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Julitta GAJEWSKA[1], Piotr JACAK[1]
and Leszek BABIŃSKI[2]

INFLUENCE OF ANOXIC CONDITION ON THE COMPOSITION OF MICROORGANISMS COLONIZED A CONTEMPORARY WOOD SAMPLES IN ARCHAEOLOGICAL SITE IN BISKUPIN

WPŁYW WARUNKÓW BEZTLENOWYCH NA SKŁAD MIKROORGANIZMÓW ZASIEDLAJĄCYCH WSPÓŁCZESNE DREWNO NA STANOWISKU ARCHEOLOGICZNYM W BISKUPINIE

Abstract: From 2003 year a research project was initiated in Biskupin with monitoring of physical, chemical and microbiological parameters of deposed archaeological wood in wet peat soil on sp1 and sp4 sites. In this paper colonization by microorganisms of a temporary oak wood (*Quercus* sp.) and pine wood (*Pinus sylvestris* L.) deposed during four years in the similar anoxic conditions in flooded sites were observed.

The results of the performed investigations showed on an interesting microbial community as colonizators of wood samples, outside and inside them, including a lignocellulotic bacteria and microscopic fungi, responsible for wood decomposition, and pathogenic, toxinogenic microorganisms, eg: *Pseudomonas aeruginosa*, *Aeromonas hydrophila / caviae*, *Clostridium perfringens* bacteria and *Aspergillus fumigatus*, *Penicillium* spp., and *Candida* spp. fungi.

Keywords: bacteria, microscopic fungi, wood colonization, physical and chemical parameters, Biskupin

Since many years ago on archaeological positions of defensive settlement from VIII century BC of Lusatian culture in Biskupin, physical, chemical and microbiological investigations were provided, on the basis which conservatory works of archaeological wood oak and pine were done. In the aim of recognition the threats, the archeological wood on what be subject in Biskupin, the identification of pine and oak wood

[1] Department of Microbial Biology, Faculty of Agriculture and Biology, University of Life Sciences – SGGW, ul. Nowoursynowska 159, 02–776 Warszawa, Poland, phone: +48 22 593 26 35, email: gajewska3@wp.pl

[2] Department of Conservation, Archaeological Museum in Biskupin, 88–410 Gąsawa, Poland, phone: +48 52 302 50 37, email: leszek.babinski@wp.pl

colonizing bacteria and microscopic fungi in anoxic conditions was conducted [1–4]. This work was widened on oak and pine contemporary wood, kept by 2 years in peat soil on sp1 and sp4 sites, in similar conditions like archaeological wood is still resting [1–3, 5, 6].

The objective of this work was isolation and identification of microorganisms colonized a contemporary oak wood (*Quercus* sp.) and pine wood (*Pinus sylvestris* L.) and estimate the degree of wood colonization, after four year period of deposition in similar conditions to those of the archeological wood: in wet peat soil and in anaerobic condition in sp1 and sp4 measuring stations in Biskupin.

