Substrate heterogeneity and microfauna in soil organic ‘hotspots’ as determinants of nitrogen capture and growth of ryegrass

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Abstract

In this study we simultaneously manipulated the patchiness of complex organic resources and the composition of microfaunal populations (protozoa and nematodes) in soil, to influence microbial mineralization processes and to elucidate the underlying mechanisms of nutrient acquisition from decomposing plant residues by ryegrass plants.

Hotspot treatments of decreasing patchiness were established by filling laboratory microcosms with defaunated soil and adding labelled (¹³C, ¹⁵N) grass residues as 1-layer, 4-layer or completely mixed within the soil. Microfaunal treatments were set up by inoculation of the soil with either protozoa or bacterivorous nematodes, a combination of both or neither (control). The microcosms were planted with surface sterile ryegrass seedlings.

Growth of ryegrass plants was enhanced by both, increasing patchiness of the organic matter in soil (1-layer > 4-layer > mixed) and microfloral–microfaunal interactions (protozoa + nematodes = protozoa > nematodes > control). The presence of microfauna enhanced the decomposition of hotspot material. Protozoan grazing in particular increased the availability of N in soil and leaching water and led to a concomitant increase in plant growth. While root foraging in organic hotspots enhanced the spatial coupling of mineralization and plant uptake, microfaunal grazing increased the temporal coupling of nutrient release and plant uptake. Consequently the greatest plant biomass was found in treatments combining aggregation of organic material in patches and the presence of microfauna. ©2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Modern farming practice relies on high fertilizer inputs to ensure high rates of nutrient capture by crops. However, there are increasing concerns due to high nutrient losses, especially of nitrogen and its negative consequences to the environment (Byrnes, 1990; Addiscott et al., 1991). Therefore, the knowledge of

processes that regulate nutrient release and plant uptake in soil is an essential prerequisite for sustainable agricultural management.

Even though detailed studies on the mineral nutrition of plants under controlled conditions have revealed exact knowledge of the chemical balances of plant nutrients in water culture systems, efforts to translate this knowledge to soil systems fail to predict element fluxes in the rhizosphere. It is essential to appreciate that in soil most nutrients are bound in organic form and the amount of nutrients available for plant growth depends on complex interactions between plant

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roots, microorganisms and soil animals. In addition, the distribution of nutrients in soil often is heterogeneous. Consequently, plants have evolved specific strategies, such as ‘root foraging’ (Hutchings and de Kroon, 1994; Robinson, 1994) to exploit nutrient rich ‘hotspots’ in soil. However, as an organic hotspot is degraded and nutrients made available from it by microbial extracellular decomposition, element uptake by plants is simultaneously accompanied by severe competition for available nutrients with the microbial community. Although the mechanisms are still poorly understood, it is generally accepted that microorganisms are superior competitors (Hayman, 1975; Newman, 1985; Jackson et al., 1989; Chang et al., 1997; Wang and Bakken, 1997a,b) and microfaunal grazing is required to enhance significantly microbial turnover and subsequently the release of nutrients for plant uptake (Coleman et al., 1984; Gerhardtson and Clarholm, 1986; Ritz and Griffiths, 1987; Griffiths, 1994; Zwart et al., 1994).

Only little is known about biological processes regulating microbial decomposition and plant uptake of mineralized N. We hypothesized that the supposed mechanism of root foraging by plants would be most effective where organic matter is aggregated in patches, but inefficient where organic matter was homogeneously mixed with the soil. In addition, we expected a high microbial activity on the freshly decomposing organic matter with microfaunal grazing increasing microbial turnover and subsequently the release of nutrients for plant growth. Therefore, in our experiment we simultaneously manipulated both the patchiness of organic resources in soil and microfaunal–microfloral interactions in order to influence subsequent microbial mineralization processes and to elucidate the underlying mechanisms of nutrient acquisition by ryegrass plants.

2. Materials and methods

2.1. Microcosms

Laboratory microcosms based on those described by Wolters (1989) and Scheu (1989), with modified lids for CO₂ collection, were used in this experiment. The microcosm chambers consisted of perspex tubes (150 mm height, 60 mm diam) which were placed on

ceramic plates. The chambers could be drained under natural conditions of soil matric potential by lowering the atmospheric pressure in a box below the plates (Fig. 1).

The chambers were filled with experimental soil to a depth of 140 mm, leaving an air-space of ca. 30 cm³, and each planted with a single, sterile *Lolium perenne* L. seedling (as described further). The stem of the seedling was led through a central hole (10 mm diam) in the lid of the chamber and sealed with kneading mass (Teroson, Teroson GmbH, Heidelberg, Germany). The lids thus separated the soil and rhizosphere atmosphere from the shoot. Tubes on the lid allowed watering and were connected to a continuous air-flow system for aeration and to trap respired CO₂ from soil in order to follow decomposition processes from labelled organic material and root respiration in the rhizosphere.

Air was pumped through a gas washing flask containing 2M KOH to remove CO₂, then through another flask containing sterile demineralized water, and subsequently led into a dispersal system connecting all experimental chambers. A constant low air-flow of 6 ml min⁻¹ was passed through individual microcosms, and led through a separate gas washing tube containing 5 ml 1M KOH to trap the CO₂ resulting from soil and rhizosphere respiration. Alkali solution was changed at regular intervals and evolution of CO₂ was determined titrimetrically from 0.5 ml aliquots (Macfadyen, 1970). In addition, ¹³CO₂ was analysed from 1 ml of the alkali solution, as described further.

2.2. Soil, hotspot and microfauna

Garden soil (0.2% N, 3.1% C), collected at the wall-garden of the University of York, UK, in May 1997 was defaunated with chloroform (Alphei and Scheu, 1993) and mixed with autoclaved sand (1:1) giving a low nutrient soil (0.1% N, 0.4 at.% ¹⁵N, 1.5% C, 1.08 at.% ¹³C). The soil was kept in darkness at room-temperature until use, and is the same soil used in hotspot studies by Hodge et al. (1998).

