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Carbon and nitrogen stabile isotope ratio and heavy metals in *Leccinum aurantiacum* in a hybrid aspen plantation in agricultural land initially fertilised with biogas production residues, sewage sludge, and wood ash

ARTA BĀRDULE[1]*, DAGNIJA LAZDIŅA[1], KRISTĪNE ZADVINSKA[2], LAUMA BUŠA[2], ARTURS VĪKSNA[2] AND ANDIS BĀRDULIS[1]

[1] Latvian State Forest Research Institute 'Silava', Rigas str. 111, Salaspils, Latvia, LV-2169
[2] University of Latvia, Jelgavas str. 1, Riga, Latvia, LV-1004

** Corresponding author: arta.bardule@silava.lv, phone: +371 27119666*

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Abstract

Edible mycorrhizal fungi can be harvested in the fourth year after establishment of a hybrid aspen plantation in previous agricultural land at hemiboreal conditions. It is important to understand the role of fungi in element cycling at the ecosystem level as well as the amounts of elements, including heavy metals, that are accumulated in fruitbodies of edible fungi in the context of food safety. Therefore we evaluated the carbon and nitrogen stable isotope ratio and content of heavy metals (Cr, Pb, Mn, Ni, Cd, Cu and Zn) in *Leccinum aurantiacum* (Bull.) Gray in a juvenile hybrid aspen (*Populus tremuloides* Michx. × *Populus tremula* L.) plantation in agricultural land initially fertilised with biogas production residues (digestate), sewage sludge and wood ash, which are potential sources of pollution of heavy metals. The research site was established in the spring of 2011, but the natural occurrence of fruitbodies of *L. aurantiacum* was observed in 2014. The mean isotopic data for fruitbodies of *L. aurantiacum*, collected in the 2014–2018 monitoring period, ranged between -27.4 and -24.5‰ for $\delta^{13}C$ and between 7.8 and 10.1‰ for $\delta^{15}N$. The mean content of investigated heavy metals in fruitbodies of *L. aurantiacum* were up to 129 mg kg^{-1} for Zn, up to 99 mg kg^{-1} for Cu, up to 30 mg kg^{-1} for Mn, up to 1.5 mg kg^{-1} for Ni, up to 1.7 mg kg^{-1} for Cd, up to 1.1 mg kg^{-1} for Cr and up to 0.6 mg kg^{-1} for Pb. We concluded that the application of digestate, sewage sludge and wood ash as fertilisers to improve soil quality in hybrid aspen plantations in agricultural land did not result in increased heavy metal accumulation in fruitbodies of *L. aurantiacum*, but N isotopic compositions were altered (^{15}N-enriched) for a period of up to five years after application of the organic fertilizers (digestate and sewage sludge).

Keywords: agricultural land, hybrid aspen, *Leccinum aurantiacum* (Bull.) Gray, heavy metals, stable isotope ratio

Introduction

A healthy and stable tree-dominated ecosystem largely relies on the ectomycorrhizal relationship and the community of ectomycorrhizal fungi (EMF) in terms of nutrient cycling (Hou et al. 2012). In general, the soil nitrogen cycle in forests has been thought to be governed by mineralisation processes driven by bacteria. However, there is increasing evidence that macrofungi, particularly those involved in mycorrhizal symbioses, play important roles in N and C cycling in temperate and boreal forests, suggesting that the N cycle in these systems is more complex than previously thought (Trudell et al. 2004). Many studies have shown that mycorrhizal fungi, as mutualistic root extensions, obtaining carbon sources (plant photosynthates) from their plant partners, improve mineral nutrition uptake for plants (Trudell et al. 2004, Mayor et al. 2009, Hou et al. 2012). As well many species of fungi take part in geochemical cycle of metallic elements and their subsequent transfer to plants, small and larger animal species (e.g., deer), and people (Falandysz et al. 2012).

Stable isotopes are naturally occurring, non-radioactive forms of elements that have the same number of

protons, but a different number of neutrons, causing differences in the atomic masses of the isotopes (for example, carbon-12 and carbon-13). Stable isotopic composition has been widely used in ecological and element cycling studies to trace the geochemical cycling of nutrients and contaminants moving through the natural environment. Isotope values in fungi provide a form of ecological information independent of phylogenetics, soil excavation or molecular sequencing. When information on isotopic composition is combined with one or more of these other techniques, provides definitive evidence of the nutritional ecology of specific fungi (dietary pattern) and trophic relationships within ecosystems (Mayor et al. 2009). The isotopic composition of a fungus generally reflects the climatic and geographical conditions in which it grew (relative humidity, temperature, amount of precipitation, distance to the nearest sea and other sources of evaporation, altitude, latitude), but also reflects the trophic types and nutrient sources (Piao et al. 2004, Yang et al. 2007, Hou et al. 2012, Romulus et al. 2017). Thus, habitats or ecosystems with different nutrient inputs and plant communities can show large differences in overall $\delta^{13}C$ and $\delta^{15}N$ values (Stapp et al. 1999), making it essential to obtain background information about natural abundance levels in soil, vegetation and litter (Griffith 2004). In fungal ecology, the use of stable isotope analysis has largely been restricted to the macrofungi which form tissue masses greater than 10–20 mg of fresh weight (corresponding to ca. 100 µg N), although EA (elemental analysis)-IRMS (isotope ratio mass spectrometry) measurements are often made on sample masses containing between 10 and 100 µg N (Griffith 2004).

