml^{-1} (Herring, 2002).

In such a prokaryotic / eukaryotic symbiosis the host provides the luminous bacteria a sheltered environment with nutrients as well as oxygen. The bacteria in turn provide a continuous source of light (Ruby & Nealson, 1976). Additional to the light that becomes available to the non-luminous host, it has been suggested that luminous bacteria also provide metabolic products such as pyruvate to the host (Tebo et al., 1979).

The role of bioluminescence is diverse and different groups of luminous organisms may use it as means of offence, defence or communication. The attraction of prey by light is one of the major advantages that a luminous organism has or a symbiotic non-luminous host gets from cultivating luminescent bacteria. Bright, brief flashes of light may frighten another organism and can hence be used as an offence or defence mechanism (Hastings, 1995). Further, bioluminescence may provide camouflage for an organism against light, whereas otherwise it would form a dark silhouette. The bobtailed squid *Euphrymna scolopes* projects light downward from its light organs to camouflage itself against the moonlight (Graf,

2007). Light may also be used in intraspecies communication. A specific pattern of emitted light may serve as a mating signal and can be recognised by the opposite sex (Hastings, 1995).

However, bacteria have no means for detecting photons. The advantage for free-living planktonic bacteria to be able to emit bioluminescence is not well understood (Czyz et al. 2000). It has been suggested that the free-living planktonic state in the ocean is a secondary habitat produced by overflow of the symbiotic habitat and serves as a reserve future inoculum (Hastings, 2004). Czyz et al. (2000) further proposed that bioluminescence of *V. harveyi* may have developed to repair DNA damage caused by UV radiation. However, it remains unclear whether UV levels for organisms, whose habitat is free-living in the water column of oceans, marine sediments and within the intestinal system of marine symbionts, are high enough to cause serious DNA damage.

Maximum light emission of a single bacterial cell is approximately 10^4 photons s^{-1}. Hence, for the light to be visually noticeable, the bacterial cell density must be about 10^9–10^{10}, which is a sufficiently dense population to trigger autoinduction (Hastings, 2004; Herring, 2002).

3.1.1.3 Mechanisms of bioluminescence

Bioluminescence is an enzyme-catalysed form of chemiluminescence. In contrast to fluorescence and phosphorescence light emission is not dependent on the initial absorption of light. Luminous systems share the same basic mechanism of oxidation of a substrate, called luciferin (literally "light bearers" [Hastings, 1996]) by molecular oxygen and the enzyme luciferase with the production of an excited state. This results in the emission of a photon, as shown in the following equation:

$$\text{Luciferin} + O_2 \xrightarrow{\text{luciferase}} (P)^* \rightarrow P + h\nu$$

Where P is a product molecule and P* is its electronically excited state (Hastings 1983).

All known luciferases are oxygenases, which "are enzymes that catalyse the incorporation of oxygen from O_2 into organic compounds" (Madigan et al., 2003). However, the enzymes of different light-emitting systems have no homology to each other and also the substrates are chemically unrelated (Hastings, 1998). Bacteria have two substrates, luciferin, which is a reduced flavin mononucleotide ($FMNH_2$),

and a long chain (7–16 carbons) fatty aldehyde (RCHO). An external reductant via flavin monooxygenase oxidoreductase catalyses the reduction of flavin mononucleotide (FMN) to $FMNH_2$. The reduced flavin ($FMNH_2$) binds to the enzyme and reacts with O_2 to form a 4a-peroxy-flavin intermediate. This complex interacts with the aldehyde (RCHO) to form the corresponding acid (RCOOH) and a highly stable luciferase-hydroxyflavin intermediate in its excited state, which decays slowly. Upon return to ground state it emits blue-green light with a maximum emission at about 490 nm (Hastings, 2004; Hastings, 1995; Meighen, 1991). This luciferin reaction is extremely slow, at 20°C one single catalytic cycle takes about 20 seconds (Hastings, 1995). The whole reaction cycle is shown in figure 38 and a simplified reaction can be described as follows:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + h\nu\,(490\text{ nm})$$

(adapted from Meighen, 1991)

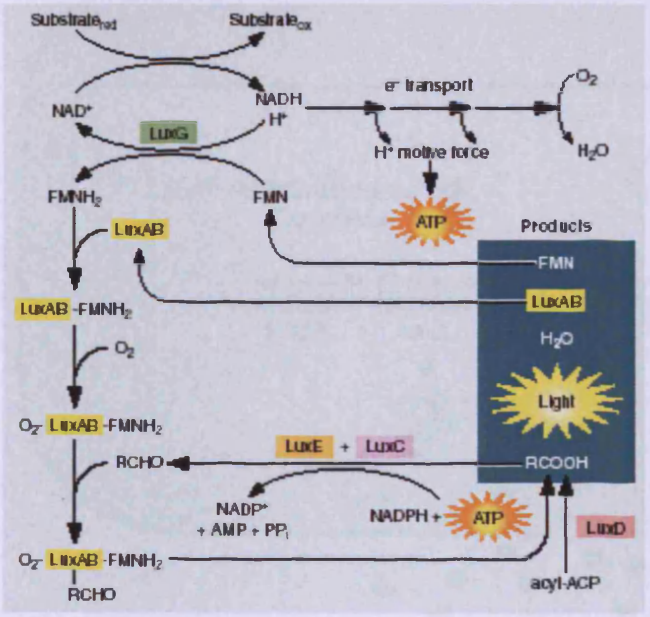


Figure 38 Biochemistry and physiology of *V. fischeri* bioluminescence
LuxAB: luciferase; LuxC: reductase for aldehyde tetradecanal synthesis; LuxD: transferase for aldehyde tetradecanal synthesis; LuxE: synthease for aldehyde tetradecanal synthesis; LuxG: FMN reductase (Stabb, 2005).

A phenomenon, called auto-induction when it was first reported by Nealson et al. (1970), but now commonly referred to as quorum sensing, describes a mechanism that allows the *lux* genes to be transcribed only at high cell densities. Bioluminescence of *V. fischeri* was one of the first, or possibly the first intraspecies communication mechanisms described in bacteria (Nealson et al., 1970). The autoinducer is a N-3-oxohexanoyl-L- homoserine lactone (OHHL), which is an acyl-

homoserine lactone (AHL) signalling molecule encoded by the *luxI* gene, is produced by the bacterium and excreted from the cell. As the population grows, the concentration of external autoinducer accumulates in the medium surrounding the cells and reaches a threshold, which is in the micro-molar range. Consequently, the efflux of autoinducer from the cell becomes balanced by an influx. This allows the interaction with the autoinducer receptor protein. This autoinducer-receptor protein complex is believed to bind to the luciferase promoter and hence luciferase is synthesised (Pappas et al., 2004). This regulation mechanism provides the advantage that at low cell densities, where too few photons would be produced for the light to be visible, luciferase is of no value and hence not synthesised (Hastings, 2004). Consequently, in a free-living state, where luminous bacteria are largely dispersed in sea water, they do not produce light.

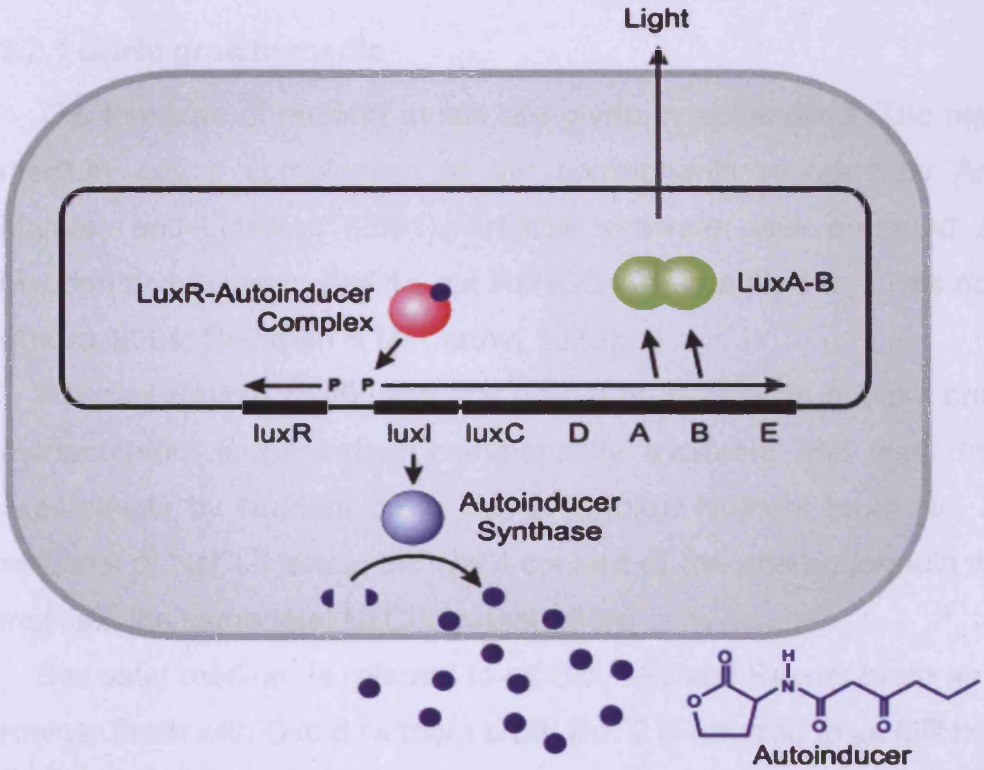


Figure 39 Quorum sensing system of *V. fischeri*
The LuxR-LuxI quorum sensing system of *V. fischeri.* Autoinducer synthase, encoded by *luxI*, synthesizes N-3-oxohexanoyl-l-homoserine lactone (OHHL) from S-adenosylmethionine and acyl-ACP. OHHL diffuses across the cell envelope and accumulates intracellularly only at high population density. It binds the LuxR receptor protein, and LuxR-OHHL complexes activate transcription of the *luxICDABE* operon, resulting in bioluminescence (Pappas et al., 2004).

3.1.2 Aims of the development of a system to test sub-lethal and lethal effects on bacteria

The aim of this section was to develop a system that could reliably assess fast acting sub-lethal perturbation of microorganisms. Sub-lethal perturbation is an important parameter for the development of innovative synergistic antimicrobial treatment methods of low eco-toxicity, which are discussed and tested in chapter 4. To this end a reliable, non-invasive high throughput system was needed that could assess the sub-lethal perturbation of a model bacterium. It should later be used to assess each component of a potential synergistic antimicrobial method, which by itself cannot kill the model organism but perturb it.

3.2 Materials and methods

3.2.1 Basic growth media

The formulae of nutrient media are given in appendix 3. The recipe for seawater medium was a combination of the components reported by Andre (2004) and Madden and Lidesten (2001). Artificial seawater was prepared as suggested by Madden and Lidesten (2001), but $NaHCO_3$ was omitted as it was not invariably used (Andre, 2004; Goldman & McCarthy, 1978).

"Michael Rayner broth" was first prepared with Bacto nutrient broth (Difco). Bacto nutrient broth is no longer commercially available and was replaced in recent experiments by Nutrient broth No. 2 (Oxoid). Nutrient broth No. 2 contains small amounts of NaCl. Hence, the NaCl content of the altered formula was decreased to maintain the same total NaCl concentration.

Seawater medium is referred to as SM, Michael Rayner broth as MR and Michael Rayner Broth with Oxoid Nutrient broth No. 2 is referred to as MR new.

3.2.2 Culture preparation

Unless otherwise noted, 25 ml medium in 100 ml conical flasks, plugged with either a plastic sponge or cotton wool and capped with aluminium foil were sterilised by autoclaving for 15 min at 121°C. Each flask was inoculated with 0.6 ml *V. fischeri* NRRL-B-11177 (Agricultural Research Service Culture Collection Peoria, Illinois, USA) cell suspension with a turbidity of A_{400nm}= 0.05. The inoculum was prepared by transferring luminescent *V. fischeri* from agar plates into sterile artificial seawater (for

peptone tests) or sterile water containing 2.5% (w/v) NaCl (for nutrient broth composition tests). Unless otherwise mentioned, the inoculum for all other experiments was prepared by transferring *V. fischeri* from agar plates into sterile growth medium and incubating them till bright luminescence occurred. Incubation was performed at 22°C (±2°C) under constant shaking at 200 rpm. The incubation time varied according to the application as indicated.

3.2.3 Turbidity measurement of batch cultures

Turbidity was measured with a Unicam SP 1800 Ultraviolet Spectrophotometer at 400 nm, slit width of 0.46 mm and a band width of 1.5 nm. Cuvettes containing 0.8 ml *V. fischeri* suspension were diluted with 3 ml starvation buffer (see appendix 3). The instrument was zeroed with starvation buffer.

3.2.4 pH measurement of batch cultures

pH was measured using a pH meter from RS Components Ltd., Stock No 610-540. Where sterility had to be maintained, an aliquot was taken from the culture. A volume of 0.8 ml was the smallest volume in which the pH electrode could be fully immersed.

3.2.5 Luminosity measurement of batch cultures

Luminosity assessment for the tests to determine the influence of peptones on bioluminescence was performed by visual observation. Values between 0 (no bioluminescence) and 3 (bright bioluminescence) were attributed to individual cultures. The aim was to identify the peptone that provided the brightest and longest lasting bioluminescence and therefore, it was sufficient to use this subjective, non-quantitative method.

For tests on the effect of nutrient broth components, bioluminescence was measured with a Varian Cary Eclipse Fluorescence Spectrophotometer. To prevent overload of the spectrophotometer, 0.8 ml *V. fischeri* culture was diluted with 3 ml starvation buffer. The instrument was used in bio- / chemiluminescence mode. emission at 490 nm was measured with a gate time of 200 ms, an emission slit of 10 nm with open emission filter and high photomultiplier detector voltage. The instrument was zeroed before each light measurement using starvation buffer.

3.2.6 The effect of peptones on *V. fischeri* bioluminescence in batch culture

Seawater Medium was prepared with different kinds of peptones. Incubation was performed as described in 3.2.2 for 4 days. Four replicates of each of the following media were tested for their effect on bioluminescence:

1. Artificial seawater (75% v/v), water (23.9% v/v), glycerol (0.3% v/v), yeast extract (Oxoid) (0.3% w/v), Tryptone soya broth (Oxoid) (0.5% w/v)
2. Artificial seawater (75% v/v), water (23.9% v/v), glycerol (0.3% v/v), yeast extract (Oxoid) (0.3% w/v), pancreatic casein (Solabia) (0.5% w/v)
3. Artificial seawater (75% v/v), water (23.9% v/v), glycerol (0.3% v/v), yeast extract (Oxoid) (0.3% w/v), pancreatic gelatine (Merck) (0.5% w/v)
4. Artificial seawater (75% v/v), water (23.9% v/v), glycerol (0.3% v/v), yeast extract (Oxoid) (0.3% w/v), Bacto nutrient broth (Difco) (0.5% w/v)
5. Artificial seawater (75% v/v), water (23.9% v/v), glycerol (0.3% v/v), yeast extract (Oxoid) (0.8% w/v)

3.2.7 The effect of nutrient broth components other than peptones on *V. fischeri* bioluminescence in batch culture

SM and MR broth (appendix 3) as well as variations thereof (table 3) were tested for their effect on bioluminescence of *V. fischeri* and pH changes of the culture. The media differed in the kind of peptone, pH buffer, additional carbon source and electrolyte supplements additional to sodium chloride, such as magnesium chloride, magnesium sulphate, calcium chloride and potassium chloride, all of which are contained in seawater. Four replicates of each of the medium variations were tested. The incubation time was 21 days. Light emission and pH were measured daily.

Table 3
Growth media tested for the role of nutrient broth composition on bioluminescence.

	Medium	Variation to original formula
1	MR	no variation
2	MR	with 0.5% $CaCO_3$
3	MR	without glycerol
4	MR	with 0.3% glycerol
5	SM	no variation
6	SM	with 0.5% $CaCO_3$
7	SM	without glycerol
8	SM	with 1% glycerol
9	SM	without yeast extract
10	SM	pH adjusted to 7.6
11	SM	with PO_4 buffer for pH 7.6

3.2.8 The effect of temperature on *V. fischeri* bioluminescence

For experiments in batch culture, 250 ml MR broth without glycerol was inoculated with 10 ml *V. fischeri* cell suspension. The culture was incubated at 20°C for approximately 24 h until bright bioluminescence was emitted. After this initial incubation time, the culture was divided into different flasks, each containing approximately 25 ml of cell culture. These brightly luminescing *V. fischeri* cultures were subsequently incubated at 15°C, 20°C, 25°C or 30°C. Bioluminescence was measured 2 and 5 h after incubation at individual temperatures. After 5 h all cultures were incubated for 20 h at 20°C to determine if changes in luminescence could be restored. The tests were performed four times each with duplicates.

For continuous monitoring of changes in luminosity upon temperature variations, the fermenter with brightly luminescing *V. fischeri* cultures (chapter 3.2.9) was transferred to a temperature-controlled incubator. A temperature range between 15 and 36°C was tested. The incubation temperature was decreased or increased in small increments of approximately 2°C as well as through larger changes of approximately 5°C.

3.2.9 Continuous culture device

The different components of the continuous culture device are shown in fig. 40. The working volume of the fermentation vessel (maximal capacity: 1 l) was maintained at approximately 500 ml. Oxygenation of the culture was provided by pumping air with an aquarium pump (Rena Air 300) through an autoclavable Whatman HEPA–Vent filter (0.2 µm pore size) at a flow rate of 600 ml/min, controlled by an air flow meter (Gap Meter R6/CD, Gap Basingstroke) to the bottom of the

fermentation vessel. Stirring was provided by a magnetic stirrer flea driven by a control (LH engineering, 502) from the bottom. A stirring rate of 350 rpm provided homogenous distribution of substrates and cells within the culture, while not causing significant foam formation. MR new medium was pumped (Watson-Marlow 503S Issue 1) through silicone tubing into the fermentation vessel. The overflow was driven by the pressure created by the air inflow. The height of the stainless steel tube, connected to the waste container, determined the volume of the culture within the fermentation vessel. The culture was pumped (Watson Marlow 502S) through silicone tubing (0.29 ml/min) through a light-tight box for luminosity and turbidity measurements. Decreased bioluminescence due to O_2 depletion was avoided by the use of O_2-permeable silicone tubing. A high pump rate was chosen to minimise the risk of biofilm formation within. Turbidity was measured in a flow cell using a photodiode (Opto OPT301M) collecting pulses from a light-emitting diode transmitted through the culture passing through this flow system (LED intensity minus transmitted light). Values between flashes of the LED were 0 V, hence light emitted by the bacteria did not interfere with turbidity measurement. Luminosity was measured by leading the silicone tubing (2.5 mm bore, 1.0 mm wall) in front of a photomultiplier (PMT) (Hamamatsu R7467). The temperature probe was connected to a custom-made digital thermometer. A Mettler Roledo pH combination electrode type 3030 connected to a LH Fermentation 505 pH control was used to monitor the pH of the culture. This meter was calibrated after the system was autoclaved by sampling from the fermenter and measuring pH using a second reference pH meter (RS Components Ltd., Stock No. 610-540) and adjusting the pH meter of the fermenter. The concentration of dissolved oxygen within the culture was measured using an oxygen electrode (Uniprobe Instruments LTD, 400 Series, type G2), connected to a Biolab Oxygen meter (B. Braun, D.O.M.I 2/6200). The maximum oxygen concentration, 20.9% for atmospheric air, was determined by bubbling air into the un-inoculated culture until no further increase in oxygen concentration was observed. This value was set to 100%. For experiments, culture was pumped into a glass vessel, equipped with a magnetic stirrer, air supply, PMT and a sample introduction line. All measurement signals were recorded in 5 s increments (unless otherwise noted) using LabView 7.1 (National Instruments) with a PCI6023E data

acquisition card and a SCB68 Connection box. The data acquisition application records and plotted luminosity, turbidity, LED signal, temperature, pH and oxygen concentration in real time.

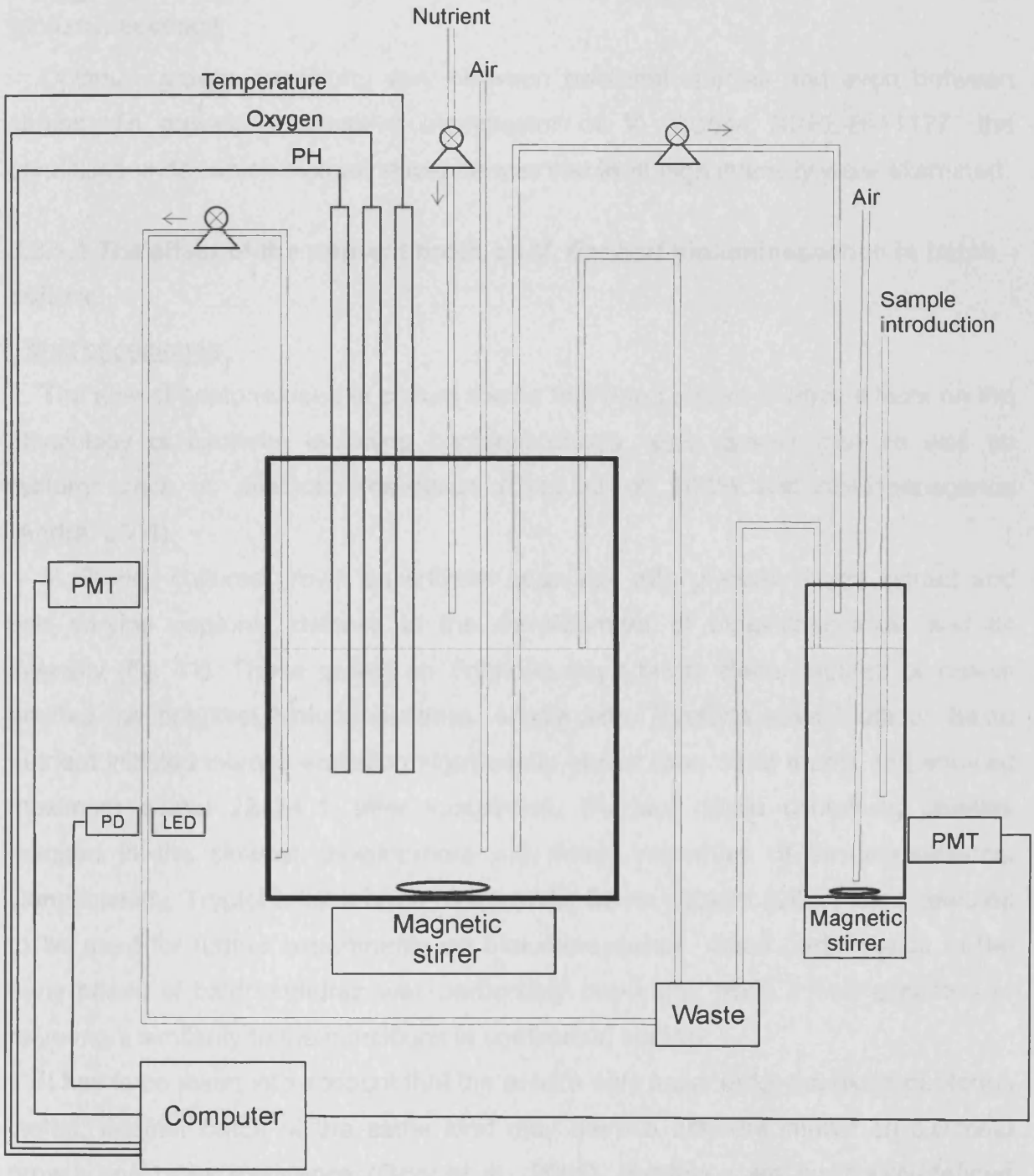


Figure 40 Fermenter for continuous *V. fischeri* luminescence
Schematic diagram of the fermenter with supporting instruments. PMT: Photomultiplier tube, LED: Light emitting diode, PD: Photodiode.

3.3 Results and discussion

3.3.1 Nutrient broth composition and culturing conditions that optimise bioluminescence

Optimum growth conditions vary between bacterial species and even between strains. To provide a sensitive bioindicator of *V. fischeri* NRRL-B-11177, the conditions under which bioluminescence was stable at high intensity were examined.

3.3.1.1 The effect of the nutrient broth on *V. fischeri* bioluminescence in batch culture

Effect of peptones

The type of peptone used in culture media has been shown to have effects on the physiology of bacteria, including bacterial shape, size, growth rate as well as features such as antibiotic resistance (Gray et al. 2005) and bioluminescence (Andre, 2004).

V. fischeri cultures grown on artificial seawater with glycerol, yeast extract and one varying peptone, differed in the development of bioluminescence and its intensity (fig. 41). Those grown on Tryptone soya broth, Bacto nutrient or casein emitted the brightest bioluminescence. Media with Tryptone soya broth or Bacto nutrient initiated intense emission significantly earlier than other media and showed maximum output 22–24 h after inoculation. Nutrient media containing gelatine resulted in the slowest development with least intensities of bioluminescence. Consequently, Tryptone soya broth (Oxoid) and Bacto nutrient (Difco) were selected to be used for further experiments on bioluminescence. Good performance in the early phase of batch cultures was particularly important, since it was expected to have more similarity to the conditions in continuous culture.

It has to be taken into account that the results only account for the exact peptones tested; another batch of the same kind may have a different impact on bacterial growth and bioluminescence (Gray et al., 2005). Peptones are the poorly-defined product of hydrolysed proteins from variable natural sources (Lawrence, 2000). Andre (2004) found that there was a great difference in *V. fischeri* luminescence between cultures grown on the same kinds of peptones provided by different suppliers as well as peptones from two different batches by the same supplier.

However, there was no statistical difference between the group of gelatine based culture media and the group of casein based culture media. This indicated that variations in the manufacturing process of peptones may affect the quality of the peptone more than the source from which it was obtained. A good batch should be obtained in quantities sufficient for an entire project.

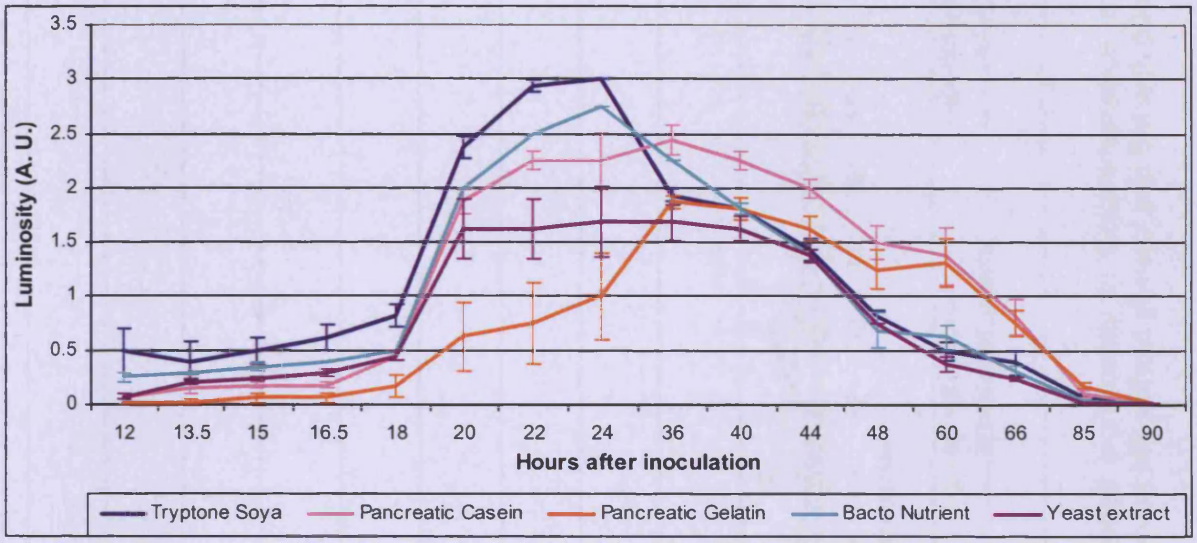


Figure 41 Bioluminescence of *V. fischeri*
Bioluminescence of *V. fischeri* cultures grown on artificial seawater with glycerol, yeast extract and one varying peptone. Luminosity was measured in arbitrary units determined by visual appearance. Error bars are the standard error of mean of 4 replicates.

Effect of media components other than peptones

The intensity of bioluminescence and its duration varied widely in batch cultures of *V. fischeri* grown on media with different components. Maximum light intensities were observed on the first or second day after inoculation (fig. 42). Cultures with low glycerol content performed better, as did those containing a pH buffer.

