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REGULAR ARTICLE

ANALYSIS OF FUNGI OCCURRENCE IN ENERGY CHIPS PILES

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ABSTRACT

This article is focused on the analysis of fungi in energy chips piles. At the same time factors affecting the formation and evolution of fungi were analyzed. The bases for the experiment were nine experimental chips piles produced from wood species: European beech (*Fagus sylvatica*), European aspen (*Populus tremula*) and Norway spruce (*Picea abies*). Wood chips of each particular tree species have been produced from a fresh biomass, biomass stored in half-shade and in opened area. The greatest number of fungi was found in piles 3 - spruce wood chips produced from biomass stored on the meadow, 7 – chips from aspen stored in half-shade and 8 - beech wood chips produced from biomass stored in half-shade. The most represented species were *Aspergillus niger* and *Mucor spinosus*. These two species were recorded in seven of nine piles. Presence of *Trichoderma koningii* was identified only in two piles of chips, which were made from spruce and aspen biomass stored in the opened area.

Keywords: forestry, wood chips, storage, fungi, relative humidity, temperature

INTRODUCTION

In the use of renewable energy, Slovakia lags behind highly developed foreign countries. In 2009, very slow growth of production and domestic consumption of forest fuel

dendromass in the form of fuelwood and energy chips continued. Current share of fuel dendromass as the most important renewable energy source in total consumption of primary energy sources is about 2.1%, from which the proportion of forest fuel dendromass is 1%. Total exploitable potential of fuel dendromass is up to 9%, especially in the production of heat and electric power (**Green Report, 2010**). One of the reasons for such low use of forest dendromass for energy purposes may be the poor public awareness about this renewable energy source, about its positive aspects and advantages for its use, but also about the risks associated with its processing. Wood chips for energy purposes are the most produced from forest biomass. In the process of their production and subsequent handling, the workers are threatened by various risks and harmful factors. Work injuries and occupational diseases represent the major risk in energy chips processing. Harmful factors such as noise, vibrations and environment dust are eliminated by suitable technique and technology. The biggest dangers in the handling of wood chips represent fungi, which act as pathogens on the human body.

Fungi which are formed on stored chips when handling are getting into ambient air. Inhalation of organic dust contaminated by dangerous microorganisms has been recognized as an occupational hazard for those working with decompositing plant material (**Pratt, May, 1984**). The production of spores is huge and according to some authors represents up to $1{,}8.10^{12}$ sporangia per 1 kg of absolute dry chips (**Thörnquist, 1983**). There are presumptions that the so-called Mycotoxins are carcinogenic, teratogenic and mutagenic.

Sebastian *at al.* (2006) analyzed the microbial dustiness of baled straw (cultivated both conventionally and ecologically) and of wood chips from piles that had been stored outdoors for up to 11 months by using total spore counting, cultivation, and measuring of endotoxin and chemical markers of fungal biomass, lipopolysaccharide, and peptidoglycan. The bacterial dustiness of straw was much greater than of wood chips whereas the fungal dustiness did not differ much. In general, samples taken from the inner part of each biofuel material were dustier than samples taken from the surface, except for fungal and bacterial biomass in wood chips and total fungi and fungal biomass in ecological straw. A considerable increase of bacterial dustiness occurred during storage over summer. In summary, biofuels represent sustainable energy resources of growing economic importance but might at the same time pose significant health problems.

Softwood tops and branch fuel chips with high moisture contents were subject to biological heating in storage (**Miller *et al.* 1982**). This was due primarily to infestations of mesophilic [ca. 5×10^4 CFU/g (Colony Forming Units per gram) dry weight wood] and

thermophilic (ca. 1.6×10^6 CFU/g dry weight wood) fungi. Loading chips into a home fuel-chip furnace resulted in the distribution of fungal propagules throughout the basement and upper floors. Many of the species isolated are human allergens and pathogens.

When assessing the influence of fungi, not only their amount is important, but also their kind. According to a research (**Scholz, 2005**), from the total of 6-8 analyzed thermophilic fungal species, in samples of chips big fractions 2 to 4 and in chips small fractions 4-8 potential human pathogens, toxic or allergenic species that occurred at least temporarily during one year of storage were found. The fungus *Aspergillus fumigatus* with the greatest pathogenic impact on humans was present throughout the whole period of storage. Its occurrence was recorded in small and medium-sized chip fractions in 93% and in large chip fractions in 76% of the total 756 analyzed samples.

