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Short- and long-term tannin induced carbon, nitrogen and phosphorus dynamics in Corsican pine litter

KLAAS G.J. NIEROP*, JACOBUS M. VERSTRATEN, ALBERT TIETEMA, JOKE W. WESTERVELD and PIET E. WARTENBERGH
*IBED-Earth Surface Processes and Materials, Universiteit van Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands; *Author for correspondence (e-mail: k.g.j.nierop@science.uva.nl; phone: +31-20-5256957; fax: +31-20-5257431)*

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Abstract. Pine litter amended with either tannic acid (TA) or condensed tannins (CTs) was studied to assess the effects on C, N and P mineralization in relation to the fate of tannins by incubation experiments during various time intervals. TA induced a rapid short-term effect resulting in high C respiration and net N and P immobilisation. After one week of incubation, TA was decomposed and net C, N and P mineralization and net nitrification resembled that of the control (non-amended litter). CTs exhibited effects on net mineralization on longer terms, i.e. after several weeks of incubation until the end of the experiment (84 days). While net N and P mineralization were greatly reduced, net nitrification was only slightly affected. Most likely CTs formed complexes with organic N of the substrate thereby reducing net N mineralization, while such complexes were not involved in net nitrification processes. The reduction of net P mineralization is due to the lack of need for P by microbes when they cannot get access to N. The fact that decreasing amounts of extractable CTs were accompanied by increasing effects on mineralization processes with incubation time strongly suggests that CTs were incorporated into the litter in such a way that they were inextricable by the common solvents needed to measure tannins, such as for the Folin–Ciocalteu and HCl–butanol assays.

Abbreviations: CT – Condensed tannin; DIN – Dissolved inorganic nitrogen; DOC – Dissolved organic carbon; DON – Dissolved organic nitrogen; HT – Hydrolysable tannin; ICP-OES – Inductively Coupled Plasma-Optical Emission Spectrometer; NMR – Nuclear Magnetic Resonance; Nt – Total nitrogen; PC – Procyanidin; PD – Prodelphinidin; TA – Tannic acid; THM – Thermally assisted Hydrolysis and Methylation

Introduction

Tannins are secondary metabolites occurring in virtually all higher plants. Principally, two types of tannins can be distinguished: condensed tannins (CTs) and hydrolysable tannins (HTs). Gymnosperms and monocots contain only CTs, while in about 15 of the 40 dicot orders HTs can be found (Harborne 1997). CTs are flavan-3-ols that are linked through carbon–carbon bonds (Figure 1a). The most common linkage is the C4–C8 bond, but C6–C8

connections have also been reported. Monomers, and therefore also the CTs, differ in hydroxylation pattern (procyanidin (PC) vs. prodelphinidin (PD)) and C2–C3 stereochemistry. HTs contain a sugar core to which gallic acids or hexahydroxydiphenic acids are ester-linked. The most common HT is tannic acid, which is composed of a glucose unit to which gallic acids are connected (Figure 1b).

Tannins exhibit many functions in ecological and biogeochemical processes, such as herbivore defence, pedogenesis, metal complexation, organic matter decomposition and nutrient dynamics (Kraus et al. 2003a, and references cited therein). With regard to organic matter decomposition and subsequent nutrient dynamics, they exhibit a variety of effects due to the essential property of tannins, i.e. the complexation with proteins. First, they can be toxic directly to soil microbes Field and Lettinga 1992). Second, the activity of microbial enzymes, which are also proteins, can be inhibited by tannins (Goldstein and Swain 1965; Juntheikki and Julkunen-Tiitto 2000), and as a consequence, organic matter decomposition decreases in general (Handley 1961; Benoit and Starkey 1968a; Horner et al. 1988). Third, they can form complexes with protein-containing organic matter through which these moieties become resistant to decomposition (Hättenschiler and Vitousek 2000).

As a consequence of their effect on nitrogen transformations, they also affect the fate of other organic matter elements, such as carbon and phosphorus. Kraus et al. (2004) found a higher CO_2 respiration after addition of CTs and mixtures of CTs and HTs to forest A horizons suggesting that tannins can act as a C source for microbes. P fixation in soils can be limited by tannins by competing for binding sites such as Al, Fe and their (hydr)oxides (Northup et al. 1998). As far as we know, it is less well recognized if, and to what extent P mineralization is limited in forest litter material by tannins similarly to N

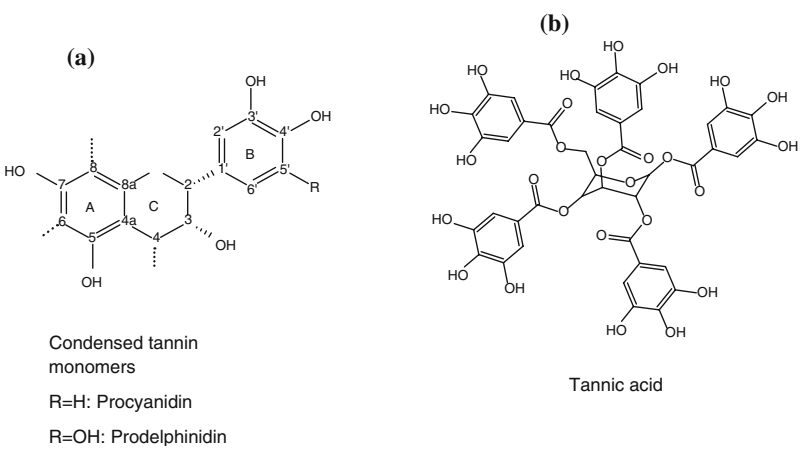

Figure 1. Structures of epicatechin and epigallocatechin, monomers of condensed tannins, and of Tannic Acid (TA).

mineralization. Apart from nucleic acids, which is the smallest fraction of soil organic P compounds (Stevenson 1994), no biomolecules contains both P and N. As a consequence, different effects of tannins on their respective mineralization will be expected.

To date a number of incubation studies have been carried out with tannin amendments in relation to C and N dynamics (Bradley et al. 2000; Fierer et al. 2001; Kraus et al. 2004). The results were not always consistent, which was obviously due to the different tannins used that were added to different litter or soil substrates at different rates. Another feature is that most studies look at the nutrient status of the incubated samples after only one time interval: at the end of the experiment and compared with the initial situation, so that the dynamic behaviour of C and N did not become clear. In the present study we examine the dynamics of C, N and also P during various time intervals, not only in relation to the start of the incubation but also during various periods along the incubation experiment. We also consider the changes in nutrient concentrations after a second addition of tannins as in nature tannin release is not a momentary event but proceeds continuously. Tannins were added to Corsican pine litter at the start of the experiment and after 28 days of incubation to investigate the C, N, P and tannin dynamics induced by the two main tannin types, HTs (Tannic acid, as a pure HT) and CTs (extracted from Corsican pine needles).

