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Heteroorganic molecules and bacterial biofilms: Controlling biodeterioration of cultural heritage

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Dedicated to Prof. Jacek Młochowski on the occasion of his 80th anniversary

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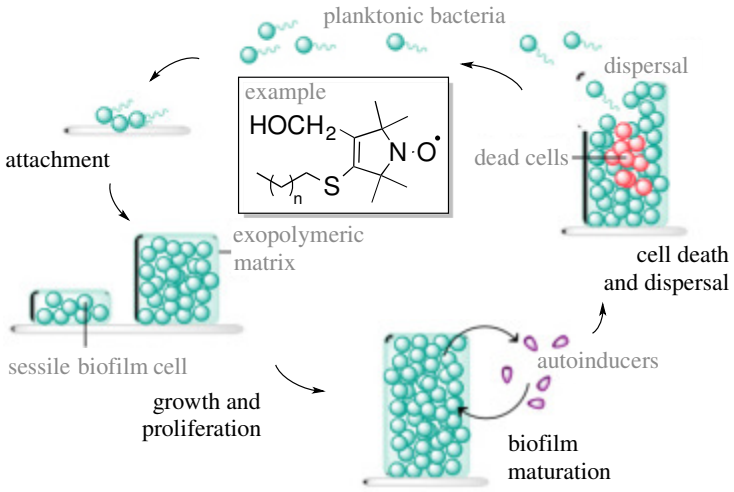
Abstract

In this review we describe the mechanisms of biodeterioration, the challenges faced by heritage conservators in treating biodeterioration caused by bacterial growth and metabolism, and outline current remediation techniques found to inhibit the growth of bacterial biofilms and induce their dispersal.



Keywords: Biodeterioration, biofilm, dispersal, anti-biofilm, nitric oxide, nitroxide

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1. Introduction

Biofouling and biodeterioration of materials caused by bacterial biofilms are significant problems that impact many sectors of society. Critical examples include the biofouling of turbines and ship hulls resulting in increased drag and reduced hydroelectricity generation and maritime fuel efficiencies; and chronic systemic infections and impaired wound healing, especially in diabetic patients, affecting the quality of life of millions of people worldwide.[1,2] In addition, considerable aesthetic and structural damage to culturally significant materials and monuments – such as the 12th-century Hindu Temple at Angkur Wat (Cambodia) – can be caused by the growth of biofilms and the production of harmful metabolites by microorganisms.[3] These processes usually begin with bacterial colonisation of a substrate leading to the formation of a biofilm.[4,5] Research suggests that the biofilm mode of bacterial growth modifies the substrate, thereby providing nutrients for successive colonisers such as mould, diatoms, algae and invertebrates.[4,5] The dispersal of single-celled planktonic bacteria from biofilms is an important mechanism by which the cycle of colonization and infection continues.[6] Although more motile in the planktonic form, bacteria leaving the physical protection of a biofilm are 1000-times more susceptible to exogenous pressures such as antibiotics and biocides.[7] As such,

much research has focused on the development of preventive techniques that inhibit biofilm formation, and remediation techniques that induce biofilm dispersal (anti-biofilm agents), with the anticipation that they will increase eradication efficacy and decrease chemical load when used in combination with low doses of biocides.[8]

Free radical and redox processes within bacterial biofilms are critical to the life cycle of the biofilm because they trigger events that include cell proliferation and survival, enzyme inhibition, cell death and cell transformation.[9] These processes often rely on "redox shuttling" aided by polymers within the biofilm matrix itself.[10] As such, these electron transfer events can be considered to be "chemical signalling processes" that result in changes to the biofilm, including growth and dispersal. One example is the free radical nitric oxide (NO) that at low (nM) non-toxic concentrations is an important signalling molecule that induces biofilm dispersal.[11-13] Encouraging biofilms to disperse by beneficially interrupting these chemical signalling processes lies at the heart of solving biofilm-related problems in areas such as materials science, energy efficiency, hygiene and cultural materials conservation.

To fill a current gap in the literature, this review aims to outline the theory of bacterial biofilms and recent key discoveries towards the development of novel small molecule and free radical-based remediation strategies as they relate specifically to the interdisciplinary field of heritage conservation.

2. Biodeterioration of Cultural Materials

Culture is 'a source of identity, innovation, and creativity'.[14] In a United Nations Educational, Scientific and Cultural Organization (UNESCO) report, cultural industries were estimated to have contributed more than US$1.3 trillion to the global economy in 2005 alone, accounting for more than 7% of global gross domestic product (GDP).[14] While cultural heritage represents a subset of this industry, strategies towards its conservation are a vital activity given its fragile nature, and economic and social importance. Government and industry reports have consistently acknowledged that risks to, and long-term security of, this heritage are not clearly understood or quantified.[14-17] Since 2001 physical degradation, poor accessibility, lack of research and lack of relevant industry training have been identified as key threats.[18]

'*Culturally significant materials*' is a term used to describe all manner of art, artefacts and objects upon which our society places a particular historic, aesthetic, scientific, or ethnological value. Our global cultural heritage is extremely vast, with an estimated 1,032 World Heritage sites in 163 countries,[19] 55,000 museums in 202 countries,[20] and an abundance of art galleries and small heritage collections. Not surprisingly, the amazing multitude of culturally significant materials within these sites and collections comes with an extensive range of ecological habitats and chemical compounds which provide a source of nutrients and energy for an equally extensive array of microorganisms.[21] As a consequence, microorganisms are able to colonize all types of cultural materials from wall murals to ancient parchments to stone monuments, often causing extensive and irreversible aesthetic and structural damage.[5,21,22] Biodeterioration, a term first coined by Hueck in 1965, is *'any undesirable change in the properties of a material caused by the vital activities of organisms'*.[23,24] A key aim for conservation science is to advance our understanding of the processes leading to biodeterioration and ultimately develop new conservation-driven biodeterioration treatments.

The early visible aesthetic signs of biodeterioration such as pigment discolouration and staining are frequently the consequence of both assimilatory and dissimilatory biodeterioration and often result in successive and complex interactions between a community of organisms and the physio-chemical

environment provided by these materials *in situ*.[5,22] Assimilatory biodeterioration is the most commonly understood form of biodeterioration and involves the microorganisms using the material as a nutrient source. Dissimilatory biodeterioration involves the chemical degradation of a material through the chemical and structural interactions of organisms with the material without the direct use of the material for nutrition.[22] The type of changes occurring in the material as a consequence of both forms of biodeterioration can be described as either biophysical or biochemical. Biophysical deterioration encompasses all manner of physical changes on the material such as superficial losses due to surface attachment and detachment of microorganisms during their growth and movement. Additionally, penetration and exertion pressure result in mechanical/structural damage.[25]

Biochemical deterioration is the most complex form of biodeterioration and occurs by the direct action by microorganisms' metabolic processes on the material substrate. This may involve a bio-corrosion process whereby acidic and pigmented organic and inorganic products are excreted which can etch and stain the object, weakening the matrix of the material, leading to more favourable conditions for further attachment and growth and therefore continuing to add to the degradative process.[4,26] Microorganisms may also secrete chelating agents which form complexes and sequester metallic cations from paint pigments or metallic objects.[25,27] For example, cadmium is known to be accumulated in a large number of microorganisms as cell-bound cadmium hydrogen phosphate ($CdHPO_4$).[28] Intracellular leaching of the cadmium cations, from commonly used cadmium-based pigments (cadmium yellow, orange or red), presumably as a bacterial detoxification mechanism,[29] will result in permanent colour alteration. Another example is the chelating affinity of siderophores for iron.[30] As a common metal used in sculpture and a metal cation in many pigments (e.g. Prussian Blue, Yellow Ochre, Red Ochre, Burnt/Raw Sienna, Raw Umber), the establishment of a concentration gradient from the immobilization of iron cations by siderophores and the continual iron leaching process will be deleterious to iron-based cultural materials contaminated with microorganisms. Metal cations are also capable of being bound by the electronegative phosphoryl groups of lipopolysaccharides and phospholipids on the outer membrane of bacteria.[31] While immobilizing toxic metals and preventing entry into the cell, the fate of the metal is closely tied to the fate of the cell and therefore these metal cations are capable of migration, deposition, accumulation and thus colour alteration of the painted surface as the cell undergoes cell movement, metabolism and apoptosis.[29] Biochemical deterioration may also occur enzymatically, with enzymes secreted by colonizing microorganisms catalyzing the degradation of organic molecules such as cellulose fibres in paper or collagen in parchment.[32] Whatever the mechanism, the interactions between cultural materials and colonizing microorganisms that lead to biodeterioration are numerous, complex and influenced by a number of factors. These include:[5,33]

i) The bioreceptivity of the material to colonization, which encompasses the chemical composition of the artefact and the totality of its associated properties (e.g. porosity, pH, surface roughness);
ii) The environmental conditions, such as relative humidity, temperature and air quality as well as the physical and chemical influences of cleaning, display and storage;
iii) The biochemistry of the individual colonized organisms, which include nutritional requirements of, and metabolites produced by, the colonizing microorganism.

An awareness of the mechanisms involved in biodeterioration is required in order to develop a careful and informed approach to the conservation of fragile biodeteriorated materials. Detailed discussions of biodeterioration processes on particular material types have been thoroughly reviewed elsewhere,[5,32-35] and it has been firmly established that the chemical and physical processes of microorganisms on cultural materials

leading to biodeterioration is vast and complex with intertwining chemistries between organism, material, and environment.

2.1 Current biodeterioration treatment practices in conservation

Biodeterioration treatment and control in regards to art, artefacts and archival materials often results in high expenditure for the institutes whose job it is to protect and care for our cultural heritage.[22,34-38] Two methodologies towards the control of biodeterioration currently dominate – preventive and remedial. A preventive treatment methodology encompasses the control of relative humidity, temperature and air quality within recommended limits in a non-invasive manner in order to create an unfavourable environment for microorganisms to flourish.[36,37,39,40] While this non-invasive mantra is likened to the idiom '*prevention is better than cure'*, preventing biodeterioration is not always practical, for example in immovable heritage, nor is it always possible. Conservators may then turn to a remedial conservation methodology, which involves interventive treatments of biodeteriorated materials to eliminate degradation products induced by microorganisms and wherever possible, delay reoccurrence. The effectiveness of remedial treatment techniques depends on the methods and products utilized as well as conservation technique, and for objects not within a controlled environment routine treatment is often necessary. Of course the appropriateness of an interventive treatment must always be evaluated taking into account the identity of the biodeteriogens, degree and type of damage, safety of a treatment towards the constituent materials of the object, risk for the conservator and possible environmental impacts (toxicology and ecotoxicology), in addition to the intangible attributes of the objects. The ethical considerations associated with preservation and conservation of cultural material are complex and as numerous books are dedicated to the subject these considerations will not be discussed further.[37,41-45] Current remedial and interventive techniques to treat biodeteriorated materials are mechanical, physical and chemical in nature and any given conservation treatment may require the combination of two or more techniques. These methods will be discussed briefly below.

