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EUROPEAN COMMISSION RESEARCH - DIRECTORATE-GENERAL
Directorate I - Preserving the Ecosystem: Environmental Research
"City of Tomorrow and Cultural Heritage"

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Picture made by Ing. Bureau Rotterdam.

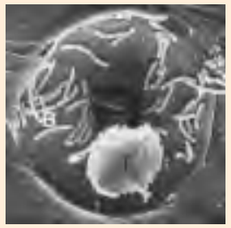
Preserving cultural heritage by preventing bacterial decay of wood in foundation piles and archaeological sites

BACPOLES

An EC funded program (Energy, Environment and Sustainable Development - "The City of Tomorrow and Cultural Heritage") EVK4-CT-2001-00043

PERIOD: 2002 – 2005

Cross section of Pine with bacterial degradation (Liese 1950).



Bacteria in a bordered pit of a pine tracheid (Peek & Leise 1979).

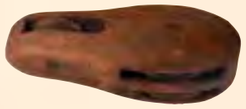
Submerged wood was previously considered safe from decay due to low oxygen content under water. About 3 decades ago it became clear that bacteria can cause degradation in such wood. Degradation under waterlogged conditions is a slow process and for wooden piles under monumentale buildings or for archaeological wood the exposure time is typically very long which makes this cultural heritage vulnerable for degradation by bacteria.

WOOD DEGRADING BACTERIA IDENTIFICATION

Little is known about the process of bacterial degradation and until now it has not been possible to isolate or identify any of the wood attacking bacteria. One main target that is crucial for the project is the identification of the bacteria by an innovative approach of new isolation techniques in combination with molecular DNA technology.

IN SITU MEASUREMENTS

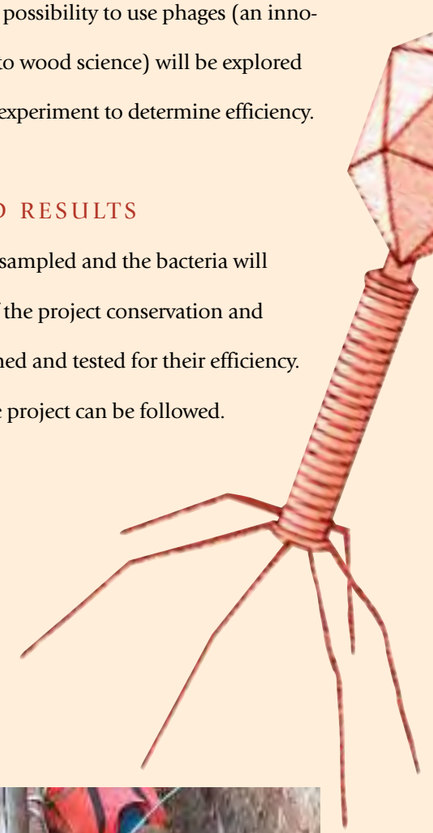
The second target is to asses the relationship between environmental conditions and wood quality in foundation piles as well as archaeological wood from marine and terrestrial sites. Equipment for measuring oxygen at very low concentrations will be developed and used at all sampling sites. Laboratory trials will be used to identify the key factors of bacterial degradation of wood. Foundation piles on more than 10 locations all around Europe will be sampled or fully extracted. Also European archaeological terrestrial sites and ship wrecks will be sampled. All sample site conditions will be correlated with the degradation of the wood and the identified bacteria. On the base of these results protection strategies will be developed based on the ecology of the bacteria and the possibility to use phages (an innovative medical technique but new to wood science) will be explored and tested in a running laboratory experiment to determine efficiency.



An oak clamp for holding a rope of the Stora Sophia, a Danish admiralty ship sunk outside Gothenburg, Sweden 1645.

TIME SCHEDULE AND RESULTS

In the first 1.5 year all sites will be sampled and the bacteria will be identified. In the second half of the project conservation and preservation strategies will be defined and tested for their efficiency. On the web-site the progress of the project can be followed.



Site showing position of piles from a medieval house. Only the outlines of the piles can be seen in the sandy soil, no wood is preserved.

Medieval foundation pile in sandy soil. Most of it has disappeared because of degradation processes in the soil.

Excavated foundation piles in Amsterdam.

Foundation inspection pit in Haarlem (the Netherlands), piles are measured and sampled.

Preserving cultural heritage by preventing bacterial decay of wood in foundation piles and archaeological sites

Final report

EVK4-CT-2001-00043

Preserving cultural heritage by preventing bacterial decay of wood in foundation poles and archaeological sites
EVK4-CT-2001-00043

Bacpoles

Final report
(with eight appendices)

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Wageningen 2005

Front cover: (picture left: foundation piles under family house (1937) Koog aan de Zaan, the Netherlands; upper picture middle row: foundation piles under Ponte Balbi, Venice, 16th century, Italy; middle picture middle row: foundation Medieval Castle, Travenhorst, Germany; lower picture middle row: Roman ship and embankment piles, Leidsche Rijn, the Netherlands; picture right: foundations under Parliament Stockholm, 1894, Sweden

A project for the Fifth Framework. Thematic programme IV: Energy, environment and sustainable development; Key action 4: City of tomorrow and Cultural heritage, Sub action 4.2.1: Improved damage assessment on cultural heritage

Summary

From February 2002 to January 2005, the European Commission funded a scientific project with the title “Preserving cultural heritage by preventing bacterial decay of wood in foundation piles and archaeological sites”, EU number “ EVK4-2001-00043” with the acronym or work name “BACPOLES “.

For a long time it was believed that storage in water or use below the ground water table prevents wood from biological degradation. However in the 1990ies serious problems with Dutch wooden foundation piles, were caused by bacteria. This shows that bacterial wood degradation should be regarded as a serious problem when dealing with wooden constructions under water and that even wooden objects other than foundations could be affected. In contrast to fungal degradation where the economical impact was realised a long time ago, research on bacterial degradation had until now low priority and therefore little knowledge was available on the process.

Objectives
One of the main objectives of BACPOLES was to provide basic knowledge on the impact of bacterial degradation on wood stored under different environmental conditions like foundations and archaeological wooden remains. Another issue was to protect wood under water against bacterial decay and to develop practical preservation methods for wooden foundations. In the last decades, scientists from all over the world did not succeed in identifying wood degrading bacteria. Yet, to understand the process of degradation the identification is crucial. Hence, the isolation and identification of wood attacking bacteria was one of the main challenges of the project.

Contributions
Experts on wood and soil sciences as well as microbiologists, archaeologists and geophysicists from Germany, Great Britain, Italy, The Netherlands, and Sweden collaborated in this project. The universities and research institutes concentrated on fundamental research whereas the companies were responsible for the in-situ investigation of wooden foundations and the incorporation of new technologies for wood preservation into existing techniques.

Methods
In all five collaborating European countries inquiries were made on the impact of bacterial degradation on wood stored in sediment. Based on scientific and local reports as well as on interviews with archaeologists and wood constructors it has to be concluded that bacterial wood degradation is not recognised as an important threat to wood stored in sediments, in 3 of these 5 countries. Mainly in the Netherlands the importance of this type of wood degradation is realised.
A standard procedure was developed to study the impact of bacterial degradation in different environments. Research was carried out at 27 sites in six European counties. There were 13 piling sites, of which two were older than 250 years, five marine sites and 9 archaeological sites. It was assured that bacterial wood degradation could be expected. At all sites wood, soil, and water samples were taken and analysed and an environmental characterisation was made based on visual observations and field measurements. During the project it became evident that seasonal dynamics could be of importance and therefore two long-term-field-measurements were carried out additionally throughout a period of 12 months.
Wood samples were taken to isolate and identify wood degrading bacteria. Because no standard techniques were available, the main effort of the microbiologists was the development of new methods.
Already in the beginning of the project microcosms were installed to simulate bacterial wood degradation in the laboratory to learn more about the process of degradation by manipulating the sediment conditions.

Results

The results from the 27 sampled sites indicate no justification to ignore the impact of bacterial wood degradation for piling constructions and archaeological remains. The concern for wood foundation as appeared in the Netherlands and a little bit in Sweden, should also be aware in the rest of Europe. Especially in Venice the role of bacterial wood degradation is considerably underestimated.

With new techniques it was possible to isolate consortia of bacteria which cause wood degradation. It became obvious that there is a wide variety in novel species and that these species belong to the CFB (*Cytophaga-Flavobacterium-Bacteroides*) complex. The bacteria are mobile by gliding. The shape of wood degrading bacteria varies, they can be short and thick as well as long and thin rods, but they can also be round. Each of the wood degrading bacteria species seems to have its own environmental amplitude, suggesting that bacterial wood degradation occurs under a wide range of soil conditions which was actually confirmed by the presence of bacterial wood degradation in all sampled sites. Additional research showed that wood degrading bacteria are present in all watery environments. Consequently, the infection (always from the outside inwards) with wood degrading bacteria is not the important factor, but the intensity of the degradation. The characteristic factor for wood degrading bacteria is that they can live or even need to live in environments with low nitrogen and oxygen contents. The natural nitrogen content in wood is typically low and in combination with the absence of oxygen, wood degrading bacteria are able to adapt to these circumstances as one of few micro-organisms. Although no relation was found between the degree of degradation and the sediment, shortage of nutrients seems to promote the degree of bacterial wood degradation. Furthermore it is evident that the intensity of degradation differs with timber species. Wood structures with a low resistance against water transport like alder, poplar and the sapwood of pine and oak are much more susceptible to bacterial decay than wood structures with a high resistance like spruce, and the heartwood of pine and oak. The reason behind this is not fully understood while it seems that the process of degradation is not only stimulated by a unidirectional water stream through the wood but also by an oscillating water flux inside the wood. Beside permeability it was already known that also lignin content determines whether a wood species is susceptible to bacterial decay. Pine and spruce have higher contents than alder and poplar. Furthermore it was proven that oak heartwood is very resistant against wood degrading bacteria.

Outlook

The methods and knowledge built up as well as promising preservation techniques tested already under laboratory conditions in BACPOLES enable us to start preservation tests in the field. There are three promising approaches and for all the approaches it is necessary to make a full description of the field to be treated. The site hydrology as well as the identification of the bacteria consortium, which causes bacterial degradation, is most important. Conventional as well as in this project developed methods should be used. Based on these inquiries specific mixtures of phages can be made and tested in the field on its generality and efficiency using monitoring techniques as developed in this project. A second approach is related to the hydrology. It became clear that bacterial wood degradation is mainly active when there is a water flux through the wood. In order to investigate a static situation, either the hydrology can be manipulated or the wood can be impregnated in the field to close its structure or both strategies can be combined with such a result that in addition the strength of the wood is improved. As the efficiency of all conventional wood preservatives against bacteria is not reliably proved, a third approach should be tried using an active product, which is not toxic but affects the already weak competition position of wood degrading bacteria by promoting others.

It can be concluded that the impact of bacterial wood degradation for our European cultural heritage is underestimated and methods are available to carry out a test program with promising preservation methods in the field.

Keywords
Wood degrading bacteria, wooden foundations, archaeological wood, DNA identification, phages, wood conservation, wood preservation

Content

Chapter 1

Introduction

(by Klaassen)

This project was funded by the European Commission and was carried out from February 2002 till January 2005.

Submerged wood was previously considered to be safe from any form of fungi decay due to the absence of oxygen. In the eighties of last century it became clear that bacteria could be active in these conditions and although the process of bacterial wood degradation is slow it is a threat for timber which is stored in the soil under the ground water level over a longer time. This is the case for wooden foundation piles but because time is almost unlimited within archaeology, the process of bacterial wood degradation in this field forms a special threat for wooden remains.
Wooden foundations have traditionally been used in areas with weak soils to support buildings, many of which are of historic importance. Well-known examples are the buildings in Venice (e.g. Santa Maria Della Salute church on 1.150.657 piles; Rialto bridge on 120.000 elm piles), Sint Petersburg (Hermitage), Berlin (Reichstag), Scandinavia (Gothenburg, Stockholm, Helsinki) and the Netherlands (Palace in Amsterdam on 14.000 piles).
Over the last three decades more and more wooden foundations (in different locations throughout Europe) were found where piles were seriously damaged by biological attack like in Stockholm (Sweden) and Haarlem (The Netherlands). If this process of bacterial degradation can continue over decades many monumental buildings standing on wooden piles might need expensive foundation repair or even be severely damaged. An inventory was therefore needed to know what the impact is of this relatively new form of decay for the European situation.
Archaeological wood is conserved either in dry or very wet environments (under surface water or ground water) and is of great importance because it provides us with information about the life style of our ancestors and sometimes even about the past climate (tree ring sequences, Bailey 1993). Not all archaeological sites found are automatically excavated because of missing funding or the lack of appropriate techniques to do a full analysis on the whole site. In order to save the information, which is inside the wood, it has to be protected against bacterial degradation.

Before the start of this project the knowledge on the process of bacterial degradation was limited and wood attacking bacteria had not been isolated nor identified. All information available was based on empirical results of the morphology of the bacteria and the patterns of degradation in the wood.
The main aim of this project was therefore to isolate and identify wood degrading bacteria and to get an idea of the impact of bacterial degradation as threat for wooden foundations under monumental buildings and for wood in archaeology. To understand the ecology and the mechanisms of degradation of the bacteria laboratory experiments were carried out and many sites spread all over Europe were sampled and the environment in which bacterial wood degradation occurred was characterised.
Based on all gathered information environmentally friendly concept conservation techniques were developed and tested on laboratory scale.

In chapter 2, as a state of the art an overview is given of the present knowledge of the impact of bacterial wood degradation in the countries of the participants of the project, i.e. Germany, Great Britain, Sweden, Italy and the Netherlands. Chapter 3 describes the standardised sampling and analyses method, which was used for 27 sites. Each site is characterised and based on the results of the wood, soil and water analyses general trends are discussed. Chapter 4 is dealing with new developed isolation and identification techniques and describes the environment of the bacterial species found in relation to wood degradation. Several microcosm experiments with wood degrading bacteria are described in chapter 5 and conclusions on the environmental interactions with wood degrading bacteria are discussed. Chapter 6 deals with water flow through wooden piles. It describes the experiments and discusses the results in relation to velocity of bacterial wood degradation. As the behaviour of sulphur in the soil seems to be linked in one way or another with the

process of bacterial wood degradation some background information is given on this element in chapter 7. In the final chapter 8 general conclusions are made and concept conservation techniques are proposed.

Chapter 2

Bacterial wood decay in Europe

(by Abrami, Björdal, Eaton, Gelbrich, Huisman, Klaassen, Nelemans, Nilsson)

2.1 Introduction

Participants of BACPOLES made a survey of the present knowledge of the process of bacterial decay and of the impact of bacterial decay for foundations and archaeological wooden remains. The aim of the chapter is to combine all information and get a general idea of the impact of bacterial degradation on the European level.

2.2 General overview of the European situation

This general overview is based on all available information from Great Britain, Sweden, Germany, Italy, and the Netherlands (see paragraph 3).
In the last few decades, especially in Germany and Sweden, fundamental research was carried out on the process of bacterial decay. The pattern of degradation was recognised and conditions under which it appeared were studied. Until BACPOLES no reliable identifications were made and no general characterisation of the environment in which bacterial decay appears could be made.

The significance of bacterial decay for piling constructions became obvious after 1990 in the Netherlands and in the last decade a general impression was achieved of the actual state of the Dutch foundation constructions. It became clear that within 100 years piling constructions, which were always underneath the groundwater table could be severely degraded and that some cities are more susceptible than others. Although wooden foundation constructions are well known in Sweden and Venice, the problems appear to be not so prominent in both areas. In Sweden bacterial decay in wooden piles was already recognise in 1970 but it was regarded as of minor importance for the quality of the buildings. However Venice has to deal with severe settlement problems, but bacterial degradation was not seen a possible cause of this problem.
In Great Britain and Germany wooden piling construction are of less importance, although some locations are known with buildings on wooden piles.

Beside some fundamental research was carried out on archaeological wood, bacterial degradation is not an item in the archaeology, in any of the five countries. However all available information shows that bacterial degradation is the major threat to the wooden cultural heritage stored under water or in soils. As under fungal and animal activity wood is degraded fast and if the environment allow these types of degradation no wood is conserved after 100 years and no archaeological wooden remainders will be found. There are records of wooden remaining over a long period of more then 2000 years. For archaeologists the state of the wood sample is important and is related to the possibility to recognise the original sample shape and size. In wooden remains degraded by bacterial, the outline of the original sample is often intact and although it is soft its wood quality is regarded as good. It is known that after drying the wood structure will collapse and specify treatments are developed to remove the water and retain the original sizes.

Based on all experiences and fundamental research the following statements on bacterial degradation can be made:

In general
If wood is well conserved (no bacterial degradation appears) it can last for centuries and retain its strength properties;
It is believed that in combination with fungal activity, bacterial wood degradation is more aggressive;
The significance of bacterial degradation for piling constructions and archaeological wood is underestimate in Germany, Great Britain and Italy.

Related to wooden foundations:
All wooden foundation piles that have been in service for about 100 years and that are situated under the groundwater level show bacterial decay at least in their outermost layer;
The degradation process starts from the out-side and gradually decreases towards the centre of the pile;
Spruce seems to have a higher resistance against bacterial degradation than pine sapwood and alder. Pine heartwood seems to be quite resistant;
In contrast to the general trend, situations were recorded where 100 years old Spruce piles were severely degraded and in other situations 100 years old Pine piles were without without any degradation;
The degree of bacterial degradation depends on the location. There are Dutch cities were the sapwood of the pine piles is fully degraded in a period of 70 years. Whereas in other cities the pine sapwood is less degraded in the same period. The degraded peal in Spruce piles is much more limited and in about 100 years this peal is less than 5 mm in some cities or can be as wide as 20 m in other cities.
Polluted environment with nitrogen / phosphorus seems to increase the degree of degradation and a clear relationship was found between the degree of degradation and nitrogen / phosphorus concentration in the wood;
Oxygen levels around the piles are supposed to be low;
Severe bacterial attack seems to appear more in permeable soils (such as sand), whereas the attack is less in non-permeable soils such as clay and peat;
Boron treatment is used to conserve piles but no result are available on its efficiency.

Archaeology
In the archaeology Pine and oak are regard as most durable species in contrast to alder, willow, beech, birch, and lime.
In contrast to the situation around piles, archaeology wood underneath the groundwater surface, degradation seems to be less fast in sandy soils than in peat or clay soils.
In archaeological wood there seems to be no relation between degree of attack and age;
With increasing excavation depth the degree of decay seems to be decreasing.
Although excavated wood seems often sound, this is not case because precautions are almost always necessary in order to avoid collapse of the wood structure and excavated wood seems to be susceptible for fungal attack.

It can be concluded that the impact of bacterial wood degradation in Europe is underestimated especially in the archaeology.

2.3. Description of the individual countries

2.3.1 The Dutch situation

(by Nelemans, Klaassen & Huisman)

2.3.1.1 Foundations

2.3.1.1.1 History

The soil profile of the Western part of the Netherlands is built up with (very) soft soil layers (see figure 1). The right side of the profile is more or less the middle of the Netherlands. There the Pleistocene sand layers reach the ground level. Going to the west, in the direction of the North Sea, the Holocene layers of clay and peat increase with depth. Behind the dunes soft soil layers are found up to 15-meter depth.

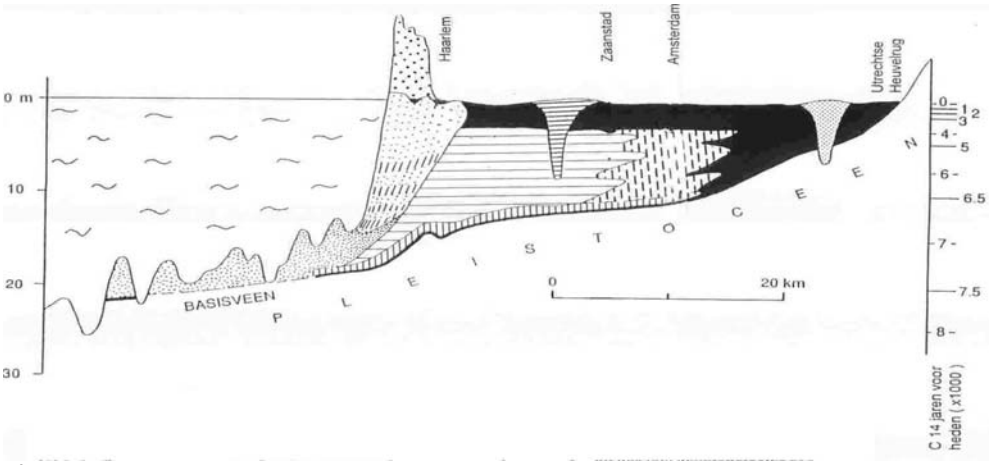


Figure 1

Due to the soft soil conditions wooden foundations are used since the 11th century. At Amsterdam, based on archaeological excavations site, the following steps can be recognised in the evolution of wooden foundations.

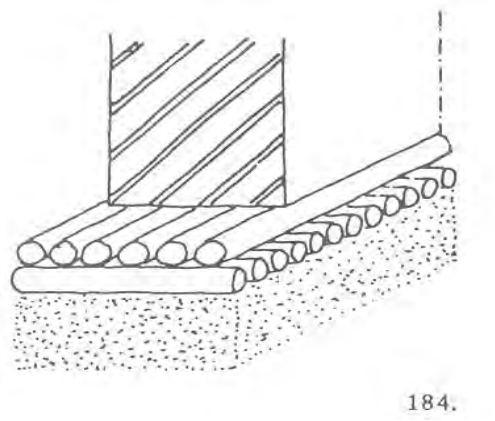


Figure 2: *foundations without piles (spread foundations).* This system was constructed with cross wise layers of thin wooden trunks.

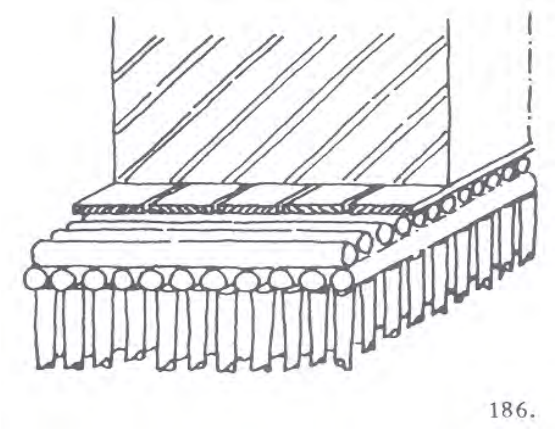


Figure 3 *many short piles.* At this stage many short piles were used below a crosswise layer of small wooden trunks. The length of the piles is approx. 3 to 4 meters.
From a soil mechanic point of view this system acts as a combination of friction piles and soil improvement.

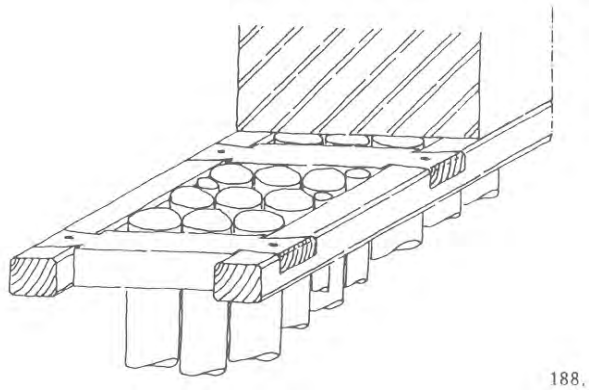


Figure 4: one of the problems with the system of figure 3, besides considerable settlements, was the stability of the piles at the edges. Therefore a system was developed as shown in figure 4, where a framework prevents the horizontal displacements of the piles.

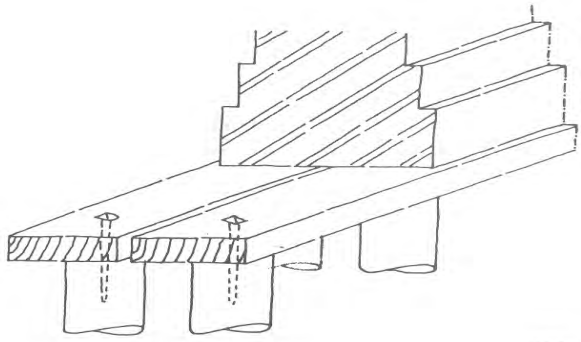


Figure 5: *longer (friction) piles*
Around 1300-1400 longer piles with a higher bearing capacity were used (length 7 to 8 meter). These piles act as friction piles, while they still did not reach a suitable bearing stratum. Due to the higher bearing capacity a system with longitudinal beams was developed. Main problems were stability and settlements.

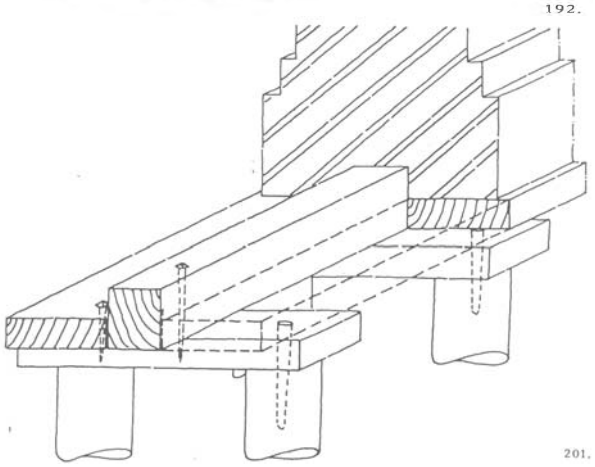


Figure 6: *long piles founded in the sand layers*
This system (figure 6) has been developed from figure 5. The system with cross- and longitudinal beams gave a very stable construction. The piles were founded in dense sand layers (end bearing piles) and were quite reliable. This system has been used up to the first part of the 20th century.

In other parts of the Netherlands variations of above are found, depending on the thickness of the soft layers. There are two main types of wooden foundations in the Netherlands. They are known as an “Amsterdamse fundering”, used in and around Amsterdam and a “Rotterdamse fundering”, used in and around Rotterdam. An Amsterdam foundation is a foundation with cross beams and pair of piles (see figure 7). A Rotterdam foundation is a system of stand-alone piles and only longitudinal beams (see figure 8).


Figure 7: Amsterdam foundation


Figure 8: Rotterdam foundation

Due to the Industrialisation a rapid expansion of the cities started at approx. 1875. From that year on to 1960 large volumes of wooden piles were used. The length and bearing capacity of the piles were based on engineering judgement and trial and error methods, as systematic soil investigations and geotechnical engineers did not exist.

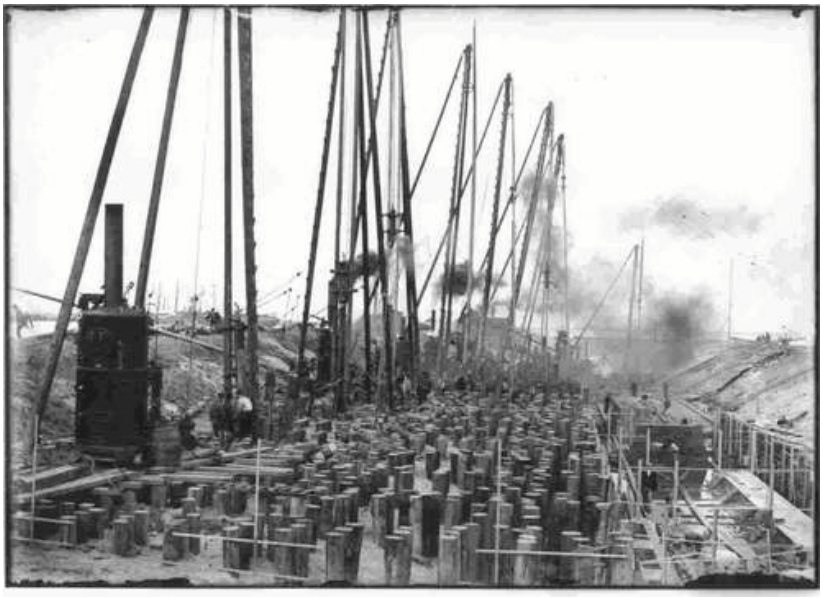


Figure 9


Figure 10

2.3.1.1.2 Problems with existing wooden foundations

In many cities, buildings with foundation problems are located in the quarters built from 1875 up to 1940. Most times foundation problems are recognised by the settlement behaviour of the buildings, which is illustrated in figures 11 and 12.

The house of figure 12a has a differential settlement between right and left wall of more than 20 centimetres. This causes such a high distortion that cracks in the brickwork occur, causing structural damage to the house (see figure 12b).

Figure 11.

Figure 12b

Figure 12a

Prior to buying or renovating one of these buildings the question rises, what the quality of the foundation is. Foundation replacement or -repair is very expensive. Costs involved can be up to 50% of the total renovation costs.
Therefore it is recommended to inspect foundations in order to make a sound judgement on buying, renovating or demolishing a building.
Main causes of foundation problems are:
Bearing capacity foundation is too low (piles too short or discontinuity in soil conditions);
Loads and/or negative skin friction are too high;
Mistakes in foundation construction design;
Decay of piles due to softrot (groundwater table below top of piles; see figure 13) and/or bacterial degradation (see figure 14).

Figure 13: softrot.

Figure 14: bacterial degradation

Especially the degradation of piles causes serious problems. Submerged wood was previously considered safe from decay. Therefore extensive investigations are carried out nowadays to determine the quality of foundation piles at many residential area's.

2.3.1.1.3 Sampling, testing and inspection of foundations
The last 10 years work on existing foundations has been increased for Fugro and consists nowadays of: surveying of (differential) settlements of buildings (approx. 3000 per year); inspections of existing piled foundations (approx. 300 per year); consultancy regarding the quality of wooden piles (maintaining, repairing or replacing).
Due to the large quantities of inspections and the fact that several companies were involved in the investigations, the method of investigation is more or less standardised. An uniform investigation makes interpretation of the results more reliable. Also comparison of an investigation executed by different persons or companies is possible. In a national protocol "foundation inspections" a detailed description is given of the method of inspection, the equipment that should be used and minimum requirements of how the results should be reported.

According to the protocol the sampling is done by using an increment borer with a inside diameter of 10 mm. From each pile a core is taken from the bark to the pith approximately 500 mm below the pile head. Immediately after boring the core is put with some water in a plastic tube (see figure 15 to 18), sealed on both sides and send by post to the laboratory. In the laboratory the core is split into sub-samples of 15 mm (figure 20). For each core the species and the type and degree of degradation is determined by microscopic analysis. The moisture content and the wood density is determined by weighting and drying for every sub-sample. After having investigated a huge number of whole pile disks (figure 21) it was possible to study the relationship between wood density and moisture content and the compression strength of a pile in more detail. A simple model is calculated to estimate compression strength from the quotient of wood density and moisture content. Figure 22 shows this relationship for 100-year old Pine piles with 93% regression fit.


Figure 15


Figure 16

Figure 17

Figure 18

Figure 19

Figure 20

Fig 15 – 20: showing the sampling strategy with an increment borer, post in a sailed plastic tubes and investigated in segments of 15 mm.

Figure 21: Pile head used for estimation of compression strength; heartwood stained red.

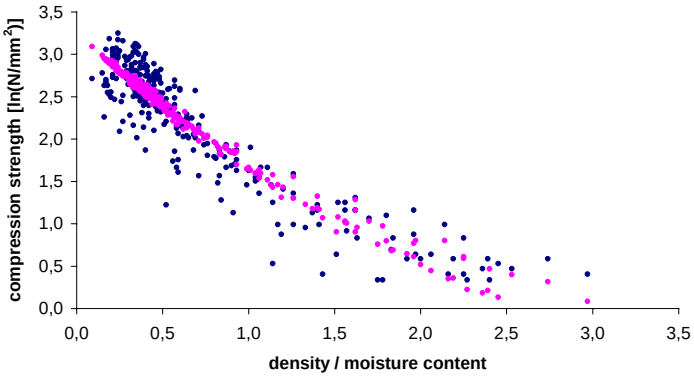


Figure 22: Relationship between compression strength and the quotient of density end moisture content for Pine. Blue dots are measured data, pink dots are estimated data (see text).

In the foundation pith the quality of the piles are determine by using a Penetrometer or wood test hammer. Figure 23 shows a Pilodyn wood test hammer (comparable with a concrete or Schmidt Hammer).

Figure 23: Pilodyn wood test hammer

Before use, the pen of the hammer is pushed inside. When a button is pushed in the pen comes out and penetrates the wood with a uniform force and the penetration depth is determined. At least 3 measurements are carried out at each pile (spread over the circumference of the pile) at approx. 150 mm below the pile head. The average penetration depth is an indication of the degree of the decay.
The advantage of this apparatus is that the equipment is simple and not operator related. In the past the thickness of the soft skin of a pile was measured with screwdrivers, keys, awls and sometimes other types of hammers. It depended on the strength of the person who carried out the test, how deep the equipment used penetrated the softwood. Therefore results were not reliable and could not be compared with other persons or companies. This is not the case with the Pilodyn hammer and therefore this hammer is now standardized in combination with the already mentioned Protocol for foundation inspections.

In general an investigation starts with a visual inspection and some survey work. To measure the differential settlements of a building, an indication is obtained of the settlement behaviour of the foundation. This relative simple (and therefore cheap) method gives a good indication of possible defects of the foundation.
At locations with large differential settlements additional investigations are executed, such as excavations of inspection pits and quality tests of the wooden piles.

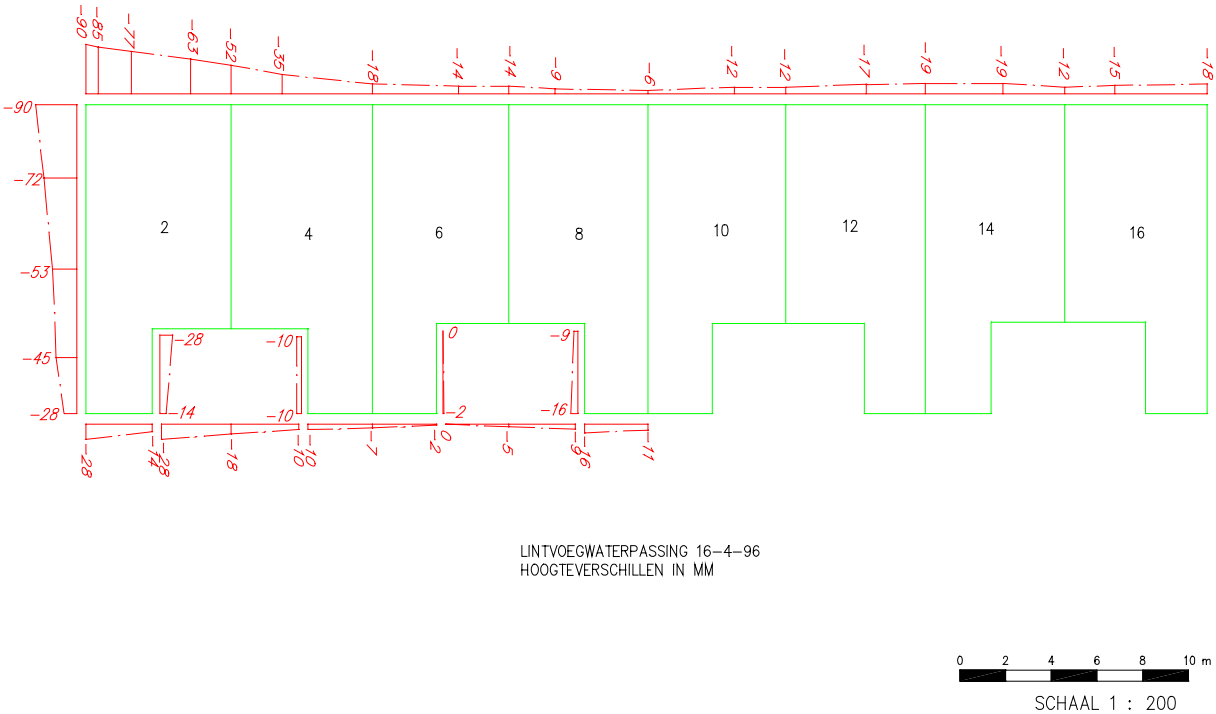


Figure 24

In figure 24 an example of the leveling results of a horizontal joint in the brickwork is presented. The results are an indication of the total settlements occurred since the building has been constructed. When the foundation is acting properly no differential settlements are measured (see the right side of the drawing). But in this case, at the left side differential settlements are measured up to 90 mm. It's an indication that the foundation is not acting properly (not enough bearing capacity or a defect?). In this case additional investigation is recommended at house number 2.
If the survey work can only be executed at the front side of the houses and more information is required, leveling of the ground floor is carried out. The results give a good indication of the settlement behavior of the house and shows if there are any differential settlements between front, back, left or right side of the house (see figure 25).

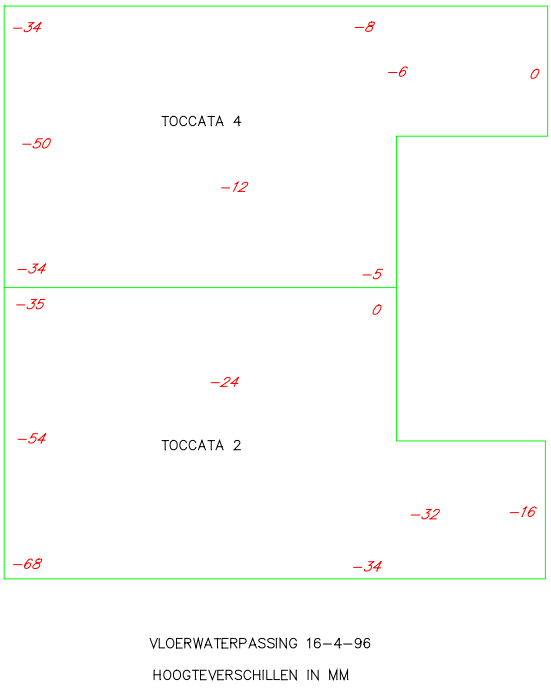


Figure 25

Two examples are given how inspections are carried out in the Netherlands. The first example is about a sound foundation and the second is about a foundation with degradation. Figures 26 and 27 give an impression for the first location where the pit has been excavated. In figure 27 it is seen that it is not always easy to excavate close to the building. A dewatering system was necessary to lower the groundwater table.

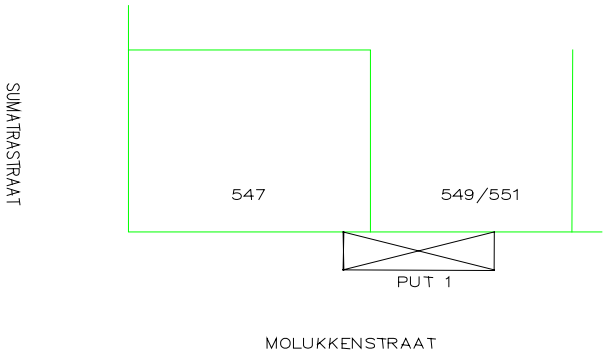


Figure 26

Figure 27

After 90 years the foundation construction is still in perfect condition (see figure 28 and 29).

Figure 28

Figure 29

In figure 30 a drawing is presented with all the inspection results, such as diameters of the piles, dimensions of the beams, distances in between, a cross section, the groundwater level (well above the wooden structures) and the soil profile found in the excavation pit.

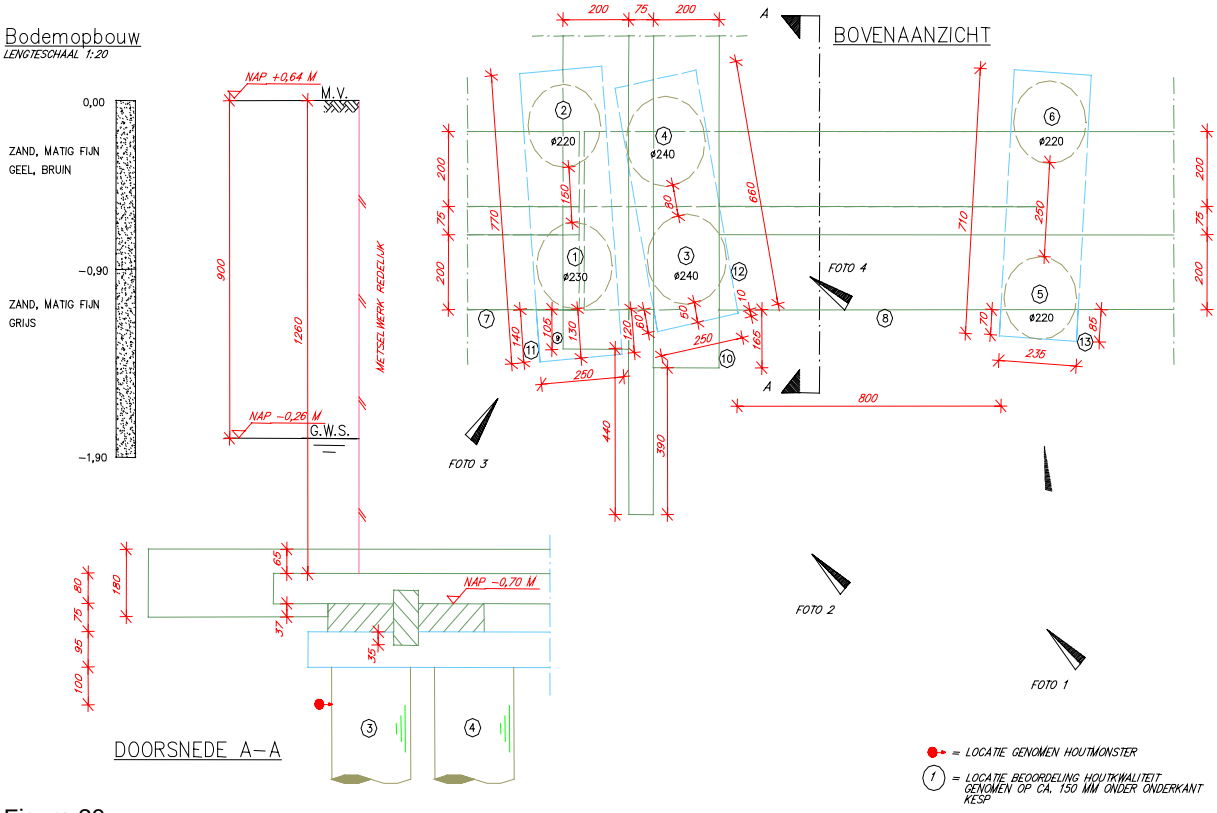


Figure 30

The mean penetration with the penetrometer was 15 mm, which was in agreement with the results of the microscopic research and the quality of the foundation was regarded as sound.

The figure 31 – 36 gives an impression of the second example of an inspection with a poor foundation. Due to a groundwater table, which is below the level of the wooden structures, softrot in combination with bacterial degradation of the wood, caused a terrible condition of the crossbeams and the top of the piles. In such a case foundation replacement is the only option to preserve these houses.

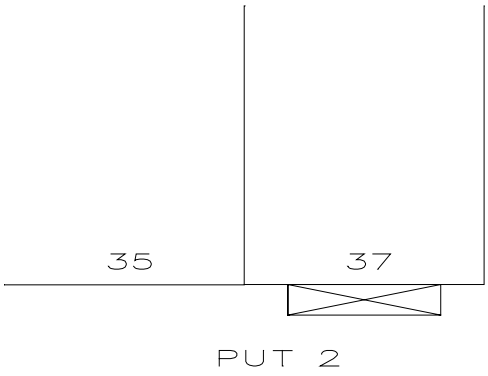


Figure 31

Figure 32

Figure 33

Figure 34

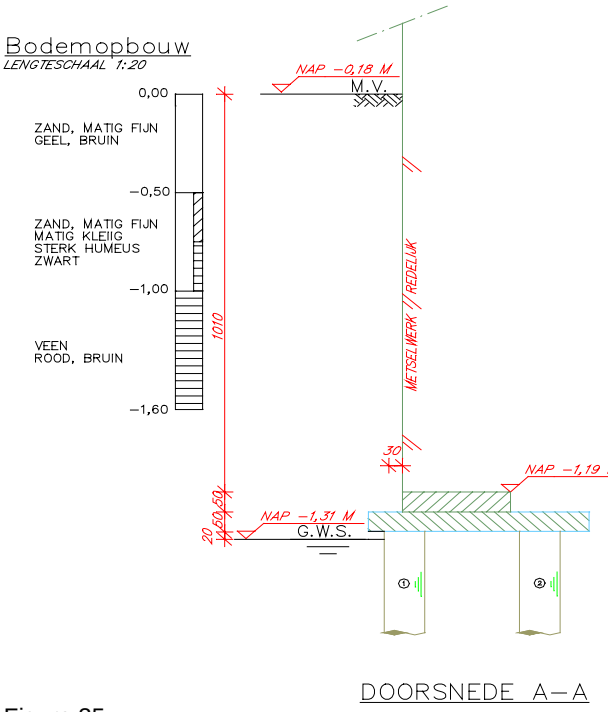


Figure 35

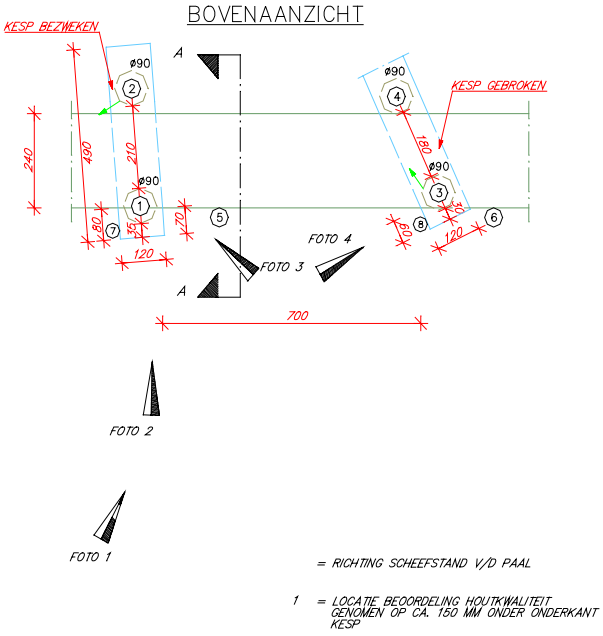


Figure 36

2.3.1.1.4 Results of wood research on foundation piles

In the period from 1997 to 2004 SHR analysed more than 4000 wood sample originated from foundation piles from different cities in the Netherlands. Spruce and Pine were the most common species that were used; in addition some Alder and Fir piles was found (figure 37). The majority of the samples originate from three cities namely Amsterdam, Dordrecht and Haarlem (figure 38). Wooden piles in Haarlem mainly suffer from bacterial decay, whereas many problems in Dordrecht also occur due to changes in ground-water level with the consequence of decay by soft-rot fungi. Amsterdam is dealing with both problems.

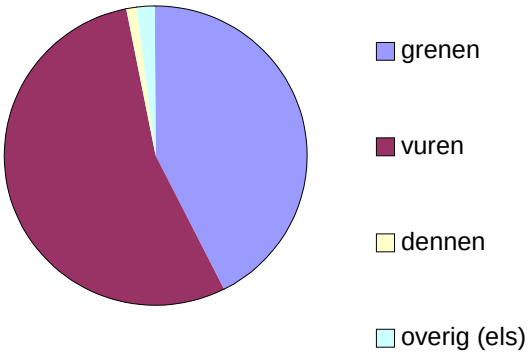


Figure 37: species distribution found in 4345 wooden foundation piles.

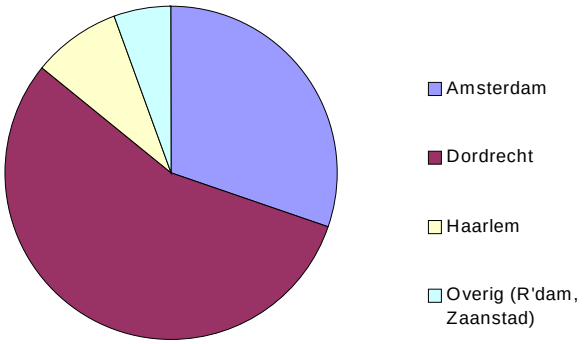


Figure 38: origin of samples from 4354 wooden foundation piles.

The data base that has been compiled on wooden piles during the last eight years allowed us to investigate the relationship between the most important parameters: wood density, moisture content and compression strength.

In the figures 39 to 42 the ranges of moisture content and density as well as the relation between both parameters are shown for the four most common timber species. We found the moisture content ranging between 100 and 600% and density values between 200 and 600 kg/m^3. Both parameters are related in the three softwood species, whereas no relationship can be seen in Alder. At the same time Alder shows, a higher moisture content and density. Possible reasons for these differences will be discussed later.

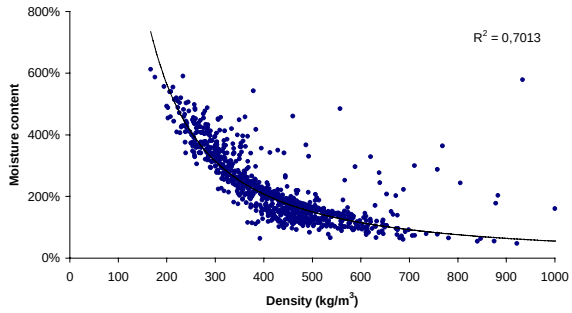


Figure 39: moisture content and density of all Pine samples with calculated model fit (n=1391).

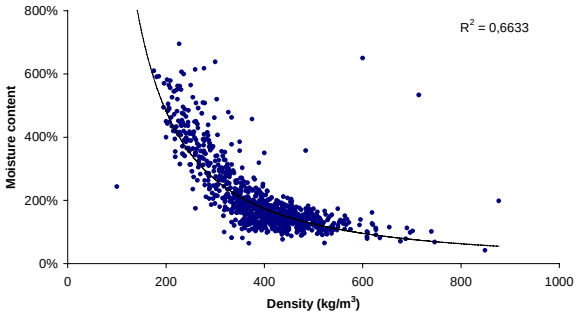


Figure 40: moisture content and density of all Spruce samples with calculated model fit (n=1384).

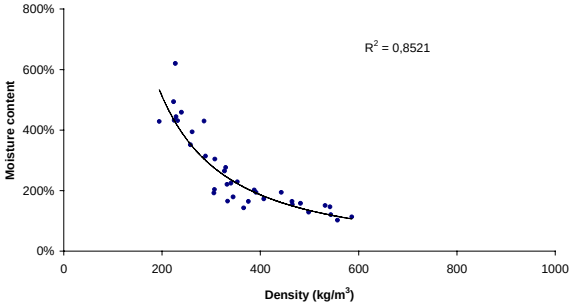


Figure 41: moisture content and density of all fir samples with calculated model fit (n=33).

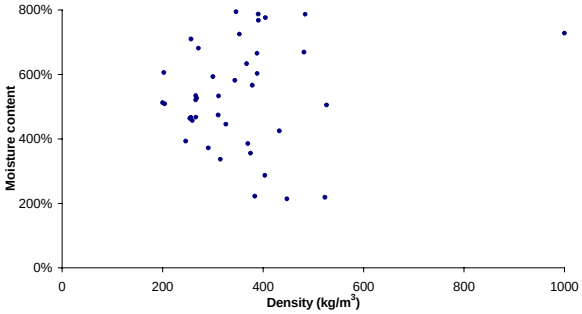


Figure 42: moisture content and density of all alder samples (n= 29)

For evaluation of decay in wooden foundation piles four categories ranging form sound (non-decayed) to total decayed wood were defined by SHR. The evaluation is done under a light microscope using radial sections.
In figure 43 the relationship is given between these categories of decay and both moisture content and density for the two most common timber species used as foundation piles.
The general pattern in Spruce and Pine is comparable for both density and moisture content and both parameters show a strong relationship with the degree of decay.
To get an idea about differences in degradation between the four main species that have been used as wooden pile we calculated the mean degree of degradation (given in four categories) across the radius of the piles (figure 44).

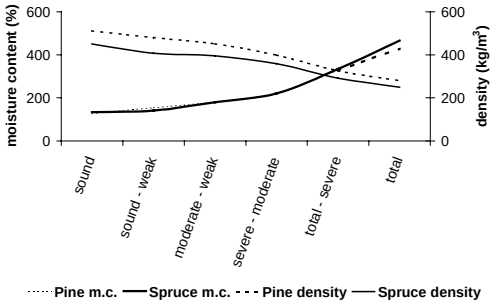


Figure 43: relationship between categories of decay and moisture content and density for Pine and Spruce.

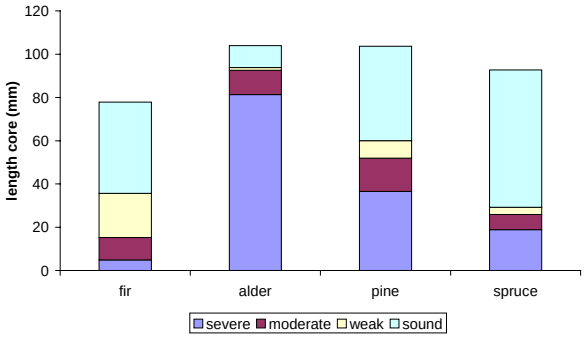


Figure 44: mean degradation across the pile radius based on 859 piles for the most common species.

It can be seen that most of the Alder piles are severely degraded across almost their whole radius. However, it has to be considered that Alder piles were not used during the last 100 years. This means that the Alder hardwood piles are probably a lot older than the softwood piles. If comparing Pine and Spruce it is seen that the severely degraded radius in Pine is in average as twice as thick as in Spruce. Fir seems to be less affected by bacterial decay. However, it has to be admitted that these rely on only a few samples.

The pattern of degradation can be studied in more detail by using frequency diagrams. These are based on the evaluation of the degree of degradation (four categories) in 10 mm segments from the bark to the pith. The results for the two common wood species are summarised in figures 45 to 46.
Figure 45 shows that less then 5% of all Pine piles are free of degradation and that 90% of all piles are severely degraded in the outermost 10 mm. Half of all Pine piles have a severely degraded outermost peal of approximately 40 mm which is a lot considering that the mean pile diameter is about 100 mm. The situation of Spruce is better: 10% of the Spruce piles are free of degradation and 70% of all piles have a severely degraded peal of 10 mm. Half of the Spruce piles show severe degradation on the outermost 20 mm.

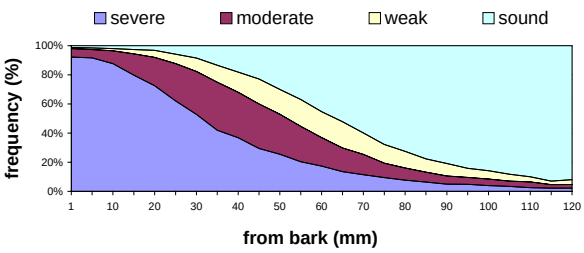


Figure 45: frequency diagram based on 803 Pine piles.

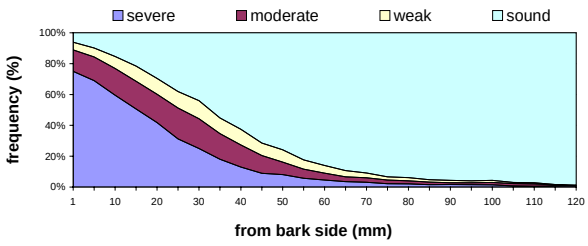


Figure 46: frequency diagram based on 789 Spruce piles.

2.3.1.1.5 Relationship between wood degradation in foundation piles and different peat types

In the western part of the Netherlands different types of peat can be recognised (see figure below). Depending on the nutrients in the environment, the peat was formed by different plants. *Spagnum* peat (oligotrophic) is found at large areas north and south of Amsterdam. This peat is nutrient poor and supplied by rainwater and up welling groundwater. *Carex* peat (mesotrophic), is found in Haarlem, close to the dunes along the North Sea. This peat contains more nutrients, due to continuous flow of nutrients-rich water from the dunes.

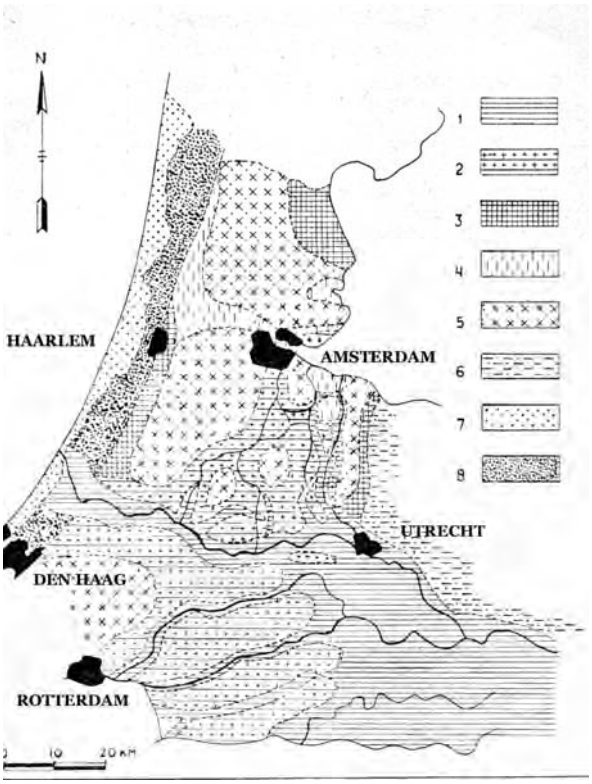


Figure 47. Peat map of the western Netherlands. 1 = river clay; 2 = forest peat; 3 = *Carex* peat; 4 = *Phragmites – Scirpus* peat; 5 = *Sphagnum* peat; 6 = pleistocene sand; 7 = young dunes; 8 old dunes

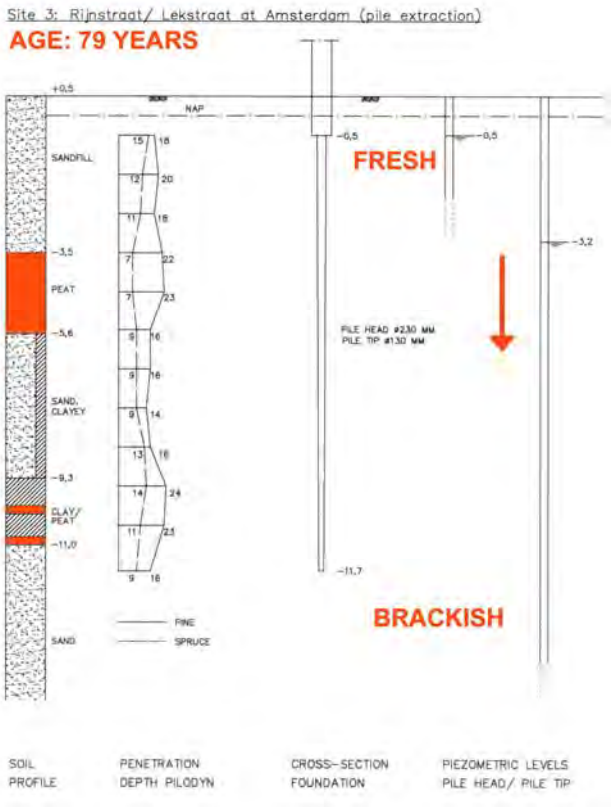


Figure 48: soil profile BACPOLES site 3 (Amsterdam), water in pile is fresh, peat over ¼ of pile length moderate decay

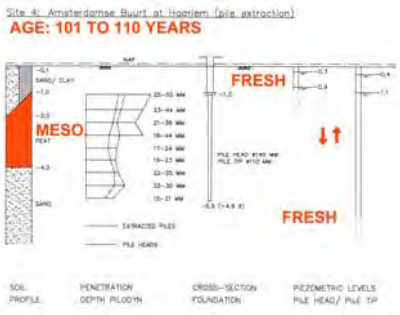


Figure 49: soil profile BACPOLES site 4 (Haarlem), water in pile is fresh, peat over 2/3 of pile length, severe decay

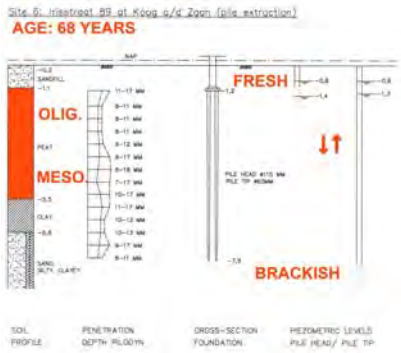


Figure 50: soil profile BACPOLES site 6 (Koog aan de Zaan), water in pile is brackish / fresh, peat over 2/3 of pile length, weak decay, severe decay in pile head only moderate decay

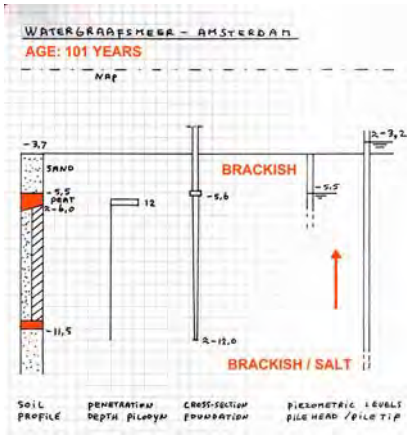


Figure 51: soil profile Watergraafsmeer (Amsterdam), water in pile is brackish / salt, peat over 1/8 of pile weak decay

The up-welling water is quite frequently brackish or even salty. In figure 49 – 51 four situations of bacterial wood degradation, water pressure and salinity of the groundwater are presented. At locations where peat contains more nutrients the intensity of the bacterial wood degradation was higher. Furthermore at locations with brackish or salty water, medium to weak bacterial wood degradation is found in contrast to severe degradation where the water quality is fresh. Finally the difference in ground water pressure between the sallow and deeper areas seems to be positive related to the degree of bacterial wood degradation.

2.3.1.2 The archaeological point of view

Literature on bacterial decay of archaeological wood is scarce. Internationally, there are a few papers. In the Netherlands, no literature is to be found on this subject. Still, since the Netherlands consist for a large part of wetlands, there is a large amount of experience with archaeological wood in various phases of degradation. We therefore decided to replace the literature-review with a series of interviews with these specialists, hoping that the combined experiences of these practical specialists provide “circumstantial evidence” on the bacterial decay of archaeological wood. The people interviewed are mainly senior (field) archaeologists and paleobotanists. The main conclusions are reviewed below.

2.3.1.2.1. Beneath groundwater

There is a general consensus that wood is preserved best when it is permanently beneath the lowest groundwater level. This reflects the strong effects of oxygen on the degradation of wood. Some specialists indicate that it should not be too close beneath the lowest groundwater level. It is not clear whether this effect is caused by diffusion of oxygen into the groundwater, causing the upper groundwater to be oxic, or that it is an effect of sporadic extreme low groundwater levels exposing the wood to oxygen.
Assuming that degradation under the influence of oxygen is at least partly mediated by fungi, we will concentrate on wood that is preserved below groundwater. Under these conditions, degradation processes are purely bacterial.

2.3.1.2.2. Soil texture
From experience, most people would assume that wood is less well preserved in sandy soils and better preserved in clayey or peat soils. However, this is mostly due to the fact that sandy soils (at least in the Netherlands) usually have lower groundwater tables. Moreover, a large part of the sandy soils are in infiltration areas, where the groundwater is oxic to greater depths than in exfiltration areas. If we only look at suboxic or anoxic conditions, there are few indications for differences in preservation of wood between clayey and sandy soil.

2.3.1.2.3. Wood species
Two species are known to be preserved very well, pine and especially oak. There are numerous examples where oak or pine are preserved, but where wood from other species, although buried under the same conditions, are very strongly degraded. Such species include beech, birch, alder, willow and lime. However, there are reports of well-preserved alder wood. This complies with Vitruvius, who wrote
" The alders, which is produced close by river banks, and which seems to be altogether useless as building material has really excellent building qualities. [..] in swampy places, alder piles driven close together beneath the foundations of buildings [..] remain imperishable forever. "(trans. Morris Hickey Morgan)
It is striking to see that oak sapwood, heartwood and bark are all preserved in cases where whole trees were buried in a short period of time, especially tree-falls in peat environments. In several cases, recognisable artefacts are recovered that were made from twigs (wicker walls, fish traps). These usually have lost their strength and part of their cohesion, but embedded in clay they are still recognisable.

2.3.1.2.4. Age
Surprisingly, there is a general consensus that there is no discernible effect of age on the preservation state of wood. From several excavations, complete trees of oak and pine have been recovered in excellent state, after having been buried for 6 000 to 10 000 years.

2.3.1.2.5. Visual characteristics archaeological wood in different phases of degradation
Well-preserved freshly excavated oak in most cases has a yellow to brown colour. However, within hours of exposure to air, the outside turns black. The core of thick pieces of wood tends to retain a yellow colour, even after exposure to air (e.g. after cutting).
One deviating report is that hollowed-out oaks, used as wells, are black when excavated and become covered in a dew-like white powder within hours or even minutes after exposure. The wood tends to become greyish in colour after drying-out. It is not clear whether this is specific for wood used in wells, or that it is a general characteristic of certain soil/water conditions.
The core of buried alder has a light-pink colour (like that of fresh wood) that turns dark-purple when exposed to air. The core of ash is yellow to orange when freshly excavated, and willow is white. However, both darken on exposure. Birch does not discolour.

2.3.1.2.6. Structural properties, cohesion and strength
The strength and structural stability of well-preserved old wood sometimes is surprising. The most striking examples for this are from the archaeological record. There are now two archaeological sites cases known, where old, buried, well-preserved wood was re-used as building material. One case, location Tjonger, is from the Bronze Age (approx. 3500 years ago), the other, location Borssele, from roman times (approx. 2000 years ago). In both sites, construction wood was found that was several thousands of years old at the time of use. Apparently, driven by a shortage of wood, people used wood from trees that had become exposed by the erosion of peat deposits. This indicates that this wood was still strong enough to be used as construction material after thousands of years of burial.

2.3.1.2.7. Other characteristics

Although the strength of this wood is retained, several properties of preserved wood indicate that there have been changes in the composition of the wood.

Firstly, most well preserved wood tends to crack and strongly deform upon drying. Sometimes cracking is so severe that it results in complete disintegration of wooden objects. After exposure, keeping the wood moist or wet does not prevent this type of deterioration.

Secondly, the wood is extremely hard; sawing or cutting ancient wood is more difficult than cutting recent timber; several specialists reported cases where saws were blunted or broken because of the hardness of the wood. Interestingly, similar effects are sometimes reported for roof beams from medieval buildings.

Thirdly, the wood tends to burn less well. It is difficult to light, smoulders or burns with small blue flames and it keeps burning for a long time.

2.3.1.2.8. Causes and effects

There is a whole range of effects that result from anaerobic bacterial-induced decay of wood. The best-known change in decaying wood is the disappearance of cellulose and hemicellulose from the wood structure. This is accompanied by a relative increase in lignin, and in an increase in porosity. Some of the effects we can ascribe to a larger or lesser extent to these changes in composition and structure:

Chemical change:

The colour change after exposure to air indicate that the decay-process produced chemical substances that are sensitive to oxidation (sugars?). On exposure to oxygen, they degrade fast, or they promote microbial activity that subsequently causes increased deterioration of the woody material still present. It is not clear what happens with wood that turns grey from a general black colour.

The relative increase in lignin content is probably also the reason why archaeological wood and “mummified” wood from the geological record does not burn well.

Porosity change

The loss of cellulose and hemicellulose increases the porosity of the wood. As a result, upon drying the wood will probably lose more water than fresh, undegraded wood. This is a possible explanation for the problems with tools upon sawing archaeological wood. This also explains why similar problems can arise when beams from age-old buildings have to be sawn; they are probably not degraded, but dried out very extensively.

2.3.2 The Germany situation
(by Gelbrich)

Since the beginning of the 1950th investigations of sub fossil woods, submerged and stored for hundreds of years, revealed new information about wood degradation (Müller-Stoll, 1951), but the questions for possible perpetrators could not satisfactory replied.
In the following decades some observations of water stored wood (J. Liese, 1950; Boutelje und Kiessling, 1964; Harmsen und Nissen, 1965) and telegraph poles (W. Liese, 1955) were done and the symptoms of attack were assigned to a mixed flora of fungi and bacteria which were clearly different from decay patterns of the knowing wood destroying fungi.

For clear diagnostics concerning the causer of this new kind of cell wall degradation laboratory experiments with water stored wood were done by Courtois (1966), Liese und Karnop (1968) and Liese (1975), which showed that bacteria entail wood degradation. Because of these results the question of economic relevance raised.

In natural system the wood degradation by bacteria occurs only under certain wet conditions. So water stored logs, foundation poles, wood of water cooling towers and archaeological wood can be attacked. In the following citations the bacterial wood degradation of wood in mentioned usage is evaluated as marginal:

- “Despite of the considerable aggressiveness of bacteria their decomposition activity is compared to the decomposition intensity of fungi very low” (Courtois, 1966).”But it could be hardly not reckon, that, even with intensive research within this thematic, commercial important, up to now unknown damage influences because of bacteria will be found. For that, the possible decomposition is too small-sized and to slow” (Liese und Karnop, 1968).
- The cognition, that certain bacteria are able to degrade wood, even if very slowly, was not accepted by researchers until the 90th. The microscopic investigations of foundation poles from different buildings (Palace Giffhorn, Nikolai-Church Bodenwerder, Castle Lethe near Bremen, Magni-Church Braunschweig) were interpreted: “that none of the poles showed degradation caused by herbal or animal organisms….apart from the controversial possibility, that the degradation of cellulose and partly lignin is caused by bacteria, an acid depends hydrolysis of the wood, especially of cellulose, has to be accounted” (Böttcher, 1989). The results of microscopic and chemical analysis of water storage archaeological findings of different wood species (Hoffman and Jones 1990; Fengel 1991) were also evaluated as an effect of a-biotic chemical decomposition process.

For foundation piles which exist in many German areas and cities like Berlin, Bremen, Hamburg and Leipzig, bacterial degradation is up to now no cause for damages. The foundation piles of the Berlin Reichstags building were investigated for bacterial attack in 1997. The log wood samples, which were between 66 and 111 years old, were degraded heavily only in the outer 0.5 to 1.5 cm of sapwood, the heartwood was not attacked (Grinda, 1997).
For archaeological wood the bacterial degradation seems to be more relevant. The bacteria cause an extensive degradation of cellulose and hemicellulose resulting in losses of structural integrity. The fragile wood residue can be easily damaged or totally destroyed if not handled properly or treated with appropriate procedures. Therefore new information about the wood degrading mechanism of bacteria should be helpful to assess the condition of waterlogged woods, help to define the degradation process and be useful to ascertain appropriate conservations (Hoffmann, 1993).
The condition of foundation piles of buildings near the opencast-pits of the Rheinisches Braunkohlerevier in the Western part of Germany, was subject of different studies. Because of the opencast-mining it is necessary to lower the ground water level. Therefore some foundation piles are not completely waterlogged any more and can be damaged very fast

because of fungal attack. The costs related to the damages because of the decreased ground water level have to be paid by the opencast company. In order to investigate, if the damages are caused by fungal attack, which is caused by the reduced groundwater level, the foundation piles were examined by the Fraunhofer Institut Braunschweig. These investigations proofed that some of the damage cases are not caused by a reduced ground water level. In case of a negative detection of fungal hyphae, the damage is assigned to bacterial or hydrological wood degradation (Lukowsky, 2004 verbal).

2.3.2.1. Enquiry

For getting an actual survey about the German situation of bacterial attack different wood research institutes, centres of archaeologist, institutes of civil engineers and pile foundation companies were addressed to ask about the experiences with this topic. The following table shows the results of the poll by numbers.

Number	institution	reply	negative	positive
14	Wood research institutes	10	8	2
15	Archaeology	6	4	2
12	Under water archaeology	7	3	4
13	Civil engineer institutes	5	4	1
17	Pile foundation companies	6	6	0
		34	25	9
71		47,9%	35,2 %	12,7 %
		100 %	73,5 %	26,5 %

Nearly 50% of the positive replies are from under water and ship archaeologists, the others were from wooden foundation piles and archaeological wood.
Most of the positive replies did not identify the bacteria attack. They only know by literature that under the wet conditions of their wood samples it is possible that bacteria degrade wood. Because of the damaged wood structure and the loss of strength it is believed that bacteria degradation exists but there are no specific microscopic studies to back up this idea. Only two positive replies contain a professional name of the wood degrading bacteria groups.

2.3.2.2. Conclusions

Compared to the situation in the Netherlands in Germany the foundation piles show no strength losses with economical significance. Additional to this there is no agreement about the topic, that bacterial wood degradation could be the cause of damages. Therefore in most cases of bacterial degradation the damages were assigned to other causes.
Most archaeologists are not very interested in cause studies, but more in damage control by efficient preservations or treatments. So the interest in the reasons of wood degradation decreases if an efficient preservation method has been found.

2.3.3 The situation in Great Britain

(by Eaton)

In Great Britain the importance of historical monuments, heritage sites and maritime archaeology has taken on a new importance in recent years. In addition to input from central and local government and local interest groups we have seen a resurgence of public awareness and interest in heritage matters and issues through the media, particularly via radio and television programmes. This revitalised enthusiasm for the subject has been encouraged by organisations such as English Heritage and Historic Scotland which are key to maintaining continued efforts in conserving the cultural heritage of the nation.

Whilst undertaking a survey of 'BACPOLES activities' in Great Britain, the British partners were personally unaware of ongoing projects or studies into the condition of piling timber beneath buildings constructed on low-lying land that were outside their own local area. Discussions with local archaeologists and conservators indicated that the best sources of information at the national level were the staff of English Heritage so staff at regional centres were contacted. The responses to this enquiry revealed relatively few known examples of buildings erected on driven timber piles.

Overall, it was thought that London and the River Thames estuary would be the richest source of timber piles, but no specific examples were given other than the statement that timber piles would only be under Roman buildings in London. In this country there are numerous remains which date back to the Roman era (55BC – 450AD) and many are associated with cities and towns that were built on wet or waterlogged ground because of their close proximity to rivers, estuaries and the sea. The strategic military importance as well as the need for trade through these places is well-documented and Chichester on the south coast is a good example. Further inland, in other parts of southern England, it was stated that apparently Salisbury Cathedral (13th Century) was built on wooden piles and that Winchester Cathedral, which pre-dates Salisbury, might also be built on wooden piles.

In other parts of England there was again little information forthcoming. In Yorkshire, the city of Kingston upon Hull situated on the estuary of the River Humber was considered to be probably the best source. Hull has 17th and 18th century buildings that are built on rows of driven timber piles, as is the Citadel and certain sections of the town wall. A number of these piles were exposed in the summer of 2004 and samples were taken for dendrochronological analysis. A little way south of Hull is Boston in Lincolnshire, which has two sites of interest. The first is the 15th Century brick built Hussey Tower approximately twenty metres high. Although the walls are of appreciable thickness and appear to be in relatively good condition, there is some vertical cracking. Since it is thought that the tower was built on timber piles it has been suggested that the timbers may be decaying because the ground is drying out leading to settlement of the foundations. A second site is to be found in the mud banks of the River Witham. Visible at low tide are a series of timber posts, protruding from the mud at a slightly inclined angle. It has been suggested that these might be the piled foundations of a crane used to construct the modern (19th Century) town bridge.

One region in England that has recently been invigorated by the establishment of an integrated programme for the protection and conservation of historic heritage and maritime archaeology is the Solent. The Solent is the coastal region of Central Southern England bounded to the south by the Isle of Wight and to the north by the mainland where the cities of Southampton, Portsmouth and Chichester are situated. The Solent is a unique, nationally important focus for Maritime Heritage and Archaeology.

Out of a total of 42 sites around the coast of the UK, 6 protected wrecks are within the Solent. In chronological order these are the Grace Dieu (1439), the Mary Rose (1545), the Yarmouth Roads wreck (1567), the Hazardous (1706), the Invincible (1759) and the Pomone (1811).In addition, there are over 800 archaeological sites currently recorded in the Solent and Wight waters. There are several prehistoric sites, a dozen Roman sites, more than twenty Medieval and post-Medieval (1550 – 1800) sites plus many 19th and 20th Century sites. The region is a rich source of heritage and archaeology, both on land and underwater.

Much of this activity is recorded on the following web-site: http://www.solentforum.hants.org.uk/herarch/sgsherarch.htm and describes three particular aspects which are of interest in strategic guidance for the Solent. These are: (i) historic buildings and structures associated with the maritime history of the region. The Solent contains the most important concentration of coastal defence heritage features in the UK, important sites associated with the history of commercial activity and coastal settlement, and a large number of shipwreck sites below high water mark;
(ii) submerged archaeological evidence of past landscapes and land uses at times of lower sea level;
(iii) archaeological remains and historic structures under particular threat, including those put at risk by coastal erosion.

The Solent Forum entry on this web-site, in partnership with Hampshire County Council, describes the status of archaeological and historic sites and points out that of the nationally important archaeological sites, some are recognised as Scheduled Monuments. Scheduled sites only make up a small percentage of the overall total and sites that are not scheduled are protected through local authority planning systems as Sites and Monuments Records. The most important historic buildings are recorded as Listed Buildings and suburban areas of historic importance as Conservation Areas. Thus each type of building or site is offered statutory protection through the planning system.

In recent times the levels of awareness and interest in maritime archaeology have increased significantly with the raising of King Henry VIII's flagship the Mary Rose from the Solent seabed in 1982. This one event sparked an expansion of activity in seabed excavations around the coasts of the British Isles, including the Newport ship in South Wales, the Stirling Castle (1703) on the Goodwin Sands, Kent and the Royal Anne off the coast of the Lizard Peninsula in Cornwall. Over the centuries shipwrecks have not been an unusual or exceptional occurrence in the coastal waters of Britain to the point where there is a wealth of historic heritage waiting to be examined and studied.

Unlike the situation on land where statutory protection is in place, underwater archaeological sites are not subject to the same rules and are very dependent on voluntary codes of practice being established between interested parties and key organisations. In 1991 the Hampshire and Wight Trust for Maritime Archaeology was formed to provide a point of focus for research and advice within the Solent. Although central and local governments have taken a keen interest in aspects of marine archaeology, a voluntary approach has been adopted. In 1995 the Code of Practice for Seabed Developers was produced by the Joint Nautical Archaeological Policy Committee (JNAPC) in conjunction with key industrial bodies. Another example of joint action reported in the Solent Forum web-site was the initiative by the Royal Commission on the Historical Monuments of England and the Nautical Archaeological Society. In this instance amateur divers are enabled to report wrecks and other man-made objects that they encounter on the seabed and this information is then entered into a national database.

In summary it is encouraging to report that current interest in historic heritage and maritime archaeology appears to be flourishing, particularly in the Solent region, through the activities of certain key organisations. Amongst the lead organisations in this respect are English Heritage, the Royal Commission on the Historical Monuments of England, Hampshire and

Wight Trust for Maritime Archaeology and local county and city councils in the Solent area. Other key organisations include central government departments e.g. Department of Culture, Media and Sport, Ministry of Defence, Crown Estate, local borough and district councils, harbour conservancies and commissioners, universities and local clubs and societies.

2.3.4 The Italian situation

(by Abrami)

The use of wooden piles to consolidate the foundations of a building or other important construction is not common in Italy. After the Neolithic period, when pile-dwellings were built on lagoons and lakes and on riversides, this type of construction disappeared almost completely. In the Lagoon of Venice however, where building on water become part of an almost unique way of life, pile constructions stay important. After the collapse of the Roman Empire, many settlements developed within the different lagoons of North Adriatic Sea. One of these, located on and around the islands of *Rivo Alto* and *Olivolo*, gave rise to a real town: Venice.

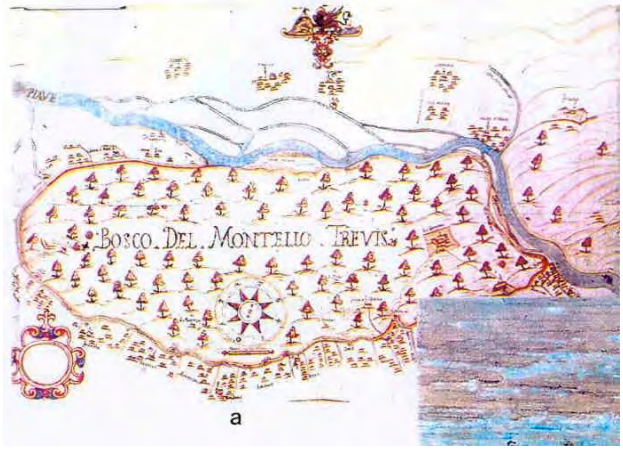


Figure 52a: The famous oak forest of the *Montello*, a hilly area of the Veneto region at the foot of the Alps, with the river *Piave* in the back. (A mup of the 17th century, *Treviso*

Figure 52b: A water-colour of Basilio Fasinio (1825) depicting a raft made of five sections of piles floating on the river *Piave*, near *Nervesa* (*Treviso*).

Until the end of the medieval period, most of the piles needed for the foundations were obtained from forests on the near mainland. Common houses built on the marshes were made by wood and straw or reeds. Through Venice's growing commerce with northern Italy, salt and other merchandise were exchanged for stone and marble as well as logs that were brought from the Alps floated down rivers.

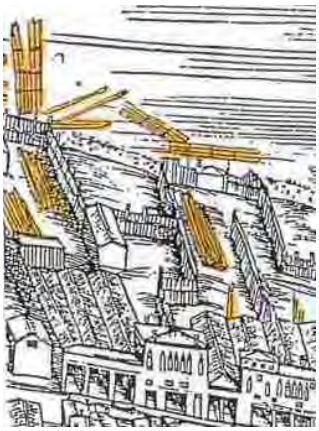


Figure 52c:
Wooden piles floating in the lagoon or stored near the Arsenal of Venice. (From the mup of de Barbari, 1.500).

Figure 52d:
A pictorial view of Venice (17th century) with the skyline of the Alps mountains. The tree vegetation in evidence by green colour.

Figure 52e:
Some of the remaining woods on the mainland near Venice (middle of 19th century) from a mup of Giovanni Battista Gorlato, Venice 1847. (In evidence the woods by green colour).

After the conquest of the Veneto region by the Venice Republic, as well as of most of the Istrian and Dalmatia coasts (beginning of 15th Century), logs were almost exclusively taken from hilly and mountain forests. From this time Venice's buildings were almost all built of brick and of white Istrian stone sustained by wooden foundation piles. These techniques were applied, with small improvements, until the last century. Millions of piles are now lying under the mud, clay and sand of the lagoon. They support the City of Venice and the settlements on other islands.
In which conditions are they at present? Few studies have been made on the different aspects of wood decay and on the endurance of the piled foundations.

2.3.4.1. State of the art of Italian knowledge on wooden foundations piles used

Recent research in Italy has mainly focused on four fields of investigation:
a) Dendrochronology, using tree-ring and radiocarbon analysis.
b) Decay, due to xylophages, like the *Mollusca Terenidae* and the *Crustacea Limnoridae,* acting at the intertidal zone in aerobic conditions.
c) Decay, due to bacteria and fungi acting often in anaerobic conditions.
d) Decay, due to physical-chemical processes.

Dendrochronology has witnessed great development, mainly in connection with archaeological studies. For instance oak chronology, related to *Quercus robur* and *Quercus cerris*, was applied using the "wiggle – matching" method of tree – ring analysis and radiocarbon dating on pile dwellings of settlements of Middle Bronze Age (between B.C. 1630 and 1637) near Lake Garda (Martinelli and Kromer, 1998). Other sites have been investigated within the Polesine region (Marzatico and Salzani et al., 1996). A review on dendrochronological investigations for the Alpine area is given by Martinelli (1996).
Within the city of Venice excavations were carried out recently on different sites, including the foundations of the *Malibran* theatre and the ex-cinema *San Marco* (Martinelli, personal communication) (Fig. 53). The samples came from piles and planks made of elm and oak. Using the wiggle – matching method, the samples were dated at the 7th Century (between A.D. 658 and A.D. 687). This is a period in which the Lagoon of Venice was sparsely settled.
According to the work of DENDRODATA s.a.s., which carried out most of the dendrochronological research in Venice on behalf of national Authorities and private Agencies, most of the piles used for the foundations, were of *Quercus robur*, while for upper building structures larch (*Larix decidua*) was preferred, at times substitute by silver fir (*Picea alba*) or spruce fir (*Picea excelsa*).

Figure 53a: The oldest foundation piles found recently within the basement of the building of the *ex-cinema San Marco.*

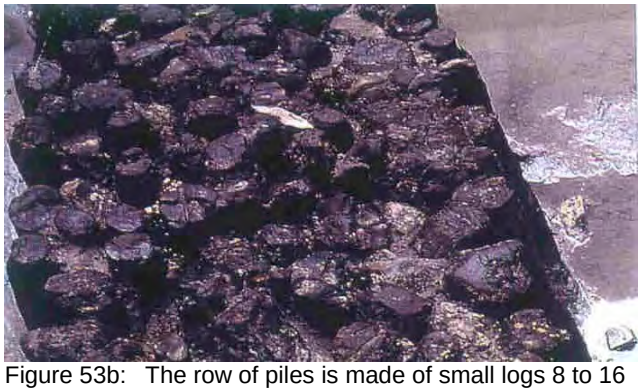


Figure 53b: The row of piles is made of small logs 8 to 16 cm width.

Research on decay of wooden piles due to xilophages living in the intertidal range, including mollusc of the genre *Teredinidae*, and crustaceans such as *Limnoia tripunctata* and *Chelura terebrans,* have been made by the Consorzio Venezia Ricerche, an enterprise comprised of public and private Agencies.
The aims was to find a way to classify the state of decay of archaeological wood (personal

communications, 2002). Most of the researches done in Italy in the last decades have been on the decay of wood find in archeological findings, especially relicts of ships. For instance Abbate Edlman et al. (1989) of the *Istituto per la Ricerca sul Legno* of the C.N.R. in Florence, made an indirect scanning electron microscope study on bacterial and chemical attak on a relict of a ship of 17th century B.D. finded near the *Isola del Giglio* (Tuscany). D'Urbano et al. of the *Istituto Centrale per il Restauro*, Rome, made and indirect evaluation through a biochemical analys with the Hoffman metod, of the decay of the wood of a relict found near *Torre Flava* (Ladispoli, Rome).
An attempt to study wood decay to find the best way to preserve pile foundations and other wooden structures in Venice, was made by a group of enterprises and researchers within the EUREKA Project, EU 1069, in co-operation with the local enterprise INSULA S.p.a. (Venezia Care, 1995). The study was done in connection with the renewed cleaning of the small canals of Venice. Several samples were taken on the foundations of the *Ex-Convento San Giovanni*, along the *Rio Novo* and at the *Convento Le Terese*.

2.3.4.2. Venice: 15 centuries experience with wooden construction in and on water

A few centuries after the collapse of the Roman Empire, different settlements were growing here and there within the North Adriatic lagoons, on small islands often surrounded by large areas of salt marshes. These included *Grado*, *Eraclea*, *Torcello*, *Rivo Alto*, *Olivolo*, and *Malamocco*. At the middle of A.D. 800 *Rivo Alto* and *Olivolo*, slowly becoming a real town. This was organized along a principal water way, the *Canal Grande* an old river-bed. Many more little canals and natural marsh channels (locally called *ghebi*) were dug, making them suitable for navigation, at the same time feeding a process of land reclamation that was active for centuries. A clear demarcation between land and water was obtained after raising the soil level. The perimeter of each settlement was consolidated with tight rows of sticks that included fascines of reed (Cassiodorus, A.D. 537). The inhabitants lived in wooden houses roofed with thatch. Only the most important public buildings, such as the Doge's Palace, the church of *San Marco* and a few others, were built of brick and stone.
From the 14th century, after repeated outbreaks of fires, building were commonly built of more solid materials. The town became one of the most populous in Europe. As space was limited, houses that were originally for the most part two stories high, had to be raised up to five floors. The foundations had to be replaced or reinforced, while the architecture sometime shows the superimposition of different styles.
In particular, a final stage of foundation construction techniques (These techniques, to some extent, where well known since Vitruvius, the Roman father of Architectura, who wrote in his Ten Books (charter 9, par.10): "p*iles driven close together beneath the foundations of buildings take in the water that their own consistency lacks, and remain imperishable forever, supporting structures of enormous weight and keeping them from decay…One can see this at its bests in Ravenna; for there all the buildings, both public and private, have piles of this sort beneath their foundations*."), with several rows of big piles, solid debris fitted within them and a *Zattaron* of planks laying over, was reached in the 16th century. Little changed after this time.

2.3.4.2.1. Venice: lagoon environment (water, soils saltmarshes)
In the North East of Italy the climate ranges between continental and sub-Mediterranean values with an average temperature in July of 23° C (max 29° C) and in January of 4° C (max. 12° C). In this area, the Lagoon of Venice is the largest among the many that once were present from the Po Delta to the carsick hills of *Trieste*.

This lagoon is a body of shallow water large about 55,000 hectares, separated from the sea by a small range of sandy dunes. Salt water is brought in through three mouths two time a day, generating a tidal range of about 60 cm. The salinity within the lagoon varies from 34‰ near the mouths to 23‰ near the river estuaries, with an average of 32‰ within the City of Venice. Water temperature within the city channels, where there exist a continuous

discharge of the sewers, ranges from a maximum of 25° C in July – August, to a minimum of 6°C in January-March.
Concentrations of some common pollutants of these waters are given as following:

N – $NO_3^{(-)}$	13,46 mg/l
P – PO_4	0,43 mg/l
Si-SiO_4	9,15 mg/l
S	31,2‰.

The highest tide, to date, followed the ruinous flood of 1966 when a level of 1,94 m above median water level was recorded.
The formation of the lagoon was the result of an interaction between the sediments discharged from the river, mainly the *Brenta* and *Piave* rivers, and the action of the sea. The rivers' contribution to the morphology of the area has been in terms of a large amount of silt and clay that tended to fill up the water body, while the sea has brought some sandy transport. The sea action is expressed mainly in terms of erosion and the subsequent washing out of the soil to the Adriatic Sea.

This in fact is the prevailing trend in the current conditions of the lagoon, where man made transformations of the morphology and hydrology (dikes and groin constructions, excavation of a large shipping channel, etc.) have had a serious impact on the survival of this kind of environment. Subsidence and sea level rise are other concomitant factors that have generated a loss in the ground level of Venice of 23.5 cm in the last century alone.
The marshes land that once (early 19th century) covered large part of the surface of the lagoon, at present survives only in small area (Fig. 54). Soil erosion affects first of all these deposits where halophytic vegetations play an essential role in trapping floating sediments. The flatter bottom of the lagoon is suffering from erosion too as the water depth, once around 70-80 cm is now, reaching points with more than - 1.20 cm.

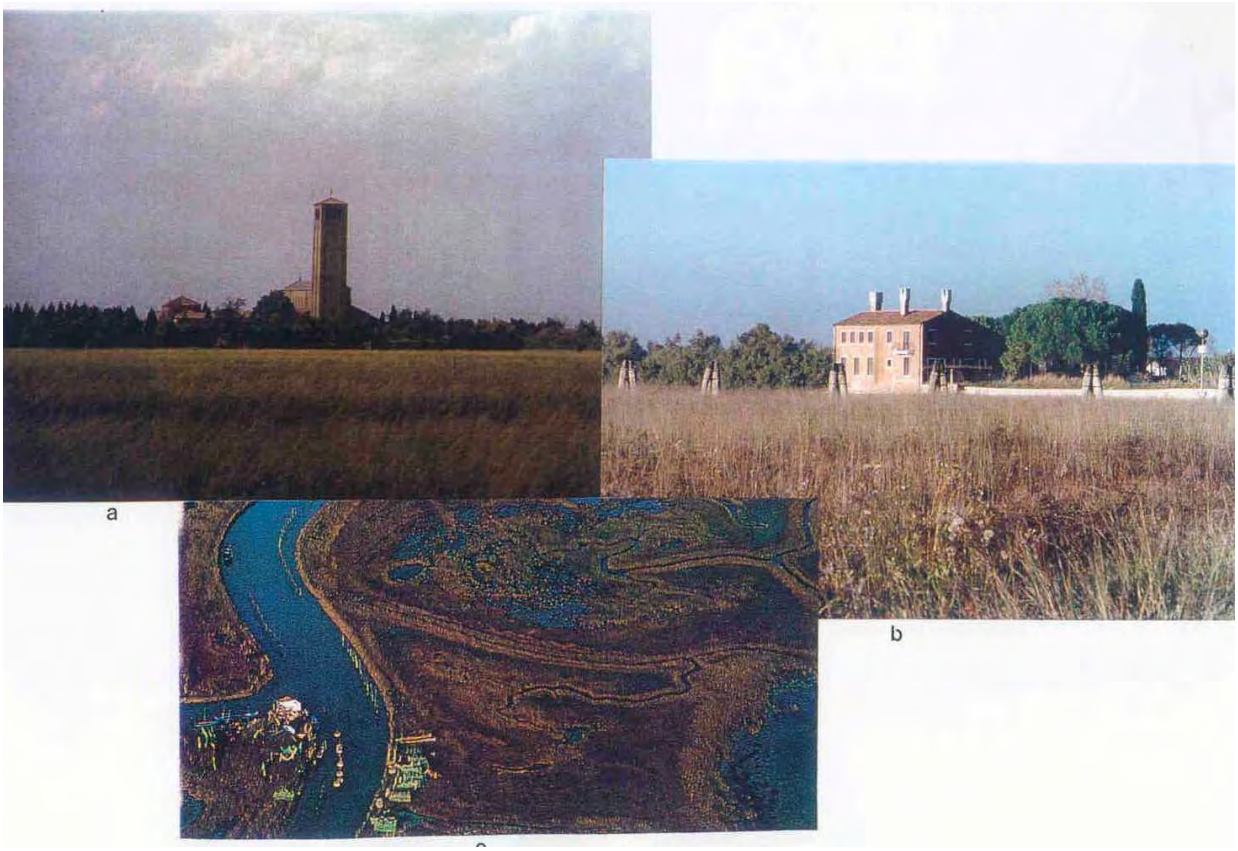


Figure 54: Some views of typical marsh lands (*barene*) near the island of *Torcello*. (a and b. Photo Abrami, 2001).

By the end of the 13th century, the city of Venice was a true metropolis with a population of nearly 120.000. Organised along its canals so that each principal building and the main public open spaces had their water as well as land access. The urban structure maintained a close physical relationship with the natural marshes of the surroundings.
This is well described by the Barbari's map of 1500 (Fig. 55), showing that gardens and vineyards were common in the periphery of the city, especially within the Monasteries.
During the first centuries of its existence Venice was a semi–independent province of *Byzantium*, and thus very much influenced by Byzantine architecture, as seen in the cathedral of *Torcello* founded in A.D. 639 and in the church of *Santi*

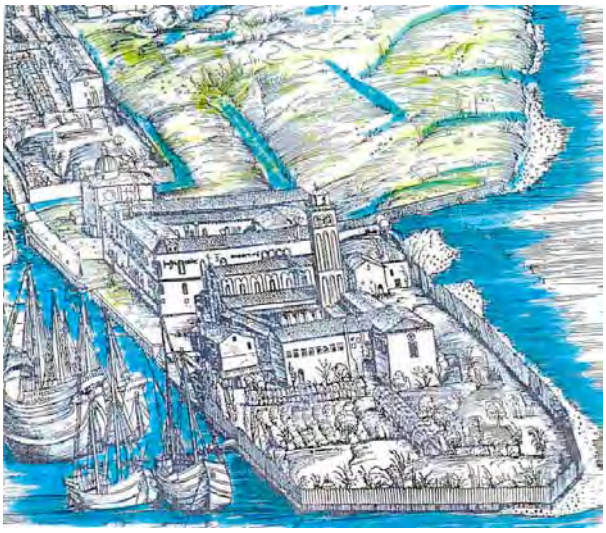


Figure 55a.
A view of a Monastery settlement in the northern part of the city of Venice from Jacopo de Barbari's maps (1.500). Clearly it appears the marsh area, yet to be reclaimed for new constructions.

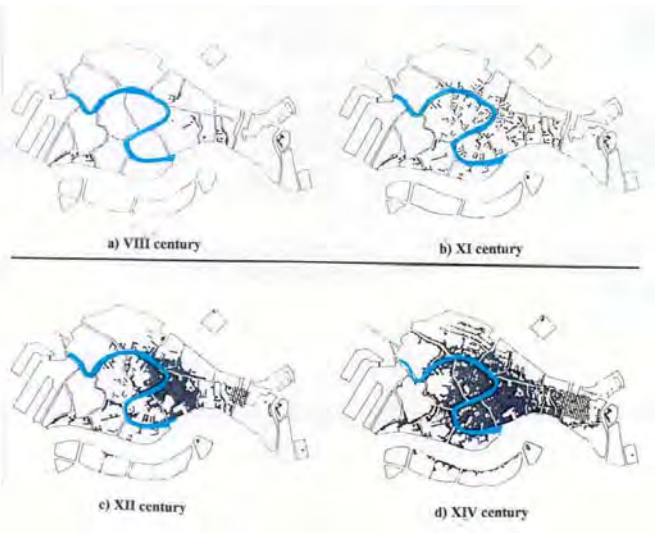


Figure 55b: The urban growing of Venice, from A:D: 700 till 1.300 (Maretto, 1969).

Maria e Donato of Murano of the 7th century. The *Basilica* of *San Marco* itself, whose original structure was built in the middle of the 9th century, for long time the private chapel of the *Doge*, is a variation on the Byzantine theme of the Greek cross supporting a large central dome.
Some early data about Venice (1346) reveal that there were more than 70 parishes situated on different islands connected by bridges. In this way the city was already a compact "land-mass" of buildings framed only by each others. A number of the original canals have then been filled into make streets *le calli* or closed courtyards, and their presence is recorded by name like *piscina* or *rio terà.*

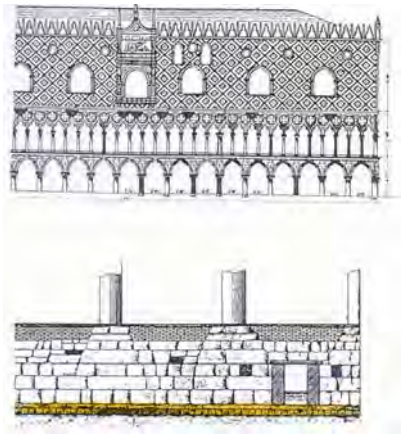


Figure 56a:
The foundation of the Doge Palace made of Istria stones laying over a *Zattaron* of timber planks (Malvezzi, 1874).

Figure 56b:
The excavations within the archaeological site of *S. Alvise*, with the foundations dating 13th – 14th century.

Figure 56c:
The long wall of Istrian stones, with its foundation piles beneath, dating 16th century, found on the island of *Giudecca*.

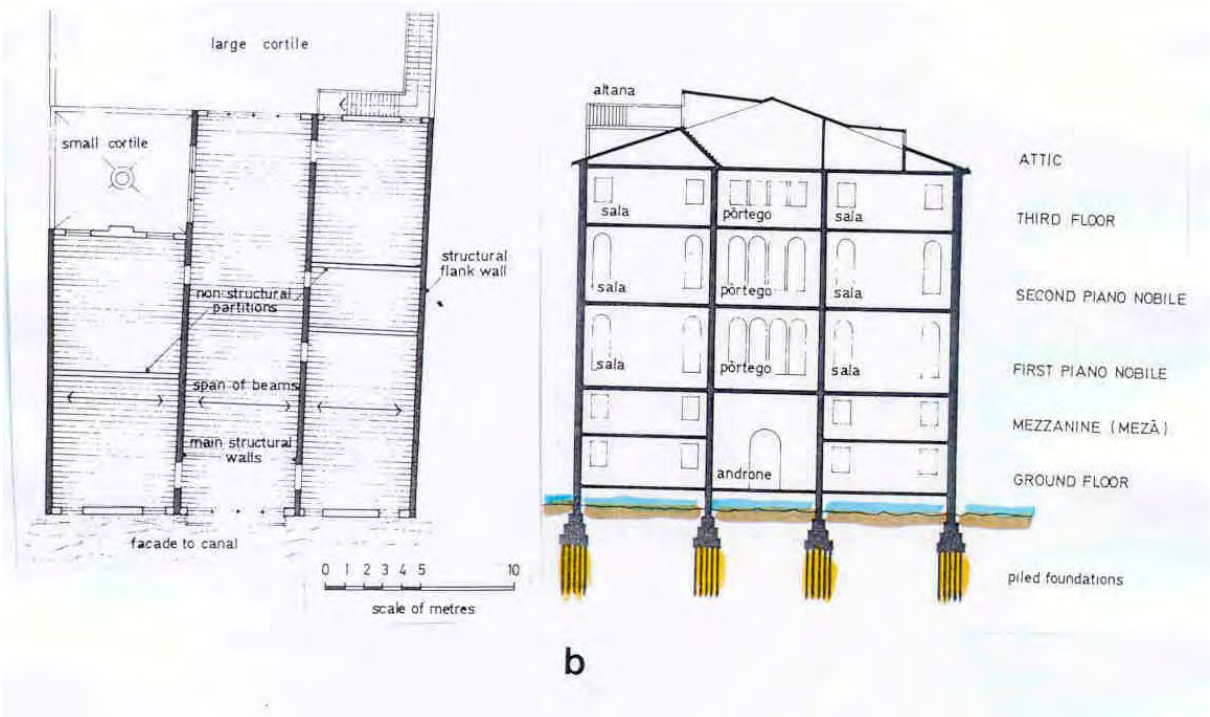
Today we recognise that the fragility of Venice and other settlements within the lagoon, is due mainly to the following factors:

a) the slow sinking of the ground, normally around 1 cm each year, that is worsening by the sea level rise;
b) the humidity and salinity absorbed by the walls;
c) the mechanical action of the waves on walls created by motor-boats on canals, especially along the intertidal surface, the most exposed to erosion;
d) the pollutants present in the water coming from the sewers, which discharge in to the canals;
e) the bacterial and other organisms that attack the wood of piled foundations and of other wooden structures of the buildings.

2.3.4.3. Construction techniques for walls and palaces

Considerably changes in the way Venice was built are related to time, to the different loading capacity of the ground and to the importance of the buildings. (Fig. 57a and 57b)

Figure 57a. Examples of the main facade of a city palaces facing the *Canal Grande* in Venice.



57b. The system of construction of the largest palaces in Venice with a structure based on wooden foundation sustaining the main walls

Some of the oldest settlements reveal a technique of land reclamation from marsh areas, these include settlements found near the old mouth of the *Brenta* river, at *Fusina*, datable around AD 800-1000. The area was enclosed by different layers of fascines strengthened by small piles of alder (*Alnus glutinosa*), oak (*Quercus robur*), elm (*Ulmus minor*), and the European hophornbeam (*Ostria carpinifolia*). All these species could be found at that time in the nearby forest of the Po plain. In 1999, during the work of restoration of the *ex-Cinema San Marco*, a foundation was found, composed of several rows of rather small piles of 8-14 cm in diameter (Fig. 53). The construction is dated at AD 1300.
The excavations undertaken recently near *Sant'Alvise* in *Cannaregio*, on the northern margin of Venice (Fig. 56), has shown how the settled area was enlarged using a sequence of embankments made of oak piles connected horizontally, at the intertidal level of the lagoon, by planks fixed with wooden nails. The ground was then filled with mud dug from the surrounding canals, together with old building material and other types of debris. In another interesting finding, on the *Giudecca* island, an old fondamenta (a pathway located at the margin of water, buildings or squares) built between AD 1300 – 1500., was recovered after a casual excavation. The oldest part of the wall is made of a small type of bricks, the latest of Istria stones. The foundations beneath are made of piles of black poplar (*Populus nigra*) and of European white birch (*Betula alba ssp. pendula*), while the large planks over the piles are made of oak and larch.

The typical structure of a palace facing a larger canal in Venice is that represented in fig.57. The pile foundations interest only the main structural walls. The piles were driven into the soil by a handy system or more recently by a machine like that represented in fig. 58.

Figure 58a.
A system for driving wooden poles by hands.

Figure 58b.
As late as in 20th century a machine called *Campanella* was still in use.

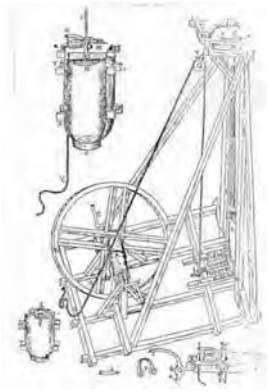


Figure 58c:
A special machine used to drive piles.

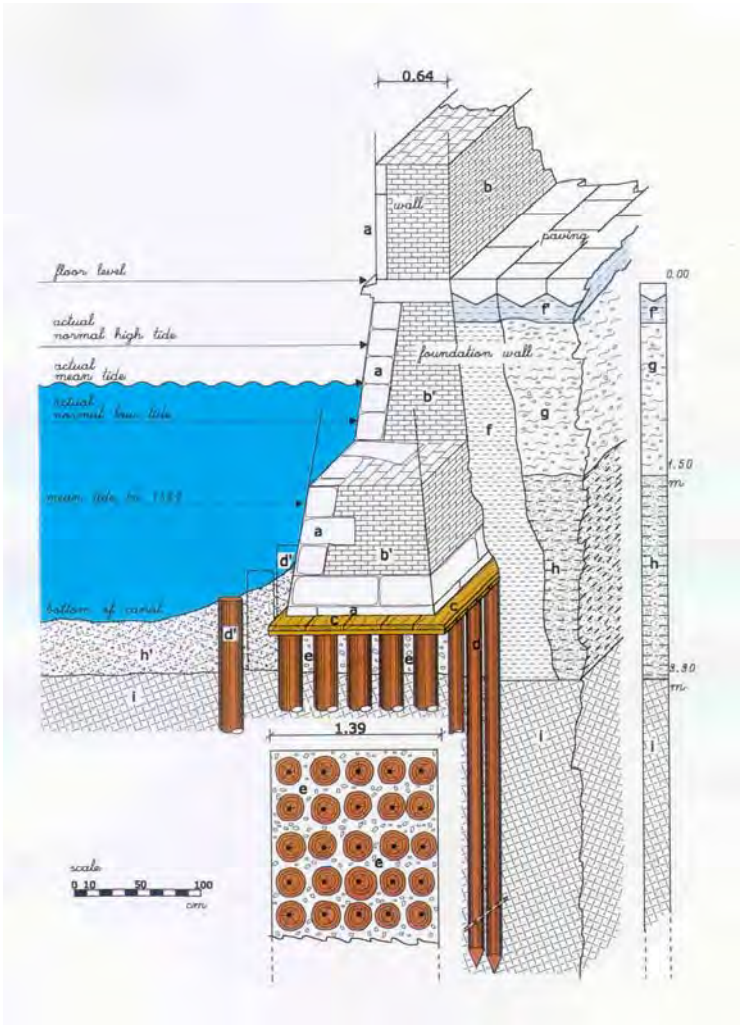


Figure 59. Schematic representation of a piled foundation of a large city palace in Venice. (After Sansovino, 1562).

a) Speeding courses of Istrian stone.
b) Brick load bearing wall, approx 64 cm thick.
B') Brick foundation, approx 130 cm thick at the base.
c) Deck or *Zattaron* made of two or three layers of timber planks, 25 to 50 mm thick, usually of larchor oak, occasionally walnut or pine.
d) Timber piles of the footing 120 to 200 mm thick, two to four metres in length, obtained from different plant species, more often oak, also elm, alder, hornbeam, poplar, birch and other species.
e) Spaces between the piles, filled with broken stones and other material.
f) Layer of clay lining the inner face of the footings.
F') Beaten earth, with mortar.
g) Layer of debris, mud and other material.
h) First natural layer of silty-clay, with organic matter.
i) Layer of sandy-clay with shells.

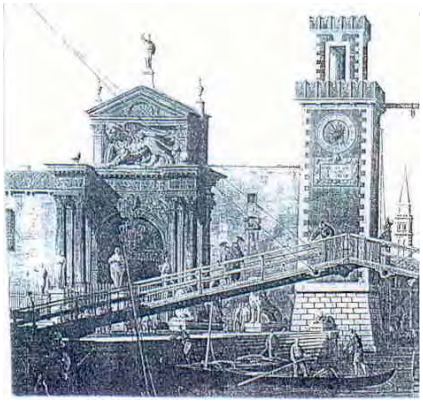


Figure 60a.
A 18th century print of the entrance of the *Arsenale*, the ship-yard of the Republic of Venice for more than a thousand years.

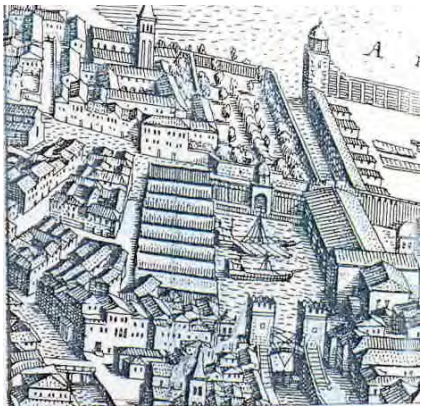


Figure 60b.
A view of the *Arsenale* from de *Barbari's mup* (1500).

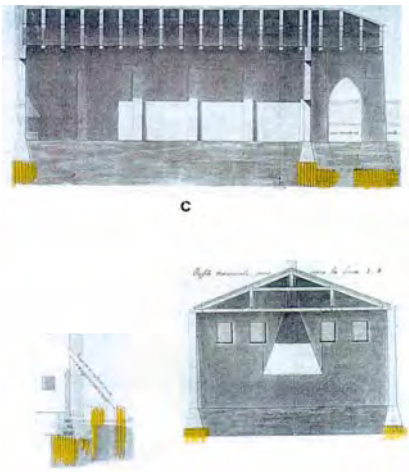


Figure 60c.
The piled foundation of some building of the *Arsenale* (constructions of 1700-1800).

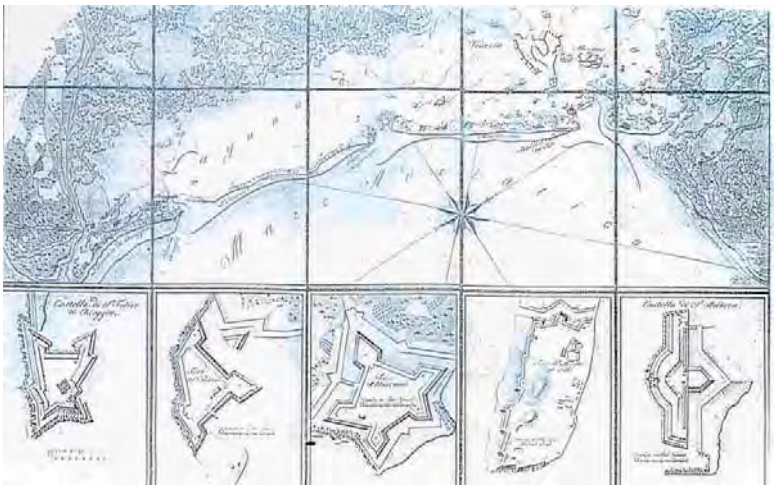


Figure 61: The fortified islands and many fortress built to defend Venice at the time of the *Serenissima Repubblica* and during the Napoleon and Austrian occupations. Figure 11a. A map with the fortification along the littoral.

Figure 61b:
the *Sant'Andrea* fortress design by Michele Sanmicheli (1543) near the mouth of *Lido*.

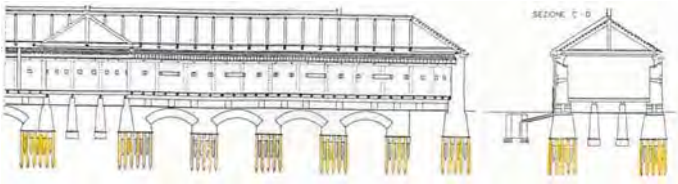


Figure 61c.
A particular of the piled foundation of the Sanmicheli fortress.

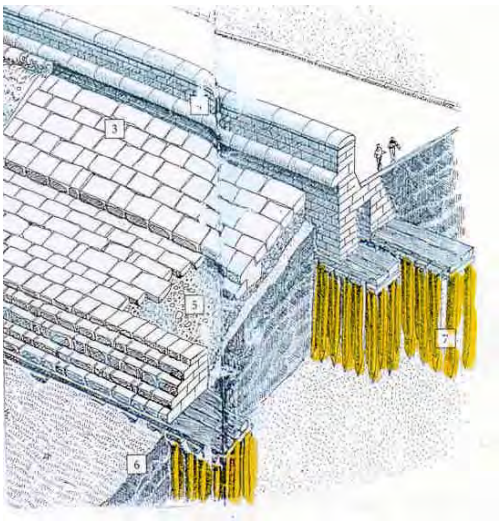


Figure 62a:
The embankment of Istria stone, called *I Murazzi*, made to protect the thin sandy dune range along the littoral of the Lagoon of Venice with its piled foundation (end of 18th century).

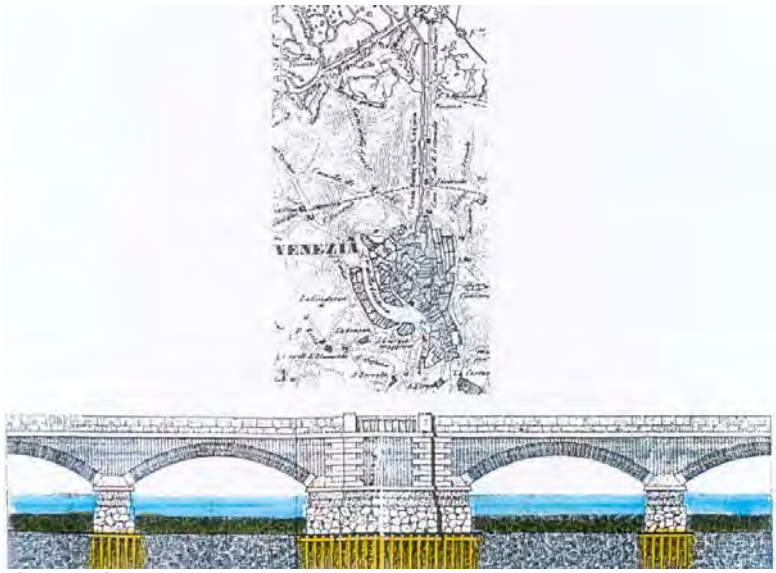


Figure 62b:
The wooden foundation of the *Ponte della Libertà*, the bridge built by the Austrian that connects Venice with the mainland (Gorlato, 1841).

Figure 63a, b and c.
The tower of *S. Marco*, that collapsed in 1902, had a foundation made of many courses of Istrian stones and a Zattaron made of two thick layers of plank lying on a few rows of wooden piles.

