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RELATIONSHIP BETWEEN *COGNETIA SPHAGNETORUM* VEJD. (OLIGOCHAETA, ENCHYTRAEIDAE) AND SOIL MICROORGANISMS: A MICROCOSM EXPERIMENT

ABSTRACT: Changes in densities of microorganisms were analysed in *Cognetia sphagnetorum* Vejd. cultures at normal and low numbers of microorganisms. Different abundance of microorganisms was achieved by using captan and oxytetracycline. During 14 weeks, the following variables were measured: total numbers of bacteria and fungi by plate method, the biomass of microorganisms by PLFA method, content of ions, and activity of acid and alkaline phosphatase and urease. Numbers of enchytraeids increased with numbers of fungi. The presence of animals reduced the activity of soil enzymes and the content of Cl, SO₄, Na, Ca, and Mg ions in soil leachates. An increase in the content of N-NO₃ and N-NH₄ was not significant.

KEY WORDS: *Cognetia sphagnetorum*, soil activity, fungi, microbial biomass, microbial numbers, ion content

1. INTRODUCTION

Two trophic chains involved in decomposition process are active in the soil: “fast”-bacterial one and “slow”-fungal one (Coleman *et al.* 1983). Their organization and interrelationships are one of the important problems in modern soil ecology (Niemi and Setälä 2001). Microbi-detritivorous

enchytraeids belong to both these chains. It was found that predatory omnivores can destabilize soil systems (Lawler and Morin 1993, Mikola and Setälä 1999), but microbi-detritivores active at lower level can regulate the functioning of trophic chain by transferring a larger stream of energy through one of these two chains.

Cognetia sphagnetorum Vejd. is one of the important enchytraeid species living in boreal forests. Many authors call it a keystone species in these sites (Bond 1994, Huhta *et al.* 1998). It can graze on fungi (Standen and Latter 1977), affecting the length and respiration of fungal hyphae (Hedlund and Augustsson 1995). In some microcosm experiments with trophic chains, the „functionally irreplaceable” *C. sphagnetorum* can determine nutrient supply for plants though not in all experiments (Sulkawa *et al.* 1996, Laakso and Setälä 1999, Liiri *et al.* 2001).

The objective of this paper was to determine the effect of this species on soil microorganisms and mineralization rate of organic matter under different abundance of microorganisms. The biocides were used to control selectively the bacteria and fungi in experimental microcosms (see also Nowak *et al.* 2005).

2. METHODS

C. sphagnetorum was cultured in the soil in the presence of bacteria and fungi occurring in different abundance and proportions. The ratios of bacteria to fungi were modified using one of the two biocides: captan that reduces fungi or oxytetracycline that reduces bacteria. The kind and concentration of biocides were applied after Beare *et al.* (1992): 8.5 mg oxytetracycline per kg d.w. of the soil, or 3.3 mg of pure captan per kg d.w. of the soil. In total, 162 microcosms were established. These were PCV tubes 3.6 cm in diameter and 14 cm long (volume 142 cm³), with a 0.4 mm mesh screen at the oblique bottom. These containers were filled with soil taken from 0–10 cm soil layer in a pine forest. The soil was sieved through a sieve with mesh size of 1 cm in diameter to separate bark, branches, and cones. It was air-dried for two weeks. After this treatment, enchytraeids were absent from the soil, whereas natural microflora survived. Soil pH was 4.3, the content of organic matter was 13.3%, and the maximum water capacity was 43.6%. The tubes were filled with 50 g of dry soil so that the volume was about 70 ml to make sure that a similar content of organic matter was present in each tube. The soil was moistened to a constant weight of 70 g with a deionized water (because of chemical analyses). Two days after moisturing, 25 enchytraeids were released to half of the containers. This number corresponds to a density of about 25 10³ ind. m⁻², it is similar to the mean density of all enchytraeids in the habitat from which the soil was taken (18 10³ ind.), and to the maximal densities of *C. sphagnetorum* in some samples (Nowak 2001). The culture was stored in a dark room with a nearly constant temperature of 17.3°C (weekly fluctuations did not exceed 3°C) for 14 weeks. Over that time, soil was destructively sampled on three occasions. Each time one-third of the samples were taken for chemical and microbiological analyses. Estimating the numbers of animals was done in 5 to 8 samples of each fraction. Constant moisture was maintained by supplementing the liquid to a constant weight two times a week. In one-third of the samples it was oxytetracycline solution (OX), in one-third – captan (CA), and in one-third

water (control C). During four weeks, 9 ml of the liquid was used for each sample.

