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Bacteriological Characteristics of Contemporary Wood During Soil Deposition in Anoxia Conditions at the Biskupin Archaeological Site (Poland) – Petrifilms™ as an Alternative for Conventional Bacteriological Media

Charakterystyka bakteriologiczna współczesnego drewna zalegającego w glebie na stanowisku archeologicznym w Biskupinie (Polska), w warunkach anoksji – Petrifilmy™ jako alternatywa względem konwencjonalnych podłoży bakteriologicznych

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Abstract: The paper presents results of quantitative and qualitative analyses of the composition of saprophytic and pathogenic microflora colonising contemporary wood of Scots pine (*Pinus sylvestris* L.) and oak (*Quercus* sp.), deposited for eight years in peat soil, under anoxia conditions at two measuring stations located at the fortified settlement of the Lusatian culture in Biskupin. Qualitative determinations of bacteria were performed based on the analyses of their cultural, morphological, physiological, and bio-chemical characteristics. Petrifilms™ and conventional bacteriological media were used for quantitative analyses. Bacteria in the examined wood and surrounding soil were identified as belonging to species, including those of the genera *Pseudomonas*, *Clostridium* (*Cl. butyricum/beierinckii, Cl. perfringens*), *Bacillus*, *Corynebacterium*, bacteria from the family Enterobacteriaceae, as well as aerobic and anaerobic cellulolytic bacteria. An important correlation between the standard pour plate method and Petrifilm™ was observed. Petrifilms are an effective alternative, in comparison with traditional methods, for the determination of total bacterial counts for wood samples.

Keywords: wood, degradation, archeological site, bacteria, pour plate method, Petrifilm™

Streszczenie: W pracy przedstawiono wyniki ilościowych i jakościowych analiz składu mikroflory saprofitycznej i patogennej zasiedlającej współczesne drewno sosny zwyczajnej (*Pinus sylvestris* L.) i dębu (*Quercus* sp.), zalegające przez okres ośmiu lat w glebie torfowej, w warunkach anoksji, na dwóch stanowiskach pomiarowych umiejscowionych na terenie grodu obronnego kultury łużyckiej w Biskupinie. Oznaczenia jakościowe bakterii przeprowadzono na podstawie analizy ich cech makro- i mikro-morfologicznych, a także fizjologicznych i biochemicznych. Do oznaczeń ilościowych użyto produktów Petrifilm™ i konwencjonalnych pożywek bakteriologicznych. Bakterie w badanym drewnie i otaczającej je

glebie zidentyfikowano do gatunków, reprezentujących m.in. rodzaje *Pseudomonas*, *Clostridium* (*Cl. butyricum/beierinckii, Cl. perfringens*), *Bacillus*, *Corynebacterium*. Wykryto również bakterie z rodziny Enterobacteriaceae oraz tlenowe i beztlenowe bakterie celulolityczne. Zaobserwowano istotną korelację między wynikami otrzymanymi z wykorzystaniem standardowej metody płytkowej i użyciem techniki wykorzystującej produkty PetrifilmTM. Te ostatnie okazały się być skuteczną alternatywą względem tradycyjnych metod oznaczania całkowitej liczby bakterii w próbkach drewna.

Słowa kluczowe: drewno, degradacja, stanowisko archeologiczne, bakterie, metoda płytkowa, Petrifilm™

Introduction

Representatives of many disciplines of science have investigated the fortified settlement of the Lusatian culture, located in Biskupin. Preserved remnants of wooden building structures of the settlement, deposited in soil, are exceptional sources of information on material culture. However, the valuable archaeological site needs to be protected against numerous destructive factors. One of the greatest threats for wood accumulated at the site is posed by cellulolytic microorganisms. By colonising the remnants of the settlement and synthesising the cellulolytic enzymes they cause degradation of the historical organic material. Undertaken preservation works should thus be preceded by an analysis of the number and composition of microflora colonising the examined wood and an assessment of the degree of its degradation. Bacterial characteristics as well as physical properties, chemical composition, and changes in the microscopic structure of the Biskupin archaeological wood were presented in several publications (Gajewska, Borkowski and Babiński 2006, 665-669; Waliszewska et al. 2007, 11-22; Zborowska et al. 2007b; Babiński 2009a, 149-172; Gajewska, Borkowski and Babiński 2009a, 209-216; Waliszewska, Zborowska and Kudela 2009, 189-205; Zborowska et al. 2009, 175-185; Ważny 2016, 48-53).

