

**A POLYSACCHARIDE EXTRACTED FROM SPHAGNUM  
MOSS AS ANTIFUNGAL AGENT IN  
ARCHAEOLOGICAL CONSERVATION**

by

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## ABSTRACT

On the basis of the well-known preservative properties of *Sphagnum* moss, a potential opportunity to use moss polysaccharides (*Sphagnan*) in art conservation was tested. Polysaccharides were extracted from the moss (*S. palustre* spp.) in the amount of 4.1% of the *Sphagnum* plant dry weight. All lignocelluloses were removed from this extract as a result of the treatment of the moss cellulose with sodium chlorite. The extracted polysaccharide possessed a strong acidic reaction (pH 2.8) and was soluble in water and organic solvents. The extract was tested on laboratory bacterial cultures by the disk-diffusion method. The antibacterial effect was demonstrated for *E. coli* and *P. aeruginosa* (both gram-negative) while *Staphylococcus aureus* (gram-positive) was shown to be insensitive to *Sphagnum* polysaccharides. The antifungal effect of *Sphagnum* extract was tested by the disk-diffusion method on the spores of seventeen fungal species. These fungi were isolated from ethnographic museum objects and from archaeological objects excavated in the Arctic. Twelve of these isolates appeared susceptible to the extract. The inhibiting effect of the extract was also tested by the modified broth-dilution method on the most typical isolate (*Aspergillus* spp.). In this experiment, in one ml of the nutritious broth, 40 $\mu$ l of 3% solution of polysaccharides in water killed 10,000 fungal spores in 6 hours. The inhibiting effect was not connected to the acidity or osmotic effect of *Sphagnum* polysaccharides. As an example of the application of *Sphagnum* polysaccharides in art conservation, they were added as preservative agents to conservation waxes. After three weeks of exposure of microcrystalline wax to test fungi (*Aspergillus* spp.), 44% of wax was consumed. When, however, ~ 0.1% (w/w) of *Sphagnum* extract was mixed with wax, the weight loss of wax was only 4% in the same time interval. On the basis of this study it was concluded that *Sphagnum* moss and *Sphagnum* products can be recommended for use in art conservation as antifungal agents.

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# Chapter 1. Introduction.

Natural polysaccharides derived from some plants, algae and lower organisms have attracted great interest as antimicrobial agents. It has been found that polysaccharides extracted from a number of medicinal plants and mushrooms have antimicrobial, immunomodulatory and anti-tumor activities (Mizuno, Sakai, and Chihara 1995; Tzianabos 2000; Smith, Rowan, and Sullivan 2002; and many others). In the last decades, based on the methods of biotechnology, a series of novel polysaccharide-related antimicrobial agents were developed. They are now used in various applications, for example in the food industry to substitute traditional preservatives, and in bacteriology as inhibitors of bacterial growth (Chang *et al.* 2000).

In recent years there has been significant progress in understanding the molecular structures of plant polysaccharides and their derivatives. The antimicrobial properties of natural polysaccharides are based on their chemical structure where the presence of a highly reactive carbonyl group was detected. The carbonyl group is able to bond primary amines to produce a stable combination of polysaccharides with proteins (glycolconjugates). Bonding of exo-enzymes of saprogenic microorganisms by reactive polysaccharides is likely the reason for their antimicrobial activity (Painter 1991a).

The introduction of polysaccharide-based antimicrobial agents into conservation and museum practice has promising implications for conservation science. In conservation, we often confront the destructive role of microorganisms, on artifacts, on museum spaces and storage facilities, and on human health. The biodegradation of the organic materials comprising heritage objects and conservation materials is of urgent concern to conservation scientists. There are three main types

of microorganisms participating in this process: bacteria, fungi, and yeasts. Of these, bacteria are the least dangerous as they require a significant amount of water to digest organic molecules and can therefore only degrade wet organic specimens, for example, freshly excavated archaeological objects. Most bacterial species grow best at 100% relative humidity (RH) and temperatures of 30°C (Prescott, Harley, and Klein 2002). In the controlled museum climate, i.e. RH between 35 and 65% and temperature of 18-21°C, bacteria cannot grow (Tumosa *et.al.* 1996; Nitterus 2000; Padfield 2005). The development of bacterial decomposers in museums is possible only in extreme conditions, for example, during floods or water disasters after fire.

Fungi and yeasts function under considerably wider climate limits, including normal museum conditions. Hence they are potentially the most dangerous organisms for museum artifacts and archaeological collections. Fungal spores are able to survive in a dormant state at prolonged conditions of low temperatures (from 0 to -40°C) and low humidity, up to RH 11%. In addition, spores are easily transported in museum interiors through ventilation systems. As a result, all organic materials in museums become more or less contaminated by fungal spores that are normally in a dormant state. The increase of museum humidity to 70% RH and higher and temperatures of more than 21°C may trigger spore germination and mould bloom within 24 hours (Dicus 2000). Consequently, the efforts of museum conservators have to be directed mostly at the deactivation or eradication of fungal spores to prevent their germination (Florian 2002).

A number of methods to prevent fungal spore germination are employed in museum practice. They include mechanical removal of spores from the surfaces of the objects and from museum air, maintaining museum climate to prevent spore germination, decreasing of free water content

in the biocontaminated material making it unavailable for microorganisms, and treatment of objects with toxic chemicals (biocides) (Nitterus 2000; Florian 2002; Valentin 2003). Using biocides is the most effective measure against fungi because only biocides eradicate fungal spores. However, biocides have considerable disadvantages: they often react with treated materials causing objectionable conversions (discoloration, browning, brittleness). In addition, their toxicity renders the process of artifact disinfection potentially harmful to museum staff (Florian 2002). Biocides are often retained on the surface of treated artifacts for a long time making them potentially dangerous for handling (ibid.).

Finding effective and non-toxic substitutes for commonly used biocides is an important goal in conservation science. Some natural polysaccharides that possess antimicrobial properties can be used for treatment of artifacts or as conservation materials. Recently, *Chitosan*, the polysaccharide-based biopolymer widely used in the food industry, was tried in paper sizing as a strengthening and antifungal agent. *Chitosan* (poly-D-glucosamine) present in the shells of all crustaceans and insects was found to be active against bacteria and fungi (Rhoades and Roller 2000; Plascencia-Jatomea *et al.* 2003). *Chitosan*-sized paper showed considerable resistance against fungi *Alternaria alternate*, *Aspergillus* spp. and *Penicillium* spp., when compared with the paper regularly treated with cellulose ethers (Ponce-Jiménez, Toral, and Gutierrez-Pulido 2002).

Several plants containing naturally occurring antimicrobial ingredients also deserve attention. One such plant possessing polysaccharide-based antimicrobial activity is *Sphagnum* moss. *Sphagnum* moss that belongs to the phylum *Bryophyta* is the inhabitant of “peatlands” or ombrotrophic (rain fed) bogs that dominate northern ecosystems. The most remarkable evidence

of antimicrobial properties of *Sphagnum* moss is the extremely slow decomposition of *Sphagnum* dead plant material, resulting in the accumulation of partially decomposed moss remains in the form of peat deposits. A convincing example of the antimicrobial properties of *Sphagnum* moss is the existence of “bog bodies”. Bog bodies are human remains buried in *Sphagnum* peat for centuries without considerable decomposition (Turner and Scaife 1995; Menon 1997; Chamberlain and Pearson 2001). The antimicrobial properties of living *Sphagnum* plants and *Sphagnum* peat have been known for centuries (Schofield 1989). A tar extracted from peat moss was used as a weak antiseptic for the external treatment of eczema and other skin diseases (Bown 1995; Saxena and Harinder 2004). Dried *Sphagnum* moss was widely employed for wound dressings during World War I (Riegler 1989).

At the present time, freshly harvested *Sphagnum* moss is recommended in Canadian Arctic archeology as a packing material for the transport of excavated objects (Newton and Logan 1992; Scott and Grant 2007). The excavated artifacts packed in *Sphagnum moss* keep their natural humidity. Therefore, they are protected against fast drying and subsequent deformation of the objects. *Sphagnum moss* also serves as a natural antibiotic preventing microbial colonization of these objects.

The slow decomposition of dead moss and foreign organic materials in *Sphagnum* bogs is explained by the low microbial activity in the bog environment. This is attributed to their acidic, anoxic, cold, and nutrient-limited environmental conditions, and to the antimicrobial ingredients contained in the *Sphagnum* plant (Wieder and Vitt 2006). It was also found that the upper layer of peat bog litter contains a considerably lower concentration of microorganisms than other grass litters (Waksman 1930; Chastukhin and Nikolayevskaya 1967).

At the end of the nineteenth century, *Sphagnol*, the phenolic component of the *Sphagnum* plant, was considered to be the main antimicrobial agent present in the peat bogs (Czapek 1899). Later, polysaccharides contained in cell walls of *Sphagnum* moss were found to play the most significant role in the antimicrobial activity of the *Sphagnum* plant (Painter 1991a). These compounds are worthy of considerable attention as antimicrobial agents and are of particular interest to the present study.

It was found in the 1960s by Clymo that *Sphagnum* plants mainly consist of polysaccharides containing glucose and the pectin-like biopolymer, polyuronic acid (Clymo, Turnen, and Tolonen 1963; Clymo 1964). Polyuronic acid is a water-soluble part of the *Sphagnum* plant cellulosic composition (holocellulose). Structurally, polyuronic acid is connected to plant hemicelluloses. Polyuronic acid (D-galacturonic acid) units have a cyclic structure where the CH<sub>2</sub>OH side chain at C6 is replaced by a carboxylic acid group. Carboxylic acid has a highly reactive carbonyl group, which takes up mineral nutrients (Ca, Mg etc.) needed for plant growth. As a result of this reaction, hydrogen ions liberate into the ambient water (Clymo 1964). This is an important reason (but not the only one) for the acidification of bog water up to pH 3.4–5.6 (Kulzer *et al.* 2001).

The search for the main antimicrobial agent contained in *Sphagnum* moss was undertaken by T. J. Painter with colleagues in the NTNU (The Norwegian University of Science and Technology, Trondheim, Norway, NTNU; <http://www.ntnu.no/english>). Based on the antimicrobial activity of *Sphagnum* moss, Painter pursued an idea to pack fresh fish in *Sphagnum* moss on trawlers during transportation of the catch to consumers (Painter 1998). The prospect of being able to extend the freshness of fish with *Sphagnum* moss usage to several days without adding scent,

color, or smell was appealing in a country with abundance of this moss and to an industry with limited refrigeration facilities.

On the basis of the works of Clymo, Painter proposed that highly reactive carbonyl groups contained in polyuronic acid completely determine the antimicrobial properties of *Sphagnum* moss (Painter 1991a). He called these reactive constituents of polyuronic acid *Sphagnan* (Painter 1991a; Ballance *et al.* 2003). According to Painter, there are three possible mechanisms governing the antimicrobial activity of *Sphagnan* towards protein-containing materials in peat bogs.

(a) *Locking up minerals* (mostly  $\text{Ca}^{2+}$ ) *essential for microbial growth*. Based on an ion-exchange mechanism, living and dead mosses are able to hold minerals until the plant tissue is disintegrated. At the same time, decomposing microorganisms need these minerals for their growth. As a result of deprivation of essential cations by *Sphagnum* plants, a depressive environment for microbial growth is created in peat bogs.

(b) *Binding of nitrogen*. The reactive carbonyl group existing in the structure of *Sphagnan* reacts with the primary amines of proteins, resulting in the formation of stable glycoconjugates. By this mechanism, exo-enzymes excreted by saprogenic bacteria are condensed and consequently their activity in peat bogs is decreased.

(c) *Tanning of protein-containing materials*. It was noticed long ago that the skin of bog bodies is tanned and therefore inaccessible for microbial digestion (Glob 1967). Although polyphenols are the most probable tanning agents contained in *Sphagnum* moss, Painter countered that

polysaccharides (*Sphagnan*) in particular caused the tanning reaction with amino-groups of skin collagen, a process known as the Maillard reaction\*.

To prove these ideas, Painter extracted the reactive constituents of *Sphagnum* moss: *Sphagnum* holocellulose and *Sphagnan*, and tested their antimicrobial activity (Børsheim, Christensen, and Painter 2001a). *Sphagnum* plants, acetone-treated plant leaves (the intermediate product of the extraction), and *Sphagnum* peat were also tested along with *Sphagnan* (ibid.). The test organisms were naturally occurring bacteria grown on different fish products. *Sphagnum* holocellulose was also tested on gram-negative and gram-positive bacterial cultures grown on liquid nutrition media. Wood cellulose and reduced *Sphagnum* holocellulose were tested as controls. The treatment of holocellulose by reductants (sodium borohydride or ammonium carbonate) deactivated the reactive carbonyl groups of holocellulose. As a result, it was proposed that the antimicrobial effect of obtained holocellulose should be eliminated.

All tested *Sphagnum* substances showed an inhibiting effect on both naturally occurring bacteria and model bacterial isolates. In contrast, the controls, i.e. wood cellulose and reduced holocelluloses, did not show any antimicrobial effect. All *Sphagnum*-treated fish products turned brown but in the controls the color was not changed. Scanning electron micrographs of the fish skin surface showed that the skin collagen for *Sphagnum*-treated products was cross-linked (tanned). On the basis of these experiments authors concluded that the

***“...preservative and tanning property is exclusively associated with the carbonyl groups in the holocellulose component, and that no other component, monomeric or polymeric, soluble or insoluble, contributes significantly to the total preservative effect”*** (Børsheim, Christensen, and Painter 2001a).

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\* This non-enzymatic reaction between reducing sugar and amino-acid resulting in the formation of caramel-like brown substances is known in the food industry (Martins 2003). Painter suggested that in peat bogs, collagen fibers may create a dense cross-linked structure and expel water, making tanned collagen unavailable for saprogenic organisms.

The results of Painter's work are of obvious relevance to art conservation. First, they provide an experimental basis for using *Sphagnum* moss as a packing material for wet archaeological artifacts. Painter showed that for fish products embedded in fresh *Sphagnum* moss, bacterial growth was inhibited for approximately two weeks. This result is in agreement with the observation of field conservators. Artifacts packed in *Sphagnum* moss did not change their appearance after two weeks of transit from the archaeological site to the museum storage, and the moss remained fresh as well (Newton and Logan 1992; Sutherland-personal communication).

In addition, polysaccharides extracted from *Sphagnum* moss are worthy of consideration, both as conservation materials or as additives to other conservation materials. Painter tested *Sphagnum* polysaccharides only against bacteria, but in conservation and museum practice, fungi are potentially more dangerous organisms than bacteria. Since hyphomycetes such as *Aspergillus* and *Penicillium* spp. are the most widespread and destructive microorganisms in the museum environment, the testing of *Sphagnum* polysaccharides against these species would be of considerable relevance to the goals of art conservators.

On the basis of what is presently understood about the processes by which *Sphagnum* polysaccharides inhibit bacterial activity, it is believed that they can also inhibit fungal activity.

(a) The low population and low biodiversity of both bacteria and fungi in bog litter has been detected (Waksman 1930). It was shown experimentally that *Sphagnum* polysaccharides cause a depressive influence on a variety of bacterial species (Børsheim, Christensen, and Painter 2001a). It was also shown that in many cases fungi are the principal decomposer microbes in

peat bog litter and dominant bacteria (Latter, Cragg, and Heal 1967; Williams and Crawford 1983; Thormann, Currah, and Bayley 2001). At the same time, their population is also depressed in peat bogs, most probably because *Sphagnum* moss inhibits a vital function of fungi as well.

(b) The depressive influence of *Sphagnan* on bacteria and fungi seems to be the same because the digestive mechanism for both organisms is also the same, i.e. enzymatic degradation of dead plant material. In addition, both organisms need essential metal cations for their growth. Therefore *Sphagnan* is supposed to be able to depress fungal growth in the same way it depresses bacterial development.

The main task of the present research was to examine the possibility of the use of *Sphagnum* moss in archaeology and in art conservation, especially, as an antifungal agent. The experiments with *Sphagnum* moss were carried out at Queen's University (Department of Art, Department of Biology and Department of Chemistry) and at Brock University (Laboratory of Biotechnology). *Sphagnum* moss for test experiments was collected at a site 40 km north of Kingston, Ontario. It was identified as *Sphagnum palustre*. The same species was used by Painter for preparation of reactive polysaccharides and consequent bacterial assay of obtained *Sphagnum* products.

In the present work, *Sphagnum* polysaccharides were isolated closely following the procedure described by Painter and following his personal recommendations (Börsheim, Christensen, and Painter 2001a; Painter 2002). Therefore, it is believed that the product obtained was the same as Painter's product *Sphagnan*. Antibiotic properties of *Sphagnum* polysaccharides (*Sphagnan*) were tested on both bacteria and fungi. The test bacteria included three common pathogens: *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The test fungi were

sampled from archaeological objects freshly excavated at the site of Nunguvik, northern Baffin Island, Canada; from ethnographic objects stored in the conservation laboratory at Queens University; and from the archaeological site storage at Kaman- Kalehöyük, central Turkey. *Aspergillus* species were the most common fungal organisms isolated from these three sources. Biological tests showed clear antimicrobial influence of *Sphagnum* extracts on the majority of examined bacterial and fungal organisms. The positive results of these tests encouraged the author to find a practical application of *Sphagnum* extracts to conservation needs. This additional part of the experimental work is briefly explained below.

The author of the present work participated in the excavation of the Japanese Institute of Anatolian Archaeology at Kaman- Kalehöyük (Turkey). In 2003-2004, the archaeological site storage at Kaman- Kalehöyük was drastically impacted by moisture, leading to the outbreak of microbial growth. As a result of this disaster, the protective wax coating on the majority of stored bronze artifacts was biodegraded. The penetration of moisture and acidic microbial metabolites through the degraded wax film triggered an intensive corrosion of the bronze artifacts known as “bronze disease”. This exposure to moisture and biocontamination provided an opportunity to investigate the response of wax coatings to such extreme conditions. It was also an opportunity to test the obtained *Sphagnum* extracts as antimicrobial additives to conservation waxes. *Sphagnum* extract (*Sphagnum*) was added to conservation waxes that were exposed to mature fungal lawn (*Aspergillus* spp.). The addition *Sphagnum* extract to conservation waxes decreased the rate of their fungal consumption up to 10 times. Detailed explanations of all observations and experiments and their results are included in the experimental chapters of this thesis; the raw counting data are included in the Appendix.

## Chapter 2. Literature Review.

This chapter consists of three parts. The first part illustrates the important role of fungi in infestations of museum objects with focus on the methods of the prevention of fungal growth. The next part builds a knowledge base of antimicrobial properties of *Sphagnum* moss. The last part describes the susceptibility of the conservation wax coatings to microbial attack. This was included in the Literature Review to give an impetus to use of *Sphagnum* polysaccharides as inhibiting additives to conservation waxes.

### 2.1. Fungi in the museum environment

Microbiological deterioration of artefacts is an urgent problem in conservation science. A diversity of microorganisms including bacteria, yeasts, and fungi participate in the decomposition of heritage and museum objects. Most cultural heritage and museum objects are comprised of organic materials that are subject to microbial deterioration. These include materials of outdoor heritage assets and a wide spectrum of modern and ancient museum materials such as wood and paper, bone, amber, parchment, leather, glue, and textiles (Florian 2002); composite materials such as easel and mural paintings (Ciferri 1999; Gorbushina *et al.* 2004); a variety of construction materials (Andersson *et al.* 1997; Gravesen *et al.* 1999); and conservation polymers and resins (Koestler 2000).

Microorganisms can also colonize a variety of inorganic materials. Stone monuments and old buildings, glass, ceramics, the plaster base of frescos, gypsum, inorganic pigments and others can be deteriorated by microbes. In outdoor conditions microorganisms have been reported to contribute to the weathering process of these materials (Caneva, Nugari, and Salvadori 1991;

Ciferri, Tiano, and Mastromei 2000). Microorganisms often cannot degrade certain materials directly. However, on the surface of attacked objects they create slime-like biofilms\* that cause subsequent degradation of such materials. For example, biofilms can be seen on the surface of archaeological glass as pitting spots. Biofilms may also trigger microbiologically influenced corrosion (MIC) of metals and alloys (Little, Wagner, and Lewandowski 1998). Microbial metabolites (exo-enzymes, organic and inorganic acids, volatile compounds such as ammonia or hydrogen sulfide) initiate electrochemical reactions leading to corrosion of ferrous and copper alloys (Beech and Gaylarde 1999). Bio resistance of conservation polymers and resins is a concern of conservation science. These materials should not be the cause of microbial colonization. At the same time, many conservation polymers and resins including petroleum and polyethylene waxes (Zaitseva 2005b), acrylic lacquers (McNamara *et al.* 2004), silica resins, and materials containing polyvinyl acetate (PVA) and polyvinylchloride (PVC) (Webb *et al.* 2000) are susceptible to microbial attack\*\*. Although some polymers are not attacked directly, they usually collect organic debris, which can be easily colonized by biofilm-forming microorganisms. Metabolites excreted by these organisms can in turn cause deterioration of polymers itself. Therefore, the protection of conservation polymers from bio-colonizers is an important goal of conservation science\*\*\*.

\* Biofilms or microbial mats are complex microbial structures where cells are closely packed and firmly attached to each other, as well as to a solid surface of the material. In biofilms, colonies of microbial cells are assembled with extracellular polymeric substances (EPS). EPS are mostly polysaccharides serving for adhesion of microbial cells to a substrate. They also store organic carbon and water, essential for microbial development (Lappin-Scott 2003). Biofilms alter the original color of materials by their pigmented cells and deteriorate materials by microbial metabolites as well. They can be formed on any inorganic materials by primary colonizers such as algae and cyanobacteria. Cyanobacteria need for their growth only light and CO<sub>2</sub>; some cyanobacteria can fix atmospheric nitrogen. Therefore, they do not need an organic substrate for their growth. For example, the nitrogen-fixing *Nostoc* found in Italian frescoes indicates that cyanobacteria may colonize materials in which nitrogen is absent (Ciferri 1999). In museums, the primary colonizers can start to grow on deposited dust particles with subsequent biofilm formation. Biofilms in turn can be the source of carbon for further fungal colonization (Tomaselli 2003).

\*\*The relative bioresistance of some conservation polymers and resins is summarized in several studies (Heyn, Petersen, and Krumbein 1995; Koestler 2000).

\*\*\*In the present work, a natural antibiotic (*Sphagnan*) was added to conservation waxes. The increasing of wax bioresistance was demonstrated (see section 5.4).

### **2.1.1. Fungi as the most hazardous microorganisms in museum practice**

In an indoor environment fungi are the most hazardous microorganisms responsible for the biocontamination of museum collections. Unlike bacteria whose growth in museums is limited by their need for water, fungi are able to grow at relatively low temperature and humidity levels such as those usually maintained in museums (National Research Council 1986; Appelbaum 1991; Lull and Banks 1995; Ogden 1999; Florian 2002). Even in quite harsh conditions including dryness and low temperatures, fungal spores are able to survive for a long time. For example, at temperatures of about 4°C commonly maintained in refrigerators, fungi are still able to grow (Florian 2002). In favorable conditions such as high humidity, increased temperatures, and in the presence of light fungal colonization can cause aesthetic and structural damage to all organic materials in museums.

Fungi are more effective decomposing organisms than bacteria because they degrade organic materials both chemically and mechanically. Extracellular enzymes excreted by fungi and their metabolites, often acidic in nature, cause chemical alteration of the structure of the material under attack. Fungal hyphae can pierce materials to find more easily digestible components, resulting in loss of durability and delaminating and cracking of the material. Some museum materials such as tanned leather are indigestive for bacteria because in tanning, cross-linkage of fibers has occurred and water required for bacterial growth is expelled from the collagen (Florian 2002). In contrast, fungal hyphae can penetrate between collagen fibers to utilize sugars and free amino-acids contained in the collagen molecules (ibid.).

Finally, fungal spores and their volatile metabolites (VOCs) are hazardous for humans (Pasanen, Lappalainen, and Pasanen 1996). Some fungal species found in the museum environment may cause allergies, respiratory problems and other diseases in museum staff (Florian 2002). These are the main reasons why the prevention of fungal colonization in museums is worthy of attention by conservators\*.

### **2.1.2. Fungal species found in museum storage**

Fungi have a cosmopolitan nature, i.e. they are restricted neither by climate or region, nor by substrates which they utilize at ambient conditions (Bennet, Wunch, and Faison 2002; Florian 2002). However, different fungal species show more or less selectivity towards certain substrata. Thus, *Aspergillus* and *Penicillium*, which are common inhabitants of dwellings, are soil-originated. At the same time, one of the most common fungi in the world, *Rhizopus stolonifer*, grows mostly on bread and soft fruits (Alexopoulos, Mims, and Blackwell 1996). The majority of fungal species found in museums are the conidial fungi (Cole and Kendrick 1981; Florian 2002). Conidia are asexual fungal spores with diameters of 3-30  $\mu\text{m}$  that are often released in the ambient air. Conidia are electrically charged and adhere to dust particles to create bioaerosols. Bioaerosols consist of spores, small inorganic particles, living and dead microorganisms or their parts, and fungal metabolites such as antigens and toxins (Burge 1995; Burge and Otten 1999). Bioaerosols enter museum buildings through ventilation systems and are passively spread through the museum's interior by airflow.

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\*It has to be noted, however, that under favorable conditions (humidity, temperature, light, and, to a lesser extent, pH) fungi live in symbiosis with bacteria and algae. For example, in the case of such combined materials as frescoes, especially those located underground (in crypts, tombs, and grottoes), fungi are considered to be only secondary colonizers (Ciferri 1999). The primary colonizers are supposed to be sulfur-cycling bacteria and eukaryotic algae (ibid.). Microorganisms isolated from frescoes include both bacteria (*Bacillus*, *Pseudomonas*, *Arthrobacter*, and *Streptomyces*) and fungi (*Penicillium*, *Aspergillus*, *Cladosporium*, *Chaetomium*, and *Alternaria*) (Ciferri 1999; Gorbushina *et al.* 2004).

When bioaerosols land on damp organic material, fungal spores may colonize this material in a period from one to twelve days. It is known that at high relative humidity (more than 80%) spores are able to produce mycelia in a period shorter than three days (Florian 2002). For hygroscopic materials this time can be even shorter (ibid.). Other sources of fungal spores in a museum interior are acquisitions (for example, newly excavated archaeological artifacts) and incoming or returning biocontaminated objects. The most typical fungi found in indoor air are *Penicillium*, *Aspergillus*, and *Cladosporium* (Hyvärinen *et al.* 2001). Bioaerosols accumulated in air-conditioning systems in museums mostly include two common genera, *Aspergillus* and *Penicillium*. Other fungi commonly found on air filters are *Alternaria*, *Cladosporium*, *Fusarium*, and *Trichoderma* (Florian 2000; Florian 2002). All these fungi originate from the natural environment. *Aspergillus* and *Penicillium* are widespread in soils and *Alternaria* and *Cladosporium* colonize the leaf litter (Khalid *et al.* 2006). These genera do not include all fungi growing in dwelling interiors. For example, in indoor environments in Finland, almost 500 pure microbial cultures (bacteria and fungi) were isolated and more than 80 taxa identified (Salkinoja-Salonen *et al.* 1998). In Canadian schools suffering from mould, a variety of fungal species were found including species that indicate contamination: *Acremonium* spp., *Aspergillus fumigatus*, *A. niger*, *A. ustus*, *A. versicolor*, *Chaetomium* spp., *Paecilomyces variotii*, *Penicillium brevicompactum*, *P. aurantiogriseum* complex, *P. variable*, *Phoma* spp., *Stachybotrys chartarum*, *Trichoderma harzianum* and *T. viride* (Rand 1998). Certain fungi, such as *Stachybotrys*, *Fusarium*, *Trichoderma*, and *Aspergillus versicolor*, are the indicators of increased wetness of the interior (Samson *et al.* 1994). The same fungi colonize museum interiors as well as museum collections and other heritage objects. For example, Dicus identified *Penicillium* and *Chaetomium* spp. colonizing both museum building interiors and artifacts, while *Aspergillus* was found only on walls and furniture and *Cladosporium* only on artefacts (Dicus 2000). The same

genera were isolated from deteriorated paper: *Penicillium*, *Aspergillus* and *Stachybotrys* spp. (Ricelli *et al* 1999).

Florian lists the most complete data of various fungi isolated from heritage objects (Table 2.1). Four genera, *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*, were found on all tested materials. *Stachybotrys*, which is referred to as "black mould", is less common in museums, but it often causes deterioration of wet dwelling interiors (Baughman and Arens 1996; Peltola *et al.* 2001). These five genera reproduce via conidia and belong to mould or filamentous fungi (hyphomycetes). Of these fungi, *Aspergillus* and *Penicillium* are the most widespread and destructive biodegrading agents in museum practice.

*“They are the prevalent genera found on canvas paintings, mural paintings, leathers and paper. Their relative amounts vary with geographic location: Aspergillus is the highest in warm climates and Penicillium the highest in temperate climates”* (Florian 2002).

Fungus	Oil on canvas	Water colours	Wall paper	Old books and parchment	Fresco, Wall Painting	Leather
<i>Alternaria</i>	*	*	*	*	*	*
<i>Aspergillus</i>	*	*	*	*	*	*
<i>Aureobasidium</i>	*		*	*	*	
<i>Botrytis</i>	*	*			*	
<i>Cephalosporium</i>					*	*
<i>Chaetomium</i>	*			*	*	
<i>Cladosporium</i>	*	*	*	*	*	*
<i>Fusarium</i>		*		*	*	
<i>Geotrichum</i>	*					
<i>Monilia</i>				*		
<i>Mucor</i>	*	*			*	
<i>Neurospora</i>				*		
<i>Papularia</i>	*	*			*	
<i>Penicillium</i>	*	*	*	*	*	*
<i>Rhizopus</i>	*	*			*	*
<i>Rhodotorula</i>						*
<i>Sepedonium</i>		*			*	
<i>Stachybotrys</i>			*	*		
<i>Stemphylium</i>	*	*		*	*	
<i>Trichoderma</i>			*			*
<i>Trichothecium</i>	*	*			*	
<i>Verticillium</i>	*	*	*		*	

**Table 2.1.** Fungi isolated from different groups of museum materials (Florian 2000; Florian 2002). Data compiled from museums in Canada, USA, Italy, Egypt, and Kuwait.

A number of fungi that are relatively rare in museums but which commonly colonize archaeological wood are white-, brown-, and soft- rot fungi. Analysis of the decay of historic and archaeological woods worldwide\* showed that soft-rot fungi are the major degrading organisms found on the examined objects (Blanchette 2003; Blanchette *et al.* 2004). However, some regional preponderance was distinguished. Soft-rot fungi dominated in cold polar environments. White- and brown-rot fungi, the common wood degraders in nature, predominantly occupied temperate and tropical ecosystems (Blanchette 2003). White- and brown- rot fungi are taxonomically placed in the Basidiomycetes and soft- rot fungi are classified as Ascomycetes or Deuteromycetes (Fungi Imperfecti).

### **2.1.3. Chemical compounds related to fungal activity**

Fungi degrade organic materials mechanically, by the growth of hyphae, and chemically, with a number of enzymes, and metabolites that are excreted into the substrate\*\* (Griffin 1994; Alexopoulos, Mims, and Blackwell 1996). The common fungal metabolites are water, carbon dioxide, specific pigments (melanin-derived black pigments, colored pigments), organic acids (acetic, citric, oxalic acids, and others), volatile organic compounds –VOCs (aldehydes, alcohols, and ketones), nitrogen-containing compounds (beta-glucans, amino-acids), lipids and polyols (glycerol and others), and secondary metabolites such as antibiotics and mycotoxins. The influence of fungal metabolites on different museum organic materials is not reviewed in the present work. These effects are discussed in many studies (Florian 2002 and others).

\*Plant materials from Anasazi Great Houses (10<sup>th</sup> century A.D.) in New Mexico, USA; wooden artifacts from ancient Egyptian tombs (8<sup>th</sup> century B.C.), in Gordion, Turkey; historical wood from first Polar stations built on Ellesmere Island, Arctic, and Ross Island, Antarctic.

\*\*Fungal degradation of different materials comprising paper documents, as well as a wide spectrum of materials for conservation has been studied intensively (Ogden 1999; Ciferri, Tiano, and Mastromei 2000; Florian 2002; Saiz-Jimenez 2003; and many others). The results of these studies are beyond of the scope of the present work.

**Enzymes** are the special exo-proteins excreted by fungal organisms for the utilization of organic compounds. Fungi can utilize only relatively small molecules. Using enzymes, they degrade complex organic molecules of a substrate to more simple ones. In this way fungi break down the chemical bonds between carbon and other elements of these molecules. For example, the proteins comprising parchment, leather, and glue are hydrolyzed into amino acids (Florian 2002). For the degradation of large macromolecules like collagen, fungi also use enzymes of symbiotic bacteria. In the process of fungal degradation of plant materials, the simpler structures such as soluble sugars and amino acids are utilized first. Then fungi subsequently utilize starch, pectin, cellulose, and waxes (Maijala 2000). The least degradable plant structure is lignin (lignocelluloses). For degradation of these ingredients to occur, a complex of enzymes is excreted correspondingly: hydrogenase and cellulase for the degradation of sugars and cellulose; amylase and protease for the degradation of starch and amino-acids; and phenol oxidase for the degradation of lignin (ibid.).

**Organic acids** are the intermediate products of fungal metabolism excreted into the substrate in the process of digesting carbohydrates (Alexopoulos, Mims, and Blackwell 1996). During utilization, carbohydrates are transformed into pyruvic acid which enters a cyclic conversion (named Krebs cycle) resulting in a subsequent series of metabolized weak organic acids. They are citric, oxalic, succinic, glutamic, gluconic, glucuronic, lactic, fumaric, maltic and acetic acids (Florian 2002). Like enzymes, acidic fungal metabolites may cause chemical and structural alterations in attacked materials, e.g. they have been found in fungal foxing spots on old paper (ibid.). Acidic metabolites also provide bio fixation of trace metals by fungi, which need trace metals for their growth. Bio fixation of trace metals occurs when chelating complexes form metal cations such as iron, copper, lead, zinc, calcium, magnesium etc. (Beech and Gaylarde

1999; Florian 2002). Deprivation of metals from the substrate in the process of bio fixation may change the chemical structure of attacked materials. For example, fungal removal of calcium and other metal ions from mural paintings leads to peeling of lime ground as well as crumbling and discoloration of paint (Petushkova and Lyalikova 1986; Ciferri 1999; Heyrman *et al.* 2003).

***Proteins, amino-acids, beta-glucans and lipids*** are presented in different fungal structures such as exo-enzymes and fungal biofilms. According to the available data, proteins vary from 7.3% to 25.5% of the dry weight of hyphal cell walls and conidia for different fungal species (Florian 2002). Beta-glucans, which are referred to the fungal slime and biofilms, are considered to be the major product of fungal activity in the substrate. Lipids, which vary between 5.5% and 17.7%, undergo oxidation of attached proteins to soluble amino-acids. They may also account for discoloration of materials (*ibid.*).

***Sugars (trehalose and glucose) and glycerol*** are fungal metabolites. The main drawback of these compounds is their ability to retain water in the vicinity of fungal colonies and thus produce slime-like substances. When moisture supplies are limited, water vapor can be absorbed by these compounds from the ambient air, providing fungal growth (Florian 2002). Water absorbed by fungal slime in combination with organic acids can cause corrosion of metal objects and also support bacterial activity on the surfaces of materials, contributing to their subsequent degradation. For example, fungal degradation of wax coating on bronze artefacts in combination with slime formation can cause intensive corrosion of the underlying metal and the appearance of “bronze disease” (see section 5.2).

*Melanins* are brown or black pigmented phenolic compounds present in dark hair and skin. They are copiously deposited in fungal cell walls. Melanin is associated with the protection of fungal conidia and hyphae. The protective mechanism of melanin remains unclear, although it has been shown that melanin protected fungal organisms against antimicrobial oxidants (Youngchim, Hay, and Hamilton 2005).

*Coloured pigments* are present in fungal hyphae and conidia. Filamentous fungi accumulate brown pigments in their hyphae, green or black pigments in their conidia, and different coloured pigments (yellow, red, blue, and others) which are excreted into the substrate (Florian 2002). Fungal melanin and colored pigments cause mostly aesthetic changes to artefacts, for example, they may cause staining on old papers (ibid).

*Volatile organic compounds –VOCs* and *mycotoxins* do not affect museum materials. However, these compounds are excreted by fungi into the ambient air and may cause negative health effects on people working with infested artifacts.

#### **2.1.4. Temperature and water requirements for fungal growth**

Fungi require certain conditions for their growth including the nature of the attacked material (i.e. bioavailability of the chemical structure of the material, moisture content, pH, and physical properties of the surface of the material) and environmental conditions (including light, the presence of oxygen, dust and fungal spores in the ambient atmosphere, relative humidity, and the presence of a microclimate that may cause condensation) (Alexopoulos, Mims, and Blackwell 1996; Valentin 2003). The influence of all these factors on fungal growth is an immense topic,

which is beyond the scope of this study. Some of these factors, including temperature, water, and nutrient availability, influencing fungal growth is reviewed by Dix and Webster (Dix and Webster 1995). However, among these factors, temperature and the presence of water in the substrate are considered the most important ones (ibid.). Temperature and water requirements for vital activity of fungi are traditionally used to control these organisms in museum practice. Their influence on fungal growth is reviewed below.

### ***Water requirements***

The parameter used in museums for the control of fungal growth is the relative humidity (RH) of the museum air. The relative humidity indicates how much moisture is in the air. It is defined as the ratio of the partial pressure of water vapor in air and its partial pressure at the dew point (i.e. to the saturated vapor pressure) at the given temperature:  $RH = p/p_{\text{saturat}} \times 100\%$  (Perry and Green 1997). For example, the saturation vapor pressure at 20°C is 17.54 mm Hg (ibid.). If at 20°C the measured vapor pressure is 1.754 mm Hg, the relative humidity is 10%. For a given dew point and its absolute humidity, the partial pressure of water decreases with temperature, i.e. RH depends on temperature of the ambient air.

The control of the relative humidity of the indoor air is not sufficient to control fungal growth. It was shown that spore germination is determined by the moisture content within a material and on the surface of the material, not by the relative humidity of the ambient air\* (Block 1953).

\*There are several reasons explaining this phenomenon. The availability of water in the material for fungal growth depends on the strength of the bond between water molecules and the microstructure of the material. Florian recognizes three types of water in the material, depending on their location and bond strength: bound water, free (multilayer) water, and condensed water (Florian 2002). Bound water is bonded to molecules of a material by strong covalent bonds. Free multilayer water is connected to molecules of a material by weak hydrophilic bonds and stored in capillaries less than 30 µm in diameter. These types of water are rarely available for microorganisms. Condensed water is free water stored in capillaries more than 30 µm in diameter and is readily available for microorganisms. Only condensed water quickly reaches equilibrium with the water content in the ambient air. Therefore, the total amount of water contained in a material can exceed that part of the water content, which is in equilibrium with the humidity of the ambient air (ibid.).

Even if the amount of condensed water at a certain location in the substrate is not enough for fungal growth, fungal hyphae can reach the regions with higher water content and transport water along hyphae to the fruiting body. Under dry conditions, fungi can also recycle their own metabolic water and produce polyols. Polyols are glycerol-containing compounds working as osmo regulators to absorb sufficient amounts of water from the ambient air and the substrate (Griffin 1994).

The quantity of water available for microorganisms in the substrate is characterized by water activity ( $a_w$ )\*. Water activity is defined as the ratio of the vapor pressure of water in the substrate to the vapor pressure of pure water at the same temperature (Ayerst 1969; Griffin 1981). Pure distilled water has a water activity of exactly one. The risk of fungal attack can be better evaluated by the value of water activity rather than by the value of relative humidity. In nature  $a_w$  ranges from 1.0 to 0.01. At 1.0, i.e. for pure water, growth of most fungal species cannot occur (only a few aqueous species are known). The majority of fungi require  $a_w = (0.60 - 0.98)$  for their growth, and bacteria need  $a_w > 0.98$ . The values of  $a_w$  that will support fungal growth vary in the literature, depending on the material: from 0.62 to 0.98 (Florian 2002) and from 0.70 to 0.85 (Saiz-Jimenez 2003). The value  $a_w = 0.7$  is considered a general limit for mould growth because fungal exo-enzymes are altered at drier conditions (Florian 2002).

\*The potential for fungal growth on the given material has been attributed also to moisture content (MC) defined as the ratio of “free water” contained in the material to the dry weight of the material. Free water is the water held in the pores due to physical absorption (capillary or van der Waals forces). It is opposite to the water of hydration, which is chemically bound to the material and cannot be evaporated by heating. For example, Block found that fungal growth for different materials including wood, leather, wool and others started when the MC increase above 0.1 (Block 1953). *Aspergillus* and *Penicillium* strains started growing in cellulytic materials (paper, textiles) at  $MC > 0.08$ . These conditions occur in paper at 63-65% RH. Paper exposed to 50% RH has a  $MC \sim 0.07$  at 22°C and these conditions are considered to be ideal for storage of paper objects (Saiz-Jimenez 2003). Control of water activity rather than moisture content is important in the food industry as low water activity prevents microbial growth (increasing shelf life).

According to the water requirements, fungi can be hydrophilic, i.e. water-loving/live in water; xerotolerant, i.e. live at  $a_w=(0.97-0.98)$ ; and xerophilic, i.e. live at  $a_w=(0.62-0.90^*)$  (ibid.). A number of *Aspergillus* and *Penicillium* species common in museums are classified as xerophilic (dry-loving) fungi, i.e. their conidia survive even in quite dry museum climates. For their growth, *Aspergillus* spp. needs a lower water activity and a higher temperature than *Penicillium* spp. and therefore is potentially more dangerous for museum materials (Florian 2002).

Despite many limitations as a parameter controlling fungal growth, relative humidity is still widely used in museum practice because its temporal variations can be easily recorded by hygrometers. The recommended museum climate requirements are still based on values of relative humidity and temperature. Today, decreasing the relative humidity of museum air is considered to be the main measure controlling fungal growth. Nitterus reports several recommended values for RH: 50-60%, 65-70%, and 75% (Nitterus 2000). Florian suggests that to prevent fungal growth in museums, the RH should be less than 70% at 20°C (Florian 2002). However, among indoor fungi there are a number of xerophilic species such as *Aspergillus versicolor* and *Penicillium auranteogriseum* that function at a relative humidity of less than 60% (Baughman and Arens 1996). Some *Aspergillus* and *Penicillium* species found on indoor dust are reported to grow even at a RH of 11% (ibid.).

### ***Temperature***

In general, fungi can grow at temperatures ranging from 0°C to 40°C. Below 0°C, fungi survive but cannot grow; above 40°C, they cannot survive for a long time (TenWolde and Rose 1993; Dix and Webster 1995). However, this is a general statement only (Florian 2002).

\* Cole Parmer company reported that a few moulds and osmophilic yeasts grow on dried fruits, caramel, coffee, and honey at  $a_w=0.60$  ([http://www.foodtechsource.com/rcenter/tech\\_data/td\\_water.htm](http://www.foodtechsource.com/rcenter/tech_data/td_water.htm)).

Some heat tolerant fungi are able to grow at temperatures up to 60°C. Despite the fact that the vital processes of microorganisms are reduced at decreased temperatures, fungi often colonize food products in refrigerators at temperatures of -4°C and some fungi can survive even at -7°C. Only freezing for at least 72 hours at -20°C is considered to be sufficient for a mortal effect on fungi because at this temperature all water condensed in the material will be converted to ice crystals (Florian 1997). For this reason, the temperature of -20°C is the general storage temperature for frozen food products. In nature, however, fungi can survive even at such cold conditions. Thus, soil temperatures in cold Arctic and maritime Antarctic environments can reach -20°C\* in the cold season (Robinson 2001). The survival of soil fungi in this temperature range has been shown from many sources. For example, a total of 40 species from 21 fungal genera were isolated from soil samples collected in Franz Joseph Land (Bergero *et al.* 1999). Fungi were found in glacial ice from various locations: on Lake Vostok, Antarctic (Abyzov, Mitskevich, and Poglazova 1998; Price 2000; Price 2001) in Greenland (Willerslev *et al.* 1999; Ma *et al.* 2000), and Norway (Sonjak, Frisvad, and Gunde-Cimerman 2006). Ma found viable fungi (*Penicillium*, *Cladosporium*, *Ulocladium*, *Pleurotus* and others) in Greenland ice cores that were 140,000 years old, and Sonjak found a diversity of filamentous *Penicillium* spp. in Arctic glaciers (Svalbard, Norway). A diverse group of organisms were found to coexist on glaciers between Washington State and Alaska (Napolitano and Shain 2004). Analysis of the temperature dependence of microbial metabolic rates:

*“supports the view that, far below the freezing point, liquid water inside ice and permafrost is available for metabolism”* (Price and Sowers 2004).

Fungi can survive in cold environments because they are able to tolerate low temperatures by a special mechanism (Widden and Parkinson 1978).

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\*Mean daily (September 1993 to July 1994) soil temperatures from 3 cm depth at a polar semi-desert site at Ny-Alesund, Svalbard, Norway, 79°N. Soil temperature rarely exceeds 0°C in the warm season at this site.

The mechanism of cold adaptation of Arctic and Antarctic fungi is reviewed by Robinson (Robinson 2001). It was suggested that fungi inhabiting the Arctic and Antarctic become adapted to temperatures from  $-6^{\circ}\text{C}$  to  $5^{\circ}\text{C}$ \* because they produce special antifreeze proteins and enzymes, as well as melanin which protects their hyphae from extreme temperatures (Robinson, Page, and Bainbridge 1997). Most of these fungi should be active only for short periods, which are interspersed with periods of prolonged dormancy. Only a few of them may be expected to show continuous slow growth (Bergero *et al.* 1999). Survival of fungi in the Arctic soil is the reason for high biocontamination of archaeological objects excavated at Arctic sites (Sutherland -personal communication). However, due to slow vital processes at low temperatures, the rates of biodegradation of Arctic artifacts are slow as well, i.e. these artifacts were often found in fairly good condition.

#### **2.1.5. Methods to control fungal growth in museum storage**

In museums, the most hazardous factor relating to fungal activity is their spores because spores represent a threat of formation of new fungal colonies. Spores are distributed by dust particles and bioaerosols for long distances. By air currents, they can disperse through the ventilation system and infest clean museum interiors and collections. Eventually settling on the surface of a new object, spores can germinate if the environmental conditions are favorable for it (Florin 2002). Therefore, any measures against fungi in museums have to be directed mostly towards the deactivation (or eradication) of fungal spores.

\*It has to be noted that fungi can probably tolerate even lower temperatures because in some materials such as fresh collagen, water does not freeze completely even at temperatures as low as  $-50^{\circ}\text{C}$  (Florin 2002). This means that fungi are potentially able to live even at this temperature. The low temperature limit for survival is generally considered to be  $-70^{\circ}\text{C}$  (Robinson 2001). Fungal spores, however, are able to live at lower temperatures. To ensure long-term viability of the spores of filamentous fungi, it is recommended to store them at temperatures of liquid nitrogen ( $-196^{\circ}\text{C}$ ) in 10% glycerol (Simione and Brown 1991; Smith and Onions 1994; Atlas 1997).

Concerning mature fungi, it is considered that their vegetative parts, i.e. fruit body and mycelium mat, can be relatively easily removed from the object by mechanical methods (Nitterus 2000). Mechanical removal of spores by brushing, aspiration and vacuuming is the simplest measure against fungal colonization of museum artefacts. However, it is almost impossible to remove all spores, especially from porous materials (ibid.).

In museum practice, fungi are traditionally deactivated by physical methods (exposure to X- and gamma-rays, drying and freezing, heating, and creating anoxic atmospheres), and chemical methods (fumigation and treatment with non-volatile biocides) (Ciferri, Tiano, and Mastromei 2000; Nitterus 2000; Salvadori 2003). There is no single point of view on the application of these methods to museum practice. In the past, toxic chemicals were commonly used to eradicate microorganisms in the museum environment. Now the aim of measures against microorganisms is more the prevention and control of their growth than total eradication of these microorganisms (Valentin 2003).

The preventive strategy is to inhibit or slow down the growth of fungal organisms through modification of both the museum environment and the stored artefacts to make them unavailable for fungal growth (Ciferri, Tiano, and Mastromei 2000). In most cases this strategy is based on decreasing water activity in organic materials comprising museum collections (Florian 2000; Florian 2002). It can be done by climate control and other physical methods such as freezing, drying and freeze-drying of artifacts, binding free water in the material with glycerol or sugar, and maintaining anoxic atmospheres (Nitterus 2000).

Some indirect methods such as air ventilation are also quite effective in preventing fungal growth (Valentin 2003). Often, the first step is to find the source of the humidity (for example, the location of water leakage or of the condensation of water vapor) and stop it by drying the interior and the collections without any chemical treatment of artefacts. Ventilation is a simple and effective method to avoid fungal growth (Valentin *et al.* 1998). It was found that fungi cannot grow at slow but constant air flow even if the humidity is relatively high. Airborne spores are inactive at humidity as high as 80% RH but with stable aeration (Valentin 2003).

The observations of the author of the present work confirm the effectiveness of this simple measure. Aeration was applied to mitigate fungal growth at Kaman-Kalehöyük in a non-heated storage area after a flood (see section 5.1). The infested ceramics and bone were washed with water and dried in the open air while the interior of the storage area was well aerated. Forced-exhaust ventilation was established working continuously to maintain a weak air flow in all storage areas. These measures stopped new microbial growth, but the level of spore contamination was still high. Further observations showed that even at the conditions of high humidity during the winter-spring season (up to 83% RH according to the records) and high concentration of airborne spores in the interior, these spores did not germinate.