When the microcosms were being filled with soil, air-dried, defaunated (by chloroform fumigation) and chopped, labelled (¹³C, ¹⁵N) plant material of *L. perenne* was added, forming ‘hotspots’ of additional organic matter. Each microcosm received 1.27 g dry

Fig. 1. Diagram of the top section of the microcosms.

ryegrass shoots (1.9% N, 48.6 at.% ^{15}N , 41.2% C, 2.8 at.% ^{13}C , C/N 21.8) and 0.83 g dry ryegrass roots (0.4% N, 33.6 at.% ^{15}N , 43.6% C, 1.7 at.% ^{13}C , C/N 11.0) as hotspot material, giving an overall composition of the hotspot of 27.4 mg N, 12.5 mg ^{15}N , 885 mg C and 21 mg ^{13}C . The hotspot was added as three treatments of increasing patchiness: (i) organic substrate homogeneously mixed into the soil (mix), (ii) organic substrate in four evenly spaced layers in the soil column (4-layer), (iii) organic substrate in one layer in the center of the soil column (1-layer).

Protozoa (naked amoebae, flagellates, ciliates) were isolated from the experimental soil and cultured in Petri dishes in sterile nutrient broth (NB, Oxoid, UK) in Neff's Modified Amoebae Saline (NMAS, Page, 1976) at 1:9 v:v, with *Escherichia coli* OP50 and native soil bacteria as food source. Nematodes were isolated from a permanent pasture on a clay-loam soil near the Scottish Crop Research Institute. To obtain protozoa-free cultures, single nematode specimens were washed in sterile NMAS and transferred to Petri dishes containing 1% agar in NB-NMAS streaked with *E. coli* OP50 as food source. The nematode cultures consisted mainly of the bacterial feeding species *Coactadara cystilarva* and *Panagrolaimus* spp. Protozoa and nematodes were added to the experimental soil within a 2 ml suspension of their culture fluid. Control treatments received the same amount of culture fluid, rendered fauna-free by filtration through a 4 μm filter.

2.3. Experimental design

The experimental design was two-factorial with different hotspot and faunal treatments. The hotspot treatments were (1) labelled organic substrate homogeneously mixed in soil (mix), (2) soil with the same amount of labelled substrate in four equal layers (4-layer) in the soil column, each layer separated by ca. 2.5–3 cm soil, (3) same amount of substrate in one layer in the middle of the soil column (1-layer).

The faunal treatments were (1) soil without microfauna (defaunated soil + filtered culture fluid) as control (Ctrl), (2) soil inoculated with nematodes (Nema), (3) soil inoculated with protozoa (Prot), (4) soil inocu-

lated with nematodes and protozoa (N $\times$ P). This gave 3 $\times$ 4 treatments with four replicates of each, resulting in 48 microcosms.

Seeds of perennial ryegrass (*L. perenne*) were surface sterilized by washing with 96% ethanol for 2 min and 5% calcium hypochloride for 5 min under vacuum (–1 atm) (Hensel et al., 1990). The seeds were kept separately until germination in 96-well microtiter plates in 100 μl NB-NMAS and checked for sterility. Sterile seedlings were transferred in cylindrical perspex tubes (10 mm height, 10 mm diam) filled with defaunated soil and grown for 1 week (average biomass 0.007 ± 0.003 g dry wt) before being transferred into the microcosms. The perspex tubes, leading through the microcosm lid and the air-gap, connected the plants with the soil surface (Fig. 1).

The microcosms were kept in a greenhouse maintained at 20°C for 16 h with supplementary sodium lighting (day), and at 15°C for 8 h with no supplementary lighting (night). The chambers were watered with 20 ml of sterile deionized tap water every 2 days. Leachate was sucked off regularly and stored at –20°C before analysis.

2.4. Analyses

A first harvest of the plants was carried out after 4 weeks when the shoots (shoot-1) were all cut 5 cm above the level of the chamber lid. The final harvest was carried out 2 weeks later when the shoots had regrown to their previous height. Harvested plants were separated into roots, shoot base (shoot up to 5 cm from the roots), shoot-1 (shoots sampled at first harvest) and shoot-2 (shoots sampled after 2 weeks regrowth).

Roots were washed from adhering soil and hotspot material. The maximal root length, number of main and lateral roots in 5 cm intervals from the base to the root tip, maximum shoot length and number of shoots at the shoot base were counted. The plant material was dried at 60°C for 24 h, milled and analysed for $^{14}\text{C}/^{13}\text{C}$ - and $^{14}\text{N}/^{15}\text{N}$ -content by continuous flow isotope ratio mass spectrometry (CF-IRMS) using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyser with ANCA-NT Solid/Liquid Preparation Module. This was operated in the dual isotope mode, allowing d^{13}C and d^{15}N to be measured simul-

taneously on the same sample. After separation of the roots, the remaining soil and hotspot material of each microcosm was mixed thoroughly and stored at 5°C for further analysis.

At the end of the experiment concentrations of mineral N (NH_4^+ , NO_3^-) in the pooled leachate were determined in a segmented flow autoanalyser (Skalar Analytical, Breda, The Netherlands). The amount of leached ^{15}N and ^{13}C was determined from 3 ml of freeze dried soil water analysed by CF-IRMS.

Microbial biomass C was determined by the chloroform fumigation-extraction technique (Vance et al., 1987; Ritz et al., 1992). Samples of fresh soil (10 g dry wt) were fumigated with ethanol-free chloroform for 24 h at 25°C, whilst control samples were stored at 5°C. After removal of the chloroform, soils were extracted with 40 ml 1M KCl on a roller bed (Wheatley et al., 1989), followed by centrifugation ($2500 \times g$ for 10 min) and filtration through a Whatman GF/F filter. The filtrate was analysed after digestion with UV radiation in a segmented flow autoanalyser. Amounts of NH_4^+ and NO_3^- absorbed in the soil matrix were determined in KCl extracts from non-fumigated soil samples.

Total numbers of protozoans (i.e., active and encysted forms) were enumerated by a most probable number technique (Darbyshire et al., 1974) in which 5 g soil were dispersed in 50 ml NMAS on a rollerbed for 20 min. 8×0.1 ml aliquots were added to microtitre plates and diluted twofold in 50 μl sterile NB-NMAS. The microtitre plates were incubated at 15°C and the flagellates, ciliates and amoebae were counted after 4, 7, 10, 14 and 21 days. Numbers were calculated according to Hurley and Roscoe (1983). The remaining soil suspension was fixed with formaldehyde (8% final concentration) and used for nematode extraction by flotation in colloidal silica (Griffiths et al., 1990).

