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Original article

# The priming effects induced by earthworm mucus on mineralization and humification of plant residues

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ABSTRACT

The aim of this research was to determine priming effects (PEs) triggered by mucus of earthworms (*Aporrectodea caliginosa*, Savigny) in relation to plant residue mineralization and humification. The influence of mucus was compared with that of glucose and ammonium ($NH_4Cl$) as some easily available substrates added in amounts equivalent to the amount of organic carbon (glucose) or ammonium ($NH_4Cl$) in the initial mucus. To verify real PEs direct connected with plant residue turnover, fresh leaves fragments (*Elytrigia repense*) were mixed with quartz sand devoid of organic matter. The plant residue mineralization expressed in loss of organic carbon was stimulated (from 11% to 20%) by single-pulse inputs of all primers whereas humification expressed in increase (from 20 to 39%) of humic substances (HS) and humic acids (HA) contents was triggered only by earthworm mucus and ammonium addition. Thus, the real PE induced by earthworm mucus was confirmed for the first time. The greatest yields of humic acids as well as the greatest optical density of HA (HA aromatization) were found in samples treated by earthworm mucus. Hence, not only amount but the quality of soil humus was affected by earthworm mucus priming. The duration of the mucus-mediated priming effects may be delayed during 30–90 days depending upon soil parameters under study. The results highlight the importance of excretory activity of earthworms in quantitative and especially qualitative changes of humus.



## 1. Introduction

In soil biology the term priming effects describes the changes in the turnover of soil organic matter (SOM) mediated by adding organic or mineral substances: plant rhizodeposition, easy decomposable organic substances, mineral-N fertilizers [1–11]. Substances released by soil fauna were also found to produce a rapid priming response. Within the soil organisms, earthworms are in term of biomass and activity among the most important detritivores in terrestrial ecosystems [12]. The daily loss of carbon due to mucus excretion from the body surface and in casts of endogeic earthworms (*Octalasion lacteum*) in soil was calculated as 0.2–0.5% of total animal carbon [13]. One g of earthworms can produce, on average, 5.6 mg of mucus (dry weight) in 24 h [14]. The earthworm mucus is a water-soluble mixture of low molecular weight carbohydrates, aminoacids, glycosides and a glycoproteins [15,16]. Earthworm mucus can cause priming effects by stimulating microbial activity [15,17] and mineralization processes in casts and in soil profile over a few years scale [18].

Generally, many authors provide information on the influence of earthworms on transformation of plant residues when they are ingested. They did not, however, address the role of earthworms for priming effects inducing by earthworm mucus. It is not yet clear how affects single-pulse input of earthworm mucus on PEs in soils, when the ingestion is excluded. The pulse mucus impact can occur in making by the earthworms burrow walls (drillosphere). This is specifically true for mineralization and humification of plant residues. In terms of the effect of earthworm mucus on humification, information regarding induced by earthworm mucus changes in humic substances which were newly formed during plant residue decomposition is lacking. Humus is one of the important constitutes of soils affecting soil properties as well as global C cycle. For this reason, experimental model with earthworm mucus is crucial to develop both PE dynamic study and understanding of mechanisms related to mineralization and humification of plant residues.

The aim of this study was to investigate whether the mucus earthworm can cause priming effects on plant residue mineralization and humification. Individuals of most abundant in grasslands and agricultural ecosystems endogeic earthworm species *Aporrectodea caliginosa* were included in the experiment. We investigated the priming effects of earthworm mucus on (1) residue C dynamics, (2) the changes in content of humic substances (HS)

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and humic acids (HA) formed during plant residue decomposition, (3) the changes in degree of aromatization (maturity) of HA. The effect of earthworm mucus was compared with the effect of the well examined primers, such as glucose and ammonium.

