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Microcalorimetric investigations of microbial activities: decomposition of needle litter under laboratory conditions

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Abstract

Microcalorimetric experiments on litter degradation included in a forestal amelioration program are reported. Abolition of water limitation in needle litter induced a strong initial peak in heat evolution. This transitory activation is due to easily degradable substrates utilized equally well by fungi and bacteria as shown by the addition of selective inhibitors. Degradation proceeded in three phases of changing activity depending on individual additives (lime, ammonium chloride, cellulose). Addition of cellulose alone stimulated degradation while nitrogen supply had no significant effect. Addition of lime always reduced the degradation rate. Particularly in combination with nitrogen addition, lime strongly inhibited the microbial activity by a release of ammonia, total weight loss after seven months being also reduced. No physical reactions were found interfering with true metabolic heat production, thus proving the validity of the method.

1. INTRODUCTION

The determination of actual biomass in complex systems like soil or litter is hampered by the physical and chemical heterogeneity of the substrate and the difficulty to distinguish between inactive organic material and active biomass [1]. On the other hand, several methods such as measurements of CO_2-production or O_2-uptake [2-5], of the electron transport system [6] or metabolic heat production [7] can be applied to monitor total microbial activity in soils. Detailed studies of Sparling who compared microcalorimetric determinations of activity with other conventional methods found remarkably good

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correlations between the rate of heat output and biomass in different soils [8-9]. One fundamental difficulty that all these methods have in common, is that the actual metabolic activity of microbial populations within natural substrates like forest litter depends on uncontrolled environmental factors, e.g. the specific layer, the local moisture content, carbon- and nitrogen availability and pH value. These variables make it rather difficult to estimate the reaction of microbial soil communities on forestal amelioration treatments as in the liming project of a pine stand in the present case.
The aim of the investigation of needle litter decomposition was, therefore, to estimate under controlled laboratory conditions the influence of a lime fertilization and of an increased or reduced nitrogen content (lowered or increased C/N value) on the microbial activity.
Microcalorimetry turned out to be a useful supplement to the conventional determination of respiration rates, especially because overall activities can be detected by this method including fermentative processes not related to oxygen consumption. Heat production rates of microbial metabolism were used here as a direct measure to follow the time sequence of microbial decomposition of organic components in the needle litter.

2. MATERIAL AND METHODS

Heat production rates were determined by means of a differential microcalorimeter E. Calvet with 4 vessels of 15 ml each. The calorimeter was run under isoperibolic conditions at a temperature of 22 °C. The calorimetric sensitivity in both diagonales amounted to 67.8 ± 0.5 mV/W and 62.5 ± 0.6 mV/W, respectively.

2.1. Samples

The investigations were carried out with needle litter (*Pinus sylvestris*) collected in a forest of Berlin (Grunewald, Jagen 63). Needles were dried and coarsely milled to pieces of 1 to 3 cm length. Subsequently, culture flasks (Kolle, DURAN-SCHOTT, Wertheim) were filled with 14.25 g of needles.
At the beginning of an experiment the culture flasks were inoculated by spraying their content with a stock suspension of microorganisms obtained by washing freshly sampled needles with water. Addition of water was adjusted to 250 % of dry weight, corresponding to an optimal water content of about 70 % for biodegradation. This value was kept constant during the whole incubation period (7 months at 24° C in the dark).
Each series of culture flasks received one of 5 different additions: (i) Nitrogen (NH_4Cl), 0.84 g per flask (final pH 3.6) for the N-series; (ii) Lime ($CaCO_3/K_2SO_4$, 42:1 w/w), 2.1 g per flask (final pH 6.0) for the L-series; (iii) Cellulose, 14.25 g per flask (Whatman filter paper, ash and nitrogen free, Cat. No. 1001917) in pieces of 1.5 x 6 cm (final pH 3.6) for the C-series, (iv) Lime and Nitrogen for the L/N-series and (v)