2.5. Statistical analyses

The two-factorial design with hotspot (1-layer, 4-layer, mix) and soil fauna (Ctrl, Nema, Prot, $\text{N} \times \text{P}$) treatments was analysed using the SAS software (SAS Institute Inc., 1993). A factorial ANOVA was

performed to test for significance between means of hotspot and fauna treatments. Comparison of the means for the individual treatments was done at the 5% probability level with a Tukey-test (Tukey's honestly significant difference, HSD).

3. Results

3.1. Plant growth

There were significant differences in plant growth after 4 weeks. Shoot-1, Ctrl and Nema treatments had significantly ($P < 0.001$) smaller shoots than the Prot and combined $\text{N} \times \text{P}$ treatments (Table 1). At the final harvest, 2 weeks later, the order in growth of the new shoots had not changed between the treatments (Fig. 2). The biomasses of shoot base and roots in the Ctrl treatment were significantly smaller than those in the Nema, Prot and $\text{N} \times \text{P}$ treatments (Table 1). Compared to control plants with a total biomass of 121 mg, plant biomass increased in the faunal treatments with Nema, Prot and $\text{N} \times \text{P}$ by factors of 1.2, 1.7 and 1.8, respectively. However, the increase was only significant for the protozoan treatments.

The patchiness of the organic matter in soil also significantly influenced plant growth. The smallest plants were found in treatments where the hotspot material had been homogeneously mixed with the soil (mix) (Table 1). Consequently, the smallest plants were found in mix-treatment without protozoa and the biggest plants in microcosms with 1- and 4-layer in Prot and $\text{N} \times \text{P}$ treatments (Fig. 2).

Differences in plant biomass were also reflected in plant morphology. Shoots in treatments with protozoa (Prot, $\text{P} \times \text{N}$) were ca. 10 cm taller than in Nema and Ctrl treatments. The average shoot length was also ca. 10% higher in the 4-layer treatment compared to the 1-layer and mix-treatment (Table 2). In addition, plants in the mix-treatment had fewer emerging shoots than in the 4-layer and 1-layer treatments (Table 2). There was no significant difference in the number of shoots emerging between the faunal treatments. No differences between treatments were observed with respect to the maximum root length. However, plants in the Prot and $\text{N} \times \text{P}$ treatments had more roots compared to plants in the Nema and Ctrl treatments. The number of roots decreased in

Table 1

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on plant growth, and sum of squares explained by the model (% SS explained)^a

	1 st shoot	2nd shoot	Base	Root	Plant
<i>F values</i>					
Model	20.2 *** ^b	18.0 ***	4.4 ***	4.4 ***	14.7 ***
Patch (P)	48.9 ***	24.4 ***	13.9 ***	9.5 ***	33.8 ***
Fauna (F)	40.8 ***	48.8 ***	5.2 **	8.0 ***	30.7 ***
P × F	0.3	0.4	0.9	1.0	0.3
% SS explained	86.0	84.6	57.6	57.6	81.8
<i>Treatment means</i> (g dry wt)					
Ctrl	0.038 b	0.027 b	0.034 b	0.023 b	0.121 b
Nem	0.044 b	0.033 b	0.042 ab	0.031 ab	0.150 b
Prot	0.067 a	0.054 a	0.045 a	0.034 a	0.200 a
N × P	0.071 a	0.060 a	0.048 a	0.038 a	0.217 a
mix	0.037 b	0.032 b	0.032 b	0.024 b	0.125 b
4-layer	0.065 a	0.049 a	0.046 a	0.034 a	0.194 a
1-layer	0.063 a	0.048 a	0.049 a	0.035 a	0.195 a

^a Mean plant biomass values and comparison among means of the Faunal treatments: animal-free control (Ctrl), nematodes (Nem), protozoa (Prot), nematodes and protozoa (N × P); and among means of the Patch treatments with organic matter mixed into the soil (mix), organic matter in four layers (4-layer), and one layer (1-layer). The means are for biomass of shoots at the first harvest cut 5 cm above shoot base (1st shoot), shoots of the final harvest cut 5 cm above shoot base (2nd shoot), shoot base (0–5 cm height) of the final harvest (base), roots of the final harvest (root) and biomass of the whole plant (plant; 1st shoot + 2nd shoots + base + roots). Different letters indicate a significant difference between means ($P < 0.05$, Tukey-test).

^b *** $P < 0.001$, ** $P < 0.01$.

Table 2

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on plant shoot length and number of shoots and roots^a

	Shoot _{max} (cm)	Shoots (no.)	Roots-0 (no.)	Roots-5 (no.)	Roots-10 (no.)	Roots-15 (no.)
<i>F values</i>						
Model	10.2 *** ^b	2.6 *	2.9 **	1.6	2.3 *	2.2 *
Patch (P)	3.4 *	5.8 **	4.6 *	1.8	2.6 (*)	4.5 *
Fauna (F)	33.0 ***	2.4 (*)	5.1 **	2.6 (*)	5.7 **	3.5 *
P × F	1.0	1.6	1.2	1.1	0.5	0.7
% SS	75.7	44.2	46.7	33.4	41.1	39.7
<i>Treatment means</i>						
Ctrl	21 b	15 a	15 b	11 a	7 b	5 b
Nem	22 b	17 a	17 ab	13 a	7 b	7 ab
Prot	31 a	19 a	19 a	13 a	9 ab	7 ab
N × P	31 a	19 a	19 a	14 a	10 a	9 a
mix	25 b	15 b	15 b	12 a	8 a	6 b
4-layer	28 a	18 a	18 ab	13 a	9 a	7 ab
1-layer	26 a	18 a	18 a	13 a	9 a	8 a

^a Shoot_{max}: maximal shoot length; Shoots: number of shoots at root base; Roots –0: number of roots at root base; Roots –5: number of roots at –5 cm; Roots –10: number of roots at –10 cm; Roots –15: number of roots at –15 cm. Details as in Table 1.