Thörnquist and Lundström (1982) indicated that with the length of chips storage from 3 to 6 months, the production of fungal spores may lead to hygiene problems, respiratory diseases and allergic reactions.

Chronic granulomatous disease is characterized by recurrent infections that result from an inability of phagocytes to kill organisms effectively. **Conrad *et al.* (1992)** describe a patient with this disease who developed *Aspergillus pneumonia* after shoveling moldy cedar wood chips. Despite aggressive therapy, the patient's condition deteriorated and he died. At autopsy, the lungs revealed diffuse granulomas, all of the same age, with aspergillus organisms confined to the granulomas. They propose the term "microgranulomatous aspergillosis" for this response, which does not conform to the commonly described aspergillus syndromes. They conclude that susceptible immunosuppressed patients should be advised to avoid occupational situations where high spore concentrations are generated.

The aim of this article is to analyze and evaluate the occurrence of fungi in chips piles produced from selected species of wood biomass.

MATERIAL AND METHODS

Sample preparation

For the analytical purposes of fungi occurrence in energy chips piles three types of wood were selected: European beech (*Fagus sylvatica*), European aspen (*Populus tremula*) and Norway spruce (*Picea abies*). From each tree species three piles of biomass were prepared: biomass of trees harvested during vegetation period, which was divided into two

parts. One pile of this kind of biomass was stored in opened area and the second pile was placed in half-shade next to the stand. Third biomass piles were prepared from trees that were harvested during vegetation resting period. From the prepared biomass energy chips were produced. The base pile consists of wooden square base with a footprint of 4x4 m. The chips were sprinkled; respectively piles have been arranged in the shape of a pyramid. The piles are located on the site Hrabiny, which is the part of the University forest enterprise of TU Zvolen.

Microbiological analysis

To achieve the aim of this article, which is the identification and quantification of fungi present in energy chips piles, 9 samples were collected. Samples were collected after 6 months of wood chips storage period. From each pile one sample from the middle was collected. These were stored in airtight plastic bags until the analysis took place. As the Department of forest harvesting and mechanization does not have appropriate instrumentation necessary to carry out the analysis, microbiological examination to identify specific fungi was processed by the test method: standard operating procedure (SOP) 15, in an accredited testing laboratory in the department of environment microbiology RPHA (Regional Public Health Office) located in Poprad with the accreditation certificate SNAS Reg. No. 126/S-140 from 21st October 2011. Quantification of fungi (CFU.g^{-1}) was analyzed by the method which is in accordance with the norm **ISO 21527-2**. Identification of fungi was carried out by **Fassatiová (1979)**.

From the suspected colonies of potentially toxin producing fungi a pure culture using the method of slide culture was prepared: On a sterile slide glass, a warm selective agar was dripped, the glass was held over the flame using tweezers and with a slight tilting the drop spilled itself on the surface at a distance of 1.5 cm from the edge. On the solidified agar a drop of spore suspension was inoculated and covered with at least two glass cover slips. We placed it into a Petri´s bowl (on which bottom there is a little sterile water or moistened cotton to maintain humidity) on a curved tube and cultivated for 7 to 10 days at the temperature of 25ºC. After several days, the grown culture is examined under the microscope. The morphological features of the culture are being monitored.

Enumeration of yeasts and molds according ISO 21527-2

The determination is carried out in accordance with ISO 21527-1, 2 Microbiology of food and feed. Horizontal method for enumeration of yeasts and molds: Part 1: The method of counting colonies in products with water activity higher than 0.95; Part 2: The method of counting colonies in products with water activity lower or equal 0.95.

Determination of moisture

At the same time, relative humidity of chips and later recorded temperature in each pile is evaluated in this article. Temperature, relative humidity and wood type crucially influence the formation and development of specific types of fungi. Relative humidity measurement was carried out by the drying method. Samples were dried at temperatures 104 °C ± 2 °C to constant weight. After reweighting on laboratory scales with the accuracy of 0.01 g, relative humidity values at different sampling sites were calculated. Relative wood humidity is the rate between the weights of water, which the wood contains to the wet wood weight, expressed in %. Temperature measurement in the pile is realized using thermometers installed in plastic tubes, which also serve to sample collecting.