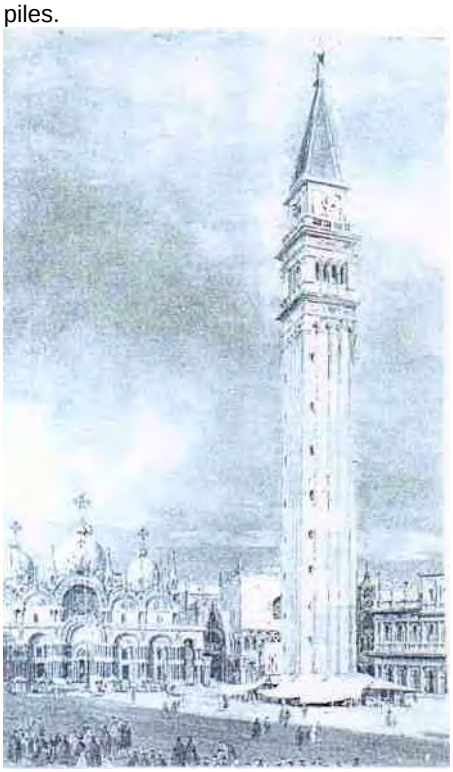


Figure 63a

Figure 63b

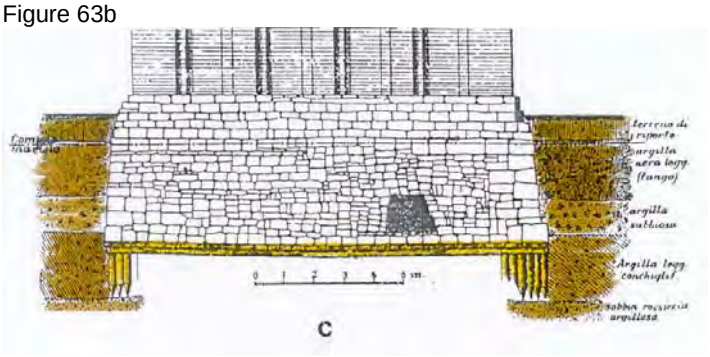


Figure 63c

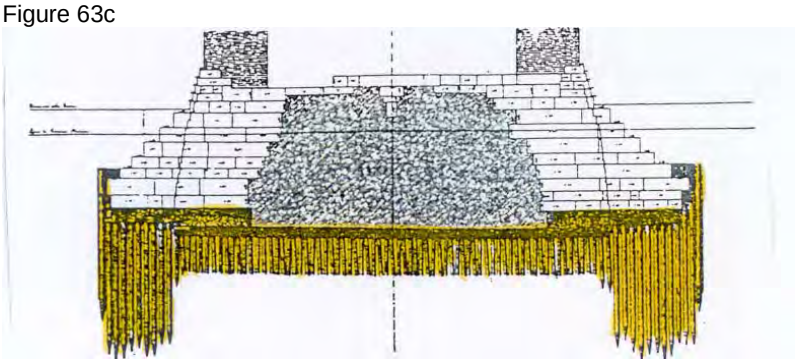


Figure 63d: After the disaster a new foundation have been built adding many rows of piles.

2.3.5 The Swedish situation

(by Nilsson & Björdal)

When the wreck of the warship Vasa was recovered 1961, bacterial degradation of wood was virtually unknown. Examination of some of the timber revealed an unusual form of decay that clearly was not of fungal origin. It was referred to as possible bacterial or chemical corrosion. No further attempts were made to identify the cause of degradation. Much later our studies here (SLU) revealed that bacteria were responsible for the attack.

During the 1960s and 1970s, studies on wooden foundation pilings in Sweden (Stockholm and Gothenburg) were carried out by Bravery, Boutelje and Göransson. They concluded that the degradation observed, was caused by bacteria. Only little attack by soft rot fungi was seen, mostly in the perpheral parts of the pilings. Isolations yielded a large number of different species of bacteria such as *Micromonospora*, *Plectridium*, *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Flavobacterium*, *Spirillum*, *Cellvibrio*, *Cytophaga* and *Cellulomonas*. Attempts to reproduce bacterial attack on wood in the laboratory were not successful.

It was observed that the peripheral parts were the most degraded and that attack gradually decreased towards the centre of the pilings. Pine heartwood was found to be quite resistant and there was some evidence that spruce pilings were slightly better than pine.
The attack resulted in significant losses in compression strength and depletion of the wood carbohydrates. The effect on DP of remaining cellulose was marginal.

Environmental analyses indicated pollution of the ground water with elevated levels of nitrogen and phosphorus. Oxygen levels were in most cases low. The most severely attacked foundations were surrounded by porous fillings (gravel, stones, soil, mortars of lime etc.), whereas the less degraded pilings were surrounded by clay. A distinct correlation between the amount of organic nitrogen and phosphorus in the wood and the degree of degradation.

At the end of 1970s we were able to demonstrate that bacteria could in fact degrade wood in the laboratory. This was done by creating small microcosms simulating piling conditions. Later a new group of wood-degrading bacteria was discovered, the tunnelling bacteria (Nilsson and Holt 1982, Nilsson and Daniel 1983). The new findings stimulated the interest in wood-degrading bacteria and lead to a series of electron microscopical studies (Nilsson and Singh 1984, Daniel and Nilsson 1985, Daniel and Nilsson, 1986, Daniel, Nilsson and Singh 1987, Singh, Nilsson and Daniel 1990, Nilsson, Singh and Daniel 1992). Attack by erosion bacteria was first described from horticultural wooden posts in New Zealand (Nilsson, 1984). It was soon realised that erosion bacteria also were responsible for most of the degradation in waterlogged archaeological wood. In the laboratory we managed to establish mixed consortia of bacteria, which could be used to reproduce attack by erosion and tunnelling bacteria. A very large number of pure strains of bacteria were cultured on wood, but none of them caused any attack.

Later a detailed study on the degradation of waterlogged archaeological wood was undertaken (Björdal, Nilsson and Daniel 1999,Björdal 2000). Erosion bacteria were found to be responsible for the main part of the degradation. No correlations were found between severity of attack and the age of wood. It was difficult to evaluate the effect on degradation by environmental conditions, except for the fact that depth of burial was an important factor. Degradation in most cases decreased with increasing depth (Björdal, Daniel and Nilsson 2000).

There is little doubt that bacterial degradation of wood represents a problem also in Sweden. Waterlogged archaeological wood is slowly degraded by bacteria, but little is known about the rate of attack. Ongoing reburial experiments may provide some insight at a few sites.

Wooden foundation pilings are used in Stockholm, Göteborg, Malmö and Uppsala. Most of the occurring problems are dealt with by private enterprises. Thus, there is no accessible information. We have been involved in the problem with the ca 15.000 pilings supporting the parliament building in Stockholm. The extracted piles were degraded by erosion bacteria along their full length (ca 7 m) to a depth of ca 10 mm. A dam is now being built that will increase the water table ca 0.7 m in order to decrease the rate of degradation. We have also examined samples of two piles from a property in Uppsala. Here we found attack by erosion bacteria and also by white rot.

Boron treatments of ground water surrounding pilings have been carried out in Sweden by Kjessler & Mannerstråle Byggteknik AB. A boron solution referred to as “Jerbor” was used. We got no information on the number of sites that have been treated and we have not been able to find any evidence that the treatment prevented further bacterial attack. We have heard that raising of the water table has been tried in order to slow down or prevent further attack. However, we have not been able to find any information on sites or results.

Chapter 3

Sample sites

(by Gelbrich, Huisman, Kretschmar, Klaassen, Keijer, Lamerdorf)

3.1 Introduction

Chapter 2 summarises the present knowledge of the impact of bacterial wood degradation for constructions and archaeology all over Europe. This information is limited and the aim of this project is to get and objective impression of the conditions under which this type of degradation occurs. The 27 sample sites are therefore sampled and analysed in such a way that the results are comparable. Furthermore the site are chosen in such a way that main variation in time, timber species and environment is covered.
Based on the results from all sites the knowledge on the process of bacterial wood degradation should be improved.
Because it was realised that soil and groundwater parameters can show a wide variation over time, long-term soil measurements over a period of 1,5 year were carried out in two sites with more or less comparable conditions but with a difference in degree of bacterial wood degradation. These long-term measurements should validate measurements of physico chemical parameters, though minimisation of installation disturbance by a one year measuring period. Furthermore they should provide more reliable information on the role of oxygen in relation to bacterial decay and to seasonal variation of physico chemical parameters.

3.2 Field and laboratory work

The table below summarises all site sampled. Besides the location and the type of the construction and estimation of the ages is given.

Location	Location description	Age			location
1	foundation	100		pile heads	Netherlands, Amsterdam
2	foundation	100		pile heads	Netherlands Dordrecht
3	foundation	100		whole piles	Netherlands, Amsterdam
4	foundation	100		Whole piles	Netherlands, Haarlem
5	foundation	100		whole piles	Netherlands, Rotterdam
6	foundation	100		whole piles	Netherlands, Koog a/d Zaan
7	foundation	100		whole piles	Netherlands, Haarlem
8	terrestrial archaeology	2000			Netherlands, Dokkum
9	terrestrial archaeology	2000			Netherlands, Dokkum
10	terrestrial archaeology	2000			Netherlands, Borsele
11	terrestrial archaeology	2000			Netherlands, Vleuten de Meern
12	marine archaeology	300		ship wreck	Netherlands, Waddensea
13	marine archaeology	300		ship wreck	Netherlands, Waddensea
14	terrestrial archaeology	650	Kemlade castle	whole piles	Germany, Travenhorst
16	foundation / archaeology/	2500		Lake Village	Great Britain, Glastonbury
17	terrestrial archaeology	2500		wooden road	Great Britain, Harter's Hill
18	foundation	500		pile heads	Italy, Venice
19	foundation	500		pile heads	Italy, Venice
20	marine archaeology	300	Stora Sophia	ship wreck	Sweden, Baltic sea
21	marine archaeology	300	Mollösund	ship wreck	Sweden, Baltic sea
22	foundation	100	parliament	whole piles	Sweden, Stockholm
23	foundation	100		pile heads	Netherlands, Leeuwarden
24	foundation / archaeology	250			Sweden, Bergen
25	fresh water archaeology	300	harbour construction		Sweden, Linkopingen
26	foundation / archaeology	2000	2000	pile head	Netherlands, Elst
27	reburied non protected ship wreck	300	300	ship wreck	Netherlands, Flevoland
28	reburied protected ship wreck	300	300	ship wreck	Netherlands, Flevoland

In order to avoid a mixed up of the samples a strict numbering system was used:

Position	description
First	sample site
Second	sample type: tc (timber core); td (timber disk); te (timber extraction pile); ta (archaeological timber); s (soil); w (water).
Third	pile number /pit or wooden object number /excavation
Fourth	distance to the pile head
Fifth	replica number

Before sampling took place, for each site information was collected about the soil profile, the hydrological conditions and the building.

3.3 Method

Soil measurements (by Kretchmar & Lambersdorf)
Before digging or excavating oxygen measurements, sediment water sampling and Redox measurements were conducted if possible in the undisturbed sediment. Oxygen is measured with oxygen optodes (PreSense, Regensburg, Germany). The Optode principle is the dynamic quenching of luminescence measuring the luminescence lifetime of a luminophore immobilised in a sensor foil (Klimant et al. 1995). A circle of 3 mm diameter of the sensor foil (PSt3-PSUP-YOP) is attached with silicone to the outer site of a polymer optical fibre fixed inside a stainless steal rod of 1 to 1.50 m which can be connected to the measurement device (Fibox 2-AOT). The tip to the so prepared optode is sensible to damage by stones etc. Therefore oxygen measurements are conducted using the following technique: first a stainless steal rod with the lower end closed by silica tape inserted in the sediment. With a metal rod is the silica tape is perforated and the stainless steal rod is flushed with nitrogen. Then the optode is inserted into the stainless steal rod and lowered until the sensor tip rest in the sediment. An especially developed borer to place the optodes in the sediment without damaging the sensor tip was not found to operate optimal. Before installation, the optodes were calibrated using water saturated air as 100% and water saturated N2 as 0%.
With mobile lysimeter probes, with a ceramic suction lysimeter head ground water was taken from which the pH was measured. The oxygen concentration in the sediment were measured every 10 cm starting approximately 50 cm above until approximately 50 cm below the pile head / archaeological sample. Three water samples were taken around the location of the pile head / archaeological sample (+50, 0, -50 cm). The Redox potential was measured in four depth approx. 20 cm above, at, 20 below and 50 cm below the pile head / archaeological sample.
After the pit or the excavation place was opened the sampling procedure was repeated and the soil was described.
Before sampling the wood, four soil and three water samples (glass bottles of 100 ml- 180 ml) were taken around the wooden object. The bottles are stored at a temperature of less than 4°C and kept in the dark.
All water samples were analysed on pH (repeated), conductivity, DOC (dissolved organic carbon), C_t (total carbon), P_t, DON, NH_4^+, NO_3^-, PO_4^{3-}, Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Al^{3+}, SO_4^{2-}-S, Cl^-.
The C and N contents in the water samples were determined by an automated C and N analyzer (Vario EL, Heraeus Elementar, Hanau, Germany). Dissolved organic carbon (DOC) was measured using a DOC-analyzer Total Organic Carbon Analyser TOC – 5050 (Shimadzu Corp., Tokyo, Japan). Total dissolved nitrogen (TDN), NH_4^+-N, and NO_3^--N were determined by a continuous flow system (SANplus Segmented Flow Analyzer, Skalar, Erkelenz, Germany) with photometrical detection. Prior to the photometrical detection, TDN was treated with alkaline persulphate digestion and UV digestion to convert both NH_4^+-N and dissolved organic nitrogen to NO_3^--N, afterwards all nitrate was reduced to nitrite by passing a column containing a cadmium-copper granulate.

Similarly, NO_3^--N was measured after its reduction to nitrite. DON was computed as total dissolved nitrogen subtracted by nitrogen and ammonium content.
Cl^- was determined by a continuous flow system equipped with an Ag/AgCl ion selective electrode. The pH was measured with a digital pH-meter (WTW GmbH Wesl-Germany) and the conductivity employing a conductivity meter (WTW GmbH, Germany). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany). SO_4 and PO_4 were derived from ICP S and P measurements respectively.

All soil samples were analysed on soil type, pH repeated (in water and in $CaCl_2$), N_t (total nitrogen), C_t, exchangeable cations (Ca^{2+}, Na^+, K^+, Mg^{2+}, Al^{3+}, Fe^{2+}, Mn^{2+}), S_t, P_t after HNO_3 digestion.
Sediment samples were oven-dried at 40°C, sieved (2 mm) and ball-milled. Total organic C and total nitrogen (N_t) contents of the samples were determined by an automated C and N analyzer (Vario EL, Heraeus Elementar, Hanau, Germany). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany) after pressure digestion in 65% concentrated HNO_3. Sediment pH was measured with a digital pH-meter (WTW GmbH, Germany) in water and 1 mol L^{-1} KCl (1:2.5). Sediment texture was determined after sieving and using the pipette method.

Long-term measurements
Because it was realised that soil and groundwater parameters can show a wide variation over time, long-term soil measurements over a period of 1,5 year were initiated. On the basis of an extensive study by FUGRO and SHR in 2000 – 2001 were across a north –south line through out Amsterdam many foundations were inspected, two locations with similar soil conditions were chosen, were remarkable differences in degree of decay appeared within the same wood species.
The total amount of foundation inspections in relation to the north-south subway in this area is 52. After a further selection on the type of wood (pine) and the presence of 0.5 m sand below the measuring level of the wood test hammer, 33 inspections remained. The data of these 33 foundation inspections showed that in the older, northern part of the area, which was developed between 1875 and 1885/1890, the average measured penetration depth of the wood test hammer was smaller than in the newer, southern part, which was developed between 1885/1890 and 1900. As penetration depth can be regard as standard for degree of decay it can be concluded that without differences in soil profiles, significant difference in degree of decay in wooden piles can occur.
A remarkable difference between both areas is that shallow foundations seems to be heavier degraded than the deeper foundations.
Differences were thought to be related to the amount of available oxygen in the groundwater or to the presence of a horizontal groundwater flow. In the northern part of the area the average groundwater level is about NAP -0.4 m and probably there is hardly any horizontal flow. In the southern part, specially near the Sarphatipark, the average groundwater level is deeper, at least NAP -0.6 m and increasing to NAP -1.0 m. The cause of the deeper groundwater level is the presence of this park, in which a low water level of NAP -2,0 m is maintained. So there is a horizontal flow of groundwater through the sandfill in the direction of the park.
From the 33 foundation inspections two locations have been chosen to perform long term measurements, one in the northern part (little decay) and one in the southern part (severe decay). Location 1 in the northern part of the area: Ferdinand Bolstraat 10 / corner 1^e Jacob van Campenstraat. Location 2 in the southern part of the area near the Sarphatipark: 1^e Jan van der Heijdenstraat 129.
In both locations a full set of above mentioned measuring techniques were installed together with 4 two meter long pine sapwood stakes which were brought underneath the groundwater table. The aim of the long term measurements is to get an idea of the gradient in time and in the vertical and horizontal soil profile of oxygen and other relevant organic and anorganic parameters.

At both locations, the measuring equipment has been installed under the pavement in steel boxes with dimensions of 1.0 by 0.7 m, for reasons of protection against the weather and vandalism. The ground inside of the boxes was excavated until 0.6 m below the pavement. The steel cover of the boxes lies at the level of the pavement.

Two locations have been used to execute the long term measurements:

- Location 1 (less degradation) in the northern part of the area: Ferdinand Bolstraat 10 / corner 1e Jacob van Campenstraat.
- Location 2 (more degradation) in the southern part of the area near the Sarphatipark: 1e Jan van der Heijdenstraat 129.

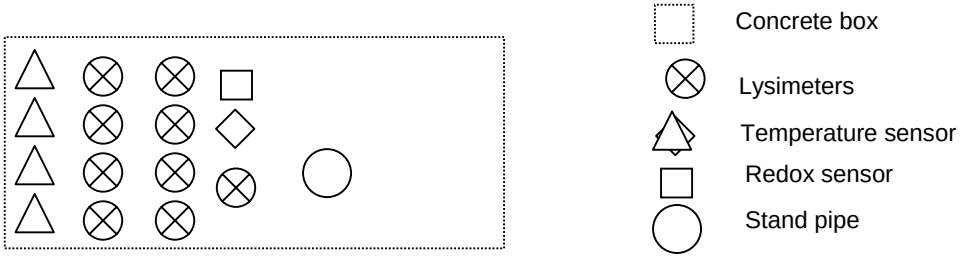


Figure 1: Overview of the sensors placed in the concrete box

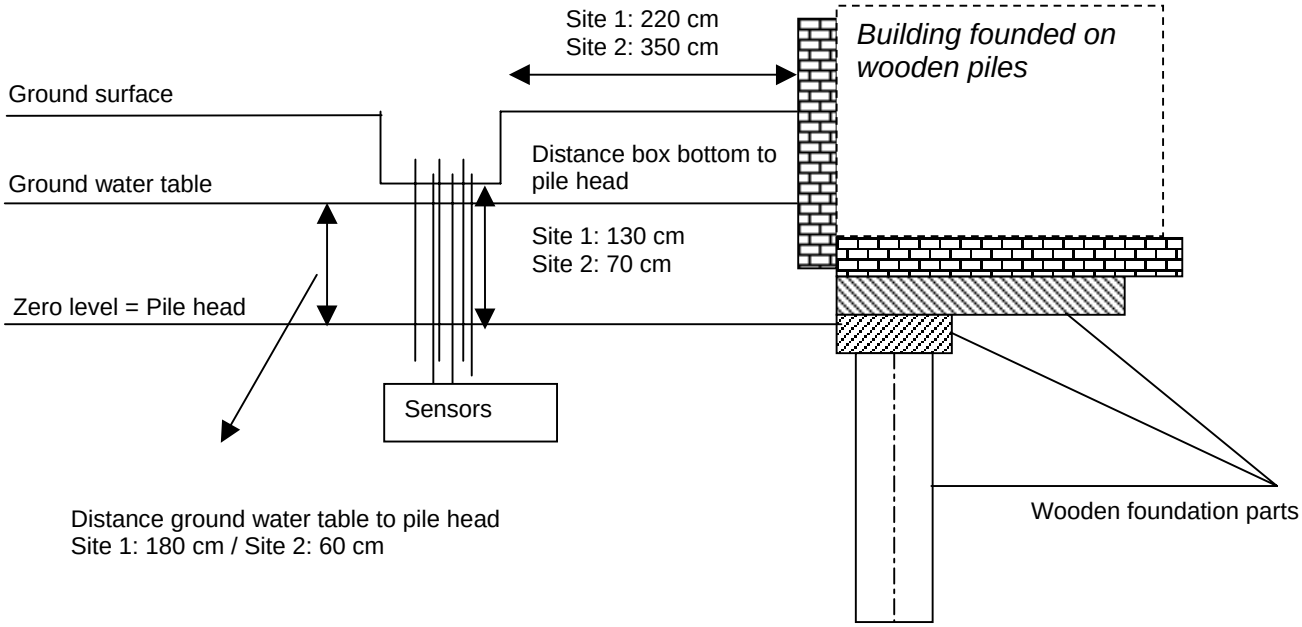


Figure 2: side view of the concrete box and sensors respective to the wooden foundation, for sensor length see table 1.

Comparison of the physical and chemical conditions surrounding decayed and very little decayed wooden foundation. Two sites with comparable sandy sediment were chosen situated not far form each other in the Ferdinand Bolstraat in Amsterdam near Sarphatipark. The wooden foundation consists of pine piles. The measurement period was one year from October 2003 to September 2004.

The measuring equipment has been installed under the pavement for reasons of protection against the weather and vandalism. Therefore steel boxes with dimensions of 1.0 by 0.7 m have been installed at both locations. The ground inside of the boxes was excavated until 0.6 m below the pavement. The steel cover of the boxes lies at the level of the pavement. The location of the boxes was chosen to be as close as possible to the wooden foundation.

Into the sediment under the open concrete box all the oxygen optodes and other sensors were installed. For the installation scheme see Fig. 1 and 2.
The oxygen sensors are installed in stainless steel tubes to facilitate installation and protected the sensible sensor tip from stones, for installation depth see Tab. 1. To minimise installation disturbance the tubes were flashed with nitrogen before inserting the oxygen sensors. The horizontal distance between concrete box and wooden foundation is about 2.5 m at site 1 and about 3.5 m at site 2. In addition to the measurement equipment at both sites 2 sawn pine pile (2 meter long) were installed and removed after 1,5 year and checked in the laboratory on decay.

Table 1: Sensor installation depth in relation to pile head at both sites, Site 1 pile head 130 cm deeper as box bottom, Site 2 pile head 70 cm deeper as box bottom

Oxygen	Temp.	Site 1 Redox Potential	Soil Solution	Wood Samples
+50		+50	+50	+50
+30				
-5*	-5	-5	-5	-5
-10				
-15		-15		-15
-30				
-50		-50	-50	-50
Oxygen	**Temp.**	**Site 2 Redox Potential**	**Soil Solution**	**Wood Samples**
+30		+50	+30	+50
+0				
-5*	-5	-5	-5	-5
-10				
-15		-15	-15	-15
-30				
-50		-50	-50	-50

*triplicate measurements

Visual inspection and wood sampling (Keijer & Nelemans)
After the pit or excavation was opened, the position and quality of the wood was determined. For foundation wood the hardness was measured with a Pilodyn hammer. Only if possible, the whole wooden objects were extracted and if they were too long they were sawn into discs of about 500 mm long. If extraction was not possible a disc (500 mm long) of the upper part of the wooden object was cut off (piles heads at foundation constructions). If this was also not possible cores were taken with an increment borer with a diameter of 10 mm. All wood samples were storied at a temperature of 4 °C and send to SHR were they were split, sealed in plastic and divided over the consortium fore further analyses.

DNA and phages analyses
From all wood samples, DNA spectrograms were made on the University in Portsmouth and phages were extracted by PhaGen (see chapter 4).

Dating of wooden objects by using Dendrochronology (Saß-Klaassen &Vernimmen)
Dendrochronology is a method to determine the age, growing period, felling date and origin of trees that have been used as building timbers.
Age determination of the trees is done by ring counting. If possible ring counts are corrected for missing rings due to incomplete radii, e.g. removed sapwood, rotten core or distance of sample from tree base.
The *exact dating of tree ring sequences and the determination of felling dates* gives an indication about the time when a wooden construction or a wooden object was build.

When the construction date of e.g. a historical building is documented the determination of the felling date provides *information about time of storage or transportation* of the timber before it was used in a construction.
If a tree-ring pattern is datable is also possible to get an indication about its origin. *Dendroprovenancing,* i.e. the determination of the area where trees grew, allows to reconstruct trade connections in historical periods and enables research about the relationship between wood quality and site/growth conditions of trees that have been used as building timber.

Bases of Dendrochronology
Tree-ring widths patterns, i.e. sequences of wide and narrow annual rings, of samples are measured. These mainly climate-induced tree-ring series are compared to a set of so-called master chronologies. These are mean tree-ring series build up from hundreds or sometime thousands of tree-ring sequences from trees of the same species, from a geographically well defined area (Schweingruber 1988). If successful, every tree ring on a piece of wood is assigned to a calendar year.

In the Netherlands, as in most of Northwestern Europe, the dendrochronological method has been successfully applied mainly to oak (*Quercus robur/petraea;* Jansma 1995). However, recently the dating of softwoods from monumental buildings has been proved possible (Sass-Klaassen 2000).

Sample collection, sample quality and sample preparation
The standard requirements for dendrochronological sampling are:
1) collecting several samples; combined tree-ring series from different samples enhance the chance of dating;
2) collecting samples from species that are datable, i.e. of which master chronologies are available for dating (ash, birch are not datable),
3) collecting samples from the same context or building phase,
4) samples should at least contain 50 tree rings and, if possible, sapwood and the last formed tree ring under the bark.
Tree-ring measurements can be either taken from stem disks or cores taken with an increment corer. At least two cores should be taken from one sample unit to account for variability of the tree-ring pattern around the stem circumference. Before measuring the tree-ring widths the transversal plane of the sample is surfaced with a stanley knife or razor blade to make the tree-ring boundaries clearly visible. The contrast can be enhanced by using chalk powder on the surface.

Tree-ring width measurement and analysis
Tree-ring widths of the consecutive tree rings are measured with a precision of 1/100 mm using a stereo microscope connected to a measuring table (*Lintab* by Rinntec, Heidelberg, Germany) and a computer. The measurements are recorded and analysed with the program TSAP (Rinn 1996). Two to four radii are measured on each sample to account for intra-tree variation in tree-ring pattern and to detect possible missing rings. Missing rings may occur (in softwoods and diffuse-porous hardwoods) in years where growth conditions were extremely unfavourable for the tree. The resulting tree-ring series are visually and statistically checked (programs TSAP, Rinn 1996; and COFECHA, Holmes 1983). Subsequently the single radii are averaged into mean tree-ring series for every sample. If possible tree-ring series of different samples from the same sample site are combined into site chronologies.

(Cross)Dating with regional master chronologies
Then all (mean) tree-ring series are ‘cross-dated’ (=compared) with regional master chronologies from different regions in Europe.

The RING foundation holds a database with in total about 100 regional master chronologies for oak, mainly from The Netherlands, Germany, Belgium, France, Great Britain, the Baltics including Poland and Scandinavia.

About 50 master chronologies for dendrochronological dating of softwoods (pine, spruce and fir) are available from Scandinavia (20), Germany/Switzerland (25) and Poland (4) (Jansma *et al*., 2002).
Since no softwood chronologies existed yet for the Netherlands five pine (*Pinus sylvestris)* chronologies have been established during this project. They represent the following regions in the Netherlands: Ede/NoordGinkel, Ommen/Eerde, Amerongen and Mattemburgh/Bergen op Zoom.

Cross-dating was performed statistically, by using programs *TSAP* (Rinn 1996) and COFECHA (Holmes, 1983). Different statistical parameters are used to detect and statistically justify a match:
t-test: Baillie (1982*):* t-value, should be greater than 3,5 to indicate match;
Gleichlaufigkeit (=Coefficient of parallel variation, Eckstein & Bauch 1969): indicates the % of parallel changes in tree-ring width values from year to year.
Running correlation: calculation of correlation between a time series and a chronology from other samples of the same collective in 50-year overlapping segments.

Precision of dating – Felling year
Often, the last tree ring(s) or even the whole sapwood is missing on a sample. This holds especially true for samples from archaeological context, which are sometimes seriously decayed. In this case it is possible to date the remaining heartwood rings of the sample and to use so-called sapwood statistics to estimate the number of missing sapwood rings in oak. The number of sapwood rings in oak varies depending on the site conditions and region where the oaks grew. Hollstein (1978) presented a sapwood estimation for oak from Central-West Germany. He calculated that 16 ± 5 sapwood rings can be expected for oak trees up to 100 years old, 20 ± 6 for 100 to 200 year-old oak trees and 26 ± 8 for oak tree older than 200 years. The average number of missing sap rings for oak from the Baltic is a little less than that of oak from Western Europe: 15 (+9/-6) (Wazny 1990). If no sapwood is present and an unknown number of heartwood rings is missing dendrochronology can provide a *terminus post quem* date (after…). This means that the earliest possible felling date can be indicated by taking the last dated heartwood ring and adding the estimated number of sapwood rings. Pine wood (*Pinus sylvestris*) has clearly distinguishable sapwood. However, the number of sap rings in trees from different sites and also among trees from the same site is very variable; no estimation of missing sapwood rings can be made. Hence only the presence of waney edge, i.e. the last tree ring under the bark, indicates the exact felling date.

Precision of dating - Felling season
Only if the last tree ring is present the exact felling date and even the felling season can be determined. Every tree ring can be divided in two parts by using wood-anatomical characteristics. First, the earlywood consisting of thin-walled tracheids (softwoods) or wide vessels (hardwoods), which is formed in (early) spring and early summer and second, the latewood with thick-walled tracheids (softwoods) or small vessels (hardwoods). Even if earlywood (EW) and latewood (LW) are not always well distinguishable, like in some softwoods, it is mostly possible to determine if the last ring is complete (EW+LW formed) or incomplete (only EW present) with the latter indicating that the tree was felled in the spring or early summer season. A complete last tree ring indicates either a felling campaign in summer, autumn/winter of the calendar date assigned to the last ring or in the winter/spring of the following year.

Dendroprovenancing
Dendroprovenancing is a relatively new discipline in dendrochronology. An indication of the origin of the timber can only be get if a network of regional chronologies is available for a certain species. The spatial resolution depends, of course, on the number of chronologies that are available for e.g. NW Europe but also on the diversity of environmental site conditions in a certain region that may cause 'typical' growth patterns. For this study regional chronologies from Great Britain, Scandinavia, Belgium, France, Poland/Baltics and five, especially for BACPOLES constructed, pine chronologies for the Netherlands were available.

Pattern of decay, density, compression strength and moisture content (by Klaassen)
The analyses were carried out along two radii If possible. Along a whole core taken with an increment borer or along a quarter sawn wooden strip (approximately 10 mm broad and 10 mm wide) thin section were made with a microtome of about 25 µm thick. The sections of softwoods were stained with *Picrine-aniline* blue and those of hardwoods were stained with *safranine-astra-blue* and investigate under the microscope. On the basis of the wood structure the species was determine and the type and degree of degradation was classified according to a five class system. For softwoods and hardwood a different descriptions of the degradation classification was used.

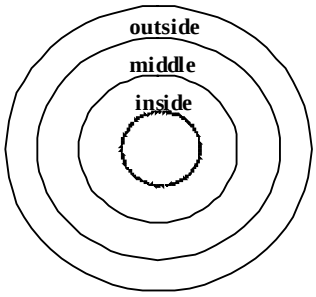
classification	**Description of the decay pattern in oak**
total disintegration cell wall structure	The cell wall structure is disintegrated but individual cell types - as well as the cell wall pitting and the vessel perforations - are still recognisable. The thick secondary wall of the fibres is missing or fully eroded and transformed in a granular structure. Under polarised light no or sporadic isolated single fibres are birefringent; in the latter cells the secondary wall is less eroded. The wall of the vessel and rays cells are sometimes weakly birefringent. The compound middle lamella around all cell types is present.
severe	As full degradation but the fibre wall disintegration in general is less extensive. More isolated single fibres are birefringent under polarised light and the fibre areas are weakly outlined as islands from the rest of the woody tissue.
moderate	A pattern of degradation is visible in the cell walls. Spots occur within the sound cell wall material, consisting of micro linear eroded areas following more or less the angle of the microfibrils. Under polarised light most fibre cell walls are weakly to clearly birefringent. The contrast with the axial parenchyma cells and the fibre tracheids - which are less birefringent – causes the fibre tissue to stand out as islands within the wood tissue as a whole. All fibre cell wall are swollen and degraded from the inside out, which causes the staining colour in the secondary wall to change form red to blue.
weak	As moderate degradation but the degree of degradation is less extensive. As a result, the fibre tissue is not outlined as islands in the wood tissue as a whole. Degradation is mainly found in the parenchyma and fibre tracheids.
sound wood	no patterns of cell wall degradation visible

classification	**Description of the decay pattern in softwoods**
total disintegration cell wall structure	All cell walls are eroded, non of the trachieds are birefringent. However all individual anatomical features are still recognisable.
severe	Most of the cell walls of the late wood are eroded, only small areas sharply demarcated from the degraded cell wall, is sound. Most of the early wood cell walls are eroded and show a micro-linear appearance.
moderate	In both late and early wood tracheids cell wall erosion is present. In early wood cells the pattern of erosion is restricted to specific areas within the cell wall and has a micro-linear appearance, whereas the pattern in the late wood cells consists of areas with eroded cell wall material in a shape triangles (a mixture of several small and enlarged ones). At least in the late wood the decay is more prominent in the tracheids adjacent to the rays and is restricted to individual cells.
weak	in some individual late wood tracheid adjacent to the rays, spots of cell wall erosion (on radial surface triangle shapes) visible
sound wood	no patterns of cell wall degradation visible

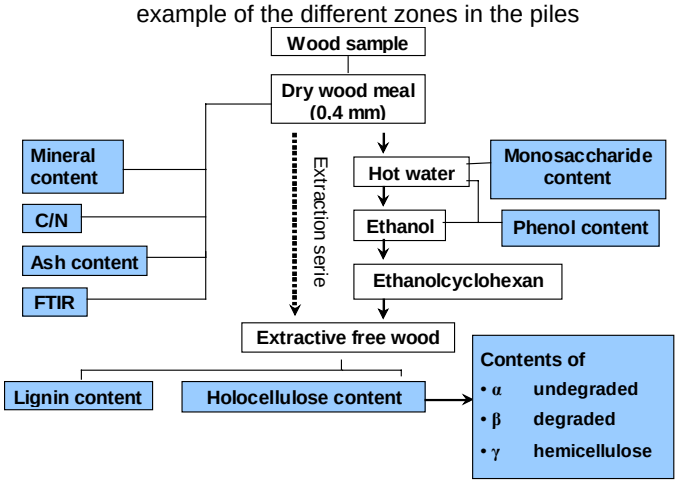
During sectioning the woody strip or core was divided in pieces of about 15 mm and the weight and volume of each of these pieces was determine before and after drying at 103 °C for 24 hours. From these data the moisture content and density – specific gravity was calculated. The judgement for the presence of heartwood was based on a staining reaction for pine and was made visually for oak.

Samples with an axial length of more then 30 mm were used for determine the compression strength on a wood testing bank. From each sample blocks of 20 x 20 mm and 30 mm in length were sawn. Before testing on the bank their exact dimensions, weight and volume was determine. Within 2 minutes after the compression strength was established on the wet bock the maximum compression strength was achieved. After the test the blocks were dried at 103 °C for 24 hours and afterwards their weight and volume was determine.
Based on all these measurements the compression strength (N/mm^2), moisture content (%) and density (kg/m^3) was calculated. For all pile samples the blocks were sawn over 4 radii and mean values were calculated for each of the parameters for the outer most layers of the stem (approximately the outermost 25 mm) for the second layer (approximately between 25 and 50 mm), for the third layer (50 –75 mm) and if possible for additional layers. Because of the way of sawing the number of replica within the inner more layers, decrease from 4 towards 1.

Chemical wood analyses (by Gelbrich & Militz)
Bacterial wood degradation starts at the outer parts and goes to inwards. Therefore the wood samples were divided in different zones from outside to inside. The number of zones depends on the dimension of the samples but there were not more than three zones, outside middle and inside, used. Each zone was used as one sample. The archaeological samples were mostly to small for dividing in different zones.
The Wood samples were ground with a cutting mill (Retsch SM 2000) and pass through a mesh-40 (0.4 mm) sieve to obtain homogenous particle sizes and remove fine particles, according to TAPPI T264 (1997).



example of the different zones in the piles



Flow diagram of chemical wood analyses

Extractives of wood are a group of cell wall components mainly consisting of fats, fatty acids, fatty alcohol's, phenols, terpenes, steroids, resin acids, waxes, etc. These components should be removed by different solvents to avoid errors in the calculation of lignin and holocellulose contents. The extractive free wood is the basis for wet chemical wood analyses. In order to determine the content of reduced sugars and phenols the order of solvents following the extraction series described in TAPPI T264 was changed. At first samples were extracted with hot water, then with pure ethanol and afterwards with a cyclohexan-ethanol mixture (2:1).All extraction steps were carried out for 6 hours.

After each step of the extraction series, the content of the extractives was determined. The solvents were removed by rotary evaporator Rotavapor RE 111 (BÜCHI) and the remained extractives were dried at 105±3 °C and weighed. The calculation based on the weight of the wood meal sample and the amount of used solvents.

Hot water is used to extract tannins, gums, sugars, starches, and colouring matter (TAPPI T264, 1997). Therefore this extract was used to determine the content of reduced sugars as an evidence of the degree of bacterial wood degradation. The DNS (3,5- dinitrosalicylic acid) assay was carried out according to Miller (1959). DNS solution (3 ml) was or water extract (1 ml) and vortexed. Then the mixture was incubated for 15 minutes in a boiling water bath. After that deionised water (4 ml) was added and the mixture was vortexed again. Reduced sugars were measured at absorbance of 550 nm. The calculation of the sugar concentrations based on an appropriate standard sugar curve.

The determination of the phenol content was carried out according to the method of Folin-Denis (Pritchard et al., 1997). This method is based upon photometric determination of phenol contents in extracts. In this *study water* and ethanol extracts of 3 ml were used. Folin-Ciocalteau-reagent (Folin-Denis solution) was added at a rate of 1:1 to the extracts and these mixtures were vortexed. Then Na_2CO_3-solution (53 g/500 ml) was added in the same volume (rate 1:1:1). Because of gas formation these solutions have to be shaken carefully. For a complete reaction the samples were kept at room temperature for two hours, shaken every 15 minutes and measured at 725 nm. The concentrations were calculated based on an appropriate Catechin calibration curve.

Lignin and carbohydrates are the main components in the wooden cell walls. Wood normally contains 20-30 % of lignin. Lignin can be removed or modified by different types of fungi and apparently also by bacteria. According to TAPPI T222 (1998), Klason lignin was determined in extractive free wood samples. The acid soluble wood components were solved with sulphuric acid. Supernatants were decanted and Klason lignin was filtered, dried at 105±3 °C and weighed. Lignin content was calculated based on oven dry weight.

In the cell wall the combination of cellulose and hemicellulose is called “holocellulose” and usually accounts for 65-70 % of the wood dry weight. These polymers are made up of simple sugars, mainly D-glucose, D-mannose, D-galactose, L-arabinose, D-glucuronic acid and lesser amounts of other sugars such as L-rhamnose and D-fucose. These sugars are the first accessible nutrients for microorganisms. Any biological decay affects the amount of holocellulose in the wood. The determination of holocellulose was carried out according to Wise *et al*. (1946). About 2 g extractive free wood was put in 100 ml Erlenmeyer flasks and 80 ml demineralised water was added. 0.25 ml acetic acid (100 %) and 0.75 g sodium chlorite ($NaClO_2$) were added too and the Erlenmeyer flasks were closed with a glass. After 1 hour of incubation at 80 °C in a water bath, the same amount of acetic acid and sodium chlorite was added to the Erlenmeyer flasks. These additions of the two chamicals were repeated three times for hardwood and four times for softwood as a whole.

The flasks were finally cooled in an ice-water bath, the contents filtered with a glass filter and washed with 100 ml demineralised ice-water and 25 ml acetone respectively. The filtered samples were dried at 105±3 °C and weighed.

Cellulose is the main component of plant cell walls. This carbohydrate is influenced by fungi and bacteria during wood degradation. In general, the α-Cellulose is that part of undegraded, higher-molecular-weight cellulose. The β-Cellulose is degraded cellulose with a reduced degree of polymerisation and the γ-Cellulose consists mainly of hemicellulose. Oven dried holocellulose samples were used for α-Cellulose determination according to TAPPI T203 (1998). As much a possible of the dried holocellulose was collected, weight and put in an in 100 ml Erlenmeyer flask. 75 ml Sodium hydroxide (17,5 %) was added to the Erlenmeyer flasks and kept at 20 °C for two hours, shaken every 15 min and then filtered by glass filter. Under these conditions α-Cellulose forms an insoluble fraction. Washing of the filtrate was carried out with 25 ml sodium hydroxide (NaOH 17,5 %), 150 ml dimineralized water, 25 ml acetic acid (10 %) and 25 ml acetone respectively. Finally, they were dried at 105 ± 3 °C and weighed. The supernatants of the α-Cellulose determination were used for the β-Cellulose determination because β-Cellulose is the soluble fraction, which is precipitated by acidification of the solution. 50 ml of 3 N sulphuric acid were added to 50 ml of the supernatant and mixed thoroughly. The mixtures were submerged in a hot water bath at about 70°-90° C for few minutes to coagulate the β-Cellulose. γ-Cellulose is that cellulose fraction remaining in this supernatant. The solutions were kept overnight for settling of the precipitate. After that the samples were filtered, dried at 105± 3 °C and weighed.

Mineral content and C/N ratio was determined by drying the wood samples at 60°C and either ball-milled or consecutively milled by an ultra-centrifugal mill and a ball-mill. C and N contents were determined by an automated elemental analyzer (Heraeus Elementar Vario EL). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany) after pressure digestion in 65% concentrated HNO_3.

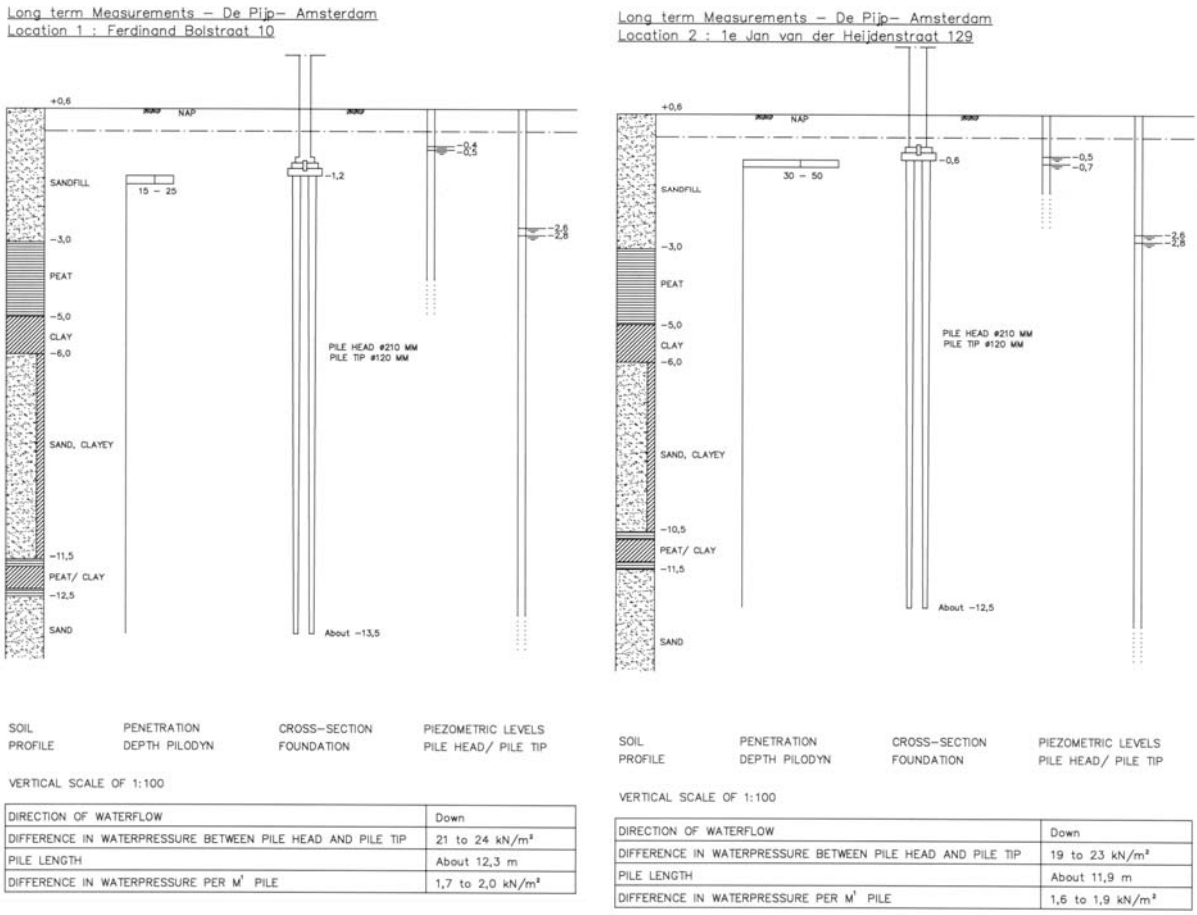
The Ash content was determine according to TAPPI T211 (1993) the milled wood samples were ignited in a muffle furnace at 525° C. Separate wood samples were analysed to determine the moisture content. The resulting ash weight and moisture content in the sample were used to calculate the ash content based on a moisture-free sample.

Determine specific cell wall components by the use of IR spectroscopy (FTIR Fourier transformation infrared spectroscopy). All chemical bonds undergo stretching and bending, resulting in absorptions at characteristic frequencies in the infrared region of light. A requirement for measuring IR-absorption are the changes in the electrical dipole moment of the stretching or bending structure. Functional groups with high permanent dipole moments, such as carbonyl and hydroxyl groups, give strong absorption, whereas non-polar bonds absorb IR radiation only weakly. Because the functional groups can be assigned to wooden cell wall components, with this procedure we get first results about the composition of the wood samples. For this method dry milled wood was used only in sample sizes not exceeding 1 g oven dry mass. The IR-measurements were done with a FT-IR VECTOR 22 (Bruker).

3.4 Results

In appendix 1 from chapter 3 all site descriptions are given. In appendix 2 - 4 all individual data the wood and soil analyses are given, receptively the data on pattern and degree of decay in combination with moisture content, density and compression strength and the data on the wood chemistry. In appendix 4 the individual dendrodating results are given and in appendix 5 the individual data of the soil and groundwater analyses are given.
Below the results are summarised per site.

Long-term measurement sites (Keijer & Kretschmar)
Groundwater and soil conditions are shown in the figure below.



In Tab. 2 the chemical sediment composition for both sites is shown. Sampling with the hand auger further down the sediment profile proofed difficult, therefore only one sampling depth per site is presented. Regarding most of the elements both sites are very similar. Nevertheless, differences in the sulphur and calcium concentration as well as small differences in the carbon concentration exist and values are higher at site 1.

Table 2: Sediment chemistry, total concentrations, mean values (n=4)

	Depth	pH	C	N	C/N	P	S	Na	K	Ca	Mg	Mn	Fe	Al
	cm	H_2O						mg/g						
Site 1	80	7.5	15.6	0.44	35.1	0.35	3.5	0.29	1.7	17.6	1.4	0.15	12.6	6.9
Site 2	75	7.6	12.1	0.36	33.7	0.25	0.49	0.25	1.6	9.0	1.1	0.10	9.8	6.2

The mean sediment water composition per site can be seen in Tab. 3. No ammonia was measured with two exceptions at site 1 instead all inorganic nitrogen was present as nitrate. At site 1 the mean annual total nitrogen concentration of 6.0 mg N/L is significant ($p<0.05$) higher compared to 1.7 mg N/L of site 2 (see also Fig. 6).

Table 3: Soil water chemistry at the two long-term measurement sites, mean values over the sediment profile, Site 1 n=3, Site 2 n=4 and mean annual values per site (dl.=detection limit).

		Site 1				**Annual**	**Site 2**				**Annual**
		Autumn	Winter	Spring	Summer	**mean**	Autumn	Winter	Spring	Summer	**mean**
Conduc-tivity	[µS/cm]	988	956	1164	1090	**1050**	628	770	1438	1099	**984**
pH		8.2	7.7	7.8	8.0	**7.9**	8.1	7.9	7.7	7.9	**7.9**
DOC	[mgC/L]	13.1	19.6	10.2	11.5	**13.6**	7.0	23.8	<dl.	<dl.	**7.7**
NO_3^-	[mgN/L]	3.0	5.3	2.7	6.2	**4.3**	0.8	1.8	1.4	1.8	**1.4**
NH_4^+	[mgN/L]	1.3	0.6	<dl.	<dl.	**0.5**	<dl.	<dl.	<dl.	<dl.	**<dl.**
N_{org}	[mgN/L]	0.48	0.36	0.49	0.20	**0.38**	0.25	0.20	0.21	0.27	**0.23**
N_t	[mgN/L]	4.7	5.7	6.7	7.0	**6.0**	1.0	2.0	1.6	2.1	**1.7**
PO_4^{3-}	[mgP/L]	0.34	0.41	0.36	0.39	**0.38**	0.38	0.41	0.38	0.35	**0.38**
SO_4^{2-}	[mgS/L]	1.6	2.1	1.7	1.7	**1.8**	5.1	12.2	33.3	24.7	**18.8**
Fe^{2+}	[mg/L]	0.33	0.14	0.33	0.52	**0.33**	1.1	0.4	0.02	0.03	**0.38**
M_B	[mmol/L]	9.8	9.7	11.7	11.5	**10.7**	6.4	9.2	16.7	12.9	**11.3**

Fig. 3 shows the mean annual conductivity profile at the two sites. The two sites are very similar but there seems to be a depth gradient at site 1. In Fig. 4 is the conductivity within the profile at the four seasons separately plotted for site 2. The conductivity in the sediment water displays a seasonal variation with higher values in spring and summer compared to autumn and winter which is statistically significant ($p<0.05$).
Fig. 5 displays the mean annual phosphate profile for site 1 and 2.

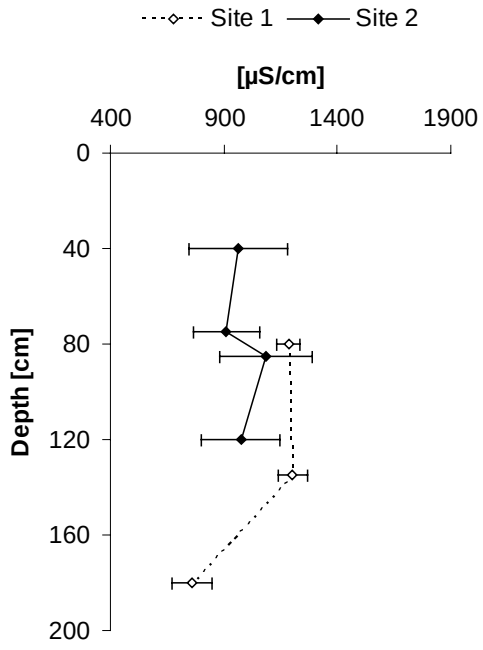


Figure 3: Mean (±SD, n=4) annual conductivity profiles for sites 1 and 2

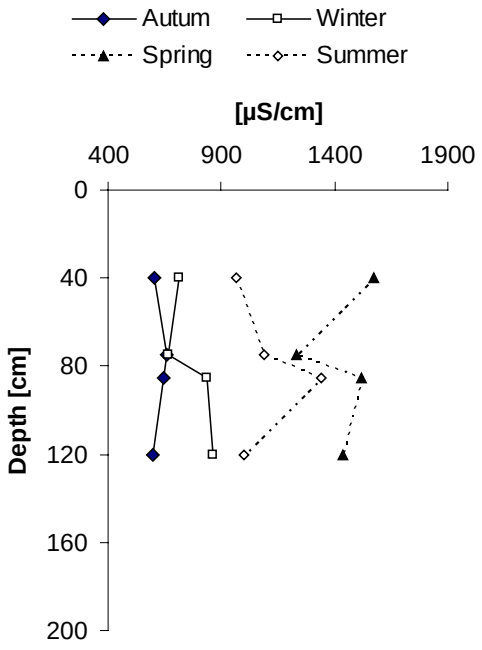


Figure 4: Conductivity profiles at the different seasons for site 2

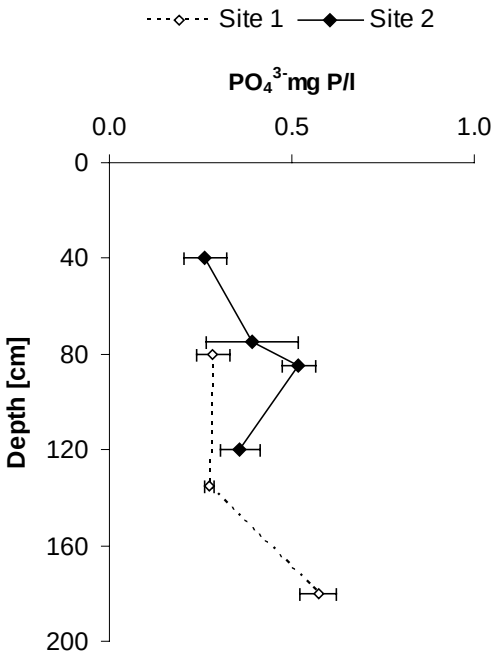


Figure 5: Mean (±SD, n=4) annual phosphate profiles for sites 1 and 2

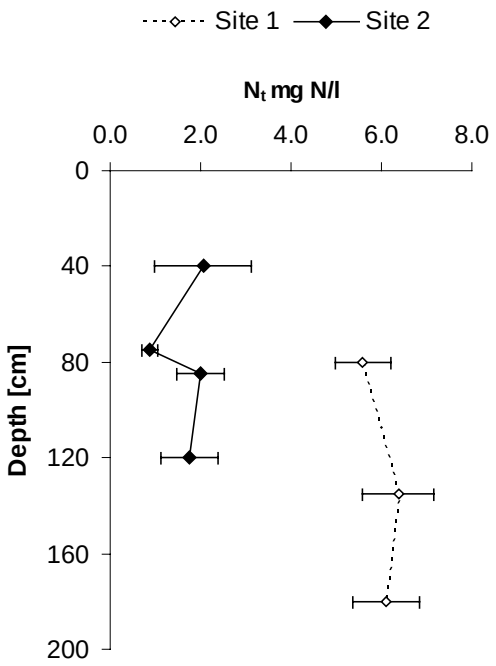


Figure 6: Mean (±SD, n=4) annual total nitrogen concentration profiles for sites 1 and 2

Fig. 7 shows the mean annual dissolved organic carbon concentration profile for the two sites. At site 1 the DOC concentration displays little depth variation. At site 2 is the DOC concentration similar to site 1 but the values vary more during the seasons what causes the high SD. For the mean annual sulphate concentration profile see Fig. 8. The sulphate concentration is at site 1 lower and less variable compared to site 2. Nevertheless there is no obvious trend in the seasonal variation of the sulphate concentration.

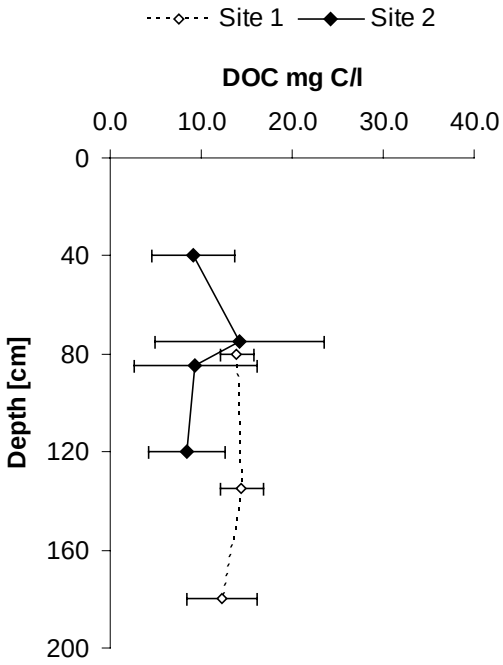


Figure 7: Mean (±SD, n=4) dissolved organic carbon (DOC) concentration along the profile at the two sites

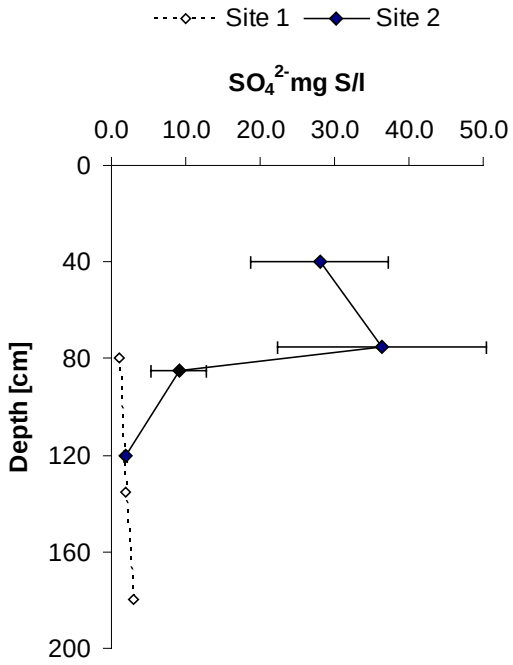


Figure 8: Mean (±SD, n=4) sulphate concentration along the profile at the to sites

The lowest soil temperature was reached in March at the end of winter with 6.1 and 8.1 °C for site 1 and 2 respectively (fig.9). The highest temperature was measured in September for site 1, 16 °C and for site 2, 17 °C. The soil temperature at site 2 is generally slightly higher as at site 1.

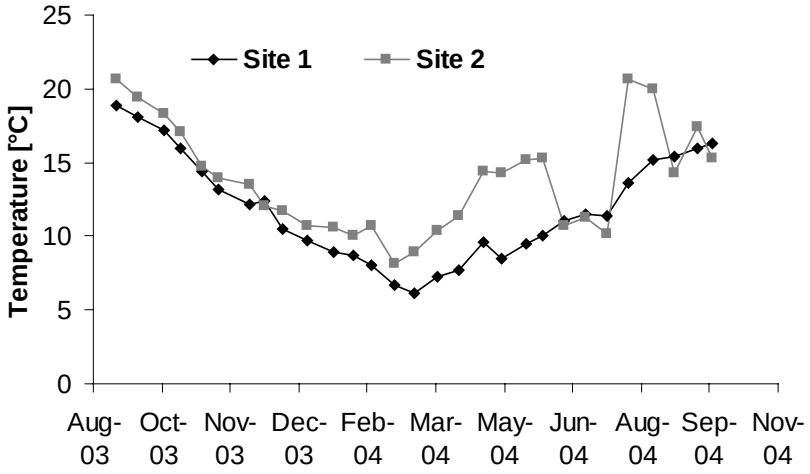


Figure 9: Sediment temperature at the two long-term measurement sites, Depth site 1: 1 m, site 2: 0.7 m from ground level

The average annual temperature (Sep. 03-Aug. 04) is 11.4° C and 13.6° C for site 1 and 2 respectively and is slightly higher then the mean annual temperature of 9.8° C in Schiphol close to Amsterdam (mean 1971-2000, Royal Netherlands Meteorological Institute).
At both sites the average groundwater table was 0.4 m below ground level as can be seen from figure 10. The lowest levels were found in summer 04 and in autumn 03. The maximum ground water table variation was 0.25 and 0.20 m for site 1 and 2 respectively.

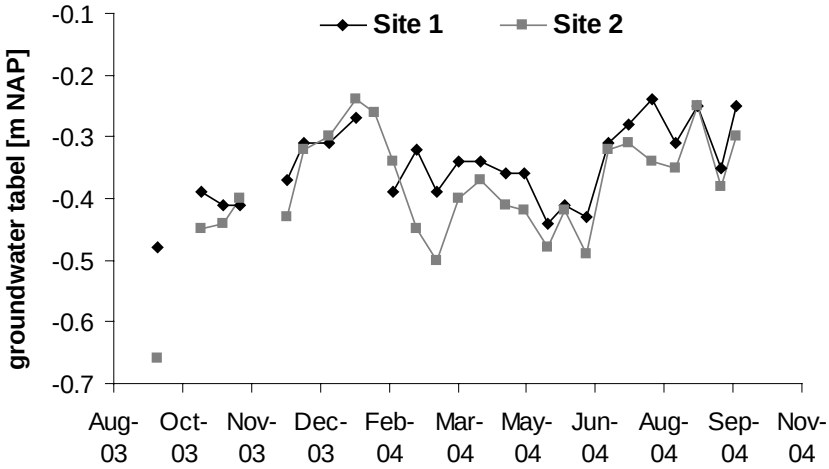


Figure 10: Groundwater table given in m NAP at the two long-term measurement sites

The ground water table fluctuation measured at this exemplary year did not give evidence of foundation exposure to unsaturated conditions. Nevertheless the ground water table was lowered at site 2 in March 04 to -0.5 m NAP which is only 0.1 m away from the pile head at -0.6 m NAP.

Table 2: Precipitation during the measurement period together with the average precipitation per month at De Bilt (Royal Netherlands Meteorological Institute).

Month	Jun-03	Jul-03	Aug-03	Sep-03	Oct-03	Nov-03	Dec-03	Jan-04	
Precipitation [mm]	34.6	29.8	9.2	51.6	84.3	39.5	96.1	123.1	
Average Precipitation [mm]	*72*	*70*	*58*	*72*	*77*	*81*	*77*	*67*	
Month	Feb-04	Mar-04	Apr-04	May-04	Jun-04	Jul-04	Aug-04	Sep-04	Oct-03 – Sep-04
Precipitation [mm]	79.4	42.0	33.1	31.2	69.2	122.1	127.2	54.1	901.3
Average Precipitation [mm]	*48*	*65*	*45*	*62*	*72*	*70*	*58*	*72*	*803*

The precipitation in the measurement period (tab. 2) was from Dec 03 to Feb 04 with 299 mm much higher as the average precipitation in that period with 192 mm as well as from Jul 04 to Aug 04 were it rained nearly twice as much (249 mm) as normal (128 mm). In contrast Mar 04 to May 04 was dryer (108 mm) compared to the average year (172 mm).
The Redox potential measurements agree very well at the different depth for site 1 (fig. 11) with values from 0 to -200 mV. At site 2 the measurement of the shallowest sensor deviates from the other tree depths which display readings around -200 mV. At the end of summer 03 (Aug 03 to Oct 03) and in spring 04 (Mar 04 to Apr 04) the shallowest sensor at site 2 shows values about +200 up to +400 mV. At site 1 increase the Redox potential at the end of summer (Aug 04) from -200 mV to around 0 mV. This was as well measured at the end of summer 2003. From Nov 03 till Jan 04 the shallowest sensor increased slightly to values around -100 mV. Between the sites the Redox potential is comparable.

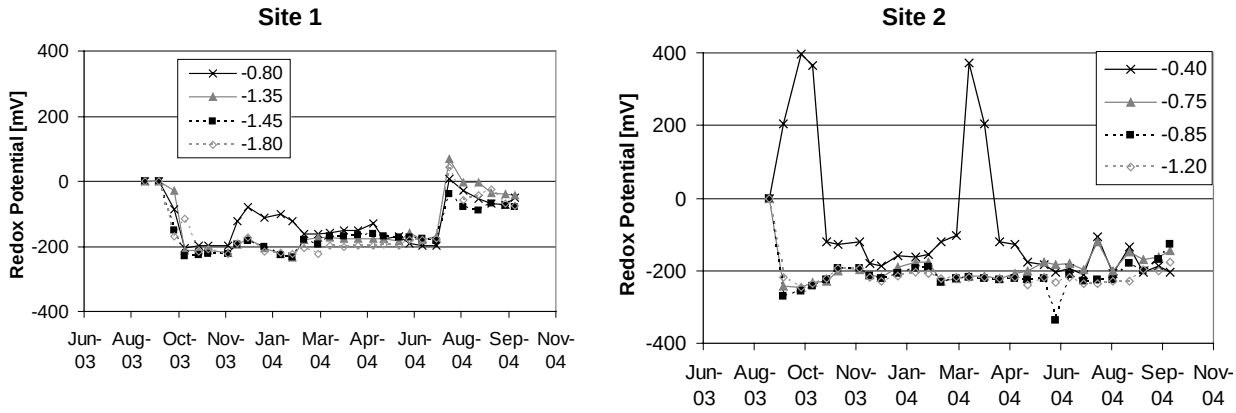


Figure 11: Redox potential at the two sites in four different depths during the measurement period (n=1). Depth [m] from box bottom site 1: 0.1 m NAP, site 2: 0.0 m NAP. Pile head site 1: -1.2 m NAP, site 2: -0.6 m NAP.

Due to high variation of the oxygen concentration at the measurement start the oxygen sensors were reinstalled in Dec 03. This resulted in a marked decrease in the oxygen concentration at all sensors at both sites (figure 12 and 13). Only the second deepest sensor at site 1 (fig. 6) does not seem to be affected by reinstallation as showing constantly 1 to 2 mg L^{-1} oxygen.

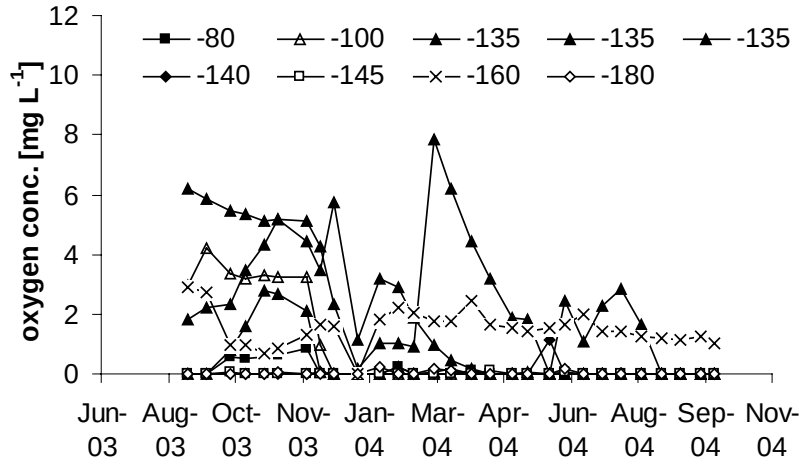


Figure 12: Site 1: Oxygen concentration in different depth [cm] from box bottom (0.1 m NAP), pile head at: -1.2 m NAP.

One of the triplicate sensors at site 1 displays in spring 04 a high increase of the oxygen readings up to 8 mg L^{-1} then decreasing and increasing again finally reaching zero oxygen in Aug 04. From April 04 to Sep 04 the other seven oxygen sensors in various depths at site 1 measure zero oxygen.

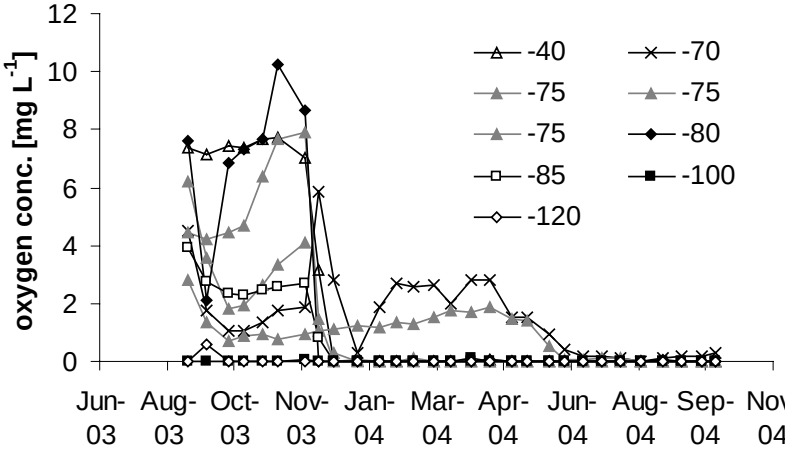


Figure 13: Site 2: Oxygen concentration in different depth [m] from box bottom at 0.0 m NAP, pile head at -0.6 m NAP.

At site 2 (fig. 7) the second shallowest sensor increases again after reaching zero after reinstallation and measures zero from Jun 04 on. One of the triplicate sensors at pile head was not affected by reinstallation increasing slightly from 1 to 2 mg L^{-1} oxygen but showing zero oxygen from June on. The other seven sensors in various depth at site 2 measured zero oxygen from spring on.
For the reproducibility of the oxygen measurements it can be derived that it is very dependent on the installation. If air oxygen can penetrate towards the sensor tip the measurements do not represent the prevailing soil conditions. From the triplicate measurements at foundation height two are showing the same reading where as one deviates widely in case of site 1 or slightly and then joining the other two readings in case of site 2.

At site 1 the direction of the ground water flow is towards Northwest (tab. 3) and at site 2 towards Northeast.

Table 3: Ground water flow direction and velocity at pile head at both sites

	Site 1		Site 2	
Date	Direction of water flow [^{0}] *	Velocity of Water flow [m day^{-1}]	Direction of water flow [^{0}] *	Velocity of Water flow [m day^{-1}]
11.06.04	324	0,27	47	0,19
11.08.04	324	0,24	23	0,22

*Direction: 0^0=North; 90^0=East; 180^0=South; 270^0=West

The direction of the water flow at site 2 is as expected towards the Sarphati Park. The velocity of the horizontal water flow at site 1 is slightly higher than at site 2. This does not fit with the hypotheses that the amount of decay is directly proportional to the water flow velocity. In contrary the decay is severe at location 2 and light at location 1.
All pine piles, which were in the soil for 18 month, show no bacterial decay over the whol length. Only in the upper 10 cm, softrot decay was found in the outermost layer (< 0.5 mm) in all four piles.

Discussion long-term measurements

The mean temperature difference between the two sites could either mirror the installation depth differences of 0.3 m between the sites or more likely is a sensor artefact, as the sensor at site 2 was replaced in May 04 after experiencing problems. The mean temperature is slightly higher than the mean annual temperature for the region because of the proximity of the sites to houses, its pavement and the shallow measuring depth. In general bacterial activity increases with increasing temperature therefore higher temperatures might have a favouring influence on bacterial wood decay. Otherwise the bacterial community can alter with changing temperature which complicates simple statements.
The annual precipitation for the measurement period was 100 mm higher as the average annual precipitation. At site 2 it might therefore be possible that in very dry years the ground water table is about or even lower as the head of foundation.
The presented Redox potential measurements are from one sensor only although Redox measurements are variable and best measured in triplicate. Therefore the emphasis should be given to Redox ranges rather then single values. Oxygen can exist from a Redox potential higher as 220 mV and aerobic respiration can be found at a Redox potential between 800 to 450 mV. Hence at site 2 0.3 m above the pile head conditions do not thermodynamically rule out the existence of oxygen in two incidents during the measurement period. The oxygen measurements did not measure oxygen 0.3 m above pile head at site 2. This is possible as oxygen can exist from a thermodynamic point of view and possibly be causing the increase in Redox potential. But it is very likely that the penetrating oxygen is rapidly consumed, as anoxic environments are ample supplied with reduced components which are rapidly oxidised upon the availability of oxygen.
The increase in the Redox potential of the shallowest sensor at site 2 coincides with low ground water table readings but does not occur in August 04 were equally low ground water tables were measured. July 04 experienced heavy rainfall which might be the cause for the slight increase of the Redox signal of the shallowest sensor at site 2 in August 04. The infiltrating rain water transports oxygen towards the ground water but considerable quantities of rain water are needed to influence ground water composition. The Redox potential increase of the shallowest sensor at site 1 in winter 04 could as well be explained with heavy rain as from Dec 03 to Feb 04, 299 mm precipitation occurred (average for that period 192 mm). It is interesting to note that the heavy rainfall periods in winter 03 and summer 04 only affected the Redox readings at one of the two sites at a time. Ground water table fluctuations do not alter the Redox potential around the pile head at site 1 significantly in comparison to site 2. Because at site 1 the height difference between pile head and mean ground water table is much higher as at site 2.
The oxygen measurements were complicated by air oxygen intrusion. Therefore the data before reinstallation do not mirror seasonal but disturbance variation. After reinstallation no oxygen was present at site 2 and hence no seasonal variation found. At site 1 variations of two sensors occurred which could not be related to seasonality.

The Redox potential measurements agree with the oxygen measurement of zero oxygen. Redox potential variations were explained by precipitation variation and its impact on ground water table fluctuations.
The results presented from the long-term measurements suggest that oxygen concentration can not explain the differences in decay intensity at the two measurement sites. The ground water flow velocity differs at the two sites but is slightly lower at site 2 as at site 1. If a lower flow velocity favours bacterial wood decay ground water flow velocity could explain the differences in bacterial decay intensity at the two sites. The only parameter possibly explaining the decay intensity difference at the two sites is the ground water hydrology influencing the Redox potential at the two sites. It appears that the distance of the pile head to the average groundwater level is the best explanation for the differences in bacterial wood decay at the two sites.
At site 1 and 2 the sediment pH is basic (7.9) which is mirrored also in the sediment water pH. Basic conditions were also found at two other sites in Amsterdam. Anoxic systems are well buffered in a basic pH range however basic sediment conditions can also be caused by natural chalk contents and building materials introduced to the sediment during house construction. In the sediment solution most (70-90%) of the total carbon was inorganic.
The sediment water composition differed between the two sites in the total nitrogen and sulphate concentration with higher values for nitrogen at site 1 and for sulphate at site 2. The inorganic analysed at the two sites was mostly in the form of nitrate. The presence of nitrate and the measured low Redox potentials at both sites are contradictory. The basic pH and relatively high DOC concentrations in the sediment solutions from both sites might have triggered nitrate formation after sampling. Therefore it can not be ruled out, that the measured nitrate was a sampling or storage artefact. Nevertheless total N was analysed to be highest at site 1.
The slightly higher carbon concentration at site 1 compared to site 2 is probable influencing the also higher sulphur and calcium concentrations. Sulphur and calcium in sediments are often associated with organic matter. However the sulphur concentration difference between the two sites is much higher as the carbon one. Focusing on the sediment solution composition the sulphate variation at site 2 can not be explained by DOC variance and is more likely caused by different sulphate ion concentration in the ground water stream.

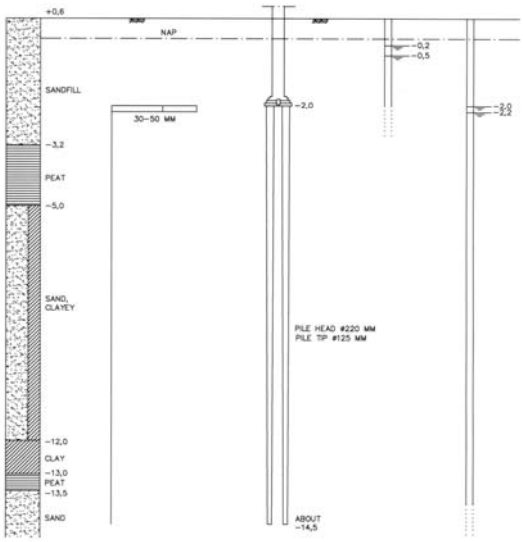
Conclusions long-term measurements
Although the oxygen measurements varied before reinstallation, the measurements were consistent afterwards and did not measure oxygen in all depth at both sites except for two sensors. The oxygen measurements worked well after reinstallation and settling time. Measured differences between the two sites were not detected by employing oxygen optodes. The Redox measurements were in agreement with the oxygen measurements indicating anoxic conditions at both profiles although some exceptions were observed. The dynamic displayed by the Redox measurements at both sites could be explained by ground water table variations caused by rain events. The ground water table fluctuations and the soil temperature both show a clear seasonal pattern. The average soil temperature measured is slightly higher as the mean annual temperature for the Amsterdam region because of the urban site situation. The physical parameters measured can not account for bacterial decay intensity difference at the two sites. The two sites only differ with respect to sulphur, calcium and carbon sediment matrix concentration. At site 1 the sulphur concentration is seven times, and the calcium concentration two times higher as compared to site 2. Only slightly higher is at site 2 is the carbon concentration of site 1. The same holds for the carbon concentration but the difference is smaller. The total nitrogen concentration in the sediment water was higher at site 1 compared to site 2. Nitrate was the dominant form but this is contradictory to the low Redox potentials measured and may be a sampling or storage artefact. In contrast to the sediment matrix composition the sediment water sulphate concentration is higher at site 2. The sediment water composition was at site 2 more variable than at site 1. A seasonal variation was observed in the sediment water conductivity at site 2. However, other chemical constituents did not exhibit seasonality.

Site 1

Site characteristics

A large block of attached houses consisting of 4 or 5 stories with an attic, Spaarndammerbuurt at Amsterdam, The Netherlands, erected in 1918. The foundation is an "Amsterdamse fundering" (a double row of wooden piles at the head connected with a short horizontal wooden beam 90 mm thick). Above longitudinal wooden beams with a thickness of 80 mm are used, supporting the masonry of the house. Of 5 piles disks and cores were sampled, with diameters of the pile heads between 180 -280 mm, the sample height was approx. 0.25 m. Groundwater and soil conditions are shown in the figure below.

Site 1: Spaarndammerbuurt at Amsterdam (foundation inspection)



DIRECTION OF WATERFLOW	DOWN
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	15 TO 20 kN/m²
PILE LENGTH	12,5 m
DIFFERENCE IN WATERPRESSURE PER M² PILE	1,2 TO 1,6 kN/m²

Site characterisation soil and water chemistry

- Soil: Poor sand, alkaline, very low carbon content, no nitrogen, low phosphorus, low base cations, low sulphur, low iron
- Soil solution: slightly alkaline, no dissolved organic carbon, medium nitrogen, nitrate and ammonium at the same time present, low phosphate, low base cations, low to medium sulphate, medium iron, medium conductivity, slight sea water influence
- Oxygen, Redox: 1 – 3.5 mg/L oxygen, 450 – -50 mV Redox potential,

Comment: extracted ground water had a different composition as soil water taken with lysimeters. Groundwater more brackish (higher conductivity, higher sulphate, chloride), oxic conditions (high nitrate, no ammonium), Oxygen and Redox probably sampling artefacts as for digging a vigorous dewatering was necessary which facilitated air oxygen intrusion

Dendro-dating

Ages 39-58 years, no date.

Summary degradation patterns

Five pile heads : 4 pine and 1 spruce.

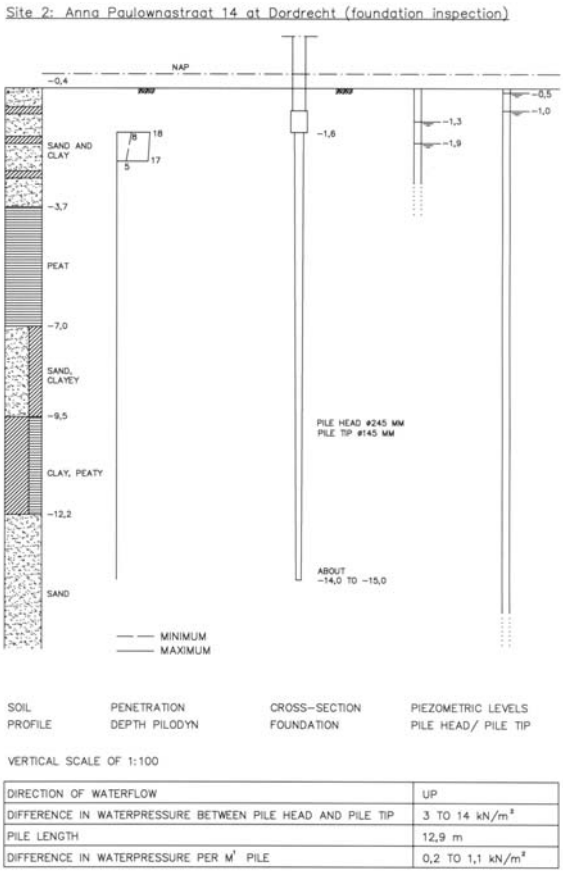
All pine piles degraded in the sapwood (55 mm) only by bacteria. Decay gradient (from severe to weak) varies but in general half severely and half moderate - weak degraded.

Spruce severely degraded in the outermost layer of 25 mm only by bacteria.

In almost piles: wood colonising fungi in the outmost layer of the degraded area.

Summary of the chemical analyses
The pile heads show a strong increased lignin content (48 -60%) in the outside zones. In the middle zones lignin is slightly increased (32 - 38 %) whereas the inner parts have normal lignin content because of the non attacked heartwood. In depths of 120 mm under pile head the outer parts show a normal or only slightly increased lignin content (32 - 38 %).
Active erosion bacteria
Present in 6 samples (1.a.td.1.100; 1.a.td.1.360; 1.b.td.3.100; 1.b.td.3.320; 1.c.td.1.350; 1.d.td.3.70), absent in 2 samples (1.c.td.1.1201.d.td.3.300).

Site 2
Site characteristics
House (no. 14) part of a block of 6 attached houses (Anna Paulownastraat 4 to 14, Dordrecht The Netherlands), with each 2 stories and an attic, erected in 1931.
The foundation consists of a single row of wooden piles, on top of the piles longitudinal concrete beams are used supporting the masonry of the house.
Twelve pile heads were sampled, with diameters between 220 - 265 mm and length of the pile heads varies 60 – 100 cm. Groundwater and soil conditions are shown in the figure below.

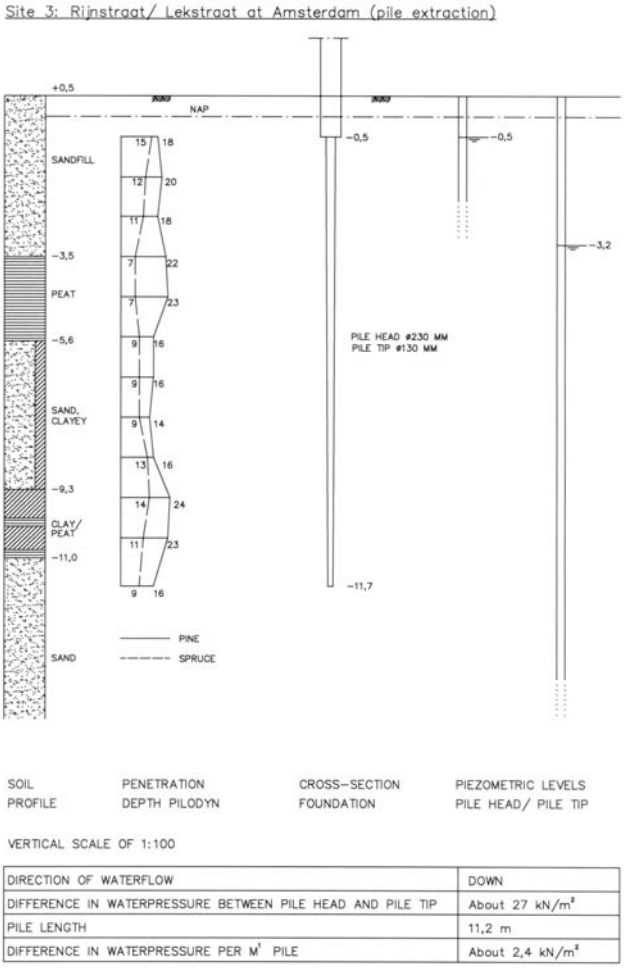


Site characterisation soil and water chemistry
- Soil: Loamy sand, slightly alkaline, low carbon content, medium nitrogen, medium to high carbon/nitrogen ratio, medium phosphorus, low base cations, low sulphur, medium iron
- Soil solution: slightly alkaline, medium nitrogen, nitrate in upper layers, ammonium in lower layers, medium phosphate, low base cations, low sulphate, low iron, low conductivity
- Oxygen, Redox: 5.3 – 0,8 mg/L oxygen from +0.3 m above pile head to 1.7 m below pile head, Redox measurements not available

Comment: the Redox probe was out of order

Dendro-dating
Age 55-113; felling date: summer – autumn 1929; origin: 2 piles from Gotland Sweden, 7 piles Germany.
Summary degradation patterns
Twelve pile heads, 11 spruce, 1 pine.
In spruce the bacterial decay varies: 2 pile with severe degradation in thin layer (< 10 mm); 8 pile with decay gradient (severe–weak) in 20 – 30 mm layer; 1 pile (no. 6) with decay gradient (severe–weak) in thick (60 mm) layer.
In pine bacterial decay (¼ severe, ¾ moderate-weak) in sapwood (80 mm) only.
Wood colonising fungi in 8 piles (including pine) in the outmost layer of the degraded area.
In 4 spruce pile initial softrot decay
Active erosion bacteria
Present in 9 samples (2.td.1.0-620; 2.td.2.0; 2.td.2.400-840; 2.td.3.400-850; 2.td.6.50; 2.td.7.100; 2.td.8.0-700; 2.td.9.0-650; 2.td.10.0-600), absent in 5 samples (2.td.3.0; 2.td.4.70; 2.td.5.100; 2.td.11.0-650; 2.td.12.0-760)

Site3
Site characteristics
Demolished church (built in 1926) at the corner of Rijnstraat and Lekstraat at Amsterdam, The Netherlands. The foundation consisted of wooden piles in a single row. On top of the piles longitudinal concrete beams were used supporting the masonry of the church.
Four piles were extracted. The diameter of the pile heads with diameters between 205 - 255 mm, at the tips between 120 - 145 mm. The length of the piles was about 11.2 m.
Groundwater and soil conditions are shown in the figure below.

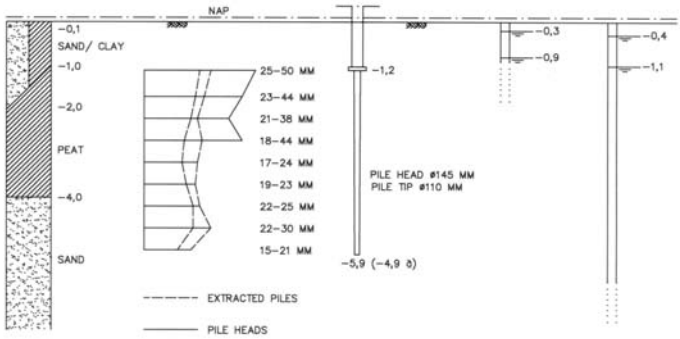


DIRECTION OF WATERFLOW	DOWN
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	About 27 kN/m²
PILE LENGTH	11,2 m
DIFFERENCE IN WATERPRESSURE PER M' PILE	About 2,4 kN/m²

Dendro-dating
Age pine 69-81; spruce 45-59 years; felling date: winter spring 1923-1924; origin: 2 pine piles from southern Finland, 2 spruce piles from southern Germany.
Summary degradation patterns
Four extracted piles of about 1 m: 2 pine and 2 spruce.
In pine bacterial decay (gradient from severe to weak) in sapwood (40 – 50 mm) only.
In spruce moderate bacterial decay (sometimes weak, occasionally severe) in outer most layer of 20 (–40) mm.
No decay gradient over the length of all four piles.

Site 4
Site characteristics
Two demolished blocks of attached house with 2 stories, built in 1904 (Dyserinckstraat) and 1895 (Vooruitgangstraat) Amsterdamse Buurt Haarlem, the Netherlands. The foundation is a "Rotterdamse fundering"(a single row of wooden piles, connected with longitudinal wooden beams supporting the masonry of the houses).
Four pile heads were extracted in the Dyserinckstraat, with diameter between 110 - 165 mm and the length between 130 – 140 cm. Three piles were extracted at the Vooruitgangstraat, with diameter of the heads between 130 - 160 mm and of the tips between 95 - 125 mm; pile length between 370 - 470 cm. Groundwater and soil conditions are shown in the figure below.

Site 4: Amsterdamse Buurt at Haarlem (pile extraction)



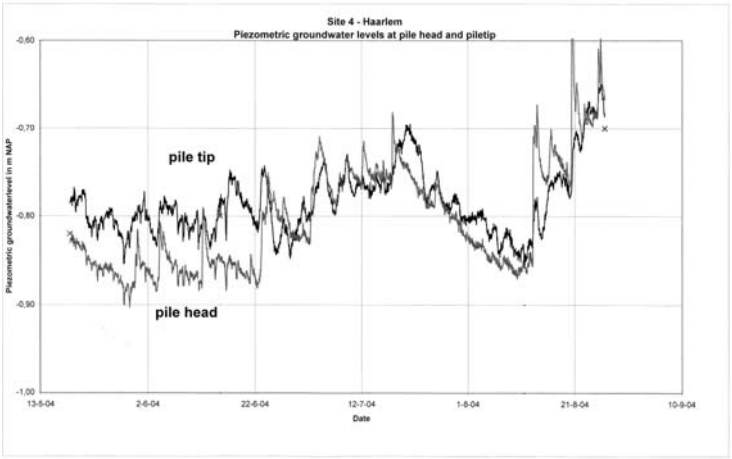
SOIL PROFILE | PENETRATION DEPTH PILODYN | CROSS-SECTION FOUNDATION | PIEZOMETRIC LEVELS PILE HEAD/ PILE TIP

VERTICAL SCALE OF 1:100

DIRECTION OF WATERFLOW	DOWN (UP)
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	8 TO (5) kN/m²
PILE LENGTH	(3,7) AND 4,7 m
DIFFERENCE IN WATERPRESSURE PER M' PILE	1,7 TO (1,1) kN/m²

Measurements of groundwater
The piezometric groundwater levels near the pile heads were measured with a diver in standpipe B4. The piezometric groundwater levels at pile tips were measured with diver in standpipe B3. Measuring period and weather type is shown in the table below.

Period / Date	**Weather**
18 / 5 till 23 / 6	quite dry
23 / 6 till 17 / 7	rainy
17 / 7 till 13 / 8	almost completely dry
13 / 8 till 26 / 8	very rainy



Earlier measurements in standpipes in the surroundings indicated a small difference in water pressure between pile head and pile tip (water flow 8 kN/m^2 downwards to 4 kN/m^2 upwards). The results of the measurements in 2004 are as follows:

Period / Date	Piezometric groundwater level at		Difference in water pressure kN/m^2	Direction water flow
	pile head m NAP	pile tip m NAP		
18 / 5 till 23 / 6	-0,85	-0,80	0,5	upwards
23 / 6 till 17 / 7	-0,77	-0,79	0,2	downwards
17 / 7 till 13 / 8	-0,81	-0,78	0,3	upwards
13 / 8 till 26 / 8	-0,71	-0,73	0,2	downwards

Considering the extremely wet summer of 2004 the results of the measurements in this period fit reasonable with the earlier measurements in standpipes in the surroundings. The direction of the water flow is more often upwards than expected.
The Horizontal water-flow is shown in the table below.

Pile head level		
Date	**Direction of waterflow in ^{0} ***	**Velocity of waterflow in m/day**
11 / 6	11	0,12
11 / 8	45	0,08
Pile tip level		
Date	**Direction of waterflow in ^{0} ***	**Velocity of waterflow in m/day**
11 / 6	242	0,23
11 / 8	252	0,15

* 0^0=North; 90^0=East; 180^0=South; 270^0=West

The velocity of the water-flow at pile tip level is higher than at pile head level. The direction of the water-flow at pile tip level is opposite to what we expected; not from the dune but towards the dunes.

Site characterisation soil and water chemistry

- Soil: Loamy sand, alkaline, low carbon, medium nitrogen, low carbon/nitrogen ratio, low to medium phosphorus, low base cations, low sulphur concentration, low iron concentration
- Soil solution: alkaline, high dissolved organic carbon, high nitrogen, nitrate increase with depth, ammonium increase with depth, both present at the same time, medium phosphate, medium base cations, low sulphate, high iron, low conductivity
- Oxygen, Redox: both measurements not available

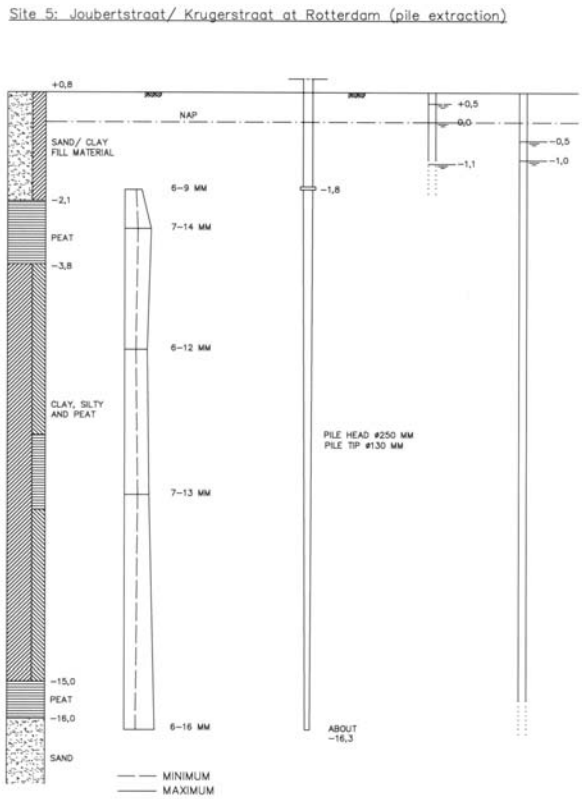
Comment: Redox reference electrode was stained at installation, strong smell of hydrocarbons.

Dendro-dating
Age piles 39-46 years; felling date: piles 1-2 in summer-autumn 1904, piles 5-7 summer-autumn 1897& 1894, origin Gotland Sweden and Ede, The Netherlands.
Summary degradation patterns
Seven piles, all pine, 3 extracted and 600 cm long, 4 piles head of 150 cm.
In all piles bacterial decay appears in sapwood only. In 2 piles the decay was severe, in 5 other piles decay gradient appear in sapwood (¾ severe and ¼ moderate-weak).
No decay gradient over the length of all piles.
Wood colonising fungi throughout the degraded area in all piles and in 1 pile additional softrot decay was found. It is believed that the fungal activity started during storage and continues in some areas of the piles afterwards.
Summary of the chemical analyses
The outside zones of the investigated pile heads have lignin content higher than 50 % sometimes higher than 60 %. The middle zones show slightly increased lignin content (38 %). The inside zones (Ø ≈ 40 mm) are sound because of a normal lignin content.
The N-content of the outer parts is higher (3,8 mg/g) than that of the middle zones (1,8 mg/g).
Active erosion bacteria
Present in 6 samples (04.te.1.0-64; 04.te.2.0-69; 04.te.3.0-45; 04.te.5.0,00-0,50; 04.te.6.0,00-0,50; 04.te.7.0,00-0,50), absent in 1 sample (04.te.e.0-67).

Site 5
Site characteristics
A complete block of houses (erected between 1901 and 1905) has been demolished at the corner of Joubertstraat and Paul Krugerstraat and the Bloemfonteinstraat at Rotterdam.
No original construction drawings are available of the original foundation (archives were destroyed during World War II) and the foundation construction was already demolished when BACPOLES got involved. A “Rotterdamse fundering” is expected (a single row of wooden piles, connected with longitudinal wooden beams supporting the masonry of the houses) The pile head level was measured at NAP -1.88 m.
Three piles were extracted with diameter at the heads of 250 mm and at the tips between 125 - 170 mm and with length of about 1450 cm. Groundwater and soil conditions are shown in the figure below.
Site characterisation soil and water chemistry
- Soil: Clay over peat, slightly alkaline, high carbon content in peat, in clay medium carbon content, high nitrogen, low carbon/nitrogen ratio, low phosphorus, medium base cations, medium sulphur, medium iron
- Soil solution: slightly alkaline, medium dissolved organic carbon, high nitrogen, low nitrate and high ammonium, medium phosphate, medium base cations, low sulphate, low-medium iron, low conductivity
- Oxygen, Redox: 1.37 – 0.02 mg/L oxygen, Redox measurements not available

Comment: the clay was bluish grey – typical for reducing conditions, there was little time for oxygen measurements as one unsupported wall of the measurement pit collapsed. The oxygen measurements increase further down there peat was found. The Redox probe was out of order.
Dendro-dating
Age 53-98 years; no date.
Summary degradation patterns
Three extracted piles, 1 fir and 2 spruce, 14 m long.
All piles degraded by bacteria in an outermost layer of < 10 mm over the whole length.
At 1 pile-point locally additional softrot degradation was found probably originated from storage.

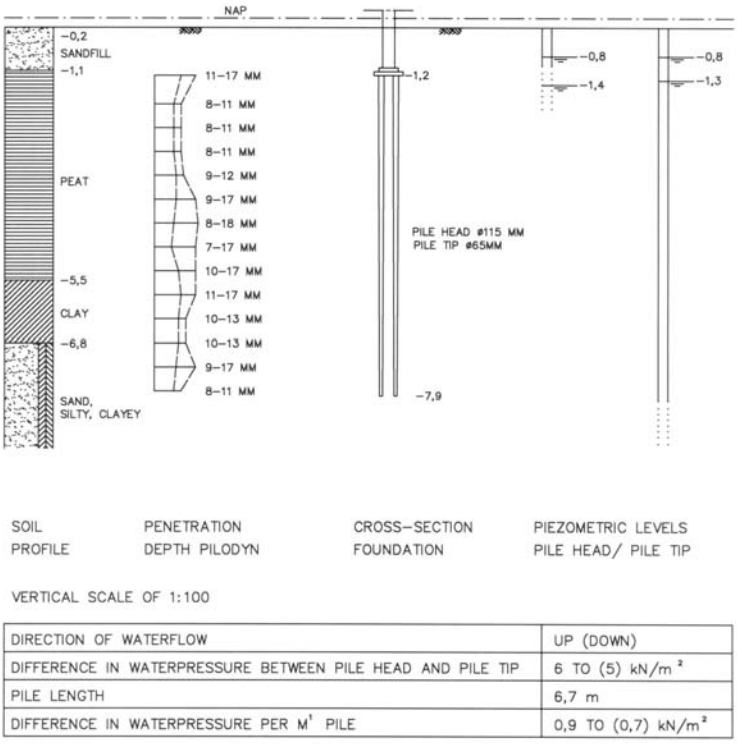
Summary of the chemical analyses
The samples of pile 1 and 2 show normal lignin contents in all three zones. The lignin contents of pile 3 are increased in all zones. From outer parts to inner parts there is a gradient from drastically increased (59 %) to slightly increased (36 %) lignin content.
The N-content of all site samples is limit like the references under the detection.
Active erosion bacteria
Present in all samples (5.te.1.12000; 5.te.1.13400; 5.te.1.1400; 5.te.1.2000; 5.te.1.4000; 5.te.1.65; 5.te.1.8000; 5.te.1.punt; 5.te.2.1000; 5.te.2.4000; 5.te.2.50; 5.te.2.8000; 5.te.2.punt; 5.te.3.1000; 5.te.3.50; 5.te.3.8000; 5.te.3.punt).



DIRECTION OF WATERFLOW	DOWN
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	0 TO 10 kN/m²
PILE LENGTH	14,5 m
DIFFERENCE IN WATERPRESSURE PER M' PILE	0 TO 0,7 kN/m²

Site 6
Site characteristics
The site was located at the side facade of the house Irisstraat 89, Koog a/d Zaan, The Netherlands. Due to the limited bearing capacity of the existing foundation, underpinning (foundation replacement) was executed in 2002. Therefore it was possible to extract the existing piles, as they did not have any function anymore. The house was built in 1937 and consists of 2 stories and an attic. It is a “Amsterdamse fundering” (a double row of wooden piles at the head connected with a short horizontal wooden beam 40 mm thick). Above longitudinal wooden beams with are used supporting the masonry of the house. Three piles were extracted with diameter at the head between 100 - 120 mm and at the tips between 55 - 75 mm and with length of about 6.7 m. Groundwater and soil conditions are shown in the figure below.

Site 6: Irisstraat 89 at Koog a/d Zaan (pile extraction)



DIRECTION OF WATERFLOW	UP (DOWN)
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	6 TO (5) kN/m²
PILE LENGTH	6,7 m
DIFFERENCE IN WATERPRESSURE PER M' PILE	0,9 TO (0,7) kN/m²

Measurements of groundwater

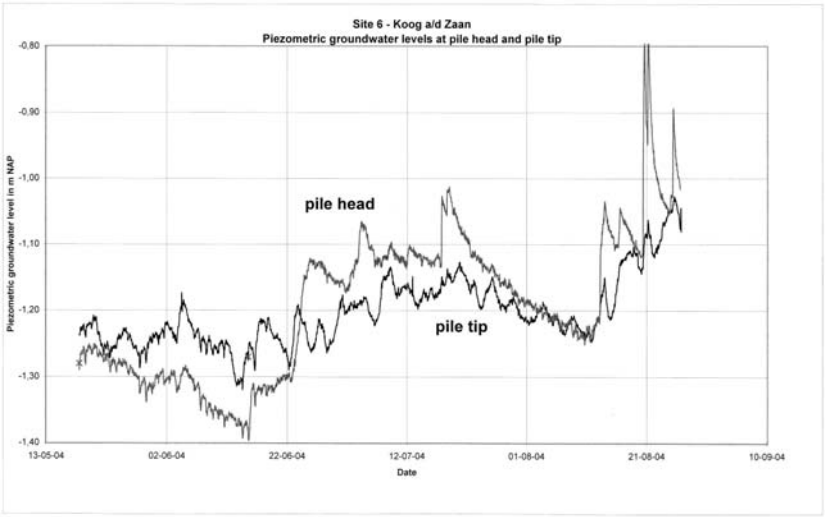
The piezometric groundwater levels near the pile heads were measured with a diver in standpipe B2. The piezometric groundwater levels at pile tips were measured with diver in standpipe B1. Measuring period and weather type is shown in the table below.

Period / Date	**Weather**
18 / 5 till 23 / 6	quite dry
23 / 6 till 17 / 7	rainy
17 / 7 till 13 / 8	almost completely dry
13 / 8 till 26 / 8	very rainy

Earlier measurements in standpipes in the surroundings indicated a small difference in water pressure between pile head and pile tip (water flow 5 kN/m^2 upwards to 3 kN/m^2 downwards). The results of the measurements in 2004 are as follows:

Period / Date	**Piezometric groundwater level at**		**Difference in water pressure kN/m^2**	**Direction waterflow**
	Pile head m NAP	**pile tip m NAP**		
18 / 5 till 23 / 6	-1,31	-1,24	0,7	upwards
23 / 6 till 17 / 7	-1,12	-1,19	0,7	downwards
17 / 7 till 13 / 8	-1,17	-1,19	0,2	downwards
13 / 8 till 26 / 8	-1,03	-1,11	0,8	downwards

Considering the extremely wet summer of 2004 the results of the measurements in this period fit with the earlier measurements in standpipes in the surroundings and with the hypotheses that the amount of decay is small when the difference in water pressure between pile head and pile tip is small.



Site characterisation soil and water chemistry

- Soil: Sand over peat, slightly alkaline, medium to high carbon, low nitrogen, very high carbon/nitrogen ratio, medium phosphorus, medium base cations, medium sulphur, high iron
- Soil solution: slightly alkaline, medium dissolved organic carbon, medium nitrogen, little nitrate and medium ammonium, medium phosphate, low base cations, low sulphate, low iron, low conductivity
- Oxygen, Redox: 1.98 – 1.66 mg/L oxygen, -150 – -250 mV Redox potential – very reducing conditions

Dendro-dating

Age 38-42 years; felling date: summer-autumn 1935; origin: Ede The Netherlands.

Summary degradation patterns

Three extracted piles, all pine and about 6 m long. One horizontal beam originated from the head of the piles (kesp)

All piles degraded by bacteria in sapwood only. In the pile heads a decay gradient appears from severe-weak. Until 4 meters in depth all sapwood is degrade but with decrease degree. Below 4 meter, sapwood is weak or not degraded.

Wood colonising fungi appear throughout the degraded area in all piles. One pile has in the lower part additional softrot. It is believed that the fungal activity started during storage and continues afterwards.

Horizontal beam was pine heartwood and not or weakly degraded by bacteria.

Summary of the chemical analyses

The pile heads of samples 1 and 3 have slightly increased lignin content (35 %). In a depth of 4000 mm under pile head these samples show normal lignin content. Pile 2 has high lignin content (41 %) in pile head sample and slightly increased (34 %) lignin content in the deeper parts.

The N-contents were measured only for the pile heads. They have relative slightly increased values between 0,15 and 0,21mg/g, but the pile 2 has the highest one.

Active erosion bacteria

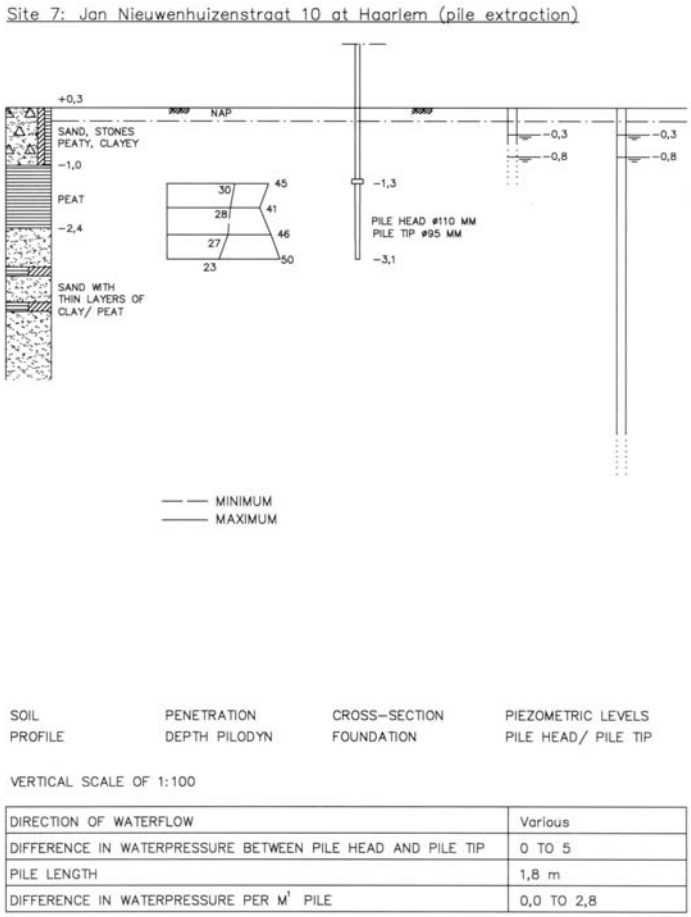
Present in all samples (6.te.1.2000-2500; 6.te.1.4000-4500; 6.te.1.6000-6650; 6.te.2.0-500; 6.te.2.2000-2500; 6.te.2.4000-4500; 6.te.2.6400-6900; 6.te.3.0-500; 6.te.3.2000-2500; 6.te.3.4000-4500; 6.te.3.6000-6900) but one (6.te.1.0-500).

Site 7

Site characteristics

A one-storey house with attic, built at about 1900 located at Jan Nieuwenhuijzenstraat 10 at Haarlem. A few days before the investigation the house on the site has been demolished. The reason for demolishing was a large (continuously) settlement of the foundation.

The foundation is a “Rotterdamse fundering”(a single row of wooden piles, connected with longitudinal wooden beams supporting the masonry of the houses).
Six piles were extracted with diameter of the heads between 95 - 140 mm and at the tips between 80 - 100 mm and with lengths about 180 cm. Groundwater and soil conditions are shown in the figure below.



DIRECTION OF WATERFLOW	Various
DIFFERENCE IN WATERPRESSURE BETWEEN PILE HEAD AND PILE TIP	0 TO 5
PILE LENGTH	1,8 m
DIFFERENCE IN WATERPRESSURE PER M' PILE	0,0 TO 2,8

Site characterisation soil and water chemistry

- No soil and water analysis took place as the site was hydrocarbon contaminated.
- Oxygen, Redox: -140 - -250 mV Redox potential,

Comment: the shallowest depth had the lowest Redox potential, as the hydrocarbon contamination was highest (determined by smell and look).

Dendro-dating

No age indication and date.

Summary degradation patterns

Six pile heads were taken, all poplar and about 1 m long.
All piles were over the whole diameter severely degraded by bacteria with in 5 pile heads .
No gradient in decay over the pile length (approximately 1 meter).
Wood colonising fungi throughout all piles and in 5 piles additional softrot decay.

Active erosion bacteria

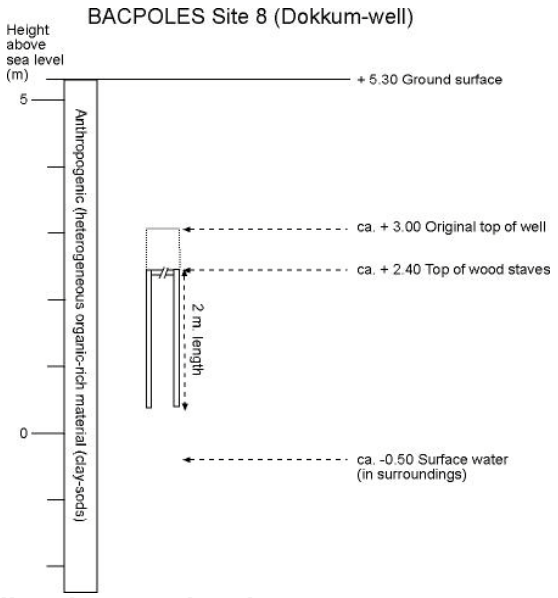
Present in all samples (7.te.1.500-750; 7.te.2.500-750; 7.te.3.850-1100; 7.te.4.1370-1680; 7.te.4.300-550; 7.te.5.1330-1730; 7.te.5.200-450; 7.te.6.430-680).

Site 8

Site characteristics

Archaeological site *well* (Dokkum, NL) Site on a so-called *terp*: a man-made mound, which prevented the town from flooding by the sea. Wood was sampled from a square well or cesspit of oak in the middle of the excavation pit, probably from the 15th or 16th century.

The *terp* material consists of clay sods mixed with large amounts of organic material and manure. Groundwater levels are unknown. Groundwater and soil conditions are shown in the figure below.



Site characterisation soil and water chemistry

- Soil: Loam, slightly alkaline, medium to high organic matter content, high nitrogen, medium carbon/nitrogen ratio, high phosphorus, high base cations, low sulphur, high iron content, very eutrophic
- Soil solution: alkaline, high dissolved organic carbon, high nitrogen, little nitrate and high ammonium, high phosphate, high base cations, low sulphate, low iron, high conductivity, slight seawater influence
- Oxygen, Redox: 0.0 – 0.3 mg/l oxygen, +270 mV at 0.1 m depth (sampling disturbance) further down +120 – -100 mV Redox potential, in deeper layers anoxic

Comment: High potassium content together with low chloride content possible release of potassium from the debris, ground water mixed with rain water had very high sulphate, nitrate and medium iron content and was different from the soil water composition.

Dendro-dating

No age indication possible; felling date around 1480, origin, Germany (central and south) and the Netherlands (east, central and north).

Summary degradation patterns

Five piles heads, all oak.

All piles are degraded by bacteria. In 2 piles the decay is severe, in 2 other piles a decay gradient (severe-moderate) appears and in 1 pile half of the diameter is severe-moderate degraded and the rest is sound. The presence of wood colonising fungi ranges from absent to throughout the whole sample.

Summary of the chemical analyses

The lignin content of all samples is increased, sometimes drastically (55 %), sometimes slightly (up to 38 %). The N-content show the same relationship like lignin content. Samples with a high lignin content show higher N-content than samples with lower lignin content.

Active erosion bacteria

Present in all samples (8.td.1.600; 8.td.2.300; 8.td.3.350; 8.td.4.600; 8.td.5.150).

Site 9

Site characteristics

Archaeological site *Barrel or casket* (Dokkum, NL). Site on a so-called *terp*: a man-made mound, which prevented the town from flooding by the sea. Wood was sampled from an oak barrel that was uncovered in the wall of the excavation pit, probably from the 15th or 16th century. The *terp* material consists of clay sods mixed with large amounts of organic material and manure. Groundwater levels are unknown.

Site characterisation soil and water chemistry

- Soil: Loam, slightly alkaline, medium to high organic matter content, high nitrogen, low carbon/nitrogen ratio, high phosphorus, high base cations, low sulphur, high iron content, very eutrophic
- Soil solution: alkaline, medium dissolved organic carbon, high nitrogen, no nitrate, high ammonium, high phosphate, high base cations, low sulphate, low iron, high conductivity, slight seawater influence
- Oxygen, Redox: 2.6 – 1.8 mg/l oxygen, +190 – -100 mV Redox potential

Comment: High potassium content together with low chloride content possible release of potassium from the debris.

Dendro-dating

No age indication possible; felling date around 1480; origin: Poland, the Baltic, Germany (north west) and the Netherlands.

Summary degradation patterns

Four oak samples over the whole diameter severely degraded by bacteria. Wood colonising fungi throughout all samples.

Summary of the chemical analyses

All samples have strong increased lignin content of about 50 %.

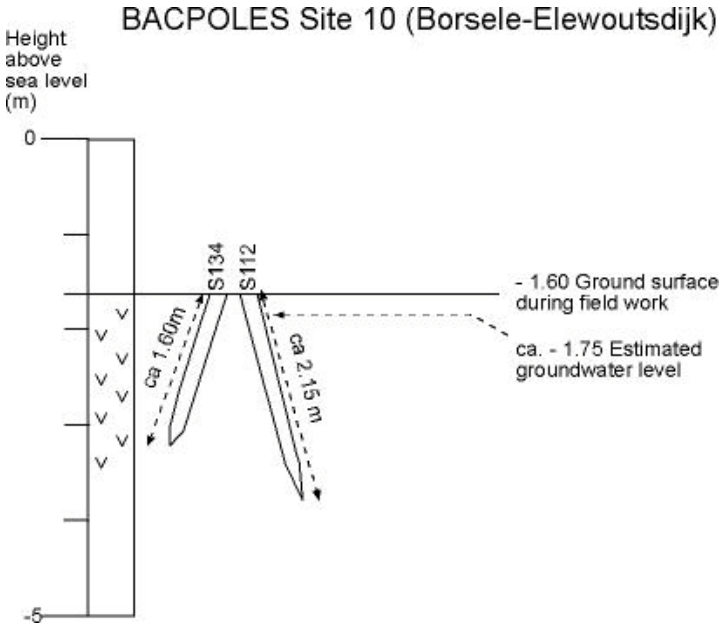
Active erosion bacteria

Present in all samples (9.ta.1; 9.ta.2; 9.ta.3; 9.ta.4).

Site 10

Site characteristics

Archaeological site NL (Borsele) Roman settlement next to the Westerschelde estuary. Two oak piles were extracted as wood samples. The pre-excavation groundwater level could not be determined due to building activities on site; during excavation it was within 15 cm of the surface. The soil consists of peat overlying clay. This is also site where prehistoric (“fossil”) wood was re-used as building material. Groundwater and soil conditions are shown in the figure below.



Site characterisation soil and water chemistry

- Soil: Peat over clay, slightly acidic, very high carbon content, high nitrogen, very high carbon/nitrogen ratio, low phosphorus, medium base cations, high sulphur, low iron content
- Soil solution: alkaline, high dissolved organic carbon, high nitrogen, no nitrate, high ammonium present, high phosphate, high base cations, high sulphate concentration, medium to high iron, high conductivity, seawater influence
- Oxygen, Redox: 1.7 – 0.0 mg/l oxygen, zero oxygen from 0.5 m depth on, +20 – -150 mV Redox potential

Comment: peat was at the surface dried out, deeper layers had H_2S smell.

Dendro-dating

No age indication possible; felling date around spring 100, origin, the Netherlands (central).

Summary degradation patterns

Two piles (Ø 160 – 200 mm), both oak.
Both piles degraded by bacteria. 1 pile severely decay over the whole diameter, other pile severe decay in sapwood and weak-sound in heartwood. No gradient over the length of the pile (30 cm).
Wood colonising fungi sometimes present in degraded wood (in outermost 20 mm, over whole length).

Active erosion bacteria

Present in 1 sample (10.td.2.120), absent in others (10.td.1.480; 10.td.1.830; 10.td.2.850).