^b *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, (*) $P < 0.1$.

Fig. 2. Biomass of ryegrass: shoots at first harvest (a); shoots and roots at final harvest (b) from microcosms with various faunal treatments [animal-free control (Ctrl), nematodes (Nem), protozoa (Prot), nematodes and protozoa (N × P)] and patch treatments [with organic matter mixed into the soil (mix), organic matter in four layers (4-layer), and one layer (1-layer)]. Means with the same letter on the top of the columns are not significantly different ($P > 0.05$).

the following order $N \times P > \text{Prot} > \text{Nema} > \text{Ctrl}$ and also in the treatments $1\text{-layer} = 4\text{-layer} > \text{mix}$ (Table 2).

3.2. C and N in plant tissue

The nitrogen content of plant tissue increased, compared to the control, in treatments with protozoa (Table 3). In the presence of protozoa the N-concentration of roots, shoot-base, 1st shoot and 2nd shoot increased by factors of 1.2, 1.1, 1.1 and 1.1, respectively. In contrast, the presence of nematodes alone did not significantly alter N-concentrations compared to the control. The percentage of N in the 2nd shoot and shoot

base was significantly reduced in the 4-layer treatment compared with the mix-treatment (Table 3).

The concentration of ^{15}N in plant tissue strongly depended on both the hotspot and faunal treatments. The hotspot treatments accounted for 42–89% of the variance in ^{15}N -concentrations of plant tissue. The concentration of ^{15}N in the plant was increased in the mix-treatment ($1.5 \times$ the 1-layer treatment) and the 4-layer treatment ($1.2 \times$ the 1-layer treatment, Table 3). This pattern was consistently found to be repeated in all faunal treatments. The faunal treatments accounted for 3–44% of the variation in at.% ^{15}N in plant tissue. The highest concentrations of ^{15}N were always found in protozoan treatments and increased in

Table 3

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on element contents in plant tissue: N, ^{15}N of shoots of the first harvest cut 5 cm above shoot base (1st shoot), shoots of the final harvest cut 5 cm above shoot base (2nd shoot), shoot base (0–5 cm height) of the final harvest (base), roots of the final harvest (root)^a

	1st Shoot		2nd Shoot		Base		Root	
	N (%)	^{15}N (at.%)	N (%)	^{15}N (at.%)	N (%)	^{15}N (at.%)	N (%)	^{15}N (at.%)
<i>F values</i>								
Model	2.7 *	42.0 ***	3.2 **	16.9 ***	3.4 **	37.6 ***	3.0 **	18.2 ***
Patch (P)	1.3	220.6 ***	4.2 *	46.6 ***	3.2 (*)	98.2 ***	1.5	57.0 ***
Fauna (F)	1.8	4.5 *	6.4 **	26.9 ***	6.9 ***	65.4 ***	5.7 **	25.7 ***
P × F	3.6 *	1.3	1.3	2.0 (*)	1.8	3.5 **	2.1	1.5
% SS	44.9	92.8	49.7	83.8	51.2	92.0	47.6	84.8
<i>Treatment means</i>								
Nem	3.98 a	11.9 a	3.57 a	13.5 a	1.94 a	12.3 a	1.09 a	10.7 a
Ctrl	4.04 a	13.0 ab	3.99 ab	15.3 b	2.32 ab	13.4 b	1.17 a	11.9 a
N × P	4.14 a	13.7 b	4.16 b	17.7 c	2.51 b	16.4 c	1.26 ab	14.3 b
Prot	4.35 a	13.6 b	4.42 b	18.5 c	2.56 b	17.1 c	1.37 b	14.6 b
mix	4.01 a	18.7 a	4.3 a	19.0 a	2.5 a	17.5 a	1.20 a	15.5 a
4-layer	4.13 a	11.4 b	3.8 b	15.7 b	2.2 b	14.2 b	1.18 a	12.3 b
1-layer	4.25 a	9.1 c	4.0 ab	13.9 c	2.3 ab	12.7 c	1.28 a	10.8 c

^a Details as in Table 1.

all plant parts in the order Ctrl < Nema < Prot = N × P (Table 3). This resulted in increased concentrations of ^{15}N in whole plants by factors of 1.1, 1.3 and 1.4 in the Nema, Prot and N × P treatments compared to control plants, respectively.

Average carbon content in plants ranged between 35 and 40%, and there were only significant treatment effects at the first harvest in the hotspot treatments. At this time plants from the mix, 1-layer and 4-layer treatments contained 38.0, 39.7 and 40.2% C, respectively. The concentrations of ^{13}C varied slightly but not significantly between treatments with average values between 1.075 and 1.105 at.‰ ^{13}C .

The faunal treatments significantly affected the C/N-ratio of plants (Table 4), which was always lower in protozoan treatments than in the Ctrl and Nema treatments. There was no clear trend of C/N-ratio with respect to the hotspot treatments.

The total contents of C, ^{13}C , N and ^{15}N in the faunal treatments increased in the order Ctrl < Nema < Prot < N × P (Table 5), with the protozoan treatments being significantly greater than the non-protozoan treatments. The amount of ^{15}N incorporated in plant biomass corresponded to 3.3, 4.2, 8.2 and 8.9% of ^{15}N originating from the labelled hotspot material in the respective Ctrl, Nem, Prot and N × P treatments. Overall, plants in the mix-treatment had lower total

contents of C, ^{13}C , N and ^{15}N than in the layered treatments. However, there was a strong fauna × hotspot interaction and the effects of protozoa on ^{15}N -content in plants depended on the distribution of organic matter in the hotspot treatments. The ^{15}N -content of plants in the presence of protozoa was approximately doubled compared to Nema and Ctrl treatments. The highest amounts of ^{15}N were found in plants from the mix- and 4-layer treatments in the presence of protozoa and the lowest in the 1-layer treatment. The opposite effect was observed in treatments without protozoa, where the lowest contents of ^{15}N were found in plants of the mix-treatment and the highest in the 4-layer and 1-layer treatments (Table 5).