RESULTS AND DISCUSSION

Result of the wood chips samples analysis for the presence of fungi, was the quantification of units (fungi) present in wood chips in summary. Simultaneously, also specific fungi on individual samples taken from test piles were identified. Results of the fungi quantification are listed in the table 1.

Table 1 Results of fungi occurrence analysis according to **ISO 21527-2**

Number of sample	**Temperature of incubation [°C]**	**Amount of units** [CFU.g^{-1}]	**Uncertainty U [%]**
sample 1	25°C	9,1 x 10^4	21
sample 2	25°C	3,0 x 10^5	31
sample 3	25°C	1,8 x 10^4	36

sample 4	25°C	5,4 x 10^5	25
sample 5	25°C	6,9 x 10^4	23
sample 6	25°C	2,9 x 10^5	31
sample 7	25°C	>7,5 x 10^5	<23
sample 8	25°C	2,4 x 10^4	32
sample 9	25°C	2,9 x 10^4	30

The total amount of microorganisms (fungi) in samples of energy chips ranged from 1,8x10^4 CFU.g^{-1} (sample 3) to 7,5 x 10^5 CFU.g^{-1} and more (sample 7). Sample in which most fungi were quantified, consisted of wood chips made from aspen poplar biomass, stored in half-shade next to the stand for the period of approximately 4 months. The second largest amount of colony-forming units was detected in the sample of chips made of beech biomass harvested in the vegetation resting period, which was processed without storage. The sample with the smallest number of fungi consisted of chips produced from spruce biomass, which was harvested in the vegetation period and then stored for 4 months in the opened area (meadow). After the subsequent verification of the information introduced above it will be possible to recommend principles for production and storage of raw materials for energy chips production in Slovak forestry. In terms of health and hygiene risks, the impact of fungi - especially their spore, the best are the piles of chips with the lowest number of fungi.

Temperature and environment humidity are the main factors influencing the process of fungi creation. For illustration on following charts, the development of relative humidity (chart 1) and temperature (chart 2) in the chips piles is indicated. The maximum relative humidity is maintained in the pile of spruce chips. This is caused due to the storage conditions of biomass - entire trees, thus with assimilation organs, from which chips were made. We assume that these results were crucially influenced by the structure of individual fractions of chips. Fine fractions of spruce assimilation organs filled the spaces between the coarser fractions of chips and reduced the proportion of air in chips piles. The course of moisture in this case is the most balanced one. In the last period (4 months), the lowest values of moisture were found in the chips piles made from aspen biomass stored in half-shade. Despite the balanced course of relative humidity in the last four months, during the entire reporting period, the largest difference of humidity values (ca 40 %) among all analyzed experimental piles was recorded within this pile. **Laurila and Lauhanen (2010)** analyzed the moisture content of Norway spruce stump wood at clear cutting areas and roadside storage sites. In this study the moisture content of Norway spruce stump wood was examined immediately after

harvesting at the clear cutting area and after different drying times at the roadside storage sites. Immediately after stump harvesting the average moisture content (wet basis) was 53%. The stump wood dried fairly fast during spring and summer. One month after stump harvesting, the average moisture content was about 31%. **Garstang *et al.* (2002)** state that after 50 days in chips storage the core layers dried, reaching a static moisture content of approximately 30% and the piles' moisture content correlates tentatively with the average monthly rainfall.