Materials and methods

Litter collection, pre-treatment and general composition

Litter, i.e. material from the F1 horizon, was collected from a pine forest named Luizenberg, which is situated in the Amsterdamse Waterleiding Duinen, a coastal dune area with a partly decalcified sandy soil at the south of Zandvoort, west of Amsterdam (The Netherlands). The trees, Corsican pine (*Pinus nigra* var. *maritima*), were planted at Luizenberg in 1935. The collected litter was air-dried at 30 °C for 24 h and subsequently sieved over 2 cm to remove course fragments and to obtain a rather homogenous substrate, which was stored at 2 °C before experiments and analysis. The initial composition was 500 g C/kg, 15.9 g N/kg, and 0.53 g P/kg, resulting in an atomic C/N ratio of 36.6 and an atomic C/P ratio of 2508. The initial microbial content was 2.4 g C/kg, 0.28 g N/kg and 0.13 g P/kg, meaning an atomic C/N ratio of 10.1 and an atomic C/P ratio of 47.4.

Tannin collection

Condensed tannins (CTs)

Needles from Corsican pine were collected by cutting branches from the trees in May 2003. Needles were dried under vacuum on the same day and were

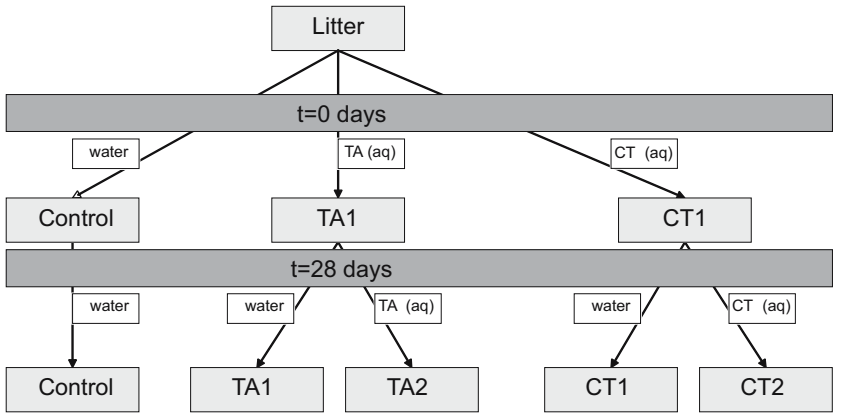

Figure 2. Schematic presentation of incubation series including the amendments at $t = 0$ and $t = 28$ days.

subsequently ground to pass a 2 mm sieve and stored at 2 °C before further treatment. Tannins were obtained following the method of Preston (1999). The ground needles were extracted with hexane using a Soxhlet device to remove lipids. The residual needles were subsequently extracted by a mixture of water/acetone (30:70, v/v) for 24 h. After centrifugation, the dark green solution was stored at 2 °C and the extraction/centrifugation sequence was repeated twice with the solid residue. All three solutions were then combined and the acetone was removed under vacuum. The resulting aqueous solution was subsequently extracted by dichloromethane for 3–5 times to remove remaining lipids until the organic phase was colourless, followed by extraction using ethyl acetate to give an orange to brown solution. After freeze-drying the crude tannins obtained were loaded on a Sephadex LH-20 column and eluted by methanol/water (50:50, v/v) to remove low molecular weight phenolics and tannin monomers. After switching the eluate in water/acetone (30:70, v/v), the tannins were obtained from the Sephadex LH-20 column. The collected and combined water/acetone solution was rotary evaporated to give an aqueous solution, which was freeze-dried to yield purified CTs. Tannins were characterized by solution ^{13}C-NMR, having a PC content of 37%, an average chain length of 6.6, and 85% *cis* configuration (Nierop et al. 2005).

Hydrolysable tannins (HTs)

As HTs are found in half of all dicots, which generally contain also CTs, they usually cannot be obtained solely. Therefore, tannic acid (TA), a commercially available tannin, is often used as a HT. Although TA is rather reactive compared with all other tannins and as such not a very representative HT (Kraus et al. 2003b), it is used by many researchers and therefore it serves as an excellent useful tannin for comparison. Tannic acid of Merck was used, which

has an average molecular weight of 1700, which equals 10 gallic acid units linked to the sugar core.

Incubation experiment

Figure 2 sketches the set up of the incubation experiment. Five gram (on absolute dry basis) of air-dried litter was weighed into petri dishes, after which 5 ml of water was added to give a moisture content of 100% (w/w). In total 248 petri dishes were filled and stored in an isothermal room (20 °C) for 4 days to acclimate. Next, a series of control samples, TA-containing samples and CT-containing samples were prepared as follows:

To control samples, another 5 ml of water was added to give litter material with a moisture content of 200% (w/w). To the TA series, 5 ml of a solution of 20 g/l TA was added, which gave litter material with the same moisture content as the control samples, and in addition 100 mg of TA per 5 g litter (on a dry weight basis). For the CT series, the same procedure was followed as for the TA samples, but using 5 ml of a solution of 20 g/l CT. Per time step (t = 1, 2, 4, 7, 14, 21 and 28 days) during the incubation series four replicates were used for each experiment (control, TA and CT). Consequently, apart from the initial situation (t = 0 days, only control samples), for each time step 12 dishes were prepared as described before, which accounted for 88 dishes in total in this first series.

After 28 days, a second addition of 100 mg TA or CT per petri dish was carried out in order to study the effect of such a second input of tannins on nutrient dynamics. For this second addition, not only the aforementioned control series was used, but also samples to which TA and CT, respectively, was added at t = 0, while at t = 28 only water was added, similar to the control samples. All together, this yielded per time step 20 samples (each situation included four replicates: control samples, samples with only 1 TA addition (TA1) and only 1 CT addition (CT1) at t = 0, respectively, and samples with 2 TA additions (TA2) and 2 CT additions (CT2) at t = 0 and t = 28) so that for all time steps together (t = 29, 30, 32, 35, 42, 56, 70 and 84 days) a total amount of 160 dishes for this second series of incubations was prepared.

CO_2 and N_2O production

To determine CO_2 respiration and N_2O denitrification rates, as a consequence of the acidic pH range of the litter (3.9–4.0 in 0.5 M K_2SO_4 extracts) during the incubation experiments, 5 g of dry litter was weighed into air-tight glass jars with a volume of 400 cm^3, and similar to the incubation experiments 5 ml of water was added to allow acclimatisation. After 4 days, the control, TA- and CT-amended samples were created by adding 5 ml of water, of a 20 g/l TA solution and of a 20 g/l CT solution, respectively.

CO_2 production was determined from the concentration of CO_2 in the headspace of the jars collected during various time intervals after the addition of water, TA and CT. 200 μl of headspace gas was injected on a Carlo Erba 4200 gas chromatograph equipped with a 2 m $\times$ 1/8″ stainless steel Poropak Q column (80/100 mesh) at a constant temperature 70 °C and a Thermal Conductivity Detector (TCD) operating at 140 °C. Helium was used as carrier gas, with a flow of 25 ml/min. The jars were ventilated between various time intervals to prevent decreased decomposition as induced by too high CO_2 concentrations in the headspace (Tietema and Verstraten 1992).

