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# Soluble phenolics' dynamics during litter decomposition in a montane Common beech forest ecosystem

## Динамика на растворливите феноли во текот на деградацијата на мртвата органска материја во планински буков шумски екосистем

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Secondary metabolites have a specific role in the organic matter and nutrient cycling in forest ecosystems, especially in the process of litter decomposition. Decomposition of Common beech (*Fagus sylvatica* L.) leaf, branch, and acorn litter was followed by litter-bag experiments in the Common beech ecosystem Calamintho grandiflorae-Fagetum in Mavrovo National Park (Macedonia) for almost 7 years. The main goal of the present study was to analyze the dynamics of soluble phenolic compounds during litter decomposition. We analyzed total phenolics, flavonoids and anthocyanins content in the course of the decomposition of leaves, branches and acorns.

The concentration of total phenolics, flavonoids and anthocyanins in leaves decreased following a simple exponential model. All of these compounds followed a 3-step dynamics in branch and acorn litter. The decrease in absolute mass of all compounds in all three litter fractions was described by simple exponential or logarithmic models.

Total phenolics, flavonoids and anthocyanins were found in low concentrations. These low concentration point out to a new hypothesis, yet to be proved, that the phenolics do not slow down the decomposition process and contribute to the low limit value for this forest ecosystem.

**Key words**: total phenolics, flavonoids, anthocyanins, litter decomposition, Common beech (*Fagus sylvatica*), leaves, branches, acorns.

Секундарните метаболити имаат специфична улога во кружењето на материјата во шумските екосистеми, а особено во деградацијата на мртвата органска материја. Деградацијата на мртвата органска материја (листови, гранчиња и буклинки) беше следена со помош на огледни вреќички за деградација во буковиот екосистем Calamintho grandiflorae-Fagetum во Националниот парк „Маврово" (Македонија) во период од скоро 7 години. Главна цел на овој труд беше да се испита динамиката на фенолните соединенија во текот на деградацијата на мртвата органска материја. За реализација на целта на овој труд беше извршена анализа на концентрацијата и количеството на вкупни растворливи феноли, флавоноиди и антоцијани.

Концентрацијата на вкупни феноли, флавоноиди и антоцијани во листовите се намалуваше според прост експоненцијален модел. Во гранчињата и буклинките, сите испитувани фенолни соединенија покажаа 3-степена динамика во текот на деградацијата. Вредностите за вкупното количество (маса) феноли, флавоноиди и антоцијани во сите три испитувани фракции покажаа континуирано намалување, кое најдобро се опишува со прости експоненцијални или логаритамски модели.

Резултатите од ова истражување се во согласност со претходните констатации за ниската гранична вредност на деградацијата на мртвата органска материја во буковиот екосистем во Националниот парк „Маврово". Добиените резултати за релативно ниска концентрација на вкупните феноли, флавоноиди и антоцијани веројатно ј поддржуваат хипотезата дека овие секундарни метаболити не го забавуваат процесот на деградација.

**Клучни зборови**: вкупни феноли, флавоноиди, антоцијани, деградација на мртвата органска материја, бука (*Fagus sylvatica*), листови, гранчиња, буклинки.

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## Introduction

Litter decomposition in forest ecosystems is one of the crucial ecological processes that impact the carbon and nutrients' cycles in the biosphere. It is a major source of carbon (C) for the atmosphere and it provides available soil nutrients at an ecosystem level (Trofymow et al. 1995; Berg and McClaugherty 2008). Humus formation is also part of litter decomposition, which produces a steady pool of soil nutrients that determine key ecosystem processes like primary productivity and cycling of nutrients (Swift et al. 1979; Berg and McClaugherty 2014). Microorganisms with an ability to decompose plant tissues are the principal actors of litter decomposition, and they are accompanied by soil fauna with varying roles in different forest ecosystems (Hristovski et al. 2014).

The limit value of litter decomposition determines the amount of humus to be produced in the soil. In some Common beech (*Fagus sylvatica*) forest ecosystems in Europe it was found to be about 50% of the initial litter mass (Berg et al. 1996). However, litter in the Common beech forest ecosystem in Mavrovo National Park decomposed almost completely without a calculable limit value (Hristovski et al. 2014). The high concentration of manganese (Mn) and low nitrogen (N) level might be one of the reasons for this limitless decomposition. Mn has been proven to be an essential part of the peroxidase enzymes that degrade lignin in plant tissues (Hatakka and Hammel 2011). High N concentration in later stages tends to retard the decomposition rate by suppression of the activity of ligninases (Kirk 1980).

Soluble litter compounds and the ones with a low molecular weight are the first to decompose. This is followed by decomposition of hemicelluloses, celluloses and, finally, lignin (Aber et al. 1984; Berg and McClaugherty 2008).

