14. Biogenic Impact on Materials

Materials as constituents of products or components of technical systems rarely exist in isolation and many must cope with exposure in the natural world. This chapter describes methods that simulate how a material is influenced through contact with living systems such as microorganisms and arthropods. Both unwanted and desirable interactions are considered. This biogenic impact on materials is intimately associated with the environment to which the material is exposed (Materials-Environment Interaction, Chap. 15). Factors such as moisture, temperature and availability of food sources all have a significant influence on biological systems. Corrosion (Chap. 12) and wear (Chap. 13) can also be induced or enhanced in the presence of microorganisms. Section 14.1 introduces the categories between desired (biodegradation) and undesired (biodeterioration) biological effects on materials. It also introduces the role of biocides for the protection of materials. Section 14.2 describes the testing of wood as a building material especially against microorganisms and insects. Section 14.3 characterizes the test methodologies for two other groups of organic materials, namely polymers (Sect. 14.3.1) and paper and textiles (Sect. 14.3.2). Section 14.4 deals with the susceptibility of inorganic materials such as metals (Sect. 14.4.1), concrete (Sect. 14.4.2) and ceramics (Sect. 14.4.3) to biogenic impact. Section 14.5 treats the testing methodology concerned with the performance of coatings and coating materials. In many of these tests specific strains of organisms are employed. It is vital that these strains retain their ability to utilize/attack the substrate from which they were isolated, even when kept for many years in the laboratory. Section 14.6 therefore considers the importance of maintaining robust and representative test organisms that are as capable of utilizing a substrate as their counterparts in nature such that realistic predictions of performance can be made.

14.1 Modes of Materials – Organisms Interactions

A number of interactions result from the contact between living systems and materials some of which result in either biodeterioration or biodegradation. The word *deteriorate* comes directly from Latin and means *to make worse*. The term biodeterioration was adopted in the late 1950s to early 1960s for the study of the deterioration of materials of economic importance by organisms. However, this definition should probably be expanded to include not only the deterioration of materials, but also of constructions (e.g. structual timber as part of a building) or processes (e.g. a paper mill) of economic importance.

Of course, the interaction between biological systems and materials is not always undesirable. In contrast to the above, the word *degrade* also comes directly from Latin and means *to step down*. Thus, biodegradation could mean activities of organisms which result in the breakdown of materials either to man's detriment or benefit. Many essential geochemical cycles (e.g. the nitrogen and carbon cycles) are almost wholly dependent on biological (indeed microbiological) processes and of course in current times the term *biodegradable* is considered an essential property of many manufactured materials to ensure that they can be recycled effectively at the end of their service life. So, although biodegradation may be seen by man as a direct opposite to biodeterioration, it is actually biologically exactly the same process and it is impossible to make a scientific distinction between them. Indeed, they are usually the same processes, changed in meaning and significance solely by human need.

A subdivision of the manifold effects of a *biogenic impact* on materials may either be made according to materials or to organisms. Changes of materials or their service properties may be caused by microorganisms, higher plants as well as by insects and other animals. Among the microorganisms bacteria, yeasts and algae play an important role as do many molds and higher fungi and basidiomycetes. The dominating animal species that impact on materials are among the insects with termites and members of the orders coleoptera and lepidoptera having an especially great destructive potential. But higher animals such as rats, mice and birds also have a significant impact on the service life of many materials. In the marine environment molluscs and crustaceans are usually considered the main deteriorating organisms. Although studies on the interaction between material and biological systems need to be holistic in their approach whether this be to determine the degree of protection required by a material in service or to examine the impact of a material on an ecosystem, they are usually subdivided into specific areas to afford a more manageable route to their execution.

Materials primarily of natural origin, such as timber, pulp, paper, leather and textiles are particularly susceptible to deterioration by biological systems. However, many modern materials such as paints, adhesives, plastics, plasters, lubricating materials and fuels, technical liquids, waxes etc. can support microbial growth. Even the properties of inorganic products, such as concrete, glass, minerals and metals may suffer from biological attack. There are also many examples which demonstrate that not all breakdown of materials is undesirable as particular microorganisms are used for beneficial purposes, e.g., in extracting and processing raw materials such as alcoholic fermentation, antibiotics, flax retting, leaching etc. and even in extracting certain minerals in mining operations (e.g., bioextraction of uranium from mining residues).

14.1.1 Biodeterioration/Biocorrosion

A natural phenomenon of organisms, especially of microorganisms, is adhesion to surfaces of materials. For example, in the course of their proliferation a slimy matrix is produced by microbial communities at the interface with a material called a biofilm. In technical systems *biofouling* occurs. Drinking or process waters become contaminated, often by biofilms, and further propagation of biomass results in blockages of filter systems, pipings and heat exchangers. Economic damage results from the decrease in performance of technical processes (such as loss of efficiency in a heat exchanger) and can even result in equipment or facilities coming to a complete standstill. Losses amounting to billions of Euros every year are attributable to the effects of unwanted biofilms.

Another prominent example of the impact of biological systems on materials can be seen in the interaction between the organisms employed in a process with the materials used to contain it in the microbial deterioration of concrete in sewage systems caused by acidic fungal excretions. The same mechanism is also responsible for the deterioration of historic frescos and monuments. Even seemingly inert materials, such as the glass in optical devices, for instance binoculars and microscopes, are susceptible to etching which impairs and ultimately destroys their optical properties.

Biological processes may also produce discoloration and bad odors of liquids such as paints, glues, lubricating and technical liquids without actually affecting the performance of the material. Of course, they may also induce changes in consistency and impair the serviceability of such products or result in complete failure of the material. With plastics, biodeterioration can result in loss of mass and changes of technical characteristics, such as elasticity and tensile strength.

Effects of biodeterioration produced by organisms can range from damage caused by inorganic or organic acids, complexation, organic solvents, salt stress, influence of H_2S, NO_3, and NO_2 as well as enzymatic alterations or degradation. Biocorrosion of metals and metal alloys is also know to occur under a wide variety of conditions. The first reports on the corrosive properties of sulfate-reducing bacteria date back to the middle of the past century. Corrosion failures in oil refineries, pipelines and harbor facilities induced intensive research and investigations of the damage mechanisms. Spectacular accidents, such as the crash of a jet airplane due to corrosion of its aluminum fuel tanks illustrated the dangers of *microbially induced corrosion* (MIC). In most cases both bacteria and fungi were found to be responsible for such damage. The corrosion processes were induced either by their metabolites, such as acids, ammonia and hydrogen sulfide or by electrochemical circuits associated with the terminal electron acceptors of anaerobic metabolism. The capability of these microorganisms to form adhesive films on the surface of metals exacerbated the problem as below such films, anaerobic conditions prevail where corrosion-inducing oxygen concentration cells are produced. Similarly, hydrogen embrittlement may be also attributed to the production of hydrogen by microorganisms and its uptake by the metal surface.

14.1.2 Biodegradation

The manifold metabolic processes of organisms have been utilized by man in the course of his evolution. Long before the term biotechnology was coined, people knew how to produce foods by exploiting microbial processes. Agricultural applications included flax retting and waste straw upgrading. Important medical applications such as the production of antibiotics emerged. For a number of microbiological metabolic processes the following synonyms for biodegradation became established; biotransformation/bioconversion, implying the biological transformation of materials as an alternative to chemical processes. Bioleaching: metal extraction (especially of copper and uranium) from poor ores and mining spoil, the extraction of which would not be profitable by metallurgical processes. Biotreatment, for example, kaolin for producing porcelain but stained by iron oxides may be successfully bleached by reducing or complex-forming metabolites of microorganisms. Bioremediation, for example, TNT and a number of other explosives may be reduced to harmless substances and microbial processes are being used increasingly to decontaminate toxic substances in the environment.

14.1.3 Summary

As discussed above, biodeterioration can be defined as a decrease in the economic value of materials caused by biological organisms. From a physical point of view, biodeterioration can be defined as the transition of a material from a higher to a lower energy level or (chemically) from a more to a less complex state. From a biological point of view it is important to be able to relate biodeterioration and biodegradation events to the cycle of changes of materials which characterize the natural world. Thus, a material may be required to be stable towards biological attack while in service but needs to degrade in the environment to substances that are harmless to and totally integrated with the environment once the service cycle has ended. Many materials, constructions and processes must therefore be looked at in a true *cradle to grave* context to ensure that the economic benefits at one point do not lead to adverse environmental (and economic) impact at a later stage in the cycle of that material's *lifespan*.

14.1.4 Role of Biocides

Many methods exist worldwide for examining the relationship between organisms and both man-made and natural materials. Much of the emphasis of these methods is related to the spoilage, deterioration or defacement of materials whether these are foodstuffs, structural woodwork or water-based coatings [14.1]. Most of the testing technology is focussed on determining both the susceptibility of materials to attack and to the efficacy of agents intended to either prevent or limit this attack. In many cases, these tests are used to form the basis of claims about how well a certain material, additive or technology may be expected to perform when exposed to biological challenges. Often this information is used to make commercial comparisons between either different final products or additives as

well as to attempt to predict whether a material will comply with a certain specification (e.g. service life).

Biocides

Much of the technology mentioned above depends on the use of biocidal agents to prevent growth in association with the material to be protected. In other disciplines, such agents are employed to either limit the growth of or kill organisms within a process, possibly to prevent them from impacting on materials they may come in contact with. A good example of such agents is the additives employed in the water treatment industry. These agents are used in applications such as cooling and humidification systems and paper mills [14.1]. They are introduced to both eliminate health risks associated with the uncontrolled growth of microorganisms (e.g., prevention of the growth of *Legionella* spp. in calorifiers) and limit the impact that they may have on structural components within the process (e.g. corrosion, loss of heat transfer efficiency) and the products of the process (e.g. foul odor in air handling systems, defects in paper resulting from bacterial slimes).

Biocides are also employed to remove populations from either within the matrix of a material or on the surfaces of a material. These agents are often applied as washes or rinses and are used to either sterilize/disinfect or at least reduce either part or all of any population that may be present [14.2]. Such disinfection processes can also take the form of an addition of an biocidal agent to a matrix which contains a population (e.g., the reduction of microbial contamination in metal working lubricoolants, the treatment of timber infected with eggs/larvae of wood-boring beetles). Often this will be combined with the introduction of protection against further growth [14.3].

Biocidal agents may also be incorporated into a material to protect it in service. For example, preservatives are used in coating systems to protect the material from spoilage while in its wet state (so-called in-can protection) as well as to exhibit a biocidal effect in the finished film, preventing mold and/or algal growth on the surface of the coating once applied. Similarly, plasticized polyvinyl chloride (PVC) may be formulated with the addition of a fungicide to protect it from attack by microfungi and so protect the plasticizer and prevent loss of elasticity in service [14.4]. Wood may be impregnated with fungicides and insecticides prior to sale for structural applications.

In most of the situations described above, the treatment of a material is intended to either prevent deterioration of it, maximize the protection of the material or remove a population from a system prior to its use. The biocides employed for such purposes and the approaches taken to achieve effective disinfection and preservation have been reviewed extensively elsewhere [14.1, 5]. However, in recent years a new form of interaction between a formulated material and biological populations has emerged. In part, this can be viewed as either an extension of the degree of protection provided to a material by the inclusion of a biocidal agent into it or as the transfer of the properties of external treatments of a material into the material itself. The inclusion of the biocidal agent is not simply to protect the material from deterioration but to exert a biological effect either to the immediate surroundings of that material or to items that come into contact with it. These effects may range from the prevention of growth of undesirable microbial populations on a material to which they pose no physical, chemical or biological threat, the immediate destruction of individual microbial cells as they come into close association with a surface (possibly without even coming into direct physical contact) or to the inclusion of insecticidal agents into netting intended as a barrier to mosquitos [14.6]. In all cases the effect is external to the material from which the article is constructed and is not merely present to protect either the material or the item itself. However, it is possible that the effect may take place within an item constructed from a modified/treated material. For example, one can imagine an air filter constructed of paper into which antimicrobial properties have been introduced which is intended to kill bacteria pathogenic to man which impact on it [14.7]. Similarly, a polyethylene foam sponge may be impregnated with an antimicrobial agent which is intended to prevent the growth of bacteria associated with food poisoning in man. This sponge may not be intended to disinfect surfaces on which it is used but simply to prevent it from becoming a reservoir of such bacteria in a food preparation environment.

Clearly, there are some complex situations when the effects intended by treated articles/treated materials are to be considered and this will impact on the suitability of the methods used to measure them. In general however, the effect of a treated item/treated material can be considered to be external to it. The effect is not concerned with either preservation or protection of the material/item itself and is not achieved by the application of a disinfecting agent after the material has entered service.

Finally, when considering suitable test methodologies, the scale and duration of the effect may need to be considered with respect to the claim made. For

example, will the material/item be able to exert the effect claimed for the effect to have any realistic benefit? Similarly, will the scale of the effect be sufficient to provide the benefit either claimed or implied? It is unlikely that data to support such claims would be available from a single test and it is likely that ageing and weathering studies would be needed in addition to tests which provide basic proof of principle and demonstrate performance under conditions which simulate actual use.

Biocidal Activity

Biocidal activity is a generic term but in the context of this chapter we are essentially considering microbiocidal, insecticidal, acaricidal and mulluscicidal activity and is considered in more detail in [14.5]. In actual use this activity is further subdivided to represent activity against one or more groups within the various classes. For example, in the case of microorganisms, this will be impacted on by the microbial types/species which are employed in testing and, to a certain extent, the type of test required and this is considered in detail in other chapters in this section. The scale of the effect will often be important in some applications but the outcome of biocidal activity will result in a reduction in the number of test microorganisms as a result of an interaction with the material through an irreversible, killing effect. Such effects may be described as

1. *Bactericidal*: the effect is limited to a reduction in the size of a vegetative bacterial population.
2. *Fungicidal*: the effect is limited to fungi. This effect may be attributed to activity against vegetative growth, spores/dormant structures or both and may require clarification depending on the intended use of the product.
3. *Sporicidal*: the effect is against the spores/dormant structures of bacteria.
4. *Virucidal*: the effect is limited to virus particles.
5. *Protisticidal*: the effect is exhibited against protozoa and their dormant stages.
6. *Algicidal*: the effect is exhibited against algae and their dormant stages.

Biostatic Activity/Repellency

In many of the cases of the protection/preservation of materials the effects required are not associated with killing a population but of either preventing its growth or preventing it coming into contact with the material. In this context most interactions between a *biocide* and microbial populations are biostatic ones. As with biocidal activity, this will be impacted on by the species which are employed in testing and, to a certain extent, the type of test required but obviously, the prevention of growth/metabolism/colonization of/by the target species should be demonstrated. It may be sufficient to demonstrate that growth is either slower or reaches a lower level than on an equivalent control material to either substantiate a claim or demonstrate a benefit. In many cases chemical microbicides exhibit both biostatic and biocidal activity with the initial impact on a microbial population being biostatic and sustained contact resulting in biocidal action. Similar relationships are found with molluscicides where presence of a toxic agent is sufficient to deter attack. In some cases limits to the efficacy of biostatic and repellency action are not related to the potency of the agent but to the durability of the effect in combination with a material (e.g., leach resistance of a fungicide preventing the germination of fungal spores that have alighted on a coating applied to the facade of a building). As with biocidal activity an equivalent subdivision of the type of activity exists, e.g.

1. *Bacteriostatic*: the effect is limited to the prevention of growth/metabolism of bacteria and possibly the germination of bacterial endospores and other dormant structures.
2. *Fungistatic*: the effect is limited to the prevention of growth of fungi and possibly the germination of fungal spores and other dormant structures.
3. *Algistatic*: the effect is limited to the prevention of growth of algae and possibly the germination of dormant structures.

Summary

Although the intrinsic activity of biocidal agents is important, of more concern is the interaction between them and the material/system which they are designed to protect. The spectrum of activity must be appropriate to the challenge the materials is likely to endure and the biocide must be compatible with the material as well as be able to provide protection for a suitable period of service. Although many of the tests described in this section are designed to examine the susceptibility of materials to biological attack, many can be adapted to examine the impact a biocidal treatment can have on that attack. With careful consideration, reliable prediction of the performance of a material equipped with a biocide can be made and this is often the main challenge when considering the negative interaction of biological systems with materials.

14.2 Biological Testing of Wood

Section 14.2 deals with the degradation of wood, wood products and wood treated with preservatives, by insects and microorganisms. It introduces the test methodology used to simulate such attack and how to estimate its impact on the material. The approach to testing differs in detail throughout the world. However, certain basic principles are commonly accepted. The approach taken in Europe will mainly be used to illustrate these principles.

Before elaborating on the specifics of attack by microorganisms (Sect. 14.2.1) and insects (Sect. 14.2.2) some general aspects concerning the testing of wooden materials will be considered.

Wood is one of the oldest construction materials. Its natural availability and omnipresence had made it the most obvious choice to build bridges, houses, ships etc. for millenia. It is relatively easy to process, has good insulating properties, has a high elasticity (compared e.g. to concrete, steel, stone) and wood with a high density is amazingly fire resistant (e.g. oak, teak).

Wood can be cut and bent to the desired size and shape. However, as a typical organic matter, its basic components and its constituents provide a nutrient source for microorganisms, molluscs, insects and other arthropods. Because of this, many species have developed natural defence systems. For example, some tree species (e.g. bongossi, teak) often produce phenolic substances which are deposited in the cells of the heartwood. These substances can considerably delay the attack by microorganisms and lend the material a degree of natural durability. Laboratory and outdoor tests have been developed to assess this natural durability for the commercially most interesting wood species used in Europe (see later: European Standard EN 350, Durability of wood and wood based products). In contrast, other wood species (e.g. pine, beech) can be rapidly degraded. To make long-term use of them in construction etc. they have to be preserved chemically with a wood-protecting biocide. Therefore, the test standards described in the following mainly deal with preservative-treated wood/wooden materials to determine the efficacy and performance of this material.

However, to determine the virulence of the microorganisms used in the different test setups, untreated wood is always incorporated. Some methods also employ so-called *reference products* which include preservatives that have shown their preserving effects on wood for decades. With the help of a reference product the severity of a method can be estimated and the results for a new preservative under test can be put into context.

Ideally, methods for determining the protective efficacy and the performance of treated or untreated timber should

- Reflect the environmental conditions to which the treated timber is subjected in service;
- Cover all relevant organisms and their succession during the time of use of the wooden commodity or construction;
- Take into account the possible methods of treatment for the wood preservative;
- Provide reproducible results rapidly;
- Be uncomplicated and easy to handle;
- Involve minimal costs.

Obstacles are

- The environmental conditions and the decaying organisms to which the timber is subjected are extremely diverse,
- The sensitivity of the decay organisms is different towards different biocides,
- The biocides applied are stressed not only by physical factors like evaporation, leaching and diffusion but;
- In the case of organic biocides, these compounds can also be utilized by organisms that are not the target of the chemical wood preservation and which may deteriorate the biocides or even use them as a nutrient source;
- Not all timber species are equally treatable.

Therefore it is necessary to simplify and to develop methods which nevertheless give sufficient certainty for the assessment of treated and untreated wood under test.

General Requirements for Resistance Against Biological Attack

More general requirements for testing procedures are outlined in the European Standard EN 350 for a natural resistance against wood-destructing organisms and in EN 599 for a wood preservative derived resistance.

- European Standard EN 350-1:
  Durability of wood and wood-based products – Natural durability of solid wood – Part 1: Guide to principles of testing and classification of the natural durability.
- European Standard EN 350-2:
  Durability of wood and wood-based products – Natural durability of solid wood – Part 2: Guide to

natural durability and treatability of selected wood species of importance in Europe.
- European Standard EN 599-1:
  Durability of wood and wood-based products – Performance of wood preservative as determined by biological tests – Part 1: Specification according to use class.
- European Standard EN 599-2:
  Durability of wood and wood-based products – Performance of wood preservative as determined by biological tests – Part 2: Classification and labelling.

The likelihood for a biological attack of wooden materials also strongly depends on the environment in which the material is used. These potential environments can be categorized into use classes, formerly known as hazard classes, according to the European Standard EN 335.

- European Standard EN 335-1:
  Durability of wood and wood-based products – Definition of use classes of biological attack – Part 1: General.
- European Standard EN 335-2:
  Durability of wood and wood-based products – Definition of use classes of biological attack – Part 2: Application to solid wood.
- European Standard EN 335-3:
  Durability of wood and wood-based products – Definition of use classes of biological attack – Part 3: Application to wood-based panel.

EN 335-1 defines the environmental compartments in which wood can be used and describes the main hazards wood is exposed to. The standard defines five use classes (Table 14.1). The European standard EN 335 is currently under review. The aim of the review is to combine all three parts to one comprehensive standard. Other countries developed similar classifications. An ISO standard titled *Durability of wood and wood based products – Definition of use classes* is basically following the same principle.

Preconditioning Methods Before Durability Testing of Treated and Untreated Wood

In order to evaluate the effectiveness of a wood preservative over time, artificial ageing of protected wood is performed before a standard test method against microorganisms or insects is carried out.

- European Standard EN 73:
  Accelerated ageing tests of treated wood prior to biological testing – Evaporative ageing procedure describes an evaporative ageing procedure, applicable to test specimens of wood which have previously been treated with a preservative, in order to evaluate any loss in effectiveness when these test specimens are subsequently subjected to biological tests, as compared with test specimens which have not undergone any evaporative ageing procedure.
- European Standard EN 84:
  Accelerated ageing tests of treated wood prior to biological testing – Leaching procedure describes an ageing procedure by leaching, applicable to test specimens of wood which have previously been treated with a preservative, in order to evaluate any loss in effectiveness when these test specimens are subsequently subjected to biological tests, as com-

Table 14.1 European Standard EN 335 *Durability of wood and wood based products – use classes: definitions, application to solid wood and wood based panels*

Use classes	General use situation	Description of expose to wetting in service	Fungi	Beetles[1]	Termites	Marine borers
1	Interior (dry)	None	–	U	L	–
2	Interior, or under cover, not exposed to the weather (risk of condensation)	Occasionally	U	U	L	–
3	Exterior, above ground, exposed to the weather	Frequently	U	U	L	–
4	Exterior in ground contact and/or fresh water	Permanently	U	U	L	–
5	Permanently or regularly submerged in salt water	Permanently	U	U	L	U

U = Universally present within Europe.
L = Locally present within Europe.
[1] The risk of attack can be insignificant according to the specific service situation.

pared with test specimens which have not undergone any ageing procedure by leaching.

- European Technical Report CEN/TR 15046 *Wood preservatives – Artificial weathering of treated wood prior to biological testing – UV-radiation and water-spraying procedure*: This method describes an ageing procedure which simulates intervals of rain and UV-radiation. Because it works with elevated temperatures, to some extent also evaporation is included with this method. The method combines the main stresses by physical factors on the wood preservative in treated wood specimens prior to fungal or insect tests.

Modern organic fungicides and insecticides are susceptible to microbiological degradation. Therefore, the above-mentioned ageing standards are not always sufficient to determine the longevity of wood preservatives formulated with these biocides. A European technical Specification (CEN/TS 15397) titled *Wood preservatives – Method for natural preconditioning out of ground contact of treated wood specimens prior to biological laboratory tests* exists. This method combines natural physical stress factors with the possible succession of naturally occurring microorganisms. This technical specification is intended to overcome the difficulties caused by the nontarget microorganisms at least for wood exposed to the general service situations defined for European use class 3.

14.2.1 Attack by Microorganisms

Microorganisms can only attack wood when water is present in its cell lumina. This state is described as *wood moisture above the fiber-saturation-point*. As a rule of thumb this state is reached at about 30% wood moisture content (related to the dry weight of the wood). Dry wood cannot be metabolized by microorganisms and needs no preservatives to protect it against them.

Above fiber saturation the main components of wood – cellulose, hemicelluloses and lignin – can be degraded by a vast number of microorganisms.

Wood-destroying fungi are the most evident and powerful wood degraders. Their species belong to the

- basidiomycetes, causing brown rot or white rot,
- ascomycetes and fungi imperfecti, causing soft rot and stains.

Wood-destroying bacteria can be present in extremely wet environments, but the speed at which they degrade wood is normally very slow and they can be neglected as metabolizing organisms of wood components. Nevertheless, with wood preservation moving away from inorganic, undegradable components towards organic compounds, the deterioration of these substances might be influenced by bacteria, leading to a failure of the wood preservative and therefore opening pathways for wood-metabolizing organisms to attack.

In relation to wood destruction mass loss and therefore strength loss of the wood are the primary issues. However, wood can also lose value through discoloration by microorganisms through molds and blue-staining fungi that cause no loss of cellulose, hemicellulose or lignin.

Detecting Wood-Destroying Fungi by Visual Means

The above-mentioned types of rot cause different appearances of the attacked wood. In many cases this can even be detected by the bare eye and can be confirmed by microscopic work. For macroscopic evaluation the wood should be dry, because checks and cracks become more obvious this way. For cutting sections to be analyzed microscopically the wood moisture should be above the fiber saturation point (above 30% wood moisture), because all strength properties of wood decrease with increasing moisture content of the wood until the fiber saturation point is reached. In other words: cutting becomes easier.

Visual Distinction of the Main Types of Wood Decay

Figures 14.2a and 14.3a show brown rot and white rot on wood macroscopically (here the wood has been cut longitudinally). Figure 14.1a shows undecayed, Figs. 14.2b and 14.3b decayed cross sections of wood as it can be seen under the microscope at a magnification of 150× to 200×. Further macroscopic and microscopic examples of wood decay can be found in [14.8].

Soft Rot. These fungi degrade cellulose and hemicelluloses. Macroscopically they cause a greyish-black rot with small cubicle cracks. Microscopically this form of decay is characterized by cavity formation inside the cell wall (Fig. 14.1b).

Blue Stains. Blue stains do not degrade lignin, cellulose or hemicelluloses and therefore cause no loss in mass or stability of the timber. They metabolize sugars deposited in the parenchymatic tissue of the wood. Blue stain fungi grow through the parenchymatic cells and spread in the

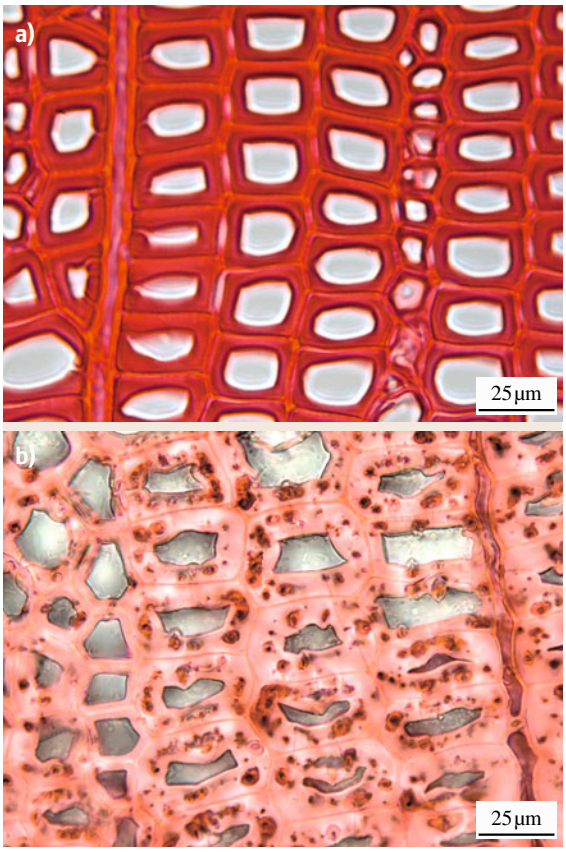


Fig. 14.1 **(a)** Cross section of sound undecayed wood (*Picea* sp.). **(b)** Cross section of wood (*Picea* sp.) decayed by soft rot causing cavity formation in the cell wall (Courtesy of Swedish University of Agricultural Science, Uppsala, Sweden)

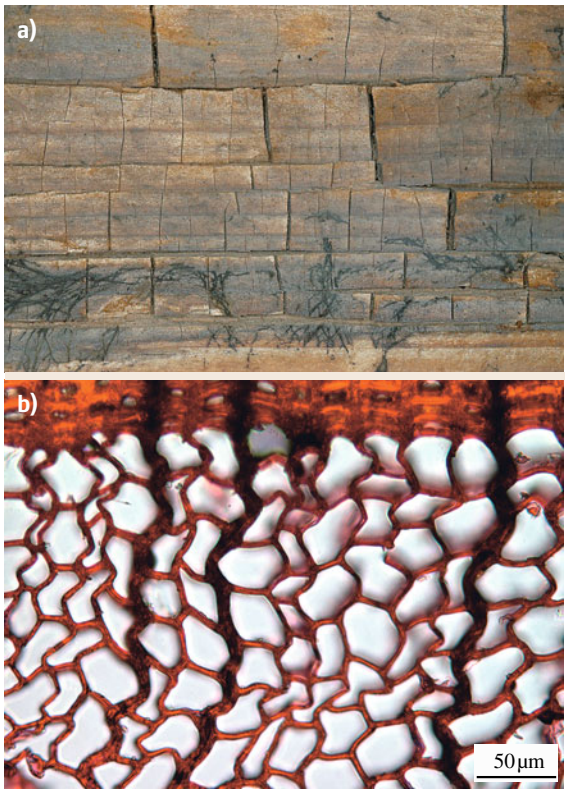


Fig. 14.2 **(a)** Longitudinal cut of wood (*Picea* sp.) decayed by brown rot causing fungi; wood surface shows cubicle cracks in wood decayed by brown rot, in the lower part of the picture mycelium of the brown rot causing fungus can be seen. **(b)** Cross section of wood (*Picea* sp.) decayed by brown rot causing fungi (Courtesy of Swedish University of Agricultural Science, Uppsala, Sweden)

wood. They stain the wood through their black-bluish hyphae and spores.

Brown Rot Fungi. Brown rot fungi metabolize cellulose and hemicellulose of the wood. Lignin can not be degraded by these fungi. The rot leaves behind cubic cracks (Fig. 14.2a) and a dark brownish tinge to the wood. The microscopic features are shown in Fig. 14.2b.

White Rot Fungi. White rot fungi metabolize all three main components of wood: lignin, cellulose and hemicelluloses.

Macroscopically white rot fungi generally lighten the color of the wood. Two types of white rot can be distinguished: a) simultaneous rot, where in pockets of decay the cellulose, hemicelluloses and lignin are completely degraded and b) the selective lignin degradation. Whereas the pockets of decay can be easily detected (Fig. 14.3a) the selective degradation of lignin, which is the more common form of white rot, can not be determined by changes of the wood surface like cracks or holes. Mass loss and change to a lighter color of the wood compared to the undecayed timber are the first signs of such an attack. The microscopic features for lignin degraders are shown in Fig. 14.3b.

Lignin-degrading fungi can be detected by the presence of a phenolic oxidase enzyme based on the Bavendam-test. This test allows to biochemically distinguish brown from white rot fungi.

Sap Stains. Fungi grow only on the surface of freshly cut timber. They do not metabolize the wood itself, but

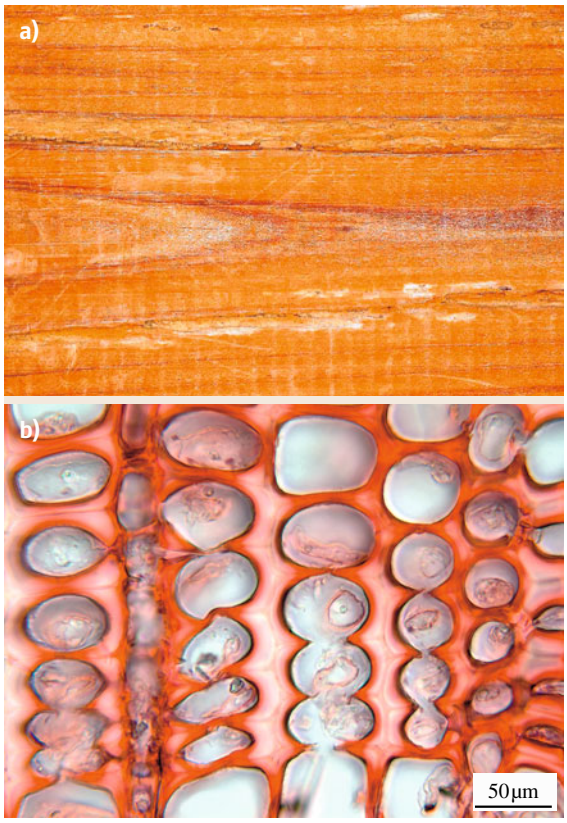


Fig. 14.3 **(a)** Longitudinal cut of wood (*Picea* sp.) decayed by white rot causing fungi; this picture shows an example for so called *pocket rot*, because white pockets of decay can be seen on the wood surface. **(b)** Cross section of wood (*Picea* sp.) decayed by white rot causing fungi (Courtesy of Swedish University of Agricultural Science, Uppsala, Sweden)

the sugars deposited in the parenchymatic tissue of the wood. The fungi can access these sugars only in cells that have been damaged (by force: felling or processing). By their metabolic products and colored spores they stain the wood and lead to loss in value.