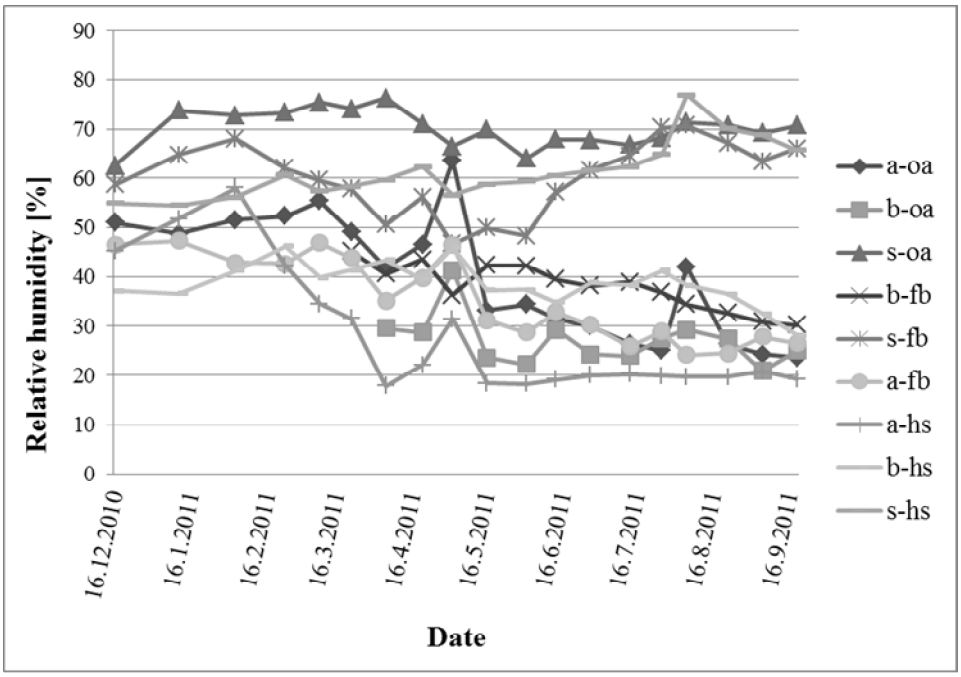

Figure 1 Relative humidity during the time of researching

Legend to the graph:
a-oa - chips of aspen biomass stored on opened area (pile 1)
b-oa - chips of beech biomass stored on opened area (pile 2)
s-oa - chips of spruce biomass stored on opened area (pile 3)
b-fb - chips of beech biomass harvested in vegetation resting period - fresh biomass (pile 4)
s-fb - chips of spruce biomass harvested in vegetation resting period - fresh biomass (pile 5)
a-fb - chips of aspen biomass harvested in vegetation resting period - fresh biomass (pile 6)
a-hs - chips of aspen biomass stored in half-shade (pile 7)
b-hs - chips of beech biomass stored in half-shade (pile 8)
s-hs - chips of spruce biomass stored in half-shade (pile 9)

The temperature in energy chips piles has relatively similar course in all chips piles except the spruce chips pile made of the biomass stored in half-shade. Temperature values in

this case are lower than in other piles. Apart from the extreme values, the highest temperature was recorded in the pile of spruce chips (34 °C). After creating piles usually for 10 to 14 days there is a sharp increase of temperature in the central part of the pile. The temperature usually reaches 65-80 °C, regardless of outside temperature (**Kuchtík, 1988**). **Garstang *et al.* (2002)** assessed the temperature development in the pile forestry residue. The results show a rapid rise to a peak of around 60 ºC in the first week followed by a decline to around 30ºC after about 6 weeks. Going into the autumn of 2001 the temperature decreased further to a winter low of around 15ºC. In the spring of 2002 the temperature began to rise again. This confirms the dependence of temperature in the pile from the ambient air temperature. **Bergman and Nilsson (1979)** found the different temperatures development in the three piles of energy chips (the first pile: from 35 ºC to 60 ºC, the second pile: from 40 ºC to 68 ºC (the first month) and to 25 ºC (the end of storage), the third pile: from 55 ºC to temperature below the freezing point) observed during a storage period of five months. **Scholz et al. (2005)** described the average temperature course in experimental piles of wood chips as a characteristic. Temperature increases immediately after the pile establishment and reaches after 10 to 30 (50) days maximum value of 60 ºC. This value substantially affects the pile volume, surface, ambient air temperature and mainly the chip size. Locally the temperature can also rise up to 65 ºC. 100-150 days after the chips storage (end of January) they achieved a significantly lower level of temperature and then gradually decrease to ambient air temperature.