Carbohydrates, lipids, proteins, chlorophyll, and nucleic acids are primary metabolites regulating the essential cell processes. In addition to primary metabolites, plant cells are able to produce secondary metabolites with a role in plant defense and protection of essential molecules, coloration, and odor of certain plant organs, and sometimes with a specific role in vital cell processes as follows: transportation, growth regulation, signalling etc. (Croteau et al. 2000; Boudet 2007). Based on their biosynthetic origins, plant secondary metabolites can be subdivided into four major groups: phenols, terpenoids, steroids, and alkaloids (Croteau et al. 2000).

Plant phenolics are secondary metabolites that encompass several classes of structurally diverse natural products arising from the shikimate-phenylpropanoids-flavonoids pathways (Ververidis et al. 2007). These compounds confer various physiological functions to plants, so that they can survive and adapt to environmental disturbances (Landolt et al. 1997; Andersen 2003; Lattanzio et al. 2009). It is known that concentration of total phenolics increases with the ageing of tree leaves (Sỳvacỳ and Sökmen 2004), which is also the case with ageing of fine roots (Watteau et al. 2002).

Amongst the phenolic compounds, flavonoids have received significant attention in the past few years because they appear to have diverse functions in plants; for example, as antimicrobial agents (phytoalexins), photoreceptors, visual attractors (flower pigments), UV protectants, feeding repellents (insect and herbivore protectants), signals in the early steps of rhizobium-legume symbiosis, regulators of auxin transport and stimulators of pollen germination (Harborne and Williams 2000; Williams et al. 2004; Zimmer et al. 2005). Flavonoids are primarily present in leaves as water-soluble glycosides in the vacuoles of epidermal cells and on the upper leaf surface in the epicuticular wax. According to the modifications of the central C-ring, flavonoids may be divided into different structural classes, such as flavanones, isoflavones, flavones, flavonols, flavanols, and anthocyanins.

Anthocyanins represent a class of flavonoids providing the colors in many flowers, leaves, fruits, seeds and other tissues (Mol et al. 1998). Nonetheless, certain anthocyanins possess antiviral, antibacterial and fungicidal activities but these effects are less effective than those of other phenolic compounds (Lev-Yadun and Gould 2009).

Plant litter is the major source of phenolics in the soil although a number of them are produced or transformed by decomposers - microorganisms. A vast array of primary organic polymers (lignin, tannins, polyphenols) and monomer (flavonoids, phenolic acids) compounds are transformed during litter decomposition into a complex mixture of aromatic structures making the substance of soil organic matter (Haider et al. 1975). On the other hand, soluble phenolics are known to influence the soil biota (Kuiters 1990) and litter decomposition rate (Horner et al. 1988). In general, phenolics (especially tannins and polyphenols) slow down the litter decomposition rate. In this context, litter with high content of tannins is less palatable to detritivores and fungi (Harrison 1971). Phenolics also suppress the nitrifying organisms, thereby lowering the decomposition rate (Kuiters 1990). The polyphenol-protein complexes are resistant to decomposition and also contain N that is not readily accessible to decomposers.

Having in mind the importance of secondary metabolites in the overall material turnover in forest ecosystems, the main goal of the present study is to analyze the dynamics of soluble phenolics during litter decomposition in the Common beech ecosystem Calamintho grandiflorae-Fagetum in Mavrovo National Park. We analyzed total phenolics, flavonoids and anthocyanins content in course of the decomposition of leaf, branch and acorn litter, by applying litter-bags.

## Study Site

The study was conducted in Mavrovo National Park in western Macedonia. The research statio-n and forest stand are situated in a well-developed, about 70-80-year-old Common beech forest in Bistra Mt., Leunovo village area, in the vicinity of Mavrovo Reservoir at 41°42' N and 20°48' E and at an altitude of 1,400 m.

The climate is mountain-continental with a Mediterranean influence (Filipovski et al. 1996). The average annual temperature is 7.1°C and the mean annual precipitation is 1,103 mm (Lazarevski 1993).

Common beech is the dominant tree species at the study site, with a density of 1,200 trees $ha^{-1}$ and a mean diameter at breast height (DBH) of approximately 16 cm. The understory is predominantly represented by Common beech and Macedonian Fir (*Abies borisii-regis*). The herb layer at the study site has low biomass with

less than 6 kg $ha^{-1}$ (Melovski et al. 2004b). Aboveground annual litter fall biomass is 4.98 t $ha^{-1}$, 3.44 t $ha^{-1}$ of which is foliar litter, 1.28 t $ha^{-1}$ branches, and 0.25 t $ha^{-1}$ acorns (Šušlevska et al. 2001); unpublished data).