Materials and methods

Investigation was conducted on a contemporary oak and pine wood, which saved in wet peat in anoxic conditions in period 4 years (September 2003–September 2007). The experimental material, cut out from the long of trunks of approximately 240 mm diameter, derived from the outer part of the heartwood zone of oak and sapwood of pine. Dimensions of wood samples were as follow: 100 (L) × 10 (T) × 10 (R) mm. These samples were buried in the archeological site sp1 (No. 72) and sp4 (No. 6) in Biskupin in the layer of peat soil at the depth of 100 cm. At this level archaeological wooden construction from the Lusatian settlement are still deposited. During the experimental period the following physical and chemical measurements were done: level of ground water at sp1 and level of water in the trench at sp4, water reaction (pH) and water conductivity, soil temperature and redox potential, according to the methods described by Babinski et al [7]. After 4 years wooden materials to microbiological examinations were taken, for comparison of microbial colonization on the surface of wood (1–1.5 mm) and internal tissue (3–5.5 mm) and in soil arrounded to wood. For isolation and identification of bacteria, actinomycetes and microscopic fungi, the following media were used: nutrient agar with/without of 10 % mutton blood medium, Bunt and Roviry medium supplemented with 1 % starch and nystatine, King's B medium, Wilson–Blair medium for *Clostridium perfringens* reduced sulphite, Dubos medium with stripes of filter paper as source of carbon, Weimer and Zeikus medium with stripes of filter paper, Copier and Barjac medium for nitrifying bacteria, medium for denitrifying bacteria with nitrate, medium for amonifying bacteria with Winogradski salts and Doebereiner's medium for nitrogen fixing bacteria, and for microscopic fungi Martin's and Sabourad's or Chapek–Dox medium. Cultures were cultivated in 28 ^{o}C, and 37 ^{o}C for mesophilic strains and 60 ^{o}C for thermophilic strains. Anaerobic cultures were placed in an anaerostat (in presence of hydrogen and carbon dioxide, palladium catalyst and methylene blue as indicator of anaerobic condition) or anaerobic station (Juan). For systematic determination of bacteria Api tests bioMeriux with computer analyses programe were used. Microscopic observations and photos documentation by Nicon's E 600 fluorescent microscope with camera and computer were done. Systematic determination of bacteria according to Bergey's Manual of Systematic Bacteriology [8] and to identify fungi – systematics according to Barnett [9] and Fassiatova [10] were used.

Results and discussion

The measurements of physical and chemical properties of water and soil were performed on sp1 and sp4 stations, where investigated samples of pine and oak wood were deposed during 4 years. The results of these observations were presented in Table 1.

Table 1

Some selected physical and chemical properties of water and soil from Biskupin

Property	Value	Place of measurement (station)	
		sp1	sp4
Water level		above samples	above samples
Water reaction (pH)	minimum	6.6	6.9
	maximum	7.6	8.8
	mean	7.0	7.8
Water electrical conductivity [mS · cm^{-1}]	minimum	0.46	0.40
	maximum	2.10	0.96
	mean	1.45	0.59
Soil temperature [^{o}C]	minimum	2.1	not measured
	maximum	19.0	
	mean	10.4	
Soil redox potential [mV]	minimum	–410	–240
	maximum	–100	–80
	mean	–240	–180

During resting on archaeological site (August 2003–August 2007), the samples of pine and oak wood always were below of groundwater level (sp1 station) and below a water level in a trench (SP4 station). The average value of water reaction (pH) was neutral, and pH value of trench water was significantly alkalic (even to 8.8). The higher conductivity of groundwater from sp1 station were observed, where average salinity was above twice higher than for a water from trench at sp4 station. The measurements of temperature only in soil of sp1 station were done. The temperature of peat on 100 cm of depth, in the wood samples left in soil, oscillated from 2.1 to 19.0 ^{o}C. The average value of redox potential was –240 mV on sp1 station and –180 mV in a bed level of a wet trench. Results indicated on the strong reducing conditions present in two wood deposited sites. The changes of water reaction (pH) and conductivity, and soil redox potential followed serial fluctuations according to changes of groundwater level and depending on the change in season of the year. The detailed results of chosen monitoring environmental parameters at wet archaeological sites in Biskupin were described by Babinski et al [7].

The results of microbiological determinations on the settlement of a contemporary oak (*Quercus* sp.) and pine wood (*Pinus sylvestris* L.) showed the high diversity of

physiological groups of soil microorganisms in strictly anaerobic conditions. Results of these investigations were presented in Table 2 (bacteria) and Table 3 (microscopic fungi). One could observe in both samples of wood a temporary and strictly anaerobic bacteria and the microscopic fungi, which could grown in vitro in aero- and anaerobic conditions, when redox potential was very low periodically from –410 mV (minimal value), to –100 mV (maximal value).