N_2O production was also derived from the concentration in the headspace of the closed jar. About 200 μl was injected on a Varian 3600 GC gas chromatograph equipped with a 2 m $\times$ 1/8″ stainless steel Poropak Q column (80/100 mesh) at a constant temperature of 50 °C and a Electron Capture Detector (ECD) operating at 300 °C. Nitrogen (N_2) was used as carrier gas, with a flow of 25 ml/min. N_2O production was determined as the difference between N_2O present in the headspace and that in the air (Tietema and Verstraten 1992).

Chemical analysis

During each sampling day, 4 (at $t = 0$), 12 (between $t = 1$ and $t = 28$ days) or 20 (between $t = 29$ and $t = 84$ days) dishes were collected. Approximately 1.3 g of litter based on the dry weight was weighed into polypropylene flasks to which in 40 ml of 0.5 M K_2SO_4 was added. The mixtures were subsequently shaken for 1 h, centrifuged and filtered over 0.45 μm filters, after which the extracts were stored at 2 °C before analysis (not longer than a week). Prior to centrifugation, pH of the extracts was measured. About 0.7 g of litter (dry weight basis) was taken from each petri dish, weighed into aluminium dishes and dried at 70 °C for 48 h to determine actual moisture contents. The petri dishes with remaining litter were stored at -18 °C to inhibit mineralization before further analysis (total phenolics, condensed tannins).

Concentrations of NH_4^+, NO_3^-, NO_2^-, *ortho* P and dissolved organic carbon (DOC) in the K_2SO_4 extracts were determined colorimetrically on a Skalar continuous flow AutoAnalyzer, and total N (Nt) was determined after reduction of organic N into NH_4^+ on the AutoAnalyzer. Dissolved organic nitrogen (DON) was calculated as DON = total N $-(NH_4^+ + NO_3^-)$ as NO_2^- was negligible. Net N mineralization rates at $t = t1$ were calculated as $(NH_4^+ + NO_3^-)_{t1} - (NH_4^+ + NO_3^-)_{t0}$. Similarly, for net nitrification and net P mineralization, the rates at $t1$ were calculated as $(NO_3^-)_{t1} - (NO_3^-)_{t0}$, and $(ortho\ P)_{t1} - (ortho\ P)_{t0}$, respectively.

Total C and N in litter were determined by an Elementar VarioEL. Total P was determined by digestion with $HF/HNO_3/HCl$ (Isaac and Kerber 1971) using a Perkin Elmer Optima 3000XL ICP-OES.

Microbial C, N, and P for the initial litter and microbial C only for samples after 7, 28, 35 and 84 days were determined according the chloroform

fumigation extraction method (Horwath and Paul 1994). About 1.3 g of dry weight based litter samples were weighed into glass flasks with caps and fumigated for 24 h. After fumigation, 0.5 M K_2SO_4 extracts were analysed for DOC, NH_4^+, NO_3^-, NO_2^- and *ortho* P similarly as for non-fumigated samples (see above). The difference in C, N and P concentrations between fumigated and the corresponding non-fumigated samples was used as a measure for microbial C, N and P. To calculate the biomass C, N and P, an extraction efficiency factor of 0.45 was used, assuming a similar extraction efficiency for P as for C and N by 0.5 M K_2SO_4 (Wu et al. 1990).

Total phenolics and condensed tannins were determined using the Folin–Ciocalteu assay and the HCl–butanol assay, respectively (Waterman and Mole 1994; Yu and Dahlgren 2000), using 0.7 g of litter (dry weight basis) which was extracted by 40 ml of water/acetone (30:70, v/v) for 24 h in the dark at 20 °C. Total phenolics in the extracts obtained were determined with tannic acid as the standard for all samples. For the HCl–butanol assay, CT derived from Corsican pine was used as the standard. Although it is recommended to use CT as a standard in case of the CT-amended series (Kraus et al. 2003b), control samples appeared to have certain levels of total phenolics as determined by Folin–Ciocalteu, but no response was found using HCl–butanol. Therefore, to allow comparison with the control samples, TA was also used as the standard for the Folin–Ciocalteu assay for the CT-amended series. After acetone/water extraction, the residual litter was air-dried to measure residual tannins by the HCl–butanol assay (Lorenz et al. 2004).

Data were statistically analysed for their differences induced by tannin additions by one-way ANOVAs ($p < 0.05$, $n = 4$).

Results

CO_2 respiration and N_2O production

Addition of tannic acid (TA) resulted in a large increase of the CO_2 respiration with a maximum approximately 2 days after TA addition (Figure 3a). In comparison with the control and condensed tannin (CT)-amended litter samples, the CO_2 respiration induced by TA was much greater suggesting that TA functioned as an alternative C source. Addition of CT also caused a higher CO_2 respiration during the first days after addition. After about a week the CO_2 respiration became lower than that of the control samples, which points to an inhibition of C decomposition induced by the CTs (Figure 3b). Overall, the cumulative CO_2 production during the course of the experiment was greatest in the TA-amended samples, about 3.7–3.8 as much as the control and CT series (Figure 3c). There were small differences between the control and CT-amended samples after CT amendment, but eventually the cumulative amounts of CO_2 respiration were similar.

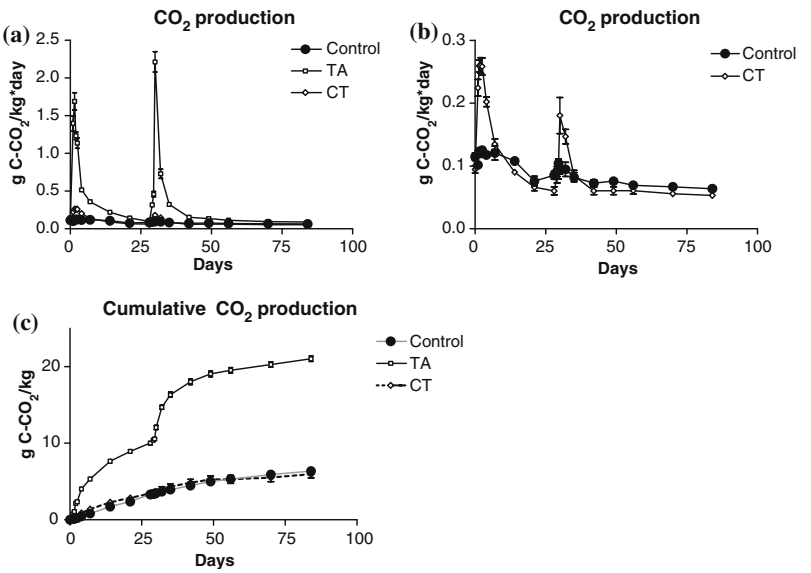

Figure 3. (a) CO_2 respiration during incubation experiment, (b) zoomed for only control and CT, and (c) cumulative CO_2 respiration.