The plant community develops on a dystric cambisol soil type (Ol-$A0_{f/h}$-A-B-(B)C-C). The forest floor is 6 cm thick on average, with the upper mineral soil very rich in humic compounds, with more than 10 % organic matter and a pH of 5.8 (Table 1). The total content of humus as well as the total concentrations of N, P, potassium (K) and Mn clearly decrease with depth, whereas those of iron (Fe) and sodium (Na) tend to increase with depth (Hristovski et al. 2014). The average litter layer organic mass is 20.6 t $ha^{-1}$, which may be subdivided into the following: unaltered leaves - 1.23 t $ha^{-1}$, fragmented leaves - 4.47 t $ha^{-1}$, amorphous matter - 10.84 t $ha^{-1}$, branches - 3.37 t $ha^{-1}$, acorns - 0.67 t $ha^{-1}$ (Melovski et al. 2004a).

## Materials and methods

*Litter collection, storage, weighing, field incubations and sampling of litter-bags.*

Decomposition of leaf litter of Common beech was measured by using 1.5-mm-mesh nylon bags (20 x 20 cm) (Bocock et al. 1960). In November 1997, newly shed leaves of Common beech were collected just after the main leaf fall. Litter material air-dried at room temperature was used, and amounts equi-valent to 10 g of absolutely dry leaves, were inserted into 123 separate bags, placed in three plots with different terrain configuration, all located within a fenced area of 1 ha (the exact moisture content was determined on separate samples, dried at 105 ºC). The litter-bags were laid on top of the litter layer and in time covered by new litter fall. Incubation took place on November 15, 1997. One bag was collected every month from each of the three plots, and after April 2000 every 3rd month. The total duration of the experiment was 6.19 years or 2,260 days.

*Determination of mass loss*

After sampling, plant remains, roots and soil particles were removed from the litter-bags. The remaining dry mass was determined by drying the samples to constant mass at 105 ºC. The mean values of mass loss (n=3) were calculated for each sampling (Hristovski et al. 2014).

*Chemical analysis*

Phenolic compounds extraction and quantification were performed as previously reported (Causevic et al. 2005; Gadzovska et al. 2007). Briefly, phenolic compounds were extracted from freeze-dried and powdered plant material (0.2–0.5 g) with 80 % (v/v) methanol in ultrasonic bath for 30 min at 4 ºC.

Total phenol content was determined when methanolic extracts were mixed with Folin–Ciocalteau reagent (Carlo Erba Reagenti, Rodano, Italy) and 0.7 M $Na_2CO_3$. The samples were incubated for 5 min at 50 ºC, and then cooled for 5 min at room temperature. Absorbance was measured spectrophotometrically at 765 nm. The concentration of total phenolic compounds was calculated applying (+)-catechin (0–10 mg $mL^{-1}$) as a standard.

The total flavonoid contents were determined in methanolic extracts (Markham 1989). The extracts were filtered through Sep-pack C18 cartridges (Waters) in order to exclude chlorophyll and carotenoid pigments (solid-phase extraction). Spectrophotometric measurements of the absorbance were made at 360 nm. Molar extinction coefficient of quercetin ($\varepsilon_{360}$ = 13.6 $mM^{-1}$ $cm^{-1}$) was used to identify total flavonoid contents.

Anthocyanin identification was conducted as described by Giusti et al. (1999). Anthocyanins were extracted from freeze-dried and powdered plant material (0.2–0.5 g) with 2 mL solution of 1 % $HCl/CH_3OH$ (15/85, v/v) ultrasonicated for 60 min at 4 ºC and then centrifuged at 20,000 x g for 30 min. The absorbance of the supernatant was measured at 530 nm. The anthocyanin content was calculated by employing the molar extinction coefficient of cyaniding-3-glucoside ($\varepsilon_{530}$ = 34,300 $M^{-1}$ $cm^{-1}$) in acidic methanol.

## Results

The initial values of total phenolics in the leaf litter were rather low (Tab. 1). The value of 19.6 mg·$g^{-1}$ is lower than the one for senescent Common beech leaves of ~80 mg·$g^{-1}$ (Bärlocher and Graça 2005).

The dynamics of total phenolics concentration in leaves, branches and acorns is presented in Fig. 1. An initial decrease of total phenolics is evident in all three litter fractions. A constant decrease of phenolics in leaf litter was observed throughout the litter-decomposition experiment. In both branch and acorn litter, the concentration of total phenolics increased and once again decreased by the end of the experiment.

The absolute mass of total phenolics decreased continuously in all three organs (Fig. 2).

The concentration of flavonoids in leaves decreased persistently. Flavonoids in branches and acorns had three-step dynamics with initial decrease, "accummulation" in the late stage, and release in the final stage of Common beech litter decomposition (Fig. 3). Similar to the case of total phenolics, the mass of flavonoids in all three organs decreased constantly. The decrease in acorns was pronounced in the initial stage of litter decomposition.