Site 11

Site characteristics

Archaeological site NL (Roman ship Vleuten de Meern) Roman embankment on the river Rhine, dated at 100 AD. Wood samples were taken from an oak pole and some oak planks that were obtained during an excavation at a later date. The soil material was sand and clay. This is also the site where a roman ship was found to be lying (next to the embankment).

Site characterisation soil and water chemistry

- Soil: Sand and clay mixed, medium alkaline, low carbon content, low nitrogen, medium carbon/nitrogen ratio, medium phosphorus, high base cations, medium sulphur, medium iron
- Soil solution: alkaline, high dissolved organic carbon, medium nitrogen, no nitrate, medium ammonium, medium phosphate, medium base cations, low sulphate concentration, low iron, low conductivity
- Oxygen, Redox: -100 – -250 mV Redox potential, very reducing

Comment: Soil sample only from one depth as sampling collided with archaeological excavation, clay bluish grey colour.

Dendro-dating

No age indication possible; felling date around spring 100; origin: the Netherlands (central part).

Summary degradation patterns

Four planking samples and one pole sample . All samples are oak.
All samples degraded by bacteria over whole diameter. Severe decay in 4 samples and decay gradient (severe-weak) in the pile sample.

Site 12

Site characteristics

Marine archaeological site NL. The Burgzand Noord 15 (or BZN - 15; the fifteenth shipwreck known from the Burgzand area in the Wadden sea) site comprises the remains of a ship - one of many - that sunk on the infamous Burgzand in the seventeenth century ad. It lies at a depth of between 10 and 15 metres at high tide. Two wood samples were taken; an oak plank from 20 to 30 cm and a pine plank from 30 to 40 cm below the surface.

Site characterisation soil and water chemistry

- Soil: Pure sea sand, alkaline, very low carbon content, no nitrogen, low phosphorus, low base cations, low sulphur, medium iron
- Surrounding water: alkaline, no dissolved organic carbon, low nitrogen, low nitrate, no ammonium, low phosphate, high base cations, high sulphate, low iron, high conductivity, seawater influence

Comment: only one water sample of the surrounding water.

Dendro-dating

No age indication possible; felling date around 1628; origin oak: Germany (north – west) and the Netherlands; pine: Flesberg Sweden.

Summary degradation patterns

Two boards, one oak and one pine. Both samples fully severely degraded by bacteria. Wood colonising fungi throughout all samples.

Summary of the chemical analyses

All samples have increased lignin content (40 %) and increased N-content (4 mg/g).

Active erosion bacteria

Present in all (12.ta.1.1; 12.ta.1.2; 12.ta.1.3; 12.ta.1.4; 12.ta.2.1; 12.ta.2.2; 12.ta.2.3) but one samples (12.ta.2.4)

Site 13

Site characteristics

Marine archaeological site NL. The Burgzand Noord 3 (or BZN - 3; the third shipwreck known from the Burgzand area in the Waddensea) is probably the wreck of a ship of the East India Company, possibly the “Rob” which is reported to have sunk in 1640. The wreck site, which was discovered in 1985, is at least 60 metres long and 20 metres wide and lies at a depth of between 6 and 9 metres at high tide. Wood samples were taken from a part of the ship’s board that has never physically been covered - like the rest of the wreck site - but had been covered with a 30 to 100 cm-thick layer of sediment for many centuries.

Site characterisation soil and water chemistry

- Soil: Pure sea sand, alkaline, very low carbon content, no nitrogen, low phosphorus, low base cations, low sulphur, medium iron
- Surrounding water: alkaline, no dissolved organic carbon, low nitrogen, low nitrate, no ammonium, medium phosphate, high base cations, high sulphate, high iron, high conductivity, seawater influence

Comment: only one water sample of the surrounding water.

Dendro-dating

No age indication possible; felling date around 1640; origin: Flesberg Sweden.

Summary degradation patterns

Four pine samples fully degraded by bacteria. Half of the samples severe and in the other half a gradient was visible from moderate to weak. Wood colonising fungi throughout all samples.

Active erosion bacteria

Present is 13.ta.1.1, 13.ta.1.4; absent is 13.ta.1.2, 13.ta.1.3

Site 14

Site characteristics

A late medieval castle situated in a wet area formed by the river Trave near Travenhorst north east of Bad Segeberg in Schlesweig-Holstein north Germany. Three piles were extracted (diameter 260- 320 mm and length 140 to 190 cm) from 100 - 110 cm peat and extent down to the sand layer underneath. The upper part of the piles was exposed for two years and had deteriorated but 10 cm under the dried out material the peat and wood was still wet.

Site characterisation soil and water chemistry

- Soil: Peat over sand, acidic, very high carbon content, high nitrogen, medium carbon/nitrogen ratio, low phosphorus, low base cations, high sulphur, very high iron content
- Soil solution: slightly alkaline, high dissolved organic carbon, low nitrogen, nitrate only in upper layer present not further down, medium ammonium present, low phosphate, low base cations, medium to high sulphate, low iron
- Oxygen, Redox: +260 – -220 mV Redox potential

Comment: It might be that peat has formed under marine or brackish conditions with high pyrite concentrations. Today there is freshwater influence resulting in pyrite-oxidation, which releases sulphate. Simple sea spray deposition can not explain this, as the chloride concentration is far to low for this.

Dendro-dating

Age 32-72 years, no date.

Summary degradation patterns

Three oak piles fully degraded by bacteria. Half of the radius (outside) severe and the other half (inner side) moderate degraded. No gradient present in depth over 60 cm.
Wood colonising fungi absent or in the outermost quarter of the degraded area (in pile head and in lower parts).

Summary of the chemical analyses

The outside zone, which was mostly not complete, shows strong increased lignin content (59 %). The massive rest of the samples have a slightly increased lignin content (33 %). The N-content of the outside parts are also very high (8 mg/g).

Active erosion bacteria

Present is most samples (14.ta,2.0,89-1,20; 14.ta.2.1,20-1,54; 14.ta.3.0,34-0,57; 14.ta.2.0,15-0,23; 14.ta.3.0,83-1,39), absent in others (14.ta.2.0,62-0,89; 14.ta.1.0.98-1.53; 14.ta.1.0.49-071).

Site 16

Site characteristics

Archaeological site: Glastonbury Lake Village, near Godney, Somerset in the United Kingdom. The site is an Iron Age wetland settlement (maximum 20 houses at any one time) that was occupied between roughly 250 BC and 50 AD. It had a wooden foundation that was enclosed within a palisade of piles of different species including oak (*Quercus sp*.), hazel (*Corylus avelana*.) and birch (*Betula sp*.). Sampling was carried out at two piles of the palisade. At that location piles were embedded in a row.

Site characterisation soil and water chemistry

- Soil: Peat, acidic, high carbon content, high nitrogen, medium carbon/nitrogen ratio, medium phosphorus, low base cations, high sulphur, low iron
- Oxygen, Redox: -120 – -220 mV Redox potential, very reducing

Comment: only two soil samples without replicates, no water samples.

Dendro-dating

No age indication possible; no date.

Summary degradation patterns

Two alder samples severely degraded. Caused by the degree of degradation no pattern visible.

Active erosion bacteria

Present in 16.ta.15.@.1; 16.ta.15.@.2; absent in 16.ta.04.@.1.

Site 17

Site characteristics

Archaeological site: Harters Hill, near Coxley (Glastonbury), Somerset in the United Kingdom. The site is a late Bronze Age ritual pile alignment that traversed an area of shallow water. The site has been dated by dendrochronology with one pile having a felling date of 1076/5 BC with other trees used for the piling at least up till 1064 BC. Sampling was carried out at three piles.

Site characterisation soil and water chemistry

- Soil: Peat, very acidic, high carbon content, high nitrogen, medium carbon/nitrogen ratio, low phosphorus, medium base cations, high sulphur, low iron
- Oxygen, Redox: +460 – -230 mV Redox potential, at low depth possibly oxygen present further down reducing

Comment: one single soil sample without replicate and not water samples received.

Dendro-dating

No age indication possible of the tree were the timber originated from; felling date around 1065 BC; origin England.

Summary degradation patterns

Two oak samples degraded by bacteria severe to moderate.

Active erosion bacteria

Absent in all samples (17.ta.1 and 17.ta.2).

Site 18

Site characteristics

Foundations of bridge “Ponte Balbi” at Venice in Italy. The bridge dates from the 16th century. The site was located along Rio de S. Zulian (Rio della Guerra). This canal is the border between the city-quarters Castello and San Marco and was set dry for restoration works. Core samples were taken from 4 piles.

Site characterisation soil and water chemistry

- Soil: Loamy sediment with debris, alkaline, medium carbon content, medium nitrogen, high carbon/nitrogen ratio, high phosphorus, high base cations, high sulphur, medium iron content
- Soil solution: alkaline, high dissolved organic carbon, high nitrogen, no nitrate, high ammonium, high phosphate, high base cations, high sulphate, low iron, very high conductivity, slight seawater influence
- Oxygen, Redox: zero oxygen, -200 – -410 mV Redox potential, very reducing conditions

Comment: wastewater influence.

Dendro-dating

Age 43-55 years, no date.

Summary degradation patterns

Three oak piles degraded by bacteria, severe in half of the radius and in the other half showing a gradient from moderate, weak to sound. One larch pile was sound with a small layer (< 10 mm) of degradation by bacteria. One fir horizontal beam was severely degraded by bacteria. Wood colonising fungi in some of the piles in the outmost layer of the degraded area.

Active erosion bacteria

Absent in all samples 918.td.4.150 and 18.td.4.150).

Site 19

Site characteristics

Foundations of the Palazzo Balbi, Venice, Italy. The age of the foundation is probably 9th or 10th century. The palace itself is dated at 15th or 16th century. The site was located along Rio de S. Zulian (Rio della Guerra) in Venice. This canal is the border between the city-quarters Castello and San Marco and was set dry for restoration works. Samples were taken from 4 piles (3 cores and 1 disk).

Site characterisation soil and water chemistry

- Soil: Loamy sediment with debris, alkaline, medium to low carbon content, low nitrogen, high carbon/nitrogen ratio, medium phosphorus, high base cations, high sulphur, medium iron content
- Soil solution: alkaline, high dissolved organic carbon, high nitrogen, no nitrate, high ammonium, high phosphate, high base cations, high sulphate, low iron, very high conductivity, slight seawater influence
- Oxygen, Redox: 1.2 – 0.0 mg/L oxygen, -120 – -330 mV Redox potential, very reducing conditions

Comment: Oxygen measurements were hindered by debris and resulted in air oxygen altering the measurements. Site was wastewater influenced.

Dendro-dating

Age 12-22 years, no date.

Summary degradation patterns

Four pine piles degraded by bacteria, severe in half of the radius and moderate to weak in the other half.

Active erosion bacteria

Absent in all samples (19.td.4.100 and 19.td.4.100).

Site 20

Site characteristics

Marine archaeological site (Stora Sophia) in Sweden

Dendro-dating

Age 113-128 years, felling date around 1634; origin oak: Germany (north – west) and Poland.

Summary degradation patterns

Two oak samples degraded by bacteria, half the sample was severe and the other half was moderate degraded. Wood colonising fungi throughout the whole degraded area.

Active erosion bacteria

Absent in most samples (20.ta.1@2; 20.ta.2.@1; 20.ta.3.@1; 20.ta.4@1) present in and 20.ta.2@2.

Site 21

Site characteristics

Marine archaeological site (Mollösund) in Sweden

Site characterisation soil and water chemistry

- Soil: Pure sea sand, alkaline and deeper layer neutral, very low carbon content, low nitrogen, medium carbon/nitrogen ratio, medium phosphorus, medium base cations, medium sulphur, medium iron
- Surrounding water: slightly alkaline, low dissolved organic carbon, low nitrogen, no nitrate, low ammonium, medium phosphate, high base cations, high sulphate, no iron

Comment: only one water sample of the surrounding water.

Dendro-dating

Age 294 and 137; felling date around 1391; origin: The Netherlands - Germany (north – west) and Poland.

Summary degradation patterns

Two oak samples degraded by bacteria, mainly moderate but ranging from severe to weak.

Active erosion bacteria

Present in 21.ta.4@1and absent in 21.ta.1.@.1.

Site 22

Site characteristics

Two piles Ø 250 mm, about 600 cm long originated from the foundation under the parliament in Stockholm approximately 100 year old. Backfillings and mixed soils.

Dendro-dating

Age 145-147; felling summer – autumn 1894; origin: Southern Sweden.

Summary degradation patterns

In both pine piles the sapwood is degraded by bacteria and in addition by softrot. There is a gradient from severe at the bark side to weak inwards. Wood colonising fungi throughout the whole degraded area. No gradient in degradation with depth (approximately 4 meters).

Active erosion bacteria

Absent in all samples (22.td.1; 22.td.2; 22.td.2.460-470).

Site 23

Site characteristics

One pile head Ø 250 mm 40 cm long originated from foundation under a 100-year-old flower factory in Leeuwarden (North part of the Netherlands). Soil is clay and peat.

Dendro-dating

Age 52-53; felling summer – autumn 1901; origin: Southern Sweden.

Summary degradation patterns

One pine pile with bacterial degradation restricted to the sapwood. Degradation mainly severe but partly moderate. Additional softrot degradation in large parts of the degraded area. Wood colonising fungi throughout the whole degraded area.

Summary of the chemical analyses

The lignin contents are clearly increased (55 %) in the outside zones. The values of the other zones seem to be normal. The N-content of the outer part is also drastically increased (5,8 mg/g).

Active erosion bacteria

Present in all samples (23.td.1.20; 23.td.1.300/500).

Site 24

Site characteristics

The wooden city of Bryggen (Bergen Norway) is built on a wooden foundation and wooden remainings of by fire destroyed earlier built houses. The soil includes a high concentration of organic material and the groundwater is influenced by the sea level. The samples are taken approximately 110 –170 cm below the street surface.

Site characterisation soil and water chemistry

- Soil: Mixture of sand, loam and organic material*, slightly alkaline, medium to high organic matter content, high nitrogen, medium carbon/nitrogen ratio, high phosphorus, high base cations, low sulphur, high iron content, very eutrophic
- Soil solution: slightly alkaline, low dissolved organic carbon, low nitrogen, little nitrate in upper layers whereas little ammonium in lower layers, medium phosphate, low to medium base cations, low sulphate, high iron, low conductivity
- Oxygen, Redox: 2.2 – 0.9 mg/l oxygen, +220 – 150 mV

Comment: lowest layer was sand, oxygen was measured there, very close to estuary but no seawater influence because layers with groundwater originating from rainwater coming from the adjacent mountains, *Site is build from wood beams and organic and other waste material in different layers, layers very heterogenic, high anthropogenic influence.

Dendro-dating

no age indication and date.

Summary degradation patterns

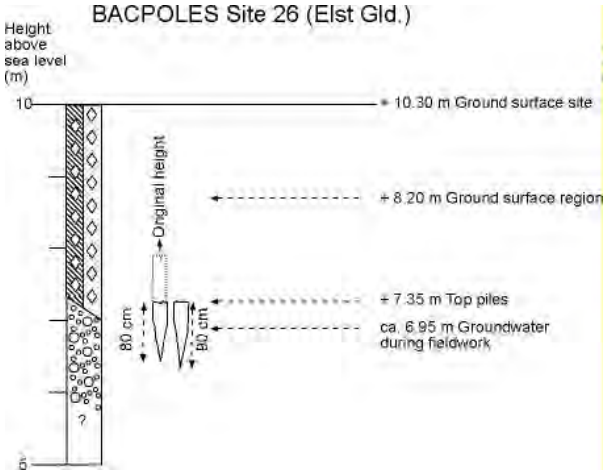
Four pine samples severely degraded by softrot fungi probably in addition with bacteria. High fungi activity throughout all samples. One sample was less degraded in burned areas.
One birch sample was totally degraded and no degradation pattern remained.

Active erosion bacteria
Present in half of the samples (24.ta.1.@4; 24.ta.2; 24.ta.3) and absent in the other half (24.ta.4 a+c; 24.ta.4; 24.ta.5).

Site 25
Site characteristics
Marine archaeological site pile construction in Lidköping habour of river Lidam (S)
Site characterisation soil and water chemistry
- Soil: Pure sand, neutral, low carbon content, low nitrogen, low carbon/nitrogen ratio, low phosphorus, low base cations, low sulphur, medium iron
- Soil solution: slightly alkaline, medium dissolved organic carbon, medium nitrogen, medium nitrate, no ammonium, low phosphate, low base cations, high sulphate, medium iron, low conductivity.

Dendro-dating
Age 72, no date.
Summary degradation patterns
One oak pile degraded by bacteria moderate to severe. Gradient present towards the lower parts of pile where a layer of 20 mm is degraded only. Wood colonising fungi throughout the whole degraded area in the pile head and partly in the degraded area in the lower part.
Active erosion bacteria
Present (25.ta.1.@.1).

Site 26
Site characteristics
Archaeological pile foundation under roman temple in Elst (NL) Foundation of Roman temple (1^{st} - 2^{nd} century AD). Oak foundation poles were sampled from the circumference wall of the temple terrain. The tops of the poles were roughly on groundwater level. The sediment in which the poles were buried is sand, overlain with clay and debris. One corner of the excavation-pit showed traces of pollution from an oil-tank. Groundwater and soil conditions are shown in the figure below.



Site characterisation soil and water chemistry
- Soil: Sand overlain by clay and debris, slightly alkaline, low carbon content, low nitrogen, medium carbon/nitrogen ratio, medium phosphorus, medium base cations, low sulphur, low iron
- Soil solution: slightly alkaline, medium dissolved organic carbon, medium nitrogen, medium nitrate, medium ammonium, high phosphate, medium base cations, medium sulphate, low iron, medium conductivity, slight seawater influence
- Oxygen, Redox: 100 – 10 mV Redox potential, reducing.

Dendro-dating
Age 46-121; felling date around 106 AD; origin: Germany.
Summary degradation patterns
Three oak samples degraded by bacteria moderate at the outside and severe near the pith. Wood colonising fungi throughout all samples
Summary of the chemical analyses
The samples show increased lignin content of 40 %, the N-content is like the reference under the detection limit.
Active erosion bacteria
Present in 26.te.1.0-90 and 26.te.30-74 absent in 26.te.2.0-78.

Site 27
Site characteristics
Ship wreck graveyard (NL) The 16th century fishing vessel (type: "Waterschip") on lot GZ 80 sank on the Zuiderzee and was embedded in the clay layer at the bottom. The sea has been converted into land in 1967. The site was covered later with sand. Four horizontal oak planks were sampled; the upper ones above, the lower ones below the groundwater level.
Site characterisation soil and water chemistry
- Soil: Sand over Clay, slightly alkaline, low carbon content, medium nitrogen, medium carbon/nitrogen ratio, medium phosphorus, high base cations, medium sulphur, no iron
- Soil solution: slightly alkaline, high dissolved organic carbon, high nitrogen, high nitrate, high ammonium, medium phosphate, medium base cations, medium sulphate, medium iron, high conductivity, seawater influence.

Dendro-dating
Age 41-118; felling date around 1549; origin: Germany (northwest).
Summary degradation patterns
Five oak samples degraded by bacteria: in three samples degradation mainly severe but partly moderate, additional softrot decay, wood colonising fungi throughout the samples. In one sample the degradation is mainly severe and partly moderate. In one sample the degradation is moderate to weak only.
Summary of the chemical analyses
The lignin content (48 %) is very increased whereas only for one of the two samples shows increased N-content (3 mg/g).
Active erosion bacteria
Present in all samples (27.ta.1 – 27.ta.5).

Site 28
Site characteristics
Ship wreck graveyard (NL) The wreck on lot KZ 47 (3 km South-Southeast of GZ 80) sank in the Zuiderzee. The sea has been converted into land in 1967. After discovery, the site was physically protected by a mound of fine sand covered with plastic foil. During the BACPOLES fieldwork, the burial environment was wet and reducing. Two wood samples were taken; a beam from the portside and deck planking above the fish well.
Site characterisation soil and water chemistry
- Soil: Sand over Clay, slightly alkaline, low carbon content, medium nitrogen, medium carbon/nitrogen ratio, medium phosphorus, high base cations, medium sulphur, high iron
- Soil solution: slightly alkaline, high dissolved organic carbon, high nitrogen, no nitrate, high ammonium, medium phosphate, medium base cations, medium sulphate, low iron, high conductivity, seawater influence

Dendro-dating
Age 20-60; felling date around 1519; origin: unknown.

Summary degradation patterns
Both oak samples degraded by bacteria: One sample degradation severe. In the other sample is the degradation moderate with additional softrot decay. Wood colonising fungi throughout both samples
Summary of the chemical analyses
These samples have very increased lignin (47 %) and N-contents (7mg/g).
Active erosion bacteria
Present in 28.ta.2, absent in 28.ta.1.

Reference values sound or fresh timber
For each species 5 sound stems are analysed
Compression strength decreases towards the pith

	Moisture content	**Density (dry)**	**Compression strength**
	Sapwood / heartwood		**Mature / juvenile wood**
Alder	110-130	500-550	23-27
Douglas	120 / 40-70	475	23-25
Larch	100 / 50	600-650	30-35
Oak	110 / 90-100	650-700	30-36
Pine	120-140 / 50-70	400-450	20-22
Spruce	130-140 / 80-100	400-450	17-20 (

3.5. Conclusions

(by Junga, Gelbrich, Huisman, Keijer, Klaassen, Kretschmar & Lamersdorf)

Although it is hardly possible to compare all 27 sites with each other because of their large variety in soil type, groundwater level and age, it can be concluded that wood degrading bacteria exist in a wide range of environmental conditions (under ground water, under salty sea water and in fresh water) and is active in timbers from different ages ranges from 65 – 3000 years. Although the process of degradation is not fully understood it is clear that velocity is environmental and species depended. In the sites 4 and 7 the whole pine sapwood layer or the whole diameter of poplar (> 60 mm wide) can be degraded within 65 years. From the long-term measurements site we learn that in two different Amsterdam environments (one more active then the other one) pine sapwood is not degraded by bacteria within a period of 18 months. Whereas degradation in a lower level is found only after 300 years under salty conditions in site 12 or after 600 years in Venice.
In order to look for general trends in degradation, the relatively young sites were separated from the old and very old sites and a division was made between the foundation sites and the archaeological sites.

Regarding the foundation sites only, the following general trends can be seen:

1. There seems to be no relation between the degree of decay and the surrounding environment;
2. There seems to be a relation between the nitrogen content in the wood (and phosphors content) and the degree of degradation;
3. The transport of nitrogen (and phosphorus) to the inside of the wood seems to be crucial for active bacterial wood degradation. The origin of nitrogen could be explained by (or a combination of) A) already available in the wood before installation of the piles; B) active accumulation by bacteria (e.g. nitrogen fixating bacteria); C) water flux through the piles; D) diffusion
4. Because bacterial wood degradation is found under oxygen free circumstances and without a “continuous” stream of oxygen supply, it seems that oxygen is not a crucial factor to initiate and support the process of bacterial wood degradation. However an increasing oxygen concentration seems to speed up the process of degradation as showed in the microcosm experiment (chapter 4. But this could be caused by secondary wood degrading fungi, who feed themselves with bacterial debris and stimulate in this way the bacterial activity. Wood under these circumstances shows more “colonising fungi”, which could be actually secondary wood degraders. Therefore the role of oxygen is unclear, but it is probably of minor importance while high degradation velocities were found in deep soil layers, where no oxygen can be expected.
5. It is hypothesed that a water flux through the wood supports the process of bacterial wood degradation because of the processes as explained at point 3 and 4. From chapter 5 we learn that water transport through the wood is possible in axial direction only. It can be imaged that the dynamic situation of the pressure differences between the deeper and shallow ground water causes a dynamic water flux in the wood. The size of this water flux depends on the openness of structure of the wood species and of the pressure differences. The higher degree of degradation in pine compared to spruce under the same conditions (e.g. site 3 Amsterdam) can be explained by the flux theory. But also the high degradation in relative short pine piles, in situations without a difference over longer time in water pressure between the deeper and shallower groundwater, shows that specific events (e.g. temporary lower groundwater, heavy rain) causes a water flux in the wood which is probably enough to support bacterial wood degradation.

6. The degradation velocity within Spruce piles is lower than that of pine but within 100 years a peal of 1-20 mm is weakly degraded by bacteria. These differences are clear by comparing the degree of bacterial wood degradation of those sites were whole piles were extracted. The pine-line: In Amsterdam (site 3, pile length 11 m), Zaandam (site 6 pile length (6) and Haarlem (site 4, pile length 5 m) pine piles were extracted and all piles are degraded over the whole length and only in the sapwood. Haarlem: severe degradation both at the head and at the tip; Amsterdam severe to weak degradation both at the head and at the tip; Zaandam severe to weak degradation at the head and weak to sound at the tip. The Spruce line: Amsterdam (site 3, pile length, 11m) weakly degraded in the outmost 20 mm in both the head and the tip; Rotterdam (site 5, length 16 m) weakly degraded in the outermost 0-10 mm in both the head and the tip.

Regarding the archaeological sites site only, the following statements can be made:
1. Because of the large diversity no specific conclusions could be made;
2. Because the sites were chosen with the expectation that bacterial wood degradation would occurs, sites with oxygen were ignored. If oxygen in measurable concentrations occurs fungal activity will appear and destroy wood structures in relative short periods. At all sites, except for Bryggen, the main cause of the degradation was bacterial activity, although often “colonising hyphes were observed;
3. Almost all samples, ranging in age from 300 – 2000 years, were over full diameter degraded by bacteria. Although the degree degradation is age independent, in most of the samples the wood degrading bacteria were still active. This means that not the infection, but the velocity of bacterial wood degradation is regulated by the conditions under which the wood is storied. As no environmental (soil chemistry) dependency was found it is also here supposed that the presence of a water flux could be crucial.

3.6 Survey of all sample sites clustered by the type of sample sites

Site	Species	Decay degree	Sound wood	Age (year)		Direction waterflow pile, difference water-pressure m^1 pile kN/m^2		Diameter (mm)	Soil type	N, P in soil	N, P in soil water
Foundation sites											
1	Pine/spruce	50%S / 50%M	heartwood	80	30- 50	Down	1.2-1.6	180 - 280	Poor sand	very low	med. N, low P
2	Spruce	25 mm S-W	80 mm	70	5-18	Up	0.2-1.1	220 - 265	Loamy sand	medium	medium
3	Pine Spruce	S-W 30 mm W	Heartwood 85 mm	80	7-15 spruce 14-24 pine	Down	2.4	230			
4	Pine	S	heartwood	100	38-50 top 17-30 point	Up/down	0-1.1 / 0-1.7	140	Loamy sand	medium	high N, med. P
5	Spruce/fir	< 10 mm W	240	100	6-14	Down	0-0.7	250	Clay over peat	high N, low P	high N, med. P
6	Pine	S-W	heartwood	65	7-18	Up/down	0-0.9 /-0-0.7	115	Sand over peat	low N, med. P	medium
7	Poplar	S	--	105	23-50	Up/down	0-2.8	110			
18	Oak	80%S-W	15%	400			?	120-190	Loamy, debris	med. N, high P	high
19	Pine	S-W	--	400			?	120-190	Loamy, debris	low N, med. P	high
22	Pine	S-W	heartwood	100			?	250			
23	Pine	S	heartwood	100			?	250			
Marine archaeological sites											
12	Oak	S	--	400			--	20	Poor sea sand	low	low
13	Pine	S-W	--	350			--	20	Poor sea sand	low	low N, med. P
20	Oak	S-M	--	350			--	20			
21	Oak	M	--	700			--	20	Poor sea sand	low N, med. P	low N, med. P
25	Oak	S-M	--	800			--	200 (pile)	Poor sand	low	med. N, low P
Terrestrial archaeological sites											
8	Oak	S (-M)	--	500			--	120 (pile)	Loam	high	high
9	Oak	S	--	500			--	20	Loam	high	high
10	Oak	S	Heartwood only	2000			--	160–200 (pile)	Peat over clay	high N, low P	high N
11	Oak Oak	S S-W	-- --	2000 2000			-- --	20 270 (pile)	Sand and clay	low N, med. P	medium
14	Oak	S-M		1000			--	240 (pile)	Peat over sand	high N, low P	low
16	Alder	S	--	2250			--	100 (branch)	Peat	high N, med. P	
17	Oak	S-M	--	3000			--	120 (pile)	Peat	high N, low P	
18	Oak	80%S-W	15%	400			?	120-190	Loamy, debris	med. N, high P	high
19	Pine	S-W	--	400			?	120-190	Loamy, debris	low N, med. P	high
26	Oak	M-S	--	1900			?	250 (piles)	Clay over sand	low N, med. P	med. N, high P
27	Oak	S (-M)	--	400			?	20	Sand over clay	medium	high N, med. P
28	Oak	S + M	--	??			?	20	Sand over clay	medium	high N, med. P

Chapter 4

Identity of wood degrading bacteria

4.1 Isolation of bacteria

(by Nilsson & Björdal)

4.1.1 Introduction

Isolation of erosion bacteria was divided into a three-step process.
1. Obtaining a successful inoculation of erosion bacteria from the original BACPOLES sample.
2. Purification of the erosion bacteria consortia.
3. Selection of samples for molecular analyses.

4.1.2 Inoculation of erosion bacteria from original BACPOLES samples

General method

All original BACPOLES samples that arrived were processed as soon as possible (within a few days). The first samples that arrived were used to find suitable culture conditions. Sampling was, where possible, intentionally made from areas judged to be in an intermediate stage of decay. From very degraded samples, sub-samples were taken from the interior part.

Fig. 1. Original BACPOLES samples incubated in inverted jars with sterilised media, kapok and small pine samples.

The procedure was basically to take a small wood block (approx. 1 cubic centimetre) from the wood. This was done with sterilised tools. Each sample was transferred to a flask or jar with mineral salts medium plus fresh pine wood and ca 20 mg of kapok (Fig. 1). The flasks/jars were sterilised in an autoclave. Incubation occurred at ambient room temperature (22°-24°C). The cultures were checked for erosion bacteria attack on the wood or kapok fibres at irregular intervals during a period of up to 32 months. Prior to examination, one wood block or a few kapok fibres were removed from the jar under sterilised conditions. Thin sections were cut by hand with a razor blade from the wood block and stained in order to highlight the microbial attack. Kapok fibres were stained directly without processing. Both fibres were examined carefully for microbial attack on the cell wall by erosion bacteria, using light-microscopy (Fig. 2,3). The first step in the attack was attachment by the bacteria to the available surfaces of the substrate, followed by erosion of the surface. The bacteria always seemed to align themselves along the cellulose microfibrils. Thus, bacteria degrading the outer part of kapok fibres were oriented transversely to the fibre axis, whereas bacteria in the lumen were oriented along the fibre axis. Microscopy suggested that the bacteria moved over the substrate by gliding.

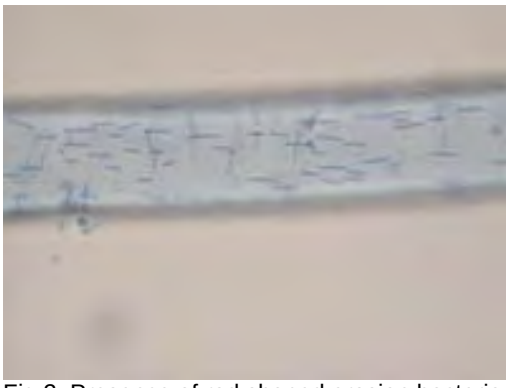


Fig 2. Presence of rod shaped erosion bacteria attached to the cell wall along the microfibrillar orientation of the kapok fibre.
X630

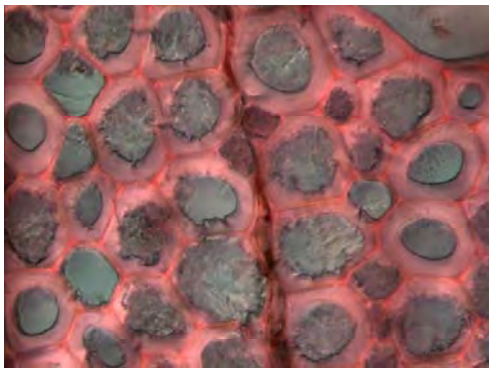


Fig. 3. Initial degradation of the secondary cell wall of pine samples by erosion bacteria. Residual slime material is left behind in the cell lumen. Some cell walls remain seemingly intact.

Media and oxygen supply
Initially, BACPOLES erosion bacteria were cultured following the procedures earlier used to isolate terrestrial bacteria (not published). A large number of different media were tested (table 1). Thus, the first cultures were incubated at conditions that permitted good access of air (media contained in flasks with cotton plugs). All cultures failed to provide any evidence of erosion bacteria attack.
Later it was discovered that restricting the access of air was necessary for obtaining attack by erosion bacteria. Restriction of air was achieved by filling glass jars to ca ¾ of their volume and by using tight caps. The jars were also inverted to make sure that air could not penetrate easily into the jars (Fig. 1). This culturing technique proved to be very successful.
A medium previously used at the department was found to be most suitable for the BACPOLES erosion bacteria. The composition of this medium, BAK-7, is given in Table 2.
The consortia of bacteria present quite rapidly reduced the oxygen in the jars, whereby anaerobic or near anaerobic conditions were created. This was clearly seen as decolourisation of the resazurin. In most cultures growth and activity of sulfur reducing bacteria (SRB) was evident from the strong smell of hydrogen sulfide when the jars were opened.

Results
The continuous improvement of the isolation technique for the BACPOLES erosion bacteria, lead to growth of active erosion bacteria in cultures from 72 % of all BACPOLES samples, representing 84 % of all BACPOLES sites (Table 3).
It is worth noting that erosion bacteria could be isolated from very ancient wood samples, such as the 2000 years old wood samples from BACPOLES site 26 and site 10. This suggests that erosion bacteria may be active in wood over centuries, if not millennia.
In some jars we noted activity of SRB without any signs of erosion bacteria attack. This suggests that SRB do not attack wood. Thus, erosion bacteria do not belong to the SRB group.

4.1.3. Purification of cultures
Kapok and pine
In most jars, fresh pine wood and kapok fibres were used as substrates for attack of erosion bacteria. Transfer of degraded pine wood or degraded kapok to new inoculation jars led to new subcultures of erosion bacteria (Fig. 4). Microscopic observations revealed that attack on pine always coincided with attack on kapok. This strongly indicated that the bacteria observed attacking pine wood were identical to the bacteria degrading kapok fibres.

Additional examination by the use of scanning electron microscopy (SEM) also showed strong morphological similarities in the decay pattern between kapok and pine fibres (Fig. 5,6). The decay pattern obtained in purified sub-samples of pine (fig.6) was seemingly identical to the erosion bacteria decay pattern in original BACPOLES samples (Figs. 7a,b).

Fig. 4. Sub-cultures of kapok attacked by erosion bacteria in new flasks containing sterilised media, kapok and pine.

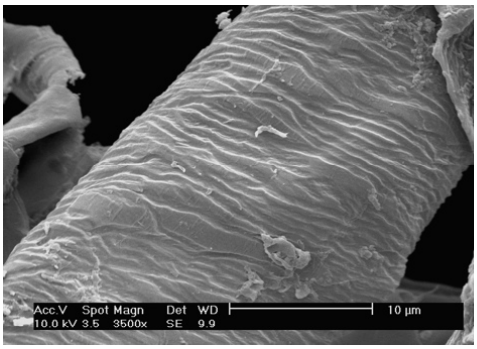


Fig. 5. Showing the typical decay pattern observed on kapok fibres in purified subcultures of erosion bacteria. Troughs are formed by the bacteria during decay and are orientated across the fibre axis following the microfibrils of the outer cell wall layer. SEM x3500

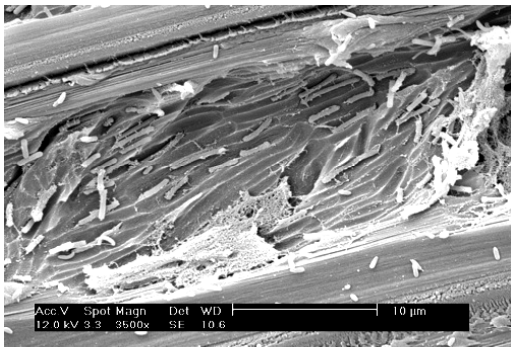
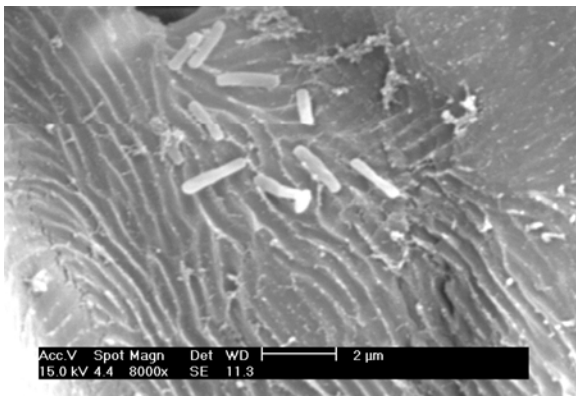
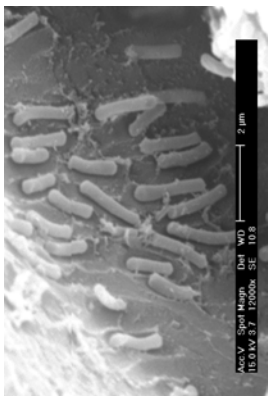


Fig. 6. Showing active erosion bacteria degrading sound pine samples inoculated in purified sub-cultures. Bacteria troughs follow the microfibrillar orientation of the secondary cell wall (S2). Note the similarities in morphology between troughs in kapok (fig. 5) and pine (fig. 6).





Figs. 7 a,b. Showing active erosion bacteria degrading original BACPOLES foundation pile from site 1, Amsterdam. The rod shaped bacteria are attached to the wood cell wall within their respective troughs orientated along the microfibrils.

Aerated conditions

In order to test the ability of erosion bacteria to growth in a more aerated environment, sub-cultures of erosion bacteria attacking kapok fibres were used to inoculate flasks with cotton plugs, to provide better access of air. However, no activity of erosion bacteria was observed under these conditions.

Reduction in the number of bacteria species

Several cultures of erosion bacteria were sub-cultured using new jars with fresh media and sterilised pine and kapok. It was evident macroscopically that SRB were lost during this process. Microscopical observations revealed that the total number of bacteria as well as the number of species around the kapok fibres was successively reduced.

Cellophane

Cellophane (regenerated cellulose foil) was introduced as substrate to some of the active erosion bacteria cultures in order to further purify the cultures. We observed that the cellophane was rapidly consumed in some cultures of erosion bacteria whereas it lasted much longer in other cultures. We assume that the rapid degradation was due to activity of typical cellulolytic bacteria. In cultures with slow degradation, microscopy revealed bacteria morphologically similar to erosion bacteria forming erosion troughs in the cellophane. Several of these bacteria appeared to be strongly attached. Variation in orientation of erosion troughs created morphological patterns that varied due to the origin of the cultures.

Our interpretation of the observations was as follows: Cultures inoculated with wood from a sample will contain a high number of species of bacteria. During transfers several species are lost and in most cases these species are unknown and not noticed. The absence of hydrogen sulfide and the highly reduced activity on cellophane suggests that SRB and typical cellulolytic bacteria are lost during transfers. Thus, the slow degraders of cellophane probably represent erosion bacteria. This is also supported by the fact that pieces of cellophane could be used to start new cultures of erosion bacteria with wood and kapok as substrates.

The fact that erosion bacteria were firmly attached to the cellophane was exploited in attempts to purify erosion bacteria cultures. The procedure was as follows: Cultures with slow degradation of cellophane were selected. The cellophane was removed from the jar, washed thoroughly to remove loosely attached bacteria. Microscopic examination of the washed cellophane indicated presence of a few rod shaped bacteria, presumably erosion bacteria (Fig.7). The washed cellophane was cut using a sterile razor blade into minute pieces (ca 1-2 square mm). They were then used to inoculate fresh media containing wood and kapok fibres. After incubation, erosion bacteria were observed in ca 25 % of the inoculated jars. Molecular analyses (UoP) analysis indicated that one of the purified cultures only contained ca 4 species of bacteria.

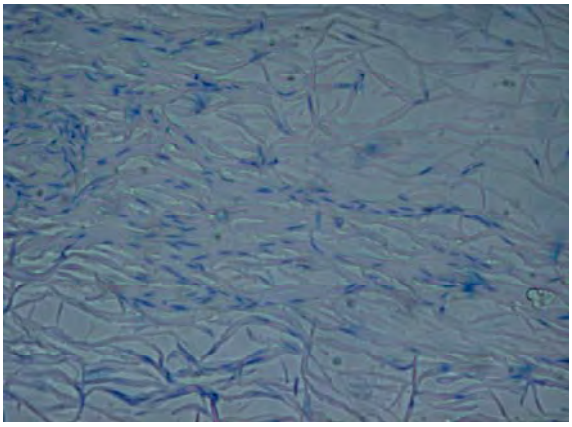


Fig. 8. Cellophane is used as substrate in the last step of purification. Erosion bacteria are firmly attached in their “dykes”, whereas other microbes have been washed away.

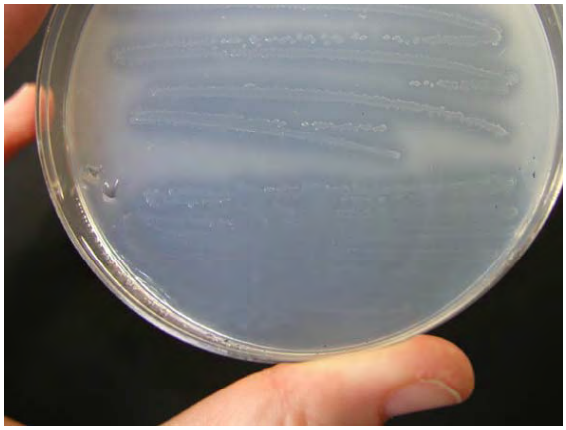


Fig. 9. Cellulolytic bacteria cause clearing zones on cellulose agar. These bacteria are not able to degrade wood, but are present in the microbial community surrounding erosion bacteria.

Traditional culturing on agar media

A large number of bacteria were also isolated on various agar media. Some were isolated on cellulose agar media where they caused clearing zones suggesting cellulolytic activity (Fig.9). A number (ca 16) of pure strains were sent to UoP for identification. Most of the bacteria isolated were also tested for degradation of wood and kapok fibres, but all pure strains of bacteria tested failed to show any signs of degradation.

Dilution
Dilution in liquid media or by streaking on agar failed to lead to purer cultures. This is easily understood if the erosion bacteria adhere strongly to their substrate.

4.1.4 Results
Microscopical examination of bacteria degrading kapok fibres indicated that several morphologically different bacteria were present. All were rod shaped, but the size and the precise form varied. Degradation patterns in kapok and in cellophane also varied. These observations suggest that erosion attack may be caused by a large number of bacteria species. From some purified cultures of erosion bacteria growing on kapok and cellophane, it was possible to study the variation in bacteria species present by molecular analyses (UoP). Results from one such purified culture demonstrated that three related species of Bacteriodetes were present. This shows that even a seemingly pure population may represent different species.
By repeatedly sub-culturing of erosion bacteria attached to kapok, it was possible to reduce the total number of different bacterial species within each mixed culture to a minimum while maintaining active degradation. One example is that the SRB appeared to be removed by this method since no hydrogen sulfide could be detected. This also demonstrates that erosion bacteria are unable to reduce sulfate. The cellophane method described above, made it possible to reduce the number of species further. This was, however, the final stage of purification with positive results.

4.1.5 Selection of samples for molecular analysis
Kapok samples
Pine wood and kapok fibre samples with a large number of erosion bacteria were sent to UoP for molecular analysis. A total of 90 samples were sent. We also suggested that FISH (fluorescent in situ hybridisation) could be done on this material. It was discussed whether the lignin present in the kapok fibres was expected to cause problems for FISH due to the fluorescence of the lignin. In order to overcome this problem we also sent cellophane with attack by erosion bacteria to be used for FISH.

Separation of single bacteria by advanced laser-technique
From selected BACPOLES sub-samples of kapok and wood, where microscopic examination revealed active erosion bacteria in combination with a very purified consortia, advances laser technique were tested in order to separate single bacteria. Two method were tested:

1. Optical tweezers at Umeå University, Dept. of Physics in cooperation with Ass.prof. Erik Fällmann.

2. Laser dissection microscopy at a Leica demonstration at Karolinska Institutet in Stockholm.

About 10 vials were sent to UoP for identification.

1. By use of the optical-tweezer-technique single bacteria with morphological similarities to erosion bacteria were selected from a macerated fibre droplet one by one with help of a micro-injection-needle (Figs. 10,11). The method was time consuming but very successful. Each vial for molecular analysis contained between 4 and 20 single bacteria.

Fig. 10. Advanced optical tweezer system in work at Dept. of Physics, University of Umeå.

Fig. 11. The process of trapping single bacteria and their way into the microinjection needle could be followed on the monitor.

2. With the laser dissection method, a smaller fibre area with presence of erosion bacteria attached to the kapok cell wall, were cut out by laser “knife” (Fig. 12). The section was collected automatically in a PCR-vial. Consequently, each vial contained a larger amount of erosion bacteria, mixed with other secondary bacteria present in the kapok lumen.

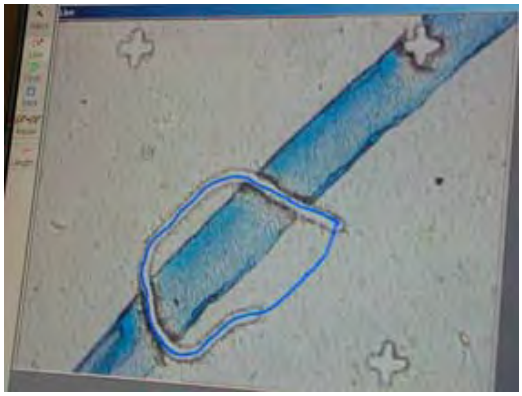


Fig. 12. By using the laser dissection technique it was possible to “cut out” a smaller area on a single kapok fibre for molecular analyses. Photo showing the laser knife in process following the marked line.

4.1.6 Conclusions

- Erosion bacteria can easily be cultured from pilings and archaeological wood.
- Erosion bacteria attach firmly to their substrate and appear to move by gliding.
- Positive cultures contained a large number of different bacteria
- Typical cellulolytic bacteria were often present
- Culturing was successful even for marine samples and also very ancient archaeological samples.
- Culturing was dependent on reduced access of oxygen.
- Positive cultures became anaerobic with time and production of hydrogen sulfide occurred initially in most cultures.
- Some cultures with hydrogen sulfide lacked erosion bacteria.
- Hydrogen sulfide was not detected in the most purified cultures
- BACPOLES erosion bacteria differ from terrestrial erosion bacteria by requiring reduced oxygen levels
- All isolated strains of bacteria failed to attack wood or kapok fibres.
- It is possible that there is a number of different species of erosion bacteria present in each BACPOLES sample.
- Optical tweezer revealed a novel pathway for more exact molecular analysis on single bacteria.
- Laser dissection could be an efficient and useful tool in future work.

4.1.7 Hypothesis

BACPOLES erosion bacteria require specific conditions for being able to degrade wood or kapok. It appears that the presence of other bacteria is required. Their prime role is to create conditions suitable for erosion bacteria attack. One mechanism could be the reduction in the level of oxygen.

Table 1. Media used for isolation of Bacpoles erosion bacteria.

Medium used for isolation	Lab name	Liquid or Agar	Comments	Successful ?
No.1	BAK-7	Liquid	CaCO3	positive
No.2	BAC 02-1	Liquid	Marine	
No.3	BAC 02-2	Liquid	Fyris, 8 variations	
No.4	BAC 02-4	Agar	Dubos media, 3 variations	
No.5	BAC 02-5	Liquid	Dubos media	
No.6	BAC 02-6	Liquid	Fyris	positive
No.7	BAC 02-7	Liquid	Fyris + cellubiose	
No.8	BAC 02-10	Liquid	Dubos media, 4 variations	
No.9	BAC 02-13	Agar 2-layers	Cellulose	
No.10	BAC 02-18	Agar	Casitone	
No.11	BAC 02-19	Liquid	Fyris + N,P	
No.12	BAC 02-20	Liquid	Fyris + N,S,P	
No.13	BAC 02-22	Liquid	Ref. Aumen	
No.14	BAC 02-23	Liquid	Casein	Positive
No.15	BAC 02-24	Liquid	Na- thioglucolate+ Casein	
No.16	BAC 02-25A	Liquid	FXAG-medium	
No.17	BAC 02-25B	Liquid	Compost-extract	positive
No.18	BAC 02-26	Agar	yeast, CaCl2	
No.19	BAC 02-27	Agar	Cellulose	
No.20	BAC 02-28	Liquid	Cellulose	Positive
No.21	BAC 02-29A	Agar 2-layers	Na-thioglucolate+Cystein	
No.22	BAC 02-29B	Agar 2-layers	Cystin	
No.23	BAC 02-30	Liquid	Sulphate reduc. Bact. Media	
No.24	BAC 02-31A	Liquid	Mod. Bak-7, CaCl2	Positive
No.25	BAC 02-31B	Liquid	Na-thioglucolate	positive
No.26	BAC 02-31C	Liquid	Na-thioglucolate + Cystin	Positive
No.27	Bac02-40	Liquid	Coco	positive

Table 2. Contents of BAK-7 media, the most successful media for isolation of erosion bacteria from the BACPOLES-samples

amount	type
0,2g	$NaNO_3$
0,4g	K_2HPO_4
0,2g	$(NH_4)_2SO_4$
0,1g	$MgSO_4$, $7H_2O$
0,05g	KCl
0,01g	$FeSO_4$,$7H_2O$
5ml	Trace elements
0,1g	Yeast extract
0,1g	Casein hydrolysat
1000ml	dem. H_2O

Table 3. Showing all BACPOLES samples that was cultured in order to isolate erosion bacteria. Most samples were found to have active attack of erosion bacteria.

Bacpoles number	Site	Active erosion bacteria
1.a.td.1.100	Amsterdam	Positive
1.a.td.1.360	Amsterdam	Positive
1.b.td.3.100	Amsterdam	Positive
1.b.td.3.320	Amsterdam	Positive
1.c.td.1.120	Amsterdam	
1.c.td.1.350	Amsterdam	Positive
1.d.td.3.300	Amsterdam	
1.d.td.3.70	Amsterdam	Positive
2.td.1.0-620	Dordrecht	Positive
2.td.10.0-600	Dordrecht	Positive
2.td.11.0-650	Dordrecht	
2.td.12.0-760	Dordrecht	
2.td.2.0	Dordrecht	Positive
2.td.2.400-840	Dordrecht	Positive
2.td.3.0	Dordrecht	
2.td.3.400-850	Dordrecht	Positive
2.td.4.70	Dordrecht	
2.td.5.100	Dordrecht	
2.td.6.50	Dordrecht	Positive
2.td.7.100	Dordrecht	Positive
2.td.8.0-700	Dordrecht	Positive
2.td.9.0-650	Dordrecht	Positive
04.te.1.0-64	Haarlem, NL	Positive
04.te.2.0-69	Haarlem, NL	Positive
04.te.3.0-45	Haarlem, NL	Positive
04.te.e.0-67	Haarlem, NL	
04.te.5.0,00-0,50	Haarlem, NL	Positive
04.te.6.0,00-0,50	Haarlem, NL	Positive
04.te.7.0,00-0,50	Haarlem, NL	Positive
5,te.3.punt	Rotterdam	
5.te.1.12000	Rotterdam	Positive
5.te.1.13400	Rotterdam	Positive
5.te.1.1400	Rotterdam	Positive
5.te.1.2000	Rotterdam	Positive
5.te.1.4000	Rotterdam	Positive
5.te.1.65	Rotterdam	Positive
5.te.1.8000	Rotterdam	Positive
5.te.1.punt	Rotterdam	Positive
5.te.2.1000	Rotterdam	Positive
5.te.2.4000	Rotterdam	Positive
5.te.2.50	Rotterdam	Positive
5.te.2.8000	Rotterdam	Positive
5.te.2.punt	Rotterdam	Positive
5.te.3.1000	Rotterdam	Positive
5.te.3.50	Rotterdam	Positive
5.te.3.8000	Rotterdam	Positive
5.te.3.punt	Rotterdam	Positive
6.te.1.0-500	Koog aan de Zaan	
6.te.1.2000-2500	Koog aan de Zaan	Positive

Bacpoles number	Site	Active erosion bacteria
6.te.1.4000-4500	Koog aan de Zaan	Positive
6.te.1.6000-6650	Koog aan de Zaan	positive
6.te.2.0-500	Koog aan de Zaan	Positive
6.te.2.2000-2500	Koog aan de Zaan	Positive
6.te.2.4000-4500	Koog aan de Zaan	Positive
6.te.2.6400-6900	Koog aan de Zaan	Positive
6.te.3.0-500	Koog aan de Zaan	Positive
6.te.3.2000-2500	Koog aan de Zaan	Positive
6.te.3.4000-4500	Koog aan de Zaan	Positive
6.te.3.6000-6900	Koog aan de Zaan	Positive
7.te.1.500-750	Haarlem	Positive
7.te.2.500-750	Haarlem	Positive
7.te.3.850-1100	Haarlem	Positive
7.te.4.1370-1680	Haarlem	Positive
7.te.4.300-550	Haarlem	Positive
7.te.5.1330-1730	Haarlem	Positive
7.te.5.200-450	Haarlem	Positive
7.te.6.430-680	Haarlem	Positive
8.td.1.600	Dokkum	Positive
8.td.2.300	Dokkum	Positive
8.td.3.350	Dokkum	Positive
8.td.4.600	Dokkum	Positive
8.td.5.150	Dokkum	Positive
9.ta.1	Dokkum	Positive
9.ta.2	Dokkum	Positive
9.ta.3	Dokkum	Positive
9.ta.4	Dokkum	Positive
10.td.1.480	Borselle	
10.td.1.830	Borselle	
10.td.2.120	Borselle	Positive
10.td.2.850	Borselle	
12.ta.1.1	Waddensea 15	Positive
12.ta.1.2	Waddensea 15	Positive
12.ta.1.3	Waddensea 15	Positive
12.ta.1.4	Waddensea 15	Positive
12.ta.2.1	Waddensea 15	Positive
12.ta.2.2	Waddensea 15	Positive
12.ta.2.3	Waddensea 15	Positive
12.ta.2.4	Waddensea 15	
13.ta.1.1	Waddensea 3	Positive
13.ta.1.2	Waddensea 3	
13.ta.1.3	Waddensea 3	
13.ta.1.4	Waddensea 3	Positive
14.ta.1.0.49-071	Travenhorst. D	
14.ta.1.0.98-1.53	Travenhorst. D	
14.ta.2.0,15-0,23	Travenhorst. D	Positive
14.ta.2.0,62-0,89	Travenhorst. D	
14.ta,2,0m89-1,20	Travenhorst. D	Positive
14.ta.2.1,20-1,54	Travenhorst. D	Positive
14.ta.3.0,34-0,57	Travenhorst. D	Positive / rhizoid fungi

Bacpoles number	Site	Active erosion bacteria
14.ta.3.0,83-1,39	Travenhorst. D	Positive
16.ta.04.@.1	piles GB	
16.ta.15.@.1	piles GB	Positive
16.ta.15.@.2	piles GB	Positive
17.ta.1	piles GB	
17.ta.2	piles GB	
18.td.4.150	Venice	
18.td.4.150	Venice	
19.td.4.100	Venice	
19.td.4.100	Venice	
20.ta.1@1	Stora Sofia	Positive
20.ta.1@2	Stora Sofia	
20.ta.2.@1	Stora Sofia	
20.ta.2@2	Stora Sofia	Positive
20.ta.3.@1	Stora Sofia	
20.ta.4@1	Stora Sofia	
21.ta.1.@.1	Mollösundvraket	
21.ta.4@1	Mollösundvraket	Positive
22.td.1	Parliament, Stockholm	
22.td.2	Parliament, Stockholm	
22.td.2.460-470	Parliament, Stockholm	
23.td.1.20	Leevworde	Positive
23.td.1.300/500	Leevworde	Positive
24.ta.1.@4	Bryggen	Positive
24.ta.2	Bryggen	Positive
24.ta.3	Bryggen	Positive
24.ta.4 a+c	Bryggen	
24.ta.4.	Bryggen	
24.ta.5	Bryggen	
25.ta.1.@.1	Swedish	Positive
26.te.1.0-90	Roman Temple	Positive
26.te.2.0-78	Roman Temple	
26.te.30-74	Roman Temple	Positive
27.ta.1		Positive
27.ta.2		Positive
27.ta.3		Positive
27.ta.4		Positive / patchy
27.ta.5		Positive
28.ta.1		
28.ta.2		Positive

4.2 Identity of Wood Degrading Bacteria

(by Landy, Hotchkiss, Mitchell, Eaton)

4.2.1 Introduction

Research data produced from the BACPOLES project indicates that tunnelling, erosion and cavitation bacteria are well documented as the bacteria involved in the decay of wood in Europe (Jeewon *et al* 2003). In searching for those microbes with a specific role in wood degradation, it must be emphasised that the majority of bacteria cannot be cultured using standard methods (Fuhrman & Campbell 1998). The lack of bacterial isolates capable of causing wood degradation similar to that observed in natural environments is a major limitation in the development of preventative treatments. The availability of 16S rDNA sequences from a wide range of organisms has made it possible to identify microbes without the initial bias of culturing. Linking environmental signals, such as the 16S rRNA/rDNA sequences, with those from cultured organisms provides an identification framework to target those organisms that might be involved in the degradation process.

4.2.2 Objectives and Research Strategy

In this section of the report we outline the research conducted to address two of the objectives for the University of Portsmouth, as outlined in the original BACPOLES proposal:
To develop molecular techniques for the characterisation and identification of erosion bacteria isolated from and present in sampled pilings
To establish a DNA database for wood degrading bacteria for use in future work
Two basic strategies were used for the molecular characterisation of bacterial 16S rDNA and rRNA signals associated with wood pilings and submerged archaeological wood within a European sampling zone. A third strategy was used which involved the development and application of FISH (fluorescent *in situ* hybridisation) techniques for *in situ* observation and identification of bacteria in the samples. A flow diagram outlining the strategies adopted in this study is shown in figure 13.
The first strategy (fig. 13) involved extracting nucleic acids directly from wood sections followed by 16S rDNA and rRNA PCR-amplification using universal bacterial primers. Amplified fragments representing the bacterial populations were recovered by cloning or after DGGE analysis. Sequences from these fragments formed the basis of the database and bacterial group identities were made after BLAST sequence homology searches of public databases.
The second strategy (fig. 13) implemented in this study concerned the molecular identification of bacteria cultured from wood pilings and uncultured bacteria present in consortia growing on, or in the presence of, cellophane or kapok fibres. Cultures were provided by partners SLU and PhaGen. Wood and soil samples from the GoT microcosm studies were also included this analysis. The 16S rRNA gene from independent cultures was PCR-amplified and sequenced. This gene was also amplified from the mixed consortia, separated into phylotypes after molecular cloning/ DGGE analysis and sequenced. Preliminary group identities were made after BLAST searches, and the sequences compared with those obtained from the environmental study (strategy 1). Genetic links were made after phylogenetic analysis.
Each step in these strategies required the development and modifications of techniques, particularly those used to analyse environmental samples. Modifications to the techniques used up to the beginning of May 2003 (Jeewon *et al.* 2003) were necessary to increase the output of results from this project (see Landy *et al.* 2003, 2004a, 2004b). First, an increase in the yield of DNA extracted from bacteria present in low biomass in a complex substrate was achieved via appropriate selection of portions of wood for the freeze-drying of samples upon arrival at the UoP. Second, Clean-up of extracted DNA using a Qiagen Nucleotide Clean-Up kit and the manipulation of the annealing temperature used in PCR protocols led to an increase in the yield of PCR products (16S rDNA genes) for subsequent analysis.

Third, manipulation of conditions used during DGGE revealed a more suitable gradient for separation of 16S rDNA genes from wood. Finally, molecular cloning steps were included in the strategies so that estimates of sequence recovery could be made and in order to preserve rare signals. Cloning of PCR products also allowed larger DNA fragments to be isolated and sequenced, thus allowing greater scope for analysis.
Phylogenetic analyses of sequence data was used to make genetic links between all the sequences and thus provided information on the uniqueness of sequences and relationships to other organisms. Multivariate analysis generated knowledge on the occurrence sequences within different wood species and geographical regions thus giving information on the diversity of bacteria associated with various samples.
The last strategy (fig. 13) used in this study involved the development and application of fluorescent *in situ* hybridisation (FISH) techniques to identify bacteria in direct contact with wood sections, cellophane or kapock fibres. This section was included in the last 6 months of the project to provide a means of matching microscope observations with sequence information obtained from the environment (strategy 1) and cultured/ consortia bacteria (strategy 2).

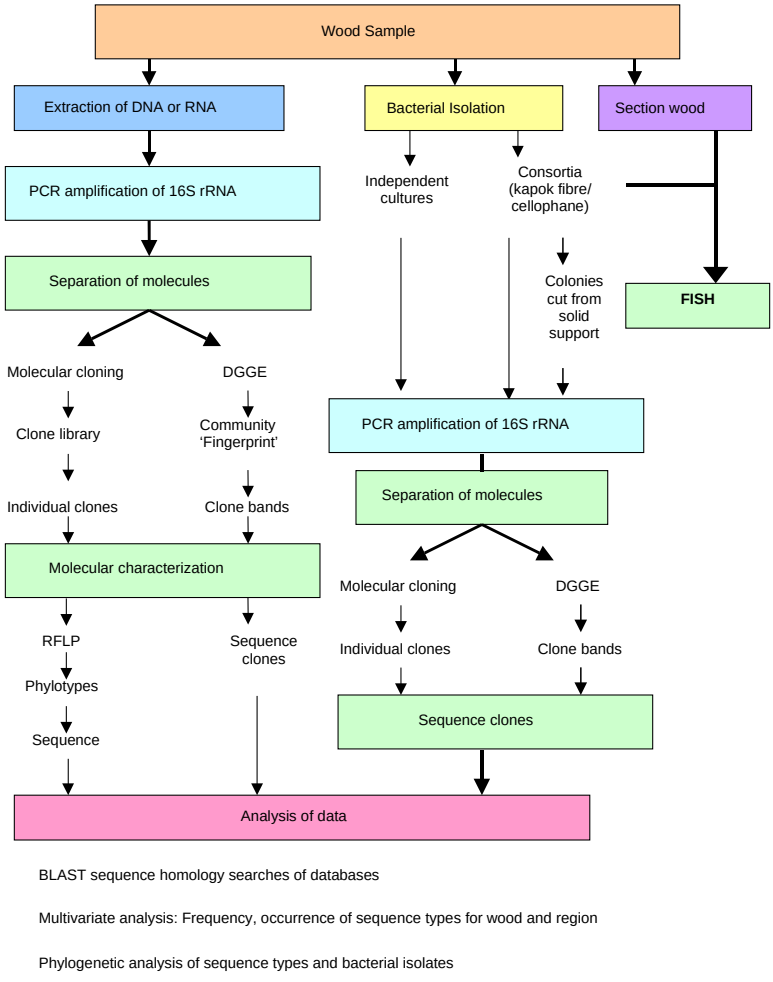


Figure 13. The molecular strategies adopted in the study.

4.2.3 Materials and Methods

This section outlines the methodology used in the molecular characterisation of bacterial 16S rDNA and rRNA and estimation of bacterial diversity up until December 2004. A Full description of all procedures used is outlined in Appendix 6.

Table 4. Sample information for all wood (a) and bacterial (b and c) samples collected for the BACPOLES project. Information given on sample site locality and wood details. Reference codes for samples also given.

(a) UoP	Reference BACPOLES	Wood species	Wood Age	SLU Reference
1w	10.td.2.950	Oak	1900	
2w	10.td.1.930	Oak	1900	
3w	24.ta.4a+c	Pine	-	
4w	24.ta.3	Pine	-	
5w	22.td2.4700-4760	Pine	100	
6w	22.td.2.2420-2460	Pine	100	
7w	9.ta.4	Oak	590	
8w	22.td.1.6600-6660.3.1	Pine	100	
9w	24.ta.1	Pine	-	
10w	24.ta.5.1	Pine	-	
11w (Hw)	22TD1 2420-2460 3.1	Pine	100	
12w	10.td.1 5801	Oak	1900	
13w	24.ta.2	Pine	-	
14w	9.ta.2	Oak	590	
15w	24.ta.4b	Pine	-	
16w	9TA3	Oak	590	
17w	1DTD 3 110	Spruce	-	
18w	1CTD 1-320	Spruce	-	
19w	1.d.td3.340	Spruce	-	
20w	12.ta.1.2	Oak/Pine	376	
21w	12.TA.2.1	Oak/Pine	376	
22w	12.ta.2.2	Oak/Pine	376	
23w	12.td.1.3	Oak/Pine	376	
24w	12.td.1.4	Oak/Pine	376	
25w (Fw)	12.TA.1.1	Oak/Pine	376	
26w (Ew)	12.ta.2.3	Oak/Pine	376	
27w	12.ta.2.4	Oak/Pine	376	
28w (Gw)?	1ATD1 180	Spruce	-	a27
29w (Gw)?	1ATD1320	Spruce	-	
30w (Uw)	1BTD 3140	Spruce	-	a29
31w	1.b.td.3.310	Spruce	-	a30
32w (Rw)	1CTD 1140	Spruce	-	
33w (Dw)	8TD1680	Oak	535	a17
34w	8.td.2.380	Oak	535	a16
35w	8.td3.440	Oak	535	
36w	8.td4.680	Oak	535	
37w	8.td5.210	Oak	535	a14
38w	9.ta1	Oak	590	a43
39w	13ta1.3	Oak/Pine	360	
40w	13.ta4	Oak/Pine	360	
41w	13.ta1.1	Oak/Pine	360	
42w	13.ta.1.2	Oak/Pine	360	
43w (Bw)	23.td.1.80	Pine	100	
44w	23.td.1.300	Pine	100	a23
45w	td.2.100-110.6	-	-	
46w	td.3.100-110.6	-	-	
47w	slu-ieb			
48w	a73-au1			
49w	27ta1	Oak	450	a187
50w	2td3400-850	Spruce	75	
51w	2.td.1.0-620	Spruce	75	
52w (Tw)	2.td.2.400-840	Spruce	75	a86b
53w (Ow)	2.td.3.400-850	Spruce	75	
54w (Qw)	2.td.8.0-700	Spruce	75	a87
55w	2.td.9.0-650	Spruce	75	a88
56w	2.td.10.0-600	Spruce	75	a89
57w	2.td.11.0-650	Spruce	75	a90
58w (Cw)	2.td.12.0-760	Spruce	75	a91
59w	a63p			
60w	a70p			
61w	a73p			
62w	5te1	Fir/Spruce	-	a99
63w	6te10-500	Pine	70	a113
64w	7.te1.500-750	Poplar	-	a121
65w (Mw)	6.te.2 0-500	Pine	70	a113
66w	7.te.2.500-750	Poplar	-	a122
67w	5.te.2.50	Fir/Spruce	-	a100
68w	6.te.3.0-500	Pine	70	a117
69w	7.te.3.850-1100	Poplar	-	a123

(a) UoP	Reference BACPOLES	Wood species	Wood Age	SLU Reference
70w	5.te.3.50	Fir/Spruce	-	a106
71w (Nw)	7.te.4 300-500	Poplar	-	a124
72w	7.te.2.200-450	Poplar	-	a122
73w	7.te.6 430-600	Poplar	-	a128
74w	21ta2@1	Oak	610	
75w	21ta3@3	Oak	610	
76w (Aw)	25.ta.1.a.4	Oak	-	a132
77w	2td4150	Spruce	75	
78w	2td5200	Spruce	75	
79w	2td6130	Spruce	75	
80w	2td7140	Spruce	75	
81w(Ww1)	28.ta.2	Oak	480	a193
82w(Pw)	14.ta.he.3.034-l57	Oak	-	a167
83w(Sw)	2td64,150	Spruce	75	
84w(Vw)	10td2 220	Oak	1900	
85w(Gw)	1atd1	Spruce	-	
86w(Jw)	28ta1	Oak	480	
87w(Kw)	27ta5	Oak	450	a191
88w(Lw)	27ta3	Oak	450	a190
89w(Aw)	25ta1a4	Oak	-	a132
90w	E1st.te.3 0-74 26	Oak	1900	a171
91w	14ta+te1049-071	Oak	-	
92w	26te10-78	Oak	1900	a169
93w	s4326te10-90	Oak	1900	
94w	27ta2	Oak	450	
95w	4td20-069	Pine	100	
96w	4te70,0-0,5	Pine	100	
97w	16ta15q01	Alder	-	
98w	16ta15q02	Alder	-	
99w	16ta04q01	Alder	-	
100w	4te60.00-0.50	Pine	100	
101w	4.te.5.0.00-0.50	Pine	100	a199
102w	4td4 0-0,67	Pine	100	
103w	4td3 0-0.45	Pine	100	
104w	4td1 0.064	Pine	100	
105w	17ta01q01	Oak	3050	
106w	17ta023501	Oak	3050	
107w	14 ta+te 20-, 62 - 0,89	Oak	-	
108w	14 ta+te 3 0, 34- 0,57	Oak	-	a168

(b) SLU	BACPOLES	Wood Type	Wood Age
a51	water sample Bryggen,	-	-
a73	Norway	-	-
a79	Lake Botan, Sweden	-	-
a93	Sediment near Vasa,	Fir/Spruce	-
a94	Sweden	Fir/Spruce	-
a95	5te11400	Fir/Spruce	-
a96	5te12000	Fir/Spruce	-
a97	5te14000	Fir/Spruce	-
a98	5te18000	Fir/Spruce	-
a101	5te112000	Fir/Spruce	-
a102	5te113400	Fir/Spruce	-
a104	5te2100	Fir/Spruce	-
a105	5te24000	Fir/Spruce	-
a107	5te2punt	Fir/Spruce	-
a108	5te350	Fir/Spruce	-
a110	5te38000	Pine	70
a111	5te3punt	Pine	70
a112	6te12000-2500	Pine	70
a114	6te14000-4500	Pine	70
a115	6te16000-6500	Pine	70
a116	6te22000-2500	Pine	70
a118	6te24000-4500	Pine	70
a119	6te26400-6900	Pine	70
a120	6te32000-2500	Pine	70
a125	6te34000-4500	Poplar	-
a126	6te36000-6900	Poplar	-
a127	7te51370-1680	Poplar	-
a134	7te5200-450	-	-
a143	7te51330-1730	-	-
a144	driftwood Acores	-	-
a149	Oskarshamn Cog stored	-	-
a183	25 years in a tub	-	-

Reference (a) UoP	BACPOLES	Wood species	Wood Age	SLU Reference
a184	Do	-	-	
a189	Bog water Varmland	Oak	-	
a197	Sweden	Pine	100	
a207	Archaeological Wood,	-	-	
a82	Biskupin, Poland	Spruce	75	
a141	Do	-	-	
a185	27ta3	-	-	
a208	4te30-45	-	-	
	laboratory simulation			
	sample after 5 years			
	2td20			
	Lake Valloxen Fresh Water			
	Arch wood, Biskupin, Poland			
	Acores Marine			

(c) Type of Sample	SLU and UoP	Notes
Laser Cut	P1 a17/833	stained a test 5-7 bacteria in tube
	P4 a51/813	unstained, max 2 bact. long rods
	P6 a51/813	stained, max 5 bacteria
	P9 a208/796	unstained, max 15 bacteria - good sample
	P10 a141/767	
Laser Trapped	Lotte c – lotte l	unstained, max 13 bacteria mixed culture at least 2 different types of bacteria, different, mobile, thick

108 wood samples were provided for this research (table 1). Information on sample location, wood type, age and labelling is given in Table 3.2.1a.
77 mixed bacteria samples were provided by SLU. Information regarding these samples is given in Tables 3.2.1a and 3.2.1b
A small number of laser cut and laser trapped bacterial samples were also provided by SLU (Tab. 3.2.1c).
DNA Extraction from Wood Samples
Wood Samples were split using a bench vice and a rubber headed mallet. A boring gouge was used to extract approximately 1-2g wood from 3 points along the longitudinal surface (see fig. 14) and from a point on the piling surface. Wood samples were transferred to sterile screw capped bottle and then freeze dried for 1 week at -80^{o}Celsius.
DNA was extracted from the wood samples using a variety of techniques (see Appendix 6). For rapid analysis of many samples the Qiagen DNAeasy Plant Mini kit was used and the nucleic acid purified using a Qiagen Nucleic acid Clean-up kit (www1.qiagen.com) according to the manufacturers protocol.

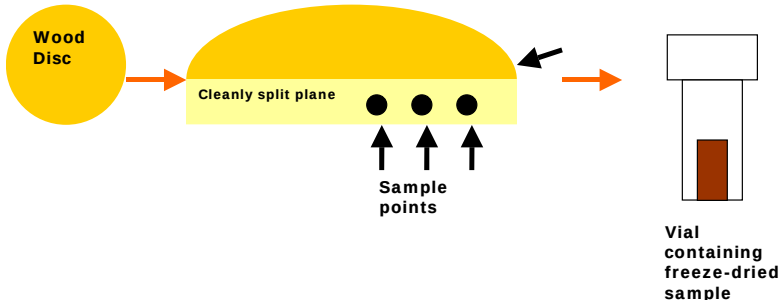


Figure 14. Sampling of wood from wood discs

PCR (Polymerase Chain Reaction) Amplification of 16S rDNA.
Different sets of primers were used for 16S rDNA amplification (Tab 2) using PCR protocols outlined in Appendix 6. Amplifications were performed using an Eppendorf Gradient Mastercycler. For wood samples and bacterial cultures, universal bacterial primers 8F-1492R were used. For amplification of rDNA for DGGE, primers UNV2F-UNV3R with a GC clamp on UNV2F were used.

Reamplification of DNA fragments recovered from the DGGE gel was done using UNV2F-UNV3R without a GC clamp. For amplification of plasmid DNA for cloning the primer set M13F-M13R was used.
Primer sets were chosen based on the preliminary work by Jeewon *et al.* (2003) and suitable annealing temperatures determined using gradient PCR.
PCR products were separated electrophoretically using 1.5 or 2% agarose gels and visualised using an Ethidium Bromide stain under UV. When necessary, DNA fragments were recovered from the gel using a QIAEXII Gel Extraction Kit (www1.qiagen.com) and sent for sequencing.

Table 5. Details of primer sets used

Primer Set	Annealing Temp.(^{o}C)	PCR Product Size	References
8F-1492R (5'ATRGTTTGATCCTGGCTCA3'-5'CGGCTACCTTGTTACGACT3')	53^{o}C	1400 base pairs	Bocklemann *et al.* 2000 Jeewon *et al.* 2003
M13F-M13R (5'GTAAAACGACGGCCAG3'-5'CAGGAAACAGCTATGAC3')	55^{o}C	Depends on insert size	Bocklemann *et al.* 2000 Jeewon *et al.* 2003
UNV2- (GC CLAMP) UNV3 (5'ATTACCGCGGCTGCTGG3'-5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG3')	56^{o}C	Approx 220 base pairs	Bocklemann *et al.* 2000 Jeewon *et al.* 2003
UNV2- UNV3 (5'ATTACCGCGGCTGCTGG3'-5'GCACGGGGGGCCTACGGGAGGCAGCAG3')	56^{o}C	Approx 220 base pairs	Bocklemann *et al.* 2000 Jeewon *et al.* 2003

DGGE (Denaturing Gradient Gel Electrophoresis) of PCR products
30μl of PCR product was applied to a denaturing gradient gel using an Ingeny phor U system. A polyacrylamide gel with a denaturant gradient of 30% - 70% urea:formamide was used and electrophoresis was performed at 60^{o}Celsius, 100V for 16 hours. A nested PCR protocol was used to obtain suitable sized PCR products for separation under DGGE. Extracted DNA was first amplified with 8F-1492R primers to produce 1400bp products, which were cloned. The subsequent amplification used the UNV2-UNV3 primer set attached to a GC clamp for DGGE analysis. This generated a product of approx. 220 bp. DNA fragments (bands) were recovered from the denaturant gradient gel and were re-amplified using the UNV2-UNV3 primer set but with the GC clamp omitted. DNA was visualised using an Ethidium Bromide stain gels under UV. Where necessary, DNA fragments were recovered from the gel using a Qiagen QIAEXII Gel Extraction Kit (www1.qiagen.com) and sent for sequencing. For full DGGE protocol please see Appendix 6.
Cloning
PCR products were inserted into plasmid vectors to produce a library of clones for subsequent analysis (see Appendix 6). The cloning process allowed for several copies of the PCR product to be produced and increased the amount of DNA for analysis. Molecular cloning was carried out using a TOPO TA cloning kit (www.invitrogen.com) following the protocol outlined by the manufacturers. Clones were screened for positive inserts using the standard X-Gal and Ampicillin resistance.

Table 6. Restriction Enzymes used to differentiate between bacterial species incorporated in wood samples and isolated as clones

Restriction Enzyme	Cut Site	Reaction Conditions	References
AluI	5'-AG ↓ CT-3' 3'-TC ↓ GA-5'	37^{o}C React 1 buffer	Jasalavichi *et al.* 2000
HaeIII	5'-GG ↓ CC-3' 3'-CC ↓ GG-5'	37^{o}C React 2 buffer	Jasalavichi *et al.* 2000
RsaI	5'-GT ↓ AC-3' 3'-CA ↓ TG-5'	37^{o}C React 1 buffer	Jasalavichi *et al.* 2000

Restriction Enzyme Analysis.
Ten clones per wood sample, with positive inserts, were digested with restriction enzymes to analyse for inserts of different lengths i.e. sequences from different species. The restriction enzymes used (see table 6) provide information on the possible number of species per wood sample. The HAEIII endonuclease was generally used as this enzyme is appropriate for digestion of larger PCR products with longer base pair sequences i.e. 1400 bp) Two other endonucleases, ALU1 and RSA1, were also used but were less efficient. (For full protocol please see Appendix 6).
Restriction digest products were separated on a 3% agarose gel and visualised with an Ethidium Bromide stain and visualised under UV light.

DNA from clones shown by the restriction digest to represent individual species was cleaned up using the S.N.A.P. Miniprep kit (www.invitrogen.com) according to the manufacturer's protocol and sent for sequencing.
RNA analysis
A small number of samples were chosen for analysis based on the extraction of RNA rather than DNA. The methodology used for RNA was similar to that outlined above for DNA but with a few modifications (see Appendix 6 for full protocol). RNA is not as robust as DNA and breaks down easily. Rnases are also ubiquitous and care must be taken to perform all protocols under strict sterile conditions.
RNA Extraction and Cleanup
RNA was extracted using Rneasy Mini Kit (www1.qiagen.com) and cleaned up using Rneasy Mini Protocol for RNA Cleanup (www1.qiagen.com) according to the manufacturers protocol. Extracted RNA was stored at –80^{o}Celsius for limited periods when necessary, due to its rapid breakdown, but was generally amplified using RT-PCR (Reverse Transcription PCR) as soon as possible. (For full protocol please see Appendix 6).
RT-PCR
DNA digestions were performed on all RNA samples to ensure that no DNA contaminants were present. RT-PCR was subsequently used to produce cDNA from the RNA samples and a concurrent PCR was run to identify any DNA not removed by the digest (See Appendix 6 for protocol). cDNA being more stable than its RNA counterpart is then used for the remainder of the analysis.
PCR and RT-PCR products were separated on a 1.5% agarose gel and visualised using UV and an ethidium bromide stain. Clean RTPCR products were re-run on a 1.5% agarose gel and cDNA fragments (approx 450 bp) recovered using Qiagen QIAEXII Gel Extraction Kit (www1.qiagen.com).
Cloning and Restriction Enzyme Analysis.
cDNAs were cloned to produce more copies using a TOPO TA cloning kit (www.invitrogen.com) and analysed with Restriction Enzymes Digestion (see relevant sections above).
Sequencing and Identification
Any 16S rRNA genes that were isolated from the samples were sent to the Sequencing Service at the University of Dundee for analysis (www.dnaseq.co.uk).
Returned sequences were then run through a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) for identification. Possible identities being based on sequence comparisons with known taxa already loaded in the database. The search returns information on the percentage similarity of matches between sequence comparisons.
Phylogenetic, rarefaction and coverage analyses
Sequences from isolates, environmental samples, DGGE bands and clone libraries were aligned with those representing known bacterial taxa deposited in databases. Alignments were constructed using CLUSTAL X and manually adjusted in text editors (Se-Al and PAUP). Data matrices representing the major bacterial groups were prepared using 50 to 500 taxa for 1000 nucleotides. These matrices were used to perform phylogenetic analyses under parsimony and distance criteria implemented in PAUP.

Analyses were performed on unrooted data and when *Aquiflex pyllophilus* was used as the outgroup. Under general parsimony criterion, heuristic searches were made with random sequence addition for 10 replicates and trees constructed using the TSB algorithm. Genetic distances were calculated using the Kimura-2, log-det, GTR and maximum likelihood evolutionary models and trees constructed via the nearest neighbor method.

FISH (Fluorescent *in situ* Hybridisation)
Transverse (TS) and longitudinal (LS) sections of selected wood samples were fixed in 4% Paraformaldehyde (PFA) and 96% ethanol prior to hybridisation or storage at 120 ^{o}Celsius. Fixed samples were mounted on gelatine coated slides and hybridised in the dark at 46^{o}C for 2 hours. A 30% hybridisation buffer containing the appropriate oligonucleotide probes (Tab. 4) and a blocking reagent to counteract the autofluorescence of the wood was used. (see Appendix 6 for full protocol)
Oligonucleotide probes were designed to target the 16S rRNA of any bacteria present in the wood samples. Two probes were used (Tab. 4). EUB 338 is a general bacterial probe and C319a is a probe specific to the CFB group of bacteria. Samples were also mounted in a medium containing DAPI, which binds to DNA and fluoresces under UV light.

Table 7. Details of oligonucleotide probes used for FISH

Probe	Fluorophore attached	Excitation wavelength of fluorphore	Emission wavelength of fluorophore	References
Eub 388				Amann *et al.* 2000,2001
	5’	494 nm	518 nm	Bocklemann *et al* 2000
5’-GCT GCC TCC CGT	Fluorescein			Uphoff *et al* 2000
AGG AFT –3’-				Schonholzer *et al.* 2001
				Falkentoft *et al.* 2002
C319a				Amann *et al.* 2000,2001
	5’ CY3	550 nm	570 nm	Bocklemann *et al* 2000
5’ -TGG TCC GT (AG)				Uphoff *et al* 2000
TCT CAG TAC -3’				Schonholzer *et al.* 2001
				Falkentoft *et al.* 2002

Samples were viewed under a Nikon Eclipse E800 epi-microscope with the appropriate using filters.

4.2.4 Results

DNA and RNA analysis were successfully carried out on a range of wood, bacterial, soil and water samples. All extracted DNA and RNA, PCR products, purified DGGE bands and clones that are referenced in this report are databased and stored at The University of Portsmouth, UK.

BACPOLES Wood Samples
Molecular analysis was carried out on a total of 108 wood samples, representing 23 of the BACPOLES sites.
DNA was successfully extracted from 98 of the 108 wood samples, representing 22 of the sites.
DNA extraction and subsequent PCR amplification was unsuccessful for site 11 (Oak pile of a Roman embankment, Vleuten de Meern, the Netherlands)
DGGE analysis was successfully applied to wood from 18 of the BACPOLES sites. Figure 3 (below) of an annotated DGGE profile is representative of the information obtained. A full listing of profiles, identities and associated information is given in Appendix 8.

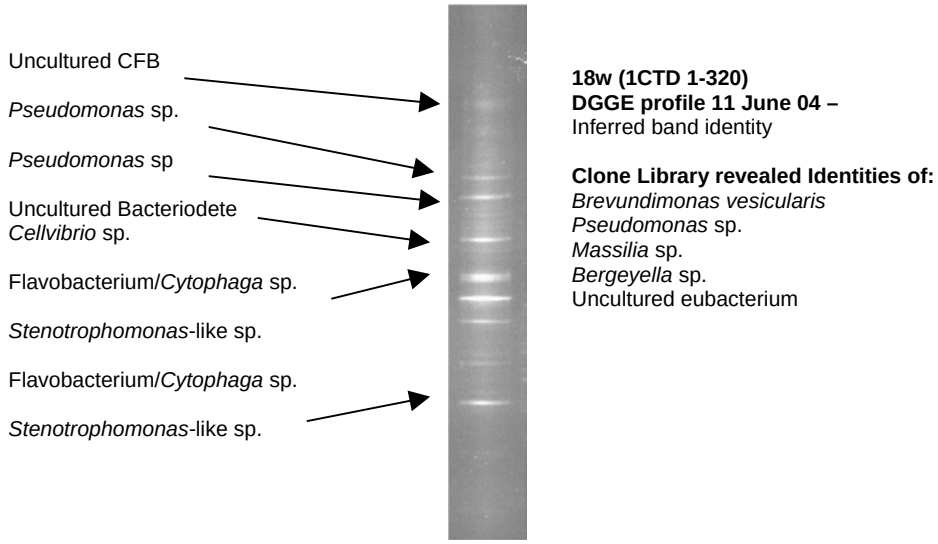


Figure 15. DGGE profile of wood sample 18w (1CTD 1-320) showing inferred identities of bands.

A total of 17 recovered DGGE bands were successfully sequenced and bacterial identities matched with the NCBI database (Tab. 5a).
A total of 68 clones form 17 sites and representing unique RFLP types, were successfully sequenced and bacterial identities matched with the NCBI database (Tab. 5a).
A cloned library was constructed: 400 clones were analysed from 17 sites and sub-divided into 68 unique RFLP types.
RNA was successfully extracted from 2 wood samples of different wood types (oak and spruce). RNA analysis on different wood types is still in progress.
5 clones were successfully sequenced (Tab. 5b)
A full listing of sequences and identities is given in Appendix 7, SLU Bacterial Cultures
Molecular analysis was carried out on 25 mixed bacterial cultures, 12 laser cut/trapped samples from UMEA and 4 samples received in March 2004.
DNA was successfully extracted and PCR amplified from all mixed bacterial cultures.
DGGE was carried out in triplicate for all 25 samples. Figure 4 (below) of an annotated DGGE profile is representative of the information obtained. A full listing of profiles, identities and associated information is given in Appendix 8.

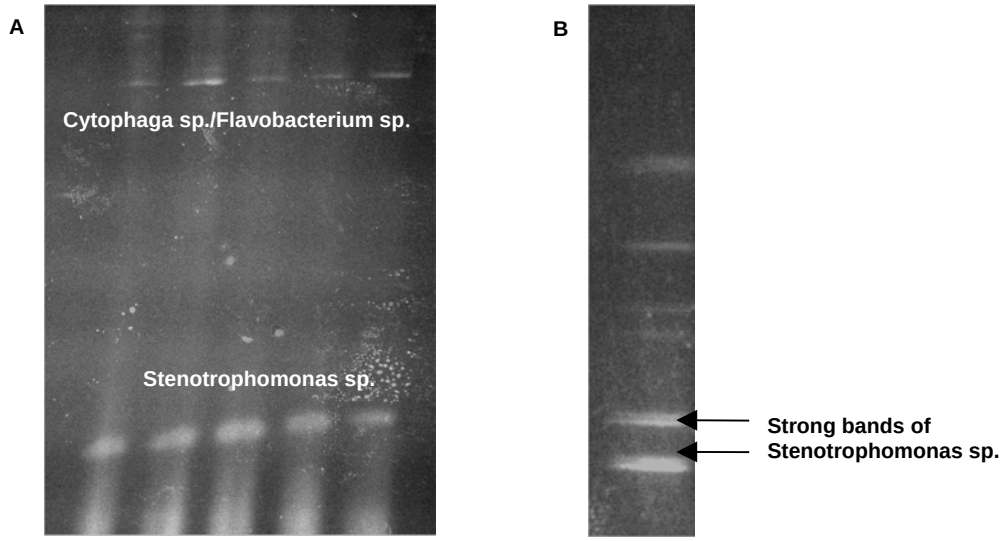


Figure 16. A: DGGE gel of five SLU UMEA samples (from left to right, A208, 796, P6, A17, and 833 (P1 D)) showing faint upper bands of Cytophaga sp and Flavobacterium sp and darker lower bands of Stenotrophomonas sp. B: DGGE gel of SLU water sample showing multiple contamination bands.

Recovery of bands, re-amplification of DNA and subsequent sequencing was variable but 28 recovered DGGE bands were successfully sequenced and bacterial identities matched with the NCBI database (tab. 8a).
DNA was extracted from one laser trapped/cut UMEA sample and 3 DGGE bands successfully recovered and sequenced (tab. 8a).
A full listing of sequences and identities is given in Appendix 7.
Stenotrophomonas sp. was identified as a serious contaminant in the water of SLU samples. It occurred at high levels in all laser trapped/cut samples (fig. 16b).
A total of 19 clones were successfully sequenced and bacterial identities matched with the NCBI database (tab. 8a).
Preliminary DNA analysis was also carried out on 30 bacterial isolates as part of the protocol development (Jeewon *et al.* 2003). Sequence and phylogenetic analyses revealed that these isolates belonged to the following bacterial groups: *Pseudomonas*, *Paenibacillus*, and *Cellvibrio*.
RNA extraction and PCR amplification from SLU bacterial cultures on cellophane and kapok has been unsuccessful to date. Further analysis is in progress.
RNA has been successfully extracted from a laser trapped UMEA bacterial sample, further analysis is ongoing (tab. 8b).

Table 8. Bacterial Identities from Wood Samples and SLU mixed bacterial cultures, best identities from BLAST comparisons of sequences. A) based on DNA b) based on RNA. Note samples beginning with number are wood samples, samples beginning with A are SLU cultures.

A) BACPOLES Sample Reference	**Clone No/ DGGE band.**	**Best Identities (% sequence match)**
1CTD 1-320,	18w1 cl. 1	Pseudomonas (99)
1ATD1 180/SLU A27r1	6	Oxalobacteriaceae (98)
(1025)	7	Chryseobacterium sp/CFB (97/96)
1.d.td.3.340	8	Pseudomonas sp (99)
1.atd.1	9	Pseudomonas sp (99)
1.btd.3140/SLU A29 (986)	10	Brevundimonas sp (97)
	18w1 cl.	β-proteobacterium/Herbaspirillum sp (97)
	18w1 cl. 6b	Chryseobacterium sp (100)
	19w1 Cl. 8_2	CFB(<95)
	28W1 Cl. 1	Chryseobacterium sp/Flavobacterium sp
	2	(100)
	4	CFB (97)
	85w1 cl.1	CFB (100)
	A27r1 dgge a	Acinetobacter (96)
	b	CFB (97)
	c	Flavobacterium sp (95)
	d	Flavobacterium (96)
	e	α-proteobacterium/Brevundimonas sp
	A29 cl. 1	(96/95)
	A29 cl. 1	α-proteobacterium/Brevundimonas sp (96)
	A29 cl.	Brevundimonas sp (100)
	A29 dgge	Brevundimonas sp/Caulobacter sp (97)
	A29 B cl.8	Brevundimonas sp/Caulobacter sp (97)
	A29 dgge a	α-proteobacterium/Brevundimonas sp (99)
		Brevundimonas sp/Caulobacter sp (100)
		Pseudomonas sp (100)
2.td.1.0-620	14w dgge a	Stenotrophomonas sp (98)
2.TD.2.400-840/SLU A86	51w1 cl. 1	Rhizobium sp/Agrobacterium sp (100)
2.TD.3.400-850	4	Oxalobacteraceae (97)
2.td.11.0-650	Cl. 6_5	α-proteobacterium/Brevundimonas sp
2.TD.12,0-760	52	(100)
2.td.7 140	53w1 cl. 5_1	
2. td.8-700/A87	25_1	Pseudomonas sp/Flavobacteriaceae (100)
	25_2	Pseudomonas sp (99)
	25_9	Rhodococcus sp (95)
	57w1 cl. 2_2	Rhodococcus sp (94)
	80w1 cl. 1	CFB
	3	α-proteobacterium (100)
	4	Rhodobacter sp (98)

A) BACPOLES Sample Reference	Clone No/ DGGE band.	Best Identities (% sequence match)
	A86 cl. 1 A87 cl. 3 6	Unidentified Bacillus sp (100) Brevundimonas sp/Caulobacter sp (100) Sphingomonas sp/Zymomonas sp (96)
4. te.5.0.00-0.50	101w DGGE c e f h k l	β-proteobacterium (91) Oxalobacteracea (86) β-proteobacterium (96) Acidovorax sp/Comamonas sp (96/95) Brevundimonas sp (96) Acidovorax sp/Comamonas sp (96)
5.te.1 5.TE.2.50 5.te.11400/SLU A93 (994)	62w cl 2 2b 67w1 cl. 3 4 A93 dgge a b	Pseudomonas sp (98) Pseudomonas sp (99) Γ-proteobacterium (98) Pseudomonas sp (100) Pseudomonas sp (89) β-proteobacterium (86)
6.te.10-500 6.te.1.2000-2500/ SLU A110/A114 6.te.1.4000-4500/SLU A111/A115	63w1 cl.3 5 A110 cl. 5 A110 a A114 cl. 1 A114 cl. 1 A111 cl. 1 A115 B cl. 4 A118 A cl. 8	Sphingomonas sp (100) Sphingomonas sp (98) Mycoplana sp/Brevundimonas sp (98) Pseudomonas sp (100) Bacteroidete (99) Flavobacteriaceae (90) Sphingobacterium sp (98) Brevundimonas sp/Caulobacter sp (100) CFB (100)
7.TE.4 300-500 7.te.6.430-600	71w cl.3_{10} 24_3 24_8 73w1 Cl. 4_3 4_7	α-proteobacterium/Oxalobacter sp (96/95) β-proteobacterium (95) Oxalobacteraceae/Janthinobacterium sp (97) Acidovorax sp (99) Janthinobacterium (98)sp
8. td.1680/SLU A17	A17 dgge A17 dgge	Pseudomonas sp (98) Pseudomonas sp (99)
9.TA.4	7w1 cl.1	unidentified
12.TD.1.3 12.ta.2.3 12.ta.2.4	23W1 Cl. 1 2 4 5 26w1 dgge.d f 27w 1	Acinetobacter sp/Cellulomonas sp (100) α-proteobacterium/Loktanella sp (100) Psychrobacter sp (100) α-proteobacterium (100) α-proteobacterium (94) α-proteobacterium (98) α-proteobacterium (96)
13.TA.4	40W1 Cl. 1 3	Bacillus sp (100) Oxalobacteriaceae /Novosphingobium sp (100)
14.ta.he.3.0,34-1,57	107w DGGE B b	CFB (95) CFB (98)
19.TD.4.100 A4-2 Bac 15 A3-2 Bac 10	Bac15 cl. 1 2 3 5 BAC10 cl.5	Rhodobacter sp/Paracoccus sp (98) Rhodobacter sp/Paracoccus sp (98) Rhodobacter sp/Paracoccus sp (99) Unidentified Unidentified (95)
2.1.ta.3@3	75w1 DGGE a	α-proteobacterium (96)
23.TD.1.80	43w1 cl.2 44w1 44w1 dgge	Chryseobacterium sp (96) Chryseobacterium sp (96) Bacteroidete/Chryseobacterium sp (97)
24.TA.5.1, 24.TA.3	4W1 10w1 Cl. 12_1 12_8 12_9 9w DGGE a b	Acidovorax sp (100) Janthinobacterium sp (98) β-proteobacterium /Janthinobacterium sp (98), β-proteobacterium (96) unidentified unidentified
25.TA.1.A.4	76w1 cl. 1_2	Oxalobacteracea (97)

A) BACPOLES Sample Reference	Clone No/ DGGE band.	Best Identities (% sequence match)
	1_6	β-proteobacterium/Oxalobacteraceae (99/97)
26. te.10-78/SLU A169(993) 26.Elst.te.3.0-7.4/SLU 171	A169 DGGE A169 DGGE a A169 cl. 1 2 8 A171 dgge A171 DGGE b	β-proteobacterium/Comamonadaceae (86) α-proteobacterium/Brevundimonas sp (86) Bacillus sp (100) Bacillus sp (100) Brevundimonas sp/Caulobacter sp (98) Pseudomonas sp (99) Pseudomonas sp (99)
27.TA.3	88w1 cl.3 4 5 8 9 LW1 cl.4	Rhizobium sp/Agrobacterium sp (100) Unidentified Cellvibrio sp/Pseudomonas sp (99) Sphingomonas sp (97) CFB (99) Cellvibrio sp/Pseudomonas sp (100)
28ta2 28ta1	81w cl.11_3 11_9 20_8 20_9 86w1 Cl. 13_1 13_2 13_3	Janthinobacterium sp (98) Chryseobacterium sp (99) β-proteobacterium (96) unidentified Flavobacterium sp/Cytophaga sp (99) CFB/Oxalobacteraceae (98) β-proteobacterium/Oxalobacteraceae (98)
SLU A208 (796) P9	A208 (P9) dgge a	α-proteobacterium/Xanthomonas sp (97)
SLU A301	A301 dgge a b c	Rhizobium sp (100) Rhizobium sp (98) Brevundimonas sp/Caulobacter sp (95)
SLU A51	A51 dgge A51 dgge a	Acidovorax sp (100) β-proteobacterium/Acidovorax sp (97)
SLU UMEA 1	Dgge d Dgge e F h	Unidentified () β-proteobacterium β-proteobacterium/Oxalobacteriaceae() Bacillus sp. ()
B)……..BACPOLES Sample Reference	Clone No/ DGGE band.	Best Identities (% sequence match)
1.ctd.1-320	18w1 cl. 1 2 3	α-proteobacterium (98) β-proteobacterium /Duganella sp (99) Bacteroidete (98)
9.ta.1	38w1 cl. 1 2	α-proteobacterium /Novosphingobium sp (86). Flavobacterium sp (99)

PhaGen Bacterial Isolates

DNA was extracted and successfully sequenced for 10 bacterial isolates from (Tab. 9). PCR products were purified and sent directly for sequencing.

With the exception of *Microbacterium* sp (site 1) and *Delftia* sp (site 8) the same taxa were identified from comparable BACPOLES wood samples. *Microbacterium* sp has been identified as a possible match for sequences but generally below the 90% threshold. Although *Delftia* sp has not previously been identified, other members of the Comomonadaceae like Acidovorax sp and Comamonas sp have been.

Table 9. Bacterial Identities from PhaGen isolates, best identities from BLAST comparisons of DNA sequences

Bacterial Isolate	BACPOLES Site Reference	Best Identities (% sequence match)
B 1:13	18.td.4.210.2	Microbacterium sp (98)
B 2:33	8.td.3.440	Comamonadaceae/Delftia sp (98)
B 3:13	1.A.td.1.320	Microbacterium sp (99)
B 3:41	1.B.td.3.310	Flavobacterium sp (99)
B 3:71	1.D.td.3.110	Brevundimonas sp/Caulobacter sp (100)
B 3:82	1.D.td.3.340	Chryseobacterium sp (98)
B 4:23	9.ta.2	Chryseobacterium sp (99)

Bacterial Isolate	BACPOLES Site Reference	Best Identities (% sequence match)
B 5:7	24.ta.3	Flavobacterium sp (98)
B 11:71	28.ta.2	Flavobacterium sp (96)
B 4:1	9.ta.1	Bacillus sp (99)

Microcosm Samples

DNA/RNA analysis was carried out on 18 wood samples, 8 soil samples and 4 water samples

DNA was successfully extracted and PCR amplified from 1 water sample MC22c and three DGGE bands were excised and sent for sequencing. The identities are given below (tab. 10a).

DNA was extracted from the remaining 17 samples but further analysis was unsuccessful. Sufficient DNA was extracted for some DGGE profiles, but not enough for successful recovery of bands. Cloning failed in all cases despite 3 attempts.

RNA was extracted and PCR amplified from 2 wood samples and a total of 15 clones successfully sequenced (tab. 10b).

RT-PCR amplification was unsuccessful for 7 other wood samples but analysis is ongoing or the remaining 3 samples.

Table 10. Bacterial identities from microcosm samples, A) based on DNA, B) based on RNA extraction and subsequent molecular analysis

A. Sample Reference	**Description of treatment**	**Clone No/ DGGE band.**	**Best Identities (% sequence match)**
MC22	+ Air	MC22c dgge 12e MC22c 12f MC22c 12g	Bacillus sp (86) Bacillus sp. (89) α-proteobacterium/ Sphingomonas sp (92/91)
B. Sample Reference	**Description of treatment**	**Clone No/ DGGE band.**	**Best Identities (% sequence match)**
MC18 708		MC18 708 cl.7_1 7_3 7_4 7_5 7_6 7_{10} 7_{15} 7_{17}	Γ-proteobacterium (96) Mycobacterium sp (99) Γ-proteobacterium (90) Pseudomonas sp (93) β-proteobacterium (97) α-proteobacterium (98) α-proteobacterium (96) unidentified clone (4)
MC22 879		MC22 879 cl. 6_2 6_6 6_7 6_9 6_{17} 6_{20} 6_{19}	Acinetobacter sp. (100) Pseudomonas sp (94) Δ-proteobacterium (95) Γ-proteobacterium (98) Flavobacterium sp (98) Γ-proteobacterium (94) Δ-proteobacterium (95)

Analysis of Bacterial Communities

Patterns of occurrence of different bacterial taxa in samples was analysed using the multivariate statistical package PRIMER as outlined in Appendix 6.

Wood Type

In general there was high variability in bacterial taxa both within and between (tab. 11) groups of sample sites with different wood types.

Bacterial communities associated with each of the six different wood types were different (Tab 8). % similarity between wood types was low and ranged from 0% (ie. No taxa in common) to 38%.

Pine and Poplar had the most similar bacterial communities, albeit low (38%)

Silver Fir/Spruce and Oak/pine had no taxa in common, 0% similarity (tab 11).

Samples from sites with single wood types *i.e.* pine, spruce, oak and poplar were in general more similar to one another (14% to 26% similarity) than the mixed sample sites *i.e.* silver fir-spruce and oak-pine (0% to 18% similarity). It must be noted however, that the oak-pine samples were all taken from marine environments. A small number of oak samples were also marine but in general sample sites were all non-marine in nature.
% similarity between sample sites with the same wood type was generally low and ranged from 0% (oak-pine group) to 14% (pine and oak groups). Similarity was higher (61%) between samples in the spruce group. Although the oak-pine sample sites were both marine, they were from different marinas. No measure of variation could be given for the Silver Fir/Spruce and Poplar groups as there was only one sample site per group.
Each wood type has a distinctive bacterial community (as shown by SIMPER analysis), which is defined by up to 7 taxa (tab. 12). These taxa are important in defining the community as they are not necessarily abundant but are representative of most samples within a particular group (Appendix 6).
Some taxa are fairly ubiquitous *eg.* α-proteobacterium, Pseudomonas sp. and Oxalobacteraceae, others are more characteristic of their wood type *eg.* Acidovorax sp. in pine.

Table 11 Taxa richness for each wood type. Taxa ranked in order of abundance, highest first.

OAK	**SPRUCE**	**PINE**	**POPLAR**
Oxalobacteriacea	Caulobacteracea	B-proteobacterium	B-proteobacterium
CFB (Bacteroidete)	Brevundimonas sp.	Comamonadales	Sphingomonadales
Pseudomonas sp.	CFB (Bacteroidete)	Sphingomonadales	Acidovorax sp.
A-proteobacterium	Pseudomonas sp.	Chryseobacterium sp.,	Janthinobacterium sp.
Bacillus sp	Flavobacterium sp.	Unidentified	Oxalobacteraceae
Pseudomonadales	A-proteobacterium	Brevundimonas sp	Unidentified
Chryseobacterium sp	Oxalobacteraceae	Oxalobacteraceae	
Flavobacterium sp	Rhizobiales	Acidovorax sp.	
Sphingomonadales	Actinomycetales	Bacteroidete	
Unidentified	Unidentified	Caulobacteriaceae	
Caulobacteraceae	Acinetobacter sp.	CFB (Bacteroidete)	
Rhizobiales	Bacteroidete	Flavobacteriales	
B-proteobacterium	Chryseobacterium sp.	Flavobacterium sp.	
Janthinobacterium sp	Flavobacteriales	.	
	Bacillus sp.		
	Sphingomonadales		
SILVER FIR-SPRUCE		**OAK-PINE**	
Pseudomonas sp		A-proteobacterium	
Γ-proteobacterium		Pseudomonadales	
B-proteobacterium		Rhodobacterales	
		Bacillus sp	
		Oxalobacteriaceae	
		Unidentified	

Table 12. Taxa listed in order of importance in characterising the microbial community from SIMPER analysis. Note: = denotes that taxa are of equal importance. No information given on Poplar or Silver Fir/Spruce where there was only one sample site.

OAK	**SPRUCE**	**PINE**	**OAK/PINE**
A-proteobacterium	Oxalobacteraceae =	Acidovorax sp. =	A-proteobacterium
CFB (Bacteroidete)	Pseudomonas sp. =	B-proteobacterium =	Pseudomonadales =
unidentified	Rhizobiales =	Bacteroidete	Rhodobacterales =
Pseudomonas sp.	Unidentified =	Brevundimonas sp.	Bacillus sp =
Oxalobacteraceae =	Actinobacteria =		Oxalobacteraceae =
B-proteobacterium =	A-proteobacterium =		
	Brevundimonas sp. =		

Region

In general there was high variability in bacterial communities both within and between groups of sample sites from different regions (Tab. 13).

Bacterial communities associated with each of the five different regions were different and % similarity between regions was low. % Similarity ranged from 0% (ie. No taxa in common) to 38%.

The Swedish, Norwegian and German sites had no taxa in common (0% similarity) however, it must be noted that these regions were represented by single sample sites.

Bacterial communities were most similar, albeit very low, between Norway and the Netherlands, marine and non-marine, sites. % Similarity was 22% and 24% respectively.

% similarity between sample sites from the same region was low, 15% and 21% for Netherlands marine and Netherlands respectively. The remaining groups each comprised samples from one site only and hence no measure of variability can be given.

Both Netherlands regions have diverse bacterial communities in comparison with the other regions but this probably reflects the imbalance in sampling regime. SIMPER analysis showed a number of taxa to be important in characterising both groups of samples, Oxalobacteraceae, Pseudomonas sp, α-proteobacterium and Bacteroidete. Non-marine sites were however the more diverse and were charcterised by a larger suit of taxa (tab. 14).

Table 13. Taxa richness for each wood type. Taxa ranked in order of abundance, highest first.

NETHERLANDS	NETHERLANDS MARINE	NORWAY
Pseudomonas sp	CFB (Bacteroidete)	Comamonadaceae
Caulobacteraceae	Oxalobacteraceae	β-proteobacterium
β-proteobacterium	Pseudomonadales	Janthinobacterium sp.
CFB (Bacteroidete)	Chryseobacterium sp	Acidovorax sp.
Oxalobacteracea	α-proteobacterium	unidentified
unidentified	unidentified	
α-proteobacterium	Bacillus sp	
Acidovorax sp/Comamonas sp	β-proteobacterium	
Flavobacterium sp	Janthinobacterium sp	
Rhodobacterales	Rhizobiales	
Sphingomonadales	Sphingomonas sp	
Bacillus sp		
Bacteroidete		
Brevundimonas sp		
Chryseobacterium sp		
Rhizobiales		
Janthinobacterium sp		
Acinetobacter sp		
Flavobacteriaceae		
Γ-proteobacterium		
Sphingobacterium sp		
GERMANY	SWEDEN	
CFB (Bacteroidete)	α-proteobacterium	

Table 14. Taxa listed in order of importance in characterising the microbial community from SIMPER analysis. Note: = denotes that taxa are of equal importance. No information given for Germany, Sweden and Norway where there was only one sample site.

NETHERLANDS	**NETHERLANDS MARINE**
Pseudomonas	Oxalobacteraceae =
β-proteobacterium	Pseuomonadales =
Oxalobacteraceae	α-proteobacterium =
Brevundimonas sp	unidentified
α-proteobacterium	CFB (Bacteroidete)
unidentified	
Bacteroidete	
Flavobacterium sp	
Sphingomonadales	
Rhizobiales	

When considering all regions, bacterial diversity differed between marine and non-marine sample sites (only 18% similarity).
The characteristic diversity of the non-marine sites is essentially the same as that of non-marine sites from the Netherlands (which comprise the majority of samples collected) as most of the taxa found in the Swedish, Norwegian and German samples are also found in the Netherlands
The same 6 taxa were important in defining approximately 80% of characteristic community of non-marine sites from the Netherlands and non-marine sites in general. The remaining taxa varied between the 2 groups of samples and reflected regional differences.
Bacterial diversity was highly variable within each group of sample sites with marine and non-marine sites only showing 15% and 18% similarity.

Table 15. Taxa listed in order of importance in characterising the microbial community from SIMPER analysis. Note: = denotes that taxa are of equal importance.

NON-MARINE	**MARINE**
Pseudomonas	Oxalobacteraceae =
β-proteobacterium	Pseuomonadales =
α-proteobacterium	α-proteobacterium =
Oxalobacteraceae	Unidentified
Unidentified	CFB (Bacteroidete)
Brevundimonas	
Acidovorax	
Janthinobacterium	
CFB (Bacteroidete)	
Bacteroidete	

Phylogenetic Analysis
Phylogenetic analysis of the sequence data provided a means of determining the relationship of the various phylotypes or ribotypes to other bacteria.

Sequences of the various environmental phylotypes or ribotypes represented a broad group of bacterial taxa (Figure 17).
Few of these sequences formed clades (related groups) after phylogenetic analysis indicating that these sequences represented unique bacterial isolates.
These environmental sequences represented taxa such as Rhodobacter and Rhizobium from the alpha-proteobacteria, which are known inhabitants of the rhizoshpere and are not thought to be involved in wood degradation.
Other environmental sequences belonged to taxonomic groups that were present from a number of wood samples. These groups included the Oxalobacterieaceae, Pseudomonas and CFB.
Environmental sequences representative of the Oxalobacteriaceae included those of Zoogloea. These bacteria are common inhabitants of aquatic environments and are possible water contaminants although they are active acid-producers.
Some environmental phylotypes, although each having a unique sequence composition, formed related clades after analysis. These phylogenetically related sequences were present within the Pseudomonas and CFB groups.
The environmental sequences representative of the CFB group were of particular interest because they formed related groups with sequences recovered after DGGE analysis of consortia (mixed cultures) and bacterial isolates (Figure 18).
Within the CFB group three groups of phylogenetically related groups were identified: Two within the Flavobacterium/Cytophaga group and one within the Chryseobacterium group. These sequences formed clades (groups) that are distinct according to their genetic distances and presumably represent novel isolates. The isolates that match the environmental sequences include Phagen cultures B3,41, B3.82, B4.23, B5.7 and B11.71.

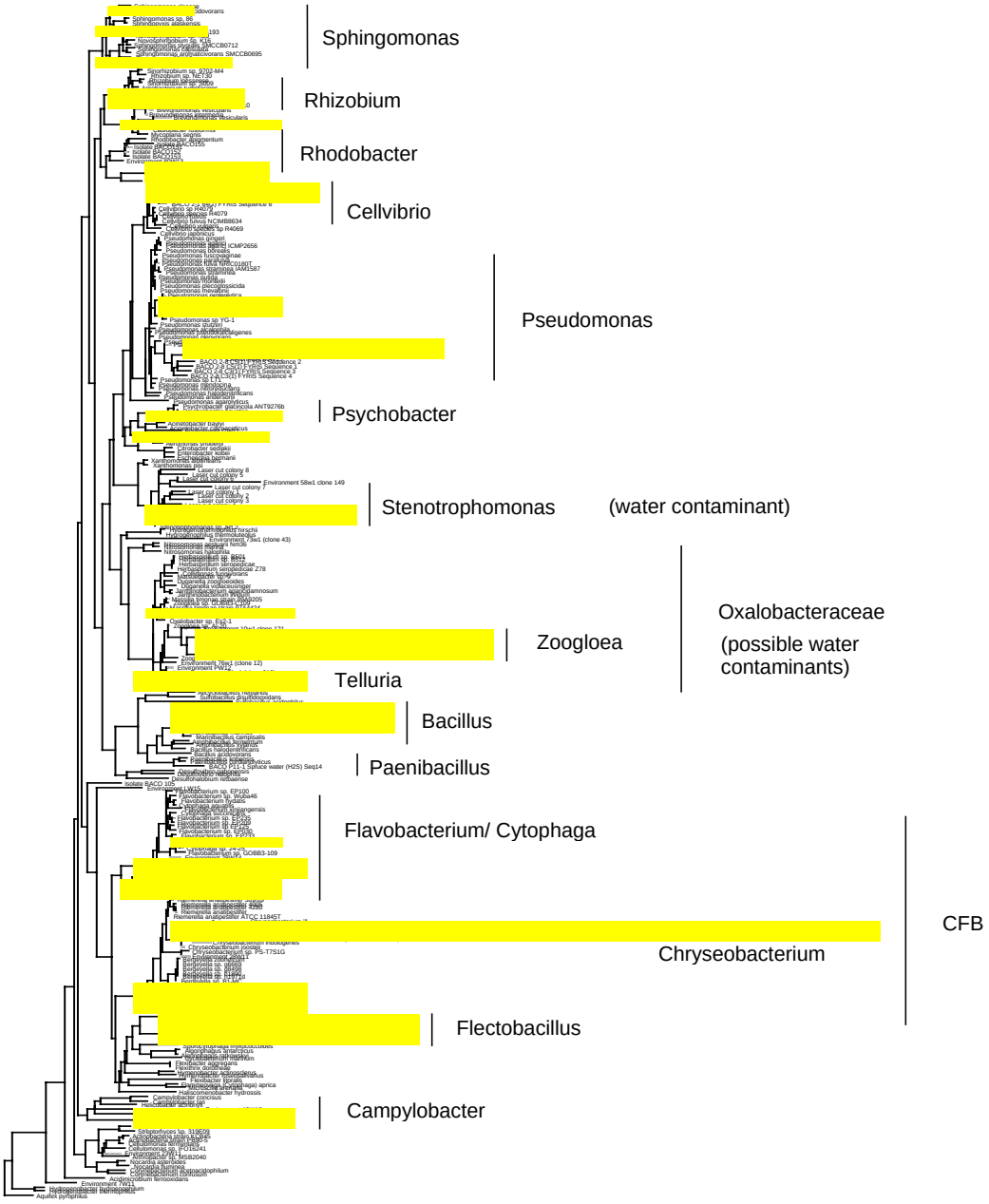


Figure 17. Phylogram of some 16S rDNA environmental sequences. The data matrix comprised 500 bacterial taxa. The yellow (highlighted) sequences show the position of the environmental sequences.