## 2. Materials and methods

### 2.1. Earthworm mucus collection

Endogeic earthworm *A. caliginosa* Savigny were collected from umbric albeluvisols near Biological Research Institute of Saint-Petersburg State University, Peterhof, Russia. Mature earthworms with an individual biomass of 0.4 ± 0.1 g were selected for collection of mucus. The earthworms were rinsed with distilled water at least four times. The gut content was removed by storing adult specimens on moist filter paper during three days at 6 °C in the dark. The evacuation of the gut was determined visually, as the absence of dark soil particles. Then earthworms with empty gut were rinsed with distilled water and placed into ten Petri dishes with 20 ml distilled water (3 specimens per dish) for 24 h at the same temperature in the dark. After that, the earthworms were removed from the Petri dishes. The mucus solutions from ten Petri dishes were collected and thoroughly mixed for further input of the mixture in sand.

### 2.2. Experiment layout

To verify real PEs direct connected with plant residue turnover, the plant mass was thoroughly mixed (in 1:10) with quartz sand that was previously ignited at 700 °C so as to devoid of organic matter. Plant material for incubation was fresh leaves of couch grass (*Elytrigia repense* L.) with C-to-N ratio 24.7:1. The fresh plant leaves were cut into 2–3 mm pieces. The initial organic carbon content in dry plant-sand mixture was 13.4 mg C $g^{-1}$. At the start of the experiment, the mixture was once treated with mucus. 20 ml of the mucus solution derived as described above was thoroughly mixed with the samples. With the mucus were introduced $C_{org}$, 7 μg, and $NH_4^+$-N, 1 μg, per g dry samples. The effect of earthworm mucus was compared with the effect of easily available substrates, such as glucose and ammonium ($NH_4Cl$). Both glucose and ammonium are well examined primers which have been commonly used in basic PEs research [4,6]. As aqueous solution they were mixed with sand samples in amounts equivalent to the amount of organic carbon (glucose) or ammonium in the initial mucus. The sand samples were adjusted to 60% of the maximum water holding capacity with distilled water and were incubated in open plastic pots (165 g per pots) for 15, 30, and 90 days at 20–22 °C. The procedure allows to analyse of remaining C and therefore to determine real PEs expressed in both (i) mineralization rate changes and (ii) quantitative and qualitative changes of humus induced by different primers. There are the advantages as compared to measurements of $CO_2$ efflux (as in the most other studies) because the last technique may characterize only PEs expressed in changes of plant residue mineralization rates.

### 2.3. Analysis

The determination of humic substances (HS) based on the properties of solubility in the alkaline aqueous solutions used as extractants [19]. The term humic acids (HA) is used to indicate the humic substances soluble in dilute alkali but insoluble in dilute acid. The organic carbon content and humus composition of the samples was analyzed using Ponomareva and Plotnikova procedure [20]. HS were extracted with 0.1 mol $L^{-1}$ NaOH solution from 10 g of samples; HA – with 0.1 mol $L^{-1}$ NaOH solution after precipitation by 1.0 mol $L^{-1}$ $H_2SO_4$ solution. The total organic carbon content was determined by Tyurin dichromate-oxidation method [20]. In this procedure, 0.2 mol $L^{-1}$ potassium dichromate ($K_2Cr_2O_7$) solution was mixed with concentrated $H_2SO_4$ (1:1). Then the mixture was added to between 0.1 and 1.0 g (depending on organic carbon content) of sand samples. The samples and extraction solutions were gently boiled at 150 °C for 20 min. The addition of heat to the system leads to a complete digestion of the organic carbon in the samples. Titrimetric method was used for determination of organic C in samples [20]. In the method, excess dichromate was titrated with standardized 0.2 mol $L^{-1}$ Mohr's salt solution. The degree of aromatization (maturity) of HA ($Ec^{mg/ml}$) were calculated as follows [20]: $D/(C \cdot l)$, where $D$ – the optical density of solution at 440 nm in 0.1 mol $L^{-1}$ NaOH; $C$ – carbon concentration in the same solution, mg $ml^{-1}$; $l$ –optic way, sm. In this study, the optic way was 1 sm. As demonstrated [21,22], the measures for aromaticity of organic matter at 440–465 nm allows to avoid interfering influence induced by non-specific organic compounds of soil in comparison to measures at UV.