Table 1. Initial concentration of total phenolics, flavonoids and anthocyanins in the Common beech litter in Mavrovo National Park.

| Phenolics | Leaves | Branches | Acorns |
|---|---|---|---|
| Total phenolics (mg·$g^{-1}$) | 19.6 | 13.5 | 23.5 |
| Flavonoids (mmol·$g^{-1}$) | 2.5 | 1.6 | 2.3 |
| Anthocyanins (µg·$g^{-1}$) | 63.5 | 37.3 | 69.4 |

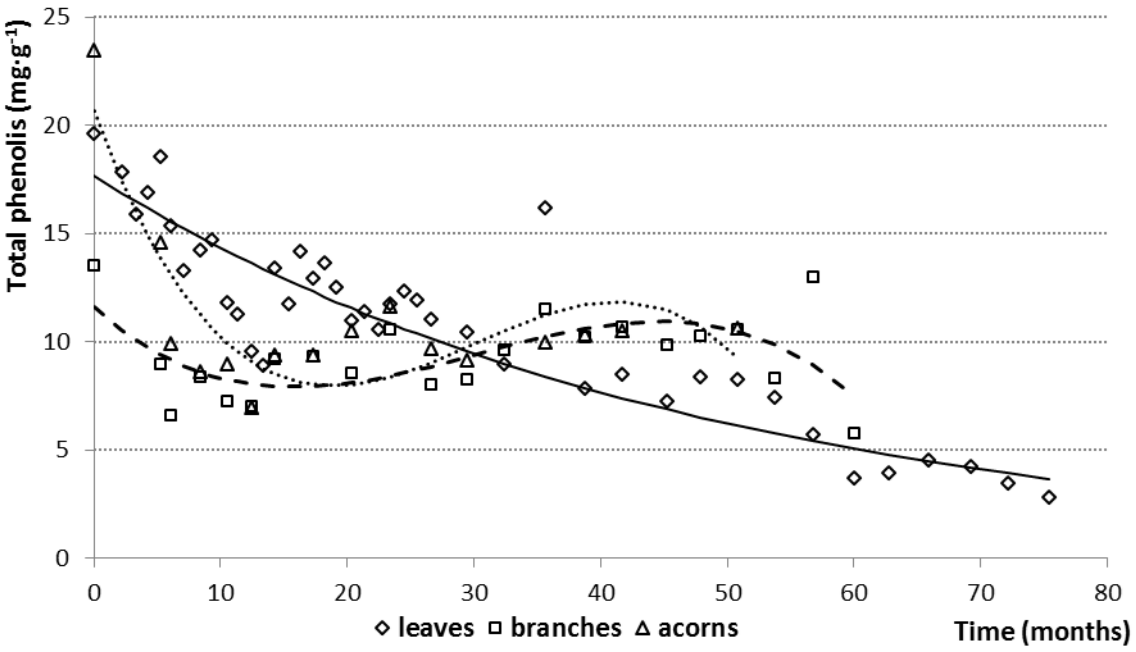


Figure 1. Total phenolics content in leaves, branches and acorns in the course of litter decomposition (The mathematical model of total phenolics in leaves is presented with full line, the one for branches with dashed line and the one for acorns with dotted line).

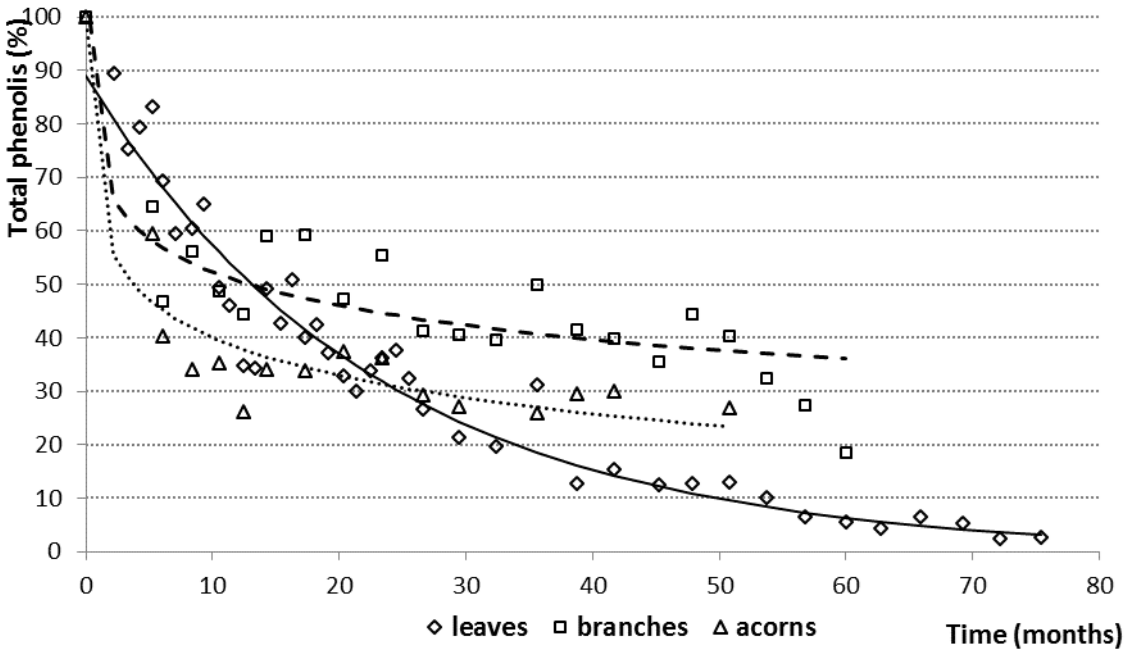


Figure 2. Absolute mass of total phenolics (in %, relative to the initial mass) in leaves, branches and acorns in the course of litter decomposition (lines as in Fig. 1).