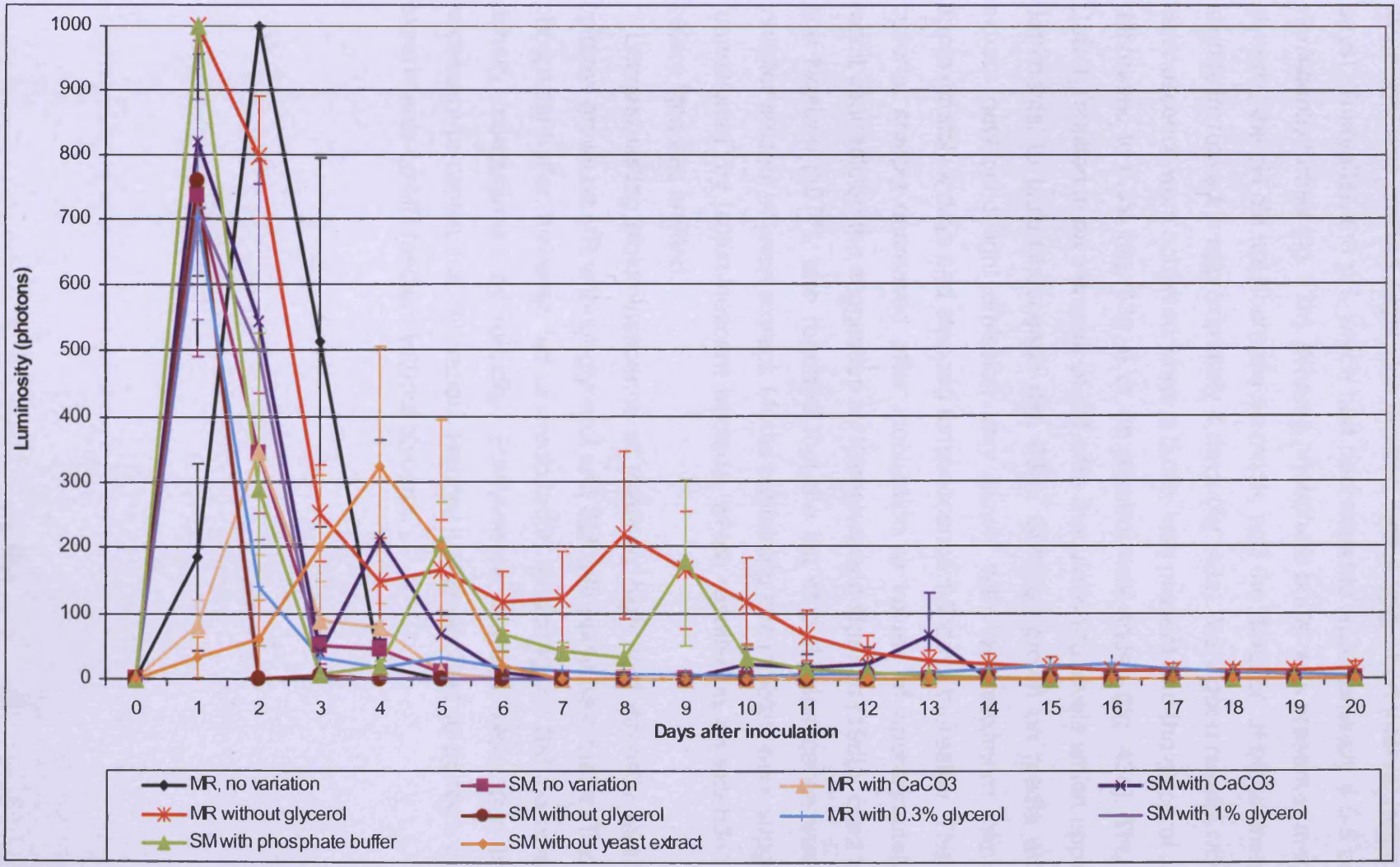


Figure 42 Influence of nutrient broth on *V. fischeri* bioluminescence

V. fischeri cultures grown on nutrient broth with different components varied significantly in bioluminescence intensity. Longest lasting bioluminescence at relatively high light intensity was emitted by cultures grown on MR without glycerol and SM with phosphate buffer Error bars are the standard error of mean of 4 replicates.

Glycerol (1%) in the nutrient medium led to decreased culture pH during the time when light was emitted. Light was emitted over a very short interval (approximately 2 days). Thereafter, the pH, which had decreased to approximately 4.5-5 did not drop significantly further (fig. 43h). When a phosphate buffer was present in media with 1% glycerol, the pH did not decrease as much, and the duration of bioluminescence was slightly increased to approximately 4 days (fig. 43a). Very good results with long-term luminescence were achieved when a buffer was present and the glycerol content was decreased to 0.3% (fig. 43g, i) or no glycerol was added (fig. 43e). The addition of $CaCO_3$ resulted in an increase of pH after inoculation to levels which appeared to be detrimental to bioluminescence (fig. 43d). Cultures grown on media without yeast extract developed light emission very slowly with low maximum intensities after approximately 4 days and stopped luminescence 1-2 days thereafter. The pH of such cultures sharply decreased after inoculation to values of approximately 5.5. This result contradicted the suggestion by Kempner and Hanson (1968), cited by Hastings and Nealson (1977), who reported that the lag of bioluminescence was due to an inhibitor present in yeast extract. Media containing this inhibitor were suggested to be "conditioned" by bioluminescent bacteria, which resulted in an extended lag phase before light was emitted.

Longest lasting bioluminescence at relatively high light intensity was emitted by cultures grown on MR without glycerol and SM with phosphate buffer. The addition of phosphate buffer, however, led to precipitation rendering the SM too opaque for cell density measurement by turbidity. Furthermore, it might block thin bore tubing, necessary in continuous cultivation. Hence, it was decided to perform the following experiments on MR medium without glycerol.

Figure 43 Correlation between pH and *V. fischeri* bioluminescence in batch cultures
The pH on day 0 (blue column) was pH at inoculation. Green columns indicate glowing cultures and black ones those where bioluminescence had ceased. Error bars are the standard error of mean of 3 replica.

3.3.1.2 pH limits for *V. fischeri* bioluminescence

pH appeared to be one of the factors that determine the intensity of *V. fischeri* bioluminescence. To be able to provide stable bioluminescence for continuous culture it was essential to examine the pH range favourable for bioluminescence in more detail. Whether changes in pH were an indirect symptom resulting from the accumulation of toxic metabolic products or if the culture pH itself was the determining factor required investigation. The influence of pH on bioluminescence was tested by adding either 0.5 N HCl or 0.5 M NaOH to a brightly luminescing *V. fischeri* culture until bioluminescence terminated. The pH changes were subsequently reversed by adding the acid / alkali until the initial pH value was reached.

The initial pH of the cultures was approximately 8. The addition of small amounts of acid did not result in visually noticeable changes in luminescence. Light intensities started decreasing below pH 5 and decreased rapidly thereafter to terminate below pH 4. A rapid increase in pH by the addition of NaOH resulted in restoration of bioluminescence, however at a significantly lower light intensity than the initial luminescence. If the cultures were kept for several minutes at approximately pH 4, luminescence could not be restored by the addition of an alkali. Likewise, cultures to which NaOH was added initially showed no decrease in luminescence. The first visually noticeable signs of decreasing luminescence started at pH 9, and were enhanced with further increasing pH. At pH 11.4 luminosity terminated. Again, light emission could be restored only if the highly alkaline conditions were maintained for very short time. Cultures with restored bioluminescence emitted significantly less light than before the experiment. Microscopic examination showed no motility of cells from cultures that could not restore bioluminescence. Transfer of those cultures into fresh medium with subsequent incubation for 24 h did not result in the development of bioluminescence. Cultures whose luminescence was restored by reversing pH changes developed bright luminescence after incubation in fresh medium. This suggested that pH extremes killed *V. fischeri* cells. After a short time at extreme pH not all cells were killed, therefore luminescence could be restored. It is unclear if bioluminescence was lower after pH restoration because the viable count was lower or if the perturbed cells emitted fewer photons. The pH extremes beyond which bioluminescence decreased and the thresholds beyond which light emission terminated were greater than the pH values, which correlated with luminescence

cessation of *V. fischeri* batch cultures determined in chapter 3.3.1.1. This indicated that for batch cultures the pH was an indirect indication for conditions, such as the accumulation of toxic waste products, which affected bioluminescence. In continuous culture these metabolic waste products are diluted by the constant introduction of fresh medium and should therefore not present a problem for the maintenance of bioluminescence.

3.3.1.3 The effect of temperature on *V. fischeri* bioluminescence

Temperature is another factor that has often been reported to influence the quantity of light emitted by bioluminescent bacteria (Correia & Snook, 2004; Waters & Lloyd, 1985). The abundance of various luminescent bacterial species in their natural habitats has been reported to be dependent on the water temperature of the habitat (Hastings & Nealson, 1981). Reichelt and Baumann (1973) reported that none of the *V. fischeri* strains they examined was able to grow at or below 4°C or over 40°C, however, all grew at 25°C, most (92%) were able to grow at 30°C and more than half of the *V. fischeri* strains (67%) could grow up to 35°C. Nishiguchi (2000) found that various *V. fischeri* strains grew significantly faster at 26°C than at 18°C and Fidopiastis et al. (1998) observed that the luminescence of *V. fischeri* was optimal at temperatures above 24°C. Andre (2004) reported that the shortest lag phase and the fastest replication rate of *V. fischeri* were reached at 30°C. However, at this temperature *V. fischeri* was not bioluminescent. The activity of *V. fischeri* luciferase has been reported to be stable up to 30°C; above this the enzyme rapidly loses activity (Rupani et al., 1996).

Effect of incubation temperature on *V. fischeri* batch cultures

Ideal temperature conditions for the continuous cultivation of *V. fischeri* are those that provide a slow growth rate for the economic utilisation of nutrient broth with high intensities of luminescence. Monitoring luminosity and turbidity of *V. fischeri* cultures at different incubation temperatures showed that growth increased with increasing temperature (fig. 44), while bioluminescence was most intense at 20-25°C (fig. 45).

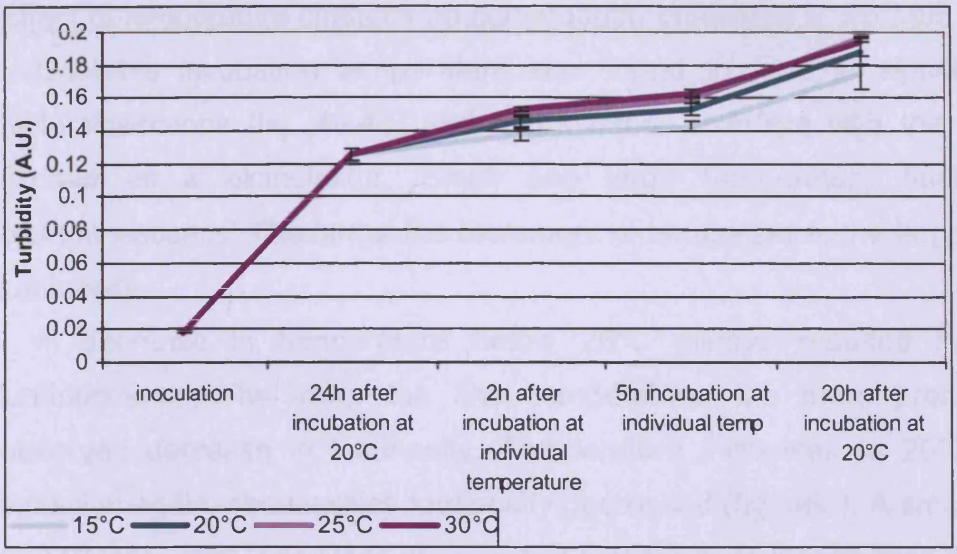


Figure 44 Influence of temperature on cell density of *V. fischeri* batch cultures
The first 24 h after inoculation all cultures were grown at 20°C. Subsequently, the cultures were incubated at 15, 20, 25 or 30°C, which influenced cell density. After 7 h incubation at individual temperatures, they were returned for 20 h to 20°C. Error bars show the standard error of mean for triplicate assays.

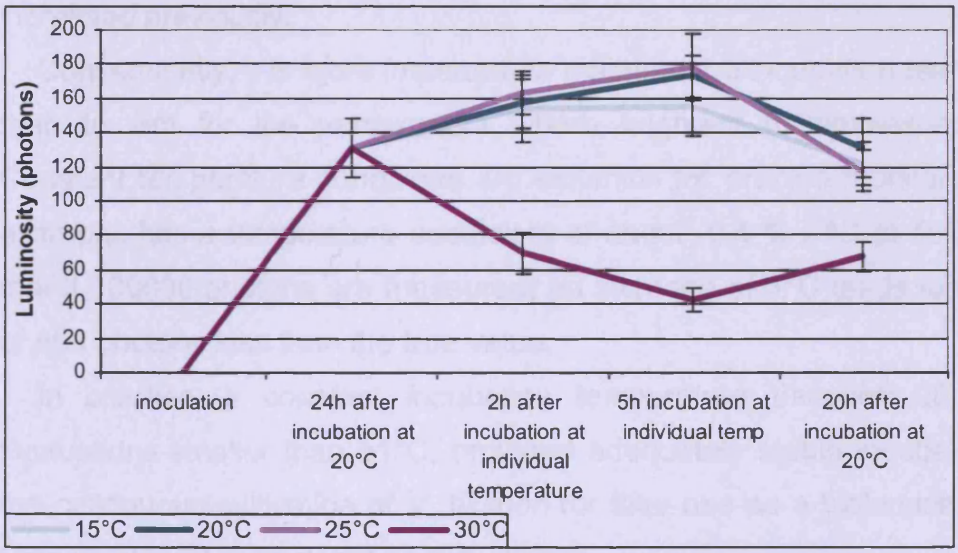


Figure 45 Influence of temperature on bioluminescence of *V. fischeri* batch cultures
The first 24 h after inoculation all cultures were grown at 20°C. Subsequently, the cultures were incubated at 15, 20, 25 or 30°C, which influenced intensity of bioluminescence. After 7 h incubation at individual temperatures, they were returned for 20 h to 20°C. Error bars show the standard error of mean for triplicate assays.

Effect of temperature changes on continuously cultivated *V. fischeri*

Unstable incubation temperature was found to lead to significant changes in bioluminescence (fig. 46-48) and might hence interfere with the application of *V. fischeri* as a bioindicator. Small and large temperature fluctuations affected bioluminescence. The larger the increment of temperature, the larger the deviation in luminosity.

A decrease in temperature below 26°C always resulted in a decrease in luminescence. The lower the final temperature, the more pronounced was the observed decrease in luminosity. Temperature increases to 26°C enhanced light emission again, above which luminosity decreased (fig. 46a). A small change from 26 to 27°C (fig. 46a) led to less pronounced luminosity reduction than that due to a large temperature shift above 27°C (fig. 46b). Stable bioluminescence before the temperature change could be restored when the temperature was brought back to the initial value (fig. 46c), regardless of whether the temperature had been decreased or increased previously.

Consequently, it is more important to maintain the incubation temperature constant than to aim for the temperature where brightest bioluminescence is observed. Constant temperature conditions are essential for precise monitoring. The PMT, for example, has a temperature coefficient of about -0.4 % / °C at 490 nm. This means that if 100000 photons are measured, an increase of 1°C leads to the measurement of 400 photons less than the true value.

In practice, a constant incubation temperature between 20 and 26°C, with fluctuations smaller than ±1°C, provided adequately stable incubation conditions for the continuous cultivation of *V. fischeri* for their use as a bioluminescence biosensor for assessment of bacterial perturbation.

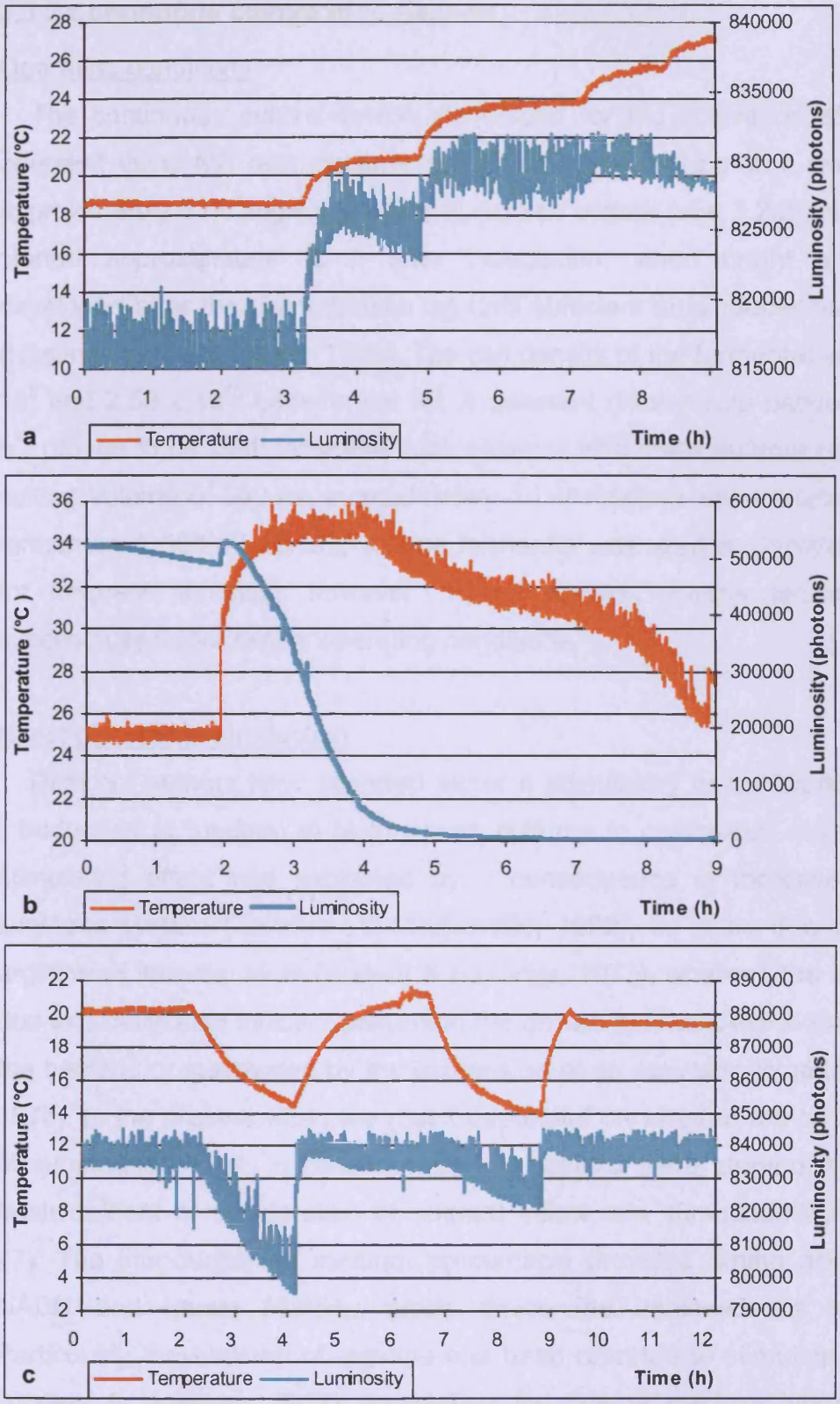


Figure 46 Influence of temperature variations in continuous cultivation of *V. fischeri*. Small increases in temperature between 18 and 24°C resulted in increased bioluminescence. Above 24°C bioluminescence decreased (a). Large increase in temperature above 25°C resulted in large decrease in bioluminescence (b). Decreases in bioluminescence could be restored with the restoration of favourable temperature conditions. The temperature variations were achieved by placing the fermenter inside an incubator.

3.3.2 Continuous culture of *V. fischeri*

Operating conditions

The continuous culture device, developed for the cultivation of *V. fischeri,* was operated using MR new medium (see 3.2.1) without glycerol. It was inoculated with approximately 5 ml brightly glowing *V. fischeri* culture (see 3.2.2). Nutrient inflow was started approximately 12 h after inoculation, when bright luminescence had developed after the characteristic lag until sufficient autoinducer had been produced (Hastings and Greenberg, 1999). The cell density of the fermenter was between 9.5 x 10^9 and 2.53 x 10^{10} bacteria per ml. A constant dilution rate between 0.08 and 0.09 h^{-1} proved to be ideal for stable luminescence with small nutrient consumption. For a culture volume of 500 ml, approximately 1 l of medium was introduced daily into the fermenter. A 500 ml working volume fermenter was used to provide sufficient culture for frequent sampling, however, 10 ml working volume fermenter was tested successfully under similar operating conditions.

Effect of nutrient introduction

Different authors have reported either a stimulatory or an inhibitory effect on the introduction of medium to bioluminous cultures in continuous culture systems. The stimulating effect was explained by a consequence of increased $FMNH_2$ in the luminous system (Zavoruev & Mezhevikin, 1982), or to be due to the addition of arginine or its precursors (Waters & Hastings, 1977), whereas the inhibition might be due to a dialyzable inhibitor present in the growth medium, which may be removed by the bacteria or inactivated by the expression of an activator (Wardley-Smith & White, 1975). In the present work, the result depended on whether the culture was depleted of substrates or not. In cases where the bacteria were starved, the introduction of fresh nutrient or acceleration of nutrient inflow rate stimulated light production (fig. 47). The introduction of medium presumably provided amino acids as sources of NADH and hence $FMNH_2$, which drives the luminescence system (fig. 38). Particularly the addition of arginine has been reported to stimulate bioluminescence (Waters & Hastings, 1977). Increasing the rate of medium influx after cultivation under favourable conditions resulted in decreased luminescence. The increased inflow of medium caused a decline in biomass, which was observable in a decrease in turbidity. As fewer cells produce fewer photons, the overall luminosity was lower.

With stable nutrient input the pH remained between 7.8 and 8, which was within the pH values where bioluminescence was not affected. This showed that the constant inflow of fresh medium and outflow of culture was adequate to prevent the accumulation of metabolic by-products, which may alter the pH.

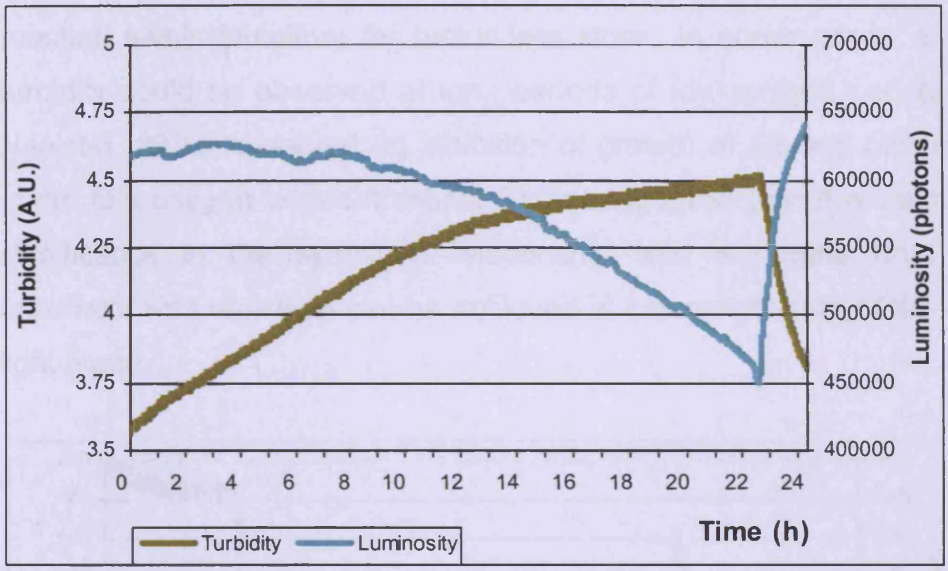


Figure 47 Influence of nutrient supply in continuous cultivation of *V. fischeri*
Cessation of nutrient input (at time 0) led to an increase in turbidity and a decrease in luminescence. Introduction of medium after approximately 22 h reversed the changes.

Effect of air introduction

The arrest of air supply resulted less light emission (fig. 48). Likewise, starting or increasing the air inflow after oxygen depletion resulted in a sharp increase in luminosity. Sometimes a characteristic "overshoot" was observed. This "overshoot" phenomenon, during which very high light emission was transiently observed, has been reported to be due to the accumulation of reduced cofactors NAD(P)H and $FMNH_2$ of the luminous system under anaerobic conditions. As soon as oxygen is available, high light intensities can be produced until the excess substrates have been utilised (Hastings, 1983; Hastings & Nealson, 1977). The dependency of bioluminescence on oxygen concentration has been established very early by Sir Robert Boyle in 1667 (Hastings, 1983) and luminescent bacteria have long been known to be able to emit light at oxygen concentrations of as low as 0.093 Pa (Harvey & Morrison, 1923). However, there is an upper limit of oxygen concentration, above which it becomes inhibitory for the luminous system. Ulitzur et al. (1981) reported that bioluminescence of cells grown at lower oxygen concentration was

greater than that of cells grown in a 100% oxygen environment, although the total luciferase content was the same under both conditions. They further demonstrated that intracellular levels of reduced flavin ($FMNH_2$) and long chain aldehyde were higher at lower oxygen concentrations. Nevertheless, the dissolved concentrations of oxygen in the culture, supplied by 600 ml ambient air (20.9% O_2) per min, never reached levels inhibitory for bioluminescence. In some cases a slight decrease in turbidity could be observed at long periods of low oxygen concentration. Ruby and Nealson (1976) observed an inhibition of growth of several bioluminescent bacteria under low oxygen concentrations. They suggested that this might be of ecological significance in the symbiotic relationship with a marine host organism, where maximum light emission can be achieved at low growth rate of the bacteria within the light organ.

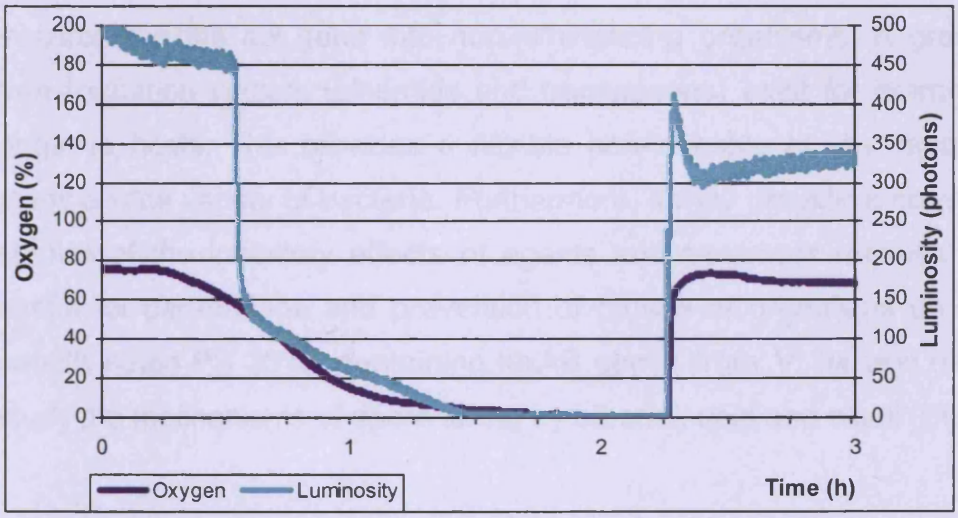


Figure 48 Influence of air supply in continuous cultivation of *V. fischeri*
After stopping the air supply (at 30 min), oxygen concentration (% of atmospheric oxygen) and luminosity decreased. The restoration of air supply after 2 h and 15 min resulted in a sharp increase in luminosity and oxygen concentration with a characteristic overshoot of luminescence after oxygen depletion.

3.3.3 Conclusion

A simple nutrient medium was developed that provided bright and stable bioluminescence of *V. fischeri* NRRL-B-11177. Stable luminosity of *V. fischeri* NRRL-B-11177 in continuous culture can be achieved by operating the fermenter at a dilution rate of 0.08–0.09 h^{-1}, an air supply of 600 ml/min, a stirring rate of 300–350 rpm and an incubation temperature at a constant value within the range of 20 and 26°C. As the pH value of *V. fischeri* cells grown in continuous culture with the

indicated growth medium was very stable, pH monitoring and control is not an essential component of the continuous culture device. Under these conditions, continuous culture of *V. fischeri* NRRL-B-11177 could be maintained for up to 8 weeks. After several weeks, usually after 3–4 weeks, the culture started to decrease light emission to very low light intensities, possibly due to the selection of a mutant. It has been demonstrated that non-luminous luxA mutants of *V. harveyi* may out-compete wildtype *V. harveyi* when cultivated together without selective pressure for luminescence (Czyz et al., 2003).