A good overview on different forms of decay is also given by *Wilkinson* [14.9], and a more detailed description of macro- and microscopic observations can be found, e.g., in *Anagnost* [14.10].

As outlined above (see on *sap stain*, *blue stain*) some microorganisms cause only discoloration of wood while others also lead to mass loss of the wooden substance. Mass loss and therefore density loss is the more critical parameter since it is related to strength loss [14.11] which is especially undesirable for a construction and building material. Therefore laboratory methods determine the mass of a wooden specimen before and after exposure to fungi which have been selected as aggressive wood-deteriorating organisms under laboratory conditions.

To measure mass loss at a set point in time requires the drying of the wooden material to 0% wood moisture content. This drying process kills living fungal cells and can lead to severe cracking of the wood structure. The rheological properties of the wood also lead to irrevocable physicochemical changes while drying. Therefore the determination of mass loss by weighing is not a nondestructive method.

Nondestructive Testing Methods to Detect Fungal Decay

Nondestructive methods are required when the changes of wood structure have to be monitored over a longer time period or when the timber to be tested is already part of a construction. *Bodig* [14.12] listed the following nondestructive methods as examples.

- Sonic stress wave
  Stress waves are generated either through an impact or by a forced vibration. Usually, with this method either the speed of sound or the vibration spectrum is measured. The dynamic modulus of elasticity (MOE) can be calculated from these measurements.
- Deflection method (static bending technique)
  The deflection is measured at a safe load level which does not lead to rupture of the test piece. The static MOE can be calculated from these measurements.
- Electrical properties
  The products of fungal metabolism are carbon dioxide and water of which the latter leads to a higher moisture content in the wood. This method is based on the relationship between moisture content and electrical resistance of wood.
- Gamma radiation
  is tool for quantifying decay. It is also employed as a tracing method for quantifying the distribution of preservatives in wood. One of the limitations of this method is the regulations associated with the use of a radioactive source.
- Penetrating radar
  This method is currently being developed for wood products. The method bears the potential to detect and quantify degradation at inaccessible locations.

- X-ray method
  is mostly used in the laboratory or in production lines due to the bulky nature of the x-ray source and the measuring equipment.

Similar techniques (transverse vibration techniques, static bending techniques) are listed in a review on nondestructive testing for assessing timbers in structures by *Ross* and *Pellerin* [14.13].

Also infrared, x-ray and gamma-ray computerized tomography have been employed to visualize microbiological attack in timber. However, the techniques are mostly very cost intensive.

The oldest nondestructive test method is the visual estimation with the bare eye followed by rating the decay or discoloration of the specimens. This inexpensive method provides the expert with a lot of information. It is mainly applied when large numbers of specimens in a test field have to be assessed. An experienced evaluator will be able to rate the intensity of decay as well as to determine which type of decay has infested the wood.

Testing Wood for Different Use Classes

While the environmental conditions in use classes 1 and 2 do not provide the necessary amount of water to allow growth of microorganisms, the use classes 3 and 4 are the more relevant for testing microbiological decay above ground and in ground contact.

Testing Wood for Use Class 3.

Field Tests. Use class 3 is a very complex class. Depending on the local climatic conditions, the dimensions of the cross section of construction parts and their actual location (near to the ground, mostly covered under a roof etc.) it may reach from nearly use class 2 to use class 4. Many test methods have been developed for use class 3, intended to accelerate the attack by microorganisms and thus to give results in relatively short times. Some of them even use additional artificial wetting regimes. But all of them are simply reflecting different situations in use class 3. They are not accelerated test methods, except when they are used under extremely severe tropical conditions.

Three of these methods shall be described exemplarily.

- EN 330: *Wood preservatives – Field test method for determining the relative protective effectiveness of a wood preservative for use under a coating and exposed out of ground contact: L-joint method.*
  With slight modifications the AWPA E9-06 *Standard Field Test for the Evaluation of Wood Preservatives to be Used in Non-Soil Contact* is comparable to this method. Stylized corners of window frames with mortise and tenon (L-joints) are treated with a wood preservative by a method recommended by the supplier of the preservative (double vacuum, dipping or others). After drying of the preservative the mortise members are sealed at the cross section opposite to the mortise and the whole L-joints are coated with an alkyd reference paint or a paint system provided by the supplier of the preservative. Then the specimens are exposed in the field on racks in a position slightly leaned backwards. Prior to exposure the top coat will be broken at the joint by opening and reclosing the joint. In at least annually intervals the L-joints are visually examined for occurrence of wood-disfiguring and wood-destroying fungi. For the assessments the joints are taken apart in order to check the situation within the joint. The fungal attack is rated according to a 5-step rating scale reaching from 0 (sound) to 4 (failure). After 3 and 5 years of exposure additionally exposed specimens are assessed destructively by cutting the joint members lengthwise as to detect interior rot in the wood. The mean service-life of a series of L-joints will be determined by adding the service-life of the individual members of the series after the last member is rated *failure* and dividing that number by the number of parallels in the test.
- ENV 12037: *Wood preservatives – Field test method for determining the relative protective effectiveness of a wood preservative exposed out of ground contact – Horizontal lap-joint method.* AWPA E16-09 *Field Test for Evaluation of Wood Preservatives to be Used Out of Ground Contact: Horizontal Lap-Joint Method* uses the same method with only slight modifications. Objective of the method is to evaluate the relative effectiveness of the preservative, applied to jointed samples of pine sapwood by a treatment method relevant to its intended practical use. In contrast to the L-joint method, the wood preservative is applied without subsequent surface coating. Bound together with cable straps the jointed specimens are exposed on racks outdoors not touching the ground. The joint functions as a water trap, thus providing optimal wood moisture conditions for the attack by wood-destroying fungi for relatively long periods. Again the specimens are examined visually at least annually using a rating scale for the fungal attack.
- AWPA E18-06 *Standard field test for evaluation of wood preservatives intended for use in category 3B*

applications exposed, out of ground contact – Uncoated ground proximity decay method. Test specimens of pine or other softwood species, measuring $125 \times 50 \times 19\,mm^3$ are treated with a wood preservative according to the recommendation of the supplier of the preservative. After drying of the preservative the specimens are exposed outdoors, lying horizontally on concrete blocks measuring $40 \times 20 \times 10\,cm^3$ which are placed on the ground. The arrangement is covered by an open frame with a horticultural shade cloth on top. The distance between specimens and cloth is about 3 cm. The cloth is intended to protect the specimens from direct sunlight. It also reduces the drying of the specimens and provides an increased relative humidity within the frame. The specimens are checked for fungal attack at fixed intervals. The attack is rated according to a rating scale.

The purpose of these methods is to expose the treated specimens to the complete range of microorganisms occurring under natural conditions. That means, all possible microorganisms, like bacteria, yeasts and fungi get to attack the wood. According to the local climatic conditions the specimens are subjected to changing temperatures, precipitations and relative humidity. All microorganisms metabolizing wood have their specific optimum temperatures and wood moisture content. Therefore a natural succession of microorganisms occurs which cannot be achieved in the laboratory. To some of the organisms the active ingredients of the wood preservatives may be poisonous, while other microorganisms may detoxify them or in the case of organic substances may even use them as a nutrient source.

All these methods provide data on the performance of wood preservatives. But as the local conditions of temperature and precipitation of the exposure sites can be extremely different even at relatively small distances, the performance data can also vary extremely. As experiments in a European research project (FACT project) have shown, even untreated lap-joints may not be attacked in Northern Europe within three years but be heavily attacked within one year in the tropics. And because it is more or less accidental which decay organism attacks the specimens at which time, the tests are not sufficiently reproducible. Therefore the results cannot be used for approvals of wood preservatives, where reproducible efficacy data are needed which give a certain overall reliability for the consumer [14.14–16].

Laboratory Tests.

- EN 113 *Wood preservatives – Method of test for determining the protective effectiveness against wood destroying basidiomycetes – Determination of the toxic values* works with pure cultures of different basidiomycetes that cause brown or white rot. The preservatives are incorporated into the wood at different concentrations under vacuum conditions. The treated specimens are then exposed to the fungi for 16 weeks at an optimum temperature. The mass loss (%) is determined at the end of the test.
- CEN/TS 839 *Wood preservatives – Determination of the protective effectiveness against wood destroying basidiomycetes – Application by surface treatment* is designed to assess whether a wood preservative is suitable to protect the surface of timber constructions from decay and to prevent the penetration of fungi into the interior parts of timber which are not impregnated with the wood preservative.
- EN 152-1 *Test methods for wood preservatives; laboratory method for determining the protective effectiveness of a preservative treatment against blue stain in service; part 1: brushing procedure* and
- EN 152-2 *Test methods for wood preservatives; laboratory method for determining the protective effectiveness of a preservative treatment against blue stain in service; part 2: application by methods other than brushing* are methods that are applied partly in the field as well as in the laboratory. After a natural weathering period of 6 months (between April to October) on outdoor racks the timber specimens are taken into the laboratory where they are inoculated with blue stain fungi. After 6 weeks of incubation the discoloration of the brushed/coated timber surfaces is evaluated. Optionally the outdoor weathering can be replaced by an artificial weathering in a weathering device with UV-light, condensation and rain periods (see: Preconditioning methods before durability testing of treated and untreated wood).

Testing Wood for Use Class 4.

Field Tests. While in use class 3 the local climates play the decisive role in start and progression of microbial attack of wood, the type of soil is the decisive factor in use class 4.

An example for a test method for this use class is

- EN 252 *Field test method for determining the relative protective effectiveness of a wood preservative in*

ground contact. The AWPA E7-07 *Standard method of evaluating wood preservatives by field tests with stakes* follows the same principle.
EN 252 uses stakes of Scots pine sapwood (dimensions: $500 \times 50 \times 25\ mm^3$) which are treated with the preservative under test by a vacuum-pressure process. The stakes are exposed in the test field, buried half of their length in the ground. Annually the stakes are examined visually for fungal decay using a rating scale. In addition, the remaining strength of the stakes is probed by a gentle kick against the stakes when still buried in the ground. Stakes treated with a well-known reference preservative are exposed simultaneously. The efficacy of the test preservative is determined by comparing the performance of the test preservative with the performance of the reference preservative.

Methods of nondestructive testing (see above) can also be applied at this point. For instance, measuring the MOE in a static or dynamic manner of the wooden stakes before they are exposed in the field and once every year does give an indication of the performance of the preserved or unpreserved material over time [14.17].

Laboratory Tests.

- Prestandard ENV 807 *Wood preservatives – Determination of the effectiveness against soft rotting micro-fungi and other soil inhabiting microorganisms* gives a basis for assessing the effectiveness of a wood preservative against soft rot-causing fungi. The source of infection is the natural micro-flora of biologically active soil, which may also contain other microorganisms such as bacteria and other fungi. The data obtained from this test provides information by which the value of a preservative can be assessed. Nevertheless it has to be supplemented with other test data for use class 4 to provide a more complete picture.

Testing Wood in Aquatic Environments (Part of Use Class 4 and Use Class 5). The fresh water (use class 4) or marine (use class 5) environment, is a very complex environment in which bacteria, fungi and molluscs can lead to wood destruction. Other organisms like algae might settle on the wood and will help to establish a biofilm on the wood that enhances fouling. Standardized laboratory methods for wood treated with preservatives or tested for their natural resistance against decay are not known to the author. The methods known are all field tests, which implies the setup of the test specimens into open waters.

Only a few marine organisms shall be mentioned in this context specifically: The mollusc borers *Toredo navalis*, and *Bankia* sp., commonly called *shipworms* and the crustacean borers *Limnoria*, *Cherula* and *Sphaeroma*. These organisms actively bore into the wood and therefore affect wood stability.

14.2.2 Attack by Insects

Several industrial und household materials and construction devices of organic matter, especially those of biogenic origin, are endangered by pest insects. Susceptible to insect attack are mainly materials of plant origin made up of lignin and cellulose, namely wood, or products of wood origin [14.8]. These materials provide a habitat with shelter, food, and breeding sites for a manifold of different pest insect species [14.18]. Most are beetles (*Coleoptera*) (Figs. 14.4 and 14.5) and termites (Fig. 14.6). In most cases the developmental stages of the pests feed on the materials causing their destruction or contaminate it in such a way that their intended function is irreversibly altered.

The rate of destruction caused by insects is strongly influenced by various factors like climate, moisture content of the material, its nutritional value, and the infestation density. Additionally, the respiratory activity of a heavy insect infestation generates heat and moisture and affects the microclimate, favoring the growth of fungi, yeasts, and bacteria which further increase the overall decay rate of the material.

Preventive protection and, more important, regular inspections of potentially endangered materials are essential to guarantee the safety and the serviceability of, for example, wooden constructions, paper-based insulation materials, tools, furniture, and historic artefacts. The sooner an infestation is detected, the better is the chance for a remedial measure. The signs of destruction are generally material specific but may also be pest specific, allowing target-specific corrective and control actions.

However, the degree of damage, especially the one caused by wood-destructing insects, can easily been overseen, for larvae of wood-boring beetles and some termite species excavate wood only from the inside and leave a shallow surface layer fully intact. Nevertheless, a number of indices may point to a pest infestation, and no particular equipment for the detection is necessary.

Preventative control action against material insect pests is usually achieved through the application of

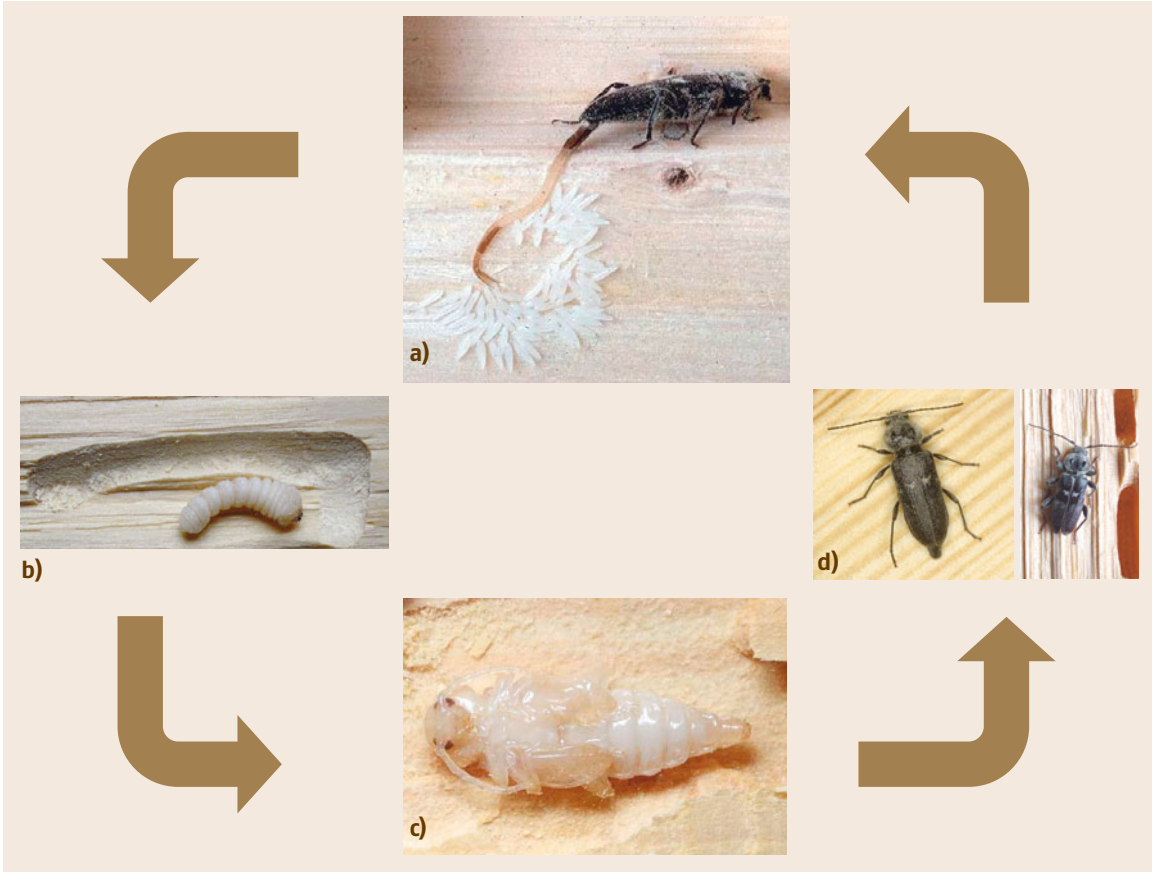


Fig. 14.4a–d Development stages of wood boring beetle using the old house borer *Hylotrupes bajulus* as an example. All stages except adult beetles are inside the wood and usually not visible from the outside. **(a)** Egg laying female; **(b)** full grown larva (3 to 6 years old) **(c)** pupa; **(d)** adult beetles (female *left*, male *right*)

residual pesticides. The effectiveness of theses insecticides can be evaluated in several laboratory test methods.

Methods for Detecting Insect Attack

The most important types of insects that attack constructive timber are beetles and termites. Occasionally, a few ant species, wood wasps or horntails, wood-boring moths and a solitary bee may be of some relevance. Numerous indices on the surface wood may directly point to an attack by wood-feeding insects. Prominent signs allow differentiating between the possible pests.

Various inspection methods are available to check for the presence of wood-boring insects: visual, auditory, x-ray, infrared, and even the use of tracker dogs.

Visual Inspection. The simplest check-up of materials potentially endangered by insect attack is visual inspection of the material's surface, streaming debris from the material and all signs of insect presence (Table 14.2).

Flight holes: Flight or emergence holes are the exit sites of emerged adult insects after having completed their larval stage inside the wood. They appear round or oval and sharp-edged, not to be confused with screw- or nail holes. Broadly oval holes are characteristic for cerambycid beetles, whereas a round shape of these holes points to powder-post or anobiid beetles. However, the mere existence of flight holes is no final proof for an ongoing attack, since the completion of beetle development and the infestation may have occurred a long time previous. Additional information is required. New emergence holes appear bright to light yellow in color, like freshly-sawed wood, and indicate recently emerged beetles with possibly more larvae in the material to complete their development. The longer an infestation is extinct

Fig. 14.5 Wood boring beetles *Anobium punctatum* adult

the more dust is accumulated and oxidation processes darken the powdery inner edges of the holes over time. Paint sealed holes from a previous coating may also indicate an already extinct infestation when no fresh holes are evident. In case of doubt, existing emergence holes should be marked and the material be rechecked after time for additional holes to have occurred. The material may also be tightly wrapped or sealed with paper, for new emerging beetles will penetrate the wrap and indicate developmental activity by leaving their exit holes in the wrap.

The flight holes of dry-wood termites, which may be confused with the exit holes of anobiid beetles, are first signs of their presence. They live in small colonies of up to some hundred individuals entirely in wood that is moderately to extreme dry. They require no contact with the soil. Because of their concealed life, colonies can go undetected for many years inside timber. Often it becomes visible only when already considerable damage was produced.

Fig. 14.6 Termite *Mastotermes darwiniensis*; 2 worker termites *at the bottom* and 3 soldier termites *at the top*

Big round holes, 12 mm wide, mainly outdoors, are the nest entrances of carpenter bees. The wood below the hole often shows yellowish fecal streaks. The entrance is usually guarded by the female bee, giving a humming sound.

Horntails also emerge through round-shaped flight holes. Their size can vary between 4 to 6 mm in cross section and fresh holes occur exclusively in recent cut and build-in timber during the first three years. This is because development is completed from eggs which had been laid by female horntails in the forest on trees declining or dying from fire, disease or insect damage or other natural causes. They also infest newly felled and freshly sawed lumber. Reinfestation of dry structural timber is most unlikely.

Appearance of the wood surface: The larvae of wood-boring insects usually start their tunnelling in the most peripheral parts, leaving a paper-thin layer of the wood surface untouched. The frass produced by growing larvae occupies a greater volume than the wood from which it was produced, and this causes the surface of the infested wood to have a blistered or rippled appearance. Occasionally the surface will break, and frass may fall out through the fine cracks and accumulate on the floor beneath.

Little mud tubes, so-called galleries, extending from the ground over exposed surfaces to a wooden food source are good indicators of the presence of subterranean termites. The tubes are either round or flat and usually measure at least 8 mm. These termites live in colonies which can contain thousands to millions of individuals and are closely associated with the soil habitat where they tunnel to locate water and food, namely wood.

Termites excavate galleries or tunnels in wood as they consume it, leaving nothing more than a thin wooden layer. These areas are easily crushed with a hard object (knife, hammer or screwdriver). In the case of extreme damage partly collapsed wood at bearing points may pinpoint to internal excavation. Noninfested wood gives a sound resonance when pounding the surface, damaged wood sounds hollow.

Slitlike openings called *windows* with some frass directly beneath are positive signs for carpenter ant activity. Usually this frass contains fragments of ants and other insects mixed with the wood fibers, because unlike termites that consume wood, carpenter ants scavenge on dead insects, insect honeydew and other materials.

Table 14.2 Characteristics damage by wood infesting insects (modified from [14.18])

Infested material	Signs of infestation			Most likely pest
	frass	holes	galleries, tunnels, feeding tubes	
Seasoned sapwood of hardwoods or softwoods (rarely in heartwoods)	Fine powder with elongate lemon-shaped pellets, loosely packed	Exit holes circular 1.6 to 3 mm	Up to 3 mm circular in cross section, numerous and random	Anobiid powderpost beetle
Sapwood of hardwoods primarily, minor in softwoods	Fine to coarse powder, tightly packed, tend to stick together	Exit holes circular 2.5 to 7 mm	1.6 to 10 mm circular in cross section numerous and random	Bostrichid powderpost beetle
Sapwood of ring and diffuse porous hardwoods only	Fine flour-like, loosely packed in tunnel	Exit holes circular 0.8 to 1.6 mm	1.6 mm circular in cross section, numerous, random	Lyctid powderpost beetle
Seasoned sapwood of softwoods	Very fine powder and larger cylindrical pellets, tightly packed in tunnels	Exit holes oval 6 to 10 mm	10 mm oval in cross section, numerous in outer sapwood, ripple marks on walls	Hylotrupes bajulus
Unseasoned wood under bark only, inner bark and surface of sapwood only	Coarse to fine powder, bark coloured, tightly packed	Exit holes circular 1.6 to 2.5 mm	Up to 2.5 mm circular in cross section, random	Bark beetles
Seasoned dry soft- or hardwood	Hard elongate pellets of uniform size, less 1 mm, six flattend or Concavely depressed sides	None	Hollow tunnels beneath a thin wooden layer, sometimes filled with fine frass	Dry wood termites
Wood coated with mud galleries	Fine lamellae of late wood remain intact	None	Hollow tunnel beneath a thin wooden layer, mud tubes leading to the wood	Subterranean termites
Seasoned wood	Piles of coarse wood shavings with insect parts	Slitlike *windows*	Clean smooth galleries	Ants, carpenter ants
Softwood or softer hardwood with no bark	Fine wooden debris with yellowish faecal streaks	Entrance holes large and circular 12 mm	Smooth walled 12 mm circular in cross section, very regular	Carpenter bees
Fresh to slightly seasoned softwood, very rarely hardwood	Fine coarse, usually not outside the wood	Exit holes circular 4 to 6 mm	Strong variation in size, circular in cross section, tightly packed with frass	Horntails
Unseasoned wood or occasionally damp	Fine in texture with granules being circular	Narrow oval ragged margins	Indistinct, less than 3 mm in cross section	Weevils
Unseasoned wood with bark or occasionally damp	Fine, stacked between bark and sapwood	Numerous small entrance and larger exit holes	Bark is lifted up by frass deposits	Warf beetles

Lamellar degradation of the cut end of foundation beams point to the activity of the wood ant *Lasius fullginosus*. This small ant in general starts to attack wood at the ground level, where it is in contact with moisture and therefore is susceptible for fungus decay. This is a precondition for an initial attack. Later, by autonomous moisture intake, together with this symbiotic fungus the ants progress deeper into the wood thus possibly causing substantial damage.

Occurrence of frass (bore dust): While feeding, beetles often push out powdery frass from holes which they have constructed in the infested wood. The frass is piled below the holes or in cracks of the structures. However, those piles are not indicative off an active attack, as concussions can cause a release, after an infestation has already ceased. Furniture or other wooden objects with past infestations will sometimes be suspected to be reinfested when frass or insect parts fall out in the process of handling or moving. By placing a dark paper beneath nonmoved objects to detect the appearance of fresh frass will clarify whether the infestation is active or not.

If the wood surface is probed where tunnelling is suspected, the powdery borings may be located. The consistency of the frass ranges from very fine to coarse, depending on the pest. The size and shape of larval frass are often species specific. Larger cylindrical frass pellets

like those produced by the old house borer, *Hylotrupes bajulus*, are typical for cerambicid beetles, whereas round frass pellets with tip-point edges indicate the presence of anobiid larvae like *Anobium punctatum* for example. Flour or talc-like frass points to the presence of powderpost beetles. This will fall out of the emergence holes while tapping the wood with a hammer. A magnifying lens should be used for a reliable inspection of frass pellets.

Small fecal pellets generally found in the close vicinity of their wooden habitat are good indicators for the presence of drywood termites. The pellets can vary in color, depending on the wood that has been consumed. They appear hard, elongate, of uniform size, less than 1 mm in length, with round ends and six flattened or concavely depressed sides. The piles do not contain any other debris such as insect parts or fiber.

A pile of wood shavings outside a hole or opening is a hint for the presence of carpenter ants. The wood shavings are coarse and insect parts and bits of insulation will be mixed among them. These shavings may also be found in spider webs and window sills close to the nest site.

The frass produced by carpenter bees is very similar to those of carpenter ants regarding color and size. It usually lacks insect fragments.

Damaged wood: The larvae of most wood-boring beetles develop for several years inside the inner portion of seasoned wood. Tunnelling is most extensive in sapwood, but it may extend into the heartwood, especially when it is partly decayed. The size and shape of feeding tunnels may be a good indication for the causing pest. However, small tunnels produced by young larvae of cerambycid beetles at an incipient decay can easily be confused with those from an old infestation by anobiid beetles. Therefore, other indices like the shape of frass pellets are needed for a final proof. The frass in the tunnels may be loosely to tightly packed and does not tend to fall out freely from the wood. Some wood-boring species only attack softwoods, like the old houseborer, others specifically infest hardwoods like most bostrichid and lyctid beetles. Some anobiid species will attack both hardwoods and softwoods. Defrassing of suspected infested timber may expose the feeding tunnels and thus ease inspection.

Unseasoned hardwood with bark or wood in damp environments like pit-shafts or seasides may be attacked by wood-boring weevils or wharf beetles (also known as wharf borers). Weevil infestation can be differentiated from those of anobiids by the bore dust and frass which are finer in texture and the individual granules being more circular. The feeding tunnels are smaller and the exit holes are narrow oval with ragged or indistinct margins. Wharf beetles usually deposit the frass between the bark and the outer sapwood portion, which lifts and loosens the bark. The larval feeding tunnels are covered with ambrosia fungi staining the wood slightly dark. The wood surface may be covered with circular small larval entrance and larger adult emergence holes. Warf beetles are, next to submerged marine wood degraders like shipworms and certain crustaceans (which do not belong to the insects and are therefore dealt with elsewhere), economically the most important pests in the ship-building industry.

Wood damaged by carpenter ants (*Camponotus* spp.) contains galleries that are very clean and smooth. Ants do not eat wood, but tunnel into wood to make a nest. Wood ants like *Lasius* spp. preferentially excavate the early wood layers, leaving a lamellar set of late wood untouched.

Some soil-inhabiting termites (e.g., those of the genus *Coptotermes*) decay wood from the surface (erosive decay). They coat it with wide mud galleries usually underneath and feed on the early wood. Fine lamellae of the late wood remain almost untouched. Others (e.g., of the genus *Reticulitermes*) intrude into the wood and hollow out all but a thin surface layer. Drywood termites simply excavate tunnels and chambers within the timber, which can be filled with frass. They prefer softwoods and the sapwood of hardwoods, but they have been recorded to attack heartwoods as well.

Technical devices can assist in the inspection of possible infestation sites: the use of an endoscope supplies additional information about the degree of damage; a moisture-meter is especially useful for detecting termites in their cavities.

Insects, insect parts: Occasionally, the obvious presence of adult beetles, wasps, bees or termites will be noted. As adult beetles emerge in confined structures, they often are attracted to lights or windows. Membranous insect wings in great number around windows or beneath lamps are an indication for termite activity. Insect manuals and determination keys may allow the identification of the pest. Sometimes insect fragments found in the tunnelled wood or in the frass (wings, legs, cuticle fractions) may be sufficient for identification, however, professional entomological education and good magnifying devices are required.

X-ray and infrared: Hidden infestations inside the wood or concealed parts of a building may be recorded with x-ray machines or infrared cameras. However, the use of x-ray is very limited due to the lack of safe-

to-use portable x-ray devices and the high costs of this technique when applied stationary. Infrared cameras record the heat generated by living organisms. They may be very useful to pinpoint large cryptic infestation hotspots by termites. The accuracy of the recording, however, depends on the building insulation and other potential heat sources. In most cases, cost-benefit considerations do not justify the use of the infrared technique.

Auditory Inspection. Sounds, generated by the insects' interactions with their substrate, may be a hint for an active infestation. Under certain circumstances, especially during the quiet nightly hours, auditory inspection and acoustic detection of wood-infesting insects is possible. Computer-based devices have been developed to facilitate the prospect of success [14.19, 20].

Gnawing sounds of beetle larvae: Even in the early stages of an infestation, the rasping or ticking sounds made by the larvae while boring can be heard. This sound may be detected from a distance of 1–2 m, day and night, at infrequent intervals. The amplitude and frequency spectra of the feeding sound appear to be species specific.

Tapping sounds of adult beetles: Adult beetles of the death-watch beetle tap their heads on the wood as mating signals. The tapping noise is made by both sexes and can be heard unaided. It can be imitated tolerably well, at least to the extent of stimulating surrounding beetles to themselves start tapping.

Running termites: With the help of high-resolution contact microphones attached to the wood to be tested the sound of termites running in their tunnels may be detected.

Alarm signals of carpenter ants: An active colony may produce a dry rustling sound, similar to the crinkling of cellophane. After identifying a potential nest site, tapping against it with a screwdriver may cause a response clicking of alarmed ants. A listening device, such as a stethoscope, may be useful when conditions are quiet and outside noises are at a minimum.

Swarmers. The occurrence of swarming insects is evidence for the presence of ants or termites. Because of the consequences for possible remedial action, it is essential to know the major differences between those two insect groups. Ants, like most hymenoptera, have much larger forewings than hind wings. Termite wings all are of the same size; they break off easily. The antennae of ants are kinked, those of termites are straight. The thorax including the first abdominal segment (first abdominal tergum) and the rest of the abdomen in ants are joined by a narrow waist, while the thorax of termites is broadly joined to the abdomen.

Swarming termites in the exterior only indicate a termite infested area and not necessarily a termite attack. If, however, the swarmers are observed flying out of the structure from around windows, doors, porch columns or other wood constructions, then there might be some concern. Indoor swarmers point to the presence of either soil-inhabiting termites underneath the structure or drywood termites, which live in the house framework or in wooden furniture. An entomologist should be consulted for identification of the pest species, because control measures are specific for the different insect groups.

Termite Dog. Specialized dogs, trained to smell the trail odors of termites, are used to detect termites in- and outside of properties. According to an investigation at the University of Florida, the success rate is up to 96% [14.21].

Sticky Traps. Sticky traps, baited with the female sex pheromone, mostly used for detecting and monitoring beetle populations, are marketed for *Anobium punctatum*. It only has limited use for mass-trapping by setting out large numbers of traps in infested areas to catch a large number of beetles and thus reduce the population. As the traps only attract males and trap attractiveness has to compete with the natural pheromone of female beetles, the number caught may be too low to prevent mating. Therefore, pheromone traps are mostly used for detecting and monitoring beetle populations only.

Control. Knowledge about the particular insect species responsible for the impact may determine the control measure. Even prior to a thorough investigation of the dwelling and probable consequent treatments, the significance of a possible infestation has to be considered. A differentiation between wood-feeding and wood-breeding insects can assist in estimating the degree of a damage.

The impact on timber by wood-breeding insects can mostly be neglected, as they only attack wood for completing their development. They are only active in green timber. Debarked wood and seasoned lumber is never infested. Prominent examples are the following.