Due to the limited possibility of collecting samples from historical Biskupin structures, some microbiological analyses were conducted on contemporary wood. The degradation dynamics of contemporary wood was analysed at many monitored archaeological sites (Powell et al. 2001, 165-173; Gregory et al. 2002, 213-223; Hogan et al. 2002, 187-212; Brenner, Krieg and Staley, 2005; Fojutowski et al. 2011, 17-30). Fresh undegraded experimental material was buried at the site, in the immediate vicinity of the preserved wooden fragments of the settlement, to ensure a similar composition of microflora to that on the archaeological wood.

The aim of this study was to provide bacteriological quantitative and qualitative characteristics of contemporary pine and oak wood, after eight years of deposition in peat soil under anoxia conditions. Classical, but still widely used methods of bacterial count determination have been used alongside relatively modern one – Petrifilm™ assay.

Our research gives significant information for the assessment effect of the soil medium on the wood microflora composition. Identification of threats for archaeological wood from the microbiological point of view is also explored. Moreover, we conducted an evaluation of Petrifilms, as an alternative way of determining total bacterial counts for wood samples.

1. Materials and methods

1.1. Experiment localisation and sample collection

Analyses were conducted on samples of soil, as well as samples of contemporary sapwood of Scots pine (*Pinus sylvestris* L.) and heartwood of oak (*Quercus* sp.), deposited for eight years (from August 2003 to August 2011) in waterlogged peat soil at measuring stations SP1 and SP4, located at the fortified settlement of the Lusatian culture in Biskupin. Characteristics of macroscopic structure and physical properties of

wood used in the analyses were presented in earlier publications (Babiński et al. 2006, 9-21; Zborowska et al. 2007a, 13-26; Babiński 2009b, 299-313).

Conditions, under which wood samples were deposited at the site, were evaluated on the basis of the redox potential of soil. Measurements were taken, using a CX-401 measuring device (Elmetron, Poland), coupled with a platinum electrode, described by Faulkner and co-workers (Faulkner, Patrick, and Gambrell 1989, 883-890), and an RL-200 reference calomel electrode (Hydromet, Poland). Redox potential was given in relation to the standard hydrogen electrode (E_H, 244 mV at 25°C), taking into consideration soil temperature at the depth of sample deposition. Measurements were recorded every two weeks in the years 2004-2006, and every four weeks in the years 2007-2010.

Two soil (S) samples were collected for analyses (S1 – from station SP1; S4 – from station SP4), as well as two samples of pine (P) wood and two samples of oak (O) wood of 150 × 10 × 10 (L × T × R) mm. Samples for microbiological analyses came from outer (o) (0-1 mm) and inner (i) (approx. 5 mm) layers of wood samples excavated from the site. The following samples were collected from wood: Po1 – the outer layer of pine wood from station SP1; Po4 – the outer layer of pine wood from station SP4; Pi1 – the inner layer of pine wood from station SP1; Pi4 – the inner layer of pine wood from station SP4; Oo1 – the outer layer of oak wood from station SP1; Oo4 – the outer layer of oak wood from station SP4; Oi1 – the inner layer of oak wood from station SP1; Oi4 – the inner layer of oak wood from station SP4.

1.2. Quantitative analyses of bacteria

Quantitative analyses of bacteria, performed using pour plate method by surface inoculation of diluted suspensions of soil and wood samples, were conducted on the following microbiological media: nutrient agar and Columbia agar to determine total numbers of bacteria, and Endo agar to determine counts of bacteria from the family Enterobacteriaceae. Analyses were also performed using Petrifilms™ by 3M Poland. To determine the total numbers of bacteria, the Petrifilm Aerobic Count Plate (AB) test was applied, while to determine counts of bacterial microorganisms from the family Enterobacteriaceae – a Petrifilm Enterobacteriaceae Plate (EB) was used. The amount of 1 ml diluted bacterial suspensions from analysed samples of contemporary pine and oak wood, and adjacent soil, were transferred onto the surface of Petrifilms. Inoculated tests were incubated for 24 h at 30°C. Results recorded using both methods (pour plate method and using Petrifilms) were listed and compared.

The Most Probable Number (MPN) of cellulolytic bacteria (aerobic bacteria in the Dubos medium and anaerobic bacteria at the Weimer-Zeikus medium) and bacterial sulfate-reducing microorganisms (at the Wilson-Blair medium) were determined using the tube method.