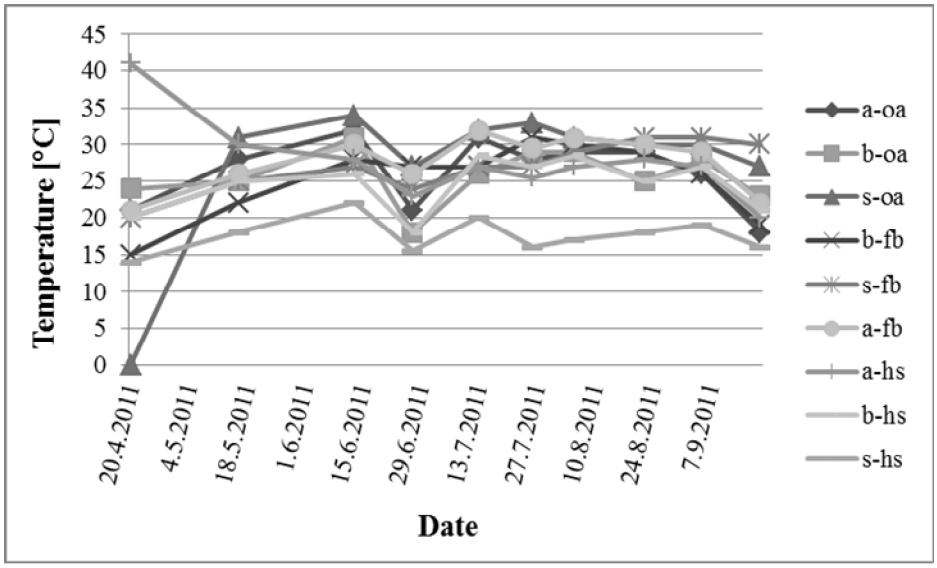

Figure 2 Temperature development in the chips piles

The results of specific fungi identification analysis are following findings: In piles of energy chips five species (three genus: *Trichoderma, Mucor* and *Aspergillus*) of fungi were identified: *Trichoderma koningii*, *Aspergillus niger* and three species of the genus *Mucor*: *Mucor spinosus*, *Mucor globosus* and *Mucor hiemalis*. **Bergman and Nilsson (1979)** identified in the piles of wood chips different kinds of *Ascomycetes* (*Aspergillus sp., Trichoderma sp., Penicillium sp*. and other), *Basidiomycetes*, imperfect fungi and *Zygomycetes* (*Rhizopus arrhizus Fischer)*. **Garstang *et al.* (2002)** determined the number of spores from air samples taken in handling the chips. They have identified by the analysis 267 CFU/m^3 for thermophilic *Actinomycetes* and 50 CFU/m^3 for *Aspergillus fumigatus*.

Table 2 Occurrence of fungi in piles

Species	*Trichoderma koningii*	*Aspergillus niger*	*Mucor spinosus*	*Mucor hiemalis*	*Mucor globosus*
Presence in piles	P1, P3	P1, P2, P3, P4, P5, P7, P8	P2, P3, P4, P5, P6, P7, P8	P4, P5, P6, P7, P8, P9	P2, P3, P6, P7, P8, P9

P – pile

In Table 2 we can see the occurrence of specific fungi species in each pile. The largest amount of fungi was identified in piles 3, 7 and 8. The lowest amount of fungi was evaluated in piles 1 and 9. The most represented kind is *Aspergillus niger* and *Mucor spinosus*.

Aspergillus niger occurs in all the piles except of pile 6 and 9. Pile 6 consists of aspen chips from biomass harvested during vegetation resting period. Pile 9 is characterized by high relative humidity of chips and the lowest temperature recorded in piles. *Aspergillis 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. Aspergilla found primarily in soil and on plants and vegetables. The incubation period lasts 1-3 weeks. The disease manifests itself most often in the disseminated pulmonary form or lung aspergiloma. In addition to lower respiratory tract paranasal sinuses, eyes, CNS and myocardium can be affected. Diagnosis is determined by serology. Prognosis is unfavorable in the pulmonary form (13% mortality) and infaust in cerebral and cardiovascular form. Involvement of the eyes causes a rapid loss of vision. Amphotericin B is used for the treatment (**Buchancová *et al.*, 2003**). *A. niger* is one of the most common fungus causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane. *Aspergillus niger* is a fungus

and one of the most common species of the genus *Aspergillus*. Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins (**Abarca *et al.*, 1994**), but other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A (**Samson *et al.*, 2001; Schuster *et al.*, 2002**).

Mucor species are often found in soil, dead plant material, horse dung, fruits and fruit juice. It is also found in leather, meat, dairy products, animal hair, and jute. A *Zygomycetes* fungus may be allergenic (skin and bronchial tests). This organism and other *Zygomycetes* will grow rapidly on most fungal media. They may cause mucorosis in immune compromised individuals. The sites of infection are the lung, nasal sinus, brain, eye, and skin. Infection may have multiple sites (http://www.moldremoval.com/mucor-sp.html).