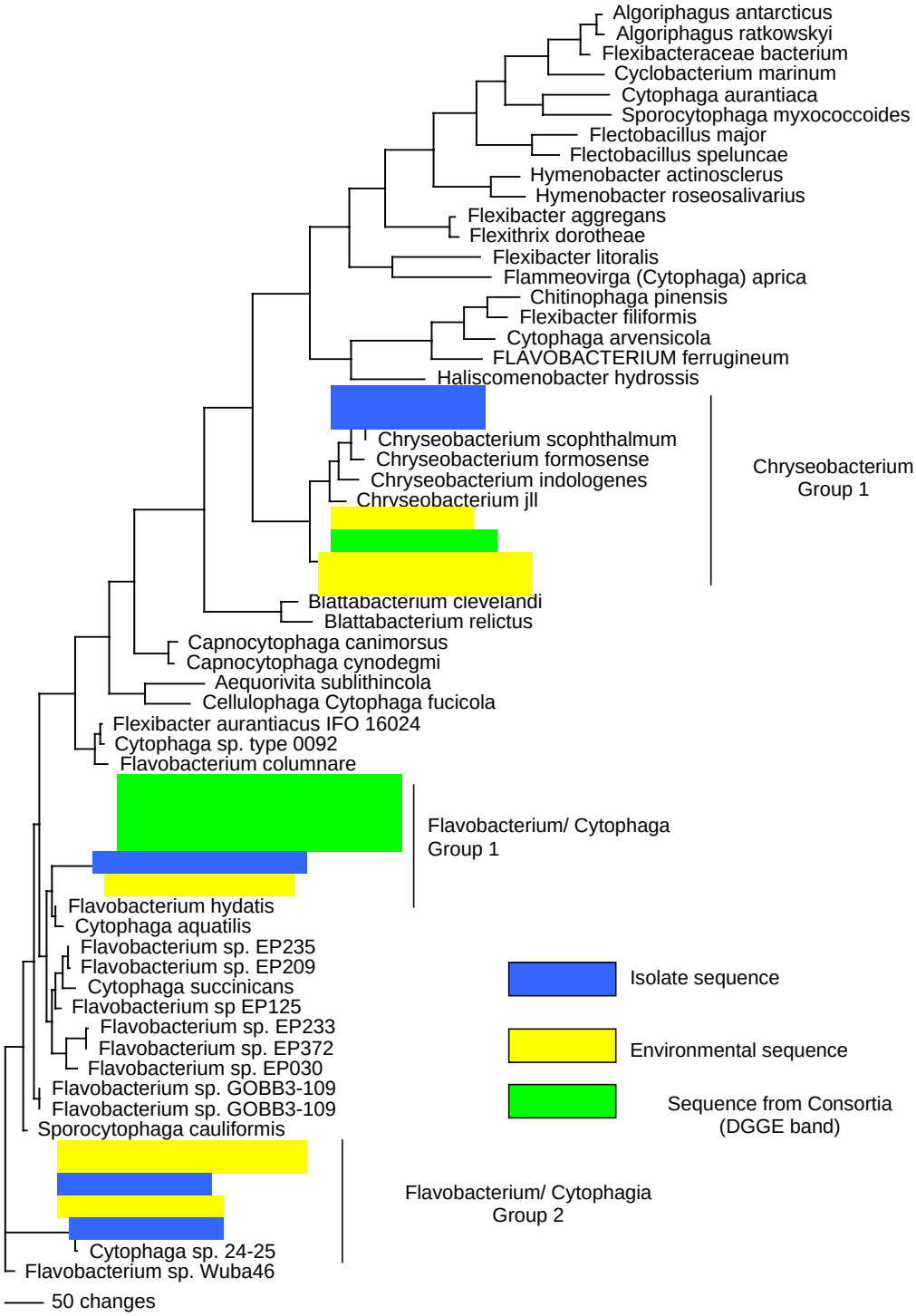


Figure 18. Unrooted phylogram of environmental [highlighted yellow], isolate highlighted blue] and DGGE band [highlighted green] sequences related to the CFB group of proteobacteria

FISH (Fluorecent *in situ* Hybridisation)

Fish was applied to a variety of samples including bacterial cultures (fig. 19), wood sections (fig. 19 & 20), cellophane cultures (fig. 21) and kapok cultures (fig. 21). The BACPOLES project is the first to apply the FISH technique to wood samples.

Initial observations were generally positive although further modification of the technique is required to optimise the usefulness of FISH for use with wood samples.

Probe specificity of commercially available probes EUB388 (general bacterial probe) and C139a (specific probe for CFB complex bacteria) was successfully tested using *Eschericia coli* as the target organism for EUB388 and SLU isolate A27R1 (1025) belonging to the CFB complex as the target organism for C139a (Figs 19, 20, 21).
Two probes JFC1 and JRCB1 were designed and successfully tested against CFB complex target organisms (Fig 19c).
Background fluorescence proved problematic during initial tests (Fig 19e-g). This was reduced using a blocking agent (Cat. No. 1096176, Roche Diagnostics) to levels where bacteria could be observed (fig. 20).
Autofluorescence of lignin is well documented and is thought to have contributed to the high levels of background fluorescence in the wood sections. Various techniques are currently being tested to quench the autofluorescence of lignin.
FISH was also tested on bacterial cultures growing on cellophane to eliminate the problem of lignin autofluorescence but levels of background fluorescence were still high in comparison with that of the bacteria present.
FISH appears to work well in situations where bacterial numbers are high but when bacterial abundance is low or the sample comprises a dense background matrix (such as wood, cellophane or kapok) the fluorescent signals of the bacteria are difficult to detect.
Further modification of the technique is required to optimise the fluorescent signals of the bacteria and to further reduce background fluorescence.

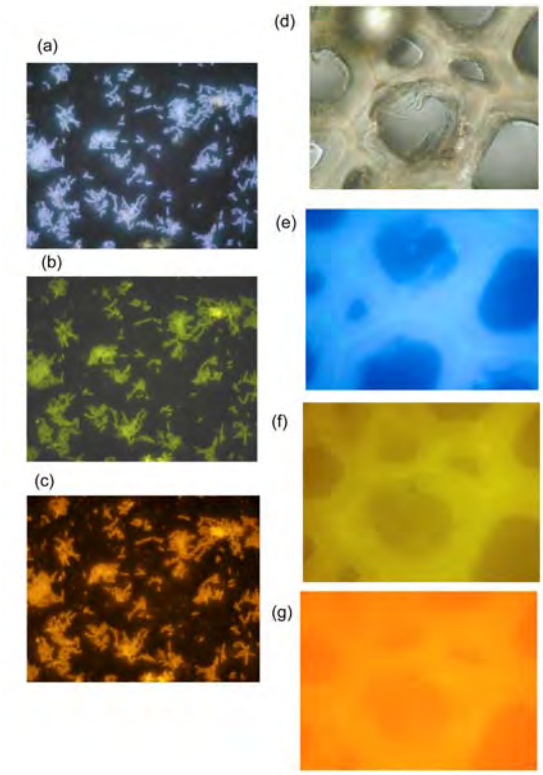


Figure 19. (a-c) Images from FISH probe specificity tests on SLU isolate A27R1 (1025) using a) DAPI stain, b) EUB388 with Fluorescein tag and c) JFC1 probe with CY3 tag. (d-g) using FISH on thin wood sections. NOTE: high levels of background fluorescence in d-g.

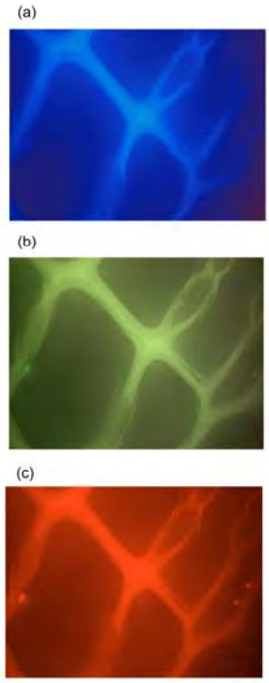


Figure 20. Wood sections probed with EUB388 and C139a with a blocking agent added using (a) DAPI stain, (b) EUB388 with Fluorescein tag, (c) C139a probe with CY3 tag. NOTE: Rings indicate areas of higher fluorescence where CFB bacteria are present. Levels of background fluorescence reduced when compared to fig. 19.

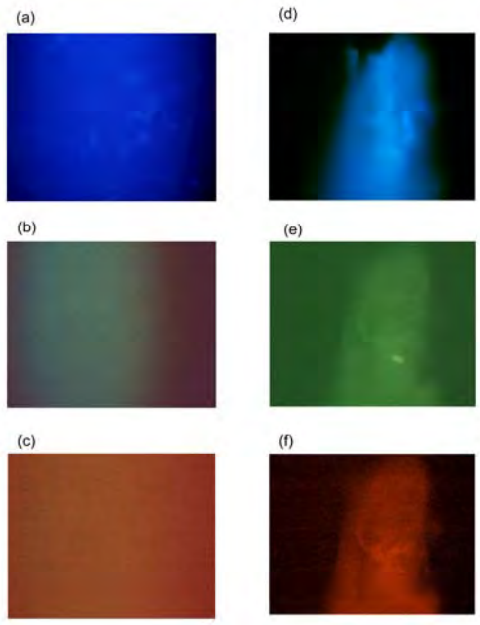


Figure 20. Images of FISH applied to cellophane (a-c) and kapok fibres (d-f) using DAPI (a & d), EUB388 with Fluorescein tag (b & e) and C139a probe with CY3 tag (c & f).

4.2.5 Discussion

Little attention has been given to the effects of bacteria on the deterioration of wood despite them being amongst the most common wood inhabiting micro organisms, and initial colonisers of wood (Clausen 1996). The BACPOLES project is the first to use molecular biology techniques to link molecular environmental signals directly from archaeological wood where degradation has occurred to cultured bacterial isolates. Such a link represents a first step in defining the types of organisms responsible for wood degradation. Protocols have been developed through the introduction of novel techniques and modification and refinement of existing methods. This has allowed the direct extraction of DNA and RNA from degraded wood samples and subsequent molecular analysis through to sequence identification and phylogenetic comparisons.

This research has used Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and molecular cloning of ribosomal Ribonucleic acid (rRNA) sequences directly from wood samples to assess the diversity of living/active bacteria associated with foundation poles without using conventional culturing techniques. The application of these techniques to environmental wood samples required optimisation on a sample-by-sample basis as wood typically contains high levels of contaminants that interfere with the PCR-DGGE process.

This part of the research strategy was essentially two fold with techniques being applied i) directly to the wood and ii) to bacterial cultures isolated from similar but independent wood samples. The results generated a small database from which environmental site comparisons and sequence matches with cultured organisms were made. This database also provides a foundation from which more in depth studies of certain bacterial groups can be made.

Although the presence of bacterial associations and wood degradation has been demonstrated (Eslyn & Moore 1984, Daniel et al. 1987, Line 1997, Björdal et al. 1999, Björdal et al. 2000), as yet, the majority have proven hard to isolate and culture successfully (Fuhrman & Campbell 1998).

A number of wood degrading isolates have been successfully cultured by SLU as part of the BACPOLES project but these represent a very small percentage of the overall bacterial diversity identified using molecular techniques. Although not all bacteria present in wood will necessarily be erosion bacteria, the results suggest that erosion bacteria potentially represent a genetically broad group of organisms with no single isolate or strain responsible for degradation.

In general, the rRNA diversity associated with wood samples was low (<20 taxa present), particularly for samples recovered from marine sites but there was high variability. Statistical analysis and phylogenetic comparisons of rRNA sequence diversity between poles from different environmental sites revealed little commonality. Levels of molecular diversity varied between different wood types and between samples from different regions as would have been expected considering the variation in environmental factors between sites. Spruce, Oak and Pine poles housed a greater number of sequence types than those of Poplar and mixed wood samples however the sampling regime was imbalanced and this result may reflect the variation in number of samples.

Despite the variability, rRNA signals representing particular bacterial groups were detected across a variety of wood types. Sequences representing bacteria from the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex and the *Pseudomonas* group were commonly recovered, with others related to the *Cellvibrio* and *Brevundimonas* groups also present. Phylogenetic comparisons showed that a number of environmental sequence types fell within the CFB complex and some of these types matched sequences from cultured isolates from independent wood samples. Defined phylogenetic groups were observed.

These observations are the first to provide a molecular link between environmental samples where degradation had occurred and cultured organisms, although the precise role of the bacterial strains remains obscure. The environmental sequences recovered for each of these groups were novel, suggesting that they came from organisms that have not as yet been characterised molecularly. Research by SLU strongly supports the molecular analysis, with cultured isolates exhibiting morphological and physiological characteristics of bacteria belonging to the CFB complex. It was cultured bacteria from this complex that matched the molecular profiles obtained from environmental sources and the mixed consortia.

Both of the common bacterial groups recovered molecularly, the CFB complex and the *Pseudomonas* group, represent organisms that are metabolically diverse. Members of the *Pseudomonas* group are strict aerobes although some species involved in denitrification are facultative anaerobes. Others belonging to this group have also been implicated in sulphur and iron metabolism. The group, however, is renowned for the presence of a variety of metabolic pathways capable of degrading complex organic compounds including xenobiotics. The CFB complex encompasses a broad assemblage of bacteria present in soil and aquatic environments (Hengstmann et al., 1999; McCammon and Bowman, 2000; O'Sullivan et al., 2002), as well as animal and fungal symbionts (Barbieri et al 2000; Lau et al., 2002). Members of the *Cytophaga*, *Flavobacterium* and *Flexibacter* groups are chemoorganotrophs; metabolism is usually respiratory using molecular oxygen as the electron acceptor, but variation can exist within these groups. Some *Cytophaga* species may undergo fermentative respiration producing acetate, propionate or succinate end products. Other *Cytophaga* species may use nitrate as an alternative electron acceptor. Although these organisms tend to be strict aerobes, many of them are facultative anaerobes capable of living in a reducing environment (Llobet-Brossa, E. et al., 1998; Okabe et al., 2003; Ferrera et al., 2004).

CFB organisms display a high abundance and diversity in marine and freshwater environments; where they are considered to have relevance in the degradation of organic matter (Cottrell and Kirchman, 2000a and b). *Cytophaga* species, in particular, are known degraders of complex polysaccharides, such as agar, cellulose and carboxymethylcellulose or chitin. They exist in a variety of morphological forms including short, flexible rods to unbranched filaments, and they move by gliding. Normally they are associated with particulate matter, although they can exist as planktonic forms (Fandino et al 2001).

Cytophaga species, along with others from the CFB group, have been implicated in the degradation of phytoplankton (Höfle and Brettar, 1996), associated with nitrifying bacteria in autotrophic biofilms (Kindaichi et al 2004); and the mobilisation of lignocellulose material in mine drainage wastewater treatment (Clarke et al., 2004). Fluorescent *in situ* hybridisation (FISH) techniques were also applied to a variety of BACPOLES samples including sections of wood, cellophane and kapok fibres in order to observe and identify wood inhabiting bacteria *in situ*. Although this work is still ongoing, initial observations are generally positive. The hybridisation probes chosen have been successfully tested for target organism specificity and CFB bacteria have been observed in thin wood sections. However, further modification/refinement to the FISH technique is required to remove background fluorescence and optimise the fluorescent signals emitted by bacteria.

4.2.6 Conclusions

The BACPOLES work to date has provided a baseline study from which future research can be directed.

A database of sequences and organisms is now available for continued molecular investigation.

Erosion bacteria most likely fall within the Cytophaga-Flavobacterium-Bacteroides (CFB) complex although the Pseudomonas, Cellvibrio and Brevundimonas groups are also common and their presence also requires further investigations.

As the molecular sequences identified here represent novel types, a better understanding of the physiology and ecology of these bacteria must be developed, along with their molecular characterisation.

Identification of the enzymes and pathways involved in the bacterial degradation of wood is of key importance to confirm the involvement and control of organisms in the process.

Further development of the FISH techniques using specific probes for the bacterial groups outlined above should prove a useful tool for rapid screening of wood samples for the presence of potential wood degrading bacteria.

4.2.7 Future Work

Develop genetic probes for isolates shown to match environmental sequences so that the fate of these organisms can be followed in the environment.

Biochemical and physiological characterisation of the matched isolates.

Further development of FISH techniques to allow identification of erosion bacteria in situ.

Characterisation of genes coding for lacases and other polyphenol oxidases, cellulases that are involved in the process of wood degradation.

Such a characterisation will allow the identification of molecular targets for future treatments.

Continuation in the development of environmental RNA techniques to help identify ‘active’ bacteria. This could involve the use of real time PCR techniques.

To make molecular diversity estimates of lacases and cellulases recovered from wood under bacterial attack.

4.3 Ecology of wood-degrading bacteria
(by Eaton)

4.3.1 Introduction
Many sites of archaeological significance and importance as well as those identified as heritage sites are located in areas subject to the influence of freshwater, saturated soils and sediments and/or seawater. The use of wooden poles as piling timber to support buildings has been used for centuries as a means of establishing city conurbations in low-lying areas of land throughout Europe. Many of these locations are of maritime importance for strategic and commercial reasons and are situated in river estuaries and coastal lagoons. In addition, these are areas where wooden shipwrecks are often to be found on the seabed at inshore sites. Timber exposed at these locations is therefore subject to degradation by lignolytic freshwater micro-organisms and marine organisms that are active in fully saline and also brackish water conditions.

The major agents of damage to archaeological timbers in oxygenated marine environments are the marine wood-boring animals that destroy the wood over relatively short periods of time. In European waters, these animals are predominantly the shipworms (Teredinidae) and gribble (Limnoriidae) (Eaton & Hale, 1993). In contrast, microbial decay of wood in these situations is slow and progressive and in anoxic environments such as sediments, it may take centuries for degradation of the polysaccharide components of the wood cell walls to occur in large timber structures. As with attack by animals, the speed of microbial degradation will depend on the natural durability of different wood species, the greater resistance of heartwood versus sapwood, plus environmental factors including water temperature, oxygen availability and salinity. A further consideration in this regard is the role of micro-organisms in the digestion of wood fragments in the gut of marine boring animals and the symbiotic relationship that exists between some members of these two groups of organisms. This will be considered in more detail later, but suffice to say that the accepted view that wood biodeterioration in the sea and in brackish water is a marine-borer problem may have underestimated the importance of micro-organisms in the overall process.

4.3.2 Microbial Successions In Decaying Wood
For many years, bacteria have been recognised as early colonisers of damp and wet wood (Levy et al, 1974). In terrestrial situations they are regarded as the first phase of a consortium of decay micro-organisms that invade wood in soil contact and can include bacteria with nitrogen-fixing properties. This latter observation was considered to be of some significance, since wood has very little nitrogen naturally present, but still supports a succession of decay organisms that are dependent on available nitrogenous sources in the substratum. Most of these later invaders have greater lignolytic capabilities than the bacteria and these include the soft rot fungi (Ascomycota and mitosporic fungi) and the brown and white rot fungi (Basidiomycota).

4.3.3 Wood as a substrate and a substratum
In aquatic and waterlogged environments, submerged wood is colonised quite rapidly and then decayed by bacteria and soft rot fungi, but colonisation by basidiomycetes is less common. Bacteria and soft rot fungi are more tolerant of the low oxygen conditions in saturated wood, and in wood that is buried in sediments bacterial decay tends to predominate. One important observation that was reported in the 1970s in Ireland, was the ability of bacteria to increase the porosity of saturated wood. Studies into the ponding of softwood logs prior to treatment with creosote preservatives revealed that over a relatively short period of time a bacterial flora had established in the tracheids and rays of the outer sapwood band. The bacteria were part of a micro-flora naturally present in bodies of water where logs were stored and had invaded the wood over a matter of months.

Microscopic examination of the sapwood showed that pits in the wood cell walls had been destroyed resulting in an increase in wood porosity and a consequent increase in preservative uptake. This work focussed on spruce logs that are notoriously difficult to treat with preservative solutions because the bordered pits become aspirated during drying, preventing free movement of fluids. In this instance, bacterial pit breakdown can have a positive outcome, but it also showed that the breakdown of pit structure by bacteria is one of the first stages in the microbial invasion of woody tissue. This highlights the fact that as the succession of invading micro-organisms builds up, the physical and chemical status of the substratum is modified in different ways.

One important aspect of wood colonisation by micro-organisms is the type of wood being invaded and its susceptibility to infection. A major distinction can be made between the less durable outer sapwood band of a log and the more durable inner heartwood. The heartwood has greater natural resistance to invasion by virtue of the significantly higher levels of chemical extractives in the tissue. Wood species also exhibit different levels of natural durability based on the performance of heartwood exposed in ground contact. The time taken to fail in soil contact provides an index of durability class ranging from very durable to perishable species. One further characteristic of a wood species that is related to its natural durability classification is its density, hence the extensive use of oak through the ages for a range of purposes. Thus the choice of wood species for exposure in situations and locations that can now be described as EN Hazard Classes 4 and 5, where wood is at greatest risk, has a significant bearing on its longevity in these environments.

Wood is a suitable substrate for breakdown by enzymes released by invading lignolytic micro-organisms because it is in effect, an open network of microscopic tubes and galleries. It is composed of axial and radial elements made up of different cell types. The axial elements impart strength to the living tree and to converted timber, but the strength is dependent on the thickness of cell walls in the axial system. Wood cell walls have an outer primary and an inner secondary wall layer. The secondary wall is also made up of 3 layers – the outermost S_1, the S_2 and the innermost S_3, which is adjacent to the cell lumen.

The three major components of wood cell walls are cellulose, hemicelluloses and lignin, but of these only cellulose performs a structural function by providing strength to the material. Cellulose is a long-chain homo-polymer composed of thousands of glucose monomers bonded together by regular β1-4 chemical linkages. The molecular organisation of cellulose in wood cell walls is based on aggregations of cellulose chains into micro-fibrils that adopt a helical orientation within the cell wall. The angle of the helix is different in different cell wall layers, but in the S_2 layer the angle is particularly steep. In addition the S_2 layer is thicker than the other layers and is extremely cellulose-rich. Unlike cellulose, hemicelluloses are short-chain, branched hetero-polymers composed of several different monosaccharides as well as glucose units. The branched nature of the molecules, which are about 200 units in size, requires three different types of bonding and hemicelluloses are considered to be part of the matrix in which cellulose micro-fibrils are embedded. However, the enzymatic breakdown of hemicelluloses bears close similarity to that of cellulose degradation through the activity of hydrolase and oxidase enzymes.

In contrast to the wood polysaccharides, lignin is described as a three dimensional polymer composed of phenyl propane units. It is a recalcitrant molecule that is degraded slowly in nature and along with the hemi-celluloses, is a major component of the matrix material in wood cell walls. Lignin is a complex molecule and in addition to the phenyl propane monomers has up to 15 different types of chemical bond to hold the polymer together. Its distribution within the wood cell wall is not uniform and lignin is found in greatest concentration in the middle lamella region and the cell corners. Hence the progressive destruction of wood cell walls leaves these more highly lignified regions as the final remnants of the structure.

Clearly the physical and chemical composition of woody materials has a direct bearing on the ecology of the micro-organisms that destroy them. The lignin matrix is in some measure a barrier to the activities of cellulolytic and hemi-cellulolytic enzymes except in the case of basidiomycete white rot attack where all three components of wood cell walls are broken down. However in the thick cellulose-rich S_2 layer, where the lignin content is very low indeed, cellulase enzymes are able to function freely. All wood-destroying micro-organisms are capable of degrading cellulose, but for some their status as lignin-degraders is still unclear. This is especially so for lignolytic bacteria, although tunnelling bacteria have been visualised traversing lignin-rich middle lamellae under TEM (see below).

4.3.4 Location and site conditions

In addition to the structure and chemical composition of wood, the environmental conditions operating at each exposure site will influence the severity of the damage caused to the material. Some general principles are widely accepted in microbiology to describe groups or categories of micro-organism based on their growth responses to factors such as temperature, pH, oxygen levels, salinity, osmolarity and available nutrients.

Temperature has a very obvious effect on the growth of micro-organisms in piling timber and shipwrecks and although this will show seasonal variation, the temperature ranges will reflect the line of latitude of each site. Thus the warmer waters of the Mediterranean Sea throughout the year in contrast to the colder conditions in the Baltic Sea are more likely to support the growth of mesophilic micro-organisms as against mesophilic/psychrophilic forms in cooler locations. The same will apply to terrestrial sites. This raises a question about the diversity of wood-degrading bacteria and fungi that exists between sites where certain environmental conditions may be quite different from one another.

It is generally accepted that the optimum pH conditions for the growth of bacteria are slightly more alkaline than those for fungi, although there are exceptions to this rule. Most bacteria grow best in conditions where the pH is 7 – 8, but there are examples of bacteria that will grow in much more extreme pH conditions. Sulphur oxidisers, including bacteria in the genus *Thiobacillus,* are able to grow at pH. 2 or less and have the reputation for destroying stone and concrete. Micro-organisms that grow in different pH conditions are described as either acidophilic, neutrophilic or alkalophilic. In aquatic environments or saturated terrestrial situations, such extreme conditions do not occur, but situations can arise where mildly acidic or mildly alkaline conditions might prevail. This is more likely to happen in soil or sediment systems and once again will have a direct bearing on the composition of the microbial communities in these systems and their potential to invade wood under these conditions.

Perhaps the environmental factor that has a greater importance than any other, when considering archaeological and piling timbers, is oxygen availability. For some time bacteria have been categorised as being either obligate aerobes, facultative anaerobes, aero-tolerant, micro-aerophilic or obligate anaerobes. These groupings span the range of responses to oxygen shown by bacteria from those that will only grow in the presence of oxygen to those that will not grow when oxygen is present. In between, the facultative anaerobes will grow in the presence or not of oxygen, because they are capable of oxidative respiration or fermentation of available substrates. Aerotolerant forms do not utilise oxygen, but are unaffected by its presence, whilst micro-aerophilic bacteria grow better in limiting oxygen conditions. Fungi have always been considered to be obligate aerobes, but more recently it has been shown that a group of primitive fungi inhabit the guts of ruminants, clearly under anaerobic conditions.

The conditions prevailing in soil or sediments, particularly underwater, would obviously favour the establishment of a population of anaerobic bacteria inhabiting timbers below the surface. In the absence of oxygen, or at least under very low oxygen tension, the rate of bacterial growth is slow. It is interesting to speculate whether these organisms are truly obligate or facultative. Furthermore, such anoxic conditions promote the release of sulphide compounds from other anaerobes such as the sulphate-reducing bacteria.

4.3.5 Wood Degrading Micro-Organisms

Early reports identified unicellular bacteria in foundation piling and shipwreck timbers (Boutelje and Kiessling, 1964; Harmsen and Nissen, 1965; Boutelje and Bravery, 1968), but more recently three bacterial decay types in wood cell walls are now recognised – erosion, tunnelling and cavitation bacterial decay. Examples of the micromorphology of each of these decay types in archaeological timbers have been elegantly illustrated in recent publications by Bjordal, *et al.,* (1999); Bjordal, *et al.,* (2000); Blanchette, (2000); Kim and Singh, (2000) and Powell *et al.,* (2001). Descriptions of each type of microbial decay process are given below.

4.3.6 Erosion bacteria

In a study by Bjordal *et al.* (1999), erosion bacteria were the dominant microbial degraders of archaeological, waterlogged wood ranging in age from 400 – 9,000 years. The oldest wooden samples were removed from marine sediments, but wood was examined from seawater and also terrestrial sites. It is now accepted that waterlogged wood maintained under conditions of minimal oxygen availability is decayed primarily by erosion bacteria (Bjordal, *et al.,* 2000; Blanchette, *et al.,* 1990; Blanchette, 1995; Kim and Singh, 1994; Kim, *et al.,* 1996). Although some attack by soft rot fungi and tunnelling bacteria can also be found in these timbers, it has been suggested that the very low oxygen conditions are periodically alleviated for this to occur (Daniel and Nilsson, 1997; Singh and Kim, 1997).

Decay by erosion bacteria has been reported in different wood species resulting in the utilisation of wood polysaccharides; the extent of lignin modification is not known (Blanchette, 2000). This type of degradation is characterised by the formation of erosion troughs or grooves on the lumen surface of wood cell walls and is most effectively visualised using scanning electron microscopy (SEM). The eroded areas are associated with single-celled bacteria which are typically rod shaped and decay is progressive from the lumen surface towards the compound middle lamella of the cell wall. Initially a population of erosion bacteria can be observed on the lumen surface. Holt (1983) noted that rod-shaped bacterial cells were orientated in a parallel fashion following the alignment of the cellulose microfibrils. Grooves excavated in the S_3 layer of the wall by individual bacterial cells were more or less the same size as the cells, indicative of restricted enzyme lysis of the wall surface around each bacterium.

Attachment to the lumen surface is through the formation of a glycocalyx and as progressive breakdown occurs, the secondary cell wall is converted into amorphous material mixed with bacterial cells and bacterial slime (Bjordal, *et al.,* 1999). Daniel and Nilsson (1986) concluded that extracellular mucilage was important in bacterial cell adhesion, motility and enzymatic degradation of the cell wall. In softwood timbers, attack of the cell wall by erosion bacteria can also result in the formation of angular cavities in the S_2 layer and this is probably caused by interaction between the orientation of cellulose microfibrils and extracellular bacterial enzymes. So far, identification of erosion bacteria from field specimens has not been successfully accomplished. A greater understanding of their physiological requirements in laboratory culture and the optimum conditions for promoting adhesion and decay at cell wall surfaces is necessary for this to be achieved.

4.3.7 Tunnelling bacteria

Decay of wood cell walls by tunnelling bacteria is found in wood exposed in the sea. Eaton and Dickinson (1976) first reported a previously un-described decay type observed in pine timber exposed in seawater for up to 2 years. This type of decay was also recorded in saturated wood exposed in the warm conditions of industrial water cooling towers. In both situations oxygen was limiting, but did not approach the near-anaerobic conditions of burial in silt and sediments. Later studies using transmission electron microscopy (TEM) and SEM confirmed that this type of cell wall attack was caused by tunnelling bacteria (Nilsson and Daniel, 1983; Daniel and Nilsson, 1985; Singh and Butcher, 1991). The presence of tunnelling bacteria in wood in soil contact is indicative of the ubiquitous nature of these organisms. In addition their ability to decay preservative-treated wood and durable heartwood is further evidence of the aggressive capabilities of these wood decay organisms.

Tunnelling bacteria remove the polysaccharide components of wood cell walls and some of the lignin (Blanchette, 2000). The appearance of the decayed wood is soft and darkened. Longitudinal sections of decayed wood viewed with a transmission light microscope, display regions of granulation in softwood tracheids. Fine tunnels are found at the margin of these regions and with appropriate staining and good microscope optics, single-celled bacteria can be seen at the ends of tunnels inside the wood cell walls. The granulation zones develop from a loose, irregular network of fine tunnels following bacterial penetration into the cell wall. Under polarised light, the zones of granulation show loss of birefringence confirming the breakdown of crystalline cellulose in these infected areas. But not all examples of tunnelling bacterial decay show exactly the same pattern of attack. In some cases the tunnels appear wider, are fewer in number and appear to radiate more precisely from a central point producing an ‘ice-fern’ pattern (Bjordal, *et al.,* 2000). Decay patterns associated with tunnelling bacteria are not uniform and differences in their appearance may be influenced by environmental factors, wood species or indeed by the bacteria themselves, since to date none have been identified.

The penetration of these bacteria into wood cell walls is initiated by adhesion to the lumen surface via an extracellular glycocalyx. The bacterium lyses the S_3 layer and is then able to tunnel in the S_2 and S_1 layers. The direction of tunnelling is apparently random and TEM shows bacteria penetrating through the primary wall and middle lamella into adjacent cell walls. In badly attacked regions, the cell wall is riddled with tunnels, showing bacteria at their distal ends and a series of concave transverse layers along the tunnels (Nilsson and Daniel, 1983; Daniel and Nilsson, 1985). These transverse layers or cross walls are thought to be composed of mucopolysaccharide deposited by the advancing bacterium and have been described as chambered tunnels by Mouzouras, *et al.,* (1986). These observations suggest that cell wall lysis is therefore not a continuous process.

From recent studies into the condition of waterlogged archaeological wood from a range of sources, the fact that attack by tunnelling bacteria and soft rot fungi is more concentrated in the outer layers and erosion bacteria predominate deeper in the wood, indicates the latter are much less oxygen dependent.

4.3.8 Cavitation bacteria

Bacterial attack of wood cell walls that produces cavities was described by Holt (1983) and was further documented by Mouzouras, *et al.,* (1986). These authors described in detail two types of bacterial decay involving tunnelling - one chambered (see above) and the other non-chambered tunnelling. This was observed in different wood species exposed for up to 12 weeks in seawater. In a review of the bacterial degradation patterns in wood cell walls, Singh and Butcher (1991) indicated that it was not clear whether the decay pattern described by

Holt was the same as that studied by Nilsson and Singh (1984) which they had described as cavitation attack. The main distinction appears to be whether cavities are orientated with the axial direction of wood cells (Holt, 1983) or are perpendicular to the cell axis (Nilsson and Singh, 1984).

Cavitation attack was described from preservative-treated pine posts in horticultural soils. Microscopically, the decay pattern was similar to that described by Boutelje and Bravery (1968), working on ancient softwood piling timber in Stockholm. Decay occurs in the wood cell wall producing angular, often diamond-shaped cavities. Attack is initiated at the lumen surface where bacteria attach themselves via extracellular slime (Singh and Butcher, 1991). Following penetration of the S_3 layer, the underlying S_2 is attacked resulting in the formation of small cavities. It is assumed that one bacterial cell or a small group enlarge the cavity and as cavities increase in size they begin to coalesce. Although the bacterial population in the cavities will increase, it is assumed that enzyme breakdown of the cell wall is non-localised and that cells produce diffusible substances similar to those observed with brown rot basidiomycete decay. It is clear from the literature that any definitive diagnosis of cavitation attack by bacterial cells can only be achieved with the aid of SEM and TEM equipment.

4.3.9 Bacterial symbionts of shipworm

Apart from recent observations of bacterial endosymbionts associated with the marine boring pholad *Xylophaga*, the most detailed studies of symbiotic associations in marine boring animals has focussed on the Teredinidae. Electron microscopy pinpointed the Gland of Deshayes, a specialised region of the shipworm gill, as the site where bacterial symbionts reside (Waterbury, *et al,.* 1983). Unlike many symbiotic associations, the bacteria can be isolated from the animal and cultivated in the laboratory and so far symbionts from seven shipworm host species are maintained in culture (Sipe, *et al,.* 2000). The bacteria produce cellulolytic and proteolytic enzymes, plus nitrogenase for atmospheric nitrogen fixation allowing the shipworm to survive principally on a wood diet that is naturally nitrogen-depleted. Investigations into the identities of bacterial symbionts from four shipworm species representing three different genera, revealed a phylogenetically common bacterial strain *Teredinibacter turnerae,* a member of the gamma sub-division of the *Proteobacteria* (Distel, *et al.,* 1991). However, later studies on the identity of the bacterial symbiont from *Bankia setacea,* which was not included in the previous investigation, found that this teredinid harboured a different bacterial symbiont (Sipe, *et al.,* 2000). This work also showed that acquisition of the symbiont by successive generations of *B. setacea* was through a vertical mode of transmission, the symbiont cells being present in the host ovary and offspring.

As a footnote to this work, the gut contents of *Limnoria* have been examined using SEM but no microorganisms were found associated with wood particles even though the wood ingested by the limnoriids was decayed by soft rot fungi and tunnelling bacteria. It has been suggested that ingested microbes are digested by the animals. Subsequently, work by Dymond *et al.,* (2003) isolated a cellulase enzyme from the mid-gut diverticulae of *Limnoria quadripunctata.* The role of a wood degrading bacterial gut flora is therefore very questionable in this group of marine wood boring animals.

4.3.10 Identification Using Molecular Techniques

Molecular techniques for studying microbial community diversity are well documented (MacGregor 1999, Amann *et al* 2001, Giraffa & Neviani 2001, Schäfer & Muyzer 2001). These techniques make use of 16S rRNA or its encoding gene (rDNA) as molecular markers for bacteria. 16S rRNA sequences can be used to identify bacteria and infer phylogenetic relationships based on comparative analysis with known sequences stored in databases. Bacterial research has generally focussed on aquatic systems or enrichment cultures but a number of studies on fungal diversity of environmental samples such as soils and wood have been documented (see Vainio and Hantula 2000 and references therein).

The BACPOLES project (Landy *et al.* 2004) is one of very few studies that have employed such techniques to identify the bacterial diversity of archaeological or waterlogged wood (see also Helms *et al.* 2004).

Although it is widely accepted that bacteria are primary degraders of archaeological and waterlogged wood (Clausen 1996, Tiano 2002, Blanchette 2000) taxonomic clarification of these bacteria still remains problematic. To date, most studies have used traditional microbiological techniques and microscopy to demonstrate the presence of bacterial associations and wood degradation (Eslyn & Moore 1984, Daniel *et al*. 1987, Line 1997, Björdal *et al.* 1999, Björdal *et al.* 2000) and as yet the majority have not been cultured successfully (Fuhrman & Campbell 1998). The current status of knowledge is essentially based on morphological studies and patterns of decay.

A recent study by Helms *et al.* (2004) and BACPOLES research by Landy *et al.* (2004) have used molecular techniques to identify groups of bacteria from archaeological wood samples. Whilst in both studies the identification of any bacteria present in the wood was based on molecular analysis of extracted DNA, Helms *et al.* (2004) still used traditional culture techniques to cultivate bacteria from the wood before any molecular analysis was carried out. Landy *et al.* (2004) however extracted DNA directly from wood samples.

Applying molecular techniques to environmental samples such as wood and soils can be problematic. Extraction of DNA from bacteria is in itself a straight forward process but when the bacteria are contained within a matrix of other material, the process can be hampered. Successful molecular analysis requires clean DNA but environmental samples typically contain high levels of contaminants that affect the extraction and purification process. Polysaccharides and secondary metabolites found in wood affect the purity of DNA and subsequent molecular treatments (Csaikl *et al.*1998). Soils contain contaminants such as tannins, polysaccharides and humic acid all of which inhibit accurate quantification of DNA and interfere with enzyme activity during the extraction process (Porteous *et al* 1994, Yeates *et al* 1998). DNA “clean-up” steps have thus been introduced to molecular protocols where such contamination is likely (Chandler *et al.* 1997, Vainio & Hantula 2000, Helms *et al.* 2004, Landy *et al.* 2004).

A range of protocols is available for the extraction and purification of DNA fragments but those used for environmental samples are typically harsh. Physical methods such as grinding of freeze dried material or with liquid nitrogen (Cubero *et al.* 1998, Landy *et al.* 2004), grinding with abrasive sand (Vainio & Hantula 2002) and beads (Chandler *et al.* 1997, Helm *et al.* 2004) serve not only to homogenise the samples but also promote bacterial cell lysis. Although commercially available kits are now used, the CTAB (cetyltrimethylammoniumbromide) method for DNA purification (Chandler *et al.* 1997, Cubero *et al.* 1998, Landy *et al.* 2004) is most commonly adapted for extraction of DNA from a range of plant materials. Extraction with CTAB buffer removes polysaccharide contaminants from the extracted lysate.
Another problem encountered when using molecular techniques to analyse bacterial diversity in wood is that the bacteria and hence DNA may be present in very low quantities. Optimisation of the extraction protocol is essential if the DNA extracted is to be representative of all bacteria present, however techniques are available to increase rare or low quantities of DNA for subsequent analysis. PCR (polymerase chain reaction) and cloning techniques are commonly used. PCR is used to amplify target DNA sequences using specific oligonucleotide primers and a thermostable DNA polymerase (MacGregor 1999). Target DNA can also be cloned into plasmids and then replicated by subsequent insertion into bacterial cells that rapidly divide and reproduce.

Extraction of DNA from environmental samples generally results in a heterogenous mix of DNA fragments (PCR products) that act as “fingerprints” of bacterial diversity. The most commonly used protocol for separating these fragments and screening for heterogeneity is PCR-DGGE (denaturing gradient gel electrophoresis).

DGGE exploits the variation in melting properties of different bacterial DNA molecules and separation is brought about using polyacrylamide gels containing denaturing gradients. Gradients of urea:formamide or temperature are generally used (Schäfer & Muyzer 2001). Separated DNA fragments can be recovered from DGGE gels and used for sequence comparisons. DGGE has been successfully used to estimate bacterial diversity in aquatic systems and environmental samples such as soils, sewage sludge and biofilms (Muyzer 1999, Schäfer & Muyzer 2001 and references therein) (Muyzer 1999) and recently in archaeological wood (Landy *et al* 2004).

Helms *et al.* (2004) constructed a clone library of 21 clones from bacterial DNA extracted from archaeological wood, 15 of which were successfully sequenced and affiliated with known databased sequences. All of the identified clones belonged to bacterial groups that are known to exist in soil or bog environments and utilise cellulose as an energy source (Helms *et al.* 2004). Clones were affiliated to the *Spirochaeta, α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria* and *Geobacteriacea.*

Landy *et al.* (2004) recorded 56 independent molecular fingerprints from samples of different archaeological wood across Europe. These were predominantly affiliated with the bacterial groups *Flavobacterium-Cytophaga, Janthinobacterium-Pseudomonas, Oxalobacteriaceae, Sphingomonas* and *Cellvibrio.* There is documented evidence to show that the bacterial groups present are either capable of degrading wood or utilising cellulose and lignin as energy sources, or are known associates of microbial communities that do so (Landy *et al.* 2004).

Recent work has shown that molecular techniques can be successful in estimating the diversity of bacteria inhabiting complex materials such as wood and soil. The bacterial groups that have been affiliated with DNA fingerprints show that although diversity is generally low, a wide range exists. If these bacteria were to be isolated using traditional culturing methods, the experimental design would at least have to factor in the potential presence of anaerobes, aerobes, soil bacteria, freshwater bacteria, marine bacteria and nitrogen fixers. Relatively little is still known about wood degrading bacteria and there is huge scope for continued research but molecular methods undoubtedly offer a rapid and effective way forward.

In conclusion, it is difficult to pinpoint precisely the environmental conditions which optimise decay of wood by erosion bacteria. The fact that this form of decay occurs in wood which has been exposed in marine sediments as well as wood buried in wet terrestrial sites clearly indicates the broad diversity within this group of organisms which we refer to as erosion bacteria. Nevertheless, it is apparent that there is a requirement for the wood substratum to be in a saturated state. This may be necessary for the movement of bacterial cells over wood cell wall surfaces if these organisms are shown to be gliding bacteria and are dependent on extracellular mucilage for adhesion and enzyme release as well. However saturated wood, particularly wood buried in sediments, creates conditions with very limited available oxygen allowing growth of anaerobic bacteria only, probably facultative anaerobes. In addition, these organisms can be described as neutrophilic with respect to their pH requirements and mesophilic with respect to their temperature requirements.

4.4 Final conclusions

(by Björdal, Eaton, Mitchell, Nilsson)

Molecular analyses of original BACPOLES wood samples showed a large number of different bacteria species to be present in the wood material.

Wood degradation of BACPOLES samples by bacteria was observed in the laboratory and these bacteria were successfully isolated and purified. It was proposed that these bacteria belonged to the CFB complex (*Cytophaga-Flavobacterium-Bacteriodes*), based on:

- Their common occurrence in BACPOLES samples;
- literature surveys that described the CFB group as abundant in very diverse environments in nature, including anoxic sediment;
- Morphological studies showing the isolates to have similarities with bacteria from the CFB group (gram negative rod shaped bacteria, motile by gliding, absence of flagellae, and size);
- Subsequent molecular analysis confirmed that the isolates did belong to the CFB complex and that these bacteria were common in many samples.

Erosion bacteria were isolated from quite varying environments and the different molecular sequences that were identified suggested that several different species of erosion bacteria probably exist, even within the CFB complex. The identity of active erosion bacteria must therefore be documented on a site-by-site basis before any treatment can occur. Further, if phages are to be used as a biological control for wood degradation then a number of phages will have to be produced.

Future work is necessary to find the most common erosion bacteria and also to gain more knowledge on the individual species and their physiological requirements for culturing under laboratory conditions.

With increased access to more pure cultures and isolates it will be possible to take a closer look at their general physiology but also the enzymatic systems required for the degradation of lignocellulosic materials. As yet these systems are not understood.

FISH (Fluorescent *in situ* hybridisation) appears to offer a valuable technique for fast *in situ* observation and identification of potential erosion bacteria prior to phage-treatment. However, further refinement of this technique is required.

Chapter 5

Microcosm experiment

5.1 Microcosm Experiment

(by Kretschmar & Gelbrich)

Material and methods

Figure 1 shows the microcosm (MC) set up. In the gas inflow and outflow CO_2, N_2O and CH_4 were continuously monitored using a GC-ECD and FID (Shimadzu, Tokyo, Japan) described in Loftfield et al. (1997). Four treatments were used aiming to vary the oxygen availability in the sediment by altering the gas supply to the microcosm. The gas inflow was: 1. Air (A), 21% vol. O_2, 2. Air and oxygen (A+O_2), 50% vol. O_2, 3. Nitrogen (N_2), 0% vol. O_2. In the fourth variant water circulation by pumping the extracted water from the bottom to the top of the sediment column was combined with air inflow (A+C).

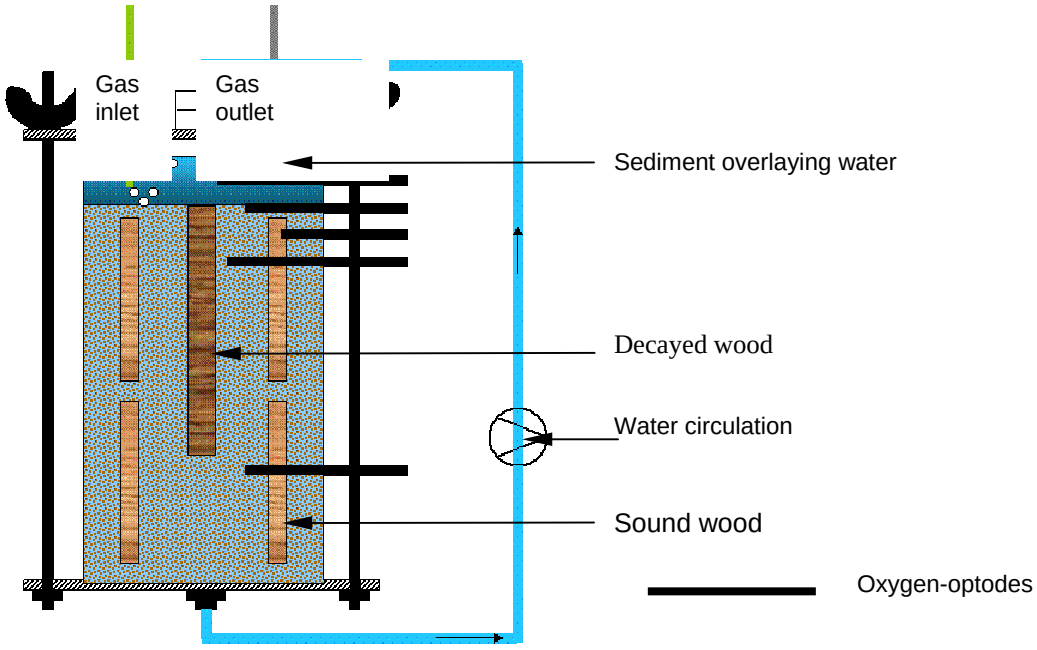


Figure 1. Microcosm scheme with gas supply, water circulation and oxygen-optodes.

Additionally to the sediment 20 sound pinesap wood sticks decay control together with a moderately decayed pine sap wood stick as bacteria source were included. The moderately bacterial decayed pinewood originated from a foundation pile extracted from a house in Koog an de Zaan/ in the Netherlands. Controls for the sediment gas production were sediment columns without wood subject to the same gas supply treatments. For the treatment variants four replicates, for the control with air treatment three replicates and for the other treatments controls only one MC were used. For basic chemical composition of sediment, water, sound and infected wood used in the experiment see table 1.
The microcosms (MCs) were incubated in the dark at 20 C. The MC were harvested consecutively after 120, 150, 195 and 350 days and 1, 1, 2, 2 MCs per treatment were opened after the incubation time respectively with the exception that for the air treatment (A) after 195 days only one MC was harvested. Harvesting took place in a glove box under nitrogen atmosphere by extracting the former sound wood together with sediment and water samples.

In the first stage of the Microcosm experiment sound pine sapwood samples with dimensions of 5 x 10 x 100 mm were water saturated and in two layers (top and bottom) arranged in the sediment of the MC. In each layer 20 samples were located in two circles as shown in figure 2.

Tabel 1. Chemical characteristics and amount of the sediment, wood infected and sound and water included in the microcosms (MCs).

	Sediment [‰]	**$Wood_{infected}$**	**$Wood_{sound}$**		**Water [mg L^{-1}]**
C_{org}	3.5	452	485	DOC	56.5
N_t	0.1	1.3	1.2	N_t	3.6
P_t	0.2	0.053	0.033	PO_4^{3-}	0.5
S_t	0.2	1.8	0.034	SO_4^{2-}	21.6
PH	8.3			pH	8.1
				Conduct.	658 [µS/cm]
Dry mass [g]	6990	5.3	237.7	Volume [L]	1.75

Figure 2. Distribution of the samples of one layer in the Microcosm. (At this stage in the middle of the MC is a plastic stick to keep the space for the attacked wood, added later)

In each MC 2 samples of the inner and 2 of the outer circle in both layers were investigated using light microscopy. Each wood sample was examined at 3 heights (top, middle and bottom) with transverse and tangential sections.
It was checked that three cutting levels are sufficient for precise assignment of bacterial attack degrees by investigating 8 wood samples from one MC per treatment in 10, one cm high layers.
Thin transverse- and tangential sections were cut by hand using a razor blade. Sections were stained with either 1 % w/v safranin in ethanol to highlight the micromorphology of the wood, or 0,1 % w/v aniline blue in 50 % lactic acid to stain fungal hyphae and bacteria. Polarized light was used to demonstrate the remains of crystalline cellulose.

The microscopical studies were executed by one person only. During the microscopic examination the sample identity was unknown to ensure unbiased observations. The bacterial attack degree assignment was conducted following the questions listed in table 2.

It is impossible to judge initial bacterial attack from one section only. Therefore it is necessary to compare all sections from one sample to get a rough picture of the degree of bacterial attack in this sample. In the following figure two examples of different degrees of bacterial attack can be seen. Each example is not assigned to a defined degree class because that depends on the other sections. If all sections show nearly the same patterns, the second degree class can be used. If the example is the only section or only some sections show such decay patterns, the lower first degree class have to be used.

The evaluation of bacterial attack is based on a comparison of the samples. With crosses it should be shown in which Microcosm a higher or lower degree of bacterial attack exists. It is important to mention that the differences between the classes are not always the same. That is why the classes were characterised by crosses and not by numbers which feign a higher accuracy. However, for further bacterial attack intensity data analysis with other MC exp. results numerical values are needed. Therefore we transferred the numbers of crosses in to digits (+ = 1; (+) = 0.5).

Table 2. Steps for evaluating bacterial wood decay degrees

Transverse section	Longitudinal section
• **Is there a positive attack?** • **What is attacked?** – rays only (Figure 1a) – cell parts (Figure 1b) – whole cells (Figure 1c) • **How many cells are attacked?** **(**only an estimation in **none, some, middle, many or all cells)** • **Do we have the same results in all sections?**	• **Is there a positive attack?** • **What is attacked?** – only cell parts (Figure 1d) – whole cells (Figure 1e) • **How many cells are attacked?** **(**only an estimation in **none, some, middle, many or all cells)** • **Do we have the same results in all sections?**

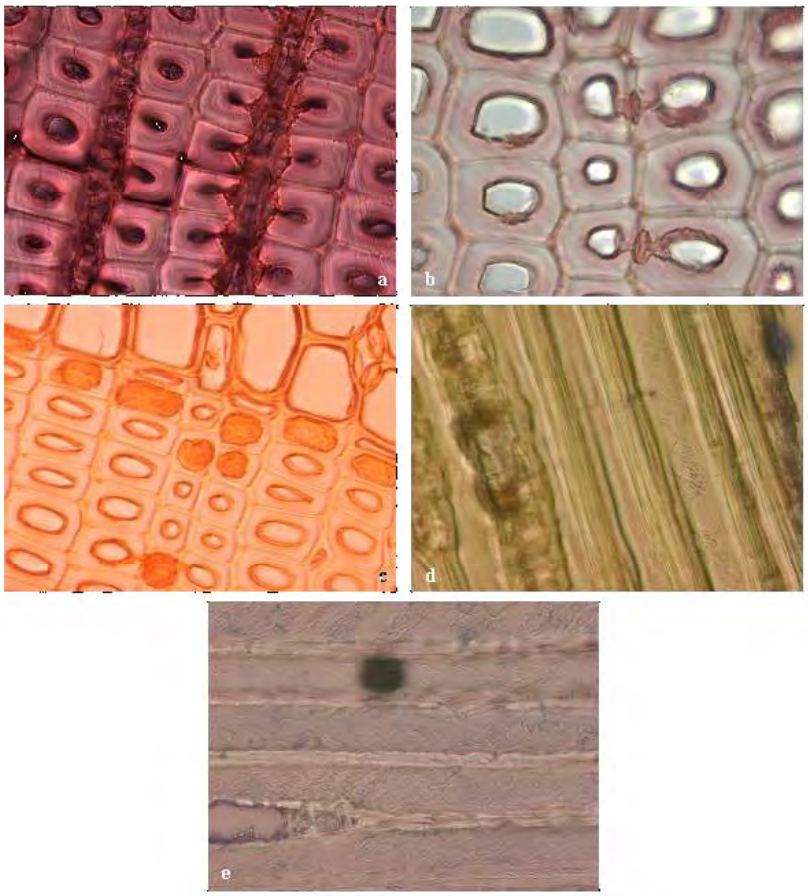


Figure 3. Different stages of bacterial degradation in transverse and tangential sections. **a:** The first sign of bacterial attack is a degradation of the rays. The bacteria move through the rays into the wood and there they start the degradation of the cell walls. **b:** Progress of the initial attack. Now the bacteria are in cells, not only next to the rays, and start cell wall degradation from the lumen. **c:** Advanced level of decay creates a typical pattern of apparently sound tracheids adjacent to heavily degraded cells. **d:** Characteristic local decay pattern of initial decay in longitudinal sections. **e:** The decay pattern in progressed attack shows orientation with the microfibrills.

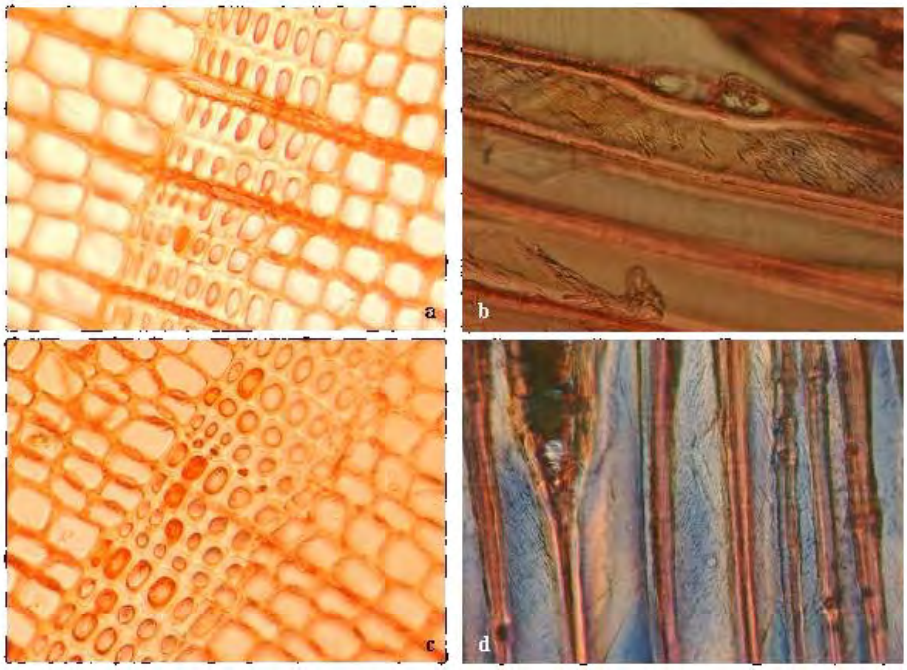


Figure 4. Examples for two different degrees of bacterial attack. **a, b:** Initial attack characterised with "(+)" or "+". **c, d:** Progressed attack, graded with "++" or ++(+).

Sediment and water sampling

The microcosms were filled with sediment and water from a heavily decayed pine pile foundation site in the south of Amsterdam (for basic sediment and water characteristics see Table 1). The ground water saturated sediment was taken as bulk sample in buckets at the height of the head of foundation. The water was ground water extracted with a pump and transported together with the sediment in lidded buckets. Before the start of the experiment the sediment and water samples were stored in buckets at 4° C. The microcosms were packed by hand and shaken when a certain height was reached to ensure even sediment compaction. The wood samples where inserted during filling of the acrylic glass cylinders with sediment. The sediment is overlain by water to maximise gassing efficiency by applying the aeration into the overlying water hence circumventing the gas to liquid diffusion barrier.

Sediment, water and wood analysis

Sediment samples were oven-dried at 40°C, sieved (2 mm) and ball-milled. Total organic C and total nitrogen (N_t) contents of the samples were determined by an automated C and N analyser (Vario EL, Heraeus Elementar, Hanau, Germany). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany) after pressure digestion in 65% concentrated HNO_3. Sediment pH was measured with a digital pH-meter (WTW GmbH, Germany) in water and 1 mol L^{-1} KCl (1:2.5). Sediment texture was determined after sieving and using the pipette method.

The C and N contents in the water samples were determined by an automated C and N analyser (Vario EL, Heraeus Elementar, Hanau, Germany). Dissolved organic carbon (DOC) was measured using a DOC-analyser Total Organic Carbon Analyser TOC – 5050 (Shimadzu Corp., Tokyo, Japan). Total dissolved nitrogen (TDN), NH_4^+-N, and NO_3^--N were determined by a continuous flow system (SANplus Segmented Flow Analyser, Skalar, Erkelenz, Germany) with photometrical detection. Prior to the photometrical detection, TDN was treated with alkaline persulfate digestion and UV digestion to convert both NH_4^+-N and dissolved organic nitrogen to NO_3^--N, afterwards all nitrate was reduced to nitrite by passing a column containing a cadmium-copper granulate. Similarly, NO_3^--N was measured after its reduction to nitrite. DON was computed as total dissolved nitrogen subtracted by nitrogen and ammonium content.

Cl^- was determined by a continuous flow system equipped with an Ag/AgCl ion selective electrode. The pH was measured with a digital pH-meter (WTW GmbH Wesl-Germany) and the conductivity employing a conductivity meter (WTW GmbH, Germany). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany). SO_4 and PO_4 were derived from ICP S and P measurements respectively. Wood samples were oven-dried at 60°C and either ball-milled or consecutively milled by a ultra-centrifugal mill and a ball-mill. C and N contents were determined by an automated elemental analyser (Vario EL, Heraeus Elementar, Hanau, Germany). The elements P, S, Na, K, Ca, Mg, Mn, Fe and Al were analysed by ICP-AES (Spectro Analytical Instruments, Kleve, Germany) after pressure digestion in 65% concentrated HNO_3.

Oxygen measurements

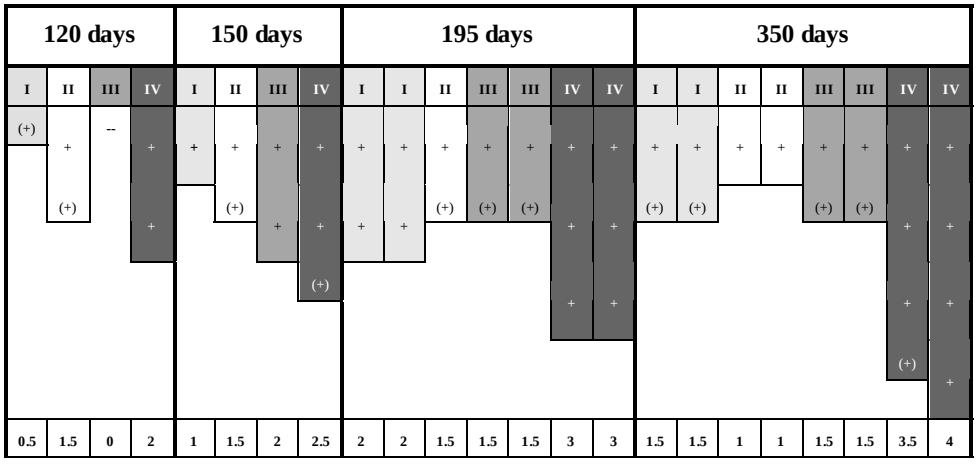
During the experiment the oxygen concentration in selected microcosms was measured with oxygen optodes (PreSense, Regensburg, Germany). The Optode principle is the dynamic quenching of luminescence measuring the luminescence lifetime of a luminophore immobilised in a sensor foil (Klimant et al. 1995). A circle of 3 mm diameter of the sensor foil (PSt3-PSUP-YOP) is attached with silicone to the outer site of a closed plane end of a glass rod. The so prepared glass rod is inserted into the microcosms with silicone rubber tubing as gasket. For measurements a polymer optical fibre connected to the measurement device (Fibox 2-AOT) is hold from the outside of the glass rod against the sensor foil, which is inside the microcosm. Before installation, the optodes were calibrated using water saturated air as 100% and water saturated N_2 as 0%. Long-term stability was checked by re-calibrating a spare optode kept in the same room at similar conditions.

Data analysis

Data were analysed using Microsoft Office Excel 2003 or STATICTICA 6.1. The non-parametric Mann-Whitney U-test was performed for comparison of means of the cumulative CO_2-C production and the calculated wood derived CO_2-C production. Linear regressions were carried out to analyse the relationship between the bacterial decay intensity and the incubation time, the CO_2-C production.

Results

Degradation



I = N_2 **II** =A (Air) **III** = A+ O_2 (Air + Oxygen) **IV** = A+C (Air and water circulation)

Figure 5. Degree of attack (length of the columns or rather number of crosses) per Microcosm treatment after different time periods. The numeral values were used for statistical analysis with other results of the Microcosm experiment

We got in all treatments bacterial attack (see figure 5). In the beginning the attack increased with the time, except at the treatment with air, which had constant values. The increase at the nitrogen and circulated air treatment is relative continual. The bacterial attack in the oxygen added treatment starts relative late, but then with a sudden increase.

The investigations showed no differences in the bacterial attack between samples of the inner and outer circles. Nevertheless sometimes some differences between the upper and the lower layer could be observed. In these cases the degree of attack was always higher in the upper layer than in the lower one.
The best results of bacterial attack we get in the treatment of circulated air. But in this treatment soft rot attack could be observed sometimes too. These fungi attacked only wood from the upper layer and were first detected after 195 days.

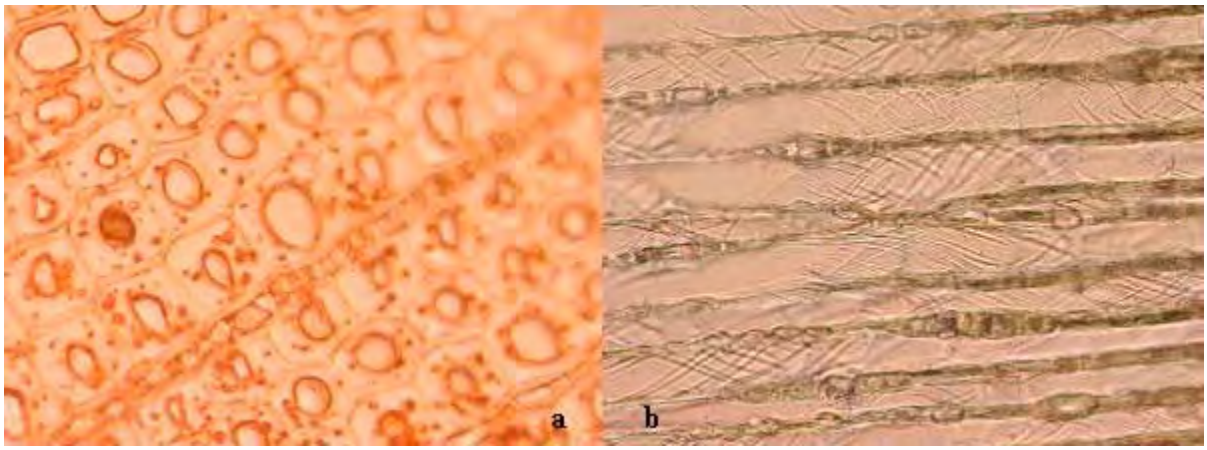


Figure 6, Soft rot attack in MC 15 (circulated air) after 195 days a: transverse section b: longitudinal section

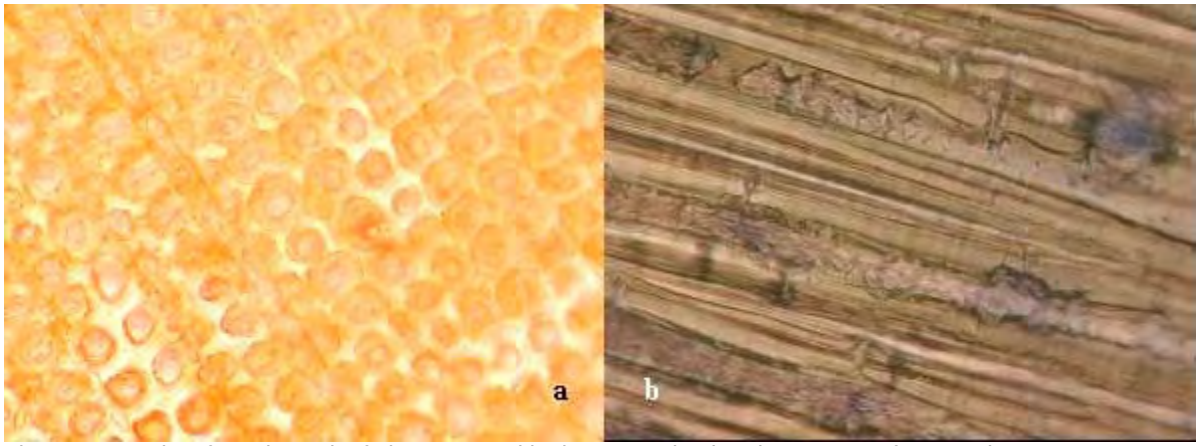


Figure 7. Heaviest fungal attack of pine sapwood in the water circulated treatment after 350 days. **a:** In transverse section nearly all cells are completely degraded **b:** In this stage of attack the characteristic cavities of bacterial degradation could be observed in the longitudinal sections.

That soft rot fungi and erosion bacteria can be seen in one section is shown in the following figure. But this is no evidence that both wood degraders can live at the same conditions. The soft rot could be detected not before the third harvest date (195 days), but the degradation patterns of the erosion bacteria (EB) could be observed even after 120 days. So it could be that the EB degrade the wood at first, than the conditions changed (see oxygen content) and the soft rot was able to degrade the wood.

Oxygen profile in the microcosms (MCs)

In figure 9 the oxygen profile in the MCs resulting from the different gas supply treatments is shown. In the nitrogen treatment, no oxygen was detectable at all depth. For the other treatments a steep gradient at the sediment water interface from oxygen concentrations corresponding to the solubility of oxygen in the overlaying water to zero at 1 to 2 cm depth in the sediment were measured. The air and water circulation treatment resulted in oxygen being measured further down in the sediment. Even at 6 cm depth in the sediment about 1 mg/L oxygen occurred. It was noted that the reproducibility of the oxygen concentration was low for the four MC monitored per treatment.

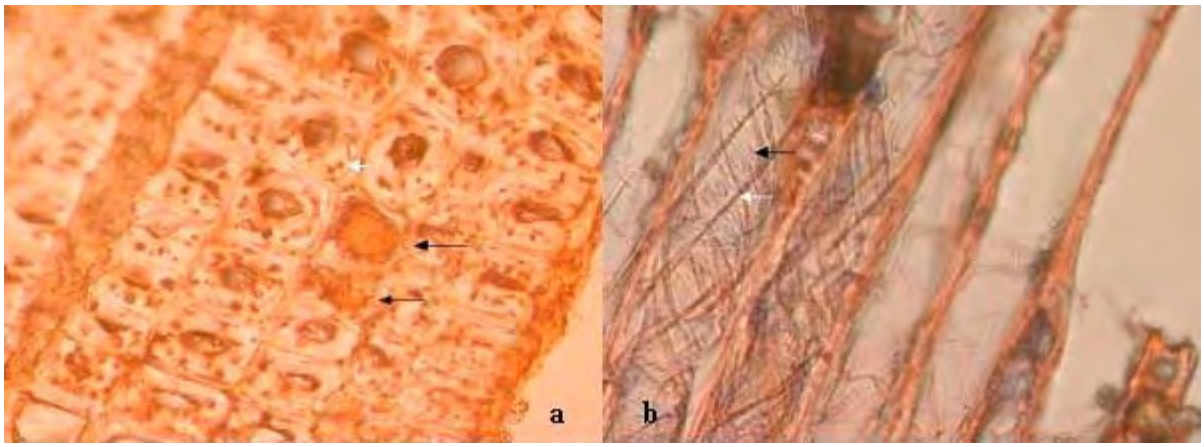


Figure 8. Decay pattern of erosion bacteria (black arrows) and soft rot (white arrows) together in a: transverse section b: longitudinal section

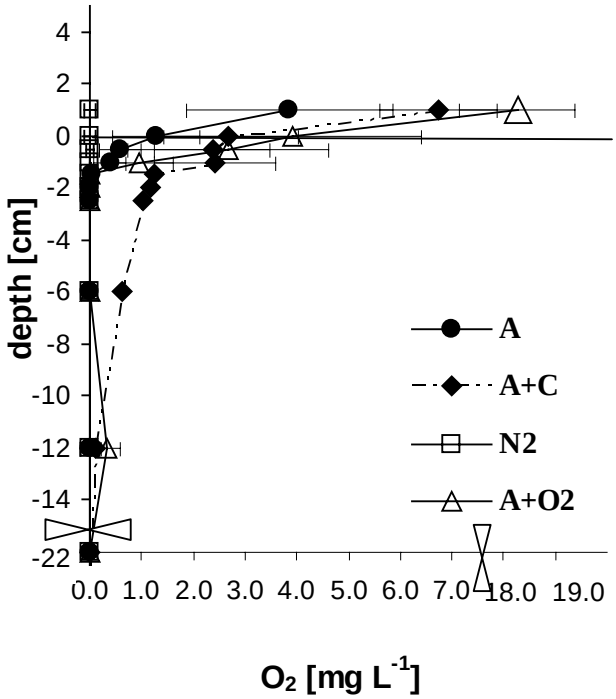


Figure 9. Mean (±SE) vertical gradient of oxygen concentration in the MC, positive values are within the overlaying water, negative values within the sediment core, n=4 for depth +1, 0, -0.5, -1.0, -12 cm and n=1 for depth -1.5, -2.0, -2.5, -6.0, -22.0 cm, A=Air, A+O_2=Air enriched with Oxygen, A+C=Air and water Circulation, N_2=Nitrogen, Note the O_2 value in the overlying water for A+O_2 is 18.32 mg L^{-1} and the last measured depth -22.0 cm.

Figure 10 shows the development of the oxygen concentration in MCs of the A+C (air and circulation) treatment at five different depths during the time course of the experiment. The standard error is not shown for clearness of the picture the mean and (min max) values are 1.34 (0.00 2.45). The highest oxygen concentration was always measured in the sediment overlying water. First nearly saturation concentrations of oxygen at 18°C occur. After the onset of the water circulation the oxygen concentration decreases and increases again after five month. At the start of the experiment only at the sediment water interface oxygen was detectable. After two month in 1 cm depth oxygen was measured as well. After six month, even 12 cm deep in the sediment oxygen was present. This shows how during the experiment oxygen penetrated deeper into the sediment. The same occurred in two of the four monitored MC for the A+O_2 treatment. At two O_2 treatments a marked increase in the CO_2 production coincides with a deeper penetration of O_2 into the MC.

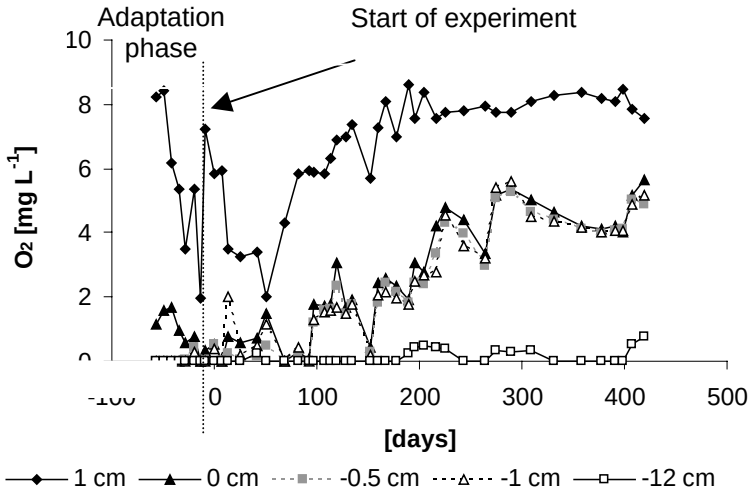


Figure 10. Mean oxygen concentration in the air and circulation (A+C) treatment at different depth during the experiment (n=4, 0=start of the experiment with water circulation and gas measurements), (SE not shown for clearness of the picture, see text).

Gas production

Mean CO_2-C production for all microcosms per treatment as average over all replicates is shown in figure 11. The production differs between the different gas supply treatments. The control (without wood) of all treatments shows, with some exceptions, lower CO_2-C production then the corresponding treatment. The A+C treatment displays the highest CO_2-C production. The difference between the other treatments is not so pronounced. The CO_2-C emission varied considerably between the replicates and with time. No pattern was found for the variation with time therefore no trend analysis was performed.

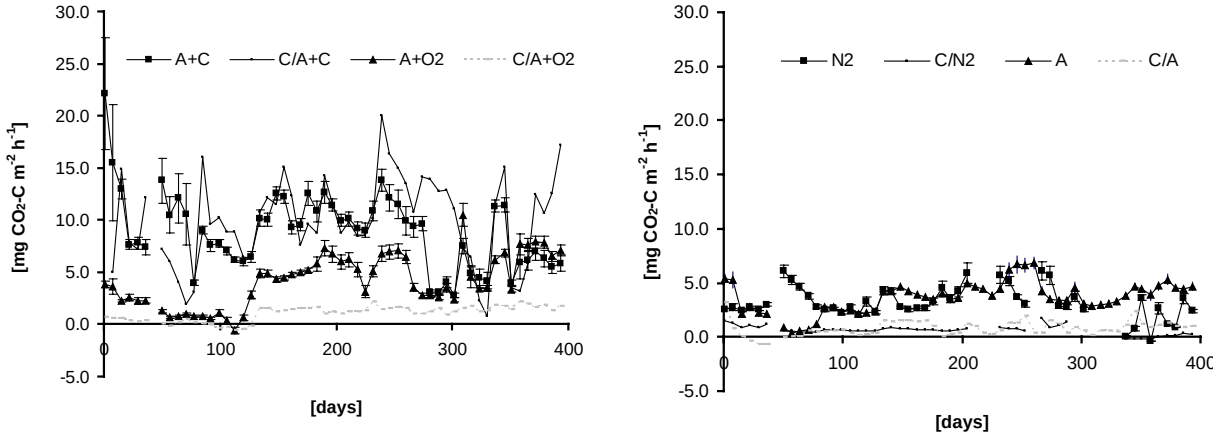


Figure 11. a + b: Mean (±SE) CO_2-C production in the different treatments, (weekly average, treatments up n=12 at 0 d, n=11 at 120 d, n=10 at 150 d, n=8 at 195 d, n=6 at 350 d, Control Air (C/A) n=3, Control air and water circulation (C/A+C), air and oxygen (C/A+O_2) and nitrogen (C/N_2) n=1). Arrows indicate harvesting times and consequently change in n.

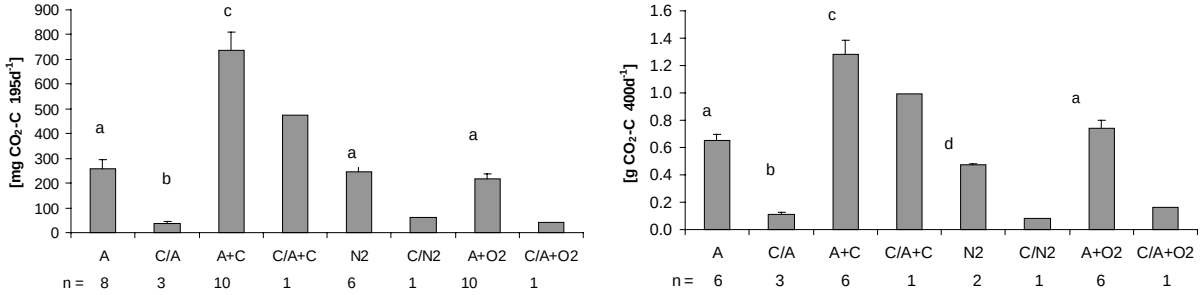


Figure 12. Mean (±SE) cumulative CO_2 emission for different MC treatments, additionally to treatment abbreviations n as number of replicates is given at the diagram bottom. Different letters indicate significant differences between treatments at α<0.05. Figure 5a, after 195 days in mg CO_2-C, Figure 5b: after 400 days in g CO_2-C.

Figure 12 shows the cumulative CO_2-C emission for the different treatments and the corresponding controls after 195 days. There is no significant difference between the Air treatment and the A+O_2 and N_2 treatments. Since the controls of the A + C, N_2 and A+ O_2 treatment had no replicates, it was not possible to verify statistically differences between the treatment and their corresponding control. After 400 days (Figure 5b) the CO_2 emission of all treatments is significantly different. The highest wood effect, i.e. the difference of treatment with wood with the control without wood, on the CO_2 emission occurs at the A and A+O_2 treatment.

Table 3. Mean (±SE) cumulative C emission (CO_2-C + CH_4-C), percent of total C in MC (sediment, wood and water), sediment and wood derived C after 400 days. Different letters indicate significant differences between treatments at α<0.1.

	Total C emission [g C]	Percent of total-C [%]	Percent soil derived C [%]	Percent wood derived C [%]
A	0.44(0.06)	0.28 a	0.06	0.21 ab
A+C	1.27(0.09)	0.79 b	0.62	0.17 b
N2	0.52(0.04)	0.33 a	0.05	0.28 b
A+O2	0.49(0.07)	0.31 a	0.01	0.21 a

A C-budget was calculated using the cumulative CO_2-C and CH_4-C gas production after 400 d (table 3). The controls without wood supplied the sediment derived C emission no statistic was possible, as no replicates were available. The percentage of the C emission at the total C content is shown together with the wood derived C emission. The percentage of the C emission is the highest in the air and circulation treatment but the wood effect is less pronounced compared to the other treatments. The wood derived C emission is the highest in the nitrogen (N_2) followed by the air (A) and air and oxygen ($A+O_2$) treatment. The CH_4-C emissions are only in the N_2 treatment up to 30 percent of the CO_2-C emissions in all other treatments they are 1 to 20 percent.

Discussion

There is bacterial attack in all treatments regardless of whether air, oxygen added air or nitrogen was added. Therefore it could be supposed that the bacterial degradation of wood is independent of oxygen. But all the only gas added treatments had in the soil oxygen contents below the detection limit. Only the air added and circulated water treatment oxygen was detectable in the upper parts of the soil. So it could be that the wood degrading bacteria need low levels of oxygen. Another possibility is that water flow plays a crucial rule for transportation of nutrients or waste.

In this treatment soft rot was found because of the relative high oxygen values in the upper sediment layers. In figure 8 decay patterns of soft rot fungi and erosion bacteria could be seen. However that does not show that both wood degraders live together. Erosion bacteria could be detected after 120 days, whereas soft rot fungi could be detected not before 195 days. So we suppose that the erosion bacteria were the first wood degrader before the soft rot fungi appeared. The oxygen concentration in one cm depth of the sediment rose in the circulation treatment (A+C) after 100 to 120 days to 1 to 2 mg/L oxygen. The oxygen penetration deeper into the sediment enabled the soft rot growth in the circulation treatment (A+C). After 350 days incubation soft rot pattern were detected without any signs of erosion bacteria. Because soft rot fungi are better and faster wood degrader than erosion bacteria it is supposed that the erosion bacteria were out competed by the soft rot fungi or more likely that bacterial erosion patterns were obscured by soft rot decay patterns.

Figure 13 shows the correlation between the mean CO_2-C emission rate and the decay intensity. Only for the A+C treatment a significant correlation of r^2=0.47 existed. No correlation between the mean CO_2-C production rate and decay intensity exist for the other treatments.

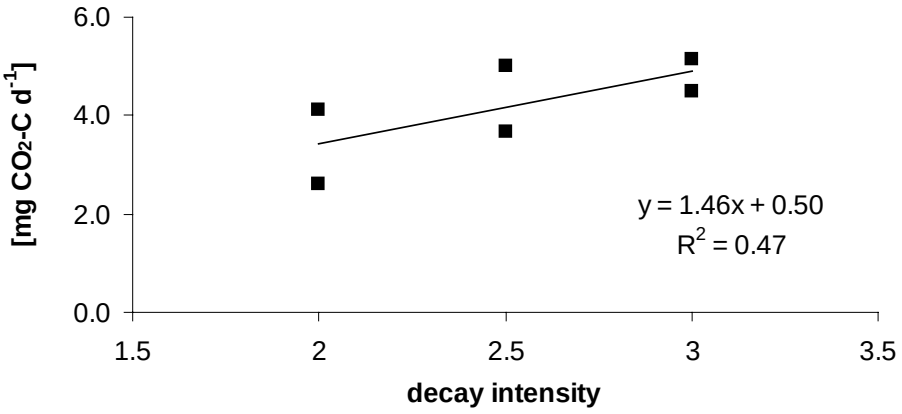


Figure 13. Mean CO_2–C emission of the air and circulation treatment (A+C) versus bacterial decay intensity

The chosen set up of the experiment did not alter considerable the oxygen concentration in the sediment in depth were the wood was located. Only the air and circulation (A+C) treatment transported oxygen deeper into the sediment. This might possibly be the reason why the cumulative CO2-C production and the bacterial wood decay intensity did not vary significantly between the treatments except for the A+C treatment.

The monitoring of the oxygen concentration in the MCs with oxygen optodes succeeded. The installation of the optodes with silicone rubber tubing as gasket caused in probable air oxygen penetration through this pass way. This resulted in the nitrogen (N_2) treatment in CO_2-C production rates significantly higher in MC with optodes as in MCs without. In the other treatments this effect was not significant.
The increase of the bacterial wood decay intensity with increasing CO_2-C production rate in the A+C treatment could possibly be explained with increased oxygen penetration and hence increased CO_2 emission and increase bacterial wood decay. This might suggest that more oxygen increases bacterial wood decay. The other treatments (A, A+O_2 and N_2) showed comparable bacterial wood decay intensities which could be related to the limited availability of oxygen. This might as well suggest that either the N_2 treatment did have sufficient oxygen availability or that bacterial wood decay can proceed without free oxygen present. Further more it might suggest that the CO_2 production is not a suitable indicator for bacterial wood decay.
We suggest that bacterial wood decay can proceed without free oxygen present but that it is more intense if oxygen is present.
At the beginning of the experiment, easy degradable organic matter breakdown utilises oxygen and prevents deeper oxygen penetration into the sediment. After some time the organic matter breakdown slows down upon depletion of easy degradable substrate. Therefore, oxygen diffusion into the sediment results then in oxygen measured in some depth in the sediment.
Within the sediment, gas accumulates and results in an increase in the volume the sediment occupies. The methane escapes from the sediment mostly not constantly but accumulates until released though bubbles. Therefore, the time course of the methane concentration is very heterogeneous and difficult to measure even with four measurements a day. The air and circulation treatment prevented the gas accumulation within the sediment presumable though enhanced gases exchange though preferential water flow channels.

5.1.1 Additional experiment in smaller jars

Material and methods
Glass jars of 500 ml volume with modified twist off lids were used in the second microcosm experiment (figure 14). The microcosms (MC) were connected to an automated GC-ECD and FID (Shimadzu, Tokyo, Japan) described in Loftfield et al. (1997) for continuous CO_2, N_2O and CH_4 measurements and aerated at a constant rate (approx. 20 ml/min) for 155 day (22 weeks) at 20°C in the dark (figure 15).

Figure 14. Glass jar and twist off lid equipped with in and out flow valve and septum closed cylinder for water addition

Figure 15. MC filled with sediment and water, and connected to the aeration and GC system

Tabel 4. Different treatments, their abbreviations (abbriv.), chemicals added and their aimed concentrations in the sediment

Abbriv.	**Treatment**	**Addition**	**Concentration [mg/g]**		
			N	**P**	**S**
S	Sediment (pure)	-	0.1	0,15	0,25
M	Mixture of 50 % sediment and 50 % silica sand	-	Half		
SS	Silica sand	-	< dl.		
S+A	Sediment with ammonium addition	NH_4Cl	1 mg N/g		
S+N	Sediment with nitrate addition	KNO_3	1 mg N/g		
S+P	Sediment with phosphorous addition	K_3PO_4	0.5 mg P/g		
S+Su	Sediment with sulphate addition	K_2SO_4	0.5 mg S/g		

dl. = detection limit (0.1 mg/g)

For the treatments the MC's were filled with sediment, sound and infected wood and water saturated with additional approx. 1 cm overlying water. Before the filling the weight of each component was determined. The aeration was applied into the overlying water to circumvent the air-water-diffusion barrier. Each treatment had a control were no wood samples (sound and infected) were included. For the different treatments chemically modified sediment from Amsterdam was used together with the non modified water from Amsterdam. Table 4 shows the different chemical compositions of the sediment in the treatments. For each treatment 4 replicates were applied.

To lower the sediment nutrient concentration it was "diluted" with silica sand (treatment M for mixture) or pure silica sand (SS) was used. The silica sand was prepared by firstly washing it with strong NaOH and then with HCl before rinsing it several times with distilled water. To study the effect of nutrient addition to the degree of bacterial wood decay nitrate and ammonia were added to the sediment as nitrogen compounds as well as phosphorus. The last treatment had a sulphate addition to the sediment to assess the bacterial wood decay prevention potential of the chemical reactions in the sediment triggered by this addition.

At the end of the incubation time the MC's were opened, the wood samples extracted and the pH in the overlying water measured.

The samples and analyses of sediment and water sampling were done as described for the bigger micro-corms.

Wood samples
Because of the reduced size of the microcosms (MC) smaller samples of sound pine sapwood were used. The wood samples had the dimensions of 10 to 5 to 30 mm. Like in the bigger MC the samples were water saturated and added to the soil. In each MC 10 wood samples were arranged in one circular layer. To insure rapid infection a moderately bacterial decayed piece of pine sapwood (10 to 5 to 30 mm) was placed in the middle of each MC with wood addition.

Per Microcosm 5 wood samples were investigated at 2 heights (top and bottom). At each height four to six single sections were analysed using light microscopy. The methods of cutting and staining are the same as for the bigger MC.

In addition the degree of bacterial decay (-, + or ++) was assigned to every single section and not per se as a mean of all sections for the top or bottom part. All sections of a sample were summarized as an averaged value for the top and bottom part of the sample and for the whole sample. For further data evaluation values were then transferred to numerical, respectively mean values for the whole MC (table 5).

Table 5 Example of the wood sample evaluation from one MC of the second stage of the experiment, sample gives the number of the wood sample from the MC. This evaluation was conducted for the top and bottom part of each sample.

sample	Evaluation of the sections				Numeral average of the sample
1	x	-	-	-	0,25
3	-	-	-	-	0
5	-	x	-	x	0,5
7	x	x	x	x	1
9	xx	x	xx	x	1,5
average					0,64

Results
In contrast to the bigger MC only little or no bacterial attack has been assigned. Following the evaluation scheme treatments with pure sediment (S), silica sand (SS) or their mixture (M) show bacterial wood decay of the (+) degree in most of the samples. Furthermore treatments, in which phosphorus (PO_4) and sulphur (SO_4) was added to the sediment, did not show any sign of attack after 155 days. However in one single MC of the S+P treatment (added PO_4) some slight signs of bacterial attack were observed.

Table 6. Intensity of bacterial decay ("mean" intensity of 5 wood samples encompassing top and bottom values from five sections).

150 days																												
S				SS				M				S+A				S+N				S+P				S+Su				
(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	

The following rank order of bacterial wood decay (transferred to numeric) was found (table 7): Silica sand (SS), followed by pure sediment (S) and mixture (M). However, the transfer of bacterial decay degrees into numerical values might suggest a higher accuracy but further interpretation should be taken with caution.

Nevertheless a high correlation (R^2=0.87) was found between the bacterial wood decay intensity in the top and the bottom part of the wood samples and the bacterial wood decay intensity appears to be slightly higher in the bottom part of the wood sample.

Table 7. Results with the more detailed evaluation of the samples and assigned numerical values.

150 days																												
	S				SS				M				S +A				S + N				S + P				S + Su			
Top	0.6	0.6	0.62	0.75	0.8	0.8	0.55	1.25	0.8	0.48	0.8	0.55	0.6	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0
Botto m	0.8	0.6	0.82	1.05	0.8	0.8	0.5	0.75	0.8	0.8	0.8	0.5	0.6	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0
MC	0.7	0.6	0.72	0.9	0.8	0.8	0.53	1	0.8	0.64	0.8	0.53	0.6	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0
Treat ment	0.73				0.78				0.69				0.15				0				0.1				0			

In the second stage of the Microcosm experiment the evidence of bacterial attack was mostly in single cells (figure 16). Attacked neighbouring sells were scarcely observed.

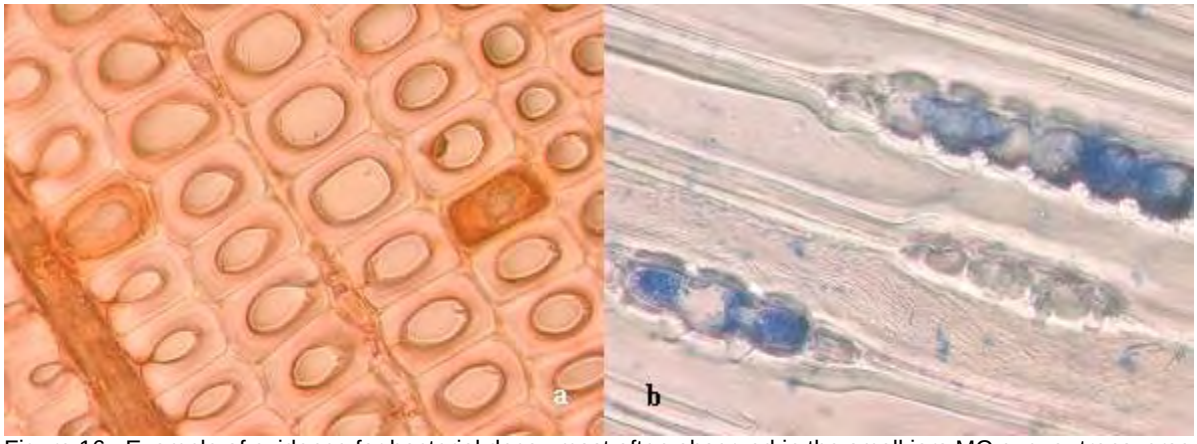


Figure 16. Example of evidence for bacterial decay most often observed in the small jars MC exp. **a:** transverse section **b:** longitudinal section (magnification 400times)

Fungal wood decay

Table 8. Soft rot decay in the four different MC's per treatment given as mean value encompassing the top and bottom part of the sample consisting of four to six different sections. The number of the parallels is given with n and the minimum and maximum values with min and max.

n	S	M	SS	S+A	S+N	S+P	S+Su
1	0.0	0.0	0.0	2.0	0.0	0.0	0.0
2	1.0	3.0	1.0	0.0	0.0	0.0	0.0
3	4.2	3.0	0.0	0.0	0.0	0.0	3.5
4	4.0	3.6	0.0	3.5	3.0	0.0	0.0
Min	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Max	5.0	4.0	1.0	4.0	4.0	0.0	5.0
Mean	**2.3**	**2.4**	**0.25**	**1.4**	**0.75**	**0.00**	**0.88**

During the experiment gas was produced in the sediment of the MC which resulted sometimes in wood samples being pushed out of the sediment. The wood was then exposed to the aeration in the overlaying water which provided sufficient oxygen for fungal wood decay. Therefore there was not only bacterial but also fungal (soft rot) wood decay (table 9). The mean values are only used for statistical analysis and should not be mistaken to indicate a high accuracy.

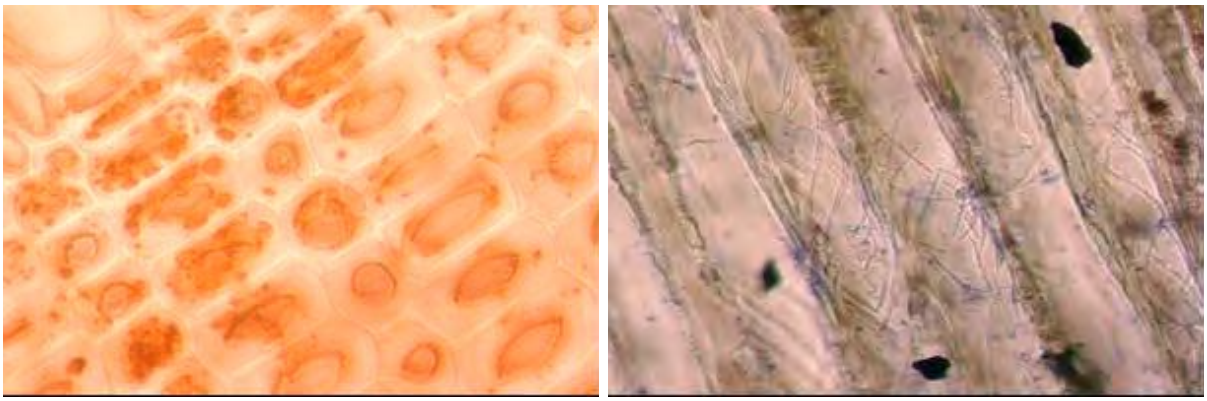


Figure 17. Typical soft rot decay in transverse (left) and longitudinal (right) section (magnification 400times)

Chemical sediment composition

In table 10 the chemical sediment composition resulting from the element additions can be seen. The sediment pH at the beginning was considerably raised by the phosphorus addition and slightly lowered by the ammonia addition. The silica sand (SS) had a very low pH of 5.4 at the beginning. The sediment pH at the end of the experiment for the different treatments was different from the one at the beginning. The silica sand showed the same pH as the pure sediment, the ammonia addition lowered further the pH whereas the phosphorous addition resulted at the end in a less basic pH compared to the beginning.

Table 9. Chemical sediment composition in the different treatments at the beginning of the experiment (total element concentrations) and sediment pH at the start and the end of the experiment, dl. = detection limit (N=0.1 mg/g)

Treatment	C [mg/g DW]	N	P	S	pH start	pH end
S	4.2	0.11	0.16	0.25	8.3	7.9
M	2.2	<dl.	0.11	0.21	8.4	8.0
SS	0.0	<dl.	0.07	0.17	5.4	8.0
S+A	4.6	0.62	0.19	0.38	7.4	6.7
S+N	4.3	0.96	0.18	0.42	8.2	8.9
S+P	3.9	0.12	0.48	0.20	10.1	9.3
S+Su	4.1	0.12	0.14	0.45	8.2	8.5

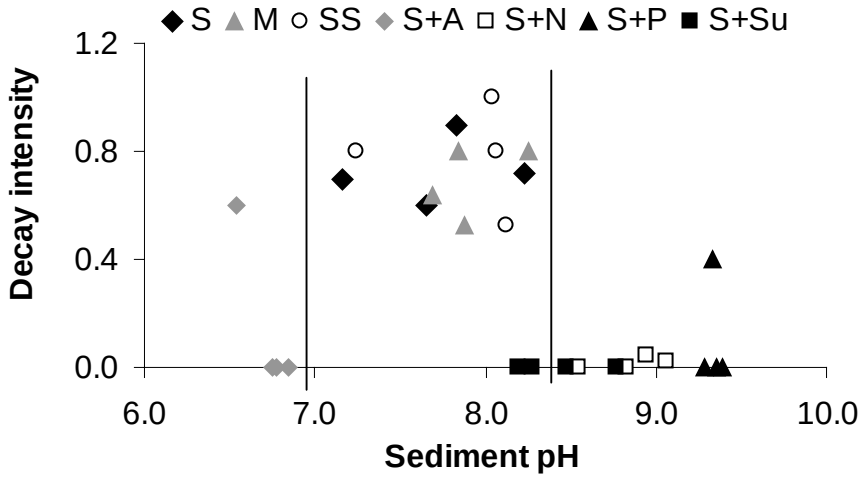


Figure 18. Bacterial wood decay intensity versus sediment pH at the end of the experiment. Vertical lines may indicate region of pH range suitable for bacterial wood decay

The bacterial wood decay intensity appears to be highest at the sediment pH range from 7 to 8.5 in the treatments without chemical addition to the sediment (figure 18). The addition of chemicals to the sediment did affect the sediment pH and it can therefore not be distinguished between the pH effect and the chemical effect of the addition. Furthermore is the evidence not conclusive as, as well at the lowest as at the highest sediment pH some bacterial wood decay was detected in one MC each. Nevertheless these two values seam to be outliers out of four parallels.
Figure 19 shows the bacterial wood decay intensity. Results are arranged according to increasing sediment nitrogen concentrations. It appears that bacterial wood decay intensity decreases with increasing sediment N concentration.

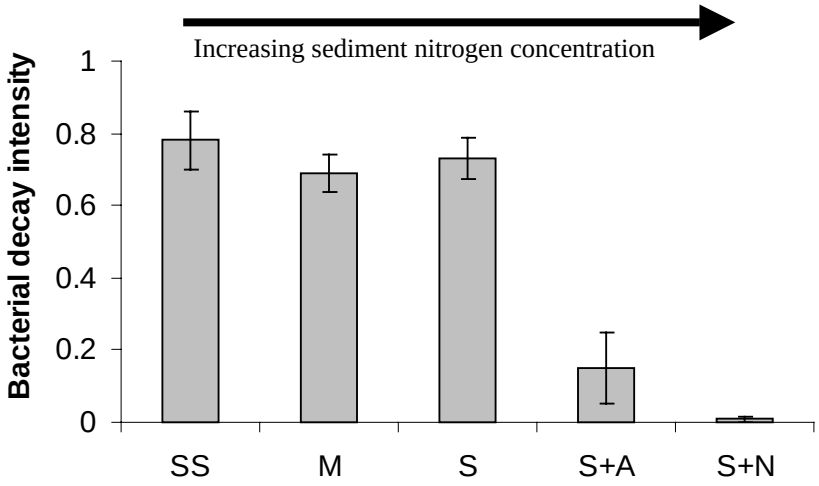


Figure 19. Relation between the mean (±SE, n=4) bacterial wood decay intensity and the sediment N concentration per treatment

CO_2 gas emission
In figure 20 the CO_2-C emissions are shown. The difference between the treatment and their corresponding control is for all treatments statistically different. All treatments did emit CO_2 whereas from the controls all but silica sand (SS) and pure sediment (S), which absorbed it, did the same. Mean values of the treatments vary only slightly between the different treatments and are not statistically different. In the control (no wood) the ammonium addition (S+A) resulted in the highest measured CO_2-C emission, followed by the phosphate addition (S+P) and the mixture treatment (M). For treatments (sediment+wood) phosphate addition (S+P) emitted the highest amount of CO_2-C followed by ammonia addition (S+A) and the mixture treatment (M).

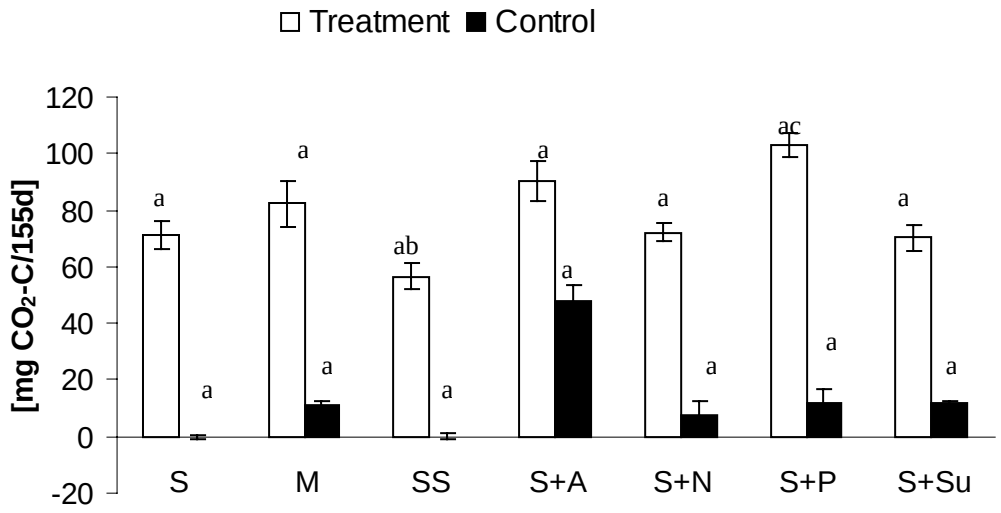


Figure 20. Mean (±SE, n=4) cumulative CO_2-C emissions after 155 days of the different treatments (sediment+wood) and controls (no wood). All treatments differ significantly ($p<0.05$) from their corresponding controls. Different letters indicate statistical difference at $\alpha=0.05$. For the controls no significant differences were found between treatments.

To assess the effect the wood addition has on the CO_2-C production from the treatments the corresponding control was subtracted. The wood derived CO_2-C production can be seen in figure 21. Statistically the values belong to the same population but the lowest value of S+A and the highest of S+P are significant different between each other.

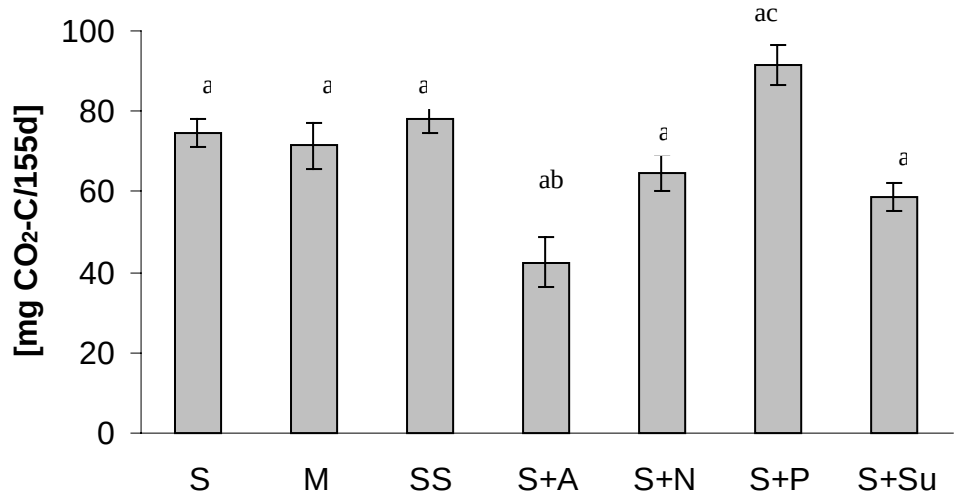


Figure 21. Mean (±SE, n=4) cumulative wood derived CO_2-C emissions after 155 days. Different letters indicate statistical difference at $\alpha=0.05$.

In figure 22 the wood derived cumulative CO_2-C production is plotted against the bacterial wood decay. The values from the S+A and the S+P treatment do not fit the line. Both treatments had the lowest respectively the highest sediment pH at the end of the experiment. If these two values are interpreted as out layers and are ignored a high correlation ($R^2=0.88$) exist. For fungal decay no such correlation was found.

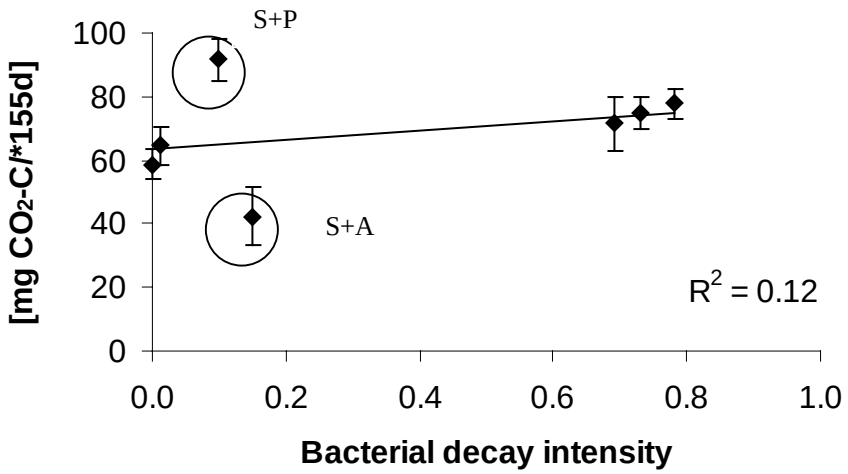


Figure 22. Correlation between the mean (±SE, n=4) cumulative wood derived CO_2-C emissions after 155 days and the median bacterial wood decay intensity.

CH_4 gas emission

The cumulative CH_4-C gas emission from the MC did not show such a distinctive patter as the CO_2-C emission (figure 23). All controls (without wood edition) but the silica sand (SS) one absorbed CH_4 from the aeration and only the treatments with sediment (S), silica sand (SS) and their mixture (M) emitted CH_4 whereas the other treatments absorbed CH_4 as well. The CH_4-C emissions [in ng] are six orders of magnitude smaller than the CO_2-C emissions [in mg].

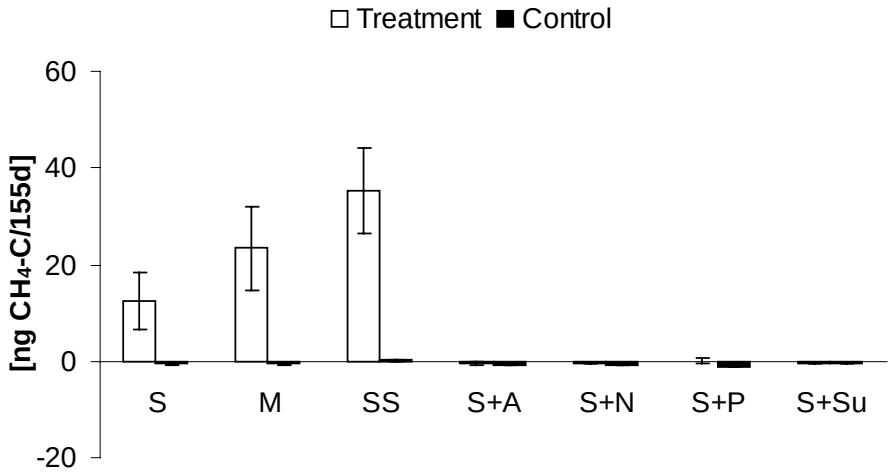


Figure 23. Mean (±SE, n=4) cumulative CH_4-C emissions after 155 days of the different treatments and the corresponding controls (without wood addition). The figure is shown with different scales to take into account the difference of the values.

The treatments with chemical addition to the sediment resulted in higher CH_4-C up take in the corresponding controls then in the treatment itself. The SE of the cumulative CH_4-C up take is high due to the nature of the methane release in bubbles.

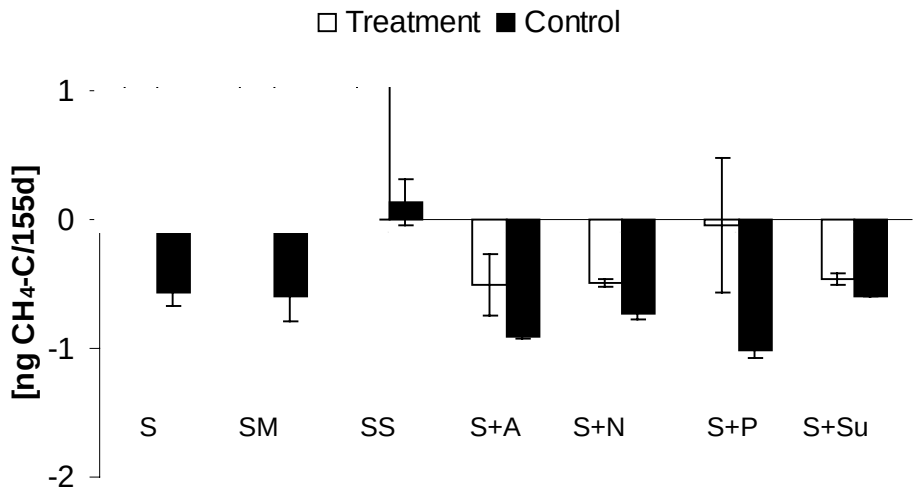


Figure 24. Mean (±SE, n=4) cumulative CH_4-C emissions after 155 days of the different treatments and the corresponding controls (without wood addition). In all treatments the control differs statistically from the corresponding treatment but only at α=0.1.

Conclusions of the experiment with the small jars

A correlation between the wood derived CO_2-C production and the bacterial wood decay was found. Hence the wood derived CO_2-C production might serve as an indicator for bacterial wood decay intensity in laboratory studies. However, the CO2 production does not give any insight into ongoing CO_2 producing processes. In contrast the N_2O and CH_4 production could not be related to bacterial wood decay intensity.

Unintended the sediment pH varied considerably between the treatments with chemical additions. Nevertheless the sediment pH seams to effect bacterial wood decay intensity when it is outside an "optimal" range, i.e. below pH 7 and above pH 8.5. As in general bacterial activity is highly influence by the pH in the growth media it is likely that the bacterial wood decay intensity was affected by pH together with nitrogen and phosphorus concentration variation in the sediment. Conclusions related to the sediment pH are based on values measured at the end of the experiment. It is assumed that end sediment pH was approached shortly after the experiment commenced. However, further research is needed to separately identify pH effects and impacts of nutrient additions on bacterial wood decay.

There are indications that a negative relation between bacterial wood decay intensity and sediment nitrogen concentration exist if presuming that sediment pH obscured but not totally governed bacterial wood decay intensity. This might indicate that in sediments with very high C/N ratio or generally very low carbon and nitrogen concentrations the high C/N ration of wood is still "interesting" for the bacteria as food source. Moreover might the finding that bacterial decayed wood has high N concentrations be related to the attractiveness of wood with high nitrogen concentrations and consequently lower C/N ratio for bacterial wood decay. Following this thought might lead to the hypothesis that under nitrogen or generally nutrient poor conditions wood is more susceptible to bacterial wood decay then under nutrient rich conditions. Concluding, a as hypothesis for further research might serve:

Our recent results may indicate that wood in nitrogen poor and water saturated sediment is more likely to be affected by bacterial wood decay then under conditions where sediments are relatively nitrogen enriched.

The sulphate addition to the sediment resulted in no bacterial decay in the wood samples but this occurred also in the nitrate addition to the sediment. Thus there is only weak evidence that sulphate addition is a possible prevention strategy. To consolidate these findings the sulphate sediment addition was further tested.

5.1.2 Preservation strategies

Material and Methods

The big microcosms (MC) with the same treatments (A=Air, A+C= air and water circulation, N_2=Nitrogen and A+O_2=air and oxygen (50 vol%)) were used. The different addition treatments are listed in table 10. From the remaining big MC's four were equipped with oxygen optodes. In three MC five optodes and in one ten optodes were installed. The incubation time was 4 weeks and two replicates per treatment (in total 8 replicates per addition treatment) were used.

Table 10. Different treatments, their abbreviation (short), the chemicals added and the number of optodes installed in the two MC's per treatment

Short	Treatment	Addition
C	Pure sediment as control	-
G	Sediment with glucose addition	20 mg glucose
G+S	Sediment with addition of glucose and sulphate	20 mg glucose + 20 mg K_2SO_4

Application of chemicals

Glucose and K_2SO_4 were dissolved in 200 ml water from Amsterdam. The solution was inserted into the sediment with a syringe. First a 30 cm long stainless steel cannulae, which was protected from clogging with sediment by an inserted thin metal rod, was pushed 25 cm deep into the MC sediment. Then, the metal rod was removed and the syringe connected to the cannulae. Applying the solution the cannulae was slowly lifted out of the sediment to reach an evenly solution distributed over the MC height. For the control the same procedure with water from Amsterdam was used. In some MC's it was necessary to remove some overlying water first before new solution could be applied.

Kapok samples

In order to gain bacterial wood decay in short time kapok fibre was used as a surrogate for wood. Kapok is the seed enveloping fibre from the *Ceiba pentandra* tree. For indepth information about kapok fibre refer to chapter 3.1. Approximately 5 mg of kapok fibre was filled in bags made of polyester mesh sealed together by heating it. To facilitate recovery a string was attached to the bags. Per MC four kapok bags were inserted. The MC's were opened and with the help of tweezers the kapok bags one after another, were pushed approximately 2 cm deep into the sediment. The kapok bags were positioned between the inner wood samples and the infected wood from the MC at the corners of a square. Kapok bags were recovered after four week by pulling at the string. In some cases the string failed and tweezers were used instead. Although not all four kapok bags could be extracted in some cases.

The bacterial kapok decay was light microscopically detected. From each bag one kapok sample was examined. Bacterial decay degrees ranging from – to +++ were assigned.

Results

Gas production

In the MC sediment with glucose addition methane was produced vigorously. The concentrations were outside the GC measuring range and together with water caused a system failure. Therefore no record of the CO_2 and CH_4 production exists.

The methane could not escape immediately from the sediment and accumulated within. Hence, the sediment volume increased considerable, which reduced the MC headspace. In all MC's with glucose addition, water was removed to prevent it from entering the GC-system. Later on, in some MC's the sediment overlying water filled space in the sediment former occupied by released gas. This resulted in the drying out of the sediment surface, which was noted at the end of the incubation time during kapok bag removal.

Kapok decay

See figure 25 for bacterial kapok decay. The control treatment resulted in six out of eight MC in third degree bacterial kapok decay. In one MC of the A+O_2 treatment only first degree bacterial kapok decay existed. In two MC no bacterial decay was found whereas the kapok in three MC was decayed by fungi. The addition of glucose resulted in bacterial kapok decay of first to second degree in three out of eight MC. The addition of glucose and sulphate caused only in one MC second degree bacterial decay and in one fungal decay whereas six MC did not exhibit any bacterial kapok decay. The fungal decay was caused by soft rot and was found in four MC's from the water circulation (A+C) treatment and also in one from the air treatment (A). The decay degrees for the kapok bags from one MC mostly agreed.

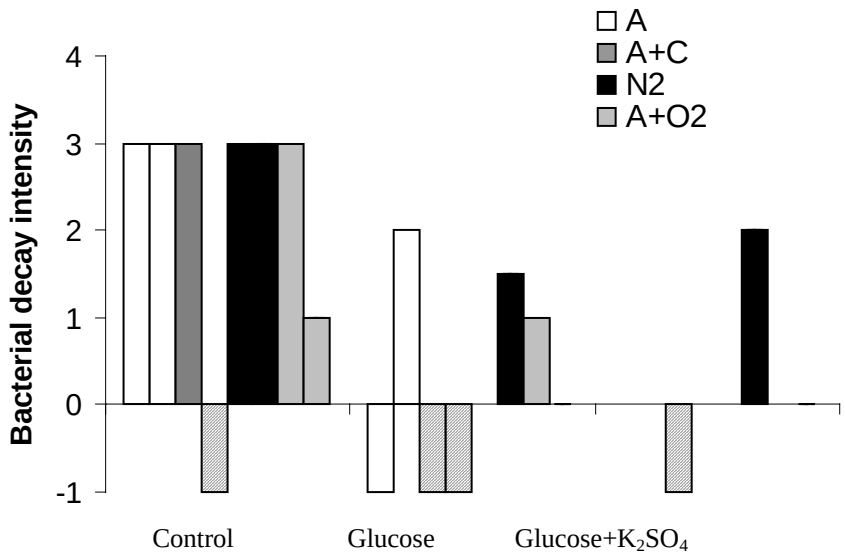


Figure 25. Mean kapok decay per MC, no value is no attack whereas negative values indicate fungal decay.