1.3. Bacteria identification

Qualitative determinations of bacteria were conducted based on the analyses of their cultural, morphological, physiological, and biochemical characteristics. The shape and arrangement of bacterial cells, the results of Gram staining, and the presence of endospores were assessed under a Nikon E600 microscope, coupled with a camera. Biochemical profiles of bacteria were identified using respective tests (applying commonly used liquid and solid media), consisting in the determination of the following capacities: starch hydrolysis, growth at 6.5% NaCl concentration, acetoin formation (the MR-VP test), the formation of acid from arabinose, indole from tryptophane, synthesis of catalase and oxidase, fermentation of lactose, hydrolytic degradation of urea, production of hydrogen sulphide, and decarboxylation of lysine. For this purpose, the following media were used: McConkey agar with methyl violet by GrasoBiotech, Christensen, *Salmonella/Shigella* by LAB AGAR™, starch medium, agar, 6.5% NaCl,

media for MR-VP tests, for sugar fermentation analyses, for detection of decarboxylation capacity of lysine by microorganisms, as well as tryptophane water. In order to classify bacteria from the genus *Clostridium* to species, the API 20A test by bioMerieux was used (Gajewska, Jacak and Babiński 2009b, 331-342). Biochemical profiles were interpreted based on the latest edition of Bergey's Manual of Systematic Bacteriology (Brenner, Krieg and Staley 2005; De Vos et al. 2009).

1.4. Statistical analysis

Counts were transformed into $\log_{10}$ and reported as $\log_{10}$ cfu/g of d.m. Data were analysed by one-way analysis of the variance (ANOVA), with three replicates. The mean values were compared using the Tukey test. Differences were considered statistically significant at $p \leq 0.05$. The correlation analysis was carried out to measure the level of the relationship between variables. The Pearson coefficient of correlation (r) was calculated and considered statistically significant at $p \leq 0.05$. All statistical analyses were done with Statistica 12.5. Software (StatSoft).

2. Results and discussion

The redox potential of soil, in which the tested samples of contemporary wood were deposited, is presented in Table 1. At both measuring stations, the mean redox potential was similar, and fell within the range from -190 to -160 mV. Only in the first year of measurements (2004), lower values of E_H were recorded at station SP1, while in the last year of the study (2010), higher E_H values were observed at station SP4. Apart from single cases (SP1 in 2008 and SP4 in 2006 and 2010), the highest values of soil redox potential did not exceed -100 mV. The reason for the observed differences, in the values of the first and last measurement year, is difficult to state. It cannot be excluded that variable intensity of rainfall in Biskupin influenced the soil redox potential; however, it is only a matter of supposition.

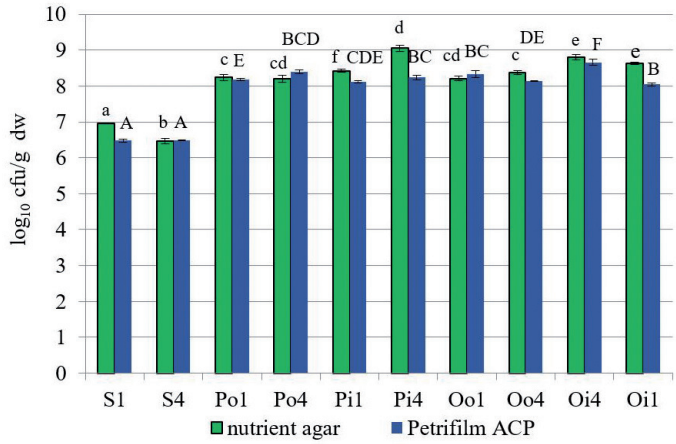
Based on the measurements, it was shown that soil, in which samples of contemporary pine and oak wood were deposited, is characterised by a low redox potential. Patrick and Mahapatra (Patrick and Mahapatra 1968, 323-359) defined the environment, with a redox potential between -300 and -100 mV, as highly reduced. Conditions found in such an environment are close to anaerobic and biological degradation of organic materials, and is slow. In the course of the experiment, wood samples were always located below the ground water level.