Mucor spinosus occurs in all the piles as well except of pile 1 and 9. First pile consists of chips made of aspen biomass which was stored in opened area. *Mucor spinosus* - 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 are released which can grow another fungus. Zygomycosis is a rare fungal infection of the skin, lungs, blood vessels or intestine, occurring after trauma. *Mucor* may also invade the ear canal causing otomycosis. These fungi, however, are more commonly found in soil, on dung and spoiled food. Magnification: x245 at 6x7cm size (http://sciencephoto.com/media/13529/view).

Next two species of genus *Mucor* occurred in six piles, e.g. in 67% of piles.

Mucor globosus does not occur in piles 1, 4 a 5. The piles 4 and 5 consist of chips made of biomass (beech and spruce) harvested in vegetation resting period (fresh biomass).

Mucor hiemalis does not occur in the first three piles, hence in piles which were made of biomass stored in opened area. It occurred in chips piles made of biomass of all three tree species which were stored in half-shade and from biomass harvested during vegetation resting period. *Mucor hiemalis* is a fungal plant pathogen. *M. hiemalis* grows in expanding gray colonies. It grows branched sporangiophores that yielding yellow to dark brown sporangia which can mate to form black-brown, spiny zygospores. *M. hiemalis* is nitrate positive and requires thiamin to grow (http://en.wikipedia.org/wiki/Mucor_hiemalis). *Mucor hiemalis* is a fungus which may be allergenic (skin and bronchial tests). There may have been scattered reports of individuals who have been infected by this fungus through wounds. These were reported as a causation agent of a primary cutaneousmycosis in an otherwise healthy person. This organism may cause an infection called "mucorosis" in immune compromised

individuals (i. e., transplant recipient, herpes, and common cold). The sites of infection are the lung, nasal sinus, brain, eye, and skin. Infections may have multiple sites (http://www.mold-help.org/content/view/421/).

The presence of the species ***Trichoderma koningii*** was identified only in two piles: 1 and 3. Those are chips piles of wood stored in opened areas before their chipping. *Trichoderma koningii* is among the most commonly cited species in the genus. The morphological species *Trichoderma koningii* can be considered to be stereotypical of *Trichoderma*, viz. conidiophore with a more or less conspicuous main axis from which often paired lateral branches arise, the branches increasing in length with distance from the tip of the main axis and themselves branch in the same manner. Several species of the well-known saprophytic genus *Trichoderma* have been identified as the cause of infections in immunosuppressed humans (**Samuels *et al.*, 2006**).

Information on the effects of temperature, humidity and type of chips for the presence and quantity of fungi, integrated with knowledge on the aggressiveness of particular fungi species on the human body are an essential prerequisite for the elimination of health and hygiene risks in forestry of SR.

CONCLUSION

Aim of this article was to analyze the presence of fungi in energy chips piles after some storage period. The influence of specific chips kinds, hence the origin of biomass, from which chips were made, was also analyzed.

The biggest amount of fungi was found in piles 3 - spruce wood chips produced from biomass stored in the meadow, 7 - aspen chips stored in half-shade, and 8 - beech wood chips produced from biomass stored in half shade. The smallest amount of fungi was evaluated for piles 1 - aspen chips from biomass stored in opened area and 9 - spruce wood chips produced from biomass stored in half-shade. The most represented species are *Aspergillus niger* and *Mucor spinosus*. These two species were recorded in seven of nine piles. Two of the identified types of fungi occurred in six piles and one kind in two piles. *Aspergillus niger* was presented in all the piles except of piles 6 and 9. *Mucor spinosus* occurred, in all the piles as well, except of piles 1 and 9. Pile 9 consisted of chips made of spruce biomass which was stored in half shade. This pile was characterized by high relative humidity and the lowest environment temperature from all of observed piles. Two other species of the genus *Mucor* occurred in six piles, e.g. 60% of piles. *Mucor hiemalis* did not occur in first three piles. It occurred therefore

in chips piles produced from biomass of all three types of wood stored in half-shade and in the biomass produced in the vegetation resting period. *Mucor globosus* was absented in piles 1, 4 and 5. The piles 4 and 5 consist of chips made of biomass harvested in vegetation resting period Presence of *Trichoderma koningii* was identified only in two piles: 1 and 3. Those are piles of aspen and spruce wood chips stored in opened area before chipping.

Based on the obtained results we can conclude that prevention in the fields of production, processing and storage of energy chips should not be underestimated. The analysis results show that after the half-year of storage, five kinds of fungi that can be, based on the research, characterized as pathogenic, were identified.

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