2.1.1 Mechanical techniques. The removal of biodeteriogens such as bacteria and fungi through mechanical means - scraping, abrasion, scrubbing etc. - are widely utilized in conservation treatments due to their simplicity and immediacy of results. However as colonization is rarely superficial, complete elimination of biodeteriogens and associated metabolites by mechanical action alone is difficult to achieve without damaging the object. Results are generally short lived, and for this reason mechanical methods must be combined with physical or chemical techniques to ensure the longevity of the treatment.[25,35,46,47]

2.1.2 Physical techniques. Physical methods such as electromagnetic radiation (microwaves, ultra violet and gamma rays),[48-63] anoxic treatments,[64,65] and extreme temperatures,[66-70] have biocidal activity towards the biodeterioration-inducing organisms. This may be through direct interaction with genetic material or alteration of cellular structure and function. The use of physical methods are not yet wide spread due mainly to the associated cost and to possible damage of the materials to which the treatment is applied.[33,71] Regardless, physical methods do not provide any level of prevention from subsequent, and in theory, immediate re-colonization.

2.1.3 Chemical techniques. The most popular interventive technique towards the control of biodeterioration is the use of chemical biocides (also known as disinfectants, bactericides or antimicrobial agents). Biocides are

usually employed in liquid form (brushed, sprayed or in a bath) or as gaseous fumigants, and work by disrupting bacterial or fungal membrane function or inhibiting vital cellular processes thereby leading to cell death.[33] A vast range of commercial products and formulations based on both organic and inorganic compounds such as pentachlorophenol, tributyltin oxide, ethylene dioxide, methyl bromide, prussic acid, and arsenic and mercuric derivatives have been utilized in the past, however due to the significant toxicological risks associated with many of these compounds and the limited knowledge of their compatibility with historic materials, their use has become infrequent.[72,73] Current commercial biocides used in conservation are composed of a wide variety of chemical classes, such as quaternary ammonium salts, halogenated compounds, organometallics, aromatics and isothiazolinones, with the choice of biocide depending primarily on material type, microorganism type and biocide availability.[33,74-80] There is a growing lack of biocidal efficacy however, which encourages the use of excess concentrations of chemical agents resulting in increased expenditure, increased chemical load to the contaminated cultural material and an increase in the toxicological risk for conservators applying the treatment. Furthermore, this chemical blanket approach may lead to the development of tolerance by biodeterioration-inducing microorganisms rendering the treatment ineffective in the long term.[81] There is also growing evidence that bacteria in particular respond specifically and defensively to antimicrobial treatments forming surface adherent microcolonies with reduced cellular growth and respiration that are physically protected from biocidal activity by a communal existence.[82-86]

3. Biofilms are Primary Colonizers in the Biodeterioration Process

A great variety of microorganisms have been found on culturally significant materials.[5,46] However research into the mechanisms of biodeterioration on these types of materials indicates that bacterial microorganisms – such as prototrophs, which do not require organic material for growth – play a significant role as primary colonizers. They do so by modifying substrates and providing through their growth, movement, metabolic processes and cell mass, organic nutrients for successive colonizers such as fungi.[4,5] The proliferation and persistence of these organisms and their potential to cause biodeterioration, either directly or through successive colonization, is the establishment of surface-associated assemblages of microorganisms, known as biofilms which provide enhanced resistance to physical and chemical stress through a multicellular existence.[87] The formation and survival of bacterial biofilms on various surfaces is well documented. Although still not fully understood, through microscopic and molecular techniques, it has been revealed that biofilm formation is a complex process regulated by both genetic and environmental factors.[88] While many species of bacteria are able to form biofilms, further discussions on structure, function and treatment of biofilms will focus primarily on the ubiquitous gram-negative bacterium, *Pseudomonas aeruginosa*. Identified in association with biodeteriorated cultural material, *P. aeruginosa* remains one of the most extensively studied model organisms for bacterial biofilm formation.[5,38]

3.1 Attachment

Common to many motile bacterial species, biofilm growth can be seen as a stagewise process (Figure 1). Planktonic, or single-cell bacteria travel to the surface of an artwork by diffuse, convective and flagellum-mediated transport,[89] where they may then attach reversibly.[90] The first bacterial colonists to adhere to a surface initially do so by weak van der Waals forces. After enough cells attach to a solid surface, the genes that allow a biofilm to grow and proliferate are activated and the new colonizers begin to secrete

exopolymeric substance (EPS) (also known as the glycocalyx) in order to anchor themselves more permanently.[91] The EPS is composed of a variety of biomolecules mainly, polysaccharides, proteins and nucleic acids secreted by the colonizing bacteria.[87] These biopolymers aid in sticking the cells to the material surface and their adhesive properties contribute to the formation and cohesion of biofilms, forming a matrix in which the cells are embedded. Cell debris and inorganic material absorbed from the colonized surface, also contribute to the EPS.[92] Furthermore, the anionic nature of exopolymers that maintain a hydrated, fibrous extracellular matrix, also strongly adsorb cations, minerals and dissolved organic molecules from the external environment and can stabilize airborne dust particles and spores.[92,93]

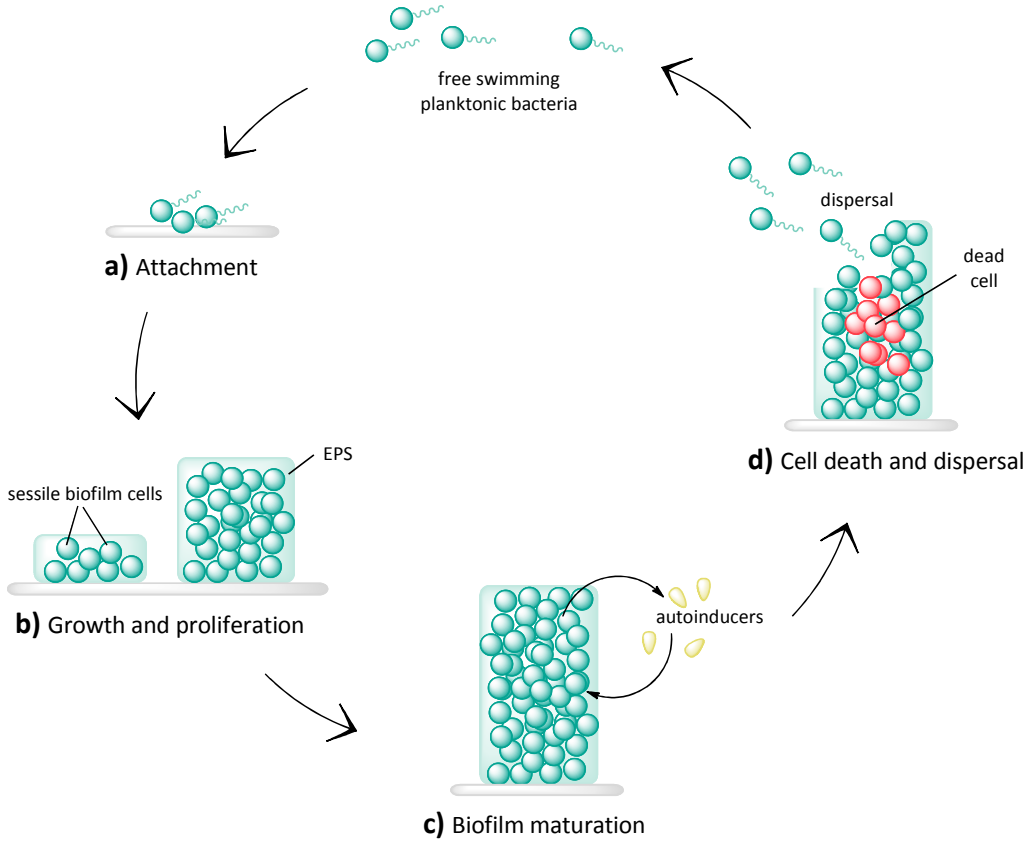


Figure 1. Pictorial representation of biofilm development. Planktonic bacteria may associate reversibly to a surface (**a**) or they may adhere and undergo growth and proliferation (**b**). Extracellular polymeric substances (EPS) produced by biofilm cells reduce the vulnerability of cells to physico-chemical pressures, including biocides. Biofilm microcolonies undergo maturation (**c**) and autoinducers (bacterial chemical signaling molecules) signal sessile biofilm cells to be released from the biofilm via dispersal (**d**), returning to their planktonic state.

3.2 Biofilm maturation

With the protection of the EPS serving as an enclosed microenvironment, the biofilm can develop and mature.[87,94] Early maturation of the biofilm is often observed by the physiological changes from planktonic cells to sessile biofilm cells which are manifested as the biofilm structure becomes three dimensional in space (Figure 1b and c).[91] As the biofilm fully matures, characteristic morphological and topographical features become evident. Mushroom cap formation[95] and unique pillar shapes protrude from the biomass allowing for maximization of nutrient adsorption and waste disposal, while cavities or hollow water-filled channels throughout the biofilm form and provide the biofilm with the transport necessary to deliver nutrients deep

within the complex cellular community.[96 97] These channels also allow the biofilm community to expel planktonic bacteria in a process known as dispersal.[87,98]

3.3 Biofilm detachment and dispersal

Bacteria within a biofilm often undergo regulated and coordinated dispersal events in which sessile biofilm cells detach from the biofilm matrix and convert to free swimming planktonic bacteria (Figure 1d).[99,100] Detachment initiation has been hypothesized to occur in response to specific endogenous or exogenous cues such as high cell density or changes in nutrient levels that trigger starvation and eventual cell death.[101-104] Free radicals and redox processes are involved in the communal behaviour of bacteria, providing the triggers for biofilm formation that often results in biofouling and biodeterioration.[83,87] While it has been established that these processes are important in regulating key events in the biofilm life cycle, the exact mechanism governing detachment and dispersal events are complex and still poorly understood.[105,106] Regardless of how the specific detachment cues are detected, the phenotypic changes they initiate are evident. Induction of a cascade of signalling pathways may result in an increase in matrix-degrading enzymes, a decrease in EPS[107] and the evacuation of the interior of microcolonies, forming hollow vacuoles (Figure 2), which release and disperse planktonic bacteria into the environment to form new colonies on a distal, nutrient-rich surface.[99,102,108,109]

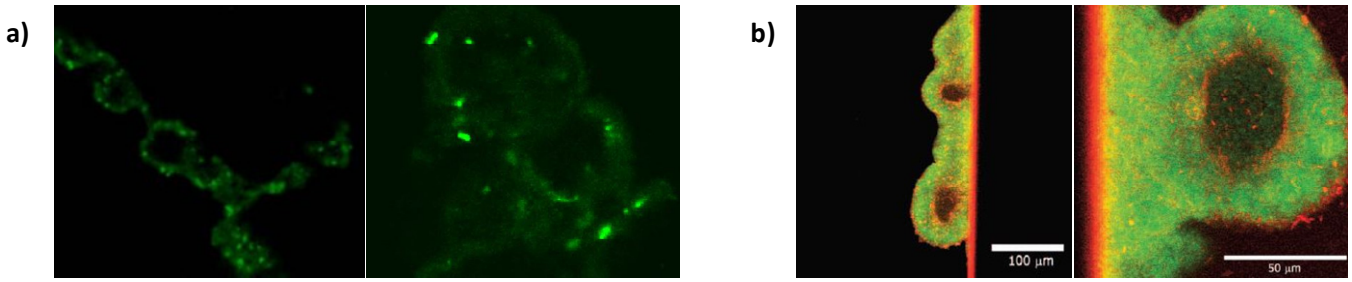


Figure 2. A biofilm undergoing cell lysis and dispersal showing hollow cavities filled with highly motile cells that are released and dispersed upon opening of the channels. **a)** Cells are stained with SYTO 9;[110] **b)** Cells contain green fluorescent protein and are counterstained with rhodamine B (red).[102]

Lee, Li and Bowden[111] showed that a surface protein releasing enzyme mediates the release of cells from *S. mutans* biofilms, while Boyd and Chakrabarty[112] showed that degradation of the exopolysaccharide alginate in *P. aeruginosa* through the over expression of alginate lyase induces increased detachment. Cell-signalling through the release of autoinducers has also been found to be negatively correlated with cell aggregation,[113] the reduction of biofilm biomass and a loss of EPS.[107] In order to detach efficiently, the morphology of the cells must also change. In a coordinated series of events called the '*launch sequence*', type IV pili are required for suitable orientation towards the biofilm surface and flagella are required to break loose and swim away.[114] Thus both motility appendages, flagella and type IV pili, are equally crucial for efficient detachment and dispersal.