The plate method was used to estimate total numbers (ind g dw⁻¹ of soil) of heterotrophic bacteria on TSA substrate and total numbers of fungi on Czapek-Doxa substrate. Bacterial and fungal biomass was estimated by using the phospholipid fatty acids (PLFA) method. The PLFA method (Frostegård *et al.* 1991) relies on the isolation of lipids with the solution of chloroform, methanol and citrate buffer, and their fractioning to separate phospholipides. The method is considered as the reliable method for biomass (expressed as nmol g⁻¹ organic matter) assessing of bacteria and fungi in soil. After methanolysis and methylation of phospholipides, methylene esters of fatty acids were isolated and identified by using gas chromatography/mass spectrometry. The bacterial PLFA (Frostegård and Bååth 1996) comprises the following acids: i15:0, a15:0, 15:0, i16:0, 16:1 ω 7t, i17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0. Fungal biomass estimate was based on the acid 18:2 ω 6.

The activity of three soil enzymes was estimated too: acid and alkaline phosphatases and urease. Phosphatase activity was estimated after Beck. This method is based on estimating phenol that was released after the 2-hour hydrolysis of sodium phenylphosphorane at 37°C. Acetic buffer (pH=5.0) was used to estimate acid phosphatase, and borate buffer (pH=10) to alkaline phosphatase. Phenol in soil solution was estimated by using 2,6-dibromochinochloramid. The intensity of coloration was measured by using a colorimeter at a wave length of 600 nm. The concentration of N-NH⁴⁺ released during estimating the urease activity was determined using a colorimeter at a wave length of 630 nm.

Concentrations of cations N-NH₄⁺, Na⁺, Ca²⁺, Mg²⁺ and anions Cl⁻, N-NO₃⁻, SO₄²⁻ were determined in soil leachates in two separate analytical series by ion chromatography (Methrom IC System 690). Prior to the analyses, the samples were filtered through teflon filters of 0.45 μ resolution (Machery-Nagel, Germany). The supernatant was obtained after a 4-h exposure of 50-g soil samples in 0.5 l of deionized water.

Enchytraeid worms were extracted by the method of wet O'Connor funnels.

The following statistics were applied: ANOVA and Tukey's HSD post hoc test, principal component analysis (PCA), correlation coefficient, and linear regression.

3. RESULTS

The results of the experiment were tested by three-way ANOVA (Table 1). The effect of three factors were analysed: time, biocides, and animals. Time consisted of three dates (4, 9 and 12 weeks) of sampling, or of two dates in the case of biomass and some chemical analyses. No significant differences were found in microbial biomass between two successive samples. Other measurements varied with time. Biocides affected all the measured variables, as they inhibited the activity of specific microbes. They had a small effect on the ratio of fungi to bacteria, which was 0.10 for control samples (C), that is, treated with deionized water, 0.11 for samples treated with captan (CA), and 0.08 for samples treated with oxytetracycline. The effect of the third, most important factor, that is, enchytraeids reduced statistically significant the content of anions and cations in the leachate, activity of the two enzymes, and numbers of bacteria.