Lime and Cellulose for the L/C-series, respectively. A control series was run without additions besides water (nitrogen content 0.931 %, pH 3.6). Samples of each series were measured microcalorimetrically after 10 hours and after 4, 8, 11, 18, 25, 39, 53, 67, 88, 109, 130 and 154 days of incubation (in some cases also between these dates).
A total of 21 culture flasks were used in these experiments: two for each series and 9 further ones for additional investigations of L/N supplementation (2 L/N and 1 control flasks), sterile tests (2 control flasks) and L/N tests with inhibitors (4 L/N flasks). At the start of the experiment the C/N-values resulting from the different additions amounted to 60 for the control, to 125 for C, to 24 for N, to 63 for L to 113 for L/C and to 25 for L/N.
The results of chemical analyses referring to details of substrate decomposition are not included here, but will be published elsewhere.

2.2. Microcalorimetry

The sample vessels were filled with 1.5 to 2.0 g of needle litter (fresh weight), closed, cleaned with ethanol on the outside and placed in the calorimeter. Temperature equilibration took place in the calorimeter. The total heat released during a constant time period of 10 h was calculated from the area under the power-time-curve determined by means of a polarplanimeter (HAFF, No.315 E, Pfronten). Weight specific activities were calculated on the basis of dry weight. This was determined at the end of each microcalorimetric run. All measurements were run with at least two parallel samples.

2.3. Inhibitors

Experiments on selective microbial inhibition were run in the L/N-series (Fig. 7). To supress bacterial activity, standard solutions of penicillin/streptomycin (10.000 u/10.000 µg/ml SERVA) were used in a final concentration of 2.5 mg/g d.w. [2,3,10,11]. Actidione (cycloheximide, SERVA) was applied in a final concentration of 4 mg/g d.w. to supress fungi and other eucaryotes [11]. In a third series actidione and penicillin/streptomycin were combined in the concentrations mentioned above. A L/N sample without inhibitors served as reference.

2.4. Sterility test

The untreated needle litter was autoclaved for 20 min at 121° C for sterilisation. Subsequently, one part was inoculated with a microbial suspension from fresh needle litter, while sterile water was added to the other part. Duplicates of both samples were measured in parallel for heat production.

2.5. Microscopic investigations

Samples of the different experimental series were monitored

microscopically for any detectable effect on microbial cell populations. Particular attention was paid to quantitative and qualitative changes in the distribution of fungi and bacteria in the "control-", "L/N-" and "L-" series during the first two days after inoculation, i.e. until the maximum of microbial activity was reached. At this stage the number of cells/g d.w. was determined in appropriate counting chambers. The inhibition tests were run in the same manner.

3. RESULTS

3.1. Abolition of water limitation

The activity of microbial biomass in the forest depends on climatic factors (temperature and precipitation) and by these on the degree of water limitation of the soil. The more appropriate these factors, the higher the heat production rates in laboratory experiments.
In the present study, water limitation was abolished by the inoculation of litter samples with calculated amounts of microbial suspensions. This is clearly demonstrated by the strong increase of heat production at the beginning of experiments (Fig. 1). Even where substrate supplementation was negligible as in the control and L-series, the addition of