This continuous culture device is a sensitive system for monitoring the kinetics of perturbation of bacterial metabolism. It has the potential for the investigation of a variety of physical and chemical agents. At this stage, where the functionality of the system had to be proven, it was operated with *V. fischeri* cultures. However, tests with luminescent biosensors may be extended to other organisms by genetically transforming the lux gene into non-luminescing organisms. A growing selection of transformation vectors (plasmids and transposons) exist for gram-positive or gram-negative hosts. This provides a flexible non-invasive *in vivo* monitoring system to study a wide variety of bacteria. Furthermore, it may provide a convenient system for studies of the inhibitory effects of agents and treatment regimes that might prove useful for perturbation and prevention of growth of organisms on stone. In fact, *B. subtilis* strain PS 3379, containing luxAB genes from *V. harveyi* have been used to study the mechanisms of spore killing by ethanol, acid and alkali (Setlow et al., 2001).

Chapter 4
Antimicrobial treatments and their assessment

4.1 Introduction to antimicrobial measures for cultural heritage objects

Antimicrobial measures may become necessary for various reasons. While aesthetical motives must be evaluated extremely critically, the removal of a specific microbial population or the entire microflora may become important to protect the object or uncontaminated artefacts in its close vicinity from irreversible damage.

Actions against microbial growth can be divided into four major categories: (1) alteration of environmental conditions; (2) mechanical removal of biodeteriogens; (3) chemical eradication methods (biocides), and (4) physical eradication methods, which will be discussed in chapter 5. Biological control against stone decay with antibiotics, peptides, parasitic organisms that feed on others, or microorganisms that kill insects (e.g. *Bacillus thuringensis* that produces a toxin that kills a large variety of insects, Ramírez et al., 2005) have been used in medicine and agriculture, however, its application on cultural heritage objects is still some distance in the future.

The removal of the microbial community from a surface is an intervention that must be carefully evaluated. It may give rise to a new succession of microorganisms, which in some cases might be more damaging than the original microbial populations (May, 2003) and the inhibition of specific groups of microorganisms may favour the growth of others (Warscheid & Braams, 2000; Frey et al., 1993). Furthermore, physical and chemical eradication treatments may damage the artefact materials (Tretiach et al., 2007; Blazquez et al., 2000; Frey et al., 1993; Webster et al., 1992). The approach to control biodeterioration must be a polyphasic, interdisciplinary one that is integrated into the entire conservation concept. It must consider the history and condition of the artefact as well as physical and/or chemical effects on the material.

4.1.1 Alteration of environmental conditions

Biodeterioration can usually not be prevented completely; it is an important ubiquitous process. However, if the living conditions for microorganisms are made as hostile as possible, the degradation process can be limited to an insignificant level. Approaches towards "good housekeeping" and climate control should be the first step in the control of biodeterioration. Good housekeeping implies a limitation of possible

nutrients for microorganisms as well as providing environmental conditions (e.g. temperature, humidity and light) that are unfavourable for microbial growth. In an indoor environment, such as a museum or an archive, this includes regular dusting (without the use of water to provide stable RH conditions) and restrictions to eating and drinking for visitors and staff. Further, good housekeeping includes building maintenance (e.g. avoidance of leaks, maintenance of properly functioning heating and ventilation systems).

For outdoor monuments the approach is more difficult and often impossible. The possibilities of altering the environmental conditions (e.g. exposure to rain and sunlight) have to be evaluated from case to case (fig. 49).

Figure 49 Protective roof on a north wall at the Mayan pyramid of Edzna, Mexico
Directly underneath the roof (left), where rain protection was the best, very little microbial biofilm developed. Underneath the final, less protected metre of the roof (middle) more biofilm formation could be noticed and in completely unprotected areas (right) severe development of biofilm was visible.

4.1.2 Mechanical removal of the causative microorganisms

Generally, the approach of removing of microorganisms rather than their killing without any cleaning process is to be preferred. Dead microorganisms may still pose a health hazard and aesthetic compromises. Furthermore, excreted microbial substances can continue damaging the artefact after cell death and dead microorganisms may serve as nutrients for others. Mechanical removal of

biodeteriogens, performed by the conservator, may entail the use of tools including scalpels, scrapers, brushes, air abrasion or vacuum cleaners (Caneva et al., 1991; Tiano, 2002). However, mechanical removal cannot eliminate all microorganisms. Therefore, the mechanical removal of microbial structures should be combined with another antimicrobial strategy; preferably with the alteration of the environmental conditions to prevent further proliferation of the remaining microflora.

4.1.3 Chemical eradication methods

"Biocide" is a general term for all toxic chemicals that can kill living organisms. Biocides for microorganisms can be more or less specific, killing only a certain microbial group or the entire microbial flora. However, most biocides cannot discriminate between microorganisms detrimental to artefacts and non-target organisms, e.g. soil microbes.

Biocides often exhibit harmful effects on the stone e.g. discolouration, oxidation/reduction of stone compounds, alteration of the surface properties of the stone (e.g. "greasy" films) and salt formation with subsequent crystallisation upon drying, leading to exfoliation (Blazquez et al. 2000; Warscheid & Braams, 2000; Kumar & Kumar, 1999; Cameron et al., 1997; Frey et al., 1993; Caneva et al., 1991). The ecotoxicity of commercial biocides may make them poor candidates for use in outdoor environments and many countries have prohibited the use of some of the previously most common (and effective) ones. Certain biocides, e.g. those containing carbon and nitrogen, may even serve as nutrients for resistant surviving or newly attaching microorganisms, particularly after the biocide has undergone decomposition and degraded into less harmful components (Warscheid & Braams, 2000).

When testing the minimal biocide dose needed for the treatment of an outdoor monument, it is important to consider that there is a significant discrepancy between their effect on isolated organisms grown on laboratory media and those grown in environmental biofilms (Warscheid, 2003; Von Rege & Sand, 1998). Biofilms provide a physical barrier that protects microorganisms from detrimental substances, such as biocides (see 1.5.1). Therefore, the dose needed for a microbial community on outdoor stone monuments might be much higher than the concentration needed to successfully kill isolated organisms growing on agar plates. The dose further depends on the absorptive capacity of the substrate and environmental condition during the application (e.g. temperature, RH, air flow).

If a biocidal treatment is unavoidable, the aim must be clearly defined: (1) short-term control during temporary unfavourable conditions (e.g. humid archaeological objects directly after excavation [Warscheid, 2003]; humidification during a conservation treatment); (2) long-term control for artefacts, where environmental conditions cannot be significantly improved. The desired degree of reduction of the viable microbial community needs to be defined (complete sterilisation versus diminishing the population to a level that was defined by the conservator and microbiologist together as acceptable). Appropriate methods to assess the biocidal efficiency have to be found, as this is a significant source of error for the interpretation of the success.

Despite all the disadvantages of chemical biocides, they have been extensively used on cultural heritage monuments. Biocides may be applied as an aerosol, with brushes, by injection, by immersion, or as a paste. They range from simple inorganic compounds like sodium or calcium hypochlorite to organometallic compounds; complex organic compounds such as quaternary ammonium, aromatic compounds such as formaldehyde, phenol and its derivates, urea derivates, halogenated compounds such as chlorine and iodine; pyridine derivatives, nitrogen containing compounds and isothiazol derivates (Frey et al., 1993; Warscheid, 2000; Nugari & Salvadori, 2003a; Tiano, 2002; May, 2003). Alternative substances include metallic salts and oxides (e.g. carbonates and oxides of copper and zinc); acetic or salicylic acid; borax; p-hydroxybenzoic acid (PHB) esters and ethereal oils. These substances are of lower toxicity and thus usually require longer application times (Warscheid, 2000). However, Gorbushina et al. (2003) reported that in a study of 40 tested commercially available biocides, none of acceptable toxicity level to humans or the environment demonstrated sufficient success in eliminating damaging microbial biofilms, which demonstrates the urgent need for research towards alternative antimicrobial methods.

Quaternary ammonium compounds (quats) have been the most frequently used biocides in cultural heritage preservation, as they have high efficacy and bear relatively small hazard to the substrate (Nugari & Salvadori, 2003a). Molecules of quaternary ammonium salts contain a hydrophobic and a hydrophilic group. They act as cationic surfactants, reduce surface tension at interfaces and are attracted to negatively charged surfaces. Quats may disrupt the microbial cytoplasmic membrane (i.e. disruption of nutrient flow and discharge of waste) and denature proteins (Buck,

2001; Noecker, 2001; Warscheid, 2000; Falbe & Regitz, 1995). They are effective against a wide range of microorganisms including species of bacteria, fungi and algae. Their effect on lichens is controversial (Nugari & Salvadori, 2003a). Quats have been reported to potentially cause corrosion to mineral iron in the stone matrix Warscheid & Braams, 2000). Detailed information on differences in various kinds of quats can be found in Buck (2001).

Organometallic compounds particularly tin organic compounds (TOC) have been used frequently against microbial biodeterioration. They contain one or more direct linkages between a carbon atom and a metal atom. They inhibit the energy metabolism of microorganisms. Different TOCs vary in the degree of toxicity. Tetraorganotins have relatively low toxicity to humans, however they can decompose into triorganotin, which are the most toxic TOCs. Diorganotins have low toxicity and are not sufficiently effective against microorganisms and monoorganotins have no effect as microbial biocides. TOCs are strongly adsorbed by soil, which may cause an environmental hazard in outdoor applications (Department of the Environment and Heritage, Australia, 2005). They have long-term effectiveness against bacteria, fungi and algae. However, they have been reported to have low stability to UV light (Warscheid, 2000). An application of TOCs on outdoor monuments is therefore questionable.

Metal oxides and salts, such as carbonates may inhibit microbial growth. Copper and zinc oxides and salts have been used on outdoor stone monuments. Their action is biostatic rather than biocidal. Both metals, once taken up by the microbial cell form complexes with proteins and nucleic acids, which disrupts the cell function. When present in excess within the cell, they lead to the limitation of micronutrients available to the cell and their subsequent deficiency.

Aldehydes bind to the outer cell layers resulting in an inhibitory effect on RNA, DNA and protein synthesis. Glutaraldehyde has been used as a biocide as well as the odourless alternative ortho-phthalaldehyde, which is effective over a wide pH range (Buck, 2001). Glutaraldehyde has a broad spectrum and rapid activity against microorganisms. It has largely replaced formaldehyde due to its higher sporicidal action and lower toxicity. (Gorman et al., 1980).

Isothiazoline derivates are known to be efficient against a broad range of microorganisms (Curri, 1979). They react with amino acids and other reactive groups

within the cells. There is little known about uptake mechanisms or their precise targets (T. Heuer, Troy Chemie GmbH, personal communication, 2005).

Halogens such as iodine and chlorine are powerful oxidising agents. Iodine irreversibly changes the secondary and tertiary structure of proteins (Frey et al., 1993). Chlorine forms hypochlorous acid in aqueous solution (Buck, 2001).

Phenolic compounds disrupt the plasma membrane and inactivate proteins and nucleic acids (Frey et al., 1993).

Alcohols, most commonly ethanol and isopropanol, have a broad variety of toxic effects and act therefore non-specifically against a wide range of organisms. They may denature and coagulate proteins, disrupt the cytoplasmic membrane, dissolve lipids and cause cell lysis (Buck, 2001).

Peroxygen compounds, such as hydrogen peroxide have great activity against a wide range of microorganisms. Generally, they are more effective against gram-negative bacteria than gram-positive ones and anaerobes are particularly sensitive as they do not usually possess catalase. They are very strong oxidants and kill microorganisms by peroxidative attack of membrane lipids or DNA (Buck, 2001). They break down into oxygen and water and can hence be considered environmentally friendly while highly effective against target organisms. However, as strong oxidants with bleaching effects, they have to be tested carefully before they can be used on cultural heritage objects.

Anoxia treatments are a very gentle method to effectively kill insects. By the introduction of nitrogen or argon into well-sealed containers at oxygen levels lower than 0.05% insects are effectively killed, however, most microorganisms are not sensitive and this treatment may sometimes even lead to accelerated growth (Pinzari et al., 2003).

Surveying the literature of case studies on antimicrobial treatments of outdoor stone monuments, it becomes clear that the results are not comparable and sometimes even contradictory (Tretiach et al., 2007; Gorbushina et al., 2003; Wessel, 2003; Ascaso et al, 2002; Tomaselli et al., 2002; Blazquez et al., 2000; Pinck et al., 2000; Frey et al., 1993; Strzelczyk, 1981; Curri, 1979). The tested organisms are often described as if they were giant homogenous groups, such as "the bacteria" or "the phototrophs", which certainly cannot portray their individual character nor their sensitivities against specific treatment methods. The tests are often carried out under

laboratory conditions, which cannot entirely imitate environmental conditions (Blazquez et al., 2000; Pinck et al., 2000). Ascaso et al. (2002) emphasised the importance of performing *in situ* tests. However, *in situ* experiments on artefacts are not reproducible, as every artefact is unique and environmental growth conditions cannot be reproduced for replicate tests.

4.1.4 Aims of antimicrobial treatments and their assessment

The aim of this section was to investigate a variety of methods to evaluate chemical antimicrobial treatments against *V. fischeri*. *V. fischeri* was selected, as mentioned in chapter 3, as a model organisms to develop synergistic eradication methods. It may not be the most appropriate organism to study biocides for outdoor stone monuments. Therefore, the efficiency of the antimicrobial agent was not considered in the evaluation process. The goal was to determine the reliability of the protocols to evaluate the success of an antimicrobial treatment.

4.2 Materials and methods

4.2.1 Biocides

The following biocides were selected to test a variety of evaluation methods for antimicrobial treatments against *V. fischeri:*

Preventol R 50 (Lanexess, Newbury, UK) contains a 48–52% solution of alkyl benzyl dimethyl ammonium chloride (common name: benzalkonium chloride) in water. It has a broad activity spectrum against filamentous fungi, yeasts, bacteria, algae and lichens. The supplier recommends a 1.5–3% solution for substrate treatment.

Parmetol DF12 (Schülke & Mayr UK Ltd., Sheffield, UK) contains 0.8–1% 2-Octyl-2H-isothiazol-3-one, 0.2–0.3% mixture of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one, 9–11% Urea,N,N'-bis-(hydroxymethyl)-reaction products with 2-(2-butoxyethoxy)-ethanol, ethylene glycol and formaldehyde, 10% 2-(2-butoxyethoxy)-ethanol. The supplier recommends a dosage of 2–5% for surface sanitation.

Troysan S97 (Troy Chemical Corporation, Macclesfield, UK) was called Mergal S97 until 2004. It contains 50–100% 1,2-propanediol; 7–10% 2-octyl-2H-isothiazol-3-one; 3–5% benzene sulphonic acid, 4-C10-13-sec-alkyl derivates / dodecylbenzene

sulphonic acid 2-benzimidazole carbamic acid, methyl ester; 0.25–0.5% 5-chloro-2-methyl-3(2H)isothiathiazolone mixed with 2-methyl-3(2H)-isothiazolone. It is effective against filamentous fungi, yeasts and bacteria. Due to its low water solubility, it provides durable protection. Troysan S97 is recommended for exterior systems in a final concentration of 1–2 %.

Mergal K14 (Troy Chemical Corporation, Macclesfield, UK) is a combination of chlormethylisothiazolinone / methylisothiazolinone and a formaldehyde releasing agent (7–10% ethylenedioxy dimethanol; 0.5–1% 5-chloro-2-methyl-3(2H)isothiathiazolone mixed with 2-methyl-3(2H)-isothiazolone). For aqueous in-can applications the manufacturer recommends a final concentration of 0.1–0.4%; concentrations for solid surfaces are not specified.

Ucarcide Tm 50 (The Dow Chemical Company, London, UK) contains 50% glutaraldehyde. A 2% aqueous alkaline solution of glutaraldehyde has been reported to have a killing time of <1 min for vegetative bacteria and <3 h for bacterial spores.

Furthermore, ethanol and hydrogen peroxide (H_2O_2) were tested for their antimicrobial efficiency. The positive control was treated with sterile water and the negative control was treated with sterile water and subsequently autoclaved.
The above mentioned substances were tested in the following concentrations:

Table 4
Concentrations of biocides used for the tests.

Biocide	Biocide concentration	Active ingredient(s) concentration	Final biocide concentration	Final active ingredient(s) concentration
Preventol R50	4%	2%	0.4%	0.2%
	2%	1%	0.2%	0.1%
	1%	0.5%	0.1%	0.05%
	0.5%	0.25%	0.05%	0.025%
	0.25%	0.125%	0.025%	0.0125%
	0.125%	0.0625%	0.0125%	0.00625%
	0.0625%	0.013125%	0.00625%	0.0013125%
	0.013125%	0.0065625%	0.0013125%	0.00065625%
Parmetol DF12	100%	21%	10%	0.21%
	50%	10.5%	5%	1.05%
	25%	5.25%	2.5%	0.525%

	12.5%	2.625%	1.25%	0.2625%
	6.25%	1.3125%	0.625%	0.13125%
	3.125%	0.65625%	0.3125%	0.065625%
	1.5625%	0.328125%	0.15625%	0.0328125%
	0.78125%	0.1640625%	0.078125%	0.01640625%
Troysan S97	100%	13–20%	10%	1.3 – 2%
	50%	6.5-10%	5%	0.65-1%
	25%	3.25-5%	2.5%	0.325-0.5%
	12.5%	1.625-2.5%	1.25%	0.1625-0.25%
	6.25%	0.813-1.25%	0.625%	0.081-0.125%
	3.125%	0.406-0.625%	0.3125%	0.041-0.063%
	1.5625%	0.203-0.313%	0.15625%	0.020-0.031%
	0.78125%	0.102-0.156%	0.078125%	0.010-0.016%
	0.390625	0.051-0.078%	0.0390625	0.005-0.008%
	0.1953125	0.025-0.039%	0.01953125	0.003-0.004%
Mergal K14	100%	10%	10%	1%
	50%	5%	5%	0.5%
	25%	2.5%	2.5%	0.25%
	12.5%	1.25%	1.25%	0.125%
	6.25%	0.625%	0.625%	0.0625%
	3.125%	0.3125%	0.3125%	0.03125%
	1.5625%	0.15625%	0.15625%	0.015625%
	0.78125%	0.078125%	0.078125%	0.0078125%
Ethanol	100%	100%	10%	10%
	70%	70%	7%	7%
	50%	50%	5%	5%
H_2O_2	10M	10M	1M	1M
	1M	1M	100mM	100mM
	100mM	100mM	10mM	10mM
Positive control	100% sterile H_2O	100%	10%	10%
Negative control	100% sterile H_2O, autoclaved	100%	10%	10%

4.2.2 Device to determine the minimum inhibitory biocide concentration

The first attempt to determine the approximate minimum inhibitory concentrations for several biocides on *V. fischeri* cultures was by continuous measurement of the light emission under steadily decreasing biocide concentration. The biocide-treated culture was mixed with a constant inflow of untreated, luminescing *V. fischeri* culture from a fermenter. An outflow was attached to retain the volume constant at 10 ml. Continuous light measurement recorded any increase in light emission as an effect of the dilution of the biocide. At the time when light emission started to increase, the biocide concentration was expected to be the MIC for *V. fischeri*. Similarly, the concentration when light emission was fully restored to the value before biocide treatment, was expected to be the minimum concentration that perturbed *V. fischeri*.

4.2.3 Light emission of *V. fischeri* during biocide introduction

A brightly luminescing *V. fischeri* culture (9 ml) was sampled from the fermenter and placed in a sterile glass container, which was viewed through a fibre optic cable with a PMT. The culture was continuously stirred with a magnetic stirrer. The introduction of 1 ml biocide (table 4) was performed in a darkroom. Recording of light emission was started 30 s before biocide introduction and stopped 90 s after biocide introduction. Light emission signals were recorded in 50 ms increments as described in 3.2.9.

4.2.4 Plate count of biocide-treated *V. fischeri* cultures

Biocide-treated *V. fischeri* cultures (see 4.2.3) were diluted with 2.5% NaCl in a 10-fold dilution series between 10^{-1} and 10^{-8}. To avoid an underestimation of bacterial cell numbers due to the aggregation of organism, all bacterial suspensions were vortexed before each dilution step.

The diluted cultures (15 µl) were plated in triplicates on Petri dishes containing MR new broth with 15% agar, according to the Miles and Misra method (Miles & Misra, 1938). CFUs were counted after 2 and 3 days and checked for bioluminescence to rule out contamination.

4.2.5 Subculturing of biocide-treated *V. fischeri* cultures

Brightly luminescing *V. fischeri* culture, sampled from the fermenter, was mixed in a 9:1 ratio with various concentrations of different biocides (table 4). The biocide was allowed to react on the culture for 1 h. After that, a 1 ml aliquot was transferred into a 100 ml conical flask containing 19 ml sterile MR new broth. Positive control samples, consisting of untreated *V. fischeri* cultures were subcultured in the same fashion. Incubation was performed for 5 days as described in 3.2.2. The assessment of the growth of subcultured biocide-treated *V. fischeri* was performed by turbidity and light emission. Turbidity was measured directly after subculturing and every 24 h thereafter for 5 days by the same method as described in 3.2.3 with one modification: 1 ml of biocide-treated, subcultured *V. fischeri* culture was diluted with 2 ml MR new broth to prevent over-saturation of the instrument. Light emission of biocide-treated, subcultured *V. fischeri* was measured by placing the flask containing the culture in front of a PMT inside a light-tight box. The light intensity was measured with the sample program for H7467 series Version 1.02 (Hamamatsu Photonics K.K.).

4.2.6 Visual evaluation of biocide-treated *V. fischeri* at X1000 magnification

4.2.6.1 Light microscopy

Biocide-treated *V. fischeri* cultures (see 4.2.3) were examined in transmission light at x1000 magnification. The percentage of motile and non-motile bacteria was recorded for 100 cells. To avoid a bias during the assessment the samples received a number, the actual sample (biocide-type, concentration) was identified only after the assessment.

4.2.6.2 Epifluorescent microscopy employing fluorophores

Biocide-treated *V. fischeri* cultures (see 4.2.3) were assessed for viability by employing an epifluorescent microscope with a mercury lamp for excitation and a broad emission (full visible range). Both, planktonic and washed (1.5 ml culture in Eppendorf microtubes were centrifuged for 10 min at high speed [exact speed was not specified by supplier], the pellet was re-suspended in sterile 2.5% NaCl solution) biocide-treated *V. fischeri* cultures were tested for UV stimulated fluorescence

differences for live and dead cells. The cultures were mixed on a microscope slide with the appropriate fluorophore and incubated according to recommendations of the supplier before assessment at x1000.

The following fluorophores were tested:

Rhodamine-123 (Molecular Probes, Invitrogen Ltd. Paisly, UK) is a cationic dye that can enter a viable cell if it performs active metabolism and a membrane potential has established. The uptake is rapid, within a few minutes.

$DiBac_4(3)$ (Molecular Probes, Invitrogen Ltd. Paisly, UK), an anionic oxanol III dye is based on the same principle of indicating active metabolism through the presence of a membrane potential. However, since $DiBac_4(3)$ is negatively charged it can only enter and subsequently stain the cell if there is no membrane potential indicating dead or possibly dormant cells. It is non-toxic to the cell.

Acridine orange (AO) (Molecular Probes, Invitrogen Ltd. Paisly, UK) is one of the most commonly used fluorescent dyes. When it complexes with single-stranded nucleic acid it fluoresces red to orange (650 nm) and green (525 nm) when bound to double-stranded nucleic acid. As the amount of RNA is a measure of cell activity, those fluorescing red / orange are considered active, while green fluorescing cells are assumed to be inactive or dead (McFeters, 1995).

Fluorescein diacetate (FDA, Sigma) was one of the first probes used to indicate cell viability (see 2.1.5). When excited with light at 495 nm it emits fluorescence at 520 nm.

Propidium iodide (PI, Sigma) stains by intercalating without sequence preference into DNA and RNA. It has a double positive charge, which generally excludes it from viable cells with intact cell membranes (Maraha et al., 2004). However, stained cells may be either dead or damaged (Maraha et al., 2004) and those that did not let PI enter may not necessarily be viable (Shapiro, 2004). Once the dye has bound to nucleic acid, its fluorescence is enhanced approximately 10-fold, the excitation maximum is shifted 30–40 nm to the red and the emission maximum approximately 15 nm to the blue (Molecular Probes, 2006).

4.3 Results and discussion

4.3.1 Device to determine the minimum inhibitory biocide concentration

This device employed biocide-treated *V. fischeri* cultures, which were constantly diluted with untreated, brightly luminescing *V. fischeri* cultures. The level of toxicity was determined by the intensity of the restored bioluminescence. The method was based on precise measurement of the concentration of the biocide, which was only possible by accurate quantification of the volume within the reaction chamber and the amount of untreated *V. fischeri* culture that was used to dilute the sample. However, the introduction of the biocide resulted in foam development within the sample, either as a direct result of the biocide (in the case of a surfactant, such as Preventol R50) or as a result of cell lysis due to the lethal effect of the biocide. Hence, the volume of the sample within the reaction chamber was altered, rendering the system useless. An antifoam agent could not be used as it might have an influence on the toxicity of the biocide. Therefore, this method could not be used as an automated system to determine the minimum inhibitory and minimum perturbing concentration of *V. fischeri* or other bioluminescent bacteria.

4.3.2 Light emission of *V. fischeri* cultures during biocide introduction

The introduction of a biocidal solution into a luminescing *V. fischeri* culture led to an initial strong increase in light emission, with a high peak of approximately 200-300% of the initial luminosity (fig. 50a-f). This luminosity overshoot terminated after 1-2 s. Higher concentrations of toxic substances in general resulted in a lower luminosity overshoot than those of lower toxicity or water. This could clearly be seen for Preventol R50, Parmetol DF12 and ethanol (fig. 50c-e), however Mergal K14, Troysan S97 and hydrogen peroxide did not follow this trend as strictly. The reason for this characteristic luminosity peak remains unclear. The bacterial culture was constantly stirred during the whole treatment, therefore an overshoot due to oxygen supply after its depletion (fig. 48) can be ruled out. Furthermore, similar luminosity peaks could be observed upon the sudden perturbation of *V. fischeri* cultures, such as temperature changes, increase or decrease of flow velocity of cultures in silicon tubing, changes in pH, etc (fig. 46b).

The intensity of light emission after the initial peak depended on the toxicity of the inserted substance. In the case of a non-toxic substance, such as H_2O, light emission remained for several minutes (approximately 2-5 min) at a higher intensity than before its introduction and decreased subsequently to approximately the same value as before the introduction (data not shown). The decrease of luminosity of samples with toxic substances followed an exponential curve with an initial rapid decline. Luminosity declined more slowly after the introduction of solutions of low toxicity and levelled out to a value above that of samples with highly toxic anti-microbial agents. After a highly toxic treatment, the gradient of the luminosity decline was very steep and the final value was close to 0. The final value, 90 s after the introduction, reached lower and lower with rising toxicity of the introduced substance. However, total inhibition of light emission was not always a sign that the organisms had become non-viable (data not shown).