### 2.4. Statistics

The experiment consisted of four independent measurements of the parameter investigated: four soil (plant) samples were taken from four pots. Data were subjected to analysis of variance procedures (one-way ANOVA). Statistical significances of the means were determined by Student–Newman–Keuls (S–N–K) test at $P < 0.05$. Effect of primer (with three treatments – earthworm mucus, ammonium, glucose) on mineralization and humification rates were assessed by two-way ANOVA with the primer addition ("primer") and time of exposure (samplings after 15, 30 and 90 days, "time") as factors. When the ANOVA documented a significant primer effect, data were analysed separately using an ANOVA for each primer treatment (earthworm mucus addition, ammonium addition, glucose addition). All statistics were performed using IBM SPSS Statistics, version 19.

## 3. Results

### 3.1. Plant residue mineralization rates

Greater mineralization of fresh plant residue was observed during shorter first 15 days period, where nearly three times as much organic C was mineralized than between days 15 and 90 (Fig. 1A). The addition of primer as three treatments (mucus, glucose, and ammonium) significantly affected the mineralization rate (Table 1, Fig. 1A). Glucose and $NH_4Cl$ additions accelerated on average by 20% plant residue mineralization on the 15th days (GLUCOSE × TIME interaction, AMMONIUM × TIME interaction: $P < 0.001$, Table 2; Fig. 1A), whereas earthworm mucus increased the index by 11% on the 30th days after start of experiment (EARTHWORM MUCUS × TIME interaction: $P = 0.016$, Table 2; Fig. 1A) compared to the control without addition. Thus, there was observed the delay in the mucus effect as compared to glucose and ammonium. At ninety day after treatment the loss of organic C induced by all primers under study disappeared.

### 3.2. Plant residue humification rates

The greatest yields of HS and HA were found at 15–30 days whereas at the end of the experiment, the HS and HA contents decreased about by 2-times compared with that at 30 day (Fig. 1B). The addition of primer with three treatments (mucus, glucose, and ammonium) significantly affected the HS and HA contents (Table 1, Fig. 1B, 2A). Both HS content and HA content were strongly

increased by the earthworm mucus or ammonium addition and were not affected by glucose addition (Table 2; Figs. 1B and 2A). Ammonium addition increased the HS content on the 15th day after treatment by 39% and on the 30 days by 20% (AMMONIUM × TIME interaction: $P < 0.001$, Table 2; Fig. 1B), earthworm mucus addition increased the index at the same times on average by 21% (EARTHWORM MUCUS × TIME interaction: $P < 0.001$, Table 2; Fig. 1B) compared to control without addition. Ammonium stimulated HA content enhance by 45% on the 15th day (AMMONIUM × TIME interaction: $P = 0.003$, Table 2; Fig. 2A) whereas earthworm mucus – on the 30th day on average by 29% (EARTHWORM MUCUS × TIME interaction: $P = 0.001$, Table 2; Fig. 2A) in comparison with no treated control at the same times. No significant differences in the HS and HA contents were detected on the 90th day after beginning of the experiment (Figs. 1B and 2A). Throughout the experiment, the optical densities of HA significantly increased by all primers (Tables 1, 2; Fig. 2B). However, the highest values of the index were found for earthworm mucus treatment (at 30 and 90 days) that induced increase in optical density by 20% compared to the control without addition.