- Green wood beetles: Their larvae feed in the cambium, the thin layer of plant tissue between bark and wood. They usually groove the sapwood. At

the end of their development, old larvae tunnel into the wood and pupate. The emerging beetles leave through those tubes. On the surface of plane timber the flight holes may be visible, which can be confused with those from wood-feeding species.

- Bark beetles: Their larvae also feed in the cambium zone of living trees, fallen trees and logs. They excavate a characteristic tunnel called a gallery usually parallel to the grain and may penetrate superficially into the sapwood. Timber is not attacked unless of a high moisture content.
- Wood wasps: Wood wasps, also known as horntails, are capable of penetrating solid wood, especially of debilitated, dying and freshly felled trees, in which the eggs are laid. The damage is characterized by round boreholes densely packed with frass. Emergence holes are circular in cross section and up to 8 mm in diameter.

Methods for Testing Insect Resistance

The resistance of wooden materials against insect attack can be material-specific or generated through the modification of the wooden matrix or the application of wood preservatives, namely insecticides. Several general and specific standard testing procedures are available which allow resistance data obtained in the laboratory to be transferred to field situations. Specific standards may differ for either preventive or curative measures. The test organisms used in these testing standards represent the economically most important pests. *Hylotrupes bajulus* represents a softwood-infesting cerambicid beetle, *Lyctus brunneus* an exclusively hardwood-infesting beetle, and *Anobium punctatum* an opportunist. Tests with termites are usually carried out with subterranean species like *Reticulitermes santonensis* or *Coptotermes formosanus* or others.

Specific Tests for Resistance Against Wood-Boring Beetles (Preventive Measures). The eggs of wood-boring beetles are deposited in cracks and crevices of the wood. Larvae of wood-boring beetles therefore hatch inside the wood and begin tunnelling immediately. This fact was generally taken into account when test procedures were designed. The natural resistance of wood against insect attack may also be tested applying the following standards.

- European Standard EN 20: Determination of the preventive action against *Lyctus brunneus* (Stephens) – Part 2: Preservatives application fully impregnated wood treatment (laboratory method).
- European Standard EN 21: Determination of toxic values against *Anobium punctatum* (De Geer) by larval transfer (laboratory method). This standard describes a laboratory test method which gives a basis for assessment of the effectiveness of a wood preservative against *Anobium punctatum*. It allows the determination of the concentration at which the product prevents the survival of *Anobium punctatum* larvae in impregnated wood of a susceptible species. Although an infestation normally starts from egg-laying, a larval transfer test is applicable when considering the situation of treated wood being put into contact, during repair work, with wood that might be infested.
- European Standard EN 46-1 and EN 46-2: Wood preservatives – determination of the preventive action against recently hatched larvae of *Hylotrupes bajulus* (Linnaeus) (laboratory method). These standards make it possible to determine whether recently hatched larvae are capable of boring through the treated surface of a susceptible wood species and of surviving in the untreated part of the test specimen. For this purpose, the procedure seeks to reproduce normal egg-laying conditions existing in cracks in the wood, which provide the principal egg-laying sites. It takes account of the fact that, if larvae pass through the treated surface, they will then tunnel in the direction of the least protected regions of the wood.
- European Standard EN 47: Determination of the toxic values against recently hatched larvae of *Hylotrupes bajulus* (Linnaeus) (laboratory method). This standard specifies a laboratory test method which gives a basis for the general assessment of the effectiveness of a wood preservative against *Hylotrupes bajulus* by determination and comparison of the concentration at which the product prevents their survival in totally impregnated wood of a susceptible species.
- European Standard EN 49-1: Determination of the protective effectiveness against *Anobium punctatum* (De Geer) by egg-laying and larval survival – Part 1: Application by surface treatment (laboratory method). This part of EN 49 describes a laboratory test method which gives a basis for assessment of the effectiveness of a wood preservative, when applied as a surface treatment, against *Anobium punctatum*. It allows the determination of the concentration at which the product prevents the development of infestation from egg laying. The method simulates conditions which can occur in practice on timber

which has been treated some time previously with wood preservative applied by dip, brush or spray and on which eggs of *Anobium punctatum* are laid.

- European Standard EN 49-2: Determination of the protective effectiveness against *Anobium punctatum* (De Geer) by egg-laying and larval survival – Part 2: Application by impregnation (laboratory method). In contrast to part 1 of this standard, this method simulates conditions which can occur in practice on timber which has been treated some time previously with a deeply penetrating wood preservative and on which eggs of *Anobium punctatum* are laid.

Specific Tests for Resistance Against Wood-Boring Beetles (Curative Control Measures). When susceptible wood is infested with beetle larvae at low density, the infestation can be cured before structural damage occurs. This work requires to be done by experts only. The wood preservative is usually applied by surface application through brushing or spraying and has to penetrate deep enough for contact with tunnelling larvae.

- European Standard EN 1390: Determination of the eradicant action against *Hylotrupes bajulus* (Linnaeus) larvae (laboratory method). This standard describes a laboratory test method which gives a basis for assessment of the eradicant action of a wood preservative against *Hylotrupes bajulus*. It allows determination of the lethal effect of a surface application of a preservative product on a population of large larvae previously introduced into the test specimens. The method simulates conditions in practice where a stake is treated which is only slightly attacked and where insect tunnels have been exposed by cutting away. This represents a valid test of the product.
- European Standard EN 48: Determination of the eradicant action against larvae of *Anobium punctatum* (De Geer) (laboratory method). This standard describes a laboratory test method which gives a basis for assessment of the eradicant action of a wood preservative against *Anobium punctatum*. It allows the determination of the lethal effect of a surface application of the preservative on a population of larvae already established in the test specimens. The method simulates conditions which can occur in practice where a length of wood such as an affected stair tread is treated, which is still free from exit holes and in which certain of the faces are inaccessible, thus constituting valid test conditions.
- European Standard EN 370: Determination of eradicant efficacy in preventing emergence of *Anobium punctatum* (De Geer). This standard describes a laboratory test method which gives the basis for the assessment of the eradicant efficacy of a wood preservative in preventing emergence of *Anobium punctatum*. It determines the lethal effects, on beetles attempting to emerge through treated wood surfaces, of an insecticidal product deposited by surface application.

Specific Test for Resistance Against Termites. Termite control is not achievable by curative treatment of infested wood. Usually, the damage caused by termites is already too severe by the time termite-infested wood is detected. Wood preservative action against termites focuses on preventive treatment of the endangered material or the creation of biocidal or physical soil barriers. The test species may vary depending on the geographical situation.

- European Standard EN 117: Determination of toxic values against European *Reticulitermes* species (laboratory method). This standard describes a laboratory test method which gives a basis for assessment of the effectiveness of a wood preservative against *Reticulitermes* species. It allows the determination of the concentration at which the product completely prevents the attack by this insect of impregnated wood of a susceptible species.
- European Standard EN 118: Determination of preventive action against European *Reticulitermes* species (laboratory method). This standard describes a laboratory test method which gives a basis for assessment of the effectiveness of a wood preservative, when applied as a surface treatment, against *Reticulitermes* species.
- American Society for Testing and Materials ASTM D 3345: Laboratory evaluation of wood and other cellulosic materials for resistance to termites. This method covers the laboratory evaluation of treated or untreated cellulosic material for its resistance to subterranean termites. This test should be considered as a screening test, for treated material and further evaluation by field methods is required.
- American Wood-Preservers' Association Standard E1-72: Standard method for laboratory evaluation to determine resistance to subterranean termites. This method provides for the laboratory evaluation of treated or untreated cellulosic material for its resistance to subterranean termites. This test should be

considered as a screening test for treated material and further evaluation by field methods is required.

- Australian Standard 2178: Protection of buildings from subterranean termites – detection and treatment of infestation in existing buildings. This standard sets out methods for the detection and treatment of subterranean termite infestation in existing buildings and also sets out methods for the prevention of reinfestation.
- Japan Wood Preserving Association Standard 11(2): Method for testing the effectiveness of surface treatments of timber (brushing, spraying and dipping) with termiticides against termites (2) field test. This standard describes a field test method for evaluating effectiveness of surface treatments of timber such as brushing, spraying and dipping with termiticides against termites.
- Japan Wood Preserving Association Standard 12: Method for testing the effectiveness of pressure treatment of timber with termiticides against termites. This standard describes laboratory and field test methods for evaluating effectiveness of pressure treatment of timber with terniticides against termites.
- Japan Wood Preserving Association Standard 13: Method for testing the effectiveness of soil treatment with termiticides against termites. This standard describes a test method for evaluating effectiveness of soil treatment with termiticides against termites.
- Japan Wood Preserving Association Standard 14: Qualitative standards for termiticides, preservative/termiticides and soil-poisoning termiticides. This standard describes qualitative standards for termiticides, preservative/termiticides and termiticides for soil treatment.

14.3 Testing of Organic Materials

Polymeric materials (plastics) are used in all sectors of life as very durable products with tailor-made properties. They provide a combination of easy thermoforming (most plastics, if not cross-linked) and excellent use properties. For some applications, such as paints, items in the automobile industry or for use in buildings, they have to maintain their properties for a long period of time, often decades. The long-lasting exposure to environmental factors often results in a change of material properties such as roughening of the surface, embrittlement, a loss in mechanical strength or just a discoloration. It has been observed in some cases that also the presence of microorganisms such as bacteria, fungi or algae can cause or enhance changes in plastics, although most plastics are supposed to be inert to attacks of microorganisms (*they do not rot*). All these effects usually are not desired and are denoted generally as biocorrosion or as biodeterioration, if the material falls apart due to the ageing process. A number of test methods have been developed to characterize these phenomena.

However, the increasing stability of many plastics against environmental ageing in combination with the intense use of modern plastics generated serious problems with plastic waste in the last decade, especially from plastic packaging. Alternative waste management strategies to landfilling such as incineration or plastics recycling are not always optimal and the subject of very controversial discussions. On this background intensive attempts were made since the early nineties to develop novel plastics which combine a good performance, comparable to conventional polymers, with a controlled susceptibility to microbial degradation. This new class of materials is usually called *biodegradable plastics*. Applications of theses materials are, e.g., bags for collecting biowaste, packaging (disposed via composting), or mulch films in agriculture.

For these novel materials which are claimed to be environmentally friendly, it is demanded to prove their environmentally safe biodegradation using scientifically based and generally accepted methods. Concerning the parameters used for monitoring biodegradation, the testing procedures applied and the evaluation criteria for biodegradability of plastics significantly differ from testing biocorrosion phenomena of plastics. Hence, an own system of test methods and evaluations criteria is being developed for this kind of materials.

14.3.1 Biodeterioration

In contrast to the biodegradation of plastics, where a near complete conversion of the material components into naturally occurring metabolic products of microorganisms (e.g., water, carbon dioxide, methane, biomass etc.) occurs (see later), for biocorrosion or biodeterioration processes only a change in the polymer structure or the plastics composition is observed in many cases [14.22].

General Mechanism of Biodeterioration

Since usually mechanical properties of plastics are predominantly determined by the length of the polymer chains in the material, scission of polymer chains (reduction of the average molar mass) is one major reason for changes in mechanical properties. This effect is especially dramatic if the cleavage of the polymers occurs statistically along the chains (endo-degradation) and not at the ends (exo-degradation). Even just a single endo-cleavage in a polymer chain can reduce the molar mass to 50%, and hence cause significant changes in mechanical properties.

Embrittlement (= loss in elasticity), however, can also occur when plasticizers are removed from the plastic materials by microorganisms. Especially for polyvinylchlorides (PVC), which in some cases contain high amounts of plasticizers (e.g., low-molecular-weight esters), biocorrosion due to this phenomenon has been reported in earlier times [14.23–25]. A similar effect is observed in polymer blends or (block)-copolymers when single components are selectively degraded by microorganisms. An example for this mechanisms of biodeterioration are mixtures of (unmodified) polyethylene and starch. Here the starch basically can be metabolized by microorganisms, leading to a weakening of the entire material and finally result in fragmentation [14.26].

If the attack of microorganisms only concerns side groups attached to the polymer main chains, this usually results in a change of the chemical characteristic and hence, in the material properties. The cleavage of ester bonds in side chains of cellulose esters (e.g., cellulose acetate) results in the formation of charged chemical groups (at suitable pH values) increasing the hydrophilicity of the material (this can be followed by an increased water uptake and swelling of the material). If a sufficiently high amount of esters has been transformed into hydroxyl groups the entire material can become accessible to direct microbial attack [14.27,28]. Similar effects can be caused by oxidation phenomena, where new polar carbonyl and carboxylgroups are formed in the polymer (or at the surface of the polymer).

Coloring of plastics can be caused due to the formation of new chromophoric chemical groups, but also the release of pigments from microorganisms can cause changes in the color of plastic materials.

Strictly speaking, real biodegradation involves the direct action of enzymes (biocatalysts) on the plastic material itself. The hydrolysis of ester bonds in polyesters by hydrolases is such a case [14.29–31]. However, in biodeterioration processes microorganisms mostly indirectly contribute to changes in the plastic materials. During microbially induced oxidative degradation of plastics (in nature, for instance present in degradation of lignin or latex [14.32]), the oxidative enzymes do not directly act on the polymers, but produce low-molecular-weight oxygen compounds which diffuse into the polymer material and there cause chemical reactions. Microorganisms can also indirectly induce hydrolytic degradation processes by changing the pH in a microenvironment at the surface of the material (e.g. in biofilms, see below) or through the excretion, e.g., of organic acids such as lactic acid or acetic acid as products of their metabolism.

In real environments, however, corrosion phenomena of plastics are often a mixture of physical, chemical and biological processes. Chemical hydrolysis caused by water which has been diffused into the polymer and oxidation induced by light (photo-oxidation) or by increased temperatures (*thermal oxidation*) in many cases play an important role during biocorrosion and biodeterioration. In fact, the expression *environmental corrosion* would be an expression, better meeting the point than *biocorrosion*.

The mechanism and the impact on the plastics differ basically between chemical or physically processes and the direct action of enzymes on the polymer. While water and oxygen are small molecules which are in principle able to penetrate into the entire plastic material, enzymes are too large to diffuse into the polymer bulk, and thus, can only act at the surface, causing a typical erosion process, where the material is affected layer by layer from the surface. This is illustrated in Fig. 14.7 for the chemical (bulk degradation) and enzymatic hydrolysis (surface erosion) of e.g. a polyester.

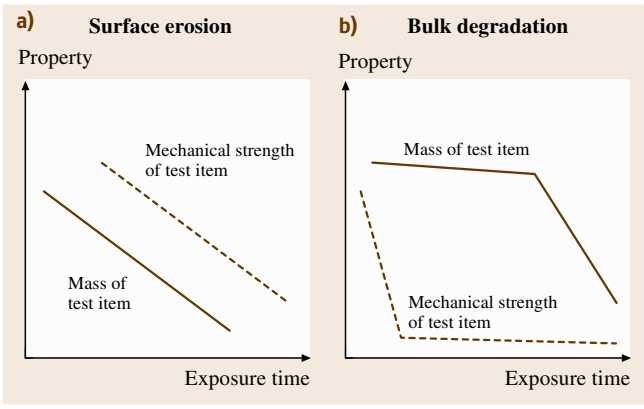


Fig. 14.7a,b Influence of surface erosion **(a)** and bulk degradation **(b)** on the material properties of plastics

While for the enzymatic action only a small part of the material at the surface *knows* about the degradation which proceeds slowly from the surface, chemical hydrolysis caused from water contained in the plastics, affects the entire material right from the beginning. As a consequence, changes in mechanical properties and mass loss due to degradation run in parallel for the enzymatic degradation. In contrast, chemical hydrolysis causes chain scissions in all polymer chains simultaneously, resulting in an instant decrease in mechanical strength, while a mass loss of the material is only observable at a later stage of degradation, where polymer chains become so short to be water soluble.

Biofilms

One important phenomenon in correlation with biodeterioration and biocorrosion is the forming of biofilms on material surfaces, also known as biofouling [14.22]. Formation of biofilms is not only restricted to polymer materials, but also plays a central role in corrosion of, e.g., concrete, stones (buildings) and metals. Such biofilms consist of a combination of various microorganisms which is highly complex and variable in time and includes the microorganisms themselves and additionally a number of extracellular polymeric substances, e.g., polysaccharides. Biofilms represent an own microenvironment at the surface of the materials providing optimal living conditions (humidity, pH, nutrient concentrations) for the microorganisms inhabiting the biofilm and protecting them from external attacks, e.g., from other bacteria or fungi, and also to a certain extent from biocides.

In biofilms the concentration of polymer-degrading enzymes, but also, e.g., of oxidating agents formed by the microorganisms can substantially be higher than in a liquid environment, where such substances can diffuse away from the surface. Also concerning the pH value, biofilms can present totally different conditions to the material surface than be measured in the liquid environment. Thus, the presence of such biofilms on polymer surfaces can substantially influence biodeterioration phenomena of plastics.

Standards for Evaluation of Biodeterioration

As mentioned above biodeterioration is usually a very complex process, involving the direct or indirect action of diverse microorganisms forming often a biofilm on the material surfaces and also including in many cases nonbiotic actions such as irradiation or thermal oxidation. As a consequence, the corresponding standard procedures for testing biodeterioration and biocorrosion phenomena of plastics are correlated to different topics. On the one hand, a number of materials different from plastics such as steel, concrete, textiles or paints are covered, on the other hand, also nonbiotic factors (light, heat, oxygen, moisture, chemicals) are regarded in such tests exclusively or in combination with biotic influences. Thus, only a limited number of tests strictly deal with *pure* biocorrosion or biodeterioration mechanisms of plastics. Those tests, only focussing on biotic effects usually use a number of defined test organisms (Table 14.3). However, in many cases simulation or field tests are used which combine the action of biotic and nonbiotic factors. These standards often are correlated with the expression *weathering* – a selection of such standards is also included in Table 14.3.

14.3.2 Biodegradation

At the beginning of the nineties a novel group of polymers were developed which were intended to be degradable by microorganisms in a controlled manner, but at the time were no adequate methods and criteria available to evaluate the property of biodegradability. First tests carried out at that time (e.g., by using the growth of microorganisms on the surface or a certain loss in mechanical properties such as the tensile strength as indicators for biodegradation) originated from the field of plastics biocorrosion and biodeterioration (see above). However, these evaluation methods proved to be unsuitable to characterize biodegradable materials. A first generation of modified polyethylenes, claimed to be biodegradable based on these tests, did not meet the expectations of the users and caused to some extent a general negative image of biodegradable plastics at the time. As a consequence, the development of suitable testing methods and evaluation criteria for biodegradable plastics started and resulted in a number of standards of various national and international standardization agencies during the past 15 years. This process still continues, since the number of different environments, where plastics can be degraded, make it necessary to establish a quite complex and extended system of testing methods and evaluation criteria for biodegradable plastics.

General Mechanism of Biodegradation

When talking about biodegradation of plastics usually one is reffering to the attack of microorganisms on water-insoluble polymer-based materials (plastics). Because of the lack of water-solubility and the length of the polymer molecules, microorganisms are not able to

Table 14.3 Standard test methods for biocorrosion phenomena on plastics

Action of microorganisms on plastics	
ISO 846 – 1997	Plastics – Determination of behaviour under the action of fungi and bacteria. Evaluation by visual examination or measurement of changes in mass or physical properties
ISO 16869 – 2001	Plastics – Assessment of the effectiveness of fungistatic compounds in plastics formulations
EN ISO 846 – 1997	Plastics – Evaluation of the action of microorganisms
ASTM G21-96	Standard practice for determining resistance of synthetic polymer materials to fungi
ASTM G29-96	Standard practice for determining algal resistance of plastic films
DIN IEC 60068-2-10 – 1991	Elektrotechnik; Grundlegende Umweltprüfverfahren; Prüfung J und Leitfaden: Schimmelwachstum; (Identical with IEC 60068-2-10:1988)
IEC 60068-2-10 – 1988	Elektrotechnik; Grundlegende Umweltprüfverfahren; Prüfung J: Schimmelwachstum
Weathering of polymers	
ISO 877:1994	Plastics – Methods of exposure to direct weathering, to weathering using glass-filtered daylight, and to intensified weathering by daylight using Fresnel mirrors
ISO/AWI 877-1	Plastics – Methods of weathering exposure – Part 1: Direct exposure and exposure to glass-filtered daylight
ISO/AWI 877-2	Plastics – Methods of weathering exposure – Part 2: Exposure to concentrated solar radiation
ISO 2810:2004	Paints and varnishes – Natural weathering of coatings – Exposure and assessment
ISO 4582:1998	Plastics – Determination of changes in color and variations in properties after exposure to daylight under glass, natural weathering or laboratory light sources
ISO 4665:1998	Rubber, vulcanized and thermoplastic – Resistance to weathering
ASTM D1435-99	Standard practice for outdoor weathering of plastics
ASTM D4364-02	Standard practice for performing outdoor accelerated weathering tests of plastics using concentrated sunlight

transport the polymers directly through their outer cell membranes into the cells where most of the biochemical processes take place. To be able to use such materials as a carbon and energy source, microorganisms have developed a special strategy. The microbes excrete extracellular enzymes which depolymerize the polymers outside the cells. This means that biodegradation of plastics in its first step is usually a heterogeneous process.

If the molar mass of the polymers has been sufficiently reduced and water-soluble intermediates have been generated, these small molecules can be transported into the microorganisms and introduced there into the metabolic pathways. As a final result new biomass and natural metabolic end-products such as water, carbon dioxide and methane (for degradation processes in the absence of oxygen = anaerobic degradation) are formed.

The excreted extracellular enzymes usually have a molar mass of some ten-thousand Daltons and hence, are to large to penetrate deeper into the polymer material. In consequence, the enzymes only can act on the polymer surface of the plastics and erodes the material layer by layer – biodegradation of plastics is usually a surface erosion process, only affecting a relative small fraction of the entire polymer material at one time. In many cases the enzymatic catalyzed chain length reduction of the polymers is the process in biodegradation determining the rate of the entire process.

In parallel to the enzymatic attack, nonbiotic chemical and physical processes such as oxidation, irradiation (photodegradation), thermal degradation or chemical hydrolysis can affect the polymer and contribute to the degradation process. In some cases an abiotic degradation mechanism is exclusively responsible for the first step of molar mass reduction.

Some materials, claimed to be biodegradable, directly used such effects to induce the biodegradation process. For instance, poly(lactic acid) is at first hydrolyzed into oligomeric esters by an autocatalytic chemical hydrolysis or sunlight in combination with heat then generates in pro-oxidant-modified polyethylene short hydrophilic intermediates which are considered to be assimilated by microorganisms. Because of the coexistence of biotic and nonbiotic processes in many cases the entire mechanism of polymer degradation could also be called an environmental degradation. Both, biotic and abiotic processes have to be considered in tests evaluating biodegradation of polymers.

Environmental factors do not only influence the plastics themselves, but have also a crucial impact on the microbial population and on the activity of the different microorganisms involved in the polymer

degradation. Parameters such as humidity, temperature, pH, concentration of salts, the presence or absence of oxygen and the supply with different nutrients have an important effect on the microbial degradation of polymers and must be adequately considered when testing the biodegradability of plastics.

A further complicating factor when dealing with biodegradation of plastics is the complexity of plastic materials with regard to their possible compositions, structures and morphologies (Fig. 14.8).

Different monomers can be combined in one polymer chain, where these elements can be distributed statistically along the polymer chains (random copolymers), strictly alternate (alternating copolyesters) or build longer blocks of each structure (block copolymers). Different polymers can be mixed physically in the melt or solution forming polymer blends. Depending on the chemical structure of the components and the formation process, mixtures of different characteristics can be formed (e.g., homogeneous mixtures for miscible polymers, small domains of component A in a continuous phase of component B or penetrating networks of both components). Furthermore, in many cases low-molecular-weight additives (e.g., plasticizer, antiblock agents, nucleation agents) are added to a polymer to adjust properties such as flexibility or processibility.

Different structures, even with the same overall composition of a polymer, can directly influence the accessibility of the material to the enzymatically catalyzed polymer chain cleavage significantly. Another important structural characteristics of polymers are their average molar mass, their molar mass distribution and possible branching of the chains or the presence of networks (crosslinked polymers).

The structural characteristics of the polymer have a crucial impact on higher ordered structures of the material (crystallinity, crystal morphology, melting temperature or glass transition temperature) which in some cases have been shown to control predominately the degradation behavior of many polymers [14.30, 31]. Finally, the crystallinity and crystal morphology depends on the processing conditions and can change with storage time of the material.

All these factors described above have to be taken into account when measuring biodegradation of plastics and interpreting the results. This makes testing of biodegradable plastics a typical interdisciplinary work. General statements such as *polymer xyz is biodegradable* can not be made since the specific properties of the polymer have to be taken into account. Thus, a detailed description and identification of a material to be tested must be a basic prerequisite for any testing protocol.

Definitions

Standardized evaluations of biodegradable plastics must always be based on definitions as to the meaning of the term biodegradation with regard to plastics. The various national and international standardization agencies and organizations have published a number of different definitions (Table 14.4) which vary significantly. While the definition for *biodegradable plastics* established by the ISO only refers to a chemical change

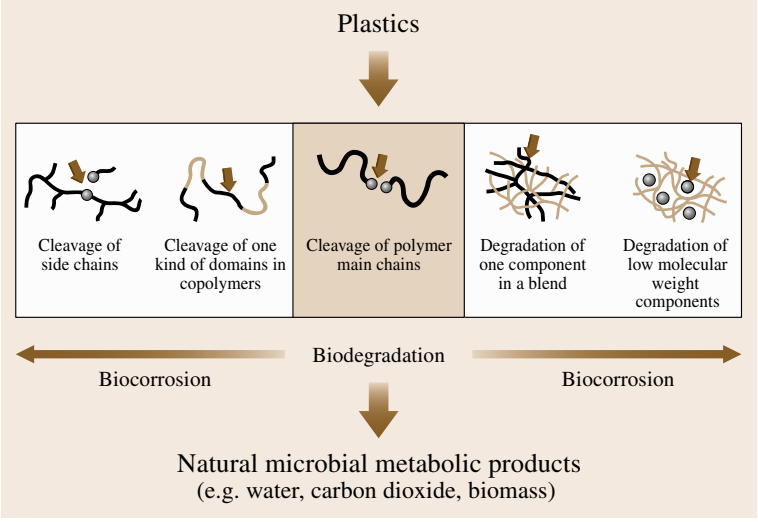


Fig. 14.8 Biodegradation- and biocorrosion phenomena in plastics

Table 14.4 Biodegradation and biodegradable plastics – definitions established by different standardization organizations

Definitions of *Biodegradation*	
DIN	Biodegradation is a process, caused by biological activity, which leads under change of the chemical structure to naturally occurring metabolic products
CEN	Biodegradation is a degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of a material
Definitions of *Biodegradable plastics*	
DIN	A plastic material is called biodegradable if all its organic compounds undergo a complete biodegradation process. Environmental conditions and rates of biodegradation are to be determined by standardized test methods
ASTM	A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae
JBPS	Polymeric materials which are changed into lower molecular weight compounds where at least one step in the degradation process is through metabolism in the presence of naturally occurring organisms
CEN	A degradable material in which the degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide and/or methane and a new cell biomass
ISO	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification. The change in the chemical structure results from the action of naturally occurring microorganisms
Definition of *Inherent biodegradability*	
CEN	The potential of a material to be biodegraded, established under laboratory conditions
Definition of *Ultimate biodegradability*	
CEN	The breakdown of an organic chemical compound by microorganisms in the presence of oxygen to biodegradability carbon dioxide, water and mineral salts of any other elements present (mineralization) and new biomass or in the absence of oxygen to carbon dioxide, methane, mineral salts and new biomass
Definition of *Compostability*	
CEN	Compostability is a property of a packaging to be biodegraded in a composting process. To claim compostability it must have been demonstrated that a packaging can be biodegraded in a composting system as can be shown by standard methods. The end product must meet the relevant compost quality criteria

of the material (e.g. oxidation) by microorganisms, the European Standardisation Organisation CEN and the German DIN, in contrast, consider biodegradation of plastics as a final conversion of the material into microbial metabolic products. Other definitions listed in Table 14.4, such as *inherent biodegradability* or *ultimate biodegradability*, are adapted from according considerations for the degradation of low-molecular-weight chemicals, but can also be applied for polymers. Generally, the definitions do not specify any particular environment nor time frames; these are defined in corresponding standards specifying different degradation environments and processes.

Additional definitions have been set up for plastics classified as *compostable*. In the definition of *compostability* biodegradation of the polymeric material is only one requirement and further demands such as a sufficient compost quality after the composting process with plastics are included in the definition.

However, despite of the quite inconsistent definitions the different standards and evaluation schemes are surprisingly congruent.

General Test Methods for Biodegradable Plastics

The evaluation of the degradability of chemicals in the environment has become important as one crucial aspect of their ecological impact. First regulations and according test methods were established for products reaching the wastewater and for pesticides. In this respect a large number of standardized tests have been developed for different environments applying different analytical methods [14.33]. Nowadays, the evaluation of biodegradability, as one aspect of an environmental risk assessment, has become a standard procedure for any new chemical product intended to be marketed. However, testing methods developed for this purpose do not consider the special features (see above) of plastics materials.

Testing methods focussed on the effect of microorganisms on polymers already existed long time before biodegradable plastics started to be developed. It had been shown that conventional plastics, although they are quite resistant to environmental influences, can be attacked in some cases by microorganisms, causing un-

desired changes in their material properties, e.g. in the color or in mechanical properties such as flexibility or mechanical strength and according tests were developed [14.34, 35]. However, for these biocorrosion phenomena a generally different question underlies the corresponding test compared to the process of real biodegradation (as e.g. defined in CEN definitions listed in Table 14.4). While biocorrosion tests aim to characterize changes in the material properties (which can even be caused by minor chemical changes in the polymers such as extraction of plasticizer or oxidation, etc.), biodegradation tests for plastics have to prove that the plastic material is finally transformed into natural biological products.

Despite the large number of standardized degradation tests it turned out to be necessary to develop special testing methods when dealing with biodegradable plastics.

The testing methods published for biodegradable plastics during the last decade [14.36] are predominantly based on principles used for the evaluation of low-molecular-weight substances, but have been modified with respect to the particular environments that biodegradable plastics are exposed to and with respect to the fact that plastics are often complex materials and degrade mainly by a complicated surface mechanism.

The Dilemma in Choosing the Right Degradation Test

When testing degradation phenomena of plastics in the environment one has to face a general problem concerning the kind of tests applied and the conclusions which can be drawn. In principle the degradation tests can be classified into three categories: field tests, simulation tests and laboratory tests (Fig. 14.9).

Field tests such as burying plastic samples in soil, to place samples in a lake or river, or full-scale composting performed with the biodegradable plastics represent ideal practical environmental conditions, but there are some serious disadvantages of such kinds of tests. One is that environmental conditions such as temperature, pH, or humidity, can not be efficiently controlled in nature and secondly, analytical methods for monitoring the degradation process are very limited. In most cases it is only possible to evaluate visible changes of the polymer samples or to determine the disintegration by measuring the weight loss, and even that may not be feasible if the materials disintegrates into small fragments which have to be quantitatively recovered from the soil, the compost or the water. Analysis of residues and intermediates is complicated due to the complex and undefined environment. Since a pure physical disintegration of a plastic material is not regarded as biodegradation in the sense of most definitions (see above), these tests alone are not suitable to prove whether a material is biodegradable or not.

To overcome these problems at least partially, various laboratory simulation tests have been developed. Here, the degradation takes place in a real environment (e.g. compost, soil or sea water), but the exposure to the environment is performed in a laboratory reactor. This environment is still very close to reality, but important external parameters which can affect the degradation process (e.g. temperature, pH, humidity, etc.) can here be controlled and adjusted, and analytical tools are better than in field tests (analysis of residues and intermediates, determination of CO_2 production or O_2

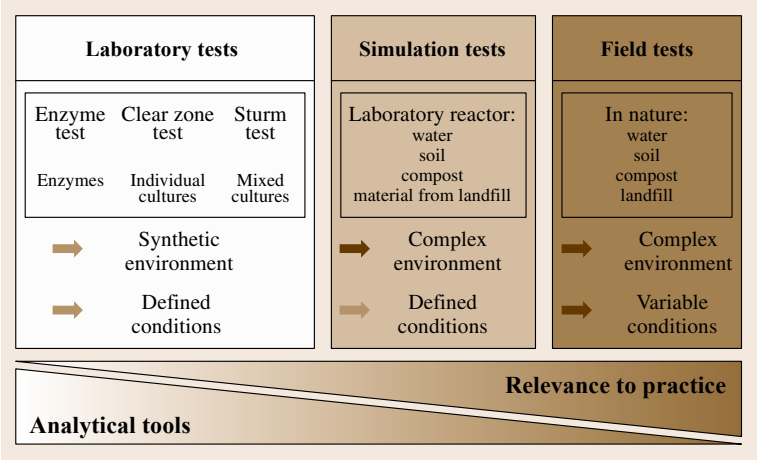


Fig. 14.9 Classification of test methods for biodegradable plastics

consumption). Examples for such tests are the soil burial test [14.37], the so-called controlled composting test [14.38–42], test simulating landfills [14.43–45], or aqueous *aquarium tests* [14.46]. To increase the microbial activity, nutrients are sometimes added in these tests with the aim to accelerate degradation and to reduce the duration of the degradation tests.