Bacteria in each stage of biofilm development - attachment, growth and proliferation, maturation, and detachment - have been found to be physiologically distinct from cells in other developmental stages. Davies and co-workers[99] characterized biofilm developmental stages using protein analysis and showed that there was a difference of 29-40% in detectable proteins between stages. The identified proteins are important in cellular functions such as metabolism, phospholipid and lipopolysaccharide biosynthesis, membrane transport and secretion as well as adaptation and protective functions.[88] While bacteria within each of the stages of

biofilm development are generally believed to be physiologically distinct from cells in other stages, in a mature biofilm all stages of development may be present concomitantly and by not competing for the same chemicals and nutrients these *'diverse cooperators'* reduce competition for resources, thus benefiting the entire biofilm micro-community.[115]

4. Cell Motility

Cell motility is instrumental in biofilm formation, driving its shape and architecture.[114] It enables cells to escape local stresses, move to better nutritional environments and efficiently invade a host, be it living tissue or a non-living art work.[116,117] Motility of a subpopulation of cells has also been suggested to be linked to dispersal of single organisms from biofilms.[99,109,118] Microorganisms capable of biofilm formation usually exhibit one or more of the three main types of motility - swimming, twitching and swarming - which depend on flagella and type IV pili (or frimbriae) (Figure 3).[119-121] *P. aeruginosa*, is one of the rare bacterial species that possess the capability of all three types of motility which is thought to be controlled by four chemotaxis-like signal transduction pathways.[122] The Pil-Chp system regulates twitching motility;[123,124] the Che and Che2 systems regulate flagella-mediated chemotaxis;[125-128] and the Wsp system controls expression of Cup fimbria[129] and Pel and Psl polysaccharides are implicated in *P. aeruginosa* biofilm formation.[130-134]

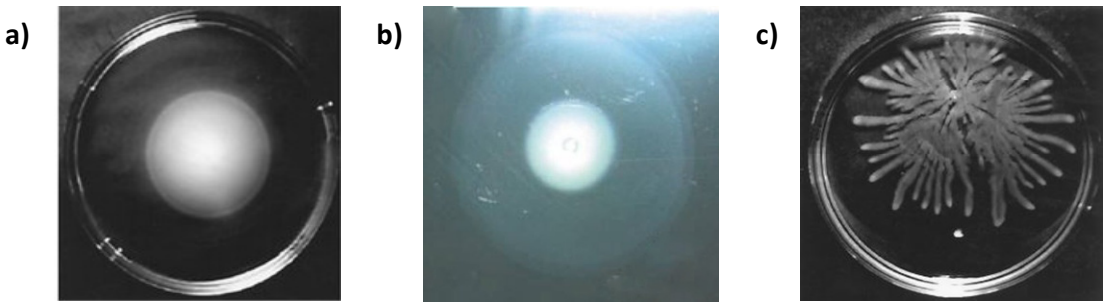


Figure 3. Macroscopic examples of: **a)** swimming;[135] **b)** twitching;[136] and **c)** swarming[135] motilities.

4.1 Swimming motility

Swimming enables bacteria to move towards favourable environments and away from unfavourable ones, and on a surface takes place under highly aqueous conditions (eg. <0.3% agar) when the fluid film is sufficiently thick and the micromorphology unorganized.[116] Dependent on flagella, swimming is based on random individual cell movements rather than the community coordinated movements of twitching or swarming motility (see sections 4.2 and 4.3). Rotation of the flagellum alternates between counter clockwise (smooth swimming) and clockwise (tumbling)[93] moving the bacteria through water-filled channels creating concentric rings (Figure 3a).[116] In the presence of introduced chemical agents, the probabilities of smooth and tumbling swimming is altered, resulting in movement away or towards the chemical agent; this process is known as chemotaxis.[116] Flagella-dependent swimming motility is the dominant form of motility for microorganisms existing in a planktonic mode of growth. As well as being required for biofilm dispersal, swimming motility is thought to be required in early bacterial attachment by functioning as an adhesion factor to overcome the repulsion between the bacteria and the material surface.[99,137,138]

4.2 Twitching motility

Twitching motility is suggested to be involved in the early formation of microcolonies.[101,138] With a surface-regulated switch from flagella-based swimming motility to type IV pili-mediated twitching motility, microorganisms are propelled across the surface in a twitchy manner via the extension and retraction of the type IV pili.[95,136,138-142] In addition to initiating movement, each type IV pilus has adhesion on the distal tip that is able to stick to a variety of organic and inorganic surfaces from glass, to plastic, to the surfaces of art works, as well as to each other, thus initiating cell aggregation and aiding to strengthen surface attachment.[114,143] As a means of bacterial transport across surfaces with low water availability as opposed to free flowing fluids,[136] twitching motility may have a variety of macroscopic manifestations depending on bacterial species. In *P. aeruginosa* for example, twitching organisms form flat, rough colonies with the twitching edge consisting of a thin layer of cells (see Figure 3b).[141] These '*rafts*' or '*trails*' of cells move radially outwards, always in cell-to-cell contact, leading to a dynamic lattice-like network (Figure 4).[141,144] Biofilm formation ensues once a mature lattice with less motile cells and a higher three dimensionality begins to develop.[141]

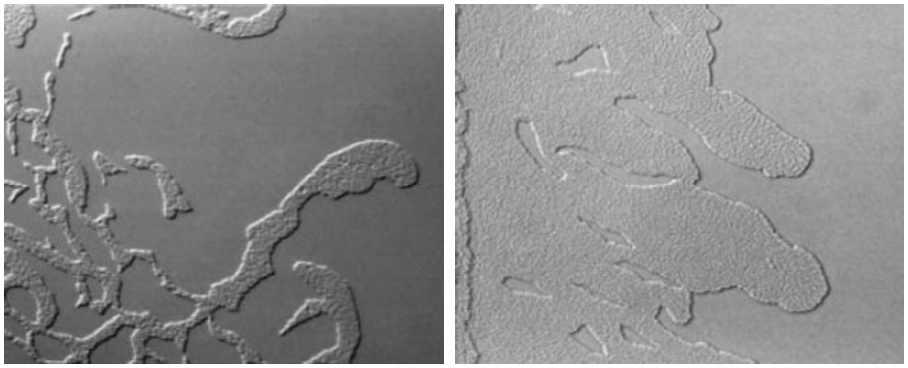


Figure 4. Examples of microscopic twitching motility in *P. aeruginosa.*[144]

While twitching motility may initiate biofilm formation, alternatively overstimulation of twitching motility may also lead to detachment of cells from biofilms.[145,146] It is important to appreciate that this highly organized mechanism of bacterial translocation can be employed both to bring cells together to form biofilms or to promote rapid movement away from a communal existence. These processes are regulated by complex signal transduction systems involving community-wide changes in gene expression.[136,147,148] For example, Welsh and co-workers[149] showed that twitching was stimulated by the chelation of iron by lactoferrin. This causes a significant decrease in cell cluster and biofilm formation, a phenotype in part attributed to the upregulation of rhamnolipids, a class of biosurfactant composed of L-rhamnose and 3-hydroxyalkanoic acid, that increases twitching motility and alters mature biofilm formation.[150,151]

Apart from its role in biofilm formation and dispersal, functional type IV pili-mediated twitching is also required for a wide variety of other critical processes, ranging from DNA transformation[152] to electron transport.[153] For additional information about type IV pili-mediated twitching, see reviews by Mattick,[136] Burrows,[142] and Shi and Sun.[154]

4.3 Swarming motility

Distinct from swimming and twitching, swarming motility represents a complex multicellular adaptation to viscous (semi-solid, 0.5-0.7% agar) surfaces.[135] Usually elongated and hyperflagellated, swarmer cells are thought to differentiate from vegetative cells by flagellum-assisted surface viscosity sensing or in response to

nutritional signals.[93,135,155] In contrast to swimming, swarming is a rapid (several μm s^{-1})[156] and highly organized movement with extensive cell-to-cell contact.[150] Microscopically, the elongated swarm cells are found mainly near the swarm-advancing edge lined up in parallel directing cell movement.[93] This elongation of cells has an effect on the macroscopic appearance of swarm colonies, producing highly coordinated movement and forming extensive lateral tendrils, which branch, eventually creating a characteristic dendritic pattern (Figure 3c).[116,156,157] Multiple flagella were also found to be crucial for swarming since *E. coli* mutants lacking hyperflagellation were unable to swarm.[158] The biosynthesis of flagella is thought to be controlled by over 50 genes that can be either negatively or positively expressed depending on the input signal.[156] In comparison to other swarming bacteria, such as *E. coli*,[158] *S. marcescens*, and *P. mirabilis*,[93] that only require flagella to swarm, *P. aeruginosa* also requires type IV pili.[95,116,135,150]

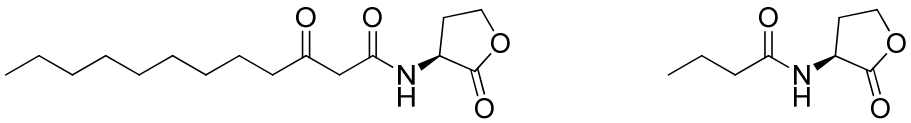
Efficient colonization of a surface is aided by the production and secretion of biosurfactants which act as wetting agents to overcome strong surface tension and provide an appropriate micro-viscous environment.[95,116,135,150] Research by Köhler *et al.* suggests that rhamnolipids are not only important biosurfactants in twitching motility,[149] but are also involved in swarming motility.[135] Cell density and critical cell mass has been proposed to account for the increase in biosurfactant production that is needed for swarming.[159] Controlled by cell-to-cell signalling molecules, *N*-acyl-homoserine lactones (AHLs), the production of biosurfactants such as rhamnolipids determine the pattern of the swarming colony.[160,161] In addition to their role as signalling molecules, AHLs may also have a direct function by acting as surfactants themselves, promoting surface translocation.[162] As described in the next section, evidence that the shift between migrating and sessile surface behaviour is regulated via a method of cell-to-cell communication know as quorum sensing is accruing.