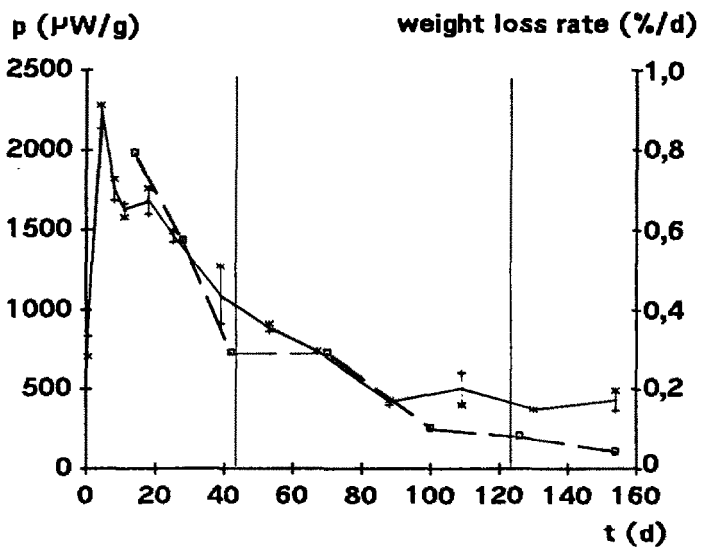


Figure 1. Heat production rate, p, and weight loss rate of the control-series as a function of time, t, in days. p is given per g dry weight of organic matter, the weight loss rate in %. All points are mean values of parallel determinations.
—— heat production rate, ---- weight loss rate

water to the previously dried sample led to mixing and diffusion effects and as a consequence to an increase in the availabity of soluble substrates.
It was demonstrated that this initial activity burst was of true metabolic origin (see sterility test) and not due to heat production related with abiotic physicochemical processes.
After this transitory activation of metabolism a sequence of three further activity phases (Fig. 2 to 4) was detectable in the different series:

- the first phase (up to 42 days) was initiated by a once more increasing heat production rate with a maximum output of approximately 2.0 to 2.5 mW/g of organic dry substance, probably due to a catabolism of easily utilized compounds;
- the second phase (42 to nearly 120 days) was characterized by medium metabolic activities of approximately 1.0 mW/g and a decrease to about 0.5 mW/g, originating from the secondary utilization of substances which are more difficult to metabolize;
- the third phase (120 to nearly 210 days) showed low activity levels of approximately 0.3 to 0.5 mW/g suggesting slow decomposition rates typical for the more recalcitrant polymers.

A clearly reduced efficiency was established for the L-, L/N-, and L/C-series as compared with the control.
In contrast to the inhibitory effect of lime, additional nitrogen fertilization had no measurable effect. N-series showed an activity pattern very similar to that of the control series, indicating that nitrogen was not a limiting nutrient.

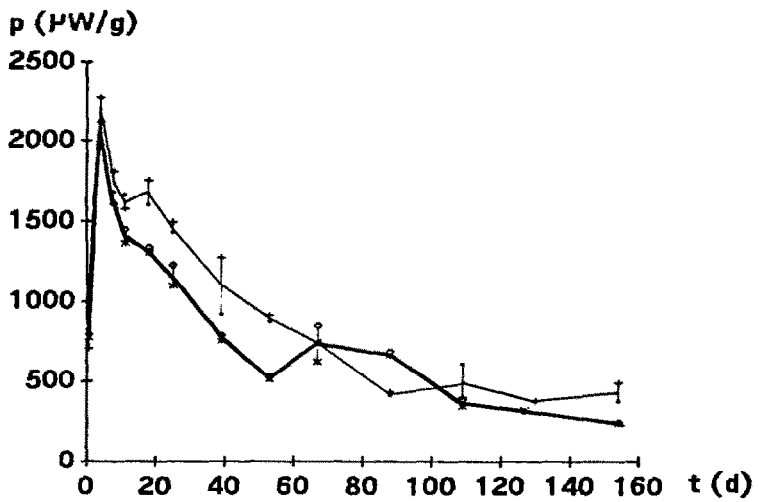


Figure 2. Heat production rate, p, as function of time, t, in days. p is given per g dry weight of organic matter. All points are mean values of two parallel determinations.
—— control-series, ——— L-series