## 4. Discussion

### 4.1. Plant residue mineralization

Real priming effects are changes of SOM turnover whereas apparent priming effects are accelerated turnover of microbial biomass that is unconnected with SOM turnover [4]. In this study, plant residues were added to the sand devoid of organic matter during ignition. Therefore, the observed increase in soil C losses characterized the plant residue mineralization. Hence, there is evidence that accelerated decrease in carbon contents after primer

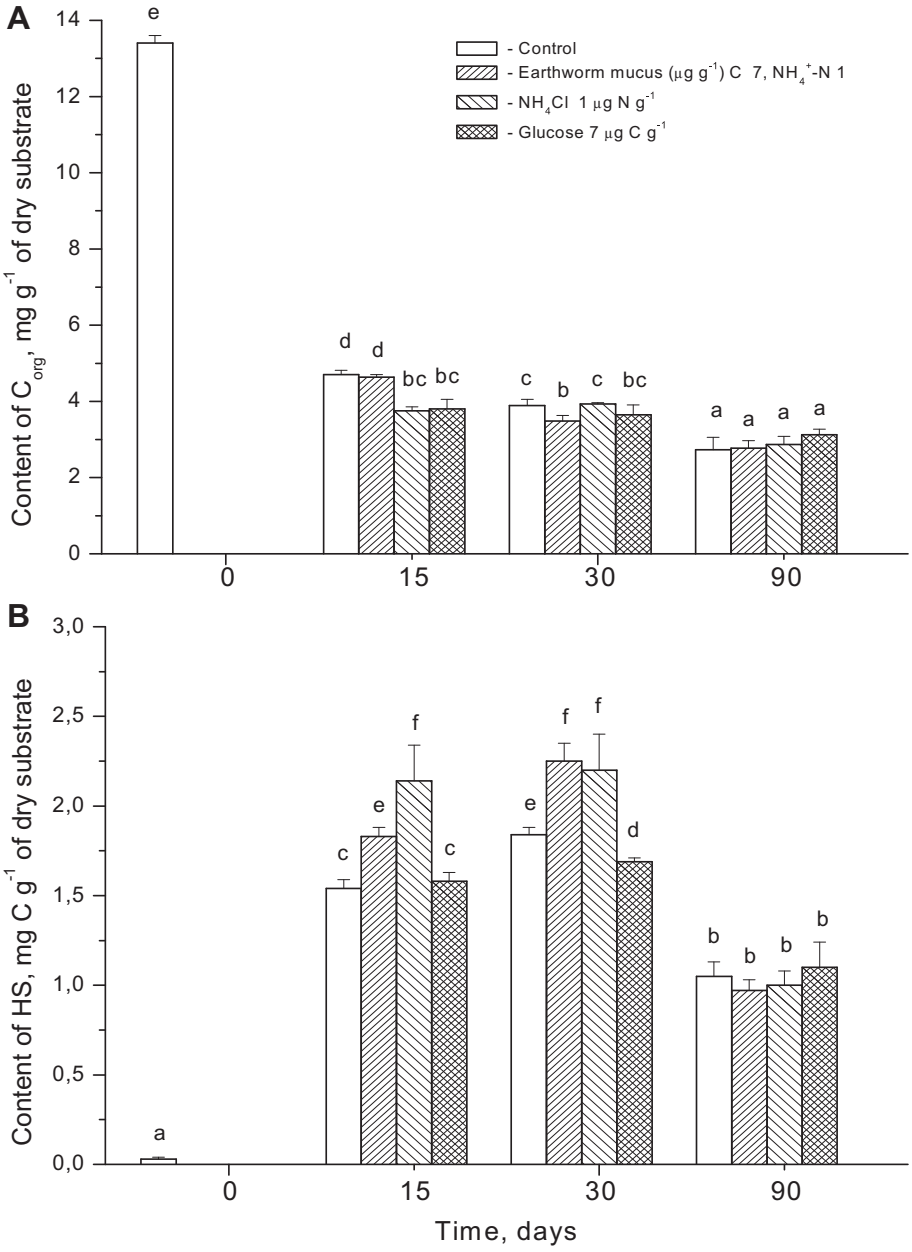


**Fig. 1.** Contents of organic C (A) and humic substances (HS) (B) during incubation of plant residues treated with mucus of *Aporrectodea caliginosa*, glucose or ammonium (mean ± SD). Bars sharing the same letter are not significantly different (Student–Newman–Keuls test, $P < 0.05$, $n = 4$).