The variables measured in this experiment were intercorrelated, and this must be considered when analysing the results. The correlation between bacterial biomass and their numbers is described by the equation:

$$\text{Number of bacteria (10}^5 \text{ ind.)} = -73.75 + 0.095 \text{ nmol of bacterial biomass,} \quad (1)$$

and it is significant at $P < 0.009$. This is a weak correlation with Pearson coefficient $r = 0.52$. The same correlation for fungal biomass was:

$$\text{Number of fungi (10}^3 \text{ ind.)} = -340.1 + 5.03 \text{ nmol of fungal biomass, } r = 0.57, P = 0.004. \quad (2)$$

A stronger correlation was found between enzymatic activity and respective biomass. The correlation coefficient of the activity of acid phosphatase with fungal biomass was $r = 0.80$, $P < 0.0001$. The equation was:

$$\text{Acid phosphatase activity (}\mu\text{g phenol g}^{-1} \text{ dw h}^{-1}\text{)} = -110.4 + 3.23 \text{ nmol of fungal biomass.} \quad (3)$$

Alkaline phosphatase was even more strongly correlated with fungal biomass: $r = 0.84$, $P < 0.0001$,

$$\text{Alkaline phosphatase activity (}\mu\text{g phenol g}^{-1} \text{ dw h}^{-1}\text{)} = 14.36 + 1.02 \text{ nmol of fungal biomass.} \quad (4)$$

Urease activity was weakly correlated with bacterial biomass; $r = 0.54$, $P = 0.007$,

Table 1. Statistical significance of differences between variants of *C. sphagnetorum* culture ANOVA ($F_{2,71}$) multiple regression (R), ** $P < 0.00001$, * $P > 0.01 < 0.05$

	Microbial biomass	Number of bacteria	Numb. of fungi	Enzymes activity	Nitrogen	Anions	Cations
1.Biocides	R 18.5 **	F 402 **	F 171 **	R 259 **	R 53.8 **	R 162 **	R 10.8 **
2.Time	-	F 30.9 **	F 47.9 **	R 121 **	R 102 **	R 23.6 **	R 19.6 **
3.Animals	-	F 5.6 *	-	R 61.6 **	-	R 3.4 *	R 3.7 *
1,2	-	F 31.7 **	F 18.2 **	R 15.7 **	-	-	-
1,3	-	F 8.0 **	-	R 11.4	-	-	-
2,3	-	F 14.4 **	F 14.4 **	R 17 **	-	-	-
1,2,3	-	F 13.0 **	F 19.8 **	R 12.2 **	-	-	-

Urease activity ($\mu\text{g N-NH}_4 \text{ g}^{-1} \text{ dw h}^{-1}$) = $11.06 + 0.007$ nmol of bacterial biomass. (5)

The effect of enchytraeid worms was analysed in time and in different variants (Table 2). The two correlated variables, biomass and numbers of bacteria, increased in the presence of animals, though not significantly. The change was about 1% for numbers and about 16% for biomass (Table 2A). The latter was determined by changes in the control variant (C, with deionized water), where at the beginning of the experiment numbers of bacteria increased in the variant with animals (39.5×10^5 to $74.3 \times 10^5 \text{ g}^{-1}$, $P < 0.001$), remained unchanged in the second period (41×10^5 , $45 \times 10^5 \text{ g}^{-1}$), and even decreased (27×10^5 , $17 \times 10^5 \text{ g}^{-1}$, $P = 0.0078$) in the third period. In the two remaining variants, OX and CA, bacterial abundance was low, that is, $2.8 \times 10^5 - 7.1 \times 10^5 \text{ g}^{-1}$, and it did not change in the presence of animals. The animals reduced fungal biomass and numbers (biomass by 2% and numbers by 13%, both

not significantly). Also in this case, the effect of animals was pronounced only in control variant C with the highest fungal abundance. This effect varied with time. In the first period, fungal numbers decreased from 200 000 in the control to $48\,000 \text{ g}^{-1}$ in the variant with enchytraeids ($P < 0.0001$). The numbers increased in the second period from 200 000 in the variant without animals to 425 000 with animals ($P < 0.0001$), and decreased again in the third period (from 377 500 to 190 000). Although fungal numbers differed by a factor of 10 between variants CA and OX, no enchytraeid effect on their numbers was found in any of them.

