Analysis of Fungi in Wood Chip Storage Piles

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There are health and safety risks involved in the production and storage of forest biomass. Fungi that are formed in the stored piles of wood chips pose a high potential risk for human health. Three experimental piles, containing wood chips from three species of trees, were created. They included European beech (*Fagus sylvatica*), common aspen (*Populus tremula*), and European spruce (*Picea abies*). The piles created were in the shape of a pyramid with the base measuring 4 m x 4 m. In each pyramid, 3 points of measurements were established at 0.5 m, 1.0 m, and 1.5 m above the ground. Temperature, relative moisture, and the number of microscopic fungi colonies were monitored at each point of measurement in the period between 13th December 2011 and 6th June 2012. The highest relative moisture content was recorded in the pile with the European spruce. The aim of the experiment was to identify the genus and species of fungi that are formed in the chip piles during long-term storage and which pose a potential risk for human health. In total, 5 species and 8 genera of fungi were identified in the collected samples, whereby there was significant growth only during the first 4 to 6 months of storage.

Keywords: Wood chips; Moisture content; Fungal activity; Storage; Biomass

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INTRODUCTION

As the utilization of biomass for energy purposes becomes more intensive, the importance of its quality parameters increases as well. The quality is in specific cases determined by the method of production and the conditions associated with storage (Cavalli and Grigolato 2010). Health and safety risks are involved in the production and storage of biomass. Fungi that grow on stored wood chips are released into the air during handling (Suchomel and Belanová 2012). This may pose some risk for those working with decomposing botanic material (Pratt and May 1984). The production of spores is immense and reaches up to $1.8x10^{12}$ spores per 1 kg of dry matter of chips (Thörnquist 1983). The activity of fungi is also influenced by the temperature inside chip piles, and there is a significant risk of spontaneous combustion (Jirjis 1995; 2005). When stored between 3 and 6 months, the production of spores can cause health problems, such as respiratory illnesses and allergic reactions (Thörnquist and Lundström 1982; Suchomel *et* *al.* 2009). The development of fungi is also influenced by the rise in temperature and relative moisture content (Wei-hong *et al.* 2005; Highley 1999). These factors also influence the process of degradation of stored wood chips. In the course of the first year of storage, a loss of energy value of biomass as high as 25% to 55% can be caused by increased relative moisture and degradation of stored chips (Thörnquist and Lundström 1982; Huber 2009).

In the piles of chips the increase in exterior and interior temperature is of great importance. The maximum internal temperature in the centre of a pile is commonly reached after 10 to 30 days and can reach up 80 °C, regardless of the external temperature (Kuchtík 1988; Nurmi 1999). These values are influenced by the volume of the chip pile, the terrain upon which it is placed, the outside temperature, and the size of the chip fraction. After 100 to 150 days of storage, the temperature in the piles is much lower than 80 °C (Scholz *et al.* 2005).

The aim of the present study was to analyse the increase in temperature, moisture, and the occurrence of fungi in experimental piles of wood chips produced from three wood species: European beech (*Fagus sylvatica*), common aspen (*Populus tremula*), and European spruce (*Picea abies*). The result is a subsequent overview of the development in the fungi colonies harmful to humans. This study also examines the colonies that destructively influence the quality of wood chips with given development of temperature and moisture. The aim was to identify the fungi genera and species that form in chip piles during long-term storage and which pose a potential risk to human health. The results should provide information on the development of harmful phytopathogens that can negatively affect the health of workers during chip production, but mostly during manipulation and storage.

EXPERIMENTAL

Material and Experimental Piles

The piles of wood chips designed for the experiment were produced from three species of wood: European beech (*Fagus sylvatica*), common aspen (*Populus tremula*) and European spruce (*Picea abies*). Chips from biomass of these species were produced right after the trees were cut down (13th December 2011 winter dormancy). Wood chip piles were located in the area of "Hrabiny" within the University Forest Enterprise near the town Zvolen. Three piles were created, one out of each species in the shape of pyramid with the base of 4 m x 4 m (Fig. 1). Temperature measurement was carried out by means of the electronic probe "SFK" placed at three height levels: 0.5 m, 1.0 m and 1.5 m. Each probe was connected to "Datalogger" serving for storing the data in one-hour intervals. The capacity of the Datalogger is 10,000 data. The experiment was carried out between 13th December 2011 and 6th June 2012 (176 days).