In figure 26 the total mean kapok decay intensity over the three addition treatments is shown. There is a statistical difference between the control and the addition of glucose and glucose and sulphate. However comparing the two additions no different bacterial kapok decay resulted.

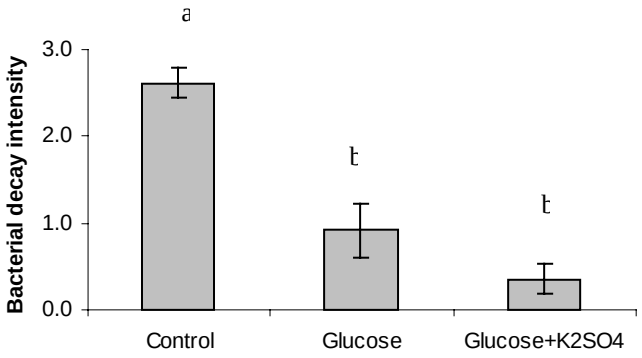


Figure 26. Mean (±SE, n=8 MC's) kapok decay per addition treatment hence 8 MC from 4 different aeration treatments, the fungal decayed samples are not taken into account. Different letters indicate statistical difference at (α<0.05)

Conclusion of the preservation experiment
Sediment Glucose addition reduced bacterial kapok decay. Further sulphate addition totally inhibited bacterial decay with one exception. Within the time frame of the experiment of only four weeks glucose and sulphate addition was able to hinder initial bacterial kapok decay.

5.1.3. Final conclusions
Bacterial wood decay was induced under laboratory conditions and was more intense when oxygenated water was circulated through the wood containing sediment. Even in the nitrogen gassing treatment providing anoxic conditions bacterial wood decay was present. The CO_2 production from the sediment was found to correlate with bacterial wood decay although the underlying CO_2 producing processes are divers and not fully understood.
The nitrogen and phosphate addition to the sediment did not promote bacterial wood decay. On the contrary the bacterial wood decay intensity could be interpreted as being negatively related to the sediment nitrogen concentration. The nutrient addition to the sediment revealed a sediment pH dependency of the bacterial wood decay intensity. In sediment with sulphate addition, bacterial wood decay was not found after 155 days. The addition of glucose to sediment addition repressed bacterial decay intensity. In sediment with glucose and sulphate addition nearly no bacterial decay was found.
Although no strong evidence was presented there are hints that the sulphate addition to sediment might as least for 155 days inhibit bacterial wood decay. When glucose is added to the sulphate containing sediment nearly no bacterial decay was found.

5.2 Enzymatic wood degradation caused by fungi

(by Gelbrich & Militz)

Introduction

The major wood cell wall constituents involved in the decay process are cellulose, hemicellulose and lignin. Decay occurs primarily from the enzymatic activities of a few groups of specialised fungi. White-, brown- and soft-rot are broad categories for different types of decay caused by fungi.

White-rot fungi

White-rot fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin within lignocellulosic substrates. The name white-rot derives from the appearance of wood attacked by these fungi, where lignin removal results in a bleached appearance of the substrate. Most known white-rot fungi are basidiomycetes, although a few ascomycetes' genera within the *Xylariaceae* are also capable of white-rot decay.

Brown-rot fungi

The brown-rot fungi are also capable of degrading cellulose and hemicellulose but are unable to digest the lignin component of wood. In this case, the lignin remains intact and appears as a brown, crumbly matrix. All of these species are members of the Basidiomycota. The brown-rots are most prevalent in conifer woods throughout the northern hemisphere.

Soft-rot fungi

This group contains species that are capable of degrading cellulose and hemicellulose and may partially digest lignin. The soft-rots are particularly prevalent at the early stages of wood decay and in conditions of high moisture and increased nitrogen content. They therefore play an important role in the decomposition of fence posts, building timbers, window frames, and other structural components of homes. Wood affected by soft rot may appear wet, spongy, or pitted. There are over 300 species of known soft-rots. These include many filamentous micro fungi such as Cephalosporium, Acremonium, and Chaetomium.

In the following, the decay characteristics caused by these groups of are summarized. In addition is a prefacing survey of the degradation mechanisms of each decay type given.

Table 11: General survey of decay characteristics caused by the three fungal wood decay types

Decay type	***White-rot***	***Brown-rot***	*Soft-rot*
Decay organism	Basidiomycetes	Basidiomycetes	**Asco-/ Deuteromycetes**
Wood colour	± bleached	± brown	**brown or (bleached)**
Wood texture	fibrous	fibrous texture lost early, cross-checking	**usually on surface, some fibrous texture lost, cross-checking in some cases**
Chemistry of decay	all cell wall components degraded, lignin mineralization by ring cleavage	primarily carbons lost, lignin modification	**carbohydrates preferred, but some lignin modification**
Cellulose degradation	completely by enzymes	by enzymes + non-enzymatic agent	**depending on species like white- or brown-rot**
Hemicellulose degradation	completely by enzymes	by enzymes + oxalic acid	**completely by enzymes**
Lignin degradation	**by H_2O_2 using enzymes**	**demethoxylation of lignin**	**demethoxylation of lignin**

Cellulose degradation

Cellulose is a linear homopolymer of β-1,4- linked glucose units. For stereo chemical reasons the repeating unit in cellulose is the disaccharide cellobiose. Cellulose is difficult to degrade because cellulose molecules often form tightly packed, extensively hydrogen bonded regions called crystalline cellulose (Tarchevsky and Marchenko, 1991; Heiner and Teleman, 1997). The crystalline regions are believed to be separated by less ordered amorphous regions, but these still contain many hydrogen bonds. Cellulose is insoluble in water and oligomers of six or more glucose residues are also insoluble.

Many fungi are able to degrade modified cellulose products but native, high crystalline cellulose (cotton, ramie and wood pulp) can be decomposed only by a limited number of so called *cellulotytic* fungi. These have a complete enzyme system with endo- and exocellulases for extracellular degradation of crystalline cellulose and normally belong to the Ascomycetes, Deuteromycetes or Basidiomycetes (Eriksson and Johnsrud, 1982).

Brown-rot Basidiomycetes can also degrade crystalline cellulose but exocellulases have not been detected (Ljungdahl and Eriksson, 1985).

Cellulose degrading organisms produce a large numbers and types of cellulases which may act synergistically. Currently three major classes of cellulose depolymerising enzymes are accepted: endo cellulases, exocellulases and β-glycosidases. In addition many organisms produce a variety of isoenzymes and cellulases attacking in different, stereospecific ways (Eaton and Hale, 1993).

One type of cellulases alone will not degrade crystalline cellulose beyond 5 % no matter how many enzymes are active (Wilson and Irwin, 1999). From the current available data it can be postulated that synergism occurs in cellulose hydrolysis when two or more cellulase types attack different sites of the cellulose molecules. As a result of such a multi-site attack by different cellulose types new sites are created for each other. For example, endocellulases create reducing and non-reducing ends with each cleavage. So they can synergise exocellulases which specifically attack both end types. A non-reducing end attacking exocellulases possessively cleaves a cellulose chain by disrupting the hydrogen bonds to neighbouring chains. This generates new sites for endocellulase attack and more reducing ends. Thus exocellulases synergise with both endocellulases and reducing end attacking exocellulases (Wilson and Irwin, 1999). In experiments with synergistic mixtures containing endo- and exocellulases it has been shown that hydrolysis by endoglucanases and exocellulases stimulate each other to the same extent. This agrees with a study by Nidetzky et al. (1995) in which pre-treatment of cellulose with an endocellulase increased the degradation rate by an exocellulase.

White-rot fungi:

This group of fungi is rather heterogeneous, but all have the capacity to degrade all of the structural wood components (cellulose, hemicellulose and lignin). They also have in common the extracellular production of enzymes which oxidise lignin related phenolic compounds. This property has been used since long for the identification of white-rot fungi (Bavedamm, 1928, Davidson et al., 1938). The wood polysaccharides are degraded during the so called primary metabolism (growth) whereas lignin is degraded only in the secondary metabolism of the fungus (Keyser et al., 1978; Kirk et al., 1978). *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) is the most used white-rot fungus in studies on enzymes involved in cellulose degradation and their regulation. The enzyme mechanism of this fungus was extensively investigated by Eriksson (1978).

For *Phanerochaete chrysosporium* Eriksson and Pettersson (1975 a, b) identified five endo-1,4-β-glucanases and one exo-1,4-β-glucanases and two 1,4-β-glycosidases could be determined by Deshpande et al. (1978). Multiple cellobiohydrolase-like enzymes were subsequently characterised (Uzcategui et al., 1991).

It is well established that the degradation of cellulose by white rot fungi is carried out by a multicomponent enzyme system in which the individual components interact synergistically. Endo-β-1,4-glucanases attack randomly the β-1,4-linkages of the exposed cellulose microfibrills surface. So theses hydrolytic glycoproteins split the cellulose chains into oligosaccharides and open up non-reducing ends for continued action of exoenzymes like exo-glucanases (Eriksson and Wood, 1985; Ljungdahl and Eriksson, 1985). The non-reducing termini are hydrolysed by exo-1,4- β glucanases producing cellobiose and/ or glucose units. The glucose units are directly absorbed by the hyphae whereas the cellobiose concentrates at the outer hyphal wall (Zabel and Morrell, 1992). For further cellobiose decomposition three possibilities do exist.
Cellobiose may be cleaved by β-1,4-glycosidase, yielding directly glucose. β-glycosidases hydrolyse compounds containing β-D-glycosidic linkages by splitting off terminal β-D-glucose residues (Eriksson et al., 1990).
Cellobiose is also transformed into cellobionolactone by cellobiose oxidase which requires molecular oxygen for this reaction. Cellobionolactone is oxidised by lactonase to cellobionic acid (Westermark and Eriksson, 1974). In the enzyme system, glucose oxidase also exists as a regulator controlling glucose release (Zabel and Morrell, 1992). Similar to cellobiose oxidation gluconic acid is formed with gluconolactone as intermediate product.
Cellobiono acid can also result from the action of cellobiose:quinone oxidoreductase (CBQ). CBQ transforms cellobiose into cellobionolactone which reacts with lactonases. CBQ is important for both cellulose and lignin degradation but this enzyme does not affect phenoxyl radicals produced by lignin peroxidase (LiP) or phenol polymerisation catalysed by LiP in vitro as proposed by Westermark and Eriksson (1974) (Odier et al., 1988).

Cellobiose oxidase and CBQ are extracellular flavocytochromes and belong to the enzyme group of cellobiose dehydrogneases (CDHs). CDHs are oxidoreductases (Henriksson et al., 2000) that means this enzyme oxidises cellobiose to cellobionolactone followed by transfer of electrons to an electron acceptor. If this acceptor is dioxygen, than this CDH is cellobiose oxidase. CDH is called CBQ when quinone is the electron acceptor. CBQ reduces quinones formed by phenoloxidases during lignin degradation. This reduction seems to be an important feature because quinones have been shown to be very toxic to the organism and inhibitory to its enzymes (Buswell et al., 1979). CBQ have been found in several other white-rot fungi as well as in species of Fungi Imperfecti and Ascomycetes (Westermark and Eriksson, 1988).

CDH is able to react with a variety of wood components such as cellulose, hemicellulose and lignin (Mansfield et al., 1997, Vallim et al., 1998, Henriksson et al., 2000) It is believed that the function of CDH is to induce depolymeration of wood components by generating highly reactive hydroxyl radicals *via* a Fenton-like reaction (Mansfield et al., 1997, Henriksson et al., 2000). In this scenario, cellobiose is oxidised with the concomitant reduction of ferric ions. The resulted ferrous ions would then react with hydrogen peroxide to generate the required hydroxyl radicals according to the Fenton's chemistry (Mansfield et al., 1997).

Cellulase regulation in fungi has not yet been fully elucidated. The mechanism of cellulase induction is still unknown. The most generally accepted view of the induction process is that fungi produce a basic level of cellulases. This constitutive amount of enzyme produces soluble hydrolysis products of cellulose which function as inducers (Highley and Dashek, 1998). Many cellulolytic organisms use two different mechanisms to regulate cellulase synthesis: induction by cellobiose and repression at high availability of a carbon-source (Wilson and Irwin, 1999).

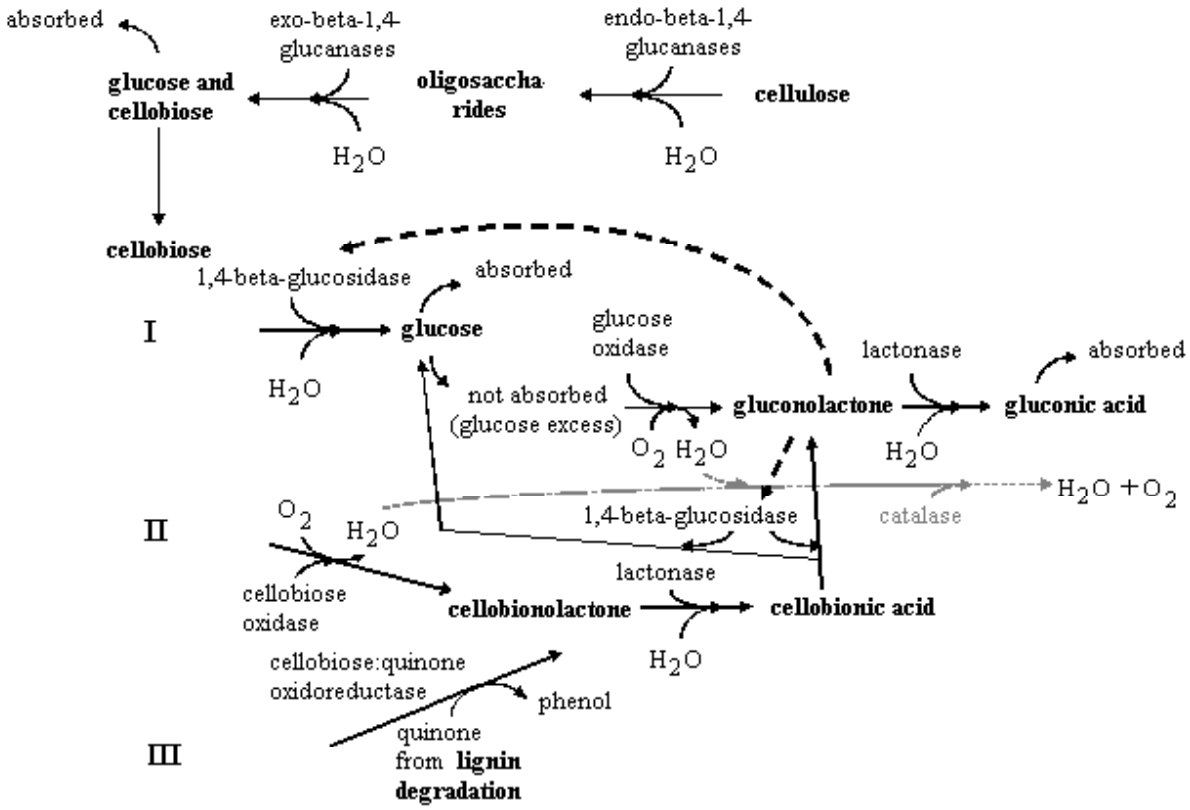


Figure 27: Enzyme mechanisms involved in degradation of cellulose by the white-rot fungus Phanerochaete chrysosporium (Inhibition).

In *Phanerochaete chrysosporium* cellobiose as one product of cellulase action both induces and inhibits cellulases (Eriksson and Hamp, 1978). Cellobiose is an inhibitor of exoglucanases but normally it is removed by β-glycosidase forming glucose (Stephen et al., 1989). In this fungus a series of reactions appears to regulate the enzymatic cellulose decomposition and so controls the rate of glucose release to the capacity of the fungus to utilize it. An excess of glucose inhibits the release of the 1,4-β-endoglucanases and possibly the exoglucanases by catabolitic repression. Excess of glucose induces also the formation of glucose oxidase (Zabel and Morrell, 1992). As by-product hydrogen peroxide is formed. The cellobionic acid formed in oxidation of cellobiose by the two oxidative enzymes can be hydrolysed by β-glycosidase but the product of this reaction, gluconolactone strongly inhibits β-glycosidase (Deshpande et al., 1978). Lactonase may decrease the inhibitory effects of both glucono- and cellobionolactone on β-glycosidase and is therefore a component of synergistic attack of this system (Highley and Dashek, 1998). Lactonase transforms gluconolactone in gluconic acid which can be absorbed by the hyphae and utilized. When β-glycosidase is inhibited cellobiose can not be degraded into glucose and thus cellobiose accumulates. Then transglycosylation and the formation of storage products (e.g. dextrin) may occur by endoglucanases. An excess of simply sugars may also inhibit the release of hemicellulases (Zabel and Morrell, 1992).

Brown-rot fungi

In decayed wood brown-rot fungi leave a brown residue (hence the name) of partially demethylated lignin. These fungi are unique among cellulose degrading organisms because they are the only known microbes degrading wood cellulose without prior removing lignin (Highley and Dashek, 1998). It is a fact that brown-rot fungi depolymerise cellulose rapidly during early stage of wood decay but the mechanism of degrading crystalline cellulose is unknown.

Isolated enzymes of brown-rot fungi show nearly no activity to degrade crystalline cellulose. They only attack the present cellulose derivates (Highley, 1988, Enoki et al., 1988). This group of fungi apparently lacks a synergistic endo-exo-glucanase cooperation system.

They can only produce and utilise endoglucanases (Highley, 1973, 1975, Ritschkoff et al., 1992 a). Because no other enzyme systems are known with the capacity to degrade crystalline cellulose other explanations are needed to clarify the mechanism of cellulose degradation by brown-rot fungi.
The production of a non-enzymatic depolymerising agent has been proposed because the pore sizes in cell walls of brown-rotted wood are too small for enzymes to pass through (Cowling and Brown, 1969, Srebotnik and Messner, 1991, Flournoy et a., 1991). However, it could be suggested that lignin repolymerizes after the physical pathways have been opened for enzyme penetration (Eaton and Hale, 1993). It was demonstrated by Cobb (1982) that in brown-rot fungi a non-enzymatic cellulose mechanism has to exist. He used an ultrafiltration membrane which did not allow passage of enzymes. This membrane was placed between the fungal culture and the cellulose substrate but in spite of this enzyme barrier cellulose degradation occurred.

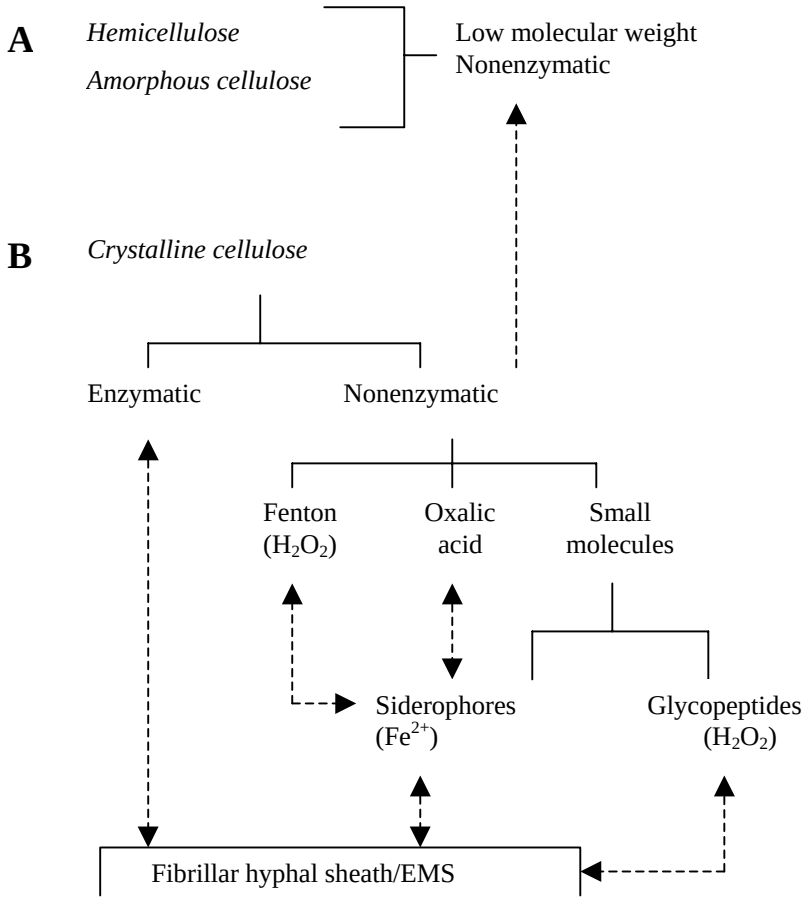


Figure 28: Proposed mechanisms of brown-rot decay. EMS = extracellular membranous structures. (Green and Highley, 1997).

Halliwell proposed at first the possible existence of a non-enzymatic cellulolytic system involving peroxide and iron. In 1965 he described the degradation of cotton cellulose by Fenton's reagent, an H_2O_2/ Fe^{2+} system which generates hydroxyl radicals or similar oxidants (Highley and Dashek, 1998). Koenigs (1972, 1974 a, b, 1975) demonstrated that brown-rot fungi are more powerful H_2O_2 producer than white-rot fungi. Since wood contains sufficient amounts of iron the cellulose in wood can be depolymerised by Fenton's reagent. But in more than twenty years research on the mechanism of by brown-rot fungi a definite and satisfying result could not be found (Highley and Flournoy, 1994). There are conflicting reports even by the same scientist. So, Highley (1980) observed that in *Poria placenta* the cellulose degradation decreased as a result of addition of ·OH and H_2O_2 quenching agents.

Is this the evidence that H_2O_2 might be involved in cellulose degradation? In later work (1982) Highley found out, that only one of six studied brown-rot fungi produced significant amounts of extracellular H_2O_2 (Eriksson et al., 1990). By the use of sensitive methods extracellular H_2O_2 could be detected in different brown-rot fungi e.g. *Coniophora puteana, Seprula lacrymans* and *Tyromyces placenta* (Ritschkoff et al., 1990, 1992 b, Ritschkoff und Viikari, 1991, Backa et al., 1992, Tanaka et al., 1992).

Different prepared cellulose depolymerisation products were compared with brown-rotted cellulose. Beside by Fenton's reagent oxidised cellulose acid hydrolysed (HCl) and HIO_4/Br_2-oxidised cellulose were chemically characterised in the same way as the cellulose depolymerisation products after brown-rot decay. Only the Fenton's system imitated the brown-rot fungi system in nearly all measured characteristics (Kirk et al., 1989, 1991). But there was no correlation between the oxidation of cellulose and its depolymerisation. So it is still unknown whether oxidation is a result of post-depolymerisation modification, whether oxidation of cellulose precedes depolymerisation or if oxidation and depolymerisation are coupled (Flournoy, 1994).
In further studies on the decay mechanism of brown–rot fungi a model is proposed involving Fe^{2+}, H_2O_2, oxalic acid and the production of hydroxyl radicals (Schmidt et al., 1981, Tanaka et al, 1991). Oxalic acid is said to play a role in reduction of Fe^{3+} to Fe^{2+} and thus increasing the cellulose decomposition by the Fenton system. However, it was shown that higher concentrations of oxalic acid inhibit cellulose degradation by the Fenton reaction. Green et al. (1992) showed that brown-rot fungi do accumulate oxalic acid in wood.
The role of H_2O_2 is thought to be the generation of hydroxyl radicals in reaction with metal or metal chelates (Green and Highley, 1997). Hydroxyl radicals were detected by various methods in liquid media, agar media or in wood (Highley, 1982, Vaness and Evans, 1989, Illman et al., 1989, Backa et al., 1992). As hydroxyl radicals are very reactive within a very short life time they have to be formed at the sites of action. A low molecular weight phenolate chelator is produced by *Gleophyllum trabeum* and can be detected within decayed and undecayed cell wall area (Jellison et al., 1991). Lu et al. (1994) proposed that this chelator produces radical species within the wood cell wall when iron and H_2O_2 are present.
The ability of brown-rot fungi to oxidise 2-keto-4-thiomethylbutryric acid (KTBA) to ethylene is reported by Enoki et al., (1989). This conversion occurs by one-electron oxidants and is correlated with weight loss but not with cellulose depolymerisation. An extracellular protein was isolated from cultures of *G. trabeum* (Enoki et al., 1990. It is an iron-containing glycoprotein, which requires H_2O_2 and has the ability of KTBA oxidation. The authors suggested the existence of "a unique wood-component degrading system that participates directly or indirectly in the fragmentation of cellulose as well as of lignin in wood and oxidises KTBA to give ethylene". Similar glycoproteins were isolated from white-rot and soft-rot fungi (Enoki et al., 1991).
In other studies brown-rot fungi decomposed cellulose using a system of Mn (II) and hydroxyl radicals (Illman et al., 1988 a, b, 1989, Messner and Srebotnik, 1989). Here the cellulose should be depolymerised in small fragments only by the radical system. The wood susceptibility to brown-rot decay was correlated with increasing Mn^{2+}signals shown by ESR over a period of 4 week. In brown-rot resistant wood species no or little Mn^{2+} increase could be observed.
However, after separating of cellulose chains in the crystalline zone by a non-enzymatic mechanism endo-1,4-β-glucanases cleave randomly the cellulose molecule and 1,4-β-glucosidases convert the cellobiose to glucose. The cellulases of brown-rot fungi are not very substrate specific, e.g. purified β-glucosidase from *Lenzites trabea* also hydrolyses β-xylosides, purified endoglucanase from *Polyporus schweinitzii* also shows both mannanase and xylanase activity, but endoglucanase from *Poria placenta* appears to be even more non-specific.

It is able to hydrolyse polysaccharides like xylan, mannan and carboxymethyl-cellulose and several glycosides (Eriksson et al., 1990). Because these enzymes are multifunctional in cellulose degradation of brown-rot fungi no inhibiting mechanism compared with white-rot fungi exists. That is why glucose and cellodextrins excesses are present in brown-rotted wood (Zabel and Morrell, 1992).
From the above work it can be concluded that a non-enzymatic mechanism has to be involved in depolymerisation of crystalline cellulose by brown-rot fungi. It is obvious that much more work is needed until an exact cellulose degradation mechanism can be described.

Soft-rot fungi
The microscopic characteristics of soft-rot decay are chains of biconical and cylindrical cavities in the secondary wall. Most of the soft-rot fungi additionally produce erosion from the lumen towards the middle lamella (Eriksson et al., 1990).
This group contains species that are capable of degrading cellulose and hemicellulose and may partially digest lignin. The soft-rots are particularly prevalent at the early stages of wood decay and in conditions of high moisture and increased nitrogen content.
These fungi resemble to both the white-rot fungi in having similar hydrolytic enzyme systems and to the brown-rotters with limited on lignin. Because of these differences, soft-rot fungi now are defined roughly as non-basidiomycete decayers. They are a cosmopolitan group of fungi with rather diverse mechanisms for degrading cellulose (Zabel and Morrell, 1992).
For degradation of highly ordered cellulose soft-rots like *Myrothecium verrucaria* and *Phialophora malorum* produce a complete setup of enzymes. They possess endoglucanases, exoglucanases (cellobiohydrolases) and 1,4-β-glucosidases. However, not all soft-rot fungi produce a complete enzyme complex. In same fungi e.g. *Chaetomium globosum* no exoglucanases could be detected. That is why they are capable of degrading only the more disordered, amorphous celluloses (Eriksson et al., 1990).
Many studies of soft-rot enzyme systems are based on *Trichoderma spp.* The most of decay of *Trichoderma spp.* varied in different studies in birch. *Trichoderma* showed only erosion decay, cavity formation or none of both. Thus, the interpretation of this species as a soft-rot fungus has to be regarded with caution (Eaton and Hale, 1993).
Trichoderma reesei is one of the most important cellulose degrading organisms (Eriksson and Wood, 1985) with a complete enzyme complex. The fungus excretes large amounts of cellulases. It is well established that this fungus produces two cellobiohydrolases (CBH I and CBH II) (Eaton and Hale, 1993) but the number of endoglucanases produced is less clear. In addition to hydrolytic enzymes, oxidative breakdown of cellulose by *T. reesei* has been reported by Vaheri (1982). The isolated enzyme requires molecular oxygen acting on crystalline cellulose and produces cellobionic and gluconic acid.
The reasons for such differences in the enzymatic system within one group of fungi producing the same microscopic characteristics are not clear. It is obvious that much more work is needed to understand the mechanism (probably the mechanisms) of cellulose degradation by these fungi.

Hemicellulose degradation
The structure of hemicellulose is more complex than that of cellulose. Hemicelluloses are a group of homo- and heteropolymers with numbers of substituents. The most frequent hemicellulose types in wood are 1,3- and 1,4-D-galactans, 1,4-β-mannans and 1,4- β-xylans. There is a difference between hemicelluloses of hardwood and softwood. In hardwood the major hemicellulose is xylan (O-acetyl-(4-O-methylglucurono)-xylan) whereas in softwood hemicelluloses mainly consist of mannan (galactoglucomannan) (Schmidt, 1994).
The specific degrading enzyme system has to be as complex as the hemicellulose structure. Therefore at first a universal characterisation of xylan and mannan degrading enzymes is given which is valid for all of the three decay types. Afterwards some typical features in hemicellulose degradation by white- and brown-rot fungi are described.

The xylanolytic system is a complex of several hydrolytic enzymes with diverse specificities and modes of action (Berg et al., 2001). To this system belong the main chain-cleaving enzymes xylanases and β-xylosidases. Side-chain cleavage is performed by acetylxylan esterases, α-glucoronidases, galactosidases, ferulic acid esterases and α-arabinofuranosidases (Belancic et al., 1995). All these enzymes act synergistically to convert xylan into its constituents sugars (Silva et al., 2000).
Similar to cellulases both exo- and endo- β-xylanases exist but the endo-type is more common (Eaton and Hale, 1993). Other authors describe only endo-xylanases Levin, and Forchiassin, 1998, Coughlan et al., 1993, Beg et al., 2001). However, exo-xylanases would only be expected to attack on heteroxylans to a limited degree, because their action would be terminated at each branch point encountered (Dekker, 1989). Endo-xylanase splits off oligomers from the xylan backbone. β-xylosidase hydrolyses these oligomers to xylose. α-arabinosidase removes α-arabinose side chains and glucuronidase separates 4-O-methylglucuron side chains from the backbone releasing glucuronic acid units (Zabel and Morrell, 1992). Acetyl esterase and ferulic acid esterase attack the non-glycosidic appendages of the heteroxylan. They are specific for hydrolysis of O-acetyl and feruloyl groups, respectively (Dekker, 1989).

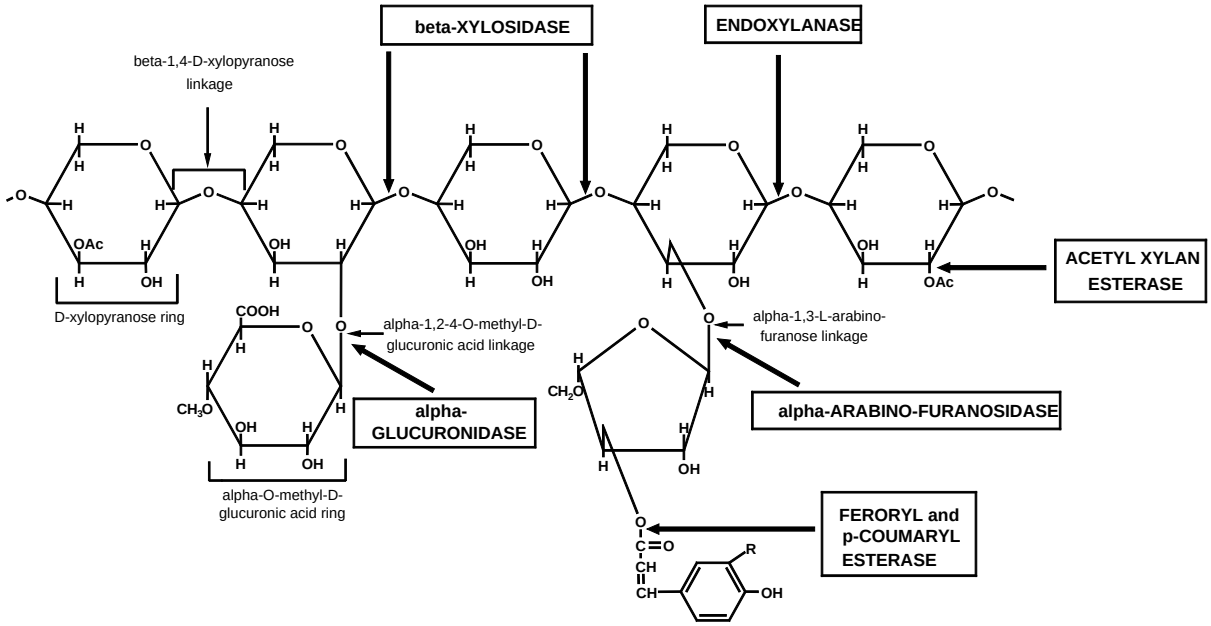


Figure 29: A hypothetical plant xylan structure showing different substituent groups with sites of stack by microbial xylanases (Beg et al., 2001).

During xylan hydrolysis, synergism has been observed between backbone and side chain cleaving enzymes. The side arms ultimately impede hydrolysis through steric hindrance and their removal is necessary if degradation of xylo-oligomers is to proceed to completion (Cairney and Burke, 1998). For example: efficient degradation of acetylated xylan exists because of the synergism between acetyl xylan esterase and endo-xylanases. The release of acetic acid by esterase increases the accessibility of xylan backbone for endo-xylanase whereas it creates shorter acetylated polymers which are preferred substrates for esterase activity (Biely et al, 1986, Coughlan et al., 1993). Another possibility of synergism exists between endo-xylanases and β-xylosidase. β-xylosidase stimulates endo-xylanases activity by relieving the end-product inhibition of endo-xylanases (Bachmann and Mc Charty, 1991). Cellulolytic fungi frequently produce high levels of both xylanases and cellulases when cultured with substrates containing both cellulose and xylan or with cellulose alone but they may produce only xylan degrading enzymes when cultured on xylan (Coughlan and Hazelwood, 1993).

The mannan degradation is also a combined action of several enzymes because mannans are heteropolymers. They are degraded in a similar way like xylans. Endo-mannanases release shorter substituted and non-substituted oligomers, mannobiose and mannose and β-mannosidase converts these oligomers and dimers from the non-reducing end to mannose (Puls and Schuseil, 1993). The glucose and galactose substituents are released by α- and β-glycosidases acting in synergism with endo-mannanases and mannosidases (Eriksson et al., 1990).

White-rot fungi

The degradation of hemicelluloses by white-rot fungi occurs in a similar manner as described above. Similar to cellulases, simple sugars repress the production of most hemicellulose degrading enzymes by white-rot fungi (Highley and Dashek, 1998). The regulation of the enzymes involved in xylan degradation appears to be controlled by induction of suitable carbon sources. This was demonstrated for the white-rot fungus *Trametes trogii* by Levin and Forchiassin (1998).

The most effective substrate for endoxylanase and β-xylosidase production was found to be xylan plus crystalline cellulose followed by xylan used as the only carbon source. However, the highest growth rate of *T. trogii* was obtained with glucose, starch and cellobiose as carbon source. An increase in xylan concentration promoted growth as well as xylanase production. Endoglucanases activity increases with xylan concentration whereas the β-xylosidase revealed a maximal production at 5 g xylan/ l (Levin and Forchiassin, 1998).

Brown-rot fungi

These fungi produce a number of hemicellulases and the mechanism of hemicellulose breakdown appears similar to that of the white-rot fungi. The hemicellulose degrading enzymes that have been isolated from brown-rot fungi until now are not substrate specific. Brown- and white-rot fungi differ in their regulation of hemicellulose degradation. Compared to white-rot fungi many brown-rot fungi show high cellulase and hemicellulase activities growing on simple sugars (Highley and Dashek, 1998).

Oxalic acid may also be involved in the solubilisation and hydrolysis of hemicellulose. Due to acidic conditions, depolymerisation of hemicellulose and amorphous cellulose occurs and wood porosity increases so that cellulose is accessible for enzymatic depolymerisation (Green et al., 1992).

Lignin degradation

Lignin serves several functions in the extracellular matrix of plants: it lends the cell wall mechanical support, it serves as a barrier against microbial attack and it decreases the hygroscopicity of wood (Schmidt, 1994). Lignin has a random, high irregular three dimensional structure with no typical repeating unit exists (Schoemaker et al, 1991 Betts et al. 1991).

This biopolymer is composed of three different oxygenated phenylpropane units: p-coumaryl alcohol (p-C), coniferyl alcohol (C) and syringyl alcohol (S) (Dean and Eriksson, 1992, Boudet, 2000). There are several various C-C and C-O bonds within the polyphenolic macromolecule which are very stable and non-hydrolysable (Evans, 1991, Betts et al, 1991 Piontek et al., 2001). Different degrees of methoxy substitution on the aromatic rings determine the type of lignin as guaiacyl (softwood) and syringyl (hardwood) (Evans, 1991, Boudet, 2000).

The lignin polymer is stereo irregular and the structure is comprised of inter unit carbon-carbon and ether bonds. That is why the lignolytic system consists of extracellular which have lower substrate specificity than typical enzymes and the degradative mechanism has to be oxidative rather than hydrolytic (Kirk and Cullen, 1998).

White-rot fungi:

Lignin is mineralised in an obligatory aerobic oxidative process yielding no net energy gain, and so lignin is not a substrate in primary metabolism. Rather, it is degraded during secondary metabolism in order to access wood polysaccharides locked in lignin-carbohydrate complexes, so providing an energy source to which other organisms do not have access.

White-rot fungi are the only known organisms, which are capable to completely mineralise lignin to carbon dioxide and water. Lignin biodegradation has been recognised as an extracellular, mainly oxidative process, characterised by aromatic ring cleavage, aromatic hydroxylation and formation of aromatic carboxylic acid, cleavage of aryl propyl side-chains, demethoxylation and other types of ether bond cleavage (Schoemaker et al., 1989).

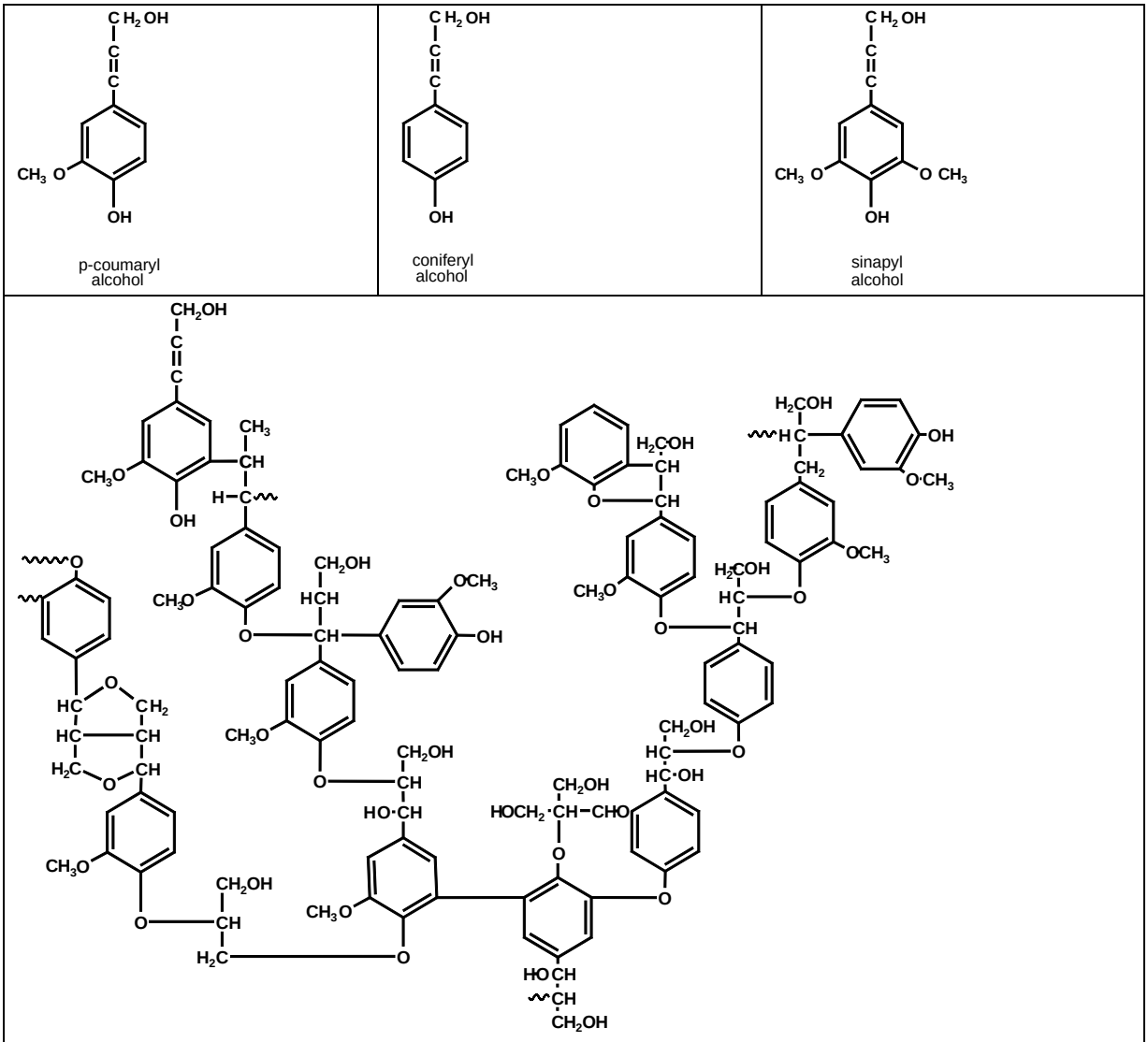


Figure 30: Phenylpropanoid precursors of lignin (left) and a model of a softwood lignin (right) (Betts et al., 1991).

In lignin degradation by white-rot fungi three groups of enzymes are involved. The first group consists of phenoloxidases (lignin peroxidases (LiP), manganese-dependent peroxidase (MnP), laccase, horseradish peroxidase (HLP)) and dioxygenases (protocatechuate-3,4-dioxygenase, 1,2,4-trihydroxybenzene-1,2-dioxygenase, catechol-1,2-dioxygenase). The second group of enzymes only cooperates with the first enzyme group. These enzymes are superoxid dismutases (SODs) and glyoxal oxidase (GLOX) and they never attack wood on their own.

The third group consists of the so called feed back enzymes (glucose-1-oxidase, aryl alcohol oxidases, pyranose-2-oxidase, cellobiose:quinone oxidoreductase and cellobiose dehydrogenase). These enzymes play a key role in combining different metabolic reactions. All of these enzymes may act separately or in cooperation with each other (Leonowicz et al., 1999).

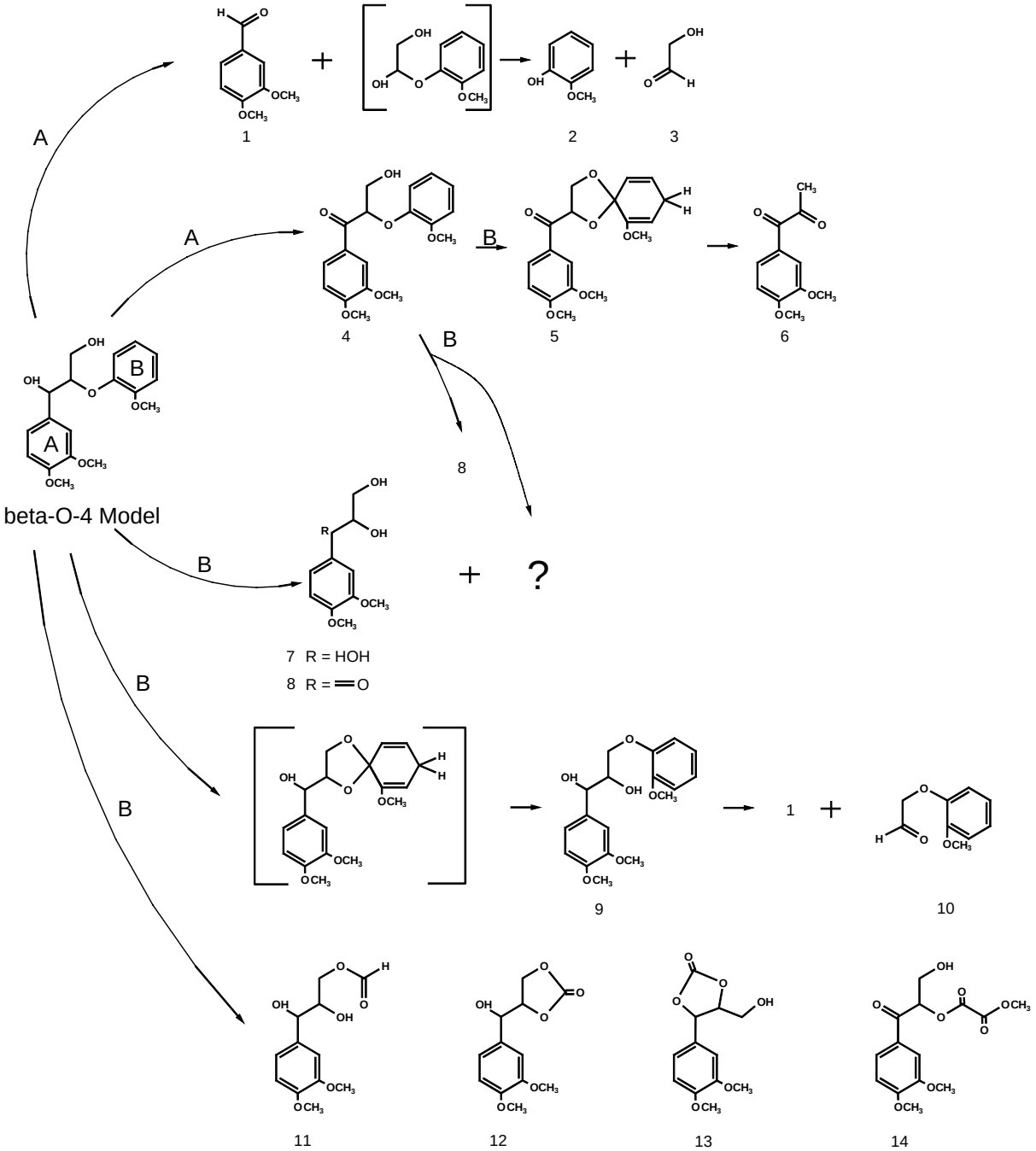


Figure 31: Lignin peroxidase/ H_2O_2 oxidation of a β-O-4 model lignin substructure compound leads to a variety of products. The one-electron oxidation can be either in ring A or ring B, as shown. (Kirk and Cullen, 1998).

Peroxidases and Phenoloxidases

Peroxidases and oxidases act non-specifically via the generation of lignin free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions. White-rot fungi secrete unique combinations of peroxidases and oxidases (Perie and Gold, 1991, Pointing, 2001).

Peroxidases are heme-containing enzymes which mediate the oxidation of a variety of molecules using hydrogen peroxide as an electron acceptor. Lignin peroxidase (LiP) and manganese- peroxidase (MnP) play a leading role in lignin depolymerisation using H_2O_2 to produce reactive intermediates (Cairney and Burke, 1998).

LiPs contain a single ferric protoporphyrin IX heme moiety. Within lignin degradation, LiP catalyses oxidation of lignin structures by a one electron transfer with formation of a cat ion radical. This radical subsequently forming a wide variety of degradation fragments. The major reaction is C_α-C_β cleavage but oxidation can also result in cleavage of aryl- C_α bond, aromatic ring opening, phenolic oxidation and demethylation. (Evans, 1991, Kirk and Cullen, 1998).

MnPs are peroxidases, which require manganese as a cofactor. They catalyse H_2O_2-dependent oxidation of lignin and other phenols but only in presence of Mn^{2+}. The primary reducing substrate of MnP is Mn^{2+}, which is oxidised to Mn^{3+}.
In many fungi MnP is thought to play a crucial role in the primary attack of lignin because Mn^{3+} is a chelated form may act as a diffusible oxidant (Cohen et al., 2002). MnP does not oxidise non-phenolic lignin structures during the normal reaction with H_2O_2 and Mn^{2+}. Many fungi lack LiP but have MnP which has been shown to degrade non-phenolic lignin structures. These structures are slowly co-oxidised when MnP peroxidises unsaturated fatty acids (Kirk and Cullen, 1998). It has been suggested that lipid peroxidation plays a role in fungal lignolysis by MnP (Kapich et al. 1999).
Laccases are copper containing polyphenol oxidases which are inhibited by halides in the order F^->Cl^->I (Garzillo et al., 1998). They catalyse the one-electron oxidation of *ortho*- and *para*-diphenols, aromatic amines and other electron rich substrates by removing an electron and a proton from a hydroxyl group to form a free radical. It oxidises the phenolic units in lignin and so catalyses the alkyl-phenyl and C_α-C_β cleavage (Higuchi, 1990). Laccases also catalyses the demethoxylation of several lignin model compounds (Eriksson et al., 1990).

After catalysation of four consecutive one-electron oxidations laccase transfers the electrons to molecular oxygen reducing it to water and returning the enzyme to its native state. So the oxidation activity is accompanied by the reduction of dioxygen to water. (Kirk and Cullen, 1998, Cohan et al., 2002). Laccases can also oxidise non-phenolic lignin model compounds in the presence of certain auxiliary substrates. ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonate) (Bourbonnais and Paice, 1990), 3-hydroxyanthranilate (Eggert et al., 1996) and 1-hydroxybenzotriazole (HBT) has bee described as low molecular weight mediators which transfer charge for the enzyme into the cell wall matrix.

Peroxid-generating enzymes

An essential component of all white-rot lignolytic systems is a source of H_2O_2. MnP itself can form hydrogen peroxide from molecular oxygen when NADH, NADPH or gluthathione are present (Highley and Dashek, 1998) but a few extracellular oxidases have been regarded as more likely candidates.
One of these is glyoxyl oxidase (GLOX). GLOX oxidises any of a variety of small aldehydes including glyoxal and methylglyoxal, which are extracellular metabolites. Then they transfer the electrons to dioxygen generating H_2O_2. Many white-rot fungi produce GLOX but not all (Hatakka, 1994). In *P. chrysosporium*, GLOX is secreted and produced co-ordinately with LiP and MnP isoenzymes and is activated by the presence of LiPs and their aromatic substrates (de Koker and Kersten, 2002).
Other candidates are superoxide dismutases (SODs). They are almost universally distributed enzymes containing iron (FeSOD), manganese (MnSOD) or copper and zinc (CuZnSOD) in their active sites. SODs catalyse dismutation of superoxid anion radicals ($O_2^{\cdot\cdot}$, SORs) to oxygen and hydrogen peroxide (Lehman et al., 1996, Leonowicz et al., 1999).

Metabolic combining enzymes

In this group there are also peroxide generating enzymes but this production of H_2O_2 combines lignin biodegradation with another process in the biodeterioration of wood.

Glucose-oxidase reduces the β-D-glucose excess within cellulose degradation process yielding D-glucono-1,5-lactone and H_2O_2. Pyranose-oxidase catalyses the D-glucose oxidation yielding 2-dehydro-D-glucose and also H_2O_2 (Leonowicz et al., 1999).
Aryl alcohol oxidase (AAO) is an extracellular flavoenzyme, which is in some white-rot fungi involved in lignin biodegradation. This enzyme catalyses the extracellular oxidation of aromatic alcohols to the corresponding aldehydes using molecular oxygen as an electron acceptor yielding H_2O_2 as a by-product (Cohen et al., 2002). In *Pleurotus ergynii* it was observed that laccase and AAO were simultaneously produced and that this enzyme penetrates the cell wall (Guillen et al, 1992).
Cellobiose: (acceptor) oxidoreductases play an important role in both cellulose and lignin depolymerisation. According Fenton's chemistry, very reactive hydroxyl radicals may result from the activity of these enzymes, which are involved, in the lignin degradation. The special enzyme cellobiose: quinone oxidoreductase reduces the toxic quinone formed by phenoloxidases during lignin degradation.
Brown-rot fungi
Until now, the most favoured mechanism is that brown-rot fungi only strongly decrease the methyl content of lignin in wood. During this demethylation aromatic hydroxyl groups are formed (Eriksson et al., 1990). But recently more and more studies showed that some species of brown-rot fungi may have a greater lignolytic capacity than previously thought. Kim et al. (2000) showed in a microscopic study that *Coniophora puteana* caused degradation of hardwood cell walls, including lignin-rich middle lamella and Score et al. (1997) and Lee et al. (2001) evidenced that this fungus produces extracellular laccase which is only active on hardwood, not on softwood. This wood type specifity could be possibly related to the difference of lignin monomer composition between hardwood and softwood.

Another laccase producing brown-rot fungus is *Merilus lacrymenas* (Evans, 1991) and in *Polyporus ostriformes* Del et al. (1991) even confirmed lignin peroxidase but on a low level compared with that in white-rot fungi.
Highley et al. (1985) proposed that there exist two types of brown-rot fungi; some have the capacity to degrade and metabolize all cell wall components, including lignin whereas others merely modify the structure of lignin.
Soft-rot fungi:
As a group, soft-rot fungi show considerable variation in their attack on cell wall components. In their lignin degrading capabilities they appear to be intermediate between the white- and brown-rot fungi (Zabel and Morell, 1992). They degrade or modify lignin in a greater extent than brown-rot but more slowly than white-rot fungi (Eriksson et al., 1990).
Degradation of wood by soft-rot fungi was mainly studied with *Chaetomium globosum* in beech wood in which were observed that at high weight losses, the lignin loss correlated with the loss of methoxyl groups but, to date, demethylation could not been observed for isolated lignins (DHP or Kraft lignin) (Eriksson et al., 1990).
In conclusion, many soft-rot fungi are able to degrade or modify lignin to some extent but the mechanism and the enzymes involved in soft-rot attack of lignin are still not known.

Table 12: Survey of enzymes involved in lignocellulolytic degradation

Recommended name	Systematic name	EC number	Reaction
Endo-1,4-β-glucanase	1,4-(1,3; 1,4)-β-D-Glucan-4-glucanohydrolase	3.2.1.4	Endohydrolysis of 1,4-β-D-glucosidic linkages
Exo-1,4-β-glucanase	1,4-β-D-Glucan cellobiohydrolase	3.2.1.91	Hydrolysis of 1,4-β-D-glucosidic linkages releasing cellobiose
	1,4-β-D-Glucan glucohydrolase	3.2.1.?	Hydrolysis of 1,4-β-D-glucosidic linkages releasing glucose
β-Glucosidase	β-D-Glucoside glucohydrolase	3.2.1.21	Hydrolysis of terminal non-reducing β-D-glucose residues with release of β-D-glucose
Endo-1,4-β-xylanase	1,4-β-D-Xylan xylanohydrolase	3.2.1.8	Endohydrolysis of 1,4-β-D-xylosidic linkages in xylans

Recommended name	Systematic name	EC number	Reaction
β-Xylosidase	1,4-β-D-Xylan xylanohydrolase	3.2.1.37	Hydrolysis of 1,4-D-xylans to remove successive D-xylose residues
α-L-Arabinofuranosidase	α-L-Arabinofuranosidase arabinofuranohydrolase	3.2.1.55	Hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides
Acetylesterase	Acetic-ester acetylhydrolase	3.1.1.6	An acetic ester + H_2O = an alcohol + acetate
α-Glucuronidase	α-D-Glucuronoside glucuronosohydrolase	3.2.1.?	α-D-glucuronoside + H_2O = an alcohol + D-glucuronate
Endo-1,4-β-mannanase	1,4-D-Mannan mannohydrolase	3.2.1.78	Rndom hydrolysis of 1,4- β-mannosidic linkages in mannans
β-Mannosidase	β-D-Mannoside mannohydrolase	3.2.1.25	Hydrolysis of terminal, non-reducing β-D-mannose residues in β-D-mannosides
α-Galactosidase	α-D-Galactoside galactohydrolase	3.2.1.22	Hydrolysis of terminal α-D-galactose residues in α-D galactosides
Ligninase		1.11.1.14	Catalyses several oxidations in alkyl side chains, C-C cleavage in side chain of lignin, cleavage of aromatic ring, oxidation of benzyl alcohols to aldehydes
Manganese-dependent peroxidase		1.11.1.13	Catalytically dependent on H_2O_2 and Mn^{2+} ion
Laccase	Benzendiol:oxygen oxidoreductase	1.10.3.2	4-Benzendiol + O_2 = 4-benzosemiquinone + 2 H_2O
Horseradish peroxidase	Donor:hydrogenperoxide oxidoreductase	1.11.1.7	Donor + H_2O_2 = oxidised donor + H_2O
Protocatechuic 3,4-dioxygenase	Protocatchuate:oxygen3,4-oxidoreductase	1.13.11.3	3,4-Dihydroxybezoate + O_2 = 3-carboxy-*cis,cis* muconate
Catechol 1,2-dixygenase	Catechol:oxygen 1,2-oxidoreductase	1.13.11.1	Catechol + O_2 = *cis,cis* muconate
Superoxide dismutate	Superoxide:superoxide oxidoreductase	1.15.1.1	$O_2{\cdot\cdot}^{-}$ + $O_2{\cdot\cdot}^{-}$ + 2 H^+ = O_2 + H_2O_2
Glyoxalate oxidase	Glyoxylate:oxygen oxidoreductase	1.2.3.5.	Glyoxylate + H_2O + O_2 = oxalate + H_2O_2
Glucose 1-oxidase	β-D-Glucose:oxygen 1-oxidoreductase	1.1.3.4	β-D-Glucose + O_2 = D-glucono.1,5-lactone + H_2O_2
Aryl alcohol oxidase	Aryl-alcohole:oxygen oxidoreductase	1.1.3.7	An aromatic primary alcohol + O_2 = aromatic aldehyde + H_2O_2
Pyranose-2-oxidase	Pyranose:oxygen-2-oxidoreductase	1.1.3.10	D-Glucose + O_2 = 2-dehydro-D-glucose + H_2O_2
Cellobiose:quinone oxidoreductase	Cellobiose:quinone 1-oxidoreductase	1.1.5.1	Cellobiose + quinone = cellobiono-1,5-lactone + phenol
Cellobiose oxidase		1.1.3.25	Cellobiose + dioxygen = cellobiono-1,5-lactone + H_2O_2
Cellobiose dehydrogenase	Cellobiose:(acceptor) 1-oxidoreductase	1.1.99.18	Cellobiose + acceptor = cellobiono-1,5-lactone + reduced acceptor

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5.3. Preservation strategies and technologies against bacterial wood degradation

(by Gelbrich & Militz)

Introduction

This chapter should give a survey about different possibilities to find a preservation strategy against bacterial wood degradation. The life conditions of the wood degrading bacteria are up to now not fully known. That is why not much research of preservation strategies is reported.

For this reason this report is not only a survey about preservation treatments. It is a survey of both preservatives which inhibit bacteria and which have no effect against bacteria. Some of them are not chemicals but native original wood extractives.

Natural resistance of wood due to cell wall composition

Investigations of archaeological wood of *Pinus sylvestris* showed, that various wood cell types or cell wall layers have a different resistance to bacterial attack. The secondary wall of axial tracheids and ray parenchyma is first degraded, whereas the middle lamella is mostly intact even in the most heavily degraded cells (Singh et al., 1996).

The pit borders in axial tracheids were not completely resistant but more resistant to degradation than the rest of the secondary walls in the same cells. Even having a comparable chemical composition but the accessibility of pit borders to erosion bacteria (EB) may have been restricted. A warty layer, very resistant to chemical dissolution because of its high lignin content, completely covered the inner face of the pit borders. The warty layer in the archaeological wood was coated by a dense layer likely to be rich in extractives, probably toxic to microorganisms (Singh et al., 1996). The generally accepted view, that initial pit borders are primary structures would suggest that both have nearly the same lignin content. That would explain the resistance of initial pit borders to degradation by EB (Singh et al., 1996; Singh, 1997).

The ray tracheid walls in the archaeological wood were completely resistant. Reasons therefore could be the high lignin content of ray tracheid walls and that bacterial entry into the ray tracheids may have been prevented due to the presence of an extractive layer (Singh et al., 1996).

EB have a limited capacity to degrade lignin rich areas of wood cell wall but it is somewhat greater than for soft rot fungi (Singh and Wakeling, 1993). The S_3 layer of *P. radiata* with a lignin content of about 52 % appears to be resistant to soft rot fungi but only partly so to EB (Singh and Butcher, 1991). Tunnelling bacteria (TB) can degrade all areas of wood, including the highly lignified middle lamella (Singh and Butcher, 1991).

Tropical timbers resistant against soft-rot, either because of toxic extractives or a high lignin content are not prevented against TB attack, e.g. *Alstonia scholaris*, *Callitris columellaris*, *Homalium foetidium*, *Libocedrus bidwillii*, *Tectonia grandis*, *Vitex parviflora* and *Pteridium aquilinum* (Nilsson and Holt, 1983). In the two tropical hardwood species with high lignin content *Alstonia scholaris* and *Homalium foetidium* it could be observed that all wood cell wall layers, including the middle lamella were degraded through the tunnelling action of bacteria (Singh et al., 1987).

Even the naturally durable *Eusideroxylon zwageri* wood can be attacked by TB. Both the fibres and parenchyma of this wood species are rich in extractives. The extractives are in large amounts in the lumen of parenchyma and fibre and even penetrate the fibre walls. Therefore the degradation of *E. zwageri* is markedly slower than any previously examined wood species and shows other differences (e.g. the attack starts from the S_1 layer moving inwards). In spite of this, TB are able to degrade this durable, extractive rich wood (Nilsson et al., 1992).

In water stored branch segments of *Betula pendula* parenchyma cells secreted substances which covered the pit membranes. It was darkly stained, typical for phenolic compounds.

The wood samples were incubated with a bacterial consortium of *Cellulomonas flavigena*, *Erwinia carotovora* and *Pseudomonas flurescens*, which attacked neither the secretion products nor the membranes in a period of 6 month (Liese et al. 1995).
In conclusion, the variable resistance of various components of wood appears to be related to their chemical composition as well as their accessibility to EB present in wood (Singh et al., 1996).

Wood preservatives against bacterial attack
The topic of toxicity of some wood preservatives and their components, which can prevent bacteria attack, was investigated in the 1970th in laboratory test.
Schmidt et al. (1975) investigated the bactericidal concentration of 5 wood preservation components for 4 bacteria species. Boron, chromium, copper, fluorine and pentachlorophenol (PCP) as well as CF-salt (chromium-fluorine) were examined using *Bacillus subtilis, Cellulomonas sp., Erwinia carotovora* and *Serratia marcescens*. The preservatives were examined in water, free of nutrients. As a criterion for a bactericidal effect, the lethal dose 50 (LD 50) was used, which was defined as the decrease of living cells of ≥ 50 % within 24 h incubation. In the following table 1 the comparison of the results of the LD 50 % and the 100 % bactericidal concentrations (LD 100) have shown.

Table 13: Bactericidal concentrations (%) (Schmidt et al., 1975).

	BACILLUS SUBTILIS		***CELLULOMONAS SP.***		***ERWINIA CAROTOVORA***		***SERRATIA MARCESCENS***	
Salt	LD 50	LD 100	LD 50	LD 100	LD 50	LD 100	LD 50	LD 100
$Na_2B_4O_7$*10 H_2O	1	> 5	1	1	0.1	5	0.1	1
$Na_2Cr_2O_7$*2 H_2O	0.001	> 1	0.01	0.01	0.01	0.001	0.001	0.01
$CuSO_4$*5 H_2O	0.0001	> 0.1	0.0001	0.01	0.0001	0.0001	0.0001	0.001
NaF	0.1	> 4	1	4	0.01	1	1	4
PCP-Na	0.01	1	0.0001	0.001	0.00001	0.00001	0.0001	0.0001
CF-salt	0.01	> 5	0.1	0.1	0.001	0.001	0.01	0.01

Boron and fluorine were less active, but chromium, copper, PCP and CF-salt showed medium to strong toxicity. *Bacillus subtilis* was the most resistant species.
The limiting concentrations were generally lower in water than those obtained earlier in nutrient liquids (table 2). That means that the compositions of the culture medium influence the tolerance considerably (Schmidt et al., 1975).

Table 14: Bactericidal concentrations (LD 100) in water and synthetic nutrient liquids in % (Schmidt et al., 1975).

	BACILLUS SUBTILIS		***CELLULOMONAS SP.***		***ERWINIA CAROTOVORA***		***SERRATIA MARCESCENS***	
	water (%)	nutrient liquid (%)	water (%)	nutrient liquid (%)	water (%)	nutrient liquid (%)	water (%)	nutrient liquid (%)
$Na_2B_4O_7$*10 H_2O	> 5	> 50	1	> 1	5	5	1	1
$Na_2Cr_2O_7$*2 H_2O	> 1	0.1	0.01	0.05	0.001	1	0.01	0.01
$CuSO_4$*5 H_2O	> 0.1	0.1	0.01	0.1	0.0001	0.075	0.001	0.005
NaF	> 4	> 50	4	0.5	1	0.5	4	0.5
PCP-Na	1	1	0.001	0.01	0.00001	0.025	0.0001	0.005
CF-salt	> 5	20	0.1	0.1	0.001	> 5	0.01	0.05

Further investigations of Schmidt and Liese (1975) showed same results added by determination of bacteriostatic concentrations of 12 preservative salts against 6 bacteria strains (table 15). A high toxicity against bacteria were obtained for PCP-Na, Cr, Cu and Hg, whereas B and As were not so effective. Compared with the extreme active compounds there was a medium toxic effect of F, Hydrogenflourid and the mixed salts.
Additional to the toxic effect of the preservative salts, two different reaction patterns of bacteria with increasing salt concentration could be observed. One pattern was called "learning strain" and was only observed for *Erwinia carotovora*. This bacteria strain evinced a certain adaptation to higher concentration. The other strains were "not learning strains" because they react on higher preservative concentrations with a lower biomass production. The reactions did not depend on the preservative but on the bacteria species. Bacills can be more resistant due to spore formation ability (Liese and Schmidt, 1975).

Table 15: Bacteriostatic concentration of different wood preservatives and preservative components in nutrient liquid in % (Liese and Schmidt, 1975).

	BACILLUS PUILUS	***BACILLUS SUBTILIS***	***CELLULOMONAS SP.***	***ERWINIA CAROTOVORA***	***PSEUDOMONAS CONVEXA***	***SERRATIA MARCESCENS***
NaF	0.5	0.5	0.5	0.5	0.25	0.5
KHF_2	0.5	0.5	0.1	0.05	0.1	0.1
$Na_2Cr_2O_7$	0.005	0.01	0.05	1	0.01	0.01
Na_2HAsO_4	0.5	5	10	15	5	10
$CuSO_4$	0.001	0.001	0.01	0.075	0.05	0.005
$Na_2B_4O_7$	1	0.5	0.5	1	0.25	0.5
$HgCl_2$	>0.0001	0.0001	0.0005	0.075	0.075	0.005
CF-salt	0.005	0.005	0.05	5	0.05	0.05
CFA-salt	0.005	0.005	0.05	0.5	0.05	0.05
CKB-salt	0.005	0.001	0.01	0.5	0.1	0.05
CFK-salt	0.001	0.005	0.01	0.5	0.05	0.05
PCP-Na	0.0005	0.0001	0.005	0.025	0.025	0.01

Nilsson and Rowell (1982) modified small blocks of ponderosa pine with butylene oxide with three different weight percent gains (8, 15 and 23.7 WPG) and exposed them for 6 weeks in unsterile soil. The samples with the two lower WPG of butylene oxide showed attack by soft rot and TB, but the wood blocks with the highest WPG showed no signs of attack.
The effect of three wood extractives (Obtsaquinone, Obtusastyrene and Lapachol) on the growth of marine micro-organisms was tested by Furtado et al. (1976). The micro-organisms tested were eight marine fungi, seven bacteria and two actinomycetes. Table 4 shows the minimal inhibitory concentration (ppm) of the extractive compounds against bacteria and ascomycetes.
Of the seven bacterial isolated all except one were susceptible to obtasastyrene at concentrations of 0-50 ppm. For lapachol 100-200 ppm were required to completely inhibit the growth of the bacteria and for obtusaquinone a concentration of 400-800 ppm showed the same results. The ascomycetes showed similar tolerance as the bacteria (Furtado et al., 1976).

The three compounds were also tested for their effect as wood preservatives in a microbial high environment. Therefore pine sapwood was impregnated with these three extractives and placed in a water-cooling tower with brackish water circulating. Under these conditions only attack by marine fungal flora (soft rot) could be observed and obtusastyrene afforded the best protection results (Furtado et al., 1976). But the authors did not discuss if the wood showed any bacterial attack because the extractives are so efficient or if the fungal flora is too dominant against bacterial decay.

Figure 32: Structures of the extractive components (Furtado et al., 1976)

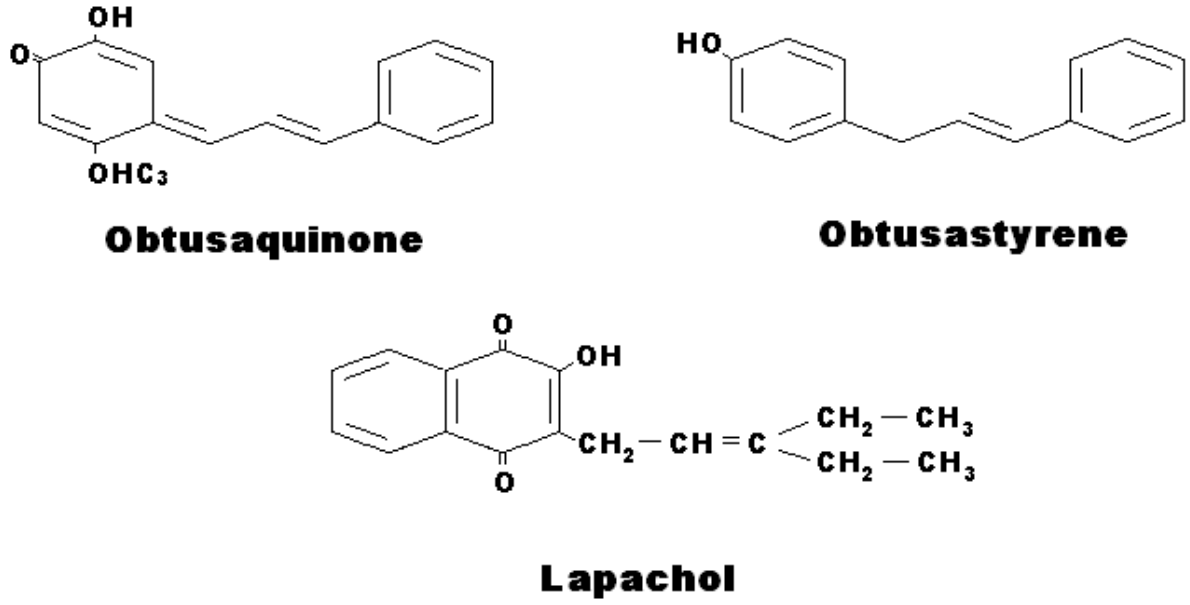


Table 16: Minimal inhibitory concentrations (ppm) of the compounds against bacteria and ascomycetes (Furtado et al., 1976)

ISOLATA BACTERIA	***ORIGIN***	***OBTUSAQUINONE (PPM)***	***LAPACHOL (PPM)***	***OBTUSASTYRENE (PPM)***
LR–1	Wood exposed at La Rochelle	800	200	200
K-1	Wood exposed at Sekondi	600	100	50
K-2	Wood exposed at Sekondi	600	100	20
F-1	Fras of *Terredo* sp.	400	100	20
F-2	Wood exposed at C.E.G.B.	400	100	50
F-4	Fleetwood Power Station	400	100	10
S-4	Wood exposed at C.E.G.B. Stella North Power Station	600	200	50
Ascomycetes				
AC-2	Wood exposed in Vancouver harbour	600	100	20
AK-4	Wood exposed at Sekondi	800	100	10

Wood preservatives without resistance against bacterial attack

Chromium and copper salts, common CCB, CF and CFA wood preservatives and quaternary ammonium compounds were strong bacterial inhibitors in laboratory tests on agar (Schmidt and Liese, 1994). The bacteriostatic, bactericidal and spore germination inhibiting concentrations are comparable with those against wood decay fungi. However, the failure of CCA-treated timbers in service shows the limitation of laboratory experiments.

In different studies it is reported, that bacteria are able to degrade CCA-treated wood for eucalypt power transmission poles (Leightley et al., 1982; Willoughby and Leightley, 1984; Willoughby et al., 1987), horticultural pine posts (Butcher, 1984; Butcher et al., 1984; Drysdale, 1984; Drysdale and Hedley, 1984; Nilsson, 1984), *Pinus sylvestris* in draught water or seawater (Eaton, 1994; Sturgess and Pitman, 1996; Brown et al., 2002), *Pinus radiata* in cooling towers (Singh et al., 1992; Singh and Wakeling, 1995, Singh, 1997a), *Pinus radiata* posts, house piles and from a retaining wall (Singh and Butcher, 1985; Singh and Wakeling, 1993; Singh et al., 1994).

A treatment of pine wood with ACQ13 (amminiacal copper quaternary ammonium formulation) up to a WPG of 22 % was not effective against attack of TB (Eaton, 1994).

Wyles and Dickinson (1987) used the thin section technique to test the protection of wood by copper based preservatives against bacteria at two different soil moisture contents (95 % and 150 % w.h.c.). The tested preservatives are shown in the following table 5.

Table 17: Used preservatives in thin section experiment by Wyles and Dickinson (1987).

PRESERVATIVES	CONCENTRATION IN % (W/W)	REMARKS
Tanalith C salt	2.36	
Tanalith NCA	1.46	Preservatives were used at
Celcure A salt	2.20	concentrations based on Tanalith C salt
Celcure A oxide	1.79	on an equivalent active element
K 33 oxide	1.40	(copper and arsenic) basis

The bacterial attack was very variable. The authors believe in pockets of bacterial populations within the soil jars because sections taken from different areas of the soil jars were attacked to varying degrees. This phenomenon probably represents the distribution and lack of mobility of the causal organisms. Decay was often present in some sections after four weeks but absent in the replicate sections at later periods. However, this study showed that bacteria are able to degrade wood treated with all tested preservatives (Wyles and Dickinson, 1987).

Biological controlling of the bacteria

It is known that there exist interactions between various wood decay fungi and bacteria. Thorn and Tsuneda (1992) tested 53 wood decaying basidiomycetes from which 38 attacked or lysed one or more of the following bacteria: *Agrobacterium tumefaciens*, *Bacillus pumilus, Pseudomonas cepacia, P. fluorescens, P. putida* and *P. tolaasii.* The attack took the form of increased hyphal branching within the bacterial colonies, often preceded by direction growth toward them. In some interactions, colonies of bacteria were lysed without directional growth, increasing branching within the colonies or even direct contact of colonies by hyphae. *Pleurotus ostreatus* attacked and consumed nematodes and all fifteen tested bacteria on agar and wood, including species of *Agrobacterium, Azotobacter, Bacillus, Erwinia* and *Pseudomonas* (Thorn and Tsuneda, 1993).

The antagonistic effect of wood decaying fungi is not directly useful to protect wood against bacterial degradation. But perhaps it is possible to find out which enzymes or other fungal ingredients enable this effect and search for possibilities to work with this isolates.
Another strategy against bacteria is to use phages as natural antagonist of bacteria. This is one way which will be investigated in the BACPOLE-project.
Up to now it was not possible to isolate the wood degrading bacteria in pure cultures but this is necessary for the rearing of the effective phages.

Outlook
For the future new wood treatments or preservatives should not only be tested against fungal decay but also against bacterial attack.
Changes in the environmental conditions are a possibility to control the bacteria in their wood degradation and have to be studied. First test with changes in the soil nutrient and phosphate content will be investigated within the BACPOLES-project.

5.4 Bacteriophages production

(by Mårch)

Introduction

A bacteriophage, or phage, is a virus, which is limited in many aspects. It infects bacteria, with a high specificity for a certain bacterial strain (host cell). The phage is an obligate bacterial parasite. The genome of a phage virus may contain as little as 10 genes and comprises single- or double-stranded DNA or RNA molecules. *By itself the virus can persist, but it cannot replicate* except within the host cell where it utilises and completely depends on the host cell genome (genetic element). A virulent virus lyses (kills) the host cell. Hundreds of viral copies are released to the environment within 20-30 min after infection. Each new virus has the capability to infect and kill new cells. Thus a virus is a genetic element that replicates in cells but is characterised by having an extracellular state, too. Bacteriophages are viruses, which constitute a means to control bacterial growth.

Bacteria play an important role in biomass degradation, wood degradation included. It is therefore tempting to believe that suitable phages can act as wood protective agents. A first and very basic requirement to test this hypothesis is to isolate and identify wood degrading bacteria. Then pure bacterial cultures are used for screening of specific bacteriophages in various fractions from wood objects, e.g., from the Bacpoles project; all such phages constitute potential wood protective agents.

One important objective for SLU and UoP in the Bacpoles project was to find, isolate and characterise wood degrading bacteria. Intimately linked to this was another objective for Phagen - to isolate bacteriophages with the capability to attack these bacteria.

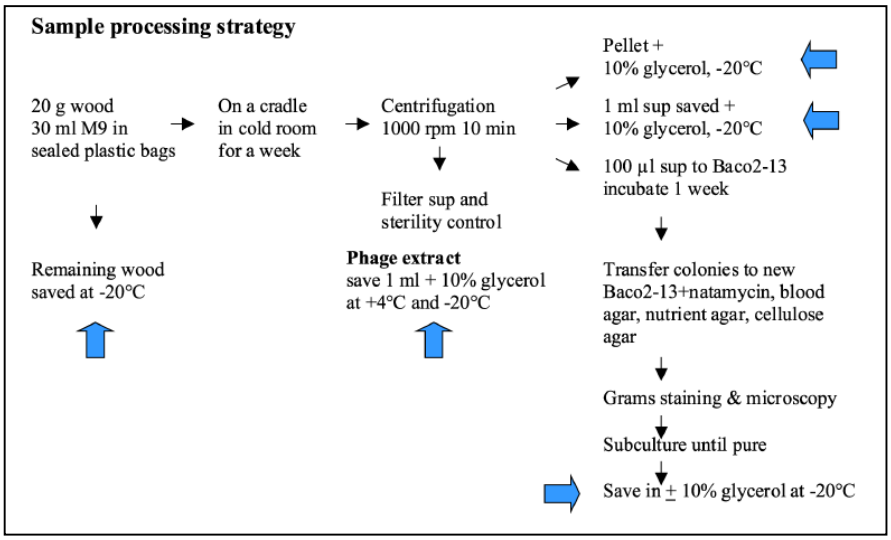
Wood objects in the Bacpoles project were derived from many different sites and were in varying states of degradation. In these samples one would expect to find a heterogeneous spectrum of various microbes, including wood degrading bacteria and viruses. All these samples were of interest to carefully investigate for potentially wood protective effects by phages already present in the samples. In order to successfully carry out this task, interesting viruses and their host bacteria had to be extracted and saved early in the project. Phagen could not rely on a successful nor extensive isolation of living bacteria by the other Bacpoles partners.

Microbes that were attached to the surface of the wood objects, as well as those within the wood, were of interest to investigate. Therefore, upon arrival to the laboratory, samples from all objects were treated according to a specified method (Figure 1). The various fractions were stored at several conditions considering possible differences in survival stability of phages as well as their specific host bacteria. Some bacteria and viruses may be stored at temperatures above zero degrees, while others upon long-term storage are more stable below zero. Often biological membranes, also in living organisms, are protected against damage when frozen by agents as glycerol. Therefore glycerol was included in many samples. With this strategy, both phages and their host bacteria were expected to be rescued until the wood degrading bacteria species were identified by other partners.

Early in the project it was evident that the isolation and identification of wood degrading bacteria were difficult and extremely slow processes and thereby constituted major obstacles for SLU and UoP. These bacteria are required for the production of specific phages; hence related phages could not be identified. Initially the work of Phagen was focused on rescuing fractions with bacteria and phages with a potential interest for future work at times when the isolation and identification problems had been solved.

The available methods of studying the wood decay process were not suitable for screening the large number of bacteria and fractions with phages that were isolated by Phagen. During the last part of the project, however, we looked for a combination of properties of the bacteria, i.e., ability to grow on cellulose agar, inherent peroxidase activity, attachment to wood, and ultimately degradation of wood. The first three tests appear rapid and convenient for a first selection of bacteria making it possible to focus on the original tasks of Phagen - to identify suitable phages for a phage-based preservative.

Figure 33. Sample processing strategy. Block arrows indicate fractions saved.



Methods

General microbiological techniques were used throughout the work. Below follows selected methods that proved useful for a successful progress of the project.

Cellulose agar:

Bacterial growth was tested on several types of cellulose, e.g., cotton and cellophane with the aim to obtain suitable host cells for phage isolation. In Phagen´s laboratory the following cellulose agar was found convenient and satisfactory for growth of many bacteria:

Cellulose *0.5 g*
NH_4Cl *0.5 g*
Na_2HPO_4 *2.4g*
KH_2PO_4 *2.2g*
$MgSO_4x7H_2O$ *0.25g*
Agar *7.5g*
Dissolve in 1000 ml and pour onto petri dishes.

Peroxidase test:

Bacteria may utilise peroxidase enzymes for degrading biomolecules, e.g., lignins.
Bacteria are incubated with *1% H_2O_2 in PBS* at room temperature.
Bubbles are formed in a positive test.
Fresh wood do not show a positive reaction, but infected wood does!
Sections of wood can be stained with peroxidase chromogens, e.g., aminoethylcarbazole (AEC) or diaminobenzidine tetrahydrochloride (DAB), to visualise infected areas of the wood specimen in the microscope.

Preparation of phages:
Isolated bacteria may be infected by phages which can be isolated from cultures of the bacteria (when testing for infection "phage extract" below should be omitted):
I. Growth of bacteria:
E-flasks, 100 ml sterile
Nutrient broth, autoclaved
Bacteria
Phage extract
32°C -shaker
Take 25-30 ml of the Nutrient broth in a flask and add approx. 1-10µl from the bacteria colonies from an agar plate (± phage extract). Incubate at 32°C for 2-3 days with shaking at 100 rpm. Continue with step II below.

II. Purification, precipitation and testing of phages: *Modified from: Molecular Cloning 1989 (Sambrook, Fritsch, Maniatis, Eds.) Cold Spring Harbor Laboratory Press.*
PPS: Phage precipitation solution, 2.5 M NaCl, 20% PEG (polyethylene glycol) 8000 in 50 ml water
$CHCl_3$
Save 2 ml of the bacteria (step I) to use for plaque assay! Then add 100µl of $CHCl_3$ to the rest and shake for 1h at 32°C.
Centrifuge 4000 rpm 30 min, at 4 °C.
Repeat centrifugation until no pellet is observed or filter the supernatant.
Let stand for aeration for 30 min in the hood
Apply a sample of the supernatant over a soft-agar confluent culture as negative plaque control onto an NA-agar plate. (Take 50µl of the first 2 ml that was taken of and add to 3 ml soft-agar, pour onto a plate and let grow). Add drops of the supernatant onto respective plate.
Then: Add 2 ml of PPS for each 6 ml of "bacteria lysate" (phage supernatant).
Mix by inverting the tube and incubate at 4°C for 48 h.

Centrifuge the phage supernatant 30 min at 15 000 rpm at 4°C in a Beckman JA-20 rotor. Handle the tubes carefully; do not disrupt phage pellet. The pellet should be hardly visible. Keep on ICE!
Gently resuspend the pellet in 1/25 (approx. 500µl) of the original volume with sterile 10mM $MgSO_4$. Apply a sample of the supernatant over a soft-agar confluent culture (from each bacteria make a mix with NB and soft-agar) as a control of precipitation. Put on a drop of the supernatant.
To keep phages for a long time, add about 5µl of $CHCl_3$, in order to avoid bacterial contamination.

Results
From hundreds of Bacpoles wood samples more than 600 extracts and 400 bacteria isolates were retrieved coded and stored (Table 1). Phagen codes were given in sequence as wood samples arrived to the laboratory. These numbers are used also to identify bacteria isolates and various fractions isolated and stored by Phagen.

In the Bacpoles wood specimens there are a vast number of microbes. Most of these were of no interest for the project. Wood degrading bacteria, however, have the capability to utilise cellulose as energy source in their environment. This is achieved by means of extracellularly located or secreted cellulases, which are specific enzymes capable of degrading the polymeric structure of the cellulose molecule to simple sugar entities. In order to limit the number of isolated bacterial candidates as wood degrading species and thereby the number of bacteria interesting to save and test for wood degrading capability, techniques of culturing bacteria on cellulose agar was used. A number of bacteria from Bacpoles samples and the microcosms were able to grow and survive under these very limited conditions (Figure 2).

Table 1. Bacteria and various fractions saved from Bacpoles specimens. Phagen´s codes and original wood sample codes from SHR are presented. Additional subgroups (B numbers) of bacteria were saved depending on various characteristics as colour of colonies, growth appearance on the various agar plates, hemolysis on blood agar etc.

ID numbers

Pha	SHR	Pha	SHR	Pha	SHR
B 1:1	18.td.4.210.2	B 4:5	10.td.1.580.2	B 8:2	21.ta.3a1
B 1:2	19.td.4.160.2	B 4:6	10.td.1.930	B 8:3	25.ta.1a4
B 1:3	20ta1a1	B 4:7	10.td.2.220	B 9:1	2.td.4.150
B 1:4	20ta2a2	B 4:8	10.td.2.950	B 9:2	2.td.5.200
B 1:5	20ta1a2	B 5:1	22.td.1.2420-2460.3.2	B 9:3	2.td.6.140
B 1:6	20ta3a3	B 5:2	22.td.1.6600-6600.3.2	B 9:4	2.td.7.120
B 2:1	8.td.1.680	B 5:3	22.td.2.2420-2460.3.2	B 10:1	14.ta.1.0.49-0.71
B 2:2	8.td.2.380	B 5:4	22.td.2.4700-4760.3.2	B 10:2	14.ta.2.0.62-0.89
B 2:3	8.td.3.440	B 5:5	24.ta.1	B 10:3	14.ta.3.0.34-0.57
B 2:4	8.td.4.680	B 5:6	24.ta.2	B 10:4	26.te.1.0-90
B 2:5	8.td.5.210	B 5:7	24.ta.3	B 10:5	26.te.2.0-78
B 2:6	13.ta.1.1	B 5:8	24.ta.4 a+c	B 10:6	26.te.3.0-74
B 2:7	13.ta.1.2	B 5:9	24.ta.4b	B 11:1	27.ta.1
B 2:8	13.ta.1.3	B 5:10	24.ta.5	B 11:2	27.ta.2
B 2:9	13.ta.1.4	B 6:1	2.td.2.100.A	B 11:3	27.ta.3
B 2:10	23.td.1.80	B 6:2	2.td.3.100.A	B 11:4	27.ta.4
B 2:11	23.td.1.380	B 7:1	2.td.1.0-620	B 11:5	27.ta.5
B 3:1	1A.td.1.320	B 7:2	2.td.2.400-840	B 11:6	28.ta.1
B 3:2	1A.td.1.80	B 7:3	2.td.3.400-850	B 11:7	28.ta.2
B 3:3	1B.td.3.140	B 7:4	2.td.8.0-700	B 12:1	0.4.te.1.0-64
B 3:4	1B.td.3.310	B 7:5	2.td.9.0-650	B 12:2	0.4.te.2.0-69
B 3:5	1C.td.1.140	B 7:6	2.td.10.0-600	B 12:3	0.4.te.3.0-45
B 3:6	1C.td.1.320	B 7:7	2.td.11.0-650	B 12:4	0.4.te.4.0-67
B 3:7	1D.td.3.110	B 7:8	2.td.12.0-760	B 12:5	0.4.te.5.0.00-0.50
B 3:8	1D.td.3.340	B 7:9	5.te.1.65	B 12:6	0.4.te.6.0.00-0.50
B 3:9	12.ta.1.1	B 7:10	5.te.2.50	B 12:7	0.4.te.7.0.00-0.50
B 3:10	12.ta.1.2	B 7:11	5.te.3.50	B 12:8	16.ta.04.01
B 3:11	12.ta.1.3	B 7:12	6.te.1.0-500	B 12:9	16.ta.15.0.1
B 3:12	12.ta.1.4	B 7:13	6.te.2.0-500	B 12:10	17.ta.1
B 3:13	12.ta.2.1	B 7:14	6:te.3.0-500	B 12:11	17.ta.2
B 3:14	12.ta.2.2	B 7:15	7.te.500-750	B 13:1	11.ta.1
B 3:15	12.ta.2.3	B 7:16	7.te.2.500-750	B 13:2	11.ta.2
B 3:16	12.ta.2.4	B 7:17	7.te.3.850-1100	B 13:3	11.ta.3
B 4:1	9.ta.1	B 7:18	7.te.4.300-550	B 13:4	11.ta.4
B 4:2	9.ta.2	B 7:19	7.te.5.200-450	B 13:5	11.ta.5
B 4:3	9.ta.3	B 7:20	7.te.6.430-680		
B 4:4	9.ta.4	B 8:1	21.ta.2a1		

Figure 34. Bacteria isolated from microcosm soil and cultured on a cellulose agar plate; Staining and microscopy showed gram-negative coccoids in this specific isolate.

In nature bacteriophages are frequently found wherever bacteria are present. Bacteria isolated from the Bacpoles samples may already be infected by specific phages. Therefore selected bacteria from our fresh isolates were tested whether they were infected. Furthermore, various "phage" extracts were tested by means of plaque assays using non-infected bacteria.

Bacteria isolates were cultured in the laboratory and phages were isolated from the cultures by PEG-precipitation techniques. These phage preparations were subsequently tested by means of plaque-assays (Figure 35). The results indeed show that several bacteria isolates initially were infected with natural phages.
In the last part of the Bacpoles project, Phagen had the task to produce a phage-based wood preservative for the microcosm experiments. No wood degrading bacteria had at this time

been isolated and identified by any Bacpoles partner. Phagen, however, received various samples from UoG and initiated bacterial and phage isolation in order to gain some information about the microbiology of the soil and water used in the microcosms. Several bacteria and phages were isolated and some results are summarised in table 19.

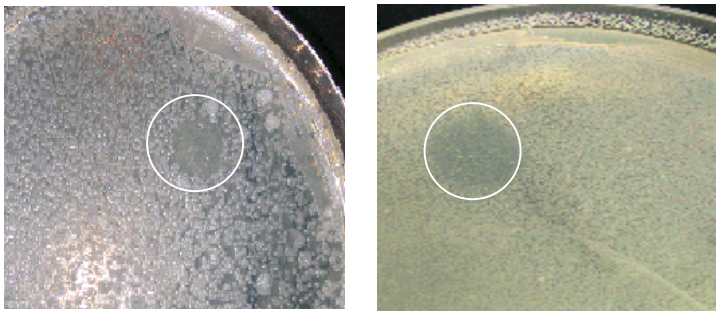


Figure 35. Plaque-assays using 5 µl of "inherent phage" preparations on bacteria from microcosm soil. Cultures were on nutrient agar plates with top agar. Clear plaques indicate lysis of bacteria induced by phage. A: gram-positive rods; B: gram-negative short rods.

Electron microscopy was used to verify the nature of phage in such a phage preparation (Figure 36).

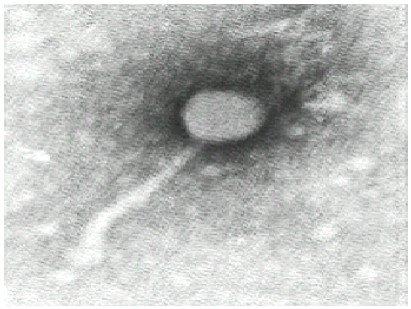


Figure 36.Electron micrograph of a phosphotungstic acid stained bacteriophage isolated from bacterium "B12:7" in Bacpoles 4.te.7.0.00-0.50 (with courtesy O Garcia, a guest scientist at our laboratory).

Table 19. Soil (MS) and water (MW) samples used in microcosms, and a sample from MIK14, were obtained from UoG. A number of bacteria were isolated and stored frozen; various characteristics as colour of colonies, growth appearance on the agar plates were recorded. C denotes ability to grow on cellulose agar, and P peroxidase positivity. Lytic phages were prepared from MS 1, 4, and 7 of these bacteria isolates.

Bacteria isolated from microcosms by Phagen

MS:		MW:		MIK:	
	1		1 C P		1 C
	2		2 C P		2 C
	3		3 P		3
	4 C		4		4
	5 C				
	6 C				
	7 C				
	8				
	9 C P				
	10 C P				
	11				

Although in the beginning of the study, none of the pure bacteria isolated in our laboratory were capable of degrading wood samples tested at SLU, the general techniques of phage isolation and phage assays were successfully tested by Phagen.
Late in the project bacteria related to *Bacteroidetes* received special attention. Selected bacteria from Phagen isolates were sent to UoP for 16S rRNA-identification. Several yellowish, colony-forming and possibly related bacteria from our saved Bacpoles bacteria isolates, were identified: *Flavobacterium* sp. (Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium) (samples B5:7, B3:41, B11:71); *Chryseobacterium* sp. (Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium) (samples B3:82, B4:23). Among these and related bacteria, B3:41, B3:71, B3:82 and B4:22, were found to be positive in subsequent peroxidase tests.

Since infection of fresh wood and culturing wood degrading bacteria appeared to be slow processes and a hindrance in the project to develop a phage-based preservative, it was important to make a strong effort to develop a "screening assay". Once wood degrading bacteria are identified and when pure bacterial cultures have been obtained, as described in our original proposal, monoclonal antibodies against bacteria can be produced. These antibodies can then be used for rapid identification of the bacteria. For obvious reasons such antibodies could not be developed. In Phagen´s laboratory we have recently started to test and made progress by using an alternative procedure to the "infection-in-water" method originally used by SLU. Thin cylinders (2-3mm height, 8 mm Ø) of fresh pine are placed on agar plates with cultures of selected bacteria. After incubation (weeks-months) the wood specimens are sectioned and stained for microscopy in order to investigate infection and degradation. The method is attractive because it is "rapid". A high number of fresh and viable bacteria on the agar plate may infect the wood, and the external environment such as composition of other nutrients, atmosphere and temperature can easily be controlled. The peroxidase reaction is important for degradation of lignin in wood, and the peroxidase-positivity easily reveals attachment of positive bacteria in the wood samples. Assays of infection of wood, peroxidase tests, utilisation of cellulose in agar, and phage isolation in various bacteria are in progress. Further evaluation of the method together with SLU will be carried out.

Conclusions and future perspectives
Successful isolation and identification of bacteria are prerequisites for specific phage isolation and hence for the development of a phage-based wood preservatives. 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.

Another aim in the project was to investigate effects of phages in the microcosm experiments. The phage isolation procedure and phage techniques proved successful. Phages were isolated from several samples from the microcosms. However, the general microflora of the microcosms was not investigated, nor were specific wood degrading bacteria successfully identified by other Bacpoles partners. The lack of identification of the bacteria of interest limited possible conclusions.
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 by UoP to assess the identity of living organisms in the samples. In addition, all isolated bacteria constitute potential reservoirs of suitable phages to be used as phage-based preservatives once the wood degrading bacteria have been identified.

During the work on the Bacpoles project a great general interest around the project have been noticed but also a special interest of the prospects of the development of a biological, phage-based wood preservative. In general, many types of phage appear extremely resistant to degradation. They are also extremely successful survivors existing in both intra- and extracellular states, all of which are great assets for a future preservative.

However, there are many practical considerations that have to be met:

- Specific phages have to be produced against the wood degrading bacteria strains that are present at the site (soil/water) to be treated. This means that if there are several types of wood degrading bacteria present, several types of phages must be produced, tested and applied.
- For archaeological objects and foundation piles, e.g., means of applying the preservative in the environment have to be investigated, as well as the duration of the phage application/ treatment etc.
- The properties of a phage-based preservative used in fresh wood surface-impregnation techniques may be different from those for archaeological objects.
- 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 from the Bacpoles project, the present results, and through the development of new methods from several Bacpoles partners for isolation and identification of wood degrading bacteria, we believe that a successful commercial phage based wood preservative is within reach.

Chapter 6

Water flow experiment

(by Klaassen with a contribution of Keijer)

6.1 Introduction

It is hypothesised that for bacterial decay (wood degradation under water), a water flux in the wood could be crucial. From the 4 sites where whole foundation piles were extracted, we learned that in 3 out of 4 cases the degree of decay at the pile head was similar to that at the pile tip. Furthermore a relationship was found between the degree of decay over the whole pile and timber species and location. In spruce severe bacterial degradation is often limited to a thin outermost layer, whereas in pine often the whole sapwood region is degraded. Differences in degree of degradation between localities could be related to ground water dynamics in combination with the soil constitution. Also archaeological wood field observations show that under permanent submerged conditions wood is more susceptible to bacterial degradation in less permeable soils than in more open soils. The idea is that a water flux through the wood occurs only when the permeability of the wood is higher than that of the soil around it.

In order to check this hypothesis, different tests were carried out to determine the water transport capability of timber species frequently found in archaeology and construction pilings. The amount of groundwater above the pile heads increases the water pressure at the pile head. In order to check whether this could be an important factor in increasing the water stream velocity through the piles, pine piles only were tested with pressures up to a water column of 250 cm.
From the long-term groundwater measurements as done in Haarlem and Zaandam (sites 6 and 7) it became clear that there is no static negative ground water pressure gradient along the piles. It is assumed that the groundwater pressure varies with specific ground layers. In order to check the possibility that piles transport water between different soil layers the radial water transport capacity in combination to the axial water transport capacity was tested. From the wood structure and based on field observations it is supposed that water transport capacity is most obvious in pine and therefore tests to check radial water transport were carried out in pine only.

6.2 Materials & Methods

Three stems of approximately 30 cm length of the following species were used for this investigation:

- Scots pine (*Pinus sylvestris*)
- Norway spruce (*Picea abies*)
- Larch (*Larix spec*.)
- Oak (*Quercus spec*.)
- Douglas fir (*Pseudotjuga menziesii*)
- Alder (*Alnus spec*.)

The stems have been supplied in lengths of 1 meter by Gebroeders Van Beek Houten Heipalen (Douglas, larch and spruce) and Staatsbosbeheer (alder, pine and oak) in June / July 2003. All stems originated from Dutch forests. Until the preparation of the experiment the stems were stored under water. After sawing (to a length of 300 mm), and debarking, the outsides of the wet stems were coated with Poly-Service Poly-Pox GT600 epoxy (Harder 455). For a good connection, this coating system needs a dry wooden surface and therefore the outside of the stems were dried with a hot air dryer for several minutes until the outermost stems layers of about 1 mm had a reduced moisture content. At the upper side of the stem a collar of Fibreglass was connected and impregnated with epoxy.

Experiment 1
About 10 mm water was put upon the cross section of the stems. The stems were put on a shelf with small rills, and in order to avoid evaporation a thick plastic sheet closed the upper part of the shelf. A tube was connected to the lowest point of the shelf to collect the amount of water in a container. Every week the container was emptied and the amount of water was measured.

Figure 1. Test set-up.

In the fourth week of 2004 the experiment started by measuring weekly the amount of water which came out at the underside of the stems. Because the water on the cross surface became contaminated by bacterial slime and algae, it was refreshed weekly from the seventh week onwards.

After the experiment the pathway of the water transport has been determined. On the cross surface of the stem a groove (20 x 20 mm) was made over the full radius and crossing the pith. The groove was filled with copper-sulphate-solution and was refilled when necessary. After 1.5 day the stem was sawn open along the axial direction, the copper solution was stained and photographs were made of the stained surfaces. From the staining reaction the pattern of the effective water transport zone was described.
From the sawn axial surface the stem diameter as well as the sapwood area was measured. The sapwood was recognised visually using differences in moisture content, differences in colour and the presence of blue stain as parameters. The sapwood area of the cross surface was used in the calculations of the water flow.
In order to find out which solution was most effective to visualise the water stream through the wood, different solutions were tested, Brilliant Blue, Safranine, Copper sulphate and Rhoadamine.. On the cross surface of water saturated pine, spruce and alder (samples size 20 x 20 60 mm) a hole (Ø 5mm, 10 mm deep) has been drilled. The stains were added in the holes, and after one week the samples were sawn length-wise open. The results are shown below. Only Copper sulphate did not block the wood structure and marked most clearly its pathway. Therefore it was chosen to use in the final tests.

Experiment 2
The effect of the height of the water column above the pile heads (pressure) was investigated by using one spruce stem and one pine stem, which had been used in experiment 1. The water column varied from 2.5 m, 1.75 m, 1.2 m and 0.75 m above the cross surface of the stems. The amounts of water which came out at the underside of the stems was measured after 1, 2, 3, and 4 hours after implementing the pressure. On the basis of these measurements the weekly water flow through the stem was calculated.

Experiment 3
Three additional Scots pine (*Pinus sylvestris*) stems, supplied by Staatsbosbeheer (Ede) in October 2004 have been stored in water until sample preparation. The stems were debarked and sawn to a length of 400 mm. The outsides of the stems were coated with epoxy in a similar way as described for experiment 1. At 95 mm below the upper cross surface a collar of fibreglass was connected and impregnated with epoxy (see figure 2). In order to avoid a water stream through the cross surface the water level in the collar was 10 mm below the cross surface. The amount of water that came out at the underside of the stems was measured daily.

Figure 2. Test set-up experiment water flow trough bark.

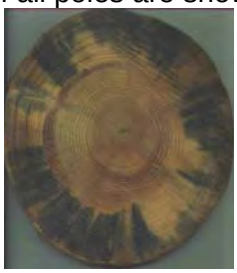
6.3 Results

Experiment 1

Below pictures of the cross surface of all poles are shown

Pine 1

Pine 2



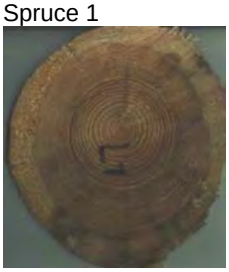
Pine 3

Blue stain!!!

Spruce 1

Spruce 2

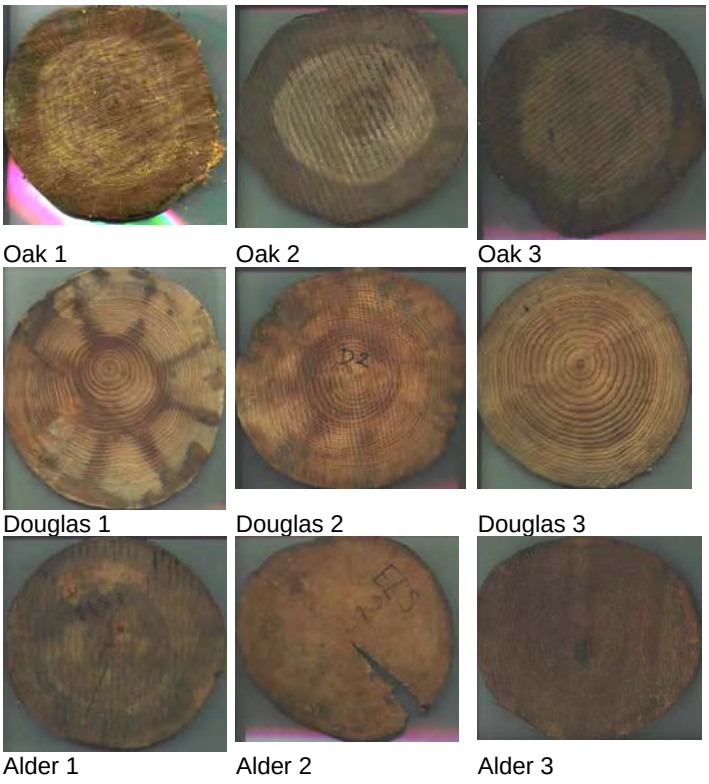
Spruce 3



Larch 1

Larch 2

Larch 3



In figure 3 the weekly water flow through the individual stems is shown. The average flow through a sample and wood species is given in figure 4.

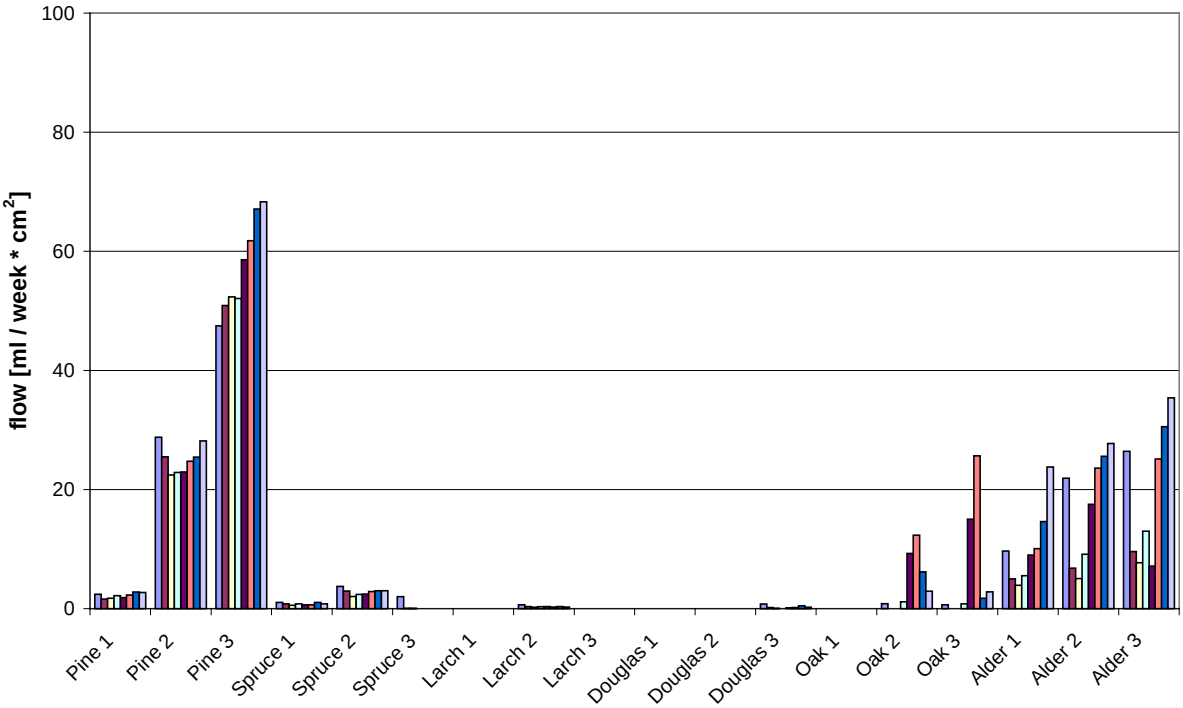


Figure 3. The weekly water flow through each of the stems during 8 weeks. The bars represent the flow for each of the 8 weeks and the flow is calculated on the basis of the whole stem diameter. After 4 weeks the water on top of the stems was weekly refreshed

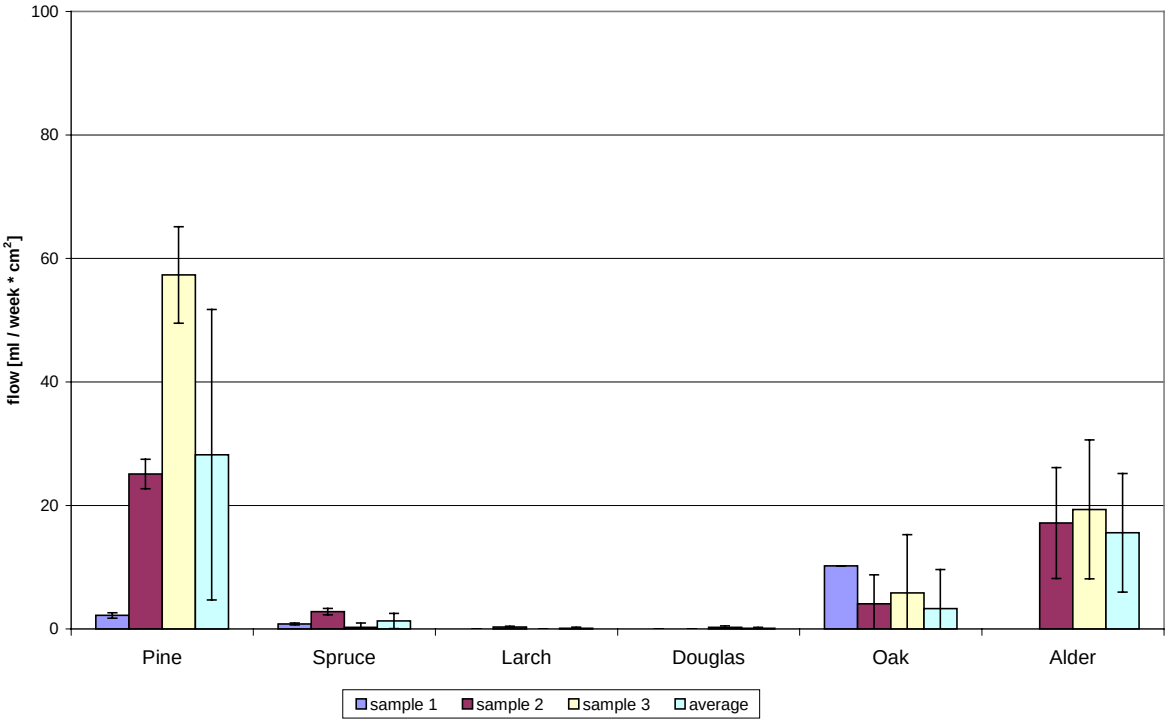
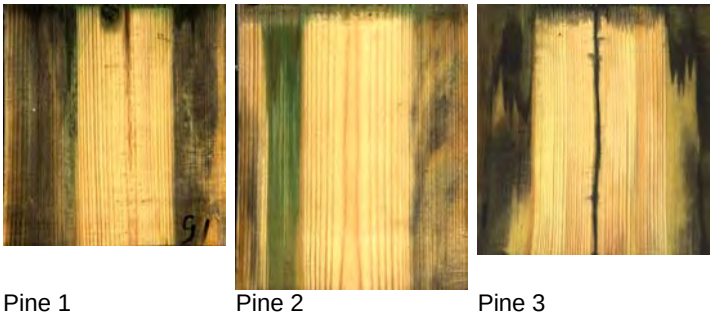


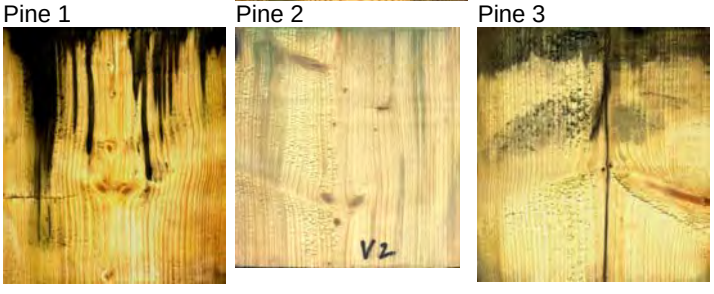
Figure 4. The average weekly water flow through each of the stems during 8 weeks. The flow is calculated on the basis of the whole stem diameter.

Below the sap flow with a stained copper solution is shown.



Pine 1 Pine 2 Pine 3

Water flow through parts of sapwood > 300mm/36 h. In area with blue stain no water flow. Water flow in heartwood < 10mm/36h. Water flow also along longitudinal pith check (pine 3).



Spruce 1 Spruce 2 Spruce 3

No differences in heart- and sapwood. Differences between stems:
Spruce 1 specific year rings are more open than others water flow 15-20mm/36h.
Spruce 3 all year rings closed water flow 20mm/36h.
Water flow also along longitudinal pith check (spruce 3).

Larch 1 Larch 2 Larch 3

Water flow through parts of sapwood > 40 mm/36 h. In area with blue stain no water flow. Water flow in heartwood < 10 mm/36h. Water flow also along longitudinal check (larch 2).



Water flow through sapwood > 100 mm/36 h. Water flow in heartwood < 10 mm/36h.

Oak 1 Oak 2 Oak 3



Water flow through parts of sapwood 200 mm/36 h. In area with blue stain no water flow. Water flow in heartwood 10-25 mm/36h.

Douglas 1 Douglas 2 Douglas 3



No difference between inner and outer part of the wood.
Water flow first through the vessels (200-300mm/h36) additional through other tissue (150mm/36h)

Alder 1 Alder 2 Alder 3

In the table below the stem diameter as well as the sapwood area, determined visually for each of the stems, is given.

	stem 1			stem 2			stem 3		
	Ø (mm)	**sapwood (mm)**		**Ø (mm)**	**sapwood (mm)**		**Ø (mm)**	**sapwood (mm)**	
pine	209	70	50	180	55	47	228	44	55
spruce	216	90	95	210	-	-	220	-	-
Douglas	215	35	25	209	38	21	206	23	24
larch	200	16	16	206	15	11	196	21	25
oak	164	26	21	156	25	25	156	21	24
alder	175	-	-	138	-	-	179	-	-

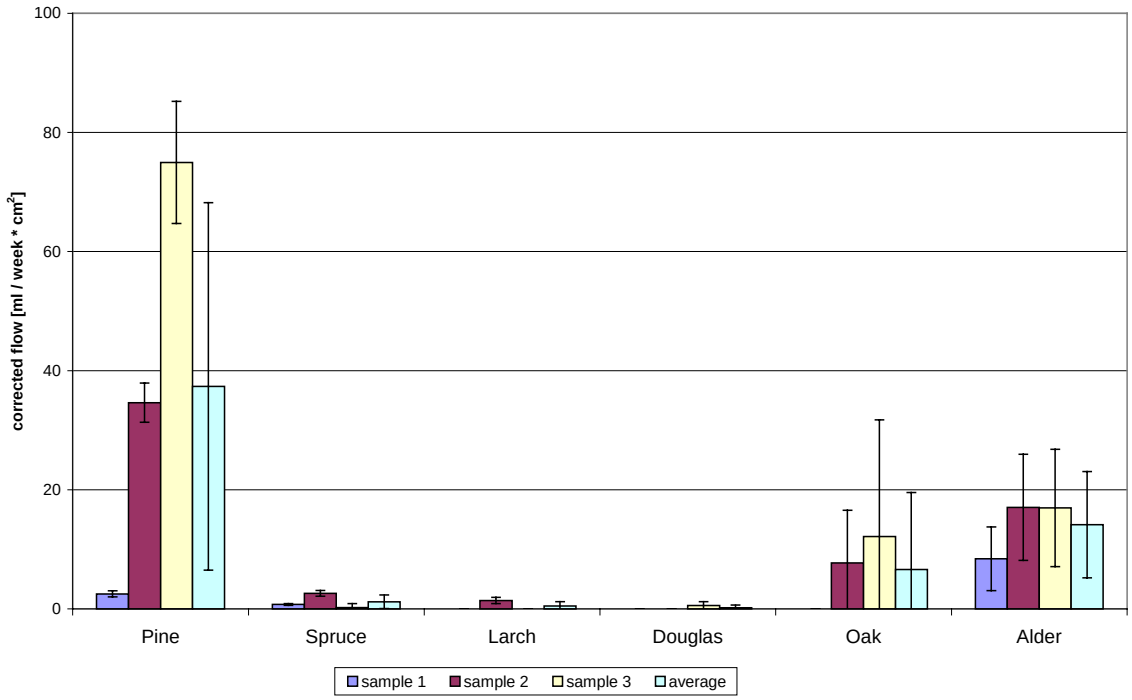


Figure 5. The average weekly water flow through each of the stems during 8 weeks. The water flow is calculated here on the sapwood areas only (as visually determined).