N_2O production for this acidic litter appeared to be negligible for the control samples and both TA- and CT-amended litter (data not shown).

N and P concentrations of litter during incubation

Net NH_4^+ production increased with time for the control samples during incubation (Figure 4a), but after 42 days, the NH_4^+ concentrations reached a maximum. After TA addition, the NH_4^+ concentration decreased sharply but increased after 7 days and also reached a maximum after about 28 days, after which no significant increase was observed. The second addition of TA at $t = 28$ days (TA2) gave a similar behaviour of NH_4^+ concentration as after the first TA addition: a sharp decrease during the first 7 days (from 28 to 35 days) followed by an increase between 35 and 84 days. Contrary to TA, addition of CT did not induce an initial rapid and sharp response, but only a smooth decrease in NH_4^+ concentration. Between 28–84 days the NH_4^+ concentration reached a minimum. In fact hardly any NH_4^+ was measured after that, which remained during the course of the experiment. The second addition of CT at $t = 28$ days did not affect the NH_4^+ concentration in comparison with CT1.

NO_3^- concentrations increased continuously with time for the control samples and also for the samples amended with CT, but at a slightly slower rate (Figure 4b). For TA-amended samples, and similar to NH_4^+, NO_3^- concentrations decreased sharply after TA addition, and then increased 7 days after

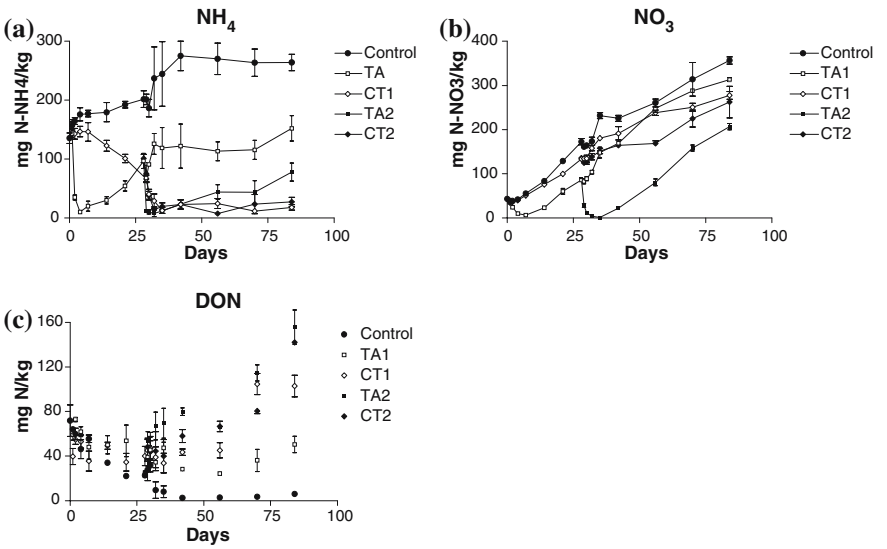

Figure 4. Concentrations in litter during incubation of (a) NH_4^+, (b) NO_3^-, and (c) dissolved organic nitrogen (DON).

addition. For TA1 the NO_3^- amounts eventually became greater than that of CT1. Similar to NH_4^+, NO_3^- concentrations were affected by the second TA addition.

The DON concentrations showed less clear trends (Figure 4c). The main point was the decreasing levels of DON in the control sets with time. The TA- and CT-amended samples also tended to decrease in DON concentration, but at the end of the experiments the DON concentrations increased heavily. The DON concentrations showed a large variability and therefore, the observed differences between treatments were not significant ($p < 0.05$).

For *ortho* P a rather similar behaviour as for DIN ($NH_4^+ + NO_3^-$) was observed (compare Figure 5a with b): a rather constant increase in *ortho* P concentrations for the control samples, a slightly less increase for the CT-amended litter, and an initial sharp decrease after TA addition(s).

Net N and P mineralization and nitrification rates

The mineralization rates as calculated after the complete experiment (84 days) indicate that both tannins had a great inhibiting effect on net N and P mineralization and net nitrification rates (Figure 6). The net N mineralization rate was highest for the control, and lowest for TA2 and both CT treatments. Net nitrification was highest for the control, followed by TA1 and CT1 series. CT2 and TA2 had the lowest overall net nitrification rates. All treatments had less

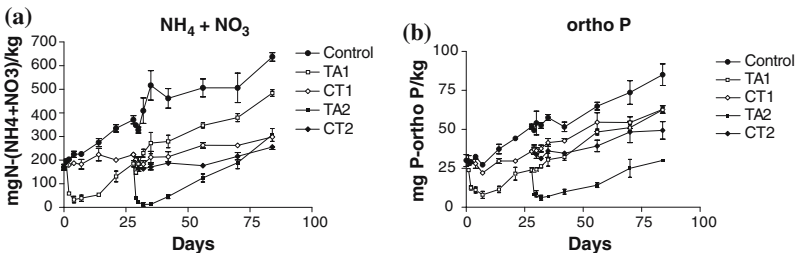

Figure 5. Concentrations in litter during incubation of (a) $NH_4^+ + NO_3^-$ (DIN), and (b) *ortho* P.

effect on net nitrification than on net N mineralization with respect to the control. Overall net P mineralization was again highest for the control, while TA1 and CT1 had similar lower overall rates, CT2 a slightly lower rate and TA2 exhibited the lowest net P mineralization rate.

However, from the NH_4^+, NO_3^-, DIN and *ortho* P concentrations (Figures 4, 5) it became clear that TA addition had very rapid and short-term effects, while CT additions showed more pronounced differences after longer periods of time. After splitting the experiment into four segments: 0–7 days (first 7 days after addition), 7–28 days, 28–35 days (first 7 days after second addition), and 35–84 days, and calculation of the rates during these periods, the dynamic behaviour of the tannin effects become easier to visualize (Figures 7, 9, 10).