3.3. N in soil and leachate

The faunal and the hotspot treatments both affected the amount of exchangeable nitrate bound in the soil matrix (i.e. nitrate leached from soil in KCl extracts), but did not affect ammonium contents. The faunal effects were responsible for 47% of the variation in NO_3 contents of soil, compared to 8% for the hotspot treatments. The exchangeable amount of nitrate decreased in the order Prot > N × P >> Ctrl > Nema, with 3.1 and 2.8 $\mu\text{g NO}_3\text{-N g}^{-1}$ soil in the Prot and N × P treatments compared to 1.9 and 1.7 $\mu\text{g NO}_3\text{-N g}^{-1}$

Table 4

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on C:N ratios in shoots of the first harvest cut 5 cm above shoot base (1st shoot), shoots of the final harvest cut 5 cm above shoot base (2nd shoot), shoot base (0–5 cm height) of the final harvest (base), roots of the final harvest (root)^a

	1st shoot	2 nd shoot	Base	Root
<i>F values</i>				
Model	3.2 **	4.5 ***	5.6	4.0 ***
Patch (P)	1.1	6.5 **	3.9 *	1.6
Fauna (F)	3.9 *	9.2 ***	13.9 ***	7.8 ***
P × F	3.6 *	1.6	2.1	2.9 *
% SS explained	49.5	58.1	63.3	55.0
<i>Treatment means (C:N ratio)</i>				
Nem	10.0 a	10.4 a	19.3 a	38.0 a
Ctrl	10.0 a	9.7 ab	16.2 b	35.6 ab
N × P	9.4 ab	8.8 b	14.2 b	31.6 bc
Prot	9.1 b	8.7 b	14.1 b	29.1 c
mix	9.7 a	8.8 a	14.8 a	35.0 a
4-layer	9.8 a	9.9 b	17.1 b	33.8 a
1-layer	9.4 a	9.6 b	16.0 ab	31.9 a

^a Details as in Table 1.

soil in the Ctrl and Nema treatments, respectively. The ammonium content of the soil ranged from 1.6 to 1.05 $\mu\text{g NH}_4\text{-N g}^{-1}$. The distribution of the hotspot material affected the amount of nitrate in soil in the order mix > 4-layer > 1-layer. With 2.7 $\mu\text{g NO}_3\text{-N g}^{-1}$ soil the nitrate content of the mix-treatment was higher than the nitrate concentration in the 1-layer treatment with 2.1 $\mu\text{g NO}_3\text{-N g}^{-1}$ soil, but neither were signifi-

cantly different from the 4-layer treatment with 2.3 $\mu\text{g NO}_3\text{-N g}^{-1}$ soil (Table 6).

90% of the nitrogen leached as nitrate. No significant differences were observed in the ammonium contents of the leached water. The faunal and hotspot treatments were responsible for 20 and 24% of the variation in NO_3 -leaching, respectively. Within faunal treatments, the highest amount of nitrate was

Table 5

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on contents of C, ^{13}C , N and ^{15}N in plant material and biomass of the whole plants^a

	C	^{13}C	N	^{15}N	Biomass
<i>F values</i>					
Model	14.2 ***	14.0 ***	29.5 ***	26.0 ***	4.7 ***
Patch (P)	36.2 ***	35.4 ***	43.3 ***	3.0 (*)	33.8 ***
Fauna (F)	26.9 ***	26.7 ***	76.1 ***	89.3 ***	30.7 ***
P × F	0.5	0.5	1.6	2.1 (*)	0.3
% SS explained	81.3	81.1	90.0	88.8	81.8
<i>Treatment means (mg)</i>					
Ctrl	47 a	0.5 a	3.6 a	0.41 a	121 a
Nem	58 a <i>x1.2</i>	0.6 a <i>x1.2</i>	4.1 a <i>x1.1</i>	0.53 a <i>x1.3</i>	150 a <i>x1.2</i>
Prot	76 b <i>x1.6</i>	0.8 b <i>x1.6</i>	6.7 b <i>x1.9</i>	1.02 b <i>x2.5</i>	200 b <i>x1.7</i>
N × P	81 b <i>x1.7</i>	0.9 b <i>x1.7</i>	7.1 b <i>x2.0</i>	1.11 b <i>x2.7</i>	217 b <i>x1.8</i>
mix	45 a	0.5 a	4.1 a	0.71 a	125 a
4-layer	74 b	0.8 b	6.0 b	0.76 ab	194 b
1-layer	75 b	0.8 b	6.1 b	0.82 b	195 b

^a Numbers in italics indicate factors of increase in element contents in faunal treatments compared to the Ctrl treatment. Details as in Table 1.

Table 6

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on nitrogen bound in the soil matrix (N-soil), nitrogen leached (N-leached), ratio ^{15}N -leached: ^{15}N -uptake (^{15}N -Ratio), accumulated microbial nitrogen-mineralization (AMM) as the sum of N in plants, N in soil and N leached^a

	N-soil (mg N per microcosm)	N-leached (mg N per microcosm)	^{15}N -Ratio	AMM (mg N per microcosm)
<i>F values</i>				
Model	3.6 **	3.2 **	7.5 ***	20.1 ***
Patch (P)	0.9	8.5 **	4.2 *	37.4 ***
Fauna (F)	10.2 ***	4.2 *	20.2 ***	48.8 ***
P × F	1.2	1.0	2.2 (*)	0.1
% SS explained	52.4	49.6	69.7	86.0
<i>Treatment means</i>				
Ctrl	1.35 ab	1.89 b	0.727 a	7.4 a
Nem	1.06 b	1.56 b	0.668 a	7.6 a
Prot	1.67 a	4.03 a	0.314 b	11.5 b
N × P	1.55 a	3.12 ab	0.286 b	11.3 b
mix	1.47 a	1.54 b	0.604 a	7.6 a
4-layer	1.42 a	2.21 b	0.435 b	9.8 b
1-layer	1.33 a	4.24 a	0.458 ab	11.0 c

^a Details as in Table 1.

leached in the presence of protozoa and in the combined treatment with protozoa and nematodes (Table 6). Within the hotspot treatments, the amount of nitrate leached from the 1-layer treatment was more than double that from the 4-layer and mix-treatments (Table 6).