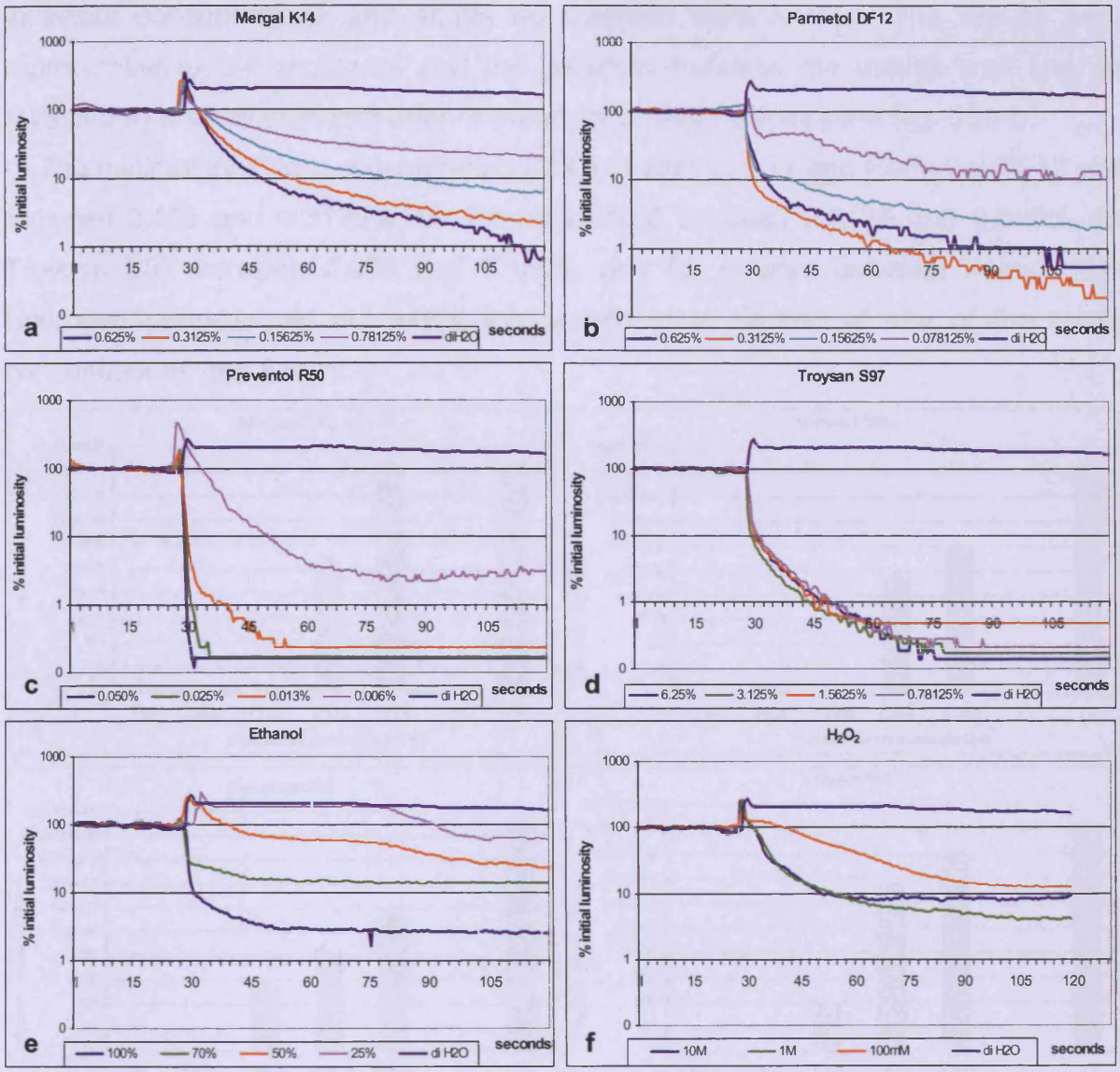


Figure 50 Dynamics of bioluminescence of *V. fischeri* during biocide introduction
Stable bioluminescence briefly increased (overshoot) and subsequently decreased upon the introduction of various concentrations of Mergal K14 (a), Parmetol Df12 (b), Preventol R50 (c), Troysan S97 (d), ethanol (e) and hydrogen peroxide (f). Each line represents the mean of triplicates.

4.3.3 Plate counts of biocide-treated *V. fischeri* cultures

The direct plate count is a sufficiently accurate method for the quantification of surviving microorganisms if their cultivation requirements (nutrient composition of the medium, incubation temperature etc.) are known. For unknown organisms, however, this method is inappropriate as the same cultivation-related problems emerge as discussed in 2.1.2.2.2.

Direct plate count of surviving *V. fischeri* after biocide treatment revealed lower numbers of surviving organisms with increasing biocide concentrations. At minimal

inhibitory concentrations and above no colonies were formed. The results were reproducible in 3-4 replicates and the variation between the results was low, as revealed by a small standard error of mean, indicated by error-bars (fig. 51a-f).

The minimal inhibitory concentration (MIC) of Mergal K14 and Parmetol DF12 was between 0.156 and 0.3125%, for Preventol R50 between 0.0125 and 0.025%, for Troysan S97 between 0.078 and 0.156% and for ethanol between 7 and 10%. Hydrogen peroxide did not inhibit the growth of *V. fischeri* at any of the tested concentrations (fig. 51a-f).

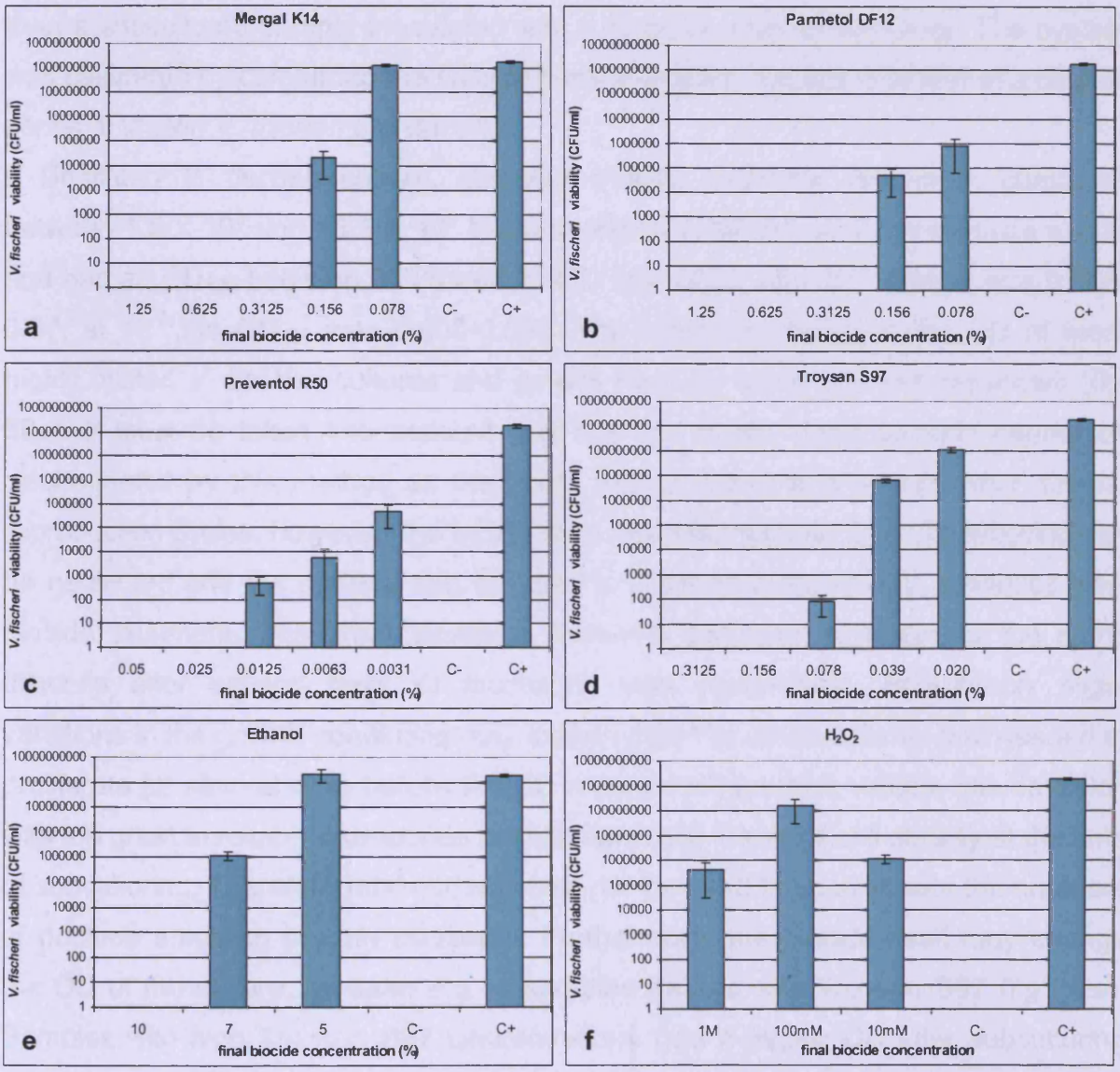


Figure 51 Survival of *V. fischeri* cultures
Colony forming units decreased with increasing concentrations of Mergal K14 (a), Parmetol Df12 (b), Preventol R50 (c), Troysan S97 (d), ethanol (e) and hydrogen peroxide (f). C- is the negative control and C+ the positive control. Error bars show the standard error of mean for triplicate assays.

4.3.4 Subculturing of biocide-treated *V. fischeri* cultures

The advantages of subculturing biocide-treated *V. fischeri* cultures into liquid medium were evaluated. It was significantly less time-consuming than the direct plate count. However, the enumeration of surviving cells was more complicated and less accurate.

Enumeration of survivors by optical density measurement

The cell density of a culture with few surviving cells would increase more slowly than a subcultured sample inoculated with a large number of survivors. The system was calibrated by comparing the ODs of biocide-treated cultures with that of a dilution series of known *V. fischeri* cell density.

Undiluted *V. fischeri* culture, sampled directly from the fermenter, contained between 1.5 X 10^9 and 13.5 X 10^9 bacteria per ml (determined by direct plate count) and had an OD_{400} between 0.495 and 0.505. The OD_{400} of a 10^{-1} dilution was 0.065-0.07, at 10^{-2} the OD_{400} was 0.005-0.022. The difference between the OD of more highly diluted *V. fischeri* cultures and growth medium alone was not significant (fig. 52a). It must be taken into account that live and intact, dead bacteria cannot be discriminated by this method as they both reduce light transmission. After several reproduction cycles, however, the influence of dead bacteria on light transmission can be neglected and the method can be used to determine the growth dynamics after biocide treatment. The great deviation observed between replicates at the same dilutions after several days of incubation was problematic. Presumably slight variations in the growth conditions may explain this. For those cultures that needed to proliferate for several days before the OD reached measurable values, this deviation was too great to reliably extrapolate back to calculate the initial cell density at the time of subculturing. Therefore this method could not be used to approximate the numbers of bacteria surviving biocide treatment. Furthermore, the biocide itself may change the OD of the culture, as seen e.g. in samples treated with Troysan S97 (fig. 52e). Samples with high Troysan S97 concentrations had a higher OD after subculturing than those treated with a low concentration of Troysan S97. The OD did not change significantly thereafter. The two lowest concentrations had an initially low OD, which increased in the following days, demonstrating bacterial growth. It was concluded that shortly after the biocide treatment and subculturing the content of biocide itself was

the most important factor influencing the OD, whereas subsequently bacterial growth was an additional factor to change the OD of surviving cultures. Non-viable cultures could be distinguished from those which proliferated after subculturing as their OD did not increase significantly during the days following subculturing (fig. 52a-g). Due to the visual presentation in logarithmic scale, the variation of the OD between replicate samples (standard error of mean shown as error bars) as well as those treated with different biocide concentrations appeared to be the highest shortly after subculturing and less significant in denser cultures.

The viability assessment by OD measurement was more sensitive than the direct plate count method, because of the greater sample size used. This allowed even very few surviving individuals to be detected. The MICs of all biocides, except for Troysan S97, were determined to be one concentration higher than revealed by direct plate count. The MIC of Mergal K14 and Parmetol DF12 was between 0.625 and 0.3125% final concentration, that of Preventol R50 was between 0.06 and 0.025% and that of Troysan was between 0.0781 and 0.0156%. High concentrations of ethanol were demonstrated to reduce bacterial viability, however after 48 h the cell density of a *V. fischeri* culture treated with the highest ethanol concentration (10% v/v final concentration) was the same as that of the control sample. Hydrogen peroxide did not have a noticeable effect on *V. fischeri* viability and the dynamics of the growth curve was the same as that of the control sample.

A disadvantage of the method was that enumeration of the surviving cells was impossible. Another difficulty was the daily removal of an aliquot from the culture to perform the OD measurement. This was not only a source for contamination, it also decreased the total amount of culture, making other experiments, e.g. bioluminescence measurements, with the same culture difficult or impossible.

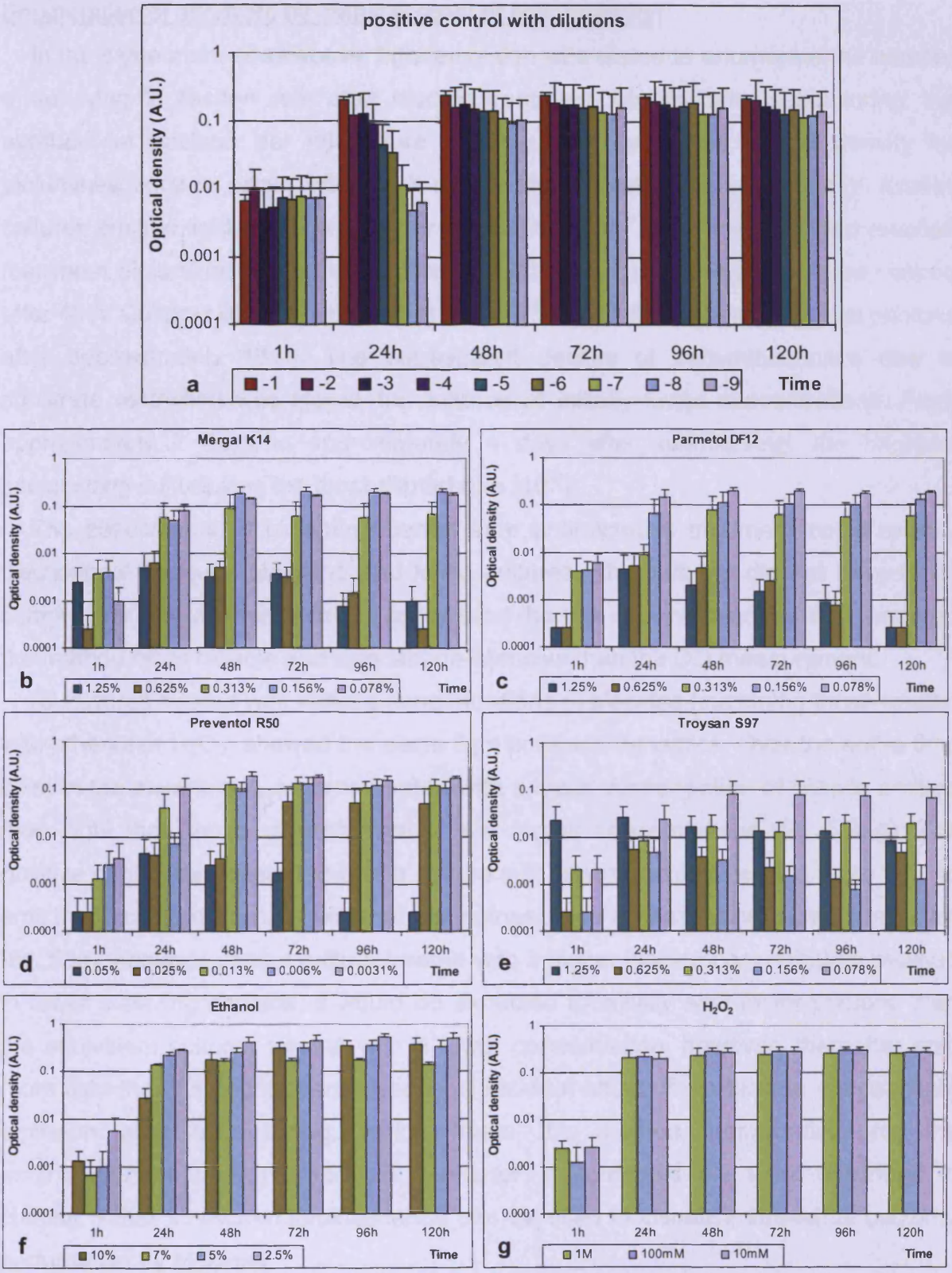


Figure 52 Cell density of biocide-treated *V. fischeri* after subculturing
Diluted (a) and biocide-treated (b-g) *V. fischeri* cultures experienced an extended lag-phase after subculturing. In some cases (e) the colour of the biocide interfered with optical density measurement. Error bars show the standard error of mean for triplicate assays.

Enumeration of survivors by measurement of light emission

In the same manner as above, light emission was tested to enumerate the number of surviving *V. fischeri* cells after biocide treatment. Directly after subculturing, the numbers of bacteria per ml culture medium was below the critical density for bioluminescence to occur. After 24 h all the tested dilutions of untreated *V. fischeri* cultures emitted light (fig. 53a). Higher populations (10^{-1} – 10^{-4} dilution) had reached maximum bioluminescence at this time; a decline in light emission could be noticed after 48 h. Cultures of lower concentrations (10^{-5} – 10^{-9} dilution) emitted most photons after approximately 48 h. The subsequent decline of bioluminescence due to substrate restriction was slower for cultures at initially lower concentrations. From approximately 2 days to approximately 4 days after subculturing, the brightest luminescing culture was the most diluted one (10^{-9}).

The assessment of bioluminescence after antimicrobial treatment could reliably discriminate between surviving and killed cultures. The cultures did not have to be sampled for the measurements; this lowered the risk of contamination and rendered the method more reliable and less labour-intensive than the OD measurement.

All cultures treated with various concentrations of biocides (excluding those treated with ethanol or H_2O_2) showed the same light emission dynamics. Over the entire time span of the experiment, cultures treated with a lower concentration of biocide emitted more light than those cultures treated with higher concentrations (fig. 53b-g). The positive control demonstrated that a culture with fewer bacteria needed more time to emit the maximum intensity and that bioluminescence declined more slowly thereafter (fig. 53a). Assuming that a culture treated with a higher biocide concentration resulted in fewer surviving bacteria, it would be expected to initially emit fewer photons than the equivalent cultures treated with a lower concentration, however, thereafter emit more light than its counterparts. Hence, a residual effect of the biocide affected light emission after subculturing, which made the method inappropriate for the enumeration of surviving bacteria. However, it confirmed the work described in chapter 3 that *V. fischeri* luminescence can be used to visualise sub-lethal bacterial perturbation by biocides.

Ethanol and H_2O_2 treated cultures reacted in the same manner as the control samples (fig. 53a, f, g). H_2O_2 was demonstrated not to affect *V. fischeri* viability significantly due to its ability to detoxify hydrogen peroxide by using its catalase (Visick & Ruby, 1998). However, it seemed that some perturbation due to the H_2O_2

occurred, as the maximum bioluminescence was approximately one order of magnitude below that of the control samples. Furthermore, the luminescence of cultures treated with the highest concentration of H_2O_2 decreased more slowly. This might suggest that some of the bacteria were killed by the treatment. However, these results should be compared to those for cell density after biocide treatment (fig. 52g), it was demonstrated that the cell density was approximately the same after 24h. Thereafter, the culture treated with the highest H_2O_2 concentration did not grow as rapidly as the lower concentrated H_2O_2 samples. This suggested that there was a residual effect that inhibited bacterial growth while enhancing bioluminescence. Light emission of *V. fischeri* cultures treated with a final concentration of 10% ethanol were slightly affected, while lower concentrations of ethanol did not seem to have any effect on bioluminescence. Cultures treated with 10% ethanol emitted the least light initially. After 2 days, however, it became the brightest culture. This corresponded well with the bioluminescence dynamics of highly diluted control cultures. This showed that there was no residual perturbing effect of the ethanol that reduced light emission and that the initial lower bioluminescence was due to a reduction in the number of bacteria.

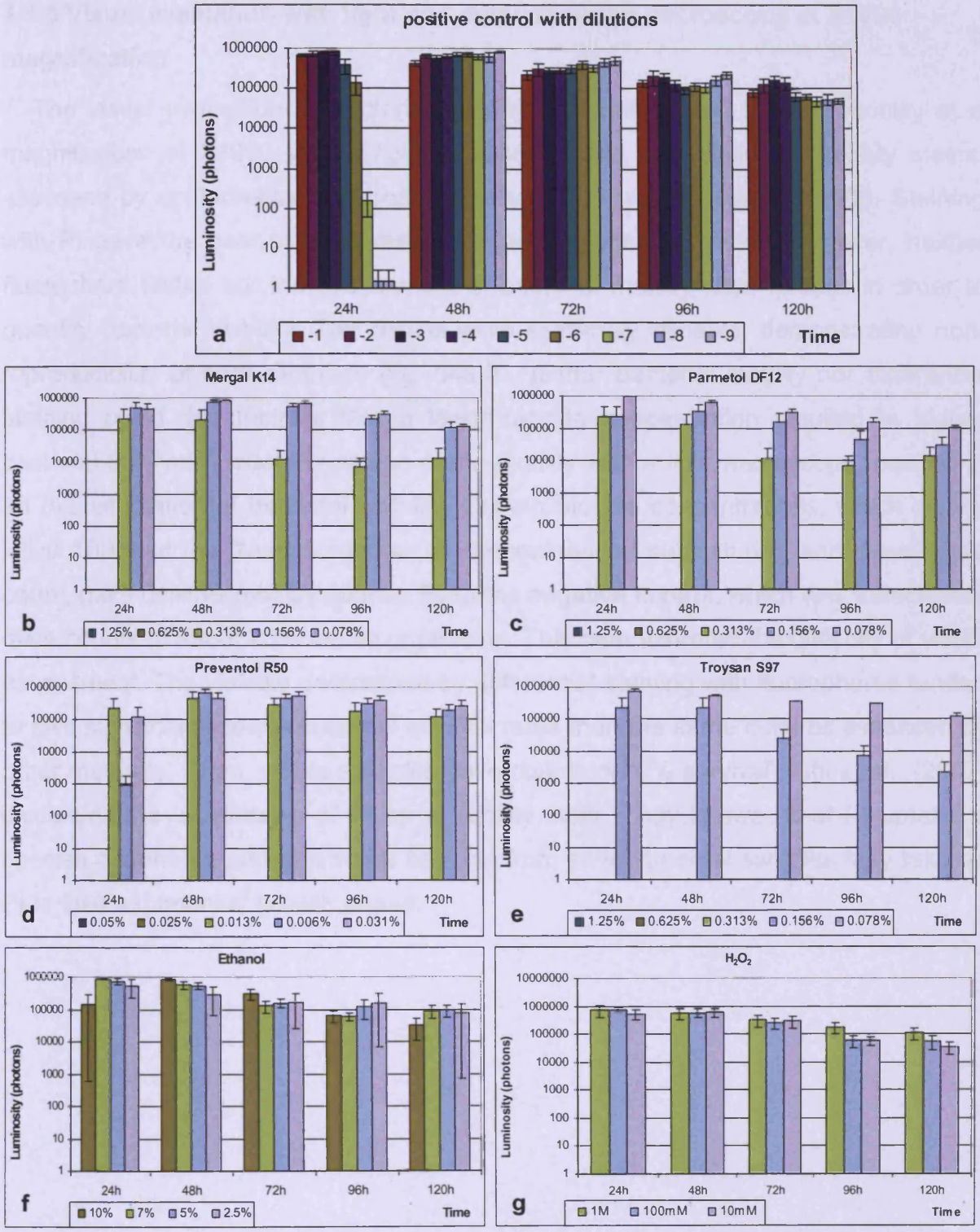
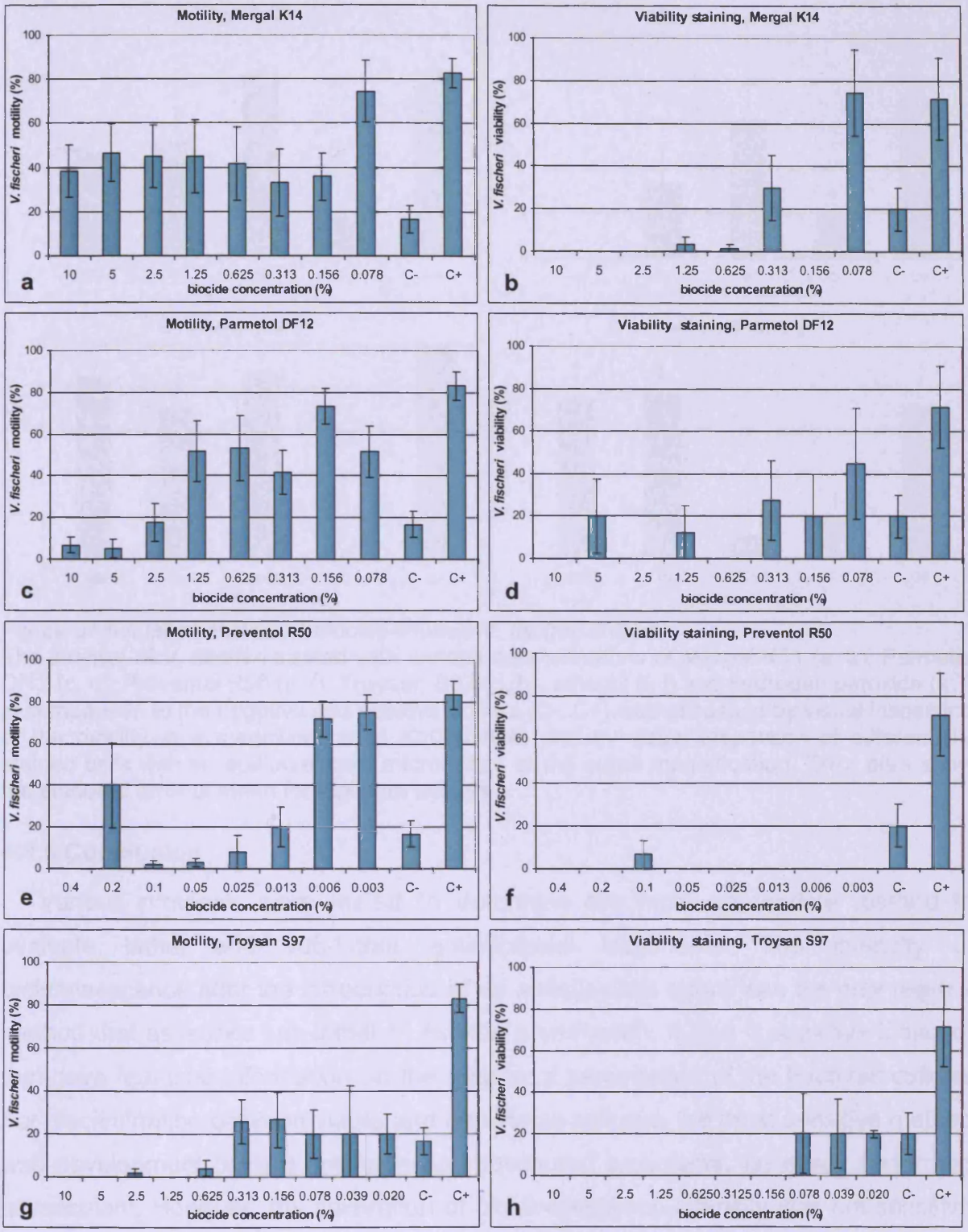


Figure 53 Bioluminescence of biocide-treated *V. fischeri* after subculturing
Highly diluted (a) cultures and those perturbed by 10% ethanol experienced an extended lag phase with bright luminescence thereafter. Perturbed but viable cultures treated with high biocide concentrations did not luminesce to the same intensity as control samples. Error bars show the standard error of mean for triplicate assays.

4.3.5 Visual evaluation with light and epifluorescent microscopy at X1000 magnification

The visual evaluation of bacterial viability was determined by their motility at a magnification of X1000 with a light microscope and the uptake of viability stains, assessed by epifluorescent microscopy, also at a magnification of X1000. Staining with PI gave the best staining results of the fluorophores tested, however, neither fluorophore tested nor the assessment of bacterial motility were reliable in order to quantify bacterial viability. The results were extremely variable, demonstrating non-reproducibility of both methods (fig. 54a-l). Neither bacterial motility nor differential staining could demonstrate that a lower biocide concentration resulted in higher bacterial survival. Visual inspection of the motility with a light microscope resulted in an overestimation of bacterial viability. Certain biocide concentrations, which clearly killed 100% of the treated culture, as determined by subculturing and direct plate count, gave positive motility counts. Even the negative control, which was autoclaved, gave counts of about 20% motile organisms. This demonstrated the difficulty of visual assessment. The viability determined by differential staining with fluorophores tended to give significantly lower bacterial survival rates than the same cultures assessed by other methods. Often, viable samples gave counts of "0% survival". Shi et al., (2007) discussed the unreliability of PI as a viability stain. They showed that PI uptake is species dependent and that some bacteria from environmental samples may take up PI in their exponential growth phase.