### ***Climate control***

The most commonly used measure for prevention of spore germination in museums is the maintenance of temperature and humidity to retain spores in a dormant condition. In general, mould growth in a museum environment starts at an RH of more than 70% (Appelbaum 1991; Lull and Banks 1995; Florian 2002). The recommended climate limits to prevent mould growth were found experimentally for indoor construction materials and museum collections. Most

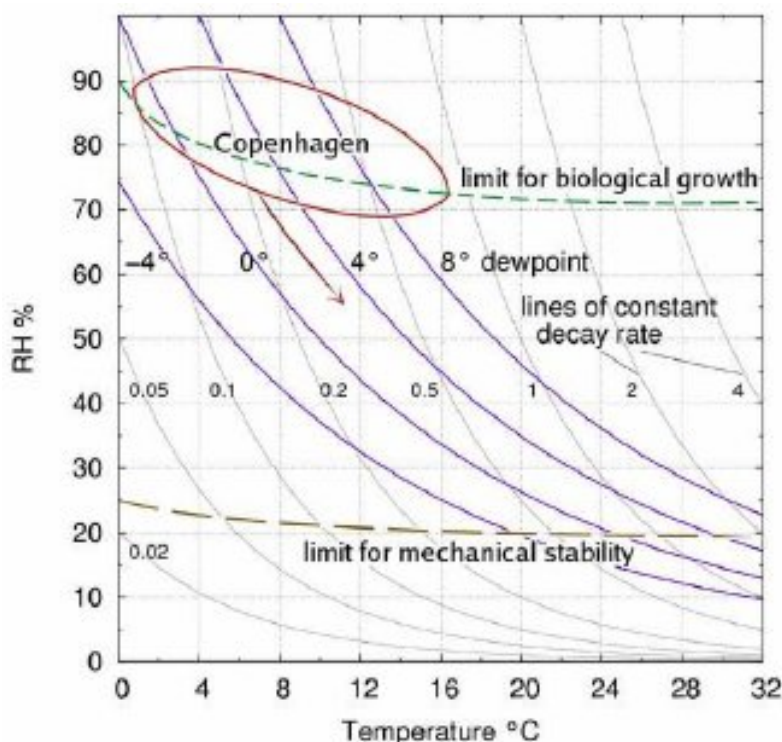
specialists are of the opinion that for the general control of fungi in museum storage it is necessary to keep the level of humidity at 50-55% RH and the temperature at 15-18°C (Florian 2002; Nitterus 2000; Ogden 1999).

On the basis of these recommendations, numerical models have been created with curves that assess the risk of fungal growth. Numerical models were formulated only for materials containing cellulose (wood and paper) and for certain fungal species that attack these materials (Rowan *et al.* 1999; Hukka and Viitanen 1999). Hukka and Viitanen found that blue stain fungi started to grow on wood within the following ranges: temperatures between 20°C at humidity > 79% RH; temperature 10°C at humidity > 82% RH; and temperature 5°C at humidity > 89% RH.

Using such data, an interesting approach to the problem of mould growth on old paper documents was proposed (Padfield 2005). Padfield analyzed the effects of temperature and humidity on mould growth in combination with the effects of natural aging of paper artefacts. As a result, the optimal museum climate for the best preservation of paper artefacts was established.

The process of natural aging of paper depends on temperature and humidity. Elevated temperatures increase the rate of chemical aging of cellulose; increased humidity accelerates the hydrolysis of cellulose. The combined effect of temperature and humidity on the rate of the natural aging of paper was expressed as a family of "isoperms", i.e. lines of equal deterioration rates (Sebera 1994). An arbitrary deterioration rate (i.e. equal to 1.0) was taken for conditions close to the "ideal" museum climate, i.e. 50% RH and 20°C.

When Padfield superimposed climate limits for the risk of mould growth on cellulose materials\* on the family of Sebera's graphs (Fig. 2.1), he demonstrated the effect of temperature and humidity on both processes: mould growth and paper aging (Padfield 2005). Obviously, at ~70% RH and above, paper is subject to mould growth and at ~20% RH the flexibility of paper is decreased. The area between these limits provides a favorable climate for the storage of paper artefacts. To demonstrate the practical value of this approach, Padfield superimposed the approximate monthly average of the outdoor climate in Copenhagen on Sebera's graph (Fig.2.1). It became clear that the climate in Copenhagen (which is typical for northern Europe) must be modified by museum air conditioning in the direction of decreasing RH. This model was formulated for paper artefacts only. Other organic museum materials can be assessed in the same way.



**Fig.2.1.** Superimposition of Sebera's isoperms (grey-colored), slightly revised by Padfield (purple-colored) on the climate limits for microbial growth and mechanical stability. The oval brown line reflects the variations of T and RH for the average climate in Copenhagen. For better preservation of paper artefacts this climate has to be corrected in museum storage in the direction of decreasing RH (arrow). Reproduced from Padfield (Padfield 2005).

\*T and RH limits for mould growth on cellulose can be taken from the models formulated for wood construction materials (for example, from Hukka and Viitanen 1999 or others).

### *Physical methods of control*

Despite its general effectiveness, climate control cannot guarantee that all fungi will become inactive. Even at relatively low humidity of the museum air, the quantities of water absorbed on the surface of organic materials can be enough for fungal growth (Florian 2002). For effective inhibiting effect, climate control should be complemented by various physical and chemical methods.

Deep freezing is one of the most common physical methods of controlling microorganisms in museum storage (see section 2.1.4). However, when artefacts are removed from the freezer, spores start to germinate intensively because temporary freezing activates their vital processes (Florian 2002). In addition, formation of ice crystals during freeze-drying may cause deformation of the material. This is the reason why freezing should be accompanied with drying. In freeze-dryers, water is removed by sublimation without causing any dimensional change to the material (ibid.). The disadvantages of this method are as follows: loss of condensed water leads to brittleness and an increase in the porosity of the material (ibid.). In museums, freeze-drying requires complicated and expensive equipment and cannot be used for mass treatment.

An alternative method is the treatment of infested artefacts with increased temperatures.

Temperatures above 40°C can kill fungal structures but will rapidly dehydrate the material, and consequently cause the deformation of the artefacts (Florian 2002). Heating is not practiced in museums because it is considered to be an aggressive method of treatment.

Another common physical method, the impregnating of organic materials (wood, leather) with sugar solutions, is recommended as an alternative to freeze-drying (Florian 2002). In this

method, free porous water contained in the material is bonded with sugar, glycerol, and polyethylene glycol (PEG) to make them unavailable for fungal growth. There are some disadvantages to this method: high quantities of sugar that are added to the material cannot be easily removed, and these artefacts cannot be used for further studies such as radiocarbon analysis. In addition, impregnating materials can serve as substrates for certain microbes. For example, PEG commonly used for leather treatment can activate fungal growth (ibid.).

Most of the fungi involved in the deterioration of museum materials are aerobic organisms. Creation of anoxic atmospheres is recommended by some authors as an effective strategy to prevent fungal colonization (Valentin, Lidstrom, and Preusser 1990; Tavzes, Pohleven, and Koestler 2001; Valentin 2003). It is also widely advertised as a progressive method to mass kill fungi in museum storage (The Sherman Fairchild Center for Objects Conservation 2002). To expel oxygen, nitrogen is used most frequently in low permeable plastic bags, showcases and anoxic chambers.

**“However, these treatment systems are expensive and difficult to handle for de-infesting objects of large size” (Valentin 2003).**

Recently, portable equipment producing large amounts of nitrogen was tested. These systems allow treatment of large objects enclosed in plastic bubbles (Valentin 2002; Valentin 2003). When inert gas is used, the atmosphere inside the bubble is very dry that can also cause mortal effect on microorganisms. Valentin suggested that reduced humidity alone is sometimes a more effective measure against fungi than creation of anoxic atmospheres (Valentin, Lidstrom, and Preusser 1990).

Generally speaking, climate control systems and other climate equipment (refrigerators, freeze-dryers, anoxic chambers, nitrogen generators, and others) are available in well-equipped

museums only. In most other cases, i.e. in provincial museums and on-site storerooms, these measures are often unattainable. In such conditions, chemical treatment may be the only method preventing mould growth. In addition, if a material once becomes contaminated, it is almost impossible to disinfect it except by chemical methods (Baughman and Arens 1996).

### ***Control of microorganisms with biocides***

A number of toxic chemicals (biocides) have been used for the treatment of museum artefacts. Although biocides are the most effective at eradicating spores and mature organisms, in many cases they do not provide residual protection from future microbial growth. In other cases, these toxic substances can be retained on the surface of artefacts, making them harmful for humans. Biocides can be simply applied in solutions, powders and as a fumigating gas.

Fumigation is a method of eradicating fungi by exposing contaminated artefacts to a toxic gas. The treated artefacts are exposed to gaseous fumigants in sealed chambers. The common gaseous fumigants are: ethylene oxide, widely used for sterilization of medical supplies such as bandages and surgical implements in the medical industry; methyl bromide, a general-purpose fumigant widely used in the past, but now in the process of being banned as an ozone-depleting chemical; sulfuryl fluoride, a widespread fumigant used mostly against dry wood termites; hydrogen cyanide, an extremely dangerous chemical warfare agent; phosphine, the only effective, rapidly acting fumigant that does not leave residues on the stored product (phosphine is widely used for farm pest control); formaldehyde, a common indoor contaminant, restricted for use in several European countries because it is a toxic, allergenic, and carcinogenic agent; and thymol, a phenol derivative which was widely used as antifungal agent in archives and libraries for the treatment of paper (Salvadori 2003; Nitterus 2000).

The advantages of gas fumigation are as follows: deep penetration into the material; effective eradication of fungal spores; fast treatment procedure; and the possibility of treating a large number of artefacts in a short time. Fumigation is necessary in case of disasters.

Thus, fumigation by formaldehyde was used on a very large scale for the treatment of 8.1 million books and documents from the collection of the Russian Academy of Sciences, St. Petersburg, after a fire and subsequent flooding (Del Rey 1990; Nitterus 2000). The total volume of treated items was over 44,000 m<sup>3</sup> and has no analogy in world practice.

A variety of non-gaseous industrial and agricultural biocides were also used for the treatment of museum collections\*. For application to artefacts, non-gaseous biocides are generally diluted in distilled water or organic solvents at low concentrations 0.1-3% (Salvadori 2003).

The primary disadvantage of most biocides is their toxicity, rendering disinfection of artefacts a potentially harmful procedure for museum staff. For example, naphthalene, formaldehyde and orthophenylphenol are listed as potential carcinogens (Sax 1981). Dangerous properties of biocides can be found in a number of sources (NIOSH 1994; Sax 1981; Sax and Lewis 1987; Bretherick 1999; Lewis 2000 and many others).

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\*The most popular biocides commonly used in museum practice (non-gaseous) are listed below (Nitterus 2000):

phenolic derivatives: pentachlorophenol (PCP), p-chloro-m-chresol (CMC), orthophenylphenol (OPP), n-phenylsalicylanilide; naphthalene; quaternary ammonium compounds (quats)-common disinfectants; poisoning agents:-sodium hypochlorite (bleach), mercury compounds such as mercuric chloride; tributyltins - used as industrial biocides (as antifungal agents in the manufacturing textiles, paper, wood pulp, breweries etc.); benzoic esters- common food preservatives marked as E-numbers (E 210-E 213)\*\*.

\*\*E-numbers are codes for food additives established in the European Union; parabens- E-214, E 216, and E 218 that are common preservatives in the cosmetic industry; antibiotics- Streptomycin, Nystatin etc.

Another problem of biocides is their chemical activity in relation to treated materials. Gaseous fumigants can cause polymerization and oxidization as well as corrosion of treated materials (Salvadori 2003). Florian reported that fumigation of protein-containing materials with ethylene oxide, methyl bromide, or methyl chloride

**“causes methylation of the proteins which changes their basic chemistry”** (Florian 2002).

Chlorinated molecules comprising the structure of many biocides can cause discoloration and brittleness of treated materials (Florian 2002; Nitterus 2000).

There is a lack of common strategy and rules for the application of different biocides to treated objects. In addition, there is also no standardized response of certain fungal species to certain biocides, which can be used to compare the efficiency of different biocides (Nitterus 2000). For these reasons the mode of their action on artefacts is often unpredictable and their concentration for the treatment of artefacts is often wrong. Finally, biocides can be retained in treated materials for a long time, representing a health hazard for the persons who handle them.

Biocides were commonly used in art conservation in the past. Nowadays due to their toxicity for humans and negative environmental effects, biocides are rarely used. It is difficult to verify where certain biocides are still in use because, in some countries, their usage is restricted or prohibited according to national standards and regulations.

However, the use of biocides is in many cases the only option to stop the rapid growth of mould after disasters in museums, during field excavations, and in poorly equipped storage areas at archaeological sites. In field conservation, biocides can be used as a temporary measure before transporting artefacts to well-equipped museum storage facilities. Among specialists, there is no

consensus about using biocides in regular museum practice. Florian considered that biocides can be used only for cleaning of a museum interior, but she contends that they should not be used for the treatment of artefacts because

**“Today we have to deal with the terrible legacy of toxic biocides still contained in heritage objects years after treatment; they present a hazard to all those who handle them” (Florian 2002).**

According to another opinion, limited usage of biocides is still possible, but only as the “last resource” (Tiano 2002).

### **2.1.6. Control of microorganisms with new products based on polysaccharides and polyphenols**

The search for effective and non-toxic substitutes for commonly used biocides is an important goal in conservation science. In recent years, using the methods of biotechnology, novel antimicrobial products have been created from natural antimicrobial compounds. These products can be incorporated into the life cycle of microbial organisms to deactivate their digestive functions. Today natural antibiotics and synthetic products made on this basis are widely used in medicine. Introducing them into conservation practice is a worthy goal, because they are effective against microorganisms as well as being non-toxic.

Many natural antibiotics are polysaccharides by their nature and derived from different lower organisms (crustaceans, medicinal plants, some microbes). In plants, polysaccharides are structurally connected to different phenol compounds comprising plant lignin. Many plant phenols possess strong antimicrobial properties and therefore they also deserve attention as possible antimicrobial conservation products.

## *Polysaccharides*

Polysaccharides with various active biological functions have been found in some medicinal plants, fungi, algae, shells of shrimp and other sea crustaceans. Polysaccharides or polysaccharide-protein complexes (glycolconjugates) in natural products serve as bioactive absorbents with special properties that allow them to bind to proteins, fats, micronutrients, heavy metals, and trace elements. The relationship between polysaccharide structures and bioactivity is still not well understood and they cannot be considered as true antibiotics\*. Most likely, polysaccharides possess a complex effect on different organisms. However, some of them, which have clear antimicrobial properties, can be used in conservation.

One natural polysaccharide made of chitin of sea crustaceans (*Chitosan*), was recently applied in paper conservation as a sizing material (Ponce-Jiménez, Toral and Gutierrez-Pulido 2002).

*Chitosan* is made on an industrial scale by deacetylation of lobster, crab and shrimp shells obtained from shellfish processing. Structurally, *Chitosan* is a glycoconjugate containing  $\beta$ -1,4-linked polymer of glucosamine, which contains cationic groups located on the polyglucosamine chain. This group can bind to negatively charged substances (Sugano *et al.* 1993).

\*Nevertheless, the medicinal effect of polysaccharides contained in different plants and mushrooms is demonstrated in many works (Chang 2002-a review; Zhu *et al.* 2005; and others). Polysaccharidic extracts and powders have been used for centuries in Chinese traditional medicine (Chang and But 1988). Now a number of natural medicinal products and food supplements are being developed with a polysaccharide base. The bioactive properties of polysaccharides can be utilized in various medicines such as antibiotics, immunomodulators, dietary fibers, fat burners, drug delivery agents, etc. Many medicinal plants and fungi have immunomodulatory and anti-tumor activities that are probably due to their polysaccharide components (Ukai *et al.* 1983; Chang and But 1988). In recent years numerous studies have demonstrated the health-promoting benefits of various medicinal mushrooms e.g. basidiomycetes (Wang *et al.* 2002; Wasser 2002; and others). Polysaccharides in *Cordyceps sinensis* found at high altitude in the Himalayas have recently attracted great interest due to their various medical properties: immunostimulating, antileukemic, antitumor, DNA synthesis inhibiting etc. (Halpern 1999; Holliday *et al.* 2004). Polysaccharides from certain medicinal herbs (*Arctium lappa* var. *herkules*, *Aloe barbadensis* and others) serve as antioxidants (Kardosova and Machova 2006). Polysaccharides extracted from *Psyllium* (the name of some members of plant genus *Plantago ovata*, an annual herb grown in India) are effective in lowering cholesterol when used as dietary supplements (Moreyra, Wilson, and Koraym 2005). They also serve as laxatives and drug-loaded hydro gels for effective drug delivery against inflammatory bowel diseases (Singh *et al.* 2006).

The bioadhesive properties of *Chitosan* were initially used for water purification: the substance was simply spread over the surface of water where it would absorb a number of toxic substances such as fats, greases, oils, and heavy metals.

Due to its cationic nature *Chitosan* also showed inhibiting activity against different target organisms. Yeasts and moulds were the most sensitive group, followed by gram-positive bacteria and finally gram-negative bacteria (Rhoades and Roller 2000). Thus, the inhibitory effect on spore germination for *Aspergillus niger* was 40% after 13 hours and 73% after 24 hours of exposure to 3 g/l of *Chitosan* (Plascencia-Jatomea *et al.* 2003).

Being cationic, *Chitosan* has the potential to bind to many different food components such as alginates, pectin, and proteins (Rhoades and Roller 2000). As an antimicrobial agent, *Chitosan* is widely used as natural preservative in the food industry, for example, in the production of antimicrobial wrapping films.

*Chitosan* was recently applied as an antifungal agent to the conservation of paper documents (Ponce-Jiménez, Toral, and Gutierrez-Pulido 2002). Fungal spores commonly found in archives and libraries (*Chaetomium globosum*, *Aspergillus niger*, *Aspergillus terreus*, *Alternaria alternata*, *Penicillium sp.*, and *Chaetomium britannicum*) were inoculated onto samples of paper sized with *Chitosan* salts and with cellulose ethers taken as controls. *Chitosan*-treated paper showed much more resistance against test organisms than paper sized with cellulose ethers. These tests confirmed the advantage of using *Chitosan* salts as antimicrobial additives in paper conservation.

### ***Phenol compounds***

Another large group of natural antimicrobial agents are polyphenols (Duke1985; Cowan 1999). Polyphenols are aromatic compounds excreted as secondary metabolites of plants (Bell and Charlwood 1980). They network with biopolymers protecting plant cells against the predation of foreign microorganisms (Cowan 1999). Polyphenols consist of a number of classes including simple phenols, phenolic acids, flavonoids, lignin, tannins and others (Swain, Harborne, and van Sumere 1979; Harborne 1980).

The largest classes of natural polyphenols are flavonoids and tannins occurring in the form of glycosides (i.e. they have one or more simple sugars attached to them). Their antimicrobial properties are realized through different mechanisms, such as binding microbial proteins, inhibition of microbial enzymes, and chelating of essential metals (Cowan 1999; Urquiaga and Leighton 2000). Tannins and flavonoids extracted from some medicinal plants have received a great deal of attention as sources of naturally occurring antimicrobial agents.

Bryophytes such as *Sphagnum* moss contain an abundant source of both groups of antimicrobial agents, i.e. polysaccharides and polyphenols. Evidence of the antimicrobial properties of *Sphagnum* moss and polysaccharides extracted from moss are reviewed below.

## 2.2. *Sphagnum* moss as a natural antibiotic

*Sphagnum* mosses are water-loving plants growing in boreal ecosystems that are characterized by a relatively cold climate and a high level of annual precipitation (Watson 1971; Shaw and Goffinet 2000). These plants are important globally due to their considerable peat-forming ability and their influence on global climate (Gorham 1991). The areas where *Sphagnum* species are dominant are also called peatlands\*. Peatlands are peat-forming ecosystems including bogs and poor fens where species of the genus *Sphagnum* dominate (Gignac and Vitt 1994). In the northern hemisphere, peatlands are located above latitude 45° N in Alaska, northern Canada, northern Europe, and Siberia (Gunnarsson 2005). In the tundra, *Sphagnum* mosses represent the major biomass, covering up to 100% of the surface (Russel 1990). However, *Sphagnum* mosses do not grow only in peatlands. A number of species are found in forests and in water-logged undergrowth\*\*.

The wide areas covered by *Sphagnum* mosses serve as a giant sink for atmospheric carbon dioxide, which is deposited in the peat (Gorham 1991; Gignac, Halsey, and Vitt 2000; Gunnarsson 2005).

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\* Peatlands are wetland ecosystems where productivity exceeds biodegradation, resulting in deposition of organic matter named peat (Williams and Crawford 1983). Basing on their hydrology, peatlands can be classified as fens and bogs. Bogs are peat-forming wetlands fed by precipitation only. Fens also accumulate peat, but they obtain additional nutrients from ground waters (Gignac and Vitt 2000). Fens are often not dominated by moss, but by sedges and grasses. “Poor” fens refer to acidic fens where vegetation is less diverse in indicator species than in “rich” fens (Gignac, Hasley, and Vitt 2000, after Sjörs 1952). Development of fens into bogs is accompanied by the invasion of *Sphagnum* species (Scheffer, Van Logtestijn, and Verhoeven 2001). *Sphagnum*-dominated bogs are subdivided into raised bogs and flat or blanket bogs (Kulzer *et al.* 2001). Raised bogs have a raised central area filled with water-saturated peat that has accumulated above the water table, while flat bogs follow the local topography.

\*\*Coniferous trees more commonly coexist with *Sphagnum* than broad-leaved trees. In Alaskan *Sphagnum* bogs grow *Tsuga*, *Pseudotsuga*, and *Pinus* spp. (Rigg 1914; Van Breemen 1995). In many cases, especially after fire, *Sphagnum* moss forms an adverse environment for vascular plants (low nutrient availability, high acidity, etc.) with subsequent displacement by *Sphagnum* species. This “aggression” is typical, for example, in forested Labrador (Foster 1984). As they invade, they can quickly transform a high-pH fen with little peat accumulation to a peat bog (Rydin, Gunnarsson, and Sundberg 2006).

The main reason for peat formation is considered to be slow decomposition of dead *Sphagnum* plants (Hasley, Vitt, and Gignac 2000). In other words, *Sphagnum* species are very resistant to microbial decay. There are a number of reasons for this phenomenon: acidification of the *Sphagnum* environment; low annual temperatures and low nutrition in *Sphagnum*-dominated areas; anoxic conditions in *Sphagnum* litter; and finally, the antibiotic properties of *Sphagnum* plants (ibid.).

For practical purposes, the antibiotic activity of *Sphagnum* moss is the most important property. Antimicrobial properties of both *Sphagnum* moss and peat are well known (Saxena and Harinder 2004; Glime 2007). A number of different materials including human and animal flesh embedded in the moss were found intact. Due to its antimicrobial properties and the ability to hold large quantities of water (up to twenty times its dry weight), *Sphagnum* moss was widely employed historically for different applications, e.g. as a bedding material, as a material for wound dressing, as an antimicrobial soil additive, and many others (ibid.). In recent decades, some studies have been undertaken to identify the chemical composition of *Sphagnum* moss and to find the ingredients responsible for its antimicrobial properties. These attempts were not completely successful and further research is required. The results of these studies, as well as *Sphagnum* ecology, chemistry and the evidence for the antimicrobial properties of *Sphagnum* moss are briefly reviewed below.

### **2.2.1. *Sphagnum* taxonomy, morphology, and ecology**

*Sphagnum* mosses are referred to as phylum *Bryophyta* and this includes mosses, liverworts and hornworts (Watson 1971; Schofield 1985; Shaw and Goffinet 2000). Mosses are non-vascular

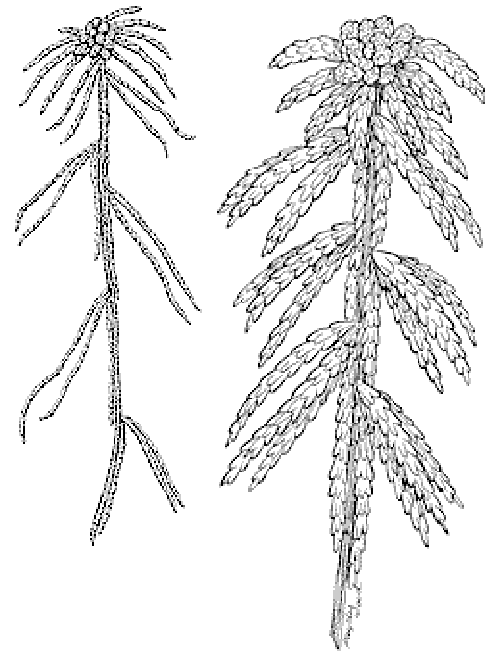
plants, i.e. they have no circulatory systems. They also have no flowers or seeds, and reproduce via spores. Different classifications of mosses have been summarized and discussed by Vitt (Vitt 2000). The genus *Sphagnum* is divided into ten sections according to the following factors: plant cell anatomy; the number of branches in a fascicle; branch shape and color; and plant habitat preference (McQueen 1990). Following Shaw, the genus *Sphagnum* includes 250-450 species, but only about fifty are important in peat formation (Shaw and Goffinet 2000; Gunnarsson 2000). In North America, 84 *Sphagnum* species are currently recognized (Hasley, Vitt, and Gignac 2000). Species typical for raised bogs of North America are *S. fuscum*, *S. capillifolium*, and *S. rubellum*. For North American flat bogs, the common species are *S. pacificum*, *S. palustre* (Fig.2.2), *S. compactum*, and *S. angustifolium*. For entirely ombrotrophic (rain-fed) bogs, *S. angustifolium*, *S. papillosum* and *S. magellanicum* dominate (Kulzer *et al.* 2001).

*Sphagnum* plants grow in very closely-knit masses and form lawns or floating mats that often form hummocks with hollows in between. Certain *Sphagnum* species dominate hummocks which are better drained while others prefer wetter hollows. These groups have different tolerances for light, wetness, and pH.

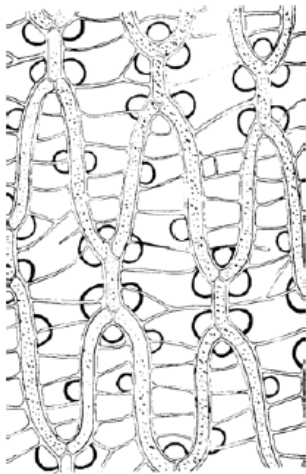
*Sphagnum* plants (Fig.2.3) grow from the top through a stem elongation. Having no true roots and leaves, *Sphagnum* plants absorb water from the water table and from the atmosphere through hyaline cells occurring side by side with green cells. Hyaline cells, which are located in plant leaves, are large, dead, hollow cells interspersed with smaller live photosynthetic green cells (Fig.2.4). Hyaline cells make up about 80% of the plant volume (Van Breemen 1995) and hold 16 to 26 times their dry weight of water (Kulzer *et al.* 2001).



**Fig.2.2.** *Sphagnum palustre* capitulum. From [http://www.botany.ubc.ca/bryophyte/stanleypark/sphagnum\\_palustre/index.htm](http://www.botany.ubc.ca/bryophyte/stanleypark/sphagnum_palustre/index.htm). UBC Dept. of Botany.



**Fig.2.3.** *Sphagnum* spp., the overview (from McQueen 1990).



**Fig.2.4.** Section of *Sphagnum* phyllode (from Kulzer *et al.* 2001). Hyaline cells contoured in grey, green cells-in black.



**Fig.2.5.** The overview of Lindow Moss, near Wilmslow, Cheshire, UK, where the “Lindow Man” was found. (<http://www.wilmslow.org.uk/wilmslow/warealindowmoss.html>).

Due to their unique architecture, *Sphagnum* plants are extremely efficient at retaining water (Rydin and MacDonald 1985). Water wicks up through the hyaline cells by osmotic pressure up to 30 cm above the water table (McQueen 1990). The older, lower portions of the *Sphagnum* plant die and collapse downwards. The remains of partly decomposed *Sphagnum* plants form the peat deposits, the thickness of which can be up to 6 m. The oldest peat deposits are up to 10,000 years old (Fig.2.5) (Clymo and Hayward 1982; Shaw and Goffinet. 2000).

*Sphagnum* bogs have a highly water-permeable and oxygen-saturated surface layer called *acrotelm*, which can be 10-40 cm thick. In this layer, living parts of *Sphagnum* plants are mostly accumulated and they often create a dense floating *Sphagnum* mat. Although *acrotelm* is water-permeable, water velocities in the *acrotelm* are low (up to 1 cm/s), and therefore lateral water transport through this layer is limited (Van Breemen 1995). The *acrotelm* is underlain by a thick (up to 6 m) slowly permeable, water-saturated layer called *catotelm*. Towards the base of the *acrotelm*, dead parts of *Sphagnum* plants bend down and become compressed. The bulk density of *Sphagnum* plant material increases from 0.02 g/cm<sup>3</sup> in *acrotelm* to 0.1-0.2 g/cm<sup>3</sup> in *catotelm* (ibid.). In *catotelm*, dissolved oxygen is depleted and only the slow anaerobic decomposition of dead portions of the moss takes place. The water velocities in *catotelm* are so extremely low (typically 10<sup>-3</sup>-10<sup>-7</sup> cm/s) that they practically block the vertical water transport of mineral nutrients through this layer (Van Breemen 1995). Therefore, both vertical and lateral uptake of mineral nutrients to the *acrotelm*, i.e. to the living portion of *Sphagnum* moss, is very limited. In addition, the raised character of hummocks isolates the upper *Sphagnum* plants from the influence of groundwater.

The chemical content of bog water was found to be close to the content of rainwater. This fact proves that nutrients enter bogs mainly by wet and dry atmospheric deposition. Therefore, in most cases precipitation (rainwater and atmospheric aerosols) is the only source of nutrition for *Sphagnum* moss (Clymo 1984; Shaw and Goffinet 2000; Kulzer *et al.* 2001).

### **2.2.2. *Sphagnum* moss chemistry**

In *Sphagnum*-dominated bogs, nitrogen (N) and phosphorus (P) are the growth-limiting nutrients (Turetsky 2003; Bragazza and Limpens 2004). Following Turetsky, the main sources of nitrogen for *Sphagnum* ecosystems are atmospheric deposition, biological fixation, and weathering (Turetsky 2003).

*Sphagnum* mosses are able to absorb atmospheric nitrogen using symbiotic organisms such as cyanobionts. Cyanobionts (cyanobacterial partners), such as the cyanobacteria *Nostoc* spp., present on *Sphagnum* plant leaves convert atmospheric N<sub>2</sub> into ammonia (NH<sub>4</sub><sup>+</sup>). Then ammonia is utilized by plant tissue as amino-acids (Turetsky 2003; Rai, Soderback, and Bergman 2000).

In general, *Sphagnum* moss is adapted to low concentrations of nitrogen and utilizes nitrogen more effectively than co-existing plants\* such as moor grass, *Narthecium ossifragum* (Malmer *et al.* 2003). Due to effective utilization of nutrients, *Sphagnum* moss produces low mineralized and decay-resistant litter.

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\*Vascular plants and *Sphagnum* mosses have different sources of mineral nutrients. Vascular plants use decaying organic matter in the litter while *Sphagnum* uses atmospheric absorption. In *Sphagnum* mosses the entire plant surface participates in the processes of photosynthesis and the accumulation of mineral nutrients. In vascular plants these two functions are divided between leaves and roots (Malmer *et al.* 2003).

*Sphagnum* mosses also effectively assimilate dissolved nitrogen in both organic and inorganic forms, without any preference being displayed (Malmer *et al.* 2003). Assimilation of inorganic nitrogen is provided by a number of litter bacteria through the processes of denitrification, nitrification, and dissimilatory nitrate reduction. Denitrification takes place in the *catotelm* where oxygen is depleted with resulting production of nitrates ( $\text{NO}_3^-$ ) and the excretion of nitric oxide  $\text{N}_2\text{O}$ . Denitrifiers include a variety of gram-positive bacteria (excluding *Bacillus*) and the *Enterobacteriaceae*. Organic forms of nitrogen are released from the microbial breakdown of organic plant matter (*ibid.*).

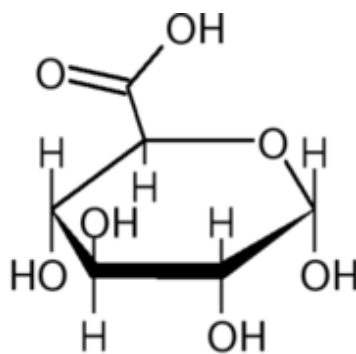
*Sphagnum* plants need micronutrients, mostly calcium, for their growth. According to Clymo, *Sphagnum* assimilates micronutrients using the mechanism of ion-exchange (Clymo 1963, Clymo 1964). Metal ions from soil water are exchanged with hydrogen ions ( $\text{H}^+$ ) contained in the hyaline cell walls. Then metal cations (e.g.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) are assimilated by the *Sphagnum* plant, while hydrogen ions are released into the ambient water.

A number of organic acids are also excreted into the ambient water as microbial metabolites during moss decay. These processes increase the acidity of bog water up to a typical pH range of 3.4-5.6 (Clymo 1964; Clymo 1984; Mullen, Janssens, and Gorham 2000; Kulzer *et al.* 2001). Acidity is one of the most important characteristics of *Sphagnum*-dominated bogs and poor fens, distinguishing them from other wetlands. The complex mixture of all these acids accumulated in peat is called humic acid. The nutrient-poor, acidic and wet conditions of *Sphagnum* bogs depress the growth of other plants in bog environments (Watson 1971; Shaw and Goffinet 2000).

The following structures were identified in the *Sphagnum* plants: carbohydrates (mono- and disaccharides; holocellulose, a mixture of cellulose and hemicelluloses; and pectin); lignocelluloses; and small amounts of amino acids, fats and waxes (Rowell, Han, and Rowell 2000; Kulzer *et al.* 2001). These structures are briefly reviewed below.

### ***Carbohydrates***

The *Sphagnum* plant mainly consists of polysaccharides, made up of glucose and galacturonic acid units, which are believed to render *Sphagnum* its high cation exchange capacity and antimicrobial activity. D-galacturonic acid (a form of polyuronic acid) is a polysaccharide produced in the cell-walls of the living *Sphagnum* plant (Clymo 1963; Clymo, Turnen, and Tolonen 1963; Clymo 1964). D-galacturonic acid is an oxidized form of D-galactose, which is a main component of plant pectin. The chemical structure of D-galacturonic acid is illustrated on Fig.2.6.



**Fig. 2.6.** The chemical structure of D-galacturonic acid (from Painter 1991a).

D-galacturonic acid has an aldehyde group at C1 and a carboxylic acid group at C6, i.e. in this structure one side chain at C6 has been replaced by a carboxylic acid group  $\text{-COOH}$ . The carboxylic acid group contains, in turn, a highly reactive carbonyl group,  $\text{C=O}$ , which confers on D-galacturonic acid its ion-exchange properties. At this position, base cations are absorbed at the

carbonyl exchange site (C=O) from ambient water while weakly-held hydrogen is easily released into the ambient water, increasing its acidity\*. This mechanism of cation exchange is used by *Sphagnum* plants to obtain macro and micro nutrients and it is different for each cation (Urban, Verry, and Eisenreich, 1995). Observations show that in a natural bog environment the majority of bivalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) from the water are absorbed by the plant while the majority of monovalent cations ( $\text{K}^+$ ,  $\text{Na}^+$ ) remain in solution.

The degree of cation retention depends on the acidity of the particular bog environment (where certain vegetation dominates). For neutral fens (pH about 7.0), the concentration of  $\text{Ca}^{2+}$  in water was detected at 40 mg/l, while for acidic *Sphagnum* bogs (pH 3.5), the concentration of  $\text{Ca}^{2+}$  was found close to analytical zero, i.e. all dissolved calcium was utilized by the *Sphagnum* plants (Mullen, Janssens, and Eville 2000).

Due to retention of metal cations, they cannot be released into the ambient water and re-utilized by the living plant as long as the dead plant tissue is not decomposing. Because of this, the peat deposits are a giant sink for essential cations.

Locking up cations is also one of the important reasons for the low productivity of *Sphagnum* moss (Clymo and Reddaway 1971; Clymo 1984). Some authors consider that cation retention is also a reason for the “sterility” of peat bogs because bonded cations become unavailable for litter microorganisms (Painter 1991a).

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\*The unique cation exchange properties of *Sphagnum* plants was considered to be an important source, but not the only one, of the acidity in *Sphagnum*-dominated bogs (Clymo and Hayward 1982). Other studies have attributed the possible sources of acidity to  $\text{CO}_2$  build-up, oxidation of reduced N and S, assimilatory cation uptake, production of organic acids during decomposition, and acid deposition (Urban 1987; Urban, Verry, and Eisenreich 1995). Potential sinks of the acidity include the decompositional release of cations, assimilatory anion uptake, dissimilatory anion reduction, and weathering reactions (ibid.).

### ***Phenol compounds***

Phenol compounds similar to vascular lignin have been identified in mosses (Reznikov and Novitskii 1975; Wilson *et al.* 1989; Van-der-Heijden 1994; Verhoeven and Liefveld 1997; Williams *et al.* 1998). However, the structure of *Sphagnum* phenolics reveal distinct differences from the structure of vascular plants due to their low content of guaiacyl and syringyl groups and to their high content of *p*-hydroxyphenyl and carbonyl groups (Farmer and Morrison 1964; Reznikov and Novitskii 1975). The hydroxyl phenyl units build a polyphenolic network, which protects *Sphagnum* cellulose against breakdown by microorganisms. Carbonyl groups are readily liberated in water-soluble form by acid hydrolysis. These water-soluble phenolics exist in the form of phenolic acid, including the genus specific *Sphagnum* acid (Verhoeven and Liefveld 1997; Williams *et al.* 1998). In some *Sphagnum* species (*S. fallax* and *S. cuspidatum*), the majority of phenolics were found in a free water-soluble form including endogenous *Sphagnum* acid, *t*-cinnamic acid, and *p*- coumaric acid. They are excreted in surrounding media as metabolites in the process of *Sphagnum* growth (Rasmussen 1994; Rasmussen, Wolff, and Rudolph 1995). Excreted water-soluble phenolic acids are accumulated in bog water as constituents of humic acid, which prevents bioassimilation of *Sphagnum* plant material. This is another explanation of the “sterility” of the *Sphagnum*-dominated bog environments.

The phenolic composition of *Sphagnum* moss is not confined to water-soluble ingredients (Verhoeven and Liefveld 1997). Like other plants, *Sphagnum* contains a particular phenol profile, from the low molecular weight phenolic acids to the high molecular weight condensed tannins. Most of these compounds have not yet been fully characterized. Higher molecular weight phenolics are present in peat bogs in hydrolysable and solid forms. Hydrolysable forms

are present in humic acid mostly as tannic acids. Solid forms are condensed tannins containing flavonoid units.

Phenolics possess important antimicrobial properties. Due to their unavailability for microorganisms, phenolics are accumulated in bog litter. In general, the higher molecular weight phenolics are more involved in linking organic matter and slowing decomposition, while the low molecular-weight phenolics are more easily degraded by microorganisms. Therefore, the deepest peat cores contain the most decay-resistant constituents of plant material (Verhoeven and Liefveld 1997).

### **2.2.3. The phenomenon of slow decomposition of *Sphagnum* litter**

The most remarkable feature of *Sphagnum* moss is its extremely slow decomposition rate. Many *Sphagnum* species have been found to decompose much slower than leaves of vascular plants (Coulson and Butterfield 1978; Johnson and Damman 1993; Aerts, Verhoeven, and Whigham 1999; Thormann, Bayley, and Currah 2001; Scheffer, van Logtestijn, and Verhoeven 2001; Limpens and Berendse 2003).

In general, the decomposition rate of *Sphagnum* litter is 10-20 % per year compared to 40-80 % per year for vascular plants (Kulzer *et al.* 2001). In the Polar Regions, *Sphagnum* moss is decomposed much slower, about 3-5 % per year or even less (Table 2.2). Several studies indicate regional and interspecific differences in decomposition rates among *Sphagnum* species (Rocheffort, Vitt, and Bayley 1990; Johnson and Damman 1993; Belyea 1996; Limpens and

Berendse 2003). For example, among three widespread species, *Sphagnum fuscum*, *S. rubellum*, and *S. balticum*, *S. fuscum* was found the most resistant to decay.

Rate, (% year <sup>-1</sup> )	Species	Location	Reference
0.04-3	Moss, different spp.	Arctic	Russell 1990
5	<i>Sphagnum fuscum</i>	Arctic	Roswall <i>et.al</i>
4	<i>Sphagnum balticum</i>		1975
7	<i>Sphagnum lindbergii</i>		
5	<i>Drepanocladus schulzii</i>		
7	<i>Dicranum elongatum</i>		
1.5	Moss, different spp.	Antarctic	Davis 1986
1.3-2.4			Baker 1972
0.1-8.3			Fenton 1980
2	<i>Chorisodontium aciphyllum</i>	Signy Island	Collins 1973
14	<i>Sanionia uncinata</i> (dry)	(South Orkney	
25	<i>Sphagnum uncinata</i> (wet)	Islands, Antarctic)	

**Table 2.2.** Decomposition rates of Polar mosses. Adapted from Bowden *et al.* 1999

The next was *S. rubellum*, and the last was *S. balticum*, a typical hollow species (Rocheftort, Vitt, and Bayley 1990). In *Sphagnum* litter, hummock species degrade slower than hollow species (Limpens and Berendse 2003). Thus, another typical hollow species, *S. cuspidatum* degrades 1.5 times faster than the typical hummock species, *S. fuscum* (Johnson and Damman 1991).

In peat bogs, the major part (up to 80%) of dead plant material degrades into *acrotelm* and this contributes to further peat formation in the anoxic *catotelm* (Clymo 1984; Vitt 2000; Malmer and Wallén 2004). Peat that contains under-decomposed brown substances and humic acids generally exhibits increased resistance to decay. In *catotelm*, *Sphagnum* decomposes with a mass loss of 0.1-0.001% per year or 2-4 orders slower than in *acrotelm* (Rocheftort, Vitt and Bayley 1990). The low decomposition rate of dead *Sphagnum* moss leads to the accumulation of peat with an average rate of approximately 2.5 cm in 40 years (Kulzer *et al.* 2001). The formation of peat is governed by complex enzymatic (biological) and abiotic (auto-oxidative) reactions that

are still not completely understood. A discussion whether peat formation was a biological or chemical process was started long ago. Several theories have been proposed to explain the mechanism of the synthesis of humic substances (Stevenson 1994). The most popular concept holds that the microbial degradation of plant remains determines the process of the peat formation (Waksman 1936). Waksman suggested that humic substances were formed as the result of microbial degradation of the plant lignin. According to this “classical” theory, microbes degrade the readily available chemical ingredients (carbohydrates) first, and finally, the less digestible ingredients of plant lignin. Decomposition processes result in much higher CO<sub>2</sub> concentrations in *acrotelm* than in the atmosphere. Increased concentrations of CO<sub>2</sub> and organic acids excreted by microorganisms contribute significantly to the acidification of bog environments. The most resistant ingredients of lignin are accumulated as soil humus.

In confirmation of Waksman’s theory, further studies indicated that the leading process in the peat formation (diagenesis) in peat bogs is the microbial assimilation of *Sphagnum* moss (Clymo 1964; Clymo 1965). Clymo attributed the phenomenon of the slow degradation of *Sphagnum* litters to the low total number and to the low vital activity of degrading microorganisms in bog environments. The opposite carbohydrate theory emphasizes abiotic reactions in humus formation (Cheshire 1979; Ikan *et al.* 1992; Ikan *et al.* 1996; Painter 1991b). According to this theory, reducing sugars contained in dead *Sphagnum* plant material and amino acids (products of microbial metabolism) are condensed together. This process of non-enzymatic polymerization (Maillard reaction) causes the formation of brown melanoidin-like substances: soluble (called “aquatic humus”) and insoluble (Painter 1991b). These substances comprise humic acid, the essential part of peat. This theory is now not widely accepted (Stevenson 1994).

\*Painter called this fraction “dark-brown chromophore”.

#### 2.2.4. Microbial decomposition of *Sphagnum* plant material

Peat bogs were shown to contain a variety of microorganisms of wide metabolic diversity, including bacteria and fungi. The variety of bacterial genera was identified in peat bogs: *Achromobacter*, *Actinomyces*, *Chromobacterium*, *Clostridium*, *Cytophaga*, *Micrococcus*, *Micromonospora*, and *Pseudomonas* (Williams and Crawford 1983). The bacterial population includes also actinomycetes\*, such as *Mycobacterium*, *Nocardia*, and *Streptomyces*. In aerobic layers of peat bogs, *Mycobacteria* have been also found worldwide. Fungal species include *Penicillium*, *Cladosporium*, *Trichoderma*, *Mucor*, *Mortierella*, *Cephalosporium*, and others (ibid.).

In fact, the population of microorganisms and their taxonomic diversity in *Sphagnum* bogs is considerably lower than in other plant ecosystems (Shaw and Goffinet 2000; Wieder and Vitt 2006). For example, the typical concentration of microorganisms (aerobic bacteria, actinomycetes and microscopic fungi) found by Waksman in the oxygen-saturated *acrotelm* of peat bogs was  $\sim 10^8$  CFU/g; that was about 100 times less than in typical grass litter (Waksman 1930). In the anoxic *catotelm*, the concentration of microorganisms was  $10^4$ - $10^6$  CFU/g, i.e. about 4 orders lower than in *acrotelm* (ibid.). The lack of decomposing microorganisms in the anoxic zone is the reason why 80% of organic matter decomposes to oxygen-saturated *acrotelm* (ibid.). At the same time, the decaying material mostly accumulates in the cold and anoxic *catotelm* where microbial populations and decay rates are low.

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\*Actinomycetes are the gram-positive soil bacteria which form long, thread-like branched filaments resembling fungal mycelia. Actinomycetes are the primary decomposers of tough plant materials such as cellulose, chitin, and lignin (<http://www.digitalseed.com/composter/biology/actinomycetes.html>).

The presence of particular degrading microorganisms in peatlands depends on a number of factors: region, climate, moisture regime, and water chemistry (oxygen and mineral level, pH). In the oxygen-saturated *acrotelm*, a diversity of decomposing bacteria exists. These bacteria use ammonia or amino-acids as nitrogen sources and carbohydrates as carbon sources (Trckova *et al.* 2005).

In the anoxic *catotelm*, the bacterial community is almost entirely represented by denitrifiers, (most commonly by *Pseudomonas*), which provide organic nitrogen in soluble forms available for consumption by *Sphagnum* plants (Turetsky 2003). In wet or submerged plant litter, dead *Sphagnum* moss is degraded by bacteria only because fungal growth is stopped at 100% water saturation of the substrate. In drier *Sphagnum* litter fungi are supposed to be commensal organisms, but the individual role of these two groups in decomposition is still unclear (Romani *et al.* 2006).

Some authors believed that fungi are not important in the decomposition of plant litters (Gilbert *et al.* 1998). At the same time, in the early sixties, Chastukhin found that some basidiomycetes and hyphomycetes (spp. *Penicillium*, *Trichoderma*, and *Torula*) decompose *Sphagnum* litter with the release of soluble nitrogenous compounds, which are essential for the growth of *Sphagnum* plants (Chastukhin 1967; Chastukhin and Nikolayevskaya 1967). Later, a variety of parasitic fungi were found on bryophytes (Redhead 1981; Williams and Crawford 1983). In recent studies, it was shown that, in acidic environments, fungi exceed bacteria in the biomass and the production of organic matter (Latter, Cragg, and Heal 1967; Collins, D'Sylva, and Latter 1978; Wynn-Williams 1982). In peat moss, the concentration of fungi (mostly *Monocillium* spp. and *Penicillium citreonigrum*) was found to be  $4.6 \times 10^7$  CFU/g. That was almost ten times higher

than the concentration of bacteria,  $0.54 \times 10^7$  CFU/g (Cormier *et al.* 1998). These studies confirm that in acidic environments such as *Sphagnum* bogs, fungi, not bacteria, are the principal decomposing organisms (Tsuneda, Thormann, and Currah 2001).

#### **2.2.5. Fungi decomposing *Sphagnum* moss**

The fungal communities in peatlands have been examined worldwide from the 1930s (see references in Thormann, Currah and Bayley 2004). However, the assemblages of fungi investigated in these early studies have never been linked to the vegetation of particular peatland environments (*ibid.*). Examined fungi were only roughly classified as cosmopolitan fungi and fungi taxonomically specific for given peatlands (Dooley and Dickinson 1971; Thormann, Currah, and Bayley 2004). In the majority of peatland environments, cosmopolitan fungi such as *Penicillium*, *Trichoderma*, *Mortierella* and *Mucor* species were identified as predominant.

Even in the last decades, fungi were rarely recognized as *Sphagnum* decomposers in bog environments (Grasso and Scheirer 1981; Redhead 1981). Only in the 1980s were provided the first proofs of fungi connected to *Sphagnum* moss (Felix 1988).

Recent studies show that there are distinct differences between fungal assemblages associated with particular dominant plants grown in bogs, fens or other types of wetlands, and their litter decomposition rates (Scheffer, van Logtestijn, and Verhoeven 2001; Thormann, Currah and Bayley 2002; Thormann, Currah and Bayley 2003).

These differences were demonstrated for *Carex*\* and *Sphagnum*-dominated peatlands in boreal Alberta, Canada and for two European *Carex*- and *Sphagnum* –dominated fens (Scheffer, van Logtestijn, and Verhoeven 2001). In both cases the observation period lasted for two years. This included laboratory and field examination of *Sphagnum* decomposition rates by fungi isolated from these areas. In the field experiments, *Sphagnum* was placed in test litter-bags that were subjected to decomposition in natural conditions (ibid.).

In both areas (i.e. Canadian and European), decomposition rates considerably differed between the two types of vegetation; in the case of *Sphagnum* domination they were much lower than for *Carex* domination. In a Canadian study, 262 records of fungi representing 55 fungal species were identified in living and decomposed *Sphagnum fuscum* (Thormann, Currah, and Bayley 2002).

The most frequently occurring genera found among these fungi were *Penicillium*, *Trichoderma*, *Mortierella* and *Mucor*. The wide spread occurrence of genus *Aspergillus* and *Penicillium* in peat bogs was explained by their ability to produce a number of antibiotics and mycotoxins that suppress the growth of other soil microorganisms in competition (Wainwright 1995; Khalid *et al.* 2006).

To investigate the phenomenon of the slow decomposition of *Sphagnum* litter, the fungal degradation of *Sphagnum fuscum* was tested in laboratory plate experiments (Thormann, Currah, and Bayley 2002). *Sphagnum fuscum* was placed in small mesh bags and inoculated in Petri dishes with selected fungi isolated from the *Sphagnum* litter.

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\**Carex* is a bog plant referred to as sedges. They are mostly found in marshes where they can be the dominant vegetation (Bruhl 1995).

From fifty-five previously identified species, nine of the most representative fungal isolates were selected. All these species decomposed *Sphagnum* plant material quite slowly; however, they showed distinctive differences in decomposition rates (Table.2.3).

Fungal taxa	Mass loss, %	
	4 weeks	8 weeks
<i>Acremonium cf. curvulum</i>	3.3	3.9
<i>cf. Bjerkandera adusta</i>	1.6	1.7
<i>Mucor hiemalis</i>	0.3	0.1
<i>Oidiodendron maius</i>	1.5	2.5
<i>Oidiodendron scytaloides</i>	3.2	3.6
<i>Penicillium thomii</i>	0.5	1.8
<i>Sordaria fimicola</i>	4.2	5.1
<i>Sporormiella intermedia</i>	3.4	3.2
<i>Trichoderma viride</i>	0.4	0.5

**Table 2.3.** Mass loss of *Sphagnum fuscum* exposed to tested fungi for 4 and 8 weeks. Moss samples were placed in polyester mesh pouches and inoculated in Petri-dishes with one of nine fungal taxa (Thormann, Currah, and Bayley 2002).