**Table 1**
*P* values from ANOVA for the effect of primer (earthworm mucus, ammonium addition, glucose) and time of exposure (samplings after 15, 30 and 90 days) on the organic C content, HS content, HA content and $Ec^{mg/ml}$ of HA.

| Main effects and interactions | Dependent variable | | | |
|---|---|---|---|---|
| | $C_{org}$ | HS | HA | $Ec^{mg/ml}$ |
| Primer (P) | 0.031* | <0.001*** | 0.007** | <0.001*** |
| Time of exposure (T) | <0.001*** | <0.001*** | <0.001*** | <0.001*** |
| P × T | <0.001*** | <0.001*** | <0.001*** | <0.001*** |

Significance levels are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

input with three treatments (earthworm mucus, $NH_4Cl$ and glucose) compared to the control without addition (Tables 1, 2; Fig. 1A) is due to real positive PE manifested in stimulation of plant residue mineralization. The highest real PEs induced by earthworm mucus was on average 11% of the control. The contribution of soil fauna, particularly protozoa and nematode, to N mineralization is estimated to be about 10–40% [6,18,23]. Thus, the activity of large soil invertebrates can affect the litter decomposition not only through reducing particle size of the litter [24], but also through their mucus remaining in the soil.

Such accelerated by primers decomposition is usually explained as an activation of microorganisms trough an increased availability of energy [2,3,25,26]. The aqueous phase of earthworm gut contents may contain more than 100 mM glucose likely derived from the hydrolysis and degradation of the mucus secreted into the alimentary canal. Large amounts of ammonium, dissolved amino acids, amino sugars, and maltose can also occur in gut contents [27]. Oleynik and Byzov [28] demonstrated that earthworm surface excreta affected the formation of soil microbial communities by direct stimulation or suppression of specific microbial populations. As the significant decrease in organic C contents primed by earthworm excreta was detected 15 days latter (on 30th day) in comparison with that primed by ammonium or glucose (Fig. 1A), it seems possible that the observed difference were at least partially due to both glucose and ammonium were a little more easily available for microorganisms responsible for the residue mineralization than earthworm mucus.

The amount of labile carbon added can affect the direction and degree of priming effects. Fontaine et al. [10] demonstrated that soil C losses increase when soil microbes are nutrient limited. If the amount of glucose C and mineral N was excess negative PE were manifested, because both K and r-strategists started to grow leading to switch of K strategists from SOM decomposition on glucose utilization [26]. A strong decrease of the decomposition rates in fresh leaves was observed when relatively large amount of sucrose (about 1 mg C $g^{-1}$) was added [29]. In our experiment, labile carbon was added in much smaller amounts ($\leq$7 μg C $g^{-1}$) than that in experiments of Chigineva et al. [29]. Thus, the new finding of our study is that the real PE can occur as a long-term effect after application of very small amount of substrate.

**Table 2**
*P* values from ANOVA for the effect of earthworm mucus addition, ammonium addition, glucose addition and time of exposure (samplings after 15, 30 and 90 days) on the organic C content, HS content, HA content and $Ec^{mg/ml}$ of HA.

| Main effects and interactions | Dependent variable | | | |
|---|---|---|---|---|
| | $C_{org}$ | HS | HA | $Ec^{mg/ml}$ |
| Earthworm mucus (EM) | 0.009** | 0.001** | 0.003** | <0.001*** |
| Ammonium (AM) | 0.021* | <0.001*** | 0.003** | <0.001*** |
| Glucose (GLU) | 0.015* | 0.987 | 0.290 | <0.001*** |
| Time of exposure (T) | <0.001*** | <0.001*** | <0.001*** | <0.001*** |
| EM × T | 0.016* | <0.001*** | 0.001** | <0.001*** |
| AM × T | <0.001*** | <0.001*** | <0.001*** | <0.001*** |
| GLU × T | <0.001*** | 0.095 | 0.067 | <0.001*** |

Significance levels are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.
The effects of some interactions the factors were not significant, and are not presented in the table.