Laboratory Analyses

Samples were taken from the piles at 3-week intervals at three distances from the ground, 0.5 m, 1.0 m, and 1.5 m (Nurmi 1999). Plastic pipes with lids were placed in the designated places (Fig. 1). The samples were also used to determine the moisture content, by drying and reweighing the sample (Jirjis 2005; Afzal *et al.* 2010). The extraction of samples was carried out in 2 to 3 week intervals (total of 18 samples from each pile and each distance from the ground between 13th December 2011 and 30th August 2012). The samples were dried at 105 °C ± 2 °C to a constant weight. After weighting on laboratory scales (accurate to 0.01 g), the values of relative moisture content were calculated. The relative moisture content in individual extraction points was calculated as a percentage value of the weight of water contained in the samples.

Fig. 1. Experimental pile

During the whole experiment, nine samples from each pile at the height levels of 0.5 m, 1.0 m, and 1.5 m, used for the analysis of fungi occurrence, were sampled. The first sample was taken after about 2 months of storing from all 3 points of measurement of each pile, and the last sample was taken 6th June 2012. The samples were stored in airtight sealed plastic bags until performing the analyses. Microbiological identification of fungi was performed by examination (ISO 21527-2) in the accredited laboratory of the Regional Public Health Authority in Poprad. The quantification of the fungi (Colony forming units per gram [cfu.g^{-1}]) was performed in compliance with standard ISO 21527-2. Different species of fungi were identified in compliance with scientific literature.

For qualitative determination of microscopic fungi, culturing on agar medium was used. A homogenised sample of 20 g was added to 180 mL of sterile water containing 0.02% of polyoxyethylene (20) sorbitan monooleate (Tween 80). For more precise release of microorganisms from the material, the sample was shaken on a horizontal shaker for 30 min. For the inoculation of wort agar, the dilutions of 10^{-1} to 10^{-4} in three repetitions were used. The wort agar was composed of 1,000 mL of beer wort, 20 g of agar, and 1,000 mL of distilled water. Agar plates were cultivated at the temperature 25 °C for 5 to 7 days. After obtaining pure microscopic cultures, their identification was performed based on morphological and cultural characteristics according to the guides listed in the literature (Hoog De *et al.* 2000; Klich 2002; Samson *et al.* 2001).

Weather Conditions

Temperature and relative air humidity was recorded during the duration of storage every day in hourly intervals by a meteorological station located directly in the area of storage piles. The air temperature during the 176 days fluctuated in the range of -21.27 °C (3rd February 2012) to 32.87 °C (2nd July 2012). The relative air humidity ranged from 100% (multiple days) to 15.05 % (26th March 2012) and averaged 78.25 % (Fig. 2).