5. Quorum Sensing - Biofilm Cell-to-Cell Signalling

The initial formation and continual maintenance of a community of microorganisms requires a method of cell-to-cell communication between the different cell types within the biofilm network. Quorum sensing (QS) is a bacterial communication system used to organize communal behaviour by coordinating population wide changes in gene expression during times of high cell density.[98, 99] This coordinated behaviour is attributed to the intracellular production and extracellular release and detection of biochemical messenger molecules called autoinducers. A substantial population density of microorganisms is needed to activate QS systems as autoinducers and must reach a critical minimum threshold in order to bind to receptors.[163] Upon binding, a signal transduction cascade follows which allows the population to function in unison and thus provide an effective team in the biodeterioration process.[163-166] The regulation of QS is highly conserved in bacteria, however its molecular mechanisms and the chemistry of the autoinducers varies between gram-negative and gram-positive bacteria. At least half a dozen types of bacterial QS systems have been described and more are almost certain to exist. For the purposes of this review a brief overview of QS in *P. aeruginosa*, a prototype biofilm-forming species, will be presented. For detailed reviews on QS the reader is referred to the literature.[163,164,167,168]

The *lux*-type QS system is the most well studied and understood mechanism for communication in gram-negative bacteria and is based on the production of, and response to AHLs.[166] This 2-component system is composed of an autoinducer synthase (e.g. LuxI), which synthesizes AHLs from S-adenyosyl methionine, and a transcription regulator (e.g. LuxR). In *P. aeruginosa* key virulence traits are controlled by two distinct yet

interrelated Lux-type QS systems – las and *rhl* – which are named after their influence on elastase and rhamnolipid production respectively.[169-173] These two QS systems operate via the autoinducers, *N*-(3-oxododecanoyl)-l-homoserine lactone (**1**, *las*) and *N*-butyryl-l-homoserine lactone (**2**, *rhl*), that are synthesized by the LasI and RhlI syntheases and bind to and activate transcription activator proteins LasR and RhlR (Figure 5). In addition to elastase and rhamnolipid, the gene products produced by these systems also activate phenotypes such as exopolysaccharide production, virulence factor production, toxin production, motility (as discussed above) and biofilm formation.[172,174,175] In fact, a seminal paper by Davies *et al.* first uncovered that QS, specifically the *las* and *rhl* systems, are involved in the differentiation stage of biofilm development in *P. aeruginosa*.[83] It was shown that the knockout *lasI* mutant was unable to form a biofilm akin to the highly structured wild type, displaying none of the characteristic morphological structures typical of a mature biofilm.[83]



1 **2**

Figure 5. *N*-(3-oxododecanoyl)-L-homoserine lactone (**1**) and *N*-butyryl-L-homoserine lactone (**2**) are *P. aeruginosa* autoinducer molecules in the *las* and *rhl* QS systems respectively.

6. Anti-Biofilm Compounds

Significant differences in structure, function and gene expression between planktonic and biofilm cells translate to a difference in biocidal and antibacterial sensitivity. Living outside the physically-protective embrace of a biofilm, planktonic bacteria are up to 1000 times more sensitive to treatment with biocides which is why microorganisms are estimated to be 90% more likely to exist with a biofilm.[7,176-178] Furthermore planktonic cells are thought to be more metabolically active and therefore more biocide susceptible than biofilm cells.[179] Anti-biofilm compounds prevent biofilm formation and/or induce dispersal events thereby moving bacteria away from their protective EPS barrier and inducing cellular change from sessile biofilm cells to the more metabolically active and antimicrobial sensitive planktonic cells. With the incorporation of anti-biofilm compounds into our biodeterioration-fighting toolbox we may be able to reduce the concentrations of conventional biocide used or abolish their need altogether. Furthermore, the manipulation of native bacterial processes in preference to biocidal mechanisms mitigates the development of resistance. Anti-biofilm compounds may provide the answer to targeting biodeterioration-inducing and pathogenic bacteria growing in biofilms.

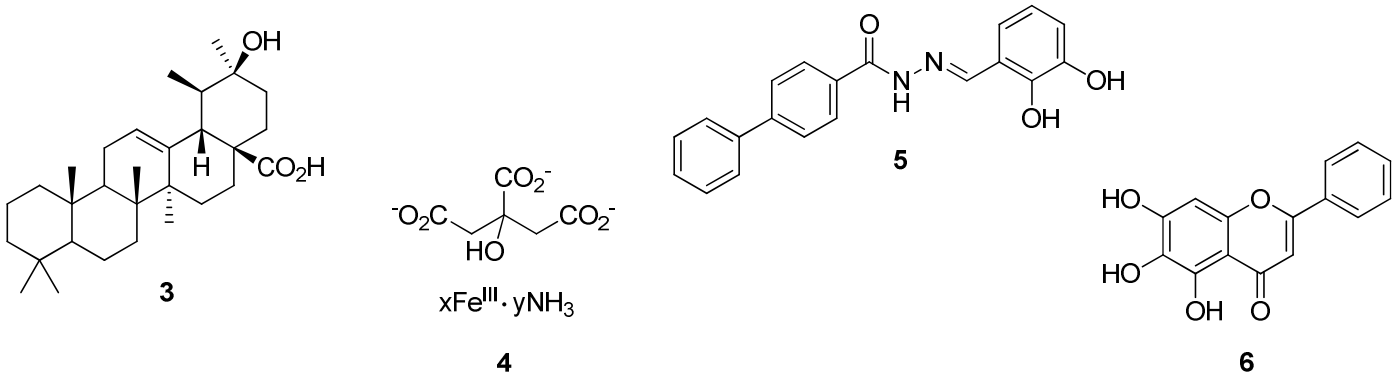


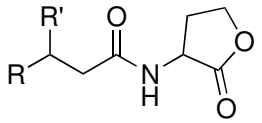
Figure 6. Anti-biofilm compounds obtained by high-throughput screening – ursolic acid (**3**), ferric ammonium citrate (**4**), N'-(2,3-dihydroxybenzylidene)-4-biphenylcarbohydrazide (**5**) and 5,6,7-trihydroxy-2-phenyl-4*H*-chromen-4-one (baicalein) (**6**).

In recent years much attention has focused on searching for novel non-biocidal, anti-biofilm compounds. Traditional methods to control biofilm formation have focused on biocidal and antibacterial approaches. The pitfalls associated with such strategies involve the development of tolerance in addition to poor effectiveness due to the refractory nature of biofilms to exogenous physio-chemical pressures. High-throughput screening is a popular methodology for discovering new anti-biofilm compounds from a library of seemingly unrelated chemical compounds.[180] Molecules such as ursolic acid (**3**),[181,182] ferric ammonium citrate (**4**),[183] N'-(2,3-dihydroxybenzylidene)-4-biphenylcarbohydrazide (**5**),[184] and 5,6,7-trihydroxy-2-phenyl-4*H*-chromen-4-one (baicalein) (**6**)[185] were all discovered, through high throughput screening methods, to inhibit *P. aeruginosa* biofilm formation (Figure 6).

A number of alternative methodologies have recently arisen that specifically explore the manipulation of native bacterial molecules and processes such as the manipulation of QS, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) signalling and activation of dispersal. These will be discussed briefly below.

6.1 Manipulation of quorum sensing

Given QS is an important and complex regulatory mechanism in biofilm formation and biofilm dispersal, anti-biofilm compounds that target QS by inhibiting the production, release, or detection of autoinducers may offer antagonistic activity to the pathways that lead to biofilm formation.[98,186,187] Mentioned previously, many gram-negative bacteria such as *P. aeruginosa* regulate gene expression by using AHLs as their QS signal molecules. Naturally occurring AHLs consist of a lactone ring with an amide linked side chain ranging from 4 to 18 carbons in length. The *N*-acyl group may be saturated or unsaturated and is usually substituted with H, OH or =O at the 3-position (Figure 7).[187]



$R = C_1\text{-}C_{15}$, R' = H, =O, or OH

Figure 7. General structure of an AHL. The identity of R and R' groups are dependent on bacterial species.[188]

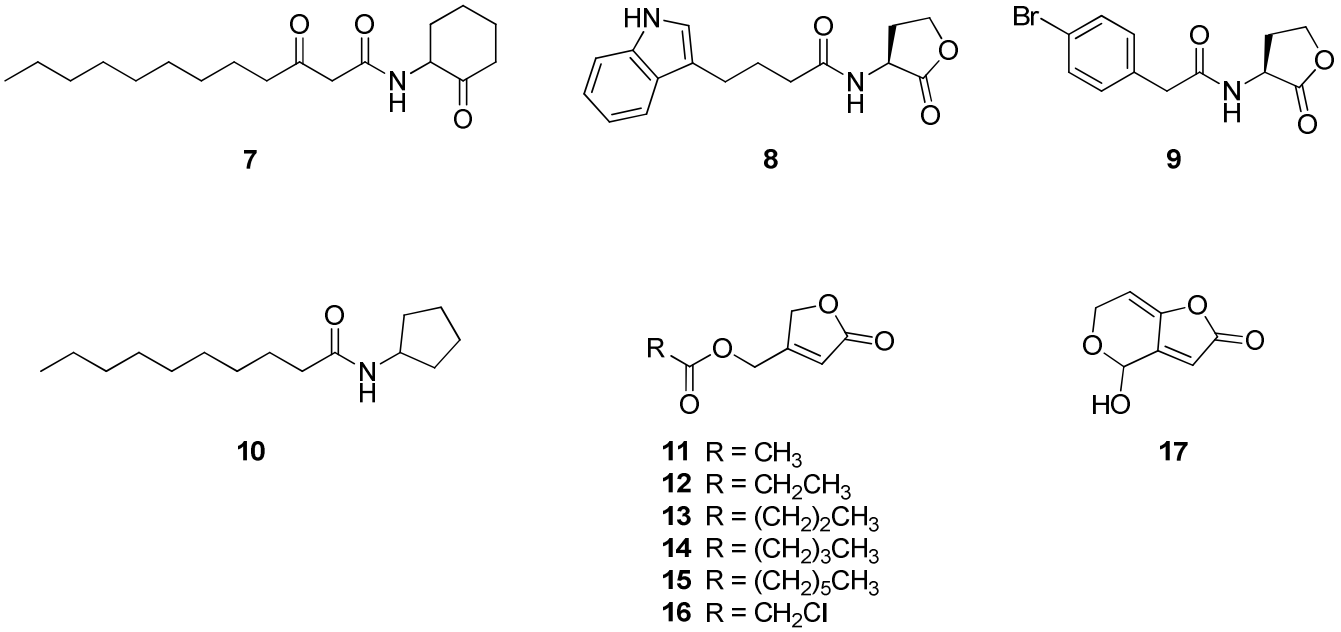


Figure 8. QS modulators derived from AHLs with anti-biofilm activity (**7**-**17**).[190,193,198,199]

Since the first study of AHL analogues by Eberhard and co-workers,[189] many groups have explored the use of various native and synthetic AHLs to both agonize and antagonize QS-behaviours.[190-197] Some biologically active QS modulators derived from AHLs were also found to suppress biofilm formation in a non-biocidal manner. A selection of these modified AHLs (**7**-**17**) are given in Figure 8.