Wild-growing fungi may accumulate considerable amounts of metallic elements, including heavy metals, and metalloids in their fruitbodies due to specificities in their physiology (Frankowska et al. 2010, Aloupi et al. 2011, Brzezicha-Cirocka et al. 2016, Kalac 2016). The polysaccharide components of the cell walls of fungi, such as chitin, can fix metallic elements on functional groups (phosphate, carboxyl, amine, etc.). These elements are quickly transported to the cell interior and then circulate throughout the entire mycelium. This translocation is favoured for the fungal special form of osmotrophic nutrition and highly efficient cellular communication (Campos et al. 2009). Furthermore, the fruitbodies of many fungal species accumulate these heavy metals much more effectively than cereals or other foodstuff growing in similar conditions (Nuorteva et al. 1986, Svoboda et al. 2002). However, significant variations have been observed in heavy metal concentrations within fungal groups (Pelkonen et al. 2006, 2008). Heavy metals such as cadmium, lead, nickel and others have severe toxicological effects on human health, even at extremely low concentrations of a few micrograms per kg. In the EC regulations (EC 2006), the threshold values are specified with the maximum levels of Cd and Pb in the fresh matter of unspecified fungi species as 0.05 and 0.10 mg kg^{-1} fresh weight, respectively. However, the legislation does not provide limits for values of the other heavy metals.

Previously, the fruitbodies of these non-green edibles have been appreciated for their texture, aroma and flavour, but currently, their usage is undergoing a paradigm shift as a lot of work has been focused on their biochemical characterisation. They are considered as a delicacy with high nutritional and functional values and are also accepted as nutraceutical foods because of the presence of numerous bioactive compounds and minerals (Lalotra et al. 2016). In Latvia, data acquired via a sociological research show that 67.6% of the inhabitants gathered fungi. Furthermore, fungi are not only one of the most significant forest plant non-wood products, but also considered as highly important for the national economy (Donis and Straupe 2011).

Leccinum aurantiacum (called Red Aspen Bolete, Orange bolete, Aspen, Common Aspen, Eurasian Aspen or Red-capped Scaber Stalk) is a species of fungi in the family Boletes. *L. aurantiacum* is an edible mycorrhiza-forming species; it is common in deciduous and mixed forests, scrublands, or parks in Europe, North America and Asia (Laiviņš 2002, Falandysz et al. 2012, Strumińska-Parulska et al. 2016). In Europe, *Leccinum* species are associated either exclusively or primarily with poplars, usually with *P. tremula*, but also with other species such as *P. alba* or the various planted hybrids and deciduous trees including beech, birch, chestnut, willow, and lime (Robinsona et al. 2011, Strumińska-Parulska et al. 2016). There are no *L. aurantiacum* in Europe associating with conifers (Strumińska-Parulska et al. 2016). Its cap can be orange, brown, or red with 3–25 cm diameter. In favourable conditions, they can grow very fast reaching a diameter of 5–8 cm in one day, and that of 23 cm – in 6 days (Strumińska-Parulska et al. 2016). Its flesh is white, burgundy at first when bruised, then greyish or purple-black. The underside of the cap has very small whitish pores that turn olive brown when bruised. The stipe is rough, 10–25 cm tall, and 2–5 cm thick; can colour blue-green when bruised. *L. aurantiacum* can be collected during summer and autumn (from June to October) (den Bakker et al. 2004, Strumińska-Parulska et al. 2016). *L. aurantiacum* is a tasty, popular and valuable wild grown fungi used as gourmet product (Falandysz et al. 2012).

Considering that food security for an ever-increasing global population will be a major challenge of the XXI century, mycologists are currently exploring ways to increase the awareness of the general public on the nutritive value of edible fungi and to accelerate the transformation from the consumption of vegetables to fungi. However, in the past three decades, research on the nutritive aspects of wild macrofungi has also exposed negative facets (Lalotra et al. 2016). This study investigates the carbon and nitrogen stable isotope ratio and heavy metal content in fruitbodies of *L. aurantiacum*, which naturally occurred in a juvenile hybrid aspen (*Populus tremuloides*

Michx. × *Populus tremula* L.) plantation in agricultural land initially fertilised with biogas production residues (digestate), sewage sludge and wood ash, which can be potential sources of pollution.