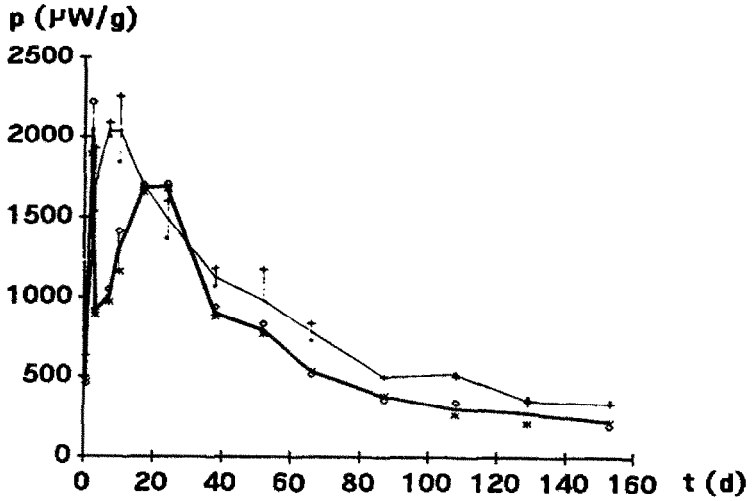


Figure 3. Heat production rate, p, as function of time, t, in days. p is given per g dry weight of organic matter. All points are mean values of parallel determinations.
—— N-series, ▬▬ L/N-series

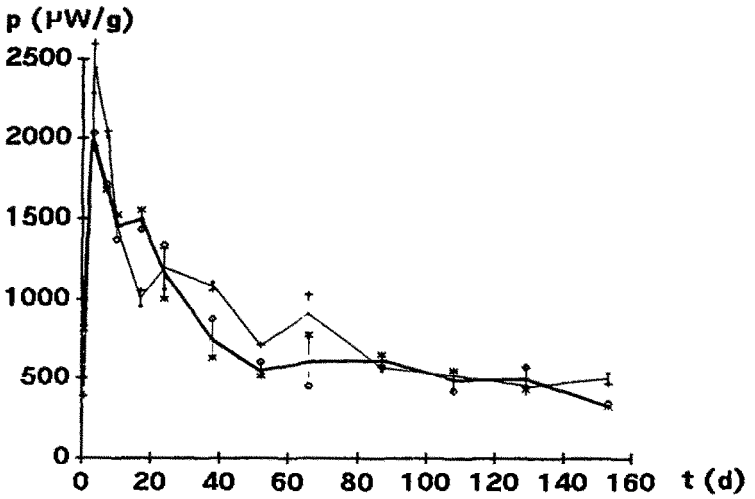


Figure 4. Heat production rate, p, as a function of time, t, in days. p is given per g dry weight of organic matter. All points are mean values of parallel determinations.
—— C-series, ▬▬ L/C-series

Remarkably, C-series exhibited a higher heat production rate than the control at first, but afterwards till day 40 a reduction to the level of the control. At the end (60 to 160 days) once more higher heat production rate was obtained for C-series than for the control.

3.2. Oxygen limitation

Two small wholes in the lid of the sample vessel were made to facilitate gas exchange during microcalorimetric measurements. Only a small decrease of activity was noticed with this device after 10 h of incubation in the microcalorimeter. Therefore, an additional gassing with oxygen at the beginning was no longer necessary.

3.3. L/N supplementary test

In the L/N-series a significantly lower activity was determined than in all other series. Therefore, this test was repeated to reexamine the microbial development especially during the first 50 hours of initial activation. The results shown in Figure 5 confirm the strong inhibitory effect of a combination of ammonium chloride and lime. Moreover, the fact that free ammonia was detected in the gas phase of the culture flasks during at least 30 days strongly suggests that ammonia is responsible for the poisoning symptoms observed in the L/N-series.
Microscopic observations revealed an extremely high development of bacteria in this L/N-series while any development of fungi was completely suppressed during the first 11 days. Bacterial counts gave values of about 1×10^{10} cells/g d.w.
The pH value of the L/N-series was in the range of 6 to 6.5 at this time and rose to 7.0 to 7.5 towards the end of the experiment while the pH value of the control started with 3.6 and rose to 6 at the end.