Three of nine fungal species that showed the lowest decomposition rates were *Penicillium*, *Trichoderma*, and *Mucor*, the cosmopolitan fungi. Their decomposition rates are comparable to those in polar regions (Bowden *et al.* 1999). The authors connected the extremely slow *Sphagnum* decomposition rates detected in these experiments with bioresistant phenol compounds present in *Sphagnum fuscum* (e.g. tannins, *Sphagnum* acid and other phenolics) (Thormann, Currah, and Bayley 2002).

In nature, the phenomenon of slow *Sphagnum* decomposition can be also explained by the disturbance in the succession of degrading organisms inhabiting bog litter (the absence of some fungi in the microbial community in peatlands). In forests and grassland plant litters, there is a

*“classical taxonomic zygomycete – ascomycete/fungi imperfecti – basidiomycete pattern of succession during organic matter decomposition”* (Thormann, Currah and Bayley 2003).

These successive fungal groups\* are able effectively to degrade all plant chemical structures, from easily digested (carbohydrates) to more resistant ingredients (lignin). From these groups, the ability to decompose *Sphagnum* moss is generally higher in basidiomycetes, e.g. higher fungi such as white-rot fungi than in ascomycetes and zygomycetes (Osono and Takeda 2006). In nature, white-rot fungi play a significant role in the recycling of lignin-containing plant tissue and other highly oxidized, very resistant compounds such as humic acid and melanoidin (Blondeau 1989; Steffen 2003; Rice, Tsuneda, and Currah 2006). The ability to degrade these compounds is thought to be dependent on a family of peroxidases secreted by white-rot fungi (Palmer and Evans 1983; Have and Teunissen 2001).

Compared to basidiomycetes, ascomycetes possess a much lower enzymatic capability to degrade phenol compounds. Their enzymes are able to modify relatively easily degradable structures such as mono- and di-saccharides, cellulose, and starch. Only a few ascomycetes (*Myxotrichceae* spp.) have enzymes, which are able to degrade lignocelluloses. The mechanism of *Sphagnum* decomposition by *Myxotrichceae* was found to be close to that of white-rot fungi (Rice, Tsuneda, and Currah 2006). In *Sphagnum* litters, instead of the classic fungal succession, co-existing zygomycetes and ascomycetes were found, while basidiomycetes did not inhabit this environment. Without basidiomycetes, zygomycetes and ascomycetes have a very limited ability to decompose resistant phenolic compounds, resulting in the accumulation of peat in these ecosystems (Thormann, Currah, and Bayley 2003).

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\*Ascomycetes are the largest group of fungi. All common fungal genera such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, and *Stschybotrys* are referred to as phylum Ascomycota. A common example of a zygomycete is black bread mould (*Rhizopus stolonifer*). Fungi imperfecti is a category of fungi that lack a sexual reproduction mechanism. They include *Alternaria*, *Cladosporium*, and *Fusarium*. Basidiomycetes are the “higher fungi” within the Kingdom Fungi. They include white-rot fungi, mushrooms, puffballs, stinkhorns, jelly and rust fungi, mirror yeast and others. In general, basidiomycetes are filamentous fungi that reproduce sexually (Prescott, Harley, and Klein 2002).

To confirm these suggestions, the enzymatic degradation of the main ingredients of *Sphagnum* plants was examined in the laboratory. Cellulose, starch, and tannic acid were degraded by nine selected fungal species isolated from natural bog litter. The presence of polyphenol oxidases (PPO) which are necessary for the degradation of phenolics, was tested by using tannic acid as a medium (Thormann, Currah, and Bayley 2002). The ability to degrade cellulose and starch was found in almost all examined species, but tannic acid was consumed only by *Oidiodendron maius* (Table 2.4). Therefore, only *Oidiodendron* spp.\* are able to excrete PPO.

Fungal taxa	Tannic acid	Cellulose	Starch
<i>Acremonium</i> cf. <i>curvulum</i>	-	+++	++
cf. <i>Bjerkandera adusta</i>	-	++	-
<i>Mucor hiemalis</i>	-	+	+++
<i>Oidiodendron maius</i>	+++	+	+
<i>Oidiodendron scytaloides</i>	+	++	++
<i>Penicillium thomii</i>	-	++	+++
<i>Sordaria fimicola</i>	-	+	-
<i>Sporormiella intermedia</i>	-	-	+
<i>Trichoderma viride</i>	-	+++	+++

+++ , strong reaction; ++, intermediate reaction; +, weak reaction; -, no reaction

**Table 2.4.** Enzymatic degradation of tannic acid, cellulose, and starch by fungal species isolated from living and decomposing *Sphagnum fuscum* growing in a southern boreal bog in Alberta, Canada (adapted from Thormann, Currah, and Bayley 2002).

These experiments provide one of the possible explanations why the decomposition rate of *Sphagnum* litter is so slow. In natural bog environments, only a limited number of fungal species is able to degrade *Sphagnum* phenolics. As long as the phenolics are intact, the other ingredients of a plant cell wall are inaccessible for degradation by soil fungi, which are depressed by starvation. Consequently, the growth of a microbial community in *Sphagnum* litter slows.\_\_\_\_\_

\**Oidiodendron* spp. are symbiotic with the roots of some plants growing in peatlands (Barron 1962). *Oidiodendron* belongs to the Fungi Imperfecti (Deuteromycetes). It is generally classified as a dematiaceous (dark-walled) fungus.

These facts clearly indicate that the phenomenon of low bioassimilation of *Sphagnum* plant material most strongly argues for its antimicrobial activity. Slow decay of *Sphagnum* litter can also be explained by the antimicrobial environment that exists in *Sphagnum* bogs (e.g. cold temperatures, acidity, lack of oxygen), as well as by antimicrobial agents excreted by moss into ambient water during its life and decay. However, specific features of *Sphagnum* cell-wall architecture, for example the presence of insoluble phenolics, can also contribute significantly to the resistance of *Sphagnum* plants against microbial attack.

These results are important to the possible use of *Sphagnum* moss and *Sphagnum* products in conservation practice as antifungal agents. A large group of *Sphagnum*-decomposing fungi, which have been identified as cosmopolitan soil fungi, also commonly inhabit interiors of museums, dwellings, as well as construction materials and museum artifacts. Five taxa that have been isolated from *Sphagnum* litter (*Acremonium*, *Aspergillus*, *Mucor*, *Penicillium*, and *Trichoderma*) make up the vast majority of fungi in museums and interiors. The evidence of their occurrence in museum and dwelling interiors, as well as their brief description and pathogenicity, are listed in Table 2.5. Some of these species are known to be pathogenic to humans. It is remarkable that in peatlands the vital activity (sporulation, growth rate) of all these taxa were suppressed. It is reasonable to suggest that products made from *Sphagnum* moss can cause the same effect on these fungi in museum environments. Therefore, *Sphagnum* moss and *Sphagnum* products (polysaccharides and phenolics) can be effective against fungi, which are a particular threat in museums\*.

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\*From tested species, only *Oidiodendron* spp. has an enzymatic system that is capable of degrading phenolics. At the same time, these fungi are not listed among museum inhabitants. Basidiomycetes, which can degrade recalcitrant *Sphagnum* phenolics, are potentially not susceptible to *Sphagnum* products. However, they are not widely present in museums.

Fungal genera/species	Description	Presence in dwellings and pathogenicity	Hazard class	References
<i>Acremonium</i> spp.	Filamentous fungi commonly isolated from plant debris and soil.	Found in school buildings. Causes opportunistic infections: arthritis, pneumonia.	B	Fincher <i>et al.</i> 1991; Rand 1998; Lugauskas <i>et al.</i> , 2004
<i>Aspergillus niger</i>  <i>A. versicolor</i>	"Black mould" -filamentous fungi ubiquitous in soil; common food contaminant.  Xerophilic- can grow at low water activity (< 0.8).	Commonly reported in damp buildings. Produces aflatoxins. Found in museums. If large amounts of spores are inhaled, a serious lung disease, aspergillosis, can occur.  Found indoors on gypsum board and damp walls, floor carpet, furniture dust, etc. Found in museums. Produces mycotoxins that can cause cutaneous disease, onychomycosis, and pulmonary disease. Mycotoxin sterigmatocystin is known as a carcinogen.	  A	Andersson <i>et al.</i> 1997; Nakagawa <i>et al.</i> 1999; Robbins <i>et al.</i> 2000; Florian 2002  Hodgson <i>et al.</i> 1998; Torres-Rodriguez <i>et al.</i> 1998; Piecková and Jesenská 1999; Engelhart <i>et al.</i> 2002; Florian 2002
<i>Mucor hiemalis</i>	Filamentous fungus found in soil, plants, decaying fruits and vegetables.	Common laboratory contaminant; found in museum environment. Causes Zygomycosis (mucocutaneous and rhinocerebral infections, renal infections, and pulmonary infections).	A	Bigby, Turney, and Matthay 1986; Kontoyiannis <i>et al.</i> 2000; Ribes, Vanover-Sams, and Baker 2000; Florian 2002
<i>Penicillium</i> spp.	Widespread filamentous fungi found in soil, decaying vegetation, and the air.	Commonly found in dwellings, including the museum environment. Produces a broad number of mycotoxins (some are potential pathogens) May cause infections, especially among immunosuppressed patients.	Most of spp. B	Piecková and Jesenská 1999; Garbino <i>et al.</i> 2001; Florian 2002
<i>Trichoderma</i> spp. including <i>T. harzianum</i> and <i>T. viride</i>	Filamentous fungi commonly found in soil and on plants as green mould. <i>T. viride</i> is used for the suppression of soil and seed diseases caused by other fungal pathogens.	<i>Trichoderma</i> spp. indicates moisture-damaged building materials. These fungi are isolated from gypsum board, water saturated wood; floor, carpet and mattress dust; paint; domestic water supply, and HVAC system air. Found in school buildings.	B	Bren 1998; Rand 1998; Groll and Walsh 2001; Florian 2002

**Table 2.5.** Fungi found on *Sphagnum fuscum* and in dwelling and museum environments.

### **2.2.6. Evidence of antimicrobial properties of *Sphagnum* moss relating to organic specimen**

There are numerous historical references to the preservative properties of *Sphagnum* moss, peat, and bog water scattered throughout literature sources (see Glime 2007, a review). However, most of them are not well documented. For example, it is known that the Vikings traditionally used water from peat moss during their voyages because peat water stayed fresh for at least a month; Scandinavian freshwater fishermen traditionally preserved their catches in peat bogs (Mellgren 2001). The whole fresh *Sphagnum* plant is considered to be antiseptic (Grieve 1931; Schofield 1989). The antimicrobial activity of *Sphagnum* moss has been known in folk medicine where moss was traditionally used as a material for dressing wounds (Saxena and Harinder 2004). Thus, ancient Scandinavian warriors healed their wounds by applying *Sphagnum* moss (Mellgren 2001). In the beginning of the twentieth century, a tar extracted from *Sphagnum* peat moss was used as a weak antiseptic and in the treatment of skin diseases such as eczema, psoriasis, and pruritus (Grieve 1931; Bown 1995).

Another piece of evidence of the antimicrobial activity of *Sphagnum* moss is the preservation of different organic materials in contact with the moss. This can be attributed to specific antimicrobial compounds excreted in pore water during moss decay. Preservation of organic materials in contact with *Sphagnum* moss deserves special attention for the potential use of *Sphagnum* moss and *Sphagnum* products for conservation needs. In peat moss, a wide spectrum of archaeological materials with different chemical structures was found. These materials are: cellulose (archaeological wooden artifacts including construction materials, kegs, etc.); vegetable protein (bread, cereal grains, and nuts); glucose and pectin (fruits and berries); animal protein (cheese); and fat (butter, tallow).

The most remarkable evidence of preservation in *Sphagnum* is the existence of dead human bodies kept intact in peat bogs for centuries. Facts of the preservation of different archaeological materials in *Sphagnum* moss and peat were collected by Painter (Børsheim, Christensen, and Painter 2001 a). All these facts can be attributed either to freshly harvested *Sphagnum* plants or to dead (decomposed) *Sphagnum* plants, i.e. peat. The most important facts from two groups are discussed below.

### ***Preservative properties of harvested Sphagnum plants***

The antimicrobial activity of *Sphagnum* moss plants, their excellent bedding properties, and their ability to retain large amounts of liquid have been recognized for many years. The most important and the best documented examples of preservation in harvested *Sphagnum* plants are: wrapping freshly excavated archaeological objects in *Sphagnum* moss; using moss as a material for dressing wounds; and using moss as an antimicrobial soil supplement in gardening.

#### **(a) Use of Sphagnum moss in field conservation**

Regarding the goal of the present study, the most interesting application of harvested *Sphagnum* moss is its use as a packing material in archeological field conservation, especially in the Arctic. One of the most urgent problems of Arctic archaeology is the fast biodegradation of excavated organic specimens (Sutherland 2002). A number of microorganisms including bacteria, yeast, and fungi inhabit Arctic soil and ice. However, during prolonged periods of freezing, i.e. most of the year, they remain in a dormant state. Organic artefacts buried in boreal latitudes are normally wet and highly contaminated by microbial spores. Nevertheless, in most cases, they have been found well-preserved due to the suppressed microbial activity in these cold environments. Once

excavated, they are exposed to new conditions that are favorable for intensive microbial growth: oxygen, increased temperatures, light, and moisture.

To prevent microbial growth, artefacts should be dried. However, accelerated drying can cause their deformation and cracking. To prevent deformation of excavated objects, they must be kept wet until they can be moved to museum storage. However, if organic materials are wet, they can be quickly attacked by degrading microorganisms. The most effective solution to this dilemma is to wrap excavated objects in freshly harvested *Sphagnum* moss because moss sustains stable moisture levels in wrapped artefacts. At the same time, moss inhibits microbial growth. In addition, *Sphagnum* moss is a cheap and accessible material at northern sites. Archaeologists in the Canadian Arctic have used *Sphagnum* moss for a long time as a packing material for the transportation of artefacts to museum storage. Employment of *Sphagnum* moss in different archaeological expeditions showed that the moss protects artefacts from microbial attack for at least three weeks (Newton and Logan 1992; Murdock and Rice). If the time is longer, the moss will start decaying. Nevertheless, in Canada, that *Sphagnum* moss is the officially recommended material for field conservation, is confirmed in the Conservation Manual for Northern Archaeologists:

*“At low temperatures, this moss is effective in inhibiting biological growth (e.g. mould) over the short-term. It is a natural cushioning material and protects the artifact from physical damage during transport”* (Scott and Grant 2007).

Following the advice in this manual, an excavated artefact should be packed in wet moss, then in plastic film to prevent water evaporation and finally, in aluminum foil. Packing of a fragile artefact in *Sphagnum* moss by this technology is shown in Fig.2.7.



**Fig.2.7.** A fragile bark net float is supported by a piece of coroplast, cushioned by a layer of *Sphagnum* moss, wrapped in plastic film to prevent drying, wrapped in aluminum foil, and finally securely taped with the identification records (photos from Scott and Grant 2007).

The acidic environment created by water-saturated *Sphagnum* might cause damage for some kinds of artefacts. For example, organic acids excreted by the moss can be a cause of corrosion of metal objects. The moss phenolics such as tannins could affect wooden artefacts, causing a black stain on their surface (Scott and Grant 2007). For this reason, Rice and Murdock advised that archaeological iron and wood should not be wrapped in wet moss (Murdock and Rice). The experience of Arctic archaeologists confirms the antimicrobial properties of *Sphagnum* moss\*. However, these recommendations should be carefully tested in the laboratory and in the field, and better documented.

*(b) Use of Sphagnum moss as dressing material in surgery*

Antimicrobial activity and the ability of *Sphagnum* moss to retain large amounts of liquid determined its wide use in the past for wound dressings. Dried moss was employed extensively to bandage wounds during World War I by both Germany and the Allies (Riegler 1989).

\*It should be noted that Arctic archaeologists usually wrap organic artefacts in any available moss. It is hard to determine whether the moss used is related to real *Sphagnum* because it is never identified by botanists. It seems that all mosses possess some antimicrobial activity, but *Sphagnum* is more active (Turetsky 2003).

At the beginning of World War I, the huge number of wounded caused a search for a suitable substitute for traditional cotton, which was then in short supply. *Sphagnum* moss as a material for wound dressing was introduced in surgical practice in the 1880s in Germany. In 1885 *Sphagnum* was also recognized as an official dressing by the French War Department. After testing in England, *Sphagnum* moss was found to help wounds heal because moss

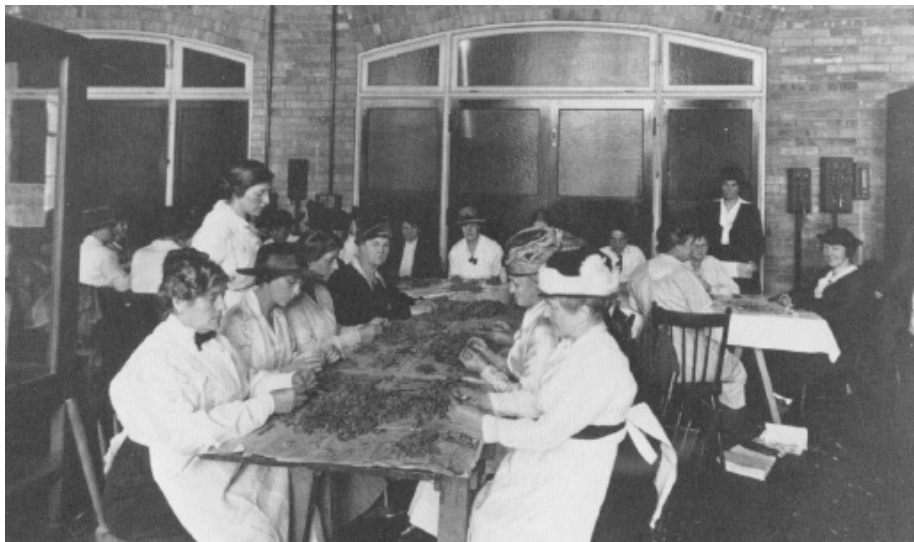
*“retains liquids much better”, “distributes liquid more uniformly”, and is “cooler, softer, and less irritating”*

than traditional cotton dressings (Riegler 1989). The experience of the manufacture and use of *Sphagnum* moss bandages was described in many sources (Porter 1917; Hotson 1921; and many others). Canada was deeply involved in the manufacturing of moss bandages. In North America, four species of *Sphagnum* moss were selected as a satisfactory raw material: *S. papillosum*, *S. palustre*, *S. imbricatum*, and *S. magellanicum*. From these four, *Sphagnum papillosum* (a relative of *Sphagnum palustre*) was found to be

*“the best for absorbency, softness, and strength”* (Riegler 1989).

In Canada, manufacturing of moss bandages was started in 1915 by women volunteers from the University Women’s Club, Toronto (Fig.2.8). Freshly harvested moss was dried, disinfected by corrosive sublimate ( $\text{HgCl}_2$ ), and put up loosely in small, flat muslin bags. Sometimes, moss was not specially disinfected. Despite its very successful employment, after World War I, *Sphagnum* moss dressings lost their importance. The probable reason was the difficulty of converting moss bandages manufacturing to an industrial basis. *Sphagnum* dressings were cheap only because they were assembled by volunteer labor. It is remarkable that no difference was noted between the effect of disinfected and not disinfected dressings. This indicates that antimicrobial activity of *Sphagnum* moss is comparable with that of disinfectant. There is also some evidence of the use of *Sphagnum* moss in surgery during World War II and afterwards (Podterob and Zubets

2002). Today it still recommended for wound dressing in survival medicine (US Army Survival Manual 1970).



**Fig 2.8.** Women preparing *Sphagnum* moss for wound dressings at the University of Toronto during World War I (photo from Riegler 1989).

(c) Use of *Sphagnum* moss for soil improvement

*Sphagnum* moss is recommended to gardeners as a soil additive for better water retention and as a dressing for plant wounds ([http://en.mimi.hu/gardening/sphagnum\\_moss.html](http://en.mimi.hu/gardening/sphagnum_moss.html)). Also, semi-decomposed *Sphagnum* plants excavated from bogs act as a first-rate soil conditioner and can be also used in seed and potting composts (Schofield 1985; Schofield 1989). The powder made from *Sphagnum* moss plants is considered to be a natural fungicide, protecting plant roots and seeds against fungal diseases. Thus, the addition of small amounts of homogenized *Sphagnum fallax* plants to the *Carex* and *Sphagnum* litters significantly decreases the decay rate of both litters (Verhoeven and Toth 1995). This effect can be explained by the antibiotic effect of chemicals leaching from *Sphagnum*. The authors suggest that one of these chemicals is *Sphagnum* acid, a phenolic compound found in all *Sphagnum* species.

### ***Evidence for preservation in peat***

There are a number of instances where a wide spectrum of organic materials has been found in a well-preserved condition in peat. They include both plant and animal materials.

#### **(a) Materials containing proteins and lipids**

“Bog bodies” or “bog people” provide the best evidence for the preservative effects of protein-containing materials in *Sphagnum* peat. Bog bodies are the mummified human bodies found in *Sphagnum* bogs in Britain, Ireland, the Netherlands, northern Germany, and Denmark. Human and animal remains were discovered in peat during peat cutting for fuel as far back as the 18<sup>th</sup> century (Turner and Scaife 1995; Menon 1997; Chamberlain and Pearson 2001). About 1000 bodies or their parts have been found in Iron Age peat in northern Europe. The oldest finds were radiocarbon-dated in northern Europe at about 2500 years old.

The degree of disintegration of bog bodies varies. In many cases, bones are reported to be almost completely gone or they became as soft as cartilage. At the same time the skin, eyes, brains, stomach, intestines as well as hair and fingernails can be intact. The skin of bog bodies is dark brown in color due to the tanning effect of the peat on the skin protein. In general, unlike most ancient human remains, bog bodies have skin and tissues found in rather well-preserved condition. Most of the bog bodies are poorly documented. Recently thirty five bog bodies from different northern European bogs were radiocarbon-dated (Van der Plicht *et al.* 2004). Wood, fur, textile and peat as material associated with bog these bodies were also examined. The most notable bog bodies are Lindow Man and Tollund Man. They were found in good preservation state. They were scientifically examined and the results of this examination were widely published (Glob 1967; Stead, Bourke and Brothwell 1986).

Tollund Man was found in 1950 in Denmark in a peat deposit at a depth of 2 m (Fig.2.9). The death date of the body was determined by radiocarbon dating to be about 350 B.C. X-ray examination of the body's tissues showed that his brain, heart, lungs, liver, stomach, and intestines were in a good state of preservation. Lindow Man was found in 1983 in Lindow Moss (Cheshire, England), which was used for the commercial cutting of peat (Fig.2.10). This body found in peat was also well-preserved. Lindow Man was examined in detail by specialists at the British Museum. Radiocarbon dating showed that his death occurred between 2 B.C. and 113 A.D (Turner and Scaife 1995). The remains of Lindow Man showed good preservation of the collagen and the non-collagenous protein (Stankiewicz *et al.* 1997; Stucker *et al.* 2001). This demonstrates the excellent conservation of dermal collagen in the bog. Lipids were also found in rather good condition, while phospholipids were degraded. Other acyllipids, for example triacylglycerols and steryl esters, remained intact (Evershed and Connolly 1988; Evershed 1990).



**Fig. 2.9.** Tollund man wearing a small fur cap (photo from Glob 1967)



**Fig.2.10.** Lindow Man appearance (photo from Turner and Scaife 1995).

In some cases, along with bog bodies, parts of their clothes have been found, e.g. tunics, stockings, caps, and shoes. They were made of different protein materials: leather, fur, and wool. Garments that were made of plant fibers were totally decomposed. One of the oldest women's caps (1400 B.C.) was found intact in peat in Bredmose, Denmark \*(Fig.2.11).



**Fig. 2.11.**Female bonnet made in sprang technique from the Bredmose bog, Denmark (photo from Glob 1967).



**Fig. 2.12.** Pesse dugout canoe (photo from Waterbolk 1981).

### (b) Plant-originated materials

These materials include archaeological wood artefacts and the contents from the guts of bog people. Wood cellulose is one of the best preserved materials found in peat bogs. The majority of the excavated wood objects were the trackways across the bogs linking up either side of ancient villages. The trackways were made from planks of wood or from thin branches that were woven together. More than 200 such ways have been found in Irish bogs (Raftery 1990). The most remarkable one was built in 148 B.C. and was one km long. It was made from oak and

\*This cap was made of wool yarn in the technique called "sprang" (Hald 1980).

birch planks, which are still well-preserved (ibid.). Five more very old (ca. 2600 B.C.) trackways were found in Ireland, England, the Netherlands, and Germany (Casparie and Moloney 1994). Among the most notable wooden artifacts ever found in peat is the Pesse dugout canoe (Fig.2.12). This 3m×44cm wooden vessel is one of three oldest boats ever found. It was carbon dated to between B.C. 8040 and 7510 (Christensen 1990). Another notable example is the Ubbena wooden disc wheel (on exhibit at the Drents Museum, Assen, the Netherlands). This disc wheel with a diameter of 75cm is one of the oldest known wheels in Europe (ca. 2700 B.C.). The last meals of both Tollund Man and Lindow Man have been identified (Turner and Scaife 1995; Menon1997). Their guts contained a number of domesticated and wild seeds found intact: barley, linseed, wild flax, buckwheat, and sunflower\*.

### **2.2.7. Antimicrobial ingredients of *Sphagnum* cell wall**

The mechanisms protecting *Sphagnum* moss against microbial degradation are still not well understood (Dickinson and Maggs 1974; Tsuneda, Thormann, and Currah 2001). There are two classes of compound contained in the *Sphagnum* cell wall that are believed to be responsible for the resistance of *Sphagnum* plants against decay: polysaccharides and phenol compounds.

However, the influence of these compounds on microorganisms is still unclear because in nature it is masked by a number of environmental factors preventing microbial growth. They are low temperatures, acidity, lack of oxygen, lack of nitrogen and other nutrients, etc.

#### ***Polysaccharides***

Polysaccharides (polyuronic acid derivatives) contained in *Sphagnum* plant tissue may depress microbial activity in the peat bog environment. Polyuronic acid can make up glycoconjugates

\*In Lindow Man's gut *Mistletoe* (poisonous plant) pollen was found, which has led to the theory that there was a Druidical association with his death.

by the reaction of nitrogen binding. As a result, the dead plant material may not be available for degrading microorganisms (Painter 1991a). Polyuronic acid derivatives are also responsible for the strong chelating properties of the *Sphagnum* plant through an ion-exchange mechanism (Clymo, Turunen and Tolonen 1963; Clymo 1964). Sequestering of metal cations by plant tissue may have a negative impact on microbial population due to competition for essential metals (Basiliko and Yavitt 2001). In addition, ion-exchange properties of *Sphagnum* polysaccharides cause acidification of peat bogs. This is an additional reason for the “sterility” of peat.

### ***Phenol compounds***

*Sphagnum* cell walls produce a number of phenolics. They were initially identified in *Sphagnum* moss by Czapek and called “sphagnol” (Czapek 1899). In the past, the presence of “sphagnol” was attributed to a specific, unusual form of lignin present in *Sphagnum* plants (Lindberg and Theander 1952; Farmer and Morrison 1964). Recent studies have shown that phenolics likely protect the amorphous components of the *Sphagnum* cell wall such as mono- and di-saccharides, cellulose, and pectin. A polyphenol network provides a physical and chemical barrier to degrading microorganisms. This is considered to be the main reason for the slow decomposition of dead *Sphagnum* plant material in peat bogs.

### **2.2.8. The hypothesis of the polysaccharide origin of the antimicrobial activity of**

#### ***Sphagnum* moss**

In previous decades, the preservative properties of *Sphagnum* moss were extensively examined by Painter and his colleagues (Painter 1991a). The goal of this work was to employ *Sphagnum* moss for preservation of fish catches in the sea without refrigeration. This promises saving

millions of dollars per year in refrigeration and transport costs. Using *Sphagnum* instead of freezing promised good financial benefits in Norway with its well developed fishery and abundance of cheap *Sphagnum* material. Preliminary experiments showed that

**“Fish that had been stored in the peat or moss stayed fresh for about a month, and bacterial growth after removal was delayed by between 7 and 12 days”**(Jones 2001).

An additional benefit was the promise that fish could be shipped to market fresh (unfrozen).

According to Painter,

**“Definitely, deep freezing and quick filleting are the best way to preserve fish ... but it is also darned expensive”, “Norway, a major exporter of fish caught by trawlers and raised on fish farms, has about 2 billion tons of peat. Little is used, so Painter was seeking industrial applications”** (Mellgren 2001).

These ideas were triggered by the observation of good preservation of bog bodies in *Sphagnum* peat moss, and particularly, by the examination of Lindow Man in the British Museum in the 1980's. Painter said:

**“I thought if it will preserve a body, it ought to be able to preserve a fish”** (Mellgren 2001).

During these studies, antibiotic ingredients were extracted from *Sphagnum* moss and their antibacterial activity was tested. After preliminary experiments, Painter did not believe the role of phenolics as antimicrobial agents in *Sphagnum* because their concentrations in mosses never exceeded 2% of the plant dry weight (Painter 1991a). To prove his assumptions, Painter extracted phenolics from *Sphagnum* moss\* and tested this extract against putrefactive bacteria *Pseudomonas aeruginosa*. Antibacterial effectiveness of phenol extract was determined by the Rideal-Walker method\*\* (Varley and Reddish 1936).

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\**Sphagnum acutifolium* collected in the north Trøndelag, Norway.

\*\*This method was suggested by Rideal and Walker in 1903; with some modifications, it was later accepted by the British Standards Institution. The method determines the Rideal-Walker Coefficient (R.W.C), the relative effectiveness of the tested disinfectant to the standard disinfectant. As a standard disinfectant, a water solution of phenol (carbolic acid) is used in known proportion. R.W.C.= 0.05-0.5 means that the tested antimicrobial agent indicates the antimicrobial effect of 5 - 50% of the effect of the standardized phenol solution. If R.W.C.=20, this means that the tested antibiotic has 20 times higher antimicrobial activity than the standard. The details of the test procedure are described in <http://nathpeters.com/rwc.htm>

The media used as well the details of experiments were not reported. The combined phenol extracts (~2% of the plant's dry weight) had quite low "phenol coefficients" of 0.05-0.5 (Painter 1991a; Painter 1991b). Later, Painter and Børsheim reported that

*“ an exhaustive search for the presence of such a compound in aqueous and ethanolic extracts of Sphagnum mosses has proved fruitless; phenolic compounds are found in such extracts, but their bactericidal potencies ("phenol coefficients") are too low to have any significant effect at the concentrations in which they would occur in bog water”* (Børsheim, Christensen, and Painter, 2001a).

As an alternative to phenol constituents of *Sphagnum* moss, Painter developed a theory of a polysaccharide origin of *Sphagnum* antimicrobial activity. This theory was completely based on the works of Clymo. Clymo found that the *Sphagnum* plant contains polyuronic (D-galacturonic) acid, a water-soluble polysaccharide polymer produced in the cell walls of the living *Sphagnum* plant (Clymo, Turunen, and Tolonen 1963; Clymo 1964). Because of high ion exchange ability, polyuronic acid is responsible for the strong retention of metal cations by the *Sphagnum* plant. The simultaneous liberation of hydrogen-ions in the ambient water was supposed to be the reason for the acidification of peat bogs environments (ibid).

Clymo's studies were mostly concerned with bog hydrochemistry and ecology. Painter developed Clymo's hypothesis by focusing on the antibiotic properties of *Sphagnum* polysaccharides. According to Painter, D-galacturonic acid comprises two inter-convertible isomeric rings, pyranose and furanose. The pyranose ring consists of the residuals of 5-keto-D-mannuronic acid (or 5 KMA), a weak acid found in bog water (Andresen *et al.* 1987; Painter 1991a). 5 KMA possesses an unstable, highly reactive carbonyl group (see Fig. 2.6) which is responsible for the antimicrobial properties of the *Sphagnum* plant. Painter called this highly reactive form of 5 KMA "Sphagnan". In *Sphagnum* moss, it comprises about 5 % of plant dry weight. In terms of cell wall architecture, *Sphagnan* is a part of plant hemicelluloses. Hemicelluloses are soluble in water; however, their solubility is low.

Following Painter's hypothesis, *Sphagnum* is slowly (for millennia) liberated from the dead moss into the ambient water by autohydrolysis of hemicelluloses. In *catotelm*, without oxygen, *Sphagnum* is converted into the brown substances of peat and humic acid. This process is called peat diagenesis (Stevenson 1994). Based on the reactivity of *Sphagnum* polysaccharides, Painter proposed two explanations for the existence of low microbial populations in bogs.

Painter's first hypothesis was that the antibacterial activity of *Sphagnum* is derived from its ability to strongly bind ions of essential metals by the ion-exchange mechanism (Painter 1991a; Painter 1995). The living *Sphagnum* plant binds these ions (e.g.  $\text{Ca}^{2+}$ ) so they are no longer available to microorganisms in competition for nutrients. As a result, microbial population and activity is inhibited. The second explanation for the antimicrobial behavior of *Sphagnum* is based on the ability of its carbonyl group to react with nitrogen contained in bogs to create carbohydrate-protein compounds (Painter 1983a; Painter 1983b; Painter 1998; Painter 2000). By this reaction, exo-enzymes of degrading microorganisms became deactivated by *Sphagnum* and therefore microbial population and activity were depressed in peat bogs as well.

### ***Binding metal ions***

The fact that in most cases calcium was partly or completely removed from the bones of bog bodies suggest that calcium is sequestered by peat. In fact, the concentration of cations in acidic bog waters is considerably lower than in other terrestrial waters, which have neutral reactivity. Among metal cations,  $\text{Ca}^{2+}$  is most closely related to pH of peat bogs (Mullen, Janssens, and Gorham 2000).

At the same time, all microorganisms need calcium for their growth. Therefore, sequestering calcium and other metal ions by *Sphagnum* can be a reason for the low populations of microorganisms in peat bogs.

To test this idea, Painter examined the response of the bacteria *Azotobacter vinelandii*, strain E\*, to calcium sequestering by *Sphagnum*, “aquatic humus”\*\*, and EDTA\*\*\*. The bacterial culture was grown in Jensen's Medium\*\*\*\*, which contains 2 g/l of calcium carbonate. The response of bacteria to the tested materials was determined by the Rideal-Walker method. Painter did not report the details of his experiment. He only concluded that bacterial growth was effectively inhibited, between the phenol coefficients from 10 to 20 (Painter 1991a). However, when excess  $\text{Ca}^{2+}$  was added to the medium, the inhibiting effect of test materials was stopped (ibid). In this experiment, Painter demonstrated that *Sphagnum* products (*Sphagnum* and aquatic humus) depressed the bacterial community due to deprivation of calcium. He suggested the same mechanism exists in a natural bog environment.

### ***Binding nitrogen***

Compared with other ecosystems, peat bogs are characterized by very low rates of denitrification and nitrogen fixation due to low microbial activity (Bragazza and Limpens 2004).

\**Azotobacter vinelandii* is an aerobic nitrogen-fixing bacteria commonly found in soil (Prescott, Harley, and Klein 2002).

\*\*“Aquatic humus” is a water-soluble constituent of humic substances present in peat. According to Painter’s theory, “aquatic humus” contains *Sphagnum*.

\*\*\*EDTA (ethylenediamine tetra-acetic acid) is the amino-acid with an ability to bind ions of metals via its carboxylate amine groups. EDTA is widely used as a chelating agent to sequester  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and other cations in cleaning, complexation of detergents, and many other industrial and environmental applications (Holleman and Wiberg 2001).

\*\*\*\*This medium is recommended for the detection and cultivation of nitrogen-fixing bacteria (Norris and Jensen 1958).

In peat deposits, the sources of nitrogen are proteins of different organisms inhabiting peat bogs (bacteria, fungi, worms, dead insects, etc.), and structural glycoproteins of dead plant material. These glycoproteins are usually associated with L-arabinose and D-galactose comprising *Sphagnum* plants (Rowell, Han, and Rowell 2000). During diagenesis, nitrogen is converted into peat-forming brown substances consisting of a water insoluble moiety, i.e. dark brown aromatics referred to as chromophore\* and a soluble moiety, i.e. aquatic humus. The latter consists of water-soluble sugars and *Sphagnum* that are partly bonded with proteins (Painter 1991a). Therefore, in aquatic humus, nitrogen in the form of amino acids and polypeptides has become covalently bonded to the carbonyl group in polyuronic acid. These assumptions are supported by the fact that in nature many proteins contain a sugar attachment. The covalently linked reaction of a carbonyl group with a protein occurs in glycoconjugates including glycoproteins and glycopeptides, which are widely used in pharmacology (JCBN 1985).

Painter suggested that if the nitrogen is bonded by *Sphagnum* reactive polysaccharides during peat formation, the nitrogen-containing enzymes (e.g. exo-proteases) secreted by microorganisms must be deactivated by the same reactions (Painter 1991a). According to Painter, bacterial exo-enzymes present in peat bogs became immobilized because they rapidly form Schiff's bases\*\* with the reactive carbonyl groups of polyuronic acid. Bacteria use these enzymes for hydrolyzing organic materials buried in peat. Consequently, bacteria cannot degrade protein in bog environments (or else they degrade it very slowly) and suffer from severe nitrogen limitation.

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\*Chromophore is the colored part of molecule. Chromophores exist in one of two forms: conjugated double bonds with amino acids and protein or metal complexes (Streitwieser and Heathcock 1985).

\*\*A Schiff base (or azomethine) is a functional group that contains a carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group (<http://goldbook.iupac.org/S05498.html>).

Therefore, *Sphagnum* -bonded nitrogen is unavailable to denitrifiers and putrefactive bacteria (Painter 1991a; Painter 1998). This is the second (and the most important) explanation of the low bacterial population and activity in peat bogs.

The studies of Lindow Man and other bog bodies showed that their unavailability to putrefactive bacteria is closely connected to the effect of the tanning of skin collagen. Molecules of tanning agents join to functional groups of collagen through hydrogen bonding. As a result, water is expelled from the interstices, and the collagen fibers became indigestible to putrefactive bacteria (Harlan and Fearheller 1977).

The tanning reaction in peat can be easily explained by phenol compounds abundantly present in *Sphagnum* plants and in bog water. However, Painter denied the role of phenolics in the preservative effect of *Sphagnum* moss. If we accept that *Sphagnum* polysaccharides are responsible for antimicrobial properties of peat moss, then the tanning effect on bog bodies should be explained on the basis of polysaccharic theory only. According to Painter, the tanning reaction in peat bogs is caused not by phenolics but by an alternative mechanism. He proposed that it is a reaction between *Sphagnum* contained in aquatic humus and the skin collagen. The reaction between sugar and protein which results in tanning of protein is called the Maillard reaction\*.

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\*The Maillard reaction is a non-enzymatic browning reaction, caused by the condensation of an amino group and a reducing sugar that changes the structure of protein. This reaction, described for the first time by Louis Maillard in 1912, is mostly applied in the food industry including the manufacturing of caramel and creating golden brown colors on cooked meat and fish. The chemistry underlying the Maillard reaction is very complex. It encompasses a whole network of various reactions including a cascade of consecutive and parallel reaction steps. The final products of the reaction depend on the chemical composition, concentration, and nature of the reactants as well as the factors controlling the reaction, i.e. pH, temperature, the presence of oxygen and metals, and the presence of reaction inhibitors. The influence of these factors on the reaction rate and final products was widely studied (Benzing-Purdie, Ripmeester, and Ratcliffe 1985; O'Brien 1998; Martins 2003; and many others).

Painter's assumptions were based on the fact that in general, periodate-oxidized polysaccharides (like *Sphagnum*) are good tanning agents,

*“because the multiplicity of aldehydic groups in these polymers offers broad scope for cross-linking”* (Painter 1991a).

Actually, recent studies confirm the tanning effect of some polysaccharides such as glutaric aldehydes (Kuznetsova *et al.* 2003; Lischuk, Plavan, and Danilkovich 2006). To support the occurrence of the Maillard reaction in bogs, Painter pointed to the presence in peat of the products of this reaction, such as organic acids, ammonia  $\text{NH}_4^+$ , and  $\text{CO}_2$ .

Painter also argued that there was a similarity between humic substances and melanoidins, which are the final products of the Maillard reaction (Benzing-Purdie, Ripmeester, and Ratcliffe 1985). In nature, the Maillard reaction was proposed as one of the possible mechanisms for the formation of humic substances created in bogs. This is the carbohydrate theory of diagenesis (Cheshire 1979; Stevenson 1994; Ikan *et al.*, 1996). Therefore, regardless of the “classical” Waksman's theory, which suggests that humic substances were formed as the result of microbial degradation of the plant lignin, Painter suggested the priority of chemical interactions, generalized as the Maillard reaction, in the peat diagenesis (Painter 1991a).

Painter tried to prove his ideas in a series of simple experiments. He investigated aquatic humus isolated from water sampled from a 1.5 m deep hole dug in the peat located in North Trøndelag, Norway. Following Painter's estimations, this humus would have been leached by rainwater from peat approximately 1000 years old. This aquatic humus was a complex glycolconjugate containing 46% of glycuronoglycan (*Sphagnum*), 38% of dark brown chromophore and 16% of nitrogen corresponding to polypeptide.

Painter tested the tanning capability of aquatic humus using precipitation of aqueous gelatin\* as a simple indicator of tanning. As a result, the aquatic humus itself showed only a weak tanning effect. However, when aquatic humus was subjected to chlorous acid (HClO<sub>2</sub>) oxidation, an abundant precipitation of aqueous gelatin by aquatic humus occurred. To explain this contradiction, Painter suggested that in the glycuronoglycan accumulated in 1000 year old water, the nitrogen binding reaction has been already accomplished, i.e. glycuronoglycan was already bonded to polypeptide. In chlorous acid, however, these bonds were released to react with gelatin.

According to Painter, this experiment suggested that glycuronoglycan (*Sphagnan*) was the original tanning agent in aquatic humus. Therefore, despite the controversial result, Painter believed that this experiment proved the existence of a *Sphagnan*- nitrogen binding reaction in natural peat bog water (Painter 1991a).

### **2.2.9. The isolation of *Sphagnan* from *Sphagnum* moss**

*Sphagnan* can be isolated from aquatic humus contained in peat bog water or from *Sphagnum* plant material. However, Painter showed that in aquatic humus, the reactive carboxyl groups of polyuronic acid were already bonded to nitrogen and therefore most of *Sphagnan*'s tanning capacity was lost. This is the reason why Painter extracted *Sphagnan* from fresh moss, not from aquatic humus. According to Painter, in natural conditions, *Sphagnan* is liberated into pore water over a long period of time, up to a millennium.

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\*There are two methods to evaluate the tannin content in aqueous solutions. They are based on either precipitation of tannin with gelatin, or its absorption on hide powder with subsequent filtration of absorbed solids (hide powder method). Both methods are based on the binding of tanning agents to protein-containing materials, gelatin and hide powder. The hide powder method is a standard method in leather manufacturing, which was elaborated at least a century ago (Tempel 1982).

Therefore, during the laboratory extraction of *Sphagnan*, this process should be compressed by a simulation procedure. It was shown experimentally that, at pH 2.0 - 4.5, hexuronic acid (which resembles 5 KMA) was able to hydrolyze glycosidic linkages in the glycuronoglycan chains by intramolecular autocatalysis (Smidsröd *et al.* 1969). In this experiment, the activation energies and temperature coefficients for glycosidic hydrolyzeation (resembling the liberation of *Sphagnan*) were determined. Painter suggested that the reaction of *Sphagnan* autohydrolysis is a first-order reaction. If the temperature of this process is increased from 20°C to 100°C and using Smidsröd's results, the time of the reaction can be decreased from 2000 years in nature to ~10 days of laboratory simulation (Painter 1991a). The high temperature autohydrolysis of *Sphagnum* holocellulose, i.e. high-temperature dissolution of soluble part of holocellulose (hemicelluloses), is the main procedure in the process of the preparation of *Sphagnan*. Therefore, for preparation of *Sphagnan*, holocellulose is extracted first, followed by extraction of hemicelluloses. To obtain *Sphagnum* holocellulose, Painter used the common procedure for the isolation of holocellulose from plant fibers. This technique is widely used for manufacturing of paper and described for example by Han and Rowell (Han and Rowell 1997). Preparation of holocellulose in the paper industry includes two basic stages: removal of extractives and removal of plant lignocelluloses by an oxidizing procedure (bleaching). After that, the water-soluble part of holocellulose (hemicelluloses) is isolated by high-temperature autohydrolysis. This fraction contains *Sphagnan* (Painter 1991a; Børsheim, Christensen, and Painter 2001a).

For preparation of holocellulose, Painter used two *Sphagnum* species, which were collected in North Trøndelag, Norway: *S. acutifolium* and *S. palustre*. Before processing, moss was either frozen at -20°C until required or dried in a current of air at 60°C. Then dry moss was milled to pass through a 1.25 mm sieve and treated with boiling acetone to remove plant waxes. The

residue was boiled in methanol to remove monosaccharide ingredients and then dried in a fume hood. Painter believed that phenolic compounds associated with plant lignin should be removed due to their evident uselessness as antimicrobial agents. For lignin removal, he employed a standard chlorite method elaborated for bleaching of pulp in the paper industry (Han and Rowell 1997). In this method, sodium chlorite  $\text{NaClO}_2$  serves as a bleaching agent. The reaction occurs at an increased temperature (70-75°C) in acidic media (acetic acid is added). The final product of this stage is delignified *Sphagnum* holocellulose. The water-soluble part of this product is *Sphagnum* hemicelluloses, which contains *Sphagnan*. For isolation of *Sphagnan* from hemicelluloses, holocellulose was converted into thick slurry in oxygen-free distilled water. The slurry was heated under a nitrogen flow to 98°C. The temperature was a little less than 100°C to avoid experimental difficulties with boiling water. Nitrogen flow prevented the oxidation of highly reactive carbonyl groups of 5 KMA. For more complete extraction of hemicelluloses, the residual solid was collected by filtration and re-suspended in another portion of water. This procedure was repeated for ten days at daily intervals. The combined liquid filtrates were concentrated in a rotary evaporator at 30°C and freeze-dried. The final product (*Sphagnan*) was a light-brown solid that was a free-acid form of 5 KMA ( $\text{H}^+$  form of 5 KMA). From 50g of *Sphagnum* dry leaves, 5g of *Sphagnan* were prepared. The final product

*“gave dense precipitates with aqueous gelatin and was absorbed on to hide powder”,*

i.e. was able to effectively bind nitrogen from aqueous solutions (Painter 1991a). Painter also reported that ~6% of the monosaccharides were liberated during autohydrolysis. He suggested that, in natural conditions, this small residue is readily assimilated by bacteria during peat diagenesis. According to Painter's theory, this relatively small amount of accessible food is the probable reason for the small microbial populations in peat.

### **2.2.10. Experimental confirmation of antibacterial activity of *Sphagnum* moss polysaccharides**

To support his proposal of using *Sphagnum* moss for the preservation of fresh fish, Painter tested the antimicrobial activity of *Sphagnan* ( $H^+$  form of 5 KMA). The test substances were applied to the following fish products: whole aquarium Zebra fish, salmon skin, and mackerel skin (Børsheim, Christensen, and Painter 2001a). Along with *Sphagnan*, freshly harvested *Sphagnum* moss, *Sphagnum* peat, extractive free *Sphagnum*, and *Sphagnum* holocellulose were tested. *Sphagnum* holocellulose is considered to be non-refined *Sphagnan*. The raw material for all experiments was freshly harvested moss *Sphagnum palustre* collected in North Trøndelag, Norway. *Sphagnum* peat was collected in the same area. The collected *Sphagnum* peat was essentially free from sedges and other biological contaminants. Samples of moss were separated from concomitant plant material and autoclaved before use to kill their own microbial flora. Extractive-free moss, holocellulose and *Sphagnan* were prepared following Painter's procedures that were described above.

All *Sphagnum* materials were applied to fish tissues in aqueous solutions. The test organisms were natural bacteria inhabiting the fish tissues. The bacterial species were not identified. In all experiments, contact with atmospheric oxygen was not excluded. Bacterial growth in aqueous media was indicated by turbidity or by plate counts. According to Painter's hypothesis, the antimicrobial activity of *Sphagnum* moss is based on the reactivity of carbonyl groups present in residues of polyuronic acid. The reduction of these groups contained in *Sphagnum* holocellulose and *Sphagnan* should completely eliminate their antimicrobial effect. To demonstrate the unique antibiotic properties of free carbonyl groups, a portion of the tested holocellulose was given a

preliminary treatment to reduce its carbonyl groups. Sodium borohydride ( $\text{NaBH}_4$ ) and ammonium carbonate ( $\text{NH}_4\text{HCO}_3$ ) were used as standard chemical reducing reagents (Gribble 1998).

To examine the role of tanning in the preservation of skin collagen, the antimicrobial activity of an industrial tanning agent, tannic acid, was also tested. Tannic acid is not a true antibiotic, but it converts the skin collagen to an indigestible state for putrefactive bacteria. Because of this, tannic acid is widely used in taxidermy for the treatment of skins against microbial degradation. Wood cellulose was selected as a control substance because it is a polysaccharide, which does not contain reactive carbonyl groups and therefore should not cause any antimicrobial action. The experiments concerning the interaction between *Sphagnum* and fish products are reviewed below.

### ***Experiments with salmon skin embedded in Sphagnum materials***

In these experiments, the objective was to preserve fresh salmon skin in *Sphagnum* moss (Børsheim, Christensen, and Painter 2001a). Atlantic salmon (*Salmo salar*) skin strips with scales measuring 5×1.5 cm were embedded centrally in wads (8×8×2.5 cm) of water-saturated solids consisting of untreated *Sphagnum* moss, solvent-treated moss, peat, *Sphagnum* holocellulose, borohydride reduced holocellulose, ammoniated holocellulose, and wood cellulose (control). *Sphagnum* was not tested in this series of experiments. Wads were enclosed in small plastic boxes with tightly fitting lids and stored for 28 days at 23°C. During storage, contact of salmon skin with atmospheric oxygen was not excluded. At time intervals, the skin samples were removed to check their odor and appearance. Then skin samples were washed in distilled water, and transferred to liquid nitrogen-free media where fish skin was the only source

of nitrogen (ibid.). Bacterial growth was monitored by the appearance of turbidity. The “lag” times, when the media solution remained transparent (no bacterial growth occurred and no turbidity observed), were determined. Results are shown in the Table 2.6.

