Chapter 8

Conclusions

(by the whole team)

In this chapter general trends, which emerged from the results of the field as well as from various laboratory tests are discussed in a wider sense and conclusions are drawn. Seven parameters (oxygen, sulphur, nitrogen, peat, ground water, hydrology and variety in species) play an important role in the process of bacterial wood degradation. Furthermore practical translations of the results towards real future preservation methods are discussed.

8.1. Identification of bacteria

Erosion bacteria are present everywhere

Literature and experience at SLU suggest that erosion bacteria are present in most terrestrial and aquatic environments. They have often been isolated from water samples, suggesting that they constitute a part of the microbial plankton. Isolations from quite varying environments and culturing experiments suggest that erosion bacteria, as a group, have a very wide ecological amplitude.

Many species of bacteria can cause wood decay

Molecular biology studies and culturing points strongly to the CFB complex (*Cytophaga, Flavobacterium, Bacteroides* and related). Morphology also supports this view.
Evidence suggests that a number of related erosion bacteria species might occur in one wood sample. Sequence types that matched other groups of bacteria were also commonly found in wood samples (*Pseudomonas, Cellvibrio* and *Brevundimonas*) but these bacteria have not as yet been successfully cultured or observed to degrade wood *in situ*.

Wood-degrading bacteria are novel species, so no information on ecology and physiology

Phylogenetic analysis of environmental sequence data suggests that these bacteria are novel as they have not been previously characterised molecularly. Further general information may be gained from what is currently known of bacteria in the CFB complex.

The successful isolation of erosion bacteria suggests that they are widespread in natural environments. This is supported by numerous reports on erosion bacteria attack in diverse environments. A more intense sampling of the BACPOLES wood samples would probably have resulted in isolations from almost all samples. Now, erosion bacteria were isolated from 84 % of all BACPOLES samples, comprising archaeological wood and wooden pilings, which suggests ongoing decay at 72 % of all sites. At SLU erosion bacteria have also been isolated from water samples.

The rod shape and the gliding ability, plus the fact that they can degrade a complex organic substrate, lignocellulose, suggest that erosion bacteria may be part of the CFB complex (*Cytophaga*- *Flavobacterium*- *Bacteroides*). This is supported by molecular analyses. Sequence types representing bacteria from the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex were commonly recovered from environmental samples where degradation had occurred. These sequence types also matched those from cultured isolates. The presence of CFB bacteria in BACPOLES wood samples and cultures has also been indicated using FISH (fluorescent *in situ* hybridisation) techniques with probes designed specifically for CFB bacteria.
Bacteria in the CFB complex are known to be abundant in very diverse environments including anoxic sediments (Borneman et al. 1996, Cottrell, M.T and Kirchman, D.L 2000, Crump et al. 1999, Eilers et al. 2001, Kirchman 2002, Reichenbach 1992, Rosselló-Mora et al. 1999, Tanner et al. 2000). Cellulolytic members of this complex are known to degrade cellulosic substrates by binding to the substrate, oriented along the cellulose microfibrils (Stanier 1942) in a similar manner as was observed here during attack on wood and kapok fibres. Cellulases of bacteria in the CFB complex are known to be strongly bound to the surface of the bacteria. Thus, clearing zones are generally not produced in cellulose agar (Coughlan and Mayer 1992). In contrast to cellulolytic bacteria, erosion bacteria are capable of degrading lignified cellulose, such as pine and spruce wood and kapok fibres. There is no information on enzyme or other systems that enable erosion bacteria to overcome the hindering effects of lignin.

The fact that the BACPOLES erosion bacteria require reduced oxygen levels and appear to degrade wood and kapok under anaerobic conditions, suggest that the bacteria are facultative anaerobes (microaerophilic facultative anaerobes?). This is in line with the observations that attack in nature may occur in the absence of oxygen (Chapter 2). Attack on wood or kapok fibres has only been observed in the presence of other bacteria. Their role is not clear. They may help in producing reduced levels of oxygen, but may also produce metabolites required by erosion bacteria. Culturing experiments demonstrate that erosion bacteria are unable of sulfate-reducing activity.