Flavonoids concentration is presented in $mmol \cdot g^{-1}$. We used the molecular weight of 417,9 $g \cdot mol^{-1}$ (Oganesyan et al. 1972) in order to calculate the mass fraction (concentration in $mg \cdot g^{-1}$) of flavonoids in Common beech litter in Mavrovo National Park. The resulting values of initial concentrations for leaves, branches and acorns were 1.0, 0.7 and 1.0 $mg \cdot g^{-1}$, respectively.

The patterns of the dynamics of anthocyanins' concentration in leaves, branches and acorns were very similar to the ones of total phenolics and flavonoids (Fig. 5). This is apparent in the case of mass dynamics of anthocyanins, too (Fig. 6).

All mathematical models (Tab. 2 and 3) describing the dynamics of analyzed phenolics in leaves, branches and acorns (concentration and mass) were statistically significant ($p<0.05$). The only exception was the concentration of anthocyanins in branches ($p<0.1$).

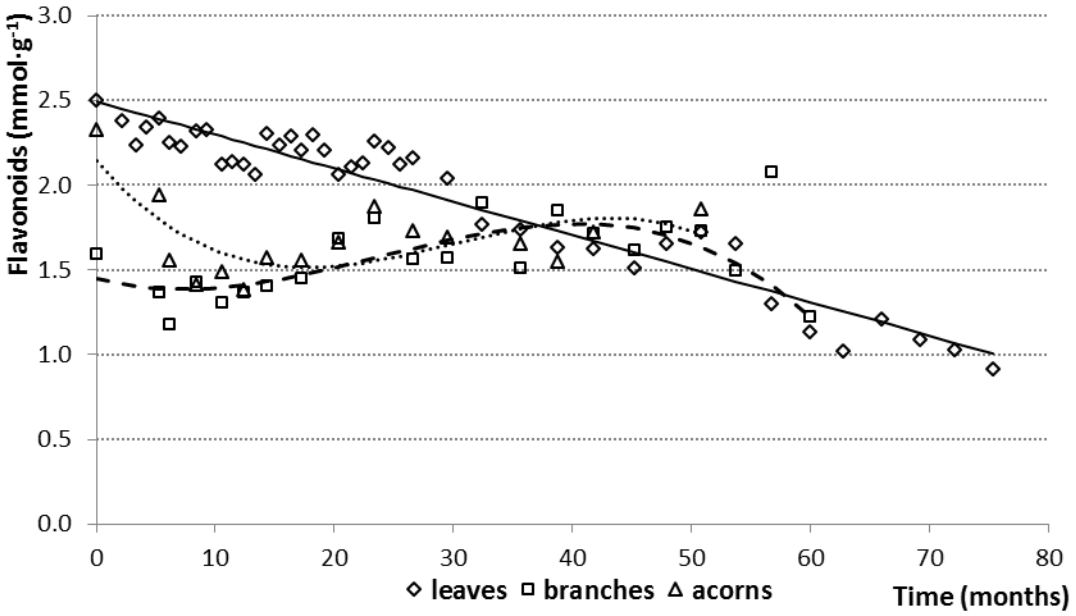


Figure 3. Flavonoids content in leaves, branches and acorns in the course of litter decomposition (lines as in Fig. 1).