It was expected that urease activity would vary like numbers (and biomass) of bacteria to which it was correlated. But it was not so. Urease activity was $16.5 \mu\text{g N-NH}_4 \text{ g dw}^{-1} \text{ h}^{-1}$ without animals and $16.2 \mu\text{g}$ – with animals; the difference being not significant ($P = 0.076$). The dynamics of changes was also different. Fluctuations in urease activ-

Table 2. Changes caused by *Cognetia sphagnetorum* (mean values for different variants and sampling periods)

A. Abundance of microorganisms and enzymatic activity

	biomass PLFA nmol g^{-1} OM		number g dw^{-1} soil		urease $\mu\text{g N g}^{-1}\text{h}^{-1}$	phosphatase $\mu\text{g phenol g}^{-1}\text{h}^{-1}$	
	bacteria	fungi	bacteria	fungi		acid	alkal.
Without animals	971	93	$15.0 \cdot 10^5$	$124 \cdot 10^3$	16.5	193.7	111.4
With animals	980	91	$17.9 \cdot 10^5$	$110 \cdot 10^3$	16.2	174.0	101.7
Significance	–	–	–	–	$P=0.08$	$P < 0.001$	$P < 0.001$

B. Ion content in soil solution (mg l^{-1})

	N-NO ₃	N-NH ₄	Cl ⁻	SO ₄ ⁻²	K ⁺	Na ⁺	Mg ⁺⁺	Ca ⁺⁺
Without animals	2.68	3.88	9.14	2.78	2.52	2.06	0.84	5.39
With animals	2.85	4.04	8.25	2.50	2.39	1.75	0.77	4.76
Significance	–	–	$P=0.004$	$P > 0.01$	–	$P=0.02$	$P > 0.04$	$P > 0.01$

ity in successive samples were small, thus it was easy to find statistically significant differences. In the first period, animals significantly reduced enzymatic activity in each of the three variants of the experiment: C, CA, and OX. In the second period, a significant decrease in urease activity was found in the variant with oxytetracycline OX, and in the

third period in the control one C. Thus, no parallel changes were found in urease activity and bacterial numbers despite their correlation. Similarly, phosphatase activity seemed to decline in the presence of animals. The difference in the activity of alkaline phosphatase between the variants with and without animals was significant at $P=0.0001$ (Table 2A).