Table 2

Colonization of a contemporary oak wood (*Quercus* sp.) and pine wood (*Pinus sylvestris* L.) after 4 years stored in wet peat soil by relative and strictly anaerobic bacteria from soil surrounded wood

Samples	Bacteria
sp1 pine outside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens*, *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Sporocytophaga* sp.
sp1 pine inside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Clostridium thermocellum*, *Sporocytophaga* sp., *Streptomyces* sp.
sp1 oak outside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens Micrococcus* sp., *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Sporocytophaga* sp.
sp1 oak inside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium butyricum/bijerienickii*, *Clostridium thermocellum*, *Sporocytophaga* sp.
sp4 pine outside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens*, *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Clostridium clostridiiforme*, *Sporocytophaga* sp., *Streptomyces* sp.
sp4 pine inside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens*, *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Sporocytophaga* sp.
sp4 oak outside	*Bacillus polymyxa*, *Pseudomonas fluorescens*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens*, *Micrococcus* sp., *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Sporocytophaga* sp., *Streptomyces* sp.
sp4 oak inside	*Bacillus polymyxa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens*, *Clostridium thermocellum*, *Sporocytophaga* sp., *Streptomyces* sp.
sp1 soil	*Bacillus polymyxa*, *Bacillus mycoides*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Pantoea* spp., *Clostridium perfringens*, *Micrococcus* sp., *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Clostridium clostridiiforme*, *Sporocytophaga* sp., *Streptomyces* sp.
sp4 soil	*Bacillus polymyxa*, *Bacillus mycoides*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Enterobacter cloace*, *Clostridium perfringens*, *Clostridium butyricum/bejerinckii*, *Clostridium thermocellum*, *Clostridium clostridiiforme*, *Sporocytophaga* sp., *Streptomyces* sp.

In the anaerobic conditions most of isolated and identified microorganisms were able to colonize a surface of oak and pine wood and they could penetrate to inside of wood (on 3–5.5 mm of depth).

Table 3

Colonization of a contemporary oak wood (*Quercus* sp.) and pine wood (*Pinus silvestris* L.) after 4 years stored in wet peat soil by yeasts and hyphal fungi from soil surrounded wood

Samples	Fungi grown in aerobic conditions	Fungi grown in anaerobic conditions
sp1 pine outside	*Aspergillus fumigatus*, *Penicillium* spp., *Candida* spp.	*Penicillium* spp., *Candida* spp.
sp1 pine inside	*Fusarium oxysporum*, *Aspergillus fumigatus*, *Rhizopus oryzae* *Penicillium* spp., *Candida* spp.	*Penicillium roquefortii*, *Penicillium* spp., *Candida* spp.
sp1 oak outside	*Penicillium citrinum*, *Candida* spp.	*Mucor piriformis*, *Penicillium* spp., *Candida* spp.
sp1 oak inside	*Mucor piriformis*, *Aspergillus fumigatus*, *Penicillium roquefortii*	*Mucor piriformis*, *Penicillium roquefortii*, *Penicillum* spp.
sp4 pine outside	*Aspergillus fumigatus*, *Candida* spp.	*Mucor piriformis*, *Fusarium oxysporum*, *Aspergillus fumigatus*, *Penicillium* spp., *Candida* spp.
sp4 pine outside	*Aspergillus fumigatus*, *Penicillium citrinum*	*Mucor piriformis*
sp4 oak outside	*Aspergillus fumigatus*, *Penicillium roquefortii*, *Candida* spp.	*Penicillium terrestre*, *Candida* spp.
sp4 oak inside	*Penicillium* spp.	*Penicillium* spp.
sp1 soil	*Fusarium oxysporum*, *Penicillium* spp., *Candida* spp.	*Fusarium oxysporum*, *Penicillium roquefortii* *Penicillium* spp., *Candida* spp.,
sp4 soil	*Phialophora bubaki*, *Aspergillus fumigatus*, *Candida* spp.	*Fusarium oxysporum*, *Candida* spp.

It was observed, that inside oak and pine wood saprophitic producing slime bacteria *Pseudomonas fluorescens* (API 90.7 %), cellulolytic bacteria and producing slime, too, a temperate anaerobic *Bacillus polymyxa*, *Sporocytophaga* sp. as well as strictly anaerobic meso – and thermophilic *Clostridium* spp. strains were present. The pathogenic bacteria were detected also, like *Aeromonas hydrophila*/*caviae* (Api 87.6 %), *Pantoea agglomerans* (Api 78.8 %), *Pseudomonas aeruginosa* (Api 99.9 %), *Clostridium perfringens* reducing sulphite as well as yeast from *Candida* sp. and *Aspergillus fumigatus*, *Mucor* sp. and *Penicillium* spp. microscopic fungi. *Micrococcus luteus* bacteria in some samples were also detected. In soil samples were present intestinal bacteria from *Enterobacteriaceae* family (eg *Enterobacter cloacae* – Api 94.3 %), which could not penetrate inside wood.