For net N mineralization, CT addition caused a slightly lower mineralization rate compared with the control and there was a large negative mineralization rate, i.e. immobilisation after TA addition (Figure 7). By contrast, between 7

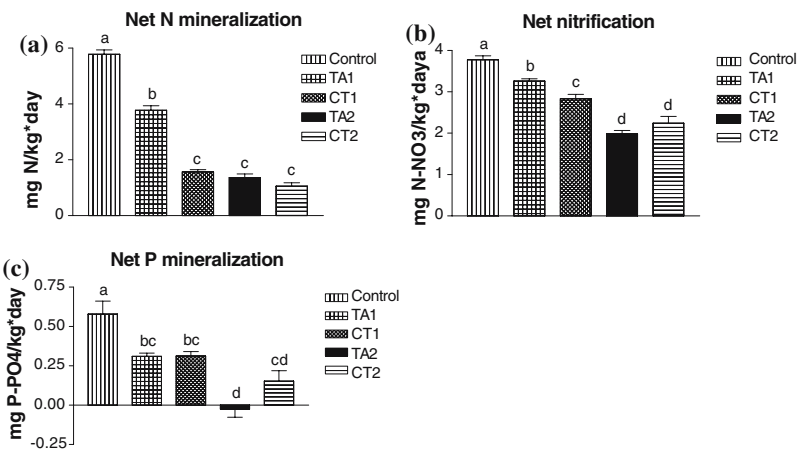

Figure 6. Overall net N mineralization rate, overall net nitrification rate, and overall net P mineralization rate after 84 days of incubation. Different letters indicate significant differences between treatments at $p < 0.05$.

and 28 days after TA addition TA had a very positive effect on net N mineralization. It exhibited a similar rate as the control, while CT induced a much lower net mineralization rate. Seven days after the second addition, TA2 had a similar effect as TA1 after the first TA addition, namely net N immobilisation. Both control and TA1 had a net N mineralization, with TA1 having a higher (but not significant) rate. Both CT amendments (CT1 and CT2) caused a small net immobilisation of N, with no significant difference between both treatments. Between 35 and 84 days, all treatments had positive net N mineralization rates. Both TA1 and TA2 had higher rates than the control, and two TA additions having more effect than only one addition. CT1 and CT2 had more than 50% lower net N mineralization rates with respect to the control.

To emphasize the importance of the dynamic behaviour of tannins, and probably of other factor such as moisture, nutrient addition etc. on mineralization we also calculated the overall net N mineralization rates during 3, 4 and 5 weeks (Figure 8). These periods were chosen as incubation experiments often last such time intervals. After 3 weeks, a net N immobilisation for TA1 was found, while after 4 and 5 weeks a small net N mineralization was observed. For CT1 in all cases net N mineralization took place, which was lower than for TA1 after 5 weeks.

For net nitrification, the rates for 0–7 days of incubation indicated that control and CT addition had net NO_3^- production with the control having slightly, but not significantly higher rates. For TA1 a large net NO_3^- immobilisation was found (Figure 9). Between 7 and 28 days, the net nitrification rate was still highest for the control, and lower but very similar for TA1 and CT1. Between 28 and 35 days, a rather similar picture as between 0 and 7 days was found for the tannin addition at $t = 28$ days. The second addition of TA caused a large net NO_3^- immobilisation, while the second CT addition induced

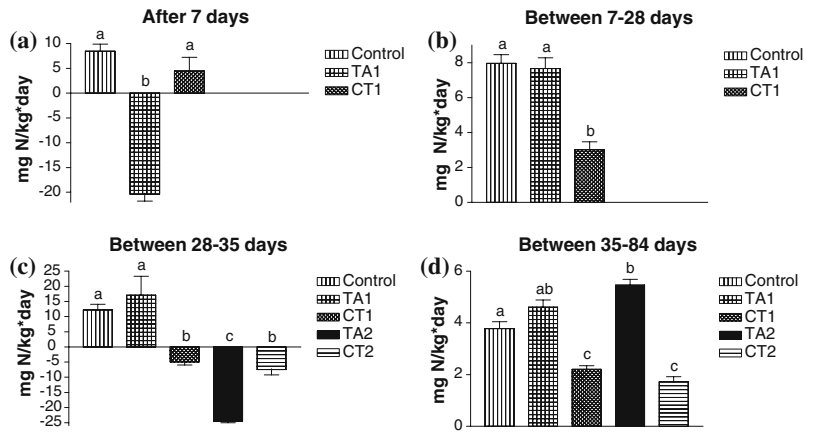

Figure 7. Average net N mineralization rates during various time intervals (0–7, 7–28, 7–35 and 35–84 days). Different letters indicate significant differences between treatments at $p < 0.05$.

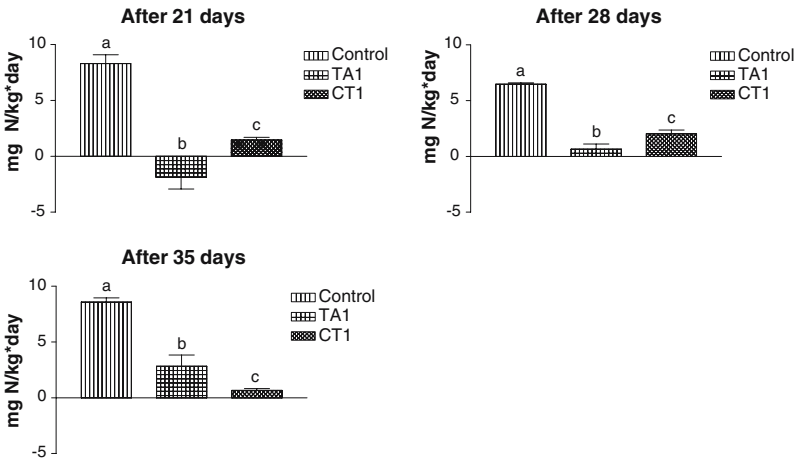

Figure 8. Overall net N mineralization rate, after 21, 28 and 25 days of incubation. Different letters indicate significant differences between treatments at $p < 0.05$.

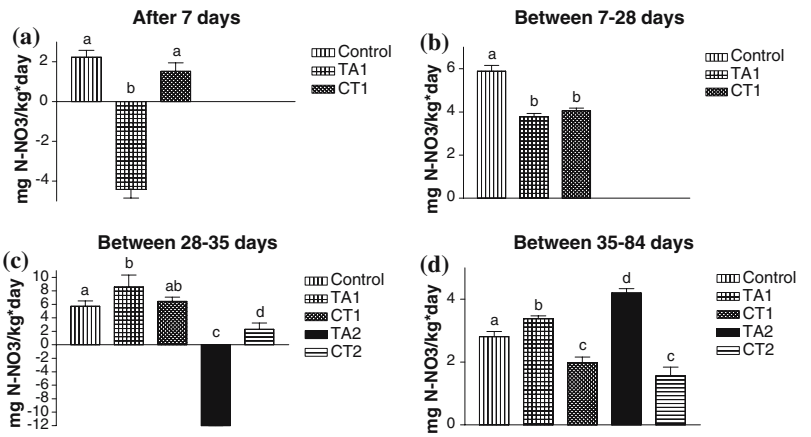

Figure 9. Average net nitrification rates during various time intervals (0–7, 7–28, 7–35 and 35–84 days). Different letters indicate significant differences between treatments at $p < 0.05$.

a much smaller decrease of the net nitrification rate. TA1, and to a lesser (not significant) extent CT1 had a higher nitrification rate compared with the control. Between 35 and 84 days, TA1 and TA2 showed the highest net nitrification rates, TA2 experiment having the highest rate, while both CT1 and CT2 had the lowest rates.