The most reproducible biodegradation tests are laboratory tests, where defined (often synthetic) media are used which then are inoculated with a mixed microbial population (e.g., from waste water or compost eluate). In some cases individual microbial strains or mixtures of some strains are used for inoculation. The organisms sometimes have been especially screened for the degradation of the particular polymer. Such tests often take place under conditions optimized for the activity of the particular microorganisms (e.g. temperature, pH etc.) with the effect, that polymers often exhibit a much higher degradation rate in laboratory tests than observed under natural conditions.

The most reproducible degradation tests directly use the isolated extracellular enzymes of the microorganisms which are responsible for the first step of the degradation process, the molar mass reduction of the polymers by depolymerization [14.29–31, 47, 48]. Also with this system it is not possible to prove biodegradation in terms of metabolization by microorganisms.

However, the shorter test durations and the reproducible test conditions make laboratory tests especially useful for systematic investigation when studying basic mechanisms of polymer biodegradation. However, conclusions on the absolute degradation rate in a natural environment can only be drawn to a limited extent.

Besides reproducibility, the shortening of test durations and minimization of the material needed is a crucial point when performing extended systematic investigations or for biodegradation testing as a tool for supporting the development of an industrial material. While degradation experiments in compost or soil often take up to one year, tests with especially screened organisms may only reqiure several weeks and enzymatic degradation can be performed even within a few hours or days. Recent reports applying polymer nanoparticles (increased surface area) indicate that enzymatic degradation tests with polyesters can be performed within seconds [14.49, 50].

As a consequence of the principle discrepancy between the analytical tools applicable in the different tests and the relevance of the test to practical degradation conditions, it will be in most cases necessary to combine different tests to completely evaluate the biodegradation behavior of a plastic in a certain environment.

Analytical Methods to Monitor Biodegradation

The analytical tools used to follow the degradation process depend on the aim of the work and the test environment used. In the following, some analytical methods are presented.

Visual Observations. The observation of visible changes of plastics can be performed in many tests. Effects which were used to describe degradation are, e.g., the formation of biofilms on the surface, changes in the material color, roughening of the surface, formation of holes or cracks or the occurrence of defragmentation. As already mentioned, these changes do not prove a biodegradation process in terms of conversion of the polymer mass into biomass and natural metabolic products (see definitions), but the parameter of visual changes can be used as a first indication of a microbial attack. More detailed information on the degradation process can be obtained from SEM- or AFM-techniques [14.51]. An example is presented in Fig. 14.10, showing SEM micrographs of a surface of a poly(β-hydroxy butyrate) (PHB) film before and after incubation in an anaerobic environment [14.52]. In the course of the degradation crystalline spherolites appear on the surface. This is caused by a preferential degradation of the amorphous polymer fraction, etching the slower degrading crystalline parts out of the material.

Especially recent developments of the AFM technique allow very detailed investigations on the degradation mechanism of polymers [14.53].

Changes in Polymer Chain Length and Mechanical Properties. Comparable to visual observations changes in material properties do not allow an evaluation of polymer degregation, since these measurements do not directly prove the metabolization of the plastic material. However, changes in mechanical properties are often used when only small effects on the material due to the degradation process have to be monitored. Properties such as tensile strength are very sensitive to changes in the molar mass of the polymers which is also often directly taken as an indicator for degradation [14.54]. While for an enzymatic attack at the surface, material properties only change if a significant loss of mass is observed (the specimen become thinner because of the surface erosion process; the inner part of the material is not affected by the degradation process) the situation is usually opposite for abiotic degradation

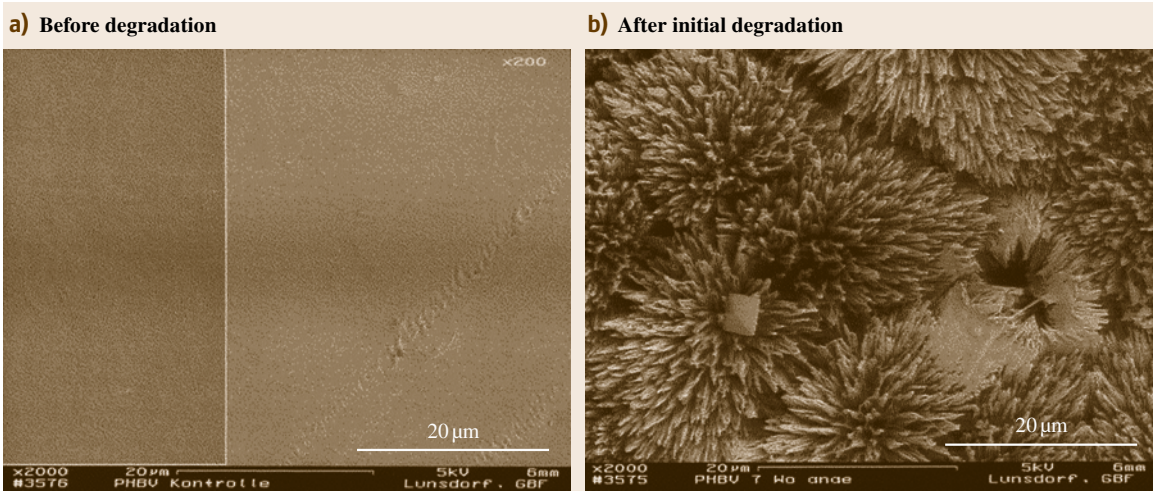


Fig. 14.10a,b Scanning electron micrographs of poly(β-hydroxybutyrate) films before (**a**) and after (**b**) incubation in an anaerobic sewage sludge. Amorphous material is degraded preferentially and spherulites of crystalline regions become visible

processes. They often take place througout the entire material (e.g., hydrolysis of polyester or oxidation of polyethylene) and the mechanical properties of the plastics already change significantly before a loss of mass due to solubilization of degradation intermediates is observed (Fig. 14.4).

Accordingly, this kind of measurement is often used for materials where abiotic processes are responsible for the first degradation step, e.g., for the chemical hydrolysis of poly(lactic acid) or oxidation of modified polyethylenes [14.55, 56].

Weight Loss Measurements/Determination of Residual Polymer. Measuring the mass loss of test specimen (films, test bars or whole items) is often applied, especially in field- and simulation tests. However, again no direct proof of biodegradation is possible from these data. Problems can arise with proper cleaning of the specimen or when the material strongly disintegrates. In the latter case the samples can be placed in small nets to facilitate recovery, a method which is for instance applied in the full-scale composting procedure of ISO 16929. A sieving analysis of the matrix surrounding the plastic samples allows for a better and more reproducible quantitative determination of the disintegration process.

The degradation of finely distributed polymer samples (e.g. powder) can be determined by an adequate separation or extraction technique (polymer separated from biomass or polymer extracted from soil or compost). This procedure always has to be carefully adapted and verified for each specific system. Together with a structural analysis of the residual material and low-molecular intermediates a detailed insight into the degradation process can be gained [14.57].

Determination of CO_2 Production and O_2 Consumption. Under aerobic conditions microbes use oxygen to oxidize carbon and form carbon dioxide as one major metabolic end-product. The determination of the oxygen consumption (respirometric test) [14.46, 58] or of the carbon dioxide formation (so-called *Sturm-test*) are good indicators for polymer degradation and the most often used methods to monitor biodegradation processes in laboratory tests. In laboratory tests using synthetic mineral media, the polymer represents the major source of carbon in the system, and only a low background respiration has to be faced. Accordingly, the accuracy of the tests is usually good. Such kinds of tests already have been used to evaluate the degradability of low-molecular-weight chemicals in water (e.g., in OECD guidelines) and now have been modified for biodegradable plastics to take into consideration the special characteristics of usually hydrophobic, nonwater-soluble materials. Also, more sophisticated experimental methods for the determination of CO_2 have been introduced to the standards. Beside the conventional entrapping of CO_2 in $Ba(OH)_2$-solution in combination with manual titration, the detection of O_2 and CO_2 concentrations in the air stream for aeration

with infrared detectors and paramagnetic O_2-detectors are often used for such experiments.

However, besides of the advantage of an automated and continuous measurement, this kind of measurements harbor also some disadvantages. The exact air flow must be known and the signals of the detectors must be stable for weeks and month. If slow degradation processes have to be monitored, the CO_2-concentration or the drop in the O_2-concentration is very low, increasing the possibility of systematic errors during such long-lasting experiments. Here, entrapping CO_2 in a basic solution ($\approx$ pH 11.5) with continuous titration or detection of the dissolved inorganic carbon [14.59] are useful alternatives. Other attempts to solve the problems with CO_2 detection use noncontinuously aerated, closed systems. Sampling techniques of the gas in combination with an infrared gas-analyzer [14.60] or a titration system [14.61] have been reported in the literature. An additional closed system with a discontinuous titration method has been described by *Solaro* et al. [14.62]. Tests using small closed bottles as degradation reactors and analyzing the CO_2 in the head space [14.63] or by the decrease in dissolved oxygen (so-called *closed bottle test*) [14.64] are simple and quite insensitive to leakage etc., but may cause problems due to the merely low amounts of material and inoculum which can be used.

The method of CO_2-determination to monitor polymer degradation was also adapted to tests in solid matrices such as compost [14.38]. Such methods are now standardized under the name *controlled composting test* (ASTM D 5338-98e1, ISO 14855, JIS K 6953). Actually the *controlled composting test* does not simulate a composting process, since in this test mature compost is used instead of fresh biowaste as a matrix. Biowaste contains large amounts of easily degradable carbon and, hence, would cause a high background CO_2-development, too high for an accurate measurement. Thus, already converted biowaste (= mature compost) is used instead of fresh biomaterials.

Monitoring polymer degradation in soil via carbon dioxide detection turned out to be more complicated than in compost. The usually significantly slower degradation rates causing on the one side very long test durations (up to two years) and a quite low CO_2 evolution compared to the background CO_2 formation in soil. Recent test developments in this area have been published by *Solaro* et al. [14.62]. Despite of the problems mentioned above, standardized methods for testing plastics degradation in soil are currently under development (ISO/PRF 17556).

In order to avoid problems with a high background CO_2 formation from the natural matrices of compost or soil, an inert, carbon-free and porous matrix has been used instead of soil or compost. The inert matrix is then wetted with a synthetic medium and inoculated with a mixed microbial population. This method turned out to be practical for simulating compost conditions (degradation at $\approx 60\,^{\circ}C$) [14.65, 66] but could not be optimized sufficiently for soil conditions up to now.

Measurement of Biogas. Analogous to the formation of CO_2 in the presence of oxygen by aerobic organisms, anaerobic microorganisms produce predominantly a mixture of carbon dioxide and methane (called *biogas*) as the major end-product of their metabolic reactions. The amount and the composition of the biogas can be theoretically calculated from the material composition with the so-called *Buswell* equation [14.67]. The biogas production is mainly used for monitoring biodegradation of plastics under anaerobic conditions [14.68–71] and also standards dealing with the anaerobic biodegradation of plastics are based on such measurements (ISO/DIS 15985, ASTM D 5210, ASTM D 5511). The biogas volume can easily be determined by a manometer method or a simple replacement of water. Additionally, the biogas composition can be analyzed, e.g., by sampling the produced gas and analysis by gas chromatograhpy [14.72].

As discussed for the carbon dioxide evolution, the basic problem is biogas formation from the inoculum. Especially for slow degradation processes this problem affects the accuracy of the testing method. Attempts to reduce the background biogas evolution were made by *Abou-Zeid* [14.68] diluting the anaerobic sludges with a synthetic mineral medium.

^{14}C-labeling. Applying biodegradable polymers which are radio-labelled with ^{14}C can avoid many of the problems mentioned above. Already very low concentrations of $^{14}CO_2$ can be detected even if carbon dioxide from other carbon sources (e.g., biowaste) is produced simultaneously. Thus, radio-labelling is used especially when slowly degradable materials are investigated in a matrix containing other carbon sources than the plastics [14.73, 74]. However, in many cases there is a problem with producing the ^{14}C-labelled materials and carrying out work with radioactive substances from the experimental point of view.

Clear-Zone Formation. The so-called *Clear-Zone test* is a very simple semi-quantitative method. In this test

the polymer is dispersed as very fine particles in a synthetic medium agar, resulting in a opaque appearance of this agar. When inoculated with microorganisms able to degrade the polymer the formation of a clear halo around the colony (Fig. 14.11) indicates that the organisms are at least able to depolymerize the polymer, which is the first step of biodegradation. The test is often used for screening of organisms which can degrade a certain polymer [14.68, 75] but also semiquantitative results can be obtained by analyzing the growth of the clear zones' diameters [14.76].

Other Methods. Some other analytical methods for monitoring biodegradation processes, especially in degradation experiments with depolymerizing enzymes have been described in the literature.

- Analysis of the amount of dissolved organic carbon (DOC) in the medium around the plastics [14.77].
- Monitoring the decrease in optical density of small polymer particles dispersed in water [14.78].
- Analysis of decrease in particle size of small polymer particles using light-scattering [14.49].
- Determination of the free acids formed in enzymatic polyester cleavage applying a pH-stat-titration [14.30, 47, 50].

Standards for Biodegradable Plastics

At the beginning of the nineties demands were stated namely by the industry to establish suitable and reliable standardized testing procedures to evaluate the biodegradability of plastics. Most of the new standards developed were based on existing tests for the biodegradation of low-molecular-weight chemicals. An overview of standard test methods for biodegradable plastics is given in Table 14.5.

First standards, mainly focused on water- and landfill environments, were published by the ASTM. A test system to evaluate the compostability of plastics was first developed by the German DIN (DIN V 54900). This standard did not only specify testing procedures, but defined also limit values for the evaluation. Other international standards from ISO, CEN and ASTM generally followed the testing strategy given by DIN V 54900, which has meanwhile been substituted by the according European standard (EN 13432).

Currently, the development of standards is focussed on degradation in soil, since there is a great interest for applications of biodegradable plastics in agriculture, e.g., as nonrecoverable mulching films or as matrices for controlled release of nutrients or pesticides etc. Compared to degradation in a compost environment, tests to evaluate degradation in a soil environment proved to be much more complex (e.g., due to different soils or wide variations in external conditions).

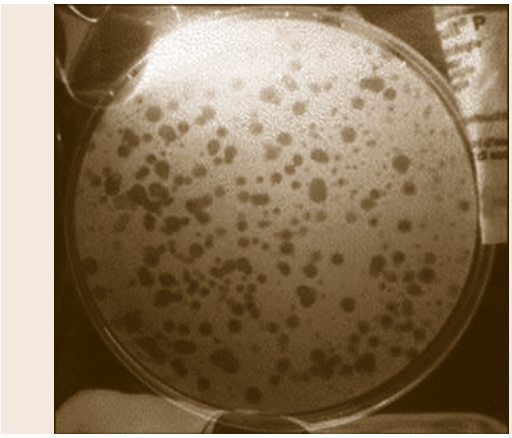


Fig. 14.11 Clear-zone-test: Fine dispersed polymer (β-hydroxybutyrate) in an microbiological agar plate results in a turbid occurrence of the agar. Microorganisms growing on the agar form clear halos (*zones*) if they are able to depolymerize the polymer

In the following, standardization agencies are listed which are involved in the development of standards for biodegradable plastics.

International:	ISO TC61/C5/WG22 *Biodegradability*
Europe:	CEN TC 261/SC4/WG2 *Biodegradability and organic recovery of packaging and packaging waste*
	CEN TC249/WG9 *Characterization of degradability*
National:	
France:	AFNOR
Germany:	DIN FNK 103.3 *Bioabbaubare Kunststoffe* (This group has been liquidated in 2002)
Italy:	UNI
Japan:	MITI/JIS
USA:	ASTM D20.96 *Environmentally degradable plastics*

A number of reviews dealing with standard testing methods for biodegradable plastics have been published during the last years [14.36, 79–81]. For some particular

Table 14.5 Standards related to biodegradable plastics

Biodegradability in different environments/simulation tests	
ISO 14851 – 1999	Determination of the ultimate aerobic biodegradability of plastic materials in an *aqueous medium* – Method by measuring the oxygen demand in a closed respirometer
ISO 14852 – 1999	Determination of the ultimate aerobic biodegradability of plastic materials in an *aqueous medium* – Method by analysis of evolved carbon dioxide
ISO/DIS 14853 – 1999	Determination of the ultimate anaerobic biodegradability of plastic materials in an aqueous system – Method by measurement of biogas production
ISO 14855 – 1999	Determination of the ultimate aerobic biodegradability and disintegration of plastic materials under controlled *composting conditions* – Method by analysis of evolved carbon dioxide
ISO 14855/DAmd 1	Use of a mineral bed instead of mature compost
ISO/AWI 14855-2	Determination of the ultimate aerobic biodegradability and disintegration of plastic materials under controlled composting conditions – Part 2: Gravimetric measurement of carbon dioxide evolved in a laboratory-scale test
ISO/DIS 15985 – 1999	Plastics – Determination of the ultimate anaerobic biodegradability and disintegration under high-solids anaerobic-digestion conditions – Method by analysis released biogas
ISO 17556 – 2003	Plastics – Determination of the ultimate aerobic biodegradability in *soil* by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved
CEN TC 249 WI 240509	Plastics – Evaluation of degradability in soil – Test scheme for final acceptance and specifications
ASTM D 5210-92(2000)	Standard method for determining the anaerobic biodegradability of degradable plastics in the presence of *municipal sewage sludge*
ASTM D 5271-02	Standard test method for assessing the aerobic biodegradation of plastic materials in an *activated-sludge-wastewater-treatment system*
ASTM D 5338-98e1	Standard test method for determining the aerobic biodegradation of plastic materials under controlled *composting conditions*
ASTM D 5511-94	Standard test method for determining anaerobic biodegradation of plastic material under *high solid anaerobic digestion conditions*
ASTM D 5525-94a	Standard practice for exposing plastics to a simulated *landfill* environment
ASTM D 5526-94(2002)	Standard test method for determining anaerobic biodegradation of plastic materials under accelerated *landfill conditions*
ASTM D 5988-96	Standard test method for determining aerobic biodegradation in *soil* of plastic materials or residual plastic materials after composting
ASTM D 6340-98	Standard test methods for determining aerobic biodegradation of radio-labelled plastic materials in an aqueous or *compost environment*
ASTM D 6691-01	Standard test method for determining aerobic biodegradation of plastic materials in the *marine environment* by a defined microbial consortium
ASTM D 6692-01	Standard test method for determining the biodegradability of radiolabeled polymeric plastic materials in *seawater*
ASTM D 6776-2002	Standard test method for determining anaerobic biodegradability of radiolabelled plastic materials in a laboratory-scale simulated *landfill environment*
JIS K 6950 – 2000	Determination of ultimate aerobic biodegradability of plastic materials in an *aqueous medium* – Method by measuring the oxygen demand in a closed respirometer
JIS K 6951 – 2000	Determination of the ultimate aerobic biodegradability of plastic materials in an *aqueous medium* – Method by analysis of evolved carbon dioxide
JIS K 6953 – 2000	Determination of the ultimate aerobic biodegradability and disintegration of plastic materials under controlled *composting conditions* – Method by analysis of evolved carbon dioxide liquidate
PR NF U 52-001PR	Matériaux de *paillage biodégradables pour l'agriculture* – Exigences, méthodes d'essais et marquage
Compostability	
ISO 16929 – 2002	Plastics – Determination of the degree of disintegration of plastic materials under defined composting conditions in a pilot-scale test
ISO 20200 – 2004	Plastics – Determination of the degree of disintegration of plastic materials under simulated composting conditions in a laboratory-scale test
EN 13432 – 2000	Packaging – Requirements for packaging recoverable through composting and biodegradation – Test scheme and evaluation criteria for the final acceptance of packaging

Table 14.5 (continued)

ASTM D 5509-96	Standard practice for exposing plastics to a simulated compost environment
ASTM D 5512-96	Standard practice for exposing plastics to a simulated compost environment using an externally heated reactor
ASTM D 6002-96	Guide to access the compostability of environmentally degradable plastics
ASTM D 6400-99e1	Standard specification for compostable plastics
UNI 10785-1999	Compostilita del materiali plastici – Requisiti e metodi di prova
Polyethylene degradation and photodegradation	
ASTM D 3826-98	Standard practice for determining end point in degradable polyethylene and polypropylene using a tensile test
ASTM D 5071-99	Standard practice for operating xenon arc-type exposure apparatus with water and exposure of photo degradable plastics
ASTM D 5208-01	Standard practice for operating fluorescent ultraviolet and condensation apparatus for exposure of photo degradable plastics
ASTM D 5272-99	Standard practice for outdoor exposure testing of photo degradable plastics
ASTM D 5510-94(2001)	Standard practice for heat ageing of oxidatively degradable plastics
Other tests	
ASTM D 5951-96(2002)	Standard practice for preparing residual solids obtained after biodegradability methods for toxicity and compost quality testing
ASTM D 6003-96	Standard test method for determining weight loss from plastic materials exposed to simulated municipal solid waste (MSW) aerobic compost environment

environments standards for biodegradable plastics will be discussed more in detail in the following.

Compostabilty of Plastics. The treatment of biodegradable plastics in composting processes is discussed as an alternative to plastics recycling or incineration. A number of standards for the evaluation of the compostability of plastics have been published, which all are, in major respects, congruent (ASTM D 6002-96/ ASTM D 6400 99e1, EN 13432 2000); an international norm (ISO CD 15986) is currently under development. Generally, demands for compostability exceed those for biodegradability; compostability usually comprises four demands [14.82].

- The organic fraction of the material must be completely biodegradable;
- The material must disintegrate sufficiently during the composting process;
- The composting process should not negatively be affected by the addition of the plastics;
- The compost quality should not be negatively influenced and no toxic effects should occur.

Taking account of this spectrum of criteria and the principle problems in testing biodegradability in complex (natural) environments discussed above, all test schemes follow for a successive, multistep evaluation strategy.

1. Chemical characterization of the test material
   These data serve for the identification of the material, provide important data for the following tests (e.g., carbon content, composition, content on inorganic components etc.) and especially give information on toxic substances such as heavy metals.
2. Evaluation of biodegradability
   The biodegradability is evaluated by laboratory testing methods monitoring the metabolization of the material by CO_2 formation or O_2 consumption. Two test methods use synthetic aqueous media (ISO 14851, ISO 14852) and allow to establish a carbon balance (besides carbon dioxide, newly built biomass is regarded as *degraded* carbon). The preferred testing method, however, is the so-called controlled composting test [14.38, 41, 42, 83, 84] with mature compost at a temperature of around 60 °C as matrix. The relevance of aquatic tests in a test scheme pertaining to a compost environment has been discussed critically [14.85]. However, disadvantages of the controlled composting test are the relatively high background CO_2 formation, which can be affected by the presence of plastics (*priming effect*) and difficulties in determining a carbon balance with sufficient accuracy. To overcome these problems, the biodegradation is calculated relative to the CO_2-release of a degradable reference substance (e.g. cellulose) or an inert solid matrix inoculated with an eluate from compost is used instead of mature compost [14.65, 66].
   In the test schemes, also limit values are given for the evaluation [14.86]. The maximum test duration is usually 6 month (1 year for radio-labelled materials

in ASTM D6400) and the degrees of degradation requested are 60% and 90% relative to the reference. The value of 60% CO_2 formation originates from the OECD guidelines which were developed for low-molecular, chemically homogeneous materials. The figure takes into account that a part of the carbon is converted to biomass. Plastics, however, are complex in their composition (blends, copolymers, additives) and, thus, the limit value of 90% had been set to ensure a complete degradability of the entire material (a 10% error range was assumed for the degradation tests). In aqueous media this limit usually can only be achieved including the biomass formed (carbon balance). In mature compost less new biomass is formed and CO_2 levels of more than 90% are observed for many polymers.

3. Characterization of disintegration in compost
   Since in real biowaste respirometric measurements are not possible with a sufficient accuracy, in a real compost environment only disintegration of the materials is evaluated. Disintegration testing can be performed in laboratory tests using controlled reactors of some hundred liters content [14.87] or tests in a real composting plant. The degree of disintegration is determined by sieving the compost and analyzing polymer fragments larger than 2 mm (in all standards a maximum fraction of 10% is allowed).
4. Compost quality/toxicity
   The quality of the final compost should not negatively be affected when plastics are composted. Tests to evaluate this requirement are defined by established national testing methods ensuring compost quality. Criteria for compost quality are, for instance, maturity, visual impurities, density, pH, content of nutrients, salts, heavy metals etc. Additionally, ecotoxicity tests focused on plant growth are part of the compost quality characterization (e.g., plant tests according to OECD guidelines (OECD 208)).
   Additional toxicity tests (e.g., tests with earthworm, luminicent bacteria, *Daphnia magna*, fish) were discussed during the development of the standards [14.41, 57, 88, 89], but due to the limited experiences with these tests in combination with compost, these tests were not generally included in the test schemes for compostability (ASTM includes earth worm test according to OECD guideline OECD 207).

Anaerobic Biodegradability. Beside *normal* composting, the treatment of biowaste by anaerobic digestion (*anaerobic composting*) is becoming more and more widespread. It has been demonstrated that the anaerobic biodegradation behavior of plastics can differ significantly from that under aerobic conditions [14.68, 69]. Thus, separate tests must become established in order to also include these conditions for biodegradation of plastics. Some standard methods for monitoring the anaerobic degradation via biogas formation do already exist (ASTM D 5511-94, ISO/DIS 14853 1999, ISO/DIS 15985 1999), based on testing protocols designed for low-molecular substances [14.90]. However, the evaluation schemes for biodegradable plastics do not mandatorily demand the proof of anaerobic degradability up to now (in EN 13432 anaerobic tests are mentioned, but are optional).

Biodegradation in Soil Environment. After focusing on degradation in landfills and then on composting processes at the first stage of standardization efforts, currently the characterization of the evaluation of plastics biodegradation in soils has become important since there is now a growing interest in applications of biodegradable plastics in agriculture (e.g., as mulching films or as matrix for controlled release of fertilizers and pesticides).

Compared to the evaluation of the compostability, standardization of testing methods focused on soil degradation face some serious problems.

- Composting is a technical process where parameters such as pH, temperature, humidity and biowaste composition are kept in certain limits to guarantee an optimal composting process. In soil, the kind of environment and the environmental conditions can vary significantly and usually can not be controlled in nature. Standards have to take these variations into account somehow.
- The higher temperatures and activity of the microorganisms involved, causes biodegradation of plastics during composting to usually be faster than in a natural soil environment.
  It is much more difficult to monitor the slow degradation processes of some plastics in soil with a sufficient accuracy. Slow degradation sometimes causes extremely long test periods (>1 y).

Currently the CEN working group CEN TC249/WG9 *Characterization of degradability* is developing an evaluation scheme for soil degradation of plastics. The structure of this scheme very likely will be generally similar to that for composting including the different aspects mentioned above. However, due to the

variability in conditions and the differences in applications the test scheme will be more complex than the standards for compostability.

First standards describing the experimental conditions for testing biodegradation of plastics in soil have been established (ISO/PRF 17556-2002). A method for CO_2 detection in a closed system with minimized amount of soil was proposed by *Solaro* et al. [14.62]; *Albertsson* et al. [14.73] published a work based on ^{14}C-labelled samples. Additionally to possible low carbon dioxide evolution rates, the determination of a carbon balance in soil is problematic and effects of the polymer added on the background CO_2 evolution have been observed [14.91] (*priming effect*). As proposed for the controlled composting test, the use of an inert matrix such as vermiculite is investigated, too.

Problems arise also for the evaluation of the disintegration behavior in soil. The influence of the soil characteristics and the environmental conditions on plastics degradation has been examined in some publications [14.92–96]. To solve this problem, degradation tests in a whole set of different selected soils or a test with a soil mixture are being discussed.

14.3.3 Paper and Textiles

Susceptibility of Paper and Textiles to Biodeterioration

Paper and textiles have been produced and used by humans for millennia. Until relatively recently the composition of both have been dominated by natural materials whether cellulose and plant fibers in the case of paper – or fibers derived from either plants or animals in the case of textiles. In the last 100 years, natural fibers have been mixed with man-made materials created either from polyolefins such as nylon or modified natural materials such as viscose and rayon and in some instances been completely replaced by them. In more recent times, synthetic materials have been used either to add new properties to paper or in some cases even replace paper in its traditional applications (e.g., tear- and water-resistant notepaper). In many cases, man-made fibers have either replaced traditional natural fibers in modern textiles or added properties which could not previously be obtained (e.g. a high degrees of elasticity). Despite this, natural fibers play an important role in both paper based and textile products.

Although it has been recognized that paper and textiles derived from natural materials can be damaged by the action of biological systems, i. e., rats and mice, insects or miroorganisms, for almost has long as they have been used, it was only in the 1940s that the study of the biodeterioration of these materials really became a scientific discipline in its own right [14.97]. Various armed conflicts in tropical regions of the Far East resulted in premature failure of items of military clothing (e.g. boots) and equipment (tents and tarpaulins) and attempts were made to predict the performance of materials under such conditions and identify treatments to prevent failure. A variety of standard tests began to emerge [14.98], mainly as part of military specifications, and many of these approaches are still used to this day. Despite the emergence of many man-made materials, the biodeterioration of textiles and paper remains a problem with microorganisms causing discoloration of/odor in finished goods and loss of functionality (e.g., destruction of plasticizers and loss of elasticity following microbial metabolism of critical components of the composition [14.99]). Similarly, microbial action in textile and paper manufacturing processes causes losses in productivity (e.g., microbial slimes resulting in defects in paper and rupturing in the manufacture) as well as function (e.g., blockage of applicators of spin finishes/weaving ancillaries by microbial growth/detached microbial biofilms resulting in either yarn being produced without antistatic agents/lubricants or areas of localized damage due to overheating on the loom).

Microbial attack of finished paper and textiles is most usually associated with fungi although a wide range of microorganisms cause problems in the manufacturing environment [14.100]. Growth occurs on the finished goods when the material is exposed to either high humidity or free water during use and storage. The resultant growth causes either marking/discoloration (Fig. 14.12) or, eventually, physical damage (Fig. 14.13). Such discoloration/spoilage might range from the small blemishes or foxing on historic documents [14.101] to the severe staining/musty odors associated with mildew on tents that have been stored without being dried properly first [14.98]. Growth of microorganisms is usually prevented by ensuring that insufficient moisture is present. In document archives, the humidity is strictly controlled to either prevent growth or to prevent further growth on material that already has some microbial damage. Not only does this ensure that the documents remain stable but also that growth of fungi and the production of fungal spores do not have a negative impact on the health of people who work in the archives [14.102]. Similarly, textiles that have heritage value are also conserved in controlled environments. Growth on other more utilitar-

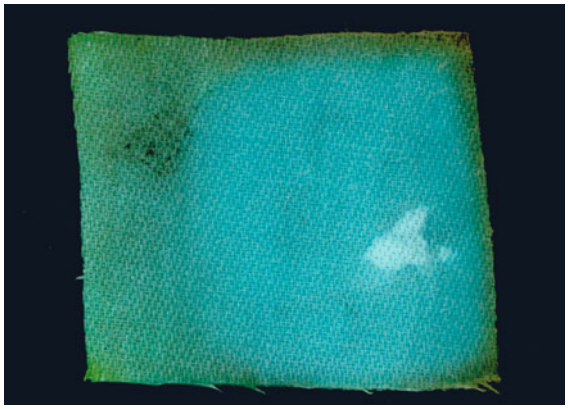


Fig. 14.12 Staining due to mould growth on a cotton based textile

Fig. 14.13 Microbial deterioration of a water damaged book

ian goods (e.g., tents and tarpaulins) can be prevented by ensuring that they are dry prior to storage and that they are stored in an environment which limits the availability of moisture. Where exposure to moisture is expected during service or where less than ideal storage conditions prevail, goods can be treated to prevent growth/spoilage [14.103].