Motility, Mergal K14
V. fischeri motility (%)
biocide concentration (%)
10 5 2.5 1.25 0.625 0.313 0.156 0.078 C- C+
a
Viability staining, Mergal K14
V. fischeri viability (%)
b
Motility, Parmetol DF12
c
Viability staining, Parmetol DF12
d
Motility, Preventol R50
0.4 0.2 0.1 0.05 0.025 0.013 0.006 0.003 C- C+
e
Viability staining, Preventol R50
f
Motility, Troysan S97
10 5 2.5 1.25 0.625 0.313 0.156 0.078 0.039 0.020 C- C+
g
Viability staining, Troysan S97
10 5 2.5 1.25 0.6250.3125 0.156 0.078 0.039 0.020 C- C+
h

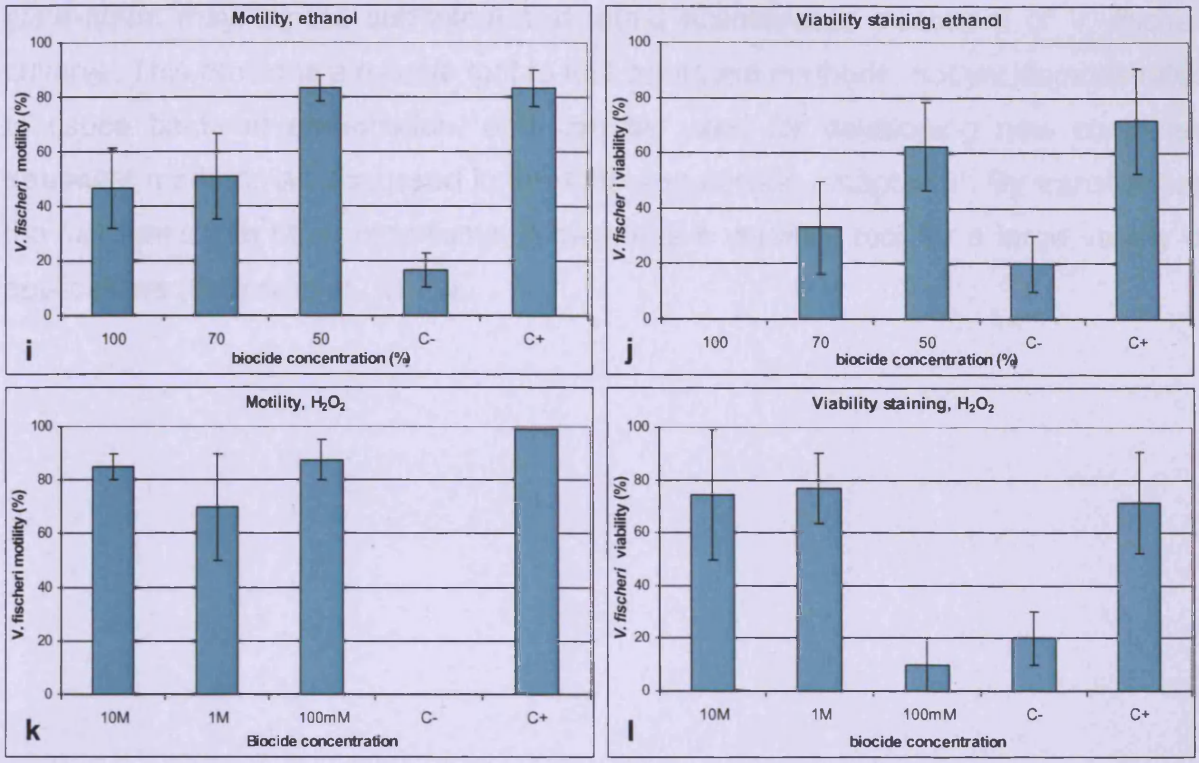


Figure 54 Visual inspection of biocide-treated *V. fischeri* cultures
The survival of *V. fischeri* treated with various concentrations of Mergal K14 (a, b), Parmetol Df12 (c, d), Preventol R50 (e, f), Troysan S97 (g, h), ethanol (I, j) and hydrogen peroxide (k, l) in comparison to the negative and positive control (C-, C+) was assessed by visual inspection of the motility at a magnification of X1000 (left) and by visual inspection of differentially stained cells with an epifluorescent microscope at the same magnification. Error bars show the standard error of mean for triplicate assays.

4.3.6 Conclusion

Various protocols were tested to determine the most appropriate method to evaluate lethal and sub-lethal antimicrobial treatments. The intensity of bioluminescence after the introduction of an antimicrobial agent was the only reliable method that assessed sub-lethal *V. fischeri* perturbation. It was a sensitive indicator that gave real-time information on the degree of perturbation of the bacterial culture. For discrimination between viable and non-viable cultures, the most sensitive method was development of light emission by subcultured organisms, an easily performed assessment. However, the calibration of bioluminescence intensity was not sensitive enough to allow enumeration of surviving bacteria. The only true quantitative method for the assessment of surviving *V. fischeri* cultures was the direct plate count. This labour-intensive method, that gives results after 2-3 days, allowed the detection even of few surviving bacteria. A combination of monitoring the bioluminescence with direct

plate count may assess sub-lethal and lethal antimicrobial treatment of *V. fischeri* cultures. This provides a reliable tool to test treatment methods, not yet demonstrated to cause bacterial perturbation, and can be used for developing new combined treatment methods as discussed in the following section (chapter 5). By transforming the *lux* genes into other organisms, it becomes a versatile tool for a large variety of applications (Setlow et al., 2001).

Chapter 5

Development of minimally-invasive antimicrobial treatments

5.1 Physical eradication methods and combined antimicrobial treatments

5.1.1 Combined antimicrobial treatments and synergistic effects

No antimicrobial treatment alone may fulfil all requirements for the ideal eradication method (efficiency only against target organisms, no hazard to humans or the environment, no effect on the substrate / artefact, ease of application, long-term efficiency). Combinations of one or more substances or treatment methods provide a wider spectrum of desirable antimicrobial criteria to achieve an effect more appropriate for a specific application. If such a combined treatment exhibits synergistic effects, the dose can be decreased, which may make the treatment cheaper and possibly more environmentally friendly. Synergistic effects exist if the interaction of two or more discrete components create an effect greater than the sum of the individual effects. If the combination is exactly the sum of the two discrete components it is referred to as "additional effect" and if it is less than the sum of their individual effects it is "antagonistic".

Countless combined treatment options may be tested for their enhanced efficiency in the eradication of microorganisms. For the application on outdoor stone monuments, the ideal method is one that can be applied locally and is highly effective in the moment of treatment. It should not present a hazard to the operator or visitors of the monument. After the treatment, sufficient toxicity should remain to prevent further microbial establishment and proliferation, however, when leached by rain the antimicrobial substance ought to be sufficiently diluted not to harm the environment. To this end, the combination of a physical and a chemical method was chosen for the investigation to develop an antimicrobial treatment of low eco-toxicity. Biocides are established antimicrobial agents for cultural heritage (see 4.1.3), however, their efficiency tends to be too low at concentrations of acceptable environmental impact and hazard to humans (Gorbushina et al., 2003). It was hypothesised that if the combination of a biocide could be enhanced with another treatment method, the

concentration could be decreased to an acceptable level. The second element of the combined treatment needed to be one that was active only at the time of treatment, such as a physical method, which is switched off after treatment.

5.1.2 Physical eradication methods

Physical methods have long been overlooked in their application on cultural heritage objects and have been labelled as requiring long treatment times and are effective only to low penetration depth (Van der Molen et al., 1980).

5.1.2.1 Ultraviolet radiation

UV radiation covers the short wavelength range of the electromagnetic spectrum between 60 nm and 400 nm or frequencies between 3×10^{16} and 7.5×10^{15} Hz. Quantum energies range from 10 eV to several hundred eV (Beeson, 1995a). Germicidal activity by DNA damage is highest between 230 and 275 nm with a maximum at 253.7 nm. UV radiation has been used in medical applications to sterilise instruments and the air surrounding patients during surgery. In cultural heritage conservation it has been used against microorganisms on renders and plasters (Nugari & Salvadori, 2003b; Tiano, 2002; Hirsch et al., 1995a). Microbial sensitivity varies with the stage of their growth phase (UV sensitivity is higher during exponential growth), cell density (the higher the cell density the lower the efficiency), relative humidity (higher efficiency at lower relative humidity levels), the properties of the substrate (transparency to UV radiation) and its surface structure, e.g. the presence of UV-protective microenvironments. A disadvantage of this inexpensive method is that it has poor penetration capacities into most substrates. Furthermore, it may damage certain materials, particularly organic ones such as cellulose and proteins, as well as pigments and dyes (Tiano, 2002; Hirsch et al., 1995a). The success of UV radiation has been demonstrated against cyanobacteria and algae, whereas heterotrophic bacteria were largely unaffected (Petersen et al., 1992; Van der Molen et al., 1980). However, a preliminary study on the removal of lichens by means of a high-intensity pulsed Xenon flash lamp gave encouraging results (Leavengood et al., 2000). More research is needed into its effectiveness in controlling a broader range of microorganisms that cause biodeterioration of stone.

5.1.2.2 Gamma radiation

Gamma radiation occupies the shortest wavelengths of the electromagnetic spectrum, generally between 10^{-11} to 10^{-13} m or above 3 EHz (exahertz or one million gigahertz [GHz]). Gamma radiation is ionising, which means that each quantum has enough energy to remove an electron from the orbit of an atom or molecule. The quantum energy ranges from thousands to millions of electron volts (Beeson, 1995b). Treatment with gamma radiation is a quick method that is effective against all living organisms. The lethal effect is most likely due to DNA damage. Thus sensitivity depends on the efficiency of DNA repair. Gamma radiation has been used for the elimination of microbial contamination in food and sterilisation of medical products (Silverman, 1983). In cultural heritage conservation it has been used on microorganisms (mainly fungi and fungal spores) on paper. Its high penetration allows mass treatment of artefacts, but serious damage to paper artefacts has been reported, including bleaching, yellowing and material degradation due to ionisation of molecules, cleavage of chemical bonds and auto-oxidation processes, which reduced the mechanical strength of the paper (Nugari & Salvador, 2003b; Wellheiser, 1992; Butterfield, 1987). Others claimed that there is no detrimental effect on paper (Rocchetti et al., 2002). The elaborate equipment needed for its safe application makes it a poor candidate for large outdoor monuments.

5.1.2.3 Temperature

Freezing, which is usually carried out at -20 – -30°C for at least 3 days, can kill insects, however, it does not kill microorganisms, but rather inhibits their growth. In fact, freezing at low temperature (in liquid nitrogen at -196°C) is used in culture collections for the long-term preservation of microorganisms.

High temperatures, however, may kill microorganisms. Enzymes are deactivated outside of their effective temperature range and all proteins are denatured (Cochran, 1958). Heat-treated cell membranes become increasingly permeable as their lipids become more liquid. Furthermore, DNA damage as well as structural damage within organelles has been reported (Adams & Moss; 1995; Anderson & Smith, 1976). Eradication of a microbial community needs to be evaluated from case to case. It

might be difficult to use heat to kill microorganisms growing on outdoor stone in hot climates. As they are periodically exposed to high temperatures, the microbial community present is likely to have high resistance to elevated temperatures.

5.1.2.4 Water blasting

The effect of high pressure water blasting on the microflora of sandstones has been tested by Warscheid et al. (1988). The authors found that there was little reduction in the microflora and shortly after the treatment the number of microorganisms was the same as before treatment or higher.

5.1.2.5 Microwave radiation

Microwave radiation is electromagnetic energy at frequencies between 0.3 and 300 GHz, or wavelengths from 1 m to 1 mm. The energy of a quantum of this frequency is 0.0016 eV (Kappe, 2004), which is not sufficient to break chemical bonds and is therefore termed "non-ionising". It is lower than the energy of Brownian motion and thus it has been concluded that quanta of this energy cannot induce a chemical reaction (Kappe, 2004).

Observed effects on irradiated microorganisms include changes of their physiology as well as structural damage. Frequency dependent increase (Banik et al., 2004, 2003; Grundler et al., 1977) or decrease (Grundler et al., 1977) of growth rate and metabolite production (Banik et al., 2004, 2003) have been reported. Furthermore, the surface of microbial cells may become pitted (Rosaspina et al., 1994) by the irradiation process and rough and swollen surfaces have been observed (Woo et al, 2000). Surface damage, however, does not seem to affect all bacteria to the same degree. While severe destruction was observed on the surfaces of irradiated *E. coli* cells, *B. subtilus* cells did not show any signs of structural damage under the same experimental conditions. Due to the fact that the viable count of both bacterial cultures decreased significantly after the treatment, the authors concluded that structural damage was not the major reason for microbial inactivation (Woo et al., 2000). The mode of action of microwaves remains a matter of speculation. Data, e.g. comparing microwave-irradiated bacterial cultures with those heated to the same bulk temperature (Sahin et al., 1998), suggested that it is not a pure heating effect but rather a combination of thermal and non-thermal effects. Heating may be caused by dielectric polarisation as well as ionic conduction (Kappe, 2004). Radiation at

microwave frequencies couples with the dipoles of a molecule, which then align within the applied electric fields. As the electric field oscillates, the dipoles attempt to realign with the alternating field. These realignments cause molecular friction; and rotational energy is released in the form of heat (Kappe, 2004). As the temperature rises due to friction of the rotating dipoles, ionic conduction also increases (Hayes, 2002). One theory, developed to explain non-thermal microwave effects, states that an additional transmembrane potential may be created by an external electric field. This transmembrane potential may be larger than the normal potential of the cell leading to pore formation and migration of solutes across the cell membrane as well as complete cell rupture (Kozempel et al., 1997). Additional to the effects of the electric field, static or oscillating magnetic fields have been demonstrated to inhibit the growth of microorganisms (Kozempel et al., 1997). The effect of microwave radiation on the substrate depends largely on the nature of the irradiated material. Microwave reflecting and transparent materials are unlikely to be altered when irradiated (Sahin et al. 1998). However, conductive heating may occur, in the vicinity of microwave absorbent material is adjacent (e.g. staples in paper). High humidity within microwave non-absorbent material may also cause this effect.

The first use of microwave radiation for cultural heritage applications was as a detection method for archaeological sites (Arnold, 1987; Arnold, 1981; Vickers & Dolphin, 1975) and as an analytical instrument to investigate the structures and/or moisture content of bricks (Visser & Gervais, 1994; Reese, 1979), stone (Derkowska, 1990), wood (Hartley & Marchant, 1988), canvas paintings (Chan et al., 1995), paper (Wan & Depew, 1995; Haberger & Baum, 1986) and leather (Odlyha et al., 2000). Its use in conservation was initially restricted to the rapid drying of materials such as paper (Brandt & Berteaud, 1987; Fischer, 1977), resin (Williams et al., 1981), photographs (Gillet & Garnier, 1989) and textiles (Oger et al., 1989). Its application against biodeterioration of cultural heritage objects has been limited to the eradication of insects (Bacílková et al., 2003; Lewis et al., 2000; Dever et al., 1990; Reagan, 1982). In the vast majority of cases microwave radiation at 2.45 GHz, the frequency of commercial microwave ovens, were used. The mode of action was most likely due to preferential heating of the biological tissue, which contained more water than the substrate.

Recently, a commercially available microwave radiation service for cultural heritage objects, called Dr. Termite, has been established in Potsdam, Germany. As the name suggests, this device has been employed mainly for the eradication of insects, however, fungal contamination has been treated successfully. This contact-free method has proven particularly useful in applications where direct access to the infested material was not possible (e.g. wooden structure covered by silk tapestry, wooden ceiling coated by plaster stucco). As the device is free-standing, there are no limits in the size of the treated objects. Furthermore, it can be used outdoors. Independent governmental examiners confirmed that all treatments resulted in 100% mortality of insects and fungi without any measurable damage to the objects (Materialprüfungsamt Brandenburg, 2003a, 2003b; Sächsisches Textilforschungsinstitut, 2003a, 2003b). Surface temperatures during exposure did not increase more than 6°C (G. Berg, personal communication, 2005). No published data could be found on the application of microwave radiation to eradicate microorganisms on outdoor stone monuments.

5.1.2.6 Ultrasound

The term ultrasound is referred to cyclic pressure waves (not electromagnetic waves) with a frequency above the upper limit of human hearing (approximately 20 kHz). For industrial applications, such as non-destructive material testing, frequencies of between 20 kHz and 10 MHz are normally used. Ultrasonic cleaning baths usually employ frequencies of 20-40 kHz.

The exact mechanisms by which ultrasonic radiation acts on materials are not entirely understood and have been discussed controversially (Calararo, 1998). In a liquid ultrasound system "cavitation" is thought to be responsible for a cleaning effect. Longitudinal waves, generated during sonication in a liquid medium, form areas of alternating compression and expansion. These pressure changes provoke cavitation, the formation and collapse of micro-bubbles, to occur (Piyasena et al., 2003). Near a solid surface (e.g. an artefact) a bubble implodes asymmetrically and hence creates a strong jet of approximately 400 km/h towards the solid surface. Subsequently, shock waves form from the cavity. This jet and the shock waves together can destroy kidney stones or remove surface particles from artefacts. During the implosion of a micro-bubble the gas is rapidly compressed, which may lead to a rapid, localised heating up to 5000°C. As this process is extremely rapid (less than a microsecond), little heat

can transfer into the liquid and therefore the bulk will not heat significantly. Furthermore, while the micro-bubble collapses, molecules may break up and form reactive fragments which will react with each other or the surrounding material (Suslick & Doktycz, 1990). Cavitation is frequency-dependent and will not occur above 2500 kHz (Alliger, 1975).

Antimicrobial treatments with ultrasound have various applications, ranging from food hygiene (as an alternative method for pasteurisation and sterilisation), waste water treatment (decontamination from unwanted microorganisms) to the medical field (treatment of wounds, implants or medical instruments). The mode of action against microorganisms is considered to be due to physical stress (localised heating, permeabilisation of cell membranes, cytological disruption of organelles), detachment of the microbial cells from their substrate and/or chemical reactions (production of free radicals) (Ananta et al., 2005; Piyasena et al., 2003, Seymour et al., 2002; Guerrero et al., 2001; Rediske et al., 1999). The effectiveness of an antimicrobial treatment depends on many variables, e.g. exposure time, ultrasound intensity (Guerrero et al., 2001; Rediske et al., 2000; Pagán et al., 1999), nature of the substrate (Seymour et al, 2002; Pagán et al., 1999), volume of the sonicated sample (Davies, 1958), water activity of the substrate (Álvarez et al., 2006) and the temperature during sonication (Guerrero et al., 2001).

Different microorganisms have varying resistance to sonication, which makes the treatment of a mixed population of unknown microorganisms particularly challenging. Gram-positive bacteria have been reported to be more resistant than gram-negative ones; this was attributed to their thicker and tighter peptidoglycan layer (Ananta et al., 2005; Villamiel & de Jong, 2000). Fungi seem to be more resistant than bacteria and protozoa have been reported to be highly susceptible, while spores, as robust survival structures, are more resistant than vegetative microorganisms (Piyasena et al., 2003; Seymour et al, 2002; Guerrero et al., 2001). However, differences in susceptibility have also been observed on the species level (Foladori et al., 2007) and even between strains of the same organism (Garcia et al., 1989).

Although ultrasound has been widely used in conservation, publications, particularly newer articles, on this topic are scarce. In hands-on conservation, ultrasound was predominantly used for the cleaning of archaeological metals (Christe, 2002; Heinrich, 2002; Organ,1959) and stone (Larson, 1990; Hempel, 1969). A comparative cleaning study on black archaeological crusts on marble

between water-based cleaning with poultices, micro-air abrasion, ultrasonic pick and lasers revealed that the ultrasound pick produced over-cleaned areas with a smoothened surface and rounded crystals, while the black crust remained in pores and cavities; the authors concluded that infrared laser cleaning was the procedure of choice (Paraskevi et al. 2003).

Literature on the application of ultrasound on organic artefacts is even rarer than on inorganic materials. A comparison of various cleaning methods on modern paper with typewriter ink revealed fibre movement, cracking and partial removal of the letter matrix of samples exposed to a ultrasonic cleaning bath (Caldararo, 1992). Archaeological leather cleaned by sonication (20 kHz) showed good results in surface areas but limited success in deeper areas due to poor penetration of the ultrasound (van Dienst, 1985). Barton and Weik (1986) reported that variously coloured feathers could be more delicately cleaned with ultrasound (40-60 kHz were tested) in an aqueous surfactant solution than by traditional cleaning methods. However, the authors themselves revised their method and considered it more critically thereafter (S. Weik, personal communication, 1997).

Beyond the treatment of artefacts, ultrasound has frequently been used for the assessment of the artefact's material. Particularly stone objects have been studied with ultrasonic techniques. The stone itself with its cracks and inclusions (Weiss & Ruedrich, 2004; Sheremeti-Kabashi & Snethlage, 2000; Weiss et al., 2000; Nappi & Côte, 1997) can be investigated as well as conservation treatments (Kamh, 2003; Sebastián et al, 1999; Nappi & Côte, 1997; Zezza et al, 1997), humidity within the material (Schwarz & Venzmer, 1995) and the influence of pollution on the stone (Bindal et al., 1987). More research is needed on potential damage to artists materials due to ultrasound exposure. Such damage may include phase changes of metal salts, removal of small fragments and initiation as well as acceleration of chemical processes (Caldararo, 1992; Suslick & Doktycz, 1990).

5.1.3 Combined antimicrobial treatment with ultrasound

While ultrasound alone has limited antimicrobial action, the combination of ultrasound with either a chemical or another physical agent is more efficient (Piyasena et al., 2003). Combinations of ultrasound with heat (thermosonication), pressure (manosonication), heat and pressure (manothermosonication) (Ordoñez et al, 1984), antibiotics (Carmen et al., 2005; Ensing et al., 2005; Rediske et al., 2000),

pH extremes or chlorination (McClements, 1995) have been suggested. Due to the lack of testing the antimicrobial effect of ultrasound alone, it was often impossible to state if the overall effect was synergistic, additive or antagonistic. The results were difficult to interpret due to the great differences in experimental setup and trends in the effect of combined treatments with ultrasound could not be established. It seemed that the antimicrobial effect was higher the more individual treatments were combined (manothermosonication was more efficient than manosonication or thermosonication, [Álvarez et al., 2006]). However, other research groups reported that elevated temperature alone was more effective than mano- or manothermosonication (Ordoñez et al, 1984).

Combined treatment of ultrasound with antibiotics is believed to have a greater effect than the antibiotic alone because two phenomena: (1) the physical effect that ultrasound facilitates the transport of antibiotics through the biofilm towards the microbial cell, (2) the indirect effect that ultrasound also enhances the transport of oxygen and other small molecules across the microbial biofilm, which increases the activity of the microorganism and makes it therefore more susceptible to the action of the antibiotic (Ensing et al., 2005). This increased metabolic state was assumed to be the reasons why growth was enhanced in various bacteria after treatment with sublethal ultrasound intensities (Pitt & Ross, 2003). The effects of a combined treatment with ultrasound and antibiotics depended on bacterial species (Carmen et al., 2005), the ultrasound parameter (Carmen et al., 2005; Rediske et al., 1999) and the antibiotic tested (Berrang et al., 2008; Carmen et al., 2005; Rediske et al., 1999).

5.1.4 Combined antimicrobial treatment for cultural heritage objects

The application of combined antimicrobial treatments for cultural heritage objects is still rare. Tiano (2002) mentioned the possibility of enhancing the effect of biocides for art objects by increasing the susceptibility of microorganisms (e.g. with EDTA or ionising radiation) before the treatment. However, he did not present experimental data. The effect of two permeabilisers of the outer membrane (ethylenediaminetetraacetic acid [EDTA] and polyethyleneimine [PEI]) and biocidal treatments with benzalkonium chloride (BC) on two *Pseudomonas* strains isolated from stone monuments was studied recently (Alakomi et al., 2006). It was found that all substances alone had some varying antimicrobial activity (PEI was the most

effective, EDTA and BC had equally little effect). A combined treatment with EDTA and BC was an addition of the antimicrobial effect of both agents alone, while PEI and BC together resulted in synergistic antimicrobial action.

5.1.5 Aims of the development of minimally-invasive antimicrobial treatments

The aim of this chapter was to test whether a combination of various chemical antimicrobial agents (see 4.2.1) in combination with ultrasound exhibits an antagonistic, additional or synergistic effect on *V. fischeri* and on the microbial community of biofilms grown on limestone.

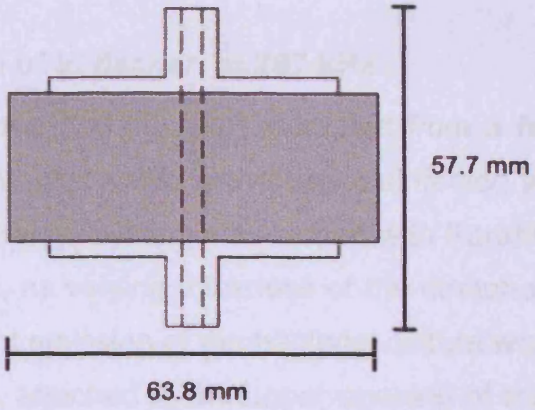
5.2 Materials and methods

5.2.1 Sonicator for microbial cultures at 267 kHz

The sonicator, used for testing the antimicrobial effect of ultrasound on *V. fischeri* cultures, was developed at Cardiff University for the disruption of microbial cells (Borthwick et al. 2005). Developmental criteria for the sonicator were the operation at an effective frequency to disrupt microbial cells, minimal heating during treatment and compactness for small sample volumes. It was designed to be used as a flow-through system to allow high through-put sample treatment. In this application for the combined treatment with biocides, however, it was used as a batch system. The sonication chamber had to be cleaned after every exposure to prevent residual biocide, which made a flow-through system, which was not equipped with a cleaning cycle, not applicable.

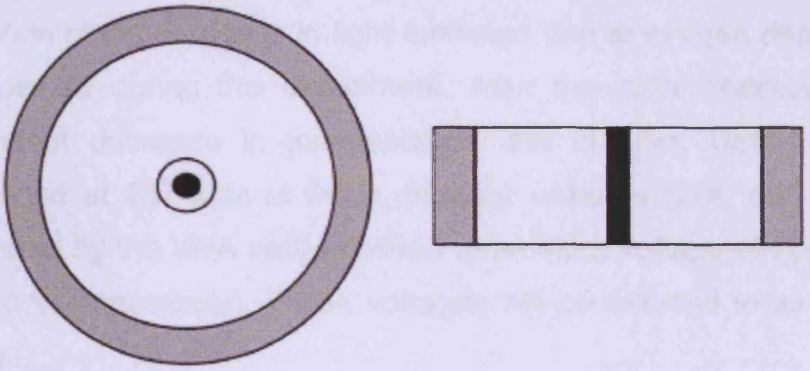
A stainless steel 0.6 ml volume exposure cylinder was surrounded by a tubular transducer (fig. 55). The ultrasound signal (267 kHz) was generated by a vector network analyser, VNA, (Hewlett Packard 33120A) and amplified (ENI 2100 L, ENI, Rochester, NY). To restrict temperature rise to a minimum it was fitted with heat sinks at the top and bottom end of the sonicator and fans on two sides along the horizontal axis. A more detailed description of the sonicator can be found in Borthwick et al. (2005).

a) Side view of tubular system.



b) Plan view of tubular system.

Elevation



Piezoceramic | Glue layer | Stainless steel | Water layer

c) Photograph of tubular system.

Figure 55 Sonicator
The side view of the tubular system with sonication chamber is represented by dotted lines (a), plan view of the device where the water layer represents the sonication chamber and a photograph of the device (c) (Borthwick et al. 2005).

5.2.1.1 Sonication of *V. fischeri* at 267 kHz

V. fischeri cultures (0.6 ml) were sampled from a fermenter and placed into the sonication chamber, which was previously disinfected with 70% ethanol. The bottom hole for the flow-through system was closed with Parafilm® and an o-ring. The upper hole was left open, as varying thickness of the stretched Parafilm® might affect light measurement. Light emission of the bacterial culture was monitored continuously with a PMT (see 3.2.9), attached to the upper opening of the sonicator. To protect it from potential splashing of the bacterial culture during sonication it was wrapped in cling film. Luminescence was recorded for 10 min before sonication applied to give an indication of the decrease in light emission due to oxygen depletion, as aeration was not possible during this experiment. After the initial characteristic exponential O_2-dependent decrease in luminescence the cultures were exposed for 3 min to ultrasound at 267 kHz at three different voltages (200 mV, 250 mV and 300 mV generated by the VNA and amplified to an input voltage of approximately 30 V, 40 V and 50 V respectively). These voltages will be referred to as low, medium and high voltage.

5.2.1.2 Combined treatment with ultrasound (267 kHz) and biocides on *V. fischeri*

The combined treatment of sonication with biocides was carried out in a similar manner as described in 5.2.1.1 with the exception that 0.54 ml *V. fischeri* culture were introduced initially into the sonicator. After 10 min, ultrasound was applied and 30 s thereafter 60 µl biocide were introduced with a pipette employing a long, transparent tip of approximately 2 mm diameter to reduce attenuation of light measurement to a minimum. The bacterial culture was mixed three times with the biocide employing the pipette. The antimicrobial substances were tested at the following final concentrations: Mergal K14 (0.1, 0.15, 0.2%), Parmetol DF12 (0.05, 0.1, 0.15%), Troysan S97 (0.02, 0.05, 0.075%), Preventol R50 (0.001, 0.0025, 0.005%) and ethanol (7, 10%). The control samples were mixed with 60 µl water or 60 µl luminescing *V. fischeri* culture.