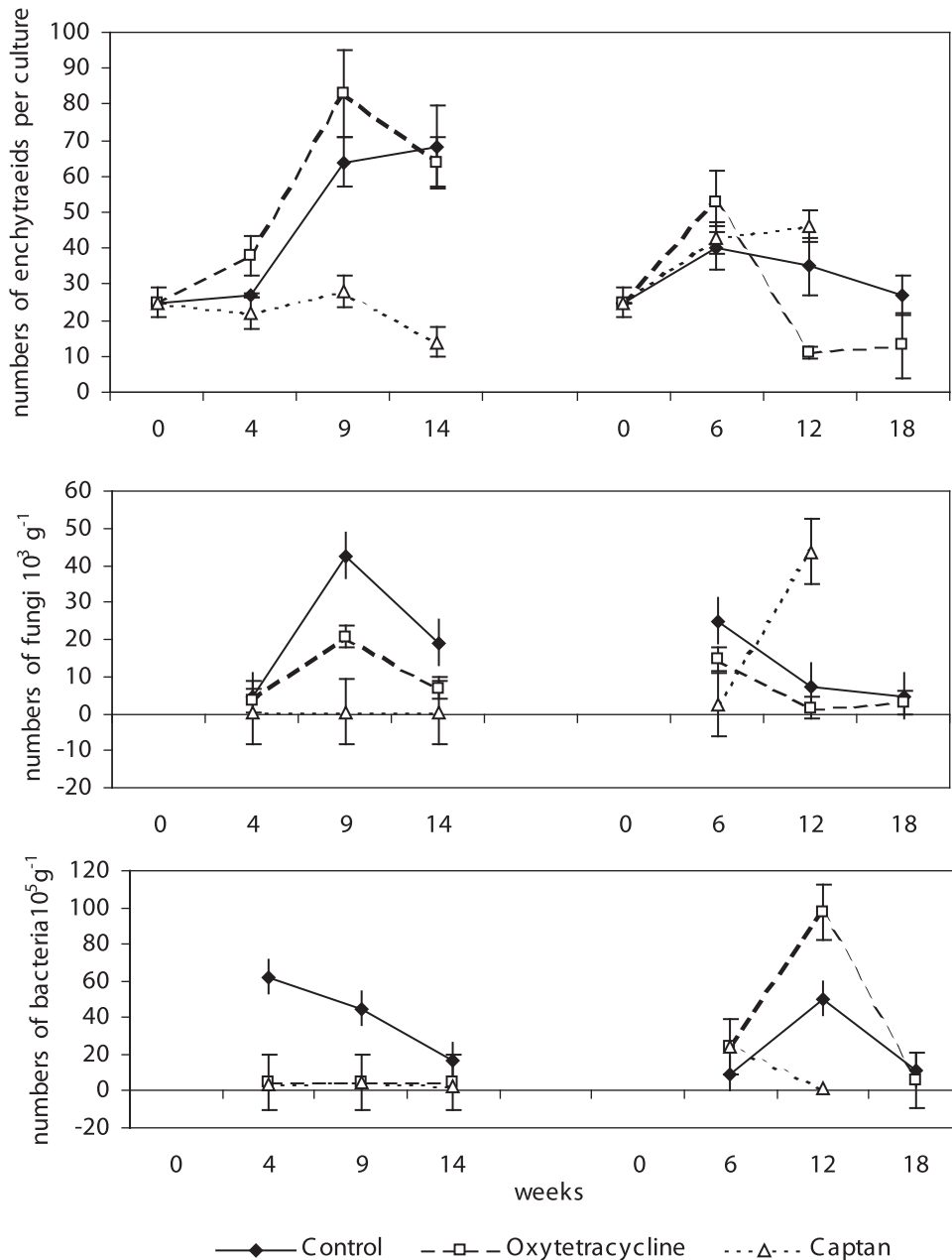


Fig. 1. Numbers of *Cognetia sphagnetorum* and microorganisms in microcosms (volume 142 cm³) in two series (14 and 18 weeks) of experiment in control and biocide variants.