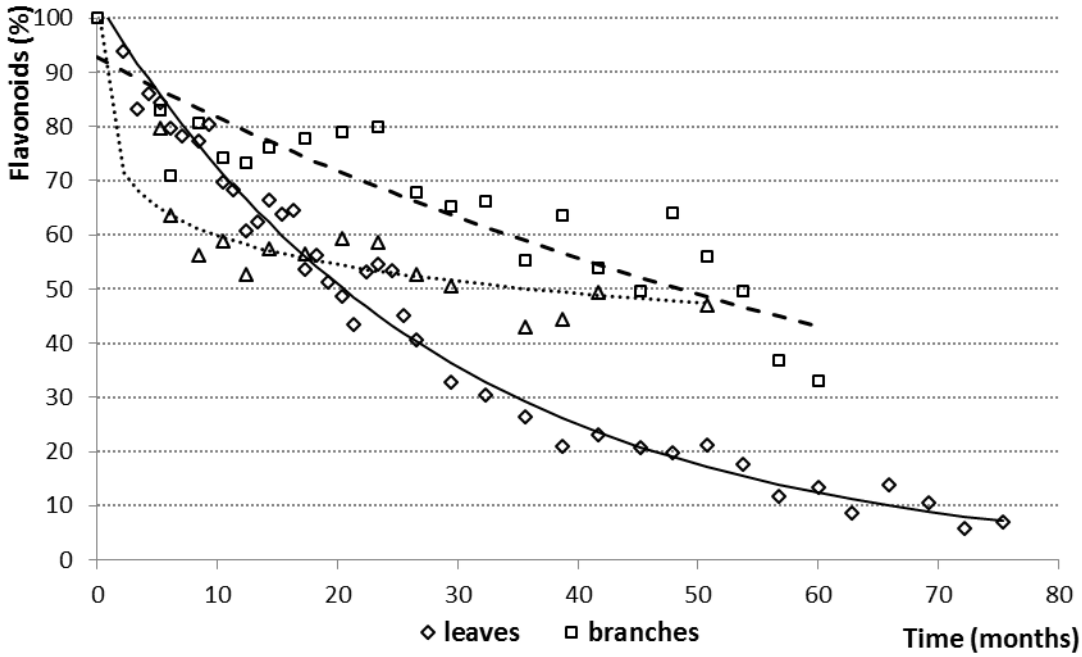


Figure 4. Absolute mass of flavonoids (in %, relative to the initial mass) in leaves, branches and acorns in the course of litter decomposition (lines as in Fig. 1).

Exponential models were employed to elucidate the dynamics of analyzed phenolics in leaves. Polynomial models of third order were used for the dynamics of phenolics in branches and acorns (Tab. 2). Our presumption is that the correlation coefficients ($R^2$) for branches are lower due to the different branch diameters in the litter-bags.

## Discussion

**Total phenolics.** It is known that the concentration of phenolics in plant leaves depends on soil properties. The concentration of phenolics is high in nutrient-poor soils. One of the hypotheses explaining this phenomenon is defense from herbivory (Nicolai 1988).

The initial concentration of phenolics in the Common beech leaves in Mavrovo National Park was 19.6 mg·g$^{-1}$. This value of total phenolics is higher than the values for two Common beech stands in Germany 12.0 and 15.1 mg·g$^{-1}$ (Nicolai 1988). Other broadleaf species (e.g. *Quercus robur, Q. rubra, Prunus serotina, Amelanchier lamarckii* and *Sorbus aucuparia*) have higher concentration of total phenolics in general, ranging from 59.6 to 98.3 mg·g$^{-1}$ (Hoorens et al. 2003).

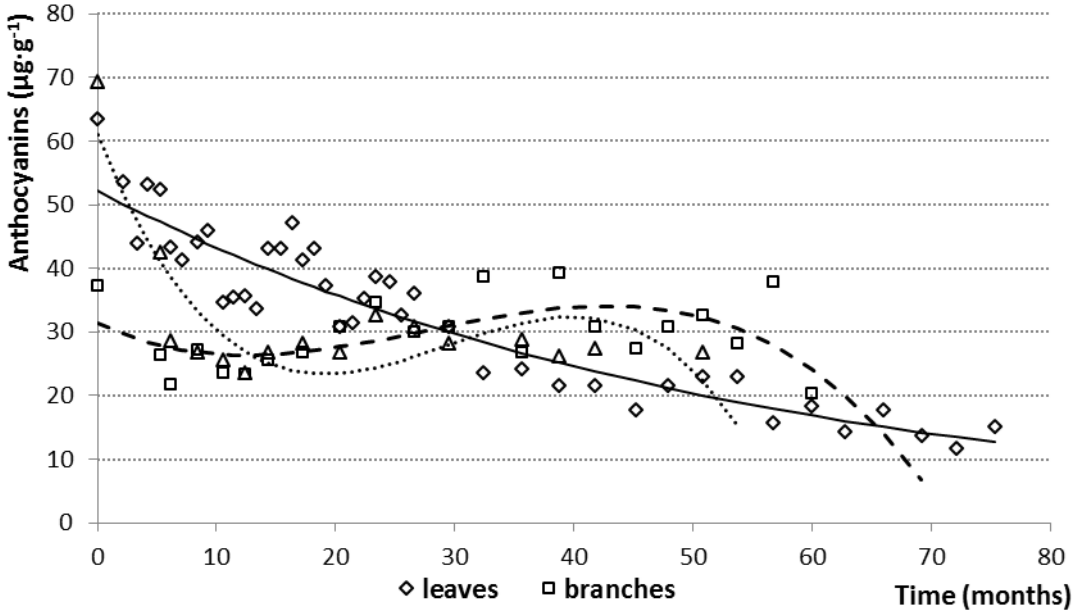


Figure 5. Anthocyanins content in leaves, branches and acorns in the course of litter decomposition (lines as in Fig. 1).