Solid substance used for embedment of a salmon skin	“Lag” time (days) in new microbial growth after embedment for different periods of time (days)				
	0	9	16	21	28
<i>Sphagnum</i> moss untreated	2	4	5	7	9
Pure <i>Sphagnum</i> peat	2	3	4	6	7
Extractive-free <i>Sphagnum</i> plant material	2	6	7	8	10
<i>Sphagnum</i> holocellulose	2	5	6	8	12
Borohydride-reduced holocellulose	2	0	0	0	0
Ammoniated holocellulose	2	0	0	0	0
Wood cellulose (controls)	2	0	0	0	0

**Table 2.6.** The “lag” times in new bacterial growth after embedment of a salmon skin in water-saturated *Sphagnum* materials. Borohydride-reduced and ammoniated holocelluloses and wood cellulose were included for comparison (slightly revised table from Børsheim, Christensen, and Painter 2001a).

All skin samples that were embedded in unreduced *Sphagnum* products showed a distinct preservative effect for up to 12 days. For any given time of embedment, all these materials indicated almost the same preserving activity. Monitoring of the appearance of the skin samples showed that they

*“retained the odor of fresh fish during the entire period of embedment, as well original dimensions, strength and resilience. With increasing time of embedment, the dermis acquired a progressively darker, coffee color. The scales retained their luster throughout, but became successively golden, copper-colored, and finally bronze”* (Børsheim, Christensen, and Painter 2001a).

It seems that during the experiments, the preserving agent was gradually liberated from the wads to the skin collagen resulting in gradual tanning of skin. The tanning reaction prevents biodegradation of skin protein, making it unavailable for bacteria. In contrast, the reduced holocellulose and the wood cellulose did not show any preserving effect on fish skin. On the skin samples embedded in borohydride-reduced or ammoniated holocelluloses, no effect of “browning” was observed and after two days putrefactive degradation started. During the next

four weeks of embedment, there was a successive graying, curling-up, shrinkage, loss of elasticity, and finally, disintegration and dissolution of skin tissue.

These results support the idea that antimicrobial activity of *Sphagnum* holocellulose is determined by the carbonyl groups contained in holocellulose. If the carbonyl groups were reduced, the antibacterial activity of *Sphagnum* holocellulose was completely lost. Experiments demonstrated that fish wrapped in freshly harvested *Sphagnum* moss could be kept intact for approximately 1.5 weeks. This provides enough time for the transportation of this fish to consumers.

It was also shown that preservation in *Sphagnum* is closely connected to the effect of “browning” of skin collagen. For untreated moss, peat, and extractive-free *Sphagnum*, the effect of “browning” can be caused by plant lignin (phenol compounds). Therefore, the presence of phenols in these *Sphagnum* products can be a reason for their antibacterial activity\*. It is remarkable that substances containing plant phenolics, i.e. untreated moss, solvent-treated moss, and peat showed almost the same preserving activity as phenol-free material. In the opinion of the authors, these results reaffirm that phenolic compounds do not contribute significantly to the total antimicrobial effect of *Sphagnum* moss.

### ***Experiments with whole Zebra fish embedded in Sphagnum materials***

In this series of experiments, the object for preservation was whole aquarium Zebra fish (Børsheim, Christensen, and Painter 2001a). Freshly sacrificed 3 cm long fish were embedded in wads of test *Sphagnum* materials saturated with sterile water (3-5% w/w).

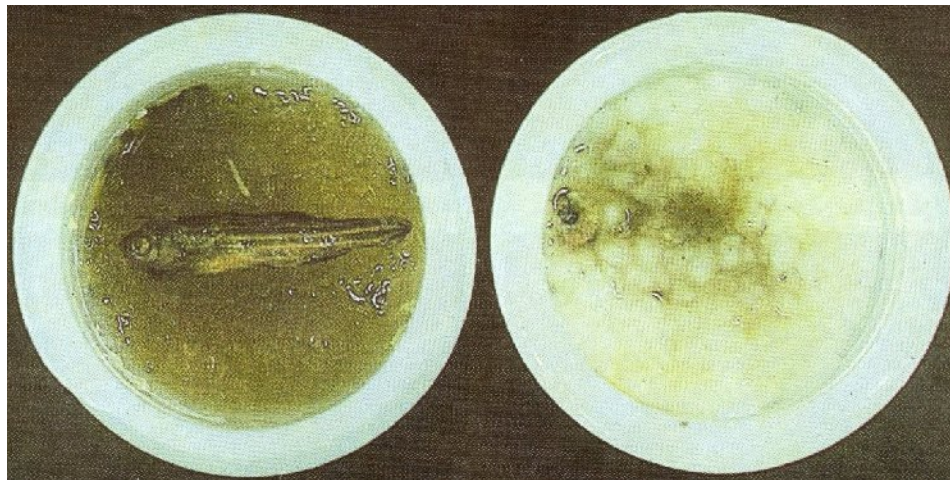
\*By Painter, the “browning” of skin treated with *Sphagnum* holocellulose is explained by the reaction between *Sphagnum* polysaccharides and skin protein (the Maillard reaction)

As in the previous experiment, wads were made of untreated moss, solvent-treated moss, peat, *Sphagnum* holocellulose, borohydride reduced holocellulose, and ammoniated holocellulose. Wood cellulose saturated with water (7.5% w/w) served as a control. In this series of experiments *Sphagnum* was also not included as a test substance. Wads with fish embedded in test materials were placed in plastic cylinders (39×29 mm diameter) with screw caps that were stored at 20°C for 14 days. At intervals, the wads were removed and the condition of the fish was monitored. The ambient water in the cylinders was sampled for microbiological assay. The concentration of bacterial cells in the ambient water was determined by plate counts.

After 14 days of embedment, the fish placed in untreated *Sphagnum* moss, solvent-treated moss, peat, and *Sphagnum* holocellulose appeared to be intact and odorless. The natural color of the scales was converted to light yellowish brown. The color of the fish embedded in the reduced holocelluloses or in the wood cellulose (control) was not changed. However, the fish appeared to be disintegrated and partly dissolved. There was also a strong odor of putrefaction in these samples.

The bacterial cells in the water surrounding the fish body grew rapidly between one and two days of embedment. Then, within an interval of 2-14 days, the cell count stabilized at ca.  $10^8$  CFU/ml for reduced holocelluloses and for wood cellulose. For other *Sphagnum* materials it was two orders lower, ca.  $10^6$  CFU/ml. No significant difference in concentration of bacterial cells for different materials was noticed. Thus, it is hard to judge which products are more effective. In general, these results were similar to the results for salmon skin. Experiments clearly showed that it was possible to keep fresh fish in moss for at least for 2 weeks before putrefaction occurred (Fig.2.12). Again, in these experiments no difference was noticed in antimicrobial

activity of phenol-containing materials (untreated and solvent-treated moss, and peat) and phenol-free holocellulose.



**Fig.2.13.** Zebra fish after embedment in *Sphagnum* moss for 14 days—on left. The same fish wrapped in wood cellulose —on right (photo from Börnheim, Christensen, and Painter 2001a).

#### ***Experiments with mackerel skin submerged in aqueous Sphagnum***

This series of experiments was slightly altered compared to the previous ones. The fresh 1×2 cm mackerel skin strips with scales removed were washed in distilled water and then put into Erlenmeyer flasks with screw caps containing 30 ml of the following test solutions: aqueous *Sphagnum* (3% w/v), borohydride-reduced *Sphagnum*, and tannic acid. Flasks were gently agitated for 24 hours. Then the skin strips were air-dried at 23°C for 12 hours. After four such treatment cycles, the skin strips were suspended in nitrogen-free culture media for 4-5 days at 23°C. After that, strips were removed and their appearance and odor were examined. The structure of skin collagen was studied by electron microscopy. The controls were mackerel skin strips which were suspended in culture media without any prior treatment. Once placed in nutrient media, untreated salmon skin and skin treated with the reduced *Sphagnum* were rapidly colonized and “*had dissolved completely within 4-5 days*”.

In contrast, the samples treated with *Sphagnum* and tannic acid “*resisted microbial attack indefinitely under the same condition*” (Børsheim, Christensen, and Painter 2001a). After four treatment cycles in aqueous *Sphagnum*, the color of the skin became coffee-brown. In tannic acid, the color was converted to light-brown, and in borohydride-reduced *Sphagnum*, it remained white\*.

In these experiments, Painter tried to show that polysaccharides (*Sphagnum*) contained in *Sphagnum* moss cause the same tanning effect on skin collagen as the traditional tanning agent, tannic acid. According to Painter, scanning electron micrographs of treated fish skin showed the similarity of the structure of collagen after treatment with tannic acid and with *Sphagnum* (micrographs are demonstrated). These results indicate that the effectiveness of two tanning agents, *Sphagnum* and tannic acid, is the same. Therefore, Painter concluded that the effect of browning of the skin of bog bodies is completely caused by *Sphagnum* polysaccharides (Maillard reaction) and not by phenol compounds (ibid.).

#### ***Immobilization of bacterial cultures by Sphagnum holocellulose***

Painter also tested the inhibiting effect of *Sphagnum* holocellulose on some bacterial cultures (Børsheim, Christensen, and Painter 2001b; Børsheim *et al.* 2005). Holocellulose was enclosed in small bags (like teabags) that were sewed from plankton gauze of 60µm mesh size.

Borohydride- reduced holocellulose and wood cellulose were used as controls. The amounts of celluloses tested were not specified. The bags were suspended separately in Erlenmeyer flasks with screw caps containing 0.9% NaCl sterile buffer solution at 20°C. Then floating cultures of bacteria cells in their stationary growth phase were introduced into the flasks and stirred mechanically.

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\*Authors also published scanning electron micrographs of skin samples treated with *Sphagnum* and tannic acid. They proclaimed the similarity in the structure of skin collagen in both cases. Therefore, they tried to prove that polysaccharides (*Sphagnum*) can cause the same tanning effect as phenolics (tanning acid).

At intervals, samples of the cell suspensions were taken out from the ambient buffer and the bacterial concentration was determined by plate count. It was shown that the concentration of the bacterial cells treated with holocellulose after 20 hours of exposure was reduced significantly to constant values, i.e. bacterial cells became immobilized. For borohydride- reduced holocellulose and wood cellulose no significant immobilization was detected. The ratio of bacterial concentration in the buffer with holocellulose added ( $C_h$ ) to bacterial concentration in the buffer with borohydride-reduced holocellulose ( $C_{rh}$ ) after 20 hours of exposure is shown in Table 2.7.

Bacterial culture	$C_h / C_{rh}$ , %
<i>Escherichia coli</i> (gram-negative)	< 0.4
<i>Bacillus</i> spp. (gram-positive, some spp. are gram-negative)	< 0.25
<i>Micrococcus</i> spp. (gram-positive)	< 10
Local isolate	< 0.1

**Table 2.7.** The immobilization effect of *Sphagnum* holocellulose on different bacterial cultures. Bacterial concentration in the treated culture after 20 hours of exposure, compared to the controls (from Børsheim, Christensen, and Painter 2001b). The local isolate was not taxonomically specified.

These results demonstrate that compared to the control (reduced holocellulose and wood cellulose) the treatment of bacterial cultures with holocellulose  $10\text{-}10^3$  times reduces the concentration of bacteria in the buffer. Holocellulose immobilized both the gram-negative and the gram-positive bacterial species, but the gram-negative species were inhibited more effectively:

*“It is therefore natural to assume that the plant Sphagnum or parts thereof possess immobilizing properties with regard to most bacteria”.*

They also considered that

*“bacterial species investigated adhered strongly to Sphagnum moss, peat and pure holocellulose from Sphagnum moss. The adhesion was strong, and occurred regardless of cell wall type such as inferred from the Gram reaction”.* (Børsheim, Christensen, and Painter 2001b).

It seems from this conclusion that *Sphagnum* products deactivate bacteria owing to an adhesive mechanism.

### 2.2.11. Phenol compounds as antimicrobial agents in *Sphagnum* moss

In recent decades it was shown that slow decay of *Sphagnum* litter is mainly controlled by the composition of the moss cell wall, where an important role is played by phenol compounds (Johnson and Damman 1993; Van-der-Heijden 1994; Verhoeven and Liefveld 1997; Williams *et al.* 1998; Tsuneda, Thormann, and Currah 2001). The main reason for the resistance of *Sphagnum* moss against decay was shown to be polyphenolic network polymers protecting readily degradable ingredients of *Sphagnum* cells, such as simple sugars, cellulose, pectin, etc. (Tsuneda, Thormann, and Currah 2001). Electron microscopy and histochemical studies of *Sphagnum* cell walls showed that phenolic network polymers provide a physicochemical barrier for exo-enzymes of the majority of degrading microorganisms. The phenolic network also protects cell walls against mechanical invasion, i.e. it makes cells impenetrable to hyphae of most hypomycetes (Nimz and Tutschek 1977; Verhoeven and Liefveld 1997; Tsuneda, Thormann, and Currah 2001).

In nature, biodegradation of plant phenol compounds occurs mainly by the enzymes phenol oxidases (PO). Few microorganisms inhabiting the bog litters produce these enzymes, i.e. in general PO appeared to be depressed in peatlands (Freeman, Ostle, and Kang 2001; Thormann, Currah, and Bayley 2002). Inactivity of PO in peat bogs is explained by acidic and anoxic environmental conditions dominating in the bogs. PO requires molecular oxygen for their activation whereas the *Sphagnum* environment is normally devoid of molecular oxygen. Also, PO activity also increased exponentially with increasing pH (Williams, Shingara, and Yavitt 2000). Therefore, at the conditions of acidity in a *Sphagnum* environment (low pH), the activity of PO became constrained.

In addition, even the activities of other degrading enzymes such as hydrolases ( $\beta$ -glucosidase, phosphatase, sulphatase, xylosidase, and chitinase), which do not normally require molecular oxygen, were found to be depleted in peatlands (Freeman *et al.* 2004). Depletion of hydrolyzing enzymes occurs because phenolic materials accumulated in a *Sphagnum* environment are highly inhibitory for these enzymes (*ibid.*). Since degradation of *Sphagnum* phenolic materials is slow, they are accumulated as peat deposits (Freeman, Ostle, and Kang 2001).

Recently, chemical fractions of fifteen *Sphagnum* species and *Sphagnum*-dominated peat samples from peatlands located between Alberta, Canada, and Ohio, USA were determined using an alkaline cupric oxide oxidation technique (Williams *et al.* 1998). As a result of the oxidation of the peat samples, the selective decay of different ingredients of the *Sphagnum* chemical profile (soluble fats, oils, and waxes, soluble carbohydrates, holocellulose, and lignin-like compounds) was demonstrated for different levels of peat deposition. The concentration of soluble fats, oils, and waxes as well as soluble carbohydrates did not vary with depth\*. On the contrary, the concentrations of holocellulose and lignin revealed strong depth dependence: lignin content increased with depth while holocellulose content decreased.

These results indicate that holocellulose (probably, its cellulosic part) is the most biodegradable constituent of *Sphagnum* plant material while lignin-like compounds are the most bioresistant constituents. Invariant distribution with depth of soluble carbohydrates (hemicelluloses) indicates that they remain intact in the process of peat accumulation. This supports Painter's idea about the bioresistance of *Sphagnum* polysaccharides.

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\*The fact that the concentrations of soluble fats, oils, and waxes did not decrease with the depth of peat deposition can indicate bioresistance of these substances. Turetsky casually mentioned that *Sphagnum* nonpolars (lipids) can inhibit decay (Turetsky 2003). However, no information was found about antimicrobial properties of these ingredients.

However, Painter proposed that only these polysaccharides (*Sphagnan*) determine the antimicrobial properties of *Sphagnum* moss. The reported results confirm that phenolics can also contribute significantly to the bioresistance of *Sphagnum* plants.

The oxidation products of harvested *Sphagnum* plants were found to be soluble phenolic acids including genus-specific *Sphagnum* acid, *p*-coumaric acid, vanillic acid, vanillin, syringaldehyde, and ferulic acid. Also the ingredients of tannic acid such as *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxyacetophenone (all in small amounts) were isolated from *Sphagnum* plant material (Williams *et al.* 1998). All these water-soluble phenolics possess a certain degree of bioactivity. Thus, many phenolic acids are known as antioxidants (ferulic acid). However, no evidence about their antimicrobial properties is known. At the same time, they participate significantly in preservation of protein in peat bogs through tanning reactions with skin collagen.

In other words, protein-containing materials became unavailable for microorganisms as a result of tanning reactions with *Sphagnum* water-soluble phenolic metabolites. Insoluble high molecular weight phenolics such as flavonoids and tannins are also present in the *Sphagnum* plant. Antimicrobial activity of flavonoids and tannins is well-documented.

The antibacterial properties of flavonoids are connected to their ability to inhibit microbial enzymes and biomembranes (Bylka, Matlawska, and Pilewski 2004, a review). Thus, flavanoids isolated from five species of mosses were shown to have antibacterial effects against *Enterobacter cloacae*, *E. aerogenes*, and *Pseudomonas aeruginosa* (Basile *et al.* 1999).

Tannins inhibit the growth of many fungi, yeasts, bacteria, and viruses because they can bind

proteins, carbohydrates, fats, and minerals, making them unavailable for microorganisms (Chung *et al.* 1998). During decay, flavonoids and tannins accumulate in peat deposits and therefore, they can be an important reason for the “sterility” of the bog environment. Since all bog bodies were found buried in peat deposits, their good preservation can be caused not only by tanning of their skin, but also by antimicrobial activity of the high molecular weight phenol compounds accumulated in peat.

#### **2.2.12. An overview of *Sphagnum* antimicrobial activity**

Historically, a number of facts attest to the inherent antimicrobial activity of *Sphagnum* moss. However, if we review these facts carefully, none of them are strong enough to prove this phenomenon. Only the sum of all observations concerning the decay of moss in nature and the decay of different organic materials in moss allows us to conclude that *Sphagnum* moss must contain antimicrobial ingredients.

The lengthy studies of *Sphagnum* plant material and humic substances derived from dead moss show that there are two classes of such ingredients: structural polysaccharides and lingo-phenols. In *Sphagnum* cell walls, polysaccharides are linked to phenol compounds by glycosidic bonds. Separation and analysis of these compounds require quite sophisticated equipment and their structures are still not completely understood. Despite these facts, Painter and his colleagues attributed the antimicrobial activity of moss only to *Sphagnum* polysaccharides (*Sphagnan*). They tried to prove experimentally that *Sphagnan* exclusively determines the antimicrobial properties of the *Sphagnum* plant.

The effect of the complete elimination of antibacterial activity of *Sphagnum* moss holocellulose by reducing compounds

*“seems to confirm that the preservative and tanning property is exclusively associated with the carbonyl groups in the holocellulose component, and that no other component, monomeric or polymeric, soluble or insoluble, contributes significantly to the total preservative effect”* (Børsheim, Christensen, and Painter 2001a).

However, the fact, that raw harvested moss, extractive-free moss, and peat (all containing lignocelluloses) and moss lignin-free holocellulose did not show any detectable difference in their antimicrobial activity suggests that the antimicrobial activity of moss is not due to polysaccharides only. It must depend on some additional compounds. Probably, these are phenolics. It is clear from recent studies that the antimicrobial activity of *Sphagnum* moss can be explained on the basis of the polyphenol theory only, regardless of the contribution of polysaccharides. At least, the role of phenolics in the *Sphagnum* antimicrobial effect cannot be negligible.

Nevertheless, Painter’s experiments showed the clear antimicrobial effect of *Sphagnum* moss and *Sphagnum* holocellulose on fresh fish protein. This effect lasted for up to two weeks. It is enough for the good preservation of fresh fish during transportation of catches to consumers.

Regarding conservation needs, Painter’s experiments proved that the use of *Sphagnum* moss in field of conservation could be effective: wrapping of artefacts in moss should stop microbial growth at least for two weeks. However, all Painters’ experiments were performed only on bacteria as test organisms. For art conservation, it is more beneficial to test *Sphagnum* polysaccharides against fungi, especially against species commonly found in museum environments. These tests have never been carried out.

Fungal tests are likely to produce a positive result because many species that are found in peat bogs are the same as those found in museums. They are common ascomycetous soil fungi. It is important to note that the growth of these species in peat bogs is depressed probably due to the antimicrobial compounds contained in the cell walls of *Sphagnum* moss. Therefore, *Sphagnum* extractives may cause not only an antibacterial, but also an antifungal effect.

In the present work, *Sphagnum* extracts were applied on fungi as the test organism (see section 4.2). Two extracts were examined: extract containing polysaccharides (*Sphagnan*) and extract contained phenolics. Also, *Sphagnan* was proposed as an antifungal additive to conservation waxes (see chapter 5). The susceptibility of conservation waxes to fungal attack is reviewed below (section 2.3).

### **2.2.13. Recent proposals for practical use of *Sphagnum* products in medicine and the food industry**

Recently, a series of innovative proposals for the practical use of *Sphagnum* or *Sphagnan* products as natural antibiotics were published and registered as patents. Thus, a hygienic tampon where *Sphagnum* was used for bactericidal and absorbing effect was described by Podterob and Zubets (Podterob and Zubets 2002). Another tampon for dental surgery where *Sphagnum* was used as absorbent material was described by Børsheim (Børsheim *et al.* 2005). The liquid absorbent made from *Sphagnum* was also proposed for manufacturing of sanitary napkins, diapers, wound dressings, nursing pads, or as desiccant for packaging materials to keep goods dry during shipping or storage (Roy and Canuel 1997).

Painter's basic proposal of the use of *Sphagnum* moss for

*“increasing of lifetime of fresh food with several days without adding scent, colour or smell, and using 100% raw material makes the Sphagnum technology platform attractive to the fresh fish industry”* (Christensen, Balance, and Granum 2004).

This proposal is now ready for commercial licensing. Two other proposals: “Oral hygiene product” and “Moss-derived antimicrobial composition” have already been patented by Painter's colleagues (Børsheim *et al.* 2005; Balance and Christensen 2007).

According to the first invention, *Sphagnum* plant powder or *Sphagnum* extract can be used with conventional fillers in the production of antibacterial tooth paste (Børsheim *et al.* 2005, WO/2005/041912). A composition for reducing the amount of bacterial flora on the teeth and in the oral cavity is also proposed (*ibid.*).

The second invention relates to a moss-derived antimicrobial agent which is a mixture of moss polysaccharides or moss holocellulose (Balance and Christensen 2007, WO/2007/077459). For production of this agent, it is planned to use any moss or moss-derived peat, but the preferable raw material is *Sphagnum* moss. Inventors proposed about a hundred different moss species including 34 *Sphagnum* species. From these *Sphagnum* species, they selected *S. cuspidatum*, *S. magellanicum*, and *S. papillosum*, which are the best for the preparation of antimicrobial agents.

Isolation of antimicrobial agents (i.e. holocellulose or polysaccharides mixture) in this invention is closely related to the procedure proposed by Painter with minor differences (Børsheim, Christensen, and Painter 2001a). For the preparation of holocellulose, harvested moss is subjected to oxidative treatment (e.g. bleaching), which essentially oxidizes aromatic compounds in the moss or peat while leaving the polysaccharide structure minimally modified.

Isolation of polysaccharides from holocellulose is performed by enzymatic cleavage or autohydrolysis with further anion-exchange fractionation of polysaccharides which are released by this cleavage. It is important, that oxidative-treated holocellulose is not only essentially *“colorless and flavorless, but also sterile”* (Balance and Christensen 2007, WO/2007/077459).

Following the invention, the mixture of polysaccharides can be used in cases where food, food waste or body exudates may serve as growth media for microbes such as bacteria, yeasts or fungi. It is proposed to use them as internally applied antibiotics, as surface disinfectants (to promote wound healing), or for the preservation of food products (ibid.). For internal treatment of a human or an animal, it is recommended to apply a dosage of 0.001 to 0.1 g of the polysaccharide per kg of bodyweight. For foodstuffs, e.g. raw or cooked fish and meat, the recommended dose is ranged from 5 to 20 mg per cm<sup>2</sup> of the product surface. The antimicrobial compositions can be used as powders, tablets, solutions, suspensions, emulsions, creams, impregnated wipes, water-impermeable backing sheets, etc. (Balance and Christensen 2007, WO/2007/077459).

The phenol-free moss holocellulose can be used as a wound dressing with a one-way contact layer and the moss holocellulose in a backing layer. It is proposed as a component in toilet paper and other tissues for absorbing moisture, especially biological fluids (ibid). Moss holocellulose can be used as an absorbent container to hold the foodstuff. It can also be used as pads, lids, or baskets (up to 5% wt) for food packaging. For example, fish fillet slices can be packaged with *Sphagnum* pads on the top and bottom side. In addition to preservative properties it was found that the smell of the fillet slices stored with the *Sphagnum* pads was deemed acceptable to consumers (Balance and Christensen 2007, WO/2007/077459).

## 2.3. Biodegradation of conservation waxes

Petroleum waxes are widely used conservation materials due to their chemical neutrality and excellent mechanical and hydrophobic properties (Moffett 1996; Bachmann 1992; Pearson 1988). Compared to many film-forming materials, petroleum waxes possess the lowest water vapour permeability (Masschelein-Kleiner 1995). They are used as adhesives and coatings to protect metals, furniture, easel and mural paintings, stone, leather, and ivory (Mills and White 1994). In conservation, waxes are traditionally recommended for treatment of archaeological bronze objects (Organ 1970; Pearson 1988; and many others). From a wide range of natural and synthetic waxes, microcrystalline waxes, originating from a family of petroleum waxes, are the current choice in art conservation. Microcrystalline waxes are considered to be essentially inert because they consist mainly of long-chained *n*-alkanes and do not contain reactive functional groups such as carboxyl, alcohol, ester, ketone, and amide groups (Atlas 1981; Leahy and Colwell 1990). Normal alkanes have a low reactivity because the C-H and C-C single bonds are relatively resistant against breakdown by microbial enzymes. In addition, excellent hydrophobic properties of all petroleum waxes provide the conditions preventing microbial growth due to repelling water from the surface of wax (*ibid.*). Despite the fact that the main ingredients of petroleum wax (e.g. long-chained *n*-alkanes) are not digestible by most microorganisms, the minor wax ingredients, e.g. residuals of crude oil\*, light alkanes and aromatics, are readily biodegradable. Biodegradation of these wax compounds has been widely studied with respect to the environmental problem of soil contamination by hydrocarbons (Atlas 1981; Leahy and Colwell 1990; Morgan and Watkinson 1993; Bennet, Wunch, and Faison 2002). These works are briefly reviewed below.

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\*Crude oil is the term for "unprocessed" oil, the stuff that comes out of the ground. Crude oil was made naturally from decaying plants and animals living in ancient seas millions of years ago.

### 2.3.1. Chemical structure of petroleum waxes

The petroleum waxes generally classified as paraffin and microcrystalline waxes are separated from the heavy fractions of crude oil in a refinery (Bennet 1975). The properties of these waxes vary widely depending on the source of the crude oil and the methods of wax processing (Warth 1947). The chemical composition of petroleum waxes is not known in detail because they are complex mixtures of molecules with different molar masses, structures, and functionality. These structures cannot be completely characterized either by gas chromatography or by other methods, such as Fourier Transform Infrared spectrometry (FTIR) and Differential Scanning Calorimetry (DSC) (Bennet 1975; Kaufman and Weisberger 1993; ASTM 1998; Knuutinen and Norrman 2000).

In general, petroleum waxes consist of complex mixtures of normal alkanes (*n*-alkanes), branched alkanes (*iso*-alkanes), and naphthenes (*cyclo*-alkanes). The difference between petroleum waxes is caused by the relative proportions of these three structures of hydrocarbons in wax and the specific molecular weights of these ingredients (Warth 1947; Bennet 1975). Paraffin wax typically contains normal alkanes ranged between C<sub>20</sub>-C<sub>40</sub> hydrocarbons and less than 20% of branched alkanes. This wax is macrocrystalline, hard, and brittle. Its melting point is in the range of 46-68°C (ibid.).

Microcrystalline waxes contain more branched and cyclic alkanes (> 60%) than paraffin waxes. In addition, they contain heavier profile of normal alkanes (C<sub>23</sub>-C<sub>85</sub> hydrocarbons). They melt between 60 and 93°C, i.e. microcrystalline waxes are generally higher in melting point than paraffin waxes. The presence of branched and cyclic alkanes in microcrystalline waxes gives them superior elastic and adhesive properties (Bennet 1975).

There are two more grades of petroleum waxes with lower molecular weights than microcrystalline waxes: slack waxes and intermediate waxes. Slack waxes contain considerable amounts of residual oil and serve as a raw material for further refining. Intermediate wax is referred to as semi-microcrystalline wax. It contains C<sub>30</sub>-C<sub>50</sub> hydrocarbons, 20-60% of branched alkanes, and has properties between paraffin and microcrystalline wax. Intermediate wax typically melts between 50 and 80°C (ibid.).

Conservation waxes represent the plastic grades of microcrystalline wax. These waxes are produced from the heavy fractions of crude oil distillation. The oil is then extracted with solvents to cause the precipitation of the microcrystalline wax (Warth 1947; Kaufman and Weisberger 1993). These grades of microcrystalline wax consist of much smaller crystals than other petroleum waxes. As a result, microcrystalline waxes have better elastic properties.

Microcrystalline waxes normally contain 1-4 % of residual oil. The molecules of residual oil are incorporated into the crystal lattice structure of microcrystalline wax; they do not migrate to the wax surface (Bennet 1975). Residual oil mainly contains two classes of small molecular weight polycyclic aromatic hydrocarbons (PAHs\*): naphthalenes and phenanthrenes, as well as alkylated PAHs such as methylnaphthalenes (Bennet 1975; American Petroleum Institute 2003). Microcrystalline waxes are readily soluble in a number of aromatic organic solvents, especially at elevated temperatures. Once introduced into the wax, the solvents are strongly retained by the crystal lattice structure (ibid.). In conservation, microcrystalline waxes, when applied as coatings, are usually mixed with solvents (benzene, toluene, xylene, and others), which evaporate from the wax coatings very slowly (Moffett 1996; Grossbard 1992). Because solvents are retained in the wax films for a long time, they normally contain traces of aromatics

\*PAHs, also called polynuclear aromatic hydrocarbons, are composed of two or more benzene rings fused together.

### 2.3.2. Biodegradation of petroleum waxes and their chemical composition

Petroleum waxes are inherently biodegradable (American Petroleum Institute 2003). Their biodegradation has been studied from the early 20<sup>th</sup> century (Rahn 1906; Söhngen 1913; Fuhs 1961; Miyamoto 1968; Miller and Johnson 1966; Yamada and Yogo 1970; and others). Although most of these works were carried out on paraffin waxes, they also referred to conservation microcrystalline waxes\*. Microorganisms including bacteria, fungi and yeast are able to metabolise the chemical composition of petroleum waxes\*\*. These studies are reviewed below.

#### ***Bacteria***

A wide group of bacteria is known for their ability to metabolise hydrocarbons (Rosenberg 1992). The most extensive bacterial degradation was observed in the light fractions from the oil refinery, i.e. short-chain alkanes (C<sub>5</sub>-C<sub>11</sub>) and light aromatics (benzene, toluene and xylene) contained in gasoline and in diesel oil (Solano-Serena *et al.* 1999; Márquez-Rocha, Hernández-Rodríguez, and Ma. Teresa Lamela 2001). The degradation of gasoline was studied in the presence of micro flora from the activated sludge sampled from a water treatment plant in the suburbs of Paris, France (Solano-Serena *et al.* 1999). After 25 days of incubation at 30°C, 94% of the gasoline was utilized by a bacterial consortium (association of partners). Aliphatic and aromatic ingredients of gasoline showed specific rates of biodegradation (*ibid.*).

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\*Taking into account that the chemical composition and physical properties of all petroleum waxes are closely related to each other, it is supposed that the mechanism of their biodegradation is also the same.

\*\*Although normal alkanes (the main ingredient of waxes) are considered to be bioresistant, petroleum waxes contain small amounts of easily biodegradable ingredients. They are various aromatics, which are normally present in residual oil, and the traces of solvents, which are used for wax dissolution. In natural environments, the microbial degradation of different petroleum hydrocarbons including wax ingredients was reviewed by Atlas (Atlas 1981).

Crude oil, which is always present in petroleum waxes, is also readily biodegradable. Utilization of crude oil by pure and mixed bacterial cultures was shown in the early 1970s (Jobson, Cook, and Westlake 1972). Later, it was shown that many aliphatic and aromatic ingredients of crude oil also can be consumed by bacteria. It was found that some bacteria (cyanobacteria *Oscillatoria agardhii* and *Anabaena sphaerica*) consume *n*-alkanes (C<sub>10</sub> - C<sub>24</sub>) and PAHs contained in crude oil (Gamila, Ibrahim, and El-Ghafar 2003). *Anabaena sphaerica* was able to mineralize up to 97.5% of PAHs present in sampled oil (ibid.). Bacteria *Burkholderia cepacia* strain 2A-12 isolated from oil-contaminated soil was shown to utilize the main PAHs ingredients: naphthalene and phenanthrene (Kim *et al.* 2003).

These results are important because biodegradation of light hydrocarbons probably resembles biodegradation of heavier ingredients contained in petroleum waxes. For example, the similarity between bacterial degradation of short chain alkanes and the initial degradation of paraffin waxes was shown (Miyamoto 1968).

The long-chain normal alkanes with highest molecular weights are the least biodegradable ingredients of the chemical components of petroleum wax. Heavier *n*-alkanes (C<sub>30</sub>, C<sub>31</sub>) showed a slower rate of degradation by a bacteria and yeast consortium than lighter *n*-alkanes, (C<sub>20</sub>, C<sub>21</sub>) (Bosecker, Teschner, and Wehner 1991). Later, it was shown that the bacterium *Acinetobacter* spp. strain M-1 is able to utilize the long-chain *n*-alkanes, C<sub>20</sub> - C<sub>44</sub> (Sakai *et al.* 1994). Recently, a bacterial pathway for utilization of long-chain normal alkanes was proposed (Wentzel *et al.* 2007).

Biodegradation of whole petroleum waxes was also tested (American Petroleum Institute 2003). Laboratory experiments simulated the aerobic utilization of waxes at 20-25°C by microbes occurring in the natural environment. The following waxes were subjected to degrading microorganisms: microcrystalline wax, paraffin waxes, intermediate wax, and slack wax. The slack wax composition consisted of C<sub>12</sub> to C<sub>85</sub> hydrocarbons, and up to 30% oil; paraffin wax consisted of C<sub>18</sub>-C<sub>75</sub> hydrocarbons and < 2.5% oil. Microcrystalline waxes with C<sub>23</sub>-C<sub>85</sub> and < 5% oil were produced by slack wax deoiling. The tested substances (13 mm glass filters with 20 mg of tested wax applied) were incubated in different liquid inoculums containing natural microorganisms: oil-contaminated soil, domestic sewage sludge, forest soil, and liquid nutrition media. Oil-contaminated soil was collected from farm land being treated by the investigators. Sewage sludge was obtained from a domestic sewage treatment plant in Pennington, NJ, USA. Biodegradation was calculated by weekly monitoring of the production of CO<sub>2</sub> as a microbial metabolite. At the end of the experiment the amounts of wax remaining on the filters were extracted with warm heptane and analyzed by gas chromatography. The specific microbes were not identified, but they were considered to be bacteria because the experiments were carried out in liquid media. Results are presented in Table 2.8.

Test substance	Inoculum	Temp., °C	Degradation % after time		
			4weeks	12weeks	18weeks
Slack wax SN 60	Activated domestic sludge	22±1	40	-	-
Slack wax Na Benzoate	Activated domestic sludge	22±1	78	-	-
Paraffin wax	Oil-contaminated soil	20±2	80	87	-
Paraffin wax	Dom. sewage sludge, soil	25±2	55	-	99
Intermediate wax	Oil-contaminated soil	20±2	66	77	-
Microcrystalline wax	Oil-contaminated soil	20±2	21	25	-
Microcrystalline wax	Dom. sewage sludge, soil	25±2	27	-	67
White mineral oil	Non-adapted dom.sewage	-	0	-	-
Technical white oil	Non-adapted dom.sewage	-	24	-	-

**Table 2.8.** Biodegradation of waxes inoculated in the liquid media (compiled from the results of the American Petroleum Institute 2003).

The microcrystalline wax showed better bioresistance than paraffin and slack wax. Nevertheless, it was quite vulnerable to bacterial attack under the conditions of the experiment. From 21 to 27% of microcrystalline wax mass was lost in one month of exposure and almost 70% was consumed in 4.5 months. Mass loss of paraffin was from 55 to 80% in one month and in 4.5 months the wax was completely consumed by microorganisms.

### ***Fungi***

It was found that certain fungi metabolize hydrocarbons, such as normal alkanes and light PAHs (Atlas 1981; Leahy and Colwell 1990; Pinto and Moore 2000). Under favorable conditions, fungi metabolize hydrocarbons rapidly (Rudd *et al.* 1996). This property was proposed for the biofiltration of volatile organic pollutants such as BTEX components of gasoline (benzene, toluene, ethylbenzene, and the xylene isomers). Biofilters of industrial air supplied with fungi converts BTEX into non-toxic fungal metabolites. Fungi were isolated from industrial air filters that were exposed to hydrocarbon-polluted gas streams (Prenafeta-Boldú, Summerbell, and Sybren de Hoog 2006). In biofiltration laboratory experiments, it was found that the fungus *Scedosporium apiospermum* grown in a bioreactor at 60% RH quickly converted toluene to CO<sub>2</sub> (García-Peña *et al.* 2001). A toluene-degrading fungus *Paecilomyces variotii* also showed high consumption rates for toluene: the fungal biomass doubled in 2-3 days (García-Peña *et al.* 2005).

Another environmental application of fungi is the bioremediation of hydrocarbon-contaminated soils. Soil bioremediation laboratory experiments focused on the ability of filamentous fungi, including wood-rotting fungi, to degrade hydrocarbon pollutants (Colombo, Cabello, and Arambarri 1996; April, Foght, and Currah 2000; and others).

It was suggested that soil fungi could contribute significantly to the bioremediation of BTEX pollution. Recently the deuteromycetes\*, *Cladophialophora* spp. strain T1, isolated from toluene, was shown to utilize some BTEX components within 15 days (Prenafeta-Boldú *et al.* 2002). However, for the utilization of other BTEX components such as benzene, fungi need indigenous soil microorganisms (Prenafeta-Boldú *et al.* 2004).

Fungi and yeast are also able to degrade crude oil. Thus, yeast cultures utilize C<sub>19</sub> - C<sub>24</sub> *n*-alkanes contained in the oil (Miller and Johnson 1966). Some common fungal cultures, *Aspergillus terreus* and *Fusarium solani*, and cultures isolated from polluted soil, *Pleurotus ostreatus*, *Trametes villosus* and *Coriolopsis rigida*, were found to consume 65-74% of crude oil hydrocarbons in 90 days (Colombo, Cabello, and Arambarri 1996). In this experiment, normal alkanes were almost completely degraded within the first 15 days of incubation. Aromatic compounds (phenanthrene and methylphenanthrenes) were also metabolized, but with slower rates of utilization (*ibid.*).

Recently, the potential of sixty four filamentous fungi, isolated from oil polluted soil (mostly *Aspergillus niger*, *Gliocladium cf. catenulatum*, *Penicillium cf. janthinellum*, and *Pseudallescheria boydii*) to degrade crude oil was tested in the laboratory (April, Foght, and Currah 2000). From these fungi, five species of *Aspergillus* and eighteen species of *Penicillium* almost completely degraded C<sub>12</sub> - C<sub>26</sub> *n*-alkanes. Six species also metabolized *cyclo*-alkanes contained in the oil. Three species were able to grow at decreased temperatures as low as 5°C.

\*This class which has no sexual reproduction is referred to as Fungi Imperfecti. Most of the 17.000 species of the deuteromycetes reproduce by conidia (Prescott, Harley, and Klein 2002).

Polycyclic aromatic hydrocarbons (PAHs) that normally are quite resistant to biodegradation also were found to be quickly consumed by some fungi. After eight weeks of inoculation in the soil, 65% of the naphthalene, 82% of the anthracene, and 76% of the pyrene were metabolized by two strains of *Aspergillus* and *Verticillium* spp. (Clemente and Durrant 2005).

All experiments enumerated here demonstrated fungal degradability of light minor ingredients of petroleum waxes in the laboratory. Biodegradation of various wax compositions was also investigated. Experiments of wax degradation have been performed in natural conditions, in relation to the problem of contamination of soil by heavy hydrocarbons. However, in these experiments, waxes were decomposed by entire microbial *consortia* including bacteria, fungi, and yeast. In this respect, they cannot be considered as pure demonstrations of wax biodegradation by fungi. However, soil filamentous fungi probably contribute significantly to the degradation of wax in these experiments.

In one series of the field experiments of the American Petroleum Institute, 2x2 cm squares of paper coated with paraffin or microcrystalline waxes were placed in nylon bags of two mesh sizes: 45  $\mu\text{m}$  or 5 mm (American Petroleum Institute 2003). The bags, each containing 20 mg of wax, were exposed for twenty six weeks in the natural leaf litter at the ambient humidity and temperatures, and in the presence of oxygen. The experiment was carried out in a state park in central NJ, USA. The authors considered that the 45  $\mu\text{m}$  mesh bags prevented the colonization of external soil microorganisms on the wax. At the same time, the soil nutrients were able to penetrate through the bag wall and support the growth of domestic microorganisms inside the bag.

The 5 mm mesh bags allowed all of the soil microbes to contribute to the wax decomposition. Samples were removed monthly from the bags, cleaned, dried and weighed. The wax residues were measured by extraction with warm heptane and gas chromatography. As a result, the paraffin-waxed paper placed in 5 mm mesh bags was completely degraded within 13 -26 weeks. In the 45  $\mu\text{m}$  bags, the process of decomposition was much slower. After 26 weeks of spring-summer exposure, 50-70% of the paraffin wax and only 40% of the microcrystalline wax was degraded. During the thirteen weeks of the autumn-winter test, the losses amounted to 60-70% for the paraffin wax and 60% for the microcrystalline wax. Comparing with bacterial degradation (Table 2.8), wax contacted freely with soil organisms (5 mm bags) degraded faster. In the case of limited access of larger organisms (fungi) to wax samples, they degraded approximately as fast as by bacterial species. These results show that fungi probably contribute significantly to wax degradation in soil. Another study carried out in The Netherlands showed almost the same results (Hanstveit 1991). In this experiment, the paper samples impregnated with the paraffin, the intermediate, and the microcrystalline waxes were placed in 5 mm mesh bags. The paraffin and intermediate wax samples were completely degraded after 24 weeks of exposure; decomposition of the microcrystalline wax samples was considerably slower.

The decomposition of quick-service restaurant wax and polyethylene-coated paper composted in soil was studied at the University of Guelph, Canada, in laboratory and in field conditions (Davie, Julien, and Varoney 1995). The heavy waxed cup stock and waxed burger wrap used in this test are normally coated with hard grades of microcrystalline wax. In the field experiments, 5  $\text{cm}^2$  paper samples isolated in 0.2 mm mesh screens were incubated in compost with a nitrogen source added (poultry manure). After eight weeks of composting, weight loss in the waxed paper was 77% on average.

In the laboratory experiments, the decomposition of waxed paper was tested in nitrogen - enriched soil at room temperature. The degradation of wax was monitored by CO<sub>2</sub> evolution. Wax residuals were analyzed by hexane extraction and gas chromatography. Waxed papers showed a 66% loss of carbon in 143 days (20.4 weeks). These results resembled the results of previous soil experiments.

Polyethylene coatings were also degraded, but twice as slowly under the same experimental conditions. In these experiments the wax degradation rate was critically dependent on two factors: the presence of nutrients in the form of nitrogen and the soil loading level on the paper. A four times increase in the soil level on the paper led to an eight times decrease of wax degradation. A possible explanation for this result is the limitation of oxygen and the exchange of fungal decomposers with rather inactive anaerobic bacterial colonizers.

On the basis of the data cited above, it can be concluded that all chemical profiles of petroleum wax composition are degraded by microorganisms (bacteria, fungi, and yeasts) in the following ideal conditions: wet environment, increased temperature, and the presence of nutrients and oxygen. The simpler the hydrocarbon molecules are, the easier they are consumed by microorganisms.

### **2.3.3. Biodegradation of wax coatings on copper alloys**

In conservation practice, microcrystalline waxes are traditionally used for protection of copper alloys against corrosion, especially for treating of archaeological bronze artefacts. The wax barrier repels water from the metal surface to ensure against further introduction of

contaminating elements by handling and exposure. Stability of this barrier in the conditions of museum storage is the most important factor for good preservation of underlying metal.

Despite their essential inertness, microcrystalline waxes are vulnerable to natural aging and microbial attack. The natural aging of wax films caused by the combined effects of ultraviolet radiation, oxygen, and moisture, increases the susceptibility of wax films to microbial growth. Microorganisms including bacteria and fungi can attack wax coatings under conditions promoting microbial activity. These are: high humidity, increased temperatures, and the presence of oxygen.

In a dry museum climate, bacteria cannot thrive, and thus they cannot play any serious role as potential wax decomposers. Fungi, on the contrary, are able to degrade microcrystalline waxes even under controlled museum conditions. In museum storage, fungal spores, together with bioaerosols and dust, can land on the surface of wax. If the conditions for fungal growth become favorable, spores will start to germinate. Growing hyphae pierce the wax film to seek degradable ingredients of the wax composition as a source of nutrients (Bennet, Wunch, and Faison 2002).

Although the heavier ingredients of microcrystalline wax are hardly digestible, the degradation of even small amounts of easily degradable low molecular weight ingredients may provoke partial loss of the barrier properties of the thin coating film. Microbial colonization causes formation of pores and cracks on the surface of the wax. At the same time, the microbial biofilm absorbed large amounts of water, creating slime on the wax surface. Absorption of water through defects on the surface of wax breaks down the dielectric properties of the wax layer and initiates the corrosion processes in the underlying metal (Mitton *et al.* 1993; Otieno-Alego *et al.* 1998a;

Otieno-Alego *et al.* 1998b; Letardi *et al.* 2002). In addition, microbes excrete aggressive metabolites that develop corrosion. Recent studies confirm that microbial metabolites such as enzymes, exo-polymers, organic and inorganic acids, and volatile compounds (ammonia, hydrogen sulphide, and others) increase the corrosion of the copper alloys (Beech and Gaylarde 1999, Yokota *et al.* 2003).

In museums and museum storage facilities with partially controlled or uncontrolled climates, microcrystalline waxes are potentially vulnerable to microbial degradation. According to the observations of the author of the present work, after a flood disaster\*, a microbial *consortium* was able completely degrade the wax coating on archaeological bronze objects in three weeks. Under favorable conditions for microbial growth and considerable initial biocontamination of the storage, whole collections of bronze artefacts can suffer from “bronze disease” (see section 5.2). For such extreme conditions, wax coatings probably require special protection against microbial colonizers.

A possible way to increase the bioresistance of conservation waxes is to add inhibiting agents, such as polysaccharides and phenol compounds extracted from *Sphagnum* moss. In the present work, *Sphagnum* polysaccharide (*Sphagnan*) was added to conservation microcrystalline and polyethylene waxes. The treated waxes were tested against fungi (*Aspergillus* spp.). Results are presented in section 5.4.

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\*This was concluded on the basis of experiments simulated the climate conditions after flood (i.e. increased temperatures and humidity). The flood occurred in Kaman Kalehöyük storage facility-see section 5.1.

## Chapter 3. Materials and Methods.

This chapter contains information about collecting of *Sphagnum* plant material, preparation of *Sphagnum* extracts, sampling and isolation of fungi, and methods of biological testing. After preparation, the physical and chemical properties of extracts and their chemical structures were analyzed. These results, including FTIR spectra, are included in the Appendix.

### 3.1. Origin of *Sphagnum* plant material used for the preparation of extracts

*Sphagnum* moss was collected from a peat bog located off Opinicon Road in the vicinity of Chaffey's Locks, 40 km to the north of Kingston, Ontario. Information about areas where *Sphagnum* moss can be found near Kingston was provided by Dr. A.A. Crowder, Professor Emeritus, Department of Biology, Queen's University. To obtain fresh *Sphagnum* moss, a one-day expedition to the Chaffey's Locks area was carried out in the middle of October 2002. The expedition was organized by Dr. H.F. Shurvell, Adjunct Professor, Art Conservation Program, Queen's University. In the bog, *Sphagnum* moss was found growing on a small island several meters from the shore. (Fig.3.1). On the island, *Sphagnum* occupied several hummocks and formed a dense green mat with a diameter of approximately two meters. Patches of moss alternated with other bog flora including sedges, living and dead shrubs, and others (Fig.3.2). The collected moss represented only one species, which was identified by Dr. A.A. Crowder as *Sphagnum palustre*\*.

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\**Sphagnum palustre* was one of several *Sphagnum* species used by Painter for the preparation and testing of *Sphagnum* extracts (Börsheim, Christensen, and Painter 2001a).



**Fig.3.1.** View of a peat bog near Chaffey's Locks. The moss was collected by Dr. Shurvell, who crossed the water by a fallen tree lying between the shore and a small island where *Sphagnum* moss was found.



**Fig.3.2.** View of *Sphagnum palustre* growing on a hummock. The dense moss mat alternates with different representatives of bog flora.

## **3.2. Preparation of *Sphagnum* extracts and determination of their properties**

The collected moss was used for the preparation of five extracts including Extract 1, Extract 2, and three water extracts: cold, hot, and boiling water extracts. Extract 1 and Extract 2 were prepared in the Department of Chemistry, Queen's University, under the supervision of Dr. W.A. Szarek. Water extracts were prepared in the Laboratory of Biotechnology, Brock University, under the supervision of Dr. A. Castle.

### **3.2.1. Preparation of Extract 1 and Extract 2**

These two *Sphagnum* extracts contain reactive water-soluble polysaccharides comprising plant hemicelluloses. They were isolated from *Sphagnum* plant material closely following the technique elaborated by Prof. Painter and following his personal description and directions. The extraction of water-soluble polysaccharides from the *Sphagnum* plant involves three principal stages: (a) removing the acetone-soluble plant constituents; (b) removing lignin to refine holocellulose; and (c) isolation of reactive polysaccharides from holocellulose.