Experiment 2

In figure 6 the effect of pressure (water column height) upon the flow is shown for spruce and pine.

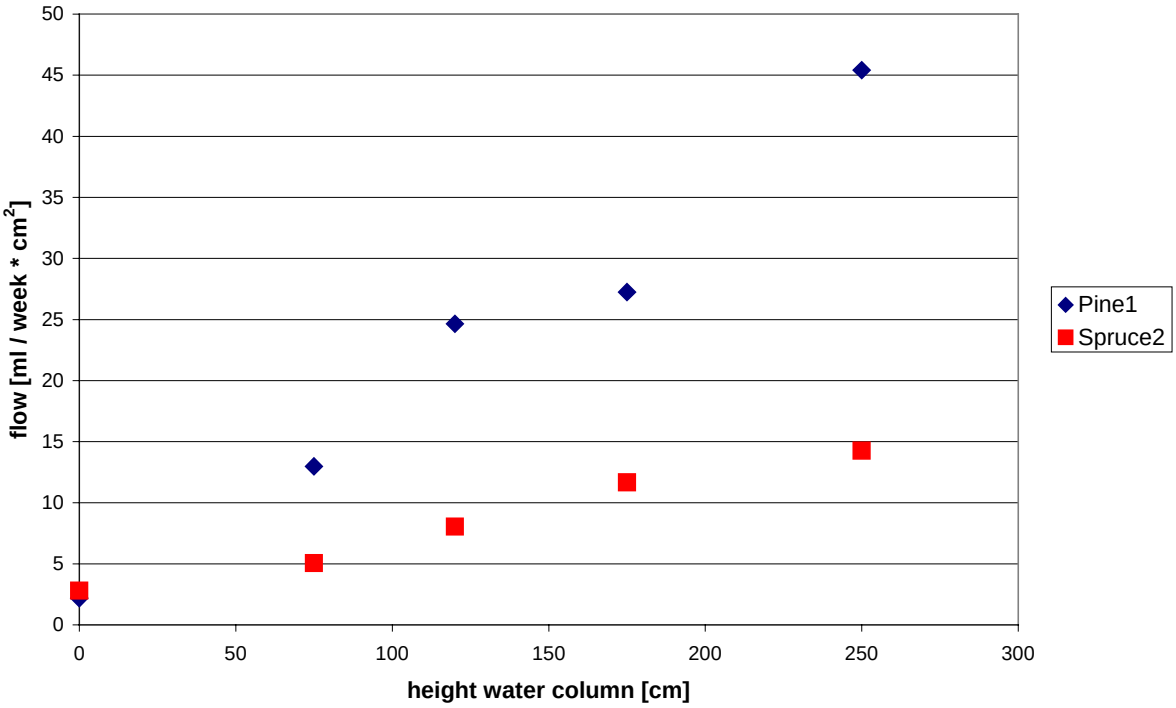
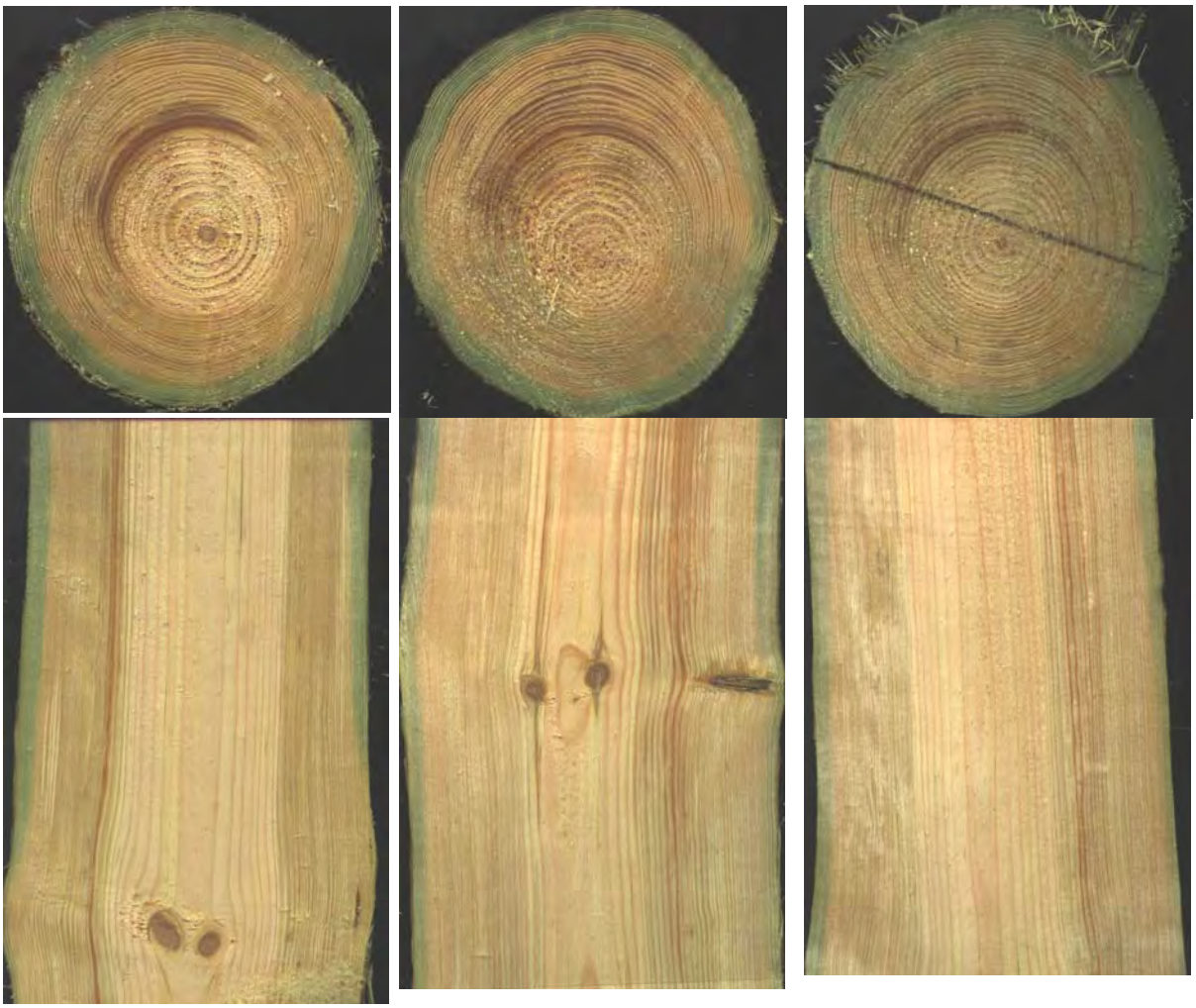


Figure 6. The effect of pressure (height water column on cross surface) on the water flow through one spruce and one pine stem.

Experiment 3
After 1-10 days no water was found which had penetrated the stem radial over a height of 90 mm, and flowed 300 mm downwards in the axial direction. After adding a stain in the water and open the stems after 15 days it became clear that there is no water flow but only a transport through diffusion. In the pictures below a cross section is shown of each of the piles just beneath the area were the stain was added. Furthermore the longitudinal section is shown.



Pile 1 Pile 2 Pile 3

6.4 Discussion

The velocity of the water flow in stems varies between and within species. The variability between wood species is caused by differences in anatomical structure.
In hardwoods the main water pathway is through the vessels and the velocity is depending on the vessel diameter, amount of vessel tissue and vessel length. Oak has long (up to 10 meters) and wide (> 200µm) early wood vessels. However, in each year ring the amount of early wood vessels is limited and within the first months of the growing session most early wood vessel can collapse and be filled with tyloses. Afterwards the water pathway depends on the latewood vessels which are smaller (< 80 µm) and shorter. After heartwood formation the wood tissue is much less water permeable owing to the increased production of tyloses and extractives. In alder, a species without heartwood, the percentage of vessel tissue is much higher on the cross surface than in oak but the vessels are smaller and shorter.

From the stained surfaces it can be seen that in alder over the whole diameter in every year ring more or less conducting tissue is present. On the other hand in oak only in the sapwood water can flow.
In softwoods, the axial pathway goes from one tracheid (length app. 3 mm) to another through the bordered pits or through ray cells by passing the cross field pits. In pine the cross field pits are simple and provide water with a low resistance pathway. In contrast, the cross field pits in spruce, larch and Douglas are picioide and offer more resistance to the water flow. The results give the suggestion that the presence of blue stain blocks the water flow through the stem. If the presence of these blue stain hyphae can block the tracheid-ray-connection it is hypothesised that these connections in pine are more important for the water flow through the stem than the intertracheid bordered pits connections. If those bordered pits would be important for the water flow, the presence of blue stain hyphae should lead to an increase rather than a decrease the water flow because they open bordered pits by degrading their membranes. However, in the blue stained wood the water flow is strongly reduced which leads to the assumption that the tracheid-ray connection could be the major pathway of water flow in pine.

A homogeneous staining of the upper layer of the stem over the whole diameter pointed towards the absence of a water conducting pathway. Water seems to be spreading through the wood only by means of diffusion through the cell walls while the wood structure is obvious blocked. This is clear in the pine heartwood, larch heartwood, Douglas, oak heartwood and for some whole stems of spruce.
Real water conduction is found in alder, and in the sapwood of pine, oak, Douglas and larch. Especially in pine sapwood the water conduction is obvious and in those parts of pine sapwood where water flow occurs, the whole woody tissue seemed to be involved. In alder, oak sapwood and in one of the spruce stems, water conduction is restricted to specific areas and the water flow is mainly through specific cell types only. In alder and oak it seems to be related to the density of the vessel tissue whereas in spruce it could be related to latewood tracheids of which it is known that they do not close their bordered pits. However the influence of the small crack can not be excluded. The observations in the softwood species and especially those in pine give the impression that the effect of the closure of the bordered pits on the water flow under atmospheric pressure conditions is negligible.
When increasing the water pressure on the stem it was seen that the water flow velocity did not only increase in the tracheid-ray-tracheid pathway (pine sapwood) but also in the intertracheid pathway (spruce). Although only one stem of spruce and pine was tested on the effect of increased water pressure, the influence was so clear for both species that groundwater dynamics should be regarded as one of the major factors that influence the water flux in stems with open as well as closed wood structures.

The velocity seems to increase in pine 3 and in oak 2-3 from week 4 onwards. Since this time the cross surface was weekly cleaned and the water was refreshed. It is suggested that debris has a closing effect on the wood structure. In both oak stems it could be related to the blocking of the wide and long early wood vessels whereas in pine the axial pith crack could be blocked. For the other stems less clear or no influences were seen. Probably the debris is too large to block small cross cut cells with diameters below around 100 µm.

From experiment 3, it became clear that the radial pathway to enter the stem offers water too high resistance to allow a axial water stream. The cross surfaces seem to be the only entrances for water in the wood in order to create a water flow through the wood.
So pressure differences of groundwater in specific soil layers are probably not connected with the water flux in the stems. Moreover, if the cross surfaces of stems in the soil are not connected to a soil water stream, no water flux in the pile will appear. In order to disable the stem water stream from the ground water dynamics the orientation of the cross surfaces of the stems should be rectangular on the direction of a groundwater gradient or the cross section should be sealed.

Where the experiments are carried out on sound wooden stems the influence of degradation on the water flow is not quantify. As the structure of degraded wood is more open it could stimulate the water movement through the wood but the debris can also have a negative effect on an open pipe system.

General hydrological aspects concerning western parts of the Netherlands

(by Keijer)

As water flow through piles is regarded as possible force behind bacterial wood degradation it has to be seen in relation with the hydrology around the pilings. As bacterial wood degradation is most prominent in Dutch foundation piles, a survey is given here of the general hydrology of the western part of the Netherlands

The general features of land and water in the Netherlands are characterised by the shaping of soil and landscape in geologically recent times. Sedimentation during the Pleistocene resulted in a vast and predominantly flat fluvial plain with mainly sandy soils, gently dipping to the Northwest. Depending on the transport capacity of the subsurface, a stream pattern developed in the course of time, which is still draining the excess water in large parts of the southern and eastern regions. Sea levels rose by several tens of metres in the Holocene age, which led to the deposition of clayey sediments on top of the Pleistocene sand in a broad coastal zone. Marshy areas originated more inland because of the rising groundwater levels, those areas being at the origin of large raised bogs with peaty soils.

Two major zones can be recognised in the Netherlands, characterised by their soil profile:

- Elevated sandy areas in the south east and geomorphologically formed during the Pleistocene;
- Low areas in the north and west where soft Holocene peat and clay deposits are present on the Pleistocene. In these areas piled foundations are applied for cities like Amsterdam and Rotterdam.

The detailed drainage system in the Holocene lowlands of the Netherlands is almost entirely artificial and based on the discharge of excess water by pumping. Most of the surface peat layers have been eroded by floods or have been excavated to supply fuel. Large lakes were created by this peat mining in the coastal regions. Many lakes were later reclaimed and made into polders, having a clayey soil.

Surface water plays an important sole in the discharge of excess water, although in the relatively elevated regions with sandy soils this role is different from that in the coastal zones. Almost everywhere in the low polder areas water levels are artificially controlled by a forced discharge, but on higher grounds the drainage of water is mostly by gravity.

The groundwater hydrology is controlled by the presence and the lithology of unconsolidated sediments, deposited in a subsiding basin. The axis of the basin dips to the Northwest resulting in the largest thickness of the Pleistocene and Holocene formations in the west of the country. Thick aquifer systems are present in the western part.

The Holocene sediments consist predominantly of clay and peat layers, deposited in a lagoonal and deltaic environment, due to the post-glacial sea level rise. They are present in a broad coastal zone and they can reach a thickness of more than 20 m near the coast.

A dune ridge has originated at the coast with an aquifer system underneath containing a fresh water lens. The shape of the lens is determined by the width of the dune zone and the rate of groundwater recharge. Land-inward, the Holocene layers thin out; they are almost absent in the eastern and southern parts of the country.

In areas covered by the Holocene deposits, the same Pleistocene aquifer system is present in the subsurface, but confined by shallow clay and peat layers. The groundwater recharge in the coastal regions consists of lateral inflow of groundwater arriving from the higher sandy areas, often in combination with a local recharge by water infiltrating from actual river beds or from former river and gully beds where the soil consists of sandy stream deposits.

The fresh groundwater underneath the coastal dunes rests on a body of brackish groundwater. The brackish groundwater is not fully stagnant; it will generally move land-inward, but mostly at a lower flow rate than the fresh groundwater above it.
The recharge of groundwater in the Netherlands is complicated because it depends on the local topographical situation. Infiltration of rainfall is the predominant form of recharge in the sandy areas.
The aquifer system in those coastal regions covered with clay or peat layers receives a relatively very small of even no recharge from local precipitation. Practically the full excess precipitation is discharged by surface flow to nearby open water courses, except for the sandy dunes. Parts of the coastal dunes have become important sites for artificial recharge by surface water transported from the rivers Rhine and Meuse to the dunes and infiltrating from ponds or canals. The groundwater flow pattern can be shown by isohypses, representing lines of equal heads. Regional isohypses valid for the Netherlands indicate the large-scale directions of the horizontal groundwater flows. Discharge areas can take the form of a river zone, where the shallow groundwater will have an upward direction and seep into open water courses. The deeper groundwater may continue to flow in the direction of draining water courses farther away and even into the coastal zone. Some of the polder areas in the western and central Netherlands discharge incoming groundwater flows, which originated in sandy areas far away from the polder. However, much of the groundwater in those polders is recharged by surface water, infiltrating at nearby riverbeds or coming from other surface waters. Prominent examples of areas receiving large amounts of seepage water are those in the western part of the Netherlands, where the groundwater level are 4 m below mean sea level. The deep polders are the focal points of regional groundwater flows.
In the Netherlands the chloride content is an important natural component when considering groundwater composition. In recent years components such as nitrate and phosphate increased in the fresh groundwater reserves due to human activities.
Consequently attention is nowadays paid to those chemical components as well.
The salt brought in by the various floodings of the sea during the Holocene period can still be recognised in the shallow soil, but the chloride concentration in the groundwater is relatively low, if compared to the chloride levels of sea water.
The salt content in the shallow subsurface was redistributed by the creation of high and low polders in the coastal region, resulting in the intensification of groundwater flow and changes in flow patterns. Shallow groundwater in the coastal cone is often brackish, but the groundwater in the sandy regions will generally be fresh.

Considering above given descriptions, hydrology proofs to be an important factor at the sites where piles were investigated / extracted. Situations with infiltration and upwelling groundwater, as well as aquifers (topsoil and deeper sand layers) with different levels of water pressure and water quality were found. The difference in piezometric level in water pressure between pile head and pile tip can extend to more than 2 meters of water column. During the project it became clear that a possible water flow through the piles could be essential for the differences in the decay at the investigated sites. This hypothesis was the basis for the water flow experiments.

6.5 Conclusions

From the tests three main results can be deduced but it has to be realised that because of the restricted amount of replica these results can only be regarded as indicative:

1. The water permeability of wooden stems is species dependent and restricted to the axial direction. The highest permeability is found for pine sapwood, less permeable are alder over the whole diameter and oak sapwood. Visible heartwood is not permeable. Spruce and the sapwood of larch and Douglas have an intermediary permeability.
2. Water pressure on the top stem surface is positively correlated with water flow through the stem.
3. Blue stain infection seems to decrease the water flow through the stems

If a water flow through the wood is related to the process of bacterial wood degradation, these results offer possibilities to stop this process. Sealing of the cross section is one possibility and using blue stain infection could be another one.

Chapter 7

Erosion bacteria and sulphur

(by Huisman)

7.1 Introduction

The presence of reduced sulphur compounds in archaeological wood poses great problems with respect to long-term *ex situ* conservation. In an oxidising atmosphere, the compounds tend to oxidise. As a result the wood becomes covered with yellow, orange and sometimes white mineral blooms. Moreover, the oxidation of the sulphur compounds produces sulphuric acid, which causes major damage to the wood structure. The most well-known example where such problems occur is the Swedish Warship Vasa, where this problem occurs on a major scale (Sandström et al., 2002). It is, however, not unknown in other places, e.g. Roskilde (Jensen, *pers. comm.*) and Lelystad (the Ventjager; *unpublished data*). Presently, a large research effort is being put into developing methods of protecting archaeological wood - i.c. the Vasa - against the oxidation of reduced sulfur species in the wood.

7.2 Sulphur in BACPOLES

For the BACPOLES project, the problem of reduced sulphur species in wood is relevant as long as it is connected to erosion bacteria. In this section, an overview will be given of what is known from literature and of the interaction of reduced sulphur species formation with erosion bacteria. The origin of the reduced sulphur species ánd the oxidation processes that occur under oxic conditions are well known from the fields of soils science ("acid sulphate soils"), geochemistry (pyrite formation and "acid mine drainage") and conservation of marine iron artefacts. The processes leading to the accumulation of reduced sulphur species in wood can be subdivided into two types: The first type are the processes that cause the formation of iron-sulphur (Fe-S) corrosion scales on iron objects (including nails) in or next to the wood. The second type are the processes of formation of sulphur compounds that occur naturally on the sea floor sediments, and that may also occur in buried wood:

Metallic iron oxidises very easily. In the presence of oxygen it will react with it to form Fe^{3+} (precipitating as Fe_2O_3 and FeOOH). In the absence of oxygen, other electron acceptors are needed to oxidise the iron. In reduced marine settings, the sulphate (SO_4^{2-}) that is abundantly available from seawater usually takes on this role. It is reduced to sulphide according to:

$$(1) \qquad SO_4^{2-} + 8\,e^- + 5H_2O \rightarrow HS^- + 9OH^-$$

However, it is not able to oxidise Fe further than Fe^{2+}:

$$(2) \qquad Fe^0 \rightarrow Fe^{2+} + 2e^-$$

Fe^{2+} and HS^- subsequently precipitate as ironmonosulfides:

$$(3) \qquad HS^- + Fe^{2+} \rightarrow FeS + H^+$$

Combining half reactions (1) and (2) with the precipitation reaction (3) gives the overall reaction:

$$(4) \qquad 4\,Fe^0 + SO_4^{2-} + 4\,H_2O \rightarrow FeS + 3\,Fe^{2+} + 8\,OH^-$$

This reaction results in the formation of a scale of black iron sulphides with sediment particles that surrounds the iron object (fig 1), or - if the corrosion has progressed far enough - a cavity in the shape of the original object. If the iron object is surrounded by wood, the precipitating iron sulphides will precipitate inside the wood (fig 2).

When no elementary iron is present, the formation of iron sulphides and other reduced sulphur species is triggered by the reduction of sulphate trough organic matter, which is traditionally described as:

(5a) $SO_4^{2-} + 2\ CH_2O \rightarrow HS^- + 2\ CO_2 + OH^- + H_2O$

The reaction of organic matter here represented as CH_2O however, is in fact more complex: It involves fermentation processes that produce hydrogen gas (H_2), methane (CH_4), and short-chain organic molecules like acetate (CH_3COOH). These components then react with the sulphate forming HS^-. The sulphide formed in this way can react with Fe^{III} minerals like goethite (FeOOH) and magnetite (Fe_2O_3) that are present in the sediment according to the overall reaction:

(6a) $2\ FeOOH + HS^- + H_2O \rightarrow S^0 + 2\ Fe^{2+} + 5\ OH^-$

Figure 1: Example of iron object - two iron chain rings - with a scale of FeS-encrusted sand. (De Meern 1)

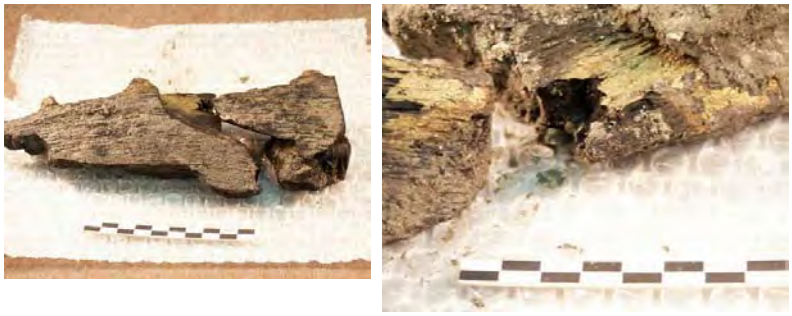


Figure 2. Fragment of wood with iron nails; iron sulfides - a lustrous gold color - have formed in a crack inside the wood.

The Fe^{2+} that is formed in this way reacts with the HS^- present (see reaction 5) to form an iron monosulphide (reaction 3). Part of the iron may also be formed by direct reduction with organic matter analogous with reaction (5) but this is not necessary. This monosulphide is usually relatively unstable. In most environments, it reacts with the elementary sulphur formed in equation (6) to form pyrite:

(7) $FeS + S^0 \rightarrow FeS_2$

Pyrite is a relatively stable mineral, and will only be attacked under reducing conditions. It is often present in a so-called framboidal shape; it then consists of groups of spheres each of which is built-up of a large number of separate pyrite crystals. However other shapes (large crystals, massive concretions) sometimes occur also.

If exposed to oxygen, reduced sulphur species will react with it to produce iron oxides and sulphuric acid according to (sulphuric acid in red):

(8) $4FeS_2 + 15O_2 + 10H_2O \rightarrow 4FeOOH + 8SO_4^{2-} + 16H^+$

The oxidation reactions for FeS and S^0 are similar and also produce sulphuric acid. While this reaction goes on, intermediate products may be formed including Fe(II,III) sulphates (e.g. $FeSO_4$) Sulfur (S^0 or S_8) and Jarosite $(K,Na,H)Fe_3(SO_4)_2(OH)_6$. In the presence of lime, gypsum ($CaSO_4$) may be formed also. Mineral "blooms" of yellow jarosite accompanied by orange-red iron oxyhydroxides and sometimes white gypsum are indicators for the occurrence of these reactions.

7.3 Conclusions

Pyrite seems to occur quite frequent in wood from both piles and archaeological sites (Klaassen and Nilsson, pers. comm). This indicates that the environment inside the wood has become sulfidic (i.e. anoxic) at some point up to the moment of sampling. It also indicates that sulphate was available inside the wood. Assuming that erosion bacteria were active during the formation of the pyrite, this suggests that erosion bacteria are at least facultative anaerobes, as oxygen is not present in sulfidic environments.
The formation of sulphides 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 sulphides towards erosion bacteria may be the basis for a potential method of inhibition of degradation; injection of sulphate in wood would potentially reduce the speed of erosion bacteria degradation. However, it would not be able to completely halt this degradation mechanism.

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.

Chapter 9

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Appendix 3

Chemical wood data

(by Gelbrich)

The investigated contents of monosaccharides, phenols, other extractives and ash show no association with the degree of bacterial attack. All investigated samples show drastically reduced contents of extractives and therefore also of phenols and monosaccharides whereas the ash content is always drastically increased. Because these results show no correlation to the degree of bacterial attack, the long period of wet storage of these samples could be a reason for these changes. The wet conditions could be the causer of wash out of monosaccharides, phenols and other extractives. The increased ash content could be a result of soil components, coated or infiltrated into the wood during the period of pile storage in the soil.

The chemical analyses of the wood show that bacterial attack results in a reduction of the holocellulose content and therefore increased percentage lignin content. Similar results were reported by Troya *et al*. (1989a, b), Blanchette *et al*. (1991) Singh and Hedley (1991) and others.

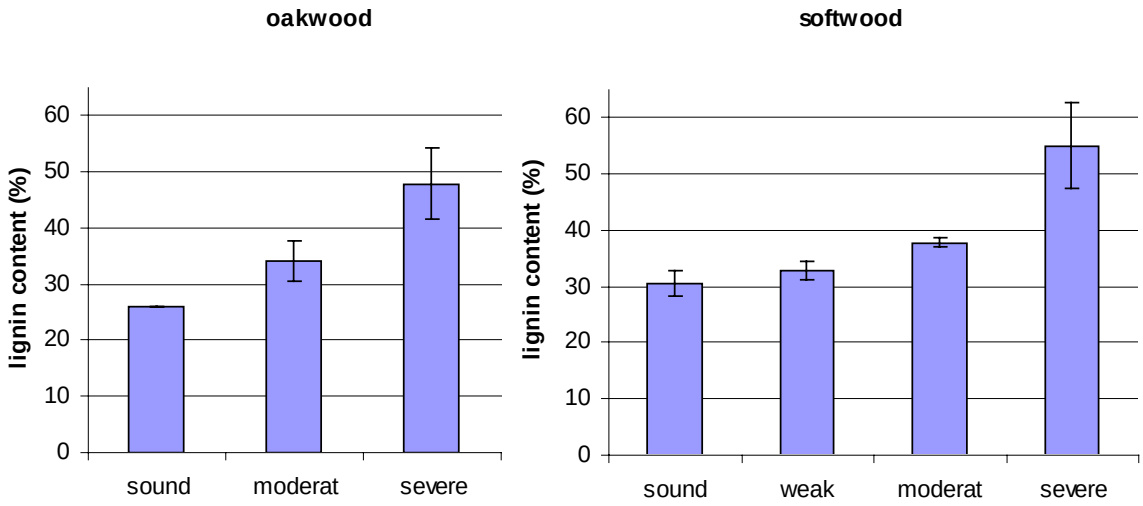

Figure: Mean lignin content (%) in relation to the microscopical classification of the bacterial attack (done by SHR) of the samples.

For the bigger softwood samples a gradient could be detected from outside zones to inside zones. The lignin content or the degree of attack respectively was in outside zones higher than in the heart of the samples. That supports the observations by e.g. Harmsen and Nissen (1965), Courtois (1966) and Grinda (1997) that bacterial degradation starts in outside zones and proceeds slowly inwards. Because of the longer storage period of the archaeological pile samples and of the restriction in the sample number, these samples were not investigated in different zones. All results of the oak pile samples were from the outside zone.

The other archaeological samples were not round wood but timer, sawn wood. Maybe because of the smaller dimensions and the long storage period, these samples were completely destroyed by bacteria. The mean lignin content of these samples is 44,6 % compared to the reference value of 25,9 %. That the sapwood of sawn wood is heavier attacked after the same storage period compared to round wood was shown by Grinda (1997) for Scots pine. Even the heartwood of sawn samples was attacked whereas the heartwood cells of the piles were never attacked.

Not only the smaller dimension of the samples is a reason for heavier attack, the fact that they are sawn results also in a higher degree of bacterial degradation.

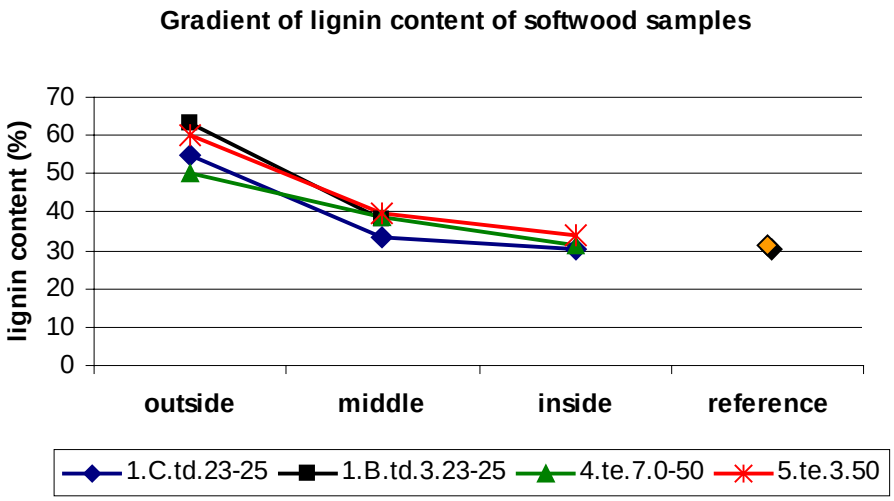

Figure: Gradient of lignin content and therefore gradient of degree of bacterial attack form outside to inside zones of softwood samples.

The relations of single cellulose components (α-, β- and γ-cellulose) of the investigated samples were not correlated with the degree of bacterial degradation. In the following figure it can be seen that samples with nearly the same lignin content (grey) have differences in the contents of the three holocellulose components.
It is conspicuous that in all samples compared to the reference the content of α-cellulose is reduced and the content of β-cellulose is increased. That means that even in samples with a normal lignin content, in which no sign of bacterial attack was found by microscopy, changes in the chemical composition occurred. In these samples the content of γ-cellulose is increased. The reason for that seems to be in the changes of the percentage ratio of the holocellulose components, because the content of α-cellulose is more reduced than the content of β-cellulose is increased. The possibility that the chemical changes results in compounds which were additional detected in the γ-cellulose fraction is improbable.
The content of γ-cellulose is decreased only in samples with heavy bacterial attack. That could be an indication that γ-cellulose, which codes the hemicellulose fraction, were degraded slower or even in a later stage of bacterial wood degradation. But the γ-cellulose content is sometimes also decreased in sound inside zone samples, which are mostly heartwood samples.
The β-cellulose is described as short chained, which means degraded cellulose fraction. It is a result of a degradation process because of its nutrient character for the wood degrading bacteria. That is the reason why the content of the degraded cellulose fraction is always only slightly increased, even in heavy decayed samples. So the DP of cellulose is only slightly reduced like described in Blanchette *et al*. (1990). Even if the degraded cellulose would not be a real nutrient, a detection of a β-cellulose accumulation would be difficult because of the wash out effect of the storage conditions.

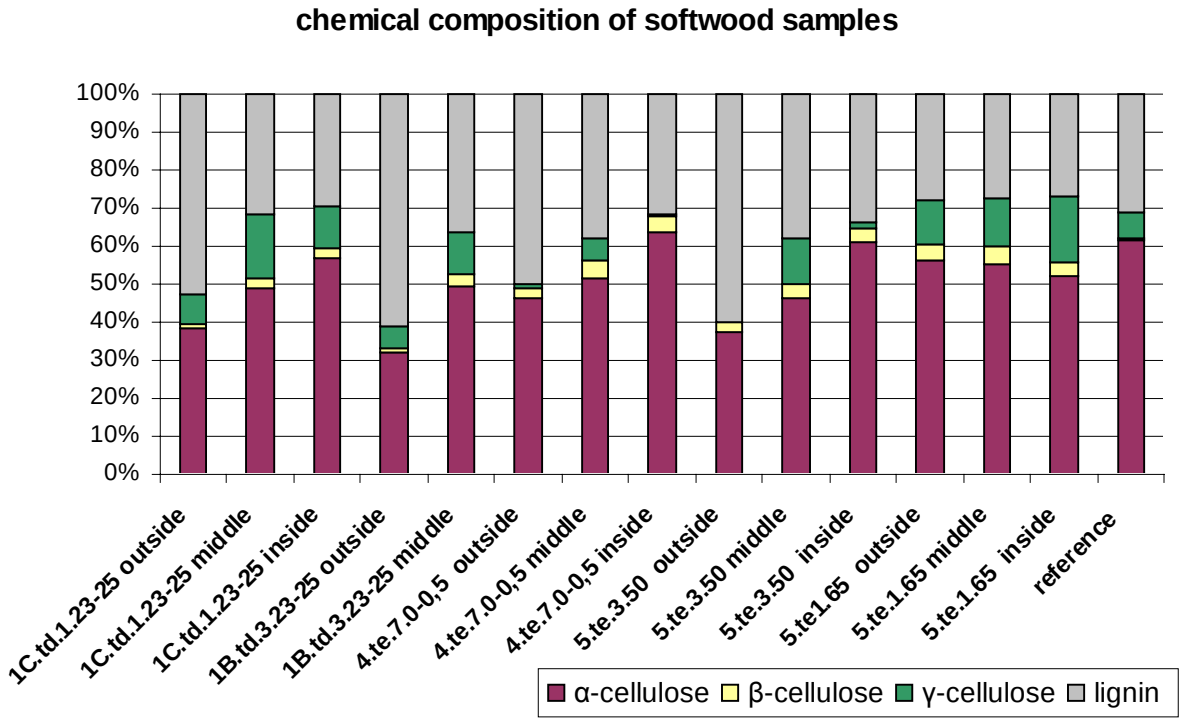

Figure: Distribution of the contents of lignin as well as α-, β- and γ-cellulose in the subdivided softwood samples

IR spectroscopy

The measurements by the relative fast method of IR spectroscopy reflect the softwood results of chemical wood analysis. With increasing of the degree of bacterial attack higher absorbance values at the lignin characterised peaks were measured. The absorbance of IR radiation depends on chemical bonds in the wood compounds. So lignin for example is characterised by some peaks because of different typical chemical bonds or groups. The results of IR-spectroscopy are only optical results in comparison to reference values, i.e. the absorption on the lignin or hemicellulose characterised peak is higher or lower than the reference. Direct quantitative statements about the content of a wood compounds are not possible. But the absorbance values are connected to the lignin content, determined chemically.

For example the absorbance between the wave numbers 1502-1506 represents C=C stretching vibration in aromatic ring in lignin or/ aromatic skeletal in lignin (Kimura *et al.*, 1992; Evans *et al.*, 1992; Pandey and Theagarjan, 1997; Rodrigues, 1998; Schulz and Glasser, 1986). The correlation of these absorbance values of the samples and their lignin content can be seen in the following figure.

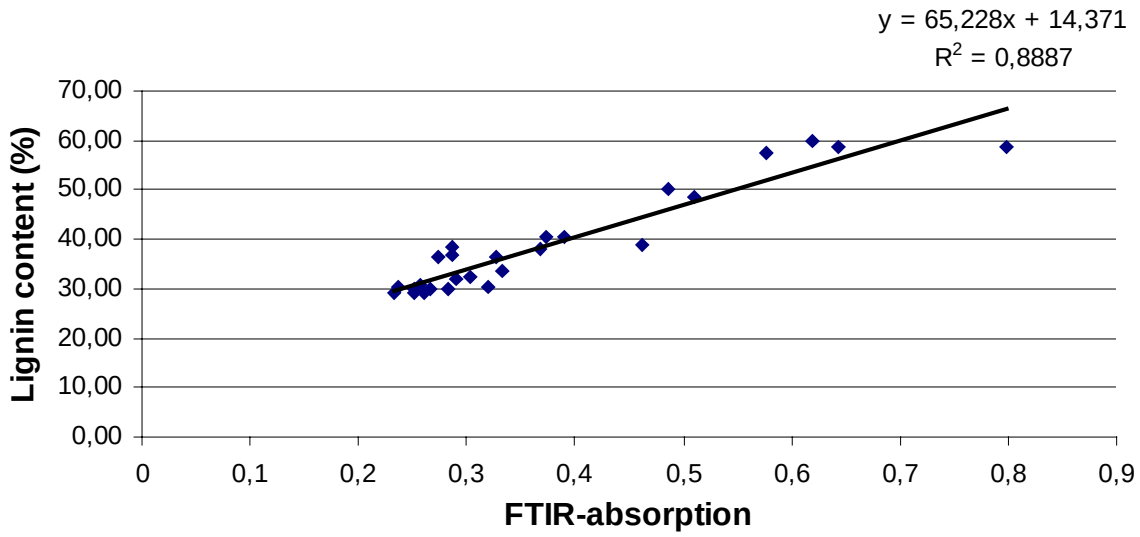

Figure: Correlation between the absorbance values at wave number 1505 and the lignin content of softwood samples.

For the oak samples no correlation could be found. A reason for that could be that all oak samples were archaeological wood. So chemical change processes are conceivable because of the very long storage period.

Element contents

The analyses of the wood elements indicate that the nitrogen content increased with the degree of bacterial attack. The phosphorus content shows a similar, but not so clear trend. These results confirm the report of Boutelje and Goransson, that the degradation of foundation piles increased with increased nitrogen and phosphorus (Blanchette *et al.,* 1990).The contents of the other elements like sulphur, sodium, calcium, potassium and ferron differ from the reference values but without a trend related to the bacterial attack.

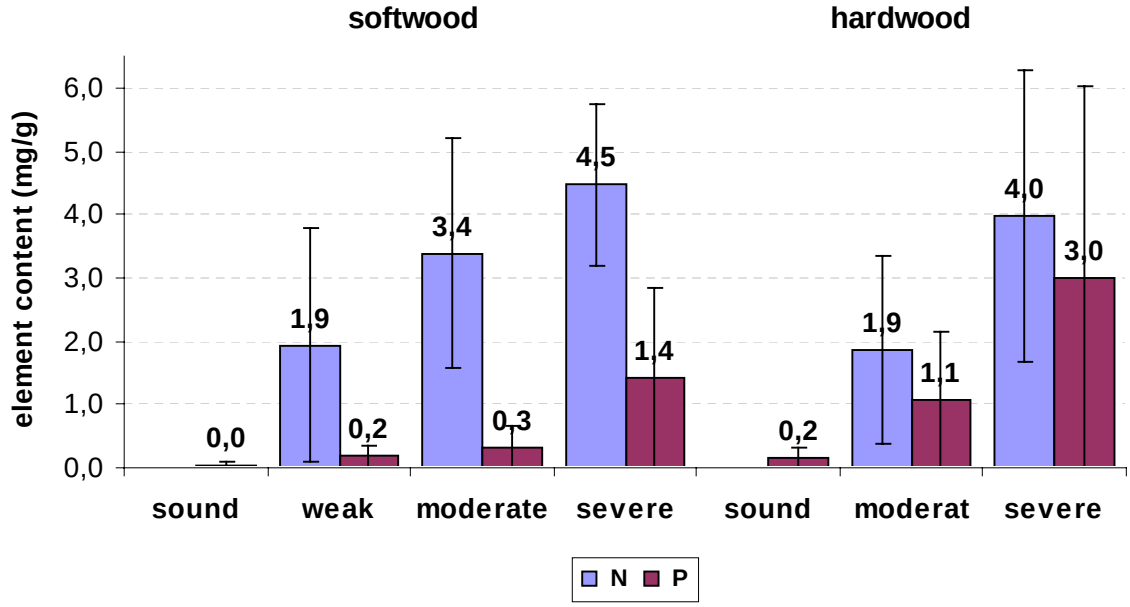

Figure: Mean content of nitrogen and phosphorus assigned to the classes of bacterial degradation of soft- and hardwood. (N content of sound wood is below detection limit.

Site 1: Foundation Amsterdam (NL)														
pine	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
1C.td.1.23-25 o	3,85	4,12	2,64	10,62	6,71	0,18	0,25	0,43	48,46	39,99	88,58	3,14	7,91	6,70
1C.td.1.23-25 m	0,26	0,64	0,18	1,08	2,87	0,08	0,37	0,45	32,04	64,45	77,44	4,64	16,34	7,89
1C.td.1.23-25 in	1,33	3,12	1,29	5,73	4,14	0,51	1,83	2,34	30,12	69,01	84,17	4,31	11,27	4,06
1C.td.1.120 out	0,42	0,07	0,00	0,49	5,38	0,34	0,60	0,94	38,45	57,29				
1B.td.3.23-25 o	4,05	3,32	2,49	9,86	11,72	0,06	1,31	1,37	59,76	34,85	89,80	4,31	5,31	11,11
1B.td.3.23-25 m	3,00	3,15	1,37	7,52	5,56	0,17	2,94	3,11	37,93	62,10	82,91	5,70	10,97	3,80
1B.td.3.120 out	0,57	0,08	0,01	0,66	6,75	0,29	0,19	0,48	32,64	65,25				
ref. sapwood	1,38	1,73	2,64	5,75	93,67	5,47	10,85	16,32	30,13	65,07	92,18	1,04	6,27	0,51
ref. heartwood	2,56	2,80	5,91	11,27	25,37	7,18	12,01	19,19	32,05	63,81	91,62	0,92	7,46	0,38

Site 2: Foundation Dordrecht (NL)														
spruce	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
2.te.3.30-100 out	0,29	0,06	0,01	0,36					36,83	61,91				
2.te.3.30-100 m	0,30	0,09	0,00	0,39					34,57	65,62				
2.te.3.30-100 in	0,29	0,16	0,00	0,45					30,16	68,97				
2.te.2.35-100 out	0,24	0,03	0,01	0,28					30,68	68,26				
2.te.2.35-100 m	0,19	0,07	0,01	0,26					29,31	69,38				
2.te.2.35-100 in	0,30	0,12	0,02	0,44					29,79	69,83				
ref. Sapwood	1,42	1,76	0,33	3,51	85,37	6,51	10,98	17,49	28,20	79,96	94,64	1,27	5,07	0,48
ref. Hardwood	1,19	2,74	0,46	4,39	34,76	7,34	10,27	17,61	31,37	78,61	92,84	1,18	6,63	0,41
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
2.td.2.35-100 out	412,01	5,39	0,47	7,71	0,44	0,82	20,21	0,83	0,10	5,18	2,59			
2.td.3.30-100 out	473,80	3,68	0,22	5,03	0,19	0,43	6,94	0,41	0,04	2,09	1,45			
ref. spruce	503,83	0,62	0,05	0,07	0,01	0,62	0,56	0,09	0,21	0,07	0,01			

Site 4: Foundation Haarlem (NL)															
pine	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**	
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**	
4.te.7.0-50 out	0,56	2,26	0,18	3,00	3,26	0,05	0,91	0,96	50,03	49,30	93,48	5,34	0,95	5,16	
4.te.7.0-50 m	0,29	4,37	0,14	4,79	4,75	0,32	1,82	2,14	38,66	60,83	86,63	7,27	5,94	3,37	
4.te.7.0-50 in	2,23	10,95	0,16	13,34	6,46	1,34	2,94	4,28	32,23	69,93	93,44	6,15	0,37	1,96	
4.te.1.0-64 out	0,88	0,36	0,01	1,25	4,12	0,61	1,09	1,7	63,81	35,05					
4.te.3.0-45 out	0,59	0,14	0,01	0,74	3,64	0,08	1,72	1,8	64,09	35,54					
4.te.3.45-92 out	0,33	1,72	0,80	2,85	3,22	0,34	0,67	1,01	57,34	45,92	87,67	5,80	6,18	2,68	
ref. sapwood	1,38	1,73	2,64	5,75	93,67	5,47	10,85	16,32	30,13	65,07	92,18	1,04	6,27	0,51	
ref. heartwood	2,56	2,80	5,91	11,27	25,37	7,18	12,01	19,19	32,05	63,81	91,62	0,92	7,46	0,38	
element contents of wood															
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**				
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g				
4.TE.7.0-50 out	489,85	4,42	2,82	6,32	0,33	0,20	5,85	0,22	0,03	0,35	0,04				
4.TE.7.0-50 m	470,02	1,85	0,29	3,58	0,26	0,16	3,29	0,11	0,01	0,08	0,00				
4.TE.3.0-45 out	495,93	3,22	0,90	1,67	0,25	0,20	4,68	0,14	0,03	0,46	0,05				
ref. pine	509,61	0,989	0,051	0,061	0,021	0,405	0,837	0,222	0,042157	0,016647	0,01458				

Site 5: Pile extraction Rotterdam (NL)														
spruce	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
5.te.3.50 out	1,14	2,26	1,29	4,69	2,87	0,26	0,46	0,72	58,68	39,34	93,29	6,57	0,19	6,63
5.te.3.50 middle	0,63	1,28	0,79	2,69	3,17	0,64	0,32	0,96	40,42	61,26	80,99	5,96	12,62	4,90
5.te.3.50 inside	0,46	1,19	0,77	2,41	2,93	0,04	1,21	1,25	36,26	70,41	93,26	5,07	1,29	0,46
5.te.1.65 out	0,91	1,53	0,08	2,52	2,98	0,03	0,26	0,29	29,05	72,31	81,98	6,04	12,28	3,19
5.te.1.65 middle	0,08	0,91	0,11	1,10	2,23	2,40	1,41	3,81	29,20	73,64	79,84	6,92	12,09	0,57
5.te.1.65 inside	1,46	1,68	0,04	3,18	2,41	1,83	4,34	6,17	29,26	73,42	76,47	5,51	17,38	0,58
5.te.1.400-450 o	1,30	1,67	0,81	3,78	1,73	0,07	0,34	0,41	28,75	76,57	93,78	29,14	5,61	4,34
5.te.1.400-450 m	1,51	1,56	0,91	3,98	1,76	1,80	0,91	2,71	28,12	75,85	58,48	35,24	6,28	1,83
5.te.1.400-450 in	1,71	2,08	1,56	5,35	1,90	3,18	2,15	5,33	28,15	75,99	78,86	26,55	5,94	0,64
5.te.2.400-450 o	2,24	2,01	1,59	5,84	2,21	0,37	0,71	1,08	29,24	72,43	78,72	10,96	4,57	3,49
5.te.2.400-450 m	2,56	1,94	1,61	6,11	2,52	0,04	2,57	2,61	28,19	71,95	61,46	35,73	6,82	0,64
5.te.2.400-450 in	2,84	3,01	1,37	7,22	2,71	1,91	3,28	5,19	30,07	69,31	83,66	18,29	5,18	0,51

ref. Sapwood	1,42	1,76	0,33	3,51	85,37	6,51	10,98	17,49	28,20	79,96	94,64	1,27	5,07	0,48
ref. Hardwood	1,19	2,74	0,46	4,39	34,76	7,34	10,27	17,61	31,37	78,61	92,84	1,18	6,63	0,41
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
5.te.1.65 out	477,09	0,00	0,07	2,88	0,13	0,06	3,04	0,18	0,04	0,18	0,16			
5.te.2.50 out	478,36	0,00	0,02	1,12	0,14	0,10	3,17	0,22	0,04	0,80	0,21			
5.te.2.50 out	455,52	0,00	0,04	1,33	0,13	0,08	2,43	0,11	0,02	0,27	0,02			
5.te.3.50 out	457,98	0,00	0,03	2,48	0,10	0,06	2,05	0,08	0,02	0,18	0,03			
5.te.3.50 m	458,15	0,00	0,02	1,78	0,12	0,05	2,12	0,10	0,03	0,16	0,00			
5.te.3.50 in	463,27	0,00	0,03	0,91	0,11	0,05	2,03	0,11	0,03	0,30	0,02			
ref. spruce	503,83	0,62	0,05	0,07	0,01	0,62	0,56	0,09	0,21	0,07	0,01			

Site 6: Pile extraction Koog a/d Zaan (NL)														
pine	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
6.te.1.0-50	0,91	0,99	0,23	2,13	7,71	0,18	1,43	1,61	36,96	64,02	73,47	5,29	17,01	2,06
6.te.2.0-50	1,05	5,42	0,24	6,71	5,47	0,34	0,57	0,91	41,33	60,39	76,50	6,49	14,29	3,49
6.te.3.0-50	1,15	2,58	0,09	3,82	3,12	1,05	1,43	2,48	34,37	68,42	73,21	5,55	16,87	1,32
6.te.1.400	2,16	0,82	0,21	3,19	1,74	0,31	0,15	0,46	31,11	76,96	72,54	39,08	6,18	1,91
6.te.2.400	1,99	0,99	0,10	3,09	1,71	0,05	1,49	1,54	34,52	76,48	64,75	40,55	5,91	2,61
6.te.3.400	2,17	0,93	0,16	3,26	2,34	0,07	0,52	0,59	32,31	77,07	68,94	23,88	6,24	2,51
ref. sapwood	1,38	1,73	2,64	5,75	93,67	5,47	10,85	16,32	30,13	65,07	92,18	1,04	6,27	0,51
ref. heartwood	2,56	2,80	5,91	11,27	25,37	7,18	12,01	19,19	32,05	63,81	91,62	0,92	7,46	0,38
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
6.te.1.50	480,69	0,00	0,17	2,64	0,10	0,03	1,40	0,16	0,05	2,51	0,05			
6.te.2.50	490,21	2,94	0,21	3,50	0,16	0,04	2,46	0,24	0,03	1,87	0,05			
6.te.2.50-100	464,85	0,00	0,09	1,45	0,14	0,07	2,02	0,11	0,01	0,06	0,03			
6.te.3.50	484,13	2,10	0,15	2,72	0,16	0,05	3,35	0,22	0,02	0,42	0,04			
ref. pine	509,61	0,99	0,05	0,06	0,02	0,41	0,84	0,22	0,04	0,02	0,01			

Site 8: Terrestrial archaeology 1 Dokkum (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
8.td.1.30	3,37	1,50	0,03	4,89	7,44	7,17	43,45	50,62	28,07	64,40	77,57	4,86	15,85	4,71
8.td.2.0	3,66	2,27	0,40	6,32	3,14	3,71	40,91	44,62	56,62	61,61	55,77	4,66	20,19	12,83
8.td.3.0	6,23	0,54	0,22	6,99	3,40	3,21	32,28	35,49	54,48	59,96	64,93	7,88	19,07	9,98
8.td.4.0	6,17	4,65	0,02	10,84	18,10	21,41	56,23	77,64	37,80	63,72	68,07	13,35	16,21	4,66
8.td.5.0	3,62	3,09	20,03	26,75	10,02	10,47	51,71	62,18	37,69	65,24	72,20	4,24	14,90	6,25
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
8.td.1.64	451,84	1,74	0,31	2,13	0,23	0,41	7,68	0,59	0,18	2,83	0,05			
8.td.2.32	415,73	6,14	6,90	13,04	0,45	0,89	16,34	1,35	0,55	19,96	1,86			
8.td.4.64	449,92	2,13	2,57	3,89	0,27	0,44	9,52	0,84	0,12	1,38	0,02			
8.td.5.45	465,78	3,58	0,99	4,28	0,43	0,44	15,20	0,60	0,24	4,43	0,29			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Site 9: Terrestrial archaeology 2 Dokkum (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
9.td.1	2,91	2,48	7,96	13,36	3,12	3,14	6,94	10,08	54,83	40,86	58,91	11,22	25,41	16,83
9.td.2	1,37	2,63	4,84	8,84	1,40	1,48	6,59	8,06	50,86	46,75	54,21	3,88	36,19	12,41
9.td.3	0,75	3,18	3,48	7,41	1,50	1,62	5,57	7,19	49,37	48,99	60,08	10,74	26,14	15,84
9.td.4	2,46	1,89	6,81	11,16	2,02	2,11	5,61	7,72	43,90	43,10	68,64	9,11	19,37	13,14
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23

Site12: Marine archaeology (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
12.td.1	1,37	1,00	4,37	6,74	6,43	6,60	3,71	10,31	40,53	57,76	76,05	18,57	5,91	16,83
12.td.2	1,81	2,09	8,19	12,09	14,81	6,87	5,58	12,45	42,12	55,78	83,58	9,21	6,27	12,41
12.td.3	0,61	1,08	1,82	3,51	3,93	4,04	0,14	4,18	36,24	53,08	77,02	20,60	5,18	15,84
12.td.4	0,76	1,67	3,81	6,24	5,54	4,67	0,14	4,80	41,76	51,56	73,77	23,99	6,07	13,14
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
12.ta.1.1	420,16	3,66	0,48	23,63	18,55	1,18	3,10	5,30	0,17	23,00	0,15			
12.ta.1.2	404,34	4,99	0,92	27,80	21,06	0,44	3,09	5,20	0,24	21,89	0,19			
12.ta.1.4	432,05	4,78	0,96	19,52	20,93	1,13	3,67	5,23	0,12	12,77	0,13			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Site 14: Kemlade Travenhorst (D)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
14.ta.1.98-153o	1,42	0,65	0,02	2,08					32,81	66,06				7,59
14.ta.2.15-23 o	1,25	0,63	0,03	1,91					34,19	65,72				5,91
14.ta.2.62-89 o1	0,77	0,19	0,17	1,13					58,84	40,58				6,36
14.ta.2.62-89 o2	1,12	0,48	0,04	1,64					31,93	66,85				8,46
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
14.ta.2.15-23	498,44	8,18	0,74	10,17	0,15	0,14	11,89	0,28	0,07	2,21	0,28			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Site 23: Foundation Leeuwarden (NL)														
pine	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
23.td.1.50 o	0,55	0,09	0,01	0,65					52,38	47,23				6,84
23.td.1.50 m	0,14	0,04	0,01	0,18					30,94	65,01				4,57
23.td.1.50 in	0,47	0,30	0,00	0,77					38,37	63,62				3,52
23.td.1.350 o	0,41	0,09	0,01	0,52					58,56	44,91				7,54
23.td.1.350 m	0,20	0,09	0,00	0,29					32,20	65,83				5,84
23.td.1.350 in	0,29	0,18	0,00	0,47					30,55	68,89				6,54
ref. sapwood	1,38	1,73	2,64	5,75	93,67	5,47	10,85	16,32	30,13	65,07	92,18	1,04	6,27	0,51
ref. heartwood	2,56	2,80	5,91	11,27	25,37	7,18	12,01	19,19	32,05	63,81	91,62	0,92	7,46	0,38
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
23.td.1.350	519,55	5,78	0,56	3,27	0,36	0,28	4,19	0,45	0,08	0,48	0,12			
ref. pine	509,61	0,99	0,05	0,06	0,02	0,41	0,84	0,22	0,04	0,02	0,01			

Site 26: Terrestrial archaeology 5 Elst (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
26.te.1.0-90	0,87	0,18	0,04	1,08					40,58	58,49				12,95
26.te.2.0-78	0,67	0,21	0,01	0,89					41,89	56,31				9,08
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
26.te.2.0-78	489,26	0,00	0,43	3,42	0,39	0,42	6,28	0,22	0,18	0,98	0,08			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Site 27: Terrestrial archaeology 6 Flevoland (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
27.ta.2	1,623	0,299	0,01	1,932					48,32	50,77				5,95
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
27.ta.3	463,80	0,00	0,97	8,44	0,63	0,30	6,81	1,13	0,51	14,73	0,39			
27.ta.4	447,08	2,79	0,51	8,65	1,18	0,72	9,61	1,56	0,53	19,58	1,38			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Site 28: Terrestrial archaeology 7 Flevoland (NL)														
oak	**content of extractives (mg/g)**				**monosaccha-**	**phenol conent (mg/g)**			**Lignin**	**Holocellulose**	**α-cellu-**	**β-cellu-**	**γ-cellu-**	**Ash con-**
sample	**water**	**ethanol**	**eth/cycl.**	**whole**	**ride (mg)**	**water**	**ethanol**	**whole**	**content (%)**	**content (%)**	**lose (%)***	**lose (%)***	**lose (%)***	**tent (%)**
28.ta.1	1,20	0,27	0,02	1,50					46,71	50,14				3,82
ref. oak	4,78	4,57	1,67	11,02	113,55	92,32	138,97	231,29	25,90	77,62	91,84	1,47	6,28	0,23
element contents of wood														
	C	**N**	**P**	**S**	**Na**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Al**			
sample	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g			
28.ta.2	477,33	6,83	1,55	10,81	1,29	0,22	5,45	1,03	0,19	11,36	0,11			
ref. oak	490,05	1,14	0,16	0,14	0,06	1,14	0,44	0,10	0,09	0,02	0,01			

Appendix 4

Soil chemistry

(by Kretchmar)

Sediment solution, mean values part 1																	
No.	Site	Depth	Depth to pile head	Type	n	Depth Redox	Redox Potential	pH	Na^+	K^+	NH_4^+-N	Ca^{2+}	Mg^{2+}	Fe^{2+}	Mn^{2+}	Al^{3+}	SO_4^{2-}-S	PO_4^{3-}-P
		cm	cm			cm	mV		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
1	Amsterdam	230	50	Lys	1	240	463	8,4	48,7	12,8	1,5	126,6	7,1	0,47	0,93	<dl	2,8	0,4
1	Amsterdam	280	0	Lys	1	280	105	8,5	25,7	10,9	1,4	144,6	6,3	0,07	0,91	<dl	2,4	0,3
1	Amsterdam	330	-50	Lys	1	310	35	8,2	30,7	10,2	1,6	152,8	8,4	0,07	0,96	<dl	2,1	0,2
1	Amsterdam	330	-50	Gw	3			7,9	29,6	11,0	<dl.	154,5	7,2	0,12	0,93	<dl	32,1	0,2
2	Dordrecht	90	30	Lys	1			8,0	19,5	3,5	<dl	66,5	3,7	<dl	<dl	<dl	14,1	0,6
2	Dordrecht	120	0	Lys	1			7,9	20,9	5,7	2,4	77,5	4,5	0,09	0,13	<dl	5,3	0,7
2	Dordrecht	170	-50	Lys	1			7,7	21,6	8,4	3,1	87,4	5,0	0,09	0,55	<dl	1,5	0,3
2	Dordrecht	60	60	Gw	2			8,3	19,7	3,5	<dl.	66,6	3,7	0,02	<dl	<dl	14,2	0,6
4	Haarlem	100	50	Lys	1			8,0	48,6	10,6	2,8	146,9	5,1	0,13	0,63	0,00	2,1	0,67
4	Haarlem	150	0	Lys	1			8,1	54,9	13,6	12,2	141,7	9,2	11,5	1,2	0,06	3,7	0,83
4	Haarlem	200	-50	Lys	1			8,1	68,6	16,4	13,1	137,3	10,9	10,8	1,1	0,12	3,4	2,6
5	Rotterdam	306	-30	Lys	1			7,8	54,9	12,5	6,2	182,3	15,8	0,49	2,99	<dl	2,6	0,7
5	Rotterdam	356	-80	Lys	1			-	88,9	6,4	12,2	216,0	24,4	0,04	1,21	<dl	3,1	1,4
5	Rotterdam	406	-130	Lys	1			-	73,2	8,4	8,4	196,8	21,4	0,03	0,03	0,04	3,5	1,2
5	Rotterdam	276	0	Gw	3			7,4	40,8	8,3	0,2	126,0	8,2	0,37	0,02	0,53	24,0	0,5
6	Zaandam	137	-37	Lys	1	140	-199	7,4	27,6	8,9	0,8	84,4	7,9	0,02	0,52	<dl	15,5	0,2
6	Zaandam	138	-38	Lys	1			8,0	28,7	7,5	1,9	76,7	11,4	0,12	0,34	<dl	2,2	1,2
6	Zaandam	185	-85	Lys	1	170	-266	7,9	40,0	10,6	3,7	88,7	17,1	0,08	0,24	<dl	2,1	1,5
6	Zaandam	90	10	Gw	3			6,9	31,8	9,6	0,2	130,0	9,6	0,21	0,29	0,05	20,2	0,3
8	Dok. Well	40	-40	Lys	2	27	105	8,3	70,6	56,6	11,6	143,9	25,7	0,02	0,34	<dl	3,4	8,1
8	Dok. Well	60	-60	Lys	1	77	-120	8,7	92,6	100,7	20,1	133,5	48,1	0,05	0,07	<dl	5,1	6,3
8	Dok. Well	100	-100	Lys	1			8,4	84,1	88,2	20,2	126,5	39,3	0,09	0,04	<dl	5,0	7,8
8	Dok. Well	0	0	Gw	1			7,8	55,2	38,9	0,2	34,7	6,3	1,01	0,03	1,28	16,9	2,8
9	Dok. Cask	40	-40	Lys	1	35	189	8,3	197,4	87,0	11,4	175,8	30,9	<dl.	0,39	<dl	2,5	11,1
9	Dok. Cask	70	-70	Lys	1	75	-66	8,2	191,2	111,4	11,7	182,3	36,1	0,01	0,89	<dl	2,3	18,0
9	Dok. Cask	100	-100	Lys	1	105	-117	8,4	159,5	185,0	34,4	173,9	73,7	0,03	0,29	<dl	5,0	15,6
10	Borsele	30	-30	Lys	1	20	98	8,1	1278,0	61,3	2,1	217,4	222,0	0,44	0,40	<dl	275,0	0,4
10	Borsele	60	-60	Lys	1	60	-170	8,2	n.a.	83,3	7,9	268,4	458,9	0,11	0,58	<dl	179,5	3,1
10	Borsele	90	-90	Lys	1	90	-168	7,9	n.a.	152,6	10,5	299,6	619,0	0,12	0,54	0,05	209,1	4,0

11	Vleuten	50	-50	Lys	1	50	-304	7,9	14,2	16,8	0,9	133,8	23,5	0,0	1,0	<dl	3,6	0,6
11	Vleuten	90	-90	Lys	1	90	-253	8,1	14,8	18,8	1,1	141,1	27,3	0,0	0,7	<dl	2,2	0,7
11	Vleuten	120	-120	Lys	1	120	-145	8,1	19,2	19,6	2,3	144,8	27,2	0,3	0,8	<dl	3,6	0,5
12	Marine Arch	12		Sea	4			-	7697	313	<dl.	301	944	0,07	<dl.	0,12	664	0,6
13	Marine Arch	13		Sea	4			-	7605	1056	<dl.	311	954	1,59	0,08	<dl.	674	1,1
14	Travenhorst	40	-40	Lys	1	40	261	-	9,2	1,1	<dl.	169,6	8,3	0,1	0,5	<dl	136,8	0,4
14	Travenhorst	80	-80	Lys	1	80	-155	7,7	7,8	0,4	0,3	151,5	8,7	0,0	0,0	<dl	91,7	0,4
14	Travenhorst	110	-110	Lys	1	110	-216	7,8	9,3	1,4	0,8	152,3	9,6	0,0	<dl.	<dl	73,1	0,4
14	Travenhorst	110		GW	3			7,3	13,0	5,6	<dl.	132,3	6,9	0,2	0,0	0,1	51,8	0,4
18	Ven. Bridge	20	-20	Lys	1	14	-229	8,1	n.a.	369,6	42,9	300,0	1052,0	0,88	0,08	0,73	241,6	4,0
18	Ven. Bridge	50	-50	Lys	1	64	-430	8,4	n.a.	338,7	55,6	236,6	956,0	0,08	0,13	0,08	117,0	5,3
18	Ven. Bridge	100	-100	Lys	1			8,4	n.a.	301,4	53,8	267,9	888,0	0,04	0,06	0,08	263,2	3,9
18	Ven. Bridge	Gw		Gw	3			8,0	n.a.	244,3	25,7	214,9	593,3	0,33	0,05	0,56	238,3	2,3
19	Ven. House	80	20	Lys	1	82	-136	8,0	n.a.	351,1	85,4	178,2	922,0	0,09	0,03	0,09	29,7	2,6
19	Ven. House	100	0	Lys	1	102	-252	8,1	n.a.	342,1	87,0	148,2	926,0	0,06	0,01	0,07	24,7	2,2
19	Ven. House	110	-10	Lys	1	122	-286	8,5	n.a.	331,1	54,7	127,3	761,0	0,05	<dl.	0,06	38,9	2,5
19	Ven. House	Gw		Gw	3			7,9	n.a.	214,9	24,4	181,0	515,7	0,30	0,04	0,31	192,1	1,0
21	Mollösund	21		Sea	3			7,6	9493	395	0,2	351,7	1053	<dl.	<dl.	0,05	769	0,7
24	Bryggen	152		Lys	1	150	133	8,0	18,0	2,9	0,5	16,8	1,7	1,04	0,06	<dl	3,0	0,8
24	Bryggen	192		Lys	1	190	123	7,9	28,0	3,0	0,4	16,2	2,3	0,32	<dl.	<dl	1,0	1,3
24	Bryggen	218		Lys	1	220	146	8,1	23,5	2,1	<dl.	10,0	1,2	1,04	<dl.	<dl	1,1	2,0
24	Bryggen	102		Gw	3			7,3	4,6	1,3	<dl.	3,1	0,5	<dl.	<dl.	<dl.	0,7	0,1
25	Lidam	25		Sea	3			7,6	34,6	8,1	<dl.	45,5	7,4	0,13	<dl.	0,26	12,7	0,1
26	Elst	1		Lys	1	20	76	7,4	26,1	75,4	<dl.	134,6	11,8	<dl.	1,3	<dl	18,4	1,2
26	Elst	2		Lys	2	60	8	7,6	32,4	78,0	0,2	171,1	15,7	<dl.	0,4	<dl.	22,6	2,4
26	Elst	3		Lys	1	90	-231	7,6	33,7	83,5	0,2	170,3	16,2	0,0	1,7	<dl.	26,0	2,2
26	Elst			Gw	1			6,8	30,2	77,7	<dl.	175,9	15,0	0,1	<dl.	0,1	24,7	2,2
27	Ship cov.	1		Lys	2			7,9	757,0	56,1	8,3	209,2	152,5	0,4	4,6	<dl.	4,7	0,9
27	Ship cov.	2		Lys	1			8,1	66,6	26,0	<dl.	334,1	33,8	<dl.	<dl.	<dl.	235,8	0,4
27	Ship cov.	3		Lys	1			8,0	1457,0	78,0	41,1	122,6	212,6	0,3	0,9	<dl.	4,3	0,7
28	Ship uncov	1		Lys	1			7,9	277,4	40,7	<dl.	71,0	105,0	0,0	0,0	<dl	2,8	0,8
28	Ship uncov	2		Lys	1			7,8	471,3	45,1	11,2	236,0	146,0	0,0	2,4	<dl	4,3	4,6
28	Ship uncov	2		Lys	1			7,3	351,1	43,3	16,2	271,4	110,8	0,1	0,9	<dl	4,1	1,0

<dl. lower detection limit

NH_4-N, NO_3-N: 0.15 mg/L

Fe^{2+}, Mn^{2+} 0.01 mg/L

Al^{3+}0.04 mg/L

Sediment solution, mean values part 2														
No.	Site	Depth	Depth to pile head	Type	n	Cl^-	NO^{3-}-N	C	Canorg	Corg.	N	Norg.	Conductivity	Base cations
		cm	cm			mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	µS/cm	[mmol/L]
1	Amsterdam	230	50	Lys	1	41,1	0,4	84,8	91,6	-	2,2	0,3	809	9,4
1	Amsterdam	280	0	Lys	1	33,9	0,2	83,1	93,8	-	1,9	0,3	770	9,1
1	Amsterdam	330	-50	Lys	1	49,6	<dl	97,1	105,7	-	2,0	0,4	911	9,9
1	Amsterdam	330	-50	Gw	3	43,2	1,9	50,2	53,0	-	0,9	0,3	1228	9,9
2	Dordrecht	90	30	Lys	1	25,4	1,6	32,4	35,6	-	2,1	0,5	462	4,6
2	Dordrecht	120	0	Lys	1	23,8	0,3	52,8	40,7	12,1	3,3	0,6	526	5,3
2	Dordrecht	170	-50	Lys	1	17,9	<dl	73,8	63,0	10,8	3,7	0,6	582	5,9
2	Dordrecht	60	60	Gw	2	25,2	1,6	26,1	33,4	-	2,1	0,4	454	4,6
4	Haarlem	100	50	Lys	1	58,8	0,00	111,9	86,0	25,8	3,29	0,48	944	10,1
4	Haarlem	150	0	Lys	1	n.a.	0,21	n.a.	n.a.	n.a.	14,5	2,1	n.a.	10,6
4	Haarlem	200	-50	Lys	1	n.a.	0,36	n.a.	n.a.	n.a.	14,7	1,2	n.a.	11,2
5	Rotterdam	306	-30	Lys	1	61,8	0,3	141,1	141,5	-	7,0	0,5	1188	13,1
5	Rotterdam	356	-80	Lys	1	84,8	0,3	n.a.	n.a.	-	13,0	0,6	n.a.	16,8
5	Rotterdam	406	-130	Lys	1	62,1	0,3	n.a.	n.a.	-	10,1	1,5	n.a.	15,0
5	Rotterdam	276	0	Gw	3	51,0	5,1	67,3	61,1	6,1	5,6	0,5	823	9,0
6	Zaandam	137	-37	Lys	1	44,7	<dl	55,2	49,0	6,2	1,1	0,3	629	6,3
6	Zaandam	138	-38	Lys	1	51,4	0,2	66,2	52,0	14,2	2,6	0,5	618	6,2
6	Zaandam	185	-85	Lys	1	51,4	0,3	83,5	69,7	13,8	4,5	0,5	769	7,9
6	Zaandam	90	10	Gw	3	45,2	1,1	64,8	51,2	13,6	1,5	0,3	820	8,9
8	Dok. Well	40	-40	Lys	2	96,4	0,2	119,3	132,7	-	13,2	1,5	1327	13,8
8	Dok. Well	60	-60	Lys	1	100,6	0,4	205,3	159,5	45,8	21,6	1,2	1511	17,2
8	Dok. Well	100	-100	Lys	1	102,5	0,5	148,5	n.a.	-	22,1	1,3	1405	15,5
8	Dok. Well	0	0	Gw	1	75,5	8,3	50,5	19,6	31,0	10,1	1,7	631	5,7
9	Dok. Cask	40	-40	Lys	1	459,8	0,2	96,0	108,5	-	12,0	0,4	2302	22,1
9	Dok. Cask	70	-70	Lys	1	452,6	<dl	103,1	121,3	-	17,7	6,0	2467	23,3
9	Dok. Cask	100	-100	Lys	1	307,9	<dl	197,0	188,8	8,1	33,7	<dl	2542	26,4
10	Borsele	30	-30	Lys	1	2587	<dl	111,3	36,7	74,6	2,6	0,6	8810	86,3
10	Borsele	60	-60	Lys	1	4886	<dl	208,9	260,7	-	6,6	<dl	16300	high
10	Borsele	90	-90	Lys	1	6924	<dl	369,1	389,8	-	9,5	<dl	22580	high
11	Vleuten	50	-50	Lys	1	7,4	<dl	134,4	110,9	23,5	1,9	1,0	867	9,7
11	Vleuten	90	-90	Lys	1	6,8	<dl	137,6	122,2	15,4	1,8	0,7	910	10,4
11	Vleuten	120	-120	Lys	1	12,3	<dl	139,5	115,9	23,6	3,2	0,9	900	10,8
12	Marine Arch	12		Sea	4	n.a.	0,4	24,3	26,8	-	n.a.	n.a.	n.a.	435
13	Marine Arch	13		Sea	4	n.a.	0,2	25,3	27,8	-	n.a.	n.a.	n.a.	452
14	Travenhorst	40	-40	Lys	1	11,8	0,7	34,7	10,1	24,6	2,1	1,3	n.a.	9,6

14	Travenhorst	80	-80	Lys	1	10,2	<dl	49,6	29,8	19,9	1,4	1,1	796	8,6
14	Travenhorst	110	-110	Lys	1	12,0	<dl	77,2	43,1	34,0	2,0	1,3	802	8,8
14	Travenhorst	110		GW	3	23,8	2,2	54,9	39,8	15,1	3,2	1,0	742	7,9
18	Ven. Bridge	20	-20	Lys	1	14917	<dl	347,6	309,4	38,2	43,5	0,6	42800	high
18	Ven. Bridge	50	-50	Lys	1	13996	<dl	431,1	374,2	56,9	56,7	1,1	40700	high
18	Ven. Bridge	100	-100	Lys	1	12974	<dl	358,9	291,9	67,0	56,1	2,3	38600	high
18	Ven. Bridge	Gw		Gw	3	10086	<dl.	220,8	200,2	20,6	27,7	1,9	29800	high
19	Ven. House	80	20	Lys	1	14362	<dl	531,9	590,6	-	94,5	9,1	41200	high
19	Ven. House	100	0	Lys	1	14997	<dl	457,4	481,8	-	93,0	6,0	44100	high
19	Ven. House	110	-10	Lys	1	12283	<dl	406,2	367,7	38,4	64,2	9,5	n.a.	high
19	Ven. House	Gw		Gw	3	6704	<dl.	199,1	181,9	17,2	26,9	2,5	26300	high
21	Mollösund	21		Sea	3	n.a.	<dl.	29,0	27,7	1,3	<dl.	<dl.	47	527,1
24	Bryggen	152		Lys	1	20,1	0,29	17,4	13,1	4,3	0,92	0,16	205	1,8
24	Bryggen	192		Lys	1	28,5	<dl	17,1	15,4	1,7	0,94	0,54	259	2,3
24	Bryggen	218		Lys	1	24,2	<dl	13,6	11,5	2,1	0,41	0,41	188	1,7
24	Bryggen	102		Gw	3	6,1	0,51	4,0	2,9	1,1	0,59	0,08	53	0,4
25	Lidam	25		Sea	3	n.a.	5,3	35,4	24,8	10,6	6,7	1,5	417	4,6
26	Elst	1		Lys	1	30,4	<dl	124,4	107,9	16,5	0,28	0,28	1000	10,8
26	Elst	2		Lys	2	49,8	0,23	135,7	124,9	10,8	0,57	0,38	1221	13,3
26	Elst	3		Lys	1	50,4	<dl	135,8	123,7	12,1	0,35	0,24	1244	13,5
26	Elst			Gw	1	59,5	2,0	143,0	130,0	13,0	2,3	0,33	1220	13,3
27	Ship cov.	1		Lys	2	1190	15,8	307,2	227,9	79,3	15,9	<dl.	5615	57,4
27	Ship cov.	2		Lys	1	88,4	0,5	92,8	45,9	46,9	1,6	1,2	1888	23,0
27	Ship cov.	3		Lys	1	2550	0,3	265,4	162,7	102,8	42,4	1,0	9410	89,0
28	Ship uncov	1		Lys	1	363,2	<dl.	313,9	180,8	133,1	<dl.	<dl.	2640	25,3
28	Ship uncov	2		Lys	1	556,0	<dl.	423,4	365,4	57,9	11,0	<dl.	4090	45,5
28	Ship uncov	2		Lys	1	412,3	<dl.	559,2	359,9	199,3	18,1	1,96	3860	39,1

Sediment analysis, mean values n=12, part 1

No.	Site	Depth	Depth to pile head	Depth Redox	Redox Potential	pH H_2O	C	N	C/N	P	S	Pressure digestion Na	K	Ca
		cm	cm	cm	mV		mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g
1	Amsterdam	230	50	240	463	7,9	6,1	0,16	38,9	0,12	0,57	0,16	1,39	15,7
1	Amsterdam	280	0	280	105	7,8	5,6	0,14	39,5	0,13	1,70	0,17	1,36	14,6
1	Amsterdam	330	-50	310	35	8,0	5,2	0,12	42,1	0,10	0,56	0,18	1,37	13,6
2	Dordrecht	90	30			8,0	14,7	0,6	25,7	0,52	0,38	0,26	2,87	17,2
2	Dordrecht	120	0			7,7	27,4	1,6	26,7	0,66	0,98	0,46	8,31	44,5
2	Dordrecht	180	-60			7,9	24,2	0,5	51,5	0,38	1,02	0,34	4,77	63,3
4	Haarlem					7,9	12,0	0,65	16,5	0,41	0,7	0,34	1,9	12,7
4	Haarlem					7,6	25,8	1,9	13,5	0,94	0,7	0,30	2,2	10,2
4	Haarlem					7,3	36,9	2,7	13,5	1,0	1,3	0,37	2,7	11,2
5	Rotterdam	306	30			7,1	119,7	6,8	17,6	0,36	11,6	0,4	4,1	36,2
5	Rotterdam	366	90			7,0	90,5	5,3	17,2	0,34	12,5	0,3	4,1	31,9
5	Rotterdam	426	150			7,1	87,4	4,8	18,1	0,33	10,6	0,4	5,9	25,9
6	Zaandam	130	-30	140	-199	7,5	81,0	1,3	62,3	0,55	1,2	0,3	1,8	14,0
6	Zaandam	160	-60	170	-266	6,5	136,6	4,2	34,4	0,82	5,5	0,6	6,6	11,8
6	Zaandam	190	-90			6,8	128,2	3,4	37,8	0,94	5,5	0,5	3,4	15,6
8	Dok. Well	7	-7	7	254	7,4	73,8	4,4	17,5	3,9	6,6	1,0	9,5	28,2
8	Dok. Well	30	-30	27	105	7,2	125,0	5,8	21,3	5,0	11,5	1,0	8,6	30,8
8	Dok. Well	60	-60	77	-120	7,6	43,1	2,8	15,5	3,0	6,8	0,9	10,8	22,9
9	Dok. Cask	10	-10			7,2	123,3	8,4	14,9	10,3	5,3	1,8	7,6	46,8
9	Dok. Cask	30	-30	35	189	7,5	60,3	3,7	16,1	6,5	2,9	1,2	9,9	34,7
9	Dok. Cask	70	-70	55	-97	7,4	62,8	4,0	15,8	6,4	3,4	1,3	9,9	33,8
10	Borsele	30	-30	20	98	6,2	453,3	11,5	39,4	0,3	39,6	9,8	0,7	16,3
10	Borsele	60	-60	60	-170	6,3	464,5	7,4	63,3	0,29	25,1	18,1	0,9	12,9
10	Borsele	90	-90	90	-168	6,5	432,7	8,5	51,4	0,26	22,1	22,8	1,7	11,0
11	Vleuten	100		90	-253	7,9	29,9	1,0	30,8	0,7	2,4	0,4	7,5	56,9
12	Marine Arch	1				8,6	2,1	<dl.	-	0,07	0,53	1,80	0,86	4,46
12	Marine Arch	2				8,7	0,0	<dl.	-	0,06	0,21	1,28	0,57	2,49
13	Marine Arch	1				8,6	2,7	<dl.	-	0,10	0,53	1,57	0,73	5,78

13	Marine Arch	2				8,5	0,0	<dl.	-	0,13	0,28	1,32	0,70	2,79
14	Travenhorst	40		40	261	4,6	354,8	18,6	19,1	0,2	104,7	0,1	0,2	21,4
14	Travenhorst	80		80	-155	4,2	237,6	13,4	18,2	0,3	126,3	0,1	1,4	16,5
14	Travenhorst	110		110	-216	5,3	128,7	7,1	18,1	0,1	115,7	0,2	2,5	12,7
16	Glastonbury	10				5,3	364,2	20,6	17,7	0,6	16,3	0,5	0,1	93,0
16	Glastonbury	5				5,6	339,2	19,8	17,2	0,9	13,7	0,7	0,1	92,4
17	Harter's Hill	40				3,6	204,3	14,9	13,7	0,7	11,7	0,5	0,6	31,5
18	Ven. Bridge	10	-10	14	-229		93,0	2,1	43,4	3,7	153,3	39,6	0,4	17,0
18	Ven. Bridge	40	-40	34	-359		89,4	1,9	46,4	2,4	147,5	43,1	0,4	19,3
19	Ven. House	82	20	82	-136		77,7	1,1	71,4	0,8	133,1	48,7	0,3	17,8
19	Ven. House	102	0	102	-252		10,6	0,6	15,4	0,4	0,0	1,0	0,9	259,5
19	Ven. House	122	20	122	-286		22,1	1,5	15,4	0,8	0,0	1,3	1,3	251,5
21	Mollösund	10				8,0	18,6	1,2	15,7	0,8	4,4	16,9	4,0	31,6
21	Mollösund	30				6,9	23,6	1,2	20,0	1,0	16,7	11,1	4,8	23,7
24	Bryggen	123				6,3	71,8	4,4	16,4	3,22	2,4	0,9	5,2	16,1
24	Bryggen	143		150	133	5,5	148,0	6,4	23,3	6,85	4,8	1,4	5,0	22,3
24	Bryggen	170		170	147	5,4	68,0	3,2	21,3	3,87	3,0	1,1	5,2	14,8
25	Lidam	10				6,9	4,5	0,4	12,3	0,4	0,7	0,6	2,2	5,3
26	Elst	25		20	76	7,9	21,2	1,0	20,9	2,2	0,9	1,0	11,8	32,5
26	Elst	50		40	107	8,6	6,9	<dl.	-	0,5	0,3	0,4	3,4	21,2
26	Elst	65		60	8	8,6	6,8	0,2	22,0	0,4	0,3	0,3	3,3	21,3
27	Ship cov	1				7,7	32,4	2,3	14,3	1,0	2,1	0,23	1,6	93,6
27	Ship cov	2				7,8	37,2	2,3	16,2	0,9	1,4	0,84	1,3	91,7
27	Ship cov	3				7,6	40,9	2,3	20,4	0,6	3,6	1,9	1,8	84,3
28	Ship uncov	1				7,8	36,1	2,3	16,0	0,7	4,0	1,0	12,6	37,6
28	Ship uncov	2				7,8	34,8	2,2	16,0	0,7	4,3	1,0	12,7	37,6
28	Ship uncov	3				7,7	42,0	2,4	17,5	0,7	7,3	1,1	11,8	29,4

Sediment analysis, mean values n=12, part2

No.	Site	Depth	Depth to pile head	Depth Redox	Mg	Mn	Fe	Al	Base cations	Cation exchange capacity: Na	K	Ca	Mg	Mn	Fe	Al	Base cations
		cm	cm	cm	mg/g	mg/g	mg/g	mg/g	[mmol/g]	mmol/kg							[mmol/g]
1	Amsterdam	230	50	240	1,08	0,06	3,5	3,7	0,92	0,88	0,46	288,4	3,22	0,00	0,41	1,8	14,7
1	Amsterdam	280	0	280	1,05	0,05	3,6	3,5	0,86	0,70	0,47	290,4	3,44	0,00	0,36	2,0	14,8
1	Amsterdam	330	-50	310	1,01	0,05	3,3	3,4	0,81	0,61	10,51	279,4	3,60	0,00	0,39	2,3	14,6
2	Dordrecht	90	30		2,35	0,24	10,4	10,2	1,1	0,62	1,07	400,2	7,08	0,16	0,57	1,3	20,6
2	Dordrecht	120	0		7,61	0,52	25,6	28,4	3,1	1,27	4,50	499,0	17,94	0,18	2,29	1,4	26,6
2	Dordrecht	180	-60		5,44	0,30	12,6	14,0	3,7	1,34	2,89	454,1	13,66	0,15	1,54	1,4	24,0
4	Haarlem				1,4	0,10	5,8	6,3	0,81	0,97	0,88	260,5	3,4	0,00	0,59	0,0	13,4
4	Haarlem				1,6	0,18	7,2	7,7	0,72	1,30	1,45	246,5	5,5	0,08	1,1	0,0	12,9
4	Haarlem				2,0	0,16	8,2	9,9	0,81	2,33	2,72	292,9	11,5	0,12	1,1	0,0	15,8
5	Rotterdam	306	30		3,7	0,4	12,5	13,9	2,2	6,8	2,0	650,7	36,2	0,3	2,2	0,0	35,9
5	Rotterdam	366	90		3,7	0,4	15,1	13,4	2,0	6,6	2,4	644,7	31,6	0,3	4,2	0,7	35,2
5	Rotterdam	426	150		4,1	0,3	15,2	19,4	1,8	7,2	2,9	578,8	29,8	0,2	2,5	0,0	31,8
6	Zaandam	130	-30	140	1,6	0,1	11,3	9,0	0,9	0,91	0,76	265,5	4,97	0,00	0,38	0,0	13,7
6	Zaandam	160	-60	170	4,0	0,3	25,9	27,0	1,1	2,80	4,45	335,8	22,05	0,04	1,30	0,0	18,8
6	Zaandam	190	-90		2,5	0,2	23,1	17,0	1,1	2,38	1,59	359,3	16,46	0,02	1,03	0,0	19,5
8	Dok. Well	7	-7	7	6,4	0,4	20,0	25,7	2,2	7,1	31,9	435,1	54,2	0,0	1,5	0,0	27,3
8	Dok. Well	30	-30	27	5,9	0,5	19,9	22,9	2,3	10,4	32,4	510,2	88,5	0,1	1,9	0,0	34,1
8	Dok. Well	60	-60	77	7,3	0,4	22,5	29,0	2,1	6,6	37,9	422,7	68,3	0,1	1,5	0,0	28,0
9	Dok. Cask	10	-10		4,7	0,7	20,0	20,8	3,0	28,3	22,7	560,2	30,5	0,1	2,2	0,0	32,3
9	Dok. Cask	30	-30	35	6,7	0,5	23,9	28,1	2,6	21,4	28,0	509,0	31,9	0,1	1,3	0,0	29,7
9	Dok. Cask	70	-70	55	6,5	0,5	23,4	27,8	2,5	22,2	28,7	513,6	32,1	0,1	1,2	0,0	30,0
10	Borsele	30	-30	20	8,2	0,2	8,9	0,8	1,9	446,4	10,2	586,8	695,8	0,2	1,8	1,1	106,3
10	Borsele	60	-60	60	9,8	0,1	0,8	0,4	2,3	758,9	17,6	431,1	724,4	0,3	1,0	1,4	114,6
10	Borsele	90	-90	90	10,3	0,1	1,5	1,9	2,4	941,6	21,2	365,3	751,7	0,2	1,1	1,7	121,6
11	Vleuten	100		90	8,7	0,5	16,8	27,6	3,8	0,5	3,9	455,1	19,5	0,1	1,9	0,0	24,5
12	Marine Arch	1			0,81	0,05	1,99	2,23	0,39	76,0	2,2	161,7	21,2	0,0	0,3	0,0	13,2
12	Marine Arch	2			0,49	0,06	1,69	1,49	0,23	45,1	1,2	75,8	13,2	0,1	0,1	0,0	6,9
13	Marine Arch	1			0,67	0,04	1,79	1,81	0,43	65,1	2,7	140,7	20,2	0,1	0,3	0,0	11,6

13	Marine Arch	2			0,59	0,04	1,73	1,77	0,26	27,6	1,4	71,9	9,2	0,1	0,2	0,0	5,6
14	Travenhorst	40		40	0,6	0,3	66,3	0,8	1,1	2,1	0,4	749,7	44,1	115,2	6,3	0,0	41,2
14	Travenhorst	80		80	1,2	0,4	58,0	5,9	1,0	2,0	0,8	527,9	34,4	159,5	4,1	16,0	29,3
14	Travenhorst	110		110	1,7	0,3	43,9	8,4	0,8	1,5	1,7	459,1	21,9	49,2	2,6	3,6	24,9
16	Glastonbury	10			5,8	0,2	0,3	0,0	5,2	6,3	1,7	1137,7	71,1	2,4	3,5	0,0	63,1
16	Glastonbury	5			6,3	0,2	0,3	0,0	5,2	7,7	1,4	1037,9	70,8	2,3	3,0	0,0	58,1
17	Harter's Hill	40			7,0	0,1	1,3	56,4	2,2	3,1	4,2	207,6	46,0	0,5	8,8	371,4	14,4
18	Ven. Bridge	10	-10	14	20,6	1,8	1,4	52,4	4,3								
18	Ven. Bridge	40	-40	34	26,8	1,7	1,7	47,6	5,1								
19	Ven. House	82	20	82	25,3	1,3	1,7	44,7	5,1								
19	Ven. House	102	0	102	3,3	0,0	0,6	0,0	13,3								
19	Ven. House	122	20	122	4,9	0,1	0,9	0,0	13,1								
21	Mollösund	10			4,6	0,2	10,7	9,0	2,8	726,4	67,5	419,2	166,7	0,1	0,2	0,0	68,0
21	Mollösund	30			4,8	0,2	21,3	11,8	2,2	409,9	13,9	405,2	66,0	0,1	0,4	0,0	43,9
24	Bryggen	123			6,6	0,4	25,5	17,4	1,5	3,2	2,1	165,9	7,7	0,0	1,3	0,0	9,1
24	Bryggen	143		150	4,8	0,4	17,3	16,2	1,7	4,3	2,4	149,2	8,4	0,3	1,7	0,0	8,4
24	Bryggen	170		170	4,5	0,3	17,0	15,0	1,3	4,3	2,1	95,3	5,7	0,3	0,8	0,0	5,5
25	Lidam	10			2,4	0,3	13,8	7,8	0,5	0,9	0,9	31,9	1,9	0,0	2,5	0,0	1,8
26	Elst	25		20	6,8	0,5	22,4	36,4	2,5	3,3	33,5	496,0	12,7	0,0	1,3	0,0	26,8
26	Elst	50		40	2,5	0,1	7,3	9,7	1,4	0,3	2,1	322,0	4,3	0,0	0,5	0,0	16,5
26	Elst	65		60	2,5	0,2	7,6	9,6	1,4	0,4	1,8	312,4	4,2	0,0	0,7	0,2	16,0
27	Ship cov	1			4,5	0,02	0,00	0,03	5,1	1,5	11,2	636,7	30,4	0,1	0,0	0,2	34,7
27	Ship cov	2			6,0	0,02	0,00	0,17	5,1	5,4	8,3	589,8	38,7	0,1	0,0	1,1	33,1
27	Ship cov	3			10,3	0,18	0,00	1,4	5,2	11,3	9,1	456,3	44,2	0,9	0,0	2,1	27,2
28	Ship uncov	1			9,5	0,8	31,3	44,0	3,0	11,5	12,8	491,0	45,6	0,0	4,1	0,0	29,1
28	Ship uncov	2			9,6	0,7	31,8	44,3	3,0	12,7	11,9	511,0	45,9	0,0	3,7	0,0	30,2
28	Ship uncov	3			8,9	0,6	31,9	41,2	2,6	17,5	11,6	493,0	52,7	0,0	2,7	0,2	30,0

Appendix 5

Dendrochronological investigation

(by Saß-Klaassen & Vernimmen)

Foundation piles of historical buildings

Tree characteristics: tree age and mean tree-ring width

The results are summarised in Table 2D and show that both the age and mean tree-ring width of the trees that were used for foundation piles varies between species and, to lesser extent, origin of the wood. However, due to the small number of samples only tendencies can be described: most of the spruce pile are made from trees between 50 and 60 years old with the exception of two spruce piles from site 2 made from two 114 and 96 year old slow-growing spruce trees with at least one originating from South Germany. The 50 to 60 year old trees show a quite consistent average tree-ring width of about 2 mm.

The sampled pine trees that were used for foundation piles differ much more in both tree age and mean tree-ring width in comparison to spruce. However, inside one sample site the material is quite homogeneous, with young pine trees around 40 years being used in sites 4 and 6 and about 80-year old trees in site 3. In general the pines show smaller annual growth rates in comparison with the spruces, e.g. below 2 mm also in young pines (site 4 and 6).

Dating results

Dendrochronology could be successfully used to determine the felling date and season of the pine and spruce trees that were used as timber for foundation piles in historical buildings (tab. 2D).

For six out of the eight investigated historical building ***dating results*** were yielded. Even in case of samples with a small number of rings (sites 4 and 6) dating was possible if several samples were available which could be combined into mean tree-ring series. The young pines that were used as piles in sites 4 and 6 could be dated by using two of the newly (for this project) constructed five pine chronologies for the Netherlands (tab. 1D).

Location			Co-ordinates		Chronology	
Site	**Nearest town**	**Forest map**	**North**	**East**	**no. trees**	**Time span**
Amerongen	Rhenen	-	52.00	5.29	15	1767-2003
Eerde	Ommen	10/17F	52.29	6.27	20	1824-2003
Edese Bos	Ede	202A	52.02	5.42	20	1750-2003
Noord Ginkel			52.02	5.45	12	1854-2003
Mattemburgh	Bergen op Zoom	-	51.27	4.19	20	1846-2001

Table D1. Tree-ring chronologies of *Pinus sylvestris* from four locations in the Netherlands (Morales 2004)

Possible reasons why no dendro-dates could be provided for the spruce samples from site 1 could be that the tree-ring series of the samples are heterogeneous, i.e. it was impossible to combine time series of the different samples into one or two site chronologies that could have been used for dating. From site 5 only one spruce samples was available and the fir samples could not combined into a mean time series either. Moreover only cores were available from site 5 whereas most other samples were delivered as complete stem disk. Stem-disk samples (in contrast to cores) enable to measure at least four radii and calculate a representative mean tree-ring series for each sample. In that way it is possible to adjust for intra-tree variability and increase the chance of dendro-dating.

The ***felling season*** can be determined by looking at the anatomical structure of the last ring. In all cases a complete last tree ring was found, i.e. consisting of earlywood and latewood and thus indicating that cutting has been taken place during a period when the trees were not growing. This is the period from late summer of the year to which the last ring was dated to early spring of the following year.

All dating results are in accordance with the information available on the construction date of the historical buildings. This means that no re-used wood is incorporated in the wooden foundations. The time of transportation and/or storage varies for the different sites from very short, i.e. several months (site 4 and 22) to about one year (site 2 and 6) up to 2 years (site 3) and in case of single stems up to about four years (site 22, td1).

Origin of wood/Dendrocprovenancing
If dating is possible it is also possible to get an indication about the origin of the wood. The last column of table 2D indicates the region that is represented by the regional master chronology that yields the best match with the (mean) tree-ring series of the sample(s). Most of the spruce originates from South Germany; two piles are from Sweden. Sites 2 contains piles originating from trees from both Germany and Sweden.
Pine that has been used in foundation piles either originates from Scandinavia or is local material from the Netherlands. At site 4 where young trees provided the wood for the piles pine from Sweden and The Netherlands is combined. Site 6 exclusively contains pine from the Netherlands whereas at sites 3, 4, 22 and 23 contain only pine from Finland (site 3) and Sweden.
Pine from the Netherlands that has been used for foundation piles derives from young, on average 45-yera old slow-growing trees.

Archaeological samples
In case of the archaeological material Dendrochronology is mainly used to get an indication about the time when a construction was erected that was later preserved in the soil.

Dating results
The majority of the archaeological samples could be dated by dendrochronology. Dendrochronologial dating worked very well for the oaks samples.
Sites 14, 18, 19 and 24 did not yield any dating results. The main reasons why dating failed are the species (site 18, 19, 24) often in combination with a small number of tree rings on the sample. There are no chronologies for dating *Larix* and only a few pine chronologies for wood older than 200 years. Tree-ring series shorter than 20 years (site 19, 24) are not datable.

Dating precision
Archaeological material often comprises damaged or degraded samples which mostly prevented dating to the year. Only at sites 10, 11 and 17 the felling date of oak could be exactly determined.

Origin of wood/Dendrocprovenancing
Dendroprovenancing becomes less reliable the further back in time the material dates. This is due to changing environmental and ecological conditions throughout the centuries in different regions that can not be traced back. However, it is well known that in the Netherlands especially during the Roman time a lot of import of building timber has been taken place (e.g. Buis 1993). From the results of sites11 and 26 it becomes obvious that timber from Germany was imported for the construction that was erected in the first century AD probably in combination with local oak (site 26: sample S41). Timber import from the Poland/Baltics are found in combianation with German or local material at the sites 9, 20 and 21 dating in the 14th and 17th century. The oak used in site 17 proved to be local.

Sample characteristics		Measurement results				Dating results and statistics				Felling year and documented building date		Origin of wood
Sample No.	species	No. rings/ tree age	Mean RW [mm]	STD RW [mm]	Last ring	Growing period	T-test	%PV	p	felling year/season	Built in	Regional chronology used for dating
1a.td.1	Piab	50	1.95	0,72	EW+LW	no date	-	-	-	-	1916-1919	-
1b.td.3	Piab	55	2.12	0,71	EW+LW	no date	-	-	-	-		-
1d.td.3	Piab	39	3.58	2,30	EW+LW	no date	-	-	-	-		-
1c.td.1	Piab	58	1.77	0,38	EW+LW	no date	-	-	-	-		-
2.td.3	Piab	55	2.31	1,28	EW+LW	1875-1929	3.59	63.9	0.04	Late summer 1929 - spring 1930	1931	Sweden, Gotland
2.td.2	Piab	65	2.05	0,68	EW+LW	1865-1929	6.89	86.7	0.0001	Late summer 1929 - spring 1930		S.Germany, Wuerttemberg
2.td.8	Piab	50	2.41	0,93	EW+LW	1880-1929	5.76	73.5	0.001	Late summer 1929 - spring 1930		S.Germany
2.td.10	Piab	60	2.13	0,93	EW+LW?	1869-1928	4.78	72.0	0.001	Late summer 1928 - spring 1929?		S.Germany
2.td.11	Piab	114	1.08	0,45	EW+LW	1816-1928	3.27	63.4	0.005	Late summer 1929 - spring 1930		S.Germany
2.td.1	Piab	55	2.23	0,83	EW+LW	1875-1929	2.79	63.9	0.04	Late summer 1929 - spring 1930		Sweden, Gotland
2.td.4	Piab	58	2,11	0,90	EW+LW	1872-1929	4.90	66.7	0.02	Late summer 1929 - spring 1930		S.Germany
2.td.5	Pisy	136	0.81	0,34	EW+LW	no date	-	-	-	-		-
2.td.6	Piab	50	2.32	1,06	EW+LW	1880-1929	3.50	65.3	0.04	Late summer 1929 - spring 1930		S.Germany, Francia
2.td.7	Piab	96	1.25	0,68	EW+LW	no date	-	-	-	-		-
2.td.9	Piab	62	1.84	1,00	EW+LW	1868-1928	4.40	74.2	0.0002	Late summer 1929 - spring 1930		S.Germany, Francia
2.td.12	Piab	69	1.91	0,65	EW+LW	no date	-	-	-	-		-
3.te.1.1	Pisy	82	1.40	1,18	EW+LW	1842-1923	via Mc 1.1+2.1			Late summer 1923 – spring 1924	1926	S.Finland
3.te.2.1	Pisy	70	1.66	0,72	EW+LW	1854-1923	via Mc 1.1+2.1			Late summer 1923 – spring 1924		S.Finland
Mc 1.1+2.1	Pisy	82	1.71	1,06	EW+LW	1842-1923	4,43	62,8	0,001			S.Finland
3.te.3.1	Piab	60	1.82	0,92	EW+LW	1864-1923	via MC 3.1+4.1			Late summer 1923 – spring 1924		S.Germany
3.te.4.1	Piab	46	2.15	0,78	EW+LW	1878-1923	via MC 3.1+4.1			Late summer 1923 – spring 1924		S.Germany
Mc 3.1+4.1	Piab	60	2.09	0,84	EW+LW	1864-1923	5,36	71,9	0,001	Late summer 1923 – spring 1924		S.Germany
4.td.1	Pisy	41	1.90	0,96	EW+LW	1864-1904	via Mc 1+2+4			Late summer 1904 – spring 1905	1904	Sweden, Gotland
4.td.2	Pisy	39	1.81	0,73	EW+LW	1866-1904	via Mc 1+2+4			Late summer 1904 – spring 1905		Sweden, Gotland
4.td.3	Pisy	34	1.81	0,60	EW+LW	no date	-	-	-	-		-
4.td.4	Pisy	41	1.61	0,87	EW+LW	1864-1904	via Mc 1+2+4			-		Sweden, Gotland
MC 1+2+4	Pisy	41	1.79	0,77	EW+LW	1864-1904	4.28	76.3	0.001	Late summer 1904 – spring 1905		Sweden, Gotland
4.te.5	Pisy	46			EW+LW	1852-1897	Via Mc 5+6+7			Late summer 1894 – spring 1895	1895	E.Netherlands (Ede)
4.te.6	Pisy	44	1.52	1,22	EW+LW	1851-1894	Via Mc 5+6+7			Late summer 1894 – spring 1895		E.Netherlands (Ede)
4.te.7	Pisy	43	1.59	1,00	EW+LW	1852-1894	Via Mc 5+6+7			Late summer 1894 – spring 1895		E.Netherlands (Ede)
MC 5+6+7	Pisy	47	1.56	0,85	EW+LW	1851-1897	6.23	80.4	0.0001	Late summer 1894 – spring 1895		E.Netherlands (Ede)
5.te.2	Abal	53	2.5	1,09	EW+LW	no date	-	-	-	-	1901-1905	-
5.te.3	Piab	51	2.56	1,24	EW+LW	no date	-	-	-	-		-
5.te.1	Abal	98	1.29	0,86	EW+LW	no date	-	-	-	-		-
6.te.3	Pisy	42	1.25	0,77	EW+LW	1894-1935	via Mc 1+2+3			Late summer 1935 – spring 1936	1937	E-Netherlands (Noordginkel)
6.te.1	Pisy	45	1.18	0,39	EW+LW	1891-1935	via Mc 1+2+3			Late summer 1935 – spring 1936		E-Netherlands (Noordginkel)

Sample characteristics		Measurement results				Dating results and statistics				Felling year and documented building date		Origin of wood
Sample No.	**species**	**No. rings/ tree age**	**Mean RW [mm]**	**STD RW [mm]**	**Last ring**	**Growing period**	**T-test**	**%PV**	**p**	**felling year/season**	**Built in**	**Regional chronology used for dating**
6.te.2	Pisy	38	1.43	0,42	EW+LW	1898-1935	via Mc 1+2+3			Late summer 1935 – spring 1936		E-Netherlands (Noordginkel)
MC 1+2+3	Pisy	45	1.28	0,46	EW+LW	1891-1935	4,86	68,2	0.001	Late summer 1935 – spring 1936		E-Netherlands (Noordginkel)
22.td.2	Pisy	145	0.77	0,50	EW+LW	1750-1894	5.84	68.1	0.0001	Late summer 1894 – spring 1895	1895-1905	S-Sweden
22.td.1	Pisy	147	0.91	0,71	EW+LW	1748-1890	8.89	77.1	0.0001	Late summer 1890 – spring 1891		S-Sweden
23.td.1	Pisy	53	2.33	0,80	EW+LW	1849-1901	5.89	72.1	0.005	Late summer 1901 – spring 1902	ca. 1900	S-Sweden
23.td.1	Pisy	52	2.23	0,78	EW+LW	1850-1901	5.81	69.6	0.005	Late summer 1901 – spring 1902		S-Sweden

Table 2D. Dating results for foundation piles of historical buildings (softwoods); results are given for sample disk closest to stem base/soil surface
Sample no.=Bacpoles sample number, Mc= mean curve; **Species**: Piab=*Picea abies*, Pisy=*Pinus sylvestris**;*** **No.rings**=**tree age**: number of measured tree rings also indicating tree age in upper pile samples; **Mean RW**=Mean ring width; **STD RW**=Standard deviation ring width; **Last ring**=characteristics of last formed tree ring under the bark, EW=earlywood, LW=latewood, EW+LW=complete ring; **Growing period**=time span of tree-ring series equals growing period of the tree, no date=tree-ring series not datable; **t-test**= The t-value resulting from a "students t-test" on the cross correlation between the measured ring-width pattern and the reference chronology at the "best match" position; **%PV**= "Gleichläufigkeit" (German) or Percentage of Parallel Variation; the percentage of rings in the measured annual ring pattern, which show identical increase and decrease in annual ring width at the position in the reference chronology which was found by dating the pattern. The significance of this percentage is a function of the length in years of the measured annual ring pattern and the reference chronology; **p**= The possibility (expressed as a fraction of 1) that the found value of %PV is just accidental; **felling year/season**=time span when the tree was felled, in case of complete tree ring (=EW+LW): after growing period (late summer) of year to which last tree ring is dated to beginning of growing season (spring) of following year; **Built in**=documented year when the building was constructed; **Region represented by chronology used for dating** =country, region or area that is represented by the regional chronology that yielded the best match with the tree-ring series and thus indicates the origin of wood.

Sample characteristics			Measurement results						Dating results and statistics					Origin of wood
Sample No.	species	find no./ element no	No. rings	No. sap-wood rings	Miss. rings until bark	Miss. rings until pith	Estim. Tree age [yrs]	Last ring	Growing period	T-test	%PV	p	Estim. felling year/ season	Region represented by chronology used for dating
8.td.1	Qusp	5	61	17-18	1 or more	0	>62	-	1402-1467	6.17	62,3	0.05	1468 AD or later	via MC timberNL
8.td.4	Qusp	6	28	6?	Ca. 14	0	42	-	no date	-	-	-	-	-
8.td.7	Qusp	7	69	16-22	?	0	>69	-	1390-1458	-	-	-	after 1458 AD	via MC 1+3
8.td.5	Qusp	2	76	36?	Ca. 1	?	>77	EW+LW	1404-1479	5.52	78.0	0.0001	1480 AD or later	C.-S.Germany
8.td.2	Qusp	1	23	8	Ca. 1	0	24	-	no date	-	-	-	-	-
Mc 1+7	Qusp	5+7	78		1 or more			-	1390-1467	6.11	67,5	0.0005	1468 AD or later	Netherlands+NW.Germany
9.td.5	Qusp	10	196	-	>15+9/-6 >26 ±8	?	>207 >214	-	1180-1375	5.74/ 5.34	63.8/ 64.9	0.0001	after 1390 AD +9/-6/ after 1401 AD ±8	C.Poland/ Netherlands+ NW.Germany
9.td.4	Qusp	9	100	Border	>20±6	?	>118	-	no date	-	-	-	-	-
9.td.3	Qusp	8	77	4?	11+9/-6	?	>82	-	1292-1368	6.13	61.8	0.04	1379 AD +9/-6	Poland-E.Pommerania
9.td.6	Qusp	6	221	-	>15+9/6	?	>230	-	1128-1348	8.49	71.1	0.0001	after1363 AD+9/-6	Poland/Baltics
9.td.1	Qusp	5	143	6	14±6	?	>155	-	1256-1398	5.53	61.3	0.01	1412 AD ±8	C.-W.Germany
10.td.1	Qusp	520 (S112)	66	13	0	0	66	EW	34-100	4.7	68.5	0.005	Spring/early summer 100 AD	C.Netherlands, wet sites
10.td.2	Qusp	521 (S134)	75	18	0	0	75	EW	22-97	4.1	72.3	0.0002	Spring/early summer 97 AD	via MC Roman way/ Vleuten de Meern
11.td.?	Qusp	V116	65	13	0	?	>65	EW	35-100	4.5	63.3	0.04	Spring/early summer 100 AD	via MC Roman way/ Vleuten de Meern
11.td.?	Ulsp	V115	92	?	0	?	>92	EW+LW	7-99	4.85	62.6	0.02	Late summer 99 – spring 100 AD	C.W.Germany
12.ta.1.3	Qusp		106	-	>20 ±6	?	>120	-	1503-1608	5.8	68.0	0.0002	after 1628 AD ±6	Netherlands+ NW. Germany
12.ta.2.2	Pisy		114	?	?	Max 10	>124	-	1513-1626	6.4	66.0	0.0005	after 1626 AD	S.Norway/ Flesberg
13.ta.1	Pisy		73	-	?	?	>73	-	no date	-	-	-	-	-
13.ta.@	Qusp	352	111	-	>20 ±6	2	>123	-	1370-1480	8.1	66.6	0.0001	after 1500 AD ±6	C.S.Germany
13.ta.@	Qusp	207	63	4	12 ±5	8	Ca. 77	-	1566-1628	6.0	75.8	0.0001	1640 AD ±5	NW.Germany, Lower Saxony
14.ta.1	Qusp	P27	32	5	0	0	Ca.38	EW+LW	no date	-	-	-	-	-
14.ta.3	Qusp	P17	72	11/12	0	0	72	EW	no date	-	-	-	-	-
14.ta.2	Qusp	P25	36	6	0	0	36	EW+LW	no date	-	-	-	-	-
14.ta.@	Qusp	Probe A	72	10	0	0	72	EW+LW	no date	-	-	-	-	-
17.ta.1	Qusp		71	?	0	ca.1	72	EW	1136-1065 BC	6.38	n.a.	n.a.	Spring 1065 BC	S.England/S.Wales
17.ta.2	Qusp		43	-	>16 ±5	ca.1	Ca. 55	-	1123-1081 BC	5.26	n.a.	n.a.	after 1065 BC ±5	S.England/S.Wales
18.td.4	Qusp		55	15?	0	0	55	EW+LW	no date	-	-	-	-	-
18.tc.1	Lade		52	?	0	?	>52	EW+LW	no date	-	-	-	-	-
18.tc.2	Lade		43	?	?	Max 5	>48	-	no date	-	-	-	-	-
18.tc.3	Lade		49	?	?	0	>49	-	no date	-	-	-	-	-
19.td.4	Pisy		12	?	1	0	13	EW?	no date	-	-	-	-	-

Sample characteristics			Measurement results						Dating results and statistics					Origin of wood
Sample No.	**species**	**find no./ element no**	**No. rings**	**No. sap-wood rings**	**Miss. rings until bark**	**Miss. rings until pith**	**Estim. Tree age [yrs]**	**Last ring**	**Growing period**	**T-test**	**%PV**	**p**	**Estim. felling year/ season**	**Region represented by chronology used for dating**
19.tc.1	Pisy		21	?	1	0	22	EW	no date	-	-	-	-	-
19.tc.2	Pisy		13	?	1	?	>14	EW?	no date	-	-	-	-	-
19.tc.3	Pisy		22	?	1	?	>23	EW?	no date	-	-	-	-	-
20.td.3	Qusp	(bottom)	128	-	>15+9/-6 >20 ±6	?	>137-142	-	1487-1614	7.53/ 6.04	68.5/ 65.4	0.0001 0.001	after 1629 AD +9/-6 after 1634 AD ±6	Poland, Wollin Germany, Friesland
20.td.2	Qusp	(lid)	113	-	>20 ±6	?	>127	-	no date	-	-	-	-	-
20.td.1	Qusp	(stave)	123	-	>20 ±6	?	>137	-	no date	-	-	-	-	-
20.td.1	Qusp	(stave)	112	-	>20 ±6	?	>126	-	no date	-	-	-	-	-
21.ta.3	Qusp		294	13	13 ±8/ 2 +9/-6	?	>290-299	-	1085-1378	8.50/ 7.95	61.1/ 65.5	0.0002 0.0001	1391 AD ±8/ 1380 AD +9/-6	Netherlands+NW.Germany/ C.Poland
21.ta.2	Qusp		137	10?	5 +9/-6	?	>136	-	1248-1384	5.16	63.2	0.005	1389 AD +9/-6	C.Poland
24.ta.1	Pisy		29	?	1	1	32	EW	no date	-	-	-	-	-
24.ta.3	Pisy		155	?	0	ca.10	65	EW+LW	no date	-	-	-	-	-
24.ta.2	Pisy		22	?	0	0	22	EW?	no date	-	-	-	-	-
25.ta.1	Qusp		72	Border?	16 ±5	1	>87	-	no date	-	-	-	-	-
26.td.?	Qusp	S46	100	Border?	20 ±6	3	>117	-	14BC-86AD	8.04	69.7	0.0002	106 AD ±6	C.W.Germany
26.td.?	Qusp	S48	46	-	>16 ±5	2	>59	-	49BC-4BC	4.56	66.7	0.025	after 12 AD ±6	C.S.Germany
26.td.?	Qusp	S47	64	-	>16 ±5	Max 5	>80	-	58BC-6AD	8.38	73.8	0.0002	after 22 AD ±6	C.W.Germany
26.td.?	Qusp	S43	121	-	>20 ±6	Ca. 3	>138	-	37BC-84AD	7.68	72.5	0.0001	after 104 AD ±6	C.W.Germany
26.td.?	Qusp	S32	107	-	>20 ±6	2	>123	-	45BC-62AD	5.17	65.6	0.0001	after 82 AD ±6	C.W.Germany
26.td.?	Qusp	S41	80	-	>20±6	ca.3	>97	-	47BC-33AD	5.41	70.9	0.0002	after 53 AD +/-6	Netherlands+NW.Germany
27.ta.1	Qusp		41	-	>16 ±5	ca.3	>55	-	no date	-	-	-	-	-
27.ta.2	Qusp		70	-	>16 ±5	ca.3	>84	-	1456-1525	5.34	69.6	0.005	after 1541 AD ±5	NW.Germany, coastal area
27.ta.3	Qusp		118	4	16 ±5	Max 5	>134	-	1416-1533	6.72	72.6	0.0001	1549 AD ±6	NW.Germany, Westfalen
27.ta.4	Qusp		55	-	>16 ±5	ca.2	>68	-	no date	-	-	-	-	-
28.ta.1	Qusp		66	-	>16 ±5	Max 10	>87	-	1503	6.44	73.6	0,0002	after 1519 AD +/-5	via MC timber NL
28.ta.2	Qusp		20	3	0	ca.2	>22	-	no date	-	-	-	-	-

Tab. 3D. Dating results for archaeological samples

Sample no.=Bacpoles sample number, Mc= mean curve; **Species**: Qusp=*Quercus spec*, Ulsp=*Ulmus spec*., Pisy=*Pinus sylvestris,* Lade=*Larix decidua;* **Find no./ element no:** sample information for archaeologists; **No.rings**= number of measured tree rings; **No. sapwood rings**= number of (remaining) sapwood rings on sample, *border* indicates sapwood/heartwood transition zone meaning that heartwood is complete; **Miss. Rings until bark**= estimated missing tree rings until bark: estim. by using diff. statistics for calculating missing sapwood rings: for C.W.Germany (trees<100 yrs: 20 ±6 yrs, trees>100: 16 ±5) and for Central Poland (15+9/-6), remaining sapwoods (see previous column) are taken into account; **Miss. Rings until pith**=estimated missing tree rings until pith; **Estim. tree age**=number of measured tree rings+number of (at least) missing tree rings until bark (calculated from sapwood statistics)+number of est. miss. rings until pith; **Last ring**=characteristics of last formed tree ring under the bark, EW=earlywood, LW=latewood, EW+LW=complete ring; **Growing period**= time span covered by tree-ring series, no date=tree-ring series not datable; **t-test**= The t-value resulting from a "students t-test" on the cross correlation between tree-ring series of the sample and the reference chronology at the "best match" position; **%PV**= "Gleichläufigkeit" (German) or

Percentage of Parallel Variation; **p**= The possibility (expressed as a fraction of 1) that the found value of %PV is just accidental; **Estim. felling year/season**=estimated felling year: calculated from last year of *growing period + missing rings until bark (column 6)*, felling season: in case of complete tree ring (=EW+LW): after growing period (late summer) of year to which last tree ring is dated to beginning of growing season (spring) of following year; in case of only earlywood present (EW): in spring of year to which last ring is dated; **Region represented by chronology used for dating** =country, region or area that is represented by the regional chronology that yielded the best match with the tree-ring series and thus indicates the origin of wood. In some cases no indication for a region could be given because the chronology that yield the best dating result is not clearly connected to a certain region in Europe, i.e *MC timber NL*=mean curve of timber found in the eastern part of the Central and Northern Netherlands and *MC Roman way/Vleuten de Meern*=mean curve from Roman Way in Vleuten de Meern.