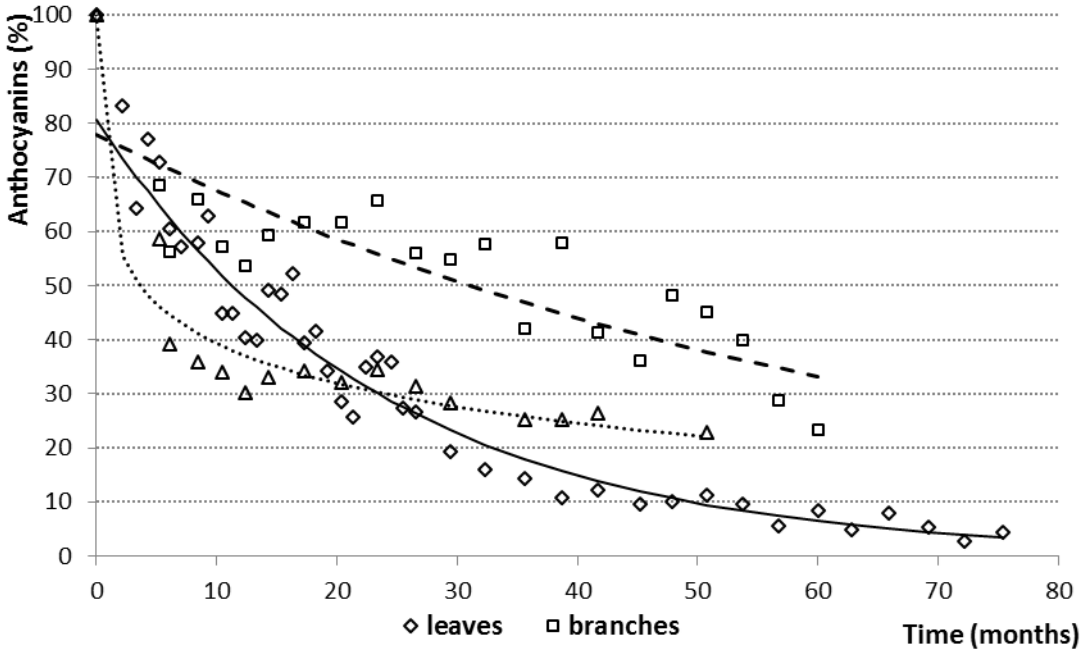


Figure 6. Absolute mass of anthocyanins (in %, relative to the initial mass) in leaves, branches and acorns in the course of litter decomposition (lines as in Fig. 1).

Nevertheless, other broadleaf tree species have lower initial phenolics concentration - *Salix exigua* of 5 $mg \cdot g^{-1}$ (Schofield et al. 1998). It appears that needle leaf litter has higher concentration of comparable phenolics than the one of Common beech in Mavrovo: 20-32 $mg \cdot g^{-1}$ in *Picea, Pinus, Abies, Tsuga* and *Pseudotsuga* (Gallet and Lebreton 1995; Hoorens et al. 2003).

The concentration and mass of total phenolics during decomposition of Common beech leaf litter decreased continuously (Figs. 1 and 2). Similar results were obtained for two Common beech forests in Germany (Nicolai 1988). In both studies, an initial rapid decrease of total phenolics was observed. This finding can be accounted for by leaching of the soluble phenolics' fraction (Kuiters and Sarink 1986). The soluble fraction of total phenolics in *Picea abies* amounts up to 10 % (Gallet 1994), yet, there are no data on Common beech leaf litter. Decomposition rate (k) for total phenolics is 0.25, which is lower than the one for whole Common beech leaf litter - 0.30 (Hristovski et al. 2014). The aforesaid implies that the disappearance of total phenolics from leaf litter is slower than the entire leaf mass.

Table 2. Mathematical models for the time (year) dependence of total phenolics ($mg \cdot g^{-1}$), flavonoids ($mmol \cdot g^{-1}$) and anthocyanins ($\mu g \cdot g^{-1}$) concentration during litter decomposition.

| Phenolic compounds | Organ | Model | A | b | c | d | $R^2$ | F | p | n |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaves | $e^{a+bt}$ | 2.496 | -0.254 | | | 89.41 | 329.29 | 0.0000 | 41 |
| **Total phenolics** | Branches | $a+bt+ct^2+dt^3$ | 11.612 | -6.471 | 3.372 | -0.452 | 38.50 | 3.76 | 0.0296 | 22 |
| | Acorns | $a+bt+ct^2+dt^3$ | 20.662 | -19.329 | 9.082 | -1.223 | 71.99 | 10.28 | 0.0012 | 16 |
| | Leaves | $a+bt$ | 20.496 | -0.241 | | | 92.17 | 471.10 | 0.0000 | 42 |
| **Flavonoids** | Branches | $a+bt+ct^2+dt^3$ | 1.451 | -0.228 | 0.224 | -0.038 | 62.69 | 9.52 | 0.0006 | 21 |
| | Acorns | $a+bt+ct^2+dt^3$ | 2.144 | -0.996 | 0.481 | -0.064 | 46.86 | 3.53 | 0.0487 | 16 |
| | Leaves | $e^{a+bt}$ | 3.955 | -0.229 | | | 90.77 | 393.36 | 0.0000 | 42 |
| **Anthocyanins** | Branches | $a+bt+ct^2+dt^3$ | 31.397 | -10.809 | 6.786 | -0.993 | 30.79 | 2.67 | 0.0786 | 22 |
| | Acorns | $a+bt+ct^2+dt^3$ | 60.928 | -56.598 | 26.566 | -3.646 | 74.86 | 11.91 | 0.0007 | 16 |