#### ***Acetone extraction***

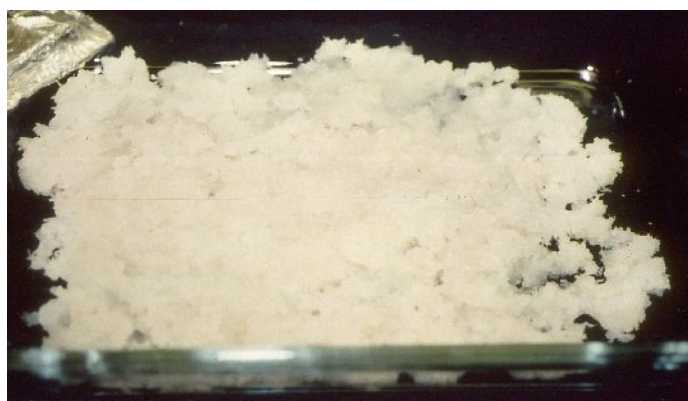
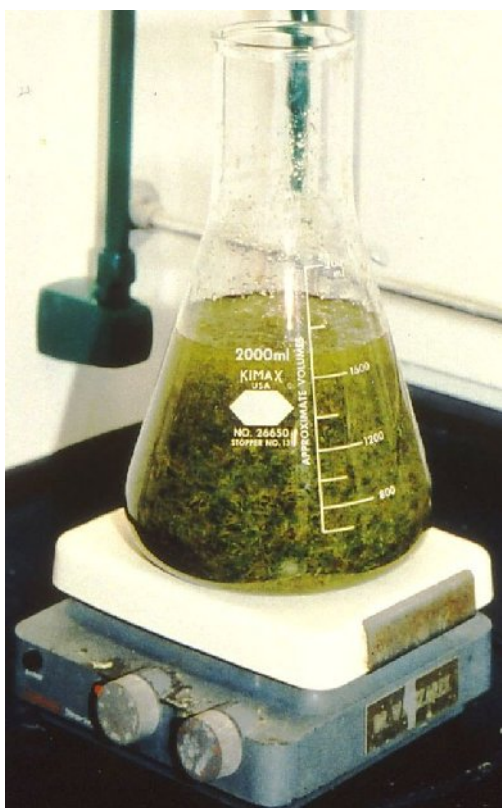
In the acetone extraction, waxes, steroids, terpenoids, carotinoids, chlorophyll and other pigments are removed from the *Sphagnum* plant material. Whole *Sphagnum* plants (Fig.3.3) were cleaned mechanically and dried overnight in a current of air at 45 °C. The dried leaves were stripped from the stems and the stems were ground and mixed with the leaves. Then 50 g of plant mixture was placed into a two liter thermo-resistant conical Erlenmeyer flask and boiled with one liter of acetone for 3 minutes. Extracted plant constituents converted the solution to a green color (Fig.3.4, left).

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\* The boiling point of acetone is  $t = 57^{\circ}\text{C}$  (Kaye and Laby 1995).



**Fig.3.3.** View of *Sphagnum palustre* which was used for preparation of extracts.



**Fig.3.4.** View of acetone extraction – on left. The product obtained after six sequential procedures of acetone extraction - on right.

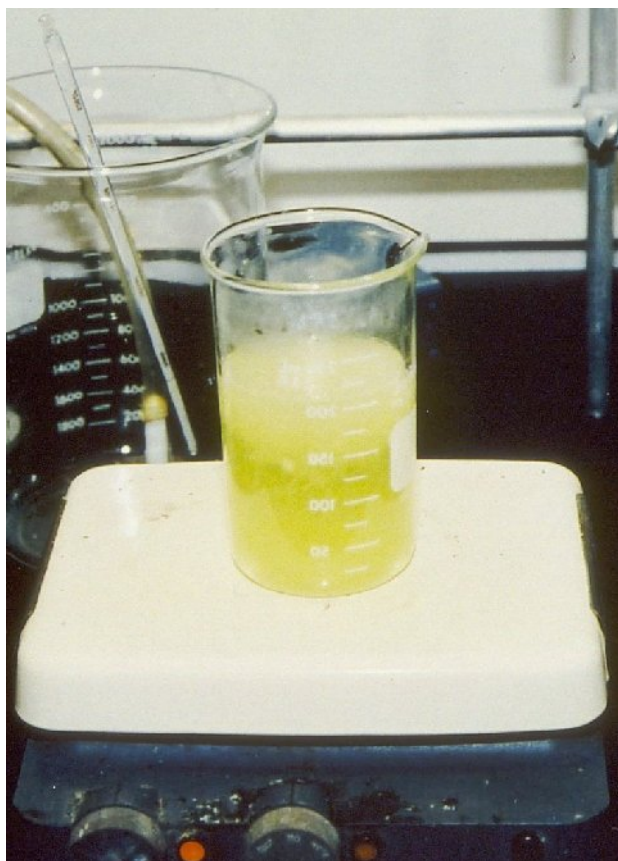
After cooling to room temperature, the solid phase of the mixture was separated from the solution by vacuum filtration through a glass filter with a pore size of 60 $\mu$ m. The process was repeated six times until the solid became almost colorless. The residue was washed once more with ethanol and air dried in a fume hood at 25°C. The final product is the lignocellulose. It was a light-grey colored solid with a fibrous structure (Fig.3.4, right). The amount of this product was 21.3 g.

### ***Isolation of lignin-free holocellulose***

After acetone extraction the residue still contains lignin-like aromatic polymers, which must be removed in the next stage. In this stage, the obtained solid was oxidized by sodium chlorite NaClO<sub>2</sub> to remove lignin-like aromatics. Delignification of lignocellulose was carried out in an acetic acid solution. The obtained amount of solid (21.3 g) was mixed with distilled water in a two-liter glass beaker in the ratio of 1:3 (v/v) and slowly heated to 75°C. When this temperature was reached, 30 ml of glacial acetic acid was added and the flask was placed in a thermostatic bath at 75°C with constant stirring. The amount, 30g, of sodium chlorite was added during the next hour in five equal portions at approximately equal time intervals. After three hours of constant stirring, the process was repeated, i.e. another 30 g of sodium chloride was added in five equal portions. After another three hours of constant stirring this procedure was repeated once more. Therefore, the process took a total of 12 hours for the three stages.

After cooling the mixture to room temperature, it was filtered using a vacuum pump through a 60 $\mu$ m pore size glass filter covered with a paper filter #4. The extracted lignin compounds colored the solution yellow (Fig.3.5, left). The bleached holocellulose collected on the filter was subsequently washed with distilled water, 0,02N hydrochloric acid and again with plenty of

distilled water until the washings became neutral. During the procedure, the color of the filtrate changed from yellow to almost colorless (Fig.3.5, right). The residue was finally washed with acetone and air dried. The water washing process took two full days to obtain the neutral washings. The final product of this stage was 9.8 g of holocellulose. It appeared almost the same as the lignocellulose, except for its clear color.



**Fig.3.5.** The extraction of lignin using the sodium chlorite method. Extracted phenolics colored the solution yellow-on left. After washing the solid with hydrochloric acid and water, the filtrate became colorless-on right.

### ***Isolation of reactive polysaccharides (Sphagnum) from Sphagnum holocellulose***

At this stage, the water soluble part of the holocellulose was subjected to lengthy high temperature autohydrolysis to obtain hemicelluloses. The waste product of this stage was the cellulosic matrix of the *Sphagnum*'s cell wall, which is not subject to hydrolysis. The critical

requirement for this process is to prevent the oxidation of reactive carbonyl groups contained in the hydrolysate. Therefore, oxygen must be removed from ambient water and the atmosphere. Finally, this isolate was concentrated to a dry water- soluble product containing a mixture of reactive polysaccharides (Extract 1 or Extract 2).

The entire amount of holocellulose (9.8 g) was mixed with 1.5 liters of degassed distilled water\* to a thick slurry and transferred into a two-liter thermo-resistant round bottom flask. The flask was placed in a thermostatic bath and slowly heated until the temperature reached 98°C. At this temperature boiling had not occurred\*\*. The mixture remained in the thermostat at 98°C under a weak flow of nitrogen for 15 days (Fig.3.6, left).

After cooling to room temperature, the mixture was filtered by vacuum through a 60µm pore size glass filter covered with a paper filter #4. The solid residue was mixed with another 1.5 liter aliquot of degassed distilled water, heated in a thermostatic bath for another three days at 98 °C and filtered under the same conditions. The combined filtrates were concentrated to 500 ml in a rotary evaporator at 30°C and then freeze-dried. The final residue consisted of 4.1 g of an amorphous light-brown colored solid with a strong and unpleasant smell. This solid was named Extract 1 (Fig.3.6, right). The procedure for the preparation of Extract 2 was generally the same as for Extract 1, including the three principal stages of the process. A minor temperature alteration was introduced at the stage of the hydrolysis of the holocellulose slurry, which was brought to a boil.

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\*Degassed water means water which is free of atmospheric air. Degassed distilled water was prepared by allowing nitrogen to flow through the distilled water with constant mixing for 30 min. This procedure removes most of the dissolved gases, excluding nitrogen, which is inert.

\*\*The water-holocellulose mixture is a colloid solution with boiling point lower than 98°C. At temperatures higher than 98°C the solution started to boil.

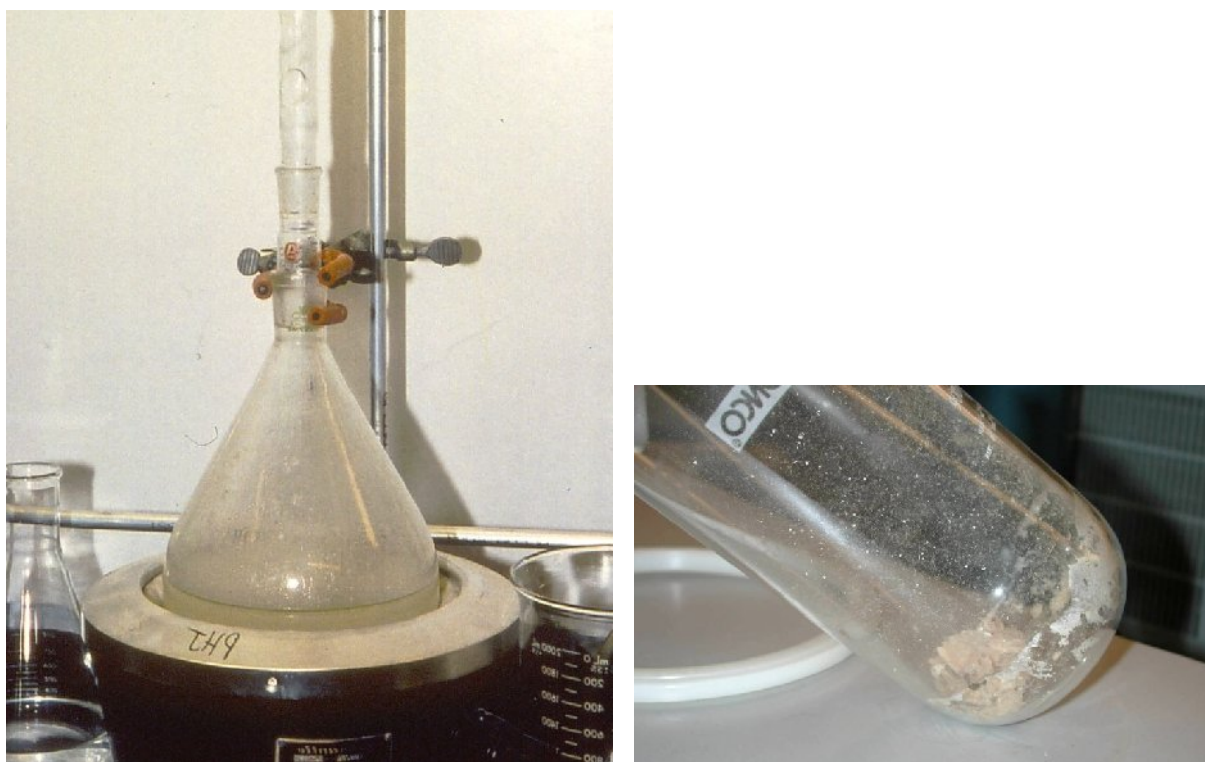
The yields of the products obtained at different stages of the preparation for Extract 1 and Extract 2 were similar and are specified below:

22.8 g of lignocellulose was obtained from 50g of dry plant material after acetone extraction;

9.8 g of holocellulose was obtained from this amount of lignocellulose;

3.7 g of final product was obtained from this amount of holocellulose.

The structure, color, smell, density, and solubility in water, alcohol, or other organic solvents of Extract 2 did not differ from Extract 1. These properties are specified in the Appendix



**Fig.3.6.** Lengthy hydrolysis of holocellulose-on left; view of the final product (Extract 1)-on right.

### **3.2.2. Preparation of water extracts**

#### ***Cold-water extract***

50 g of whole, unprocessed plants were mixed with 1.5 liters of distilled water at room temperature in a conical Erlenmeyer flask. The mixture was constantly and slowly stirred for 24 hours. During stirring, the liquid portion turned brown. This liquid portion was vacuum-filtered through a 60µm pore size glass filter covered with a paper filter # 4. The collected filtrate was freeze-dried. The obtained solid was a dark brown amorphous substance with a strong unpleasant odor.

#### ***Hot-water extract***

50 g of whole, unprocessed plants were mixed with 1.5 liters of distilled water in a conical Erlenmeyer flask. The flask was placed in a thermostatic bath with constant slow stirring at 80°C. After 24 hours the mixture was cooled to room temperature and vacuum-filtered through a 60µm pore size glass filter covered with a paper filter # 4. The collected filtrate was freeze-dried. The appearance and smell of the obtained solid substance was similar to the cold-water extract.

#### ***Boiling-water extract***

50 g of the same plant material were ground and then mixed with 1.5 liters of cold distilled water. The mixture was heated and brought to boiling for eight hours at a temperature of 98°C with slow constant stirring. After that, the mixture was cooled to room temperature and vacuum-filtered through a 60µm pore size glass filter covered with a paper filter # 4. The collected filtrate was freeze-dried. The solid was a dark brown powdered substance with an unpleasant

odor. However, the odor was less strong than in the cold- and hot-water extracts, probably due to a partial evaporation of aromatics during the boiling of the mixture.

### 3.2.3. Determination of physical and chemical properties of extracts

In the present work, only the volume weight and the solubility of *Sphagnum* extracts in alcohol and water were determined quantitatively. To determine volume weight, 2.0 ml graduated tubes\* were filled with *Sphagnum* extracts, slightly pressed, and weighed on a Sartorius analytical balance with a readability of 0.1 mg. To determine solubility, weighed portions of extracts were placed in 2.0 ml graduated tubes. Then a solvent (water, alcohol, a water-alcohol mixture, acetone, toluene and vegetable oil) was added to the tubes by Eppendorf® automatic pipette and mixed with a glass rod until the solute was almost completely dissolved\*\*. The volume of the solution was determined by weighing, using the same balance. Acidity of liquid extracts was determined by a hand-held ORION® pH-meter with pH accuracy of 0.01. All experiments were carried out at room temperature.

To characterize the difference between *Sphagnum* products, Extract 1, Extract 2, and *Sphagnum* holocellulose were analyzed by Fourier transform infrared spectroscopy (FTIR). The analysis was performed in the Art Conservation Program laboratory, Queen's University by Dr. H.F. Shurvell. The infrared spectra recorded are shown in the Appendix \*\*\*.

\*Cole-Parmer® 2.0 ml microcentrifuge tubes

\*\*All obtained *Sphagnum* extracts, including polysaccharides and the water extracts, did not dissolve completely in the given solvents. A small amount of solid phase is always present in a solution.

\*\*\*The structures of *Sphagnum* Extract 1 and the Water Extract were also analyzed by Nuclear Magnetic Resonance (NMR) Spectroscopy. The comparative NMR spectra of *Sphagnum* extracts were obtained by using the Bruker Avance 600 Digital NMR spectrometer at the Department of Chemistry, Brock University. NMR spectra are not presented in the present study.

### 3.3. Preparation of fungal material for testing of *Sphagnum* extracts

#### 3.3.1. Sampling and isolation of fungal cultures

Fungi were sampled from three sources:

- (a). Archaeological objects that were recently excavated from Arctic sites (18 isolates);
- (b). Ethnographic objects stored in the climate-controlled conservation laboratory of the Art Conservation Department, Queens University (5 isolates); and
- (c). Archaeological objects, storage equipment and building interior contaminated with a wide diversity of actively growing microorganisms in the Kaman-Kalehöyük archaeological site storage (4 isolates).

##### *(a). Archaeological objects from Arctic sites*

The first group of fungi was sampled\* from archaeological objects excavated in 2003 at the site of Nunguvik, Navy Board Inlet (northern Baffin Island, Canada). The objects belong to the culture of Dorset Paleoeskimos. All objects except one were free from any visible microbiological growth. After excavation, they were transported to the Canadian Museum of Civilization (CMC). For transportation, objects were packed in fresh moss harvested near the site \*\*. Transportation took approximately three weeks. However, no visible microbial growth was found on the objects at the time of their receipt at the conservation laboratory at the CMC.

\* This site was excavated in the 1970s and 1980s by Father Guy-Mary Rousselière (1913-1994). After his death, this work was continued by an expedition of the Canadian Museum of Civilization (CMC) under the supervision of Dr. Sutherland, Curator of Eastern Arctic Archaeology (CMC). For more details see: <http://pubs.aina.ucalgary.ca/arctic/Arctic47-3-318.pdf>.

\*\*The samples of this moss were also kindly provided by Dr. Sutherland for their morphological identification. Identification of moss species was performed by Dr. A.A. Crowder in the Department of Biology, Queen's University. The remains of many plants inhabiting peat bogs, including vascular plants, were found in the moss mass. After careful observations, eight species of moss were identified. Surprisingly, none was recognized as *Sphagnum*.

In the conservation laboratory, fungi were sampled\* from twelve objects made of wood, bone, ivory, skin (seal), and baleen. Single swabs were taken with sterilized cotton tampons from several parts of each object. The swabs were placed in sterile glass tubes and transferred to the Department of Microbiology and Immunology, Queen's University, where fungal cultures were isolated. In the microbiological laboratory, the collected fungi were plated on Petri dishes with SAB Maltose Agar (see section 3.4.3) by touching the swabs to the surface of the media. Plates were incubated in the laboratory (i.e. at room temperature and daylight) for 4-8 days, until morphological differences of colonized fungi became recognizable. Then each fungal species was inoculated once more onto three plates by a single touch of a growing colony with a sterile wooden stick. To obtain pure cultures, fungi were re-inoculated 3-4 times. Purification of cultures was performed in the Department of Microbiology and Immunology, Queen's University. Eighteen fungal species were isolated and given numbers: 15-1, 16-1, 16-2, 16-3, 17-1, 17-3, 20-1, 20-2, 21-2, 21-3, 22-1, 23-2, 26-1, 26-2, 26-3, 26-4, 26-5, and 26-5(1).

***(b). Ethnographic objects***

The second group of fungi was sampled from ethnographical objects stored in a controlled museum climate in the laboratory of the Art Conservation Department, Queens University\*\*. Objects were made from different materials, including wood, skin (caribou and seal), gut, and textile (silk and wool). The appearance of the objects was fine, without any visible microbial growth because under controlled conditions fungal growth is normally suppressed. In this case, fungi exist in the form of conidia, i.e. in a dormant state.

\*Samples were provided in 2003 by Dr. Patricia Sutherland (CMC).

\*\*These objects originated from the Arctic. They belonged to the CMC. Several years ago they were transported from the storage of the CMC to the conservation laboratory of the Art Conservation Department, Queens University.

To sample fungal spores from these artifacts, open Petri plates with SAB Maltose Agar were placed under each object for 15 minutes. Afterwards, the plates were covered with lids and, as in the previous case, incubated in the laboratory for 4-8 days. Purification of cultures was performed by the standard procedure described above for Arctic archaeological objects in the Department of Microbiology and Immunology, Queen's University. Five different fungal species were detected on the plates and named 1.1, 1.2, 3.1, 4.2, and 5.1.

***(c) Archaeological objects, storage containers, and interior materials from the severely contaminated Kaman-Kalehöyük archaeological site storage***

This group of fungi was sampled from the severely colonized interior of the archaeological site storage at Kaman-Kalehöyük (Turkey). After the flood disaster, artifacts, storage containers, and building materials were attacked by growing microorganisms including bacteria, fungi, and yeasts. Fungi were sampled from all biocontaminated materials. Spores were taken from contaminated polyethylene bags where bronze objects were stored and from paper and fabric sacks and wooden boxes for ceramic storage. Spores were also taken from wax coatings from the affected bronze objects\*. Finally, fungi were sampled from interior building materials such as furnishings, concrete walls, ceilings and others.

Spores were swabbed with sterilized cotton tampons, plated on Petri dishes with SAB Maltose Agar, and purified by the standard procedure. Purification of sampled fungi was performed at the Kaman-Kalehöyük conservation laboratory. Four fungal species were isolated.

\*Some objects were stored in conditions of increased preservation. They were packed in double plastic bags which were put in hermetically sealed plastic boxes to prevent any contact with water. These objects were found in a fine condition. For single plastic bags, their internal surfaces and the surfaces of packed bronze objects were found to be colonized by fungi. When wax coatings were affected by microorganisms, in most cases the objects were found to be severely corroded.

### 3.3.2. Selection of fungal cultures for the testing of *Sphagnum* extracts

After isolation, morphological structures of species originating from archaeological objects and ethnographical objects were examined by light microscopy. Several common morphologic forms of fungi sampled from both groups of objects were found: 1.1=26-1; 1.2=26-4; 3.1=16-1=16-2; 4.2=26-2=26-3; 5.1=21-2, and 5.2=26-1. From common cultures, species with higher viabilities of spores and rates of spore germination\* were taken for further experiments. Then, fungi from archaeological and ethnographical objects were joined in one group containing following species: 15-1, 16-2, 16-3, 17-1, 17-3, 20-1, 20-2, 21-2, 21-3, 22-1, 23-2, 26-1, 26-2, 26-4, 26-5, and 26-5(1). The inhibiting capability of *Sphagnum* Extract 1 and Extract 2 was tested on these sixteen species by the disk diffusion method (see section 3.4.1); a standard antifungal agent sodium azide was tested for comparison.

From all examined isolates, only six species showed equal or higher susceptibility towards *Sphagnum* extracts compared to sodium azide: 15-1, 16-2, 16-3, 17-3, 20-1, and 23-2 (see Table 4.1). These species were selected for further testing of the inhibiting activity of *Sphagnum* extracts in tubes\*\*. From these six isolates, species 16-2 showed the highest rate of germination. In addition, this species was found on the majority of examined artifacts/materials. For this reason, species 16-2 was used for further tube testing of the inhibiting activity of the Water Extract. The results of the tube tests of *Sphagnum* Extract 1 and the Water Extract towards species 16-2 are shown in chapter 4, section 4.2.3.

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\*The viability of spores is the relationship between the numbers of germinated spores and plated spores. The rate of germination is the time interval between the time when spores are plated and they start germinating.

\*\*In tubes, the viability of spores in broth (without Extract 1 added) was tested for all sixteen species, depending on the time of their exposure to broth.

Identification of fungi includes molecular and morphological taxonomy of a given fungal culture. Molecular taxonomy is determined on the basis of the DNA sequence of the fungal organism. Only DNA analysis enables accurate identification of fungi (Takashi and Akemi, 2004). However, this analysis is quite complicated and expensive. For this reason, in the present work only the morphological taxonomy of species 16-2 was determined. According to the author's morphological examination, species 16-2 belongs to the *Aspergillus* genera. Four cultures isolated in Kaman-Kalehöyük were tested in tubes without inhibiting agents added. One of four species showed the best rate of germination on plates. It also possessed satisfactory viability in the nutritious broth. This species was found in the majority of objects/materials examined. Hence it was selected as a degrading organism in the experiments with wax coatings protection by *Sphagnum* Extract 1. The morphology of this species was very close to that of the species 16-2 isolated from the Arctic archaeological objects, i.e. it also belongs to the *Aspergillus* genera.

#### **3.4.1. Disk diffusion method**

The disk diffusion method is widely used in bacteriology to test the susceptibility of pathogenic bacteria and to determine the minimum inhibitory concentration (MIC) for the tested antibacterial agents (Jorgensen, Turnidge, and Washington, 1999)\*.

\*This method was suggested in 1940 by Pope (Wheat and Spencer 1988). At that time the disk diffusion technique was widely used for testing of penicillin. Later, a number of antimicrobial agents with various concentrations were tested by this method on various bacterial cultures. These cultures/agents can be used as standards for newly discovered antibiotics (Gould and Bowie 1952; Bauer *et al.* 1966). Following the disk diffusion method, the tested antibacterial agent is prepared by serial two-fold dilutions\*\*. The minimal inhibitory concentration (MIC) is read as the lowest dilution of antibiotic, which produces complete inhibition of bacterial growth on nutritious agar.

\*\*A two-fold dilution reduces the concentration of a solution by a factor of two. Serial dilutions are made by making the same dilution step over and over, using the previous dilution as the input to the next dilution in each step.

In the present work, the disk diffusion method was used to test the effect of *Sphagnum* extracts on three standard bacterial cultures and sixteen fungal cultures isolated from archaeological and ethnographical objects (see section 3.3.1). Following this technique, a filter paper disk incorporating an antibacterial agent is superimposed on a nutritious agar media mixed with a bacterial culture or fungal spores. The antibacterial agent spreads in the gel medium by molecular diffusivity. After incubation, at a particular distance from the disk edge, the concentration of antibacterial agent in the media is maintained, preventing microbial growth. It appears as a circular border between the dead and growing microorganisms on the surface of the media. The dimensions of a zone without microbial growth can be used as a qualitative expression of the inhibiting effect of the agent on the tested organism.

### ***Bacterial tests***

Standardized bacterial cultures (*Staphylococcus aureus*, *Escherichia coli* EmG31, and *Pseudomonas aeruginosa* K767) were stored frozen at -28° C in 15% (v/v) Bacto Malt Extract (see section 3.4.3) in glycerol. This suspension was diluted in sterile distilled water to prepare a stock of cells. This stock was subsequently diluted up to 10<sup>-8</sup> to obtain a countable concentration of cells. The 0.1 ml aliquots of bacterial cell stock and its 10<sup>-8</sup> dilution were inoculated to Petri dishes containing Bacto Malt Extract. For even distribution of the bacterial cells in the media, 0.5 mm sterile glass beads were added in the media and distributed with a sterile glass rod. Then 20 µl of 3% Extract 1 and 3% Extract 2 were dropped onto 10 mm filter paper disks superimposed on the media.

After 18 hours of incubation, the surfaces of the plates were covered by growing colonies, excluding circular-shaped zones without growth surrounding the disks. Their dimensions were

estimated from digital photos. The antibacterial effect of *Sphagnum* extracts was compared to 0.05 % *Ampicillin*, the standard antibacterial agent\*.

### ***Fungal tests***

Sixteen fungal species isolated from archaeological and ethnographical artifacts, 15-1, 16-2, 16.3, 17-1, 17-3, 20-1, 20-2, 21-2, 21-3, 22-1, 23-2, 26-1, 26-2, 26-4, 26-5, and 26-5(1) were tested by the disk diffusion method (see section 4.2.1). The conditions of fungal tests were almost the same as the bacteria tests, with minor differences. In fungal experiments, SAB Maltose Agar (see section 3.4.3) was used. The stocks of spores in sterile distilled water were prepared by direct counting with a hemocytometer (see section 3.5). For the disk experiments, 0.1 ml aliquots of stocks were plated on the SAB Maltose Agar mixed with 0.5 mm sterile glass beads and evenly distributed with a sterile glass rod. Aliquots of 20  $\mu$ l of the 3% Extract 1 and Extract 2 in water were dropped onto 10mm filter paper disks, superimposed on the surface of the media. After incubation in the laboratory (at room temperature and daylight) for 36-48 hours, the dimensions of zones without growth were estimated from digital photos. The effect of *Sphagnum* extracts on fungal cultures was compared to the effect of 0.05%  $\text{NaN}_3$ , a standard antifungal agent\*\*.

All *Sphagnum* extracts are colloid solutions and therefore their liquid and solid phases separate on the paper disk. The solid phase is retained on the disk as a thin brown film while the liquid phase runs over the surface of the media. Since the antimicrobial activity of each phase is not known, the effect of separation may distort the result of the experiment. To avoid this experimental error, tube tests of *Sphagnum* extracts were performed.

\*This concentration was recommended by Dr. A. Kropinski, the Department of Microbiology and Immunology, Queen's University.

\*\* This concentration was recommended by Prof. A. Castle, the Laboratory of Biotechnology, Brock University.

### 3.4.2. Tube method

In the present work, the modified broth-dilution method\* was applied to fungi. Fungal spores in concentrations of 2,000 CFU/ml, 5,000 CFU/ml, and 10,000 CFU/ml were introduced into three sets of 5 ml test tubes containing SAB Maltose Broth (see section 3.4.3). These concentrations of spores were prepared using direct counting with a hemocytometer (see section 3.5). Then 20 µl of 3% Extract 1 or the Water Extract were added to each tube. After exposure at room temperature, the viability of spores was checked by plate count. The duration of the exposure varied from 3 to 24 hours. This time interval was divided into shorter 3-hour intervals. After each 3-hour interval, 0.1 ml aliquots were taken from tubes and plated to Petri dishes containing SAB Maltose Agar (see section 3.4.3). Plates were incubated in the laboratory (at room temperature and daylight) for 36-48 hours. After incubation, the numbers of grown colonies on the plates were counted. The ratio of the number of colonies to the number of spores plated characterizes the viability of spores which were exposed to *Sphagnum* extracts. If the extract effectively inhibits fungal growth, the viability of spores in tubes with the extract added should be much lower than in controls (without the extract added).

The viability of spores in the Broth (without *Sphagnum* extracts added) was tested for the following species: 15-1, 16-2, 16.3, 17-1, 17-3, 20-1, 20-2, 21-2, 21-3, 22-1, 23-2, 26-1, 26-2, 26-4, 26-5, and 26-5(1). In addition, for these species the formation of spore clusters in the Broth was observed with optical microscopy (see section 6.2). Some isolates did not survive in the broth, i.e. in water media.

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\*The broth-dilution method was suggested by Fleming as a test for bacteria (Fleming 1929). In this method, the antimicrobial agent prepared in a form of serial or two-fold dilutions, is added in broth containing the test microorganisms. After incubation, growth or absence of growth is checked in broth at the given concentration of antimicrobial agent. There are several end points indicating life or death of microorganisms: changing pH; optical density; fluorescence of bacteria; etc. (Fleming 1942; Doern 1987; Thornsberry 1984; Spencer and Wheat 1986). Our technique was modified from Fleming's method. *Sphagnum* extracts were added to the test tubes at constant concentration, while the concentrations of spores in the tubes and the time of exposure varied.

However, for the majority of the examined species, their viability started decreasing only between 18 and 24 hours of exposure. Therefore, for control samples (i.e. without *Sphagnum* extracts added), it was considered that the broth does not stimulate or inhibit the germination of spores up to 24 hours of exposure. In this interval, the number of colonies grown on the plates should be constant, depending only on the number of living spores in the broth. The results were in agreement with this assumption: the number of colonies on controls did not vary significantly between 3 to 24 hours of exposure for the majority of species. The inhibiting effect of *Sphagnum* Extract 1 was tested in tubes for the following species: 15-1, 16-2, 16-3, 17-3, 20-1, and 23-2; all were found to be susceptible to this extract\*. From these isolates, species 16-2 showed the highest degree of susceptibility. The results of these experiments are presented in chapter 4, section 4.2.3. Species 16-2 was also tested in tubes against the Water Extract and maltose and glucose (see section 4.2.4).

### 3.4.3. Media

In the present work, for bacterial tests Bacto™ Malt Extract\*\* was used. For fungal tests, Difco Sabouraud Maltose Agar\*\* (SAB Maltose Agar) media was used for the cultivation of fungal species in Petri dishes. As a nutritious media in tubes, liquid Difco Sabouraud Maltose Broth\*\* (SAB Maltose Broth) media was used. It was important to exclude bacteria from the experiments with fungal cultures. Bacteria were present in the samples taken from archaeological objects, in *Sphagnum* extracts (which were not-sterilized prior to testing) and in the surrounding air\*\*\*.

\*The inhibiting effect of Extract 1 on these six species was tested in preliminary experiments after 12 and 18 hours of exposure. In these experiments, the viability of spores in the broth was also tested in tubes for all sixteen selected fungal species.

\*\*All media products were manufactured by Difco Laboratories, Detroit, Michigan, USA ([http://www.vgdllc.com/Anonymous/Difco\\_Dehydrated\\_Media\\_BBL\\_2008.pdf](http://www.vgdllc.com/Anonymous/Difco_Dehydrated_Media_BBL_2008.pdf)).

\*\*\*The presence of bacteria may disturb the proper growth of fungal colonies and distort the results of experiments. SAB media, being a nutrient media suggested specially for fungi, prevents the development of bacterial organisms (Mather and Roberts 1998).

### 3.5. Methods of spore counting

To estimate the effectiveness of *Sphagnum* extracts, the spore density (concentration) has to be quantified. For experiments, it is desirable to operate with a proper and convenient density of spores. If the number of spores is too low, the accuracy of the experiment is decreased. If the number of spores is too high, grown colonies create a dense lawn and counting their number is a problem. Therefore, it is important to determine the concentration of spores in the stock solution prior to the experiment.

The concentration of spores can be determined using various methods (Mather and Roberts 1998). Most commonly it can be done either by successively diluting the stock of spores with subsequent plating of intermediate spore solutions on Petri dishes, or by a special cell counter such as a hemocytometer. The plating method detects only the viable spores, whereas a hemocytometer can only detect the total number of spores. The dilution method allows estimation of the concentration of living spores in the stock of cells at the moment of inoculation. However, to be counted, fungal colonies are normally grown on plates for 18-36 hours. During this time, spores in the stock can die or lose their viability and the results of a subsequent experiment can be distorted. Direct counting of spore density with a hemocytometer does not need a stage of incubation. It allows promptly adjusting the desirable number of spores in the stock. However, it is hard to distinguish by a hemocytometer whether or not the spores in the stock are viable. In the present work, the total concentration of fungal spores in stock was determined by a hemocytomete for all fungal tests. The viability of spores in tubes after exposure to *Sphagnum* extracts was estimated by plating on agar media. Counting by the dilution method was used in bacterial tests only, to determine the number of bacterial cells in stock.cells.

### 3.5.1. Counting with hemocytometer

A hemocytometer is an etched glass chamber which, in combination with a light microscope, allows counting the number of fungal spores or bacterial cells in solution (Mather and Roberts 1998). In the present work, an improved Neubauer 1/400 hemocytometer with chamber depth  $0.100\text{mm} \pm 2\%$  was used. On the bottom of the transparent chamber a grid is depicted, covering  $9\text{ mm}^2$  ( $3 \times 3\text{ mm}$ ). Boundary lines of the Neubauer grid are the center lines of groups of three. The central square millimeter is divided into 25 large squares. Each large square is divided into 16 smaller ones (the overall number of small squares is  $16 \times 25 = 400$  in 1 square mm, i.e. 1/400).

A small drop of spore solution is placed between a cover slip and the chamber bottom. The solution, having a good affinity for glass, spreads out on the chamber bottom under the cover slip. Spores were counted with light microscopy at a magnification of 10X-100X\*. The concentration of spores  $C_0$  is defined as  $C_0 = N/80 \times 4 \times 10^6$  spores/ml, where N is the number of spores counted in 5 small squares of the chamber of the hemocytometer (ibid).

The main systematic error of this technique is derived from the assumption that all counted spores are living ones. If unviable spores are present in the spore solution, the number of spores that participate in further experiments will be underestimated. However, in most cases it is possible to recognize living spores by their appearance. Live spores look perfectly round. On the contrary, dead spores have rough edges and never have a perfectly round shape.

\*The counting of spores was performed on the basis of the following recommendations: if there is more than one spore per small square, count the overall number of spores in five large squares (four corners and the center); if there are fewer than one spore per small square, count spores in all 25 large squares; if there are more than 100 spores per large square, dilute the suspension and count the diluted sample (Mather and Roberts 1998).

## Chapter 4. Results.

In this chapter, the results of the biological tests on the prepared *Sphagnum* extracts are reported. The goal of these experiments was to determine the influence of *Sphagnum* extracts on fungi as test organisms. However, to clarify the principal inhibiting efficiency of these extracts, preliminary tests were done with bacterial cultures. Bacteria have already been shown to be sensitive to various *Sphagnum* products. *Sphagnum* Extract 1 and the Water Extract were applied to bacterial cultures and fungal spores isolated from archaeological artifacts excavated in the Arctic. Spores were subjected to *Sphagnum* extracts because they are potentially the most hazardous biological objects found in a museum environment. The response of microorganisms to these extracts was determined by the disk diffusion method and the tube method. Both methods are commonly practiced in laboratories for testing new antimicrobial isolates.

Both Extract 1 and the Water Extract have relatively high acidity. The acidity of the bog environments is considered to be one of the reasons for their “sterility”. Therefore, the acidity of tested extracts can be an independent depressant of fungal growth. To determine the role of acidity in the antimicrobial effect of extracts, neutralized extracts were tested along with regular (acidic) extracts. *Sphagnum* extracts are mixtures of reactive polysaccharides (*Sphagnan*), neutral sugars, and phenol compounds. According to Painter, only the reactive polysaccharides (*Sphagnan*) determine antimicrobial activity of *Sphagnum* moss. However, other sugary ingredients can also inhibit spore germination; for example, they can cause desiccation of fungal cells by an osmotic effect. In this chapter, the possible antimicrobial effect of neutral polysaccharides was demonstrated on two model polysaccharides, maltose and glucose. They were selected because they are the common ingredients of the plant cell wall.

## 4.1. Bacterial tests

The influence of *Sphagnum* Extract 1 and Extract 2 on bacteria was tested using the disk diffusion method (see section 3.4.1). The following certified bacterial cultures were selected as test organisms: *Staphylococcus aureus* (gram-positive), *Escherichia coli* EmG31 (gram-negative), and *Pseudomonas aeruginosa* K767 (gram-negative)\*. Along with *Sphagnum* extracts, a common bacteriostatic, Ampicillin\*\*, was used for comparison. 0.1 ml aliquots of bacterial cell stock and its 10<sup>-8</sup> dilution (see section 3.4.1) were inoculated onto Petri dishes containing Bacto™ Malt Extract (Difco Laboratories, Detroit, USA). The spore slurry was distributed across the surface of the media with a sterilized glass rod. Two 10 mm filter paper disks and one 6 mm disk were placed on the surface of the media. 20 µl aliquots of 3% Extract1 and Extract 2 were dropped onto the 10 mm disks. A 20 µl aliquot of 0.05% Ampicillin was dropped onto the 6 mm disk. In this manner, twenty-seven plates were prepared for each bacterial culture, as follows:

9 plates with stock cells, with test substances applied on paper disks,  
9 plates of 10<sup>-8</sup> dilution, with test substances applied on paper disks,  
9 plates of 10<sup>-8</sup> dilution, without disks (controls).  
27 plates for each bacterial culture, i.e. 81 plates total for three cultures tested.

All plates were incubated: *E-coli* and *Staphylococcus* at room temperature for approximately 18 hours, and *P. aeruginosa* at 37°C for the same period of time. The number of bacterial colonies on the control plates was then counted.

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\*Bacterial cultures were provided by Dr. A. Kropinski, from the Department of Microbiology and Immunology, Queen's University.

\*\*Ampicillin is a bacteriostatic which belongs to the penicillin group of beta-lactam antibiotics (Lemaire *et al.* 2005). Ampicillin is active against gram-positive bacteria such as *E-coli* and *Staphylococcus*. Many gram-negative bacteria including all *Pseudomonas* are considered resistant to Ampicillin. A concentration of Ampicillin of 0.05% was recommended by Dr. A. Kropinski as this causes a visible effect on countable amounts of bacteria on a Petri dish, ca. 200 CFU/dish.

On plates where bacterial cells were inoculated from stock, bacterial colonies had grown as dense lawns. In this case, the number of grown colonies could not be counted directly. Their number was estimated by the multiplication of the number of colonies on the controls by the dilution factor, i.e. by  $10^8$ .

The number of colonies on the controls ranged between 50 and 500 per plate. The effect of *Sphagnum* extracts on the bacteria was estimated from the area around the disk where bacterial growth was suppressed (few colonies or no colonies). The results are presented below.

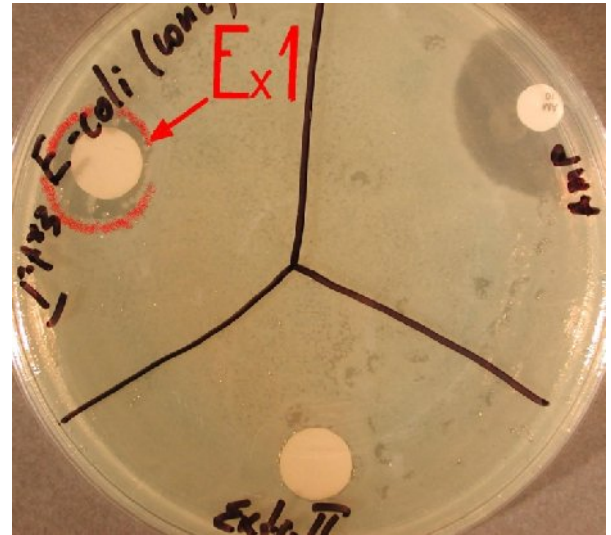
### ***Staphylococcus aureus***

After incubation, the number of colonies grown on the control plates ( $10^{-8}$  dilution) totaled 154 on average, which corresponded to the stock concentration of  $154 \cdot 10^9$  CFU/ml (Fig.4.1). The number of colonies grown in the presence of the test agents ( $10^{-8}$  dilution) was only 54 on average, i.e. three times lower than on controls. However, no distinguishable areas without growth were observed around the disks. In this case it is difficult to determine which inhibitor caused this effect: the extracts, the Ampicillin, or both. *Sphagnum* extracts also did not show any effect on the growth of bacterial cells inoculated from stock. At the same time, around the disk containing Ampicillin, an area without growth with a diameter  $\sim 29$  mm\* was formed. This indicates that in the case of the low concentration of bacterial cells, the inhibiting effect is caused by Ampicillin rather than by *Sphagnum* extracts. Therefore, *Staphylococcus aureus* was shown to be insensitive to *Sphagnum* extracts. Under the same conditions, Ampicillin showed a clear inhibiting effect on this bacterial culture.

\*In most cases the spot with no growth is round shaped. The determination of the dimensions of this area is explained in section 4.2.1.



**Fig.4.1.** The influence of *Sphagnum* extracts and Ampicillin on *Staphylococcus aureus* (stock is shown below,  $10^{-8}$  dilution at left, and control at right). On the plate with  $10^{-8}$  dilution, a decreased number of colonies were grown, compared to the control. On the plate with stock of cells, a notable area without growth around a small disk with Ampicillin can be seen. Both *Sphagnum* extracts showed no effect.



**Fig. 4.2.** The influence of *Sphagnum* extracts and Ampicillin on *E-coli* inoculated from stock is illustrated above. Notable spots without bacterial colonies are seen around disks with Extract 1 and Ampicillin. The arrow indicates the border of a spot for Extract 1.

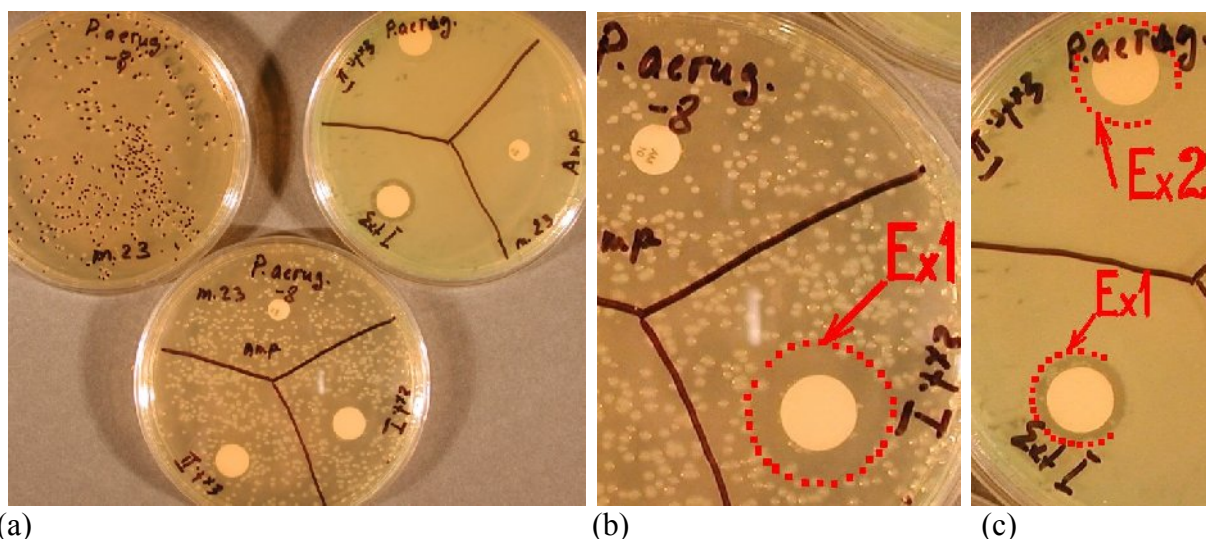
### *Escherichia coli*

After incubation, 280 colonies on average were grown on the control plates ( $10^{-8}$  dilution) (Fig. 4.2.). This corresponded to the stock bacterial concentration of  $280 \cdot 10^9$  CFU/ml. On the plates inoculated with the test agents ( $10^{-8}$  dilution), no bacterial growth was found. This means that *E-coli* are too sensitive to *Sphagnum* extracts and/or Ampicillin in terms of the experiment, i.e. at the given number of the treated bacteria and the amounts of the inhibitors. When bacterial cells were inoculated from stock (their concentration was  $10^8$  times greater) both *Sphagnum* Extract 1 and Ampicillin showed distinct areas without growth with a diameter of  $\sim 18$  mm and  $\sim 28$  mm respectively. Extract 2 did not show any inhibitory effect. The relative inhibiting efficiency  $k$  of Extract 1 to Ampicillin can be estimated:  $k = C_{ex} \cdot S_{amp} / C_{amp} \cdot S_{ex}$  where  $C_{ex}$  and  $C_{amp}$  are concentrations of Extract 1 and Ampicillin;  $S_{amp}$  and  $S_{ex}$  are the areas of their inhibiting zones. On the basis of obtained results the ratio  $S_{amp} / S_{ex}$  was estimated as  $S_{amp} / S_{ex} \approx 3$  (see Fig. 4.2).

The concentration of inhibitors was  $C_{ex}=3\%$  and  $C_{amp}=0.05\%$ , i.e.,  $C_{ex}/C_{amp}=3/0.05$  (Extract 1 was 60 times more concentrated than the Ampicillin). Therefore, the relative inhibiting efficiency of Extract 1 to Ampicillin is estimated as  $k=3/0.05 \times 3.0=180$ . It can be concluded that *E-coli* is sensitive to both Extract 1 and Ampicillin, but Ampicillin is almost 200 times more effective than Extract 1 under the conditions of these experiments.

### *Pseudomonas aeruginosa*

On the control plates ( $10^{-8}$  dilution), totals of 362 colonies were observed on average, which corresponded to the stock concentration of  $280 \cdot 10^9$  CFU/ml (Fig.4.3). On the plates with  $10^{-8}$  dilution both *Sphagnum* extracts formed approximately equal round spots without growth with a diameter of  $\sim 22$  mm. On the stock plates, extracts also produced notable spots without growth, with diameters that ranged between 16 and 18 mm. Ampicillin did not show any inhibitory influence at either concentration of bacterial spores. It can be concluded that both *Sphagnum* extracts had a distinguishable inhibiting effect on *Pseudomonas aeruginosa*, but this species is insensitive to Ampicillin even at low bacterial concentrations ( $10^{-8}$  dilution).



**Fig.4.3.** The influence of *Sphagnum* extracts and Ampicillin on *P.aeruginosa*. (a) The stock plate is at the right, the plate with  $10^{-8}$  dilution is below, and the control plate is at the left. (b) Magnified view of the plate with  $10^{-8}$  dilution. (c) Magnified view of the stock plate. Notable spots without colonies are seen around disks with Extract 1 and Extract 2 (arrow indicate the border between living and dead bacteria). No spots without growth are visible around the small disks with Ampicillin.

## 4.2. Fungal tests

### 4.2.1. Disk diffusion experiments

In the present work, the disk diffusion method was used to determine the inhibiting effect of *Sphagnum* extracts on fungi (see section 3.4.1). Sixteen fungal species were isolated using the standard procedure (see section 3.3.2). Each spore culture was converted to the individual spore slurry by repeated washing with 1 % sterile distilled water solution containing Tween<sup>®</sup> 20 surfactant\*. Each spore slurry was then dissolved in distilled water to obtain a stock of cells having a countable concentration for inoculation on Petri dishes.

The concentration of spores in stock solutions was determined by direct counting with a hemocytometer (see section 3.5.1). For the disk experiments, 0.1 ml aliquots of stocks were plated on Difco Sabouraud (SAB) Maltose Agar media\*\* and the spore solution was distributed over the surface of the media with a sterilized glass rod. For the qualitative determination of this effect, a standard inhibitor of fungal growth, sodium azide (NaN<sub>3</sub>)\*\*\*, was tested together with the extracts. The 3% Extract 1 and Extract 2 in water, and 0.05% NaN<sub>3</sub> in water were dropped onto 10mm filter paper disks, with 20 µl aliquot per disk. The disks were superimposed over the surface of the media.

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\*Tween<sup>®</sup> 20 is a commercial brand of polysorbate which is widely used as a detergent. In biological science, it is often used as a solubilizing agent for membrane proteins.

\*\*The SAB media allows the growth of fungi, but prevents development of bacterial organisms. However, the anti-bacterial properties of this media are not sufficient in some cases. To prevent bacterial growth, 5µg/ ml of Ampicillin was added to the media.

\*\*\*Sodium azide (NaN<sub>3</sub>) is a metabolic inhibitor blocking adenosine-5'-triphosphate (ATP) synthesis in microorganisms. It is a common preservative for samples and stock solutions in laboratories (Achmoody and Chipley 1978). The concentration of sodium azide (NaN<sub>3</sub>) applied on the disk was adjusted experimentally. The chosen amount of this inhibitor must have an effect on a fungal culture, comparable with the effect of *Sphagnum* extracts.