Materials and methods

Study site

Fruitbodies of *L. aurantiacum* and soil O layer samples were collected in a juvenile hybrid aspen (*Populus tremuloides* Michx. × *Populus tremula* L.) plantation in agricultural land in the central part of Latvia (lat.: 56.6919, long.: 25.1370 according to the LKS-92 coordinate system, Transverse Mercator projection, *see* Figure 1). The research site was established in the spring of 2011. Hybrid aspen container seedlings (clone No. 4 and No. 28, producer – JSC 'Latvia's State Forests' nursery 'Kalsnava') were planted with an average distance of 2.0 × 2.0 m between the trees. The experimental plot of the hybrid aspens was part of a large-scale multifunctional plantation of short-rotation energy crops and deciduous trees, with a total area of 16 ha. Four replications of four different fertilisation subplots were established as follows: control (no fertilisation), sewage sludge, wood ash and biogas production residues (digestate). The size of each subplot was 20 × 24 m. In compliance with the governmental regulations (Cabinet of Ministers 2006), Class I sewage sludge (dose 10 t_{DM} ha^{-1}) from 'Aizkraukles ūdens' (Aizkraukle Water) and stabilised wood ash from the boiler house in Sigulda (dose 6 t_{DM} ha^{-1}) were spread mechanically before planting the hybrid aspens. Digestate (as a point source fertiliser, dose 30 t ha^{-1}) from the methane reactor in the Vecauce district (Latvia) was applied immediately after planting the hybrid aspen seedlings. According to the FAO classification (FAO 2006), the soil type was classified as *Luvic Stagnic Phaeozem* (*Hypoalbic*) or *Mollic Stagnosol* (*Ruptic*, *Calcaric*, *Endosiltic*) with a dominant loam and sandy loam soil texture. Contents of heavy metals in fertilizers and soil layers immediately after fertilization in 2011 are summarized in Table 1. No significant deterioration of soil quality in the context of heavy metals has been observed as a result of application of fertilizers, although target values of Zn, Ni, As and Cd content in the soil for achieving sustainable soil quality were exceeded both in the topsoil and in deeper soil layers (Bardule 2019).

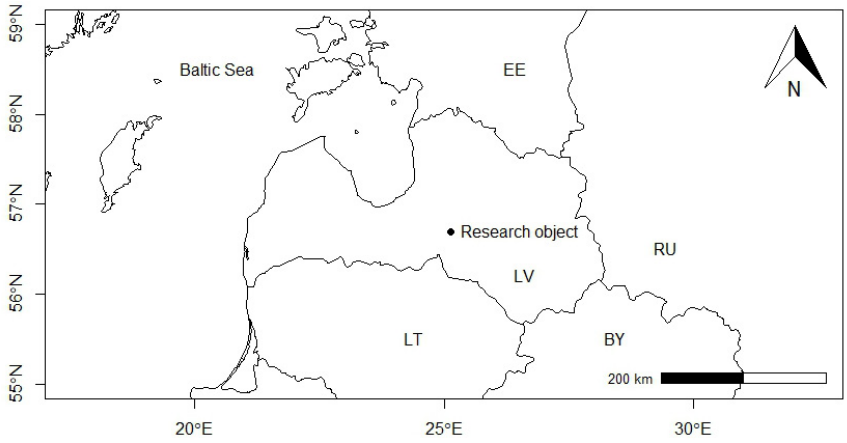

Figure 1. Location of the research site in Skriveri district (Latvia)

Table 1. Contents of heavy metals in fertilizers and soil layers immediately after fertilization in 2011

Type of fertilizer or soil layer	Cr, mg kg^{-1}	Pb, mg kg^{-1}	Ni, mg kg^{-1}	Cd, mg kg^{-1}	Cu, mg kg^{-1}	Zn, mg kg^{-1}	Mn, mg kg^{-1}
Fertilizers*							
Sewage sludge**	18 ±2	26 ±2	13 ±1	1.6 ±0.4	122 ±10	485 ±56	340 ±50
Wood ash***	138 ±104	75 ±19	25 ±23	2 ±2	149 ±17	307 ±69	3090 ±80
Soil layers****							
0–20 cm/ control	9 ±1	9.0 ±0.9	8 ±1	0.1 ±0.4	5.3 ±0.7	40 ±15	293 ±59
0–20 cm/ digestate	8 ±2	8.8 ±0.8	7 ±1	0.07 ±0.04	4.5 ±0.9	28 ±6	244 ±30
0–20 cm/ sewage sludge	6.9 ±0.3	9.0 ±0.6	6.6 ±0.8	0.05 ±0.02	4.6 ±0.6	25 ±5	226 ±22
0–20 cm/ wood ash	8 ±1	9.1 ±0.9	8.3 ±0.7	0.06 ±0.04	5.3 ±0.6	33 ±10	259 ±32
20–80 cm/ control	9 ±1	8.0 ±0.9	11 ±1	0.07 ±0.04	6.8 ±0.8	48 ±13	422 ±98
20–80 cm/ digestate	7.4 ±0.6	7.3 ±0.5	9.0 ±0.9	0.02 ±0.01	5.6 ±0.7	26 ±2	269 ±42
20–80 cm/ sewage sludge	6.6 ±0.4	6.6 ±0.4	10 ±1	0.009 ±0.006	6.1 ±0.7	23 ±2	289 ±30
20–80 cm/ wood ash	7.5 ±0.5	6.8 ±0.5	10.7 ±0.8	0.01 ±0.01	7.1 ±0.8	27 ±3	263 ±35

* Information on heavy metal content in digestate in not available.
** Information on heavy metal content in sewage sludge was provided by producer. Heavy metal content in sewage sludge determined according LVS ISO 11047:1998 in accredited laboratory in Latvia.
*** Heavy metal content in wood ash determined according LVS ISO 11047:1998 in Forest Environment laboratory at Latvian State Forest Research Institute 'Silava'.
**** Soil samples extracted with mixture of conc. HNO_3 and conc. $HClO_4$ (Bardule, 2019). Analyses were made in the Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW) in Austria. Considering the large natural variation of heavy metal content in soil in the research site, statistically significant differences in heavy metal content in soil between control and treated plots were not detected ($p > 0.05$).