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Appendix 6

Methods and protocols

(by Hotchkiss, Landy, Mitchell)

1. DNA Extraction
2. Polymerase Chain Reaction (PCR)
3. Denaturing Gradient Gel Electrophoresis (DGGE)
4. Cloning
5. Restriction Enzyme Digests
6. RNA extraction
7. Phylogenetics
8. FISH (Fluorescent *in situ* hybridisation)
9. PRIMER statistical analysis

1. DNA Extraction

Note ALL WATER IS PURITE DEIONIZED WATER UNLESS STATED AS "STERILE" OR "ULTRA" WHICH IS COMMERCIALLY SUPPLIED DNASE AND RNASE FREE

For Pure Bacteria (cultures)

On agar media

Scrape a few colonies into 35ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99^{O}C – proceed to PCR protocol A

In Liquid media

Add 3ul of an overnight culture to 32ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99^{O}C – proceed to PCR protocol A

Plasmid DNA from Clones

Add 3ul of an overnight culture to 21.45ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99^{O}C – proceed to PCR protocol B

For Environmental (wood) samples

Take 500mg of sample, place in 1.5ml microfuge tube and freeze dry at –80^{O}C for one week) Grind environmental sample using liquid nitrogen and a sterile mortar and pestle. Proceed with CTAB lysis procedure or Qiagen DNAeasy Plant Mini kit

DNA Extraction using CTAB lysis Buffer: used for BACPOLES project until August 2004

Suspend 0.015-0.025g of material in 500 ÿl 75^{O}C CTAB lysis buffer (2%CTAB, 100mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1% PVPP) and vortex for 30 seconds

Incubate Tubes @75^{O}C in water bath for 15 Min, vortexing every 5 min for 30 seconds
Allow to cool and add 0.5volume (250 ÿl) Chloroform:Isoamyl alcohol (24:1)
Vortex for 30 seconds
Centrifuge @10,000rpm for 10 min
Collect supernatant and put in fresh tube
Centrifuge again for 10 min @10,000rpm
Collect supernatant and put in fresh tube
Extract supernatant mix with 1 volume (500ÿl) Chloroform:Isoamyl alcohol (24:1)
Centrifuge again for 10 min @10,000rpm
Collect supernatant and put in fresh tube
Centrifuge again for 10 min @10,000rpm and put in fresh tube
Add 0.5 volume (250 ÿl) 30% PEG (Polyethylene glycol) 8000

Incubate on ice for 3 hours or leave overnight in –20^{O}C freezer

Pellet by centrifuge in a chilled centrifuge 4-8^{O}C @10,000rpm for 15 minutes
Pour off PEG and allow pellet to dry very well about 20 mins

Ethanol Precipitation Step
Resuspend pellet in 500 ÿl 70% Ethanol (10mM Sodium Acetate)
Centrifuge @10,000rpm for 20 minutes
Remove ethanol and dry pellet
Resuspend pellet in 100-150ml TE Buffer (10mM Tris-HCl, 1mM EDTA pH 8.0)/Sterile Water

DNA Extraction using QIAGEN DNeasy Plant Mini Kit: used for BACPOLES project from August 2004 to project completion
Full protocol given on manufacturers web page (www1.qiagen.com)

DNA Cleanup
Repeat last 4 steps in CTAB protocol (Treat 150 ÿl of DNA suspension as dry pellet) if using CTAB protocol
Use the QIAquick Nucleotide Removal Kit from QIAGEN. Full protocol given on manufacturers web page (www1.qiagen.com).

2. Polymerase Chain Reaction (PCR)

Note: EVERYTHING MUST BE DEFROSTED ON ICE AND KEPT ON ICE WHILST DISPENSING

PCR Profile

Denaturing….. 1Cycle 94^{o}C 5 Min

*Annealing…..*35 Cycles 94^{o}C 1 Min followed by 51-58^{o}C depending on primer set (see annealing temperatures in Appendix 2) 1 Min and 72^{o}C 1 Min

*Extension…..*1 Cycle 72^{o}C 10 Min

PCR Master Mix for 30 samples
Protocol A – To be used for bacterial cultures and environmental samples
150ÿl 10X Buffer
30ÿl $MgCl_2$ (25mM) - Promega/ 15ÿl $MgCl_2$ (50mM)-Invitrogen
30ÿl dNTPs (10mM)
45ÿl Forward Primer
45ÿl Reverse Primer
1050ÿl Sterile Water if using Promega bead/1065ÿl Sterile water if using invitrogen Taq
Dispense 45ÿl of mix per tube. Add add a promega bead or 0.15ÿl Invitrogen Taq polymerase plus 4.7 ÿl template DNA to each tube. Centrifuge reaction tubes at max speed for 30 seconds to ensure that all components are mixed

Protocol B – To be used for Clones (Plasmid DNA)
90ÿl 10X Buffer
54ÿl $MgCl_2$ (50mM)
15ÿl dNTPs (10mM)
15ÿl Forward Primer (20uM M13F)
15ÿl Reverse Primer (20uM M13R)
4.5ÿl Taq polymerase
Add 5.55ÿl of mix to tubes containing boiled samples. Centrifuge reaction tubes at max speed for 30 seconds to ensure that all components are mixed.

Cleanup of PCR Products
Using QIAGEN QIAquick PCR Purification kit. Full instructions given on manufacturers web site (www1.qiagen.com).

Agarose Gel Electrophoresis

PCR products are separated electrophoretically and visualized by UV light using Ethidium Bromide (EtBr) as a fluorescent label. EtBr added in concentration of 5ÿg/ml.

For looking at all DNA types:-

For long fragments (eg. 8F-1492R 1400bp products) can use two combs per gel and run for approx 1 hour at 100V

For shorter fragments (eg. UNV2-UNV3 220 bp products) must run 1 comb per gel run at 100V for 1.5-2 hrs.

1.5% = 1.5g Agarose in 100ml 1XTAE buffer – melt on high in microwave for 2 mins and then add 1.5ÿl EtBr

For looking at RFLPs:-

3% = 3g Agarose in 100ml 1XTAE buffer– melt on high in microwave for 3.5 mins and then add 1.5ÿl EtBr

Gels take approximately 20mins to set

Note: Always make sure buffer in tanks is same as buffer in gels. When running products for gel extraction, always use fresh buffer. Always run gels from black electrode to red electrode, keep lids on tanks at all time to avoid electrocution.

Cleanup of PCR Product From Agarose Gel

Using QIAGEN QIAEXII Agarose Gel Extraction Kit. Full instructions given on manufacturers web site (www1.qiagen.com).

3. Denaturing Gradient Gel Electrophoresis (DGGE)

A nested PCR is performed to obtain PCR products of the appropriate length for DGGE. Extracted DNA initially amplified with 8F-1492R primers to obtain sequences of 1400bp. These products in turn amplified with UNV2 (GC clamp)-UNV3 primers to produce smaller (approx 220bp) products

PCR products are separated using a 30% and 70% urea-formamide denaturing gradient within a polyacrylamide gel.

Stock Solutions – will last no more than 1 week at 4^{o}C

0% Denaturant

16.2ml 40% Acrylamide:Bis-Acrylamide (37.5:1)
2ml 50X TAE buffer
81.8ml ddH_2O

adjust to 100ml with ddH_2O if necessary store in a darkened container in the fridge @4^{o}C

100% Denaturant

16.2ml 40% Acrylamide:Bis-Acrylamide (37.5:1)
2ml 50X TAE buffer
42g Urea
40ml Formamide
adjust to 100ml with ddH_2O if necessary, warm to dissolve urea. Store in a darkened container in the fridge @4^{o}C and warm at no more than 40^{O}c in a waterbath to dissolve urea before use, bringing back to room temp before each run.

Note: never dissolve urea in >40 ^{o}C as gel will not set

20% Ammonium Persulfate (APS)

0.2g Ammonium persulfate
1ml ddH_2O
(prepare fresh before each run)

Preparation of Gradient:

30% Solution

17.5ml 0% stock
7.5ml 100% Stock
add 126ÿl 20% Ammonium persulfate and 12.6 ÿl TEMED before pouring

70% Solution

17.5ml 100% stock
7.5ml 0% Stock
add 126ÿl 20% Ammonium persulfate and 12.6 ÿl TEMED before pouring
also add 50ÿl gradient dye to this solution to aid visualisation of mixing of gradients

Note: make sure that you add the Ammonium persulfate and TEMED while the magnetic stirrers are going as these are the reagents which help the gel set so they must be mixed right through the solution

0% Solution

Prepare 5ml of 0% solution to top up the top of the gel where the well comb is inserted (add 25.2ÿl 20% Ammonium persulfate and 2.52ÿl TEMED keep shaking with your hand and pipette on to top of gel using 1ml piptette)
Add 50ÿl gradient dye to this solution to aid visualisation of wells when loading samples

Running and visualisation of gels

Gels are run at 60^{o}Celsius, 100V for 16hrs.
Gels are visualized using UVr and EtBr staining
Stain for 30 mins in 30ÿl EtBr:300ml dh_20
Destain for 15 mins (no longer) in 300 ml dh_20
Excise bands of interest and extract DNA

Recovery of DNA from Polyacrylamide Gels

Using the QIAEX II Gel Extraction Kit from QIAGEN (www1.qiagen.com), following the manufacturers protocol..

Post DGGE PCR and Cleanup

Recovered DNA is re-amplified using the same primer set (UNV2-UNV3) but without the GC clamp and subjected to the QIAGEN PCR purification kit.

4. Cloning

Using the Invitogen Kit: TOPO TA Cloning (www.invitrogen.com)

Ligation

It is best to make up a mastermix of the ligation reaction mixture and dispense 1.67ÿl into each reaction tube.
1.00µl Sterile water
0.33 µl Salt Solution
0.33 µl TOPO Vector
Add the 0.33 µl PCR product to be inserted (from a standard PCR reaction (Protocol A above).
Gently mix by tapping the tube (*do not* use a pipettor!) and incubate at room temperature for 5 minutes.
After incubation, place the reaction on ice (or store overnight at -20°C).

Transformation into Competent Cells:
Add 16 µl of the One Shot Chemically Competent *E. coli* cells to each cloning reaction.
Note: competent cells must be stored at -80 °C, defrosted and dispensed on ice and cannot be refrozen
Incubate on ice for 5 to 30 minutes.
Heat-shock the cells for 30 seconds at 42°C without shaking.
Immediately transfer the tubes to ice.
Add 83 µl of the room-temperature SOC medium.
Incubate tubes horizontally for 1 hour in the 37° shaking incubator at 200 rpm.
Using sterile technique, spread 40 µl of IPTG then 40 µl of XGAL onto each prewarmed plate .
Using sterile technique, spread 50 µl (do two plates) from each transformation onto a prewarmed LB plate. Let stand right-side up for about 5 minutes to allow the cells to adhere to the agar, then invert and incubate overnight at 37°C.
Note: do not incubate for longer as get an over abundance of false positives as the ampicillin is used up.

Screening of Colonies
Using sterile technique, pick 10 white colonies off the agar plates and drop into 10 microcentrifuge tubes each containing 1ml LB (with 100mg/ml ampicillin).
Note: no colony should grow unless it has an insert. The *ccd*B lethal gene in the plasmid shoÿld be disrupted if the insert is in place thus allowing the clones to grow in an ampicillin rich environment. However random mutations do sometimes occur allowing growth of other colonies. Colonies are further analysed using PCR.
Incubate colonies overnight @ 37^{o}C on a 200rpm shaker table.
Add 3ÿl of overnight culture to 20ÿl sterile water in a PCR tube, quick centrifuge and boil on Gradient PCR machine block for 20 mins.
Perform specific PCR reaction for clones (Protocol B above):
3ÿl 10X Buffer
1.8ÿl 25mM $MgCl_2$ **or** 0.9 ml 50mM $MgCl_2$
0.5ÿl 10uM dNTPs
0.5 ÿl 20uM primer M13F
0.5 ÿl 20uM primer M13R
1 unit Taq polymerase (0.7ÿl)

PCR profile recommended:
Denaturing 1 cycle 94^{o}C for 10 min
Annealing 30 cycles 94^{o}C for 1 min, followed by 55^{o}C for 1 min and 72^{o}C for 1 min
Extension 1 cycle 72 ^{o}C for 10 min
Hold @ 4^{o}C
visualise using agarose gel electrophoresis (1.5% gel). Successful clones should be approx 450 - 600bp in size whereas false positives will yield products of different sizes..
Clones that are of interest can be stored long term by adding 50 ÿl of sterile 60% glycerol and freezing at – ^{o}C.
Note: Once defrosted, clones cannot be refrozen but must be recultured by adding 10ÿl of vortexed clone culture to 1ml of LB with ampicillin (100ÿg.ml).

5. Restriction Enzyme Digests
Note: These reagents do not need to be defrosted on ice
10ÿl PCR product from clone
add 0.5ÿl restriction enzyme of choice (see table below)
7.5ÿl sterile water
2ÿl appropriate buffer (see table below)

Incubate the mix at 37^{O}C for 1 hour and then stop reaction by subsequently incubating at 65^{O}C for 10 minutes preferably in the Gradient PCR machine or in a waterbath.
NOTE: for AluI and RSAI use react buffer I, for HAEIII use react buffer II
Run RFLPs on a 3% agarose gel to visualize with a 25Kb ladder

Restriction Enzyme	Cut site	Reaction conditions
Alu I	3' – TC GA 5 5' – AG CT - 3	37 °C react 1 buffer
Hae III	3' – CC GG 5 5' –GG CC 3	37 °C react 2 buffer
Rsa I	3' – CA TG 5 5' – GT AC –3	37 °C react 1 buffer

Clean-up and send for sequencing
Grow up fresh overnight cultures of the clones to be sequenced and clean up using the Invitrogen SNAP miniprep kit (www.invitrogen.com) and send off for sequencing with the M13F and M13R primers.

6. RNA Work

All equipment (mortars, pestles and spatÿlas etc) MUST be soaked in 0.01% DEPC (1ml in 1l water) water overnight before use.
DEPC is highly toxic but degrades on autoclaving, waster DEPC water must be autoclaved before disposal down sink.
All solutions must be treated with DEPC if not Rnase/Dnase free. Some solutions are not compatible with DEPC and these must be made up using DEPC treated water
DEPC keeps for 1 month in the fridge
All pipettes must be cleaned with ethanol
Bench must be thoroughly cleaned with ethanol
Use sterile technique at all times and change gloves frequently
DO NOT attempt more than 2 (to 4) samples at a time, speed is critical
ß-mercaptoethanol must be added to RLT or RLC buffer before use. 10ÿl per 1ml buffer. This is stable for 1 month
RPE buffer must be diluted with 4 volumes of ethanol (96-100%) before use
Columns for RNA cleanup must be kept in fridge

RNA Extraction
Using QIAGEN Rneasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi (www1.qiagen.com) according to manufacturers protocol.
For wood samples: Use <100mg of sample ground to very fine powder using liquid nitrogen
For bacterial cultures: pellet and wash thoroughly in sterile water (if in media).
Resuspend in 450ÿl buffer (Step 3 of protocol)
Flash freeze in liquid N and incubate for 1-3 mins at 56^{O}C

RNA Cleanup

Using QIAGEN Rneasy Mini Protocol for RNA Cleanup (www1.qiagen.com) according to manufacturers protocol.

DNA Digestion
To make sure all DNA contaminants are removed from the samples. Using QIAGEN Rnase-Free Dnase kit (www1.qiagen.com)
Note: be sure to pipette mix all reagents as they are added and give a quick centrifuge to final mix to ensure.

Set up 2 reactions for each sample (one for subsequent RTPCR and 1 for PCR) and add the following to each:
1µl RNA sample
1µl RDD buffer
2.5µl Dnase I
5.5V sterile water
incubate at 25^{o}C for 15 mins in Mastercycler
add 1µl of 25mM EDTA
incubate at 65^{o}C for 10 mins in Mastercycler
Note: Can stop at this point and store products at – 80oC but preferable to proceed with RTPCR

RTPCR (Reverse Transcription PCR)
Note: RTPCR REAGENTS MUST BE KEPT AT APPROPRIATE TEMPERATURES THROUGHOUT THE PROTOCOL, DEFROSTED AND KEPT ON ICE WHILST DISPENSING. ALSO SPEED IS CRITICAL

A normal PCR is run concurrently with the RTPCR to check for the presence of any DNA not removed during the DNA digest step
For PCR of each RNA sample dispense the following:
5µl PCR buffer
3µl $MgCl_2$
1µl DNTPs
1µl forward primer EUB933F
1µl reverse primer EUB1387R
0.25µl Taq
27.75µl sterile water
Total 39µl
add 11µl of digested RNA sample
Perform PCR
For RTPCR of each RNA sample, dispense the following:
10µl 5XRTPCR buffer
10µl Q solution
2µl DNTPs
2µl enzyme mix
3µl forward primer EUB933F
3µl reverse primer EUB1387R
9µl Rnase free water
Total 39 µl
Add 11µl digested RNA sample
Perform RTPCR

Visualisation of PCR/RTPCR products
Run 15-20µl of product on 15% agarose gel at 100V and visualise under UV with an Ethidium Bromide stain.
Run the PCR and RTPCR products for each sample alternatively on gel for ease in identifying possible traces of undigested DNA

Recovery of cDNA for cloning
Re-run 2 lanes of each clean cDNA sample on 15% agarose gel
Extract CDNA using QIAEX II Gel Extraction Kit from QIAGEN (www1.qiagen.com), following the manufacturers protocol
Note: always use fresh buffers in the tanks and gels for best recovery of cDNA fragments

Cloning

Using the Invitogen Kit: TOPO TA Cloning (www.invitrogen.com)

Ligation & Transformation

Use one vial of One Shot cells for every three transformations.
The ligation reaction mixture:
1.00µl Sterile water
0.33µl Salt Solution
0.33µl TOPO Vector
Make up mastermix and aliquot 1.66ÿl into each reaction tube
Add 0.33 µl of fresh RTPCR product

Note: can use 1.33 µl of product rather than adding water if DNA concentration is known to be low
Gently mix by tapping the tube (*do not* use a pipettor!) and incubate at room temperature for 5 minutes.
Add 16 µl of the One Shot Chemically Competent *E. coli* cells to each cloning reaction.
Incubate on ice for 5 to 30 minutes.
Heat-shock the cells for 30 seconds at 42°C without shaking.
Immediately transfer the tubes to ice.
Add 72 µl of the room-temperature SOC medium.
Incubate tubes horizontally for 1 hour in the 37° shaking incubator at 200 rpm.
Note: do not incubate for longer than 1 hour as this allows for about 3 divisions
Using sterile technique, spread 35 µl (do two plates) from each transformation onto a prewarmed LB plate. Let stand right-side up for about 5 minutes to allow the cells to adhere to the agar, then invert and incubate overnight at 37°C.
NOTE: do not incubate for longer as get an over abundance of false positives as the ampicillin is used up.

Clone Selection & Checking

Pick off 20 white colonies and cÿlture in 1 ml of LB + ampicillin for minimum of 5 hours 200rpm @ 37^{o}C
Take 3 µl of cÿlture in 21.45 µl sterile water and boil for 10 mins
Overnight or Day 2. PCR

3µl Clone Culture
3µl PCR buffer
0.9µl $MgCl_2$
0.5µl dNTPs
0.5µl Primer M13F
0.5µl Primer M13R
0.15µl Taq
21.45µl Sterile water
TOTAL 30 µl

PCR profile as recommended for DNA cloning (section 5 above)
visualise using agarose gel electrophoresis (8 or 10 µl on 1.5% gel, 1kb marker, 1-1.5 hours @ 80V). Successfµl clones should be approx. 400-600bp in size whereas false positives will yield different sized products.

Restriction Digest and Subsequent Clean up and Sequencing

As with DNA see section 5 above.

7. Phylogenetics

Sequences from isolates, environmental samples, DGGE bands and clone libraries were aligned with those representing known bacterial taxa deposited in databases. Alignments were constructed using CLUSTAL X and manually adjusted in text editors (Se-Al and PAUP). Data matrices representing the major bacterial groups were prepared using 50 to 500 taxa for 1000 nucleotides. These matrices were used to perform phylogenetic analyses under parsimony and distance criteria implemented in PAUP. Analyses were performed on unrooted data and when Aquiflex pyllophilus was used as the outgroup. Under general parsimony criterion, heuristic searches were made with random sequence addition for 10 replicates and trees constructed using the TSB algorithm. Genetic distances were calculated using the Kimura-2, log-det, GTR and maximum likelihood evolutionary models and trees constructed via the nearest neighbor method.

8. FISH

NOTE: *in situ* hybridisation is a very sensitive technique, it is important that all reagents used must be free from all microbes. All reagents must be filtered through a 0.2 µm pore size filter into a clean bottle prior to autoclaving (for those reagents that can be autoclaved). As an insurance step, filter all reagents again immediately prior to use.

Fixation of material

NOTE: Gram-negative bacteria are fixed in paraformaldehyde (“PFA ”) and gram-positive bacteria in ethanol but both types can be fixed simultaneously in PFA if a lysozyme treatment is used. The structure of cells fixed with just ethanol is not maintained very well. The fixation protocol used will need to be tested for each sample or each individual gram-positive strain.

Reagents and solutions

1x PBS

130 mM NaCl (3.8 g per 500ml)
10 mM Na_2HPO_4 ÿH_2O (1.79 g per 500ml)
3 mM NaH_2PO_4 ÿH_2O (0.23 g per 500ml)
pH should be 7.2, adjust with NaOH or HCl as necessary
Filter through 0.22 µm filter into a sterile container, autoclave and store at 4ºC.

3 x PBS

390 mM NaCl (11.4 g per 500ml)
30 mM Na_2HPO_4 ÿH_2O (5.37 g per 500ml)
9 mM NaH_2PO_4 ÿH_2O (0.69 g per 500ml)
pH should be 7.2, adjust with NaOH or HCl as necessary
Filter through 0.22 µm filter into a sterile container, autoclave and store at 4ºC.

4% paraformaldehyde (“PFA”) in PBS

Heat (to ~ 60°C) 33 ml of high purity water (i.e. MilliQ water) that has been filtered.
Add 2 g of paraformaldehyde (work in fume cupboard!) and one drop of 2 M NaOH, and stir for about 2 min when the PFA should go into solution.
Add 16.5 m1 of 3 x PBS that has been filtered and cool the resulting solution on ice (if you are in a hurry).
Adjust the pH to 7.2 with NaOH or HCl as needed.
Store at 4°C and use within 24 hours or aliquot into small volumes (750 µl is a very convenient volume) and store at –20ºC (keeps indefinitely). NOTE: the final solution should not be filtered or autoclaved.

Oxalic acid
make a 500 mM (31.52 g per 500ml MilliQ water) solution and filter (0.22 µm).

Blocking Reagent
Roche Diagnostics cat. No. 1096176 (50g)

Proceedure

Take one ml of sample (sample size can be adjusted to suit needs, aim for 10^6-10^9 cells/ml) and centrifuge at 13000 rpm for 5 min.
Resuspend cells in 250 µl of ice-cold PBS.
Mix this suspension with 750 µl PFA fixative and incubate at 4ºC for 1-3 h.
If fixing a cell suspension that contains significant amounts of iron (Fe^{2+} or Fe^{3+}), a precipitate of iron phosphate will form. To remove this, add the oxalic acid solution in small volumes until the solution clears and proceed. Otherwise go step 5.
Pellet cells, thoroughly remove supernatant and wash the cells (use pipette to resuspend) in 1 ml PBS. Pellet cells again.
Resuspend cells in PBS to a concentration of 10^8-10^9 cells/ml and add one volume of ice cold ethanol and mix (most likely just resuspend back to ½ of the original volume with PBS and add the same amount of ethanol).
Fixed cells can be stored at –20ºC for several months (most likely years). Cells are ready to be spotted onto slides for FISH.

Preparation of microscopic slides

Reagents and solutions

Ethanolic KOH
95 ml ethanol
5 ml deionized (MilliQ) water that has been filtered (0.2 µm)
10 g KOH (this may not dissolve completely but not to worry it is only a cleaning solution.

Gelatine solution
0.15 g gelatine
0.02 g chromium potassium sulfate 12 hydrate [$CrK(SO_4)_2ÿH_2O$]
200 ml deionized (MilliQ) water that has been filtered (0.2 µm) and heated to 70ºC prior to use.

Ethanol series
3 ethanol solutions of 50% (v/v), 80% (v/v) and 95% (v/v) made with
deionized (MilliQ) water that has been filtered (0.2 µm).

Lysozyme Solution
25 µg lysozyme/ml filtered deionized water (our lysozyme has an activity of 41,100 Units/mg solid, so we are using 1027.5 U/ml)

Procedure
NOTE: Do as many as possible as they can be stored in the fridge (4^oC) for up to 6 months and warm slides to room temperature before starting

Clean slides by soaking in ethanolic KOH for 1 hour.
Rinse slides well in deionized (MilliQ) water that has been filtered (0.2 µm) and let dry.
Place slides in gelatine solution at 70°C and hold horizontally for about 10 seconds to evenly coat the slides. Let the excess solution drain back into container. Allow the slides to dry and either use immediately or store in a sealed slide box at 4º C until needed.
Spread 5 to 10 µl of fixed sample (diluted with filtered deionized water if necessary) on to a gelatine coated slide (can fit 2 samples per slide if you are careful not to allow cross-contamination) and allow the smears to air dry.
Lysozyme step
Place slide at 4C for 10 min to equilibrate at this temperature.
Add a 10 µl of lysozyme solution to each smear and incubate at 4C for 10 min.
Rinse well by immersing the slide into filtered deionized water two times.
Dehydrate the sample smears by immersing slide into a series of solutions of 50%, then 80% and then 95% ethanol, for 3 min in each.
Let slide completely dry.
The slide is ready for immediate hybridisation or can be stored in the dark at room temperature indefinitely.

Fluorescent *In Situ* Hybridisation (FISH):
NOTE: during the hybridisation process, avoid exposing the labelled probes to excess light by performing this entire hybridisation procedure in a darkened room (not completely without light) and storing the hybridised slides in the dark.

Reagents and solutions
NOTE: Autoclave all stock solutions and use to prepare hybridisation and wash buffers. Use deionized water such as MilliQ water (unless otherwise specified) and filter these solutions with a 0.2 µm pore size filter immediately before addition of formamide and use.

1 M Tris/HCl pH 7.4 (60.505g Tris Base, 440ml H_2O, 60ml HCl)

5 M NaCl (146.1g NaCl in 500ml H_2O)

10% SDS (40g SDS in 360ml H_2O adding HCl dropwise to make 400ml)

Deionized formamide (kept at -20 °C in small aliquots (1ml). **CARE: handle with gloves**

5 mM EDTA
add 36.53 g EDTA (= Na_2 ethylenediaminetetra-acetic acid) to 200 ml dH_2O
adjusting the pH to 8.0 with 10 M NaOH, the solution will clear when you approach pH 8 when the EDTA is dissolved make up to 250 ml with deionized H_2O and autoclave before use

DAPI s**olution**
1 mg 4',6-diamidino-2-phenylindole (DAPI)/ml deionized water that has been filtered and autoclaved. Store in a microfuge tube at –20ºC.

Mounting medium
Dissolve 0.233 g DABCO in 2.5 ml water,
add 500 µl of 1 M Tris (pH 9.5) and mix well,
and finally add 7 ml (8.75 g) glycerol and mix thoroughly.
Store this in small aliquots at –20ºC.
If you needed, the glycerol concentration can be altered to match the refractive index of the immersion oil.

NOTE: The amount of glycerol can affect the refractive index of the immersion oil so move up or down percents if this is a problem

Procedure

Prepare 2 ml of hybridisation buffer according to required stringency (see Table 1 below) by adding the appropriate amount of deionized formamide.
Place a tissue, soaked with about 1.8 ml of the hybridisation buffer, in a 50 ml conical test tube. Allow the tube to equilibrate in the hybridisation oven set at 46ºC for about 30 min.
NOTE: Probes and material containing probes must always be in the dark
Add 10 µl of hybridisation buffer containing 25 ng of each labelled probe used to each sample smear and cover with a coverslip.
Transfer slide, smear side up, to the equilibrated tube in the hybridisation oven and incubate at 46°C for 2 h.
Gently removed the cover slip and immerse slide for 15 min in pre-warmed (48ºC) wash buffer of appropriate stringency (see Table 2) at 48°C.
Gently rinse the slide with filtered deionized water from a squirt bottle and allow the slide to dry.
Add 1 µl of stock DAPI solution to 1ml mounting medium and smear 10µl on slide. Add a coverslip and incubate in the dark for 10 min. For long term storage, you can seal the coverslip with clear nail polish (that does not fluoresce) and keep the slide in the dark.

Preparation of hybridisation buffer

To prepare 2 ml of the hybridisation buffer mix the following and filter (0.2 µm) immediately prior to addition of formamide (or use filtered ingredients
40 µl 1 M Tris-HCl pH 7.4
2 µl 10% SDS
360 µl 5 M NaCl
X µl formamide (see below)
Y µl deionized (MilliQ) water (see below)

Table 1. Preparation of hybridisation buffer to the appropriate stringency, for hybridisation at 47ºC in the presence of 0.9 M cations (i.e. Na^+), by addition of formamide. The addition of formamide to change stringency allows hybridisation reactions to be carried out at a constant temperature when using different oligonucleotide probes. See section below for required amount of formamide

% Formamide	X µl formamide	Y µl deionized water
0	0	1598
5	100	1498
10	200	1398
15	300	1298
20	400	1198
25	500	1098
30	600	998
35	700	898
40	800	798
45	900	698
50	1000	598
55	1100	498
60	1200	398

Preparation of washing buffer

To prepare 50 ml washing buffer mix the following and filter (0.2 µm) immediately prior to use.
39.5 ml deionized water
50 µl 10% SDS
1 ml 1 M Tris-HCl, pH 7.4
0.5 ml EDTA
X µl 5 M NaCl (see below)
Y µl deionized water (see below)

Table 2. Preparation of washing buffer to appropriate stringency by altering the sodium chloride concentration to match that achieved in the hybridisation buffer by formamide (see Table 3 for required amount of formamide needed for probes). That is, for each 5% formamide used in the hybridisation buffer, you must decrease the NaCl in the wash buffer by 29.3%.

% formamide used in hybridisation buffer	**NaCl (M) needed in wash buffer**	**X µl of 5 M NaCl to add**	**Y µl H_2O to add**
0	0.9	9000	0
5	0.636	6360	2640
10	0.450	4500	4500
15	0.318	3180	5820
20	0.225	2250	6850
25	0.159	1590	7410
30	0.112	1120	7880
35	0.080	800	8200
40	0.056	560	8440
45	0.040	400	8600
50	0.028	280	8720
55	0.020	200	8800
60	0.014	140	8860

Epifluorescence Microscopy
Samples were viewed under a Nikon Eclipse E800 epi-microscope using the appropriate filter sets:-
For DAPI
UV1A Excitation 365/10 i.e. range of 360-370nm
Dichroic Mirror (DM) long pass barrier at 400nm
Barrier emission filter (BA) at 400nm
For Fluorescein
UV1A Excitation 460-500nm
DM barrier at 505nm
BA barrier at 510nm
For CY3
UV1A Excitation 510-560nm
DM is a barrier at 565nm
BA 570-610nm filter

9. PRIMER Statistical Analysis

The text below is a personal interpretation of information provided by the developers of the PRIMER software package. All information is taken from the following 2 sources:

Clarke, K.R., Gorley, R.N. PRIMER v5: User Manual/Tutorial. PRIMER-E: Plymouth. (2001).
Clarke, K.R., Warwick, R.M. Change in marine communities: an approach to statistical analysis and interpretation. Plymouth: Plymouth Marine Laboratory, 144pp. (1994).

PRIMER is a multivariate statistical package specifically developed for the analysis of complex ecological data. The underlying strategy is to highlight any patterns of similarity or dissimilarity across samples of data on the basis of their biotic composition. The results can then be compared with known or hypothesised interrelations between groups of samples, in the case of the BACPOLES research the predetermined groups are samples taken from different wood types and different regions.

PRIMER operates by comparing the extent to which two or more samples share particular taxa at similar levels of abundance. It does this by calculating a similarity coefficient (S) between every pair of samples. Similarity coefficients define a range of 0 to 100% (or 0 to 1) where S=0 when two samples have no species in common and S=100% (or 1) when two samples are identical. The most commonly used algorithm for calculating similarity coefficients is the Bray-Curtis coefficient and that has been used here. The qualities of this coefficient that make it useful for working with ecological data are that it is independent of both scale changes *(i.e.* whether data is collected per m^2 or per cm^2) and joint absences of species from the data (*i.e.* if S is dependent on the joint absence of a species then one could say that alpine and desert communities are similar because blue whales are found in neither of them!). A similarity matrix is constructed from the comparison of every pair of samples and from this a number of graphical representations of the data can be made through hierarchical clustering and ordination techniques.
Once patterns of similarity or dissimilarity between groups of samples have been identified, it is desirable to be able to test for these differences. PRIMER offers a routine, ANOSIM (analysis of similarities), which is analogous to the ANOVA (analysis of variance), a tool used for the analysis of univariate data. ANOSIM generates a test statistic (R value), which falls between 1 and 0. If R=1, then all replicates within a group of samples are more similar to each other than any replicates from different groups of samples. If R=0 then the similarity within and between groups of samples are on average the same. An important note to stress is that ANOSIM can only be applied to groups of samples that have been specified apriorly, *i.e*. before any data has been analysed.
PRIMER can also be used to identify taxa that are influential in characterising the biotic composition of groups of samples. The SIMPER (similarity percentages) routine is used to calculate the percentage contribution that each species makes to the similarity within groups of samples, based on similarity coefficients. Typical taxa that contribute highly to the similarity within groups of samples do so consistently across samples and generally in high abundance. SIMPER can also calculate the overall percentage contribution that each species makes to the average dissimilarity between groups of samples and in much the same way as outlined above, can highlight species that may be considered good discriminators between groups of samples.
SIMPER can be used to rapidly identify differences in patterns of species distribution and abundance between samples, hence its usefulness to the BACPOLES research.

Appendix 7

Sequences of Isolated Taxa

(by Hotchkiss, Landy, Mitchell)

Site1 : Amsterdam, Netherlands – Spruce of unknown age

UoP Ref: 18W1
BACPOLES Ref: 1CTD 1-320
Clone 1 closely resembled: *Pseudomonas* sp.
Seq: **UoP Ref:18W1 Clone 1**
ATTACCGCGTGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAA
AGTATTAATTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACAC
GCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCA

Clone 6 closely resembled: *Massilia* sp., *Herbaspirillum* sp., Uncultured beta proteobacterium, *Zoogloea* sp., *Matsuebacter* sp., *Oxalobacter* sp.
Seq: **UoP Ref:18W1 Clone 6**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTACCGTCATTAGATCTGG
ATATTAGCCAGACCCGTTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCAC
GCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCA

Clone 7 closely resembled: *Bergeyella* sp., Flavobacteriaceae str, *Riemerella anatipestifer, Chryseobacterium* jll, *Flavobacterium* sp., *Haloanella gallinarum*
Seq: **UoP Ref:18W1 Clone 7**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCATACTGTACCTTCAGCTACCCTCA
CGAGAGTAGGTTTATCCCAGTATAAAAGAAGTTTACAACCCATAGGGCCTTAGTCCTTCACGCGG
GATGGCTGGATCAGGCTCTCACCCATTGTCCAATATTCCTCA

Clone 8 closely resembled: *Pseudomonas* sp., Uncultured gamma proteobacterium
Seq: **UoP Ref:18W1 Clone 8**
ATTACCGCGGTCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAA
AGTATTAATTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACAC
GCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCA

Clone 9 closely resembled: Uncultured eubacterium, Uncultured bacterium clone, uncultured spirochete clone, Uncultured *Treponema* sp.
Seq: **UoP Ref:18W1 Clone 9**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTATTCGTCGGTTCATGTCATCACGCGGG
CATTCCCTCCCACGCTTATTCTTCCCCAACAAAAGGACTTTACAACCTTTCGGCCTTCGTCATCCA
CGCGGCGTCGCTCCGTCAGACTTTCGTCCATTGCGGAATATTCTTAG

Clone 10 closely resembled: *Brevundimonas vesicularis*, Glacial ice bacterium, Alpha proteobacterium, *Caulobacter crescentus*, *Asticcacaulis* sp.
Seq:**UoP Ref:18W1 Clone 10**
ATTACCGCGTGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGGTACCGTCATTATCGTC
CCCGGTGAAAGAATTTTACAATCCTAAGACCTTCATCATTCACGCGGCATGGCTGCGTCAGGCTT
TCGCCCATTGTCCAAAATTCCCCA

UoP Ref: 28w1
BACPOLES Ref:1ATD1 180 (Netherlands)
Clone 1 closely resembled: *Chryseobacterium scophthalmum, Flavobacterium* sp. *Chryseobacterium* sp.
Seq: **UoP Ref: 28w1 Clone 1**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCGTATAGTACCTTCAGCTACTCTCA
CGAGAGTAGGTTTATCCCTATACAAAAGAAGTTTACAACCCATAGGGCCGTCGTCCTTCACGCGG
GATGGCTGGATCAGGCTCTCACCCATTGTCCAATATTCCTCA

Clone 2 closely resembled: *Flavobacterium* sp.
Seq: **UoP Ref: 28w1 Clone 2**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGATCCTTATTCTCACAGTACCGTCAAGCTGATTCA
CGAATCAGTGTTTCTTCCTGTGCAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCGG
CATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCTCA

Clone 4 closely resembled: *Cytophaga* sp., *Flavobacterium* sp.
Seq: **UoP Ref: 28w1 Clone 4**
ATTACCGCGCGTTGCTGGCACGGAGTTAGCCGATCCTTATTCTTACGATACCGTCAAGCTCCTTC
ACGAAGGAGTGTTTCTTCTCGTATAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCG
GCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCTCA

Clone 5 closely resembled: *Helicobacter* sp., *Wolinella succinogenes, Helicobacter muridarum*
Seq: **UoP Ref: 28w1 Clone 5**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTACTCTGAAGGTACAATCAATACTCGATA
AATCGTGTATATTTTTCCCCTCTTACAGAAGTTTACAAGAAAAATCCCTTCATCCTTCACGCGGCG
TTGCTGCTTCAGCCTTTCGGCCATTGAGCAATATTCCTTA

UoP Ref: 85W1
BACPOLES Ref: 1.a.td.1
Clone 1 closely resembled: *Acinetobacter* sp., *Acinetobacter lwoffii*
Seq:**UoP Ref: GW1(85W1) Clone 1**
ATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGCGAGTAACGTCCACTACTCCTG
AGTATTAATCAGGGTAGCCTCCTCCTCGCTTAAAGTGCTTTACAACCAAAAGGCCTTCTTCACACA
CGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCCCCA

UoP Ref: 19W1
BACPOLES Ref: 1.d.td.3.340
Clone 8_2 closely resembled: *Flavobacterium* sp., Antarctic bacterium, *Cytophaga* sp.
Seq: **UoP Ref: 19w1 Clone 8_2**
CGGCTACCTTGTTACGACTTAGCCCTAGTTACCAGTTTTACCCTAGGCAGCTCCTTGCGGTCACC
GACTTCAGGTACCCCCAGCTTCCATGGCTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGGATCATGGCTGATATCCGATTACTAGCGATTCCAGCTTCACGGAGTCGAGTTGCAGACTC
CGATCCGAACTGAGAACGGTTTTATAGATTCGCTCCTTATTGCTAAGTGGCTGCTCTCTGTACCGT
CCATTGTAGCACGTGTGTGGCCCAGGACGTAAGGGCCGTGATGATTTGACGTCATCCCCACCTT
CCTCACAGTTTACACTGGCAGTCTTGCTAGAGTTCCCACCATTACGTGCTGGCAACTAACAACAG
GGGTTGCGCTCGTTATAGGACTTAACCTGACACCTCACGGCACGAGCTGACGACAACCATGCAG
CACCTTGTAATTTGTCTTGCGAAAGATCTGTTTCCAAATCGGTCAAACTACATTTAAGCCCTGGTA
AGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTC
CTTTGAGTTTCAGGCTTGCGCCCGTACTCCCCAGGTGGGATACTTATCACTTTCGCTTAGCCACT
CAGAATTGCTCCCGAACAGCTAGTATCCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTCGCTACCCACGCTTTCGTCCATCAGCGTCAATCGTTTGTTAGTAACCTGCCTTCGCAATTGGT
ATTCCATGTAATATCTAAGCATTTCACCGCTACACTACATATTCTAGTTACTTCACAAAAATTCAAG
CCTAACAGTATCAATGGCATTTTTTGGGTTAAGCCAAAAACTTTCACCGCTGACTTATTAAGGCCG
CCTACGGACCCTTTAAACCCAATGATTCGGATACGCTTGATCCTCCGTATACCGCGGCTGCTGGC
ACGAGTAGCGATCTTTATTCTTACAGTACGTCAGTTCCGACTTCGTCGGATGTTTCTTCTTGTACA
AAGCAGTACCACCATAGATGGCCTCATCTGACGCGCATGCTTGTCCAGCTGTCCATGACAAATTC
CTCATGGTTGCCTTCCGTAAAGAGTCTTGAATCCGG

UoP (Slu)Ref: 30W (A29 27)
BACPOLES Ref: 1b.d.t.3.310
Clone closely resembled: *Brevundimonas* sp., *Caulobacter* sp.
Seq: **UoP Ref: 30W (A29 27) Clone**
GCGGGCTCTNCGGGTACGGTCATTATCGTCCCCGGTGGAAAGAATTTTACAATCCTAAGACCTTC
ATCATTCACGCGGCATGGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCT
CCCGTAGGCCCCCCGTGCATATGNNTNNNGTAGGNCCCCAGTGCA

UoP (Slu)Ref: 30W (A29 10)
BACPOLES Ref: 1b.d.t.3.310
Clone closely resembled *Brevundimonas vesicularis*, *Caulobacter* sp

Seq: **UoP Ref: 30W (A29 10) Clone**
GGCTCTCTCGGGGACGGTCATTATCGGTCCCCGGGGGGAAAGAATTTTACAATCCTAAGACCTT
CATCATTCACGCGGCATGGCTGCGATCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGC
CTCCCGTAGGCCCCCCGTGCAGA

UoP (Slu)Ref: 30W (A29 986)
BACPOLES Ref: 1b.d.t.3.310
Clone 8 closely resembled *Brevundimonas* sp., *Caulobacter* sp
Seq: **UoP Ref: 30W (A29 986) Clone 8**
TTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGGTACCGTCATTATCGTCCC
CGGTGAAAGAATTTTACAATCCTAAGACCTTCATCATTCACGCGGCATGGCTGCGTCAGGCTTTC
GCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGAAGGGCGAATTCCAGCACACTGGCG
GCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG
TGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGT

UoP (Slu)Ref: 28W (A27r11025)
Bacpoles Ref: 1ADT1 180
DGGE band A closely resembled *Flavobacterium* sp.
Seq: **UoP Ref: 28W (A27r1 1025) DGGE band A**
ACTTATCTNAGGTACCGGTCAAGANATTACACGGTAATGATTGTTTCTTCCTGGTGCAAAAGCAGT
TTACAATCCATAGGGACCGGTCATCCTGCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGT
CCAATATTCCTCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

DGGE band B closely resembled *Flavobacterium* sp
Seq: **UoP Ref: 28W (A27r1 1025) DGGE band B**
ACTTATCTNNGGANCGGTCAAGCATCGGGACACGGTCCGGAGATGATTTCTTCCTGCTATAAAAG
CAGTTTTACAATCCATAGGCACCGTCATCCTGCACGCGGCATGGCTGGTTCAGACTTGCGATCCA
TTGACCAATATTCCTCACTGCTGCNTCCCGTAGGCCCCCCGTGCAA

DGGE band D closely resembled: *Brevundimonas* sp., *Phenylobacterium* sp., *Caulobacter* sp
Seq: **UoP Ref: 28W (A27r1 1025) DGGE band D**
GCTNTCTCNGGNACGGGTCATTATCGATCCCNGGATGANAGAATTTTACAATNCNTAANACCTTC
NTNATTCACGCGGGCATGGNTGCGCTGAGGCGTTTCGATGCATTGGNCAAGATTNNCCACTGNT
GCCTCNCNTACGNCNCNGGTGCATCCCGTAGGCCCCCCGTGCAA

DGGE band E closely resembled *Brevundimonas* sp., *Caulobacter* sp
Seq: **UoP Ref: 28W (A27r1 1025) DGGE band E**
CTCTCGGGTACGGTCATTATCGTCCCCGNGGTGGAAAGAATTTTACAATCCTAAGACCTTCATCA
TTCACGCGGCATGGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCG
TAGGCCCCCNGTGCATCTGCGTAGGCCNNCCGTGCAAAGGAA

UoP (Slu)Ref: A301
Bacpoles Ref: location unknown
DGGE band A closely resembled *Rhizobium galegae, Rhizobium* sp.*Agrobacterium* sp.
Seq: **UoP Ref: A301 DGGE band A**
CTCTCGGAACGTCATTATCTTCTCCGGTGAAAGAGCTTTACAATCCTAAGACCTTCATCACTCACG
CGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGCC
CCCCGTGCAAAA

DGGE band B closely resembled *Rhizobium galegae, Rhizobium* sp., *Agrobacterium* sp
Seq: **UoP Ref: A301 DGGE band B**
GCTCTCTCGGAACGGTCATTATCTTCTCCGGGGGAAAGAGCTTTACAATCCTAAGACCTTCATCA
CTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCG
TAGGCCCCCCGTGCAAA

DGGE band C closely resembled *Brevundimonas vesicularis*., *Brevundimonas* sp., *Caulobacter* sp
Seq: **UoP Ref: A301 DGGE band C**

CTTCTNTCGGGATACGGTCATTATCGTCTCCGGGGGAAAGAATTTTACAATCCTAAGACCTTCATC
ATTCACGCGGCATGGCTGNATCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCC
GTAGGCCCCCCGTGCAAAAAAAAA

Site 2 : Dordrecht, Netherlands – Spruce of c. 75 yrs

UoP Ref: 51W1
BACPOLES Ref:2.td.1.0-620 (Netherlands)
Clone 1 closely resembled: *Agrobacterium tumefaciens, Rhizobium* sp., *Rhizobium loessense, Azotobacter chroococcum, Reichenowia ornatae, Sinorhizobium* sp.
Seq: **UoP Ref: 51W1 Clone 1**
ATTACCGCCGTGTCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGATACCGTCATTATCTT
CTCCGGTGAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGC
TTGCGCCCATTGTCCAATATTCCCCA

Clone 2 closely resembled: *Oxalobacter* sp., *Matsuebacter* sp., *Zoogloea* sp., *Massilia* sp., *Massilia timonae, Herbaspirillum* sp., *Janthinobacterium agaricidamnosu.*
Seq: **UoP Ref: 51W1 Clone 2**
ATTACCGCGTGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTACCGTCATTAGCCCAG
GATATTAGCCTGAACCGTTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCA
CGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCA

Clone 4 closely resembled: *Massilia* sp., *Herbaspirillum seropedicae*, Uncultured beta proteobacterium, *Zoogloea* sp., *Duganella zoogloeoides*, *Matsuebacter* sp., *Oxalobacter* sp.
Seq: **UoP Ref: 51W1 Clone 4**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTACCGTCATTAGATCTGG
ATATTAGCCAAACCCGTTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCAC
GCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCA

UoP Ref: 80W1
BACPOLES Ref: 2.td.7 140
Clone 1 closely resembled: *Rhodobacter sphaeroides, Paracoccus* sp., *Paracoccus yeeii*
Seq: **UoP Ref:80W1 Clone 1**
CACGGAGTTAGCCGGGGCTTCTTCTGCTGGTACCGTCATTATCTTCCCAGCTGAAAGGACTTTAC
AACCCTAAGGCCTTCATCGTCCACGCGGCATGGCTAGATCAGGCTTGCGCCCATTGTCTAAGATT
CCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCCCCCGCCCCGCCCGCCGCGCGCGGCGGGCG
AAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCA
ATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGCTAGTGACTGG

Clone 3 closely resembled: *Rhodobacter sphaeroides, Paracoccus yeeii, Paracoccus aminophilus, Paracoccus marcusii, Catellatibacterium nectariphilum*
Seq: **UoP Ref:80W1 Clone 3**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTCTTCTGCTGGTACCGTCATTATCTTCC
CAGCTGAAAGGACTTTACAACCCTAAGGCCTTCATCGTCCACGCGGCATGGCTAGATCAGGCTT
GCGCCCATTGTCTAAGATTCCCCA

Clone 4 closely resembled: *Clostridium paradoxum*
Seq: **UoP Ref:80W1 Clone 4**
ATTCCGGCGGCTACTGGCAAGGAGTTATCAGTCCAAAAGTATAGGAACCATGTCCTTCCTCATCT
CCCCAAAAAGCACTTTACGACCCGAAGGCCTTCATCGTGCACGCGGCGTTGCTGCATCAGGCTT
TCGCCCATTGTGCAATATTCCCTA

Clone 5 closely resembled: *Pseudomonas denitrificans, Saccharospirillum impatiens, Alteromonas* sp., *Shewanella putrefaciens, Alteromonas macleodii, Psychromonas* sp.
Seq: **UoP Ref:80W1 Clone 5**
ATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTACCGTCAAAGATATCG
GTTATTAACCAACACCCCTTCTTCCCAACCTAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACA
CGCGGCATCGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCCCCA

UoP Ref: 57W1

BACPOLES Ref: 2.td.11.0-650
Clone 2_1 closely resembled: Arctic sea ice bacterium, Comamonas sp., Cytophaga sp., Uncultured Flavobacterium sp., Pseudomonas sp., Muricauda sp., Polaribacter sp., Uncultured Bacteroidetes bacterium, Porphyromonas sp., Prevotella sp., Capnocytophaga sp., Rhodovirga sp., Cellulophaga sp.
Seq: **UoP Ref: 57W1 Clone 2_1**
ATGGTTTGCATCCTGGCTCAGTCGTAACAAGGTAGCCGA

Clone 2_6 closely resembled: Pseudomonas sp., Arctic sea ice bacterium, Cytophaga sp., Wolinella sp., Streptomyces sp., Nocardia sp., Acidithiobacillus sp., Paenibacillus sp., Bradyrhizobium sp., Corynebacterium sp., Devosia sp., Methanococcus sp., Rhodopseudomonas sp., Brachymonas sp., Cellulophaga sp.
Seq: Seq: **UoP Ref: 57W1 Clone 2_6**
ATGGTTTGACTCCTTGGCTCAAACAGTCGTAACAAGGTAGCCGA

UoP Ref: 52W1
BACPOLES Ref: 2.td.2 400-840
Clone 6_1 closely resembled: *Stentrophomonas* sp., *Pseudomonas* sp., *Xanthomonas* sp.
Seq: **UoP Ref: 52W1 Clone 6_1**
ATGGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATACAAGTCGAACGGCA
GCACAGTAAGAGCTTGCTCTTACGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCT
ACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAA
GCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGT
AAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAA
ATCCAGCCGGCTAATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGC
GTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT
GGTGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGAT
CAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGGTAGTCCACGCCCTAAACGATGCGAACTGGATGT
TGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAGTTCGCCGCCTGGGGAGTACGGTC
GCAGACTGAAACTCAAGGATTGACGGGGGTCGCACAGCGGTGGAGTATGGTGGTTTATTCGATG
CACGGCGAGGAC

Clone 6_5 closely resembled: *Kocuria rosea, Pseudomonas* sp., *Halomonas* sp., Arctic sea ice bacterium, *Cytophaga* sp., *Streptomyces* sp*., Nocardia* sp., *Acidithiobacillus* sp., *Campylobacter* sp., *Paenibacillus* sp., *Bradyrhizobium* sp., *Corynebacterium* sp., *Devosia* sp., *Methanococcus* sp., *Rhodopseudomonas* sp., *Brachymonas* sp*., Gluconacetobacter* sp., *Geobacillus* sp., *Bacillus* sp., *Cellulophaga* sp.
Seq: **UoP Ref: 57W1 Clone 6_5**
ATGGTTTGATCCTGGCTCAACTATAGTCGTAACAAGGTAGCCGA

UoP Ref: 53W1
BACPOLES Ref: 2.td.3.400-850
Clone 5_1 closely resembled: Uncultured beta proteobacterium*, Halomonas* sp., *Kocuria rosea, Pseudomonas* sp., Arctic sea ice bacterium, *Cytophaga* sp., *Streptomyces* sp*., Nocardia* sp., *Acidithiobacillus* sp*., Paenibacillus* sp., *Bradyrhizobium* sp., *Corynebacterium* sp., *Devosia* sp*., Methanococcus* sp., *Rhodopseudomonas* sp., *Brachymonas* sp*., Geobacillus* sp., *Desulfiobacterium* sp., *Bacillus* sp., *Variovorax* sp., *Acidovorax* sp., *Devosia* sp., *Phyllobacterium* sp.
Seq: **UoP Ref: 53W1 Clone 5_1**
ATGGTTTGATCCTGGCTCAAACAGTCGTAACAAGGTAGCCGA

Clone 25_1 closely resembled: *Pseudomonas* sp., *Azotobacter* sp., *Rhodococcus* sp.
Seq: **UoP Ref: 53W1 Clone 25_1**
ATTACCGCGCGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACTGCAG
GGTATTAACCAGCAGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACA

CGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGG
CCCCCCGTGCCCCCGCCA

Clone 25_2 closely resembled: *Rhodococcus* sp., *Blastococcus* sp., *Streptomyces* sp., *Nocardia* sp*., Mycobacterium* sp., *Saccharomonospora* sp*., Micromonospora* sp., *Dactylosporangium* sp.
Seq: **UoP Ref: 53W1 Clone 25_2**
CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGGGGGACGACGGCCTTCG
GGTTGTAAACCTCTTTCGACCCTGACGAAGCGCAAGTGACGGTAGGGGGAGAAGAAGCGCCGG
CCAACTACGTGCCAGCAGCCGCGGTAATA

Clone 25_9 closely resembled: *Rhodococcus* sp., *Blastococcus* sp., *Streptomyces* sp., *Nocardia* sp., *Mycobacterium* sp., *Saccharomonospora* sp., *Micromonospora* sp., *Dactylosporangium* sp*. Gordonia* sp.
Seq: **UoP Ref: 53W1 Clone 25_9**
CGCCGCGCGCGGTGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGGGGGACGACGGCCTTCGGGTTG
TAAACCTCTTTCGACCCTGACGAAGCGCAAGTGACGGTAGGGGGAGAAGAAGCGCCGGCCAACT
ACGTGCCAGCAGCCGCGGTAATA

UoP Ref: 58W1
BACPOLES Ref: 2.td.12.0-760
Clone 14_2 closely resembled*: Stenotrophomonas* sp., *Pseudomonas* sp., *Xanthomonas* sp.
Seq: **UoP Ref: 58W1 Clone 14_2**
ATGGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCA
GCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTA
CTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAG
CAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTA
AAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGA
TCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAA
TCCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCG
TAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTG
GGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCA
GGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGG
GTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGCGAGTACGGTCG
CAAGACTGAAACTCAAAGGAATTGACGGGGGCCGCACAAGCGGTGGAGTATTGGGTTTAATTCG
ATGCAACGCGGAAAACCTTACCTGGCCTTGACATGTCGAAACTTTCAAGAGAATGATGTGCTCGG
GAACTCGAACCAGGTGCTGCATGGCTGTCGTCAGCTCGGTCTTGAGATGTCGGTAGTTCCGCCA
ACGAGCGCTACCTGTTCTTAGTTTGCAGC
Clone 14_9 closely resembled*: Stenotrophomonas* sp., *Pseudomonas* sp., Gamma proteobacterium
Seq: **UoP Ref: 58W1 Clone 14_9**
ATGGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCA
GCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTA
CTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAG
CAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTA
AAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGA
TCCAGCCATACCGCGTGGGTGAAGAACGTCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAT
CCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGC
AGCAGCCGCGGTAATACGAAGGTGCAGCGTTACTCGGATTACTGGGCGTAAAGCGTGCTAGTGT
CGATTAAGTCGTTGGAAAGCCCTGGGTCCACCTGGGACTGCATGAATACTGGACGACTAAGGGT
GGTTAAGGTACGGAATTCTGGGTAAATGATGCTAAAATCAGAGAAACTTCATGGGAGCATTCTGA
CAACTTGACTGAGCCAAGTGGGAACACGGATAAATCCTGTATCCGCCTAAAATGCAAATGGATTG
GGCAATGGTCGAAATCCAACCACCGTATTTCCCCTTGGGAGCCCCCCAGTAATCCAAGATCGGG
CCCACGGGGAATGGTTTTATTGGGCGGAACTT

UoP (Slu)Ref: 52W (A86)
BACPOLES Ref: 2.td.2.400-840
Clone closely resembled *Bacillus fumarioli, Bacillus* sp.
Seq: **UoP Ref: 52W (A86) Clone 1**
CAAATGGGGGCCTTTCTGGGTTGGTACAGTCAAGGTACCGGCAGTTACTCCGGTACTTGTTCTTC
CCTAACAACAGAGCTTTACGACCCGAAGGCCTTCATCGCTCACGCGGCGTTGCTCCATCAGACTT
TCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

UoP (Slu)Ref: 54W (A87a)
BACPOLES Ref: 2.td.8.0-700
Clone 3 closely resembled *Brevundimonas* sp., *Caulobacter* sp., *Phenylobacterium* sp.
Seq: **UoP Ref: 54W (A87a) Clone 3**
CACGAAGTTAGCCGGGGCTTCTTCTCCGGGTACCGTCATTATCGTCCCCGGTGAAAGAATTTTAC
AATCCTAAGACCTTCATCATTCACGCGGCATGGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATT
CCCTACTGCTGCCTCCCGTAGGAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATC
CGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGAAGCATAA

Clone 6 closely resmbled *Sphingomonas* sp., *Zymomonas* sp.
Seq: **UoP Ref: 54W (A87a) Clone 6**
CACGGAGTTAGCCGGAGCTTATTCTCCCGGTACTGTCATTATCATCCCGGGTAAAAGAGCTTTAC
AACCCGAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGGTTTCCCCCATTGTCCAATATT
CCCCACTGCTGCCTCCCGTAGGAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATC
CGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAG

UoP Ref: 75W
BACPOLES Ref: 21ta3@3
DGGE band A closely resembled: *Rhodobacter* sp., *Paracoccus* sp.,
Seq: **UoP Ref: 75W DGGE band A**
GCTCTCGCTGGTACGGTCATTATCTTCCCAGCNTGAAAAGGACTTTACAACCCTAAGGCCTTCAT
CGTCCACGCGGCATGGCTAGATCAGGCTTGCGCCCATTGTCTAAGATTCCCCACTGCTGCCTCC
CGTAGGCCCCCCGTGCAGA

Site 4 : Haarlem, Netherlands, Scots Pine c. 100 years old

UoP Ref: 101W
BACPOLES Ref:4.te.5.0.00-0.50
DGGE band C most closely resembled *Acinetobacter* sp., *Mycobacterium* sp.
Seq: **UoP Ref: 101W DGGE band C**
CGGCTNCTNGGTACGTCATTATCTCNNNTATAGNGGNNCNNNGCTTTCTTAACNGGACAANATAA
CTTNACNANCCNGANGGGCTTCNTNCTGCNCGACGGNNGTGCTGGATTAGGCTTNGNNGCNTTG
TCNAAAATTCCNCAGTGCTGCCTCCNGTAGGCCCNCAGTGCAA

DGGE band E most closely resembled *Oxalobacter* sp., *Herbaspirillum* sp., *Massilia* sp., *Zoogloea* sp., *Nitrosomonas* sp.
Seq: **UoP Ref: 101W DGGE band E**
GCTTATCTTNGGTACGGTCATTATCCCGANGATANTANCGGNNCANCGTNTTTCTTCACCGGACA
AAANANACTTTACAACCNGAAGGNCTTCNTCACTGCACGACGGNANTGCTGGATNAGGCTTNCG
NGCATTGTCCAAAATTCCNCAGTGCTGCCTCCCGTAGGCCCNCAGTGCAA

DGGE band F most closely resembles *Oxalobacter* sp., *Janthinobacterium* sp., *Herbaspirillum* sp
Seq: **UoP Ref: 101W DGGE band F**
GNGGCTNTCTTNGGTACGGTCATTAGCAGNAGATATTAGCNCCCACCGTTTTCTTCCCTGGACAA
AANAGCTTTACAACCCGAAGGCCTTCTTCACTCACGCGGCATTGCTGGATCAGGCTTGCGCCCAT
TGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

DGGE band H most closely resembles *Acidovorax* sp., Comamonas *sp., Hylemonella*
Seq: **UoP Ref: 101W DGGE band H**
GCTATCTNGGTACGGTCATGGTCCCGGGGTATTATCCNGAAACTTTTCGTTCCGTACAAAAGCAG
TTTACAACCCGAAGGNCTTCATCCTGCACGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCA
AAATTCCCCACTNCTGCCTCCCGTAGGCCCCCNGTGCAA

DGGE band K most closely resembles *Brevundimonas vesicularis, Brevundimonas* sp., *Caulobacter* sp.,
Seq: **UoP Ref: 101W DGGE band K**
GGCTATCTCGGGTACGTCATTATCGTCCCCGGATGAAAGAATTTTACAATCCTAAGACCTTCATCA
TTCACGCGGCATGGCTGCGTCAGGCTTTCGCCCATTGNGCANGATTCCCCACTGCTGCCTCNCG
TANGNCCCCCGATGCACATGCCTCCCGTAGGCCCCCNGTGCAA

DGGE band L most closely resembles *Coccomonas* sp., *Pseudomonas* sp.
Seq: **UoP Ref: 101W DGGE band L**
GGCGGGCTNTCTNGGTACGGTCATGGGCCCGCCGGTATTAGGGCAGACCTTTTCGTTCCGTACA
AAAGCAGTTTACAACCCGAAGGCCTTCTTCCTGCACGCGGCATTGCTGGATCAGGCTTTCGCCCA
TTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

UoP (Slu)Ref: A51 (A51 27)
BACPOLES Ref: unknown location
Clone most closely resembles *Acidovorax* sp.,
Seq: **UoP Ref: A51 27 Clone**
CTTCTAGGTACGTCATGGACCCCCTTTATTAGAAGGAGTCTTTTCGTTCCGTACAAAAGCAGTTTA
CAACCCGAAGGCCTTCATCCTGCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAAAA
TTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAAA

Site 5 : Rotterdam, Netherlands – Silver Fir and Spruce of unknown age

UoP Ref:67W1
BACPOLES Ref: 5.te.2. 50
Clone 3 closely resembled: *Zymobacter palmae, Pseudomonas cellulose, Alishewanella fetalis, Halomonas* sp., *Halomonas salina*.
Seq: **UoP Ref:67W1 Clone 3**
TCACGTAGTAGGTGAATGCCTTTCTCCCCGCTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCAC
ACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCAA

Clone 4 closely resembled: *Pseudomonas* sp.
Seq: **UoP Ref:67W1 Clone 4**
ATTACCGCGTGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAA
AGTATTAATTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACAC
GCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCA

Clone 5 closely resembled: unidentified bacteria
Seq:**UoP Ref:67W1 Clone 5**
ATTCCCGCCGTTGTTGGCAAGTAGTTAGCCGAGACTTATTCTGGGGATACTGTCCTTCCTCATCT
CCCCAAAAAGCACTTTACGACCCGAAGGCCTTCATCGTGCACGCGGCGTTGCTGCATCAGGCTT
TCGCCCATTGTGCAATATTCCCTA

UoP Ref: 62W2
BACPOLES Ref: 5te1
Clone most closely resembled *Pseudomonas* sp.,
Seq: **UoP Ref: 62W2 clone**
GCTATCTGTGGTACGTCAAACTGCAGGGTTATTAACCAGCAGCCCTTCCTCCAAACTTAAAGTGC
TTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCA
ATATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

DGGE band B most closely resembled *Pseudomonas* sp.,
Seq: **UoP Ref: 62W2 DGGE band B**

TNTCTGTGGTACGTCAAAACAATCACGGTATTAGGTAACTGCCCTTCCTCCCAACTTAAAGTGCTT
TACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAAT
ATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAANNNNNNNNNNNTNTNTNATATNANNAA
NAANATNNAANNANNNTATNGAANATNAAANTGGNNNNNAAAANNNGNNNNNAAATGATNAAATA
ANGNANNGANATNAAANAATAAANTNNNNTTNGNAAAANNATAAAATGNTNNNANGAANAAGANN
NTNANAAANNANNNNANNTTANNAATNATTNTAAGNNNCNTNNAANANAAANNGNGGANNAAAAT
AGTNTANNNNNNNAAAAANTNANATTACNANTNCNTNAANTNNAATTNAATNANNNNNAANTTATN
TAACTANATNGNAAANAANTANNNATNNTNNANTTNNANTNAANNNANAATANAANATTTAGNAAN
NNNATTATNTNAATAATNNNNNATTAANTANTATAANNAAANNNNANNNNAANNNNNNNAAGNNTN
NATNAAANNNATNNATNTAAAAAATANAAAAAAATNAAANNNANAANNNNGGTNNAANNAANGANN
AAAANANNANAAAAAAGNATANATANNNTTAAATNAANGNNTAANNNATAATAANATTANAANNNA
ATNNANNNGAAANNTATANAANNNAANTAAANNAATNNAAGTAGATAAATATTNGTTACTNAGTAT
ATTAATTANATTANNNNAANAAGNTAATATNTANANATANANAAAGTNGNANNATTTAAANNNATNA
NNAAATAACACNTNGTAATANANAATAANNATTANNNTANNAAAAAGATTANNAATANTTATNAATN
ANCAANANANNAAAATAANNAATCNTNAAAATTANANATNTATNTTTAAATNATANTTANNNATTNN
AAAANNAGTAGTANGGNNNNNGAAANTNNAGTGTAAATANGANTNAGNAANNNNAAAAAAAATAN
ANAAANAAAANTAAAANANANNAAANATNTTTTAANAATATGAGTAANTANANTNNATNAAAACAAA
AAAANAAAAAAAAATATTNGNGTTATAATNNNAAAGNCANCAAATNAAGANATATTNACTANANAAA
NCTNAAANTTNTAANTATAGTTANNTTGATNAGTNGAATATATTTTNATAATTAANAANANNTTANA
NNAATATNTANNNATAANAAGNTATNNTNTAANNATNGTTATTNAANAANNAATNNAANGTTGAAN
NATATTAANANACTAAGAANNAAATAAANNTAAAANAANNACACNAAANAATTATTNAATANANTNA
TAGTANAAAANNAAATTNANANNAAANANAAATATATATNAANNNNATNAGCNANNANAANANAAT
CANANNNAATAGTATGAATANTNCNNGACNATTTAGNNNTTNATACNAAATCAANANTNCNAGANT
AA

UoP (Slu)Ref: A93 (A93 994A)
BACPOLES Ref: 5te11040
Clone 5 most resembled *Pseudomonas* sp.
Seq: **UoP Ref: A93 994A Clone 5**
GAACTTCATTTTAGGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGG
ATATCTGCAGAATTCGCCCTTCCTACGGGAGGCAGCAG

UoP (Slu)Ref: A93 (A93 994)
BACPOLES Ref: 5te11040
DGGE band B resembles: uncultured beta proteo-bacterium, *Acidovorax* sp., *Variovorax paradoxus,*
Seq: **UoP Ref: A93 994 DGGE band B**
ATNACTCCCTGNTATTANAGAAANGNTTTTCGTTCCGATACNNNANCANTTTACAACCCNAAGGG
CTTCATGCTGTCACGCGCGANNGGCTGGATNANGCTTGGGNNCATTGTCCAAAATTCCCCACTN
CTGCCTCCCGTAGGCCCCCCGTGCAA

Site 6 : Koog ald Zaan, Netherlands –Scot's Pine of c. 70yrs

UoP Ref: 63W1
BACPOLES Ref: 6.te.1 0-500 (Netherlands)
Clone 3 closely resembled: *Sphingomonas* sp., *Porphyrobacter cryptus, Sphingomonas herbicidovorans.*
Seq: **UoP Ref: 63W1 Clone 3**
ATTACCGCCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCTCCCGGTACTGTCATTATCATCC
CGGGTAAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGCTTT
CGCCCATTGTCCAATATTCCCCA

Clone 5 closely resembled: *Sphingomonas* sp., *Novosphingobium* sp., *Sphingomonas stygialis, Sphingomonas aromaticivorans, Sphingomonas aromaticivorans, Sphingomonas capsulata, Sphingomonas subterranea*
Seq:**UoP Ref: 63W1 Clone 5**
ATTACCGCGTGTCTGCTGGCACGGAGTTAGCCGGAGCTTATTCTCCAGGTACTGTCATTATCATC
CCTGGTAAAAGAGCTTTACGACCCGAGGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGCT
TTCGCCCATTATCCAATATTCCCCA

UoP (Slu)Ref: 63W1 (A118A)
Clone 8 most resembled *Chryseobacterium* sp., *Flavobacterium* sp., *Riemerella* sp.,
Seq: **UoP (Slu)Ref: 63W1 (A118A) Clone 8**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCGTATAGTACCTTCAGCTACCCTCA
CGAGGGTAGGTTTATCCCTATACAAAAGAAGTTTACAACCCATAGGGCAGTCGTCCTTCACGCGG
GATGGCTGGTTCAGGCTTGCACCCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGCCCCC
CGTGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGC
TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC

UoP (Slu)Ref: 63W1 (A115B)
BACPOLES Ref: 6.te.3200-2500
Clone 4 closely resembled: *Brevundimonas* Sp., *Caulobacter* sp.
Seq: **UoP (Slu)Ref: 63W1 (A115B) Clone 4**
ATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGGTACCGTCATTATCGTCC
CCGGTGAAAGAATTTTACAATCCTAAGACCTTCATCATTCACGCGGCATGGCTGCGTCAGGCTTT
CGCCCATTGCGCAAGATTCCCTACTGCTGCCTCCCGTAGGCCCCCCGTGCAAGGGCGAATTCCA
GCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCAT
AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCC

UoP (Slu)Ref: 63W1 (A110)
BACPOLES Ref: 6.te.3200-2500
Clone 1 closely resembled *Pseudomonas* sp.,
Seq: **UoP (Slu)Ref: 63W1 (A110) Clone 1**
CACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACTGCAGGGTATTAACCAGCAGCCC
TTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATC
AGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAAGGGCGAATTCCAGCAC
ACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCT
GTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC

UoP Ref: 65W1
BACPOLES Ref: 6.te.2.0-500
DGGE band A closely resembled *Cytophaga* sp., *Flavobacterium* sp.,
Seq: **UoP Ref: 65W1 DGGE band A**
ANTNNNANAANNNNNANNTCCTTACGAAGAGTGTTTCTTCTCGTATAAAAGCAGTTTACAATCCAT
AGGACCGTCATCCTGCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCTCA
CTGCTGCCTCCCGTAGGCCCCCCGTGCAANGAANNNNNNNNNNNNNNANNGNNANNGNTNNNN
NANNA

Site 7 : Haarlem, Netherlands – Poplar of unknown age

UoP Ref: 71W1
BACPOLES Ref: 7.te.4.300-500

Clone 3_9 closely resembled: Pseudomonas sp., Arctic sea ice bacterium, Cytophaga sp., Streptomyces sp., Nocardia sp., Acidithiobacillus sp., Campylobacter sp., Paenibacillus sp. Bradyrhizobium sp., Corynebacterium sp., Devosia sp., Methanococcus sp., Rhodopseudomonas sp., Brachymonas sp., Cellulophaga sp.
Seq: **UoP Ref: 71W1 Clone 3_9** ATGGTTTGTCCTTGGCTCAACTATAGTCGTAACAAGGTAGCCGA

Clone 3_{10} closely resembled: Oxalobacter sp., Janthinobacterium sp., Pseudomonas sp., Massilia sp., Duganella sp., Ultramicrobacterium str., Aquaspirillum sp., Herbaspirillum sp.
Seq: **UoP Ref: 71W1 Clone 3_{10}**
GATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCGCGGGGCAACCTGGCG
GCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCAAGAGTGGGGGATAACGTAGCGAA
AGTTACGCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGACCGCAAGGCCTTGTGCTCCT

GGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCTACGATCAGTAG
CTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAG
GCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGGGGGCTAATATCCTCCACTAATG
ACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAAT
CCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTA
GAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGCAGCCCC
CTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACTCTGGTA
GTCCACGCTCTAAACGATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTAACGC
GTGAAGTAGACCGCCTGGGGAGTACGGTCGCAGATTAAAACTCAAAGGAATTGACGGGACCCGC
ACAGCGGTGATGATGTGGATTAATTCGATGCACGCGAAAACCTTACCTACCCTTGACATGTACGA
GCCACGAGAGATCGAGGTGTGCTCGAAGAAAATCGTACCAGTGCTGCATGGCTGTCGTCAGCTC
CGTCTGGAATGTTGTTAGTTCGCACGAGGCCAACTTGCCATAGTGCTACGAAGACCCCTTATGGA
ACTGCGTACAACCCGGAAGAAAG

Clone 24_3 closely resembled: Janthinobacterium sp., Massilia sp., Oxalobacter sp., Pseudomonas sp., Ultramicrobacterium str., Aquaspirillum sp., Variovorax sp., Alcaligenes sp., Lampropedia sp.
Seq: **UoP Ref: 71W1 Clone 24_3**
CGGCTACCTCTGTTACGACTTCACCCCAGTCACGAATCCTACCGTGGTAAGCGCCCTCCTTACGG
TTAAGCTACCTACTTCTGGTAAAACCCGCTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCG
GGAACGTATTCACCGCGACATGCTGATCCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGT
TGCAGACTGCGATCCGGACTACGACTGGCTTTATGGGATTGGCTCCCCCTCGCGGGTTGGCAAC
CCTCTGTACCAGCCATTGTATGACGTGTGTAGCCCCACCTATAAGGGCCATGAGGACTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCAACTAAATGTAGCAACT
AATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAG
CCATGCAGCACCTGTGTACTGGTTCTCTTTCGAGCACTTCCCAATCTCTCGGGAATTCCAGCCAT
GTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGG
GTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTCACGCG
TTAGCTGCGTTACCAAGTCAATTAAGACCCGACAACTAGTAGACATCGTTTAGGGCGTGGACTAC
C

Clone 24_8 closely resembled: uncultured bacteria
Seq: **UoP Ref: 71W1 Clone 24_8**
AATTGAACGCTGGCGGTTGCCTTACGCCTGCTTCTGCAAATATCCATCGGAGCTTGCTCTGGTGG
CGAGTGGTCTCGGGTGAGTATTCATCCTATACGTACCCTGGAGTGGGGTATATGGCCGTCAATTT
ACGCTAATACCACTGGCAAAACCCTGATGATACTGGGGGAATCGCCTTGTCATGCATCCCCCTTT
CCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGACTACCCTTGCCTCCATCAGCACCTGGTCT
GAGAGGAATGCCCGCCCCCTGGGAACTGACATTCGGCCCGGACTCCGAGGGGAGGACGCACTG
GGGAATTTTGGACCTTGGCCGCACCCCTGATCCCGCTCTGTCCCGTGTCTTCCCTTCGTTTCTCG
GTTGTAAAGCTCTTTTTCCCCGGAAGAAACTGTGAGGGCTAATCTCTTTTGGTAATGAATGTACCG
GATTAATGAACCCCGGCTACCTACATGTTGATTACCGCGATGATACATATGGTGCAAGCTCTACT
CGAAATTACTGGTCTCAACGCGTGCGCATGGAGTCTTGTAAGTCTGATGTGAAACTCTTGGGCTC
AACCTGGGGAATTGCATTGCACACTGCTCGGTCTATAATCTGGCATAGGGAGGTAGAATTCCACG
TGTAGCACTGTAGTGCATAGATATGTGTGAGGAACACCGATGGTGACGCAGCTCTCTGGGTCAA
GATTGACGCTCATGCTCCTAAGCGTGGCGAGCCACCAGGATTACATACCCTGATAGTCCACGCT
CACCACGATGTCTACTAGTTGTCTGGTTTTATCTAACTTGGTAACGCAGCTAACGCGTGAGTAGA
CCGCCTGGTGAGTACGGGTCTTCAATATTAAAACTCAAAGTAGTTGGCGGGACCCGCACAAGCC
GAAGCATGATGTGCATTTATGTCGATGCCACCGCGTAAAGCCTTACCTACCCTTTGACATGACTG
ATCCTCTACAGATGACGAGTGCCTCGAAGACACCCGTACACAGTGCTGCATGACTGTCATCAGCT
CCGTCTTGGGAGAGTTGGCTAAGTCCCCTCACGACCCATCCTTGCCATATGTGCTACGAAGGTCA
CCTCTAATGGAACTGCCCAGGGTACAAAGCCGGCAAGACAAAGCGGGTGGAGGCAC

UoP Ref: 73W1
BACPOLES Ref: 7.te.6 430-600
Clone 4_3 closely resembled: *Aquaspirillum* sp*., Xylophilus* sp., *Pseudomonas* sp., *Variovorax* sp*., Comamonas* sp., Uncultured beta proteobacterium.
Seq: **UoP Ref: 71W1 Clone 4_3**
CGGCTACCTTCGTTACGACTTCACCCCAGTCACGAACCCCGCCGTGGTAAGCGCCCTCCTTGCG

GTTAGGCTACCTACTTCTGGCGAGACCCGCTCCCATGGTGTGACGGGCGGTGTGTACAAGACCC
GGGAACGTATTCACCGCGACATTCTGATCCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAG
TTGCAGACTGCGATCCGGACTACGACTGGCTTTATAGGATTAGCTCCCCCTCGCGGGTTGGCAA
CCTTCTGTACCAGCCATTGTATGACGTGTGTAGCCCCACCTATAAGGGCCATGAGGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCCCATTAGAGTGCCCAACTAAATGTAGCAAC
TAATGGCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA
GCCATGCAGCACCTGTGTTACGGTTCTCTTTCGAGCACGAAACCATCTCTGGTAACTTCCGTACA
TGTCAAAGGTGGGTAAGGTTTTTCGCGTTGCATCGAATTAAACCACATCATCCACCGCTTGTGCG
GGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTCACGC
GTTAGCTTCGTTACTGAGTCAGTGAAGACCCAACAACCAGTTGACATCGTTTAGGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTACAGGTCCAGGG
GATTGCCTTCGCCATCGGTGTTCCTCCGCATATCTACGCATTTCACTGCTACACGCGGAATTCCA
TCCCCCTCTACCGTACTCTAGCTATACAGTCACAAATGCAGTTCCCAGGTTGAGCCCGGGGATTT
CACATCTGTCTTATATACCGCCTGCGCACGCTTTACGCCCAGTATTCCGATTACGCTTGCACCCT
ACGTATTACGCGCTGCTGCACGTAGTAGCGGTGCTTATTCTACGTAACGTCATGTCCCAGGTATT
ATCCGAGACTTTCCGTCGTACAAAGCAGTTACACCGAGGCTCATCTGGACGCGCATGCCTGATCA
GCCTTCGCCATGGTTCCAATTC

Clone 4_7 closely resembled: *Janthinobacterium* sp., uncultured eubacterium, *Pseudomonas mephitica*, *Aquaspirillum* sp., *Herbaspirillum* sp., *Duganella* sp., *Massilia* sp., Arctic sea ice bacterium.
Seq: **UoP Ref: 71W1 Clone 4_7**
CGGCTACCTTGTTACGACTTCACCCCAGTCACGAATCCTACCGTGGTAAGCGCCCTCCTTGCGGT
TAAGCTACCTACTTCTGGTAAAACCCGCTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGG
GAACGTATTCACCGCGACATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCAGTCGAGTT
GCAGACTACAATCCGGACTACGATACACTTTCTGCGATTAGCTCCCCCTCGCGGGTTGGCGGCG
CTCTGTATGTACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCA
TCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCAACTAATGA
CAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTGTGTACTGGTTCTCTTTCGAGCACTCCCTGATCTCTCAAGGATTCCAGCCATGTCAA
GGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTCACGCGTTAGCT
GCGTTACCAAGTCAATTAAGACCCGACAACTAGTAGACATCGTTTAGGGCGTGGACTACCAGGGT
ATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAATCTTGACCCAGGGGCTGCCTT
CGCCATCGGTGTTCCTCCACATATCTACGCATTTCACTGCTACACGTGATTCTACCCCCCTCTGC
CAGATTCTAGCCTTGCAGTCTCCAATGCAATTCCCAGGTTGAGCCCGGGGATTTCACATCAGACT
TACAAAACCGCCTGCGCACGCTTTACGCCAGTATTCGATACGCTGCACCCTACGTATTACCGCGG
CTGCTGCACGTAGTAGCGTGCTTATTCTTCAGTACGTCATAAGCAGAGAATTAGCTCTCACCGTT
CTTCTTGAACAAGAGCTTAACGCTGAGCCTCTCACTCACGCGCATGCTGGATCAGCCTTCGCCCA

UoP Ref: 64W
BACPOLES Ref: 7 te. 1. 500-750
Dgge band L most resembled: *Sphingomonas* sp.,
Seq: **UoP Ref: 64W DGGE band L**
GCTATCTCCGGGACGGTCATTATCATCCCGGGATAAAAGAGCTTTACAACCCTAAGGCCTTCATC
ACTCACGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCNCG
TAGGCCCCCCGTGCNAATGCCTNCNGTAGGCCCGCAGTGCAA

DGGE band K most resembled: *Sphingomonas* sp
Seq: **UoP Ref: 64W DGGE band K**
BGNGGGCTATCTCCGGNANTGTCATTATCATCCCGGGTAAAAGAGCTTTACAACCCTAAGGCCTT
CATCACTCACGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCT
CCCGTAGGCCCCCCGTGCAAATGNGTNTGGTANGCNNGCANTGNAAANNTANNNTAANNTTATA
ATAAGANANNAANANNAAAAANGNTNAAAAANNNTAATAAATANAAANAAATNNATNNTANATAAT
AATNTANAANNANAAATNTNNGTTNNACANNAANATNNAAGNAANAANNAANNAAAAAAAGAAAAN
TTGNAAANANAATATATTTAANTCTAANTTACTTAANTAANANATNTATAGNAAANNNGNTANAANT
ATNATAANTATNTNANNTAANNTTTANAGAAAANCNTAANNTATANTTAATNNTNGAGAAANNNAAT
TNNTATAAANANANAGAGANNANANTGNANTNNNAAANTANAAANNNTCNNANAGNTATAAANAA
NNATNNATANAAGANTTATTAAANNAGTANTTTANNTNTNNNNTAATTAAANTGNANANANANNAN
AATNANAGAATAAAACAAATATTAAATAATTNAATNANAAGTANNTTNAANNAGANTATANNTANTA

AANAAGNTTNNAAANNNAAANNNANATAAATAAGNATGNNNAANAATNNATATATAANTANAATAA
TANNAAAAAAANNANATNNAANGNAAAANTAAAAATANATANAAAAANANANNTATAATANANNAN
ANANNNATNAATNATNANACNNANNTNAANAANAANTAATCNCATNAATTNGTAAATATNACTTAAT
AAANAAATANANAANAAAAANAANANNANNGGNANTNATCNGTNNAAAAAANANAATTNTTNTNNA
ANTNANAAGTNNTATTAANNCTNAAANAAAANNNTCTTANTTATTATNANNAAATANANNNAANNAA
NTNATNTCNNANNNNATAATATAATNAAAGNTTATCATNTATAATNNNNGAGTAANANATATANAAT
AAANTNANAANATTATAANATANANATANAAAAANTTAAAAAACTNAAAAATAGTGNNAAANNNANN
TATNNNTACNACNAGNNANTATNNTAANATTAATACAANANNANANANTAAAAAAATAAANNATTAA
NAAANCAAGTNNTAANTANAANAAAANAATAATNANNAACTANTTTANGANTANTTNTAAGAGAAAT
GAANTTANATNATTACAAANNNGTCNNAANNAATTTTTNNAANAGATNAANGAGNNAANATTATAA
TTTAGANNNNTGTTAAAACANNAATNTCNNATAANANATTNTACATNNAAAAATATACATNNATAAT
AAAATAANTNATTAAAGTTANNANNCAATCAANNCANATGAATAAGAAAGATAAANNANNNNTATA
TATANTATTNATAANAATATAANTANNANTTATNNNGATTNATANANANTATANANANAATATNTAN
AATNNNTANAANATAAAAAGAATANNANTAAATTTNAATGANNATNCNTGTNATNTGANCGAANNT
AA

Site 9 : Dokkum, Netherlands – Oak of c. 590yrs

UoP Ref:7W1
BACPOLES Ref: 9.ta.4 (Netherlands)

Clone 1 closely resembled: *Desulfovibrio alaskensis, Desulfovibrio vietnamensis*
Seq: **UoP Ref:7W1 Clone 1**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCCTCTGGGGTTACCGTCAACTTCTTAC
GAAGCTTCTTCACCCCTGACAGGAGTTTACGACCCGAAGGCCTTCATCCTCCACGCGGCGTCGC
TGCGTCAGGCTTTCGCCCATTGCGCAAAATTCCTCA

Site12 : BZN, Netherlands – Oak and Scot's Pine of c. 376yrs

UoP Ref: 23W1
BACPOLES Ref: 12.td.1.3 (Netherlands)

Clone 1 closely resembled: Actinobacteria, *Cellulomonas* sp. *Streptomyces* sp. *Arthrobacter* sp.
Seq: **UoP Ref: 23W1 Clone 1**
AITACCGCGTGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTTTCGCTT
CTTCCCTGCTGAAAGGGGTTTACAACCCGAAGGCCTTCATCCCCCACGCGGCGTCGCTGCATCA
GGCTTTCGCCCATTGTGCAATATTCCCCA

Clone 2 closely resembled: *Tanella salinilacus, Tanella fryxellensis, Methylarcula* sp., *Roseobacter* sp., *Jannaschia helgolandensis, Antarctobacter heliothermus, Ruegeria* sp., *Antarctobacter* sp.
Seq: **UoP Ref: 23W1 Clone 2**
ATTACCGCCGTGCTGCTGGCACGGAGTTAGCCGGGGTTTCTTTACCAGGTACTGTCATTATCATC
CCTGGCGAAAGAGCTTTACGACCCTAAGGCCTTCGTCACTCACGCGGCATCGCTAGATCAGGCT
TGCGCCCATTGTCTAAGATTCCCCA

Clone 4 closely resembled: *Psychrobacter glacincola, Psychrobacter aquatica, Psychrobacter vallis.*
Seq: **UoP Ref: 23W1 Clone 4**
ATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGCAGCTAATGTCATCGTCTCCGG
GTATTAACCGAAGAGTCTTCTTCACTGCTTAAAGTGCTTTACAACCAAAAGGCCTTCTTCACACAC
GCGGCATGGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCA

Clone 5 closely resembled: *Tanella salinilacus, Methylarcula* sp.
Seq: UoP Ref: 23W1 Clone 5
ATTACCGCGGCTGCTGCACGGAGTTAGCCGGGGTTTCTTTACCAGGTACTGTCATTATCATCCCT
GGCGAAAGAGCTTTACGACCCTAAGGCCTTCGTCACTCACGCGGCATCGCTAGATCAGGCTTGC
GCCCATTGTCTAAGATTCCCCA

UoP Ref: 26W1 (Ew1)
BACPOLES Ref: 12.td.2.3 (Netherlands)

DGGE band D closely resembled: *Rhodobacter* sp., uncultured/unidentified sp.
Seq: **UoP Ref: 26W1 DGGE DGGE band D**
TNGGGACGGGTCATTATCATCCCNGGGGTGNAAAGAGCTTTACAACCCTAAGNCCTTCATCNCN
CACGCGGNATGGCTAGATCAGGCTTGCGCNCATTGTCTAAGATTCCCCACTGCTGCCTCCCGTA
GGCCCCCGGTGCATNGCGTANGGCNCGCGTGCAGTGCAA

DGGE band F closely resembled: *Rhodobacter* sp.
Seq: **UoP Ref: 26W1 DGGE Band F**
GGGGNTNTCGCGGAACGTCATTATCTTCCAGGTGAAGGACTTTACAACCCTAAGGCCTTCATCGC
TCCACGCGGCATGGC
TAGATCAGGCTTGCGCCCATTGTCTAAGATTCCCCACTGCTGCCTCCCGTAGGCCCCCNGTGCA
TACCCGTAGGCCCCCC
GTGCAAGNAA

Site13 : BZN, Netherlands – Oak and Scot's Pine of c. 360yrs

UoP Ref:40W1
BACPOLES Ref: 13.TA.4 (Netherlands)
Clone 1 closely resembled: *Bacillus benzoevorans, Bacillus* sp., *Bacillus megaterium, Marinibacillus marinus, Marinibacillus campisalis*
Seq: **UoP Ref:40W1 Clone 1**
ATTACCGCCGCGTTTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTAC
GAGCAGTTACTCTCGTACTTGTTCTTCCCTAACAACAGAGTTTTACGATCCGAAAACCTTCATCAC
TCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTA

Clone 3 closely resembled: *Novosphingobium* sp., *Novosphingobium subarcticum, Erythrobacter* sp., *Erythrobacter* sp., *Erythrobacter flavus, Zymomonas* sp., *Porphyrobacter tepidarius, Porphyrobacter neustonensis.*
Seq:**UoP Ref: 40W1 Clone 3**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGAGCTTATTCTCCAGGTACTGTCATTATCATCCC
TGGTAAAAGAGCTTTACAACCCTAAGGCCTTCACCACTCACGCGGCATTGCTGGATCAGGCTTTC
GCCCATTGTCCAATATTCCCCA

Site 14: Travenhorst, Germany – Oak of unknown age

UoP Ref: 82W1
BACPOLES Ref: 14.TA.HE.3. 0,34-1,57
Clone 2 closely resembled: *Herbaspirillum* sp., *Janthinobacterium* sp., *Janthinobacterium lividum, Pseudomonas mephitica, Janthinobacterium agaricidamnosum*
Seq:**UoP Ref:PW1 (82W1) Clone 2**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTACCGTCATTAGCAGGAG
ATATTAGCTCCCACCGTTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCAC
GCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCA

UoP Ref: 107W1
BACPOLES Ref: 14.TA.+ TE.20-0,62-0,89
DGGE band B closely resembled: *Cytophaga* sp. *Flavobacterium* sp.
Seq:**UoP Ref: 107W1 DGGE band B**
TTNAAANNNANNNNNNNNGGAAANTNTNTACNAANNCNNNNAAGCTNCNACACGNTCNGAATGTT
TCTTCTCGTATAAAA
GCAGTNCTANTNATCCATAGGACCGTCATCCTGCACGCGGCATGGCTGGATCAGGCTTGCGCCC
ATTGTCCAATATTCCT
CACTGCTGCCTCCCGTAGGCCCCCCGTGCAANNNANNNTNTTNTAANNNATANAAANNTATTTAA
AAANA

Site 23 : Leeuwarden, Netherlands –Scot's Pine of c. 100yrs

UoP Ref: 43W1
BACPOLES Ref: 23.td.1.80
Clone 2 closely resembled: *Sphingomonas* and *Porphyrobacter* species
Seq:**UoP Ref: BW1 (43W1) Clone 2**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCTCCCGGTACTGTCATTATCATCCC
GGGTAAAAGAGCTTTACAACCCTAAGGCCTTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTTC
GCCCATTGTGCAATATTCCCCA

UoP Ref: 44W1
BACPOLES Ref: 23.td.1.300
DGGE band closely resembled: CFB group bacterium
Seq:**UoP Ref: 44W1 DGGE band**
GCTATCGTANGGANCGTTCAGCCTACTCATCACGAGTAGATAGGGNTTTATCCCTATACAAAAGT
AAGTTTACAACCCATAGGGCCGGTCGCTCCTTCACGCGGGATGGCATGGATCAGGCTCTCACCC
ATTGTCCAATATTCCTCACTGCTGCCTCCCGTAGGCCCCCCGTGCAAGCAA

Site 24: Bryggen, Norway. Scot's Pine of unknown age

UoP Ref: 10W1
BACPOLES Ref: 24.ta.5.1
Clone 12_1 closely resembled: *Janthinobacterium* sp*., Pseudomonas* sp., *Oxalobacter* sp., *Aquaspirillum* sp., *Herbaspirillum* sp*., Matsuebacter* sp.
Seq:**UoP Ref: 10W1 clone 12_1**
ATGGTTTGATGAAGGAGGCAGATTGAACGCTGGCGGCATGCCTTACACATGCTAGTCGAACGGC
AGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTAG
AGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTACGGATGGAAGTGGGGG
ATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCT
ACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAA
TGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGG
GCTAATATCCCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTT
TTGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGA
ATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAACAC
CGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGA
CTTGGTAACTGAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
AAAGGAATTGACTGGGACCCGCACAAGCGGTGGATGATGTGGAATAATTCCGATGCAACGCGAA
AACTTACCTACCCTTTGACATGGCTGGAATCCCCGAAAGATTTGGGAAGTGTCCGAAAGAAAGCC
AGTACACAGGTGCCTGCATGGGCTTGTCGTCACCTCGGTCCGGAAATGTGGTAAGTCCCCACAC
AGCGCACCTTTGCATTTAGTTTGCTATCAAGACCCTCATAGGAACTTGCCCGGTAGACAAACCCC
TAAGATAGTGTTGGGGATAGGACACCC

Clone 12_8 closely resembled: *Janthinobacterium* sp., *Massilia* sp., *Duganella* sp., *Pseudomonas* sp., *Collimonas* sp., *Herbaspirillum* sp.
Seq:**UoP Ref: 10W1 clone 12_2**
CGGCTACCTCTGTTTACGACTTCACCCCAGTCACGAATCCTACCGTGGTAAGCGCCCTCCTTGCG
GTTAAGCTACCTACTTCTGGTAAAACCCGCTCCCATGGTGTGACGGGCGGTGTGTACAAGACCC
GGGAACGTATTCACCGCGACATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCAGTCGAG
TTGCAGACTACAATCCGGACTACGATACACTTTCTGGGATTAGCTCCCCCTCGCGGGTTGGCGG
CCCTCTGTATGTACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCAACTAAT
GACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCA
TGCAGCACCTGTGTTACGGCTCTCTTTCGAGCACACCTCGATCTCTCGTGGCTTCCGTACATGTC
AAGGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTC
CCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTCACGCGTTA
GCTGCGTTACCAAGTTAATTAAAACCCGACAACTAGTAGACATCGTTTAGGGCGTGGACTACCAG

GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAATCTTGACCCAGGGGGCTG
CCTTCGCCATCGGTGTTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGAATTCTACCCCC
CCTCTGCCAGATTCTAGCCTTGCAGTCTCCATCGCAATTCCCAGGATGAGCCCGGGATTTCACGA
CAGACTACAAACGCTGCGCACGCTTTACGCCAGTACTCGATACGCTGCACCTACGTATTACGCGC
TGCTGCACGTAGTAGGCGGTGCTTATTCTTCAGCTACGTCATAGCAGATTTAGCCCTCACGGTTC
TCCTGACAAAAGAGCTTACAGCGAGCTCTCATCACGGCATTCGATCGCTGCAAGTGCAATCCAAT
GGTCCCTCCGTAGGACCT

Clone 12_9 closely resembled: Uncultured beta proteobacterium, *Aminomonas* sp., *Methylophilus* sp., *Dechlorosoma* sp., *Methylobacillus* sp., *Azospira* sp., *Azoarcus* sp., *Dechlorosoma* sp., *Methylomonas* sp.
Seq:**UoP Ref: 10W1 clone 12_9**
CGGCTACCTCTGTTACGACTTCACCCCAGTCATGAACCCCACCGTGGTAAGCGTCCCCCTTGCG
GTTAGACTACCTACTTCTGGTGAAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGCGACATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGAAGTCGAG
TTGCAGACTTCAATCCGGACTACGATCGGCTTTCTGGGATTGGCTCCCCCTCGCGGGTTGGCAA
CCCTCTGTACCGACCATTGTATTACGTGTGAAGCCCTGGCCATAAGGGCCATGAGGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCCCATTAAAGTGCCCAACTAAATGATGGCAA
TTAATGGCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGAC
AGCCATGCAGCACCTGTGTCCACTTTCCCTTTCGGGCACCTAATGCATCTCTGCTTCGTTAGTGG
CATGTCAAGGCCAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATAATCCACCGCTTGTG
CGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTCAC
GCGTTAGCTGCGTTACTAAGAGATTTTACTCTCCCAACAACTAGTAGACATCGTTTAGGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTATTGACCCA
GGGGGCTGCCTTCGCCATTGGTATTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGAATC
CTACCCCCCTCTGCCATACTCTAGCCTTGTAGTTTCAAACGCAGTTCCCAGGTTGAGCCCGGGGC
TTTCACATCTGACTTACAAAACCGCTGCGCACGCTTACGCCCAGTATTCGATACGCTCGCACCCT
ACGTATACGCGCTGCTGACGTAGTAGCGGTGCTTCTTATCAGTACGTCATAACACAGATATCGCT
GGATCGTCCTCGTGGCAAGAGCTACACGAAGGCCTCTACTCACCGGAATGGCTGATCAGCTGGC
CCATGTTCAATCCCCAATGT

UoP Ref: 4W1
BACPOLES Ref: 24.ta.3
DGGE Band closely resembled: *Acidovora*x sp.
Seq:**UoP Ref: 4W1 DGGE band**
GCTATCTAGGTACGGTCATGGGACCCCCCTTTATTAGAAGGAGTCTTTTCGTTCCGTACAAAAGC
AGTTTACAACCCGAAGGCCTTCATCCTGCACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGT
CCAAAATTCCCCACTGCTGCCTCCCGTAGGGCCCCCCGTGCAAA

UoP Ref: 9W1
BACPOLES Ref: 24.ta.3
DGGE Band closely resembled: *Pseudomonas* sp., *Halomonas* sp.
Seq:**UoP Ref: 9W1 DGGE band**
GGGNNTTTNNTGGGNNACGNTCANNNCCACCGGGNTNATTANANAAANGAGGATTTNCGACTTC
CCGAATAAAANGTTGTTTTTACAANCCGAAGGCNCTTCATTCACAACACNTCGGTCATTGGTGGAT
NANGCCTTGCGNGCCATTGTCCAATATTCCCCACTGNTNCCTCCCGTAGGCCCCCCGTGCAAA

Site 25 : Mollosund, Netherlands – Oak of unknown age

UoP Ref: 76W1
BACPOLES Ref: 25.ta.1.a.4
Clone 1_1 closely resembled: Pseudomonas sp., Arctic sea ice bacterium, Streptomyces sp., Halomonas sp., Cytophaga sp. Nocardia sp., Acidithiobacillus sp., Microcystis aeruginosa, Staphylococcus aureus, Hydrogenophaga palleronii, Paenibacillus sp., Bradyrhizobium sp., Corynebacterium bovis, Cellulophaga sp.
Seq:**UoP Ref: 76W1 Clone 1_1**
AGGTAGCCGAGTCGTAACAAGGTAGCCGA

Clone 1_6 closely resembled: Oxalobacteraceae sp., Janthinobacterium sp.,Oxalobacter sp., Pseudomonas mephitica, Duganella sp., Massilia timonae, Ultramicrobacterium sp., Herbaspirillum sp., Aquaspirillum sp., Matsuebacter sp.
Seq:**UoP Ref: 76W1 Clone 1_2**
GATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCGCGGGGCAACCTGGCG
GCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCGA
AAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGACCGCAAGGCCTTGTGCTCC
TGGAGCGGCCGATATCTGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCATCGATCAGTA
GCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAA
GGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGTGGGCTAATATCCCATGCTAAT
GACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGTCGTGAAA
TCCCCGGGCTCAACCTGGGAATGGCGATGGAGACTGCAAGGCTAGAGTTTGGCAGAGGGGGGT
AGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCAGCC
CCCTGGGTCAAAACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCACGCCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCAGCTAAC
GCGTGAAGTAGACCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCACGCGAAAAACCTTACCTACCCTTGAC
ATGGATGCATCTTGAAAGATTGAGGAGTGCCCGAAGGAACATACACAGTGCTGCATGCTGTCGTC
AGCTCGTGTCTGAAATGTTGGTAAGTCCCGCACGAGCGCACTGTCATTAGTTGCCTACGAAAGAA
ACCTCTTATGAAACTGCGGTGACAAATCGGAGAAAAGGGGTGGGAATTAAACAGT

Site 26 : Elst, Netherlands – Oak c. 1900yrs

Note: All sequences come from DGGE bands of Thomas's consortia not actual wood

UoP (slu) Ref: 90w (A171)B
BACPOLES Ref: Elst te 3 0-74 26
DGGE band resembled: *Pseudomonas* sp.
Seq: **UoP (slu) Ref: 90w (A171)B DGGE band**
GNGGGCTTATCTGTGGTAAGATCAAACTGCAGGGTTATTAACCAGGCAGCCCTTCCTCCNAACTT
AAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCA
TTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAAAA

UoP (slu) Ref: 92w (A169 993)B
BACPOLES Ref: 26 te 10-78
DGGE band resembled: *Brevundimonas* sp.
Seq: **UoP (slu) Ref: 92w (A169 993)B DGGE band**
CTNTTGCGGGTACGGTCATTNTCGCTCNCCGGTGAAAGANTTTTANGATTCNTAANACCTTCATC
ATTCNNGCGACCATGGANNGCGTTCAGGNTTTCGCCCATTGCTGCANGATTCCCACANTTGCGT
GCCTNGCGTAAGANCCCCGTAGNNCTGCCTCCCGTAGGCCCCCCGTGCAA

UoP (slu) Ref: 92w (A169 993)
BACPOLES Ref: 26 te 10-78
Clone 1 resembled: *Bacillus* sp.
Seq: **UoP (slu) Ref: 92w (A169 993) clone 1**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGG
CAGTTACTCCGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGACCCGAAGGCCTTCATCGCTC
ACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGG
AAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGC
GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACAC

Clone 2 resembled: *Bacillus* sp.
Seq: **UoP (slu) Ref: 92w (A169 993) clone 2**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGG
CAGTTACTCCGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGACCCGAAGGCCTTCATCGCTC
ACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGG

AAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGGC
GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACA

Clone 8 resembled: *Brevundimonas* sp.
Seq: **UoP (slu) Ref: 92w (A169 993) clone 8**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGAGACTTTCTCCTGAGGTACCGTCCTTGCACCGG
ACGTTACTCCGGGTTTTGTTCTTCCCTAACCTTCTACCTTTACGACCCGAAGGTCTTCATCGCTTG
CGCCCATTTGCTCCAATTCCTCACTGCTGCCTCCCGAAGAATCGCTAAAGCTGCCTCCCTGGGAG
CCGTTAATTCCGGATCCCAGCTCGGCGTTAGCTTGGATTAATCATGGTCATAGCTGTTTCCTGTG
TCAAATTGTTATCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACTGTA

Site 27 : GZ-80, Netherlands – Oak c. 450yrs

UoP Ref: 88W1
BACPOLES Ref:27.ta.3

Clone 3 closely resembled: *Mesorhizobium* sp.,*Rhizobium mediterraneum*
Seq: **UoP Ref: 88W1 Clone 3**
ATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTACGGCTACCGTCATTATCTTCAC
CGTTGAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTG
CGCCCATTGTCCAATATTCCCCA

Clone 4 closely resembled: *Cellvibrio* sp.,*Cellvibrio ostraviensis,Cellvibrio mixtus* subsp. *Mixtus, Cellvibrio vulgaris*, Myxobacterium, *Cellvibrio fulvus, Pseudomonas fluorescens, Pseudomonas putida*
Seq: **UoP Ref: 88W1 Clone 4**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTGGGTAACATCAATTCACTCAC
GTATTAGGTGAATGACCTTTCTCCCCACTGAAAGTGCTTTACAACCCTAAGGCCTTCTTCACACAC
GCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCA

Clone 5 closely resembled: *Variovorax* sp., *Aquaspirillum delicatum, Rhodoferax fermentans, Acidovorax avenae* subsp. c*itrulli, Acidovorax avenae* subsp. *cattleyae*
Seq: **UoP Ref: 88W1 Clone 5**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTACGGTACCGTCATGAGCCCCCT
GTATTAGAGGAAGCCTTTTCGCTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCATCCTGCA
CGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCTCA

Clone 6 closely resembled: *Cellvibrio* sp.
Seq: **UoP Ref: 88W1 Clone 6**
TAAACCCCGCCCCTTTTTTTGCTGGGCACGGAGTTAGCCGGTGCTTCTTCTGTGGGTAACATCAA
TTCACTCACGTATTAGGTGAATGACCTTTCTCCCCACTGAAAGTGCTTTACAACCCTAAGGCCTTC
TTCACACACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATGTTCCCCACTGCTGCCT
CCCGTAGGCCCCCCGTGCCCC

Clone 8 closely resembled: *Sphingomonas* sp., *Sphingomonas macrogoltabidus, Sphingomonas adhaesiva, Sphingopyxis alaskensis, Sphingomonas aerolata, Sphingomonas faenia, Sphingomonas aurantiaca*
Seq: **UoP Ref: 88W1 Clone 8**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGAGCTTATTCTCCCGGTACTGTCATTATCATCCC
GGGTAAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGCTTTC
GCCCATTGTCCAATATTCCCCA

Clone 9 closely resembled: *Flavobacterium* sp., *Cytophaga aquatilis, Flavobacterium xinjiangensis, Flavobacterium hydatis, Flexibacter aurantiacus* subsp. *excathedrus, Cytophaga succinicans*
Seq:**UoP Ref: 88W1 Clone 9**
ATTACCGCGTGCTGCTGGCACGGAGTTAGCCGATCCTTATTCTTACAGTACCGTCAATCTGGCTC
ACGAGCCAGGGTTTCTTCCTGTACAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCG
GCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCTCA

Site 28: KZ-47, Netherlands. Oak c. 480 yrs

UoP Ref: 81W1
BACPOLES Ref: 28.ta.2
Clone 11_3 closely resembled: *Janthinobacterium* sp., *Pseudomonas* sp., *Aquaspirillum* sp., *Herbaspirillum* sp., *Duganella* sp., Oxalobacteraceae bacterium, *Massilia* sp., Arctic sea ice bacterium, *Collimonas* sp.
Seq: **UoP Ref: 81W1 Clone 11_3**
CGGCTACCTTGTTACGACTTCACCCCAGTCACGAATCCTACCGTGGTAAGCGCCCTCCTTGCGGT
TAAGCTACCTACTTCTGGTAAAACCCGCTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGG
GAACGTATTCACCGCGACATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCAGTCGAGTT
GCAGACTACAATCCGGACTACGATACACTTTCTGCGATTAGCTCCCCCTCGCGGGTTGGCGGCG
CTCTGTATGTACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCA
TCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCAACTAATGA
CAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTGTGTACTGGTTCTCTTTCGAGCACTCCCCAATCTCTCGGGGATTCCAGCCATGTCAA
GGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTTAATCTCGCGACCGTACTCCCCAGGCGGTCTACTTCACGCGTTAGC
TGCGTTACCAAGTCAATTAAGACCCGACAACTAGTAGACATCGTTTAGGGCGTGGACTACCAGGG
TATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAATCTTGACCCAGGGGCTGCCT
TCGCCATCGGTGTTCCTCCACATATCTACGCATTTCACTGCTACACGTGGAATTCTACCCCCCTCT
GCCAGATTCTAGCCTTGCAGTCTCCAATGCAATTCCCAGGTTGAGCCCGGGGATTTCACATCAGC
TTACAAAGCCGCCTGCGCACGCTTTACGCCCAGTATTCGATACGCTTGCACCCTACGTATTACCG
CGCTGCTGCACGTAGTAGCGTGCTTATTCTTCAAGTACGGTCATAGCAGAGATATTAGCTCTCAC
GGTCTCTGGACAAGAGCTTACACCAGAAGGCTCTACTCAGCGCATGCTTGATCAGCTTCGCATGA
CAAATCCCCATGGCTGCTTTCCGTAAGAGACTCTGG

Clone 11_9 closely resembled: *Chryseobacterium* sp., *Haloanella* sp., *Flavobacterium* sp., *Kaistella* sp., *Bergeyella* sp., *Riemerella* sp.
Seq: **UoP Ref: 81W1 Clone 11_9**
CGGCTACCTTGTTACGACTTAGCCCTAGTTACTTGTTTTACCCTAGGCAGCTCCTGTTACGGTCAC
CGACTTCAGGTACCCCAAACTTCCATGGCTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT
TCACCGCATCATGGCTGATATGCGATTACTAGCGATTCCAGCTTCATAGAGTCGAGTTGCAGACT
CCAATCCGAACTGAGACCAGCTTTCGAGATTCGCATCCAGTCGCCTGGTAGCTGCCCTCTGTACT
GGCCATTGTATTACGTGTGTGGCCCAAGGCGTAAGGGCCGTGATGATTTGACGTCATCCCCACC
TTCCTCTCTACTTGCGTAGGCAGTCTCACTAGAGTCCCCAACTGAATGATGGCAACTAGTGACAG
GGGTTGCGCTCGTTGCAGGACTTAACCTAACACCTCACGGCACGAGCTGACGACAACCATGCAG
CACCTTGAAAATTGTCCGAAGAAAAGTCTATTTCTAAACCTGTCAATTCCCATTTAAGCCTTGGTAA
GGTTCCTCGCGTATCATCGAATTAAACCACATAATCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGCTAACTTATCACTTTCGCTTAGTCTCTGA
ACCCTAAAGCCCAAAAACGAGTTAGCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TCGCTCCCCACGCTTTCGTCCATCAGCGTCAGTTAAAACATAGTGACCTGCCTTCGCAATTGGTG
TTCTAAGTAATATCTATGCATTTCACCGCTACACTACTTATTCCAGCCACTTCTACTTTACTCAAGA
CCTGCAGTATCAATGGCAGTTTCATAGTTAAGCTATGAGATTTCACCACTGACTTACAGATCCGCC
TACGGACCCTTTAAACCCAATAAATCAGGATACGCTTGCACCCTCCGTATTACCGCGGCTGCTGG
CACGGAGTAGCGGTGCTTATTCGTATAGTACTCAGCTACTCCTCACGAGAGTAGTTTATCCCTATA
CAAAAGAGGTACATCATAGGTCGTCGTCTCAGTGAATGGCTGGATCAGGTTACCATGCAAATTTC
CTATGCTGCTTCGTAGAGTCTGATTCCGGGGTTTT

Clone 20_8 closely resembled: Uncultured proteobacterium, *Oxalobacter* sp., *Janthinobacterium* sp., *Pseudomonas* sp., *Massilia* sp., *Herbaspirillum* sp., *Aquaspirillum* sp.
Seq: **UoP Ref: 81W1 Clone 201_8**
GTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCG
CGGGGCAACCTGGCGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCAAGAGTGG
GGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGCAGGGGACCGC
GAGGCCTTGTGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAGGCCTACCA
AGGCTACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACGGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGC
CGTGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGGGAGCT
AATATCTCCCACTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGC

GGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTT
GTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAAT
CTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACC
GATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTACTAGTTGTCGGGTTTAATTAACTT
GGTAACGCAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAA
GGAATTGACGGGACCCGCACAAGCGGTGGATGATGGTGGATTATTCGATGCAACGCGAAAACCT
TACCTACCTTGACATGTACGGAGCACGAGAGATCGAGGGTGCTCGAAAGAAACGTAACCAGGTG
CTGCATGCTGTCGTCAGCTCTGTTCTGGATGTGGGTAGTCCCGACCAGCCACCCTTGCATAGTTG
CTCCAAGACCCTAATGAACGTGCGGGGAAACCGGAGGAATGGTGGGGAATTAAATTTTCT

Clone 20_9 closely resembled: Uncultured Bacteroidetes bacterium, Uncultured Cytophagales, *Bacteroides* sp., Cryomorphaceae bacterium
Seq: **UoP Ref: 81W1 Clone 20_9**
CGGCTACCTCTGTTACGACTTAGCCCCAGTCACCAGTTTTACCCTAGGCCGCTCCTTACGGTTGC
AGACTTCAGGTACCCCCAGCTCCCATGGCTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT
TCACCGCGCCGTGGCTGATGCGCGATTACTAGCGAATCCAGCTTCATGAAGTCGAGTTGCAGAC
TTCAATCCGAACTGAGACCGGCTTTCGAGATTGGCATCTCCTCGCGGAGTAGCTCCCCTCTGTAC
CGGCCATTGTAACACGTGTGTAGCCCTGGACGTAAGGGCCGTGCTGATTTGACGTCATCCCCGC
CTTCCTCACAGCTTACGCTGGCAGTTTCACTAGAGTCCCCGGCATTACCCGCTGGCAACTAATGA
TAAGGGTTGCGCTCGTTATGGCACCTAAGCCGACACCTCACGGCACGAGCTGACGACAACCATG
CAGCACCTCGCAAAAAGCCATTGCTGGCTCACACCTTTCAGCGTGATTCTTCTCGCGTTCGAGCC
CAGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGTTCCTCCGCTTGTGCGGGCCCCCGT
CAATTCCTTTGAGTTTCATCGTTGCCGACGTACTCCCCAGGTGGATCACTTAATGCTTTTACTCAG
ACGCATACATTGTATCGCATACATCCAGTGATCATCGTTTACGGCGTGGACTACCAGGGTATCTA
ATCCTGTTTGATCCCCACGCTTTCGTGCCTCAGCGTCAGTACTAATTTAGTAAGCTGCCTTCGCAA
TCGGTGTTCTGTGTAATATCTAAGCATTTCACCGCTACACTACACATTCCGCCTACCTCAATTATA
CTCAAGATATTCAGTATCAATGGCAATGCTATCGTTAAGCACAGTCTTTCACCACTGACTTAAATA
CCCGCCTACGCACCCTTTAAACCCAATAAATCAGGATACGCTCGCATCCTCCGTATTACCGCGCT
GCTGGCACGGAGTAGCCGATGCTATCGTACTGTACTTCAGTATCCTCGCAGGATACAATTACCCA
GTACAAAAGAAGGTTTACACCCTTAGGCCGTCTTTCCTCCCGCGCATGCTTGTTCAGTGCCCATG
AACAAATTTCCTACTGCTGCCTCCGTAAGGATTCTGGTCCCGGG

UoP Ref: 86W1
BACPOLES Ref: 28ta1
Clone 13_1 closely resembled*: Cytophaga* sp., *Flavobacterium* sp., *Acanthamoeba* sp., Arctic sea ice bacterium sp., *Sporocytophaga* sp.
Seq: **UoP Ref: 86W1 Clone 13_1**
CGGCTACCTTGTTACGACTTAGCCCTAGTTACCAGTTTTACCCTAGGCAGCTCCTTGCGGTCACC
GACTTCAGGCACCCCCAGCTTCCATGGCTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGGATCATGGCTGATATCCGATTACTAGCGATTCCAGCTTCACGGAGTCGAGTTGCAGACTC
CGATCCGAACTGTGACCGGTTTTATAGATTCGCTCCTGGTCGCCCAGTGGCTGCTCTCTGTACCG
GCCATTGTAGCACGTGTGTAGCCCAAGGCGTAAGGGCCGTGATGATTTGACGTCATCCCCACCT
TCCTCACAGTTTGCACTGGCAGTCTTGTTAGAGTTCCCGACTTGACTCGCTGGCAACTAACAACA
GGGGTTGCGCTCGTTATAGGACTTAACCTGACACCTCACGGCACGAGCTGACGACAACCATGCA
GCACCTTGTAAATTGTCTTGCGAAAGATCTGTTTCCAAACCGGTCAATCTACATTTAAGCCTTGGT
AAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGGATACTTATCACTTTCGCTTAGCCACT
GAAATTGCTTCCAACAGCTAGTATCCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT
CGCTACCCACGCTTTCGTCCATCAGCGTCAATCAATTAGTAGTAACCTGCCTTCGCAATTGGTATT
CCATGTAATCTCTAAGCATTTCACCGCTACACTACATATTCTAGTTACTTCCTAATAATTCAAGTCT
AACAGTATCAATGGCCGTTCCACCGTTGAGCGATGGGCTTTCACCACTGACTTATAAACGCCTAC
GACCCTTTAAACCATGATTCCGATACGCTGATCTCGTATACCGCGCTGCTGCACGGAGTAGCGAT
CTTATTCTTACGATCGGTCAGGTCGAACCGTCGATGTTTCTTCTCGTTAAAGCAGTACATCAAGAC
GTCATCTGACCGGCATGCTGGATCAGCTGCACGCAACTTTTAATGTGCCTCCGTAGATCCTGATC
CGGTTCTCG

Clone 13_2 closely resembled: *Cytophaga* sp., *Flavobacterium* sp., Arctic sea ice bacterium, *Sporocytophaga* sp., Bacteroidetes bacterium, *Acanthamoeba* sp.
Seq: **UoP Ref: 86W1 Clone 13_2**
CGGCTACCTTGTTACGACTTAGCCCTAGTTACCAGTTTTACCCTAGGCAGCTCCTTGCGGTCACC
GACTTCAGGCACCCCCAGCTTCCATGGCTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGGATCATGGCTGATATCCGATTACTAGCGATTCCAGCTTCACGGAGTCGAGTTGCAGACTC
CGATCCGAACTGTGACCGGTTTTATAGATTCGCTCCTGGTCGCCCAGTGGCTGCTCTCTGTACCG
GCCATTGTAGCACGTGTGTAGCCCAAGGCGTAAGGGCCGTGATGATTTGACGTCATCCCCACCT
TCCTCACAGTTTGCACTGGCAGTCTTGTTAGAGTTCCCGACTTGACTCGCTGGCAACTAACAACA
GGGGTTGCGCTCGTTATAGGACTTAACCTGACACCTCACGGCACGAGCTGACGACAACCATGCA
GCACCTTGTAAATTGTCTTGCGAAAGATCTGTTTCCAAACCGGTCAATCTACATTTAAGCCTTGGT
AAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGGATACTTATCACTTTCGCTTAGCCACT
GAAATTGCTTCCAACAGCTAGTATCCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT
CGCTACCCACGCTTTCGTCCATCAGCGTCAATCAATTAGTAGTAACCTGCCTTCGCAATTGGTATT
CCATGTAATCTCTAAGCATTTCACCGCTACACTACATATTCTAGTTACTTCCTAATAATTCAAGTCT
AACAGTATCAATGGCCGTTCCACCGTTGAGCGATGGGCTTTCACCACTGACTTATAAACGCCTAC
GACCCTTTAAACCATGATTCCGATACGCTGATCTCGTATACCGCGCTGCTGCACGGAGTAGCGAT
CTTATTCTTACGATCGGTCAGGTCGAACCGTCGATGTTTCTTCTCGTTAAAGCAGTACATCAAGAC
GTCATCTGACCGGCATGCTGGATCAGCTGCACGCAACTTTTAATGTGCCTCCGTAGATCCTGATC
CGGTTCTCG

Clone 13_3 closely resembled: *Cytophaga* sp., *Flavobacterium* sp., Arctic sea ice bacterium, *Sporocytophaga* sp., *Acanthamoeba* sp.
Seq: **UoP Ref: 86W1 Clone 13_3**
ATAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATGCAAGTCGAGGGGTA
TAGTTCTTCGGAACTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTACCTTTTACAGA
GGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATAGTCCTGGCATCAGGATTATATTA
AAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAGCTAGTTGGTAAGGTAACGGCTTACCAA
GGCTACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAG
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCC
GCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTATACGAGAAGAAACACTCCTTCGTG
AAGGAGCTTGACGGTATCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAATAAGTCA
GTGGTGAAAGCCCATCGCTCAACGGTGGAACGGCCATTGATACTGTTAGACTTGAATTATTAGGA
AGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATACCAATTGCGAAG
GCAGGTTACTACTAATTGATTGACGCTGATGGACGAAAGCGTGGGTAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGGAAGCAATTTCAGTGGCTAAGCGAA
AGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGAACCTTACCAAGCTTAAAT
GTAGATTGACCGGTTTGGAAACAGATCTTTCGCAGACATTTACAAGGTGCTGCATGGTGTCGTCA
GCTCGTGCTGTGAGTGTCCAGGTAGGTCTTACGAGGGCACCCCTGTTGTAAGTGCAGCGAGTCA
AGTCGTACCTAACGACTGCATGCACATGGAAGAAGGTGGGGAATAACCCCAAATCATTCCGGGG
GCCC

Laser-Trapped and Laser Cut Sequences – All *Stenotrophomonas* sp.