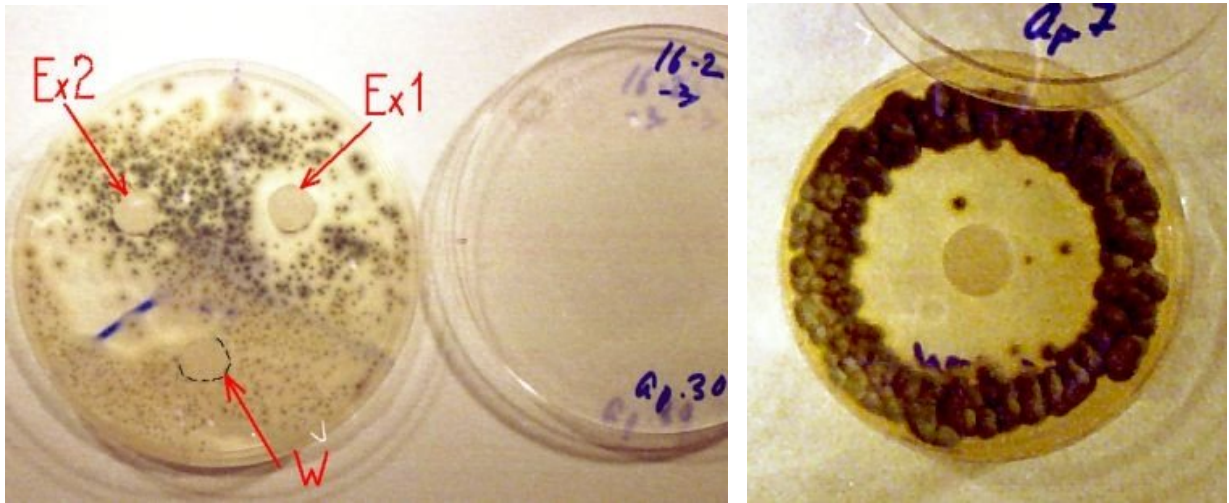
Two disks soaked with extracts were placed on one plate with the given fungal culture and a disk with  $\text{NaN}_3$  was placed on a second plate. A third plate without disks served as a control.

All tested *Sphagnum* extracts were dissolved in water, which can cause an individual inhibiting effect on fungal spores (Simione and Brown 1991). The influence of water on the viability of spores during the incubation was also tested. Disks soaked with 20  $\mu\text{l}$  of sterilized distilled water were applied to the media along with *Sphagnum* extracts. For each fungal culture the following plates were prepared:

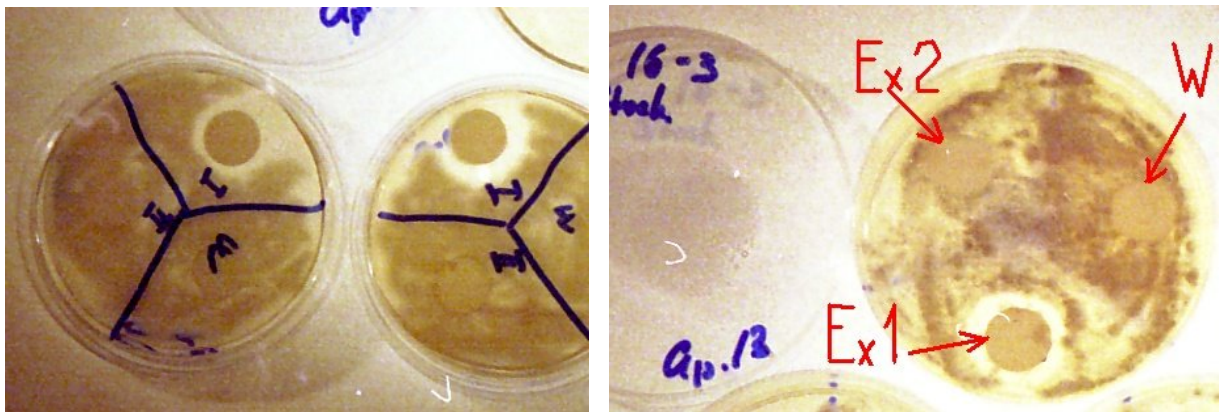
9 plates with three paper disks: Extract 1, Extract 2, and water,  
9 plates with sodium azide on one disk,  
9 control plates without inhibitors applied.  
27 plates for each fungal culture total (459 plates for seventeen isolates).

After 36-48 hours of incubation at room temperature, the number of colonies grown on the media was counted and the areas with dead spores were measured (Fig.4.4. and Fig.4.5.).

To calculate the area of the spots lacking growth, the assumption was made that the spots were ring-shaped. The area of this ring was estimated by the equation:  $S = \pi \bar{R} (D + \bar{R})$ , where D is the diameter of the paper disc (10 mm) and  $\bar{R}$  is an average width (radius) of a ring-shaped spot, i.e. width between the edge of the paper disk and the border between dead and germinated spores. Within this ring-shaped spot spores are dead. Three radii were measured for every dish using a digital camera and the value of  $\bar{R}$  was taken as the average of these three measurements.



**Fig.4.4.** Species 16-2: Extract 1, Extract 2, and water-on left; species 26-5(1):  $\text{NaN}_3$ -on right. The caps from the Petri dishes have been removed.



**Fig.4.5.** Species 20-2: Extract 1, Extract 2, and water-on left; species 16-3: Extract 1, Extract 2, and water-on right.

The inhibiting effect of any antifungal agent depends on the initial viability of the test organism. Viability of each fungal species was determined as a ratio of the average number of fungal colonies grown on control plates ( $N_1$ ) to the number of inoculated fungal spores ( $N_0$ ). The relative inhibiting effect of *Sphagnum* extracts to sodium azide is estimated as a ratio of the areas of the “dead” spots caused by the given extract ( $S_{\text{Ex}}$ ) and sodium azide ( $S_{\text{NaN}_3}$ ). The sizes of the ring-shaped “dead” spots for sixteen examined fungal species after exposure to *Sphagnum* extracts and sodium azide are shown in Table 4.1.

№ Spp.	N <sub>0</sub> , spor/pl	N <sub>1</sub> , col/pl	Via- bility, %	Extr.1 $\bar{R}$ , mm	Extr.1 S, mm <sup>2</sup>	Extr. 2 $\bar{R}$ , mm	Extr.2 S, mm <sup>2</sup>	NaN <sub>3</sub> $\bar{R}$ , mm	NaN <sub>3</sub> S, mm <sup>2</sup>	$\frac{S_{Ex1}}{S_{NaN3}}$
15-1	245	166	68	4.0	176	0	0	1.2	41	4.29
16-2	382	363	95	2.8	114	0	0	2.4	93	1.22
16.3	310	160	52	1.50	71	0	0	2.0	75	0.94
17-1	120	72	60	2.0	75	0	0	5.30	255	0.30
17-3	800	-	-	2.7	106	2.1	78	1.5	54	1.96
20-1	120	62	52	2.0	75	0.9	30	2.0	75	1.00
20-2	118	118	100	3.3	139	0	0	16.0	1306	0.11
21-2	314	270	86	3.6	151	2.0	75	15.0	1178	0.13
21-2	150	120	80	5.3	257	0	0	15.0	1178	0.22
21-3	162	160	99	0	0	0	0	0	0	-
22-1	460	120	26	1.5	52	0	0	0	0	-
23-2	360	300	83	2.0	75	2.0	75	1.5	54	1.39
26-1	850	363	43	3.3	139	0	0	13.0	939	0.15
26-2	172	113	66	2.2	85	2.6	103	6.2	313	0.27
26-4	262	241	92	2.7	106	0	0	0	0	-
26-5	735	720	98	0	0	0	0	6.8	357	0
26-5(1)	180	144	80	4.5	205	0	0	18.0	1583	0.13

**Table 4.1.** The results of the disk diffusion test for *Sphagnum* moss extracts and NaN<sub>3</sub> applied to sixteen selected fungi isolated from archaeological and ethnographic objects (see section 3.3.2).

In these experiments, the average viability of inoculated spores was 74%, i.e. rather high.

Excluding species 21-3, the tested fungi appeared to be affected by the *Sphagnum* extracts and/or by sodium azide. Both *Sphagnum* extracts showed an inhibiting effect on the test fungi. However, Extract 2 was considerably less effective than Extract 1. It did not affect the majority of cultures. Only culture 26-2 showed higher susceptibility to Extract 2 than to Extract 1, and culture 23-2 showed the same susceptibility to both extracts. The *Sphagnum* extracts and sodium azide showed differing effects on certain fungi. For example, cultures 22-1 and 26-4 were susceptible to Extract 1 and not susceptible to sodium azide while culture 26-5 was not susceptible to both *Sphagnum* extracts, but susceptible to sodium azide.

The spots without growth for Extract 1 varied from 0 to 257 mm<sup>2</sup> in squares and averaged 122 mm<sup>2</sup> (excluding species 21-3 and 26-5 where no effect was observed). For sodium azide, the spots without growth were considerably more variable, from 0 to 1583 mm<sup>2</sup> in squares with a

higher average value, at 536 mm<sup>2</sup> (excluding insusceptible species). The experiments also showed that water does not possess any inhibiting effect on the tested fungal organisms.

On the basis of these results, one of the sixteen species, 16-2, was selected as a test organism for further studies. This species showed a high viability (95%), the highest rates of growth, and a good susceptibility to both *Sphagnum* extracts. According to the preliminary taxonomic examination which was made by the author of the present work, these fungi belong to the *Aspergillus* genera. *Aspergillus* was found on most of the examined archaeological objects, which consisted of a variety of materials (see section 3.3.2). Due to the observation that species 16-2 was not susceptible to Extract 2, this extract was excluded from further experiments.

#### **4.2.2. The influence of acidity on spore growth**

To determine the contribution of acidity to the antifungal effect of *Sphagnum* extracts, the disk diffusion tests were carried out with acidic and neutral water solutions of *Sphagnum* extracts. The following *Sphagnum* extracts were tested: 3% Extract 1 in water at pH 3.0\* and 3% Water Extract in water at pH 3.4\*, and their neutralized solutions at pH 6.0. For the neutralization of *Sphagnum* solutions, a buffer of 500 mM Tris HCl\*\* (pH 7.5) was used. The test fungal organism was species 16-2. To obtain living spores, the grown lawn of this culture was washed with sterile distilled water and the washes were collected. From these washes containing the following suspension of spores, 10<sup>-2</sup>, 10<sup>-3</sup>, and 10<sup>-4</sup>, dilutions were prepared.

\*This is the regular acidity of the tested extracts.

\*\*Tris HCl (2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride) is a chemical with basic properties which can be used to buffer solutions, keeping them in the pH range of 7.0 to 9.0. Tris HCl is a common biological buffer (Sambrook and Russell 2001).

The concentrations of spores in these dilutions were counted with a hemocytometer. They were  $131 \cdot 10^5$ ,  $11 \cdot 10^5$ , and  $2.9 \cdot 10^5$  CFU/ml for  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  dilutions respectively. 0.1 ml aliquots from each of three dilutions were plated on the Sabouraud (SAB) Maltose Agar media\* and distributed over the surface of the media with a sterilized glass rod. 20µl aliquots of 3% Extract 1 in water, 3% Water Extract in water, their neutralized solutions in Tris buffer, and a pure buffer (control) were dropped onto 10 mm filter paper disks. The disks were placed on the media, in the center of the Petri dishes, one disk for one plate (Fig.4.6. and Fig.4.7.). Nine plates were prepared for each combination (9 plates per dilution×3 dilutions=27 plates), following the table:

27 plates of 3% Extract 1 in water,  
27 plates of 3% Extract 1 in buffer,  
27 plates of buffer (controls),  
27 plates of 3% Water Extract in water,  
27 plates of 3% Water Extract in buffer.  
162 plates total.

After 48 hours of incubation at room temperature the radii of the spots with dead spores were measured as previously described. The spots without growth were observed for both the Extract 1 and the Water Extract in all combinations of their acidity (normal and neutralized by buffer) and in all dilutions. For the  $10^{-4}$  dilution, the inhibiting effect was less visible, as the number of plated spores was not high enough to be evenly distributed. In this case, the radii of these zones were determined less precisely than for the  $10^{-2}$  and the  $10^{-3}$  dilutions. The measurements are recorded in Table 4.2. The visual comparison of the areas without growth, for both the acidic extracts and for the neutralized extracts, did not show a notable difference. Radii of the inhibited area for the acidic and neutralized extracts also did not differ in terms of the margin of error of the experiment (Table 4.2). The buffer did not show any inhibiting effect as well.

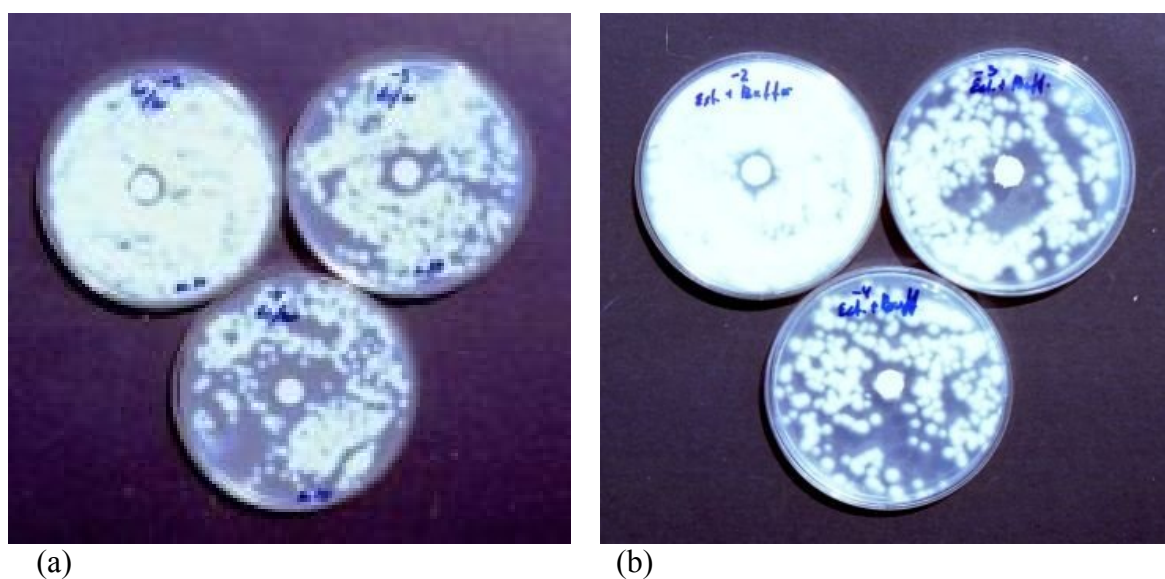
\*To prevent bacterial growth, 5µg/ ml of Ampicillin was added to the media.

The test solutions of <i>Sphagnum</i> extracts in water and in a neutral buffer	Radius of area without growth (R, mm)		
	Dilution, 10 <sup>-2</sup>	Dilution, 10 <sup>-3</sup>	Dilution, 10 <sup>-4</sup>
Extract 1 in water (pH 3.0)	2.2±0.5*	1.2±0.2	3.1±0.5
Extract 1 in buffer (pH 6.0)	1.5±0.3	2.5±0.4	3.1±0.5
Water Extract in water (pH 3.4)	2.2±0.8	1.1±0.2	1.1±0.4
Water Extract in buffer (pH 6.0)	1.1±0.5	1.4±0.4	0.9±0.2
Buffer (pH 7.5)	No effect	No effect	No effect

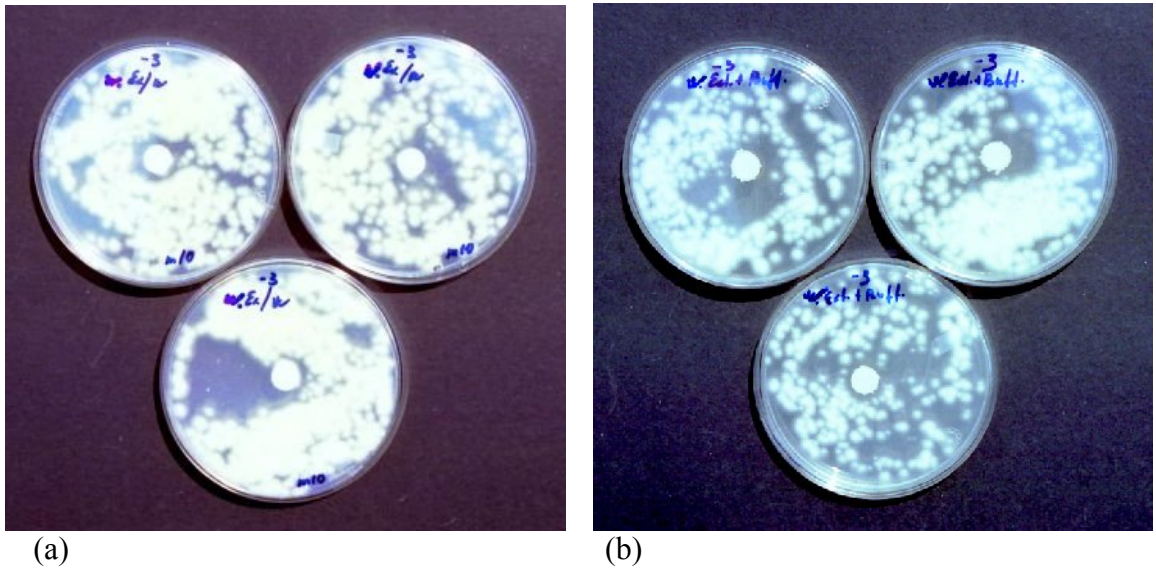
\* Standard error. The radius of a spot is characterized by the arithmetic mean  $\bar{R}$  and the standard error of the mean  $\sigma_R = \sigma / n^{1/2}$ , where  $\sigma$  is a standard deviation of the mean and  $n$  is a number of repeated measurements (Kennedy and Neville 1976). Every radius was measured three times. For a given dilution, a standard deviation of the mean was calculated for all twenty-seven radii measured in nine dishes. The number of repeated measurements of each radius was three ( $n = 3$ ) i.e.  $\sigma_R = \sigma / 1.73$ .

**Table 4.2.** Inhibiting effect of acidic and neutralized *Sphagnum* extracts on fungal spores inoculated at different dilutions. The neutral buffer is tested as a control. The concentrations of spores for the listed dilutions were  $131 \cdot 10^5$ ,  $11 \cdot 10^5$ , and  $2.9 \cdot 10^5$  CFU/ml.

The inhibiting effect of Extract 1 and the Water Extract on the tested fungi was the same at 10<sup>-2</sup> and 10<sup>-3</sup> dilutions. At 10<sup>-4</sup> dilutions, the radius of the “dead” spot for the Water Extract was 3 times smaller than for Extract 1. However, in terms of the errors of these experiments, this difference is insignificant. Therefore, it can be concluded that inhibitory properties of Extract 1 and the Water Extract towards species 16-2 were the same.



**Fig.4.6.** The effect of Extract 1 in water at pH 3.0 (a) and Extract 1 in buffer at pH 6.0 (b) on spp. 16-2. Plates with spores at 10<sup>-2</sup> dilution are shown on the left, at 10<sup>-3</sup> dilution on the right, and at 10<sup>-4</sup> dilution below.



**Fig.4.7.** The effect of the Water Extract in water at pH 3.0 (a) and the Water Extract in buffer at pH 6.0 (b) on spp. 16-2. Spores on all plates are inoculated at  $10^{-3}$  dilution.

### 4.2.3. Tube experiments

The inhibiting effect of Extract 1 and the Water Extract on fungi was also estimated by the tube method (see section 3.4.2). For tube experiments, species 16-2 was used as the test fungal organism\*. To prepare the stock of spores, spores were washed out of a fungal lawn with sterile distilled water and dissolved to a countable concentration, which was determined by use of a hemocytometer (see section 3.5.1). The stock of cells was then diluted to three concentrations: 10,000 CFU/ml, 25,000 CFU/ml, and 50,000 CFU/ml. 0.1 ml aliquots of each spore concentration were introduced into glass test tubes containing 0.4 ml of Difco Sabouraud Maltose Broth (see section 3.4.3). Therefore, the volume of spore solution in the broth was 0.5 ml/tube. The number of spores was 1000 spores/tube in the first tube set, 2500 spores/tube in the second tube set, and 5000 spores/tube in the third tube set. Thus, the concentrations of spores in these tube sets were 2,000 CFU/ml, 5,000 CFU/ml, and 10,000 CFU/ml.

\*In tubes, the viability of spores in the broth without Extract 1 added was also tested for all sixteen species selected (see section 3.3.2) and for the time of exposure between 3 and 28 hours. However, the inhibiting effect of *Sphagnum* extracts was determined only for species 16-2.

### *Extract 1*

Three 48-tube sets with spore solutions in the broth were filled (126 tubes in total). The first tube set contained 1,000 spores per tube, the second set contained 2,500 spores per tube, and the third set contained 5,000 spores per tube. In half of the tubes, i.e., in 24 tubes from each 48-tube set, 20 µl of 3% Extract 1 in water was added per tube. The rest of tubes, i.e., 24 tubes in each set containing only spores suspended in the broth, were left as controls. These three tube sets were stored for 24 hours at regular laboratory conditions, i.e., at daylight and room temperature. It was assumed that in the first 3 hours, the influence of the Extract 1 on the spores would not be detectable, and after 24 hours of exposure, the spores would die. In the time interval between 3 and 24 hours, the broth cannot inhibit the germination of spores\*. The number of colonies grown on control plates is determined only by the initial viability of spores and should not vary before 24 hours of exposure. Therefore, in the time interval between 3 and 24 hours the viability of the spores is considered to be the same. Adding Extract 1 to the broth should inhibit the viability of spores, depending on the exposure. To check the viability of the spores, they were plated onto SAB Maltose Agar media following the timetable: t=3, 5, 8, 12, 16, 18, 21 and 24 hours.

According to this timetable, 0.1ml aliquots from three tubes with broth + Extract 1 and from three corresponding control tubes (one from each of three tube sets) were inoculated onto 18 Petri dishes containing SAB Maltose Agar media\*\*. Therefore, the aliquots from each tube were inoculated to three plates. The number of spores ( $N_0$ ) plated from the first tube set was  $2000 \text{ CFU/ml} \times 0.1 \text{ ml} = 200 \text{ spores/plate}$ ; for the second tube set,  $N_0 = 500 \text{ spores/plate}$ ; and for the third tube set,  $N_0 = 1000 \text{ spores/plate}$ . After 36 hours of incubation at room temperature, the number of colonies on the plates was counted on the basis of visual observations. The ratio of the number of grown colonies to the number of plated spores indicates spore viability.

\*This was verified by preliminary experiments for species 15-1, 16-2, 16-3, 17-3, 20-1, and 23-2 with times of exposure between 3 and 30 hours. It was found that in the interval between 18 and 24 hours spores started to lose their viability. After 27 hours of exposure, spores of all examined species have died (see section 3.4.2).

\*\*5µg/ ml of Ampicillin was added to the media.

The required number of tubes/plates is as follows:

Broth, 1000 spores/tube (control)	8 time intervals $\times$ 3 tubes = 24 tubes/ 72 plates
<u>Broth, 1000 spores/tube + Extract 1</u>	<u>8 time intervals <math>\times</math> 3 tubes = 24 tubes/ 72 plates</u>
Total	48 tubes/144 plates
Broth, 2500 spores/tube (control)	8 time intervals $\times$ 3 tubes = 24 tubes/ 72 plates
<u>Broth, 2500 spores/tube + Extract 1</u>	<u>8 time intervals <math>\times</math> 3 tubes = 24 tubes/ 72 plates</u>
Total	48 tubes/144 plates
Broth, 5000 spores/tube (control)	8 time intervals $\times$ 3 tubes = 24 tubes /72 plates
<u>Broth, 5000 spores/tube + Extract 1</u>	<u>8 time intervals <math>\times</math> 3 tubes = 24 tubes /72 plates</u>
Total	48 tubes/144 plates
Total number of tubes/plates	144 tubes/432 plates

The laboratory arrangement of the test is shown in Fig.4.8.



**Fig.4.8.** A layout of a tube test in the lab (Laboratory of Biotechnology, Brock University).

The number of colonies grown from spores suspended in broth, ( $N_{Br}$ ), and from spores suspended in broth with Extract 1 added, ( $N_{Br+Ex1}$ ), for different times of exposure ( $t$ ), are listed in Table 1, (Appendix). The relationships  $N_{Br}(t)$  and  $N_{Br+Ex1}(t)$  for three concentrations of plated spores  $N_0 = 200, 500$  and  $1000$  spores/plate are shown in Figs. 4.9.-4.11.

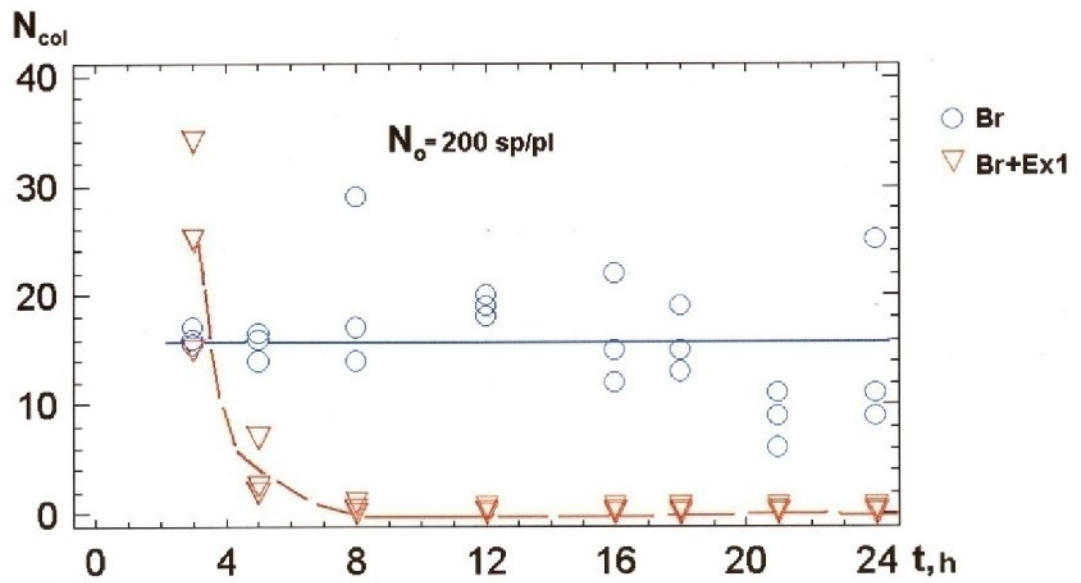


Fig.4.9. The number of colonies grown from the spores exposed to the broth ( $N_{Br}$ ) and to the broth with Extract 1 added ( $N_{Br+Ex1}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=200$  spores/plate.

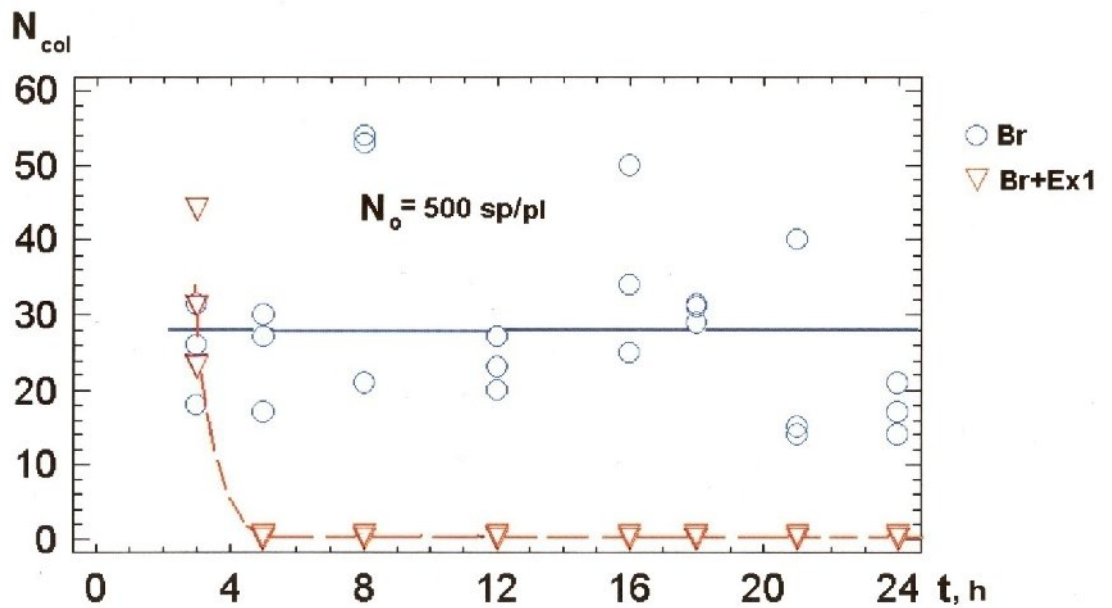


Fig.4.10. ( $N_{Br}$ ) and ( $N_{Br+Ex1}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=500$  spores/plate.

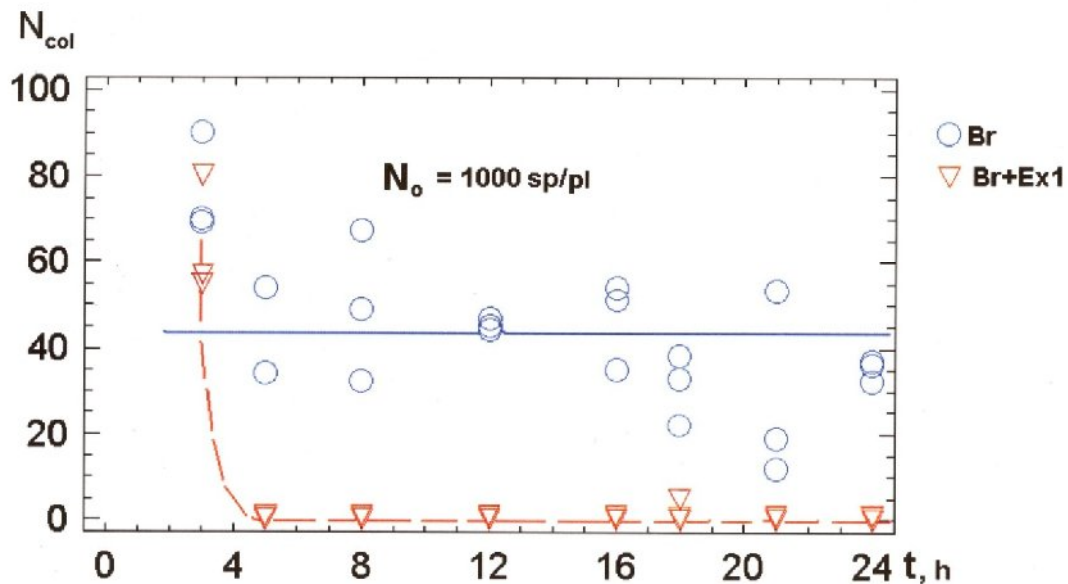


Fig.4.11. ( $N_{Br}$ ) and ( $N_{Br+Ex1}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=1000$  spores/plate.

Despite the notable scatter of the numbers of colonies growing on the controls ( $N_{Br}$ ), it did not vary significantly between 3 to 24hours of exposure for all experiments. Therefore, ( $N_{Br}$ ) can be considered constant, i.e., can be characterized by the mean ( $\bar{N}$ ) \*which is an arithmetic mean of the numbers of the colonies counted for each of the seven times of exposure. For  $N_0 = 200, 500$  and  $1000$  spores/plate, the corresponding numbers of colonies were found to be 16, 28, and 44 CFU/plate. The viability of spores ( $\bar{N} / N_0$ ) in this experiment was 8, 6, and 4% respectively, i.e. quite low. The time of exposure ( $t$ ) showed a significant influence on the number of colonies growing from the spores treated by Extract 1 ( $N_{Br+Ex1}$ ). After the first three hours of exposure, the numbers of colonies in the controls and colonies growing from the spores treated with Extract 1 were approximately the same for any given concentration of the plated spores.

\*The scatter of the number of colonies around the mean is characterized by the random errors of the experiment. Random errors occurred due to the presence of dead spores in the broth and due to the inconsistency of the spores in the test tubes. If the broth contains both living and dead spores, the amounts of both taken by pipette cannot be predicted.

After three hours of exposure, the numbers of growing colonies for spores treated with Extract 1 promptly fell to zero. The germination of spores on two sets of plates, with 500 and 1000 spores/plate, was completely inhibited at five hours of exposure. For the first set of plates, with 200 spores/ plate, four colonies were still growing after five hours of exposure. At eight hours of exposure, only one colony was able to grow and at longer exposures spore germination was completely inhibited.

### ***The Water Extract***

The experiment with the Water Extract was arranged in a similar manner to the experiment with Extract 1. The test organism was the same: species 16-2. The same numbers of spores were introduced into test tubes containing 0.5 ml of the broth (1,000, 2,500, and 5,000 spores/tube); the same amount of the Water Extract was introduced into the tubes (20  $\mu$ l of 3% Water Extract); and the same numbers of spores were inoculated onto the plates (200, 500, and 1,000 spores/plate). The only difference was a decrease in the time of exposure from 24 to 16 hours. Spores were plated after  $t=5, 8, 12,$  and 16 hours of exposure. The numbers of tubes/plates were 12 tubes and 36 plates with treated spores and 12 tubes and 36 plates taken as controls for each of three concentrations of spores, i.e., 72 tubes/216 plates in total.

The number of colonies grown from the spores suspended in the broth ( $N_{Br}$ ), and from the spores suspended in the broth with the Water Extract added, ( $N_{Br+W Ex}$ ), for different times of exposure ( $t$ ), are listed in Table 2, (Appendix). The relationships  $N_{Br}(t)$  and  $N_{Br+W Ex}(t)$  for the three concentrations of plated spores  $N_0=200, 500$  and 1000 spores/plate are shown in Figs. 4.12-4.14.

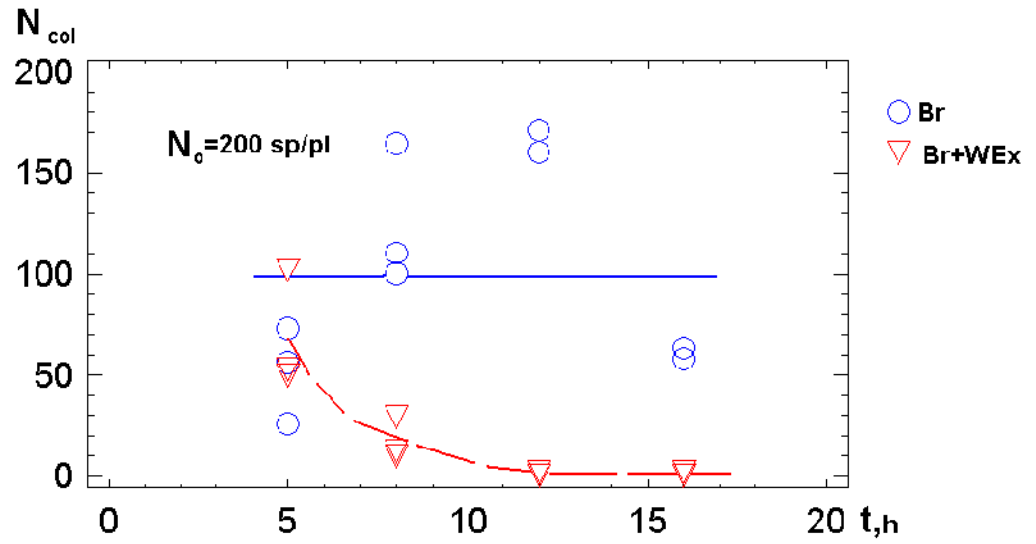


Fig.4.12. The number of colonies grown from the spores exposed to the broth ( $N_{Br}$ ) and to the broth with the Water Extract added ( $N_{Br+WEx}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=200$  spores/plate.

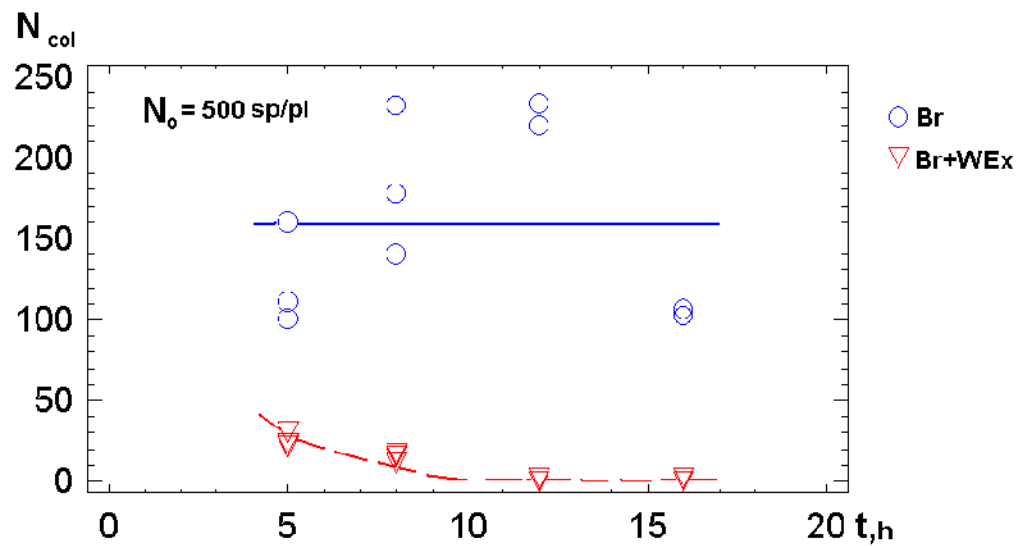
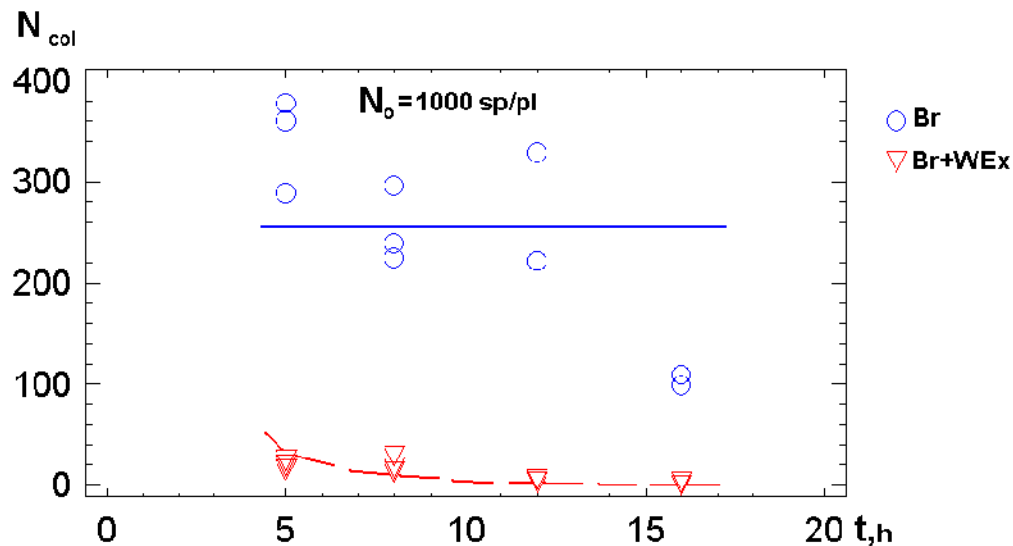


Fig.4.13. ( $N_{Br}$ ) and ( $N_{Br+WEx}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=500$  spores/plate.



**Fig.4.14.** ( $N_{Br}$ ) and ( $N_{Br+WEx}$ ) vs. the time of exposure ( $t$ ). The number of plated spores is  $N_0=1000$  spores/plate.

Similar to the previous experiment, the number of colonies grown on controls was characterized by the mean. For three sets of test tubes with the following numbers of plated spores of  $N_0 = 200, 500,$  and  $1000$  spores/plate, the corresponding numbers of the colonies were found to be 98, 158, and 254 CFU/plate. The viability of spores was 49, 32, and 25%, i.e., considerably higher than in the experiment with Extract 1.

For  $N_0 = 200$  spores/plate and at five hours of exposure to the Water Extract, the number of colonies growing from treated spores was equal to the number of colonies growing in the controls. For higher concentrations of plated spores, i.e., for  $N_0 = 500$ /plate and  $1000$  spores/plate and five hours of exposure, the numbers of grown colonies were considerably lower than in the controls. The loss of viability of these spores likely started earlier. The complete inhibition of spore germination occurred for the Water Extract after 12 hours of exposure for all concentrations of spores in the test tubes.

#### 4.2.4. The influence of the sugary nature of *Sphagnum* extracts on fungal spores

The possible antifungal effect of polysaccharides comprising *Sphagnum* extracts was tested on two model sugary substances, maltose and glucose. These sugars are the common plant polysaccharides. The design of the experiment was similar to the previous tube experiments (test organism, test tubes, nutrition broth, agar media, temperature, etc.). Two 42- tube sets containing a 0.5 ml aliquot of spore solution in the broth with two different numbers of spores were used. The first tube set contained 1000 spores per tube and the second set contained 2500 spores per tube. In half of the tubes, i.e., in 21 tubes with 1000 spores/tube and in 21 tubes with 2500 spores/tube, 20 µl of 3% maltose or glucose in water was added per tube. The rest of tubes, i.e., 21 tubes with 1000 spores/tube and 21 tubes with 2500 spores/tube in the broth, were left as controls. The viability of the spores was tested after t=3, 5, 8, 12, 16, 18, and 21 hours of exposure. According to the timetable, 0.1ml aliquots from three tubes containing the broth-polysaccharide mixture, and from three corresponding control tubes, were plated onto 18 Petri dishes containing SAB Maltose Agar media, one tube per three dishes. The number of spores ( $N_0$ ) plated from the first tube set was  $N_0 = (1000 \text{ spores}/0.5 \text{ ml}) \times 0.1 \text{ ml} = 200 \text{ spores}/\text{plate}$ , and from the second tube set was  $N_0 = (2500 \text{ spores}/0.5 \text{ ml}) \times 0.1 \text{ ml} = 500 \text{ spores}/\text{plate}$ . After 36 hours of incubation at room temperature the number of colonies grown was counted on the basis of visual observations. The required number of tubes/plates for this test is as follows:

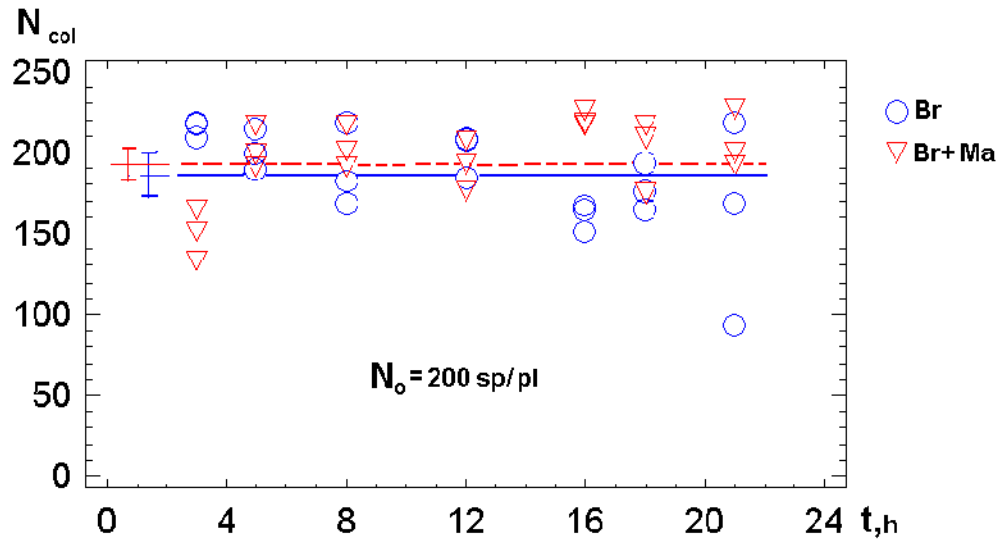
Broth, 1000 spores/tube (control)	7 time intervals × 3 tubes = 21 tubes/63 plates
<u>Broth, 1000 spores/tube + maltose/glucose</u>	<u>7 time intervals × 3 tubes = 21 tubes/63 plates</u>
Total	42 tubes/126 plates
Broth, 2500 spores/tube (control)	7 time intervals × 3 tubes = 21 tubes /63 plates
<u>Broth, 2500 spores/tube + maltose/glucose</u>	<u>7 time intervals × 3 tubes = 21 tubes /63 plates</u>
Total	42 tubes/126 plates
Total number of tubes/plates	84 tubes/252 plates

The number of colonies grown from spores suspended in the broth ( $N_{Br}$ ), from spores suspended in the broth with maltose added ( $N_{Br+Ma}$ ), and from spores suspended in the broth with glucose added ( $N_{Br+Gl}$ ), for different times of exposure ( $t$ ), are listed in Table 3, (Appendix). The relationships  $N_{Br}(t)$ ,  $N_{Br+Ma}(t)$ , and  $N_{Br+Gl}(t)$  for  $N_o = 200$ /plate and 500 spores/plate are shown in Figs.4.15-4.18.

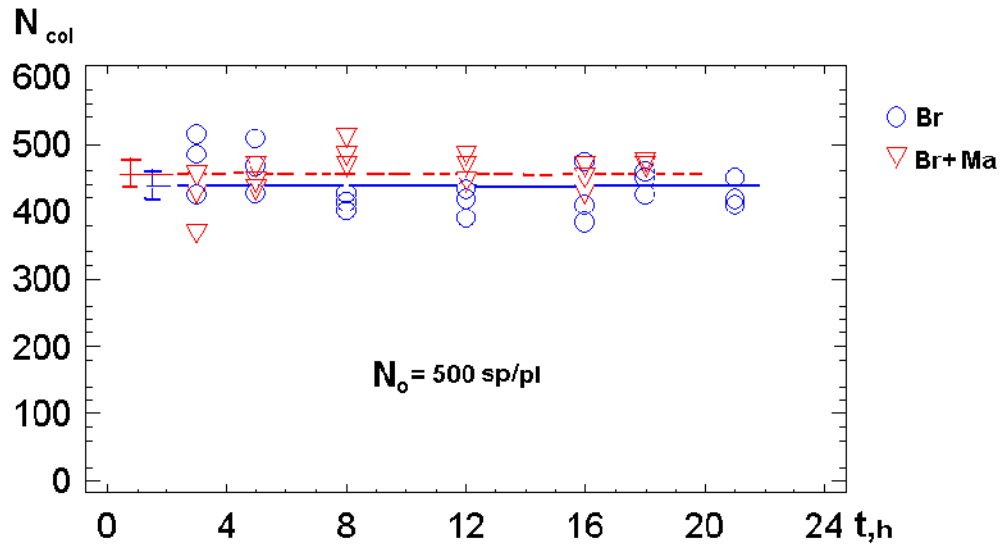
### ***Maltose***

The number of colonies grown on the controls ( $N_{Br}$ ) for  $N_o = 200$  spores/plate and for  $N_o = 500$  spores/plate did not vary significantly throughout the entire interval of exposure (3-21 hours). Therefore, ( $N_{Br}$ ) can be characterized by the mean and a standard error of the mean  $\sigma_N = \sigma / n^{1/2}$ , where  $\sigma_N$  is a standard deviation of the mean for a whole interval of exposure (3-21 hours), and  $n$  is the number of repeated measurements. In this experiment  $n=3$  and  $\sigma_N = \sigma/1.73$ . For control plates with 200 spores/plate,  $N_{Br} = 186 \pm 17$  CFU/plate and for 500 spores/ plate,  $N_{Br} = 437 \pm 14$  CFU/plate. The corresponding viabilities of untreated spores  $\bar{N} / N_o$  on the controls were 93 % and 87 %, i.e., quite high.

The number of colonies grown from spores treated with maltose was also evenly distributed in time and the relationship  $N_{Br+Ma}(t)$  also can be described by the mean and standard error of the mean. For  $N_o = 200$  spores/plate,  $N_{Br+Ma} = 196 \pm 14$  CFU/ plate and for 500 spores/plate,  $N_{Br+Ma} = 455 \pm 18$  CFU/ plate. The corresponding viabilities of spores treated with maltose were also high: 98 % and 91 %. The average number of colonies grown from spores untreated with maltose (controls) and treated with maltose did not differ in terms of the errors of the experiment. Therefore, it can be concluded that adding maltose did not change the viability of treated spores.



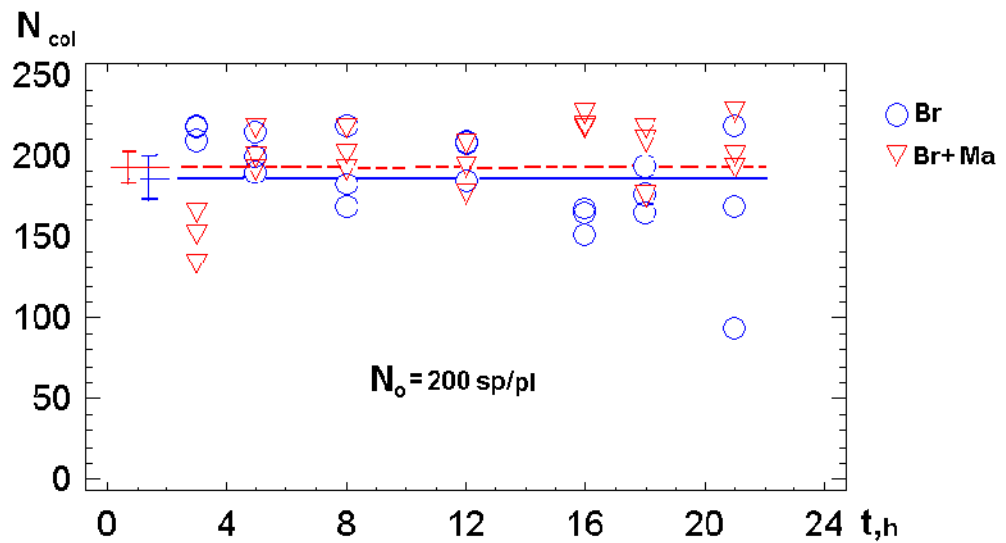
**Fig.4.15.** The number of colonies grown from spores exposed to the broth ( $N_{Br}$ ) and to the broth with maltose ( $N_{Br+Ma}$ ) vs. the time of exposure ( $t$ ). The standard errors are shown as red and blue bars on the means. The number of plated spores is  $N_0=200$  spores/plate.