Results of determinations of the total count of mesophilic heterotrophic bacteria cultured on nutrient agar (pour plate method), and on Petrifilm™ (Petrifilm Aerobic Count Plate), are presented in Fig. 1. Results for the pour plate method and

Table 1. Soil redox potential (E_H) at SP1 and SP4 measuring stations at the Biskupin archaeological site in the years 2004-2010

Year	E_H (mV)					
	SP1			SP4		
	min	mean	max	min	mean	max
2004	-370	-280	-130	-	-	-
2005	-220	-180	-100	-240*	-190*	-160*
2006	-210	-160	-100	-230	-180	-80
2007	-200	-160	-110	-190	-170	-100
2008	-200	-170	-40	-190	-160	-130
2009	-210	-180	-150	-190	-170	-130
2010	-200	-180	-150	-150	-110	-90

*Measurements were started from April 2005



Samples: S1 – soil from station SP1; S4 – soil from station SP4; Po1 – the outer layer of pine wood from station SP1; Po4 – the outer layer of pine wood from station SP4; Pi1 – the inner layer of pine wood from station SP1; Pi4 – the inner layer of pine wood from station SP4; Oo1 – the outer layer of oak wood from station SP1; Oo4 – the outer layer of oak wood from station SP4; Oi1 – the inner layer of oak wood from station SP1; Oi4 – the inner layer of oak wood from station SP4.

Figure 1. The total number of the mesophilic bacteria in examined soil and wood samples, grown on a nutrient agar medium and Petrifilm Aerobic Count Plate (ACP). Different letters within the same column colour indicate statistically significant differences between samples (p > 0.05)

Petrifilms™ were very congruent. The correlation coefficient for the two data sets was r = 0.91. The consistency of these results suggests that Petrifilms™ may be used, alternately, to the classic pour plate method.

In soil surrounding samples of contemporary pine and oak wood, deposited at both measuring stations (SP1 and SP4), counts of facultative anaerobic bacteria were lower than those in the examined wood. In all analysed layers of wood tissue (outer and inner layers of buried samples), the count of bacterial microorganisms was from approx. 10× to 100× higher than in peat soil. This means that conditions found in wood were more advantageous for the colonisation of bacterial microflora, in comparison to the periodically changing soil parameters.

Figure 2 shows counts of bacteria from the family Enterobacteriaceae cultured on the *Endo* medium and on EB Petrifilms™. It gives only the results for four samples. No bacteria from that taxon were detected in the other cases. Results obtained, using both methods, were very consistent. The correlation coefficient was r = 0.98. It means that Petrifilms™ used in this study were appropriate as an alternative for conventional bacteriological media. Bacterial counts in soils from both measuring stations were approx. 100× lower than in the outer layer of pine wood.

Results of quantitative analyses indicate that soil is the optimal environment for the development of cellulolytic bacteria, while in the inner layers of buried wood, samples were less conductive (Fig. 3). The MPN values for cellulolytic bacteria, living in soil, were the highest or close to the values recorded for surface wood layers. Determinations of all tested samples showed much higher counts of anaerobic bacteria than aerobic bacteria (10× - 10000×), which is connected with anoxia conditions found in wood and surrounding soil.

Table 2 presents the results of qualitative analyses for bacteria colonising contemporary pine and oak wood, during the 8-year deposition at the archaeological site in Biskupin. Testing results suggest that bacteria colonising contemporary wood come from the surrounding soil. All taxa detected in both inner and surface layers of the wood samples, buried at the two measuring stations, were also found in soil.

The penetration rate inside wood samples, was very high in the case of most bacteria, except for the family Enterobacteriaceae

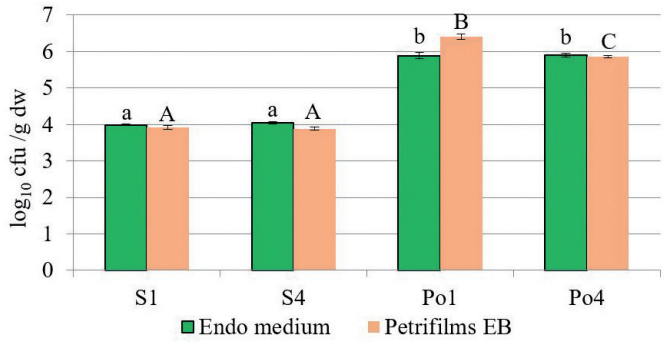


Figure 2. The total number of bacteria from the family Enterobacteriaceae in examined soil and wood samples, cultured on Endo medium and Petrifilms EB. Different letters within the same column colour indicate statistically significant differences between samples (p > 0.05). Samples: see explanations to Fig. 1.