3.4. Sterility test

A test was carried out to prove, whether the observed maximum in the heat production rate after approximately 40 hours was due to a metabolic activation after the abolition of water limmitation. To this end an experiment was run excluding any microbial activity by previous heat inactivation. Moreover, the question should be answered, if purely physical factors such as condensation of water were included in the heat balance.
The results presented in Figure 6 exhibit no activity at all in the sterile sample for at least 60 hours. On the other hand, the second sample, also sterilized but subsequently inoculated as usual exhibited the same increase in the heat production rate as the control culture with the only difference of a somewhat extended lag phase. No secondary physical effects influencing the activity measurements could be detected. Therefore, the registered thermovoltage exclusively results from the biological activities.

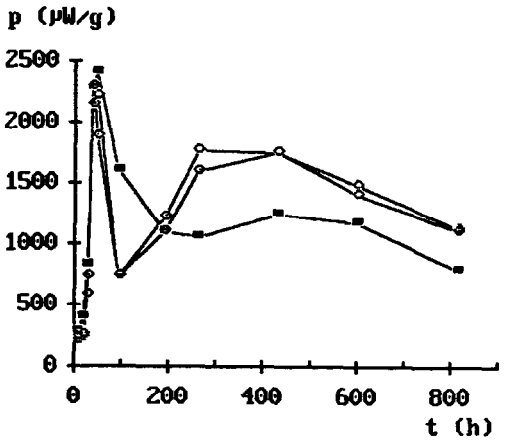
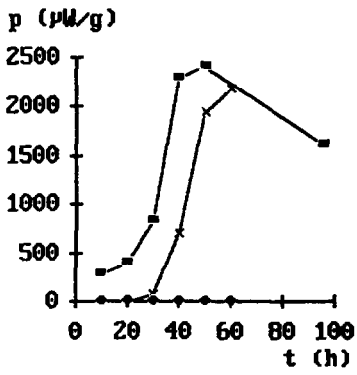


Figure 5. Abolition of wawater limitation.
Heat production rate per g dry weight of organic matter, p, as function of time t. Comparison of a control-series -■- with two L/N-series (L/N 1 -o- and L/N 2 -o-). All points are mean values of two parallels (maximal deviation 22 %).



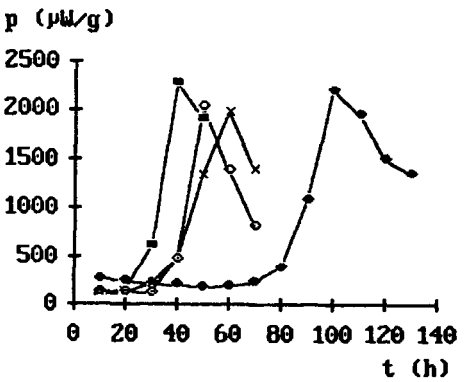


Figure 6 left. Sterility test. Heat production rate per g dry weight of organic matter, p, as function of time t. -■- untreated control-series as compared to -●- a strilized sample and -x- a reinoculated sample. All points are mean values of two parallels (maximal deviation 11 %).

Figure 7 right. Inhibition effects. Heat production rate per g dry weight of organic matter, p, of L/N-series as function of time t after the application of various inhibitors. -■- without inhibitors, -x- plus penicillin/streptomycin, -o- plus actidione, -●- plus penicillin/streptomycin and actidione. All points are mean values of to parallels.

This investigation also confirmed, that a possible modification of substrates by autoclaving had no detectable influence on the initial behavior of the inoculated microorganisms.
We thus conclude that the given water deficiency and its abolition by addition of water were the only factors determining the

measured microbial activity.