Lotte C – Clone 26_6
CGGCTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAGG
TTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAG
TTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAG
CCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGC
AACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGAC
ATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAAC
GCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGTCCA

GGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAGGAATT
CCGCTACCCTCTACCACACTCTAGTCGTCCAGTATCCACTGCAGTTCCCAGGTTGAGCCCAGGCT
TTCACAACGGGACTTAAACGACCACCTACGCACGCTTACGCCCAGTAATTCGAGTACGCTGCACC
CTTCGTATACGCGCTGCTGGCACGAGTAGCTGGTGCTTATTCTTAGGTACGTCATCCCACTGGGA
TTAGCAGCTGATCTCACAAGCTTTACACCGAGCTTCTCACTAGCGAATGCTGATCAAGGCTTGGC
GCCCCA

Lotte C – Clone 26_8

CGGCTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAGG
TTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAG
TTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAG
CCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGC
AACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGAC
ATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAAC
GCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGTCCA
GGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAGGAATT
CCGCTACCCTCTACCACACTCTAGTCGCCCAGTATCCACTGCAGTTCCCAGGTTGAGCCCAGGG
CTTTCACAACGGACTTAAACGACCACCTACGCACGCTTTACGCCCAGTAATTCCGAGTAACGCTT
GCACCCTTCGTATACCGCGCTGCTGCACGAGTAGCCGGTGCTTATTCTTTGGTACGTCATTCCAT
CGGTATTAGCCAGCTGATTCTTCCCAACAAGCCTTACACCGGAGGCTTCTTCCCCACGCGAATGC
TGATCAGCTGCCCCATGGTCCAATTTCCCCATGCTTGCCCCTCCCGTAGAGGAGTCTCTGAG

Lotte C – Straight from Vial

CCGAAAAGGGGTACGCCTAACCTTGCACGTCGAACGCGCAGCACAGTACGAGTTTGCTCTTACG
GGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTACTCTGTCGTGGGGGATAACGTA
GGGAAACTTACTCTAATACCGCATACCACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCG
CGATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCCCCAGGGCGACTATC
CGTATCTGGTCTGAGAGGATGATCACCCACTCTGGAACTGACACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGA
AGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGGTG
GGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACAAAG
GGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTG
TGAAAGCCCTGGGCTCAACCTGGGCACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGG
GTAGCGGTATTCGTGGTGTAGCAGTGATATGCGTAGAGATCAAGAGGAACATCCGTGGCGAGGG
CAGCTACGTGGACCAACACTGACACTGACGCACGAAAGCGTGGAGAGCAAACAAGATTATATAC
CGTGGTAGTCCACGCCCTACACTATGCAAACTGTATGTTGGGTGCAATTTGACACGCAATATCGA
ATCTAACGCGTTAAGTTCGCCGCGTGGAGAGTACGGTCGCAAGACTGAAACTCAAGAGATAGAC
GGGGGGCCCGCACAGCGGTGGAATATGTGGTTTTATTCGATGCAACGCAAAGACCTTTACCTGC
CCTTGACTTGTCGAGCACTCCAGAGAGGATTGGTGCTTCGGAACTCAACCACAGTTGCTGCATGC
CTGTCGTCAGCTCGTGCCGTGAAGTGTGGTTAAGTCCCGCACCAGCCCACCTTGGTCTTAATGG
CAGCGTATGCTGACTAAGACCCGGTCACCCGGAGACGGGGGGACTAGTCTATTGCCCTAGGCG
GTACCCCATTTCAGTAGACAAGGCTGAGCCGCCA

Lotte D – Clone 27_1

CGGCTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAGG
TTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAG
TTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAG
CCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGC
AACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGAC
ATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAAC
GCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGG

ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGTCCA
GGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAGGAATT
CCGCTACCCTCTACCACACTCTAGTCGTCCAGTATCCACTGCAGTTCCCAGGGTTGAGCCCAGA
GCTTTCACAACGGACTTAAACGACCACCTACGCACGCTTTACGCCCAGTAATTCCGAGTAACGCT
TGCACCCTTCGTATACGCGCTGCTGCACGAAGTTAGCGGTGCTTAATTCTTTGGGTACGTCATCC
AACGGGTATTACAAGCTTGATTCTTCCACAAGCCTTTACACCGGAGGCTTCTTACCAGCGAATGG
CCTGGATCAGCTTGGCCCCAATTGTTCAATATATTCT

Lotte D – Clone 27_8

CGGCTACCTTGTTACAACTTCACCCCAGTCATCGGCCACACCGTGGCAACCTCCCTCCCGAAGG
TTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAG
TTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAG
CCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGC
AACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGAC
ATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAAC
GCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGTCCA
GGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAGGAATT
CCGCTACCCTCTACCACACTCTAGTCGTCCAGTATCCACTGCAGTTCCCAGGTTGAGCCCAGGG
CTTTCACAACGGACTTAAACGACCACCTACGCACGCTTTACGCCCAGTAATACGAGTACGCTAGC
ACCCTTCGTATACTGCGCTGCTGCACGAGTAGCGTGCTTATCTTTGGTACGTCATCCACAGGTAT
ACAGCTGATTCTTCCCAACAATGCTTTACACTGGAGCCTCTACCAGCGAATGGCGTGATCAGGCT
GGCCCCCATTTTTTCAAATATTCCCCC

Lotte D – Clone 27_{10}

ATGGTTTGCATCCTGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGC
AGCACAGTAAGAGCTTGCTCTTACGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATC
TACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAA
GCAGGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGG
TAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
GATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGA
AATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGC
GTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT
GGACGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATC
AGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGG
GTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGC
AAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGGTGGAGTATGTGGTTAAATCG
ATGCAACGCGAAAACCTTACCTGGCCTTGACTTGTCAAGACTTCCAAAATGAATGTTGCTTCGGA
CTCGAACACAGTGCTGCATGCTGTCGTCAGCTCGGTCTGAATGTTGGTAAGTCCGCACGAGGCC
ACCTTGCTTATGCAGACGTAATGGGGACTCTTAGGGAAACCTCTGGGTCCCAATACCCGGGA

LotteD – Straight from vial

GCAGGTGGGCGGGTAAGCTAACACATGCAGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTACG
GGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTACTCTGTCGTGGGGGATAACGTA
GGGAAACTTACTCTAATACCGCATACCACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCG
CGATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCCCCAGGGCCACTATC
CGTATCTGGTCTGAGAGGATGATCACCCACACTGGAACTGACACACGGTCCACACTCCTACGGG
AGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGA
AGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGGT
GGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACAAA
GGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTATGTGGTCGTTTAACTCCGTT
GTGAAAGCCCTGGGCTCAACCTGGGCACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAG
GGTAGCGGAATTCGTGGTGTAGCAGTGATATGCATAGATATCGAGAAGAACATCCATGGCGAAC
GCAGCTACCTGGACCCACACTGACACTGACGCACGAAAGCGTGGGGAGCAAACAAGATTATATA

CCGTGGTAGTCCACGCTCTACACTATGCAAACTGTATGTTGGGTGCAATTTGGTCACGCAGTATC
AAGCATAACGCTTTAATTTCGTCCCCTGGAGAGTACGGTCGCAACACTGAATCTCAAAAGAATTG
GACGGGGCGCCCGCACAGCGGTGGAGTATGGTGTTTAATTCGTTGCAACGTGAAGACCTTTATT
TGCCGTGGACTGGTCGAAATTTTCCAAAGTGATTTGTGCTTCGGAACTTAAACAACGGTGCTGCA
TGCATGTCGACTTCTGTCTCATGAATGTTGGTTAAGTCCAGCACTAGCTCCACCCATGGCCTTAG
TGCACACCTATGCTGTATTCTAAGTAACCCCGTGCACGGAGACTGGGGAGACTGAGTCATAGCTA
AGCCTGGTACCCTGTTCATGCTAGAAAGGTGCACGGACGGTAGCCACTCC

Lotte E – Straight from Vial
TAAGTTGGCGGTTAGCCTACCATGCAAGTCGAACGGCAGCACAGTAAGAGCCTTGCTCTTGCGG
GTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTACTCTGTCGTGGGGGATAACGTAG
GGAAACTTACTCTAATACCGCATACCACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCGC
GATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCCCCAGGGCCACTATCC
GTATCTGGTCTGAGAGGATGATCACCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGAA
GAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGATAATACCCGGGTG
GGATGACGGTACCCAAAAAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACAAAG
GGTGCAAGCGTTACTCGATATTACTGGGCGTAAAGCGTGCGTATGTGGTCGTTTAACTCCGTTGT
GAAAGCTCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGGGT
AGCGGAATTCGTGGTGTAGCAGTGATATGCATAGAGATCGAGAAGAACATCCGTGGCGAGGGCA
GCTATCTGGACCAACTCTGACTCTGACGCACGAAAGCGTGGAGAGCAAACAAGATTATATACCGT
GGTAGTCCACGCTCTACACTATGCAATCTGGATGTGGGTGCAATTTGGCACGCAGTATCGAAGCT
AACGCGTTAATTTCCCCCTCTGGAGAGTACGGTCGCAAGACTGACACTCAGAGAATTAGACGGG
GGCCCGCACAGCGGTGAATATGTGGTTTATTCGATGCACGCGAAGACCTTACTGGCCTTGAATGT
CGAGACTTTCAGAAGTGATGTGCTTGGGAACTCGAACAAGGGCTGATGCTTCGACGCTGGTCAG
AATGTGTAATCCCGAACAGCCACCTTTCTAGTGCAACGAATGGCGGAATCTAAAGGACCGTACAC
CGGAGATGAGATCTAGTCATGCTAACCGGTACGTCATTGGGCAAGGTTCACGGACGGTAGCA

Lotte F – Straight from Vial
TTTTTTGGCCTCCTTAGCGGGTGGAGAGCGGCGGAGGGGTGAGGAATACATCAGAATCTACTCT
GTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGG
GGATCTTCAGACCTTGCGCGATTGAATGAGCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGG
CCCACCAGGGCGACGATCCGTATCTGGTCTGAGAGGATGATCACCCACTCTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCA
CCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCA
GCTGGTTAATACCCGGGTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAG
CACCCGCGGTAATACAAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGG
TGGTCGTTTAAGTCCGTTGTGAAAGCTCTGGTCTCACTCTGGGATCTGCAGTGGATACTGGACGA
CTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAACAGTGATATGCGTAGAGATCAAGAAG
AACATCCGTGGCGAAGGCAGCTACCTGGACCAACACTGACACTGACGCACGAAAGCGTGGAGA
GCAAACAAGATTATATACTCTGGGTAGTCCACGCCCTAAACTATGCGAACTGTATGGTGGGTGCA
ATTTGGCACGCAGTATCGATGCTAACGCTTTAAGTTCGCCGTCTGGGGAGTACGGTCGCAGACT
GAAACTCAAAGATTTGACGGGGCCCCGCACAAGCGGGGGATATGGGTTTATTCTATGCACGAAA
GACCCTTACGTGGCTTTGACATGTCGAGACTTCCAGAGTGGATTGGGCCCTTTCGGGATCTCACC
ACAGGTGCTGCATGGCTGTCTTCACCTCGTGTCGGAGATGTGGGTAAGTCCCACAAGAGCGCCA
CCTTTGCCTTAGGTGCACCCGTAATGGTGGACTCTAAGGAACCGCCGTTCACACCGGAAGAAGT
GGGGAGTAGCCCAAGTCTCATGGC

Lotte H – Clone 30_2
CGGCTACCTCTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAG
GTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGA
GTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCA
GCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTG
GCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA
CGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTC
GACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTG
TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTT
AACGCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCG

TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGG
TCCGGGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAAG
GAATTCCGCTACCCTCTACCACACCTCTAGTCGTCCAGTATCCACTGCAGTTCCCAGGTTGAGCC
CAGGGCTTTCACACGGACTTAAACGACCACCTTACGCATGCTTTACGCCCAGTATTCCGAGTAAC
GCTTGCCCCTTCGTATACCGCGCTGCTGCACGAGTTAGCCGGGGCTATTCTTGGGTACGTCATC
CACGGATTAACACTTGATTCTTTCCACAAAGGCTTACACCGGAAGCCTTCTACCCCACCGGATGC
TGAACGCTTGGCCCCATGCCAATTTTCCCCGGGCGGCCCTCCCCGGA

Lotte H – Straight from Vial
TGAATTAGGGGGGCACGGCCTGAAAGATGCAAGTCGAACGCCAGCACAGTAAGAGCTTGCTCTT
ACGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAAC
GTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGGGGATCTTCGGACCTT
GCGCGATTGAATGACCCGATGTCGGATTATCTAGTTGGCGGGGTAAAGGCCCACCAGGGCGACT
ATCCGTATCTGGTCTGAGAGGATGATCACCCACTCTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGG
TGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCTGG
TTGAGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG
AGGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCG
TTGTGAAAGCCGTGCGCTCACTCTGGGAACTGCAGTGGATACTGCGCGACTAGAGTGTGGTAGA
GGGTAGCGGAATTTCGTGGTGTAGCAGTGAAATGCGTAGAGATCAAGAAGAACATCCGTGGCGA
AGGCAGCTACCTGGCCCCAACTCTGACTCTGAGGCACGAAAGCGTGGAGAGCAAACAGGATAGA
TACCCTGATAGTCCACGCCCTACACGATGCAACTGGAATGTTGGGGTGCAATTTGGCACGCAGTA
TCAATCTAACCCTTAAGTTCGCCGCTGGGGAGTCGGTCGCAAGACTGAAACCTCAAAGGATTGAC
GGGGTCCCCACAGCGGGGAATTATTGTGTTAATTCAATGCACGAAAACCTTACCTGGCCTGAACT
GCCGAACTTCAGATGAATGGTCCTCGACTAACCAGGTCTTATGGTGCTACCTGTCGGGATTGGTA
ATCCCCACAGCAACTGCTATTGCAACATTGGGAACTAGAACGTACCCGGGAGGATCCAATTTGCC
TAGCGGTAA

Lotte I – Clone 31_3
CGGCTACCTCTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAG
GTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGA
GTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCA
GCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTG
GCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA
CGACAGCCA

Lotte I – Clone 31_9
ATGGTTTGCCCTTTGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGC
AGCACAGTAAGAGCTTGCTCTTACGGGTGGCGAGTGGCGGACGGGTGAGGAATA

Lotte I – Straight from vial
ACTAAAAAGGGTAGCCTAAGCCTGTAACGTCGTAGGCGAGCACAGTAGGAGCTTGCTCTTACGG
GTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTACTCTGTCGTGGGGGATAACGTAG
GGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCGC
GATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCACCAGGGCGACTATCC
GTATCTGGTCTGAGAGGATGATCACCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGAA
GAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGGTG
GGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACAAAG
GGTGCAAGCGTTACTCAGTATTACTGGGCGTAAAGCGTGCGTATGTGGTCGTTTAACTCCGTTGA
GAAAGCTCTGGGCTCAACGTGGGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGG
GTAGCAGATTTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAAGATCTCTTGGCAAGGGCAT
CTATCTGGACCAACAC

Lotte J– Clone 32_2
CGGCTACCCTTTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGAAGG
TTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAG

TTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAG
CCCTCTGGCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACG
TCCTCCCCACCTTCCTCCGG

Lotte J – Straight from vial
CGCTAAAGGGGGCTAGGCCTACCATGCAGTCGCACGGCAGCACAGTAAGAGCTTGCTCTTACGG
GTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTACTCTGTCGTGGGGGATAACGTAG
GGAAACTTACTCTAATACCGCATACCACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCGC
GATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACTATCC
GTATCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGAA
GAAGGCCTTCGGGTTGTAAAGCTCTTTTGTTGGGAAAGATATCCAGCTGGATAATACCTGTGTGG
GATGACTGCACCCAGAGAATAAGCACCGGCTATCTTCGTGCCAGCAGTCGCGGTAATACGAAGG
GTGCAAGCGTTACTCAGAATTACTGGGCGTAGAGCGTGCGTATGTGGTCGTTTAAGTCCTTTGTG
AAAGCTCTGGGCTCACCTGGGATCTGCAGTGTATACTGCGACGACTAGAGTGTGCTAGAGGGTA
GCGAATTCCTGGTGTAGCAGTGAATTGCATAGAGATCAGGAGGAACATCATGGCCAAGGCTGCT
ACTTGGAACAACACTGACCCTGAGCACGAGCTGGGGGACCAACAGGATCATTCCTTGGAAGTCC
CCCCTTAACATTCCAACTGGATGTGGGGCAATTGGCCCCCAATATCGAGCTTACGTTAAGTCCCC
CCTGGGGATCCGGTCGAAACTGAACCAAGGAATGCCGGCTCCCACCGGGGAATTTGGTTATTCA
TCACGGAAACTTACTGCTGACTTGCGAACTTCGATGGATTGTTCTGGACTGAACAAGGCTAGGCT
GCACCTTGCGGAAGTGGGTATTCCGACACGACACCTTGCCTATGACCACGATTGTGGGACTTAA
GACCTGGTCACCGGAAGGGGGGGAAATCAGTCATGGCCTAGGCGGGTGACCGTCATGTGGAAT
TTCACCGCAGAACATCGAACCAT

A17 (833) P1(d) – Clone 33_7
ATGGTTTGATTCAGGGTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCA
GCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGA

A17 (833) P1(d) – Clone 33_{10}
AGCTTAAACTCTTTCTTTTTATCGGAGTGTACACCCCAGTCATCGGGCCACACCGTGGCAAGCTG
CCCTCCCGAAGGTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGT
GTGCAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGACTCCGACT
TCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTC
GCCGGCTTGCAGCCCTCTGTCCCTTCCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCAT

A17 (833) Pl (d) – Straight from vial
ATTTAAGGGTAGGCCGATTTTTGTCATTCGTACGGCATCACAGAAAGAGTTTGCTCTTGCGGGTG
GCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGA
AACTTACGCTAATACCGCATACCACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGCGCGATT
GAATGACCCGATGTCGGATTATCTAGTTGGCGGGGTAAAGGCCCCCCAGGGCGACTATCCGTAT
CTGGTCTGAGAGGATGATCAGCCACACTGGATCTGAGACACGGCCCACACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAGTGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGAAGAAG
GCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGGTGGGAT
GACGGTACCCAAAGAATAAGCACCGTCTAACTTCGTGCCAGCACCCGCGGTAATACGAAGGGTG
CGAGTGTTACTCGGAATTACTGGGCGTAGAGCGTGCGTAGGTGGTCGTTTAAGTCCGGTGTGAA
AGCCGTGGTCTCCACGTGGGAACTGCAGTGTATTCTGCGCGACTAGAGTGTGATAGAGGGTAGC
GAGATTTCTGGTGTCGCAGTGAAGTGCATAGAGATCAAGAGGACATCTGTGGCGAACGCAGCTA
CCTGGACCACTCTGACACTGAGGCAGAAAGCGTGGGGAGCACACGAGATTATATTCCGTGTAGT
CACCCCTTAAACGATGCGAACTGGTTGTTGGGTGCTATTTGCCCGCATTATCGAGCTAACGCTTT
AGTTCGCCCCCTGGGTGTACGGTCGAAAAACTGAATCTCAAAGATTTGACGGGGGCCGCCCAAG
GGGGGAATATGTGGTTTTATTTCGATGCACCGAAAAACCTTACCTGGCCTTGACTGGCAGAGATT
TCGAAATGGATTGGGGCTTCGGACCTGACACTGGTCGTGGATGGTGGCTCCAC

A51 (813) – Straight from vial
GTTAAAAAGAGGTTAGCCTGAAGCATGTAACGTCGTACGCGCAGCACAGTAAGAGCTTGCTCTTG
CGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACG
TAGGGAAACTTACTCTAATACCGCATACGACCTACGGGTGAAAGCGGGGGATCTTCAGACCTTGC
GCGATTGAATGACCCGATGTCAGATTATCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGAT
CCGTATCTGGTCTGAGAGGATGATCACCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTG

AAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGTT
GGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCACCCGCGGTAATACAAA
GGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAACTCCGTT
GTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGG
GTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAAGAACATCCGTGGCGAGCG
CAGCTACCTGGACCAACACTGACTCTGACGCACGAAAGCGTGGAGAGCAAACAAGATTATATACT
CTGGTAGTCCACGCTCTACACTATGCGAACTGTATGTTGGGTGCAATTTGGCACGCAATATCGAA
TCTAACGCGTTAAGTTCGCCGCGTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATAGAC
GGGGGCGCGCACAAGCGGTAGAGTATGTGGTTTTATTCGATGCAACGCAGAAAACCATACCTGG
CCCTTGGACATGTCGAGAACTTTCCAGAGATGATTTGGTGCCTTCGGGAACTCAGAACACAGGTG
CTGCATGGCTGTCGTAGCTCGTGTCGGGAGATGTGGATTAAGTCCCACAACGAGCGACACCTTT
GTCTTAAGTTGCCACCCGTATTGGGTGACTTCTAGAACCGCGTGCACACCGGAAGAAGGGGGGG
ATGACTCAGGCTCTCTGCCTTACGCCAGGCCTACCCACGTTCAT

A208(796) – Straight from Vial
GGAAAAATGGGGGTAGCCTACCATGCAAGTCGAACGGCAGCACAGAAAGAGCTTGCTCTTGCGG
GTGGCGAGTGGCGGACGGGTGAGGAATACATCGGACTCTTCTCTGTCGTGGGGGATAACGTAG
GGAAACTTACTCTAATACCGCATACGACCTACGGGTGAAAGCGGGGGATCTTCGGACCTTGCGC
GATTGAATGAGCCGATGTCGGATTATCTAGTTGGCGGGGTAAGGGCCCCCCAGGGCGACGATCC
GTAGCTGGTCTGAGAGGATGATCAGCCACTCTGGAACTGACACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATACCGCGTGGGTGAA
GAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGG
GATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG
GTGCAAGCGTTACTCGGAATTACTGGGCGTAACGCGTGCGTAGGTGGTCGTTTATGTCCGTTGT
GAAAGCTCTGGGCTCACTCTGGGCACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGT
AGCGGAATTCGTGGTGTAGCAGTGATATGCGTAGAGATCAGGAGGAACATCCGTGGCGAGCGCA
TCTACCTGGACCCACTCTGACTCTGACGCACGAAAGCGTGGGGAGCAAACAAGATTATATACTCT
GGTAGTCCACGCTCTACACTATGCAAACTGTATGTTGGGTGCAATGTGGCACGCAATATCGAAGC
TAACGCTTTAAGTTCGCCGTCTGGAGAGTACGGTCGCAAGACTGAAT
CTCAAAAGAATTGACGGGGGCCCGCACACGCGGTGGAGTATGGTGTTTATTCAATGCAACGCAG
AGACCTTACCTTGCCTTGACATGTCGAGATTTTACAGAAATGGATTGGTGCCTTCGGAACTCAACA
CAGGGCTGATGCTGTCGTCGCTCGGTCGGGAGTGTGATAAGTCCCAAACAGCACCCTTGTCTTT
ATGCCACCGTATTGGGGACTCTAAGAACCCGTACACGGAAATGGGGGATGACTAGCTCTTGCTTA

A141 (767) – Straight from vial
GAATGGGGGGCATATTCTGTTGGTACGTCAAACTGCAGGGTATTAACCAGCAGCCCTTCCTCCCA
ACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCG
CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

A141 (767) – Straight from vial
GATCGGGTATATTCTGTTGGTACGTCAAACTGCAGGGTATTAACCAGCAGCCCTTCCTCCCAACT
TAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCC
ATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGCCCCCCGTGCAA

Sequences from DNA isolated from SLU bacterial Isolates – Incomplete List at Moment as Access to computer original sequences pre-2003 inaccessible at the moment

UoP Ref: Bac 10
BACPOLES Ref: A3-2 (From 19.td.4.100, saltwater, Venice, Italy)
Clone 5 closely resembled: Uncultured eubacterium, Uncultured bacterium clone p-131, Uncultured Clostridiaceae, Uncultured Chloroflexi bacterium, Uncultured alpha proteobacterium, Uncultured *Dehalococcoides* sp.
Seq: **UoP Ref: Bac 10 clone 5**
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTATTCATCAGGTACCGTCATTATCTTCCC
TGATAAAAGAGGTTTACAACCCGAAGGCCTTCTTCCCTCACGCGGTGTCGCTGGGTCAGGCTTTC
GCCCATTGCCCAATATTCCTTG

UoP Ref: Bac15
BACPOLES Ref: A 4-2 (From 19.td.4.100, Venice, Italy)
Clone 1 closely resembled: *Rhodobacter* sp., *Paracoccus* sp.
Seq: **UoP Ref: Bac 15 clone 1**
ATTACCGCGTGCTGCTGGCACGGAGTTAGCCGGGGCTTCTTCTGTTGGTACCGTCATTATCTTCC
CAACTGAAAGATCTTTACAACCCTAAGGCCTTCATCGATCACGCGGCATGGCTAGATCAGGGTTG
CCCCCATTGTCTAAGATTCCCCA

Clone 2 closely resembled: *Rhodobacter* sp., *Paracoccus* sp.
Seq: **UoP Ref: Bac 15 clone 2**
ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTCTTCTGTTGGTACCGTCATTATCTTCCC
AACTGAAAGATCTTTACAACCCTAAGGCCTTCATCGATCACGCGGCATGGCTAGATCAGGGTTGC
CCCCATTGTCTAAGATTCCCCA

Clone 3 closely resembled: *Rhodobacter* sp., *Paracoccus* sp.
Seq: **UoP Ref: Bac 15 clone 3**
ATTACCGCGCGTTGCTGGCACGGAGTTAGCCGGGGCTTCTTCTGTTGGTACCGTCATTATCTTCC
CAACTGAAAGATCTTTACAACCCTAAGGCCTTCATCGATCACGCGGCATGGCTAGATCAGGGTTG
CCCCCATTGTCTAAGATTCCCCA

Clone 5 closely resembled: *Rhodobacter* sp., *Paracoccus* sp.
Seq: **UoP Ref: Bac 15 clone 5**
ATTGACGCGTCTGCTGACTCGGAGTTAACCGAGGCTTCTTCTGTTGGTACCGTCATTATCTTCCC
AACTGAAAGATCTTTACAACCCTAAGGCCTTCATCGATCACGCGGCATGGCTAGATCAGGGTTGC
CCCCATTGTCTAAGATTCCCCA

Appendix 8

Microcosm Experiment Information

(by Hotchkiss, Landy, Mitchell)

1. Molecular Analysis for microcosm experiment

1.1 DNA analysis:

- To date 27/60 samples have been analysed (in red Tab. 1).
- DNA was extracted from the majority of wood and soil samples but further molecular analysis has been unsuccessful in all cases despite 3 attempts.
- DNA was successfully extracted and amplified from only 1 water sample MC22c. 3 DGGE bands were excised and sent for sequencing.
- These sequences most resembled uncultured *Bacillus* spp. and an uncultured Alpha-proteobacterium possibly *Sphingomonas* sp.

Table 1. Samples provided for DNA analysis

N2 MC 18	Air MC22	Air + O2 MC26	Air + circ MC30
682	842	1002	1162
686	846	1006	1166
691	851	1011	1171
696	856	1016	1176
698	858	1018	1178
700	860	1020	1180
702	862	1022	1182
706	866	1026	1186
711	871	1031	1191
716	876	1036	1196
718	878	1038	1198
720	880	1040	1200
Soil 18a, b	Soil 22a, b	Soil 26a, b	Soil 30a, b
Water 18c	Water 22c	Water 26c	Water 30c

MC22c DGGE band 12E

This sample most resembles an uncultured *Bacillus* sp. (Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus). Sequence match 86%
AGCACTCGCATGACGCAGTCTGAGCGAGCACTGCCGCGTGAGCTGATGAGGCCTTCGGGTCGT
AAGTTCTGTTGTTAGAGATGATCAAGTGCTAGTCGAATAGGCTGGCTCCTTCACGCTACGTAACC
ACAGAGCCACGGATAGCTACGTGCCATAGCCGCGGTAGTATATC

MC22c DGGE band 12F

This sample most resembles *Bacillus* sp. (Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus). Sequence match 89%.
ATGGACTGCGCATGACGCAGTCTGACGGAGCACTGCCGCGTGAGCTGATGAGGCCTTCGGGTC
GTAAGCTCTGTTGTTAGGGATGATCATGTGCTAGTCGAATAGGCTGGCTCCTTGACGGTACCTGC
CAGAGAGCCACGGATAGCTACGTGCCAGCAGCCGCGGTAGTATTA

MC22c DGGE band 12G

This sample most closely resembles an alpha-proteobacterium (Sequence match 92%) , possibly *Sphingomonas* sp. (Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae; Sphingomonas) Sequence match 91%.
CTGTGGAACGAGAGATAGGACGCAAGTCTGATCCAGCATGCCGCGTGAGTGATGAGGCCTTCGG
GTTGTAAAGCTCTTTCCCCGGGATGATCATGTGGGTATCGGGAGAATAGGCTCCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATAACTACTGGCCACCGCCCCCGGTATAA

1.2. Samples chosen for FISH analysis

To date FISH analysis of microcosm samples has been preliminary. 4 samples were selected for analysis as they showed visible signs of potential areas of attack (in red Tab.2). Initial observations have demonstrated the suitability of chosen probes EUB338 and Cf319a. Research is ongoing.

Table 2. Samples provided for FISH anlysis

N2 MC 18	Air MC22	Air + O2 MC26	Air + circ MC30
702	851	1036	1162
706	858		1171
720	868		1176
	878		1186
			1196
			1198

2. Molecular Analysis for Jana Gelbrich

2.1. RNA Analysis

- To date 9/12 samples have been analysed (in red Tab. 3).
- RNA was successfully extracted from 2 samples MC18 708 and MC22 879. A total of 15 clones were successfully sequenced and identified.

Table 3. Samples provided for RNA analysis

MC 18 (N_2)	MC 22 (Air)	MC 26 (O_2 added air)	MC 30 (circulated air)
699	**859**	**1001**	**1164**
708	**875**	**1015**	**1170**
719	**879**	**1035**	**1180**

MC22 879 – 7 clones were found to represent the bacterial community in this microcosm.

MC22 879 Clone 6 $_2$
This clone has a 100% sequence match to *Acinetobacter* sp. (Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter.)

GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCGCCCCTTGACA
TCCTGGGAACTCGCTAGAGATAGCTTGGTGCCGAAAGGAACCCAGAGACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGT
TGCCAGCACGTAATGGTGGGAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ACGACGTCAAGTCATCATGGCCCTCACGGGGCGGGCTACACACGTGCTACAATGGCCGGTACAA
ACGGTTGCGAGCCCGCGAGGGGGAGCCAATCCGAGAAAACCGGTCGTAGTCCGGATTGGAGTC
TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACG
TTCCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACC
AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAACCTGTCGTGCCAGCTGCATTATGA
ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTCCCTCGCTCACT
GACTCGCTGCGCTCGGTCGTTCGGGTTGCGGCGAGCCGGTATCAGCTCACTCGAAGGGCGGTA
TTTACGGTATCCCACCTGATCGAGGGAAATAGCCAGGAAAAAAC

MC22 879 Clone 6 $_{7}$
This clone most resembles (95% sequence match) an uncultured Deltabacterium (Bacteria; Proteobacteria; Deltaproteobacteria) possibly species like *Polyangium* or *Chondromyces* (Myxococcales; Sorangineae; Polyangiaceae).

GCCCGGGAACGTATTCACCGCTGCCATGCTGATCAGCGATTACTAGCGATTCCGACTTCAAAGA
GTCGAGTTGCAGACTCTTATCCGTACTGAGGCCGGCTTTTTGGGATTAGCTCCCCCTCGCGGGTT
CGCAGCCCATTGTACCGGTCATTGTAGCACGTGTGTAGCCCTGGACATAAGGGCCATGAGGACT
TGACGTCATCCCCACCTTCCTCCGACTTAAAGGTCGGCAGTCCCGTTAGAGTGCCCAACTGAATG
CTGGCAACTAACGGCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGC
TGACGACAGCCATGCAGCACCTAACTACAGATTCCCCGAAGGGCACCCCGACCTTTCGACCAGG
TTCCTGTATTTTCTAGCCCAGGTAAGGTTCTGCGCGTTGCGTCGAATTAAACCACATGCTGCACC
GCTTGTGCAAAGGCGAATTCCAGCACACTGGCGGCCGTTACTATTGGATCCGAGCACGGTATTAT
CTTGGCAGTAATCATGGTCATAGCTGTTTCCCTGTGTG

MC22 879 Clone 6 $_{6}$
This clone most resembles a Gamma-proteobacterium (94% sequence match) possibly *Pseudomonas* sp. (Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas)

CTACCTCCTATTAGGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCA
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACA
TCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTT
GCCAGCACGTAATGGTGGGAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
CGACGTCAAGTCATCATGGCCCTCACGGGGCGGGCTACACACGTGCTACAATGGCCGGTACAAA
CGGTTGCGAGCCCGCGAGGGGGAGCCAATCCGAGAAAACCGGTCGTAGTCCGGATTGGAGTCT
GCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGT
TCCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACC
AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGA
ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGGCTGCGGCGAGCGGTAATCAGCTCACTCAAAGGCGGTA
ATACCGGTTTATCCCACAGATTCAGGGGGATAACGCAAGGAAGAAACATGTGGAGCAATAAGGC
CAACCATAAGGCCAGGAACCCGTAAAAAAGGGCCGCGTTGCTGGGCGTTTTTCCATAAGGGTCC
CGCCCCCCTTGACAAACAATCAAAAAAATCGAACCTTCAAGTCCAAAGGGTGGCCAAAACCCCAC
ATGACATATAAAAGATC

MC18 879 Clone 6 $_{9}$
This clone most resembles an uncultured Gamma-proteobacterium (98% sequence match) (Bacteria; Proteobacteria; Gamma-proteobacteria)

GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCGCCCCTTGACA
TCCTGGGAACTCGCTAGAGATAGCTTGGTGCCGAAAGGAACCCAGAGACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGT
TGCCAGCACGTAATGGTGGGAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ACGACGTCAAGTCATCATGGCCCTCACGGGGCGGGCTACACACGTGCTACAATGGCCGGTACAA
ACGGTTGCGAGCCCGCGAGGGGGAGCCAATCCGAGAAAACCGGTCGTAGTCCGGATTGGAGTC
TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACG
TTCCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACC
AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAACCTGTCGTGCCAGCTGCATTATGA
ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTCCCTCGCTCACT
GACTCGCTGCGCTCGGTCGTTCGGGTTGCGGCGAGCCGGTATCAGCTCACTCGAAGGGCGGTA
TTTACGGTATCCCACCTGATCGAGGGAAATAGCCAGGAAAAAAC

MC22 879 Clone 6 $_{17}$
This clone most resembles an uncultured Flavobacterium sp. (98% sequence match) (Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium Bacteria; Bacteroidetes)

GCCCGGGAACGTATTCACCGCGCCGTTGCTGATGCGCGATTACTAGCGAATCCAGCTTCACGAA
GTCGAGTTGCAGACTTCGATCCGAACTGAGACCGGTTTTAGAGATTAGCATCTTGTCACCAAGTA
GCTGCCCTTTGTACCGGCCATTGTAACACGTGTGTAGCCCTGGACATAAGGGCCGTGCTGATTT
GACGTCATCCCCACCTTCCTCACGGTTTACACCGGCAGTTTCGTTAGAGTTCCCGGCATTACCCG
CTGGCAACTAACAATAGGGGTTGCGCTCGTTATGGGACTTAACCCAACACCTCACGGCACGAGC
TGACGACAGCCATGCAGCACCTTCACAGCAGCTATTGCTAGCTCTCCCATCTCTGGAAAATTCTC
CTGTGATTTAGCCCAGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTG
TGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTT
GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACAT
ACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGG
CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTCCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA
TCCACAGGATTCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCTAAAAGGCCAG
GAACCGTTAAAAAGGCCGCGTTGCTGGCGTTTTTACAATAGGCTCCGCCCCCCTTGACAAGCATC
ACAAAAATCGACGCTCAGGTTCAAAGGTTGCCCAAACCGACGGGACATATAAGAATACGAGGGG
TTCCCCCTTGAAGCTCCGCTCGGGGCACTCTCTTGTTTCGACCCTGACGATTACGATACTGTGTC
GACTTTCTCCTTCGGAACGGTGCGCTTTTTCTAAAGCTCAGCGCTGTAAGGTAAT

MC18 879 Clone 6 $_{20}$
This clone most resembles an uncultured Gamma-proteobacterium (94% sequence match) (Bacteria; Proteobacteria; Gamma-proteobacteria)

TTTACCTGTTTTGTGCGAGGGTTCTTTGGGCGAGTGGGCCCTCTAGGATGCATGCTTCGAGCGG
CCGCCAGTGTGATGGATATCTGAACCAATAAGGGAACTTGCACAAGCGGTGGAGCATGTGGTTT
AATTCGATGCAACGCGAAGAACCTTACCGCCCCTTGACATCCTGGGAACTCGCTAGAGATAGCTT
GGTGCCGAAAGGAACCCAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTC
TAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTC
ACGGGGCGGGCTACACACGTGCTACAATGGCCGGTACAAACGGTTGCGAGCCCGCGAGGGGGA
GCCAATCCGAGAAAACCGGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
ATCGCTAGTAATCGCTGATCAGCATGGCAGCGGTGAATACGTTCCCGGGCAAGGGCGAATTCCA
GCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAACCTTGGCGTAATCATGGTCAT
AGCTGTTTCCTGTGTGAAATTGTTATCCTCTCACAATTCCACACATCAT

MC22 879 Clone 6 $_{19}$
This clone most resembles an uncultured Deltaproteobacterium (95% sequence match). (Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Sorangineae; Polyangiaceae) possibly *Polyangium* sp or *Chondromyces* sp.(92%).

GCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCTGGGCTAGAAA
ATACAGGAACCTGGTCGAAAGGTCGGGGTGCCCTTCGGGGAATCTGTAGTTAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCGTT
AGTTGCCAGCATTCAGTTGGGCACTCTAACGGGACTGCCGACCTTTAAGTCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGTCCAGGGCTACACACGTGCTACAATGACCGGTACAA
TGGGCTGCGAACCCGCGAGGGGGAGCTAATCCCAAAAAGCCGGCCTCAGTACGGATAAGAGTC
TGCAACTCGACTCTTTGAAGTCGGAATCGCTAGTAATCGCTGATCAGCATGGCAGCGGTGAATAC
GTTCCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTA
CCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
CACAACATACGAGCCGGAAGCATAAAAGTGTAAAGCCTGGGGTGCCCTAATGAGTGAGCTAACT
CACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCCTGTCGTGCCAGCTGCAT
TAATGAATCGGCCAACGCGCGGGGGAGAGGCGGGTTTGCGTATTGGGGCGCTCTTCCGCCTCC
CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAACCTCACTCAAA
GCCGGTTATATCCGTTTATCCACAGATTCAAGGGATAACGCAAGGAAAGGAACATGTGGAGCAAA
ACGGTCAGCAAAAGGGCCAGGAACCGTTAAAACGCCCCCGTTTGCTTGGCGTTTTTTTCATAAGC

TCCGCCCCCCTGAACAAAGCACTGAATAAAATCACGCCCTCAAGTCAGAAGGTGGGCAAACCCT
AAAAGGACTTAT

MC18 708 – 8 clones were found to characterize the bacterial community in this microcosm

MC22 708 Clone 7 $_1$
This clone resembles (96% sequence match) an uncultured Gammaproteobacterium (Bacteria; Proteobacteria; Gammaproteobacteria)

TACTCATATAGGGCGATTGGGCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGAATCT
GTACAATTAACGCTTGCCCGGGAACGTATTCACCGCAGCATGCTGCTTTGCGATTACTAGCGATT
CCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACCGGTTTTCTCGGATTGGCTC
CCCCTCGCGGGCTCGCAACCGTTTGTACCGGCCATTGTAGCACGTGTGTAGCCCGCCCCATGAG
GGTCATGATGACTTGACC

MC18 708Clone 7 $_3$
This clone most resembles (99% sequence match) a *Mycobacterium* sp. (Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium)

GCACAAGCGGTGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACAT
GCACAGGACGCTGGTAGAGATATCAGTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATGTTGC
CAGCGCGTTATGGCGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAAAGGG
CTGCGAATCCGCGAGGTGGAGCGAATCCCTTGAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCA
ACTCGACCCCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT
CCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCA
AGCTTGGCGTAATCATGGTCAAAGCTGTTTCCTGTATGAAATTGTTATCCGCTCACAATTCCACAC
AACATACGAGCCGGAAGCATTAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATT
AATTGCGTTGCGCTCACTGCCCGCTTTTCCAGTCGAGAATATTGTCGTGCCATTTGCATTAATGAA
TTCGCCAACGCCTGGGGGAGAGGTGAGCTTGCGAATTGAGTCGCT

MC22 708 Clone 7 $_4$
This clone most resembles a Gammproteobacterium (90% sequence match)
ACANGATNTTAANGNNANNNCNANGGGGGAATGGGGGCNCNTCNANAAGGCAAGGCCTCGAAG
NCGGCCCGANNAGNNGATGAATGAGATATNGNGCCCCTTTTNNGGGGTTGCCCGGGNAACGTA
NTTCACCGNNAGCATGCCNCATNTGNGATTACTAGTAGNTTCCGACTTCNTGGAGTCGAGNNTG
GAGACTCCAAACCGGACNNNGACCGGNTTTCNCGNANGGGCTCNCACTCNCGGGNTNGCAGCN
GTTTGNNGNNGNGCATNGAAGACTNATGGNCCCNGCNCCNNCGAGNGGCATGATGACNTNACG
NGAGNTCNNANCTTCCACNGNCTCGCCNCNNNCGNTA

MC18 708Clone 7 $_5$
This clone most resembles an uncultured bacterium clone (93%). Possibly *Pseudomonas* sp. (Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas).

ATCCTTTTAGTGGCGATTGTTGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATAT
CTGAACAATAAAGGGGTTGCACAAGCGGTGGAGCATGTGGGTTAATTCCTTTTAACGCGAAGAAT
CTTACCGCCCCTTGGCATCCTGGGAACTCGCTAGAGATAGCTTGGTGCCGAAAGGTACTCAAAAA
CAGGTGCTGCATGGCTGTCTTCAGCTCGTGTCGTGAGATGTTCATT

MC22 708 Clone 7 $_6$
This clone most resembled (97% sequence match) an uncultured Betaproteobacterium sp. (Bacteria; Proteobacteria; Betaproteobacteria), possibly *Azospira (Dechlorosoma)* sp. (96%) (Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Azospira)

CACAAGCGGTGGAGCATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATG
TCAGGAACTTTCCAGAGATGGATTGGTGCTCGAAAGAGAGCCTGAACACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGT
TGCCATCATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG

ACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGTACAGAGG
GTTGCCAACCCGCGAGGGGGAGCCAATCCCAGAAAGCCGATCGCAGTCCGGATCGTAGTCTGC
AACTCGACTACGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTC
CCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAA
GCTTGGCGTAATCATGGTCATAGCTTGTTTCCTGTGTGAATTGTTATCCGCTCACAATTCCCACAC
AACATACGATCAGGAAGCATAAAGTGTAAAGCCTGGTGTGCCTAAATGAATGAGCT

MC18 708Clone 7 $_{10}$
This clone most closely resembles (98% sequence match) an uncultured Alphaproteobacterium (Bacteria; Proteobacteria; Alphaproteobacteria) like *Afipia* sp. (98%) (Alphaproteobacteria; Rhizobiales;Bradyrhizobiaceae; Afipia) or *Phenylobacterium* sp. (97%) (Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Phenylobacterium)

GCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAC
TCGAGTTGCAGAGTGCAATCCGAACTGAGACGACTTTTGGGGATTAGCTCACCATCGCTGGGTT
GCAACCCTCTGTAGTCGCCATTGTAGCACGTGTGTAGCCCACCTTGTAAGGGCCATGAGGACTT
GACGTCATCCACACCTTCCTCCGGCTTACCACCGGCGGTCCCATTAGAGTGCCCAACTAAATGAT
GGCAACTAATGGCGTGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
ACGACATCCATGCAGCACCTGTGTCCCAGTCCCCGAAGGCAAAGCCAGATCTCTCTGGTGGTCC
GGGCATGTCAAAAGGTGGAAAGGTTCTGCTCGTTGCTTCAAATTAAACCACATGCTCCACCGCTT
GTGCAAGGGCGAATTCAATCACACTGGCGGATGTTTACTTGTGGATCCGAGCTCGGTACCAAGT

MC22 708 Clone 7 $_{15}$
This clone most resembles an Alphaproteobacterium (Bacteria; Proteobacteria; Alphaproteobacteria) like *Bosea* sp. (96% sequence match) (Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea) or *Afipia* sp. (96%) (Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Afipia.) or *Agrobacterium* sp. (96%) (Alphaproteobacteria; Rhizobiales;Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium)

CCATAATCCTTTTAGGGCGATTGGGCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGA
TATCTGCACATTTAAAACCTTGCCCGGGAACGTATTCACCGTGGCATGCTCCATTCACAATTACTA
GCGATTCCACCTTCATGCACTCGAGTTGCAGAGTGCAATCTGAACTGAGACGGCTTTTTGGGATT
AGCTCGAGATCGCTCTTTCGCTGCCCATTGTCACCGCCATTGTAGCACGTGTGTAGCCCAGCCT
GTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCC
AAAAGTTCCCAACTGAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCC
ATCATCTCACG

MC18 708Clone 7 $_{17}$
This clone most resembles an uncultured bacterium clone (94%)

TTNNNNNNNNNNTNGGGCGAATTGGGCCCTCTGATGCATGCTCGAGCGGCCGCCAGTGTGATG
GATATCTGCCCCTTTNGCCCGTTGCACAAGCGGTGGAGCATGTGGATTAATTCGATACTAACCGA
AGAACCTTACCCAGGTTTGACATCGATCGTAAGTCCAAGAGATTGGACCCTCTTCCCAAAAGGAA
GACGTGAAGACACTTGTTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTACGGTTAAGTCCG
CCAACGAGCGCAACCCTCGTCCTATGTTGCCAGCGAGAAAGTCGGGAACTCATAGGAGACCGCC
GGTGTAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGGCAGTTACGCCTGGGGCTTC
ACACATGCTACAATGGGCGAAACAAAGGGATGCAATATCGCGAGATGGAGCTAATCCCAAAAATA
CGCCCCCAGTTCAGATTGCAGTCTGCAACTCGACTGCATGAAGGCGGAATCGCTAGTAAACGCA
GGTCAGCTATACTGCGGTGAATACGTTCCCGGGCAAGGGCGAATTCCAGCACACTGGCGGCCGT
TACTAGTGGATCCGAGCTCGGTACCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGA
AATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGTNAAGCATTAANTTGTANANGTCTGG
GGTGCCTAATGAGTGANCTACNTCACATTAATTGCGNTGCNCTCACTGCCCGCTTTCCANTCGGG
GAAANNCTGTCGTGACCATTGGCATTAA

2.2. FISH Analysis

To date FISH analysis of microcosm samples has been preliminary. 4 samples were selected for analysis and initial observations have demonstrated the suitability of chosen probes EUB338 and Cf319a. Research is ongoing.

Appendix 8

DGGE Profiles of wood and isolation cultures

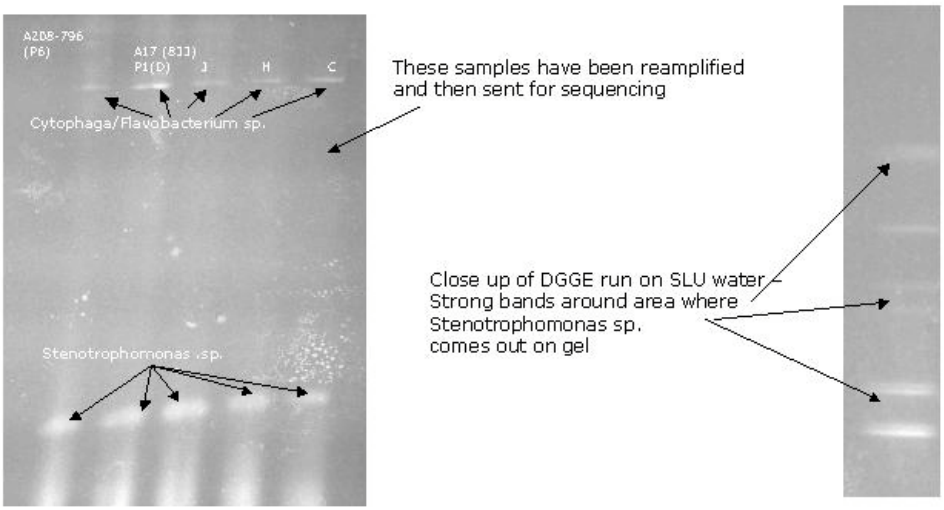
(by Hotchkiss, Landy, Mitchell)

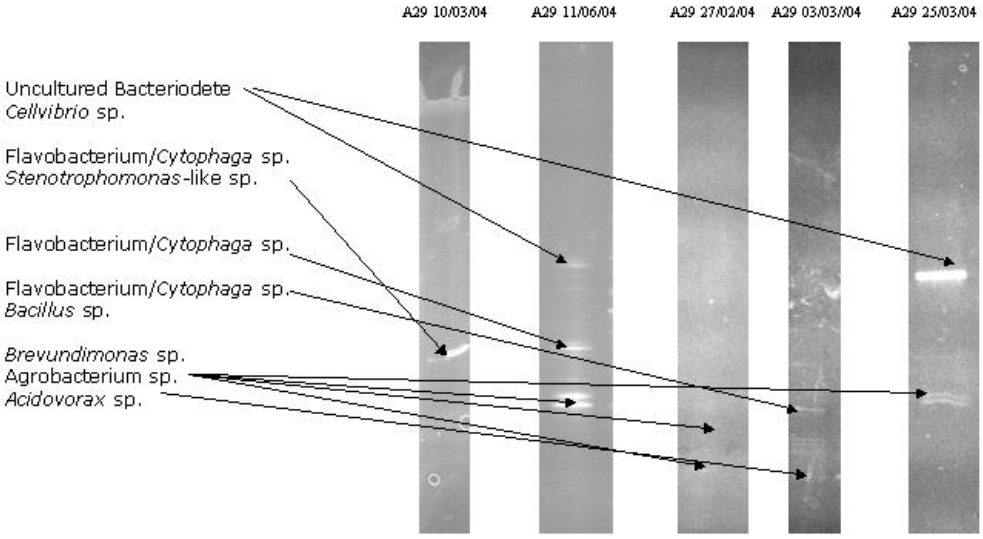
This appendix contains the best DGGE profiles of BACPOLES SLU cultures and wood samples. A Clone library has been made for all the 1400bp and 220bp sequences associated with these gels. Chimer check has been performed on all sequences, all sequences *Bona Fide*

Bacterial Species	**Gel Position (Distance from well)**	
	Full Size Gel	**Half Size Gel**
Rhodobacter sp.	1.5cm	0.75cm
Uncultured CFB	1.6cm	0.8cm
Alpha proteobacterium	2cm	1cm
Mycobacterium	2.1cm	1.05cm
Oxalobacter sp.	2.7cm	1.85cm
Comamonas sp.	3cm	1.5cm
Pseudomonas sp.	3.5cm	1.75cm
Stenotrophomonas-like sp.	3.8cm	1.9cm
Pseudomonas sp.	4.5-4.6cm	2.25-2.6cm
Uncultured Bacteriodete	5.3cm	2.65cm
Cellvibrio sp.	5.3cm	2.65cm
Flavobacterium/Cytophaga sp.	6.5cm	3.25cm
Stenotrophomonas-like sp.	6.5cm	3.25cm
Brevundimonas sp.	7.3cm	3.65cm
Pseudomonas sp.	7.3cm	3.65cm
Flavobacterium/Cytophaga sp.	7.7cm	3.85cm
Stenotrophomonas-like sp.	7.7cm	3.85cm
Brevundimonas sp.	8.1-8.3cm	4.05-4.15cm
Pseudomonas sp.	8.3cm	4.15cm
Azotobacter sp.	8.3cm	4.15cm
Flavobacterium/Cytophaga sp.	8.6cm	4.3cm
Bacillus sp.	9.6cm	4.8cm
Rhizobium sp.	9.8cm	4.9cm
Brevundimonas sp.	9.8cm	4.9cm
Agrobacterium sp.	10cm	5cm
Acidovorax sp.	10cm	5cm
Stenotrophomonas-like sp.	10cm	5cm
Brevundimonas sp.	10.7-11cm	5.35-5.5cm
Stenotrophomonas-like sp.	10.8cm	5.4cm
Brevundimonas sp.	10.9cm	5.45cm

DGGE 14/6/04 Lotte's samples from Umea

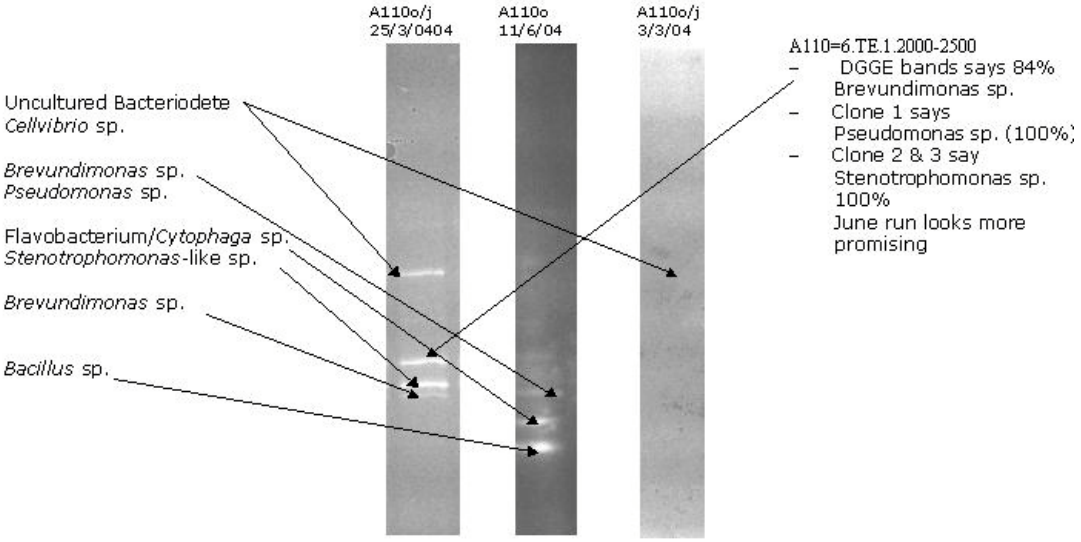
DGGE 15/5/04 Sterile autoclaved water from SLU

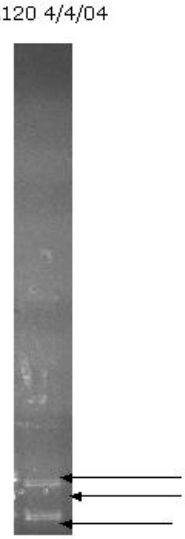




A29= wood sample 1btd31009
- 27 Feb 04 – one double band
- Clone 8 = Brevundimonas sp. or Caulobacter sp. (both 100% match)
- Clone 9 = Methanococcus sp. (98.7%) or Citrobacter sp. (98.68%)

DGGE bands suggest that both are either Brevundimonas sp. or Caulobacter sp.





A120- 6.te.6000-6900
DGGE bands reveal identities:

Stenotrophomonas sp. (98.05%)
Stenotrophomonas sp. (97.44)/Pseudomonas (97.45%)
Stenotrophomonas sp. (98.05%)

A169(993)3/3/04

A169=26.TE.3.0-74 – DGGE band has a 100% Bacillus match, Clones suggested uncultured bacterium/ Brevundimonas

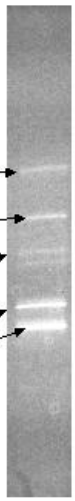
A51(951) 3/3/04

A51=Bryggen, Norway, water sample sent by Thomas (Bacpoles Site 24 – pine harbour in brackish water) one band reamplified – Acidovorax sp. – 100% match

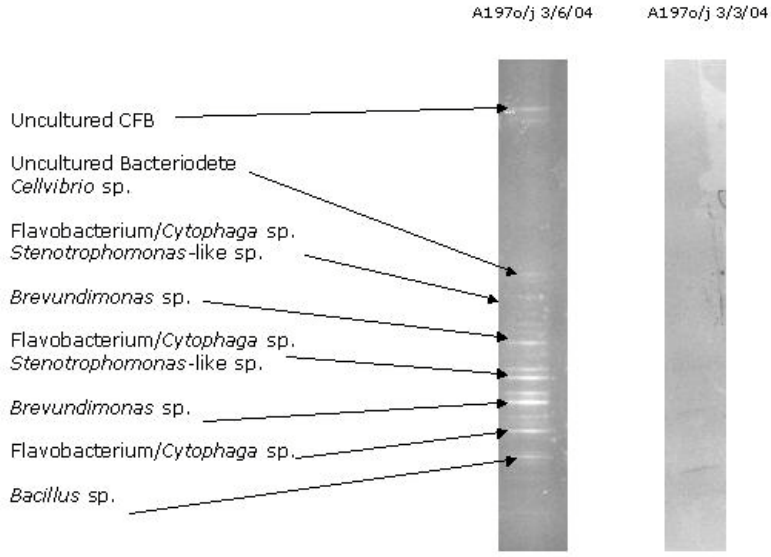
A23-13/3/04

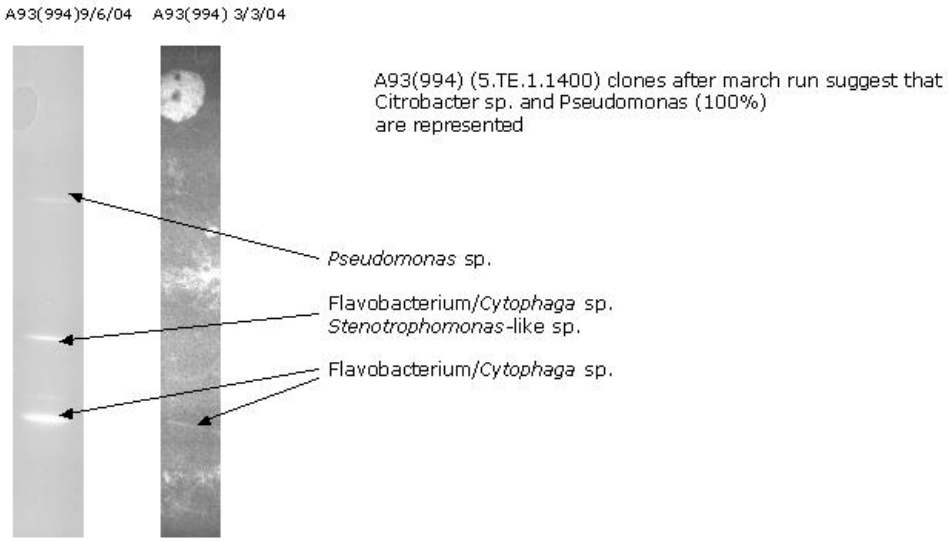
A118 25/03/03

A118 = 6.TE.3. 2000-2500
DGGE Bands not successfully re-amplified have been inferred
A118 CLONE 8 = Flavobacterium (97.35%)
A118 CLONE 3 = Stenotrophomonassp. (93.19%)
A118 CLONE 2 = Acinetobacter sp. (100%)

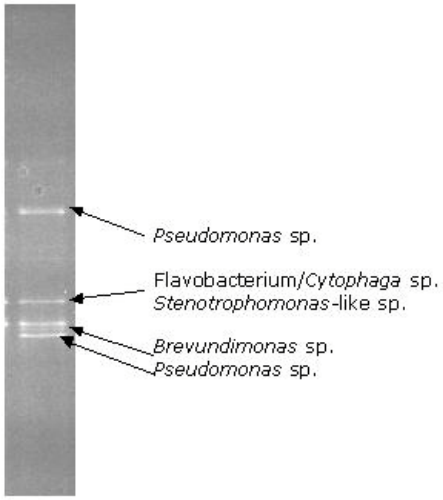


A197 4TE30-45



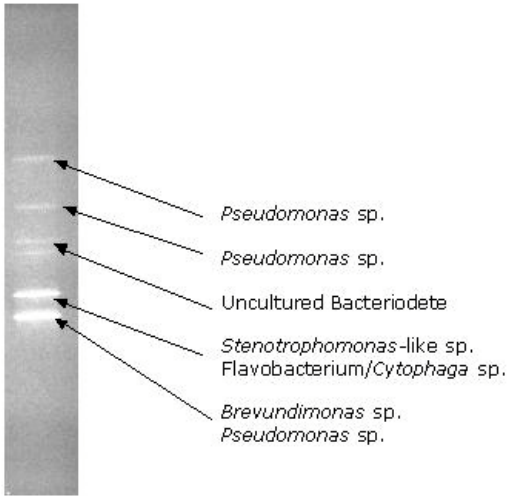


A115 25/3/04



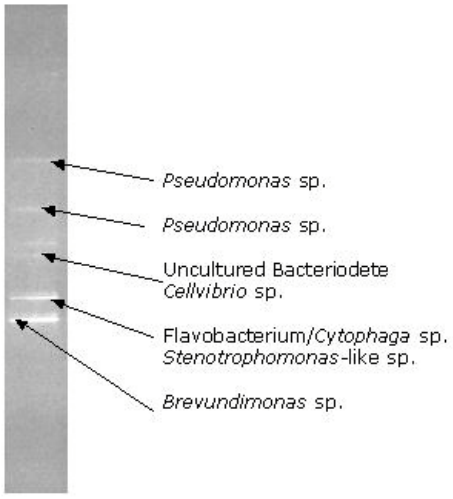
A115 – 6.TE.2.4000-4500
DGGE Bands not successfully re-amplified were inferred
A115 CLONE 4 = Phenylobacterium sp. (100%)
A115 CLONE 3 = Citrobacter sp. (100%)

A112 25/3/04



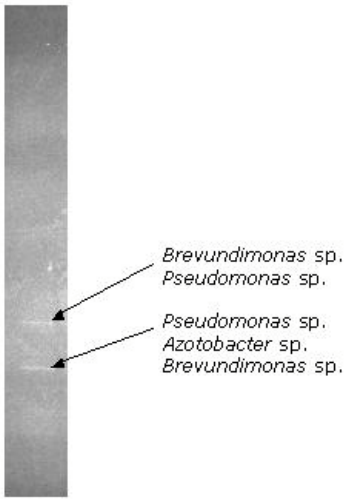
A112 = 6.TE.1.6000-6650
DGGE Bands not successfully re-amplified
A112 CLONE 3 = Citrobacter sp. (100%)

A116 25/3/04

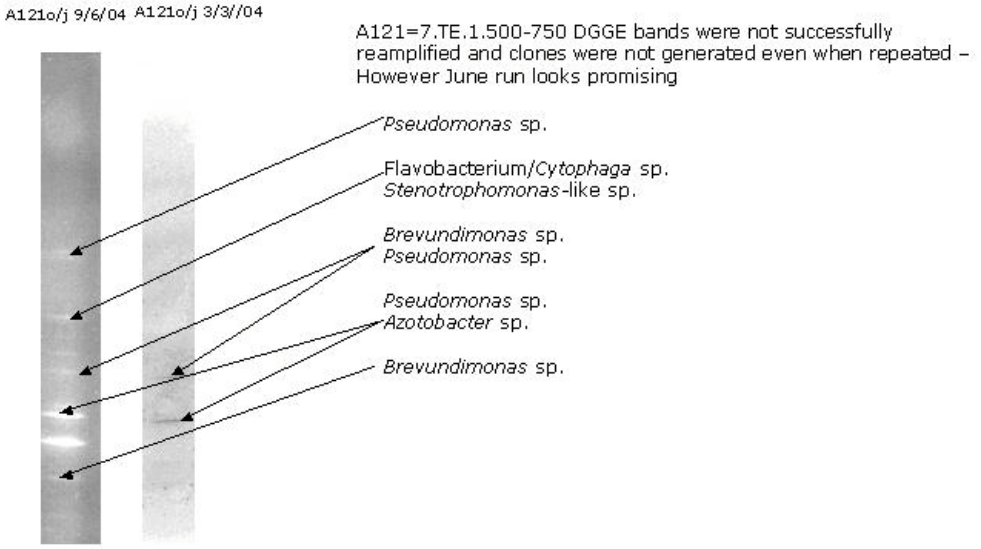


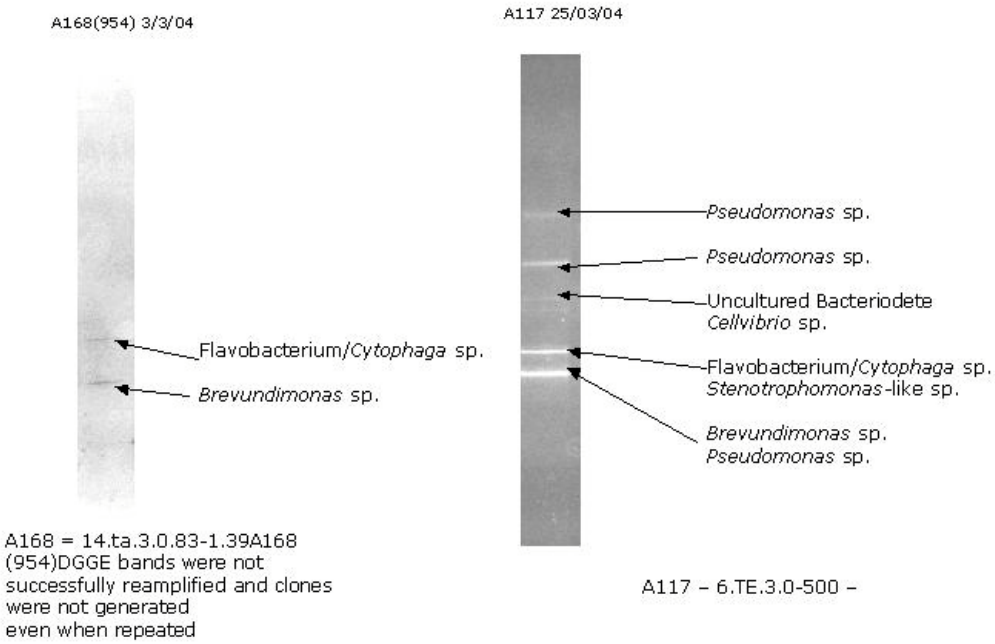
A116 – 6.TE.2.6400-6900
No information came of cloning - Bands that could not
be re-amplified have been inferred

A17-1 1/4/04

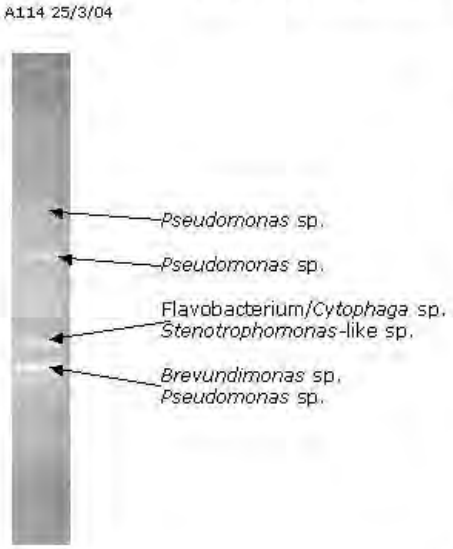


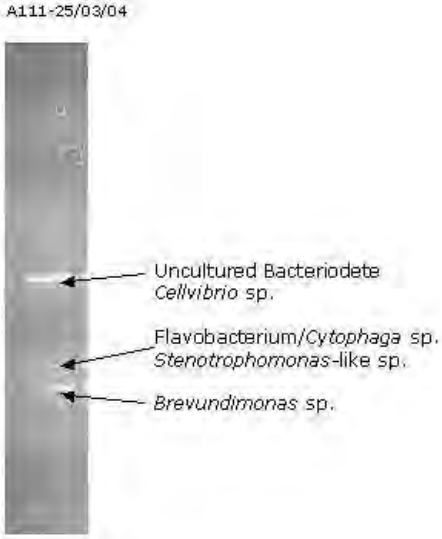
A17-1 = 8.TD.1.600– DGGE bands were not successfully
reamplified. Clone 4 says Stenotrophomonas sp. (99.49%)
Other clones yielded no more information


A121o/j 9/6/04
A121o/j 3/3//04
A121=7.TE.1.500-750 DGGE bands were not successfully reamplified and clones were not generated even when repeated – However June run looks promising
Pseudomonas sp.
Flavobacterium/Cytophaga sp.
Stenotrophomonas-like sp.
Brevundimonas sp.
Pseudomonas sp.
Pseudomonas sp.
Azotobacter sp.
Brevundimonas sp.

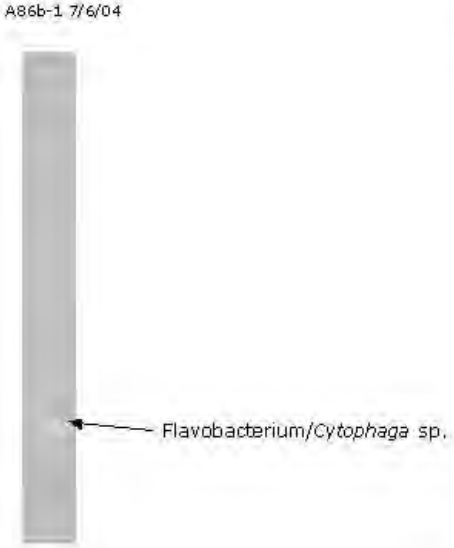

A168(954) 3/3/04
Flavobacterium/Cytophaga sp.
Brevundimonas sp.
A168 = 14.ta.3.0.83-1.39A168 (954)DGGE bands were not successfully reamplified and clones were not generated even when repeated
A117 25/03/04
Pseudomonas sp.
Pseudomonas sp.
Uncultured Bacteriodete
Cellvibrio sp.
Flavobacterium/Cytophaga sp.
Stenotrophomonas-like sp.
Brevundimonas sp.
Pseudomonas sp.
A117 – 6.TE.3.0-500 –

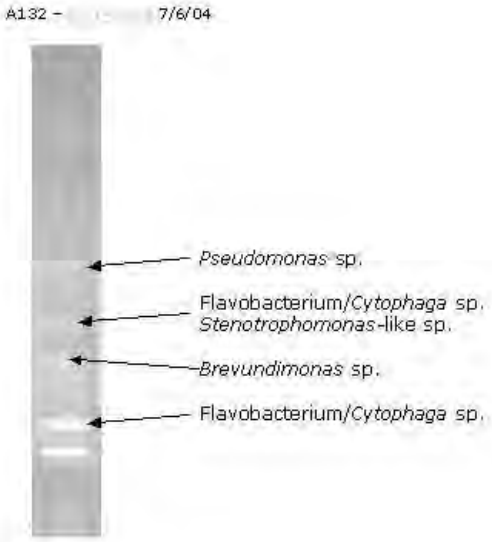
A114 – 6.TE.2.2000-2500



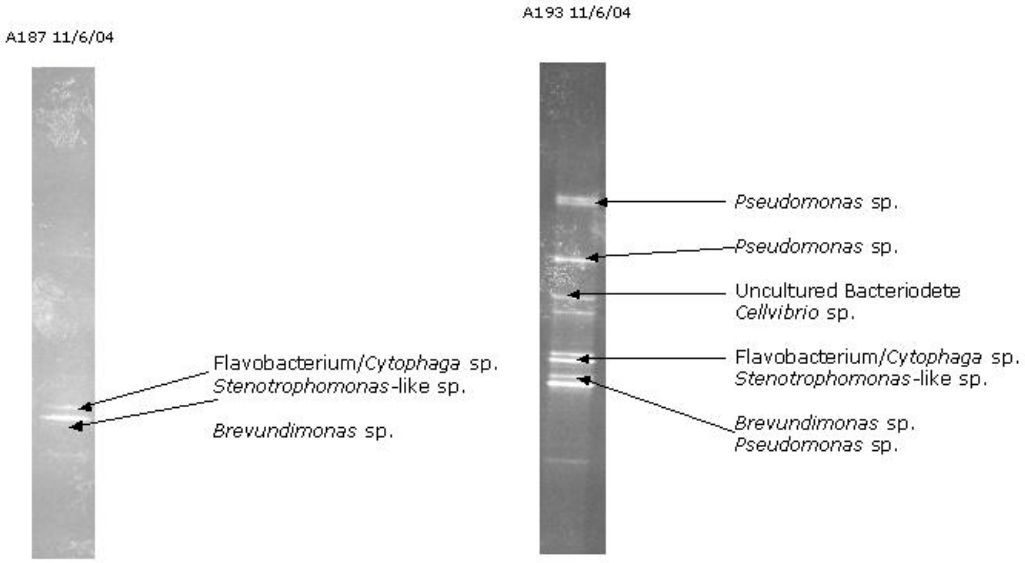


A111= 6TE1.4000-4500DGGE
Cloning was not possible on this attempt



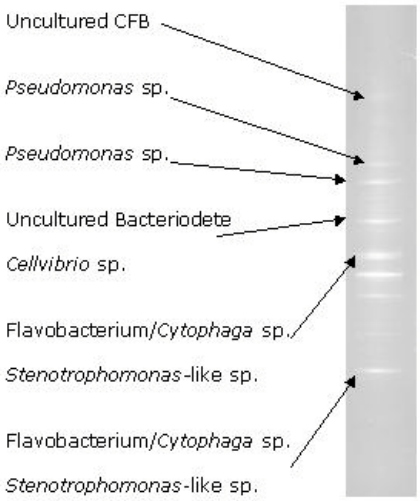


A86b-1 2.td.2.400-840



Site 1
Spaarndammerbuurt, Amsterdam, Netherlands
Spruce (unknown age) - Piles 39% of original bearing area

DNA extracted from 1DTD 3 110 (17W), 1CTD 1-320 (18W), 1dTD 3-340 (19W), 1aTD 1-180 (28W), 1aTD 1-320 (29W), 1bTD 3140 (30W), 1bTD 3310 (31W), 1cTD 1140 (32W), 1aTD 1 (85W)
Successful DGGE done on 1CTD 1-320 (18W)(once) and unsuccessful done on 1CTD 1-320 (18W)(once), 1bTD 3140 (30W)(four times) and 1bTD 3310 (31W)(once)
Extra Information came from cloning 1dTD 3-340 (19W) and 1aTD 1-180 (28W) PCR products



18w (1CTD 1-320)
DGGE successful 11 June 04 –
bands not re-amplified - but inferred

Clone Library revealed Identities of:

Brevundimonas vesicularis
Pseudomonas sp.
Massilia sp.
Bergeyella sp.
Uncultured eubacterium

Site 2
Anna Paulownastraat 14, Dordrecht, Netherlands
Spruce (c.75 yr old) - Need info on condition of wood

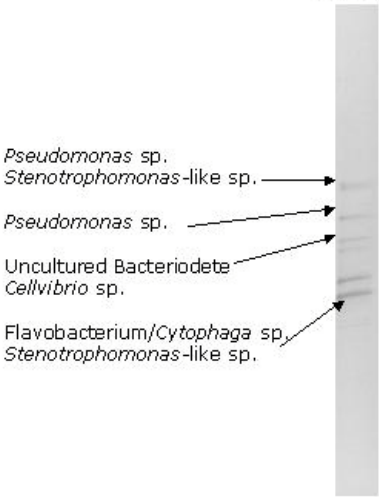
DNA extracted from 2td3 400-850 (50W), 2td1 0-620 (51W),
2td2 400-480 (52W), 2td3 400-850 (53W),
2td8 0-700 (54W), 2td9 0-650 (55W), 2td10 0-600 (56W), 2td11 0-650 (57W), 2td12 0-760 (58W),
2td4 150 (77W), 2td5 200 (78W), 2td6 130 (79W), 2td7 140 (80W), and 2td6 4 150 (83W)
Successful DGGE done on 2td10 0-600 (56W) (twice) and 2td2 400-480 (52W) (once)
and unsuccessful DGGE done on 2td2 400-480 (52W)(once),
2td3 400-850 (53W)(twice), 2td11 0-650 (57W) (once),
2td12 0-760 (58W) (once), 2td4 150 (77W) (once) and 2td7 140 (80W) (once)
Additional information yielded from Clone library of 51w, 2td3 400-850 (53W),
and 2td11 0-650 (57W) PCR products

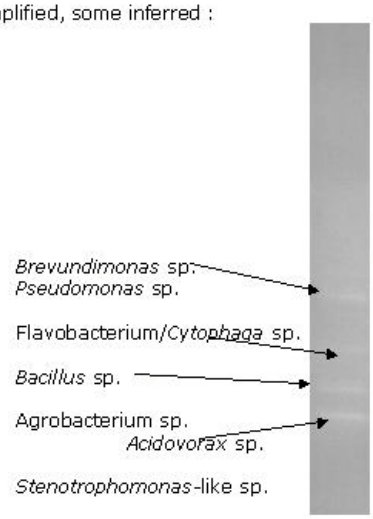
52w (2.td.4.400-840)
DGGE successful
– bands not re-amplified but inferred
Clone Library revealed Identities of:
Stenotrophomonas-like sp. (100%)
Pseudomonas sp.

56w (2td.10.0-600)
21/April/04

9th June 2004

Some DGGE bands were re-amplified, some inferred :





Cloned library was not successful for this sample

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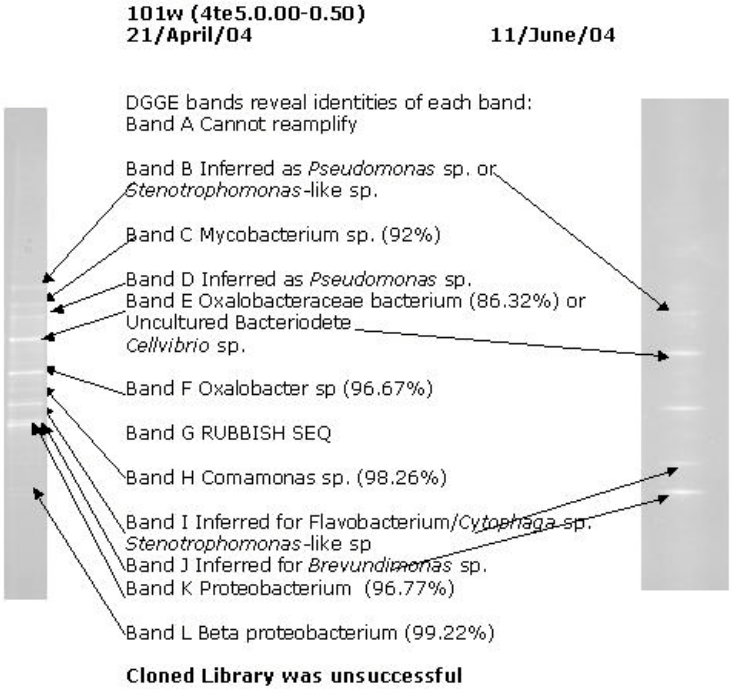
Site 4
Haarlem, Netherlands
Scot's Pine (c.100 yr old)

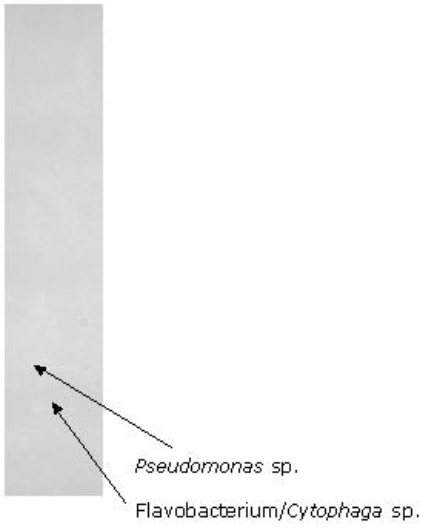
DNA extracted from 4TD2 0-069 (95W), 4TE7 0,0-0,5 (96W), 4TE60.00-0.50 (100W), 4TE50.00-0.50 (101W), 4TD4 0.00-0.67 (102W), 4TD3 0.00-0.45 (103W) and 4TD1 0.064 (104W)
Successful DGGE done on 4TE50.00-0.50 (101W) (twice)
and unsuccessful DGGE done on 4TE50.00-0.50 (101W)(once)
No additional information was yielded from Clone library of PCR products from this site



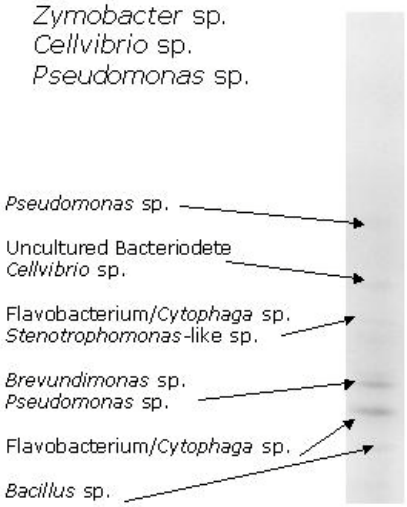
Site 5
Joubertstraat/Paul Krugerstraat, Rotterdam, Netherlands
Silver Fir and Spruce c. 100yrs - extracted piles had a degraded outmost layer of 10-15mm

DNA extracted from 5TE1 (62W), 5TE2 50 (67W), and 5TE3 50 (70W)
Successful DGGE done on 5TE1 (62W) (once) and 5TE2 50 (67W) (once)
and unsuccessful DGGE done on 5TE1 (62W) (once) and 5TE2 50 (67W) (once)
Some additional information was yielded from Clone library of PCR products from this site

62w (5.te.1) DGGE bands inferred
Clone Library revealed Identities of:
Pseudomonas Sp.

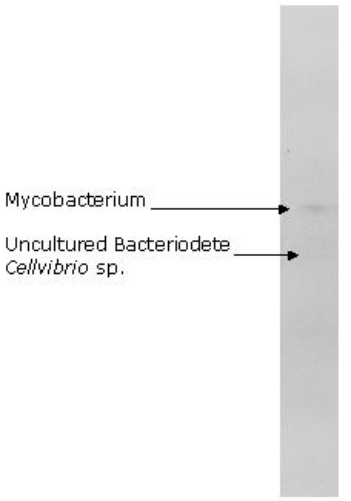


67w (5te2.50)
DGGE bands not re-amplified were inferred
Clone library revealed identities of:
Zymobacter sp.
Cellvibrio sp.
Pseudomonas sp.



Site 6
Irisstraat 89, Koog a/d Zaan, Netherlands
Scots Pine, c. 65yrs old
Wood has a degraded outer layer of 10mm

DNA extracted from 6TE 10-500 (63W), 6TE 20-500 (65W), and 6TE 30-500 (68W)
Successful DGGE done on 6TE 20-500 (65W) (once) and 6TE 20-500 (65W) (once)
and unsuccessful DGGE done on 6TE 10-500 (63W) (three times)
No additional information was yielded from Clone library of PCR products from this site

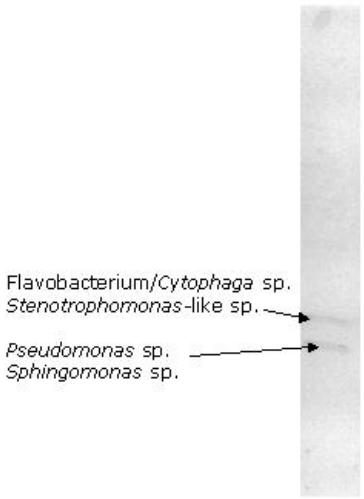


65w (6te2 0-500)
DGGE bands not re-amplified were inferred

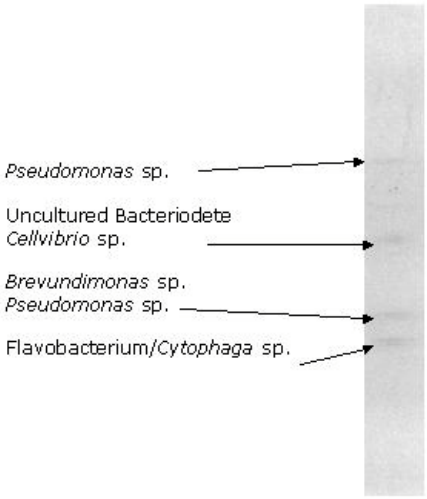
Site 7
Jan Nieuwenhuijzenstraat 10, Haarlem, Netherlands
Poplar, c.100 yrs old
Degraded outermost layer of 30-45mm

DNA extracted from 7TE1 500-750 (64W), 7TE2 500-750 (66W), 7TE3 850-1100 (69W), 7TE4 300-500 (71W), 7TE2 200-450 (72W) and 7TE6 430-600 (73W)
Successful DGGE done on 7TE1 500-750 (64W) (twice), 7TE2 500-750 (66W) (once) and 7TE3 850-1100 (69W) (once)
and unsuccessful DGGE done on 7TE1 500-750 (64W) (twice), 7TE2 500-750 (66W) (once) and 7TE3 850-1100 (69W) (once)
No additional information was yielded from Clone library of PCR products from this site

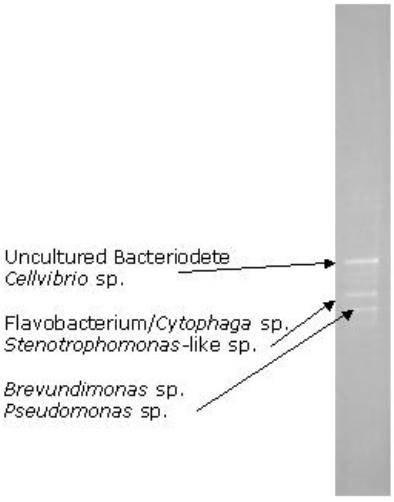
69w (7.te.3.850-1100)



66w (7.te.2.500-750)



64w (7.te.1.500-750)



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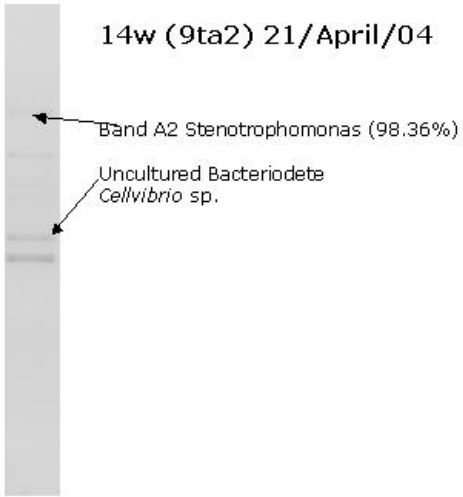
Site 9
Koningstraat, Dokkum, Netherlands
Oak (c.590 yrs old) - A 14th century wine barrell

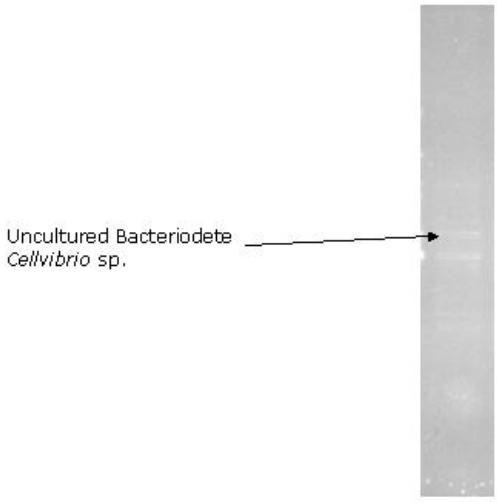
DNA extracted from 9TA4 (7W), 9TA2 (14W), 9TA3 (16W) and 9TA1 (38W)
Successful DGGE done on 9TA2 (14W) (once)
Some additional information was yielded from Clone library of PCR products from this site



Site 10
Ellewoutsdijk, Zeeland, Netherlands
Oak (c.1900yrs)

DNA extracted from 10TD2 950(1W), 10TD1 930(2W), 10TD1 5801 (12W) and 10TD2 220 (84W)
Successful DGGE done on 10TD1 5801 (12W) (once)
Some additional information was yielded from Clone library of PCR products from this site

12w (10.TD.1.0-5801)
DGGE bands not re-amplified were inferred



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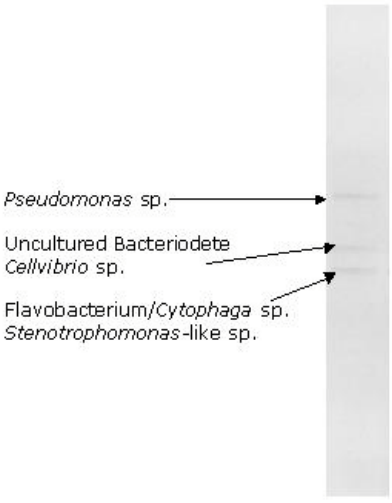
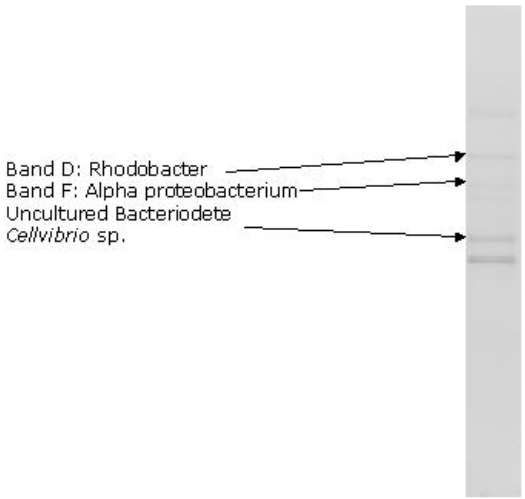
Site 12
Burgzand Noord 15
(Marine archaeological site in the Wadden Sea)
Oak and Scot's Pine (c. 376 yrs)

DNA extracted from 12TA1.2 (20W), 12TA2.1 (21W), 12TA1.3 (23W), 12TD1.4 (24W), 12TD1.1 (25W), 12TD2.3 (26W) and 12TA2.4 (27W)
Successful DGGE done on 12TD2.3 (26W)(once) and 12TA2.4 (27W) (once)
and unsuccessful done on 12TA1.2 (20W)(once)
Some additional information was yielded from Clone library of PCR products from this site

27w (12ta.4)
DGGE bands not re-amplified were inferred

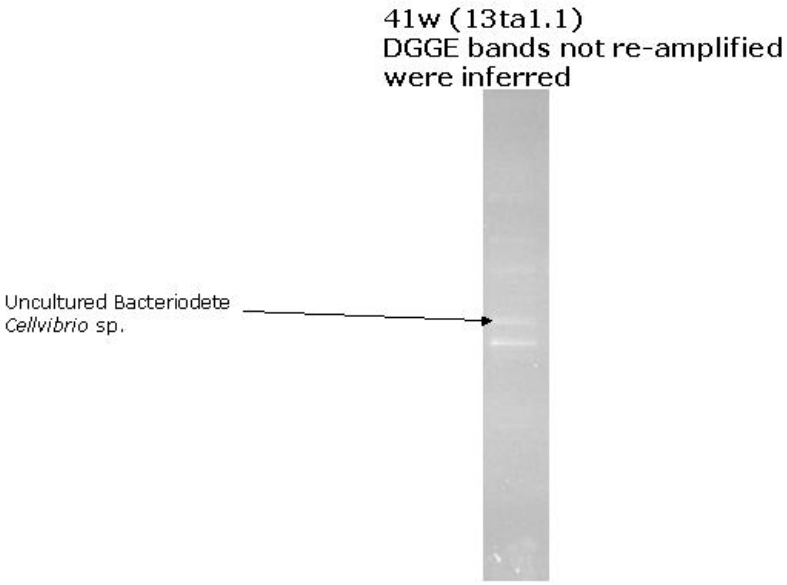


26w (12ta2.3) 21/April/04



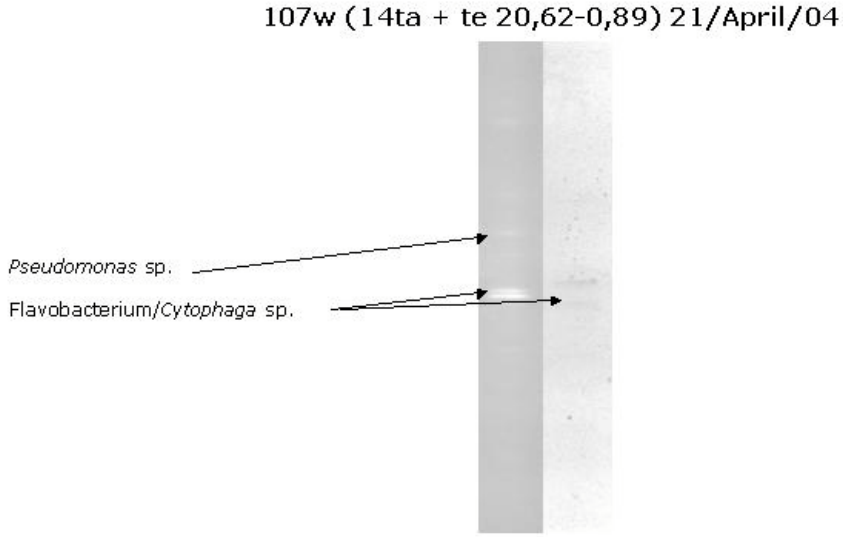
Site 13
Burgzand Noord 3
(Marine archaeological site in the Wadden Sea)
Scot's Pine and Oak (c.360yrs) possibly from the East Inida vessel 'de Rob'

DNA extracted from 13TA1.3 (39W), 13TA4 (40W), 13TA1.1 (41W) and 13TA1.2 (42W)
Successful DGGE done on 13TA1.1 (41W)(once) and unsuccessful done on 13TA4 (40W) (once)
Some additional information was yielded from Clone library of PCR products from this site



Site 14
Small Wooden Castle at Travenhorst, Germany
Oak (c.650 yrs old)

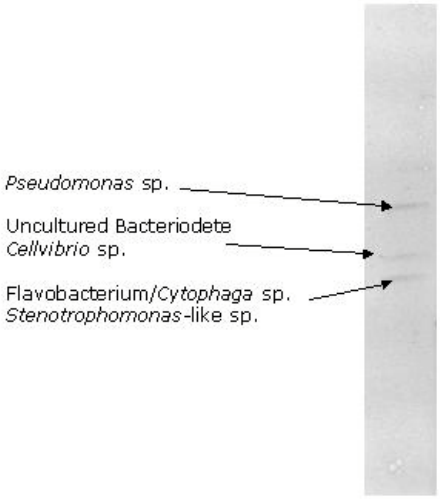
DNA extracted from 14TAHE3.034-157 (82W), 14TA+TE 1049-071 (91W),
14TA+TE 20,62-0,89 (107W) and 14TA+TE 30,34-0,57 (108W)
Successful DGGE done on 14TA+TE 20,62-0,89 (107W)(twice) and
unsuccessful done on 14TA+TE 30,34-0,57 (108W) (twice)
Some additional information was yielded from Clone library of PCR products from this site



Site 21
Kronan, Lidan, Sweden
Oak Shipwreck

DNA extracted from 21TA2@1 (74W) and 21TA3@3 (75W)
Successful DGGE done on 21TA3@3 (75W)(once)
Some additional information was yielded from Clone library of PCR products from this site

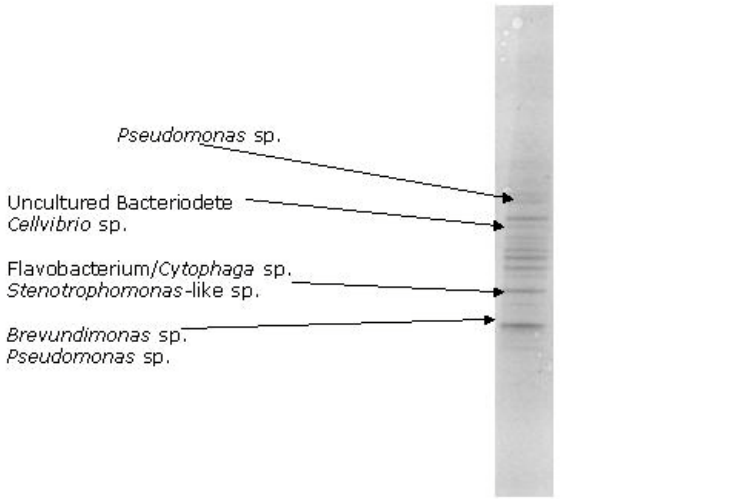
75w (21ta3@3)
DGGE bands not re-amplified were inferred
Clone Library revealed identities of:
Rhodobacter sp.



Site 22
Houses of Parliament, Stockholm, Sweden
Scot's Pine pilings (c.110yrs)

DNA extracted from 22TD2 4700-4760 (5W), 22TD2 2420-2460 (6W),
22TD1 6600-6660 (8W) and 22TD1 2420-2460 3.1 (11W)
Successful DGGE done on 22TD1 2420-2460 3.1 (11W)(twice)
Some additional information was yielded from Clone library of PCR products from this site

11w (22td1 2420-2460 3.1)
DGGE bands not re-amplified
were inferred

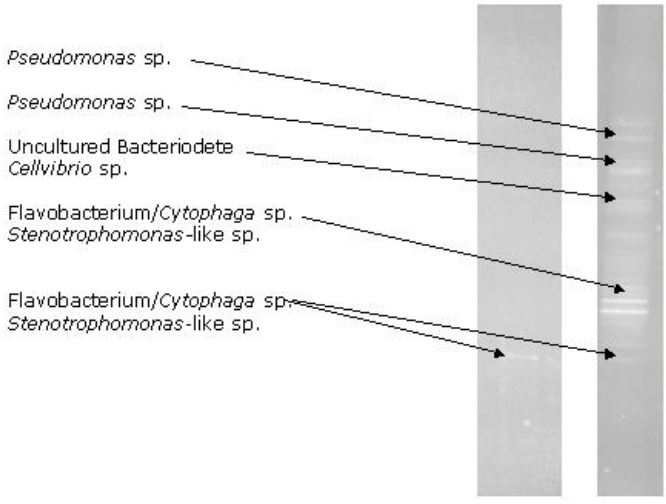


Site 23
Leeuwarden, Netherlands
Scot's Pine (c.100yrs)

DNA extracted from 23TD1 80 (43W) and 23TD1 300 (44W)
Successful DGGE done on 23TD1 300 (44W)(once) and unsuccessful done on 23TD1 300 (44W)(once)
Some additional information was yielded from Clone library of PCR products from this site

44w (23.td.1.300)
DGGE bands not re-amplified were inferred
Clone Library revealed identities of:

Chryseobacterium sp.

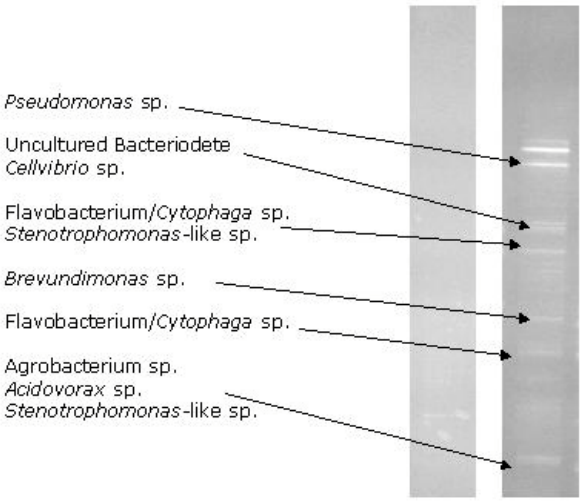


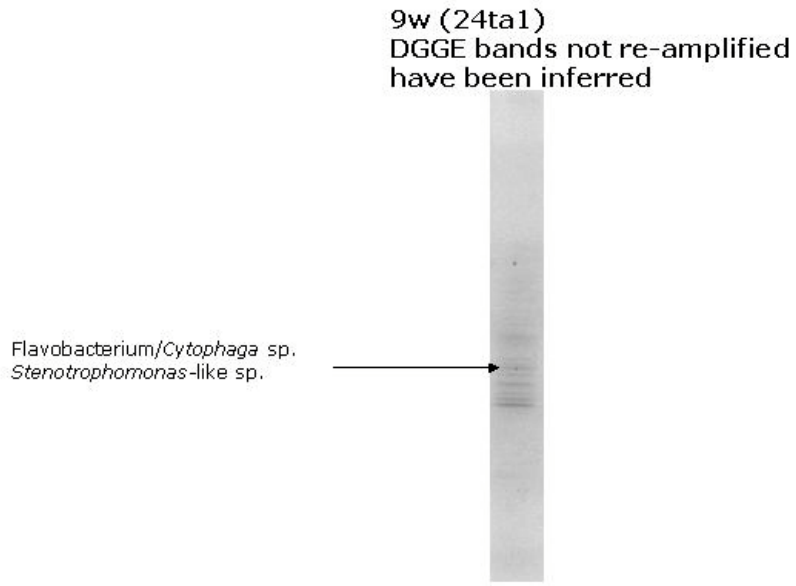
Site 24
Bryggen, Norway,
Harbour settlement (UNESCO Reserve)
Scot's Pine

DNA extracted from 24TA 4A+C (3W), 24TA 3 (4W), 24TA 1 (9W),
24TA 5.1 (10W), 24TA 2 (13W) and 24TA 4B (15W)
Successful DGGE done on 24TA 3 (4W)(once) and 24TA 1 (9W) (once)
and unsuccessful done on 24TA 3 (4W)(once) and 24TA 1 (9W) (once)
Some additional information was yielded from Clone library of PCR products from this site

4w (24.ta.3)
DGGE bands not re-amplified were inferred
Clone Library revealed identities of:

Acidovorax sp.

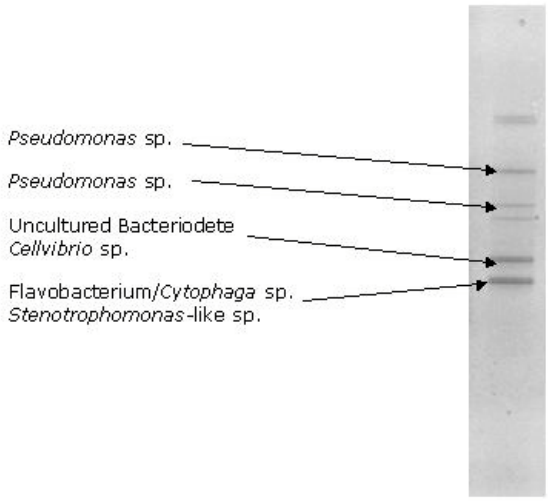




Site 25
Mollösund
Oak

DNA extracted from 25TA 1A4 (76W) and 25TA 1A4 (89W)
Successful DGGE done on 25TA 1A4 (76W)(once) and unsuccessful done on 25TA 1A4 (76W)(once)
Some additional information was yielded from Clone library of PCR products from this site

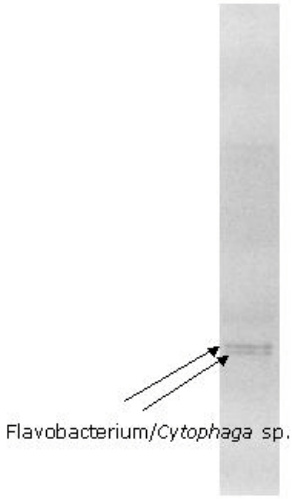
76w (25ta1a4)
DGGE bands not yet re-amplified
Clone Library revealed identities of:
Pseudomonas sp.
Janthinobacterium



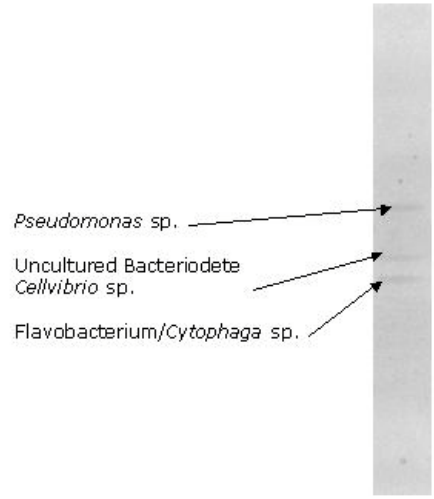
Site 26
Elst (Roman temple c. 1900 yrs old)

DNA extracted from ELST TE 3 0-74 26 (90W) and 26TE 10-78 (92W)
Successful DGGE done on ELST TE 3 0-74 26 (90W) (once) and 26TE 10-78 (92W)(once)
and unsuccessful done on ELST TE 3 0-74 26 (90W)(once) and 26TE 10-78 (92W) (twice)
Some additional information was yielded from Clone library of PCR products from this site

90w (Elst te 3 0-74)
DGGE bands not re-amplified were inferred

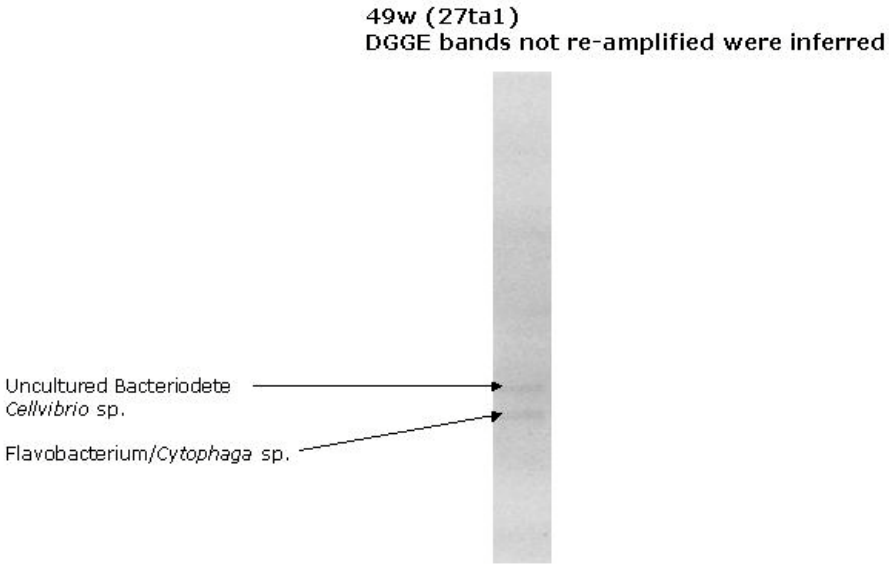


92w (26te10-78)
DGGE bands not re-amplified were inferred



Site 27
GZ-80, Netherlands
Oak

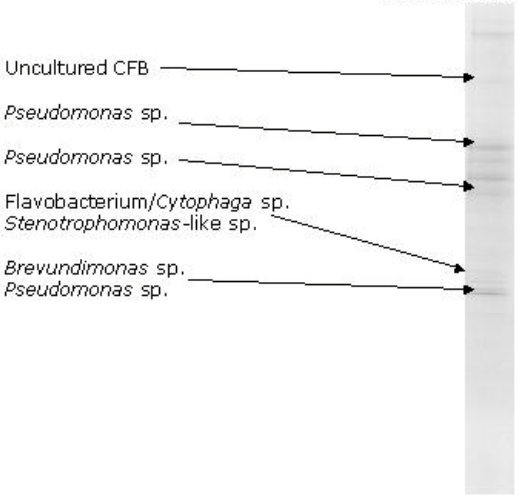
DNA extracted from 27TA1 (49W), 27TA5 (87W), 27TA3 (88W) and 27TA2 (94W)
Successful DGGE done on 27TA1 (49W)(once)
and unsuccessful done on 27TA1 (49W)(once), 27TA2 (94W) (once) and 27TA3 (88W) (twice)
Some additional information was yielded from Clone library of PCR products from this site



Site 28
KZ-47
Oak

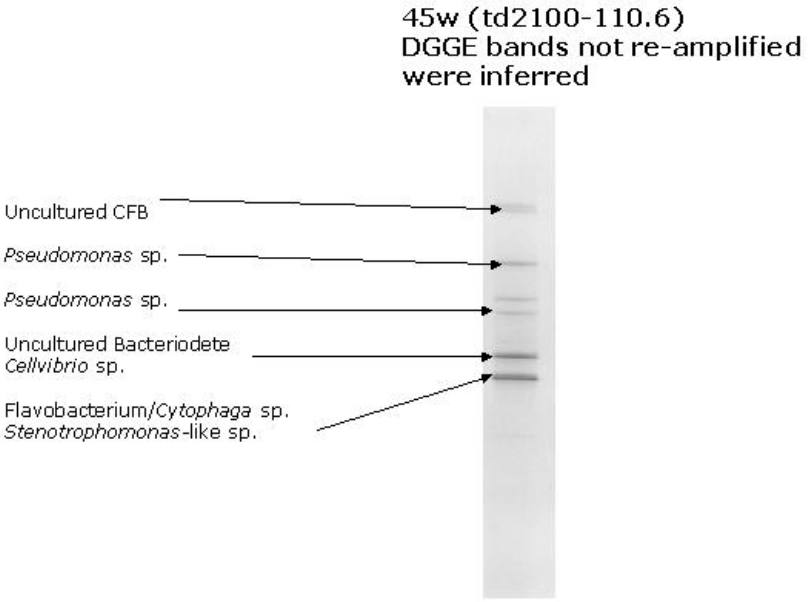
DNA extracted from 28TA2 (81W) and 28TA1 (86W)
Successful DGGE done on 28TA1 (86W) (once)
and unsuccessful done on 28TA2 (81W)(once) and 28TA1 (86W) (once)
Some additional information was yielded from Clone library of PCR products from this site

86w (28ta1)
DGGE bands not re-amplified were inferred
Clone Library revealed identities of:
Cytophaga sp.
Flavobacterium



Site ?
Amsterdam
Spruce

DNA extracted from TD2 100-110.6 (45W)
Successful DGGE done on TD2 100-110.6 (45W)(once)
Clones?
Need to check this stray one out


45w (td2100-110.6)
DGGE bands not re-amplified
were inferred
Uncultured CFB
Pseudomonas sp.
Pseudomonas sp.
Uncultured Bacteriodete
Cellvibrio sp.
Flavobacterium/Cytophaga sp.
Stenotrophomonas-like sp.