Isolation of erosion bacteria from quite varying environments and the culturing experiments suggest that these bacteria, as a group, have a very wide ecological amplitude. Molecular biology analyses showed high variability in the number of bacterial taxa found in different samples, but a few bacteria groups and the CFB complex were however common across many samples (Chapter 3.2). It is recognised that not all bacterial taxa present are erosion bacteria, however, morphological studies and culturing experiments suggest that the number of individual species of erosion bacteria is quite large. This implies that phage diversity can be expected to be high. Most species isolated within the BACPOLES project are likely to be novel. Thus, there is no information on their physiology.

Erosion bacteria start to attack solid wood from the surface. The attack then proceeds inwards. In softwoods, rays have been observed to be preferred pathways, where cross-field pits provide access to the axial tracheids. The ability of erosion bacteria to move through gliding facilitates the invasion of the wood cells. The highly variable decay rates observed for BACPOLES pilings, is most likely related to variation in decay capacity of individual species of erosion bacteria, presence of other bacteria and the environmental conditions. High levels of hydrogen sulfide generated by sulfate-reducing bacteria may slow down the attack. There is some evidence for that in the microcosm experiments (Chapter 4). Environments, where conditions fluctuate, leading to cycling of various elements may lead to higher decay rates, compared with environments where stable conditions dominate.

8.2 Soil parameters

8.2.1 Oxygen

Based on our field and laboratory measurements, wood degrading bacteria have to be regarded as facultative anaerobic. For successful inoculation of sound wood samples with these bacteria small amounts of oxygen are necessary at the beginning of the culturing. Furthermore at the tips of foundations piles (> 6 m below ground water level) bacterial degradation was often observed and thus the degradation must have continued under anaerobic conditions in situ as the surrounding ground has to be regarded as an anoxic environment. Not only deep under the ground water level but actually for all fully submerged foundation piles and (marine) archaeological remains the surroundings of the wood are regarded as mostly anoxic.

8.2.2. Nitrogen

The degree of bacterial wood degradation positively correlates with wood nitrogen concentrations. However data do not allow separating between nitrogen in the wood matrix and nitrogen incorporated in bacteria. Therefore it is suggested that the bacteria accumulate nitrogen during the time they are actively degrading the wood.

Both the microcosm experiments and the long-term measurements indicate that bacterial wood degradation is less in surroundings with high nitrogen concentrations. Also the suitable media for culturing (isolating) of wood decaying bacteria were mostly nutrient poor. It is therefore suggested that wood degrading erosion bacteria are adapted to low nitrogen concentrations.

However at the 27 sampling sites and at the long-term measurement sites bacterial wood decay was not related to sediment total nitrogen concentration. Therefore not the total concentration but the availability of nitrogen is regarded as important factor for the bacterial wood degradation process. Line (1997) isolated nitrogen-fixating bacteria that were associated with bacterial wood decay. This indicates the significance of nitrogen in the process of bacterial wood degradation.

8.2.3. Sulphur
The quite frequent observation of pyrite in bacterially degraded wood in both piles and archaeological fragments (Klaassen and Nilsson, pers. comm) indicates that the environment inside the wood has become sulfidic (i.e. anoxic) and shows that sulfate was available inside the wood. Assuming that erosion bacteria were active during the formation of the pyrite, then erosion bacteria should be at least facultative anaerobes, as oxygen is not present in sulfidic environments.
The formation of sulfides like H_2S (or HS^-) and FeS_x during wood decay by erosion bacteria - as observed in chapter 3 - indicates that erosion bacteria facilitate the activity of sulfur reducing bacteria; probably by providing CH_4 or other short-chain organic molecules. Erosion bacteria could function as the basis of the food chain of large bacterial communities. It also strengthens the suggestion that erosion bacteria are (at least facultative) anaerobes. There is not enough information to make a correlation between the degree of attack by erosion bacteria and the S-content in the wood.
The potential toxicity of sulfides towards erosion bacteria may be the basis for a potential method of inhibition of degradation; injection of sulfate in wood would potentially reduce the speed of erosion bacteria degradation. However, it would not be able to completely halt this degradation mechanism.