Many QS antagonists and anti-biofilm compounds have also been identified from other natural products, such as essential oils,[200] wheat bran,[201] garlic,[202,203] cinnamon[204] and cranberries.[205,206] Halogenated furanones, isolated from the red alga *D. pulchra* are one of the most extensively studied classes of natural QS antagonists (**18**-**22**, Figure 9).[207-211] In addition to displaying biofilm modulatory effects, Givskov and co-workers also showed that treatment of *P. aeruginosa* biofilms with synthetic furanone **23** induced a 3-fold increase in the effectiveness of antimicrobial agents, tobramycin and sodium dodecyl sulfate.[212]

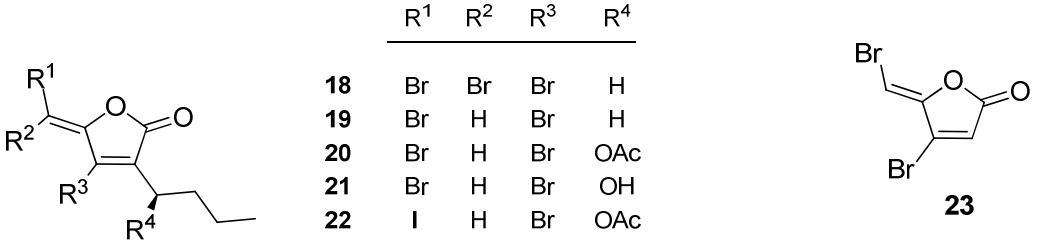


Figure 9. Halogenated furanones isolated from *D. pulchra* (**18**-**22**),[209] and synthetic furanone **23**.[212]

Like the halogenated furanones, the pyrrole-imidazole alkaloid bromoageliferin (**24**), derived from the marine sponge *A. conifera,* has inspired a library of analogues with biofilm inhibitory activity.[213-217] Bromoageliferin is a member of a class of biologically active natural products, the oroidins, that is characterized by a 2-aminoimidazole subunit.[218] This subunit is believed to be the key pharmacophore responsible for the biological activity of the oroidins.[213] Indeed biofilm plate assays and confocal microscopy

experiments with oroidin (**25**) and two analogues of bromoageliferin (**24**), **26** and **27** (Figure 10) showed that these simple 2-aminoimidazoles were able to supress biofilm formation in *P. aeruginosa*.[213,215,216]

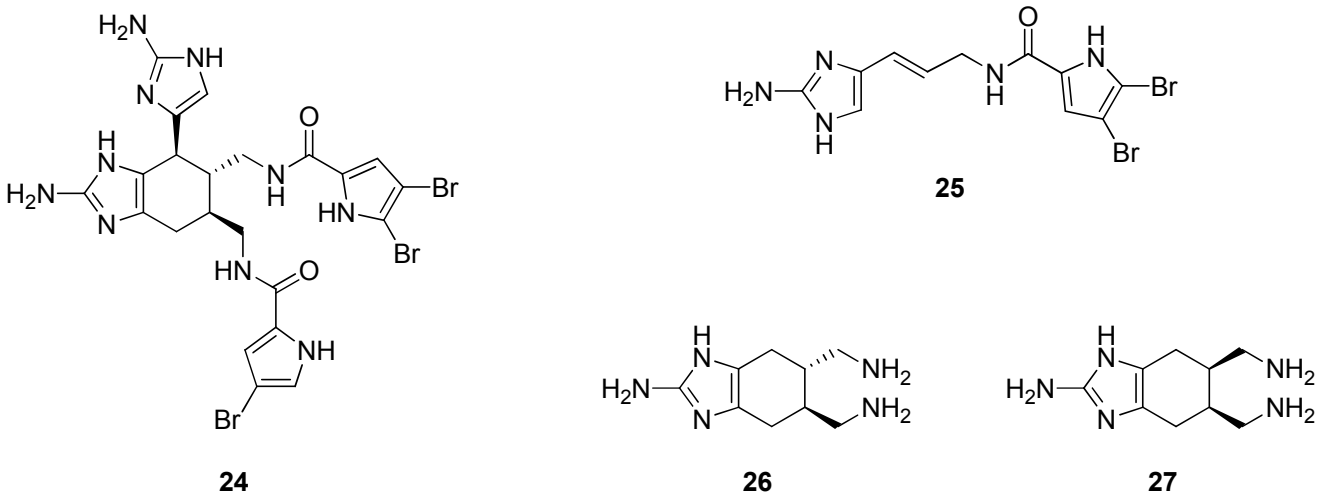


Figure 10. 2-Aminoimidazoles with biofilm inhibiting activity - bromoageliferin (**24**), oroidin (**25**) and simplified derivatives of bromoageliferin (**24**), **26** and **27**.

Modulators of QS pathways compose the vast majority of compounds investigated for biofilm control and the compounds discussed *vide supra* are simply a small selection. Other small molecules that manipulate biofilm formation in a QS-dependent manner include indoles,[219-221] fatty acids,[222-224] and certain amino acids.[225-228]

6.2 Inhibitors of c-di-GMP

Another small molecule signalling system, based on the intracellular second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP (**28**) Figure 11), has been implicated in a number of cellular functions including regulation of the cell cycle, differentiation and virulence.[229,230] Most importantly, c-di-GMP has been shown to antagonistically control the biosynthesis of adhesins and exopolysaccharides associated with biofilm formation and the ability of flagellated bacteria, such as *P. aeruginosa*, to switch from planktonic to sessile biofilm growth.[229-234]

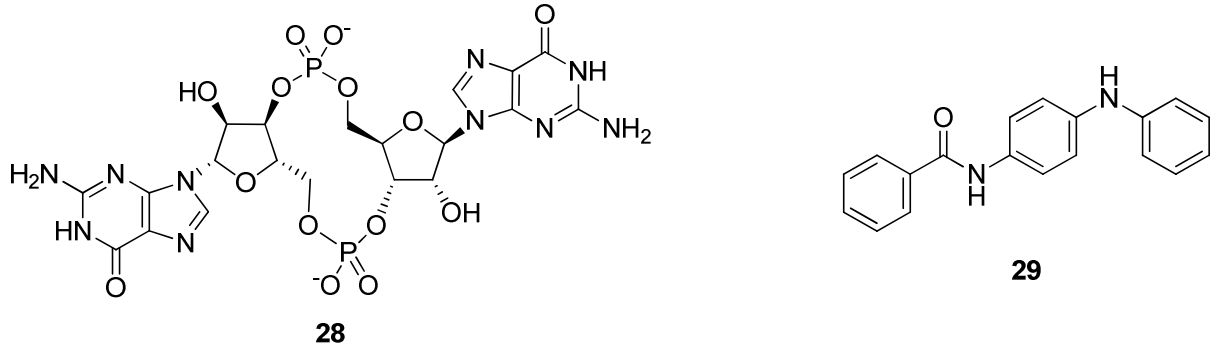


Figure 11. c-di-GMP (**28**) and the DGC antagonist *N*-[4-(phenylamino)phenyl]-benzamide (**29**).

Intracellular levels of c-di-GMP are regulated through the opposing activities of diguanylate cyclases (DGCs) and phosphodiesterases which synthesize and hydrolyze c-di-GMP respectively.[235] Modulating c-di-GMP pathways in bacteria could provide a novel way of controlling formation of biofilms on cultural material. DGCs in particular are a promising target for the development of novel anti-biofilm compounds as deletion of DGCs was found to completely abolish biofilm formation.[236,237] Recently seven small molecules that antagonize DGCs were discovered, one of which (**29,** Figure 11) was found to inhibit biofilm formation in *P. aeruginosa*.[238]

6.3 Activation of biofilm dispersal

Although the mechanisms by which biofilm dispersal is controlled are numerous, complex and have not yet been fully elucidated, dispersal is believed to be linked to certain environmental cues such as the availability of nutrients,[103,239] bacterial antagonists or fluctuations in oxygen concentrations.[240] Once these cues have been detected, biofilm cells respond by producing EPS-dissolving enzymes such as alginate lyase in *P. aeruginosa*,[112] or dispersin B in *A. actinomycetemcomitans*.[241] In contrast to the other types of anti-biofilm compounds, dispersal-inducing compounds are often active across species. For instance, the extracellular polysaccharides produced by *P. aeruginosa* were also found to induce dispersal of *S. epidermidis* biofilms.[242] Dispersal can also be induced by compounds which act to break down the extracellular polysaccharides which make up the extracellular matrix. Recently, using confocal laser-scanning microscopy, Wu and co-workers showed that the small molecule norspermidine (**30**, Figure 12), when used in combination with silver nitrate, enhances dispersal of multi-species wastewater biofilms by breaking down exopolysaccharides and disrupting the biofilm matrix.[243,244]

In the last couple of years, a number of small molecules have been identified that both inhibit biofilm formation and induce biofilm dispersal. Through high-throughput screening of a library of marine natural products, Linington and co-workers identified the biofilm-modulatory activity of antibiotic skyllamycins A – C (**31**-**33**, Figure 12) in *P. aeruginosa* biofilms.[180] These same researchers also reported the development of benzo[1,4]oxazines as biofilm inhibitors and dispersal agents against *Vibrio cholerae*.[245] In this communication, **34** (Figure 12) was reported as "the first example of a small molecule capable of inducing the dispersal of *V. cholerae* biofilms" in two hour old biofilms, placing it "among just a handful of compounds capable of inducing the dispersal of mature surface-associated biofilms".[245] Since this research, a number of other small molecules with both biofilm inhibition and dispersal properties have been observed, of which includes, 1,13-bis[((2,2-diphenyl)-1-ethyl)thioureido]-4,10-diazatridecane (**35**) which primarily acts by depolarization of the cytoplasmic membrane and permeabilization of the bacterial outer membrane.[246]

The discovery of anti-biofilm compounds that are able to both inhibit biofilm formation and induce biofilm dispersal suggests that these events may share common mechanistic elements. Regulatory signals controlling biofilm development have been found to be tightly linked to those which lead to dispersal. For example, c-di-GMP not only influences biofilm formation but also affects the extent of biofilm detachment.[247] In 2006, Barraud and co-workers demonstrated that NO, a free radical and important biological messenger, is a signal molecule for biofilm dispersal.[12,248] During this research it was suggested that the mechanism of NO-induced biofilm dispersal was through the activation of phosphodiesterase activity, resulting in the degradation of c-di-GMP, which then culminates in changes to gene expression that favour the planktonic mode of growth.[12,248-251] In addition to its association with c-di-GMP signalling, NO-induced dispersal is also linked to the accumulation of oxidative and nitrosative stress within microcolonies where localized bursts of reactive

oxygen and nitrogen intermediates create hollow vacuoles (Figure 2) within the microcolony that release and disperse planktonic bacteria from the biofilm structure.[12]