5.2.1.3 Viability assessment of *V. fischeri* after sonication and biocide treatment

After the treatment, an aliquot of the *V. fischeri* culture was sampled for the preparation of a 10-fold dilution series (10^{-1}-10^{-6}) in 2.5% NaCl (w/v) solution. Each dilution was vortexed thoroughly before preparation of the next dilution. 3 times 15 µl were plated on seawater agar and the CFUs were counted after 1, 2 and 3 days. The CFUs were checked for bioluminescence to rule out contamination. A positive control was prepared in the same manner as above without switching on the ultrasound. Before and after sonication the temperature was measured with an infrared thermometer (Raytek Ltd. UK).

5.2.2 Ultrasound cleaning bath

For the sonication of microbial biofilms on stone a larger sonicator was needed. A small volume (80 ml) ultrasound cleaning bath (Ultrasonic Wave Cleaner, Sharper Image Design) was chosen. The ultrasound bath was operated at 20 kHz with pulsed action at two power levels: low power (not further specified) and high power (70 W).

5.2.2.1 Sonication of microbial biofilms with ultrasound at 20 kHz

The samples were obtained from limestone tiles from a demolished water-tower, approximately 50 m from the sampling site of previous experiments (see 2.2.1), which showed moderate to heavy biofilm formation. The tiles were cut into 1 cm^3 cubes with a circular saw cooled with fresh water. To receive a representative mean of biofilm quantity, three cubes from three different tiles were treated and assessed at the same time. The samples were placed in a glass beaker filled with 25 ml sterile water and placed immediately in the centre of the ultrasonic cleaning bath. Sonication was carried out for 3 min at low and high power. Positive controls were prepared in the same manner without switching on the ultrasound. Negative controls were prepared by soaking the samples for 3 min in sterile water and autoclaving them for 20 min at 115°C. All samples were subsequently placed for 24 h onto sterile blotter paper in sterile Petri dishes and exposed for 12 h to fluorescent daylight lamps and 12 h darkness at 22°C.

5.2.2.2 Combined treatment with ultrasound (20 kHz) and biocides on microbial biofilms on stone

The same kind of samples (three 1 cm^3 cubes from three different limestone tiles) were treated in the same manner as in 5.2.2.1 with one exception: 25 ml biocide was used instead of sterile water. Due to limited sample availability a small range of antimicrobial substances was tested at the following final concentrations: Troysan S97 (2% and 6%), Preventol R50 (2% and 6%) and Ethanol (70%). Ethanol was only tested at one concentration, as 70% was previously determined to be the most effective concentration (data not shown). The selection was based on preliminary results of long-term inhibition of microorganisms on stone (data not shown), where Troysan S97 and Preventol R50 were the best of the group of biocides (see 4.2.1). Furthermore, ethanol was tested because of its low eco-toxicity. Hydrogen peroxide, which also has low eco-toxicity, was not selected because preliminary results demonstrated that it was not effective against the present microbial community. The limited efficiency of oxidising agents on microbial biofilms has previously been reported (Pasmore & Costerton, 2003). Although bacteria in biofilms produce significantly less catalase, the resistance against hydrogen peroxide may be over 14 times higher than that of planktonic bacteria. The authors explained that the chemical reacts with the outermost cells of the biofilm and rapidly lowers the reactive concentration while diffusing deeper into the biofilm. This may give the biofilm time to up-regulate the production of catalase.

5.2.2.3 Activity assessment of microbial biofilms on stone after sonication and biocide treatment

24 h after sonication and biocide treatment, the biofilm samples on limestone tiles were ground in a disinfected (70% ethanol) mortar. Suspensions of 100 mg/ml ground sample in 0.9% NaCl (w/v) were prepared. To assess the activity of surviving microorganisms their ATP content was measured. A HY-LiTE® Pen (Merck) was introduced into a well-shaken suspension. Two HY-LiTE® Pen tests were performed per sample. In cases where the results varied significantly a third test was performed. The sampling stick of the HY-LiTE® Pen was coated with a substance (undefined) that ensured the release of ATP from cells. The HY-LiTE® Pen contained a buffer with a mixture of luciferin/luciferase, which emitted bioluminescence in the presence of

ATP. Light emission was measured immediately on sample preparation with the HY-LiTE® 2 luminometer (Merck). Light intensities were directly proportional to quantities of ATP present in the sample. This method was extremely rapid (approximately 1 min per sample) and very sensitive (detection limit: 1.4 x 10^{-14} Mol ATP). The system compensates for temperature variations and can therefore be used reliably even in unstable climate conditions. In order to relate the ATP content to the biomass present, protein content was assessed in the same manner as described in 2.2.7.

5.3 Results and discussion

5.3.1 Effect of ultrasound (267 kHz) and biocides on *V. fischeri*

Effect of biocides alone on *V. fischeri* viability

All tested antimicrobial agents decreased the viability of *V. fischeri* significantly (fig. 56, table 5). The viable count declined with increasing biocide concentration. The only exception was ethanol, which had significantly more antibacterial activity against *V. fischeri* at 7% than at 10%. This was an interesting result, as it contradicted those from section 4.3.3, where a higher ethanol concentration resulted in higher *V. fischeri* mortality. The reason may be the time between antimicrobial treatment and assessment. Whereas previously 4 h were allowed for the ethanol to act on the bacterial culture, in this experiment the assessment was performed approximately 5 min after treatment. It is a well established phenomenon for the disinfection of solid materials that 70% ethanol has higher antimicrobial activity than absolute ethanol. It has been explained by the denaturation of proteins, which in the presence of water happens more readily (Buck, 2001). However, it was a surprising discovery in the case of a bacterial culture, where the final concentration was 7 and 10%. This suggested that the mode of action of 70% ethanol versus absolute ethanol is more complicated.

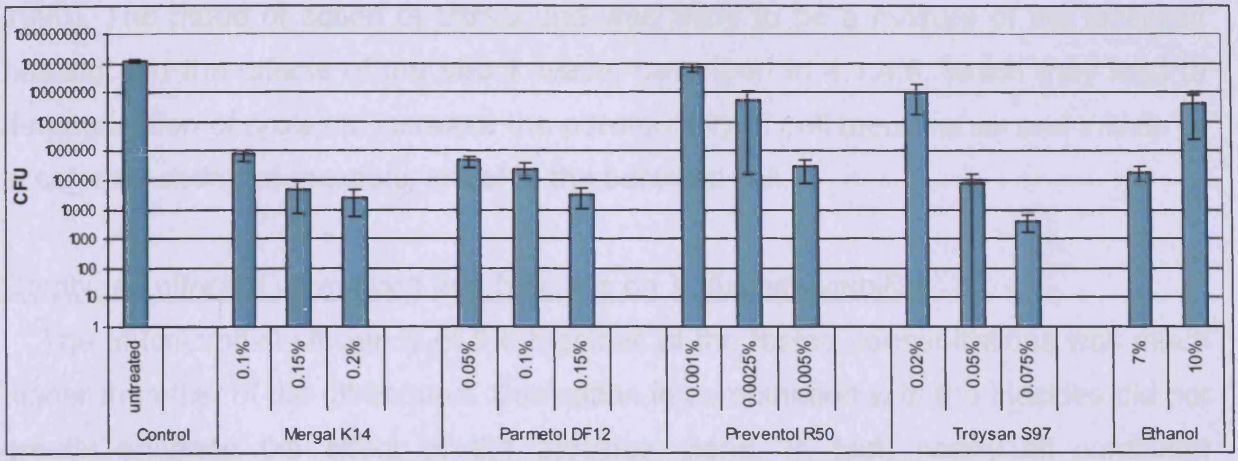


Figure 56 Reduction of *V. fischeri* viability after biocide treatment without ultrasound
Higher concentrations of antimicrobial agents, with the exception of ethanol, resulted in higher reduction of *V. fischeri* viability. Error bars show the standard error of mean for triplicate assays from 3 different batches.

Table 5 Reduction of *V. fischeri* viability after biocide treatment without ultrasound
The table shows the percentage of viability of *V. fischeri*, determined by direct plate count, after the antimicrobial treatment with biocides at different concentrations.

Biocide	Concentration	% viability after treatment	Biocide	Concentration	% viability after treatment
Mergal K14	0.1%	0.0605	Parmetol DF12	0.05%	0.0409
	0.15%	0.0036		0.1%	0.0197
	0.2%	0.0013		0.15%	0.0025
Preventol R50	0.001%	57.2410	Troysan S97	0.02%	8.0875
	0.0025%	4.4203		0.05%	0.0053
	0.005%	0.0233		0.075%	0.0002
Ethanol	7%	0.0145	Ethanol	10%	3.3291

Effect of ultrasound alone on *V. fischeri* viability

Low, medium and high voltages of ultrasound at 267 kHz were tested for their effect on the viability of *V. fischeri*. A 3 min-exposure of *V. fischeri* to low voltage ultrasound (30 V) did not reduce the viability of the culture to a statistically significant level (P= 0.930), whereas after a 3 min-exposure to medium voltage ultrasound (40 V) the viability was reduced to 36% (decline significant at P=0.017) and after high voltage (50 V) it was 4% of the unsonicated culture (decline significant at P=0.0034) (fig. 57a). Due to the design of the sonicator, employing heat sinks and fans, the bacterial cell death could not be attributed to a bulk heating effect. The temperature increase of the bacterial culture after 3 min sonication at high power was less than 0.5°C (data not shown). However, a localised superheating, too rapid to increase the bulk temperature has been suggested to occur during cavitation (Suslick & Doktycz,

1990). The mode of action of ultrasound was likely to be a mixture of the localised heating and the effects of the shock wave, described in 4.1.4.6, which may lead to denaturisation of proteins, increase the permeability of cell membranes and initiate or accelerate chemical reactions lethal to the bacterial cell.

Combined effect of ultrasound and biocides on *V. fischeri* viability

The antimicrobial efficiency of the biocides at the tested concentrations was much higher than that of the ultrasound. Sonication in combination with the biocides did not greatly enhance the effect of the biocides alone. In fact, nearly all combined treatments had an antagonistic effect (table App. 4.1).

While ethanol alone had a higher antibacterial effect at 70% than at 100% (final concentration 7 and 10% respectively), the reduction of *V. fischeri* viability was more efficient in combined treatment with ultrasound and absolute ethanol (fig. 57b). The viable count of the absolute alcohol sample decreased with increasing ultrasound voltage. By contrast, the sample treated with 70% ethanol further reduced the viable count only in combination with the highest voltage of ultrasound. All tested substances, with the exception of absolute ethanol, showed a peculiar behaviour upon the exposure to low voltage (and for some biocides also to medium voltage): the viable count was higher than that of the corresponding unsonicated sample. This behaviour only occurred for the combined treatment and not for sonicated *V. fischeri* without an antimicrobial agent. Therefore, a sonication-produced dispersion of naturally occurring cell aggregates, which lead to erroneous dilution series and CFU formation, must be disregarded. It could neither be explained by the increased metabolic activity at sub-lethal sonication (Ensing et al., 2005; Pitt & Ross, 2003), because this should also be observed at ultrasound exposure without the presence of a biocide. Possibly, the combined action of low voltage ultrasound together with mild biocidal treatment produced cell dispersion in a manner that neither of the components alone could not do. With high-power ultrasound the effect of cell death on the viable count of *V. fischeri* outnumbered the gain in viable count due to the dispersion of aggregates.

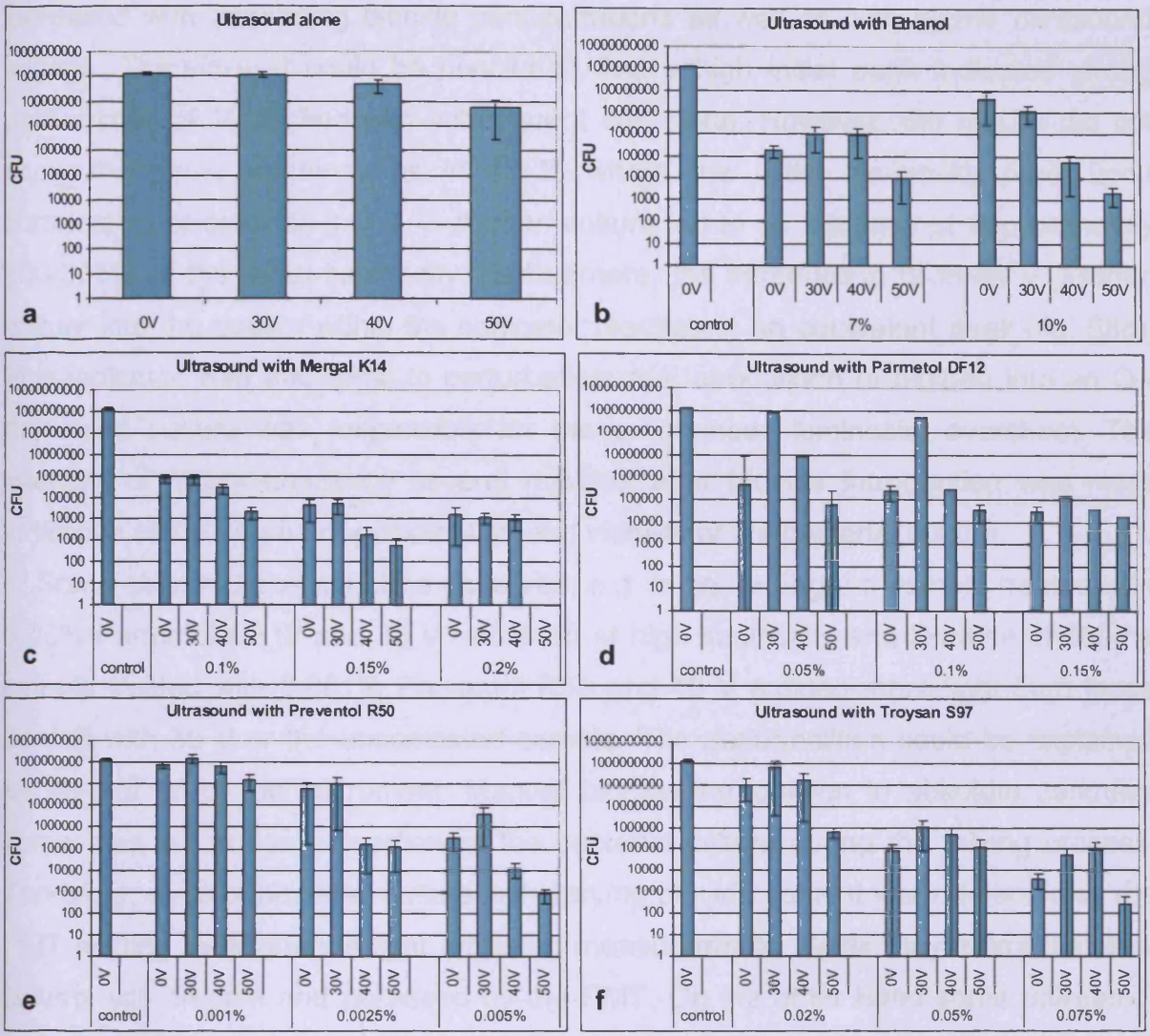


Figure 57 Reduction of *V. fischeri* viability after combined ultrasound/biocide treatment
V. fischeri viability after a 3 min ultrasound (267 kHz) exposure decreased with increasing input voltage. In combination with biocides low input voltage was less efficient than biocide treatment alone. Error bars show the standard error of mean for triplicate assays from 3 different batches.

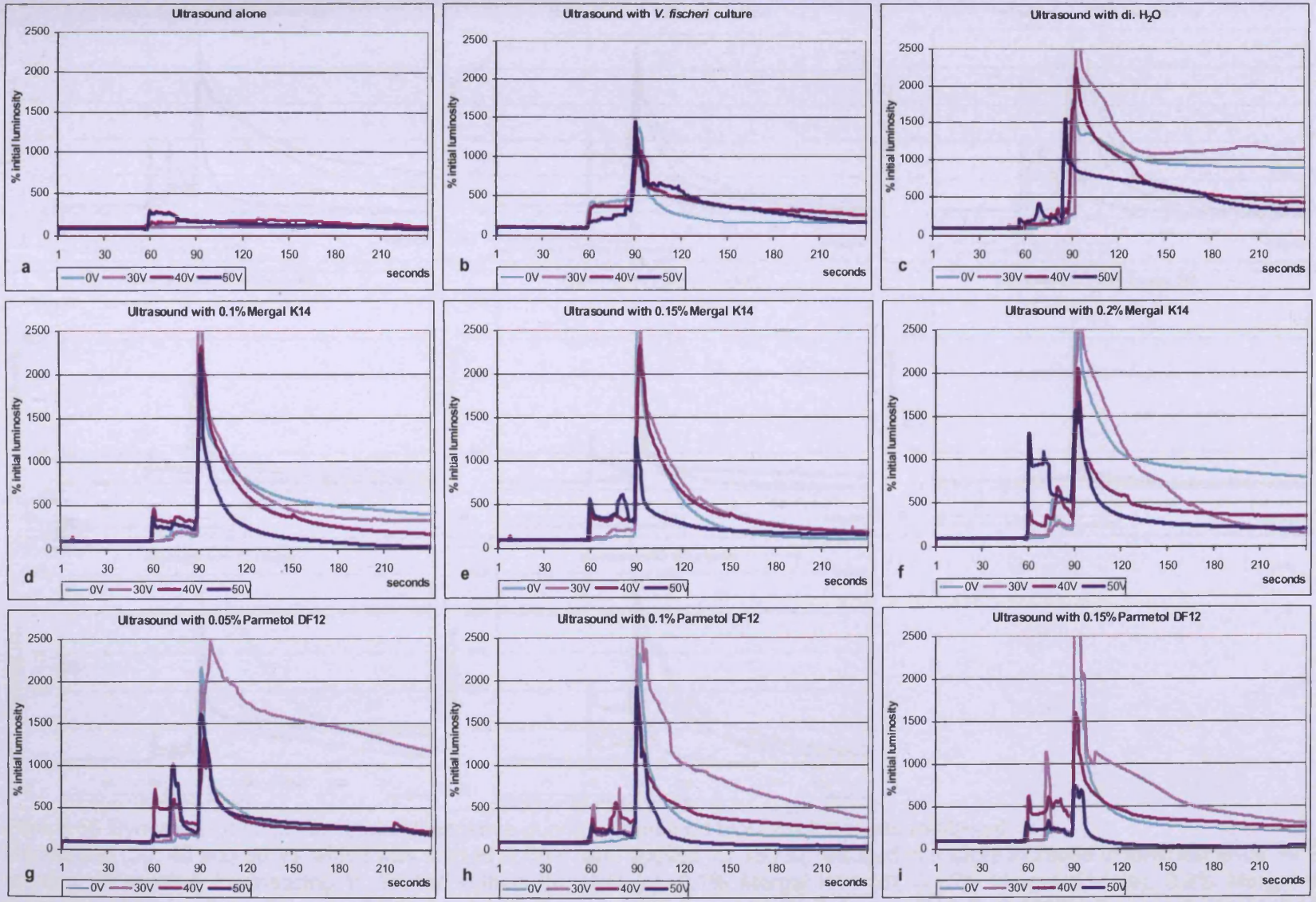
Effect of ultrasound and biocides on *V. fischeri* bioluminescence

Biocide treatments of *V. fischeri* with and without ultrasound, as well as sonication alone, resulted in a strong initial increase in bioluminescence, followed by an exponential decrease, as reported in 4.3.2. The luminescence peaks were significantly higher when the cultures were challenges by biocides (up to 3000% of the initial light emission) than by ultrasound (up to 300% upon exposure to high voltage). Higher concentrated biocides and higher ultrasound voltage tended to lead to a more pronounced luminescence peak (fig 58). This corresponded to the viability of the cultures after physical/chemical treatment. The tested biocides reduced *V. fischeri* viability significantly more than ultrasound. Furthermore, *V. fischeri* mortality

increased with increasing biocide concentrations as well as with higher ultrasound voltage. Therefore, it could be concluded that a high initial peak indicated strong perturbation of *V. fischeri* with subsequent cell death. However, the results did not show the same tendency as in 4.3.2, where the initial luminosity peak upon introduction of biocides into a *V. fischeri* culture led to an increase of approximately 200-300% of the initial luminosity. Furthermore, the introduction of fresh *V. fischeri* culture into the culture within the sonicator resulted in an equivalent peak (fig. 58b). This indicated that additional to perturbation, the introduction of oxygen into an O_2-depleated culture was responsible for the pronounced luminosity overshoot. The intensity of bioluminescence several minutes after biocide introduction was more indicative of the degree of perturbation and viability of the bacterial culture.

Some abnormalities could be observed, e.g. in figure 58g the sample treated with 0.05% Parmetol DF12 and 30 V remained at high luminosity and likewise in 58j the sample treated with 0.001% Preventol R50 and 40 V emitted more light than those treated with 30 V or the unsonicated sample. The abnormalities could be explained by the set up of the instrument. Manual biocide introduction in absolute darkness sometimes led to some overflow of the bacterial culture during the mixing process. Therefore, spills of bacterial culture with varying biocide content were detected by the PMT leading to erroneous light emission measurements. Spills may effect that less culture was present and assessed by the PMT. On the other hand some untreated, highly luminescing culture might spill directly onto the protection of the PMT, where luminosity was read. In this case the error would result in too high luminosity reading.

Despite of these abnormalities, the tendency showed that low luminescence intensity at the end of the experiment indicated low viability of the treated *V. fischeri* cultures. Samples treated with higher concentrated biocides decreased bioluminescence more significantly after the initial peak than those treated with lower concentrated biocides. This can be clearly seen for those treated with Preventol R50, Troysan S97 and ethanol (fig. 58j-q). Samples treated with biocide and the highest ultrasound voltage, 50 V, (indicated by dark blue lines in fig. 58) nearly always emitted lower light intensities at the end of the experiment than those treated with lower ultrasound voltages. This reflected well the mortality, which likewise was the lowest in samples treated with biocides and 50 V ultrasound. However, due to the experimental set up, the results have to be treated with care. To confirm the tendencies, a more reliable set up would have to be built.


Ultrasound alone
% initial luminosity
2500
2000
1500
1000
500
0
1 30 60 90 120 150 180 210
seconds
a
0V 30V 40V 50V
Ultrasound with V. fischeri culture
b
Ultrasound with di. H2O
c
Ultrasound with 0.1% Mergal K14
d
Ultrasound with 0.15% Mergal K14
e
Ultrasound with 0.2% Mergal K14
f
Ultrasound with 0.05% Parmetol DF12
g
Ultrasound with 0.1% Parmetol DF12
h
Ultrasound with 0.15% Parmetol DF12
i

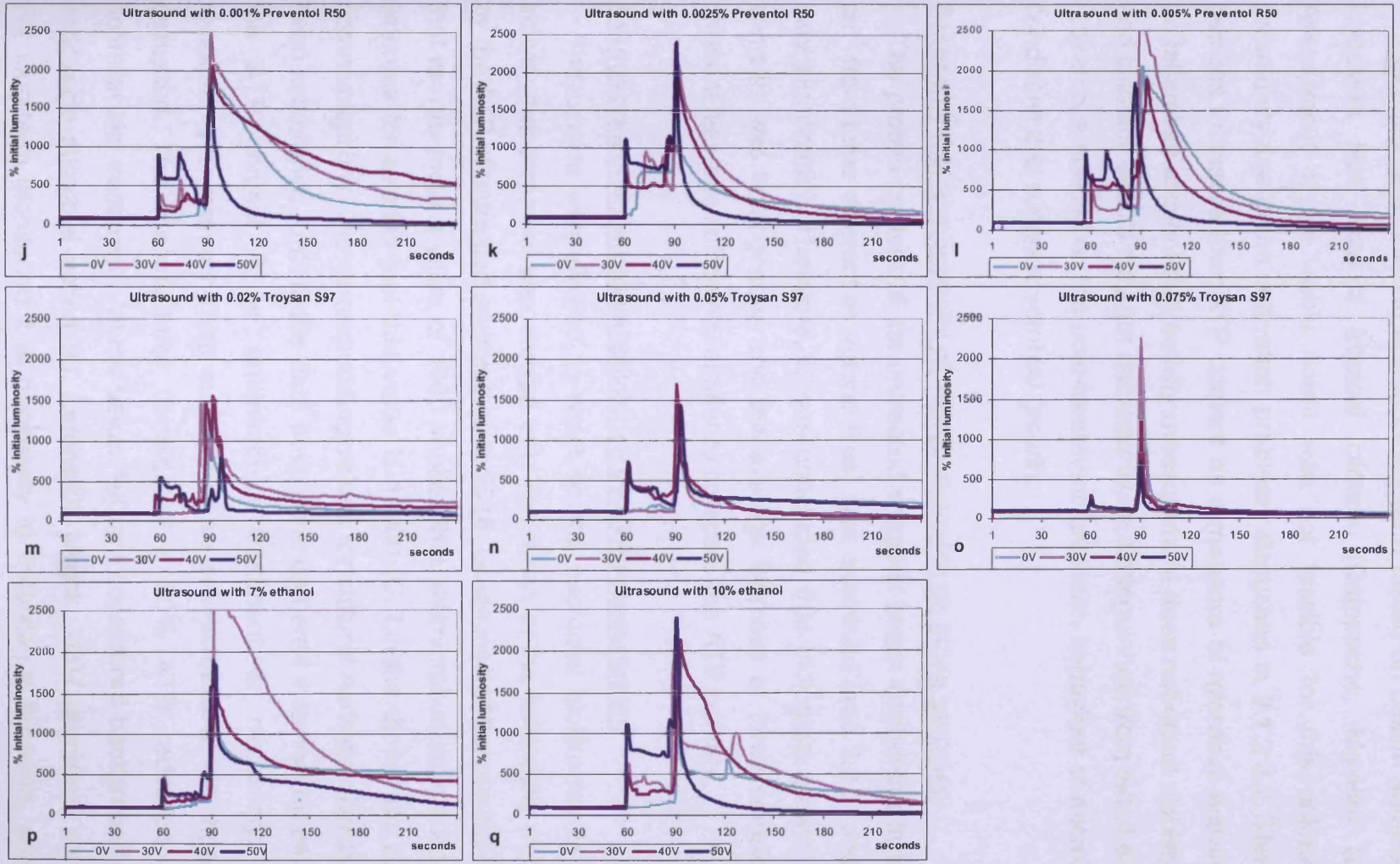


Figure 58 Dynamics of *V. fischeri* bioluminescence during a combined biocide/ultrasound treatment
Ultrasound (30, 40 and 50 V), which was started at 60 s (and applied for 180 s), resulted in a slight increase in luminescence. At 90 s (30 s after starting ultrasound) luminescing *V. fischeri* culture (b), H_2O (c), 0.1% Mergal K14 (d), 0.15% Mergal K14 (e), 0.2% Mergal K14 (f), 0.05% Parmetol DF12 (g), 0.1% Parmetol DF12 (h), 0.15% Parmetol DF12 (i), 0.001% Preventol R50 (j), 0.0025% Preventol R50(k), 0.005% Preventol R50 (l), 0.02% Troysan S97 (m), 0.05% Troysan S97 (n), 0.075% Troysan S97 (o), 7% ethanol (p) or 10% ethanol (q) was added, which resulted in a strong overshoot and subsequent decrease in luminescence. Error bars show the standard error of mean for triplicate assays.

5.3.2 Effect of ultrasound (20 kHz) and biocides on the microbial community of biofilms on stone

The effect of ultrasound and biocides were tested on biofilm samples growing on limestone tiles from a tropical climate (Campeche, Mexico) (see 5.2.2.1). Assessment of the viable count was not feasible for this unknown microbial community due to the cultivation problems discussed in 2.1.2.2.2. Therefore, it was decided to assess their ATP content as a measure of microbial activity. It needs to be taken into account that activity measurement does not equal viability assessment and biostatic effects may not necessarily be distinguished from lethal effects. Further research is needed on the post-treatment long-term behaviour of microbial activity in conditions that support microbial growth.

Assessment of homogeneity of microbial growth on stone samples

The protein content of six untreated samples (each containing three cubes of 1 cm^3 from three different limestone tiles) was examined (see 2.2.7) and found not to vary significantly. Therefore, it was concluded that biological growth on the stone samples was homogenous and the average biomass of three samples from three different tiles was sufficiently similar to compare the ATP content.