8.2.4 The role of peat type and ground water
Based on the observations around Dutch foundation piles it is assumed that bacterial wood degradation is stimulated when:
1) the free nutrients in the peat increases;
2) the water quality is changing from brackish or salty towards fresh;
3) there is an increase differenced in pressure between the shallow and deeper ground water.

8.3. The relevance of water flow through the wood
From the sites where foundation piles were investigated it became clear that pine sapwood was always degraded over its full diameter whereas the degradation in spruce piles was always restricted to the outermost layer. The Amsterdam site proved this observation because pine and spruce piles from the same location also show these differences in degradation.
Furthermore the degree of degradation seemed to be related to the locality where the species were used as pilings. Based on these two dependencies it was suggested that wood types, which have a higher resistance against water transport, are less vulnerable to bacterial degradation in areas with a dynamic hydrology.
Laboratory tests suggest that without additional pressure no water transport was possible in spruce over a length of 30 cm whereas the resistance of pine sapwood was low and water transport over the whole length is suggested. The water transport seems to be restricted to the axial direction and radial water movement is thought to be based on diffusion only. The water movement by diffusion is estimated to be 10 to 100 times slower than the transport through the axial cells.
Groundwater pressure measurements show that – over a longer time – there is no unidirectional gradient in ground water pressure between the upper soil layers, including the pile heads and the lower soil layers, including the pile tips in areas with severe degradation. However, it is suggested that the hydrology is much more dynamic in areas with severe degradation than in areas with no degradation.
It is therefore hypothesised that the dynamic of the hydrology is only reflected in relative open timber structures resulting in a continuous water circulation through the piles causing

the mixture of micro-organisms, debris and nutrients necessary for bacterial degradation activity.

8.4. Bacteriophages

Successful isolation and identification of bacteria are prerequisites for specific phage isolation and hence for the development of a phage-based wood preservative. The elusive wood degrading bacteria appeared difficult to isolate and cultivate in the laboratory environment. Due to these difficulties the objective to develop a phage-based wood preservative was delayed.

Phage isolation procedure and phage techniques from the microcosm experiments proved successful. Phages were isolated from several samples.

Phagen isolated a large number of various bacteria from the Bacpoles wood samples. The bacteria may now be employed in the 16S rRNA assays used in DNA analyses to assess the identity of living organisms in the samples. Indeed some of the bacteria were identified as members of the Cytophaga-Flavobacterium-Bacteroides group. All isolated bacteria constitute potential reservoirs of suitable phages to be isolated in future work and to be used as phage-based preservatives once the wood degrading bacteria have been identified on species level.

A great asset for a future phage-based wood preservative is that many types of phages appear extremely resistant to degradation and they are successful survivors existing in both intra- and extracellular states. However, there are many practical considerations that have to be met.

- Although it seems to be that on one location we have to deal with one bacterial species but if more species co-operate independent from each other for each of the wood degrading bacteria strains a specific type of phages has to be produced. It is quite clear that in different environments different bacteria species are active and therefore different types of phages have to be produced.
- For each of the conditions to treat the wood objects with a phage based preservative, a specific application treatment as well as the lifetime of the phages have to be investigated. Different environments can be, terrestrial archaeological, marine (fresh, salt water) objects and foundation piles.
- Any effects on the environment should be limited in space and duration. The high bacterial host specificity and the biological nature of the phage will most likely be factors that make a phage preservative meet these requirements.
- Bacteria are good survivors with well-known capability to mutate, i.e., they may transform to phage-resistant forms. Wood degrading bacteria, however, evolved early and are therefore expected to have a fairly slow mutation rate. In addition, once the bacteria are identified new phages would be found easily and can be developed in the laboratory into long-lived varieties by means of selection and modern molecular biology techniques. Phages may also mutate to overcome phage-resistant mutants among the bacteria.

In light of the new knowledge and new methods developed by the Bacpoles project, it is believed that a successful commercial phage-based wood preservative is within reach within the next 3-4 years. A possible preservation scenario should start with an investigation of the soil conditions and the wood degrading bacterial flora, using techniques as developed in BACPOLES. Based on the results a phage-cocktail is produced and a strategy determined in order to bring in the preservative. Under specific conditions this could be a pipe system which can be easily installed just above the foundation pile heads. We estimate that approximately four litre cocktail solution with suitable bacteriophages / m^2 is needs to be brought into the soil within one month. The results should be that the degradation should be strongly diminished for at least a period of 10 years.