Sampling and analyses

Fruitbodies of *L. aurantiacum* were sampled in August and September from 2014 to 2018. Ten fruitbodies of *L. aurantiacum* from each subplot were pooled to make a representative sample for chemical analyses. After being brought to the laboratory, the fruitbodies were dried at 40°C for at least 48 h and subsequently ground into powder. Soil O layer samples (three replications in each subplot) were sampled in September 2018 using a stainless-steel probe (10 × 10 cm). In the laboratory, the samples were dried and prepared for analysis according to ISO 11464 (2006).

Stable carbon and nitrogen isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) were determined using an elemental analysis isotope ratio mass spectrometry (EA-IRMS). The instrumentation consisted of an element analyser (EuroEA3024, Euro Vector), continuous flow IRMS (Nu Horizon, Nu Instruments) and the software Horizon Stable Gas Control Software (version 1.69.4). For QC/QA procedures an internal standard sample (glutamic acid) and certified inorganic reference materials (L-Glutamic acid USGS-40 and USGS-41) were used. The stable carbon isotopic composition is expressed as follows:

$$\delta^{13}C(‰) = \left[\frac{\frac{^{13}C}{^{12}C_{sample}} - \frac{^{13}C}{^{12}C_{standard}}}{\frac{^{13}C}{^{12}C_{standard}}} \right] \cdot 1000 \ ,$$

where $^{13}C/^{12}C_{sample}$ and $^{13}C/^{12}C_{standard}$ are the ratios of the sample and the reference sample (VPBD), respectively. The stable nitrogen isotopic composition is expressed by the following equation:

$$\delta^{15}N(‰) = \left[\frac{\frac{^{15}N}{^{14}N_{sample}} - \frac{^{15}N}{^{14}N_{standard}}}{\frac{^{15}N}{^{14}N_{standard}}} \right] \cdot 1000 \ ,$$

where $^{15}N/^{14}N_{sample}$ and $^{15}N/^{14}N_{standard}$ are the ratios of the sample and the reference sample (atmospheric N_2), respectively.

The levels of Cr, Pb, Mn, Ni, Cd, Cu and Zn in fruitbodies of *L. aurantiacum* and soil O layer samples were determined. Briefly, 0.25-g samples were mineralised in a closed microwave system (Start E, Milestone) using a mixture of 6 mL of concentrated HNO_3 (analytically pure, 65%, EMSURE® Reag. Ph Eur, ISO, Merck KGaA) and 2 mL of concentrated H_2O_2 (analytically pure, 30–32%, $\rho = 1.110$ g cm^{-1}, Certified ACS, Fisher Chemical). An inductively-coupled plasma mass spectrometer (8900 Triple Quadrupole ICP-MS, Agilent) was used for heavy metal quantification. The accuracy of the measurements was verified by using certified reference material (CRM) BCR-060 – Aquatic plant (*Lagarosiphon major*), Institute for Reference Materials and Measurements (IRMM), Belgium.

Statistical analysis

Data processing and all statistical analyses were performed in the R environment (R Core Team 2017). The data were divided into four groups according to the applied fertiliser. Statistical differences in carbon and nitrogen stable isotope ratios or heavy metal contents in *L. aurantiacum* between treated and control plots were analysed with the Wilcoxon rank sum test with continuity corrections; the choice of the non-parametrical statistical method was justified by the small number of repetitions in the sample (less than 30). A 95% confidence level was used for all analyses.

Results

Dispersal and biomass of fruitbodies of L. aurantiacum

Fruitbodies of *L. aurantiacum* naturally occurred in the research site in 2014 (in the third year after the establishment). Occurrence and biomass of fruitbodies of *L. aurantiacum* were monitored in September 2014 and August 2016 to determine the impacts of the initially used fertilisers (Figure 2). There was a trend of slightly higher occurrence and biomass of fruitbodies of *L. aurantiacum* in the subplots initially fertilised with digestate, and we observed a slightly lower occurrence and biomass of fruitbodies in the subplots initially fertilised with sewage sludge when compared to the control subplots, but these differences were not statistically significant ($p > 0.05$).

Carbon and nitrogen stable isotope ratio

The mean isotopic data for fruitbodies of *L. aurantiacum*, collected in a hybrid aspen plantation in agricultural land between 2014 and 2018, ranged between -27.37 ±0.05‰ and -24.5 ±0.3‰ for $\delta^{13}C$ and between 7.8 ±0.2‰ and 10.1 ±0.8‰ for $\delta^{15}N$ (Tables 2 and 3). Results show that the mean $\delta^{13}C$ values only slightly varied between treatments within year (mean $\Delta^{13}C$ is 0.6‰), while the mean variation between years within treatment was significantly higher (mean $\Delta^{13}C$ of 2.2‰). The mean variation in $\delta^{15}N$ values between years and treatments was similar (mean $\Delta^{15}N$ of 1.1‰ and 1.3‰, respectively).

The $\delta^{13}C$ values of the soil O layer for 2018 ranged from -30.0 to -29.6‰, with a mean value of -29.8 ±0.2‰, while the $\delta^{15}N$ values of the soil O layer ranged from -10.3 to -8.8‰, with a mean value of -8.1 ±0.4‰ (Table 4).