We could isolate pathogenic microscopic fungi colonizing pine and oak wood samples, eg toxinogenic and cancerogenic *Aspergillus fumigatus* strain or yeasts *Candida* sp. dangerous for people, or *Fusarium oxysporium* as plants pathogen; they can grow under an anaerobic condition. These pathogenic bacteria and fungi present in

soil surrounded wooden trenches probably can get throughout from water of Biskupin Lake.

Anoxic conditions are good for growth not only strictly anaerobes, but for a facultative anaerobic bacteria. Due to the deficiency of oxygen in flooded soils, microorganisms inhabiting flooded soils must be able to survive with little to no oxygen. Under anoxic conditions facultative microbes can use alternative (not oxygen) electron acceptors such as nitrate, ferric iron Fe(III), manganese(IV), oxide, sulfate, and carbon dioxide to produce energy and build their biomass. In 2009 Sikora and Blaszczyk [11] showed the important ecological role of Fe(III) reducing bacteria from Bacteria and Archaea domens, their classification and a biotechnological majority. According to Bagramayan et al [12], redox potential is a determinant in the *E. coli* anaerobic fermentative growth and survival. Decrease of redox potential (Eh) down to –550– –600 mV in the *E. coli* culture was observed during growth in either anaerobic or aerobic conditions.

In present experiments oak and pine wood or soil bacterial isolates classified from different physiological groups with following processes, like: cellulolise, proteolise, amonification, nitrification, denitrification, fermentation of different substrates and their assimilation, possible to recognize on basis their biochemical profiles (Api tests).

Wood colonization processes of living bacterial and fungal microorganisms, with antagonistic and symbiotic or syntrophic relations between them in strictly anoxic conditions can be very interesting studies on wood „microbial community". Anaerobic microbial processes including denitrification, methanogenenesis and methanotrophy are responsible for releasing greenhouse gase (N_2O, CH_4, CO_2) into atmosphere, and environmental factors, such as redox potential (Ex), pH, acidity, alkalinity, and salinity, are continuously changing [13]. Aditionallly, in aerobic conditions, gleying process in waterlogged soils occurred, by accumulation of Fe(II) due to reduction of ferric iron into ferrous iron [14]. Fermentative bacteria F(III) – reducing can be mainly isolated from gleyed soils. The black colour of soil is frequently observed in flooded soil. This may result from the formation of iron sulfide (FeS) and pyrite (FeS_2). The potential redox for SO_3^{-2}/S^{-2} is about: –116 mV. Disappearance of sulphate can be under Eh: –150 mV [15]. In authors own experiments, under anoxic conditions, woodden samples saved in wet soil during 4 years and soil samples, surrounded wood, were black. A strictly anaerobic *Clostridium perfringens* bacteria isolated from soil and from wood could reduced sulphite to H_2S, and caused their black colour.

A low redox potential is important for denitrification. In this process, nitrate is reduced to nitric oxide, then nitrous oxide fully reduced to dinitrogen. Microbes responsible include both organotrophs and litotrophs, and this process occurs primarily by facultative anaerobes (eg *Pseudomonas* sp., *E. coli* [2] and strictly anaerobes (eg *Clostridium perfringens* [16]. Many fermentative bacteria can reduce nitrite to ammonium, then pH value of soil can increase rapidly even to pH 10 [2].

Physiological biodiversity of microorganism under anaerobic conditions is connected with decomposition of lignocellulolytic wooden materials (eg *Clostridium* spp., *Sporocytophaga* sp., *Bacillus polymyxa).* Glucose, as end product of cellulose or hemicellulose fermentation, can be very good substrate for next colonizators of pine and oak wood saved in soil [17, 18]. In 2002 Wazny [19] on basis own and numerous

authors papers described decomposition of wood by aerobic and anaerobic bacteria isolated from archaeological wooden samples in contact with water, like *Bacillus* spp., *Cellulomonas* spp., *Cellvibrio* spp., *Corynebacterium* spp., *Ervinia carotovora*, *Clostridium* spp., *Pseudomonas* spp., and *Streptomyces* spp.