The tests used to determine both the susceptibility of materials to microbial biodeterioration and to determine the efficacy of treatments intended to prevent it range from simple agar plate bioassays through cabinet-based tests intended at simulating conditions of exposure in a more realistic manner to field exposure/soil burial trials. A range of such tests is given in Tables 14.6–14.8. The choice of test will depend upon the type of information required and the speed with which that data is needed as well as the importance of the accuracy of any predictions that are being made from it. Combined with some form of simulation of durability (e.g., leaching in water), simple plate assays can be used to screen a range of treatments to select one or two that are likely to function in service. However, such assays should be treated with caution as they do not always provide a realistic simulation of the inherent susceptibility of a material to spoilage (especially where the supporting media contain a source of nutrients) and can underpredict the protective capacity of treatments. For this reason, cabinet-based simulations (e.g. BS2011 Part 2J) and field trials should always be considered when more critical end uses are involved.

It can be seen from Table 14.6 that there is a relatively large number of tests developed to examine the resistance of textiles to fungal growth. The majority of these tests are agar plate based but some use cabinets. In the case of the agar plate methods they range from simple single species tests performed with samples being placed on a complete nutrient medium and then inoculated to multispecies tests performed on minimal media. While the former can be used to examine the dose response of a number of treatments used to prevent growth, the latter is more suited to gaining information about the inherent susceptibility of a material to microbial spoilage. The cabinet-based tests all take a similar format in which materials are inoculated with the spores of a range of fungi known to grow on textiles and are then incubated under conditions which stimulate fungal growth. Various soiling agents can be employed to increase the degree of severity of the simulation and the incubation period can be extended to simulate the durability required. In all cases growth similar to that experienced/expected under conditions of actual use should be demonstrated on an appropriate control material (in the case of BS2011 Part 2J a paper control is specified, however, the relevance of this to the material under test should be considered and an additional, relevant control included wherever possible). A similar range of fungal tests exists for paper (Table 14.7) but relatively few tests exist to simulate spoilage/biodeterioration by bacteria (BS 6085: 1992). In most instances, biodeterioration by bacteria is encompassed by soil burial trials but there are circumstances where this might be considered as too severe and work is required to develop suitable protocols in this area. In other applications, e.g. geotextiles, soil burial is probably the only sensible manner to attempt to simulate their performance in practice.

A number of tests have also been developed for materials/processes used in the manufacture of paper and textiles. For example, ASTM E1839-07 (2007)

Table 14.6 Methods used to examine the resistance of textiles to biodeterioration

Reference	Title	Description	Major principle
prEN 14119	Testing of textiles – Evaluation of the action of microfungi	The test is designed to determine the susceptibility of textiles to fungal growth. Assessment is by visual rating and measurement of tensile strength	Agar plate test
AATCC 30-1998	Antifungal activity, assessment on textile materials: mildew and rot resistance of textile materials	The two purposes of the test are to determine the susceptibility of textiles to microfungi and to evaluate the efficacy of fungicides on textiles	Agar plate test
DIN 53931	Testing of textiles; determination of resistance of textiles to mildew; growth test	The test determines the efficacy of treatments for prevention of fungal growth on/in textiles. It also allows the performance testing of a treatment after UV irradiation, leaching etc.	Agar plate test
MIL-STD-810F	Environmental engineering considerations and laboratory tests; Method 508.5 FUNGUS	The purpose of the method is to assess the extent to which a material will support fungal growth and how performance of that material is affected by such growth	Humid chamber test (90 to 99% humidity)
BS 6085 :1992	Determination of the resistance of textiles to microbial deterioration	The purpose of the method is to assess the extent to which a material will support fungal/bacterial growth and how performance of the material is affected by such growth Visual Assessment and measurement of tensile strength	a) Soil burial test, b) Agar plate test, c) Humid chamber test
EN ISO 11721-1	Textiles – Determination of resistance of cellulose-containing textiles to microorganisms – Soil burial test – Part 1: Assessment of rot retarding finishing	The test is designed to determine the susceptibility of cellulose containing textiles against deterioration by soil microorganisms. Preserved and unpreserved textiles are compared. Visual Assessment and measurement of tensile strength	Soil burial test
prEN ISO 11721-2	Textiles – Determination of resistance of cellulose-containing textiles to microorganisms – Soil burial test – Part 2: Identification of long-term resistance of a rot retardant finish	The test identifies the long-term resistance of a rot-retardant finish against the attack of soil inhabiting microorganisms. It allows to make a distinction between regular long-term resistance and increased long-term resistance. Visual Assessment and measurement of tensile strength	Soil burial test
BS 2011: Part 2.1J (IEC 68-2-10)	Basic environmental testing procedures	Mould growth test to show the susceptibility of a material towards colonization by fungi	Humid chamber test (90 to 99% humidity)
AS 1157.2 – 1999	Australian standard – Methods of testing materials for resistance to fungal growth Part 2: Resistance of textiles to fungal growth. Section 1 – Resistance to surface mould growth	Test specimens are inoculated with a suspension of spores of *Aspergillus niger* and then incubated on the surface of a mineral salts based agar for 14 d and then assessed for growth. Both leached and unleached specimens are examined. Glass rings are employed to hold the specimens in intimate contact with agar when necessary. Specimens are examined for the presence of surface mould growth	Agar plate test
AS 1157.4 – 1999	Australian standard – Methods of testing materials for resistance to fungal growth Part 2: Resistance of textiles to fungal growth. Section 2 – Resistance to cellulolytic fungi	Test specimens are inoculated with a suspension of spores of *Chaetomium globosum* and then incubated on the surface of a mineral salts based agar for 14 d and then assessed for growth. Both leached and unleached specimens are examined and exposed samples are subjected to a tensile strength test. Glass rings are employed to hold the specimens in intimate contact with agar when necessary	Agar plate test
AS 1157.3 – 1999	Australian standard – Methods of testing materials for resistance to fungal growth Part 2: Resistance of cordage and yarns to fungal growth	Test specimens are inoculated with a suspension of spores of *Chaetomium globosum* and then incubated on the surface of a mineral salts based agar for 14 d and then assessed for growth. Both leached and unleached specimens are examined and exposed samples are subjected to a tensile strength test	Agar plate test (other vessels containing media are employed for large specimens)

describes a standard test method for efficacy of slimicides used in the paper industry and E875-10 (2010) describes a standard test method for determining the efficacy of fungal control agents used as preservatives for aqueous-based products employed in the paper industry. Work is also in progress on methods to determine the performance of spin finishes and textile ancillaries by the Functional Fluids Group of the International

Table 14.7 Methods used to examine the resistance of paper to biodeterioration

Reference	Title	Description	Major principle
DIN EN 1104	Paper and board intended to come into contact with foodstuffs Determination of transfer of antimicrobic constituents	A minimum of 20 replicates subsamples (each 10–15 mm in diameter) taken from 10 samples of a batch of paper are placed in intimate contact with nutrient agar plates inoculated with either *Bacillus subtilis* or *Aspergillus niger* and incubated at 30 °C for 7 d and at 25 °C for 8–10 d respectively	Zone Diffusion Assay
ASTM D 2020-2003	Standard test methods for mildew (fungus) resistance of paper and paperboard – direct inoculation	Replicate samples (3) are inoculated with a suspension of fungal spores and then incubated on the surface of a minimal mineral salts medium to determine if they support fungal growth	Biodeterioration Test
ASTM D 2020-2003	Standard test methods for mildew (fungus) resistance of paper and paperboard – Soil Burial	Replicate samples (5) are buried in soil for 14 d and then examined for the deterioration compared with unburied samples for both physical deterioration and loss of tensile strength	Biodeterioration/ Biodegradation Test
AS 1157.7 – 1999	Australian standard – methods of testing materials for resistance to fungal growth Part 6: Resistance of papers and paper products to fungal growth	Test specimens are placed on the surface of a mineral salts based agar and then both the specimen and the agar are inoculated with a suspension of spores of a range of fungi. They are then incubated for 14 d and then assessed for growth. Growth on the specimen is assessed	Agar plate test
AS 1157.5 – 1999	Australian standard – methods of testing materials for resistance to fungal growth Part 5: Resistance of timber to fungal growth	Test specimens are placed on the surface of a mineral salts based agar and then both the specimen and the agar are inoculated with a suspension of spores of a range of fungi. They are then incubated for 14 d and then assessed for growth. Growth on the specimen is assessed	Agar plate test
AS 1157.6 – 1999	Australian standard – Methods of testing materials for resistance to fungal growth Part 6: Resistance of leather and wet *blue* hides to fungal growth	Test specimens are placed on the surface of a mineral salts based agar and then both the specimen and the agar are inoculated with a suspension of spores of a range of fungi. They are then incubated for 14 d and then assessed for growth. Both leached and unleached specimens are examined Growth on specimens is assessed. Sucrose containing media is employed where true controls cannot be obtained	Agar plate test

Table 14.8 Methods used to examine the resistance of geotextiles to biodeterioration

Reference	Title	Description	Major principle
EN 12225	Geotextiles and geotextiles-related products – Method for determining the microbiological resistance by a soil burial test	The test is designed to determine the susceptibility of geotextiles and related products to deterioration by soil microorganisms. Visual Assessment and measurement of tensile strength	Soil burial test

Table 14.9 Methods under development to examine the preservation of wipes and moist nonwoven textiles

Reference	Title	Description	Major principle
EDANA Antibacterial Preservation V8	Recommended test method: Nonwovens – Antibacterial preservation	Test designed to determine the efficacy of preservation in non-woven textiles against bacterial contamination	Agar plate test
Publication by A. Crémieux, S. Cupferman, C. Lens	Method for evaluation of the efficacy of antimicrobial preservatives in cosmetic wet wipes	Efficacy of preservative against fungi and bacteria is tested A dry inoculum is placed into the original packaging among the wet wipes. The package is then re-sealed and assessed for growth over time	Bacterial/fungal challenge test

Biodeterioration Research Group (IBRG). A number of specialized tests have also emerged in recent years to address problems that have resulted from the development of new materials. Nonwoven *textiles* have been developed to produce moist hygienic wipes for clinical and personal care (e.g., infant sanitary wipes). These products are supplied in multipacks and contain premoistened wipes. However, in some instances these

have proven susceptible to fungal growth in storage and in use and methods have been developed to both simulate the problem and help predict the performance of preservatives intended to prevent growth (Table 14.9).

Antimicrobial Textiles and Paper

Recently a number of both textile- and paper-based goods have been produced which include antimicrobial properties which are not intended merely to prevent deterioration in service but to provide an antimicrobial function in use [14.104]. These articles include items of clothing fortified with microbicides to prevent odors from being formed from human perspiration or prevent cross-infection in clinical environments.

It can be seen from Table 14.10 that there are two major forms of test for microbiological effects of

Table 14.10 Methods used to examine the antimicrobial activity of textiles (fabric, yarn or pile/wadding)

Reference	Title	Description	Major principle
ASTM E2149-10	Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions	Dynamic shake flask test. Test material is suspended in a buffer solution containing a known number of cells of *klebsiella pneumoniae* and agitated efficacy is determined by comparing the size of the population both before and after a specified contact time	Relies on either diffusion of antimicrobial mater from treated material into the cell suspension. Some activity may be due to interaction between the population and the surface of the material in suspension
AATCC 147-2004	Antibacterial activity assessment of textile materials: Parallel streak method	Agar plates are inoculated with 5 parallel streaks (60 mm long) of either *Staphylococcus aureus* or *K pneumoniae.* A textile sample is then placed over the streaks and in intimate contact with the surface of the agar and incubated. Activity is assessed based on either the mean zone of inhibition over the 5 streaks or the absence of growth behind the test specimen	Zone diffusion assay
AATCC 100-2004	Antibacterial finishes on textile materials	Replicate samples of fabric are inoculated with individual bacterial species (e.g. *Staph aureus* and *K pneumoniae*) suspended in a nutrient medium. The samples are incubated under humid conditions at 37 °C for a specified contact time. Activity is assessed by comparing the size of the initial population with that present following incubation. A neutralizer is employed during cell recovery	Cell suspension intimate contact test
XP G 39-010	Propriétés des étoffes – Étoffes et surfaces polymériques à propriétés antibactériennes	Four replicate samples of test material are placed in contact with an agar plate that has been inoculated with a specified volume of a known cell suspension of either *Staph aureus* and *K pneumoniae* using a 200 g weight for 1 min. The samples are then removed. Duplicate samples are analysed for the number of viable bacteria both before and after incubation under humid conditions at 37 °C for 24 h. A neutralizer is employed during cell recovery	Cell suspension intimate contact test
JIS L 1902: 1998	Testing method for antibacterial activity of textiles Qualitative Test	Three replicate samples of fabric, yarn or pile/wadding are placed in intimate contact with the surface of agar plates that have been inoculated with a cell suspension of either *Staph aureus* or *K pneumoniae* and incubated at 37 °C for 24–48 h. The presence of and size of any zone of inhibition around the samples is then recorded	Zone diffusion assay
JIS L 1902: 1998	Testing method for antibacterial activity of textiles Quantitative Test	Replicate samples of fabric (6 of the control and 3 of the treated) are inoculated with individual bacterial species (e.g. *Staph aureus* and *K pneumoniae*) suspended in a heavily diluted nutrient medium. The samples are incubated under humid conditions at 37 °C for a specified contact time. Activity is assessed by comparing the size of the initial population in the control with that present following incubation. No neutraliser is employed during cell recovery	Cell suspension intimate contact test

Table 14.10 (continued)

Reference	Title	Description	Major principle
EN ISO 20645	Textile fabrics – Determination of the antibacterial activity – Agar plate test (ISO 20645:2004)	Four replicate samples of fabric (25 ± 5 mm) are placed in intimated contact with a solid nutrient medium in a petri dish. The samples are then overlaid with molten solid nutrient media which has been inoculated with a cell suspension of either *Staph aureus*, *Escherichia coli* or *K pneumoniae*. The plates are then incubated for between 18 and 24 h and the plates are then assessed for growth based on either the presence of a zone of inhibition of > 1 mm or the absence/strength of the growth in the media overlaying the test specimen	Zone diffusion assay
ISO 20743	Textiles – Determination of antibacterial activity of antibacterial finished products: Absorption method	Replicate (6) samples of textile are inoculated with a standardised broth culture of either *staph aureus* or *K pneumoniae* in individual tubes and then incubated at 37 °C for 18–24 h in closed containers. Samples are analysed for the presence of viable bacteria both before and after incubation by either total viable count or the determination of total ATP. Samples are sterilised prior to testing and a neutraliser is employed during recovery. The test is validated by growth of 1 order of magnitude during the incubation period	Cell suspension intimate contact test
ISO 20743	Textiles – Determination of antibacterial activity of antibacterial finished products: Transfer method	Replicate (6) samples of test material are placed in contact with an agar plate that has been inoculated with a specified volume of a known cell suspension of either *staph aureus* and *K pneumoniae* using a 200 g weight for 1 min. The samples are then removed. Replicate (3) samples are analysed for the either the number of viable bacteria or the total ATM content both before and after incubation under humid conditions at 37 °C for 24 h. Samples are sterilised prior to testing and a neutraliser is employed during cell recovery. The test is validated by either growth of 1 order of magnitude during the incubation period or by a measure of the variability of the data obtained	Cell suspension intimate contact test
ISO 20743	Textiles – Determination of antibacterial activity of antibacterial finished products: Printing method	Replicate (6) samples of test material are either *staph aureus* and *K pneumoniae* by *printing* cells collected on a membrane filter onto their surface in a standardised manner. The samples are then incubated under humid conditions for 18–24 h at 20 °C for a specified contact time(s). Replicate (3) samples are analysed for the either the number of viable bacteria or the total ATM content both before and after incubation. Samples are sterilised prior to testing and a neutraliser is employed during cell recovery. The test is validated by either determining the survival of the inoculum on the control material	*Dry* inoculum intimate contact test
SN 195920	Examination of the antibacterial effect of impregnated textiles by the agar diffusion method	Four replicate samples of fabric (25 ± 5 mm) are placed in intimated contact with a solid nutrient medium in a petri dish. The samples are then overlaid with molten solid nutrient media which has been inoculated with a cell suspension of either *staph aureus* or *E. coli*. The plates are then incubated for between 18 and 24 h and the plates are then assessed as described in prEN ISO 20645 above	Zone diffusion assay
SN195924	Textile fabrics – Determination of the antibacterial activity: Germ count method	Fifteen replicate samples (each replicate is comprised of sufficient specimens of 25 ± 5 mm to absorb 1 ml of test inoculum) are inoculated with cells of either *E. coli* or *staph aureus* suspended in a liquid nutrient medium and incubated in sealed bottles for up to 24 h at 27 °C. After 0, 6 and 24 h, 5 replicate samples are analysed for the size of the viable population present. A neutraliser is employed. An increase of 2 orders of magnitude of the population exposed to a control sample is required to validate the test. The method defines a textile as antibacterial if no more than a specified minimum level of growth is observed after 24 h in 4 of the 5 replicate groups of samples	Cell suspension intimate contact test
SN195921	Textile fabrics – Determination of antimycotic activity: Agar diffusion plate test		Zone diffusion assay

Table 14.11 Methods used to examine the antimicrobial activity of carpets

Reference	Title	Description	Major principle
AATCC 174-2007	Antimicrobial Activity Assessment of Carpets Qualitative Antibacterial Activity	Petri dishes with nutrient media are inoculated with a single, diagonal streak (≈ 7.5 cm) of either *staph aureus* or *K pneumoniae*. An unsterilized test specimen (25 mm × 50 mm) is placed in intimate contact and transversely across the inoculum on the agar surface. The plates are then inoculated at 37 °C for 18–24 h. The front and back of the carpet are tested separately. After incubation, the plates are inspected for the presence of growth both below the specimens and for any zone of inhibition caused by the specimen is recorded. The test can also be used to test the effect of cleaning regimes. An untreated control is optional	Qualitative assessment of rate of kill and zone diffusion test
AATCC 174-2007	Antimicrobial Activity Assessment of Carpets Quantitative Antibacterial Activity	Unsterilized specimens of carpet are pre-wetted with either sterile water or a wetting agent before being inoculated with individual suspensions of either *staph aureus* or *K pneumoniae* in either a low or a high nutrient solution. The samples are then incubated in a tightly closed jar at 37 °C for a specified contact time. Cells are recovered in 100 ml of a neutraliser after 0 and 6–24 h of incubation. Activity is assessed by comparing the size of the initial population in the control (if used) with that present following incubation. A control is optional. When not employed, viable counts following incubation of the treated specimens alone are considered. The test can also be used to test the effect of cleaning regimes	Cell suspension intimate contact test

treated textiles which are not related to the prevention of biodeterioration. In the first, typified by AATCC 147, samples of textiles are placed onto agar plates which have been inoculated with bacteria and then incubated. The intention is that intimate contact between the textile and the bacteria/growth medium will result in the inhibition of growth either immediately adjacent to the textile or in an area around the textile, in case any antimicrobial agents that have been employed become dissolved in the growth medium. These methods are generally acknowledged as being nonquantitative although they can be employed as assays of certain antimicrobial products in the same manner that such techniques are used for certain antibiotics [14.105]. As with some of the biodeterioration tests described above, this could be useful as a screening tool and for investigating the effect of wash cycles etc. These methods are widely employed in the textile industry as they provide a highly graphic representation of antimicrobial activity although this can lead to misunderstandings of either the scale of effect seen (bigger zones of inhibition looking better) and the implications that mobility of active ingredient could have on service life. Although these techniques are considered to be unsuitable for *quantifying* the effect of the antimicrobial effects of treated textiles there are some disciplines in which they may provide data which is more relevant to the effect claimed than that delivered by a fully quantitative technique (e.g., predicting the effect/durability of bandages intended for use of suppurating wounds).

From Table 14.10 it can be seen that there are at least four techniques which provide quantitative data on the effect of treated textiles on bacteria. These are typified by the method described in AATCC 100-2004 in which samples are inoculated with suspensions of bacteria and then incubated for a specified time before being examined for the size of the population present (Fig. 14.14). The methods differ in the form of the suspension medium, number of replicates examined, test species and, to a certain extent, conditions for incubation. Methods AATCC-100 and JIS L 1902 appear to be the most commonly employed. The Swiss Standard SN195924 was based on AATCC 100 but was apparently modified to improve reproducibility and repeatability [14.106]. These methods show a clear potential as being suitable to determine both inherent bactericidal and bacteriostatic properties of textiles. Although primarily developed for examining effects against bacteria, they can be extended to the investigation of the impact on yeasts, fungal spores and mycelial fragments. The impact on other species of

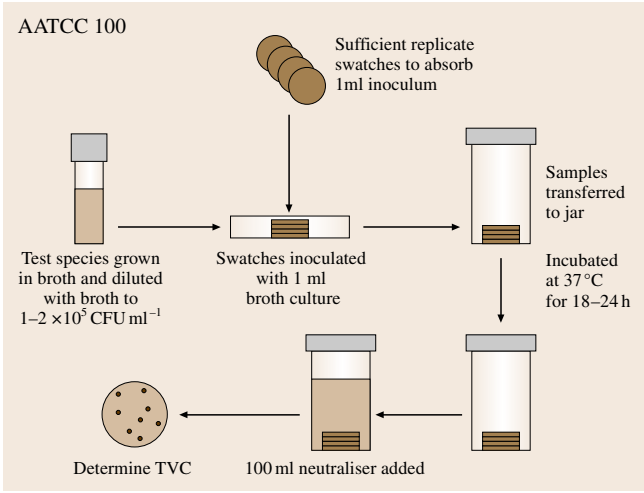


Fig. 14.14 Schematic representation of AATCC 100

bacteria can also be investigated. It is possible to envisage the method being extended to the examination of viral particles, algae and protozoa. In addition, such protocols can be combined with studies on ageing (e.g., the impact of washing cycles) to begin to satisfy at least some of the aspects associated with service life.

It can also be seen from Table 14.10 that no fully quantitative methods exist for the examination of treated textiles on fungi. All of the protocols described are zone diffusion assays of one form or another. As with antibacterial properties, these may be sufficient to substantiate certain claims (e.g., that certain fungi significant to infections on the human skin cannot germinate and grow on the textile) and methods designed for the measurement of the potential for/prevention of biodeterioration could be employed dependent on the claim being made (e.g., EN 14119: 2003 – Table 14.10) as well.

In addition to the truly microbiological methods described above, at least three methods exist which describe the performance of woven textiles [14.107] and nonwoven textiles [14.108, 109] to penetration by bacteria under wet and dry conditions. Further information is required to determine whether these have any use in evaluating treated textiles.

Although no specific standards exist for the examination of antimicrobial effects on paper and board, in many cases the tests intended for textiles can be employed. One method (DIN EN 1104) uses a zone diffusion assay to look for the presence of antimicrobial agents in paper destined for food contact applications, the intention here being to prevent the transfer of any antimicrobial agents used in both the production of and to prevent the biodeterioration of the paper/board being transferred to food.

Selection of Test Method

For assessing potential biodeterioration and the efficacy of treatments intended to prevent it, the choice of methodology is relatively straightforward and is driven either by a specification to be matched (e.g., a military specification) or by the need to simulate an effect observed in service. In some cases, microorganisms that have caused failure in the field can be isolated and utilized in laboratory tests although care should be taken to use sufficient species/strains to measure an adequate spectrum of activity. Selecting a method to simulate a nonbiodeterioration-related effect is, however, more problematic. Although many of the methods described above and in the tables below can be used to give a measure of an antimicrobial effect, it is important to ensure that this effect and the method used to measure it, is relevant to the application. For example, it may be sufficient to merely slow bacterial growth in a application intended to reduce the generation of odorous compounds from human perspiration. In this instance a test such as that described in AATCC 100 may be modified using species known to biotransform compounds in human sweat to odor-forming molecules. The presence of moisture in the system could be considered as a reasonable model of certain items of sports clothing (e.g. socks) and the temperature used/contact time employed could be altered if necessary to bring the test closer to reality. However, in many other applications such tests provide a poor simulation of the end use. For example, a method that uses a cell suspension to saturate a sample which is then incubated at 37 °C for 24 h cannot be considered as a suitable simulation of textiles used for soft furnishings and the uniforms of medical staff. In normal use these materials would be dry or at worst occasionally splashed with water/fluids containing microorganisms. Not only do the test conditions not simulate the normal environment of the textile in use, but they may artificially predict an effect where none would result in practice. Many antimicrobial agents require free water to facilitate their transfer from a material in which they are contained to a microbial cell that comes in contact with the material. Similarly, all microbial cells require free water to grow and many also require it to survive for any length of time. If either no or insufficient free water is present in an application then it is unlikely that an antimicrobial agent would migrate from a material to a cell or

that the cell would be in a suitable metabolic state to interact with the active ingredient. In some instances, the inclusion of an active ingredient might be to add potential activity which would provide function if suitable conditions were to arise (in a similar manner to the use of a fungicide in a textile intended to prevent mold growth should conditions suitable for growth occur – i. e., keeping the item dry would have the same function in most cases). In this instance care needs to be taken to understand what the effect one is trying to simulate is. If for example, it is to prevent cross infection from one patient to another by contact with the uniform of medical staff, the speed of action will be a critical factor. Not only will the level of moisture need to be selected to simulate the conditions (e.g., moisture from hand contact, a splash with a body fluid such as sputum) but the contact time will need to reflect the interval in which cross contamination might occur (potentially very short intervals in a busy clinical unit). In some circumstances, a significant level of moisture may simply never be present (e.g., during the settlement of airborne bacteria onto curtains). Methods are being developed which attempt to simulation such conditions (e.g., the printing method stage of ISO 20743) although care must be taken to ensure no artifacts are introduced in the recovery phase of such techniques. Considerable work is still required before many of the claims made for treated textiles/paper can be substantiated and their true benefit be assessed in a robust manner.

14.4 Biological Testing of Inorganic Materials

Ageing applies more or less to all materials. This natural decay process is ruled by physical and chemical interactions with the environment and can be considerably accelerated and in some special cases slowed down by the interaction with organisms and especially microorganisms. The microbes involved are wide-ranging in speciation and at the same time often very specialized in their nutritional and environmental adaptations. Adhesion to surfaces and resistance to stressed conditions are of importance as well as special biochemical pathways to furnish energy, electrons, water and mineral matter to the microbes living on and in inorganic materials. In order to understand detrimental functions and reaction chains, new methods of study and differentiation of physical, chemical and biologically induced or accelerated processes had to be developed. Quantitative data on mere physical and chemical attack in comparison to biologically induced and catalyzed biotransformations and biotransfer processes had to be compared in laboratory and field experiments. Methods of curing, protection, sterilization, biocide treatments had to be especially conceived and tested. Studies on the speed of physical/chemical deterioration as compared to biodeterioration were undertaken. In conclusion it can be stated that all inorganic materials exposed to surface conditions are more rapidly transferred and cycled biologically than under conditions of a sterile environment and atmosphere. Although water plays an eminent role in all biotransfer processes, it is shown that biologically induced accelerations of decay and ageing of materials takes place in practically all objects of industry, daily use, and of the cultural heritage. Examples of subaquatic and subaerial biofilms on inorganic surfaces and microbial networks (biodictyon) inside porous or damaged materials are given as well as the techniques to study them and eventually prevent their damaging potential.

14.4.1 Inorganic Materials Subject to Biological Attack

Generally, the decomposition of inorganic materials is related to empirical observations and even to subjective impressions. The physical, chemical and biological processes are usually not well understood. Thus everybody expects that granite, for example (or any stone) is more durable than tissue (or any other organic material). Some tombstones, however, decay so fast that the son may survive the inscriptions made to commemorate his own parents. Thus in an exclusively physical environment, it is evident that the decomposition of rock and the dissolution of rock-forming minerals proceeds much faster than the physical decay of a protein or carbohydrate. We can state that practically all materials used in the production of objects, buildings and machines will ultimately decay. Biologically induced or accelerated decay processes are, however, often underestimated. Acceleration rates of up to 10 000 × have been found in comparative laboratory experiments and field observations. The physical and chemical environment and conditioning of all objects of commercial and

cultural value will determine (1) the longevity of any object and (2) the chances of biological attack and biological catalysis of the natural physical decomposition processes. One simple example is the aggressiveness of water on a marble statue. Distilled pure water has an extremely low dissolution capacity as only a minimal amount of protons are available. Marble in the vicinity of a bakery or a restaurant, however, absorbs volatile organic compounds, which – humidity given – will be transformed to carbonic acid by ubiquitous microbes. The aggressive action of water is modified through various biological processes. Therefore, a durability scale of materials will largely depend on the environmental and biological conditions. However, we can now say more specifically which inorganic materials are more susceptible to biological attack and biodeterioration as compared to others. In the following pages we will, after a short introduction on materials, focus mainly on the organisms and biological phenomena and processes involved in the deterioration of objects made of inorganic materials. We will introduce some modern terminology, describe methods of study and ways for protection of the inorganic materials from biological hazards.

Mineral Materials

Most buildings, sculptures and objects of use are produced from rock types found and procured locally. Since the Egyptian, Greek, Hellenistic and Roman times, however, international trading of beautiful and often most durable rock types is documented and the multiple use and transfer of materials such as Pentelic and Carrara marble, Egyptian porphyry and granite stones and objects is well documented, with famous examples of porphyry columns transferred from Egypt to Rome and from Rome to Provence etc. or Egyptian obelisks being transferred to modern cities such as Paris and London. Vitruvius in his time made a survey on their durability and thus, with the exclusion of cases where cheap materials were used, the more impervious rocks were traditionally used in production of objects if the future owner was ready to pay enough money.

Calcareous Materials. Calcium carbonate is a mostly biologically deposited mineral. It is produced by skeletal macroorganisms or from the byproduct of bacterial-, fungal- and algal metabolic activities. The so-called structure-less carbonate rocks and carbonate cements of sandstones are usually biologically catalyzed deposits. Marble is a pressure-temperature metamorphosis of limestone. Traditionally, travertines and carbonate tuffs (fresh-water deposits of calcium carbonate produced by carbon dioxide uptake through algae and/or by equilibration of water highly enriched with carbon dioxide) are regarded as high quality materials because it is very easy to cut and treat them in a wet state, while they harden when drying. Many colored marbles (carbonate breccia) are of Alpine Triassic origin. The yellow to brown color of many limestone-derived marbles comes from small amounts of iron oxide admixed. These are especially susceptible to the formation of brown or red films and crusts often called *scialbatura* or oxalate films. Since Roman times, calcium carbonate has also been used as a compound in the production of mortar, stucco and mural paintings. Because the physical and biological mechanisms of decay are practically the same, we shall include carbonate-cemented sandstones in the list of calcareous materials.

Siliceous Materials. Siliceous materials can be largely classified into four groups. The magmatic intrusive class embraces granites and diorites and the very stable porphyries. The magmatic extrusive class embraces a large variety, including basalt, andesite, volcanic tuffs and natural glass (e.g. obsidian). The metamorphic class comprises a large variety of quartzites, gneisses and schists. The sedimentary or exogenic cycle derived class includes siliceous or clay-cemented sandstones and breccia but also sinters and opal, a biologically influenced arid weathering product. The ancient Egyptian architects probably found the most stable rock in the red porphyry of Upper Egypt. Columns and stones made of porphyry where used a great deal in many places. All dried, baked or sintered siliceous products such as adobe, terracotta, bricks, glassed bricks and glasses as well as enamel are included in this group. Interestingly, within this group are the so-called stabilized melts such as glass, brick and glazed brick or clinker.

Mixed Mineral Materials (Mainly Carbonate and Silicate). Astonishingly, mixtures of heated carbonates with quartz and other siliceous materials like the heated siliceous mineral compounds of (Portland) cement turned out to be extremely corrosion resistant. Opus cementicium of the Roman engineers is a well-studied example of producing almost miraculous results of stability of materials used in building and engineering. A mixture of sand and ground mollusc shells turned out to be one of the most corrosion-resistant materials of antique technology.

Metallic Materials

Pure metals and many alloys are widely distributed in buildings and objects of art. None of them are really eternally stable. The reinforcing of wood, stone and concrete by metal in buildings, the roofing by lead and copper, metallic sculptures such as the famous Marc Aurel Quadriga, the bronzes of Riace and the mysterious *never* rusting iron column in India are examples of out-door exposure of metal structures. Many metal sculptures designed for indoor exposure have, however, been subject to long periods of burial in soil or water. Patina, films, crusts and other surface changes of metallic products are partially regarded as an integral part of the artistic value of metallic objects. In some cases, artists give a finish to their products that initiates the formation of patina or can be regarded as patina from the beginning. The formation of a hydrogen layer, oxides, sulfates, carbonates on the surface of metallic objects may also contribute to their stability and longevity. All these compounds, on the other hand, contribute to the decay and loss of the original material in many cases. However, also biofilm formation may contribute to preservation although microbial attack on metallic materials is more frequent and protective action less than with mineral surfaces.