The relationship between leaching and plant uptake of ^{15}N gives a measure of the efficiency of the plants in uptake of ^{15}N from the hotspot material released by mineralization. The proportion of ^{15}N leached to ^{15}N in the plant was more than doubled in treatments without protozoa (Table 6). The effects of nematodes tended to be similar to protozoan effects, but less pronounced in the 1-layer and 4-layer treatments. In the mix-treatment only protozoa affected ^{15}N -uptake efficiency of plants significantly.

The accumulated microbial nitrogen-mineralization (AMM), the sum of N in plants, soil and leachate, was significantly enhanced by the presence of protozoa and by increasing substrate heterogeneity, which accounted for 57 and 29% of variation, respectively (Table 6). The AMM in treatments with protozoa or nematodes and protozoa was more than 30% higher compared to treatments with nematodes or control treatments and decreased in accordance with

the patchiness of the hotspot treatments in the order 1-layer > 4-layer > mix (Table 6).

3.4. Microbial respiration and biomass

Between 2430 and 3580 mg $\text{CO}_2\text{-C}$ were respired from the soil in the microcosms by the end of the experiment (Fig. 3). The cumulative amount of respired CO_2 in the faunal treatments was increased compared to the control by factors of 1.10, 1.17 and 1.21 in the Nema, Prot and N × P treatments, respectively. The amount of $^{13}\text{CO}_2\text{-C}$ respired per microcosm ranged between 29.5 and 44.9 mg (Fig. 3). Respiration of $^{13}\text{CO}_2\text{-C}$ originating from decomposition of the labelled hotspot material was closely correlated with respiration of $\text{CO}_2\text{-C}$ ($r^2 > 0.99$; all three hotspot treatments included), indicating that decomposition processes within the hotspot material were the main component of soil respiration and that root respiration was comparatively insignificant. $^{13}\text{CO}_2$ -respiration from the labelled hotspot material increased compared to the control in the Nema, Prot and N × P treatments by factors of 1.11, 1.21 and 1.27, respectively. Soil respiration did not differ among hotspot treatments.

The amount of $^{13}\text{CO}_2\text{-C}$ respired is a measure of decomposition of the ^{13}C -labelled hotspot material.

Fig. 3. Cumulative respiration (mg per microcosm) of $\text{CO}_2\text{-C}$ (a) and $^{13}\text{CO}_2\text{-C}$ (b) from microcosms with faunal treatments. See Fig. 2 for treatment abbreviations. Means are averaged since respiration did not differ between distinct hotspot treatments. Treatments with the same letter are not significantly different ($P < 0.05$).

Subtraction of the standard baseline (1.108 at.% ^{13}C) from the at.% ^{13}C measurements of CO_2 from soil respiration, gives the time course of decomposition activity in the hotspot material (Fig. 4). There was an initial increase in respired $^{13}\text{CO}_2\text{-C}$ followed by a slow decrease in decomposition towards the end of the experiment in all treatments. However, the time course and magnitude of $^{13}\text{CO}_2\text{-C}$ respiration in the Ctrl and Nema treatments differed considerably from the protozoan (Prot, $\text{N} \times \text{P}$) treatments. The rate of $^{13}\text{CO}_2\text{-C}$ respiration in Ctrl and Nema treatments peaked after 11 days with values of 0.12 and 0.13 at.% ^{13}C , respectively, while maximum decay rates of labelled substrate were reached after 14 days in the Prot and $\text{N} \times \text{P}$ treatments with 0.2 and 0.19 at.% ^{13}C , respectively

(Fig. 4). Following the first harvest of shoot material, an additional increase in $^{13}\text{CO}_2\text{-C}$ release in the protozoan treatments at the end of the experiment could be detected. The increase was absent in the Ctrl and Nema treatments.

Microbial biomass at the end of the experiment did not correlate with measures of microbial respiration. Only hotspot significantly affected microbial biomass in soil, which decreased in the order $\text{mix} > 4\text{-layer} > 1\text{-layer}$, with 504, 370 and 333 $\mu\text{g C g}^{-1}$, respectively. In the faunal treatments maximum microbial biomass was found in the presence of nematodes, but the lowest microbial biomass was present in the combined treatment with nematodes and protozoa.

Fig. 4. Time course of decomposition activity as indicated by the evolution of $^{13}\text{CO}_2\text{-C}$ from the labelled substrate. See Fig. 2 for treatment abbreviations.

3.5. Microfaunal populations

Naked amoebae were the most abundant protozoan group in the protozoan treatments. In the presence of nematodes, numbers of naked amoebae decreased significantly while numbers of flagellates and ciliates increased by factors of 1.45 and 1.9, respectively. However, these latter effects were not statistically significant. In contrast, nematode numbers significantly increased from 86 to 209 ind. g^{-1} in the presence of protozoa (Table 7). No statistically significant effects

on protozoan abundance could be detected in hotspot treatments, probably due to the high variability in protozoan numbers obtained by the MPN-method. However, increased average numbers of ciliates with 6000, 9000 and 12,000 ind. g^{-1} in the order mix-treatment < 4-layer < 1-layer, respectively, indicated a trend of increased abundance with a higher accumulation of organic matter in hotspots. Also nematode numbers tended to be higher in the 1-layer treatment. No trend could be detected for flagellate and amoebal numbers (Table 7).

Table 7

Results of a two-factorial ANOVA on the effects of hotspot configuration (Patch) and soil microfauna (Fauna) on numbers of amoebae, flagellates, ciliates and nematodes in soil^a

	Amoebae	Flagellates	Ciliates	Nematodes
<i>F values</i>				
Model	2.9 *	1.5	0.7	5.99 **
Patch (P)	2.0	0.3	0.6	2.15
Fauna (F)	7.1 *	2.8	1.7	24.9 ***
P × F	1.6	1.9	0.2	0.38
% SS explained	44.2	28.7	15.5	62.5
<i>Treatment means (ind. g^{-1} dry wt)</i>				
Nem				86 b
Prot	345,100 a	136,400 a	6100 a	
N × P	207,600 b	197,200 a	11,800 a	209 a
mix	295,500 a	187,200 a	6300 a	126 a
4-layer	328,300 a	151,100 a	8600 a	131 a
1-layer	205,200 a	161,900 a	12,000 a	185 a

^a Details as in Table 1.