Table 2. Mean $\delta^{13}C_{VPDB}$ (‰) in fruitbodies of *L. aurantiacum* (mean values ± SE)

* Statistically significant ($p < 0.05$) differences between control and treated plots within one study year.

Type of fertilizer	2014	2015	2016	2017	2018
Control	-25.89 ±0.05*	-26.64 ±0.04*	-24.89 ±0.01	-25.2 ±0.1	-24.6 ±0.2
Digestate	-26.30 ±0.06*	-26.7 ±0.1	-24.91 ±0.1	-25.1 ±0.1	-24.5 ±0.3
Sewage sludge	-25.5 ±0.2	-27.01 ±0.08*	-25.3 ±0.5	-25.3 ±0.1	-24.7 ±0.2
Wood ash	-25.88 ±0.05	-27.37 ±0.05*	-25.5 ±0.1	-25.44 ±0.09	-25.0 ±0.3

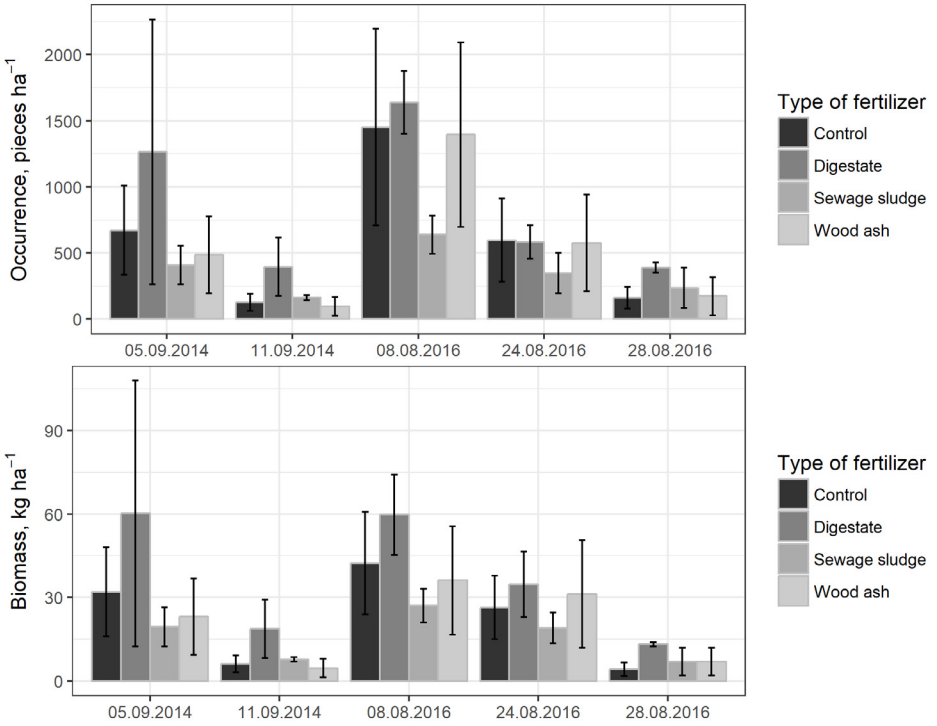

Figure 2. Natural occurrence and biomass (fresh weight) of fruitbodies of *L. aurantiacum* in the research site in September 2014 and August 2016

Table 3. Mean $\delta^{15}N_{AIR}$ (‰) in fruitbodies of *L. aurantiacum* (mean values ± SE)

* Statistically significant ($p < 0.05$) differences between control and treated plots within one study year.

Type of fertilizer	2014	2015	2016	2017	2018
Control	8.9 ±0.2	8.6 ±0.1	8.9 ±0.6	8.4 ±0.3	8.5 ±0.5
Digestate	9.9 ±0.3*	10.4 ±0.1*	10.1 ±0.8	8 ±2	9.2 ±0.2
Sewage sludge	9.7 ±0.2*	9.0 ±0.4	8.5 ±1.3	7.8 ±0.2	9.6 ±0.4
Wood ash	8.7 ±0.5	8.7 ±0.1	9 ±3	8.8 ±0.3	9.2 ±0.6

Table 4. $\delta^{13}C_{VPDB}$ and $\delta^{15}N_{AIR}$ values in soil O layer in the research site in 2018 (mean values ± SE)

* Statistically significant ($p < 0.05$) differences between control and treated plots.

Type of fertilizer	$\delta^{13}C_{VPDB}$, ‰			$\delta^{15}N_{AIR}$, ‰		
	mean	min	max	mean	min	max
Control	-30.0 ±0.3	-31.2	-29.2	-6.2 ±0.6	-8.8	-4.1
Digestate	-29.6 ±0.2	-30.0	-28.9	-8.2 ±0.5*	-10.3	-6.7
Sewage sludge	-29.7 ±0.5	-29.5	-27.9	-7.8 ±0.4*	-9.2	-6.4
Wood ash	-30.0 ±0.5	-29.7	-28.2	-10.2 ±0.7	-8.8	-4.1