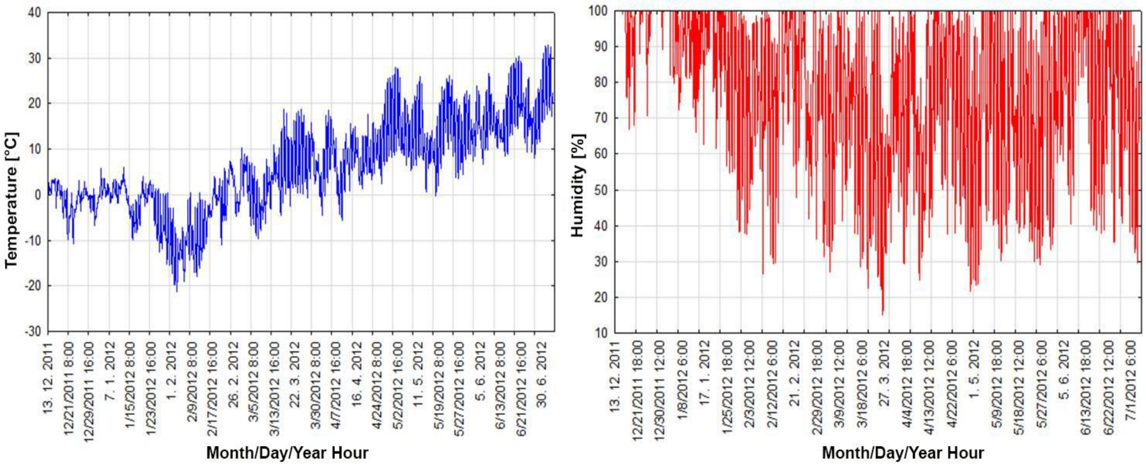

Fig. 2. Ambient temperature and humidity during the storage period

RESULTS AND DISCUSSION

Heat Development in Piles

The temperature range inside the experimental piles is illustrated in Fig. 3. The median values of temperature ranged between 18.5 °C and 26.7 °C depending on the sampling height (0.5 m, 1.0 m, or 1.5 m). In the beech pile the median values of temperature ranged between 20.7 °C and 26.7 °C. In the aspen pile, temperatures ranged between 15.9 °C and 22.8 °C. During the experiment, the temperatures never rose above 50 °C in any of the piles. The lowest value of maximum temperature (33.1 °C) was recorded in the spruce pile at 0.5 m above the base of the pyramid (2nd May 2012). Minimal temperatures were recorded immediately after the beginning of the experiment, specifically in the first week after creating the storage piles. The range of temperatures including the median values is illustrated in Fig. 3.

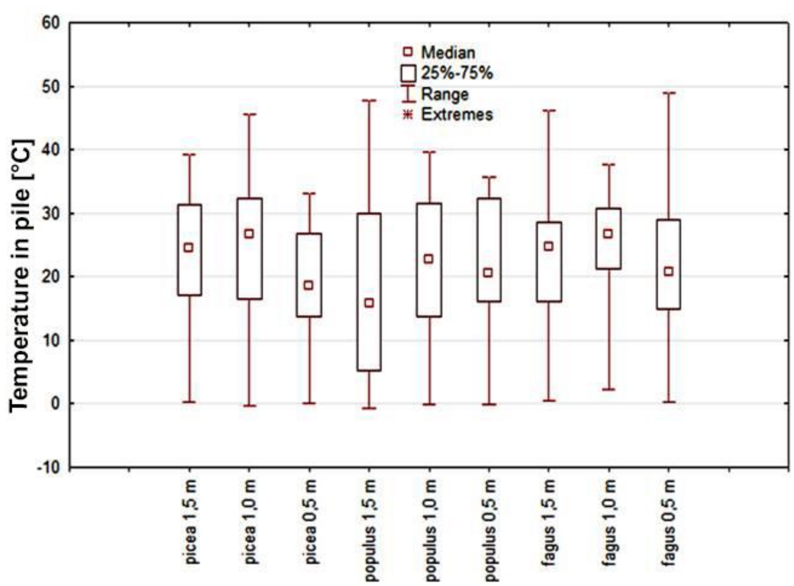

Fig. 3. Range of temperatures in experimental piles

Moisture Content

The relative moisture content in the experimental piles depended on the sampling height (Fig. 4).

Relative moisture content of the spruce chips varied depending on the sampling height at 0.5 m and ranged from 25.13% to 55.45%. At 1.0 m above ground the relative moisture content ranged from 27.6% to 55.45%, and at 1.5 m of the spruce pile the moisture was between 30.1% and 55.45%.

Overall average of relative moisture content in the spruce pile after 9 months of storage reached 41.9%. After 2 months of storage, the average relative moisture content of spruce chip was 50.2%. Compared to the same storage time of willow chip (*Salix viminalis*) it had approximately 2.8% lower value (Jirjis 2005). After 5 months of storage, the level of the average relative moisture content of spruce chip was 45.71%. When compared to the same storage time of white birch (*Betula papyrifera*), it had approximately 8.5% higher value (Afzal *et al.* 2010).