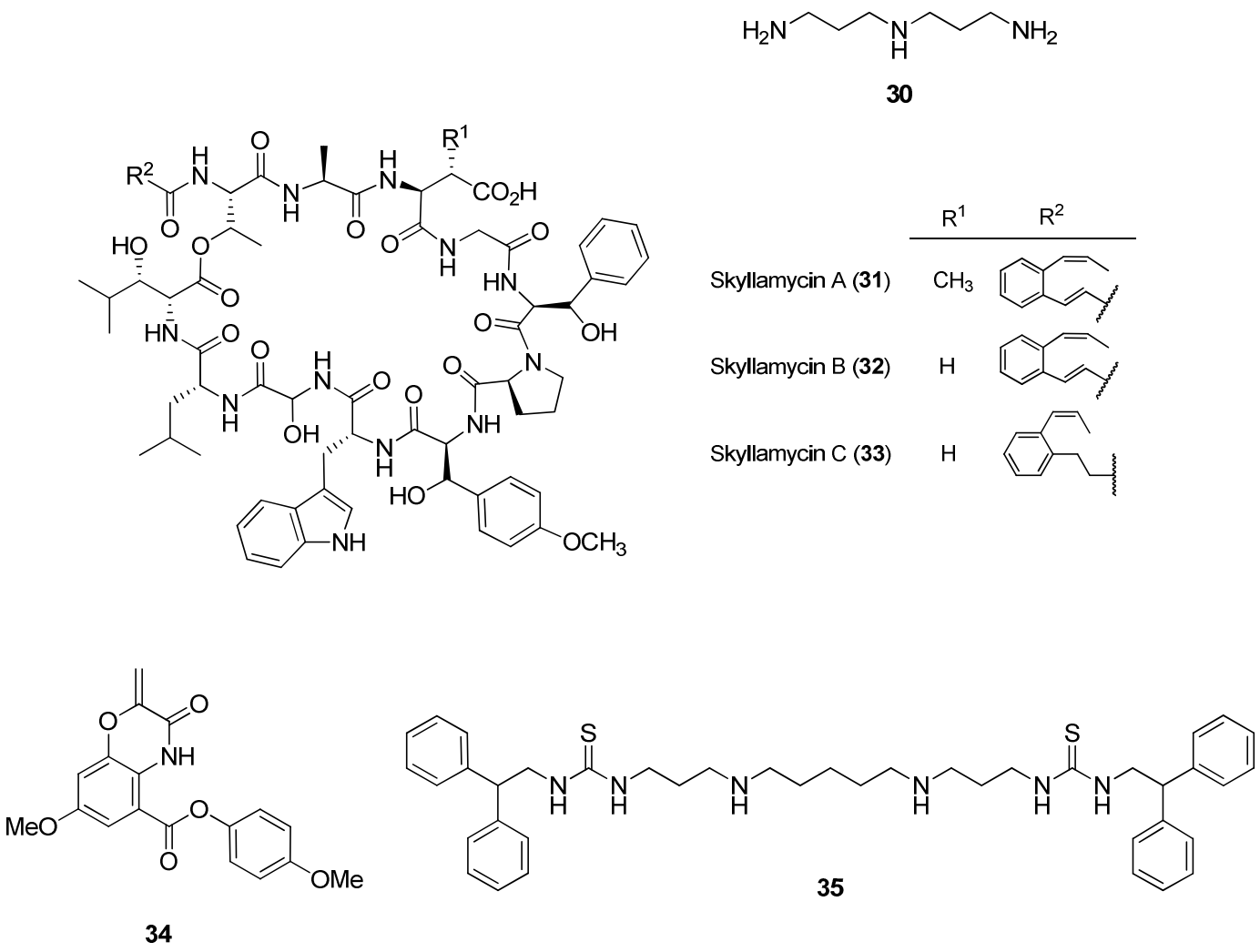
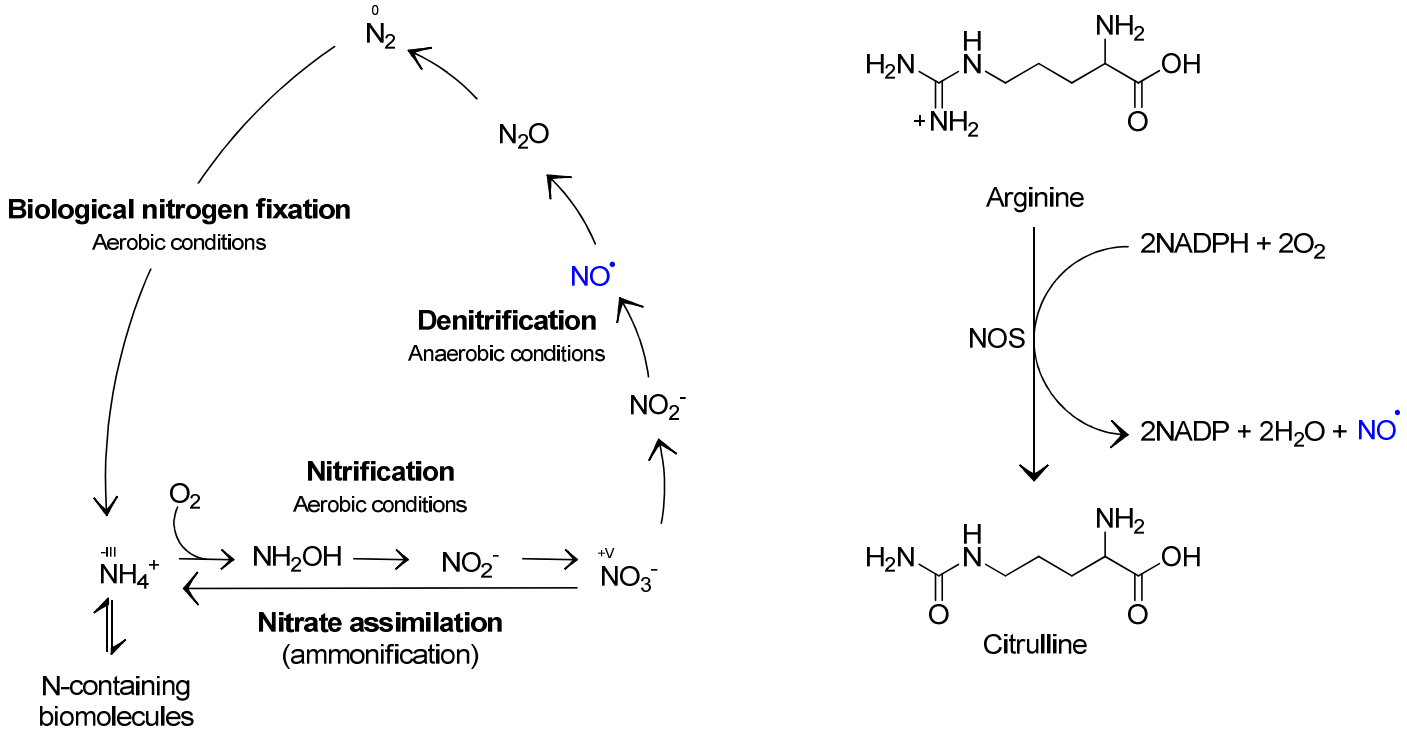


Figure 12. Molecules which activate biofilm dispersal.[180,244,245]

7. Nitric Oxide

Once of scientific interest due to its role in air pollution,[252-254] the focus of NO research has since turned to its role as one of the smallest biological mediators.[255] NO is a small molecule that contains one unpaired electron in the antibonding $2\pi*_y$ orbital and is poorly reactive with most biological molecules with the exception of other free radicals. However, its neutral charge and solubility in water (~1.7 mM) makes NO an ideal chemical signalling molecule.[9,256] Most bioenergetic NO is generated through the nitrogen cycle with nitrifying and denitrifying microorganisms playing a key role in the inter-conversion of nitrogen containing species – dinitrogen (N_2), ammonium ions (NH_4^+), and nitrate (NO_3^-) – as shown in its essential, minimized version in Scheme 1a. Enzymatic NO, on the other hand is formed from L-arginine by NO synthase (NOS) as seen in Scheme 1b. NO produced both enzymatically and bioenergetically has been the focus of intensive

investigations into diverse physiological and pathological processes over the past four decades (Figure 13) and has been shown to be relevant in understanding the pathogenesis of bacterial infections. [257]



Scheme 1. NO produced: **a)** bioenergetically;[258] and **b)** enzymatically.