Heavy metal content

The mean levels of heavy metals (Cr, Pb, Mn, Ni, Cd, Cu and Zn) in fruitbodies of *L. aurantiacum* (2014–2018) and in the soil O layer (2018), by fertilisation type, are shown in Figure 3. All heavy metal concentrations are expressed on a dry-weight basis. The contents of the heavy metals assayed in the analysed biomass of fruitbodies of *L. aurantiacum* followed the order Zn > Cu > Mn > Ni > Cd > Cr > Pb. In the dry mass of fruitbodies of *L. aurantiacum*, the mean Zn content reached up to 129 mg kg^{-1} (control plot, 2016), the mean Cu content was up to 98.6 mg kg^{-1} (control plot, 2016), the mean Mn content was up to 30 ±24 mg kg^{-1} (control plot, 2016), the mean Ni content was up to 1.5 mg kg^{-1} (plot initially fertilised with digestate, 2016), the mean Cd content was up to 1.7 mg kg^{-1} (plot initially fertilised with wood ash, 2016), the mean Cr content ranged up to 1.1 ±0.2 mg kg^{-1} (plot initially fertilised with sewage sludge, 2014/2015) and the mean Pb content was up to 0.6 ±0.6 mg kg^{-1} (control plot, 2018).

The contents of the heavy metals assayed in the soil organic O layer followed the order Mn > Zn > Cr > Ni > Cu > Pb > Cd. In the soil O layer, the highest mean Mn and Pb contents were found in plots initially fertilised with digestate (24 ±2 mg kg^{-1} and 0.37 ±0.06 mg kg^{-1}, respectively), while the highest mean Zn and Cd contents were found in control plots (5 ±1 mg kg^{-1} and 0.015 ±0.006 mg kg^{-1}). The highest mean Cr, Ni and Cu contents were observed in plots initially fertilised with sewage sludge (5 ±2 mg kg^{-1}, 3 ±2 mg kg^{-1} and 1.2 ±0.1 mg kg^{-1}, respectively). There were no statistically significant differences between mean heavy metal contents in fruitbodies of *L. aurantiacum* or in soil O layer between control and treated plots ($p > 0.05$).

Discussion and conclusions

Carbon and nitrogen stable isotope ratio

The carbon isotopic compositions of *L. aurantiacum* are in agreement with previous results reported for ectomycorrhizal fungi. For instance, Hou et al. (2012) indicated that the $\delta^{13}C$ values of EMFs ranged from -26.41 to -24.22‰, while Trudell et al. (2004) reported mean $\delta^{13}C$ value of EMFs in old-growth conifer forests of -25.3‰. However, in Romulus et al. (2017), the $\delta^{13}C$ values for edible fungi, collected in Transylvania in the 2015–2016 monitoring period, ranged between -28.6 and -19.8‰; the

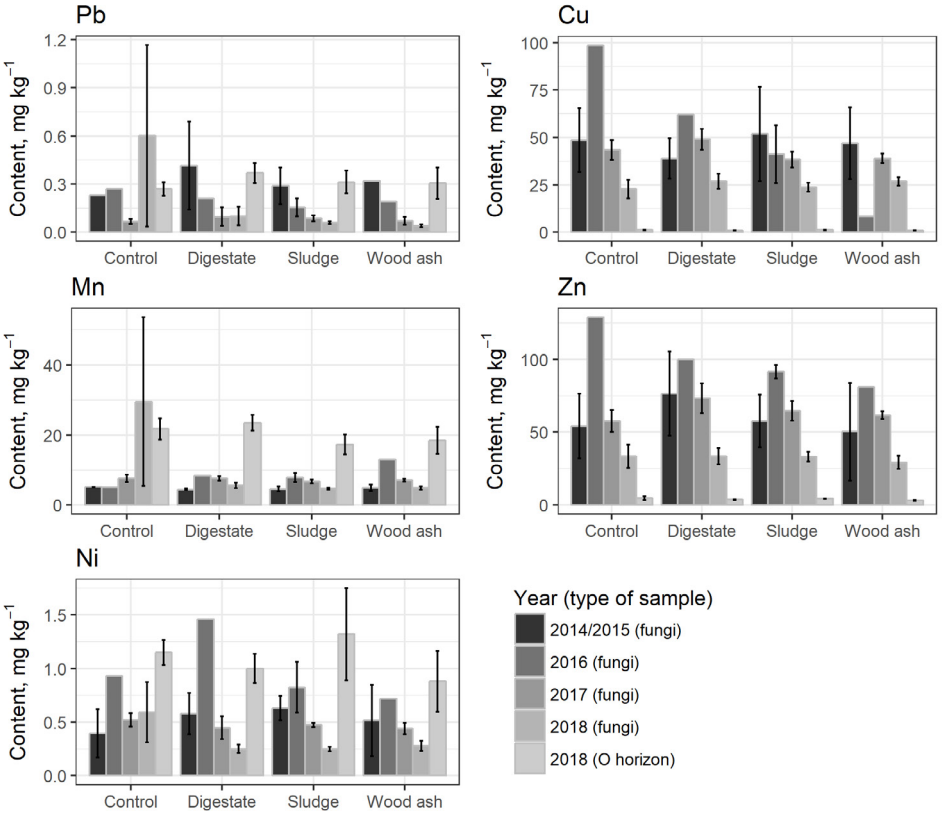

Figure 3. Mean heavy metal contents in fruitbodies of *L. aurantiacum* and soil O layer as a factor of fertilisation of the hybrid aspen plantation

$\delta^{13}C$ value varies because of isotopic fractionation during physical, chemical and biological processes (Romulus et al. 2017). The EMFs mainly obtain their carbon from live trees. Photosynthates, such as glucose and other monosaccharides, are transferred from trees to EMFs through ectomycorrhizae (Hou et al. 2012). Our results show that fruitbodies of *L. aurantiacum* (mean $\delta^{13}C$ of -25.6 ±0.2‰) were enriched in heavy carbon isotopes when compared to branches and leaves of hybrid aspen in the research site (mean $\delta^{13}C$ of -27.5 ±0.2‰ and -28.8 ±0.1‰, respectively, Bardule 2019) as well as when compared to the soil O layer (mean $\delta^{13}C$ of -29.8 ±0.2‰).