Gold, Silver, Copper, Bronze, Brass, Tin. These metals and alloys are mentioned first because they were first coming in use in a growing technological civilization. This is explained by the fact that the temperatures and techniques of processing were first invented or found and did not face humankind with serioush problems.

Iron and Steel. In order to recover metallic iron from a melt necessitates higher temperatures. Steel, in contrast to alloys such as bronze and brass needs exact supplements of carbon and other compounds such as chromium, nickel and later vanadium. Stainless steels aside more accidental findings as damascene iron are used only since the 20th Century at a larger scale and sophistication of production. The main aim was to yield highly corrosion-resistant materials. Also these, however, may undergo microbial attack by biopitting processes and other decay mechanisms.

Aluminum, Magnesium, Titanium, Platinum. These last metals have received attention as technical materials only since the onset of the 20th Century. They are very important construction materials of extremely high resistance to corrosion in cases or of importance as extremely light weight materials in the construction of airplanes, for example. Most organic or plastic replacements pose higher corrosion and decay risks then the metallic compound.

14.4.2 The Mechanisms of Biological Attack on Inorganic Materials

All inorganic materials undergo transformation and destruction, decay, disintegration and solution, even sublimation or evaporation. In many if not all cases, biological and especially microbial interaction cause acceleration of such phenomena. Thus, the natural stability of a chosen material needs to be always regarded with respect to physical, chemical and especially biological processes interfering with the standard stability data. The environment of the final *resting place* of the technical product is of great importance in aesthetical and in a physical–chemical–biological viewpoint.

Krumbein and coworkers [14.110–112] have tried to define and explain the terms frequently in use in this field. Weathering with its meteorological connotation is a somewhat awkward term not directly implying the important biological interactions in the process of rock and material decay. It should be avoided in material sciences when biological processes are involved. Erosion and abrasion involve the physical attack of solids, liquids or gases on solids with the effect of particles detached and transported. Corrosion implies a chemical transfer with surface changes as a consequence. It is used in context with industrial materials and especially with metals and alloys, to a lesser extent with stone and building materials. Degradation is a term for the combination of weathering, wearing down and erosion; it is also frequently used for the biological breakdown of organic or inorganic compounds, which serve as energy, electron or nutrient source for the organism. Also the formation of new mineral lattices and chemical compounds (rust) may be involved. Abrasion has a strong connotation with mechanical forces and material decay in moving parts of machines and other technical equipment. Deterioration and biodeterioration are terms widely used in material sciences. In the following sections we treat mainly biological actions on materials that have a deteriorative effect in terms of cultural heritage conservation efforts. The physical and chemical transfer actions will be described briefly in order to demonstrate the multitude of possible actions occurring in the ageing and decay of objects of art and technology.

14.4.3 Organisms Acting on Inorganic Materials

The durability of inorganic materials is affected by many biological activities and interactions. Biodeterioration has to be regarded as some kind of irreversible loss of information of objects of art made of mineral materials following the attack by living organisms. In this context we have to consider damages caused by man, animals, plants and microorganisms. The destructive activity of man by war, vandalism and neglect is probably the greatest of all biological effects.

Macroorganisms

All macroorganisms acting on materials are eukaryotic. Among macro organisms, roots or the climbing and adhering parts of leaves and stems of plants create a big problem to conservators because they cause partly evident aesthetic damage, partly an alteration of the stone due to (a) a mechanical action of roots, and fixing parts, (b) chemical action through ionic exchange between roots and alkali or earth alkali captions of the stone constituents, (c) vegetation shadowing slows down water evaporation [14.113].

Birds, and in particular pigeons, provoke a remarkable aesthetical and chemical damage by deposition of guano; guano, moreover can be a good growth medium for chemoorganotrophic microorganisms which, in turn, will determine a corrosive action on stone by release of acid metabolites.

There is a multitude of animals that live on and in mineral mixtures (rocks, mortar, mural paintings etc.). They construct their dwellings, seek hide and food within that environment and hereby contribute to the material change and decay as well. The most important among them are spiders, flies, mosquitoes, stone wasps, ants, mites and beetles in common terms. Insects and arthropods are the groups most frequently involved with rock dwelling and rock decay.

Mosses, lichens and algae are macroscopically evident because they cover the material surfaces with visible films of growth. Consequently, first of all an aesthetic alteration is noticed. In addition, they have a corrosive effect on the substrate by release of acid metabolites, same of which are chelating agents that determine a solubilizing and disintegrative action on the constituents. Surficial microbial mats and films and lichen colonies by their exudates (mainly sugars) act as traps of dust and particles, which in turn supply aggressive compounds and nutrients for all kinds of organisms. Lichens provoke physical as well as chemical damage: acid metabolites solubilize and disintegrate the substrate. One of the most prominent effects of lichen is the formation of pits or crater-shaped holes (Figs. 14.15, 14.16), which are produced in some cases (epilithic lichen) by the algal symbiont, in other cases (mainly endolithic lichen) by the fruiting bodies of the fungal symbiont [14.111, 112]. It is of utmost importance to note that some lichens can act protective, while others on the same rock can act destructive. Therefore cleaning action has to be considered carefully. Many algae form inaesthetic growth films, slime, which upon their degradation yields biocorrosive actions and can actively perforate the rock.

Microorganisms

It is well known that microorganisms are involved in rock and mineral decay in the geophysiological cycle of elements [14.114, 115].

A correlation between stone decay and the presence of microorganisms presents some difficulties because a great number and variety of species of microorganisms is involved. In fact, many autotrophic and heterotrophic bacteria, algae and fungi are found but a biodeteriorating role has been demonstrated only for some of them. In many cases microorganisms are directly associated to a deteriorating activity on stone materials, in others it is possible that products excreted from the cells under stress or upon lysis serve as nutrients for other heterotrophic decaying microorganisms or act directly on the material without a direct correlation to the organisms. According to terminology, among the rock- and material-damaging microorganisms we can notice that microorganisms are defined as organisms barely or not visible with the naked eye. Microorganisms can belong to the animals, the plants, the fungi, the algae and protozoa (protoctists) and to the prokaryotes (bacteria sensu latu).

Photothrophic Microorganisms

Lichens and cyanobacteria as the predominant rock dwellers are usually present in association with green and red algae and diatoms. The dominance of one or other of these groups varies both locally and regionally. Cyanobacteria (blue-green algae) and green or red algae growing on surfaces within such buildings as churches or grottoes are well adapted to survive at very low light levels. Communities' color varies with the color and growth form of the dominant forms. Cyanobacteria are predominant in the tropics and arid areas and there is no doubt that this is due in part to high temper-

atures and/or extremely high or low high air humidity as well as high or low irradiation. Cyanobacteria can endure strong illumination because their accessory pigments protect them and prevent chlorophyll oxidation in intense light [14.116].

Cyanobacteria and algae can form biofilms and crusts on rock and concrete surfaces that are deep or bright green in humid conditions and deep black, when dry. The black coatings of many rock surfaces can be explained this way. Upon extraction in polar solvents often a typical adsorption between 300 nm and 320 nm is observed besides the classical adsorption peaks in the 400 nm and 600 nm ranges. Apart from the evident aesthetic damage on stone monuments there are many evidences of significant physical and chemical deterioration of the surface by excretion of chelating organic acids and sugar-derived carbonic acids, which initiate the perforating activities of cyanobacteria, some of which are called endolithic, when they exhibit strong perforating activity. The algae have already been mentioned under the section of macroorganisms. Many of these eukaryotic phototrophs are, however, microorganisms and can also act perforating especially in connection with fungi or as symbionts of lichens. Some observations have also been made of the occurrence of anoxygenic phototrophic bacteria in decaying rocks and rock crusts.

Chemolithotrophic Bacteria

Sulfur Compound Oxidizers. Among the chemolithotrophic group, firstly the role of sulfur, sulfide and thiosulfate oxidizing bacteria has been clarified [14.117–120].

High numbers of strictly autotrophic *Thiobacillus* sp. have been found not only under surfaces of highly deteriorate stones which presented a pulverizing aspect, but also on deeper layers (10 cm) where there was no stone decay yet [14.121]. Some anaerobic species, like *Desulfovibrio desulfuricans*, are not strictly autotrophic and can sometimes utilize organic compounds as electron donors. They can find sulfate from air pollutants or from soil and produce hydrogen sulfide by the reaction

$$\text{organic acid} + SO_4^- \longrightarrow H_2S + CO_2 \,.$$

This product is a highly corrosive acid and its salts provoke on open air stone surface the formation of black and/or grey films and crusts often described as patina. Sulfate-reducing bacteria increase the decay activity of sulfur and sulfide or thiosulfate-oxidizing bacteria causing a deeper biodeteriorating action. In fact, on soil *Desulfovibrio desulfuricans* reduces sulfates to sulfites, thiosulfates and sulfur. By capillarity these compounds can reach the superficial layers of stones where sulfur-oxidizing bacteria will oxidize them to sulfuric acid. This strong acid reacts with Calcium carbonate to form calcium sulfate (gypsum), which is more soluble in water than calcium carbonate (calcite, aragonite, dolomite).

Fig. 14.15 A tear under the eye of the marble portrait of the poet Keats at the Cemetery near the Cestius Pyramid, Rome is visibly incised by black fungi and related microorganisms

Nitrifying Bacteria. The nitrifying bacteria are commonly found on deteriorated surfaces of rock materials [14.115, 117, 120–125].

Dissolved and particulate ammonia is deposited on rock surfaces with rain and wind from various sources among which agricultural sources (fertilizer, manure) are the most dramatically influential. But also bird excrements and other ammonia and nitrite sources are

Fig. 14.16 Macropitting by lichens in the Namib desert

oxidized microbially by chemolithotrophic and, in part also by heterotrophic ammonia oxidizers and nitrite oxidizers to nitrous and nitric acid.

This transformation is devided into two steps.

1. Oxidation of Ammonium by *Nitrosomonas*, *Nitrosococcus*, *Nitrosovibrio*, *Nitrosospira*

$$NH_4^+ + 1O_2 \longrightarrow 2H^+ + NO_2^- + H_2O;$$

2. Oxidation of Nitrite by *Nitrobacter*, *Nitrococcus*, *Nitrospira*

$$NO_2^- + O_2 \longrightarrow NO_3^- .$$

Both of the resulting acids attack calcium carbonate and other minerals. The CO_2 produced is utilized to form organic compounds while calcium cations form nitrates and nitrites, the latter being more soluble than the original mineral phases. Capillary zones can accumulate these products and hydrated and nonhydrated forms of such salts can create considerable damage by volume changes of the mineral phases. The characteristic symptom of the activity of nitrifying bacteria is a change of stone properties. The rock becomes porous, exfoliation occurs and fine powder may fall off, which sometimes is yellow from freshly formed iron oxides. Some evidence of organotrophic bacteria exerting nitrification has also been collected (E. Bock, private communication).

Iron- and Manganese-Oxidizing Microorganisms

The most common iron-oxidizing microorganisms which are not living exclusively in lakes and flowing water like *Gallionella* or *Siderococci* belong to the groups of fungi, chemoorganotrophic bacteria (*Arthrobacter*) or to the autotrophic group like *Thiobacillus ferrooxidans*, *Ferrobacillus ferrooxidans*, *F. sulfoxidans.* The type *Metallogenium symbioticum* has often been reported also in connection to weathering. *Krumbein* and *Jens* [14.116] have isolated numerous Fe- and Mn-precipitating microorganisms from rock varnishes and some of the isolates were closely resembling to *Metallogenium symbioticum*. *Krumbein* and *Schellnhuber* (personal communication) suggest that the organism does not really exist. The structures are fractal physical biogenic phenomena associated with fungal metabolism and iron and manganese oxide deposition outside the cell walls of the fungi. Iron-oxidizing bacteria attack directly iron rocks as well as any structure made of iron associated with stone monuments. Iron oxidation is usually rapid and is sensitive to pH and oxygen concentration. Ferrous iron is oxidized to ferric iron, which reacts with oxygen to form iron oxide (rust). This latter determines characteristic chromatic alterations on stones. These, however, are often over-emphasized as compared to organic pigments causing the same chromatic alterations [14.126].

Chemoorganothrophic Microorganisms

In recent years a constantly growing number of contributions was made about the impact of chemoorganotrophic microbiota also on the deterioration and biotransfer of materials of inorganic composition with no direct source of organic substrate. *Paine* et al. [14.127] were about the first authors to attract attention to the effects of chemoorganotrophic bacteria in rock deterioration. *Krumbein* [14.128] showed convincingly that the organic pollution within large cities might increase the abundance of chemoorganotrophic bacteria and especially fungi in rocks transferred from rural environments by a factor of 10^4 within one year. This group of microorganisms requires organic compounds as energy and carbon sources. Stone (as an inorganic material) can support chemoorganotrophic processes for several reasons. Four different types of sources of organic materials are usually present in variable concentrations on rock and mineral surfaces (mural paintings especially). These are

1. consolidating and improving products applied to the surface like wax, casein, natural resins, albumin, consolidants, hydrophobic agents,
2. dust and other atmospheric particles (aerosols) and organic vapors. The latter consist mainly of hydrocarbons from aircraft and car fuels or power plants but also of agricultural applications like manure and harvesting dusts or just pollen and other compounds excreted into the air by plants an animals. In industrial areas and cities manifold organic sources stem from industry and craft such as food factories, bakeries etc. Recently it has been calculated, e.g., that the total amount of fossil fuels in terms of organic carbon could derive from the annual production of pollen and etheric oils and volatile etheric substances (smell of flowers) that are carried into the sea.
3. Not less important is the coexistence of photo- and chemolitothrophic microorganisms with chemoorganothrophic ones in biofilms, biological patina and microbial mats encrusting the upper parts of rocks or the whole paint and mortar layer of frescoes. In this context the chemoorganotrophic bacteria and fungi can survive and reproduce themselves eas-

ily because they have all nutrients from autotrophic organisms.

4. Sedimentary rocks usually contain between 0.2% and up to 2% organic matter retained in the rock. These compounds serve as source of energy and carbon for many microorganisms.

Chemoorganotrophic Bacteria. Extremely high numbers of these bacteria have been reported on stones [14.117, 119, 128–132]. *Eckhardt* [14.115] has estimated that the rate of deposition of chemoorganotrophic bacteria from the air onto stone surface is about 10^6 cells/($cm^2 \cdot d$). *Krumbein* (personal communication [14.133]), and *Warscheid* [14.134], however, have reported that airborne bacteria are usually of different genera and species than those settling and dwelling permanently on rocks. Bacteria migrate into porous materials with the flow of groundwater and rain water washings and can reach depths of 160 m in porous rocks. Present-day information confirms, that microorganisms will invariably be detected and active at depths within the Earth's crust, which exhibit temperatures below 110 °C!

Paine et al. [14.127] found *Bacillus* and *Pseudomonas* strains on decaying stones that are also distributed in soil. Detailed investigations of *Warscheid* [14.134], however, demonstrated that the rock flora considerably differs from the soil flora composition. *Vuorinen* et al. [14.135] demonstrate a slow decay of Finnish granite due to cultures of *Pseudomonas aeruginosa*. *Lewis* et al. [14.136] isolated from decaying sandstone *Flavobacterium*, *Bacillus* and *Pseudomonas* strains that showed a severe decay activity in test cultures. *Warscheid* [14.134] isolated high numbers of gram-positive chemoorganotrophic bacteria from both, carbonaceous and quartzitic sandstones. Most of them were able to grow under oligotrophic conditions. They demonstrated that coryneform bacteria are among the main decomposers of rocks [14.134].

The chemoorganotrophic bacteria (and the fungi) will be especially detrimental and persistent on rock surfaces and within the rock pores when they produce adhesion-promoting extracellular polymeric substances such as slimes, fibrils and other means of attachment such as hydrophobic compounds. Fungi especially may form penetration pegs and hyphae, while at the surface black-stained small and resistant microcolonies occur. The true dimension of the attack is usually made visible only by staining (Fig. 14.17) or complicated thin section techniques (Fig. 14.18). Water availability and water activity in addition largely modifies the aggressiveness of chemoorganotrophic microorganisms versus mineral substrates. Mineral-bound water, surface films of nanometer-range on minerals and between the layers of hydrated clays as well as bacterially stored water have to be more considered in environments which are under permanent stress of desiccation.

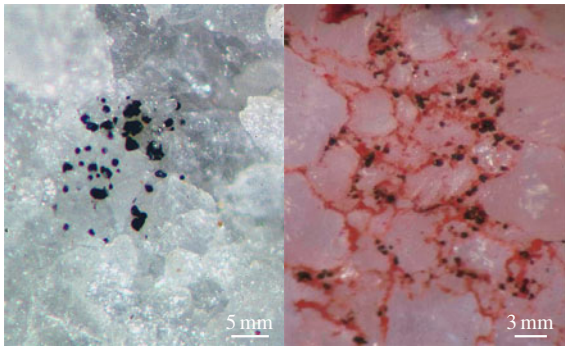


Fig. 14.17 At the surface of marble tiny black colonies and satellite colonies of black yeast are visible to the naked eye. The connecting and nourishing mycelium can only be made visible by PAS-staining for polysaccharides

Chemoorganothrophic bacteria further determine their deteriorating action by metabolic products (acidic, alkaline and gaseous) [14.128,132]. Another mechanism is the production of highly stable pigments. These in turn react with mineral elements causing an aesthetic deterioration and biogenic decay [14.126, 137].

Actinomycetes. These bacteria are thus called, because they similar to fungi form hyphae-like vegetative growth forms and produce spores similar to fungal spores. For this and other reasons their decay mechanism could

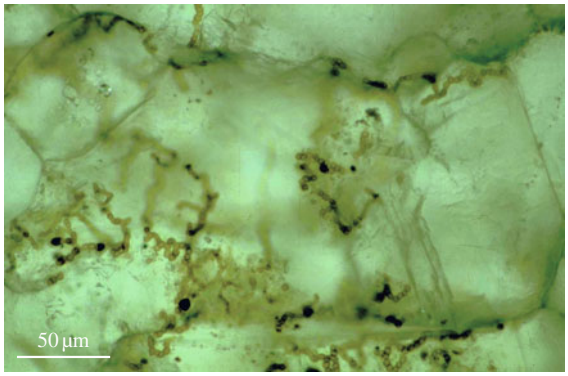


Fig. 14.18 Thin sections of marble can show as well the extent of drilling through grains and fungal penetration along grain boundaries

be the same as of fungi (hyphae penetration and acids release). Among these microorganisms, the genus *Streptomyces* is most frequently occurring in biodeteriorated stone. Penetration of the substrate by their hyphae is increased by their ability to excrete a wide range of enzymes [14.138]. They can form a whitish veil or a granulose patina on mural paintings [14.126]. They also produce water-soluble and insoluble dark pigments. Recently it was documented, however, that they rarely if ever produce noteworthy amounts of organic acids and chelates in a rock-decay environment. They may have less detrimental activities. Their presence in high numbers, however, indicates a very intense population of fungi and other microorganisms. They can perfectly well resist dryness and therefore can be regarded as excellent indicators of a progressed infection of rocks and mural paintings by other microorganisms. Early stages of rock infections usually do not exhibit high numbers of this group of actinomycetes [14.139]. On the other hand the new edition of Bergey's Manual of Systematic Bacteriology suggests placing the Actinobacteria, among which some coryneform bacteria, as already formerly the nocardioform bacteria into the group of actinomycetes or Actinomycetales. The coryneform and nocardioform bacteria, however, have been identified as frequently occurring on rock materials and producing acids from organic pollutants on building stones thus contributing considerably to biocorrosion and biodeterioration [14.134].

Fungi. Fungi are very commonly found on stone surfaces [14.118, 140]. Their deteriorating effect is due to mechanical and chemical action. The first one is related to hyphae penetration of materials that has deep-reaching deteriorating effects such as swelling and deflation as physical effects, channelling water into and keeping it in considerable depths and constant microvibrations through micromotility; the second one is due to production of acid metabolites (oxalic, acetic, citric and other carbonic acids). The latter have a chelating and solubilizing effect on many minerals [14.115, 118, 128, 141].

14.4.4 Biogenic Impact on Rocks

The phenomenological study from geological, chemical and biological groups together with many microbiological analyses of deteriorating rocks in a great number of monuments revealed various forms of deterioration such as pulverization, alveolarization, desquamation, exfoliation, efflorescences, black biofilms, films (patina), crusts and pitting [14.142–144]. Of these, pulverization and black crusts dominate sandstone and quartzitic sandstone while corrosion by biopitting and biofilms was observed more frequently on limestone and marbles. The mutual relationships and effects of biology and rock destruction phenomena as well as different corrosion levels depending on both the coating of lichens and biofilms or microbial mats and crusts were studied in many places. Environmental conditions including pollution and the mutual influence on rock biota have as well been studied. Therefore, now a selection of some characteristic examples can be described and brought in a biological context.

Pulverization

Both sandstone and quartzitic sandstone are mostly characterized by profound pulverization of the rock. This deterioration is evidenced by a reduction in cohesion and adhesion between structural components, with an increase in porosity and a reduction of the original mechanical strength leading to a spontaneous or weather or shock induced detachment of the rock material in powder form. Pulverization as a sandy surface phenomenon is also observed on limestones and marbles. In these rocks it is, however, usually by far less conspicuous than biopitting. Sometimes, this sanding changes into the formation of alveolar erosion, a deterioration of highly porous rock materials resulting in the formation of big and deep cavities. In its advanced state, it may lead to the tafonis characteristic for many desert environments [14.120, 145, 146].

In all these cases less algae, lichens and other conspicuous microorganisms are visible and involved in the biotransfer process. Chemoorganotrophic bacteria, ammonia- and nitrite-oxidizing bacteria (especially in tafonis) and masses of cocci (or coryneform bacteria) and rod-shaped bacteria forming slimes are often observed in such places and can be correlated with this type of (bio-)corrosion.

Exfoliation, Chipping and Desquamation

Exfoliation and desquamation occur in most sandstone types and granites and gneisses analyzed, but may also be observed on limestones and marbles. These corrosions are marked by a lifting, followed by the detachment, of one or more large thinner (exfoliation), small thinner (chipping), or large thicker (desquamation) rock layers.

Throughout the lichenological study of monuments, the level of this type of (bio-)corrosion is positively correlated to the lichen covering, based on observa-

tions that these structures appear more intensive, the lower the degree of lichen covering, of which particularly the lichen *Lecanora conizaeoides* as well as, e.g., *Acarospora fuscata*, *Candelariella vitellina*, *Lecanora polytropa*, *Lecidea fuscoatra*, and *Physcia orbicularis* were observed on siliceous rocks exhibiting chipping and exfoliation. Often, a significant algal layer occurs besides the lichens mentioned.

Desquamation after crust formation is often observed when an endolithic algal mat is forming under high light conditions or surficial extreme desiccation. Also when dense endolithic layers of iron-mobilizing fungi do transform parts of the cement into new and fresh reddish iron incrustations, large desquamations may occur as it was especially frequently observed in the case of Schlaitdorfer sandstone at the *Kölner Dom* (Cologne cathedral). The same phenomena have also been observed in desert environments.

Patinas and Varnishes; Crusts and Incrustations

More or less dark-colored films, rock varnishes or patinas (often called scialbatura in the Italian and art history literature) as well as thinner or thicker yellow, reddish, brown and black epilithic and endolithic crusts are very typical for many biologically attacked and degrading rocks and tombstones. We are treating these phenomena preliminary in one section because the many different phenotypes have not yet been associated with appropriate chemical, physical and biogenic chemical and physical transfers of the original rock and mineral material.

In pollution-stressed environments sandstones can be covered by a black crust, a product of the transformation of the surface of the material including dust and soot. Its chemical and mineralogical nature and physical characteristics are partly or completely different from those of the substrate material from which it may become separated sometimes by exfoliation in other cases even by pulverization underneath the black film or crust. The crust is composed of different hydrocarbons and alcohols as a result of air pollution agents and biogenic residues, especially EPS (extracellular polymeric compounds). The latter serve like flypaper as a trap and agglutinant for foundry or other ashes and dust particles when wet [14.128]. In the dry state they appear to have extremely high viscosity and serve as a migration and diffusion barrier to chemical compounds before completely rewetted. Even in the wet and swollen stage the EPS drastically reduce the diffusivity of gases and solutes into and out of the rock [14.120, 128, 134]. Gypsum as a biocorrosion product is frequently observed in these black crusts particularly in carbonate cemented sandstones, limestone and marble, but even in pure siliceous sandstones and granites. Natural basaltic rocks have also been analyzed and underneath crustose lichens biotransformation products of earth alkali containing feldspars have lead to white gypsum layers between the black basalts and the lichen crusts. The normal approach is, that acid rain especially near industrial centers and in big cities promotes transformation of limestone into gypsum crusts. This, however, is not true. Like in the case of the highly discussed damage to forests by acid rain, acid rain does not really damage building stones. In both cases other reasons are predominant: In the case of mineral transformations this is witnessed and evidenced by gypsum formation in remote desert regions, where no acid rain is documented. The view of microbiologists today is that microbial transformations of sulfur are the most active agent of transformation of calcium carbonate into calcium sulfate.

It is almost premature to analyze the biological impact on the various types of films, crusts, and patinas. Therefore, a few focal points shall be given to initiate discussion and further studies.

1. *Patinas and Varnishes (Scialbatura).* Yellow, red, brown, and black varnishes and surface films on many rock types are usually the product of repeated colonization by thin epilithic crustose lichens and/or fungal films on and beneath the surface of relatively dense rock materials such as the extremely low porosity marbles and quartzitic sandstone or silicified rock. A typical product of the biotransfer of mineral matter within these films (patina, scialbatura, rock varnish) is (1) the indicative high enrichment of manganese(IV) versus iron(III) with respect to rock derived and dust derived manganese/iron ratios (2) the often observed ring-type distribution of such films, (3) the presence of calcium oxalates (weddellite and whewellite as well as iron and manganese oxalates such as humboldtine). A recent survey on biogenic pigmentations was given by *Krumbein* [14.126].
2. *Crusts and Incrustations.* Crusts and incrustations are often brought about by the same biotransferring microbiota namely biofilms and microbial mats of algae, cyanobacteria, fungi, lichenized fungi, and often also endolithic lichens with characteristic fruiting bodies. Epilithic biofilms and microbial mats on hard substrates will form *crusts* and crustose outwards-directed growth zones. Epi-endolithic biofilms and microbial mats will form more or less deep incrustations underneath the original and trans-

ferred rock surface. In these cases the degree of light penetration, humidity regimes and other biotic factors as well as the porosity of the usually more porous rocks will regulate the thickness of the incrustation and also the question whether the physical decay phenomena will lead to exfoliation, desquamation or other effects. *Krumbein* [14.145] has given a schematic presentation of the biofilm-derived entrapping and outgrowing crusts, which will lead to an addition above the original rock surface under desquamation or exfoliation of the outwards growing crusts together with some of the rock material underneath in cases.

Incrustations and the fate of the rock material in the case of incrustation by biofilms, slimes and microbial mats forming an endolithic dense network have been first described by *Krumbein* [14.145] and schematically elaborated in a morphological and geophysiological sense by *Krumbein* [14.147]. They occur especially in Mediterranean and arid zones. In some cases also endolithic lichens have been observed to form incrustations. The physical rock development after a hardening and solidifying period is often very thick desquamation of surface parallel layers of the whole building stone or natural rock surface to a depth of up to 1 cm. After desquamation a new crust may develop in turn.

Concretioning and other types of hardening of the original rock substrate may often occur during the biotransfer process especially in crusts and incrustations. Usually ultimately the rock in question will, however, suffer more serious damage than in the case of less hardened rocks.

Colorations

A phenomenon of rock alteration (physical, chemical and biogenic physical and chemical change) as well as deterioration (worsening of the original rock material also in an aesthetical sense), closely related in most cases, however not in all, with patina, rock varnish, crust and incrustation is a general color change or coloration occurring with building stones and monuments. This is especially unwanted in the case of marble statues and sculptures in general. The surficial films and crusts may have yellow to black colors in special but also all other colors of the rainbow due to the following mostly biological or biologically catalyzed effects and products.

1. *Pigmented salts of iron and manganese.* Iron and manganese are the two most frequently occurring metals in rocks. Many of their hydroxides, oxides, sulfates, phosphates and oxalates are initiated by chemical reactions with the atmospheric environment and polluting agents but also very considerably by oxido-reduction reactions catalyzed by microbiota. The solution, complexation and precipitation of, e.g., calcium, iron and manganese leads to multicolored light-yellow to black staining on rock surfaces and within rock incrustations which may largely contribute to pigmentation and pigmentation changes in rocks [14.116].
2. *Organogenic pigmentations.* Many organogenic pigments do occur in nature and thus also in microbially influenced rocks. Naturally the mineral content of rocks and the different substratum does influence the coloring considerably. The dark-green to pitch shining black pigmentation of rocks is usually due to chlorophylls of cyanobacteria and algae growing on rock surfaces. When dry, all chlorophylls appear deep and shining black or lead-grey depending on substrate, concentration of chlorophyll per surface area and degree of wetness. Only in very rare completely wet conditions (growth period, not to be confused with the annual seasonality of, e.g., plankton blooms) the crusts will appear olive, bright, or dark green. Often one observes also brownish, grey and even black pigmentations of the biogenic crusts and biofilms when actinomycetes and some melanin-producing fungi are predominant. Protective pigments of the carotene type and other pigments may be produced and embedded into the EPS of algae and cyanobacteria as a shield against UV-light and further modify the pigmentation and coloration of rock films and crusts. Yellow to deep-brown and black stains may occur in very rare cases in which microbial nitric acid production leads to yellow to brown xanthoprotein reactions; perhaps even the caramelization effect of sugars and other organic compounds may be produced by especially (biogenic?) sulfuric acid attack. This *caramelization* or *coalification* through concentrated acids has, however, never been proved on rocks. The Maillard reaction, however, which is so well known in the cooking environment (brown sauces) may play an important role also in the natural rock decay environment [14.126]. Lastly, the possibility exists that – upon the death of any microorganism – enzymes are excreted from the protected cell interior, which may react and form melanin in the environment of decaying cell masses. These processes, however, need enormous amounts of cells and proteins. It is thus probably rarely

observed in comparison to other above-mentioned active staining processes, among which the tremendous potential of the black yeast-like fungi needs to be mentioned. *Gorbushina* et al. [14.148] gave an excellent insight into this exceptionally modern topic. The topic is modern in as much as it explains color changes in a new and logical way. The essence of this line of research is, that organic pigments attached or adsorbed to rock and soil particles may explain the color of soils and rocks in a much simpler way than any inorganic chemical pathway involving iron and manganese transformations.

Some of the aesthetically most detrimental and yet not fully understood colorations of marble, alabaster and other white rock sculptures and ornamental elements of buildings are pink, orange, cadmium and even carmine to violet red stains in many marble sculptures and mosaics. These stains have also been observed in some marbles of the Acropolis, Athens.

3. *Colored dust trapping and binding.* Certainly dust particles entrapped and agglutinated mainly by the EPS of biofilms and rock-covering microbial mats in their active growth stages or after rewetting will be other major factors of coloration and pigmentation (flypaper effect). In the northern hemisphere it is usually the black of coal soot and flight ashes. In special cases flight ashes will also produce red colors, when originating from iron foundries and cokeries of lignite (brown or low-vitrinized coal). In other regions red stains are derived from hematite desert dust, which is carried through the stratosphere to far away regions. This was observed many times by Ehrenberg in the 19th Century.
4. *Special heavy metal stains.* Special colorations (e.g., of surface films and crusts on metal containing monuments) may be derived from the (bio-)solubilization oxido-reduction and redeposition of the salts and minerals of copper, manganese, cobalt, silver, sulfur, and other elements. The most typical colors occurring on cemeteries are black and grey through lead letters, green and blue through copper alloys, red through iron and other specific stains usually biologically mediated [14.116].