Assessment of antimicrobial efficiency by ATP measurement

The biocide was allowed to react on the microbial biofilm for 24 h before the activity measurement was carried out. The mean of the luminosity (A.U.), produced by the ATP of untreated samples, was 9918. Guidelines of the manufacturer suggest that for raw meat a value of 1000 implies that antimicrobial action should be taken, whereas for aviation fuel this value is 10000 (C. Lindhardt, Merck, 2007, personal communication). The corresponding values for cultural heritage objects have not yet been established. Until these data have been gathered it cannot be determined from the ATP content if an antimicrobial treatment is necessary. Comparative measurement, evaluating the success of an antimicrobial action, however, can be assessed. The manufacturer considered a 50% ATP reduction a successful antimicrobial treatment. Values below 100 are considered background noise without noticeable microbial action (C. Lindhardt, Merck, 2007, personal communication). For long-term monitoring it is necessary to establish a baseline, which should be

obtained from a sample with "acceptable" contamination (e.g. after surface cleaning with only surface contamination present). For food products a value of 300% of the baseline is considered a "fail", however, again, for cultural heritage objects more research is needed to establish such values.

A decrease in ATP content may mean that fewer viable cells are present, however, it might also mean that the same amount of viable cells remained, however, their activity was reduced. Cell lysis, a result of antimicrobial action, releases the intracellular ATP, which can hence be detected immediately after the treatment. This may be interpreted as a false high activity level of the microbial community. However, soon after cell death phosphatases degrade the extracellular ATP. Therefore, 24 h after biocidal treatment, when the ATP measurement was performed, the sample could be considered free of extracellular ATP. As demonstrated previously on *V. fischeri* cultures, the viable count decreased after sonication (see 5.3.1). Therefore, it is more likely that a decrease in ATP content is due to cell death of part of the microbial community rather than a decrease in activity level of an unchanged viable count. However, it was demonstrated previously that the effect of ultrasound varied between species (Oulahal et al., 2007; Carmen et al., 2005). Therefore, this conclusion has to be treated with caution.

Effect of biocides on the ATP content of microbial biofilms

Treatment with all antimicrobial substances decreased the ATP content of the microbial community (fig. 59). The inhibition was statistically highly significant (Troysan S97 2% P= 0.000, 6% P= 0.000; Preventol R50 2% P= 0.0001, 6% P= 0.0001, ethanol 70% P= 0.0005). Higher concentrated biocides resulted in stronger inhibition of microbial activity. The strongest inhibition was achieved by 70% ethanol, which decreased the ATP content to 6.3%. This was followed by 6% Preventol R50 (15.5%), 2% Preventol R50 (29.3%), 6% Troysan S97 (38.1%) and finally 2% Troysan (47.0%).

Recommended concentrations of Troysan S97 and Preventol R50 for substrate treatment are 1-2% and 1.5-5%, respectively. Even at 6% neither biocide was as effective as 70% ethanol. A possible explanation why ethanol (70%) performed much better than the tested commercially available biocides may be that the small molecule penetrated better through the channels of the biofilm matrix to reach the microbial cells (Dunne, 2002). Another possible explanation is that certain functional

groups of the EPS may deactivate some biocides (Nikolaev & Plakunov, 2007; Hall-Stoodley, et al., 2004; Fux et al., 2003; Dunne, 2002; Stewart & Costerton, 2001).

On suspensions of *V. fischeri* ethanol did not perform as well as other biocides (see 5.3.1). This might be due to the dilution effect, as it is well established that the most effective antimicrobial concentration of ethanol is 70%. Furthermore, in a suspension ethanol does not have an advantage over larger molecules to pass the EPS layer surrounding the cells nor may the commercially available biocides be deactivated by functional groups within the EPS.

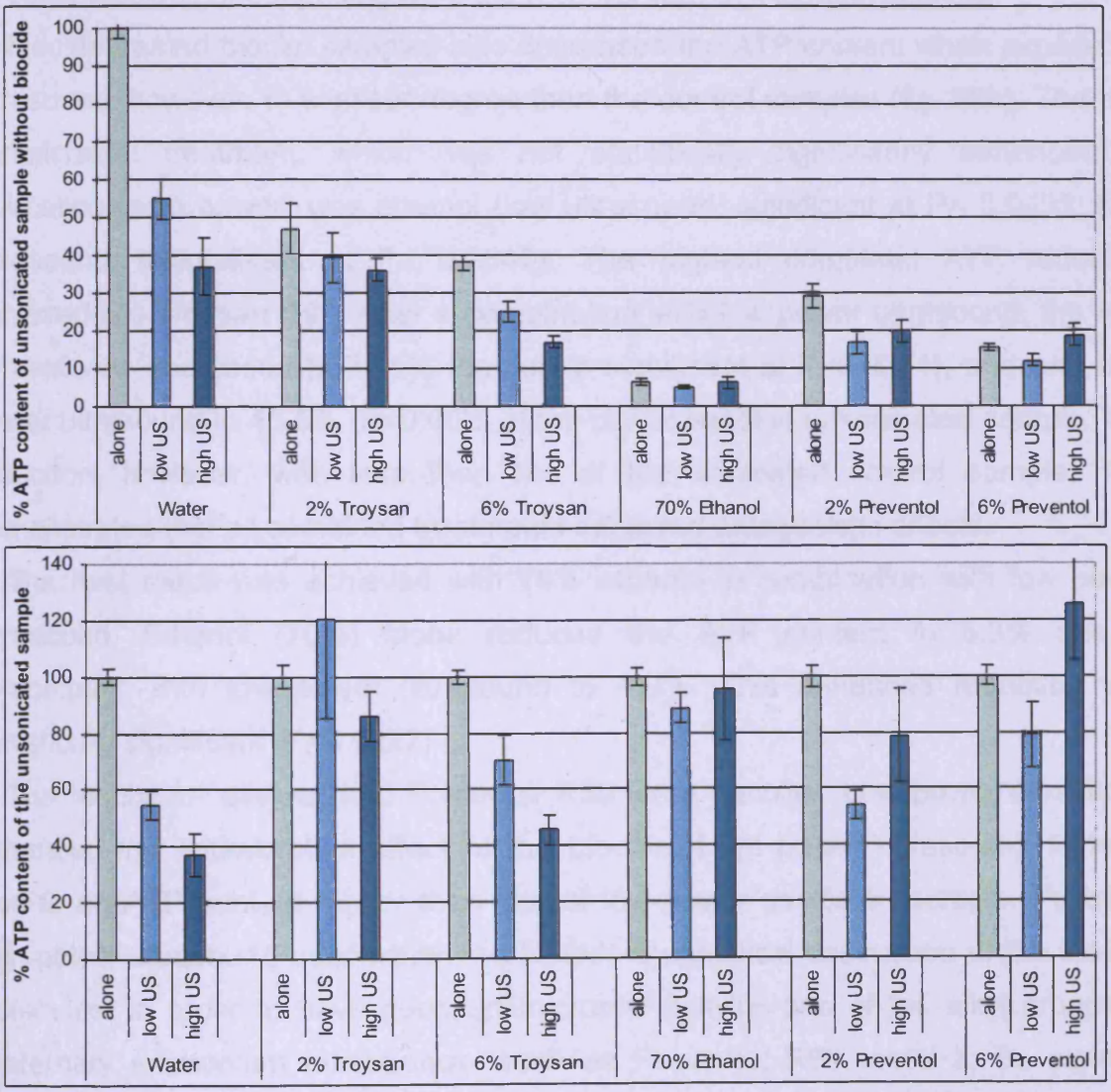


Figure 59 ATP decrease in biofilms on limestone after biocidal and ultrasonic treatment Samples treated with Troysan showed enhanced ATP reduction with higher biocide concentration and ultrasound power (low ultrasound: not specified, high ultrasound: 70 W). Ethanol and Preventol showed highest ATP reduction with low power ultrasound. (a) Values are described as % of the untreated control, (b) Values are described as % unsonicated, however, biocide treated sample. Error bars show the standard error of mean for triplicate

Effect of ultrasound on the ATP content of microbial biofilms

The exposure to ultrasound decreased the ATP content of all biofilm samples (fig. 59a, b). Interestingly, the control sample (treated with H_2O) showed the largest difference between the sonicated and the unsonicated sample (fig. 59b). After 3 min treatment with low power ultrasound the control sample decreased to 55% and after high power ultrasound to 37%. This decrease was statistically highly significant at P= 0.000 for both samples.

Effect of biocides and ultrasound on the ATP content of microbial biofilms

Biocide-treated biofilm samples also decreased the ATP content when exposed to ultrasound, however, to a lesser degree than the control samples (fig. 59b). The only antimicrobial treatment, which was not statistically significantly enhanced by sonication (high power) was ethanol (low ultrasound: significant at P= 0.0453, high ultrasound: insignificant at P= 0.2840). The highest combined ATP reduction achieved 6% Troysan S97. After a combination with low power ultrasound, the ATP content was decreased to 70.5% (decrease significant at P=0.0371), and with high power ultrasound to 45.5% (P=0.002) of the corresponding unsonicated sample. The reduction, however, was less than hst of the sonicated control sample. This demonstrated that all combined treatments exhibited antagonistic effects.

The best result was achieved with 70% ethanol in combination with low power ultrasound. Ethanol (70%) alone reduced the ATP content to 6.3% and in combination with low power ultrasound to 4.9%. This enhanced reduction was statistically significant (P=0.0227).

The results for ethanol and Preventol R50 were peculiar. Low power ultrasound enhanced the antimicrobial effect of the biocide. High power ultrasound, instead, lead to an ATP content higher than that of low-power sonicated sample. Possibly, high power ultrasound deactivated the biocide by physical destruction of the biocide molecules. In order to have good antimicrobial activity, one of the alkyl groups of quaternary ammonium compounds, such as Preventol R50, need to be eight to eighteen carbons in length (Moore & Payne, 2004). Therefore, any alteration of the molecule may result in severe reduction of the antimicrobial action. Ultrasound is known to accelerate as well as initiate chemical reactions (Suslick & Doktycz, 1990). This does not only mean that a chemical modification of the biocidal molecule can take place but also of the substrate, i.e. the artist's material. Modification of the

chemical or physical properties of the artefact is strictly to be avoided. A chemical alteration of the biocide may not only result in its (partial) deactivation but may also lead to the formation of a chemical substance with unknown effect on the artefact. Therefore, more research is needed on immediate and long-term effects of ultrasound on objects.

5.3.3 Conclusion

The effect of two distinct ultrasound frequencies (267 kHz, 20 kHz) were tested on *V. fischeri* cultures and a mixed microbial population growing in a mature biofilm on limestone, respectively. Low voltage ultrasound (267 kHz) did not kill *V. fischeri*, whereas medium and high voltage significantly reduced the viability. The higher the ultrasound voltage, the lower was the viable count. Sonication at 20 kHz, at both power levels tested, significantly decreased the ATP content of a mixed microbial population, grown in biofilms on stone. Sonication enhanced the antimicrobial effect of the biocides on *V. fischeri* as well as on microbial biofilms. However, the enhancement was small, in fact, all combined treatments, with one exception, exhibited antagonistic effects.

Both sonication experiments showed some peculiarities: (1) low voltage sonication at 267 kHz together with biocides increased the viable count of *V. fischeri*, while sonication alone did not. (2) microbial biofilms treated with ethanol or Preventol R50 and sonicated at high power ultrasound (20 kHz) had a higher ATP content than the corresponding samples sonicated with low power ultrasound. The first may have been an artefact deriving from enhanced cell dilution rather than true increase of viable biomass. The second, however, was more likely an interaction between ultrasound and the biocide leading to its partial deactivation. These results demonstrate that much research is needed on the interaction of ultrasound (with and without biocides) on microorganisms and substrates.

Plate count was a good method to quantify surviving *V. fischeri* cells. When evaluating antimicrobial treatments of unknown microbial populations, ATP reduction provided a reliable tool to measure changes in microbial activity. ATP assessment, however, is an evaluation of the total microbial activity and may not be directly proportional to cell death. It may not distinguish between biocidal and biostatic effects. ATP measurement with firefly luciferin/luciferase is a rapid and very sensitive method to assess the activity of microorganisms. The reproducibility, employing

conventional protocols, depends largely on the skills of the operator and the extraction method (Nieto et al., 1997). Therefore, the automated system employing the HY-LiTE® Pen provides a reliable, reproducible system for the assessment of antimicrobial treatments, which can be carried out by non-microbiologists. Long-term monitoring of microbial activity on cultural heritage objects, often conducted by different operators with limited microbiological experience, can be reliably performed with this system.

Interestingly, ethanol (70%) had more efficient antimicrobial action on microbial biofilms on stone than the two commercially available biocides tested. Even at a concentration largely exceeding that recommended by the suppliers, these biocides did not reduce microbial activity as much as 70% ethanol. The best result was achieved with 70% ethanol in combination with low power ultrasound. The sonication induced enhancement of the antimicrobial effect, however, was very low; ethanol alone reduced the ATP content to 6.26% of the value before treatment and in combination with low power ultrasound it was reduced to 4.93%. Therefore, it is questionable if this further decrease justifies the significantly more complicated technical set up for a combined treatment. McNamara et al. (2003) reported that no difference was detected between the bacterial community of a limestone surface that was cleaned with 50% ethanol and one that had not been cleaned for approximately 1 year. However, it seems to be essential for the success of the antimicrobial treatment that ethanol is applied at a concentration of exactly 70% (I. Nuss, personal communication, 2003). Further tests are needed to assess the antimicrobial effect of ethanol treatment of an outdoor stone object, which cannot be completely submerged into 70% ethanol. If *in situ* tests give satisfying results, 70% ethanol can be highly recommended for outdoor stone monuments. Ethanol is very cheap compared to commercially available biocides, it is available virtually everywhere and has low eco-toxicity as it is expected to evaporate completely before reaching non-target organisms. It is not known to react with stone, however, it might dissolve organic components of the stone patina. This might lead to an alteration of surface properties, such as porosity or water absorption. Again, further tests are needed to assess the effect of 70% ethanol on outdoor stone monuments. As ethanol is highly flammable, appropriate precautions are required when used in large quantities.

Although the results did not indicate synergistic interaction between biocides and ultrasound, investigation on combined treatment with other ultrasound frequencies

and biocides might reveal synergism. Other components for combined treatment against biodeterioration on stone might also provide a route into eco-friendly antimicrobial action. Preliminary results (data not shown) on the effect of microwave radiation (2.45 GHz) on *V. fischeri* demonstrated sub-lethal perturbation at low power (1 W). During irradiation, the kinetics of bioluminescence were similar to those provoked by other stress factors: an initial increase in luminescence was followed by an exponential decrease towards 0. If irradiation was stopped before bioluminescence terminated completely, light emission recovered slightly thereafter. This demonstrated that loss of light emission cannot be solely attributed to cell death but showed that sub-lethal levels of irradiation decreased *V. fischeri* bioluminescence. Hence, microwave radiation is another potential candidate to test for synergistic antimicrobial effects. Indeed, most electromagnetic oscillations of microwave and terahertz radiation, which are coherent in phase, are virtually absent from the natural electromagnetic environment and hence living organisms might have not evolved adaptation mechanisms to them.

Chapter 6
Conclusion

Many or possibly the majority of organisms contributing to biodeterioration are still unknown and even known microorganisms dwelling on stone monuments have rarely been examined for their general physiology and actual contribution to stone decay *in vivo*. A field that urgently demands more attention is the assessment and quantification of biodeterioration. The definition of criteria to distinguish if the damage of an art object was caused by biological action would greatly contribute to the development of an appropriate conservation concept. Quantification of biodeterioration is an important parameter to determine if an antimicrobial action is necessary. In fact, to my knowledge there has never been an attempt for identifying the factors that need to be examined to identify the values that indicate a critical level of biodeterioration. A potential route would be the comparison of areas in good condition with those in poor condition. This might give an insight in whether the amount of microorganisms present are more indicative of the level of biodeterioration or if the kind of organism is a more important criterion to determine the artefact's fate. Also, it might allow us a better understanding of the role that microbial activity and microbial exudates play in the overall destruction process. Long-term monitoring of different areas of the stone is another approach to assess the influence of various parameters on biodeterioration. The rate of change of the object's condition might give information which parameters are more destructive than others.

Protocols for the assessment of proteins, chlorophyll *a*, carbohydrates and microbial activity were optimised for the application on cultural heritage made of stone. Monitored together over years, they contribute to the overall conservation procedure to identify critical changes in microbial activity, composition and biomass. If the tests demonstrate that an antimicrobial treatment becomes essential for the protection of the artefact, the antimicrobial agent needs to be selected carefully. Commercial products, designed for application on outdoor stone, might not be as effective and may be more hazardous to the substrate than traditional methods, such as ethanol. Tests have to be performed for each application, as the susceptibility is expected to vary between different biofilms.

The time of application might influence the success of the treatment. It has been reported that the metabolic state of microorganisms may influence their susceptibility to biocides. Starvation has been reported to render microorganisms more resistant to biocides (Sabev et al., 2006) and high metabolic activity more susceptible (Ensing et al., 2005). This might mean that a biocide treatment could be more efficient in the rainy season, where microbial activity tends to be higher. Elevated temperature during the treatment may also increase biocide efficiency (Setlow et al., 2002). Microbial activity does not appear to vary significantly during the day. Therefore, the best time for a biocidal treatment seems to be during the rainy season at approximately mid-day, however, more work is needed to assess if the time of the antimicrobial treatment really plays a significant role in the efficiency of an antimicrobial treatment.

Combined antimicrobial treatments have long been overlooked in its application on cultural heritage objects. The tested components of ultrasound in combination with a small selection of biocides did not reveal a synergistic effect. However, a systematic, comprehensive study on chemical and/or physical methods might open new doors to a more environmentally friendly approach of microbial eradication. Electromagnetic radiation in the microwave and teraherz spectrum has great potential to contribute to a synergistic or additional effect for a combined antimicrobial treatment. The flexible, *in vivo* monitoring system employing *V. fischeri* bioluminescence demonstrated to be an excellent bioindicator to non-invasively test the effect of a wide range of sub-lethal perturbants. The system can be further developed to test a wide spectrum of perturbants in an automated manner providing high throughput for the assessment of many parameters (e.g. power and exposure time).

For the evaluation of the success of an antimicrobial treatment, ATP measurement has proven to be a simple and precise method that does not require specialised skills. The system can also be used for long-term monitoring of changes in microbial activity. For the employed method a stone sample had to be taken from the object under investigation. However, preliminary results of potential *in situ* systems employing firefly luciferase were reported (Ranalli et al., 2003). To use the suggested system as a true non-destructive method for outdoor cultural heritage monuments, the effect of the reagents on the artefact's materials have to be investigated and a light-proof housing of the area under investigation needs to be

developed. However, the system provides great potential for a non-destructive assessment of microbial activity of outdoor stone monuments with information of the extend of activity and its spatial distribution.

Another area that needs more investigation is the effect of antimicrobial treatments on the attachment strength of microorganisms on their substrate. Ultrasonic toothbrushes have recently become popular for their enhanced cleaning effect. In a similar manner, ultrasound-enhanced antimicrobial treatment in combination with a cleaning process may provide a very efficient tool for the protection of outdoor stone monuments. Furthermore, it should be kept in mind that several conservation treatments, not specifically aimed at the eradication of biodeteriogens, utilise antimicrobial agents, such as ethanol. The whole conservation concept should therefore be an interdisciplinary collaboration between the conservator and the microbiologist.

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Appendices

Appendix 1

Microorganisms isolated from calcareous stone monuments

Table App. 1 Microorganisms detected on stone monuments.

Microbial group	Family/genus/species	References
Algae	*Apatococcus, Asterococcus, Cladophora, Chlorella, Chlorococcum, Coccomyxa, Chrysocapsa, Cyanidium, Dimorphococcus, Eustigmatos, Fragilaria, Gongrosira, Heterococcus, Hormidium, Klebsormidium, Muriella, Nanochlorum, Navicula, Nitzschia, Planktospheria, Pleurococcus, Protococcus, Protoderma, Rhizothallus, Stichococcus, Trentepohlia, Ulothrix.*	Crispim et al., 2003; Flores et al., 1997; Gaylarde et al., 2001; Ohba & Tsujimoto, 1996; Ortega-Morales et al., 2000, 2005; Strzelczyk, 1981; Tiano, 2002; Tiano et al., 1995; Tomaselli et al., 2000a, b.
	Apatococcus lobatus, Botryochloris minima, Chlorella vulgaris, C. elipsoidea, Monodus unipapilla, Oocystis parva, O. marssoni, Protococcus viridis, Stichococcus baciliaris, Trentepohlia umbrina, Ulothrix punctata.	Caneva et al., 2005; Gaylarde et al., 2006; Hoppert et al., 2004; Ohba & Tsujimoto, 1996; Strzelczyk, 1981; Tomaselli et al., 2000a.
Cyanobacteria	*Arthrospira, Calothrix, Chlorogloeopsis*, Chroococcales, *Chroococcidiopsis, Chroococcus, Fischerella, Geitlerinema, Gloeocapsa, Gloethece, Hyella, Leptolyngbya, Lyngbya, Mastigocladopsis, Microcoleus, Myxosarcina, Nodularia, Nostoc,* Oscillatoriales, *Phormidium, Plectonema, Pleurocapsa,* Pleurocapsa-group, *Scytonema, Stanieria,* Stigonematales, *Synechococcus, Synechocystis, Tolypothrix, Xenococcus.*	Ascaso et al., 2002; Crispim et al., 2003; Garcia de Miguel et al., 1995; Gaylarde & Morton, 2002; Gaylarde et al., 2001, 2005; Hoppert et al., 2004; McNamara et al., 2006; Ortega-Morales et al., 2000, 2005; Tiano, 2002; Tomaselli et al., 2000a, b.
	Acaryochloris marina, Anabaena variabilis, Gloeocapsa helvetica, G. kuetzingiana, G. rupestris, Lyngbya matensiana, L. aerugineocoerulea, Oscillatoria pseudogeminata, O. terebriformis, O. subtilissima, Phormidium lignicola, Stigonema ocellatum, S. hormoides.	Caneva et al., 2005; Gaylarde & Englert, 2006; Gaylarde et al., 2005; McNamara et al., 2006; Strzelczyk, 1981.
Other photosynthetic bacteria	Chloroflexi.	McNamara et al., 2006.
	Rhodoplanes elegans.	McNamara et al., 2006.

Archaea	Halophilic bacteria: *Halobacillus, Halobacterium, Halococcus, Halomonas, Natronobacterium* Methanogenic bacteria, methanotrophic bacteria	Heyrman et al., 1999; Piñar et al. 2001; Rölleke et al., 1998, Rölleke et al., 1996 Kussmaul et al., 1998.
Chemolithotrophic bacteria	Nitrogen cycle: *Nitrobacter, Nitrococcus, Nitrosococcus, Nitrosoglobus, Nitrosomonas, Nitrosospira, Nitrosovibrio, Nitrospira.* *Nitrobacter vulgaris, Nitrosomonas ureae, Nitrospira moscoviensis.* Sulfur cycle: *Thiobacillus.* *Thiobacillus thiooxidans, T. thiosporus, T. albertis, T. neapolitanus, T. denitrificans.*	Caneva et al., 1991; Gorbushina et al., 2002; May, 2003; Spieck et al., 1992. McNamara et al., 2006; Pinck et al., 2000. Caneva et al., 1991; Flores et al., 1997; Warscheid & Braams, 2000. May, 2003; Prieto et al., 1995.
Chemoorgano trophic bacteria	Acidobacteria, *Bacillus, Clostridium, Holophaga, Melittangium, Pseudomonas,* sulfate-reducing bacteria. *Bacillus circulans, B. badius, B. licheni, B. cereus, B. licheniformis, B. barbaricus, B. thuringiensis, B. pumilis, B. megaterium,B. firmus.*	Flores et al., 1997; Gaylarde et al., 2001; Gorbushina et al., 2002; Heyrman & Swings, 2001; Kussmaul et al., 1998; McNamara et al., 2006; Ortega-Morales & Hernandez-Duque, 1998; Ortega-Morales et al., 2005; Rölleke et al., 1996; Saarela et al., 2004. Blazquez, 2000; Gaylarde et al., 2001; Heyrman & Swings, 2001; McNamara et al., 2006; Prieto et al., 1997.
Actinomycetes	*Arthrobacter, Aureobacterium, Blastococcus, Brevibacterium, Clavibacter, Geodermatophilus, Micrococcus, Microellobosporium, Micromonospora, Microphylospora, Modestobacter, Nocardia, Nocardiodes, Rhodococcus, Rubrobacter, Streptomyces.* *Arthrobacter (Micrococcus) agilis, Geodermatophilus obscurus, Kocuria rosea, Marmoricola aurantiacus, M. lylae, M. roseus, M. varians, M. halobius, M. agilis, Nocardia restricta, Saccharothrix flava.*	Aranyanak, 1992; Bassi et al., 1986; Caneva & Nugari, 2005; Caneva et al., 1991; Flores et al., 1997; Gorbushina et al., 2002; Heyrman & Swings, 2001; Hyvert, 1966; McNamara et al., 2006; May et al., 2000; May, 2003; Ortega-Morales et al., 2004, 2005; Rölleke et al., 1996; Saarela et al., 2004; Tiano, 2002; Warscheid & Braams, 2000. Blazquez et al., 2000; Eppard et al., 1996; McNamara et al., 2006; May, 2003; Prieto et al., 1995.

Fungi	*Acremonium (Cephalosporium), Alternaria, Aspergillus, Aureobasidium, Botrytis, Candida, Capnobotryella, Cladosporium, Coniosporium, Cryptococcus, Dictyodesmium, Exophiala, Fusarium, Hortaea, Lichenthelia, Mucor, Nectria, Penicillium, Phaeococcomyces, Phaeosclera, Phaeotheca, Phoma, Phialostele, Pseudotaeniolina, Rhinocladiella, Rhizopus, Rhodotorula, Sarcinomyces, Sporobolomyces, Sporotrichum, Trichoderma, Trimmatostroma, Ulocladuim.*	Allsopp et al., 2003; Blazquez et al., 2000; Caneva & Nugari, 2005; Gorbushina et al., 2002; Hirsch et al., 1995b; Monte, 2003; Prieto et al., 1995; Tiano, 2002; Urzi, 2004; Urzi & De Leo, 2001; Urzi et al., 2000; Warscheid & Braams., 2000.
	Acremonium murorum, Aspergillus niger, A. versicolor, A. wentii, Aurobasidium pullulans, Capnobotryella renispora, Chaetomium globosum, Cladosporium cladosporioides, Coniosporium apollinis, C. perforans, C. uncinatus, Exophiala jeanselmei, E. monileae, Hortaea werneckii, Phialophora melinii, Sarcinomyces petricola, Trichoderma viride, Trimmatostroma abietis, Verticillium nigrescens.	Blazquez, 2000; Caneva & Nugari, 2005; Gorbushina et al., 2002; Hoppert et al., 2004; Urzi, 2004.
Lichens	*Aspicilia, Caloplaca, Lecanora, Protoplastenia, Thyrea, Verrucaria, Xanthoria.*	Ascaso et al., 1998, 2002; Tiano, 2002.
	Caloplaca aurantiaca, C. ceria, C. citrina, C. holocarpa, C. trachyphylla, C. concolor, C. vitellina, Collera crispum, Diploicia canescens, Dirina massiliensis, Lecania rabenhorstii, Lecanora hageni, Ochrolechia parella, Phaeophysica hirsute, Tephronella atra	Ascaso et al., 1998, Frey et al., 1993; Hoppert et al., 2004; Prieto et al., 1997, 1999; Seaward, 2003.