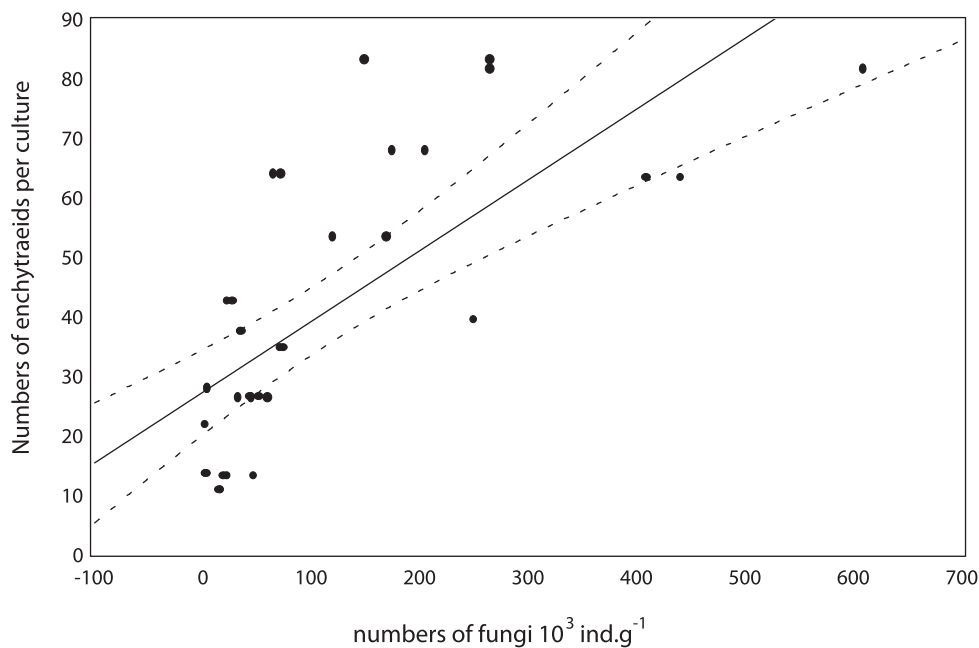


Fig. 2. Relation between numbers of *Cognetia sphagnetorum* and fungi based on all dates (regression and 95% P.).

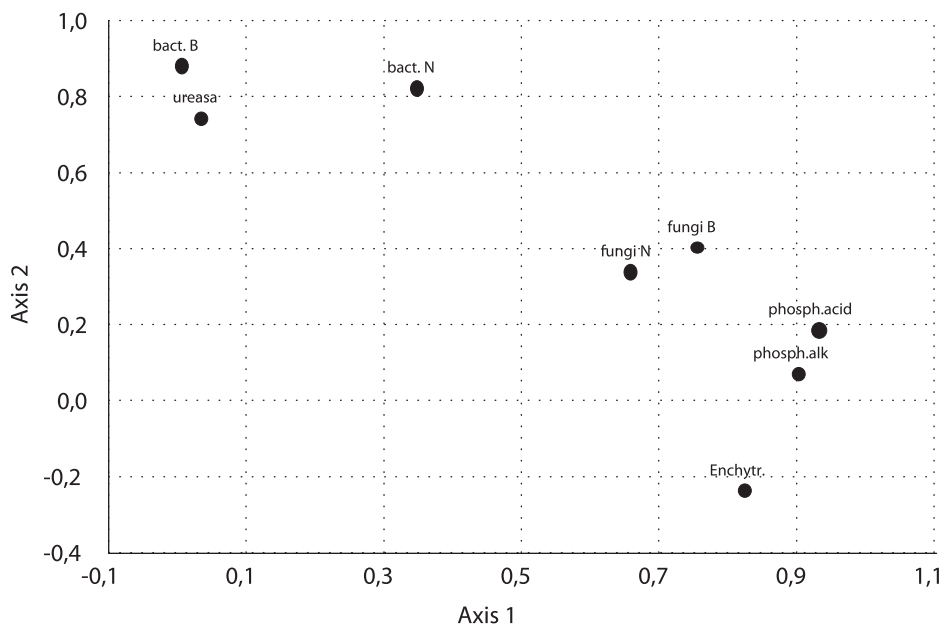


Fig. 3. Principal Component Analysis (PCA) biplot (axes I and II) illustrating pattern of variation in: numbers of bacteria (bact. N), fungi (fungi N), biomass of bacteria (bact.B), fungi (fungi B), activity of urease (ureasa), acid phosphatase (phosph.acid), alkaline phosphatase (phosph.alk), numbers of Enchytraeidae (Enchytr.).

In the variant with captan CA differences were significant on each of the tree sampling dates. In the control, they were significant on the first and second sampling dates, and in the variant with oxytetracycline only on the first date. The difference in the activity of acid phosphatase caused by animals was significant on all the sampling dates. For variant CA it was significant on the first date, and for variant OX – on the third date.

Changes of microflora abundance in the presence of animals were small, 1% for biomass and 13% – for abundance. Changes in enzymatic activity varied from 2% (urease) to 11% (acid phosphatase). Changes in the effects of microorganisms were also small (Table 2). Changes in the content of nitrogen leachate, the most important nutrient, were not significant. The content of nitrate nitrogen increased insignificantly from 2.68 to 2.85 mg l⁻¹ ($P = 0.289$), and that of ammonia nitrogen from 3.88 to 4.04 mg l⁻¹ ($P = 0.352$), what means in general about 5%. Other nutrients were significantly reduced in the microcosms with animals. The content of anions decreased by about 10%, chlorine by 10%, and sulphates by 16%. The decrease in potassium cation content was not significant (5%, $P = 0.177$), but the 8% decrease in magnesium, 12% decrease in calcium, and 15% decrease in sodium were significant (Table 2B).