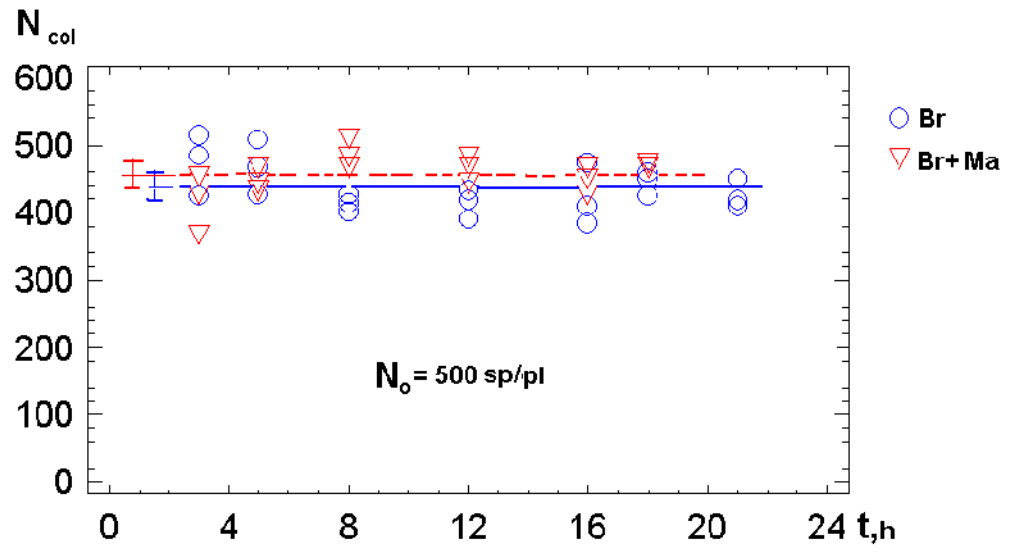
**Fig.4.16.** ( $N_{Br}$ ) and ( $N_{Br+Ma}$ ) vs. the time of exposure ( $t$ ). The standard errors are shown as red and blue bars on the means. The number of plated spores is  $N_0=500$  spores/plate.

## Glucose

The experiments with glucose showed similar results. The number of colonies in the controls for  $N_0 = 200$  spores/plate was  $181 \pm 12$  CFU/plate and for  $N_0 = 500$  spores/plate was  $421 \pm 21$  CFU/plate. The corresponding viabilities of spores in controls were 91 % and 84 %, i.e. almost the same as for maltose. After exposure to glucose, the number of grown colonies also did not vary significantly, depending on the time of exposure. Therefore,  $N_{Br+Gl}$  can also be described by the mean and a standard error of the mean. For  $N_0 = 200$  spores/plate,  $N_{Br+Gl} = 188 \pm 15$  CFU/plate and for  $N_0 = 500$  spores/plate,  $N_{Br+Gl} = 451 \pm 16$  CFU/plate. The corresponding viabilities of spores treated by glucose were 94 % and 90 %. The numbers of colonies grown from spores treated and untreated with glucose did not differ in terms of the errors of the experiment. Like maltose, glucose does not change the viability of treated spores.



**Fig.4.17.** The number of colonies grown from spores exposed to the broth ( $N_{Br}$ ) and to the broth with glucose ( $N_{Br+Gl}$ ) vs. the time of exposure ( $t$ ). The standard errors are shown as red and blue bars on the means. The number of plated spores is  $N_0=200$  spores/plate.



**Fig.4.18.** ( $N_{Br}$ ) and ( $N_{Br+Gl}$ ) vs. the time of exposure ( $t$ ). The standard errors are shown as red and blue bars on the means. The number of plated spores is  $N_0=500$  spores/plate.

## **Chapter 5. *Sphagnum* as an Additive to Conservation Waxes.**

In this chapter, the results of biological tests of Extract 1 applied as an additive to conservation waxes are reported. In the present work, the biodegradation of wax coatings in combination with corrosion of the underlying metal surface was demonstrated on several bronze objects from the Kaman- Kalehöyük archaeological site\* storage facility. The collection was subjected to extreme conditions of high humidity, high temperature and the development of microorganisms as a result of a flood. Biodegradation of wax films was also observed microscopically on model wax samples placed on glass slides. Experiments simulated the climate conditions of the site storage during the disaster. To test the protective effect of *Sphagnum* products on wax coatings, Extract 1 (*Sphagnum*) was added to two conservation waxes, Be Square 195® and Polywax 2000®, which were subjected to a mature fungal lawn. All observations and experiments described in this chapter were made in the conservation laboratory of the Kaman- Kalehöyük archaeological site.

### **5.1. The drastic biocontamination of the Kaman-Kalehöyük site storage area after the flooding disaster**

The site storage facility at Kaman- Kalehöyük was flooded in the spring of 2003-2004. During the disaster, water covered the floor of the building, and also leaked through the ceiling. Water was standing in the building until summer, when the storage facility was opened for the new excavation season\*\*.

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\* Kaman Kalehöyük is located 100 kilometers southeast of Ankara in Central Anatolia, Turkey. The archaeological findings at the site have been dated from the 23<sup>rd</sup> century BC to the 19th century AD including the Early and Middle-Late Bronze Ages, the Iron Age, the Old Hittite Kingdom Period, the Assyrian Colonial Period, and the Ottoman Empire Period (Omura 2005).

\*\*The depth of water in the storage facility during the disaster was unknown because the facility was closed in winter and spring (i.e. in time when the disaster occurred). When it was open, the floor was partly covered with water and the walls inside the facility were wet up to 1.2 m.

Following the records from the storage facility dataloggers, at that time the indoor temperatures varied between 20°C at night and 30°C during daytime and, the relative humidity was constantly 100%. Under those conditions, the interior of the building was subjected to intensive microbial attack, including the concrete floor, ceiling and walls, the steel storage shelves, and the outer surfaces of wooden and plastic boxes holding artifacts.

All artifacts located in the Kaman- Kalehöyük site storage facility had been enclosed in Ziploc® plastic bags. The bags containing ceramic shards, bone, and stone were placed in wooden boxes, which were kept on steel shelves. All small metal artifacts were enclosed in double plastic bags for increased safety. These bags were then placed into hermetically sealed archive quality plastic boxes. Every box also contained silica gel for the absorption of water vapor. The plastic boxes were stored in ice cream or Coca-Cola coolers or placed on archive quality steel bookcases with glass doors. During the disaster, water seeped into the wooden boxes and into the coolers holding metal artifacts. In the wooden boxes, plastic bags with ceramic were found filled with water because their polyethylene walls were severely degraded by microorganisms. Water was standing inside the coolers, but in most cases did not penetrate through the hermetically sealed plastic boxes and Ziploc bags with metal objects. However, the interior of all plastic bags appeared to be covered with growing microorganisms. Most probably, under the conditions of prolonged 100% humidity in the storage area, plastic locks were insufficient to prevent diffusion of water vapor through them. In time, the level of humidity in the bags became high enough to permit the germination of fungal spores retained in the bags and on objects, with consequent attack of the objects. Only the artefacts stored in double bags were found in reasonable condition. In the storage facility interior, a wide spectrum of microorganisms thrived including bacteria, fungi, and yeasts. The wooden boxes were contaminated by bacteria (wet bottom

planks) and by fungi, including basidiomycetes and hyphomycetes (Fig.5.1). The interiors of Ziploc bags containing bronze artifacts were infested by bacteria and conidial fungi whose filaments pierced the walls of these bags (Fig.5.2). The primary taxonomic analysis of these fungi carried out by the author showed that most of them belonged to *Aspergillus* genera.



**Fig.5.1.** Mould- infested wooden boxes holding ceramic artifacts in the Kaman- Kalehöyük storage site-on the left. The variety of fungal spp. on the surface of a box -on the right (magnified). In this image two fruit bodies of white rot fungi can be seen. The black spots are due to colonies of *Aspergillus niger*.



**Fig.5.2.** Polyethylene bags used to store small bronze objects after microbial attack. The black spots are due to colonies of *Aspergillus niger*. Artifacts have been removed.

## **5.2. The connection between the condition of the wax coatings on bronze artifacts and the appearance of “bronze disease” in the Kaman-Kalehöyük site storage facility**

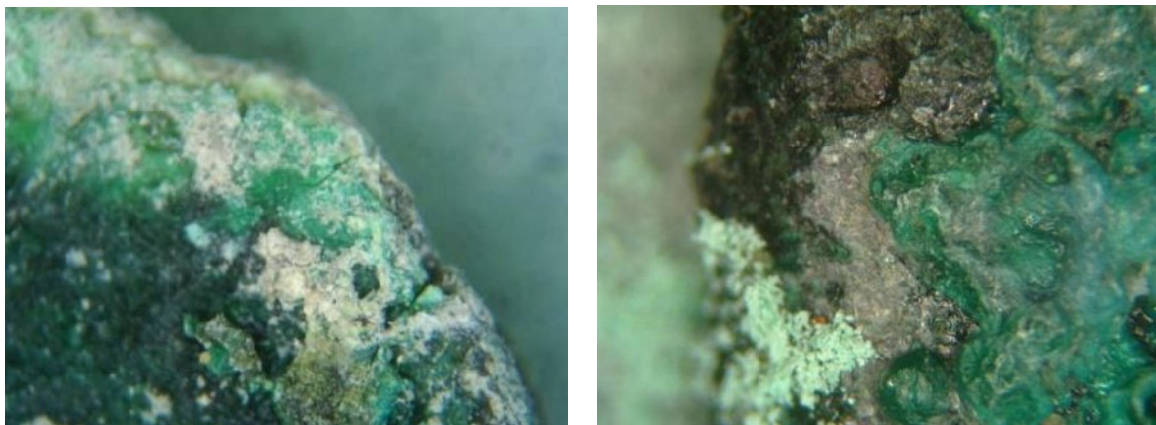
After the disaster, the condition of the collection of metal objects\* in the Kaman- Kalehöyük site storage facility was examined by optical light microscopy. It was found that the protective coating on most of them was degraded. The wax layer, which is the outer element of such coatings, determines the resistance of the whole protective composition against moisture and microbial attack. Degradation of this layer in combination with high humidity caused the intensive corrosion of the underlying bronze known as “bronze disease”. Bronze disease is a form of active corrosion of bronze artifacts that appears as a green powder or a waxy film on the surface of the metal. It disrupts the surface of the artifacts and can quickly destroy copper alloys\*\*. The majority of the bronze objects in the storage area were found to be severely corroded. Moreover, the active corrosion on the surface of the objects occurred where the wax and acrylic layers had failed.

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\*The analysis of soil at the Kaman Kalehöyük archeological site explains the rather poor preservation of the excavated metal because the objects buried in this area are normally subjected to alkaline environmental conditions (Merkel 2002). For this reason, most of excavated metal artefacts were treated in the conservation laboratory according to a procedure established by G. Wharton as the treatment protocol of the laboratory. The basic treatment for copper alloys at the Kaman- Kalehöyük archaeological site is as follows. The severely corroded objects are immersed in 3% BTA in ethanol, to inhibit corrosive processes. If objects are intact, only mechanical cleaning is recommended. The protective coating consists of two layers: the acrylic undercoating and the wax outer coating. The undercoating is a methacrylate copolymer Paraloid B-72® which inhibits the evaporation of the active anticorrosive agent, BTA, from the surface of the treated metal (BTA is highly volatile). However, the B-72 is fragile and needs additional protection against shrinkage, i.e., a wax outer coating. The basic outer coating is a wax mixture: 90% of microcrystalline wax (Be Square 195®) and 10% polyethylene wax (Polywax 2000®). The outer wax layer protects the undercoating from atmospheric moisture and oxygen, and improves the aesthetic appearance of the artifacts (Moffett 1996, La Fontaine 1981). The wax also prevents the conservator’s skin from coming in contact with BTA which is known as a volatile and toxic agent. According to Material Safety Data Sheet (MSDS) of Oxford University, BTA is harmful if swallowed or inhaled (<http://ptcl.chem.ox.ac.uk/MSDS/BE/1H-benzotriazole.html>). BTA is considered to be dermal and eye irritant (Lewis 2000). To protect the evaporation of BTA from the treated metal, some conservators add 3% BTA in ethanol to B-72 and to wax, in equal volumes.

\*\*The chemical reactions defining the process of bronze disease were reviewed by McNeil and Mohr (McNeil and Mohr 1992).

Two different types of wax degradation were observed (Zaitseva 2005a). The first type was characterized by cracking and exfoliation of the wax layer into scales on the metal surface (Fig.5.3). Spots of active corrosion covered the whole surface of the objects including the areas where the wax layer had not exfoliated. The surface of the wax films and the spots of active corrosion were dry. The second type of wax degradation was a wet sticky slime. This slime was a mixture of degraded coatings and corrosion products. In this case, the wax coating was partially lost. The metal surface around and beneath the wax film was totally covered with wet corrosion products, which penetrated through the wax layer and was present on the surface of the wax film (Fig.5.3). Microscopic examination also showed that in both types of wax degradation, the metal surface suffered from different stages of bronze disease (ibid.).



**Fig.5.3.** A severely corroded bronze artefact with degraded wax coating. Wax appeared as loose grey scales on the surface of the object. Magnification of 10X –on left. Degraded wax coating on the surface of the bronze object-on right. Wax was mixed with wet corrosion products (bronze disease). These products appeared as a sticky slime of green, light green and greyish color. Magnification of 10X.

To find a connection between the condition of the wax coatings and the degree of degradation of the underlying bronze, fifty bronze objects were selected from the Kaman- Kalehöyük site storage facility for examination (Zaitseva 2005a). The objects were examined with optical light microscopy at a magnification of 10X-100X, and photographed with a digital camera.

The examined artefacts were separated into three groups, depending on their prior condition and subsequent treatment\*. The first group included severely corroded objects. Most of them had lost their metal core and were totally mineralized. After mechanical cleaning, they were treated with BTA with subsequent B-72 and wax coating. To prevent fast evaporation of BTA, BTA was added to the B-72 acrylic lacquer and to the wax composition. The second group included objects in better condition that had only some metal core lost. After mechanical cleaning, these objects were not treated with BTA. The coating was the same as in the previous case, but with no BTA added. The last group included the artefacts that were only cleaned mechanically, without any protective coating. They were excavated in fairly good condition with their metal core intact. The results are represented in Table 5.1.

Condition, degree of corrosion	Method of treatment	N, examined in total	N, with bronze disease, (%)	N, with loss of wax coating
Poor, heavily corroded, totally mineralized	Mechanical cleaning, immersion in 3% BTA in ethanol. <u>Undercoating</u> : 5% B-72 in acetone + 3% BTA in ethanol mixed in equal volumes <u>Outer coating</u> : wax mixture* in toluene + 3% BTA in ethanol mixed in equal volumes	31	21(68%)	22
Intermediate, some metal core left	Mechanical cleaning. <u>Undercoating</u> : 5% B-72 in acetone <u>Outer coating</u> : wax mixture* in toluene	10	6(60%)	6
Fairly good, metal core present	Mechanical cleaning only, no further treatment	9	7(77%)	- -

\*Wax mixture: 90% Be Square 195® and 10% Polywax 2000®.

**Table 5.1.** The number of artefacts (N) suffering from bronze disease and artefacts with loss of the wax coating, for different methods of treatment.

The results of microscopic examination confirm that the recommended wax-acrylic coating provides anticorrosive protection for the metal surface. Even if bronze objects initially were not corroded, after the water disaster almost 80% of them appeared to be suffering from bronze disease, if they were uncoated.

\*The information about the treatment procedures of the selected artefacts was taken from the conservation reports provided in the Kaman- Kalehöyük Data Base.

On the contrary, only 60-70% of initially corroded objects were found suffering from bronze disease, if they were coated. Therefore, the appearance of bronze disease on the surface of the objects can be explained primarily by the loss of the coating. In fact, bronze disease was indicated only on those objects where the protective film was lost. Under the conditions of the water disaster, BTA did not provide any additional protection to the objects because objects treated with BTA and untreated with BTA approximately equally suffered bronze disease (Zaitseva 2005a).

The microscopic observations also indicated a correlation between the degree of degradation of the protective wax film and the appearance of active corrosion on the surface of the objects. Under the conditions of increased humidity and temperature, wax films can be degraded by hydrolytic effects and by microorganisms, which use wax as a substrate for their growth. The biodegraded wax film permits moisture and oxygen to come in contact with the metal surface, triggering the process of corrosion. At the same time, microorganisms excrete a number of metabolites such as alcohols and acids (see section 2.1.3). These reactive products increase the rates of corrosion on the surface of the objects.

### **5.3. Biodegradation of the model wax coatings**

To demonstrate the capability of microorganisms to degrade a wax film, model experiments simulating the conditions of increased temperature and humidity in combination with high bio contamination of the wax surface were performed (Zaitseva 2005a). The model coatings were processed in different combinations, according to the laboratory protocols established by G. Wharton at the Kaman- Kalehöyük archaeological site for the treatment of copper alloys (Table

5.2). Experimental films were applied to transparent glass slides by paintbrush, at a thickness of approximately 20  $\mu\text{m}^*$ .

Coating composition	Number of slides
Wax composition: 90 % Be Square+10 % Polywax 2000 in toluene	9
Wax composition + 3% BTA in ethanol mixed in equal volumes	9
5% B-72 in acetone	9
5% B-72 in acetone + 3% BTA in ethanol mixed in equal volumes	9
5% B-72 in acetone covered with wax composition	9
5% B-72 in acetone +3% BTA in ethanol mixed in equal volumes and covered with wax composition	9

**Table 5.2.** Wax and acrylic coatings applied to glass slides in model experiments.

To accelerate the bio contamination of the coating, slides were exposed for three days to an environment with high concentrations of fungal spores and bacteria. The first three slides from each set of nine were placed for three days into contaminated plastic bags where bronze artefacts had been stored during a flood in Kaman (Fig.5.2). The internal surfaces of the bags were moistened and covered by colonies of fungi and bacteria, which partly destroyed the polyethylene walls.

The next three slides from each set of nine were placed for three days on the open shelves in the Kaman- Kalehöyük site storage facility soon after the facility was opened for the next excavation season. On the first day of exposure, the humidity in the storage area was 80% RH and on the third day it was close to the outdoor humidity, i.e. between 30 and 40% RH.

After exposure, all these slides were placed in glass pans, covered with thin plastic wrap, and moved to the conservation laboratory. Filter paper soaked with distilled water was placed in each

\* The thickness of the film was estimated by the difference in weights of the glass slide with and without wax applied, the square of the slide surface, and the density of wax (the density of wax was taken from <http://physics.nist.gov/cgi-bin/Star/compos.pl?matno=213>).

pan to maintain the conditions of increased humidity. The glass pans were kept at normal laboratory temperatures for twenty days. According to the record from a datalogger placed in the pan (Fig.5.4), the relative humidity in the pan was ~75%, and temperature varied between 19 °C and 29°C during the whole period of incubation.

The last three slides from each set of nine slides were used as controls, i.e. they were not biocontaminated. These slides were kept open in the conservation laboratory for twenty days. All slides were examined on the 5<sup>th</sup>, 14<sup>th</sup>, and 20<sup>th</sup> days of the incubation.

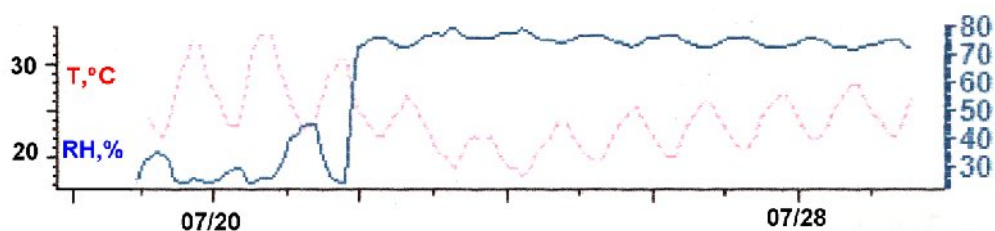
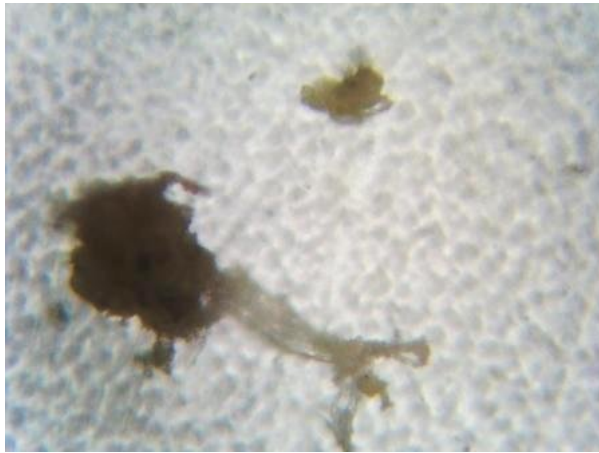
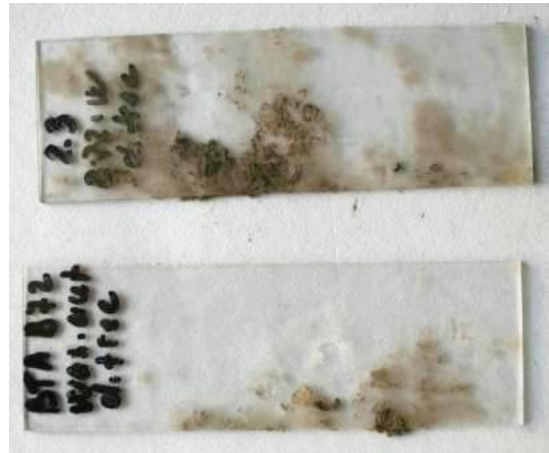


Fig.5.4. Variations of T and RH recorded in a glass pan containing the model coatings.

On controls, the surface of the wax film looked intact even by the fourteenth day of the exposure (Fig.5.5). At the same time, on bio contaminated slides exposed to increased temperatures and humidity, a number of bacterial colonies, germinated spores and growing filamentous fungi were observed, resulting in a drastic loss of the wax layer (Fig.5.6). On the fifth day of incubation, the surfaces of all contaminated slides (i.e., contaminated in plastic bags and in the storage facility) were only slightly deteriorated. Some of the conidia had started to penetrate through the wax layer (Fig.5.7). However, in general the wax coating appeared to be intact. The acrylic coating was in the same condition, but it was covered with a number of fungal spores, which had started to germinate. The combined wax-acrylic films looked like the wax film, i.e., almost completely intact. However, they were also covered with germinating spores.



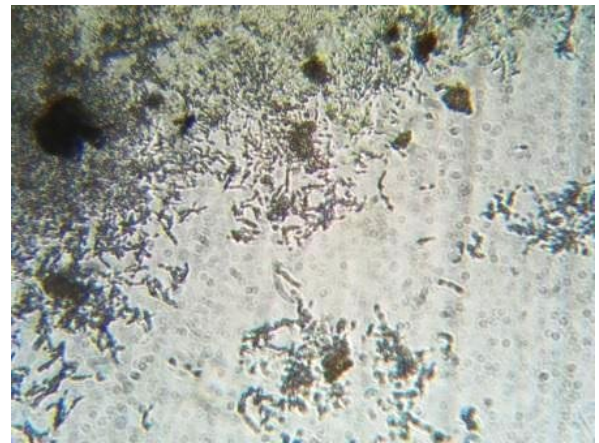
**Fig.5.5.** Wax surface of the control slide on the 14<sup>th</sup> day of incubation. Despite the presence of a few growing spores, the wax surface is intact. A cluster of germinated spores with growing filament is on the left. Magnification of 40X.



**Fig.5.6.** Biodegraded wax-acrylic coating on the glass slide after twenty days of incubation. The brown spots are the growing fungal colonies. In the centers of both slides the coating is eliminated down to the surface of the glass.



**Fig.5.7.** The view of a fungal filament piercing the wax layer on the 5<sup>th</sup> day of incubation. Magnification of 40X.



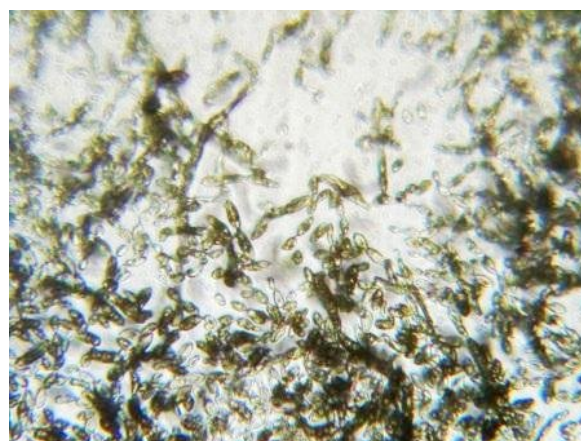
**Fig.5.8.** The view of a biodegraded wax-acrylic coating on the 14<sup>th</sup> day of incubation. On the central, bottom, and right sides, the outer wax layer is completely lost. On the left and upper side it still exists. Spores are concentrated along the edge of the degraded wax. The underlying acrylic layer is still intact, but covered by bacterial colonies and growing fungal spores. Slide was contaminated in Ziploc bag. Magnification of 40X.

On the fourteenth day of incubation, the coatings contaminated in plastic bags and the coatings contaminated in the storage facility looked quite different. Slides contaminated in plastic bags showed a dramatic loss of both wax and acrylic films. For the combined wax-acrylic coatings,

the outer wax layer was almost completely consumed by microorganisms, but the underlying acrylic layer was only partly degraded (Fig.5.8). The surfaces of slides that had been contaminated in the storage facility were covered with a great number of spores and growing conidia. Wax and acrylic coatings were partly degraded, sometimes with gaps down to the glass surface (Fig.5.9). The combined wax-acrylic films were also covered with spores and living organisms, which had penetrated through the outer wax films. However, the underlying acrylic films were still intact (Fig.5.10). In general, slides contaminated in the storage facility showed lesser loss of wax and acrylic coatings than slides contaminated in plastic bags.



**Fig.5.9.** The gap on the surface of the partly degraded acrylic layer on the 14<sup>th</sup> day of incubation. The dark border along the gap is the colonies of bacteria. Germinated fungal spores are located in the gap. The slide was contaminated in the storage facility. Magnification 40X.



**Fig.5.10.** Combined wax-acrylic coating contaminated in the storage area on the 14<sup>th</sup> day of incubation. A great number of fungal spores and filaments pierced the wax coating film. Magnification 100X.

On the twentieth day of incubation, the wax was almost completely gone in all combinations and from all slides. The acrylic films were found to be dramatically degraded, sometimes down to the glass surface. The addition of BTA in the wax and B-72 did not indicate any notable increase in the resistance of these materials against microorganisms.

The experiments with model coatings show that the thin wax, acrylic, or combined wax-acrylic films can be promptly degraded by microorganisms if environmental conditions are favourable for their growth (Zaitseva 2005a). Under the conditions of the experiment, i.e., temperatures of 19-29° C and 75% RH, the wax film with thickness of 20 µm was almost completely lost by the fourteenth day of the exposure and the acrylic film with the same thickness was degraded by the twentieth day. Combined wax-acrylic coatings were slightly more resistant. However, they were also drastically degraded in three weeks.

The initial microbial contamination of the surface of the coating is another factor that influences the rate of biodegradation of the coating material. Slides that were placed into plastic bags with a higher degree of microbial colonization showed also higher rates of biodegradation, compared with slides placed in a moderately colonized storage facility.

In these experiments, a wide spectrum of degrading microorganisms including bacteria, fungi, and yeasts participated. However, microscopic observations show that conidial fungi are the leading group in the process of degradation of the coating material.

To improve the resistance of the coating compositions against microbes, the bio resistance of wax as the outer component of these compositions must be increased. In the present work, *Sphagnum* was tested as a possible additive to conservation waxes which should make them more resistible against fungi.

#### **5.4. The effect of *Sphagnum* on the bio resistance of conservation waxes**

Extract 1 (*Sphagnum*) was added to conservation waxes Be Square 195® and Polywax 2000® to protect them against fungal degradation. Waxes were selected as the materials where Extract 1 was added because Extract 1 is soluble in organic solvents and oil (see Appendix). Waxes are readily dissolved in the same solvents (Bennet 1975). Therefore, Extract 1 can be easily introduced into waxes and the even distribution of the extract in the wax is predictable. The inhibiting effect was demonstrated by measuring the loss in weight of wax disks exposed to a mature fungal lawn for a given period of time (Zaitseva 2006).

Fungi were collected from the internal surfaces of bio contaminated plastic bags used for storage of small bronze artifacts at the Kaman- Kalehöyük archaeological site (Fig. 5.2). Fungal spores were swabbed with a sterile cotton wool tampon, wetted with sterilized distilled water, and plated with Difco SAB Maltose media. Plates were incubated at room temperature until the growth of fungal colonies occurred. In the fungal lawn, twelve species were recognized as individual organisms and purified by a number of re-inoculations (see section 3.3.1). One of them was selected for further testing. It can be suggested on the basis of the primarily taxonomic examination made by the author that this species belongs to *Aspergillus* genera. The selected culture was the dominant degrading organism for contaminated plastic bags. It also showed the best viability and the highest rate of consumption of the wax films in the preliminary tests.

To introduce *Sphagnum* extract in the test waxes, 8g of wax was slowly melted and a 0.3 ml aliquot of 3% Extract 1 (w/v) in the ethanol-toluene mixture (1:1, v/v) was introduced into the wax. The solution was added carefully into the melted wax as drops. The color of wax changed

from white to light beige. This showed that the light brown solution of Extract 1 was evenly distributed in the melted wax.

Benzotriazole (BTA)\* was introduced in the same amount of wax as a 0.3 ml aliquot, containing 0.5 % (w/v) BTA solution in the ethanol-toluene mixture (1:1, v/v). After the mixture cooled to a soft mass, 10 mm disks with thickness approximately 2 mm were cut out. The weight of the disks was measured on a Sartorius Analytical Balance with readability of 0.1 mg.

The disks were weighed and exposed to the surface of a mature fungal lawn grown in Petri dishes. Disks were superimposed directly on the lawn, 16 disks in each plate: 8 disks on one half of the plate, and 8 disks on the other half, according to the following table:

(8 disks: Be Square+3% Extract 1+ 8 disks: Be Square+0.5% BTA)×3 plates=48 disks/3 plates	
(8 disks: Polywax+3% Extract 1+8 disks: Polywax+0.5% BTA) ×3 plates=48 disks/3 plates	
<u>(8 disks: Be Square –control+ 8 disks: Polywax –control) ×3 plates=48 disks/3 plates</u>	
Total	144 disks/9 dishes

In the Petri dishes, the disks were incubated for three weeks at room temperature and in daylight. The temperatures in the laboratory varied between 15 and 25°C. The humidity at which the wax disks were incubated was not measured. However, in covered plates it is probably close to 100% RH. After 3 weeks of exposure, the disks were gently removed, washed with 1 % Tween 20 detergent (see section 4.2.1) in distilled water, and dried and weighed repeatedly. The relative loss of the disk weight  $\Delta W = (W_{\text{before}} - W_{\text{after}}) / W_{\text{before}} \times 100\%$  where  $W_{\text{before}}$  and  $W_{\text{after}}$  are the weights of the wax disk before and after incubation.

\* Benzotriazole (BTA) is commonly used for the anticorrosive treatment of bronze objects. Sometimes it is used as an additive to conservation waxes and acrylic coatings. BTA can cause an antifungal effect because it possesses acute toxicity (Lewis 2000).

The results are represented in Table 5.3.

Wax composition tested	W <sub>before</sub> , g	W <sub>after</sub> , g	ΔW, %
Be Square	8.1616	4.5666	44
Be Square+3% Extract 1	8.1123	7.7611	4
Be Square+0.5% BTA	8.8765	7.7235	13
Polywax	8.1120	7.1200	12
Polywax+3% Extract 1	8.1966	8.0112	2
Polywax+0.5% BTA	8.1241	7.2304	11

**Table 5.3.** The weight loss of wax disks exposed to fungal lawn. The weights listed in the table are an average of 24 wax disks of a given composition.

After exposure, the microcrystalline wax disks made without addition of Extract 1 (controls) appeared much thinner than before the experiment, because approximately 40% of wax was lost. The bottom surfaces of wax disks contacted with the fungal lawn were completely covered with filaments. On the upper surfaces, the filaments were located along the edges of the disk as well. In addition, the disks were subjected to considerable mechanical deformations. Some disks were cracked. Such a high rate of fungal degradation of microcrystalline wax (44% for three weeks) can be compared with the highest rates of bacterial degradation for petroleum waxes that reached 20-80% in four weeks (see Table 2.8). Experiments on the degradation of petroleum waxes by natural soil microorganisms showed similar results. 60% of microcrystalline wax exposed to natural soil litter at outdoor conditions, was lost in thirteen weeks (American Petroleum Institute 2003). Composted waxed paper was completely degraded after 24 weeks of exposure to forest litter (Hanstveit 1991).

In the experiments with wax disks, the selected fungal isolate (*Aspergillus* spp.) degraded the microcrystalline wax much faster than in experiments with wax coatings placed on glass slides. In the first case, the wax layer with a thickness of ~0.8 mm was lost in three weeks.

For model coatings, the degradation was 40 times slower, i.e. only 20  $\mu\text{m}$  of wax was lost in the same period of time. Such a big difference in the rates of degradation can be explained by the different conditions of the experiments and by differences in participating microorganisms. In the first case, the wax disks were exposed to mature fungi while model wax films were subjected to spores, which needed additional time to germinate. In experiments with model films, wax was degraded by a whole consortium of microorganisms while in the experiments with wax disks the degrading organism was a pure fungal culture. It is suggested that the degradation of wax by a given fungal species (pure culture) is more effective than by a microbial consortium (Zaitseva 2005a; Zaitseva 2006). Polyethylene wax showed a considerably lower rate of fungal degradation, in comparison to microcrystalline wax, i.e. only 12% in three weeks. This is in agreement with the results of the bio degradation of composted waxed burger wrap (Davie, Julien, and Varoney 1995). In these experiments, paper impregnated with microcrystalline wax degraded two times faster than paper impregnated with polyethylene wax.

The addition of 0.3 ml of 3% of Extract 1, i.e.  $\sim 0.1\%$  (w/w) to the tested waxes almost stopped their biodegradation. In this case only 4% of the microcrystalline wax disks and 2% of the polyethylene wax disks were consumed in three weeks.

On the basis of these experiments it can be concluded that a wax coating cannot provide an effective anticorrosive protection for bronze artifacts under conditions of increased temperatures and humidity. Under these conditions wax becomes a substrate for the growth of microorganisms which can promptly degrade a coating film.

Microscopic observations show that the degradation of wax coating on bronze objects correlates with the appearance of bronze disease on the surface of the underlying metal. This can be caused by the hydrolytic effect of microbial colonization in combination with the effect of the organic acids metabolized by microorganisms. Adding *Sphagnum* polysaccharides to the wax coatings increases the resistance of the wax film against microbial attack.

*Sphagnum* Extract 1 (*Sphagnan*) showed a clear inhibiting effect towards the selected fungal species which dominantly contaminate the bronze objects at the Kaman- Kalehöyük site storage facility (Zaitseva 2006). Therefore, using *Sphagnan* as an antifungal additive to the wax coatings showed promising results and warrants further study.

## Chapter 6. Discussion.

In this chapter, the antimicrobial effects of the *Sphagnum* extracts towards bacteria and fungi are discussed. The antimicrobial activity of *Sphagnum* extracts is governed by their chemical structures. For this reason, the structural features of extracts are also discussed.

### 6.1. Bacterial tests

The inhibiting activity of Extract 1 and Extract 2 was tested by the disk-diffusion method on three bacterial cultures, *E. coli*, *P. aeruginosa* (both gram-negative), and *Staphylococcus aureus* (gram-positive). Extract 1 showed a clear inhibiting effect on two test cultures, *E. coli* and *P. aeruginosa*. *Staphylococcus aureus* was insensitive to both *Sphagnum* extracts. These results can be compared with Painter's results for *Sphagnum* holocellulose\* (Börsheim, Christensen, and Painter 2001 a). The results of both studies are shown in Table. 6.1.

Bacterial culture (present study)	Gram stain reaction	Oxygen requirements	<i>Sphagnum</i> Extract 1	Ampicillin
<i>Staphylococcus aureus</i>	positive	Anaerobic*	No effect	+
<i>E. coli</i>	negative	Anaerobic*	+	+
<i>P. aeruginosa</i>	negative	Aerobic	+	-
Bacterial culture (Börsheim, Christensen, and Painter 2001b)	Gram stain reaction	Oxygen requirements	<i>Sphagnum</i> holocellulose	
<i>E. coli</i>	negative	Aerobic	+	
<i>Bacillus</i> spp.	?**	Aerobic	+	
<i>Micrococcus</i> spp.	positive	Aerobic	+***	

\* These bacteria are actually *facultative anaerobic* organisms. Such organisms normally use aerobic respiration. However, they can survive also without oxygen, taking energy from the oxidation of organic compounds such as carbohydrates in the process of fermentation. *Obligate anaerobic* bacteria die in the presence of oxygen (Ryan and Ray 2004).

\*\* The gram reaction of this culture is not reported. Both gram-negative and gram-positive species were found in this genus. Gram reactions of listed cultures were taken from (ibid.).

\*\*\* Only 90% of bacteria were killed after treatment with holocellulose (10% were still alive). For *E. coli* and *Bacillus* spp., the number of living bacteria after treatment was considerably (25-40 times) lower than before treatment (almost all treated organisms were killed).

**Table 6.1.** Inhibiting effect of *Sphagnum* products on selected bacterial cultures (+ effect; -no effect).

\*Holocellulose is the intermediate product in the process of *Sphagnum* preparation. Therefore, it is believed that the holocellulose in Painter's experiments possessed the same antimicrobial properties as Extracts 1 in our experiments. Extract 1 showed much better results than Extract 2. This is the reason why Extract 2 was excluded from the table.

The results of both studies demonstrate clearly antibacterial activity of *Sphagnum* polysaccharidic products. However, they cause different effects on different bacterial cultures depending on gram reaction of bacteria. Thus, gram-negative *E. coli* and *P. aeruginosa* were sensitive to *Sphagnum* products, while gram-positive cocci *Staphylococcus aureus* and *Micrococcus* spp.\* were insensitive to them. *Sphagnum* holocellulose also affected *Bacillus* spp., but Painter did not specify the gram reaction\*\* of this test culture. Our results show that the antibacterial activity of tested extracts is not as high as a standard bacteriostatic, Ampicillin. The comparison of Extract 1 with Ampicillin indicates that the antibacterial effect of Extract 1\*\*\* (i.e. *Sphagnum*) is 180 times weaker than Ampicillin towards *E-coli* (see section 4.1).

## 6.2. Fungal tests

The antifungal activity of *Sphagnum* extracts was tested by the disk diffusion method and by the tube method. Both methods are based on a loss of the viability of fungal spores exposed to liquid *Sphagnum* extracts. Extract 1 was also tested as an antibiotic additive to conservation waxes. In this case test organisms were mature fungi, not their spores. The effect was estimated by mass loss of wax, which served as a substrate.

### *Disk diffusion experiments*

In these tests, *Sphagnum* extracts were exposed to the spores of seventeen fungal species isolated from Arctic artefacts. The effect of the *Sphagnum* extracts was compared to the effect of a standard inhibitor, sodium azide (NaN<sub>3</sub>), which was applied to the same fungal isolates.

\*The fact that *Micrococcus* spp. (in Painter's experiments) showed any, even limited sensitivity towards *Sphagnum* holocellulose also deserves attention because gram-positive bacterial infections are becoming more prevalent within hospital settings and some of them, particularly, cocci, are quite resistant to antibiotics (Norrby 1995).

\*\*Gram reaction is based on the structure of the bacterial cell wall: the outer membrane of gram-negative species can react with carbonyl groups of *Sphagnum* polysaccharides, while peptidoglycan, which forms the outer layer of the cells of gram-positive species, probably prevents this reaction (Prescott, Harley, and Klein 2002).

\*\*\*The antibacterial effect caused by Extract 1 was greater than that of Extract 2.

The squares of the areas with no growth for Extract 1 ( $S_{Ex1}$ ) and sodium azide ( $S_{NaN3}$ ), as well as the ratio  $S_{Ex1}/S_{NaN3}$  are listed in Table 4.1. The ratio  $S_{Ex1}/S_{NaN3}$ , which varied from 0 to 4.29, characterizes the relative antifungal activity of Extract 1 with respect to a given culture. Having this ratio, all fungal cultures can be split into two different groups. One group includes five isolates: 26-5(1), 26-1, 21-2 (two species), and 20-2. For these cultures the effect of sodium azide appeared to be much stronger than the effect of *Sphagnum* extracts, and  $S_{Ex1}/S_{NaN3}$  varied between 0.11 and 0.22. The remaining twelve species constitute another group showing approximately the same or lower sensitivity to sodium azide as to *Sphagnum* extracts. Excluding species 26-5, 22-1, and 26-4, where at least one inhibitor showed no effect, the ratio  $S_{Ex1}/S_{NaN3}$  for this group varied between 0.27 and 4.29.

The time limits of the present work did not allow the author to explore morphological differences between these two groups of fungi. However, it has been noted that the group of isolates which were sensitive to *Sphagnum* extracts included many members of the genus *Aspergillus*. One species, 16-2, which was selected as a test organism for further studies, also belongs to genus *Aspergillus*. For this culture  $S_{Ex1}/S_{NaN3}=1.22$ ; i.e., *Sphagnum* Extract 1 and sodium azide caused similar effects on this fungus. In this case, the relative effectiveness of *Sphagnum* Extract 1 to sodium azide was easily determined. 20 $\mu$ g of 3% Extract 1 caused approximately the same effect as 20 $\mu$ g of 0.05% sodium azide. This means that Extract 1 appeared to be approximately  $3/0.05=60$  times less effective than sodium azide in terms of the experiment. Therefore, it can be concluded that the antifungal activity of Extract 1 is not as high as that of sodium azide.

When Extract 1 is applied to fungal spores, their germination can be depressed not only by Extract 1 itself, but by the water content of the extract, its sugary nature, and its acidity. These factors can be a source of random errors for experimental results. The influence of all these factors on spore germination was also tested along with Extract 1. To determine the water effect, the quantity of water contained in the test amount of *Sphagnum* extracts (20 µl aliquot) was dropped onto additional paper disks and applied on the media along with Extract 1. In this way the influence of water on all fungal isolates was investigated, but no effect was found (see section 4.2.1).

*Sphagnum* polysaccharides contained in Extract 1 are potentially able to inhibit spore germination due to their dehydration which is caused by the osmotic effect of sugar on fungal spores. Since our experiments used quite low concentrations of Extract 1, it was difficult to believe that its sugary content can cause an inhibiting effect on test fungi. However, a series of experiments has been carried out to check the role of this factor. These experiments were carried out under the supervision of Dr. A. Kropinski, at the Department of Microbiology and Immunology, Queen's University. Fungi were sampled from ethnographic artefacts (CMC) that were stored in the conservation laboratory of the Art Conservation Department, Queen's University (see section 3.3.1). Five species were isolated and named 1.1, 1.2, 3.1, 4.2, and 5.1.

Following standard procedure, 0.1 ml of each spore suspension was plated on the Difco Sabouraud (SAB) Maltose Agar media. The polysaccharidic content of Extract 1 was simulated by different sugars: Mountain Ash gum extract (prepared by Dr. A. Kropinski), polyethylene glycol (PEG), amylopectin, and xylan. In these experiments no paper disks were used.

Polysaccharides were applied to fungi as solids, not in water solutions, to reach their maximum

concentration on the surface of the media. All four polysaccharides in the amount of 0.05 mg of were placed on the same plate with one culture plated, leaving a space between solids. In this way all five fungal cultures were tested. After incubation at room temperature for 3-4 days, the plates were observed. For all plates, the surface of the media was evenly covered with germinated spores without any inhibited areas around the solids. Moreover, after 5-6 days of incubation the surfaces of all solids were also covered by newly grown colonies. Therefore, antifungal activity of *Sphagnum* polysaccharides (Extract 1) is not connected to their osmotic effect.

The water solution of Extract 1 has an acidic reaction (see Appendix), which may be a limiting factor for spore germination. Experiments were carried out on only one selected species, 16-2. Two *Sphagnum* extracts, Extract 1 (pH 3.0) and the Water Extract (pH 3.4) were tested. For comparison, portions of these extracts were neutralized with buffer and tested along with regular extracts. The area without spore germination for both extracts, acidic or neutralized, was approximately the same for all experiments. Therefore, acidity, water content or the sugary nature of the *Sphagnum* extract cannot cause the random error of the disk experiments.

The additional uncertainty of the disk diffusion method with respect to *Sphagnum* extracts may be caused by the colloidal nature of the extract solutions. Because of the difference in molecular diffusivities of the solvent (water) and the suspended matter in the extracts, both can be separated on a paper disk. As a result, the solid part of the extract can be retained on the disk and immobilized. The solids retained on the paper disk as a brown stain.

### ***Tube experiments***

In the tube test, the viability of spores depending on the time of their exposure to Extract 1 and to the Water Extract was tested. Both extracts showed similar results for the selected fungal culture 16-2. During the initial 3-5 hours of exposure, the viabilities of untreated spores and spores treated with *Sphagnum* extracts were approximately the same. During the next 3-5 hours of exposure, the viability of spores exposed to *Sphagnum* extracts fell to zero while the viability of untreated spores (controls) was not considerably changed during the entire time of the test.

The variations in viability of spores in broth can be caused by many factors. We checked two of them: dehydration of spores by *Sphagnum* polysaccharides and formation of spore clusters. The dehydration of spores followed by death can be caused by the osmotic effect of sugars contained in the *Sphagnum* extracts. Two neutral sugars, maltose and glucose, simulated the sugary content of *Sphagnum* extracts. However, introducing these polysaccharides into broth did not cause any decrease in spore viability.

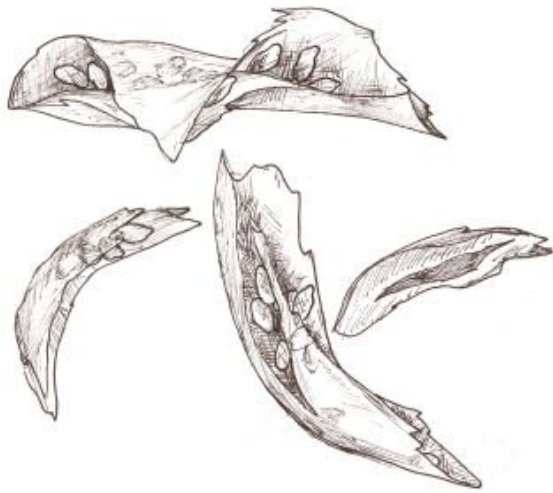
The cluster formation effect is as follows. It was assumed that if spores are submerged in water for several hours, they could electrically attach to each other to form clusters. If clusters of spores are formed, the colonies grown from these spores are located so close to each other that they are counted as one colony. As a result, many colonies are not counted; this may be interpreted as the loss of spore viability. To check this effect, the author carried out several microscopic observations of the content of test tubes. The existence of clusters was not observed in any time of exposure. Therefore, neither the sugary nature of *Sphagnum* extracts nor the formation of clusters can cause variations in viability of untreated spores in broth.

Complete killing of spores occurred after 6-8 hours of exposure for Extract 1 and after 10 hours for the Water Extract. However, taking into account the much lower viability of the spores exposed to the Water Extract compared to the spores exposed to Extract 1 (4-8% vs. 24-49%), the antifungal activity of these two extracts can be considered approximately the same.

Therefore, 40 µl of both *Sphagnum* extracts added to one ml of nutritious broth containing 2,000 – 10,000 fungal spores (20 µl of *Sphagnum* extract per 0.5 ml of broth) caused a mortal effect of these spores after 6-8 hours of exposure.

The mechanism of the inhibiting influence of *Sphagnum* extracts on fungal spores is unclear. The following observations offer some explanations for this phenomenon. A drop of spore solution in broth was microscopically examined at the sixteenth hour of exposure to the Water Extract (Fig.6.1). All spores in this drop appeared to be attached to the micro-pieces of extract. These micro-pieces floating in broth appeared like semi-transparent irregular-shaped amorphous particles with cavities. Spores were attracted to particles of extract or were trapped in the cavities.

Unlike living spores, trapped spores had rough edges and looked withered, i.e., obviously not like living objects. This trapping effect probably occurred due to the electrical charge of both *Sphagnum* extract and pre-germinated spores. Therefore, the strong electrical charge of *Sphagnum* extracts (see Appendix) can be one possible reason for their antimicrobial activity.



**Fig.6.1.** The dead fungal spores trapped in suspended micro-particles of the Water Extract –a draft from microscopic picture. The size of spores is approximately 10  $\mu\text{m}$ . The particles look like squeezed parts of leaves and stems of the *Sphagnum* plant. Spores look “skinny” and have rough edges. Probably they are already dead.

### **6.3. The search for antimicrobial agents in *Sphagnum* moss**

The antimicrobial activity of *Sphagnum* extracts was confirmed experimentally in the present study and by Prof. Painter. These experiments demonstrate the existence of antimicrobial agents in *Sphagnum* plants. It is important to identify the chemical structure of these agents for their further synthesis and production. With respect to the present study, it is important to point out that Extract 1 and the Water Extract showed approximately the same antifungal activity, even though they were quite different chemical structures. Extract 1 contains polysaccharides and does not contain phenolics, which are oxidized by the chlorite treatment. On the contrary, the hot water extract (the Water Extract) must contain phenolics.

In the present study, determination of the structure of both extracts through Nuclear Magnetic Resonance (NMR) Spectroscopy and FTIR analysis has been attempted (see Appendix). The mass spectra of Extract 1 and the Water Extract appeared to be almost the same. The only difference was the quantity of water bonded to the molecules of these extracts. This is not surprising because the detailed structure of plant hemicelluloses has not yet been determined due to their complexity (Rowell, Han, and Rowell 2000). Most often, only the ratio of polysaccharides comprising these structures is known (ibid.). NMR spectroscopy has already been used for the analysis of peat moss (Boon, Dupont, and De Leeuw 1986). However, there are serious reproducibility issues with respect to the application of the NMR technique to these studies. In FTIR analysis, the absorbance of infrared light by functional groups in molecules is detected. This technique was successfully used to discriminate *Sphagnum* peat of different geographical origins \* (Harrison *et al.* 2006) and the extent of recent decomposition of labile organic matter in the acrotelm and the catotelm (Cocozza *et al.* 2003). In peat, the major peaks associated with certain classes of chemical compounds, e.g. fatty acids, peptides, and polysaccharides, were distinguished. However, the major FTIR peaks of *Sphagnum* moss cannot be observed in much detail (ibid.). Therefore, the instrumental analysis of Extract 1 and the Water Extract showed that neither NMR spectroscopy nor FTIR analysis allowed the detection of antimicrobial structures in extracts or the discrimination of structural differences between these extracts\*\*. Nevertheless, the structures of obtained extracts need to be discussed, at least on the basis of available literature sources.

\*The main point in this study is the analysis of plant lignin. Lignin is composed of guaiacyl (monomethoxy), syringyl (dimethoxy) and hydroxyphenyl units, the proportions of which are dependent on plant species. *Sphagnum* moss does not contain true lignin, lacking components of wood lignin: guaiacyl and syringyl units. Therefore, peats with woody plant origins and peats with moss origin from different geographic areas can be distinguished. Peat with a higher percentage of *Sphagnum* moss contained lower numbers of mono- and dimethoxy phenyl units.