Hou et al. (2012) reported that the $\delta^{15}N$ values of EMFs ranged from 2.34 to 4.54‰, with a mean value of 3.79 ±0.67‰, while Trudell et al. (2004) reported a mean $\delta^{15}N$ value of EMFs in old-growth conifer forests of 5.1‰. In the current studies, the mean $\delta^{15}N$ value in fruitbodies of *L. aurantiacum* was 8.9 ±0.1‰. The EMFs obtain their nitrogen from growing substrates and appear to be key mediators of N movement in the plant–soil system, influencing isotopic patterns (Hobbie and Hogberg 2012). Ectomycorrhizal fungi can assimilate nitric-, ammonium- and protein-nitrogen with high efficiency through their widespread hyphal network, providing nitrogen to plants (their symbiosis partners) (Hou et al. 2012). Thus, EMFs provide plants with access to organic N forms that are usually richer in ^{15}N than the inorganic forms generally considered available to plants (Hobbie and Hogberg 2012). This is also confirmed by the results of this study – both in 2014 and in 2015 higher $\delta^{15}N$ values in fruitbodies of *L. aurantiacum* were detected in plots where wastewater sludge and digestate fertilizers has been initially applied if compared to control plots ($p < 0.05$). This finding indicates the impact of the organic fertilizers (wastewater sludge and digestate) on the N isotopic composition of fruitbodies of *L. aurantiacum* even 4–5 years after the application of fertilizers. In comparison with nitrogen in the soil O layer (mean $\delta^{15}N$ of -8.1 ±0.8‰), *L. aurantiacum* was enriched in ^{15}N. Brearley et al. (2005) found that the symbiotic relationship is probably the reason for the ^{15}N enrichment in EMFs, which retain ^{15}N-enriched N and transfer ^{15}N-depleted N to plant hosts (Hobbie and Hogberg 2012, Hou et al. 2012). This was confirmed by the results of the analysis of hybrid aspen branches and leaves; the mean $\delta^{15}N$ in hybrid aspen branches was 0.3 ±0.4‰, while that in hybrid aspen leaves was 2.7 ±0.4‰ (Bardule 2019).

Heavy metal contents

In general, the mycelium of fungi is designed to accumulate various elements, including heavy metals, in their fruitbodies, resulting in higher concentrations than those of the substrate where they live (Campos et al. 2009). Thus, fungi are considered environmental biomonitoring indicators to evaluate the level of the environment contamination as well as the quality of the ecosystem (Strumińska-Parulska et al. 2016). The level of the transfer of heavy metals into fruitbodies is affected by numerous factors such as fungi species (genetic characteristics), chem-

ical parameters of the substrate (substrate composition, heavy metal content, humus content, pH, although contradictory results have been reported), the mobility and availability of the metals, the age of the mycelium and, probably, the interval between fructification events (Kalac and Svoboda 2000, Gadd 2003, 2004, Rudawska and Leski 2005, Benbrahim et al. 2006, Cocchi et al. 2006, Falandysz and Bielawski 2007, Zhang et al. 2008, Arvay et al. 2014). In previous studies, it has been concluded that *L. aurantiacum* is very efficient in accumulating heavy metals in its fruitbodies (cap and stipes) (e.g., Falandysz et al. 2012, Strumińska-Parulska et al. 2016). Comparing the mean contents of heavy metals in fruitbodies of *L. aurantiacum* and in the soil O layer, we detected significantly higher levels of Cd, Cu, and Zn in fruitbodies of *L. aurantiacum*, highlighting the intensity of heavy metal accumulation. Similarly, Falandysz (2018) and Mędyk et al. (2018) also concluded that specifically Cd, Cu, and Zn are the elements to be bioconcentrated by *Leccinum* in fruiting bodies. Furthermore, there was a positive correlation between Cd and Zn content is soil O layer and fruitbodies of *L. aurantiacum* in 2018 (*r* values of 0.5 and 0.6, respectively), although in most cases, no correlation between heavy metal content in soil and fruitbodies of *L. aurantiacum* was found. Similar results have been observed by other authors (e.g., Alonso et al. 2003, Benbrahim et al. 2006). In general, our results of the heavy metal content in fruitbodies of *L. aurantiacum* are in line with previously reported findings in uncontaminated areas (Table 5).