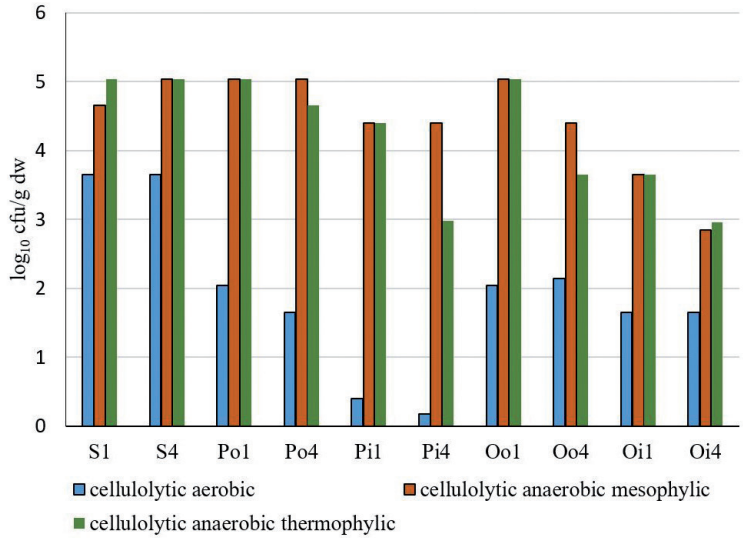


Figure 3. The Most Probable Number (MPN) of cellulolytic aerobic and anaerobic bacteria (mesophilic and thermophilic) in examined soil and wood samples. Samples: see explanations to Fig. 1.

(including also *S. liquefaciens* and *P. cacticida*), whose representatives colonised only the surface layer of pine wood from both measuring stations, not reaching the inside of samples. No expansion of bacteria *C. xerosis* outside the soil medium was observed (they were not detected in any of the tested wood samples). The other species were isolated from all layers of pine and oak samples from both measuring stations.

Our results have important methodological implications, as they provide evidence that Petrifilm™ plates can be utilised instead of standard pour plates for wood and soil samples, without loss of the credibility of our determinations. We observed high correlation between colony-forming unit numbers, obtained on the nutrient, agar, as well as using Petrifilms. Our research is consistent with previous reports. The Petrifilm™ method provides satisfactory results in the area of food products' control (Sokolov, Kashintsev and Sokolov 2011, 34-38; Park and Kim 2013, 269-272). High correlation

Table 2. Bacteria isolated from soil and contemporary wood, after eight years of sample deposition in soil, at the archaeological site in Biskupin

Sample	Bacteria
S1	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, Virgibacillus panthothenicus, Clostridium beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)*, Corynebacterium xerosis, Serratia liquefaciens, Pectobacterium cacticida,* bacteria from the family Enterobacteriaceae
S4	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)*, C. xerosis, S. liquefaciens, P. cacticida,* bacteria from the family Enterobacteriaceae
Po1	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)*, S. liquefaciens, P. cacticida,* bacteria from the family Enterobacteriaceae
Po4	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)*, S. liquefaciens, P. cacticida,* bacteria from the family Enterobacteriaceae
Pi1	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)
Pi4	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)
Oo1	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)
Oo4	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl.beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)
Oi1	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)
Oi4	*Pseudomonas* spp., *Aeromonas* sp., *A. veroni, V. panthothenicus, Cl. beierinckii/butyricum, Cl. perfringens, cellulolytic bacteria* (*Sporocytophaga* sp., *Clostridium* sp., *Bacillus* sp., *Cellulomonas* sp.)

Samples: see explanations to Fig. 1.

coefficients were observed for standard plate count techniques and Petrifilms™ for counting mesophilic aerobic bacteria in raw milk samples (Rosmini et al. 2004, 39-44). The use of Petrifilms™ on different than food matrices, were reported as well. Comparison of conventional plating methods and Petrifilms™ for the recovery of aerobic bacteria and mould from hatchery fluff, was performed with satisfactory results (Warren, Weber and Crespo 2016, 48-53). All of those studies offer evidence of equivalence between the classic pour plates and Petrifilm™ plates. Such consistency in results, speaks in favour of the Petrifilm™ system, because of its economic advantages over the standard pour plate method. The main advantage of Petrifilms™ is that they are sample-ready, and thus, do not require time-consuming preliminary media preparation. This reduces the cost of experiments, without sacrificing the accuracy of results.

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