\*\*There is a simpler approach to this problem which is traditionally used in phyto-pharmacology: a particular class of compounds contained in the plant cell wall is considered to be associated with a particular solvent which is used for the extraction of these compounds. The common solvents are water, acetone, ethanol, methanol, chloroform, dichloromethanol, and ether (Cowan 1999)

### ***Extract 1***

Painter attributed the remarkable antimicrobial effect of *Sphagnum* moss to a polysaccharidic component (*Sphagnan*), which is a reactive residual of polyuronic acid, 5-KMA. In the present study, Extract 1 has been prepared following closely the procedure described by Painter (Painter 1991a; Painter 1998; Børsheim, Christensen, and Painter 2001a; personal letter from Prof. Painter to the author). The yields of the intermediate and final products at different stages of the preparation of *Sphagnan* for both extractions are compared in Table 6.2.

<b>Dried leaves of <i>S. palustre</i>, weight (g)</b>	<b>After removal of extractives by boiling acetone, weight (g)</b>	<b>After removal of lignin by sodium chlorite, weight (g)</b>	<b>Soluble <i>Sphagnan</i> after utohydrolysis, weight (g)</b>	<b>Source</b>
50	20	10	5	Børsheim <i>et al.</i> 2001a
50	21.3	9.8	4.1	The present work

**Table 6.2.** Yields of *Sphagnum* intermediate products in different stages of the preparation of *Sphagnan*.

Taking into account the similarity of the raw material (in both cases it was *S. palustre*), the similarity of the extraction operations, and the similarity of yields in both extractions, it is believed that the final product of our extraction (Extract 1) is similar to the final product of Painter's extraction. Therefore, we can conclude that Extract 1 is also *Sphagnan*, i.e., a reactive polysaccharide containing residuals of polyuronic acid, 5-KMA.

However, the recent analysis of *Sphagnum papillosum* showed that neither *Sphagnan* isolated from this plant nor plant holocellulose contain monomers of 5KMA (Ballance *et al.* 2007). After fractionation of these products by anion-exchange chromatography, structures resembling pectin-like polysaccharides were identified. This polysaccharidic mixture contained neutral sugars rhamnose (25%), galactose (7.5%), mannose (3%), and 40% rhamnogalacturonan I-type

pectin. It was also found that rhamnogalacturonan I (the main part of *Sphagnan*) is a highly branched region of a pectin consisting of 1,4-linked D-galactopyranosyluronic acid (GalpA) and 1,2-linked rhamnopyranosyl (Rhap) residues (Inngjerdingen *et al.* 2007). (Rhap) residues are branch points for neutral sugar side chains (*ibid.*). There is also evidence that *Sphagnum* produces mono- and disaccharides such as fructan and sucrose (Marschall and Laufer 2002). The bioactivity of these polysaccharides is still unclear, but it appears to be connected to branching of their side chains (Lin 2005). Thus, there are several reports that the rhamnogalacturonan structural region, not 5KMA, is the main ingredient responsible for *Sphagnum* antimicrobial activity (Paulsen and Barsett 2005; Yamada and Kiyohara 1999).

Pectin-like polysaccharides are reported to be easily extracted from plant material with hot water (Inngjerdingen *et al.* 2007). The concentration of hot water-soluble carbohydrates in eight *Sphagnum* species ranged from 35 to 125 mg/g (Turetsky, 2003). These results are similar to our results, where the yield of Extract 1 (*Sphagnan*) was approximately 80 mg/g. In the process of preparation of Extract 1, soluble carbohydrates are partly removed from plant material by hot water during the chlorite procedure. Other amounts of soluble polysaccharides are isolated during the cleavage of the chlorite-treated holocellulose by autohydrolysis. They are collectively named *Sphagnan*. This product must contain neutral sugars, which make it sweet tasting (see Appendix). If we accept that *Sphagnan* is a pectin-like polysaccharide with rather high quantities of neutral sugars, the solubility of this composition must be quite high (Table 6.3). At the same time, the solubility of Extract 1 (*Sphagnan*) in water was found to be 3.3 g/ 100 ml (see Appendix), i.e., low compared to the solubility of neutral sugars at 16-90 g/100 ml. The solubility of Extract 1 is similar to that of vanillin, gallic and ferulic acids, i.e., phenol

compounds comprising the *Sphagnum* plant cell wall. We cannot explain this obvious discrepancy.

<i>Sphagnum</i> fiber ingredients	%	Solubility, g/100 ml at room temperature	References
<b>Soluble sugars</b>			
<i>d</i> -glucose (grape sugar, dextrose)	30	91 (25°C)	Pancoast and Junk 1980
<i>l</i> -arabinose	~5	58.9 (10°C)	Perelman 1963
<i>l</i> -galactose	~5	63.8 (9.5°C)	Perelman 1963
<i>l</i> -ramnose	<1	50	Todd *
<i>d</i> -xylose (“wood sugar”)	15	55.5	Budavari 1996
<i>d</i> -mannose	<1	15.6 (18°C)	Perelman 1963
<b>Lignocellulose</b>			
vanillyl phenols (Vanillin)		1.0 (14°C)	Perelman 1963, Esposito <i>et al.</i> 1997
cinnamic acid		4.0	Budavari 1996
<i>p</i> -coumaric acid (main component of lignocelluloses)		7.4	CAT-MED Project progresses**
Ferulic acid		1.05	Lee, Volm, and Rosazza 1998

\* Todd, A.M. Material Safety Data Sheet, [http://www.moorelab.com/iteminfo/n\\_msd\\_p\\_amt.asp?ID=192](http://www.moorelab.com/iteminfo/n_msd_p_amt.asp?ID=192)

\*\*CAT-MED Project progresses, <http://www.ics.trieste.it/catmed/PPWorkingPlan2.aspx>

**Table 6.3.** Solubility of *Sphagnum* moss constituents. The chemical composition of moss was taken from Rowell (Rowell, Han, and Rowell 2000).

Based on this discussion, it can be concluded that Extract 1 represents a mixture of polysaccharides including neutral sugars. At the same time Extract 1 is probably free of plant phenols, because all aromatics were oxidized by the chlorite procedure. This polysaccharidic mixture undoubtedly possesses antimicrobial activity. However, the chemical structure of these polysaccharides with respect to their antimicrobial properties is still unclear.

### ***Water Extract***

The Water Extract contains *Sphagnum* cell wall ingredients which are soluble in hot water. They are soluble carbohydrates (e.g. pectin-like polysaccharides), soluble lipids such as plant waxes, and soluble phenolics. According to Turetsky, *Sphagnum* litter consists of 35-135 mg/g dry wt

of water-soluble carbohydrates, 40-75 mg/g dry wt of lipids, and 1-4 mg/g dry wt of phenolics (Turetsky 2003). All these classes of *Sphagnum* biopolymers possess certain antimicrobial capabilities against attacking microorganisms. The antimicrobial function of pectin-like polysaccharides was discussed in a previous section. Plant waxes are a class of biological lipids that serve an important defensive function in plant-microbe interaction (Kolattukudy 1987). Because the temperature of the hot water extraction (80°C) is close to the melting point of most plant waxes (<http://www.cyberlipid.org/wax/wax0001.htm#4>), it is believed that the Water Extract contains some waxes. However, little is known about the antimicrobial properties of *Sphagnum* waxes and therefore they are not discussed in the present work.

Phenolics are the group of *Sphagnum* biopolymers, which have been traditionally thought of as having low bioavailability. In spite of their relatively low concentration in *Sphagnum* cells, phenolics deserve attention because their antimicrobial properties are well known. It was experimentally confirmed that *Sphagnum* moss produces endogenous water-soluble phenolics, which are excreted into the external medium as metabolites (Rasmussen 1994; Rasmussen, Wolff, and Rudolph 1995). A number of water-soluble phenolic constituents of the *Sphagnum* plant have been isolated, such as phenolic acids and soluble tannins (Williams *et al.* 1998). Phenolic acids includes genus-specific *Sphagnum* acid and *p*-coumaric acid, vanillic acid, vanillin, syringaldehyde, and ferulic acid. The *Sphagnum* plant also contains the ingredients of tannic acid, such as *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxyacetophenone (all in small amounts, *ibid.*).

All soluble phenolics can be removed from *Sphagnum* plant material by hot water and thus, it is believed that they are present in the Water Extract. The concentration of soluble phenolics

extracted from eight *Sphagnum* species with hot water was found to range from 1 to 4 mg/g of plant dry weight (Turetsky, 2003). This is thirty times less than the concentration of soluble carbohydrates (35-125 mg/g). Therefore, the solubility of the Water Extract (a mixture of phenolics and carbohydrates) should be close to the solubility of carbohydrates, i.e. should be relatively high. At the same time, the solubility of the Water Extract was found to be only 2.9 g/100 ml (see Appendix). This value is close to the solubility of lignin-like constituents of *Sphagnum* tissue (Table 6.3.), i.e. it is relatively low. It is difficult to explain the low solubility of the Water Extract and the Extract 1 on the basis of the available facts.

### ***The role of reactive polysaccharides in Sphagnum antimicrobial activity***

Our experiments show that the antimicrobial activity of Extract 1 and the Water Extract is the same. Extract 1 contains soluble polysaccharides ( $PS_{sol}$ ) and an additional portion of reactive polysaccharides isolated from oxidized holocellulose by autohydrolysis ( $PS_{react}$ ); the Water Extract contains soluble polysaccharides ( $PS_{sol}$ ) and soluble phenolics ( $PH_{sol}$ ). These equivalences can be expressed by following equation:  $a(PS_{sol}) + a(PS_{react}) = a(PS_{sol}) + a(PH_{sol})$  where  $a$  is the antimicrobial activity of the given plant ingredient. Painter and his colleagues denied the role of phenolics as antimicrobial agents in *Sphagnum* moss, i.e.,  $a(PH_{sol})=0$ . If we accept Painter's conclusion for our results, the antimicrobial activity of reactive polysaccharides must also be negligible, i.e.,  $a(PS_{react})=0$ . In this case, the antimicrobial activity of *Sphagnum* extracts is determined by the soluble polysaccharides only. As well, the extraction of additional polysaccharides by autohydrolysis is not necessary because these compounds do not noticeably increase the antimicrobial activity of the final product\*.

\*In fact, in most cases Painter used *Sphagnum* holocellulose (prepared without autohydrolysis), not *Sphagnum*, to demonstrate antimicrobial activity of *Sphagnum* moss.

On the other hand, recent publications show that phenol compounds in *Sphagnum* may be important microbial inhibitors and therefore they cannot be rejected as a negligible part of *Sphagnum* antimicrobial activity (see section 2.2.12). If this is true, the antimicrobial activity of phenolics and additional ingredients extracted by autohydrolysis should be the same.

#### **6.4. Is Painter's theory of *Sphagnum* antimicrobial activity true?**

It is of interest to discuss whether or not the recent publications and our experiments are in agreement with Painter's ideas. Many facts, such as traditional use of *Sphagnum* moss in folk medicine and the preservation of bog bodies, illustrate the antimicrobial activity of *Sphagnum* moss. Painter attributed the remarkable antimicrobial effect of *Sphagnum* plants exclusively to their reactive polysaccharides, *Sphagnan*.

In terms of plant structure, *Sphagnan* is a part of hemicellulose, which is covalently bonded to the holocellulose and cellulose of the *Sphagnum* plant cell wall. *Sphagnan* has a limited solubility in water. According to Painter, it slowly, during millennia, liberated into ambient water in the process of peat diagenesis. As it accumulates in bogs, *Sphagnan* binds the organic nitrogen of proteins and microbial exo-enzymes by the mechanism of the Maillard reaction\*. As a result of this reaction, the skin of bog bodies became tanned and was not susceptible to degradation by putrefying bacteria.

\*The condensation of amino-acids with sugars and lignin oxidation products to form humic substances have been postulated long ago (Hedges 1978). Although non-enzymatic browning is considered to be a possible pathway of the formation of humic substances (see section 2.2.3), it is slow under ambient bog conditions (Hedges 1978; Jokic 2000). The reaction between the sugar and the amino group depends on a number of factors including water activity, pH, and temperature. It was shown that normally melanoidins (final products of this reaction) are synthesized from glucose and glycine at temperatures of 70 °C - 90°C, which are obviously absent in bogs (Benzing-Purdie, Ripmeester, and Ratcliffe 1985). The latest studies also deny both purely biological processes and abiotic reactions, such as the Maillard reaction, as a major source of humic substances in bogs (Burdon 2001).

Painter tried to prove preservative properties of *Sphagnum* polysaccharides on different fish products and on some bacterial cultures. Antimicrobial effects of fresh *Sphagnum* moss, solvent-treated moss, peat moss, and *Sphagnum* holocellulose were compared. In these experiments, antimicrobial activity of all *Sphagnum* products was demonstrated. However, they do not confirm an exclusive role for polysaccharides as antimicrobial agents in *Sphagnum* moss. For example, the antibacterial effect of *Sphagnum* holocellulose was found to be almost the same as the effect of untreated *Sphagnum* moss and peat (which is rich in phenolics and is almost free of polysaccharides). These results suggest the idea that the antimicrobial activity of *Sphagnum* moss is not limited to *Sphagnan*. Also, the ability of *Sphagnan* to bind nitrogen appeared to be controversial. The bog water taken from low peat deposits (where *Sphagnan* should have accumulated) did not show a notable effect of nitrogen binding (see section 2.2.8, Painter 1991a; Børsheim, Christensen, and Painter 2001a). In addition, structural investigation of *Sphagnum* moss and *Sphagnum* products has not been accomplished for a long time.

In 2002-2005, a project was planned at the Institute for Biotechnology, Trondheim, Norway, to give *Sphagnan* a full chemical and physical description (Christensen 2002; Christensen 2006). In this project, *Sphagnum* polysaccharides were examined by different physical and chemical methods, including nuclear magnetic resonance (NMR), gas chromatography and electron ionization (EI) mass spectrometry to determine glycosyl linkages in *Sphagnum* products. Colorimetric analysis of total carbohydrates and uronic acid was also performed (Ballance and Christensen 2007). As a result of these studies,

**”no evidence was found of the presence of a previously described 5-keto-d-mannuronic acid (5-KMA) monomer in *Sphagnan*, or in the chlorite-treated leaves from which *Sphagnan* was extracted” (Ballance *et al.* 2007).**

However, these authors

**“have now surprisingly found that a moss extract, which is free of 5-KMA has particularly effective antimicrobial properties”** (Balance and Christensen 2007).

After twenty years of studies and numerous publications, this extraordinary result illustrates that the chemical structure of *Sphagnum* moss is still not understood, as well as the role of this structure in moss antimicrobial activity\*. Moreover, these authors tried to explain antimicrobial activity of *Sphagnan* by its ability to lower the pH (Stalheim, Ballance, Christensen, and Granum 2009). According to their laboratory experiments, *Sphagnan* in its sodium form at neutral pH had no antibacterial activity, i.e.

**“*Sphagnan* in its acid form can inhibit bacterial growth, but only of acid-sensitive species”.**

At the same time, our results have shown that antimicrobial effect of *Sphagnan* is not caused by its acidity (see section 4.2.2).

In all Painter’s laboratory experiments with fish skin and tissue, the antimicrobial effect of *Sphagnum* products was closely connected to “browning” or tanning of the test substance. Painter then insisted that the tanning effect of bog bodies, which occurs in natural conditions, is also caused by *Sphagnan*. At the same time, he denied the preservative role of *Sphagnum* phenolics due to their small concentration in *Sphagnum* cells. To prove the possibility of polysaccharidic tanning of skin collagen, Painter demonstrated scanning electron micrographs of mackerel skin after treatment with different tanning agents, including *Sphagnan* (Børsheim, Christensen, and Painter 2001a).

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\*A wide investigation was planned into the antimicrobial effect of *Sphagnan* on pure cultures of certified microbes (2002-2005 NTNU projects, Christensen 2002). The *Sphagnan* composition was reported to have been tested on fifty bacterial cultures over two years (Christensen, Balance, and Granum 2004). However, these results have never been published. The antimicrobial capability of *Sphagnan* has not been compared with known antimicrobial agents. Also, the minimal inhibiting concentration (MIC) of *Sphagnan* for these cultures was not determined.

Recently Painter's colleagues carried out research to examine the ability of *Sphagnum* to react with collagen resulting in tanning of collagen (Ballance *et al.* 2008). The authors concluded that the content of reactive carbonyl groups in *Sphagnum*

**“is estimated to be much less than previously thought”.**

This is a possible reason why in Painter's experiments bog water from low peat deposits did not create a colloid solution with gelatine (see section 2.2.8). The experiments with *Sphagnum*-treated hide powder collagen showed that

**“*Sphagnum* is a poor tanning agent”** (Ballance *et al.* 2008).

As a result of this research the authors concluded that the preservative properties of *Sphagnum* pectin-like polysaccharides (*Sphagnum*) are not related to tanning.

At the same time, much progress has been made in understanding the reasons for slow decomposition of *Sphagnum* moss in peat bogs. In nature, the antimicrobial property of *Sphagnum* litter is caused not only by the specific chemical structure of *Sphagnum*, but by several ecological factors and mechanisms taking place in peat bogs. For example, slow rates of *Sphagnum* decomposition/low microbial population in *Sphagnum* litter can also be explained by low temperatures, lack of oxygen, and the chemical quality of *Sphagnum* litter itself, which is generally poor in nutrients, especially in nitrogen (Turetsky 2003; Bragazza *et al.*, 2006). However, in spite of ecological factors, the antimicrobial properties of *Sphagnum* moss are determined by the structure of the moss cell wall (see section 2.2.12).

Of the compounds which constitute cell walls of mosses, phenolics play an important role. The microscopic examination of the *Sphagnum* cell wall shows that phenolics likely possess a protective function for plant cellulose, providing the physical and chemical barrier for degrading

microorganisms (Tsuneda, Thormann, and Currah, 2001). To utilize phenolics, microorganisms need specific enzymes, such as phenol oxidase. In a natural bog environment, only a limited number of microbial species are able to produce this enzyme. This is one of the reasons for a low microbial population in bog ecosystems (see section 2.2.12). Therefore, even if the concentration of phenolics in *Sphagnum* plant cells is low, they are able to protect the *Sphagnum* plant from microbial degradation.

The phenomenon of bog bodies can be also explained on the basis of the polyphenol theory, i.e. without participation of polysaccharides. Flash pyrolysis gas chromatography/mass spectrometry of Iron Age bog bodies from Lindow, UK, and Drente, The Netherlands showed that the reason for the good preservation of body tissues was a tanning reaction between amino acids of skin collagen and moss phenolics, particularly, *Sphagnum* acid (Stankiewicz *et al.* 1997). When buried in bogs, the bodies probably sank and were submerged in deep peat deposits. This media contains very bioresistant compounds, such as high molecular weight insoluble phenolics, including vascular lignin. Many of these compounds (flavonoids, condensed tannins, etc.) are known to be antimicrobial agents. These humic substances are unavailable for microorganisms and may be a main reason for the preservation of skin collagen in peat bogs (see section 2.2.12).

It seems that Painter underestimated the role of polyphenols as *Sphagnum* antimicrobial agents. Many results suggest the idea that the antimicrobial activity of *Sphagnum* moss is not limited to *Sphagnum*. Our experiments show that soluble carbohydrates and/or phenol compounds may be these additional antimicrobial ingredients in moss. After re-examination of the structure of *Sphagnum* polysaccharides by Painter's colleagues, his explanations of the antimicrobial properties of *Sphagnum* moss on the basis of polysaccharidic theory became suspended. At the

same time, our results, as well as Painter's results, prove that *Sphagnum* polysaccharides (*Sphagnan*) possess antimicrobial properties. Therefore, despite some criticism of Prof. Painter's methods, his work is worthy of appreciation. As a pioneering specialist in the carbohydrate chemistry of *Sphagnum* moss, he, in fact, created a modern theory of *Sphagnum* antimicrobial activity. Many of his ideas were confirmed experimentally and can be used for further studies of antimicrobial activity of mosses.

## **6.5. Ecological hazards of the industrial exploitation of *Sphagnum* moss**

Recent proposals for practical uses of *Sphagnum* moss suggest manufacturing of *Sphagnum* products (e.g. toothpaste with *Sphagnum* additives, *Sphagnum* food package materials, etc., see section 2.2.13) on an industrial scale. In addition, the wide use of fresh moss is planned for wrapping catches in the fish industry. Because of this, we decided to discuss ecological consequences of the industrial harvesting of *Sphagnum* moss.

It is important to note that only the thin, upper part of the moss carpet will be used resulting in the exploitation of wide areas of peatlands. The large scale of harvesting of mosses is a real threat to the northern ecosystem. It is of concern in Norway, where these proposals originated. To provide packaging materials for fresh meat and fish, two types of natural resources will be consumed: quickly renewable (fish or meat) and slowly renewable (fresh moss). Assume the following conditions: a container for packaging 1 kg of fresh fish weighs 100g, of which 5g is *Sphagnum* moss (dry weight); Norway's total catch of wild fish in 2006 amounted to 2.2 million metric tons (Norway Fishery Products Annual Report 2007); and 25% of this catch is packaged in *Sphagnum* containers: then the total annual weight of harvested *Sphagnum* moss should be

2500 tons (dry weight) or ca. 60,000 tons (raw weight) for Norway only. If we imagine a scenario where other countries started using *Sphagnum* for foodstuffs and also began adding *Sphagnum* to toothpaste and toilet paper, it is clear that the world moss population will be quickly exhausted.

It is known that northern peatlands contain about 30% of the world's carbon pool. Their surfaces typically have an almost 100% cover of mosses, and the *Sphagnum* species play an important ecological role in the capture of atmospheric carbon in peat. Anaerobic decomposition processes in peat result in the production of globally significant greenhouse gases: methane (CH<sub>4</sub>), carbon dioxide (CO<sub>2</sub>), and nitrous oxide (N<sub>2</sub>O) (Freeman, Lock, and Reynolds 1992; Aerts 1997). The stability of peatlands for carbon storage is now under threat from industrial and agricultural impacts.

Mosses are very sensitive to changes in external conditions, and their death around industrial areas indicates ecosystem contamination. Drainage of peatlands, soil fertilization, and growth in the levels of atmospheric nitrogen cause negative changes to *Sphagnum* ecosystems. Additional industrial harvesting of mosses can cause the catastrophic disappearance of mosses. As a result, carbon accumulated in peat will be released back to the atmosphere as carbon dioxide (Bragazza *et al.* 2006; Limpens *et al.* 2008). Therefore, moss ecosystems need to be strictly protected, i.e., industrial exploitation of *Sphagnum* and other mosses should be under control by national/international law. Limited harvesting without seriously affecting the *Sphagnum* population is obviously possible. It seems that using moss in Arctic archaeological expeditions can be continued because of the low number of expeditions, the limited number of excavated artefacts, and the generally small size of the artefacts. The amount of moss harvested for Canadian expeditions is very limited and cannot cause any damage to bog ecosystems.

## 6.6. *Sphagnum* moss in art conservation: the outlook

The present study provides experimental proof that *Sphagnum* moss possesses antimicrobial properties and can be used in art conservation. Antimicrobial properties of the pectin-like polysaccharides (*Sphagnan*) contained in *Sphagnum* moss have been shown in several studies. In the present work, these polysaccharides were extracted (Extract 1) and tested on bacterial and fungal cultures. This extract, known as *Sphagnan*, was free from lignocelluloses. For comparison, *Sphagnum* constituents were extracted also with hot water (the Water Extract). In contrast, this extract contained water-soluble phenolics.

Extract 1 showed an inhibiting effect on gram-negative bacteria (*E. coli* and *P. aeruginosa*) and filamentous fungi (*Aspergillus* spp.). The antimicrobial activity of *Sphagnum* extracts appeared to be not as high as that of the standard antibiotics. Extract 1 was 180 times weaker than Ampicillin towards *E-coli* and 60 times less effective than the standard antifungal agent sodium azide. However, compared to other polysaccharidic compositions, for example, *Chitosan*, the antimicrobial effect of Extract 1 was rather high. Thus, *Chitosan* in concentration 3 g/l kills 40% of the spores of *Aspergillus niger* after 13 hours of exposure and 73% after 24 hours of exposure (Plascencia-Jatomea *et al.* 2003). At the same time, Extract 1 at the concentration of only 40 µl/ml (~1g/l) killed all spores of *Aspergillus* spp. in 6 hours of exposure.

The effect of Extract 1 and the Water Extract on the test fungi (*Aspergillus* spp.) appeared to be the same for both the disk-diffusion method and the tube method. Taking into account that these extracts must contain quite different products of extraction (polysaccharides and phenolics), one can conclude that both polysaccharide and phenolic constituents of *Sphagnum* cells possess the

same antimicrobial activity. This is not surprising because in Painter's experiments, untreated *Sphagnum* moss, extractive-free *Sphagnum* moss, and *Sphagnum* holocellulose revealed almost the same antibacterial effect. Therefore, it seems that the preservative properties of *Sphagnum* moss and *Sphagnum* products are not much different from each other. This fact is important for current use of *Sphagnum* moss in field conservation and potential use of *Sphagnum* products in art conservation.

Painter's experiments with protein-containing materials (fish products) wrapped in *Sphagnum* moss can serve as a confirmation for the use of *Sphagnum* moss as a wrapping material for excavated artefacts. Protein materials were shown to be intact at least for two weeks in moss (Børsheim, Christensen, and Painter 2001a). This agrees with observations of Canadian Arctic archaeologists who noticed that artefacts packed in moss were intact for three weeks (see section 2.2.6). The disadvantage of this practice is the appearance of black staining on the surface of artefacts after prolonged (more than three weeks) contact of artefacts with moss.

Darkening of wrapped artefacts may be caused by the tanning reaction of *Sphagnum* phenolics with the material of artefacts, especially in the process of moss decay. It was noticed that when the stain spots appeared on contacted objects, the moss had already decayed. Since it is believed that the tanning effect depends on temperature and the length of time that the artefacts are packed, it is suggested that artefacts be kept wrapped for a limited time at decreased temperatures\*.

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\*The author of this work kept harvested *Sphagnum* moss in a refrigerator at 4°C for five months. All this time the appearance, smell, and colour of the moss did not change.

All *Sphagnum* products including delignified holocellulose, *Sphagnan*, and the products of *Sphagnum* water extraction (pectin-like polysaccharides and soluble phenolics) have a good potential in art conservation. The type of *Sphagnum* product to be selected, i.e. fresh or dry moss, moss extracts and their solutions, powders, etc. and the method of their application (additives, wrapping materials) depend on the specifics of the treated objects.

Water extraction is the simplest and cheapest way to obtain *Sphagnum* antimicrobial products. At the same time, water extracts contain phenolics that can cause darkening of treated artefacts. However, for certain archaeological materials such as archaeological wood, skin, or bone that are already darkened during burial in soil, the water extracts can be used. If needed, dried water extracts can be also dissolved in organic solvents (see Appendix). Phenol compounds contained in *Sphagnum* moss would be undesirable in products that come into the contact with certain groups of objects, e.g. textile or paper objects. These need to be treated with phenol-free *Sphagnum* polysaccharides (delignified holocellulose or *Sphagnan*).

Due to the equality in antimicrobial activity of holocellulose and *Sphagnan*, holocellulose can be substituted for *Sphagnan* in many cases. Holocellulose is much cheaper and more easily prepared than *Sphagnan* because the preparation of holocellulose does not require the long and labour intensive process of autohydrolysis required for the preparation of *Sphagnan*. The structure and properties of holocellulose are closely related to cellulose and therefore delignified *Sphagnum* holocellulose can be easily incorporated into cellulosic conservation products. For example, antimicrobial wrapping paper or cases for object storage shaped similar to egg cartons may be used. Ballance and Christensen proposed such a carton with the addition of 5% wt of moss holocellulose for preservation of fresh meat and fish products (Ballance and Christensen

2007). For these products, the authors also proposed *Sphagnum*-containing absorbent materials which can absorb meat and fish liquids. The presence of holocellulose in such absorbent materials may vary from 20 to 75% wt (ibid.). In particular cases, *Sphagnum* water extraction products or dry moss can be used instead of holocellulose.

*Sphagnan* dissolves in both water and organic solvents (see Appendix) and therefore it can be applied in both of these solution forms. Thus, *Sphagnan*- water solutions can be used for treatment of wet organic archaeological objects (skin, leather, wood) in cases when the presence of phenolics or solvents is undesirable. A *Sphagnan* - solvent solution (ethanol-toluene mixture) has been mixed with conservation waxes that considerably increased the bioresistance of those waxes (see section 5.4).

Despite their not very high antibiotic activity compared to standard biocides, *Sphagnum* products have many advantages for conservation practice: they are harmless to conservators and for artefacts; the raw material for their production is cheap and accessible; and they are easily manufactured. It is important for conservators that *Sphagnum* products cause a clear inhibiting effect on hyphomycetes (e.g. *Aspergillus* spp.), which are the most dangerous degrading organisms for museum collections and interiors. Besides the use of active *Sphagnum* ingredients, the possibility of introducing these ingredients to conservation materials should be explored. Recommendations for the application of these novel conservation materials to treated objects can be made after accurate testing of these materials on microbial cultures and on samples of real objects. Taking into account the positive properties of *Sphagnum* moss it can be concluded that *Sphagnum* moss and *Sphagnum* products may be recommended for further studies and consequent use in art conservation.

## Chapter 7. Summary and Conclusions.

In the present work, on the basis of well-known preservative properties of *Sphagnum* moss, a potential opportunity to use moss in art conservation was proved. The inhibiting activity of *Sphagnum* moss against fungi, the most dangerous “heritage eaters”, was demonstrated. In these experiments, polysaccharides, the active antimicrobial ingredients of *Sphagnum* moss were extracted and tested towards fungi typical for different heritage objects. The present work reports the results of these experiments. The clear inhibiting effect of *Sphagnum* extract encouraged the author to try the extract as an inhibitory additive to conservation wax. The results of these tests are also reported.

*Sphagnum* polysaccharide was extracted from the moss (*S. palustre* spp.) in the amount of 4.1% of the *Sphagnum* plant dry weight. All lignocelluloses were removed from this extract as a result of the treatment of the moss cellulose with sodium chlorite. The extracted polysaccharide possessed a strong acidic reaction (pH 2.8) and was soluble in water and organic solvents. *Sphagnum* extract also showed a strong inhibiting effect on the common bacterial pathogens *E. coli* and *P. aeruginosa*. The yield of the final product, its physical and chemical properties, and its antibacterial effect coincide with a polysaccharide (*Sphagnan*) that was isolated from *Sphagnum* moss by T.J. Painter and colleagues (NTNU). This proved that our extract was similar to *Sphagnan*.

The test fungi were isolated from ethnographic objects and archaeological objects originated from different geographical and climate zones and different soils (the Canadian Arctic and central Turkey). These artefacts were in different states of preservation, from the intact state

(ethnographic artefacts) to severely biodegraded state (artefacts from the drastically biocontaminated storage facility at Kaman- Kalehöyük, central Turkey). Samples covered a broad range of organic materials, including wood, bone, skin (caribou and seal), gut, and textile (silk and wool). During storage, the examined artefacts were exposed to different conditions of temperature and humidity: the Arctic artefacts and ethnographic objects were stored in a controlled museum climate; the artefacts from Kaman- Kalehöyük suffered from extremely high humidity and increased temperatures.

The primary criteria for the selection of fungi were the viability of fungal spores and prevalence of the given species on examined artefacts. According to the author's morphological examination, several typical species were present on the majority of samples regardless of origin, the state of preservation, and the storage conditions. The preponderant organisms among these typical species were fungi belonging to the *Aspergillus* genera. *Aspergillus* spp. presented on all examined artefacts and their spores showed the highest degree of viability.

The antifungal effect of our *Sphagnum* extract was tested on seventeen fungal isolates by the disk-diffusion method. Twelve of these isolates appeared susceptible to the extract. Most of these fungi belonged to the *Aspergillus* genera. The inhibiting effectiveness of the extract was tested on the most typical isolate by the modified broth-dilution method. In this experiment, in one ml of the nutritious broth, 40µl of 3% solution of polysaccharides in water killed 10,000 fungal spores (*Aspergillus* spp.) in 6 hours. The death of spores was shown to be not connected to the acidity or osmotic effect of *Sphagnum* polysaccharides.

As an example of the application of *Sphagnum* polysaccharides in art conservation, they were added as preservative agents to conservation waxes. In the present work, waxes were shown to

be quickly degraded by microorganisms. Thus, 20 µm films of microcrystalline wax that protected archaeological bronze artefacts were completely degraded in three weeks by a consortium of microorganisms (bacteria and fungi). Pure fungal cultures (*Aspergillus* spp.) exposed to microcrystalline wax consumed 44% of wax samples at the same time. When, however, ~ 0.1% (w/w) of *Sphagnum* was mixed with wax, the weight loss of wax was only 4% in the same time interval.

The results of the present work can be generalized as follows:

- (a) A polysaccharide with antimicrobial properties was extracted from *Sphagnum* moss. Physical and chemical properties of this polysaccharide, as well as its experimentally demonstrated antibacterial activity, proved that the extracted polysaccharide was *Sphagnum*.
- (b) Fungi typical for a number of heritage objects were isolated and identified morphologically as *Aspergillus* spp. They are also the most common and potentially the most dangerous microorganisms in museum environments. These fungi appeared to be the same on artefacts made from different materials, originating from different areas, and stored under different conditions (temperature, humidity, microbial contamination).
- (c) In the present work, a rapid degradation of conservation waxes by *Aspergillus* spp. was experimentally confirmed.
- (d) The strong inhibiting effect of *Sphagnum* polysaccharide on fungal spores was experimentally proved. Fungi were susceptible even to quite low concentrations of polysaccharide. The maximal fungicidal effect of polysaccharide was established on the selected fungal culture (*Aspergillus* spp.). The antifungal effectiveness for *Sphagnum*

polysaccharides appeared to be comparable to the effectiveness of another polysaccharide, *Chitosan*, which has already been used for the conservation of paper documents.

- (e) Adding *Sphagnum* polysaccharide to microcrystalline wax decreased the rate of fungal degradation of microcrystalline wax by 10 times.
- (f) On the basis of this study it was concluded that *Sphagnum* moss and *Sphagnum* products can be recommended for use in art conservation as antifungal agents.

Due to their solubility in water and organic solvents, *Sphagnum* polysaccharides can be used as antibiotic additives in a broad range of conservation materials. Forms of *Sphagnum* products can include powders, pastes, and solutions in water and in organic solvents. *Sphagnum* products can be used as additives to conservation polymers, for example, to conservation waxes. Since *Sphagnum* polysaccharides are related to cellulose, they can be introduced in the form of *Sphagnum* holocellulose into conservation cellulosic materials such as wrapping paper or cartons. The method of application of *Sphagnum* products to heritage objects is a subject for further research.

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## APPENDIX

**A number of fungal colonies grown from spores treated with Extract 1, the Water Extract, maltose, and glucose (row counting data for the tube test).**

t, hours	N <sub>o</sub> =200 spores/plate		N <sub>o</sub> = 500 spores/plate		N <sub>o</sub> = 1000 spores/plate	
	Broth	Broth+Ext.1	Broth	Broth+Ext.1	Broth	Broth+Ext.1
3	16	25	31	44	70	80
	17	34	18	23	90	57
	15	15	26	31	69	55
5	14	7	17	0	54	0
	16	2	27	0	34	0
	16	2	30	0	34	0
8	14	1	54	0	67	0
	29	0	53	0	49	0
	17	0	21	0	32	0
12	20	0	27	0	45	0
	18	0	23	0	44	0
	19	0	20	0	47	0
16	12	0	50	0	35	0
	22	0	34	0	51	0
	15	0	25	0	54	0
18	19	0	31	0	22	5
	15	0	-	0	38	0
	13	0	29	0	33	0
21	11	0	14	0	53	0
	9	0	15	0	19	0
	6	0	40	0	12	0
24	11	0	17	0	32	0
	9	0	14	0	36	0
	25	0	21	0	36	0

**Table 1.** The number of colonies grown from spores exposed to *Sphagnum* Extract 1 for 3 - 24 hours. The numbers of plated spores of N<sub>o</sub> = 200, 500 and 1000 spores/plate. The number of grown colonies is an average for nine plates.

t, hours	N <sub>o</sub> =200 spores/plate		N <sub>o</sub> = 500 spores/plate		N <sub>o</sub> = 1000 spores/plate	
	Broth	Broth+W.Ex	Broth	Broth+W.Ex	Broth	Broth+W.Ex
5	73	55	100	21	288	20
	26	102	111	30	377	24
	55	52	160	22	360	16
8	100	11	178	12	295	17
	110	29	232	15	239	13
	164	10	140	16	225	29
12	160	0	233	0	221	6
	171	0	220	0	329	6
16	63	0	102	0	108	8
	58	0	106	0	99	1

**Table 2.** The number of colonies grown from spores exposed to the Water Extract for 5 - 16 hours. The numbers of plated spores of N<sub>o</sub> = 200, 500 and 1000 spores/plate. The number of grown colonies is an average for nine plates.

t, hours	N <sub>o</sub> =200 spores/plate		N <sub>o</sub> =500 spores/plate		N <sub>o</sub> =200 spores/plate		N <sub>o</sub> =500 spores/plate	
	Broth	Broth+ Maltose	Broth	Broth+ Maltose	Broth	Broth+ Glucose	Broth	Broth+ Glucose
3	260	160	618	440	210	260	505	560
	251	197	510	512	242	210	481	600
	262	181	582	542	255	220	600	512
5	227	229	610	560	211	210	470	570
	257	239	560	532	216	280	445	582
	239	260	512	518	258	218	445	516
8	261	229	481	561	200	191	440	580
	201	241	511	580	230	171	462	541
	218	260	497	611	202	181	475	561
12	221	232	501	560	232	260	560	562
	248	248	469	532	213	280	481	549
	250	211	518	580	221	231	463	520
16	200	260	568	560	213	282	548	521
	197	271	460	511	251	231	542	528
	181	262	491	541	228	211	564	498
18	197	210	541	560	172	210	504	540
	211	260	550	562	198	202	521	532
	231	251	510	568	214	216	510	548
21	111	272	501	405	171	242	518	401
	201	230	492	202	191	212	522	312
	262	240	540	218	231	227	547	461

**Table 3.** The number of colonies grown from spores exposed to maltose and glucose for 3 – 24 hours. The numbers of plated spores of N<sub>o</sub> = 200 and 500 spores/plate. The number of grown colonies is an average for nine plates.

## Physical and chemical properties of *Sphagnum* extracts

### *Extract 1 and Extract 2*

Extract 1 is a light brown colored solid with an amorphous structure and a strong unpleasant smell. The volume weight of extract is 0.023g/cm<sup>3</sup>. The particles of extract are electrically charged. They scatter over metal and plastic surfaces and are strongly attracted to them. Extract 1 can be suspended in water forming a colloid solution.

The solubility of Extract 1 in water at room temperature is 0.033 g/ ml i.e. 1 g of solute is dissolved in ~30 g of solvent. Such a product can be considered to be on the edge between soluble and hardly soluble substances (Leeuwen and Vermeire 2007). The solution is acidic with pH 2.8. The boiling point of a solution of 11g of Extract 1 in 1.5 l of water is 98° C i.e. lower than the boiling point of pure water.

Extract 1 is soluble in alcohol and in a 1:1(w/w) alcohol-water mixture. The solubility of Extract 1 in alcohol is 0.028 g/ml that is close to its solubility in water. The solubility of Extract 1 in 1:1 (w/w) alcohol-water mixture is the same as in pure water, i.e., 0.033 g/ml. Extract 1 is also soluble in organic solvents such as acetone, toluene and light oil (vegetable oil), but its solubility in solvents is lower than in pure water. The appearance, smell, and physical and chemical properties of Extract 2 do not differ from those of Extract 1.

### ***Water extracts***

The cold water extract is a dark brown colored amorphous substance with light weight and strong unpleasant odor. It is more acidic than Extract 1 and 2 with pH 2.4. The volume weight of the cold water extract is 0.014g/cm<sup>3</sup>, i.e., less than Extract 1. Like Extract 1, the cold water extract forms in water a colloidal solution with a solubility of 0.029 g/ml at room temperature. It also dissolves in alcohol, acetone, toluene, and vegetable oil. Like Extract 1, the cold water extract is electrically charged. The color, smell, and the physical and chemical properties of the hot water extract were not different from the cold water extract.

The boiling water extract was a light brown colored solid with unpleasant odor as well.

However, this odor was not so strong as in the case of cold and hot water extracts (aromatics were partly evaporated). The boiling water extract was less acidic (pH=3.4) than the cold and the hot water extracts. Other physical and chemical properties of this extract were not different from the cold and the hot water extracts.

### **The results of FTIR analysis of *Sphagnum* extracts**

The infrared spectra of *Sphagnum* Extract 1 and Extract 2 (called extract II-2) and their description were provided by Dr. H.F. Shurvell. Moss holocellulose and dried moss were analyzed as well. The resume of this analysis is shown below.

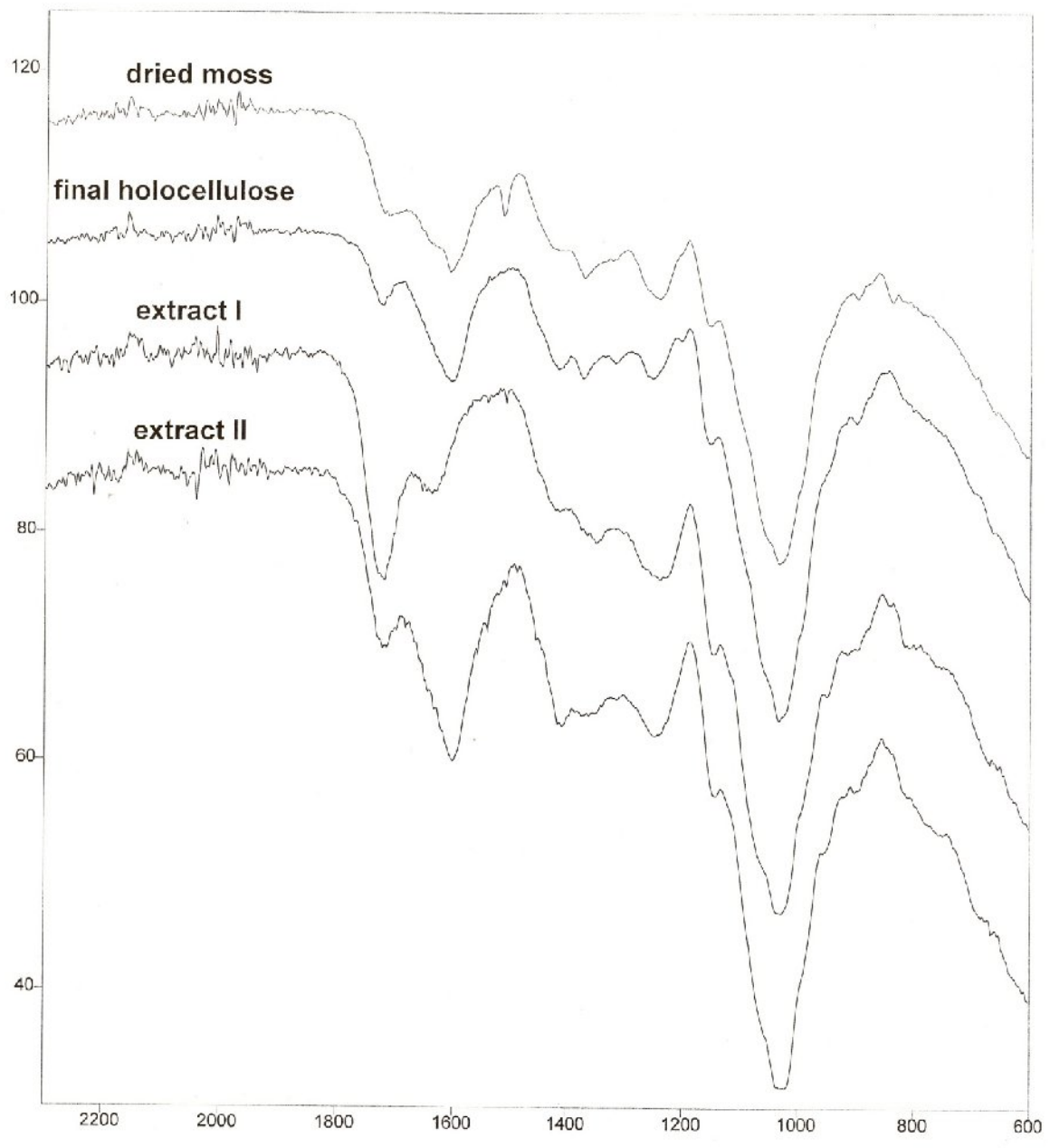
#### **Infrared spectra of final holocellulose and extracted material**

##### **Final holocellulose and extract II-2**

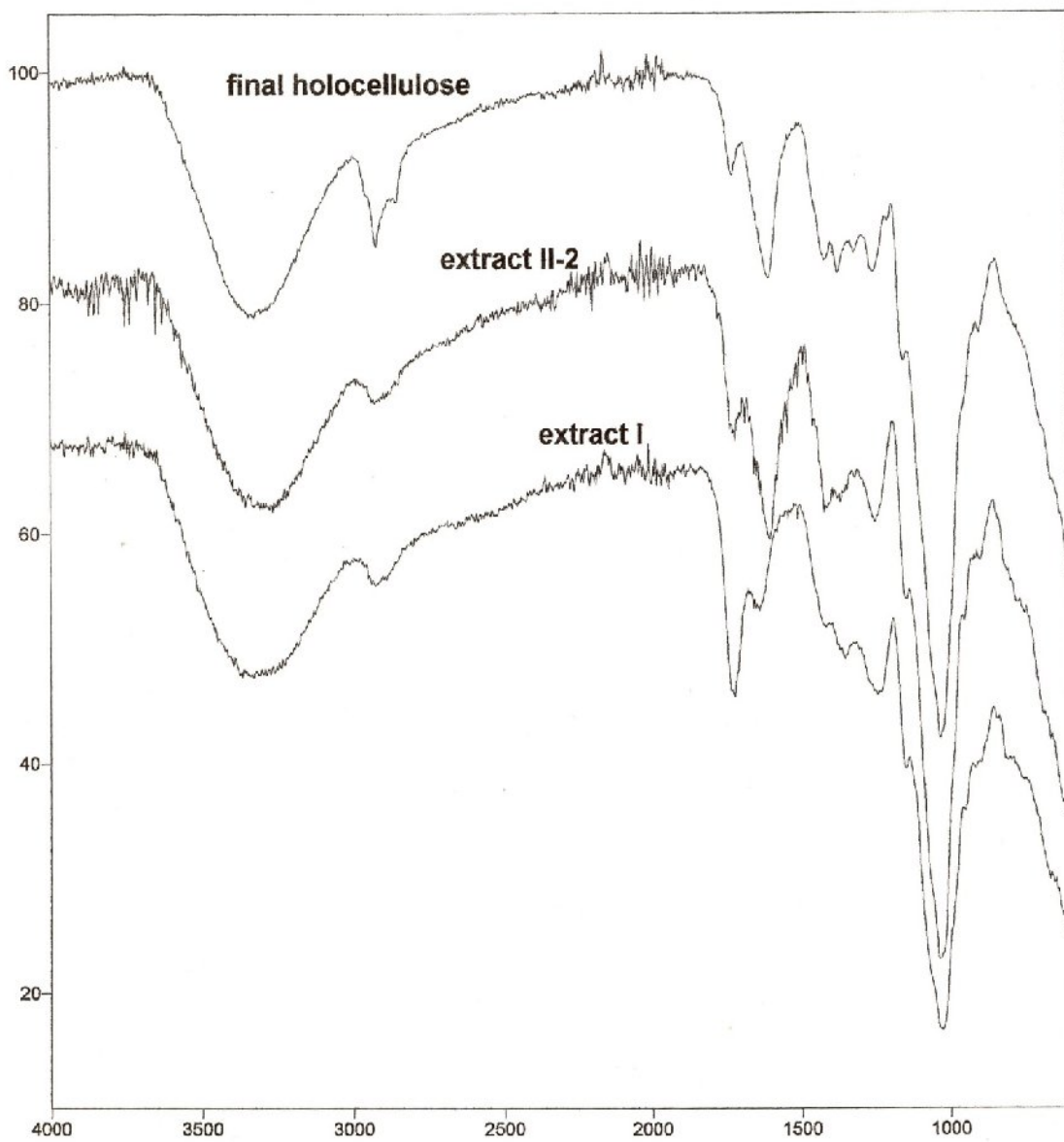
The spectra of final holocellulose and extract II-2 are very similar in the 1750 – 1550  $\text{cm}^{-1}$  region, but in the CH stretching region, the spectra differ. There is a sharp peak at 2921  $\text{cm}^{-1}$  with shoulders at 2952 and 2855  $\text{cm}^{-1}$  in the spectrum of final holocellulose, but in the extract II-2 (and extract I) spectrum the band is weak and broad with a maximum at  $\sim 2925 \text{ cm}^{-1}$  and a shoulder at  $\sim 2885 \text{ cm}^{-1}$ . Small differences in relative peak positions and intensities are seen between 1500 and 1200  $\text{cm}^{-1}$  and in the structure of the very strong C-OH stretching band centred at 1030  $\text{cm}^{-1}$ . A shoulder at 950  $\text{cm}^{-1}$  in the final holocellulose spectrum becomes a weak peak in the extract II-2 (and the extract I) spectrum.

##### **Extract I**

The spectrum of extract I differs from the other two in the 1750 - 1550  $\text{cm}^{-1}$  region. Only one fairly strong C=O stretching band is observed at 1722  $\text{cm}^{-1}$  (the band at 1638 is due mainly to water). Some other small differences are seen in the spectrum. For example the intensities of the 1408 and 1364 (shoulder)  $\text{cm}^{-1}$  peaks in the extract II-2 spectrum are reversed and shifted to 1412 (shoulder) and 1350  $\text{cm}^{-1}$  in the spectrum of extract I.



**Transmission / Wavenumber (cm-1)**



Transmission / Wavenumber (cm-1)

## LIST OF ORIGINAL PUBLICATIONS

The section 2.3. “Biodegradation of conservation waxes” and the Chapter 5 “*Sphagnum* as an additive to conservation waxes” is based on the following articles published by the author of this thesis:

Zaitseva, N. 2005a. Biodegradation of wax coating on copper alloy artifacts treated with Benzotriazole (BTA). In: *Kaman Kalehöyük 14*. Anatolian Archaeological Studies, Japan, V.14, pp.195-201.

Zaitseva, N. 2005b. Biodegradation of microcrystalline waxes. In: *Kaman Kalehöyük 14*. Anatolian Archaeological Studies, Japan, V.14, pp.203-210.

Zaitseva, N. 2006. Inhibiting effect of *Sphagnum* moss extract and benzotriazole (BTA) on conservation waxes fungal degradation. In: *Kaman Kalehöyük 15*. Anatolian Archaeological Studies, Japan, V.15, pp.269-276.