Efflorescences and *Salt Nests and Carpets*

Incrustations usually are produced by a large amount of different microorganisms and their metabolic products as well as by the water supply and modification of the pore space by biological and chemical activities. In the process of this some salts are produced and remain stable for considerable time that are quite soluble alkali and earth alkali salts and double salts with often astonishing capacities of volume changes with temperature and humidity regimes. Up to 300% volume changes have been reported for such salts [14.128]. These salts frequently are the product of the rock-inhabiting microflora [14.128]. Often, the black surface crust is thus accompanied by efflorescences of salts. These crystalline formations of soluble salts on the surface of the object are whitish in color and incoherent. They may, however, be colored by the admixture of traces of reduced and oxidized iron as already suggested in [14.120, 128, 149]. Besides the typical crystals of gypsum, chlorides and nitrates and double sulfates have been analyzed by x-ray diffraction and EDX-studies. Oxalates (whewellite, weddellite and some rarer minerals such as the iron oxalate humboldtine, the magnesium oxalate glushinskite and the yet unnamed oxalates of manganese and copper or oxammite, ammonium oxalate) have been identified as microbial mineral transformation products in crusts and efflorescences [14.147, 150]. Depending on both, the rock material and the environmental conditions, the specific composition of these salts cannot be generalized. The efflorescences may be induced below the crust, which becomes then more and more separated from the substrate material. Many of the soluble salts may come and go with the waterfronts passing through the rock. It has been suggested that the mineralization is independent from the biological activities. This is, however not true. In and underneath lichenic crusts and in and underneath biofilms halophilic and highly halotolerant bacteria were found. In their vicinity new minerals have been shown to occur, such as the phosphates apatite, struvite, dahllite, lazulite, wavellite, and even turquois or novaculite. Sometimes, the biogenic mineral formations only ephemerally play a destructive role and are later only identified by empty places of a specific mineral form near bacterial aggregates. The cleavaging and fissuring effect and the strongest efflorescences are usually limited to the lower part of the tombstones, as a result of interactions with rising water from the subsoil that also contributes nutrients and rare energy sources such as hydrogen sulfide, methane and other compounds. Special salts like jarosite have been shown in many other places to be biogenic efflorescences and recently we have found much more salt efflorescence in highly saline mural paintings. In order to complete or almost complete the list of biologically influenced efflorescence minerals the sulfates, nitrates

and even chlorides shall be mentioned that are a consequence of mainly chemolithoautotrophic bacteria but also of some chemoorganotrophs as is the case with oxalates and phosphates. Highly detrimental and often observed together with bacteria are the mixed (double-) salts of sodium, potassium, calcium and magnesium as glauberite, astrakanite, mirabilite, but also nitronatrite and nitrokalite. In special cases aluminum and silica salts and even new clay mineral formations have been reported but not yet solidly evidenced [14.115].

Biopitting

The term pitting as a synonym for smaller or larger crater-shaped cavities forming in many decaying rocks is derived from *coal and iron pit mining* of the primitive technological societies. The definition according to geological nomenclature is: *small indentation or depression left on a rock surface as a result of some corrosive or eroding process such as etching or differential solution*. We have identified and defined biopitting as the sole source of these crater-shaped cavities in many places, publications leading back to the book of Moses (Levithicus). *Krumbein* [14.145]; *Krumbein* and *Jens* [14.116], *Gehrmann* et al. [14.111, 112] and other scientists have now fully elaborated the micro-, meso- and macropitting as being caused mainly by endolithic and epilithic lichens. *Danin* et al. have substantially developed some of the work on biopitting [14.151]. On limestone and marbles biopitting, chipping, cracking and fissuring are frequently seen of which the biocorrosion by biopitting dominates. In all cases of studied biopitting the latter was correlated positively with epilithic or endolithic lichen species. This phenomenon of biocorrosive crater erosion was first introduced into literature by *Krumbein* [14.145]. *Gehrmann* et al. [14.111, 112] have classified these characteristic holes and cavities into three size groups namely (1) micro-, (2) meso- and (3) macro-pitting of the rock surface. Of these, both, micropits and mesopits were identified, upon maceration, as produced by the activities of the calcicole lichen *Caloplaca flavescens*. The specific pitting pattern indicated by the penetration of bundles of hyphae (mesopits) as well as by individual hyphae (micropits) is clearly to be seen on the rock surface. When the pits fuse they may cause kinds of alveolarization but of a significantly different morphology [14.145]. In view of the general importance of this biogenic process the present classification is given here

1. *Micropitting*. In this case etching figures are observed which correspond very much to the pencil etching described by mineralogists. They are caused by individual cells and trichomes or mycelia of fungi and are only visible by scanning electron microscopy. The diameter of these micropits is between 0.5 and 20 μm. The depths can reach several micrometers and even hundreds of micrometers in special cases.
2. *Mesopitting*. In this case we are dealing with the etching figures of the fruiting bodies of endolithic lichens, in some cases also with the *nap-shaped* grooves of cyanobacteria and/or algae underneath a crustose lichen film. They form little pockets which, upon further biocorrosion or chemical weathering have the shapes of half-ellipsoids or lens type cavities. Also fungal hyphae can be associated with this pitting type. The diameter of the craters is usually between 20–800 μm.
3. *Macropitting*. These are the typical pits and scars clearly visible on many statues and marble monuments all around the Mediterranean but also on a smaller scale in Potsdam and northern areas. A schematic model of pit formation has already been published [14.147]. In this case we are dealing with the not yet sufficiently analyzed *fusion* of several pits in which fractal physical patterns may also play an important role. Some of the macropits are also derived from deeply incising epilithic lichens of ill-defined taxonomy. The macropits usually range in diameter from 1 mm to maximally 2 cm and depths between 1 and 5 mm.

The processes of mesopitting and macropitting produce characteristic depressions, which are ovoid or circular at the rock surface, the diameter of which is usually at least double the size of the depth. The process of biopitting is well separated from the well-known, but very badly understood process of alveolization of many limestones and sandstones. Water potential and availability as well as microchemical gradients play an important role in these interesting formations observed also in many Greek temples (Akrokorinth). Alveoli usually reach sizes of 1 to several cm in diameter and are almost always deeper than their diameter.

Pitting or better biopitting thus is an exclusively biological process related to endolithic and epilithic lichens in almost all cases with a few exceptions of lichenized fungi without a defined lichen thallus and fructification.

Swelling

Another deterioration phenomenon, formerly assumed as abiogenic was called *swelling*. Swelling leads to

a gradual physical relaxation of the mineral context within the complex aggregate called a rock or a building stone. It has to be kept in mind that physically, chemically and mineralogically a rock is not something in which particles or minerals are glued together by a sort of glue or cement. On the contrary: rocks are aggregates of smaller or larger individual mineral or rock particles of which some, because they are younger or smaller are conventionally called *cementing minerals* that by physical and chemical history are intimately mixed and entangled into a more or less solid, more or less hard, more or less age and decay resistant, more or less porous aggregate. The contacts between the smaller or larger particles follow all the same physical rules of electrostatic, van der Waal or other adhesion and *stickiness* promoting forces including very thin-layered water molecule, organic molecule and gas molecule films. The best example of such a noncemented aggregate perhaps is marble with its relatively uniform calcium carbonate crystals, its micro- and macroporosity and its sudden structural disintegration. It has to be kept in mind, however, that all other rock types, albeit more complex are nothing else but densely packed particles. The weakening of the adhesive forces can be brought about by mechanical causes (less load after quarrying, earthquakes, vibrations, even storm shock waves), chemical causes (gases, liquids, redox-processes) and especially by biogenic forces and products as described before. Swelling (namely of EPS) is a very typical biogenic physical rock transfer action that contributes largely to pulverization, chipping, exfoliation and even to pitting.

A lichen crust that is often bending out before it breaks away from the surface always has some rock particles adhering. Such patterns of biogenic initial stages of desquamation is connected with epilithic crustose lichens, especially *Acarospora fuscata*, *Candelariella vitellina*, *Lecanora grumosa*, *Lecanora polytropa*, *Lecanora rupicola*, *Lecanora sulfurea* and *Lecidea fuscoatra*. Removing the lichen crust, an advanced state of incoherence of the rock surface layers is recognizable, characterized by an increase in porosity and an apparent decline in the original mechanical strength. Moreover, longitudinal sections of such lichen-encrusted sandstones reveals an extensive compact network of hyphae penetration up to 3 mm inside the rock. Often and even in the absence of lichen hyphae a very dense, slimy layer of EPS is observed in contact with the mineral grains and the uppermost layers of an endolithic or epilithic rock film or microbial mat. These slimes or EPS, among which a high percentage of polysaccharides and their acids have a phenomenal capacity of swelling with water. Many bacterial EPS (often also designated as alginates) and lower and higher eukaryote water retention systems as, e.g., *Laminaria* spp. and *Sphagnum* spp. may swell with sufficient water supply to 1600% of the dry weight and can reach more than tenfold volumina easily. These swelling and contraction stresses are some of the most dramatic biogenic physical transfers in the disintegration of aggregates such as rock, plaster, mural paintings and other materials. EPS, cellular and hyphae swelling and water retention and transport capacities are usually more damaging even as the volume changes in the alkali and earth alkali salts mentioned in the section on internal and external efflorescences.

14.4.5 Biogenic Impact on Metals, Glass, Pigments

Interaction of Microorganisms with Metals

Microbial degradation of metals is associated with a wide range of biochemical processes, including acid metabolite production and galvanic coupling. Chemolitho- and chemoorganotrophic microorganisms exert an active corrosive action on metals by production of inorganic and organic acids. *Cladosporium resinae* causes corrosion of aluminum alloys by the secretion of citric, isocitric, *cis*-aconitic, and a-oxoglutaric acids, resulting in pitting and selective removal of zinc, magnesium and aluminum, leaving copper and iron aggregates behind. This suggests that microbial acid corrosion plays an important role in pitting corrosion [14.152].

In recent years some bronze monuments have been exhibiting serious corrosion damage in the form of *patina*. It starts as a reddish-brown oxide skin to a black coating, which intermingles with green patina underneath. In this phenomenon also bacteria are involved. In fact, organic matter reacts with mineral particles (from metals in the bronze and/or deposited by the environment) and the consequence is corrosion processes. The loss of material beneath a patina surface is *just* some microns each 100 years or so, but it is these microns that represent a large part of a bronze's artistic qualities.

Bacterial extracellular polymeric substances (EPS) are a crucial factor to microbial corrosion. They strongly bind the metals, with a wide range of variations that influence its adhesion to metal surfaces and result in preferential oxidation of particular metal species [14.153]. The primary mechanism of bacterial corrosion of metal surfaces involves the creation,

within an adherent biofilm, of local physicochemical *corrosion cells*. The practical consequence of this perception is that bacteria must be in sustained contact with a metal surface, in well-organized microbial communities before the corrosion process is initiated. Bacterial corrosion seems to occur only within the biofilm! [14.154]. It often shows the same biopitting characteristics as on marble and glass, i. e., small crater-shaped holes and pits going several hundred micrometers deep through patina and material.

Interaction of Microorganisms with Glass

Biocorrosion of glass was observed on optical glass corroded in tropical climates and on church windows and other some objects [14.147, 155, 156]. Even if some authors [14.157] suggest that biodeterioration is a minor and negligible process, others had demonstrated the important role played by lichens [14.158, 159] by fungi and other microorganisms [14.160, 161]. Krumbein et al. stated that microbial growth can occur on clean glass in the presence of sufficient humidity. In addition of the supply of hydrating forces, bacteria and fungi act as physical and chemical agents. They can also metabolize, leach, accumulate and redeposit elements like K, Ca, Mg, Fe, Mn, Ag, P. On dirty glass, water supply and pollutants deposited on it act as growth-supporting substrate. As a result of the establishment of microbial communities often pits are formed and/or other etching figures that can be clearly related to microbial activities.

Interaction of Microorganisms with Pigments

Mineral, plant and animal pigments can be more or less susceptible to light (especially plant pigments), but the most important factor of their susceptability to degradation is the addition of organic substances like albumen, casein, wax, arabic gum, etc., which are an optimal growth substrate for microorganisms. Some of them provoke chromatic alterations by release of acid or alkaline products (e.g., turning to blue of malachite green). Many fungi and bacteria can cause pigment alterations and addition of detrimental and disturbing fluorescent pigments that are added to the pigments in wall paintings and thus alterating the total appearance of the mural painting.

14.4.6 Control and Prevention of Biodeterioration

The main effort in the study of biodeterioration of works of art and the interaction of macro- and microorganisms with the materials of which the objects exist is to understand the deteriorating activity and as a consequence to develop specific and specialized methods for controlling the growth of organisms responsible for and hereby preventing the biodeterioration of these valuable materials for as long as possible.

The choice of methods is related to (1) the nature of the material (stone, wood, paintings, paper, glass, etc.), (2) its location (archeological area, museum, church, library, etc.), (3) the extension and form of treated surfaces (little objects, statues, buildings, walls), (4) the processes physical/chemical and organisms involved (macro- or microorganisms). In fact, no method exists that is an overall remedy for all materials, objects and organisms. Impregnations with consolidants and water-repellent substances have been frequently proposed and used and the interactions of these compounds with microbiota were discussed [14.128, 162, 163]. The substances that have been tested and used range from silanes and siloxanes over acrylic resins and polyurethanes to epoxy materials, vinyl polymers, inorganic materials to natural oils, waxes and other substances. Very dangerous and perilous substances seem to be fluorosilicon compounds and several hydroxides that have been frequently used as well as water glass in the past centuries and in the early decades of this century.

In the context of this mainly microbiological review, however, biological methods of treatment and the microbial interaction with chemical consolidants is far more of interest. In the control of biodeteriogenic organisms growing on and in buildings or monuments, as well as other objects of art, the first step should be to eliminate the most determining factors favoring or accelerating biodeterioration: light, temperature, relative humidity (RH), nutritive factors, dust, dirt, etc. This is easier in indoor environments while it is not always possible in outdoor environments, where however, the use of particular devices like environmental recovery and protective measures can keep these parameters in a range of acceptability. Prevention of biodeterioration of indoor objects is largely determined by the conditions of the environment where the object is kept (exhibition or storage). Low RH, conditioning systems and periodic cleaning can control growth of microrganisms. *Valentin* et al. [14.164] found that the combination of low RH and low oxygen levels significantly decreases microbial activity on solid support. Previously *Curri* (pers. comm.) suggested keeping valuable sculptures made of marble under an inert atmosphere of nitrogen. Nitrogen, however, may not prevent totally microbial growth in humid conditions. Thus the preven-

tion of humidity must be added. These latter methods, however, are noninvasive, safe and relatively inexpensive and could be an important alternative in the field of conservation of art materials in museums.

As stated by the relevant Italian Committee (Normal B doc. 33/88) presently mechanical, physical, biological and chemical treatment methods are in use. These techniques have been published by *Pochon* and *Tardieux* [14.165] and are mostly still in use. Molecular techniques are amply described in *Saiz-Jimenez* [14.163]. Detail microscopy can be applied following classical direct and replica techniques. Recently we have applied also atomic force microscopy (AFM) which enables quantitative approaches to the volume of material loss by, e.g., biopitting.

The choice of methods should be taken with care because such methods affect frequently not only the organism causing deterioration but also the work of art itself or the stabilizing and consolidant additives.

- *Mechanical methods* consist in the manual removal of biological structures. This is actually what Hooke did with his valuable book infected by the imperfect fungus revealed for the first time to the eye of a microscopist. These methods, however, have not a great efficiency because they do not totally eliminate the organisms present. They have a useful function, however, in the first step of cleaning operations when it is useful to reduce the biomass and then to continue with other treatments.
- *Physical methods* have a narrow range of applications and have been used to control the growth of algae and cyanobacteria in indoor plaster and walls (MUVU) [14.166] and as deterrent for pigeons (low electricity impulses). Gamma irradiation is used to sterilize library materials and was tested at Sans Soucis in the last Century.
- *Biological methods* utilize either specific nutritive requirements of organisms or biological antagonisms of different species to eliminate undesirable growth [14.167]. Lal Gauri and coworkers have proposed a biotechnological treatment of sulfated marble (black crust) using a broth containing a mixed culture of an oxidizing bacterial biofilm producer and an anaerobic bacterium *Desulfovibrio desulfuricans* that transforms gypsum into calcite. These methods are subject to criticism of their control and incomplete removal after treatment.
- *Chemical methods* are the most frequently employed. They consist in the application or fumigation in the environment by chemical compounds with biocidal activity. The choice of biocides to be used during conservation and restoration of works of art is not easy. It involves factors like
  - specific efficiency towards the biodeteriorative agents
  - toxicity towards the person applying the technique
  - damage potential towards the work of art
  - relative ease of application and availability.

  Before applying this kind of products, e.g., on stone in objects of art they must firstly fulfil some prerequisites
  a) efficacy – it is influenced by: (1) dose expressed as amount of product/surface unit; (2) action spectrum (range of sensibility of microorganisms involved); (3) persistence of the active principle,
  b) toxicological characteristics and pollution potential – it should be considered from the view of operators care and environmental risks,
  c) noxious interference with the substrate,
  d) outdoor environments (archeological area, buildings, statues of different material).

Mineral Materials

The microbial attack and thus the necessity of treatment of rock and mineral material is usually restricted to outdoor objects, since indoor objects rarely exhibit serious microbial decay phenomena. We have, however, several times been asked to analyze serious fungal infections of statues which are stored in humid conditions in magazines and store rooms and have persistant fungal infections. Detailed recommendations on use and application of biocides on stones are described in the Document Normal 33/88 (CNR-ICR, 1988). It is stressed, as a partly general criterion, that biocides in order to avoid the interference with substrate must have no coloration, no chemical or physical reactivity capable to modify the characteristics of stone. Biocides, also, must be washed (pulled off) after their time of action. In this way risks of interference between residues of the applied products and stones are reduced to a minimum. Also the danger of damage to the personnel and visitors is reduced this way. Some indication on modern approaches to polyphasic biocidal treatments can be viewed on the Website of BIOGEMA under EU projects (http://www.biogema.de).

Tests with biocides on different kinds of stones and other mineral materials (*Groß* and *Krumbein*, personal communication) have put in evidence, that the efficiency of a biocide of a given concentration in direct

contact with the material to be treated can largely vary with lithotypes (e.g., effectless when applied to limestone, while considerable effect is stated when applied to the same flora on sandstone).

14.5 Coatings and Coating Materials

14.5.1 Susceptibility of Coated Surfaces to Fungal and Algal Growth

It is widely recognized that surface coatings can be susceptible to contamination and spoilage by microbial growth during service [14.168]. Examples of growth of fungi and algae on exterior facades are common (Fig. 14.19) and strategies to limit their growth are widely accepted in the industry. In general, these strategies rely on the inclusion of an antimicrobial agent [14.169] into the formulation and its presence inhibits the growth of microorganisms when the coating is used in areas where microbial growth is known to occur. In exterior conditions, growth is often an aesthetic problem although as it progresses, physical damage to the coating can occur. However, in interior situations, growth of certain fungi on surfaces has been implicated in human respiratory disorders [14.170]. In these circumstances limiting fungal growth on coatings has an impact on human health. Coatings for submerged surfaces such as the hulls of ships also suffer from problems associated with the growth of microorganisms (principally algae) along with colonization by species of molluscs and crustaceans.

While behavior under normal conditions of use will always be regarded as the definitive test for performance, a number of laboratory tests have been developed to provide a more reproducible and rapid means of gathering data. These tests can be combined with weathering studies ranging from simple leaching in water, through the use of weathering cabinets, to the prior exposure of coated surfaces under field conditions as is employed in studies on the efficacy of treatments on timber against blue-stain in service (Sect. 14.2).

Aside from field exposure, there are two major approaches that are employed to examine the susceptibility of surface coatings to fungal and algal growth. In the first, an artificial substrate (such as a glass fiber or paper filter) is coated and then placed onto a semi-solid microbiological growth medium in a petri dish. The coating and, in some cases, the medium is then inoculated with either single strains of test species or a combination of such strains (Fig. 14.20). This provides a highly accelerated mechanism of testing although it can be argued that the presence of a growth medium, which may diffuse into the coating, can result in the growth of species that are not true biodeteriogens of the coatings system under test. This is especially true when testing for activity against fungi and in some instances a nonnutrient medium (or at least one lacking a source of carbon) is employed such that it simply provides a source of mois-

Fig. 14.19 Growth of algae on a painted facade

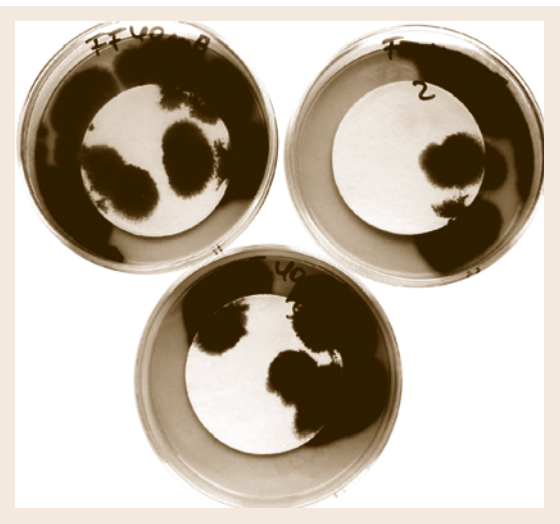


Fig. 14.20 Growth of fungi on a coated filter paper (EN15457)

ture and, occasionally, certain essential trace elements. Of course when testing algae a source of light is essential and this is usually defined in the method and in some cases reproduces a normal diurnal cycle. The major test protocols employed for surface coatings are given in Table 14.12 below, although in some instances, where particular specifications are being defined (such as powder-coated materials for military applications) methods such as ISO 846 and BS2011 Part 2J are employed. Until relatively recently many workers have based their tests on the conditions described in ASTM D5590-00. However, especially within the EU, the guidelines provided by VdL have formed the basis of so-called *filter paper tests* for many laboratories and companies. These guidelines have also formed the basis for two EN norms (EN15457 and EN15458) intended for use in providing data on the suitability of biocidal products to protect paint films in support of requirements of the Biocidal Products Directive [14.171]. While these *filter paper tests* can provide data on compatibility of biocidal products with coating formulations and may even be correlated with performance under field conditions in some circumstances, many coating companies and test institutes regard them as useful screening tests and prefer cabinet-based simulation tests. Probably the most widely employed cabinet-based tests are BS3900 Part G6 (in Europe), ASTM D3273-00 (in the USA) for fungi and the IBRG algal test (Table 14.12).

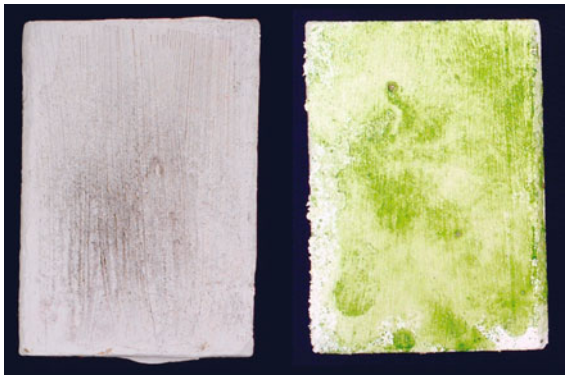


Fig. 14.21 Fungal and algal growth on panels from a cabinet test (BS3900 Part G 6 and IBRG Algal Test)

In all cases the same basic principle is applied where a combination of either fungal spores or algal cells are applied to the surface of replicate test panels. These panels are then incubated under conditions that are suitable for the growth of the species under test. After the specified interval, the panels are inspected for growth and rated and, probably most importantly, their appearance is recorded photographically (Fig. 14.21). As discussed earlier, coatings can be subjected to a number of ageing processes prior to inoculation/incubation to simulate a service cycle (e.g., paint might be aged in the can prior to application, be subjected to exposure

Table 14.12 Methods used to examine the resistance of surface coatings to fungal and algal growth

Reference	Title	Description	Major principle
BS3900 Part G6	Assessment of resistance to fungal growth	Replicate test panels coated with the test coating are inoculate with a suspension of spores of fungi known to grow on the surface of paints and related materials. The samples are then incubated under conditions suitable to support fungal growth ($23 \pm 2\,^{\circ}C$ and high humidity/surface condensation). In the published standard, condensation on the test panels is achieved by increasing the temperature in a water bath below the samples for short periods of time. Revisions are in progress which may obviate this step. The method is validated by the need for fungal growth/germination of spores to be observed on a standard coatings known to be susceptible to fungal growth after incubation for 2 weeks. After incubation growth is rated in accordance with a scale related to the percent cover with fungal growth (following visual and microscopical examination). A natural and artificial soiling agent are described in the method which can be employed when appropriate	Growth cabinet based test
ASTM D3273-00	Standard test method for resistance to growth of mold on the surface of interior coatings in an environmental chamber	Replicate test panels coated with the test coating are inoculate with a suspension of spores of fungi known to grow on the surface of paints and related materials. The samples are then incubated under conditions suitable to support fungal growth	Growth cabinet based test
ASTM WK4201	Standard test method for resistance to mold growth on building products in an environmental chamber	Replicate test panels coated with the test coating are inoculate with a suspension of spores of fungi known to grow on the surface of paints and related materials. The samples are then incubated under conditions suitable to support fungal growth	Growth cabinet based test

Table 14.12 (continued)

Reference	Title	Description	Major principle
ASTM D5590-2000 (2005)	Standard test method for determining the resistance of paint films and related coatings to fungal defacement by accelerated four-week agar plate assay		Agar plate test
SS345 Appendix B	Formal title missing at present	The bottom of glass petri dishes are coated with paint. After drying a culture of algae in a suitable growth liquid medium is placed into the dish and incubated under conditions suitable for algal growth	Liquid immersion test
EN15457	Paints and varnishes – Laboratory method for testing the efficacy of film preservatives in a coating against fungi	Coatings are applied to glass fiber discs and then placed in intimate contact with the surface of nutrient agar plates. The coatings and surrounding media are then inoculated with a mixed suspension of spores of 4 fungal species selected from a list of 10. The plates are then incubated at 24 °C for *X* d and then assessed for growth using a rating scale. The test is intended to support claims that a biocide can have an effect in a surface coating in support of its listing in the relevant use category within the EU BPD. It is not intended to assess the performance of surface coatings	Zone diffusion assay/agar plate test
AS 1157.10 – 1999	Australian standard – Methods of testing materials for resistance to fungal growth Part 10: Resistance of dried or cured adhesives to fungal growth	Test materials coated onto glass microscope slides are inoculated with a suspension of spores of a range of fungal species and then incubated on the surface of a mineral salts based agar for 14 d and then assessed for growth	Agar plate test
EN 15458	Paints and varnishes – Laboratory method for testing the efficacy of film preservatives in a coating against algae	Coatings are applied to glass fiber discs and then placed in intimate contact with the surface of nutrient agar plates. The coatings and surrounding media are then inoculated with a mixed suspension of 3 algal species selected from a list of 5. The plates are then incubated at 23 °C under illumination (16 h day length, 1000 lx) for *X* d and then assessed for growth using a rating scale. The test is intended to support claims that a biocide can have an effect in a surface coating in support of its listing in the relevant use category within the EU BPD. It is not intended to assess the performance of surface coatings	Zone diffusion assay/agar plate test
VdL RL06	Guideline to evaluate the resistance of coating materials against mould growth	Coatings are applied to paper discs and then placed in intimate contact with the surface of nutrient agar plates. The coatings and surrounding media are then inoculated with a mixed suspension of spores of *A niger* and *Penicillium funiculosum*. The plates are then incubated at 28 °C for 3 weeks and assessed for growth using a rating scale after 1, 2 and 3 weeks. Coatings for exterior use and *wet* applications are leached in water prior to testing	Zone diffusion assay/agar plate test
VdL RL07	Guideline to evaluate the resistance of coating materials against mould growth	Coatings are applied to paper discs and then placed in intimate contact with the surface of nutrient agar plates. The coatings and surrounding media are then inoculated with a mixed suspension of *Scenedesmus vacuolaris* and *Stichococcus bacillaris*. The plates are then incubated at 23 °C for 3 weeks under illumination (16 h day length, 1000 lx) and assessed for growth using a rating scale after 1, 2 and 3 weeks. Coatings for exterior use and *wet* applications are leached in water prior to testing	Zone diffusion assay/agar plate test
IBRG Algal Test	Method to determine the resistance of surface coatings to algal growth	Replicate test panels coated with the test coating are inoculated with a suspension of cells of algae known to grow on the surface of paints and related materials. The samples are then incubated under conditions suitable to support algal growth (18 ± 2 °C and high humidity/surface condensation/illumination – 1 Klx, 16 h photoperiod) for up to 12 weeks. After incubation, growth is rated in accordance with a scale related to the percent cover with fungal growth (following visual and microscopical examination)	Growth cabinet based test

with water spray and UV-light in an exposure cabinet, be soiled or abraded). Multiple inoculation events can also be employed and soiling agents applied. Such approaches have been applied to a wide range of coating applications from traditional exterior and interior coatings to powder-coated panels used in air-conditioning systems. One of the great strengths of cabinet-based tests is the ability to use a substrate appropriate to the coating under test (wood, plaster, concrete, steel etc.) and study interactions between the substrate and the coating. Modifications can even be used to explore the impact of environmental factors such as temperature and relative humidity on colonization and growth.

As discussed earlier, outdoor exposure trials are often considered to be the definitive means of testing coated surfaces (and indeed almost the only method employed for marine and freshwater anti-fouling products) however, care needs to be taken to ensure useful data is obtained. The amount of growth that is obtained on test panels differs greatly from location to location. Panel orientation (vertical, horizontal, north facing, south facing etc.), height above ground level and even the time of year in which the trial is initiated can have a highly significant impact on the outcome. This has been examined extensively in [14.172] and many companies employ multiple sites and long-term exposure periods to ensure they gain a thorough understanding of the potential performance of their systems (often in support of products developed using laboratory-based methods and already on the market).

Susceptibility of Coating Systems to Microbiological Growth in Their Wet-State

Many coatings systems are either entirely or at least substantially water-based and, without some form of protection, are susceptible to spoilage through microbial contamination of the product in its wet state [14.173]. Continued regulatory pressure is also resulting in the reduction/elimination of cosolvents that contribute to the overall concentration of volatile organic carbon (VOC). This is leading to an increase in the degree of susceptibility of many products including those which have formerly been protected by the antimicrobial properties of cosolvents present in the formulation (e.g., 2-butoxy ethanol in waterborne paints for automotive applications [14.174]). In-can preservation systems are now used in many coating formulations to prevent spoilage due to microbiological growth such as the development of foul odors, discoloration, loss of structure and the generation of gasses that might distort/damage the final packaging. The protection provided includes the interval during manufacture as well as storage both within the plant and prior to sale. The protection should be sufficient to provide a shelf-life suitable for the product and may be extended to allow storage of part-used containers by the end user.

By far the most common approach to assessing both the susceptibility of a coating formulation to microbiological spoilage and the potential efficacy of a preservation system is a microbiological challenge test. A relatively limited number of standard test protocols have been developed over the last few decades (e.g. ASTM D 2574-06) and some operators have tried to employ methods based on those described in the various pharmacopoeias for cosmetics and pharmaceutical products although these have been found to be far from satisfactory. The International Biodeterioration Resarch Group (IBRG) has been developing a test protocol for testing the in-can preservation of paints and varnishes. Although still under development it is the most common method employed by workers in the field although in many cases some modification is made to the method described [14.175]. The method uses a combination of microorganisms which have been demonstrated to grow in water-based paints to challenge a paint formulation on a number of occasions. Preincubation of samples at elevated temperatures prior to inoculation can be used to explore the interaction of biocidal products with the formulation as well as the loss of highly volatile materials and the decay of other reactive components. It has been argued that only two repeat inoculations are required to simulate the interaction of the microorganisms with a paint formulation [14.176], however, most workers in the field recommend that a minimum of three repeat inoculations (usually at weekly intervals) be applied [14.175]. However, care must be taken not to continue re-inoculation until growth is achieved in the formulation. While this could be used to bioassay the concentration of preservative within a system, it provides less information about the interaction of a preservative system and the paint formulation than a carefully structured trial including phases of ageing and relatively short campaigns of microbiological challenge. As with the number of inoculations, both the cell density and the volume of inoculum should be kept within a sensible range both to prevent the test from becoming a disinfection test and the formulation being diluted unnecessarily. Typical total bioburden applied is often in the region of 10^7 colony forming units/g with an inoculum volume of between 100–500 µl per challenge.

After inoculation, the paint is examined for the presence of viable microorganisms. The paint is examined

at least just prior to the next inoculation although in some cases analysis at intervals between the two inoculations (e.g. 1 and 3 days) can provide useful information. Many works utilize some form of semi quantitative technique to estimate the size of any population surviving after challenge using the argument that any significant growth/survival represents a failure of the in-can preservation system. The use of a fully quantitative technique (with appropriate neutralization of preservative) can be useful in some circumstances. Techniques such as impediometry and the measurement of metabolic markers like adenosine triphosphate (ATP) have been employed with success, however, care must be taken to ensure no adverse interaction between the formulation and the system results in misleading data.