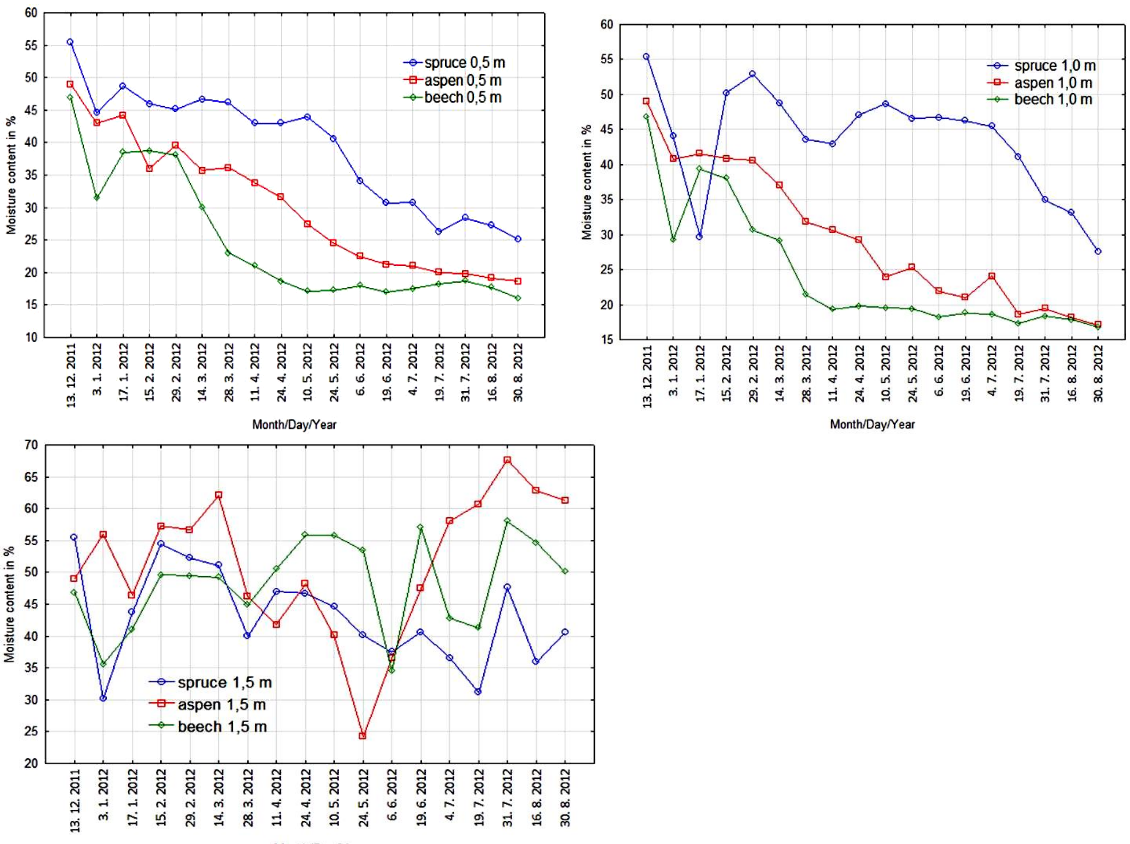

Fig. 4. Moisture content in experimental piles

The relative moisture content of the aspen chips ranged from 18.6% to 49% at 0.5 m above the ground. At 1.0 m it ranged from 17.11% to 49%, and at 1.5 m it was between 24.2% and 67.7%. On average, after 9 months of storage, the relative moisture content reached 37% (after 2 months 44.07%/ comparison with willow chip -8.93%; after 5 months 30.51%/ comparison with white birch chip -6.69%) (Jirjis 2005, Afzal *et al.* 2010). Relative moisture content of the beech pile of chips at 0.5 m above ground ranged between 46.9% and 16%. At 1.0 m above the ground it was 46.9% to 16.8%, and at 1.5 m high the moisture content ranged from 58% to 34.5%. On average the relative moisture content after 9 months of storage was 32.5% (after 2 months 42.11%/ comparison with willow chip -10.89%; after 5 months 30.82%/ comparison with white birch chip -6.38%) (Jirjis 2005; Afzal *et al.* 2010). It is apparent from the recorded data that the highest moisture content was in the pile of chips made of spruce (*Picea abies*).