Appendix 2

Statistical analysis of the conditions at the sampling sites, the composition of the samples and their activities in the sun versus shade, morning versus afternoon and dry season versus rainy season

App. 2.1 Illumination

These analyses refer to the results in chapter 2.3.2.1

Table App. 2.1 Statistical analysis of the differences in illumination
Statistical analysis of the differences in illumination between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm), in the dry season and in the rainy season.
*1 Msun: sample taken at 8 am from the sun-site
*2 Mshade: sample taken at 8 am from the shade-site
*3 Asun: sample taken at 4 pm from the sun-site
*4 Ashade: sample taken at 4 pm from the shade-site
*5 Dry season
*6 Rainy season
*7 2 sample t-test, 1-tailed
*8 Mann-Whitney test, 1-tailed
*9 Mann-Whitney test, 2-tailed
*10 2 sample t-test, 2-tailed

	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference ($\mu mol \cdot s^{-1} \cdot m^{-2}$) (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	Msun*1 D	Mshade*2 D	greater	2 st, 1-t*7	P=0.002	39.88	50.54
	Asun*3 D*5	Ashade*4 D	greater	M-W, 1-t*8	P=0.0004	1261.44	9.28
	Msun R*6	Mshade R	greater	M-W, 1-t	P=0.000	25.26	47.29
	Asun R	Ashade R	greater	M-W, 1-t	P=0.000	1384.76	5.04
Morning vs Afternoon	Msun D	Asun D	less	M-W, 1-t	P=0.0006	-1309.93	1724.72
	Mshade D	Ashade D	less	2 st, 1-t	P=0.000	-88.36	316.84
	Msun R	Asun R	less	M-W, 1-t	P=0.000	-1410.40	3042.72
	Mshade R	Ashade R	less	M-W, 1-t	P=0.000	-50.90	324.58
Dry vs Rainy	Msun D	Msun R	greater	2 st, 1-t	P=0.045	32.7	59.45
	Asun D	Asun R	not different	M-W, 2-t*9	P=0.1817	-67.8	104.87
	Mshade D	Mshade R	not different	2 st, 2-t*10	P=0.361	18.1	55.62
	Ashade D	Ashade R	greater	2 st, 1-t	P=0.012	55.5	56.98

App. 2.2 Temperature

These analyses refer to the results in chapter 2.3.2.2

Table App. 2.2 Statistical analysis of the differences in temperature
Statistical analysis of the differences in temperature between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.

	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference (°C) (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	Msun D	Mshade D	not different	2 st, 2-t	P=1.000	+/-0.00	100.00
	Asun D	Ashade D	greater	2 st, 1-t	P=0.000	+6.17	85.02
	Msun R	Mshade R	not different	2 st, 2-t	P=1.000	+/-0.00	100.00
	Asun R	Ashade R	greater	2 st, 1-t	P=0.000	+9.49	77.63
Morning vs Afternoon	Msun D	Asun D	less	2 st, 1-t	P=0.000	-13.85	150.72
	Mshade D	Ashade D	less	2 st, 1-t	P=0.000	-7.69	128.15
	Msun R	Asun R	less	2 st, 1-t	P=0.000	-16.21	161.84
	Mshade R	Ashade R	less	2 st, 1-t	P=0.000	-6.72	125.64
Dry vs Rainy	Msun D	Msun R	greater	2 st, 1-t	P=0.000	1.1	95.96
	Asun D	Asun R	not different	M-W, 2-t	P=0.0817	-1.3	103.04
	Mshade D	Mshade R	not different	2 st, 1-t	P=0.361	1.1	95.96
	Ashade D	Ashade R	greater	M-W, 1-t	P=0.0045	2.1	94.08

App. 2.3 Moisture content

These analyses refer to the results in chapter 2.3.3.1

Table App.2.3 Statistical analysis of the differences in moisture content
Statistical analysis of the differences in moisture content between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.

	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference (mg/g FW) (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	Msun D	Mshade D	greater	2 st, 1-t	P=0.002	23.41	72.04
	Asun D	Ashade D	greater	2 st, 1-t	P=0.000	18.17	76.35
	Msun R	Mshade R	not different	2 st, 2-t	P=0.250	20.04	83.41
	Asun R	Ashade R	greater	2 st, 1-t	P=0.019	22.25	71.69
Morning vs Afternoon	Msun D	Asun D	not different	2 st, 2-t	P=0.229	6.89	91.77
	Mshade D	Ashade D	not different	2 st, 2-t	P=0.757	1.65	97.26
	Msun R	Asun R	greater	2 st, 2-t	P=0.011	18.29	84.86
	Mshade R	Ashade R	greater	2 st, 1-t	P=0.002	-33.55	133.30
Dry vs Rainy	Msun D	Msun R	less	M-W, 1-t	P=0.0133	-37.1	144.29
	Asun D	Asun R	not different	M-W, 2-t	P=1.0000	-1.7	102.27
	Mshade D	Mshade R	less	M-W, 1-t	P=0.0035	-40.5	167.06
	Ashade D	Ashade R	not different	M-W, 2-t	P=0.1603	2.3	96.03

App. 2.4 Protein content

These analyses refer to the results in chapter 2.3.3.2

Table App.2.4 Statistical analysis of the differences in protein content
Statistical analysis of the differences in protein content (mg/g dry weight) between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.

	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference (mg/g DW) (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	Msun D	Mshade D	greater	2 st, 1-t	P=0.000	92.84	48.82
	Asun D	Ashade D	greater	2 st, 1-t	P=0.000	84.62	52.50
	Msun R	Mshade R	not different	M-W, 2-t	P=0.1268	10.00	91.20
	Asun R	Ashade R	not different	M-W, 2-t	P=0.3510	-1.59	101.37
Morning vs Afternoon	Msun D	Asun D	not different	2 st, 2-t	P=0.755	3.26	98.20
	Mshade D	Ashade D	not different	M-W, 2-t	P=0.6711	-4.96	105.60
	Msun R	Asun R	not different	M-W, 2-t	P=0.3115	-2.54	102.23
	Mshade R	Ashade R	not different	M-W, 2-t	P=0.1564	-14.13	113.62
Dry vs Rainy	Msun D	Msun R	greater	M-W, 1-t	P=0.0000	67.7	62.69
	Asun D	Asun R	greater	M-W, 1-t	P=0.0000	61.9	65.26
	Mshade D	Mshade R	not different	M-W, 2-t	P=0.1639	-15.2	117.11
	Ashade D	Ashade R	not different	M-W, 2-t	P=0.0213	-24.3	126.01

App. 2.5 Chlorophyll content

These analyses refer to the results in chapter 2.3.3.3

Table App.2.5 Statistical analysis of the differences in chlorophyll content
Statistical analysis of the differences in chlorophyll content between the sun-exposed and shaded sampling site in the morning (8am) and the afternoon (4pm) in the dry season and in the rainy season
*Mean difference related to dry weight is measured in µg/g and to protein content in µg/mg.

	Value related to	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference* (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	DW	Msun D	Mshade D	greater	M-W, 1-t	P=0.0000	478.21	51.07
	Protein			not different	M-W, 2-t	P=0.0620	-1.00	118.66
	DW	Asun D	Ashade D	greater	2 st, 1-t	P=0.000	270.43	69.25
	Protein			less	M-W, 1-t	P=0.0000	-1.22	123.80
	DW	Msun R	Mshade R	not different	M-W, 2-t	P=0.7225	-70.22	109.32
	Protein			less	M-W, 1-t	P=0.0127	-3.53	140.47
	DW	Asun R	Ashade R	less	M-W, 1-t	P=0.0135	-175.40	124.23
	Protein			not different	M-W, 2-t	P=0.1213	-2.44	128.57
Morning vs Afternoon	DW	Msun D	Asun D	greater	2 st, 1-t	P=0.020	97.88	89.99
	Protein			not different	M-W, 2-t	P=0.1698	0.21	96.05
	DW	Mshade D	Ashade D	less	2 st, 1-t	P=0.003	-109.90	122.02
	Protein			not different	M-W, 2-t	P=0.4653	-0.01	100.20
	DW	Msun R	Asun R	not different	M-W, 2-t	P=0.1292	29.25	96.12
	Protein			not different	2 st, 2-t	P=0.108	0.50	92.60
	DW	Mshade R	Ashade R	not different	M-W, 2-t	P=0.4338	-75.93	109.22
	Protein			not different	M-W, 2-t	P=0.3093	0.43	94.76
Dry vs Rainy	DW	Msun D	Msun R	greater	2 st, 1-t	P=0.000	224.2	77.06
	Protein			less	M-W, 1-t	P=0.0000	-1.4	126.83
	DW	Asun D	Asun R	greater	M-W, 1-t	P=0.0001	-324.2	164.96
	Protein			less	M-W, 1-t	P=0.0000	-1.1	122.28
	DW	Mshade D	Mshade R	less	M-W, 1-t	P=0.0000	155.6	82.31
	Protein			less	M-W, 1-t	P=0.0007	-1.8	128.18
	DW	Ashade D	Ashade R	less	M-W, 1-t	P=0.0009	-290.2	147.66
	Protein			not different	M-W, 2-t	P=0.0559	-1.4	121.23

App. 2.6 Carbohydrate content

These analyses refer to the results in chapter 2.3.3.4

Table App.2.6 Statistical analysis of the differences in carbohydrate content
Statistical analysis of the differences in carbohydrate content between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.
*Mean difference related to dry weight is measured in µg/g, to protein content in mg/mg and to chlorophyll content in mg/µg

	Value related to	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference* (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	DW	Msun D	Mshade D	greater	M-W, 1-t	P=0.0000	207.17	42.98
	Protein			not different	2 st, 2-t	P=0.138	0.16	91.76
	Chlorophyll			greater	M-W, 1-t	P=0.0007	0.07	80.03
	DW	Asun D	Ashade D	greater	M-W, 1-t	P=0.0039	226.88	43.88
	Protein			greater	M-W, 1-t	P=0.0001	0.44	81.17
	Chlorophyll			greater	M-W, 1-t	P=0.0000	0.13	70.64
	DW	Msun R	Mshade R	greater	M-W, 1-t	P=0.0000	116.84	64.68
	Protein			greater	M-W, 1-t	P=0.0015	1.08	70.64
	Chlorophyll			greater	M-W, 1-t	P=0.0000	0.21	55.53
	DW	Asun R	Ashade R	greater	M-W, 1-t	P=0.0039	76.43	76.79
	Protein			greater	M-W, 1-t	P=0.0008	1.42	65.82
	Chlorophyll			greater	M-W, 1-t	P=0.0000	0.19	58.74
Morning vs Afternoon	DW	Msun D	Asun D	less	2 st, 1-t	P=0.036	-40.96	111.27
	Protein			less	2 st, 1-t	P=0.001	-0.35	117.79
	Chlorophyll			less	2 st, 1-t	P=0.002	-0.07	119.28
	DW	Mshade D	Ashade D	less	2 st, 1-t	P=0.003	-21.26	113.61
	Protein			not different	M-W, 2-t	P=0.2058	-0.08	104.20
	Chlorophyll			not different	M-W, 2-t	P=0.7699	-0.02	105.28
	DW	Msun R	Asun R	not different	2 st, 2-t	P=0.942	1.52	99.54
	Protein			not different	M-W, 2-t	P=0.7134	-0.48	113.12
	Chlorophyll			not different	2 st, 2-t	P=0.973	0.00	99.77
	DW	Mshade R	Ashade R	not different	M-W, 2-t	P=0.1693	-38.89	118.18
	Protein			not different	M-W, 2-t	P=0.5796	-0.14	105.41
	Chlorophyll			not different	M-W, 2-t	P=0.2308	-0.01	105.54
Dry vs Rainy	DW	Msun D	Msun R	not different	2 st, 2-t	P=0.973	32.6	91.04
	Protein			less	M-W, 1-t	P=0.0000	-1.1	155.93
	Chlorophyll			less	M-W, 1-t	P=0.0037	-0.10	125.82
	DW	Asun D	Asun R	greater	2 st, 1-t	P=0.000	-57.8	136.99
	Protein			less	M-W, 1-t	P=0.0000	-0.7	131.14
	Chlorophyll			not different	M-W, 2-t	P=0.4350	-0.02	105.24
	DW	Mshade D	Mshade R	less	M-W, 1-t	P=0.0103	75.1	81.44
	Protein			less	M-W, 1-t	P=0.0000	-0.2	112.57
	Chlorophyll			not different	M-W, 2-t	P=0.0687	0.04	87.30
	DW	Ashade D	Ashade R	less	M-W, 1-t	P=0.0192	-75.4	142.50
	Protein			less	M-W, 1-t	P=0.0000	-0.2	110.43
	Chlorophyll			not different	M-W, 2-t	P=0.1492	0.04	87.51

App. 2.7 Unspecific enzyme activity by FDA cleavage

These analyses refer to the results in chapter 2.3.4

Table App. 2.7 Statistical analysis of the differences in microbial activity, measured by FDA cleavage Statistical analysis of the differences in microbial activity, measured by FDA cleavage, between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.
*Mean difference related to DW is measured in µg/g, to protein content in µg/mg, to chlorophyll content in µg/µg and to carbohydrate in µg/mg.

	Value related to	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference* (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	DW	Msun D	Mshade D	greater	M-W, 1-t	P=0.0000	254.11	78.18
	Protein			less	2 st, 1-t	P=0.000	-3.85	159.02
	Chlorophyll			less	M-W, 1-t	P=0.0002	-0.52	141.89
	Carbohydrate			less	M-W, 1-t	P=0.0000	-2.61	178.94
	DW	Asun D	Ashade D	greater	M-W, 1-t	P=0.0001	278.00	75.25
	Protein			less	M-W, 1-t	P=0.0010	-2.37	134.58
	Chlorophyll			less	M-W, 1-t	P=0.0023	-0.23	119.04
	Carbohydrate			less	M-W, 1-t	P=0.0000	-2.11	174.51
	DW	Msun R	Mshade R	not different	2 st, 2-t	P=0.484	41.39	96.99
	Protein			not different	M-W, 2-t	P=0.2842	-0.77	105.90
	Chlorophyll			greater	2 st, 1-t	P=0.039	0.21	89.45
	Carbohydrate			less	2 st, 1-t	P=0.000	-2.73	162.68
	DW	Asun R	Ashade R	not different	M-W, 2-t	P=0.1704	-277.13	120.01
	Protein			not different	2 st, 2-t	P=0.593	1.87	90.28
	Chlorophyll			not different	2 st, 2-t	P=0.593	0.32	97.71
	Carbohydrate			less	M-W, 1-t	P=0.0021	-1.26	124.75
Morning vs Afternoon	DW	Msun D	Asun D	not different	M-W, 2-t	P=0.2047	41.63	96.43
	Protein			not different	M-W, 2-t	P=0.9097	-0.32	104.86
	Chlorophyll			not different	M-W, 2-t	P=0.2977	0.02	98.07
	Carbohydrate			greater	2 st, 1-t	P=0.002	0.48	85.53
	DW	Mshade D	Ashade D	not different	M-W, 2-t	P=0.4373	65.51	92.81
	Protein			not different	M-W, 2-t	P=0.0828	1.17	88.74
	Chlorophyll			greater	2 st, 1-t	P=0.014	0.31	82.28
	Carbohydrate			greater	M-W, 1-t	P=0.0099	0.98	83.41
	DW	Msun R	Asun R	not different	M-W, 2-t	P=0.3268	-9.21	100.67
	Protein			not different	2 st, 2-t	P=0.681	-1.02	107.78
	Chlorophyll			not different	2 st, 2-t	P=0.303	-0.2	108.79
	Carbohydrate			not different	M-W, 2-t	P=0.3444	-0.5	110.97
	DW	Mshade R	Ashade R	not different	M-W, 2-t	P=0.3436	-327.73	124.56
	Protein			not different	M-W, 2-t	P=0.6014	0.08	99.45
	Chlorophyll			not different	M-W, 2-t	P=0.7630	-0.02	100.96
	Carbohydrate			not different	M-W, 2-t	P=0.5269	0.72	89.84
Dry vs Rainy	DW	Msun D	Msun R	less	M-W, 1-t	P=0.0000	-211.23	118.14
	Protein			less	M-W, 1-t	P=0.0000	-6.60	201.11
	Chlorophyll			less	M-W, 1-t	P=0.0000	-0.74	159.77
	Carbohydrate			less	M-W, 1-t	P=0.0000	-1.05	131.66
	DW	Asun D	Asun R	less	M-W, 1-t	P=0.0001	-262.07	123.34
	Protein			less	M-W, 1-t	P=0.0000	-7.31	206.71
	Chlorophyll			less	M-W, 1-t	P=0.0000	-0.94	177.24
	Carbohydrate			less	M-W, 1-t	P=0.0000	-2.00	170.82
	DW	Mshade D	Mshade R	less	M-W, 1-t	P=0.0000	-423.96	146.56
	Protein			less	M-W, 1-t	P=0.0006	-3.52	133.93
	Chlorophyll			not different	2 st, 2-t	P=0.923	-0.01	100.73
	Carbohydrate			not different	M-W, 2-t	P=0.2750	-1.16	119.69
	DW	Ashade D	Ashade R	less	M-W, 1-t	P=0.0000	-817.20	196.70
	Protein			less	M-W, 1-t	P=0.0001	-4.62	150.09
	Chlorophyll			less	M-W, 1-t	P=0.0021	-0.34	123.60
	Carbohydrate			less	M-W, 1-t	P=0.0008	-1.43	128.93

App. 2.8 DHA

These analyses refer to the results in chapter 2.3.4

Table App. 2.8 Statistical analysis of the differences in microbial activity, measured by INT reduction Statistical analysis of the differences in microbial activity, measured by INT reduction, between the sun-exposed and shaded sampling site in the morning (8 am) and the afternoon (4 pm) in the dry season and in the rainy season.
*Mean difference related to DW is measured in µg/g, to protein content in µg/mg, to chlorophyll content in µg/µg and to carbohydrate in µg/mg.

	Value related to	Sample a	Sample b	Statistical difference	Statistical test	P-value	Mean difference* (a – b)	Percentual difference (% of b in reference to a)
Sun vs Shade	DW	Msun D	Mshade D	not different	2 st, 2-t	P=0.367	260.88	90.37
	Protein			less	2 st, 2-t	P=0.000	-14.80	198.71
	Chlorophyll			less	2 st, 1-t	P=0.000	-1.99	171.00
	Carbohydrate			less	M-W, 1-t	P=0.0000	-8.86	216.20
	DW	Asun D	Ashade D	not different	2 st, 2-t	P=0.103	-349.06	113.00
	Protein			less	2 st, 2-t	P=0.000	-15.32	192.37
	Chlorophyll			less	M-W, 1-t	P=0.0000	-2.40	181.75
	Carbohydrate			less	M-W, 1-t	P=0.0000	-10.41	259.81
	DW	Msun R	Mshade R	not different	M-W, 2-t	P=0.1252	-333.33	115.82
	Protein			not different	2 st, 2-t	P=0.535	-5.51	128.01
	Chlorophyll			not different	2 st, 2-t	P=0.391	-0.27	108.87
	Carbohydrate			less	M-W, 1-t	P=0.000	-6.39	200.10
	DW	Asun R	Ashade R	not different	2 st, 2-t	P=0.734	-99.44	104.15
	Protein			not different	2 st, 2-t	P=0.205	-2.53	111.39
	Chlorophyll			not different	2 st, 2-t	P=0.643	0.13	96.45
	Carbohydrate			less	M-W, 1-t	P=0.000	-4.16	151.46
Morning vs Afternoon	DW	Msun D	Asun D	not different	2 st, 2-t	P=0.870	24.52	99.10
	Protein			not different	2 st, 1-t	P=0.136	-1.59	110.62
	Chlorophyll			not different	2 st, 2-t	P=0.350	-0.14	104.97
	Carbohydrate			not different	2 st, 2-t	P=0.089	1.11	85.48
	DW	Mshade D	Ashade D	less	M-W, 2-t	P=0.0094	-585.42	123.90
	Protein			not different	2 st, 1-t	P=0.236	-2.11	107.09
	Chlorophyll			not different	2 st, 2-t	P=0.328	-0.55	111.57
	Carbohydrate			not different	2 st, 2-t	P=0.492	-0.45	102.72
	DW	Msun R	Asun R	not different	2 st, 2-t	P=0.153	-287.9	113.66
	Protein			not different	2 st, 2-t	P=0.974	-2.6	113.11
	Chlorophyll			not different	2 st, 2-t	P=0.303	-0.6	121.26
	Carbohydrate			not different	M-W, 2-t	P=0.2292	-1.7	126.66
	DW	Mshade R	Ashade R	not different	2 st, 2-t	P=0.810	-53.98	102.21
	Protein			not different	2 st, 2-t	P=0.405	0.40	98.43
	Chlorophyll			not different	M-W, 2-t	P=0.5493	-0.24	107.42
	Carbohydrate			not different	M-W, 2-t	P=0.7105	0.53	95.87
Dry vs Rainy	DW	Msun D	Msun R	greater	M-W, 1-t	P=0.0010	602.8	77.76
	Protein			less	M-W, 1-t	P=0.0113	-4.7	131.18
	Chlorophyll			less	M-W, 1-t	P=0.0000	-0.2	107.65
	Carbohydrate			greater	2 st, 1-t	P=0.010	1.2	83.71
	DW	Asun D	Asun R	not different	M-W, 1-t	P=0.0522	290.4	89.19
	Protein			less	M-W, 1-t	P=0.0016	-5.7	134.13
	Chlorophyll			not different	M-W, 2-t	P=0.5253	-0.7	124.36
	Carbohydrate			not different	M-W, 1-t	P=0.3507	-1.6	124.05
	DW	Mshade D	Mshade R	not different	M-W, 2-t	P=0.7480	8.5	99.65
	Protein			not different	M-W, 2-t	P=0.0706	4.6	84.50
	Chlorophyll			less	M-W, 1-t	P=0.0040	1.5	68.54
	Carbohydrate			greater	M-W, 1-t	P=0.0002	3.7	77.48
	DW	Ashade D	Ashade R	less	2 st, 1-t	P=0.985	540.0	82.21
	Protein			less	2 st, 1-t	P=1.000	7.1	77.67
	Chlorophyll			greater	M-W, 1-t	P=0.0000	1.8	65.99
	Carbohydrate			greater	2 st, 1-t	P=0.000	4.7	72.32

Appendix 3

Nutrient media for the cultivation of *V. fischeri*

Seawater Medium

This is a combination of two recommended media for *Vibrio fischeri* by Andre (2004) and Madden & Lidesten (2001).

Seawater	750.0 ml
Peptone (Soybean Tryptone)	5.0 g
Yeast Extract	3.0 g
Glycerol	3.0 ml

Make up to 1 l with distilled water.
Autoclave for 15 min at 121°C.

Artificial Seawater

H_2O_2	1000.0 ml
NaCl	28.0 g
$MgCl_2 \cdot 6H_2O$	5.0 g
$MgSO_4 \cdot 7H_2O$	4.5 g
$CaCl_2 \cdot 2H_2O$	1.5 g
KCl	0.8 g

Michael Rayner's Photobacterium Broth

After: Lloyd, D.; Jones, G.; Rayner-Brandes, M.; Ward, M. A. A.; Lason, J.; James, C. J. Luminescence-controlled continuous culture of luminous bacteria: Design and application of a small volume bioluminostat for continuous ecotoxicant monitoring and sensitive oxygen assay. Unpublished paper.

NaCl	25.0 g
Na_2HPO_4	15.5 g
Glycerol	10.0 ml
Difco Nutrient Broth	8.0 g
NaH_2PO_4	2.0 g

Adjust to pH 7.6 using KOH.
Make up to 1 l with distilled water.
Autoclave for 15 min at 121°C.

Michael Rayner's Photobacterium Broth New

NaCl	23.0 g
Na_2HPO_4	15.5 g
Nutrient Broth No.2 (Oxoid)	10.0 g
NaH_2PO_4	2.0 g

(For media containing 0.3% Glycerol, add 3 ml Glycerol)

Make up to 1 l with distilled water.
Autoclave for 15 min at 121°C.

Seawater Agar

NaCl	15.0 g
Agar (Bacto agar)	7.5 g
Peptone (Soybean Tryptone)	2.5 g
Yeast Extract	1.5 g
$CaCO_3$	2.5 g
Glycerol	2.5 ml

Make up to 500 ml with distilled water.
Autoclave for 15 min at 121°C.

Starvation Buffer

NaCl	25.0 g
Na_2HPO_4	15.5 g
NaH_2PO_4	2.0 g

Make up to 1 l with distilled water
Adjust to pH 7.5 with KOH or NaOH or KCl

Appendix 4

The effect of the combinational treatment of biocides and ultrasound on *V. fischeri* viability and ATP reduction of microbial biofilms on stone

Table App. 4.1 Effect of combinational treatment of biocides and ultrasound on *V. fischeri* viability
The effect of the combined treatment of biocides and ultrasound on *V. fischeri* viability is expressed as % reduction of the untreated *V. fischeri* sample.

Component A	Component B	A+B	Measured reduction	Combined effect
Ethanol, 7%	30V (2.38744%)	>100%	99.91610%	antagonistic
	40V (64.13131%)	>100%	99.92785%	antagonistic
(99.98547%)	50V (95.70431%)	>100%	99.99921%	antagonistic
Ethanol, 10%	30V (2.38744%)	99.058%	99.08187%	additive
	40V (64.13131%)	>100%	99.99587%	antagonistic
(96.67092%)	50V (95.70431%)	>100%	99.99984%	antagonistic
Mergal K14, 1%	30V (2.38744%)	>100%	99.94065%	antagonistic
	40V (64.13131%)	>100%	99.97760%	antagonistic
(99.93951%)	50V (95.70431%)	>100%	99.99839%	antagonistic
Mergal K14, 1.5%	30V (2.38744%)	>100%	99.99615%	antagonistic
	40V (64.13131%)	>100%	99.99986%	antagonistic
(99.99645%)	50V (95.70431%)	>100%	99.99995%	antagonistic
Mergal K14, 2%	30V (2.38744%)	>100%	99.99903%	antagonistic
	40V (64.13131%)	>100%	99.99926%	antagonistic
(99.99869%)	50V (95.70431%)	>100%	100%	unclear
Parmetol DF12, 0.05%	30V (2.38744%)	>100%	30.81013%	antagonistic
	40V (64.13131%)	>100%	99.31989%	antagonistic
(99.95909%)	50V (95.70431%)	>100%	99.99551%	antagonistic
Parmetol DF12, 0.1%	30V (2.38744%)	>100%	99.98030%	antagonistic
	40V (64.13131%)	>100%	63.69686%	antagonistic
(99.98030%)	50V (95.70431%)	>100%	99.97872%	antagonistic
Parmetol DF12, 0.15%	30V (2.38744%)	>100%	99.99718%	antagonistic
	40V (64.13131%)	>100%	99.99621%	antagonistic
(99.99748%)	50V (95.70431%)	>100%	99.99707%	antagonistic
Preventol R50, 0.01%	30V (2.38744%)	45.14649	-24.98248%	antagonistic
	40V (64.13131%)	>100%	47.03689%	antagonistic
(42.75905%)	50V (95.70431%)	>100%	87.87289%	antagonistic

Preventol R50, 0.02%	30V (2.38744%)	97.96711	92.28021%	antagonistic
	40V (64.13131%)	>100%	99.98716%	antagonistic
(95.57967%)	50V (95.70431%)	>100%	99.99082%	antagonistic
Preventol R50, 0.05%	30V (2.38744%)	>100%	99.70434%	antagonistic
	40V (64.13131%)	>100%	99.99897%	antagonistic
(99.97668%)	50V (95.70431%)	>100%	99.99994%	antagonistic
Troysan S97, 0.2%	30V (2.38744%)	94.29997	56.59575%	antagonistic
	40V (64.13131%)	>100%	86.37829%	antagonistic
(91.91253%)	50V (95.70431%)	>100%	99.94858%	antagonistic
Troysan S97, 0.5%	30V (2.38744%)	>100%	99.93226%	antagonistic
	40V (64.13131%)	>100%	99.96903%	antagonistic
(99.99472%)	50V (95.70431%)	>100%	99.99033%	antagonistic
Troysan S97, 0.75%	30V (2.38744%)	>100%	99.99601%	antagonistic
	40V (64.13131%)	>100%	99.99238%	antagonistic
(99.99979%)	50V (95.70431%)	>100%	99.99998%	antagonistic

Table App. 4.2 Effect of combinational treatment of biocides and ultrasound on ATP content
The effect of the combinational treatment of biocides and ultrasound on ATP content is expressed as % ATP reduction of the untreated sample.
* ultrasound

Component A	**Component B**	**A+B**	**Measured reduction**	**Combined effect**
Ethanol, 70% (93.74%)	Low US* (45.22%)	>100%	95.07%	antagonistic
	High US (63.04%)	>100%	93.78%	antagonistic
Troysan S97, 2%	Low US (45.22%)	98.19%	60.63%	antagonistic
(52.97%)	High US (63.04%)	>100%	63.88%	antagonistic
Troysan S97, 6%	Low US (45.22%)	>100%	75.05%	antagonistic
(61.95%)	High US (63.04%)	>100%	83.06%	antagonistic
Preventol R50, 2% (70.74%)	Low US (45.22%)	>100%	83.37%	antagonistic
	High US (63.04%)	>100%	80.12%	antagonistic
Preventol R50, 6% (84.52%)	Low US (45.22%)	>100%	88.08%	antagonistic
	High US (63.04%)	>100%	81.18%	antagonistic