For *ortho* P, mainly a similar picture as for net N mineralization was observed: CT addition caused only a minor difference with the control samples, while TA amendment induced a large immobilisation rate of *ortho* P for the

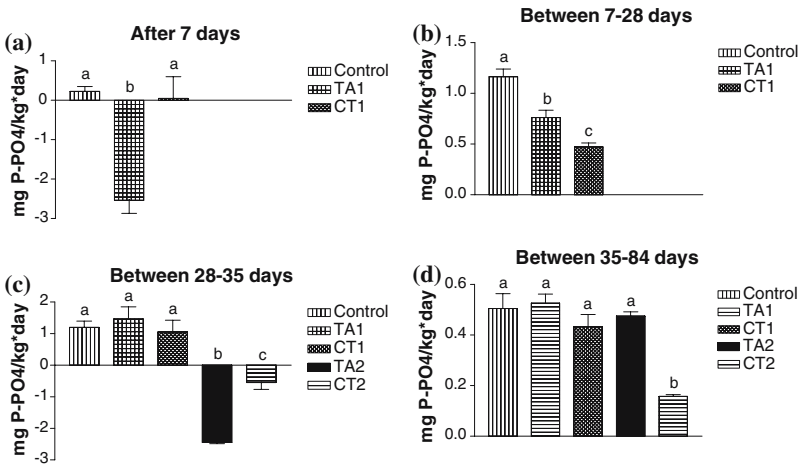

Figure 10. Average net P mineralization rates during various time intervals (0–7, 7–28, 7–35 and 35–84 days). Different letters indicate significant differences between treatments at $p < 0.05$.

period of 0–7 days after each addition (Figure 10). Between 7 and 28 days, the net P mineralization rate of TA-amended litter was higher than that of the CT-amended litter, but lower than that of the control. For the 28–35 days period, these three treatments had a similar P mineralization rate. The only difference between net N and net P mineralization was found for the second CT addition. For N mineralization, CT1 and CT2 were rather similar, but for P CT2 revealed net P immobilisation, while CT1 exhibited net P mineralization. Between 35 and 84 days the P mineralization was similar for all treatments, except for CT2 where the rate was about one third of that of the other series.

Microbial C

Microbial C was rather constant for the control samples throughout the incubation experiment, but decreased at the end of the experiment (Figure 11). For all TA-amended samples, the microbial C was higher than that of all other treatments, although for TA1 the microbial C was similar to that of the control after 84 days of incubation. The CT-amended litter exhibited microbial C amounts that were always close to the control samples. At the end of the experiment all treatments showed a decrease of microbial C, suggesting that the microbial community was deceasing irrespective of treatment.

Total phenolics

The total phenolics concentrations using the Folin–Ciocalteu assay for the control samples were rather constant throughout the whole experiment,

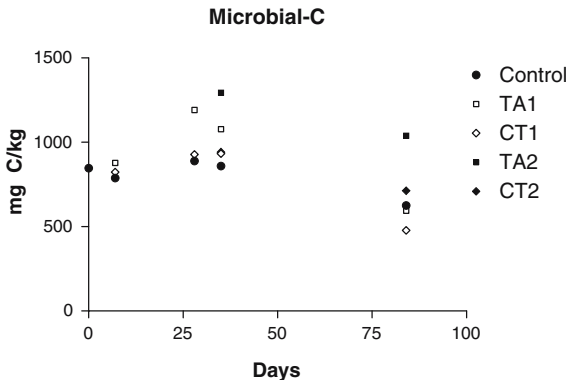

Figure 11. Microbial-C contents after various time intervals.

although a small decrease was noticeable (Figure 12a). For both the TA- and CT-amended litter, total phenolics concentrations expressed as TA equivalents decreased with time after both additions. At the end of the incubation experiment the TA-amended litter contained a small remaining total phenolics fraction that was higher than that of the control and CT-amended litter. Moreover, TA2 in its turn had a higher amount of total phenolics than TA1. CT1 and CT2 had similar amounts of total phenolics as the control at the end of the incubation.

Condensed tannins

Using the HCl–butanol assay for CTs confirmed the trend for both CT1 and CT2 series that was found by the total phenolics determination, i.e. a decrease of extractable CTs after addition and resembling those of the controls towards the end of the incubation (Figure 12b). If we use the ratio of CTs as measured by the HCl–butanol method to the total phenolics as measured by Folin–Ciocalteu (both corrected for the amounts measured for the controls), a decrease was found with time. Such decline suggests that during incubation the tannins were still recognizable as 'phenolics', but less as CTs and thus that CTs were modified with time (Figure 12c). The residual tannins in the litter after extraction hardly responded to the HCl–butanol assay (data not shown).

Discussion

Tannic acid-amended litter

Based on the respiration data for the control, only 1.2–1.3% of total C was mineralized during the 84 days of the experiment, which is relatively low. By

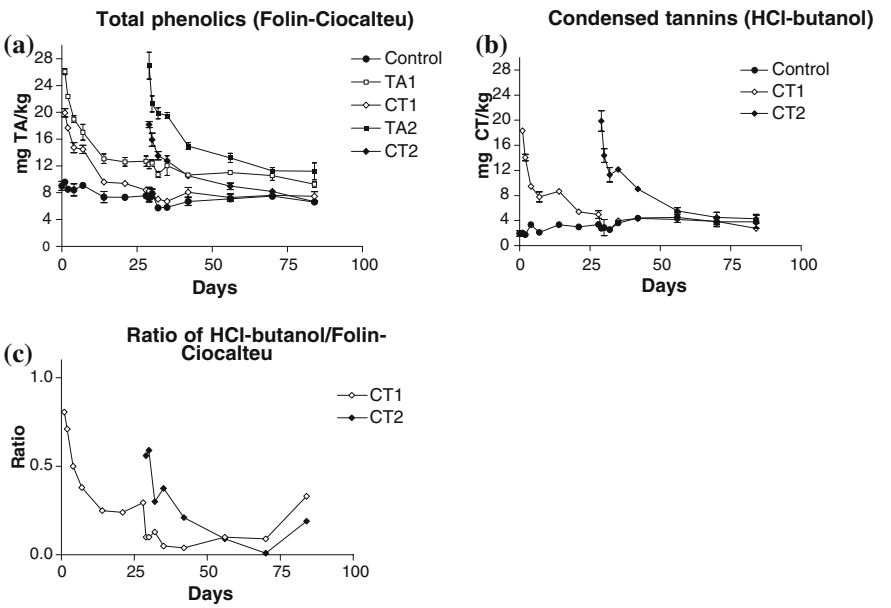

Figure 12. (a) Total phenolics concentrations (Folin–Ciocalteu assay), (b) CT concentrations (HCl–butanol assay) in acetone/water (70/30, v/v) extracts of litter during incubation experiment, and (c) ratio of CTs measured by HCl–butanol assay divided by total phenolics measured by Folin–Ciocalteu assay in acetone/water extracts for the CT1 and CT2 series.