### 4.2. Plant residue humification

Based on new analytical instrumentation, the process of biodegradation and humification cannot be separated, and the relatively resistant organic molecules are selectively concentrated into humic substances [30]. In our experiment, the plant residue mineralization in control (without additions) was accompanied with strong increase in HS and HA contents within first 30 days (humification) as well as with increase in optical density of HA (humus aromatization) (Figs. 1B and 2A). Although, in nature, humic substances are relatively degradation-resistant, a decrease in HS and HA contents (degradation) was observed between days 30 and 90 (Figs. 1B and 2A). This was probably related to the some fractions of the newly formed humic substances, which may be more available for biodegradation compared to their mature forms [31]. Fungi, especially white rot and litter decomposing fungi, play a key role in degradation and mineralization of humic materials [32].

Despite the high humification rates, when HS and HA yields were the greatest (between days 15 and 30) the formation of the humic substances was triggered by ammonium (by 20–45%) and earthworm mucus (21–29%). In addition, the earthworm mucus induced (by 20%) the greatest optical density of HA, i.e. appearance of relatively highly aromatic humic acids, that was observed at the end of the experiment (Table 2; Figs. 1B and 2). In the humification process, the rate-limiting step appears to be the oxidation of polyphenols to quinones [33]. The forming of quinine structure occurs in the presence of oxygen or polyphenol oxidase enzymes [30]. Recently, a clear increase of soil polyphenol oxidase activity in presence of *A. caliginosa* was observed [34]. This mechanism appears to be likely to explain the beneficial effect of earthworm excreta on HA formation and HA maturation rates. Thus, the results (Figs. 1B and 2; Table 2) suggest for the first time that the activity of earthworms can modify humic compounds not only through passage through the digestive tract of the earthworms [35], but also through elevated by mucus humic substance forming.

In contrast to mucus and ammonium, glucose did not affect the humification rates. It seems likely that the stimulation of plant residue humification mediated by mucus and ammonium depends at least partially on the supply of nitrogen to soil microorganisms. Previously, we found that single-pulse inputs of *A. caliginosa* mucus triggered more respiration responses of microbial community compared with glucose [36]. Moreover, no significant effect of glucose on the utilization of either HA or fulvic acids (FA) by soil saprobic microfungi was observed [37]. However, because our experimental design lacks the treatment “glucose with ammonium”, it remains unclear whether the PE caused by mucus was specific to the PE induced by the corresponding amounts of glucose with N. Further investigations are needed in the area.

Positive PE becomes evident in a release of soil-derived nitrogen, carbon or other elements while the negative PE in their immobilization [6]. In our experiment, the different directional effect of primer on plant residue transformation was observed. Within first 15–30 days, the positive total PE revealed in decrease of soil organic C content (mineralization) (Fig. 1A; Tables 1, 2) while the negative PE revealed in increase of relatively resistant humic substance content (Figs. 1B and 2; Tables 1, 2). Thus, the positive priming in relation to fresh plant residues caused by earthworm mucus can be counterbalanced by increased humification.