4. Discussion

4.1. Hotspot effects on plant growth

Plant performance was strongly affected by both the distribution of organic material in soil, i.e. the ‘hotspot’ treatments, and presence of microfauna, i.e. the ‘faunal’ treatments (Fig. 2). Biomass and N-content of the plants were significantly increased when the organic material was concentrated in distinct layers rather than uniformly distributed through the soil (Table 5). Observations of the root system at harvest indicated an increased root growth within the layers of organic matter, while numbers of main roots were 10–20% lower in the mix-treatment (Table 2). In addition, roots were found growing through the small decomposing particles of plant residues of the hotspot material. This supports the idea that localized root growth is a major mechanism whereby plants ‘forage’ for patchy nutrient supplies (Drew and Saker, 1975, 1978; Hutchings and de Kroon, 1994; Robinson, 1994; Hutchings and Wijesinghe, 1997).

Basically, plant roots and microbes strongly compete for available nutrients and this competition may even lead to reduced plant growth (Bååth et al., 1978; Newman, 1985; Jackson et al., 1989; Azam et al., 1990; Wang and Bakken, 1997a). Whether the mineralized N from the decomposing hotspot material will be available for plant uptake depends on the ability of the roots to compete with the soil microflora for this resource. In the 4-layer and 1-layer treatments, microbial extracellular decomposition processes should result in a higher concentration of available nutrients within the hotspot layers compared to decomposition processes in the mix-treatment where nutrients will be released at random in small spatially unpredictable pulses. Since the benefit of localized root growth will be progressively reduced as exploitation of the hotspot material declines with its size, plants should be better competitors if available nutrients are concentrated in patches rather than diluted within uniformly distributed organic particles in soil.

This assumption is supported by the experiments of Wang and Bakken (1997a), who introduced alternating layers of decomposing clover and straw residues in soil planted with barley. Their experiments have shown that plant roots and microbes were competing for the same N-source, but the competitiveness of plants grad-

ually increased with distance of organic patches in soil until no extra plant-N was obtained with a patch distance greater than 6 mm (Wang and Bakken, 1997a,b).

As illustrated by similar rates of $^{13}\text{CO}_2$ -respiration released from decomposing organic matter in hotspot treatments, it can be assumed that the distribution of organic material did not affect decomposition of the organic material and consequently the nutrient release for plant growth. However, increased plant growth indicated that roots had better access to nutrients when the organic material was aggregated in patches, i.e. supporting the idea of root foraging as an important mechanism for the exploitation of patchy nutrient sources. Conversely, in treatments where the organic material was uniformly mixed with the soil much of the N remaining in the soil apparently was unavailable for plant uptake.

4.2. Faunal effects on plant biomass and morphology

In contrast to hotspot treatments, microfauna affected significantly the decomposition of the hotspot material. The time-course in decomposition of the hotspot material, monitored by continuous measurements of $^{13}\text{CO}_2$ -C, showed more dynamic and increased rates of decomposition in the presence of protozoa compared to the Nema and Ctrl treatments (Fig. 4). In addition, cumulative ratios of CO_2 and release of $^{13}\text{CO}_2$ from hotspot material increased slightly in treatments with nematodes and in the presence of protozoa significantly by ca. 15%. Effects of nematodes and protozoa were additional, with maximum values of soil respiration in combined treatments with protozoa and nematodes, suggesting an increased microbial activity in the presence of microfauna (Fig. 3). Increased rates of $^{13}\text{CO}_2$ from decomposition coincided with increased amounts of KCl-extractable N, a measure of available nitrogen bound in the soil matrix, and with increased amounts of nitrogen in leaching water in protozoan treatments. These facts give strong evidence for increased microbial mineralization activity and subsequent availability of nutrients for plant growth due to microfaunal grazing.

The increased plant growth in faunal treatments show that reduced nutrient uptake from a homogeneously distributed organic source can, at least partly, be compensated for by the activity of soil microfauna, especially protozoa. These findings correspond

well with a number of investigations. Increased N-mineralization (Bååth et al., 1981; Ingham et al., 1985; Vreeken-Buijs et al., 1997) and N-uptake of plants in presence of microfauna, especially protozoa (Elliott et al., 1979; Clarholm, 1985, 1989; Ritz and Griffiths, 1987; Kuikman et al., 1990) and a concomitant increase in biomass of plants (Clarholm, 1984; Gerhardson and Clarholm, 1986; Ritz and Griffiths, 1987; Kuikman et al., 1990; Jentschke et al., 1995; Alphei et al., 1996) has been repeatedly reported. The most common explanation for increased plant growth in the presence of microfauna is based on nutrient effects due to grazing on microorganisms (Elliott et al., 1979; Clarholm, 1985; Zwart et al., 1994). Nematode and protozoan grazing can stimulate microbial turnover (Yeates and Coleman, 1982; Alphei et al., 1996) and the low C:N ratio in the microbial biomass causes microbial-feeding animals to excrete much of the ingested nitrogen, mostly as ammonia (Stout, 1973). Generally, ca. 40% of ingested microbial biomass N will be excreted by protozoa (Griffiths, 1994) and ca. 70% by nematodes (Ferris et al., 1997), leading to a constant release of nutrients readily available for plant uptake.

The smallest plants (0.08 g dry wt) were found in treatments without microfauna where the hotspot material was uniformly mixed with soil (Fig. 2). The increase in plant biomass by a factor of 2.3 in the presence of microfauna (N × P) was even higher than the 1.8-fold increase in plant biomass without microfaunal activity but when the organic material was concentrated in distinct patches. Compared to the hotspot treatments, which accounted for 34% of variance in plant growth, 47% of variation could be explained by the faunal treatments, indicating that growth of ryegrass was even more influenced in our system by microbial–faunal interactions than by root foraging. The effects of fauna and hotspots were additional, with a threefold increased plant biomass in treatments with protozoa and hotspot material in 1- or 4-layer compared to mix without microfauna. In addition, plants recovered more quickly in the presence of protozoa after the 1st shoots were harvested. In Ctrl and Nema treatments 2nd shoots gained only 70 and 76%, but 2nd shoots in the Prot and N × P treatments achieved 81 and 84% biomass of the 1st harvest shoots, respectively. It is likely that root foraging and competition with the microflora had depleted the available nutrient

pool in the absence of microfauna, resulting in a strong nutrient limitation of regrowing shoots in Ctrl treatments. This leads to the conclusion that much N was inaccessible to plants even by nutrient acquisition from distinct patches, but became available by microfaunal activity which possibly enabled a more persistent nutrient acquisition of plants. Therefore, microfaunal effects are especially important under conditions where nutrient supplies depend on the local microbial decomposition of a chemically complex, organic substrate.