8.5 Possible preservation or conservation strategies

The actual information on chemical-based preservatives is mainly related to fungal wood degradation, and their efficiency against bacterial degradation was never reliably proven. As it has to be used under water saturated conditions, water-soluble non-fixating products are not suited. Moreover, a possible preservative should fit within the strict regulations for soil and groundwater minimum toxic levels. Therefore toxic water-soluble preservatives are not regarded as realistic to use against bacterial wood decay.

There are three promising approaches defined and each of them starts with a full description of the area to be treated. The site hydrology as well as the identification of the bacteria consortium, which causes bacterial degradation, is most important.

Based on these inquiries specific mixtures of phages can be made. However field tests have to be carried out in order to get more knowledge whether a mono-phage-preservative should be used which is effective against the present bacteria consortium only, or whether it is possible to prepare a mixed-phage-preservative which is effective on a wider range of locations.

A second approach is related to the hydrology. It became clear that bacterial wood degradation is active only when there is a water flux in the wood. In order to create a static situation, either the hydrology can be manipulated or the wood structure can be closed by impregnation in the field. Probably a combination of both strategies is most efficient and could improve in addition the strength of the wood.

A third approach is based on a non-toxic active product, which affects the already weak competition position of wood degrading bacteria by promoting others. The result of this treatment should be that the number of wood degrading bacteria is diminished over longer time.

8.6 Future work and missing knowledge

Although populations of erosion bacteria in water-saturated wood make up a wide variety of species, the emergence of the CFB (*Cytophaga-Flavobacterium-Bacteroides)* group as an important component of the micro-flora requires further investigation for definite identities of pure cultures. Use of FISH (fluorescent in-situ hybridisation) techniques will pinpoint known bacterial types within a consortium. This will also provide a starting point for understanding the ecology and physiology of these bacteria. Particularly the conditions suitable for demonstrating attack on wood and kapok fibres, their carbon and nitrogen requirements, their respiration/fermentation and their response to different levels of oxygen, carbon dioxide and hydrogen sulphide, plus the effects of pH and temperature on their activity have to be further investigated. Such investigations will be tied to studies on cellulases, hemicellulases and ligninase enzymes produced by these bacteria in order to identify the optimum conditions for decay. At the same time the longevity and ecology of bacteriophages specific to isolated bacterial strains needs to be determined in natural environments.

One of the important results arising from the present work has been our improved understanding of water flow within wood. This has particular relevance to the movement of virus particles and bacterial cells in wood and raises questions about the need for a better understanding of the dynamics of water pressure in the ground, the permeability of different soil layers, the velocity of ground water flow and soil water analyses along the whole length of piles. This latter aspect of work to be done has special significance with respect to nitrogen availability and its importance in wood decay by erosion bacteria bearing in mind the limited amount of nitrogen naturally present in wood. Future work requires the establishment of field trials alongside laboratory experimentation. In the case of nitrogen, the use of ^{15}N to monitor uptake into bacteria in laboratory microcosms and the use of radioactively spiked wood to monitor uptake are just two avenues of investigation. Physiological studies of this type will determine the value of changing environmental conditions in order to inhibit/control the activity of erosion bacteria by using for instance 'lime-milk' to increase pH. Similar studies using laboratory microcosms can also be set up under very low oxygen tensions to determine the effect on erosion bacterial activity, or to measure the effect of selected phages on bacteria artificially introduced into wood samples.

In the present study, the initial base line study identified 27 sites for sampling in 6 different countries. Future fieldwork would limit the number of sites to a maximum of 4, all in the Netherlands, where full-size piles can be regularly monitored and checked. The number of timber species will be restricted to one – Scots pine, allowing a much fuller investigation of the factors influencing the rates of invasion, settlement and decay of whole wood samples (sapwood and heartwood) by erosion bacteria. All field experiments will be reproduced in the laboratory using laboratory scale wood samples.