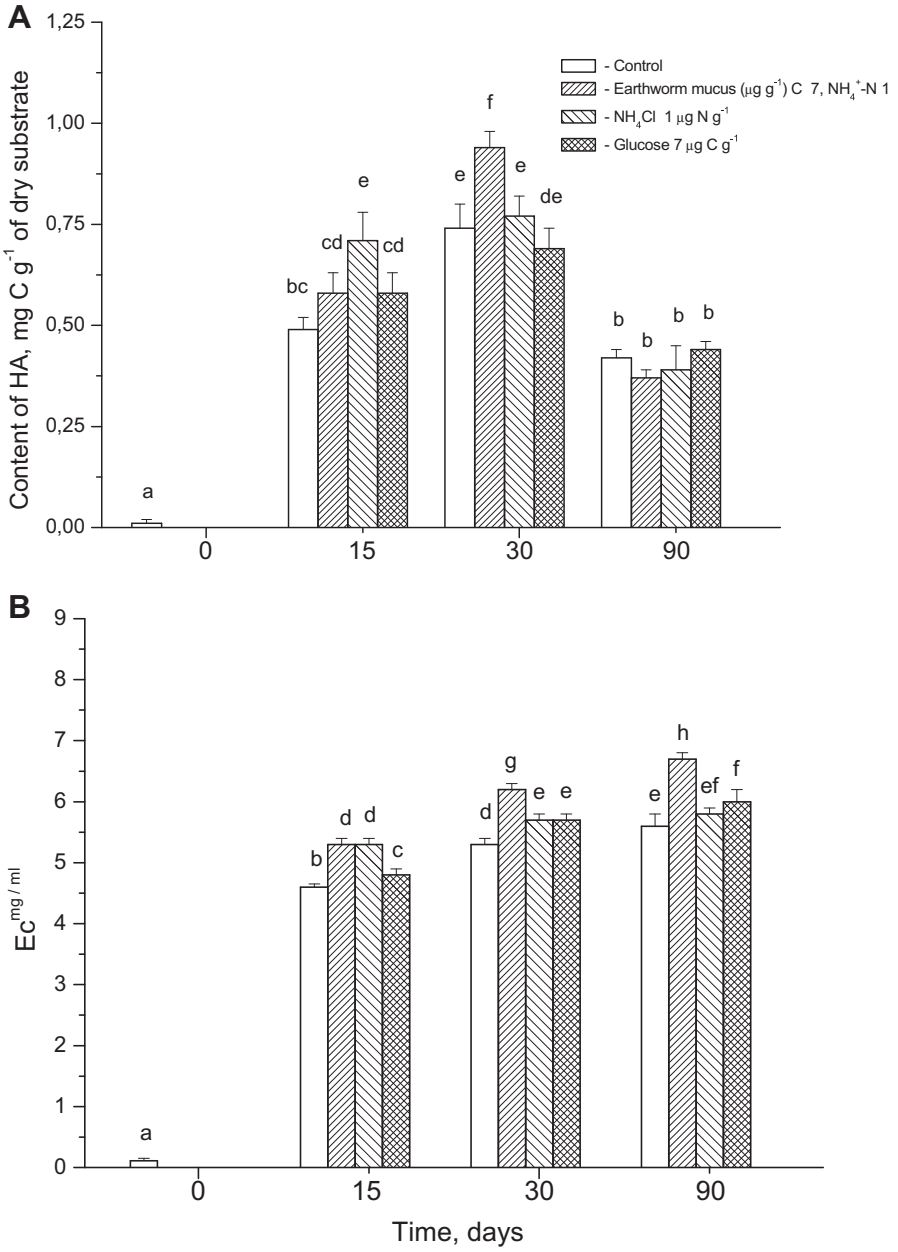


**Fig. 2.** Content of humic acids (HA) (A) and their optical density (B) during incubation of plant residues treated with mucus of *Aporrectodea caliginosa*, glucose or ammonium (mean ± SD). Bars sharing the same letter are not significantly different (Student–Newman–Keuls test, $P < 0.05$, $n = 4$).

## 5. Conclusions

In conclusion, there is evidence for the first time that earthworm mucus as a waste product of these invertebrates remaining in the soil can drive mineralization and humification of plant residues. The earthworm mucus caused strong qualitative changes in humic substance composition while the quantitative changes were rather small. In equivalent amounts, earthworm mucus cause similar or even greater priming effects in plant residue mineralization and especially humification compared with the well studied primers as glucose and ammonia. The duration of the priming effects may be delayed at least during 30–90 days depending upon soil parameters under study. Results of this study highlight the importance of excretory activity of earthworm in humus formation. It seems that earthworms can trigger this process even if a passage of the soil trough their intestines would be completely eliminated, what happens in burrow walls (drilosphere) of the invertebrates.

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