According to the World Health Organization (WHO 2010), Pb and Cd are two of the 10 elements of major public health concern. They are highly toxic and often occur in fungi, including edible ones (e.g., García et al. 1998, Cocchi et al. 2006, Yamac et al. 2007, Campos et al. 2009, Arvay et al. 2014). In our study, the mean Pb content in fruitbodies of *L. aurantiacum* (0.18 ±0.06 mg kg^{-1} dry matter or 0.018 ±0.006 mg kg^{-1} fresh weigh, considering that the mean water content in fruitbodies of fungi is 90% according to Kalac (2009)) was below the threshold value, 0.10 mg kg^{-1} fresh weight, specified in the EC regulations (EC 2006). Similarly, the mean Cd content in fruitbodies of *L. aurantiacum* (0.44 ±0.04 mg kg^{-1} dry matter or 0.044 ±0.004 mg kg^{-1} fresh weigh) was below the threshold value, 0.05 mg kg^{-1} fresh weight, specified in the EC regulations (EC 2006), although in 34% of the analysed cases, the Cd content in fruitbodies of *L. aurantiacum* exceeded the threshold value of 0.05 mg kg^{-1} fresh weight.

Commercial forests and afforested lands offer several advantages for application of bioenergy production (wood ash and digestate) and municipal waste (sewage sludge) by-products as fertilizers (e.g., Ozolinčius et al. 2005, 2007, Saarsalmi and Levula 2007, Bardule et al. 2013, Heinsoo and Dimitriou 2014, Petaja et al. 2018). Still, the presence of potentially toxic elements especially in sewage sludge and wood ash poses a risk of environment contamination, an issue that needs to be investigated in detail (e.g., Benbrahim et al. 2006, Moilanen et al. 2006, Zabowski et al. 2009). We concluded that the application of digestate, sewage sludge and wood ash with low trace element concentrations as fertilisers to improve soil quality (availability of nutrients) in hybrid aspen (*Populus tremuloides* Michx. × *Populus tremula* L.) plantations in agricultural land did not result in increased heavy metal accumulation in fruitbodies of *L. aurantiacum*, but N isotopic compositions were altered (^{15}N-enriched) for a period of up to five years after application of the organic fertilizers (digestate and sewage sludge).

Table 5. Comparison of heavy metal content in fruitbodies of *L. aurantiacum* in the research site (n = 64 pooled samples) and previously reported findings in uncontaminated areas

Heavy metal	Content, mg kg^{-1} dry matter			Range for *Leccinum* species detected in previous studies in uncontaminated areas, mg kg^{-1} dry matter	Range for different species of wild-grown fungi detected in previous studies in uncontaminated areas, mg kg^{-1} dry matter
	min	max	mean ±S.E.		
Zn	15	129	56 ±4	180–190 (*L. aurantiacum*) [19] 38–270 (*L. scabrum*) [20, 22, 24]	25 to 300 [1- 6]
Cu	8.4	101	38 ±3	10.6–136 (*L. aurantiacum*) [19, 23] 6.0–42 (*L. scabrum*) [20, 22, 24] 36 (*L. holopus*) [23]	20 to 100 [1, 3, 6]
Mn	3.5	102	8 ±2	4.6–43 (*L. scabrum*) [20, 23] 9.9 (*L. holopus*) [23]	5 to 250 [1, 3, 5-6]
Ni	0.20	1.50	0.50 ±0.05	2.25–7.1 (*L. aurantiacum*) [19, 23] 0.64–27 (*L. scabrum*) [20, 24] 4.4 (*L. holopus*) [23] 0.19-1.61 (*Leccinum* species) [21]	trace amounts to 15 [1, 5-7]
Cd	0.09	1.70	0.44 ±0.04	0.72–1.2 (*L. aurantiacum*) [19, 23] 0.57–8.8 (*L. scabrum*) [20, 18, 24] 6.3 (*L. holopus*) [23] 0.12–8.40 (*Leccinum* species) [21]	0.5 to 20 [1, 4, 6, 8-11]
Cr	0.04	2.60	0.43 ±0.08	0.54–3.33 (*L. aurantiacum*) [19, 23] 0.12–0.89 (*L. scabrum*) [20] 0.49 (*L. holopus*) [23]	0.5 to 10.0 [1, 6, 12-13] >10 [14-16]
Pb	0.02	2.30	0.18 ±0.06	0.03–0.91 (*L. aurantiacum*) [19, 23] 0.20–7.5 (*L. scabrum*) [18, 20, 24] <0.01 (*L. holopus*) [23] 0.09–4.57 (*Leccinum* species) [21]	<0.10 to 15 [1, 3, 5-6, 15, 17-18]

[1] Arvay et al. 2014, [2] Rudawska and Leski 2005, [3] Podlasińska et al. 2015, [4] Tuzen et al. 2007, [5] Giannaccini et al. 2012, [6] Ouzuni et al. 2009, [7] Chudzyński and Falandysz 2008, [8] Zhang et al. 2008, [9] Kalac et al. 1996, [10] Melgar et al. 1998, [11] Falandysz et al. 2008, [12] Demirbas 2001, [13] Konuk et al. 2007, [14] Kalac 2010, [15] Isildak et al. 2007, [16] Isildak et al. 2004, [17] García et al. 2009, [18] Kalac and Svoboda 2000, [19] Adamiak et al. 2013, [20] Zhang et al. 2013, [21] Pelkonen et al. 2006, 2008, [22] Alonso et al. 2003, [23] Koroleva et al. 2015, [24] Falandysz 2018.

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