In present work, among different wood colonizators, a nitrifying aerobic *Nitrosomonas* sp. bacteria were isolated. According to Strous et al [20], aerobic nitrifying bacteria such *Nitrosomonas* spp. might be involved in Anammox process – as anaerobic ammonium oxidation. This anaerobic ammonium oxidation, recently was described as a new process in which ammonium was converted to dinitrogen gas under anaerobic condition with nitrite as the electron acceptor, described by Vandegraaf et al in 1995 [4].

According to Badura [21] one still can not understand entirely the role of microorganisms in soil mineralization processes; certainly is difficult to recognize a real total number of living cells: fast living as eutrophic cells and slowly growing – as oligotrophic bacteria. A qualitative composition of microorganisms is not completely recognized, because during influence of manyparameters, like physical and chemical properties of soil and soil plants on microorganisms growth, which can significantly change kinds of growing microorganisms.

Most of isolated soil and wodden pathogenic or nonpathogenic microbial strains were responsible for the decomposition of a temporary and archaeological wood, deposed in wet soil. Actually we try to find the best method of conservation woodden trenches by using antibacterial and antifungal biocides, in hope, that it will be possible to demonstrate archaeological wood samples after hygienisation in Biskupin Museum, to protection of the archaeological wood monuments for future.

Conclusions

1. The contemporary oak and pine wood, kept by 4 years under anoxic conditions, during very low redox potential parameters, were colonized by different physiological groups of bacteria; oxygenic (Ammonox), a facultative and strictly anaerobic, together with a facultative and strictly anaerobic microscopic fungi.

2. The largest threat for wood can make the cellulolytic or lignocellulytic micro-organisms like: *Bacillus polymyxa, Sporocytophaga* sp, meso- and thermophilic *Clostridium* spp. bacteria and *Streptomyces* spp. actinomycetes, as well as *Penicillium* sp. and *Aspergillus* sp. microscopic fungi.

3. The present in soil bacteria from family *Enterobacteriaceae* did not colonize samples of the oak and pine wood, kept in anaerobic conditions.

4. It was observed, that *Fusarium* sp. fungi which were present in soil adhered to wood, were absent inside oak and pine wood, probably in consequence of antagonistic activity of the dominant *Pseudomonas fluorescens* strains.

5. We suppose, that maintenance of a contemporary wood and archeological wood, too, for longer time in anoxic conditions (with high concentration of sulfide and very low soil redox potential) in wet archeological site in Biskupin will have positive role in limitation of decomposition saved wood in peat and its biological corrosion in future.

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Samodzielny Zakład Biologii Mikroorganizmów, Wydział Rolnictwa i Biologii
Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

Abstrakt: Od wielu lat na stanowiskach archeologicznych obronnego osiedla kultury łużyckiej z VIII w. p.n.e. w Biskupinie prowadzone są badania fizykochemiczne, mikrobiologiczne i konserwatorskie zabytkowego drewna archeologicznego. W celu rozpoznania zagrożeń mikrobiologicznych, na jakie wciąż narażone jest to zabytkowe drewno, badania mikrobiologiczne rozszerzono o współczesne drewno dębu (*Quercus* sp.) i sosny (*Pinus sylvestris* L.), przechowywane przez 4 lata w warunkach anoksji w glebie torfowej na stanowiskach sp1 i sp4.

Stwierdzono różnorodność glebowych względnie i bezwzględnie beztlenowych kolonizatorów, np. celulolitycznych bakterii i grzybów mikroskopowych, zdolnych do rozkładu drewna, a także patogenów, np. *Pseudomonas aeruginosa*, *Aeromonas hydrophila/caviae*, *Clostridium perfringens* oraz grzybów *Aspergillus fumigatus*, *Penicillium* spp. i *Candida* spp.

Słowa kluczowe: bakterie, grzyby mikroskopowe, kolonizacja drewna, parametry fizykochemiczne, Biskupin