Fungal Activity

Overall 5 species and 8 genera were identified in the samples taken from the piles. Tables 1 to 3 show the occurrence of fungi in the samples taken from the different sampling heights of the piles. The dates of extractions were as follows 1^{st} – 15^{th} February 2012; 2^{nd} – 29^{th} February 2012; 3^{rd} – 14^{th} March 2012; 4^{th} – 28^{th} March 2012; 5^{th} – 11^{th} April 2012; 6^{th} – 24^{th} April 2012; 7^{th} – 10^{th} May 2012; 8^{th} – 24^{th} May 2012; 9^{th} – 6^{th} June 2012. The content of fungi is shown in cfu.g^{-1}. Statistical analyses did not show any notable correlation between the occurrence of fungi and the height above ground or species of wood. It is known that the occurrence of fungi depends on relative moisture as well as the temperature inside the pile of chips (Jirjis 1995). The temperature and relative moisture content inside the pile reacts to changing outside environment with some delay. The data confirm that the highest occurrence of fungi was present when the temperature inside the pile was around 20 °C and the relative moisture content was above 45%. When the temperature inside the pile and the relative moisture content were low, no occurrence of fungi was documented (Fig. 5).

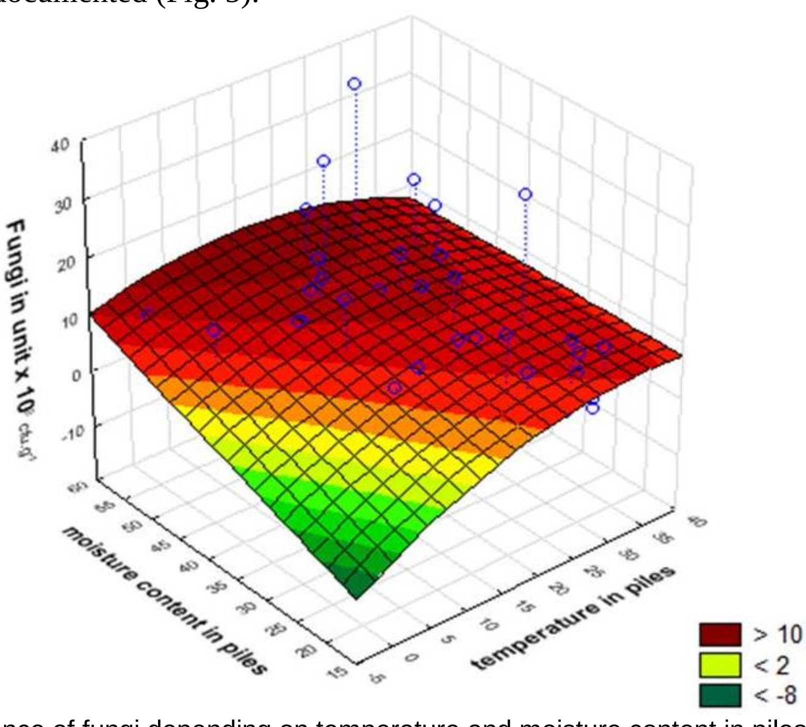

Fig. 5. Occurrence of fungi depending on temperature and moisture content in piles

The most prevalent species of fungi in the spruce experimental piles were mainly *Mucor globosus*, *Penicillium* sp., *Trichoderma koningii*, and *Aspergillus fumigatus*, regardless to the species of wood the chips were made of. Similar species of fungi were also identified in the two month long storage of the chips from osier (*Salix viminalis*) (Jirjis 2005). In the spruce pile, the prevalent fungus was *Trichoderma koningii* in the middle of the pile and it reached a maximum after 4 months of storage ($21x10^3$ cfu.g^{-1}) in the sample taken from 1.0 m above the ground.