contrast, TA-amended litter was 3.3 times higher in CO_2 respiration strongly suggesting that TA was used as a carbon source for microbes. As the microbial C was also highest for the TA-amended litter, this suggestion seems plausible. TA has a 60% C content, so the difference in CO_2 production between TA2 and control equalled 14.4 gC/kg dry litter and accounted for 60% of the total TA added (24 gC/kg dry litter). Microbial C increased by 0.45 gC/kg per TA addition, which accounted for 4% of TA added that was assimilated by microbes. Application of the Folin–Ciocalteu assay to acetone/water extracts of TA-amended litter showed that the total phenolic concentrations were higher than those of the controls at the end of the experiment. The differences of around 2 mg TA/g litter for TA1 and approximately 4 mg TA/g litter for TA2 represent 10% of TA added. The rest of the TA (26%) may be incorporated into organic matter resulting into inextricable TA moieties. Kraus et al. (2004) also measured a much higher CO_2 respiration of TA-amended soil samples, which was even higher than cellulose-amended soils. Their A horizon had a C/N ratio of 16, while our litter material had a C/N ratio of 37. The A horizon material is relatively richer in organic N compounds that could be involved in tannin complexation, which may explain their lower tannic acid respiration (16.5 vs. 60%).

For the control samples, NH_4^+, NO_3^- and *ortho* P concentrations increased with time. After TA addition, they decreased within the first 7 days after addition of TA, implying net N, NO_3^- and P immobilisation. As TA appears to

act as a C source for microbes, the decrease of NH_4^+ due to immobilisation is expected, while the apparent immobilisation of NO_3^- is less common but not unusual (Attiwill and Adam 1993; Stark and Hart 1997). Apparently the litter decomposition is limited by available C so that the large C mineralization due to TA addition induced such a large immobilisation of N that the need for N by the microbes to build their biomass could only be met when all inorganic N was used. After about 1 week the TA effect on mineralization appeared to be over (virtually no extra C release in comparison with the control). As a result, both net NH_4^+ and NO_3^- production for TA1 increased at similar or even higher rates than for the control. The slightly higher net N mineralization rates for TA1 may be caused by the somewhat higher microbial biomass (Figure 11).

Figure 8 demonstrates not only the dynamic behaviour of TA induced mineralization, but also the effect of incubation time on the conclusions of net mineralization or immobilisation of in this case N. Similarly, for nitrification and P mineralization different net rates were noticed after 21, 28 and 35 days of incubation (data not shown). As aforementioned, many studies look at mineralization rates calculated only from the difference in nutrient concentrations between the end and the beginning of the incubation experiments (Fierer et al. 2001; Kraus et al. 2004). Especially for TA, contrasting conclusions ranging from net N immobilisation (after 3 weeks of incubation) to a net N mineralization rate that is even higher than that for CTs (after 5 weeks of incubation) would be drawn.

Similar to C mineralization, the second TA addition caused similar effects on net N mineralization, net nitrification and net P mineralization as the first addition. At the time of this second addition the litter of TA1 was devoid of TA from the first addition and, apparently, it became similar to the litter of the control series. Therefore, TA2 series responded the same after $t = 28$ days as the TA1 series after $t = 0$, but at higher rates.

CT-amended litter

The CT additions induced only a very small increase in CO_2 respiration during the first week of incubation, but after that the CO_2 respiration became lower than that of the control samples. Although the differences were very small, this lower respiration occurred after each CT addition suggesting that C mineralization was inhibited. If we assume that the extra produced CO_2 was solely due to CT decomposition into CO_2 and 51% of CT is C, only 1.1% of CT was respired after CT addition. In line with the low respiration, no significant increase of microbial C was noticed during the CT incubation experiments. At the end of the experiment no extractable CTs were measured by either Folin–Ciocalteu or HCl–butanol assay. The effects of CT addition on N and P mineralization, however, were especially pronounced at the end of the experiment suggesting that the CTs must still be somewhere in the litter.

Some authors reported a decrease in CO_2 respiration upon tannin addition (Schimel et al. 1996, 1998; Fierer et al. 2001) while others noticed the opposite

(Lewis and Starkey 1968; Kraus et al. 2004). Clearly, the difference in tannin chemistry may be a reason for these discrepancies as indicated by Kraus et al. (2004). However, the quality of the substrate, e.g. rather fresh litter (F1 material) we used compared with the more humified A horizons utilized by Kraus et al. (2004), affects decomposition processes in general, and therefore also after tannin addition.

In contrast to TA, CTs had hardly any effect on net N mineralization compared with the control during the first week after addition. However, after these 7 days the net N mineralization was always less than that of the control suggesting that CTs affect net mineralization at longer terms. Overall, CTs reduced N mineralization more than TA did (Figure 6). Net nitrification rates were also lower than for the control and TA treatment. The reduction of net N mineralization was most evident during the 28–35 day interval, irrespective of the second CT addition at $t = 28$ days. Given the decreasing CT amounts in the acetone/water extracts with time (i.e. after 28 days the total phenolics and CT concentrations were similar to those of the controls), the CTs seem to have an effect on mineralization processes, especially when they are no longer extractable. Complete decomposition into CO_2 seems not to have taken place as 1.1% at most of added CTs was respired as CO_2. Such small decomposition suggests that the CTs were either transformed into extractable compounds that escaped the Folin–Ciocalteu and HCl–butanol window, or into inextricable compounds incorporated as integral parts of the litter. Reduction of N mineralization due to a toxic effect of the CTs on microbes, or binding of CTs to microbial enzymes seems unlikely as there was no decrease of microbial C. Kraus et al (2004) found similar effects of CTs on N mineralization and they suggested that N-containing organic matter compounds formed complexes with tannins that are less accessible to microbes, which slowed down N mineralization. As net nitrification was less affected, the transformation of NH_4^+ to NO_3^- was not strongly involved by this mechanism. Therefore, a possible interaction between enzymes involved in the mineralization of organic N and nitrification is likely of less importance.

DON concentrations were subject to high variability probably due to the indirect way of measuring: they are the difference between Nt and DIN, which are both high values and, consequently, lead to a high variability in the obtained DON values. The DON concentrations of all treatments during the first 35 days varied among each treatment, but after 35 days some trends became evident. The clearest trend, actually already from the beginning of the experiment onwards, was observed for the control series: a decrease of DON from around 70 mg N-DON/kg litter at $t = 0$ days to virtually nothing towards the end of the incubation. This decrease likely reflects immobilisation of easily metabolizable N by microbes. All other treatments show, after a decrease of DON from start to 25–35 days, an increase towards the end of the experiment suggesting that the tannins reduce DON decomposition (Northup et al. 1995). As production continues, DON apparently binds to tannins and these complexes may be less susceptible to decomposition. In line with this, DON

concentrations were higher for the TA2 series compared with the TA1, which is expected given the higher tannin concentrations. Similarly, the CT2 series produced generally higher DON amounts than the CT1 series.