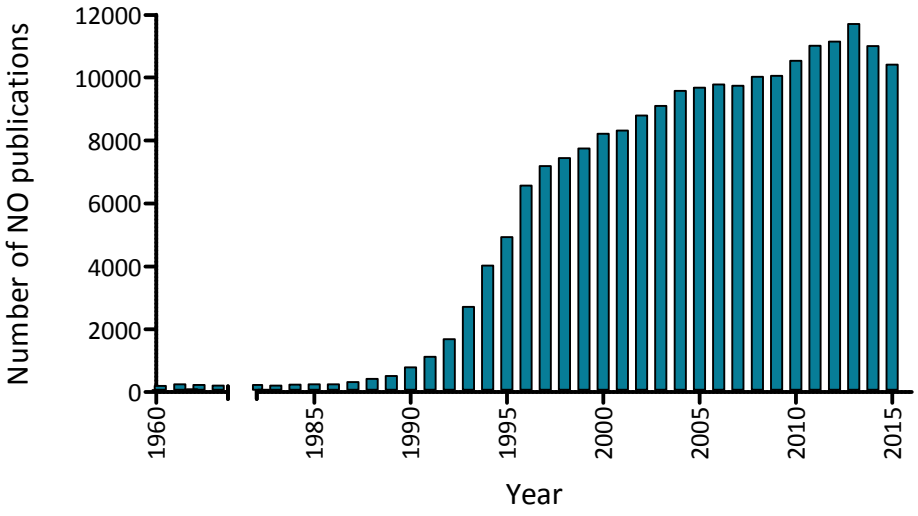
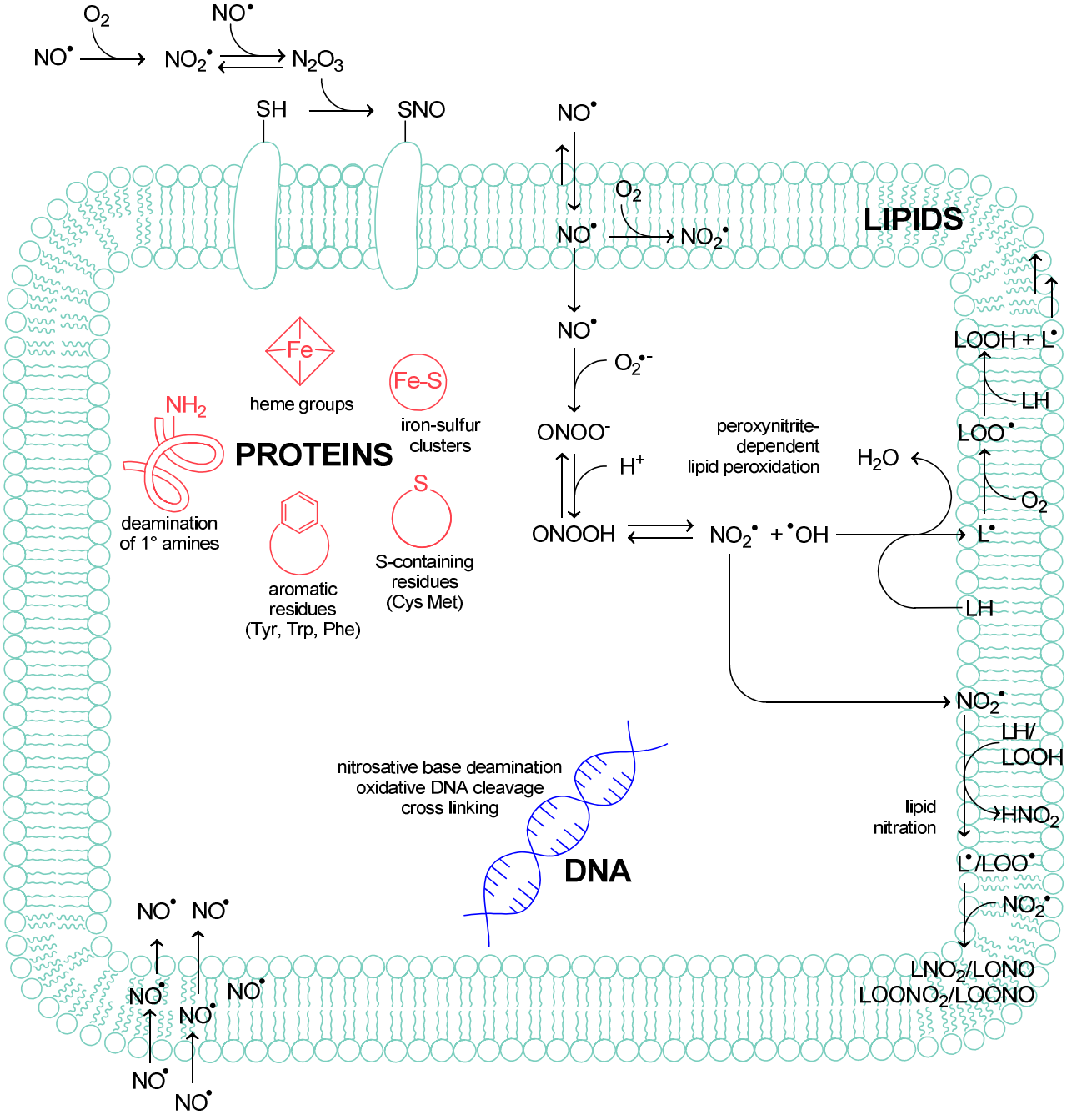


Figure 13. Growth of NO publications over the past 50 years. In 1992 the number of NO publications took a dramatic upturn, this coincided with the announcement in *Science Magazine* of NO as the '*Molecule of the Year*'.[259,260]

NO has been demonstrated to exert *in vitro* and *in vivo* microbiocidal and microbiostatic activity against a rapidly expanding list of microorganisms including yeasts, bacteria and protozoa.[255,261,263] The biological impact of NO is due to a vast array of chemical interactions with a variety of cellular targets. The particular mechanisms of its biological activities are not only concentration-dependent but also complicated by the complex reactivities of NO and the divergent reactivities of different redox states of NO (nitric oxide: $NO^{\bullet}$, nitroxyl anion: NO^{-}, nitrosonium cation: NO^{+}). The biological effects mediated by NO can be classified into two groups – those initiated at low, nanomolar 'signalling' concentrations of NO and those at high, millimolar concentrations of NO. The biological activity of low concentrations of NO is thought to be as a result of chemical interactions of NO with its biological target such as other free radicals or metal complexes.



Scheme 2. Microbial cellular targets of reactive nitrogen intermediates (RNI).[13,261,262]

High concentrations of NO however, induce nitrosative or oxidative stress events exerted by both NO and reactive nitrogen intermediates (RNI) derived from NO.[13,264,265] Reactive species such as dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$) have been shown to modify proteins, lipids and DNA through nitrosative stress events, such as thiol nitrosation, deamination of primary amines and nitrosamine formation; and oxidative stress events, such as lipid peroxidation, tyrosine nitration and oxidative DNA cleavage.[13,261,266-277] The biological impact of NO- and RNI-mediated stress events are both regulatory and deleterious with the chemical impact on cell targets being implicated in a variety of processes including cell proliferation, cell survival, enzymatic inhibition, apoptosis control, and cell transformation.[265] An overview of some of the specific reactions and interactions of NO and RNI with biological targets is given in Scheme 2. For a more detailed review see Fang,[261] and Heinrich *et al.*[9] and references therein.

In addition to its many and varied biological chemistries and reactivities, NO is also a promising anti-biofilm candidate (as discussed above). Since 2006 and the seminal paper by Barraud *et al*.,[12] much research has been devoted to NO-induced dispersal events. The use of NO donor compounds, such as diazeniumdiolates, have been a popular method of introducing NO into a biological system and have been be successfully employed to disperse *P. aeruginosa* biofilms.[100,251,278-280] NO-donor compounds offer a controlled method of NO application and are able to stabilize the developing radical until its release.[281-285] Another class of molecule that has been found to inhibit biofilm formation and induce dispersal are nitroxides.[11,286,287] Since both NO and nitroxides possess an unpaired electron that is delocalized over the nitrogen-oxygen bond (Figure 14), nitroxides are a structurally similar, sterically hindered and more stable alternative to NO when utilised for their anti-biofilm properties.

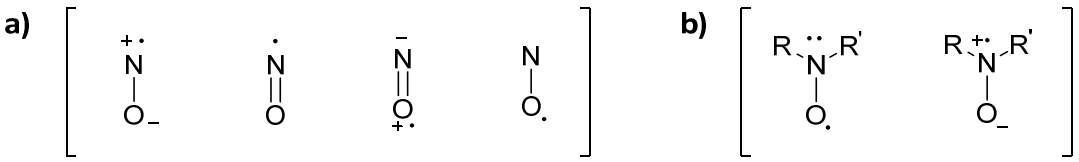


Figure 14. Resonance structures of: **a)** NO; and **b)** a nitroxide.[288,289]

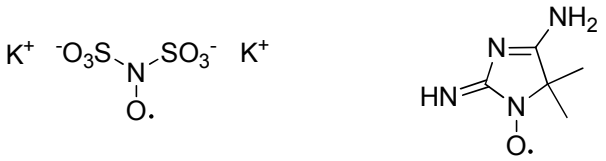
Furthermore, nitroxides are a less expensive and longer-lived alternative to NO-donors, which have half-lives ranging from seconds to hours.[281] Nitroxides are readily synthesized and functionalized and as a consequence the chemical and biological reactivity, cell permeability and solubility of any given nitroxide can be tuned depending on its desired application.

8. Nitroxides

Like NO, nitroxides (also known an aminoxyl radicals) have also been the subject of extensive research over the past five decades. With a range of remarkable physical and chemical attributes it is no wonder that nitroxides have been utilised in a variety of fields and applications including magnetic resonance imaging (MRI), electron paramagnetic resonance (EPR) spectroscopy, material and biological antioxidants, cellular metabolism, molecular mobility of proteins and lipids, and membrane structure.[290-293]

The first nitroxide to appear in the literature was Frémy's salt (or potassium nitrosodisulfonate, $K_2[NO(SO_3)_2]$ (**36**) (Figure 15), and was discovered by Edmond Frémy in 1845.[294] Frémy's salt is still widely used as a strong oxidizing agent and as an EPR standard for g-value determination.[295,296] In 1901, the first

heterocyclic nitroxide, porphyrexide (**37**) (Figure 15), was discovered by Piloty and Graf Selwerin,[297] only one year after the historical discovery of the triphenylmethyl radical by Gomberg.[298] It was noted that porphyrexide behaved in a similar manner to Frémy's salt with its high oxidizing ability, however its true identity as a radical species was only verified 50 years later, in 1951, by Holden *et al.* via EPR spectroscopy.[299]



36 **37**

Figure 15. Two of the earliest examples of nitroxides, Frémy's salt (**36**) and porphyrexide (**37**).

The next advance in this field was the development of stable paramagnetic compounds by Neiman, Mamedova and Rozantsev in 1962.[288,300,301] With this new class of nitroxide, the first reaction of a free radical compound without the direct involvement of the free valences was discovered. Since then, many different classes of nitroxides have been developed (Figure 16) and their use has expanded not only into the many facets of synthetic chemistry but also into biochemistry and medicine.[288,301,302] For a detailed review of the chemical and physical properties of nitroxides see Breuer, Aurich and Nielsen,[289] Zhdanov,[303] Keana[304] and references therein.

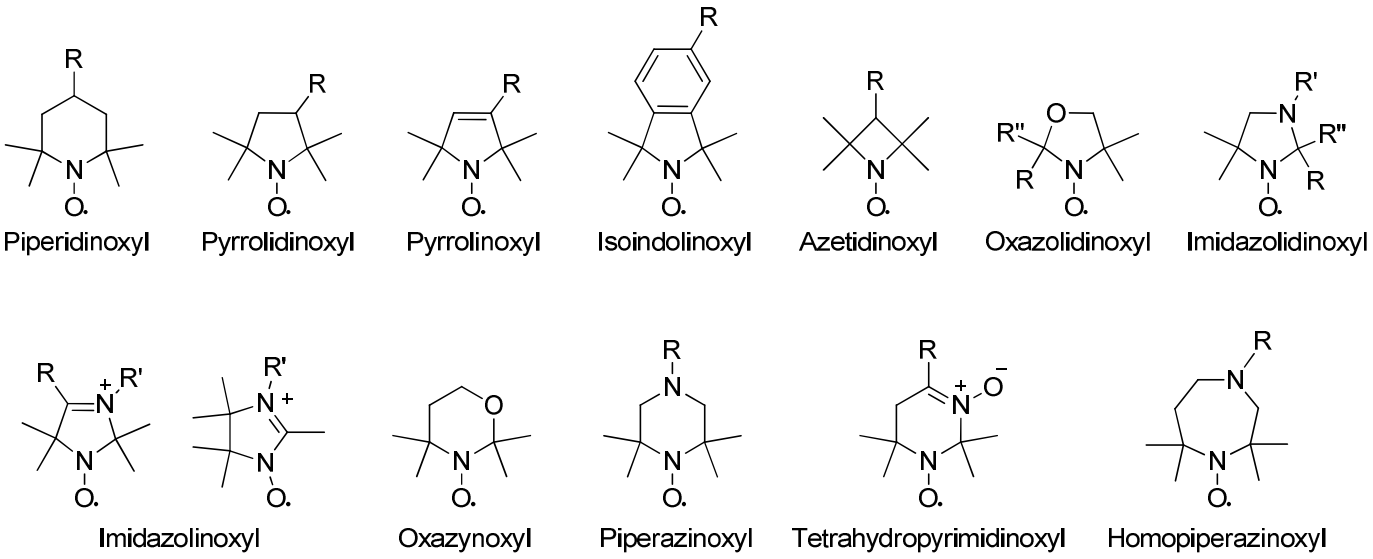


Figure 16. A selection of stable cyclic nitroxides.[289]