Table 1. Occurrence of Fungi in Spruce Experimental Pile

Picea abies **1.5 m** **Sample Nr./ species of fungi**	Occurrence of fungi x 10^3 / $cfu.g^{-1}$								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mucor spinosus	8	9		3					
Penicillium sp.					2	1	1	1	1
Scopulariopsis sp.									1
Trichoderma koningii						1	3	3	1
Aspergillus fumigatus							4	6	
Fusarium sp.									2
Picea abies **1.0 m**									
Aspergillus niger							1		
Mucor globosus	3								
Penicillium sp.			1	4	8	4	1	2	
Trichoderma koningii	6	11	21	5	6	2	1	9	2
Acremonium sp.						1	1		
Fusarium sp.				1	2		3		3
Picea abies **0.5 m**									
Aspergillus niger			1		1				
Mucor globosus	13	1	1	6	3			2	3
Penicillium sp.			1			1	3		
Trichoderma koningii							1	1	
Fusarium sp.				1					
Cladosporium sp.					1				

Table 2. Occurrence of Fungi in Beech Experimental Pile

Fagus sylvatica **1.5 m** **Sample Nr./ species of fungi**	Occurrence of fungi x 10^3 / $cfu.g^{-1}$								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Aspergillus fumigatus							1		11
Mucor globosus	2				1			3	1
Cladosporium sp.					3	1			
Penicillium sp.		1		2		1		2	
Geotrichum sp.			1						
Trichoderma koningii						1	1		
Aspergillus niger								33	
Fusarium sp.					1		1		
Fagus sylvatica **1.0 m**									
Fusarium sp.	1			2	1				
Aspergillus niger			1						
Mucor globosus	1								
Penicillium sp.			1				10		3
Rhizopus sp.									1
Aspergillus fumigatus						1			
Trichoderma koningii						1		1	
Fagus sylvatica **0.5 m**									
Fusarium sp.					1			1	
Acremoniula sp.		1							
Aspergillus niger			6						
Cladosporium sp.		1	1	1			1		
Mucor globosus	3			2	1			2	
Penicillium sp.				2	3	2	2		5
Trichoderma koningii						1			
Aspergillus fumigatus						2		9	

In the beech pile, the most numerous fungi were *Aspergillus niger* in the sample from 0.5 m extracted on 24th May 2012 (33 x 10^3 cfu.g^{-1}) and *Penicillium* sp. in the sample extracted on 10th May 2012 (10x 10^3 cfu.g^{-1}) at 1.0 m above the ground. In the aspen chip, the most numerous fungi were *Aspergillus niger* and *Aspergillus fumigatus* in samples extracted on 2th March 2013 and 11th April 2013 at 1.5 m above the ground (150 x 10^3 cfu.g^{-1}).

In the aspen and beech piles, the most abundant fungi colonies were found at the height level of 1.5 m, whereas in the pile of spruce, it was at the height level of 1.0 m. Regarding the health risks in the spruce piles, there were numerous colonies of *Aspergillus fumigatus*, *Trichoderma koningii*, and genus *Penicillium* sp. In the beech pile, the highest health risk was represented by the species *Aspergillus niger* and *Penicillium* sp. *Aspergillus niger* and *Aspergillus fumigatus* were the species found in the aspen pile.

Table 3. Occurrence of Fungi in Aspen Experimental Pile

***Populus tremula* 1.5 m**	Occurrence of fungi x 10^3 / cfu.g^{-1}								
Sample Nr. /species of fungi	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Aspergillus fumigatus				150		20	30		30
Mucor spinosus	1		1	2	1	1	3		1
Penicillium sp.								4	
Trichoderma koningii					1				
Aspergillus niger			30		150				
***Populus tremula* 1.0 m**									
Aspergillus niger									1
Mucor spinosus	10	18		12		2			2
Penicillium sp.			2					2	
Scopulariopsis sp.							1		
Fusarium sp.					1		1		
Trichoderma koningii								1	1
***Populus tremula* 0.5 m**									
Fusarium sp.				4	1				
Acremonium sp.							1		
Rhizopus sp.						1			
Mucor spinosus	2	4		1	1				
Penicillium sp.						3	3	3	
Trichoderma koningii					3		1		2
Aspergillus fumigatus						2	1		