As aforementioned, net P mineralization followed more or less the same trend as net N mineralization. However, the possible explanation of the reduced N mineralization induced by CTs, i.e. the formation of less accessible organic N-tannin complexes, does not simply explain the P mineralization trends as organic P is not expected to be present in organic N molecules (Stevenson 1994). The most probable explanation may be that if microbes are unable to mineralize organic N, it is not beneficial for them to mineralize organic P. This mineralization would cost energy, while they cannot build up biomass without (inaccessible) N. As a result, net P mineralization is also limited. However, this does not explain why the CT2 series differed from the CT1 series in net P mineralization, but not in net N mineralization. Interaction of CTs with enzymes involved in P mineralization is theoretically one obvious explanation, but as aforementioned, interactions between tannins and enzymes are not likely to occur. Determination of, for example, acid phosphatase activity during incubation of CT-amended litter may provide unequivocal evidence in the extent to which enzyme activities are affected by CTs. As yet, the papers studying N (and C) mineralization in litter as affected by tannins did not examine P mineralization. Only a few studies dealing with mineral soil samples emphasized the importance of tannins to complex with Al, Fe and their (hydr)oxides thereby competing with *ortho* P for these sites and thus reduction of P-fixation in acid mineral soils (Northup et al. 1998). This mechanism implies relative increasing *ortho* P levels in mineral soils due to tannins instead of net reduction of *ortho* P levels in litter by tannins.

Tannin transformations

All experiments using tannins exhibited a similar loss of tannins as determined by the Folin–Ciocalteu assay (for both TA and CT) and acid-butanol assay (for CT) in acetone/water extracts. The rapid loss of tannins is consistent with findings of Lorenz et al. (2000) who observed that 80% of the tannin was lost during the first year of spruce litter decomposition. Also, Schofield et al. (1998) noticed a rapid loss of tannins from willow leaves within the first few weeks with an estimated half-life of 2.4 weeks.

The decreasing TA concentrations in TA1 and TA2 experiments are in line with the large CO_2 respiration and net N and P mineralization during the first week of incubation and the subsequent reduction of TA effects on N and P mineralization after that first week when hardly or no TA was present anymore. By contrast, for CT1 and CT2, a completely different effect on C, N and P mineralization was observed: hardly any measurable CTs after 28 days of incubation, but appreciable effects of CTs on N and P mineralization.

Using the ratio of CT concentrations measured by the HCl–butanol assay divided by those as determined as total phenolics determined by the Folin–Ciocalteu assay (Figure 12c) showed that with increasing period of incubation this ratio declined. This decrease indicates that increasing proportions of the CTs are not recognized as condensed tannins, but still detectable as phenolic moieties by the Folin–Ciocalteu assay. Such a degradation pattern was also observed during adsorption/desorption experiments of the same CTs to mineral soil particles (Kaal et al. 2005). The higher ratios observed at the end of the experiment suggest that both total phenolics and CTs could not be measured anymore in reasonable amounts. Therefore, the two low values obtained yielded relatively high ratios.

The question is whether the decrease of measurable CTs in extracts is due to escaping the analytical window of the tannin assays or lack of extractability. Parfitt and Newman (2000) found that tannins from Radiata pines were chemically modified during the early stages of decomposition, after which they hardly changed. A combination of oxidation, polymerization and degradation may change the original tannin chemistry (Tiarks et al. 1989). To what extent these changes affect the responses of the tannins to the tannin assays is unclear. The extractability likely decreased, but the CT-amended litter did not show significant response to the HCl–butanol assay for residual tannins. If we assume that the tannins added were subject to chemical modifications during the incubation experiment, and Figure 12c supports this assumption for extractable tannins, this indicates that the modified tannins cannot be measured by the tannin assays (Hättenschiler et al. 2003).

Parfitt and Newman (2000) also found that tannin accumulated with needle decomposition. The B ring may be susceptible to quinone formation (Hernes et al. 2001), and quinones in their turn react with organic nitrogen to form covalent bindings (cf. Stevenson 1994) that resist acetone/water extractions. Preliminary experiments using Thermally assisted Hydrolysis and Methylation (THM) on litter amended with CTs showed that there were still significant amounts of CTs left in the litter at the end of the experiments. This suggests that CTs were inextricable by acetone/water and apparently incorporated into the organic matter, such as in complexes with polysaccharides (Benoit and Starkey 1968b). More experimental data are needed to further investigate the fate of tannins during decomposition, by work which is currently in progress.

Conclusions

TA and CT differed greatly in their effects on C, N and P mineralization, both on short and long terms. P mineralization was rather similar to N mineralization, most likely not directly by tannin-organic P interactions, but as a result of a lower N demand by the microbes.

The only resemblance between TA- and CT-amended litter during the incubation series was tannins' similar behaviour in terms of their acetone/water extractability, which decreased with incubation time. The lack of extractability of TA was because it functioned as a carbon source for microbes, and, as a consequence, was accompanied by net N and P immobilisation during the first week after TA addition. After that period of time the majority of TA was decomposed and the TA-amended litter became similar to the control. Therefore, the net N and P mineralization of TA1 followed the control. At the time of the second TA addition, the TA1 and TA2 litter was similar to the control and therefore TA2 exhibited a similar effect on C, N and P mineralization as the first TA addition, but at different rates. TA is sometimes not considered as a true tannin, and its effect on the nutrient dynamics is very different than that by CTs. However, the lack of suitable alternatives combined with the common availability makes TA an appropriate reference compound which allows comparison between different incubation studies.

The lack of CT extractability had a different cause than that of TA: hardly any CT was used as a carbon source, and most probably CT molecules were tightly bonded to the litter. Due to this different cause, also dissimilar effects on mineralization were noticed. During the first week of incubation CT-amended litter varied only slightly from the control series in terms of C, N and P mineralization. With increasing time of incubation the CT series showed a progressively more pronounced decrease in net N and P mineralization, which remained until the end of the experiment. In contrast to the TA series, net nitrification was much less affected than net N mineralization suggesting that CTs were mainly interacting with organic N of the substrate than affecting microbes or their enzymes directly. The second CT addition caused only a minor additional effect on net P mineralization, but not on net N mineralization.

From the dynamic behaviour of net N and P mineralization it is clear that the time used for an incubation experiment to determine the impact of tannins, and also for other factors (moisture, temperature, NH_4^+ addition, etc.) on C, N and P mineralization rates affects the outcome of the data and the conclusions drawn from them. Therefore, it is recommended to use either a standard period of time to compare results from other studies or to include a number of time intervals so that a more detailed picture of the dynamic behaviour will be revealed.

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