8.1 Nitroxides in biological systems

The biological activity of nitroxides was first recognized by Emmerson and Howard-Flanders in 1964 when they reported that nitroxides sensitized bacteria to subsequent treatment with radiation.[305,306] They theorized that given other free radical species such as oxygen and NO behaved as radiation sensitizers, so too must nitroxides. This is likely the very first case of nitroxides being utilized as anti-biofilm agents. Over the past 20 years, other novel applications have been proposed for nitroxide compounds after their antioxidant activity was demonstrated in a number of experimental models.[293,307-312] Nitroxides are known to protect cells against

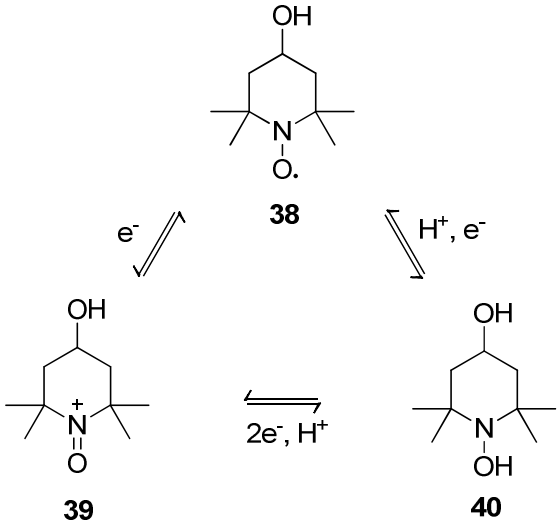
various kinds of oxidative damage, particularly from superoxide ($O_2^{-\bullet}$) and H_2O_2.[308,313-315] Studies to elucidate the mechanisms underlying the protective activity of nitroxides, suggest that nitroxides react with and detoxify deleterious species via several pathways. These include:

i) Superoxide dismutase mimetic activity – the dismutation of $O_2^{-\bullet}$ by nitroxides occurs through an oxidative/reductive catalytic cycle. Initial oxidation of nitroxide by $O_2^{-\bullet}$ to the corresponding oxoammonium cation and subsequent reduction by $O_2^{-\bullet}$ back to the nitroxide.[313-317]

ii) Radical scavenging – nitroxides are able to react with a large variety of radical species including the carbon-,[318-321] oxygen-,[322-324] nitrogen-,[325] and sulfur-centered radicals,[326-328] responsible for processes of cellular damage such as lipid peroxidation.[293,315,329]

iii) Inhibition of transition metal-mediated damage – nitroxides oxidize reduced transition metals such as Fe(II) or Cu(I) so that their potential for $^{\bullet}OH$ generation in the Fenton and related reactions is lowered.[293,308,315]

The ability of nitroxides to participate in all of the above pathways relies on redox transformations between three oxidation states - the nitroxide, hydroxylamine and oxoammonium derivatives. Scheme 3 shows the oxidation states of 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinoxyl (**38**), one of the most widely studied nitroxides in the literature.



Scheme 3. The oxidation states of 4-hydroxyl-2,2,6,6-tetramethyl-1-piperidinoxyl (**38**).

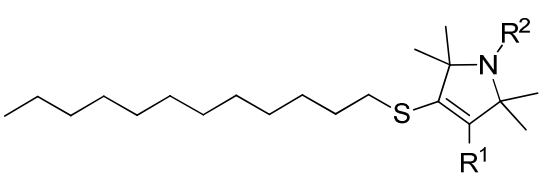
Through the interconversion between these three oxidation states nitroxides are able to confer protection from oxidative damage by acting as both reductants and oxidants, and are subsequently regenerated through spontaneous and enzymatic pathways.[330] This feature of nitroxides is also advantageous to their use as EPR spin labels and spin probes and as profluorescent nitroxide probes, a sensitive method for measuring radical formation and reactions.[331-335]

In contrast to the protective effects of nitroxides, some reports indicate that nitroxides are also able to exert mutagenic and bactericidal effects.[336-339] The exact mechanism of these effects has not yet been fully determined, however it has been suggested that some nitroxides manifest damaging pro-oxidative effects by increasing the cellular concentration of H_2O_2.[340,341] Another theory is that the highly oxidizing oxoammonium species may promote damage to vital macromolecules and deleterious effects on cell signalling via the catalytic oxidation of alcohols[342] as well chemical modification of many other organic and inorganic molecules found in biological systems.[343] Conversely Sies and Mehlhorn,[336] and Gallez *et al.*[344] attributed mutagenicity to

reactive species, such as sulfenyl hydroperoxides and sulfonate derivatives, formed from the oxidation of glutathione by nitroxides in the presence of $O_2^{\bullet-}$. Regardless of the mechanism, the anti/pro-oxidant activities of nitroxides is paralleled by those of NO.[345] Correspondingly, the concentration-dependent biological effects of NO are mimicked by nitroxides. Studies into NO-induced biofilm dispersal indicated a critical NO concentration (usually at nano- and micromolar concentrations) while at larger millimolar concentrations NO had a toxic effect.[12] Likewise Zhang *et al.* showed that nitroxides protect *E. Coli* from quinone cytotoxicity in a dose-dependent manner in the micromolar scale and potentiate cell injury at nitroxide concentrations greater than 5 mM.[346]

8.2 Nitroxides as anti-biofilm compounds

Many comparisons can be drawn between the biological effects of NO and those of nitroxides. The mediation of these biological effects lies in the free radical that is delocalized between the nitrogen and oxygen atoms in both NO and nitroxides. In fact, many of the biological effects of nitroxides are suspected to be a result of their NO mimetic properties.[345] However unlike the highly reactive NO, many nitroxides are persistent radicals because of their resistance to dimerization and disproportionation reactions.[304,347,348] Given the similar functionality between NO and nitroxides, it is not surprising that several groups (including our own) have shown that nitroxides possess anti-biofilm properties.[11,286,287] Hancock and co-workers investigated the effect of three nitroxides on biofilm formation and swarming motility in *P. aeruginosa*.[11] They showed that mutant bacterial strains lacking the gene encoding nitrite reduction, and thus lacking the ability to make NO, were unable to form biofilms and that nitroxides were able to restore swarming motility to mutant strains and mimic NO-induced dispersal. Experiments with wild type *P. aeruginosa* indicated that nitroxides did not affect swarming motility, however they were able to both inhibit the initial stages of biofilm formation and induce dispersal of pre-established wild type *P. aeruginosa* biofilms. This work suggests that nitroxides may trigger dispersal in a concentration-dependent manner via the same pathways as NO. More recently, we identified one nitroxide, 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**41**) (Figure 17), that was able to significantly suppress biofilm formation and elicit dispersal events in *P. aeruginosa* and mixed culture biofilms composed of organisms derived from cultural material.[287] In addition, we showed that when **41** was used in combination with low concentrations of biocide, these biofilms were effectively eradicated.[286] Using semi-solid agar motility assays, it was revealed that twitching and swarming motilities were enhanced by **41**, leaving the planktonic-specific swimming motility unaffected and suggesting that the mechanism of **41**-mediated biofilm modulation is linked to the hyperactivation of surface-associated cell motilities.[287] This work showed that the nitroxide moiety in **41** is likely to be important to its function, given the ethoxylamine (**42**) was less effective. Additionally, replacement of the alcohol group with an aldehyde (**43**) or ether (**44**) resulted in loss of activity.[286,287]

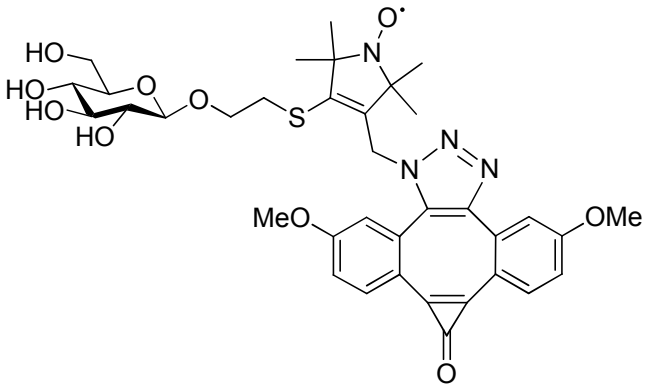


	R^1	R^2
41	CH_2OH	$O^{\bullet}$
42	CH_2OH	OEt
43	CHO	$O^{\bullet}$
44	CH_2OMe	$O^{\bullet}$

Figure 17. 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl (**41**) displays biofilm inhibiting and dispersing properties in *P. aeruginosa* and mixed culture biofilms.[286,287]

8.3 Profluorescent nitroxides as free radical probes in bacterial biofilms

Free radicals and redox processes are involved in the communal behaviour of bacteria, providing the triggers for biofilm formation that often results in biofouling and biodeterioration.[83,87] While it is well established that these processes are important in regulating key events in the biofilm lifecycle, the intricate details of how they work are still not well understood.[105,106] In an attempt to discover the function of free radicals and the role of oxidative stress in biofilm formation and dispersal, Barzegar Amiri Olia *et al.*, developed a novel profluorescent nitroxide (**45**) that detects free radical and redox processes associated with oxidative stress during *P. aeruginosa* biofilm growth (Figure 18).[349] Confocal laser-scanning microscopy and subsequent co-localization studies using digital image analysis revealed that conditions of oxidative stress occur predominantly in the EPS and in live cells during normal biofilm growth.[349] Profluorescent nitroxides (such as **45**) therefore provide a sensitive technique to detect, quantify and visualize oxidative stress conditions and alterations in the redox status of the cellular environment.[334,350] (For a comprehensive discussion of the mechanisms of profluorescence and a review of profluorescent nitroxide probes see Blinco *et al*.[334])



45

Figure 18. The first profluorescent nitroxide probe (**45**) to be used to quantify and visualize changes in redox status and associated stress conditions within the biofilm during dispersal.[349]

Conclusion

We have summarized the mechanisms of cultural heritage biodeterioration, the traditional remediation techniques used by conservation professionals to treat biodeteriorated materials, and the recent progress in the development of anti-biofilm compounds that suppress the growth, and induce the dispersal, of bacterial biofilms. We have outlined the role of free radical species such as NO and RNI in biofilm growth, metabolism and dispersal; and the role of nitroxides in novel treatment and visualization techniques.

This knowledge will be useful in developing future insight into the mechanistic detail of biofilm formation and dispersal, and in further developing novel small molecule and free-radical based anti-biofilms compounds that are not only biologically relevant in a laboratory setting, but that also hold promise as potential treatments for biodeteriorated cultural materials *in situ*.

References and Notes

[i] The following terms will be used interchangeably: culturally significant materials/objects, cultural heritage, cultural artefacts, cultural property, inherited culture, material culture. Additionally, unless otherwise stated, where the words 'material' or 'object' are used within this review, it is assumed they are of a culturally significant nature.

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