Almost all identified species of fungi represent a potential danger for human health after being inhaled, which is quite common when manipulating such chips. Commonly, they can cause morbidity or mortality of patients with hematological diseases but also other diseases that compromise the immune system (Anaissie *et al.* 2009). *Aspergillus fumigatus* can cause a pulmonary disease Aspergillosis that has a mortality of 13% (Buchancová 2003). *Aspergillus fumigatus* and *Aspergillus clavatus* can cause allergic disease. Some *Aspergillus* species cause disease of grain crops, especially maize and synthesize mycotoxins including aflatoxin (San-Blas and Calderone 2008). *Aspergillus niger* is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. Aspergillosis occurs worldwide. The disease manifests itself most often in the disseminated pulmonary form or lung aspergiloma. Besides the lower respiratory tract paranasal sinuses, eyes, CNS and myocardium can be affected as well. (Suchomel *et al.*

2012). *Mucor spinosus*, a species of this infective fungus may cause zygomycosis (Mucormycosis) and ear infection. The spiny globular structure is a sporangium containing spores and is produced at the end of a fungal hypha (thread). On bursting, spores, from which another fungus can grow, are released. Zygomycosis, occurring after trauma, is a rare fungal infection of skin, lungs, blood vessels, or intestine (Samuels *et al.* 2006; Burge 1985). Some species produce mycotoxins that can negatively impact human health or eventually cause haemorrhagic syndrome (Al-Doory and Domson 1984). The production of dangerous toxins was also confirmed in the genus *Penicillium* sp., causing allergic reactions in some people (Fassati 1979). *Trichoderma koningii* has been identified as the cause of infections in immunosuppressed individuals (Samuels *et al.* 2006).

From the obtained results, it is obvious that there is a potential health risk in the case of chip piles of all wood species studied in the experiment. *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma koningii*, and the genus *Penicillium* sp., were identified in all experiment piles and belong to the most common types of fungi. Very dangerous is *Mucor spinosus*, which was found in the most abundant amount in the aspen pile; however, spruce piles also recorded numerous colonies of this species.

CONCLUSIONS

1. Statistical analysis confirmed that the highest occurrence of fungal propagules occurred when temperatures were around 20 °C and relative moisture inside the piles above 40%. The occurrence of individual species of fungi did not significantly vary among various species of wood.
2. Similar genera and species of fungi were identified in similar studies with different wood species (Jirjis 2005). It was confirmed that when biomass is stored for extended time, there is a potential health risk to humans caused by fungi. During the experiment, 5 species and 8 genera and species of fungi were identified from samples extracted from all piles (9 samples from all levels above the ground).
3. The outside temperature and relative humidity of northern temperate zone influenced the internal temperature in piles, rising to a maximum of 49 °C, which does not pose a risk of self-combustion of stored piles. The highest temperature increase was recorded with common aspen (*Populus tremula*) during the first month, but it was followed by a notable decrease. The highest overall content of relative moisture content was recorded in the pile with chips from spruce (*Picea abies*). With common aspen measured at 1.5 m above the ground the relative moisture recorded at the end of the experiment reached 67.7%. Between May and August, its relative moisture content rose by more than 40% at this measurement level.
4. The methods used in the experiment can also be applied in further research of pathogens with different wood species.
5. It can be stated that the number of phytopathogens rises with longer storage period only within the first 4 to 6 months. After that, the longer the period of storage, the smaller the intensity of the increase of phytopathogens is. *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma koningii*, and the genus *Penicillium* sp., were identified in all experiment piles, and they belong to the most common types of fungi.

Very dangerous is *Mucor spinosus*, which was found in the most abundant amount in the aspen pile, however, spruce piles also recorded numerous colonies of this species.

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