While it is important that challenge studies on coating formulations use a relatively wide range of microorganisms (principally bacteria, but certain yeast and filamentous fungi can be relevant to certain formulations), a significant fraction of these should have been derived from spoiled formulations at some time in the past. They should obviously be maintained in such a manner that they do not lose the ability to grow in paint matrices and some form of passaging may be required to ensure this (Sect. 14.6). Ideally, at least part of the challenge microorganisms should be shown to actually be capable of growing in the unprotected formulation under examination. Although they can be relevant to the spoilage of paint, care should be taken when considering the use of endospore-forming bacteria (and the spores of some species of fungi) as the survival of spores within the system can prove difficult to interpret. In many cases, studies with these species should be performed at least alongside the main challenge studies. In many variants of the basic challenge method, the cell suspensions used to create the challenge consortia are prepared from organisms grown on solid nutrient media. However, it can be argued that organisms grown in carefully standardized liquid culture (e.g., in shake flasks) are more suitable as they better mimic the manner in which contamination is introduced in practice (i. e., through the contact of paint with contaminated wash water in a production environment and via water contained in brushes and rollers used to apply the product). The use of contaminated paint has been used as a mechanism to inoculate test products but this can be difficult to standardize, may be highly selective toward certain components of a consortium and may stimulate the formation of capsules and exopolysaccharide in the challenge species and lead to the prediction of the need for excessive concentrations of preservative.

The principle of the challenge test can be a useful tool in the prediction of both the susceptibility of a coating formulation to microbiological contamination and spoilage and the efficacy of systems designed to preserve it. Careful use of appropriate ageing, incubation conditions (temperature etc.) and challenge consortia can be used to match a method to a wide range of system types from emulsion paints to industrial electrocoat systems. In-can protection of water-based tinters have been studied with success using the principles described above, however, the simulation of growth of fungi on the surface of such systems has yet to be successfully simulated in the laboratory and is the subject of concerted international research at the time of publication [14.177].

Hygienic Coatings

Although antimicrobial activity has been a component of certain coating systems for many decades, in the last few years this activity has been extended to provide a wider spectrum of activity and coatings are now being produced which are intended to provide hygienic benefits to the surfaces coated with them [14.178, 179]. In part, these developments have been fuelled by a raised public interest in hygiene resulting from high profile food poisoning outbreaks and the current high rate of hospital acquired infections.

The control of microbial activity associated with the spoilage of coated surfaces as described above, usually depends on the use of antimicrobial agents to prevent growth in association with the material to be protected [14.169]. In traditional applications, the addition of the antimicrobial agent is intended to protect the coating either during manufacture, in storage or in service. For example, a coating system intended for use on the outside walls of a building might be formulated with the addition of a fungicide and algicide to defend it from attack by microfungi and algae and so protect the film from aesthetic defacement and surface deterioration in service. In this instance the use of antimicrobial agents in the coating is intended to prevent microbiological deterioration of the surface. However, in recent years a new form of interaction between surface coatings and microbial populations has emerged along with a plethora of other materials modified to elicit similar effects [14.180]. In part, these new materials can be viewed as demonstrating either an extension of the degree of protection provided to them by the inclusion of an antimicrobial agent or as the transfer of the properties of external treatments into the material itself. The inclusion of the antimicrobial agent is now not simply intended to

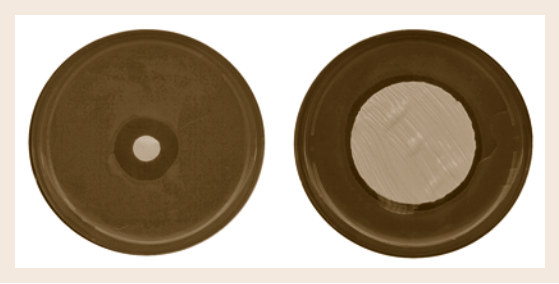


Fig. 14.22 Zone diffusion assay using liquid paint (*left*) and a coated filter paper (*right*). In both cases only an in-can preservative is present

protect the material from deterioration but to exert a biological effect either to the immediate surrounding of that material or to items that come into contact with it. These effects may range from the prevention of growth of undesirable microbial populations on a material to which they pose no physical, chemical or biological threat, (e.g., the proliferation for bacterial species such as *Listeria monocytogenes* on surfaces in a cook/chill production unit) to the immediate destruction of individual microbial cells as they come into close association with its surface (possibly without even coming into direct physical contact). In all cases the effect is external to the material and is not merely present to protect either the material or the article/surface itself. In this context, we are now dealing with treated articles [14.181]. Coatings which impart such properties on the surfaces to which they are applied can be considered to be transforming technologies. They transform objects/surfaces into treated articles. For example, a door handle coated with a powder coating which claims to have antimicrobial properties has transformed that door handle into a treated article and the testing technology needs to be able to provide data that is consistent with the claim made.

As mentioned above, there are several methods in use around the world which are employed to examine the effect of microorganisms on coated surfaces and measure the performance of additives used to protect them from microbial spoilage (mainly fungi and algae). A number of examples are given in Table 14.12. However, there are no formal tests which are intended to measure the *hygienic* effects of antimicrobial coatings although in some cases (e.g., inhibition of fungal growth), certain tests described in Table 14.12 could be employed for that purpose. A number of test methods do exist for other treated articles (e.g., nonporous polymeric materials see Table 14.13) and some of these may again prove appropriate for coated surfaces. These

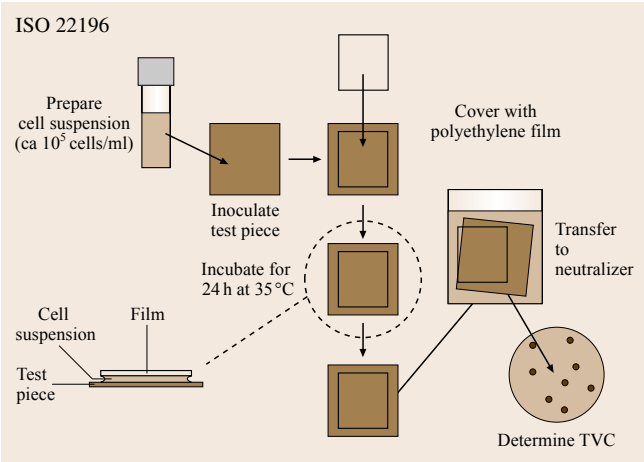


Fig. 14.23 Schematic representation of ISO 22196

methods predominantly examine the effects of such articles against bacteria but, again, these could be modified to suit other types of microorganism (e.g., yeasts and fungal spores). Although no standard methods yet exist for the determination of virucidal activity on surfaces, a test based on JIS Z 2801: 2000 has been described.

Many bacterial test assays rely on the production of growth on nutrient media to visualize their effect. Zone diffusion assays are commonly employed to investigate antibacterial agents such as antibiotics [14.182]. Their use for examining antibacterial coatings could be confusing, however, as in some systems it would be difficult to separate the residual effect of the in-can preservative from true antibacterial activity (Fig. 14.22, [14.170]). These methods also do not generate the truly quantitative data which will be required for the support of claims for treated articles.

Antibacterial activity of hygienic surfaces tends to fall into two distinct categories.

1. Surfaces which are bactericidal (i. e., a material which results in a significant reduction in bacterial numbers following a specific contact time).
2. Surfaces which are bacteriostatic (i. e., a material on which a small bacterial population did not exhibit significant growth during exposure).

A number of test protocols have been described (e.g. [14.178]) which are based on the Japanese Industrial Standard JIS Z 2801: 2000 [14.183] (Fig. 14.23). In these methods, a bacterial cell suspension is held in intimate contact with a coated surface using a sterile

Table 14.13 Methods used to examine the antimicrobial activity of nonporous surfaces

Reference	Title	Description
JIS Z 2801: 2000	Antimicrobial products – Test for antibacterial activity and efficacy	The surface of replicate sample (3 for each treatment and 6 for the blank reference material – usually 50 mm × 50 mm) are inoculated with a suspension of either *E. coli* or *Staph aureus* in a highly diluted nutrient broth. The cell suspension is then held in intimate contact with the surface by the use of a sterile polyethylene film (usually 40 mm × 40 mm) for 24 h at 35 °C under humid conditions. The size of the population on the treated surface is then compared with the size on the control surface both prior to and after incubation. A neutralizer for certain biocide types is employed. Antibacterial activity is certified if the difference between the $\log_{10}$ of the population on the treated sample and that on the control surface is > 2
ISO 22196	Plastics – Measurement of antibacterial activity on plastics surfaces	This is the current New Work Proposal at ISO created from JIS Z 2801 by the SIAA of Japan. Modification and validation is in progress in collaboration with the IBRG. Some changes are expected
XP G 39-010	Propriétés des étoffes – Étoffes et surfaces polymériques à propriétés antibactériennes	Four replicate samples of test material are placed in contact with an agar plate that has been inoculated with a specified volume of a known cell suspension of either *Staph aureus* and *K pneumoniae* using a 200 g weight for 1 min. The samples are then removed. Duplicate samples are analysed for the number of viable bacteria both before and after incubation under humid conditions at 37 °C for 24 h. A neutralizer is employed during cell recovery
ASTM E2180-07	Standard test method for determining the activity of incorporated antimicrobial agent(s) in polymeric or hydrophobic materials.	Replicate (3) samples of material are inoculated with cells of either *Staph aureus* or *K pneummoniae* suspended in molten semi-solid isotonic saline/agar. This attempts for form an *artificial biofilm* which holds the suspension in intimate contact with the test surface of inherently hydrophobic materials. Samples are then incubated at a temperature similar to that intended for the final use for a specified paeriod (usually 24 h) under humid conditions. The size of the viable bacterial populations on the control and treated surfaces is then determined using total viable count. Any effect is recorded using percent reduction calculated from the geometric means of the data. A neutralizer may be employed and sonication is used to separate the *biofilm* from the test surfaces and suspend the agar gel. Subsequent imprinting of the test surface onto solid nutrient media can be performed to look for the presence of adherent viable cells.
ASTM E2149-10	Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions.	Dynamic shake flask test. Test material is suspended in a buffer solution containing a known number of cells of *Klebsiella pneumoniae* and agitated Efficacy is determined by comparing the size of the population before and after a specified contact time.

cover (e.g., either a membrane filter, flexible polypropylene film or a glass microscope cover slip) under humid conditions. After a set contact time, the size of the residual bacterial population is compared with an appropriate control coating using standard microbiological enumeration techniques. ASTM 2180-07 has also been modified to examine such coated surfaces. These test protocols can examine both bactericidal and bacteriostatic performance.

When considering the generation of efficacy test data, it is important to note that the protocols described above rely on the presence of free water to function. It is critical therefore, to interpret the data generated with care as just because an effect is seen during testing it does not necessarily follow that activity would be seen in practice. For example, if the activity of a coating was only exhibited when moisture was present (e.g., due to the presence of a water soluble active ingredient such as silver ions or triclosan), activity would be detected in the test. If however, the coating was used in dry conditions, it is unlikely that an effect of the same scale would be exhibited and some microbial cells might remain viable on the surface (although this would probably be for a limited period only for many species [14.184]). Clearly under some circumstances (e.g., medical applications) cross-infection could still occur [14.185] despite the presence of an antimicrobial agent intended to prevent it. In this context the relationship between the environment in which the coating will be employed and the conditions under which supporting data were generated become critical factors in providing evidence of efficacy in use.

Similarly, the rate of kill would be important in many circumstances. In the control of cross-infection in clinical situations, surfaces which come into frequent contact with medical staff and patients (e.g., door furniture, bed

frames etc.) may need to be able to deactivate microbial cells very rapidly to provide a useful function. In this context, the contact time becomes a critical factor and tests in which a 24 h contact interval is employed may provide data which provides no useful information relating to efficacy in use. A slower rate of kill might be appropriate, however, to complement hygienic control on walls, flooring and difficult-to-access areas.

Although in many circumstances free water is not present, relatively few methods have been described which can simulate such conditions. Work has been published which examines the interaction of bacteria with polymeric coatings over time by spraying them onto surfaces both with and without the presence of soiling agents and holding them under differing environmental conditions. Direct vital staining and epifluorescent microscopy was employed to measure the effects [14.186]. Modifications to ISO 22196 (JIS Z 2801) have been described [14.187] which expose the inoculum to a relatively low relative humidity (65%) and measure survival at a number of intervals thereby simulating the effect on bacteria contained in an aqueous deposit coming into contact with the coating and then drying out. A number of methods are also under development in which the inoculum is presented with minimal moisture, but more work is still required before the range of effects claimed for hygienic surfaces can be investigated in a scientifically sound manner.

14.6 Reference Organisms

Methods for the determination of materials performance often require the application of test organisms. For instance, DIN EN 113 describes a test method for determining the protective effectiveness against wood-destroying basidiomycetes using a set of basidiomycetes as test strains. ISO 16869 is a method for the assessment of the effectiveness of fungistatic compounds in plastic formulations where fungal test strains are applied as spore suspensions. ISO 14852 comprises the determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium. Sludge from a sewage plant is used to inoculate the test, so all organisms inhabiting the sludge can be referred to as test strains.

For comparability of test results, the use of identical test strains is an inevitable prerequisite. These strains should therefore be specified as reference organisms.

Attempts to verify the identity of prokaryotic or eukaryotic test strains in pure culture or in an environmental sample have traditionally been performed by plating dilutions onto certain standard growth media and by assessing certain physiological, morphological and/or chemotaxonomic traits after culturing.

Only recently, through the application of molecular methods such as the polymerase chain reaction (PCR) and sequence analysis, researchers are now in a position to determine genotypic differences of phenotypic similar organisms.

As molecular methods are especially valuable for fast and reliable discrimination and identification of (micro-) organisms, this chapter gives emphasis to methods for genomic characterization of strains, species and microbial communities.

14.6.1 Chemical and Physiological Characterization

The chemical composition or the physiological potential of organisms is often used for characterizing strains and species and even for microbial communities. Methods such as gas chromatography, thin layer chromatography, high performance liquid chromatography, and various forms of spectroscopy are employed.

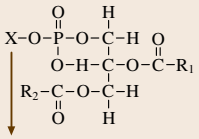
This paragraph will describe two techniques which are widely used for the characterization of strains and microbial communities.

Fatty Acid Analysis

Bacteria and fungi possess a cytoplasmic membrane as a component of their cell envelope, the composition of which is approximately 50% lipid. The membrane lipids are a diverse group of molecules which are frequently used as markers for the identification and classification of microorganisms. In particular, the amphipathic lipids (possessing hydrophilic and hydrophobic regions) have great relevance to microbial systematics.

Usually, in this approach fatty acids are released from the cells, methylated to increase volatility and subjected to gas chromatography. The fatty acid profile of an unknown sample can be compared to computer databases for identification.

Although phospholipids are the most widely known polar lipids, the cytoplasmic membrane may also con-



	Phospholipid
	R_1 and R_2 are fatty acid residues;
X	X is an additional functional group: containing phosphate
H	Phosphatidic acid
$CH_2OH \cdot CHOH \cdot CH_2OH$	Phosphatidyl glycerol
$CH_2OH \cdot CH_2NH_2$	Phosphatidyl ethanolamine
$CH_2OH \cdot CH_2NHCH_3$	Phosphatidyl methyl ethanolamine
$CH_2OH \cdot CH_2N(CH_3)_2$	Phosphatidyl dimethyl ethanolamine
$CH_2OH \cdot CH_2N(CH_3)_3$	Phosphatidyl choline
$C_6H_{12}O_6$	Phosphatidyl inositol
$CH_2OH \cdot CHNH_2 \cdot COOH$	Phosphatidyl serine

Fig. 14.24 Generalized structure of frequently encountered diacyl phospholipids (after [14.173])

tain glycolipids, polar isoprenoids, and aminolipids. The most commonly encountered lipids consist of a glycerol backbone to which either acyl groups (ester linkage) or alkyl groups (ether linkage) are attached. Polyunsaturated fatty acids generally do not occur in prokaryotes, though they do have significance in fungal characterization [14.173] Fig. 14.24.

Phospholipid fatty acid analysis has also been used as a culture-independent method to characterize microbial communities, but an important limitation of this method has to be considered [14.187]: In general, in bacteria and fungi, the types of fatty acids vary with growth conditions and environmental stresses. Consequently, if cells are cultured prior to fatty acid analysis, this has to be done under standard conditions. If microbial communities are characterized without prior cultivation, phospholipid profiles can be correlated with the presence of some groups of organisms, but they may not necessarily be unique to only those groups under all conditions, thus giving rise to false community profiles.

Carbon Utilization Profiles – BIOLOG, BIOLOG MicroLog

One of the more widely used culture-dependent methods of analyzing and characterizing microorganisms (bacteria and fungi) is the commercially available BIOLOG identification system. This system is also used extensively for the analysis of microbial communities in natural environments [14.188]. The organism/microbial community of interest is inoculated into a specialized microtiter tray with 95 different carbon sources. Utilization of each substrate is detected by the reduction of a tetrazolium dye, which forms an irreversible, highly colored formazan when reduced. The microtiter trays are read with a conventional plate reader, and the results are compared with a computer database, allowing identification.

As pointed out by *Hill* et al. [14.171], there are a number of considerations in the use of this method for community analysis. Beside the crucial step of the requirement of a standardized inoculum of *vital* cells, it has to be kept in mind that the color formation in each well is not solely a function of the number of organisms present in the sample (as is often assumed). Some strains may use a certain substrate more efficiently than others, thereby appearing to dominate the sample. In addition, the substrates found in commercially available BIOLOG microtiter trays are not necessarily ecologically relevant. Therefore, the method still suffers from similar bias problems as encountered with culture plating methods and future work with ecologically meaningful substrates should render it more suitable for the characterization of microbial communities.

14.6.2 Genomic Characterization

A major area of research in microbial characterization has been the development of molecular methods for genotyping organisms. Genotypic methods can be highly specific and sensitive and are largely independent of the physiological or growth-state of the organism. Especially the development of the PCR and PCR-based techniques provides sensitive and specific tools to detect and characterize microorganisms in the absence of growth.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a molecular method for amplifying DNA fragments. Using PCR and PCR-based techniques, rapid detection and identification of micro- and higher organisms in laboratory cultures as well as in environmental samples is possible.

The DNA fragment to be amplified is determined by the selection of appropriate primers. Primers are short, artificial DNA strands of approximately 20–30 nucleotides, that exactly match the beginning and end of the DNA fragment to be amplified. This means that the exact DNA sequence must already be known. Primers

can be constructed in the lab or purchased from commercial suppliers.

There are three basic steps in PCR (Fig. 14.25). First, the target genetic material must be denatured – that is, the strands of its helix must be unwound and separated – by heating to 90–96 °C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result is two new helices in place of the first, each composed of one of the original strands plus its newly assembled complementary strand. A cyclic repetition of denaturation, primer annealing and DNA synthesis results in the exponential amplification of the desired DNA fragment.

The development of group-specific or species-specific primers enables sensitive detection and rapid identification of selected organisms in culture and environmental samples.

ARDRA – Amplified Ribosomal DNA Restriction Analysis

In the ARDRA technology PCR-amplified ribosomal RNA (rRNA) genes are digested with restriction enzymes (enzymes that cut double stranded DNA at enzyme-specific recognition sites, Fig. 14.26) and the resulting fragments are separated electrophoretically. Comparison of patterns to those obtained from a database allows assignment of isolates to species, whereby the resolving power is depending on the restriction enzymes chosen.

This method, which can be used to screen large numbers of isolates rapidly, has gained widespread application in the detection and identification of fungi in laboratory cultures and natural substrates.

Ribosomal DNA is the molecule under investigation, because it is particularly well suited to the development of taxon-specific primers due to interspersed regions of relatively conserved (18S rDNA-, 5.8S rDNA-, 28S rDNA-gene) and nonconserved (ITS I, ITS II) sequences and a large copy number per genome [14.172, 174, 175]. Universal primers, e.g., ITS1 and ITS 4 [14.189], or primer pairs specific for higher fungi (ITS1-F/ITS4-B) or basidiomycetes (ITS1/ITS4-B) [14.190], are used for the amplification of rRNA genes and inserted ITS sequences. The PCR fragments are subjected to restriction digestion, and depending on the position of restriction sites, bands of different number and sizes appear after gel electrophoresis (Fig. 14.27). The majority of ARDRA profiles generated by any given enzyme are unique at the species level, but restriction digestion with *Alu*I, *Hpa*II, *Hae*III and *Taq*I proved to be especially useful for the discrimination of decay fungi [14.175].

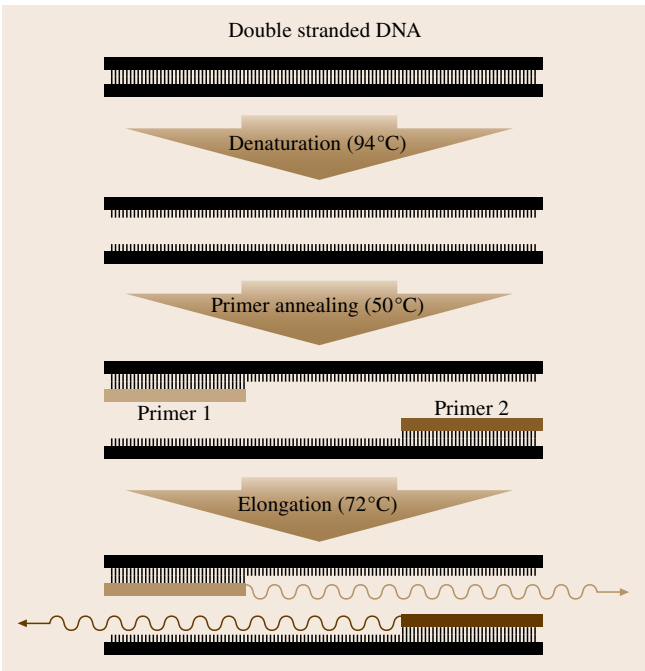


Fig. 14.25 The three basic steps in PCR

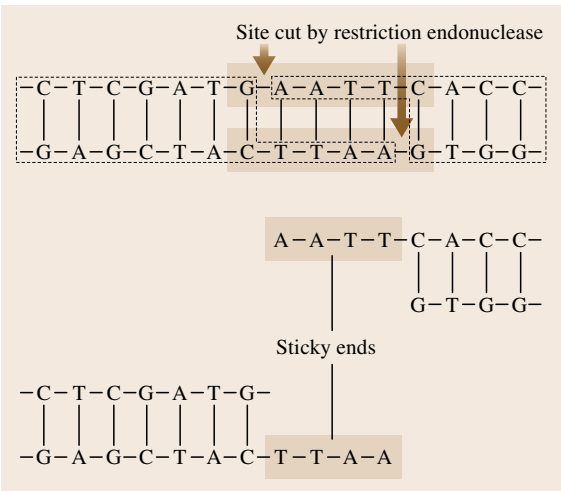


Fig. 14.26 Scheme for restriction enzyme activity. Restriction enzymes recognize a specific sequence of nucleotides and produce a double stranded DNA cut

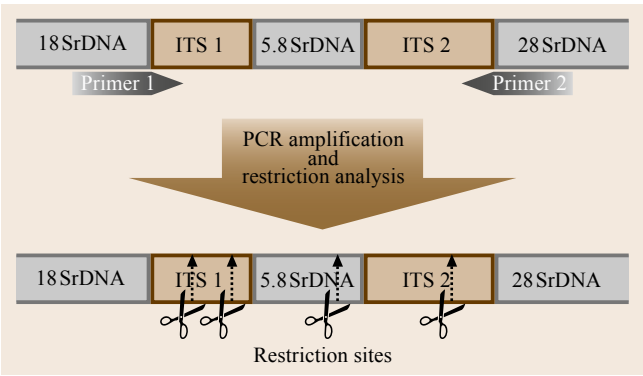


Fig. 14.27 Scheme for amplified ribosomal DNA restriction analysis (ARDRA)

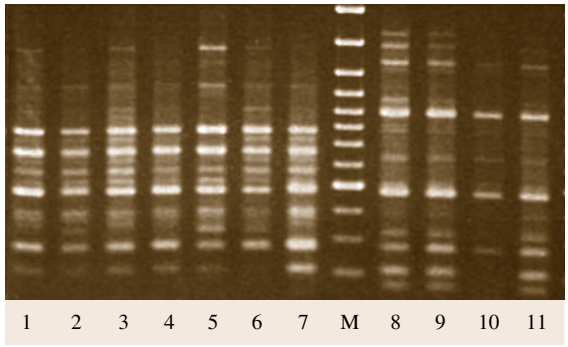


Fig. 14.28 RAPD patterns of eleven different strains of the wood degrading basidiomycete *Coniophora puteana* obtained with the same random primer. All strains were originally considered to be identical and used in the European Standard EN113. M: molecular size marker (2.0, 1.5, 1.2, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kb) (after [14.191])

RAPD – Random Amplified Polymorphic DNA (Arbitrary-Primed PCR)

Random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR) is a method that creates genomic arrays of DNA fragments (fingerprints) from species of which too little sequence information is available in order to design specific primers. It is used to identify strain-specific variations in (chromosomal) DNA [14.191] (Fig. 14.28).

Arbitrarily chosen primers are used to prime DNA synthesis from genomic sites which they fortuitously match or almost match, which results in the amplification of intervening DNA. Typically, PCR is performed under conditions of low stringency. The number and position of the primer binding sites will vary amongst different strains and consequently will lead to different strain-specific fingerprints. As a prerequisite, the primers must be within reasonable distance of each other, as the DNA polymerase must synthesize a product long enough so that it contains the site for annealing to the other primer. How soon the polymerase *falls off* the template depends on the purity and the constituents (GC-content) of the template itself.

BRENDA – Bacterial Restriction Endonuclease Nucleic Acid Digest Analysis and RFLP – Restriction Fragment Length Polymorphism

The BRENDA approach is mainly used for the characterization of prokaryotic, i. e., bacterial strains. Chromosomal DNA is digested with diverse restriction endonucleases and the DNA fragments are separated electrophoretically. Depending on the genome size and the enzymes used, the frequencies of restriction sites differ between strains, resulting in different fragment profiles. Usually, the profiles are highly complex and therefore difficult to analyze.

The number of detectable bands is reduced in the RFLP approach. Chromosomal DNA is submitted to a restriction digest and gel electrophoresis. Then, the DNA fragments are transferred and immobilized on a solid support such as a cellulose or nylon membrane. A target nucleotide sequence can be detected by a hybridization process, whereby a labelled DNA fragment (DNA probe) binds to the complementary target gene on the membrane. The presence or absence of the restriction endonuclease sites in two strains under investigation will cause differences in the length of the fragments that contain the targeted gene (Fig. 14.29). This technique, like the BRENDA approach, enables discrimination between strains [14.173].

Various systems for labelling and detection of nucleic acid probes are available nowadays. Radiolabelled probes are visualized using autoradiography, nonradioactive systems are based on the enzymatic, photochemical, or chemical incorporation of a reporter group (e.g., fluorescent dyes, marker enzymes coupled to chemiluminescence detection or to silver enhancement) which can be detected with high sensitivity by optical, luminescence, fluorescence, or metal-precipitating detection systems. For details about synthesis, labelling and detection of DNA probes (*Hames* and *Higgings* [14.192]).

As rRNA genes have some highly conserved regions (across species) which permits to use rRNA from one organism, e.g., *E. coli*, as a universal probe, these genes

are regularly used as target genes. This so-called *ribotyping* which can be regarded as a special kind of RFLP, examines differences in the restriction pattern of rRNA genes between strains. Usually, the pattern is more complex than observed after RFLP targeting other genes, because rRNA genes are present in multiple copies per genome. The resolving power of ribotyping is dependent on the species studied and the restriction enzyme chosen.

Ribotyping has been facilitated by the availability of commercial, fully automated systems such as the Ribo-Printer Microbial Characterization system (Qualicon Inc., Wilmington, DE). This molecular workstation performs the restriction digest (using *EcoR*I or other restriction enzymes) of the chromosomal DNA, separates the restriction fragments by gel electrophoresis, and simultaneously blots the DNA fragments to a membrane. DNA fragments are hybridized to a bacterial probe that is based on the conserved regions of the genes for the ribosomal DNA operon. Each fingerprint is stored in a database, so it can be accessed for future comparisons and identifications. The Ribo-Printer system is frequently used for quality control and authentication.

Gene probes addressing genes responsible for particular physiological activities (e.g., nitrification, nitrogen fixation, virulence associated genes etc.) are useful to characterize a subset of microorganisms or a microbial community, where the taxonomy or species composition of the community are of minor interest.

FISH – Fluorescence in situ Hybridization

Fluorescence in situ hybridization has been used primarily with prokaryotic communities. It allows the direct identification and quantification of specific or general taxonomic groups of microorganisms within their natural microhabitat. As whole cells are hybridized, artefacts arising from bias in DNA extraction, PCR amplification and cloning are avoided. FISH is a powerful tool that can be used not only for studying individuals within a population, but also has the potential to study population dynamics and tracking microorganisms released into the environment.

The composition of complex microbial communities is most often analyzed by rRNA-targeted nucleic acid probes: Whole cells are fixed, their 16S or 23S rRNA is hybridized under stringent conditions with fluorescently labelled taxon specific oligonucleotide probes. The labelled cells are viewed by fluorescence microscopy. Scanning confocal laser microscopy (SCLM) surpasses epifluorescence microscopy in sensitivity and allows to assess the distribution of several taxonomic groups simultaneously.

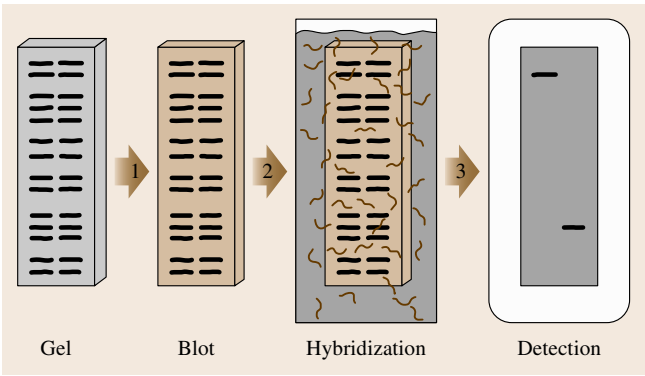


Fig. 14.29 Scheme for the RFLP approach. (1) Digested DNA of two strains under investigation is separated by gel electrophoresis, denatured and transferred (blotted) onto a nylon or cellulose membrane. (2) The blotted DNA is incubated with a labelled DNA probe which binds to the complementary target gene on the membrane. (3) After removal of unspecifically bound DNA probe, the targeted gene can be detected, e.g. by autoradiography (courtesy of S. Schwibbert)

The large amount of rRNA in most cells and the availability of huge rRNA databases for comparative sequence analysis are the major advantages of rRNA targeted nucleic acid probes. With the ARB software package, rRNA oligonucleotide probes can be designed in a straightforward fashion [14.193]. Specific organisms or groups can be selected and parameters such as probe length, G+C content, and target region can be defined and the ARB probe design tool will then search for potential target sites against the background of the full sequence data set. As the ARB database is frequently updated, old probes should not be used without re-checking the database.

Microbial groups without a common diagnostic target site should be detected with more than one probe. For increased sensitivity, as for the detection and tracking of functional genes, the application of horseraddish peroxidase-labelled oligonucleotide probes is advisable. Oligonucleotide probes labelled with a variety of fluorochromes can be purchased commercially.

DGGE – Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis is widely used in recent years for profiling microbial consortia. This method is particularly useful when temporal and spatial dynamics of the population structure are analyzed. It al-

lows the separation of DNA fragments of the same size but different nucleotide sequences by their denaturing profiles. For the characterization of microbial communities, ribosomal RNA genes are obtained directly from a bulk DNA sample by PCR and subjected to DGGE. The resulting banding pattern serves as a fingerprint of the microbial community.

During electrophoresis in an increasing gradient of denaturant (e.g. urea) or in an increasing temperature gradient (TGGE – temperature gradient gel electrophoresis), DNA molecules remain double-stranded until they reach the denaturant concentration or temperature that melts the molecules. Melting DNA branches and thus displays reduced mobility in the gel. As the melting behavior is mainly determined by the nucleotide sequence, theoretically any rDNA gene found in the mixed template DNA could be specifically amplified and resolved on a DGGE gel. Following DGGE electrophoresis, rDNA fragments can be sequenced and analyzed for similarity to other known sequences in public-domain databases. It has to be kept in mind that one band often does not represent only one species, so this approach is better suited for less complex communities.

This technique also might be limited by Dann extraction efficiency, which differs among microorganisms and type of environment (soil, mud, water). Amplification bias has also been shown to occur for templates that differ substantially in abundance, with preferential amplification of more abundant sequences [14.194]. For a detailed review of possible sources of amplification bias, see [14.195].

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