4.3. Faunal effects on decomposition and nutrient cycling

As expected, differences in plant growth were reflected in nutrient contents of the plant material. Consistently with an increase in biomass, the uptake of carbon and nitrogen was doubled in the presence of protozoa compared to Ctrl plants and plants in Nema treatments. But uptake of ^{15}N increased disproportionately by a factor of 3 in Prot and N × P treatments emphasizing the importance of protozoa in the decomposition process of the hotspot material (Table 5). The effects of protozoa depended on the type of hotspot. In plants of Ctrl and Nema treatments, ^{15}N content decreased in the order 1-layer > 4-layer > mix. The reverse effect was found in Prot and N × P treatments with an increase of ^{15}N in the order 1-layer < 4-layer < mix. The data illustrates that nutrient acquisition from hotspot material by plants was especially limited in the absence of protozoa when the organic matter was homogeneously distributed in soil.

In addition to the stimulation of microbial turnover, protozoan grazing may have changed the microbial composition leading to increased nitrogen concentrations in soil and leaching water of Prot and N × P treatments. It has been demonstrated that protozoan grazing facilitates reestablishment of certain bacterial species after fumigation. Amoebae and ciliates in particular have been shown to have a stimulatory effect on nitrifying bacteria (Griffiths, 1989; Alphei et al., 1996). In accordance with these findings protozoa strongly increased the concentration of available nitrate in soil and leaching of $\text{NO}_3\text{-N}$ was more than doubled in presence of protozoa compared to Ctrl

and Nema treatments, indicating that protozoa significantly enhance the release of N as highly mobile NO_3^- . In this way protozoa affected the mobility of nitrogen in soil and probably enabled plant roots to access N in the mix-treatment even where the roots were not growing in close contact with decomposing organic particles. Nematodes, in contrast, decreased available N in soil and leaching of NO_3^- and NH_4^+ in soil water.

The total amount of nitrogen released by microbial activity in our system, as calculated by addition of N available in soil, N taken up by plants and N leached with soil water (AMM, Table 6) increased by more than one-third in the presence of protozoa compared to the Ctrl and Nema treatments and clearly demonstrates the importance of microfaunal activity in nutrient cycling. The coupling of nutrient release and uptake is especially important for plant growth. The relationship between leaching and plant uptake of ^{15}N gives a measure of the efficiency of the plants in uptake of ^{15}N from the hotspot material released by mineralization. The ratio of ^{15}N leached/ ^{15}N plant uptake decreased by more than 50% in treatments with protozoa (Table 6), indicating that protozoa increased mineralization with a concomitant increase in the uptake of available N by plants. Thus, in addition to the spatial coupling of mineralization and nutrient uptake by plants from organic hotspots, protozoan activity enhanced the synchronization of nutrient release and plant demand resulting in a twofold increase in plant biomass in the presence of protozoa.

Recent investigations have questioned the hypothesis that increased plant growth in the presence of protozoa is solely based on nutrient effects (Jentschke et al., 1995; Alpehi et al., 1996). However, high concentrations of readily decomposable organic matter in the hotspot material in our system stimulated soil microbial activity in the rhizosphere. Hence, nutrient effects of decomposing hotspot material dominated in our system, expressed by increased N-concentrations in plants in the presence of protozoa.

4.4. Animal interactions

A closer examination of protozoa and nematode effects revealed that complex interactions did occur in our simple bacteria–bacterivore food-web. Microfaunal grazing does not ultimately result in a decreased

microbial biomass. Consistent with studies by Darbyshire and Greaves (1970) and Griffiths (1986) who have shown that bacterial production can be stimulated by bacterial feeding protozoa and nematodes, we found that the grazing pressure did not decrease, but even tended to increase microbial biomass in Prot and Nema treatments. The opposite was true in treatments with combined grazing pressure from nematodes and protozoa, where microbial biomass was reduced. Moreover, the situation in $\text{N} \times \text{P}$ treatments was complicated by interactions between protozoa and nematodes.

The twofold increase in nematode numbers coupled with a decrease of naked amoebae by 40% implies a trophic relationship between amoebae and ‘bacterial’ feeding nematodes (Table 7). This suggestion is supported by a number of studies where a significant reduction in naked amoebae concomitant with an increase in nematodes was reported (Anderson et al., 1978; Elliott et al., 1979, 1980; Woods et al., 1982). Thus nematodes may exhibit significant indirect effects on the composition of the microflora via affecting abundance and composition of the protozoan community. These findings were supported by a more detailed study of microfaunal effects on the rhizosphere microbial community structure in the mix-treatment, where not only microbial growth, but also functional diversity and the composition of the microbial population was affected by the microfauna (Griffiths et al., 1999).

The ‘microbial loop’ concept has been applied to microfaunal effects on plant growth (Clarholm, 1994). However, the data illustrates that in addition to grazing effects on microflora, interactions between protozoa and nematodes, and also among the different protozoan groups do occur. The outcome of those interactions determines whether the mineralized N will be available for plant uptake due to effects on microbial biomass, turnover and microbial catabolic ability. In addition, grazing by protozoa may alter the composition of the rhizosphere microflora from unfavorable microorganisms towards a plant growth promoting rhizosphere community (Burr and Caesar, 1984). We believe that a complex ‘microbial food-web’ is operating rather than a simple ‘microbial loop’, because the effects of microfaunal grazers cannot be fully explained by simple mechanisms affecting microbial turnover and biomass.

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