3.5. Inhibition test

To estimate the individual contribution of bacteria and fungi to the heat production, especially in the L/N-series, one or the other of both microbial groups were eliminated by means of specific inhibitors. After the addition of actidione, merely bacteria should be active and after penicillin/streptomycin merely fungi, while the addition of both inhibitors should give an estimation of the total attainable inhibition.
The results, presented in Figure 7, did not show any remarkable differences in the maximum activities. They always amounted to approximately 2.0 to 2.2 mW/g although the initial lag phase varied before activity rose. It is striking that metabolic inhibition lasted about two times longer in the samples with both inhibitors than in those with only actidione or penicillin/streptomycin addition. Microscopic observations, carried out in parallel to the calorimetric measurements, confirmed the expected selectivity of the inhibition. Since no difference in the total heat production could be observed between bacteria (fungi repressed) and fungi (bacteria repressed) and inhibition by the combined agents was rather complete, both population parts apparently can substitute each other to a remarkably high extent. This mutual substitution merely refers to the metabolization of those easily degradable substrates responsible for the initial activation peak.

4. DISCUSSION

In addition to chemical analyses of needle litter and determinations of weight loss over a degradation period of 7 months (Becker et al., in preparation), microcalorimetry was used to monitor the time course of the total microbial activity.
The highest rates of heat production were always found after the initial addition of water to the dried needle litter. Maximal values ranging from about 2.0 to about 2.5 mW/g were obtained after about two days of incubation. This initial activation peak was largely reproducible and independent of the specific solutions added (nutrients and inhibitors) and the degradation rates achieved at the end.
Any contribution to the total heat production by purely physical processes, e.g. by water condensation, could be rouled out. It is therefore concluded that the initial peak actually reflects the metabolization of a constant proportion of easily degradable substances available at once for the microflora by the abolition of previous water limitation. The selective inhibition of either bacteria by streptomycin/penicillin or fungi by actidione or both populations by combining the inhibitors induced an extended lag phase without abolishing the characteristic peak. This demonstrates that the interpretation given above is correct in the inhibition experiments. It is also remarkable in this context, that a residual heat produc-

tion level was always detectable (Fig. 7), presumably due to endogeneous metabolism of surviving dormant spores. Complete sterilization of the samples by autoclaving abolished this background activity (Fig. 6).
Characteristic patterns can be detected in the heat production rates p depending on the additives lime, nitrogen and organic carbon or combinations of them. A longlasting inhibition of microbial heat production was observed in all series containing lime (L-, L/N-, L/C-series). This agrees well with the reduction by 16 to 44 % of the final weight loss observed in these series as compared to the corresponding controls without lime (not shown). In contrast, addition of cellulose (C-series) increased the heat production rate and the final weight loss after 7 months amounted up to 50 %, in the control litter just to 40 %. In a field experiment, weighed needle litter samples exposed in litter bags exhibited nearly the same decomposition rates of 39 % within 7 months [12]. Although no direct comparison was possible between total heat production and total weight loss during the whole experiment similar trends were found. The most prominent inhibition was obtained by the simultaneous addition of ammonium chloride and lime (L/N-series). Fungal development was completely suppressed in this case (Fig. 3) for at least 11 days while the number of bacteria strongly increased at first. Because of the rise of initial pH, free ammonia was released from added ammonium chloride which is particularly toxic for eucaryotes. The collected needle litter used in this study already contained nitrogen in excess (0.931 % dry weight). Thus, it cannot be ruled out that the general inhibitory effect obtained in this study by the addition of lime was caused to some extent by ammonia developed within microenvironments. A comparable inhibitory effect of liming on litter degradation was obtained with litter bags exposed in the field [13]. Additionally, increased nitrification (ammonia oxidation by certain bacteria) and nitrate export were observed in the same area after liming [14]. Besides inhibition of most fungi by ammonia, a more specific suppression of pH-sensitive fungi may be responsible for the negative effects on degradation activities. Cellulose and lignin degrading basidiomycetes involved in the transformation of lignocellulose into humus belong to such fungi.
Summing up, the present results have shown that microcalorimetry can be used as a valuable additional tool to analyze sequential phases in complex processes such as microbial degradation of needle litter under controlled conditions.

5. ACKNOWLEDGMENTS

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