Enchytraeid cultures were established twice, and each time increase or decrease of their numbers differed and seemed to be unpredictable (Fig. 1). However, this material provides evidence for a relationship between enchytraeids and fungi. A significant correlation was found between numbers of animals and abundance of fungi ($r = 0.73$, $P = 0.001$) (Fig. 2), described by the equation:

$$\text{Enchytraeid numbers per culture} = 27.5 + 0.12 \text{ fungal numbers } 10^3 \text{ ind. g}^{-1} \quad (6)$$

A stronger correlation was obtained for percentage changes in numbers of enchytraeids and fungi ($r = 0.77$, $P < 0.0001$). Enchytraeid abundance was also correlated with percentage changes in the activity of the two phosphatases in microcosms with animals. For alkaline phosphatase it is:

$$\% \text{ change} = 76.8 + 0.31 \text{ enchytraeid numbers per culture } (r = 0.78, P < 0.0001). \quad (7)$$

For acid phosphatase the correlation was not so strong ($r = 0.62$, $P = 0.006$). These two correlations result from a strong relationship between the both phosphatases and fungal biomass.

A multiple correlation was calculated between enchytraeid numbers, on the one hand, and microbial numbers and enzymatic activity, on the other hand: $R = 0.95$, and corrected $R^2 = 0.84$ ($P = 0.0002$). Significant components of this equation comprise bacteria ($\beta = 0.71$), fungi ($\beta = 0.57$), activity of acid phosphatase ($\beta = 1.39$), and fungal biomass ($\beta = 0.43$). Principal Component Analysis (Fig. 3) of the material show the connection of *C. sphagnetorum* numbers, phosphatase activity and biomass of fungi with the first axis and numbers and biomass of bacteria, urease activity – with second axis. The first two axes explained 73% of the variation.

4. DISCUSSION

The decrease in the activity of soil enzymes and in the content of Ca, Mg, Na, Cl, and SO₄ observed in the present experiment shows that enchytraeids inhibit mineralization processes in soil. Some authors, however, report opposite results. The animals feeding mainly on microflora, such as enchytraeids (50 or 80% of the food ratio, Didden 1993, 1995), generally enhance soil respiration to a higher extent than it could be expected from their biomass. This was found by Hedlund and Augustsson (1995) and Koutika *et al.* (2001), who directly measured soil respiration, as well as by Anderson *et al.* (1983), Williams and Griffiths (1989), and others, who measured the release of nutrients into the soil. These authors describe also situations when it was not the case because of an excessive impact of animals on microflora (Hedlund and Augustsson 1995), or because of the ion absorption by the soil particles (Koutika *et al.* 2001). Hanlon (1981) has noted that animals can stimulate the growth of microflora only on a suitable substrate. In the present experiment, no statistically significant difference was found in fungal numbers between cultures with 10–40

enchytraeid individuals and with more than 40 worms, thus there was no overgrazing. The depletion of substrate, however, was possible and it could have been responsible for the reduction in both soil enzymatic activity and release of the nutrients in the described experiment with *C. sphagnetorum*.

It is worthy of emphasizing that it was the case of only one nitrogen form, thus, nitrogen mineralization was not parallel to the mineralization of other nutrients. Many authors found an increase in N content (Abrahamsen 1990, Didden 1995, Briones *et al.* 1998), ascribing a part of this increase to *Oligochaeta* metabolism (Komulainen and Mikola 1995).

Like in other experiments, also in present experiment a relationship was found between *C. sphagnetorum* and soil fungi, but fungi determined the abundance of enchytraeids rather than *vice versa*. Mikola and Setälä (1999) found that fungal biomass was not affected by the food web structure. In the present experiment, fluctuations in *C. sphagnetorum* numbers were similar to those in fungal numbers, but the latter were dependent on other factors, possibly on the substrate.

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