Table 3. Mathematical models for the time (year) dependence of absolute mass (%) of total phenolics, flavonoids and anthocyanins during litter decomposition.

| Phenolic compounds | Organ | Model | A | b | c | d | $R^2$ | F | p | n |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaves | $e^{a+bt}$ | 4.491 | -0.536 | 96.19 | 1011.21 | 0.0000 | 42 | 0.0000 | 41 |
| **Total phenolics** | Branches | $a+b \cdot \ln(t)$ | 50.537 | -9.088 | 79.11 | 75.73 | 0.0000 | 22 | 0.0296 | 22 |
| | Acorns | $a+b \cdot \ln(t)$ | 38.098 | -10.306 | 90.13 | 127.81 | 0.0000 | 16 | 0.0012 | 16 |
| | Leaves | $e^{a+bt}$ | 4.638 | -0.431 | 97.40 | 1498.91 | 0.0000 | 42 | 0.0000 | 42 |
| **Flavonoids** | Branches | $e^{a+bt}$ | 4.530 | -0.155 | 78.84 | 74.51 | 0.0000 | 22 | 0.0006 | 21 |
| | Acorns | $a+b \cdot \ln(t)$ | 58.346 | -7.702 | 86.02 | 86.16 | 0.0000 | 16 | 0.0487 | 16 |
| | Leaves | $e^{a+bt}$ | 4.392 | -0.514 | 95.66 | 881.28 | 0.0000 | 42 | 0.0000 | 42 |
| **Anthocyanins** | Branches | $e^{a+bt}$ | 4.356 | -0.174 | 71.32 | 49.75 | 0.0000 | 22 | 0.0786 | 22 |
| | Acorns | $a+b \cdot \ln(t)$ | 37.235 | 10.659 | 93.88 | 214.69 | 0.0000 | 16 | 0.0007 | 16 |

The concentration of phenolics in branches and acorns followed a 3-step dynamics (Figs. 3 and 5). The amount of total phenolics (Fig. 4 and 6) demonstrate a continual decrease, suggesting that there is no significant throughfall leachate input or secondary production of phenolics by decomposers. Slower decomposition in the late stage of decomposition should be attributed to some resistant phenolics' fraction.

**Flavonoids.** Flavonoids and some polyphenols can be regarded as antimicrobial agents, (Harborne and Williams 2000) pointing to their potential role as litter decomposition retardants. A number of studies suggest that flavonoids have a stimulative effect on certain basidiomycete fungi (Lindeberg et al. 1980) or they either stimulate or inhibit spore germination and hyphal growth of saprotrophic fungi (Hättenschwiler and Vitousek 2000). In spite of this, the concentration of flavonoids in the studied Common beech litter was considerably low ($_{\sim}$1 $mg \cdot g^{-1}$). In other plant species, higher values have been found: senescent leaves of *Morus alba* have 9.84-23.4 $mg \cdot g^{-1}$ (Zhishen et al. 1999).

The decomposition pattern of flavonoids in Common beech litter (Figs. 3 and 4) was very similar to the one of total phenolics. The continuous decrease in the flavonoids' mass (Fig. 4) implies that there is no significant secondary production of flavonoids during decomposition.

**Anthocyanins.** The role of anthocyanins in litter decomposition is not known well. Few data can be found in the literature regarding leaf senescing. The American Beech (*Fagus grandifolia*) has very low anthocyanin

concentration - old leaves have less than 50 $\mu g \cdot g^{-1}$. Other studied American species also have low concentration (*Betula, Acer, Castanea, Ilex, Populus*), while others (*Quercus, Acer, Cornus, Fraxinus, Prunus, Vaccinium*, *Viburnum*) have much higher anthocyanin concentration, with *Acer rubrum* being the highest – 1,000 $\mu g \cdot g^{-1}$ (Lee et al. 2003). Compared to these data, it is obvious that Common beech has low anthocyanin concentration.

The dynamics of concentration (Fig. 5) and mass (Fig. 6) of anthocyanins complied with the same patterns as in the case of total phenolics and flavonoids. Total phenolics concentrations in our experiment with Common beech litter contributed to continuous decomposition.

Total phenolics, flavonoids and anthocyanins were found in low concentrations. These low concentration point out to a new hypothesis, yet to be proved, that the phenolics do not slow down the decomposition process and contribute to the low limit value for this forest ecosystem.

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