European Journal of Soil Biology 69 (2015) 66–73

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

The role of bacteria and protists in nitrogen turnover in ant nest and forest floor material: A laboratory experiment

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ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form
14 May 2015
Accepted 18 May 2015
Available online 1 June 2015
Handling editor: C.C. Tebbe

Keywords:
Ammonium
Microbial loop
Bacterial biomass
Available carbon
Coniferous forest

ABSTRACT

Wood ant nests are hotspots of nutrients and microbial activity in nutrient-limited coniferous forest ecosystems, as ants accumulate honeydew and nutrients in their nests due to foraging and building activities. In this study we carried out a microcosm experiment focussing on the role of bacteria and protozoa on carbon and nitrogen turnover in nutrient-rich and nutrient-poor litter materials. Two types of litter material, (i) ant nest material or (ii) surrounding forest floor material were sterilized and inoculated either with bacteria alone (B treatment) or with bacteria and protozoa in combination (BP treatment). The litter materials were subsequently incubated in laboratory microcosms for 21 days. Respiration of the microcosms was measured during the whole incubation period and leachates were sampled every week and analysed for ammonium and nitrate. Our results showed lower ammonium leaching and increased respiration in the BP treatment, which could be explained by higher microbial biomass in this treatment. The negative effect of protozoa on ammonium leaching was independent of the type of litter material suggesting that available carbon in nest material supported fixation of nitrogen in the bacterial biomass. Our data show that protozoan grazing can play a critical role in retaining nitrogen in ant nests by increasing microbial activity and biomass, and thereby preventing nitrogen leaching.

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1. Introduction

Soils of temperate conifer forests that grow on granitic bedrock are acidic and have a low nutrient status [1]. Fulvic acids are released during the decomposition of needle litter material of coniferous trees thus acidifying the soil water and further reducing nutrient availability in litter materials as mineral nutrients are most available at neutral pH [2]. Although conifer forest ecosystems are usually nitrogen (N) limited [3], N availability is increased in nutrient-rich patches that occur in the forest floor, such as wood ant nests. However, N cycling in these nutrient-rich patches have not yet been investigated thoroughly.

Wood ant (*Formica* s. str.) nests are typical for temperate and boreal forest ecosystems [4,5]. They can occur in densities of 10–20 nests per ha [6–8], and the mound nests of wood ants are large and permanent structures, being occupied for decades [5,9]. They are constructed from plant materials, mainly needles, as well as from mineral soil particles [10]. Since ants transport vast amounts of food and building material into their nests [7,11–13], a part of these nutrient-rich organic materials afterwards succumbs to decomposition by microorganisms and, subsequently, mineral forms of nutrients are released [10,14,15]. Respiration rates per m^2 of nest mounds can be 12 times higher than of the surrounding forest floor [8], and mineral nitrogen accumulates in the nests which consecutively represent hotspots of carbon and nutrient turnover and significantly increase belowground heterogeneity in the otherwise nutrient-limited forest ecosystem. These nutrients can then be exploited for example by trees growing in nests' surroundings [16].

Although many studies deal with concentrations of nutrients in wood ant nests in comparison to the surrounding forest floor (e.g. Refs. [15,17–19]) or with carbon turnover [6,8,20], there is surprisingly little information on the nitrogen turnover in wood ant nest material. According to the general knowledge, one group of soil fauna that could have an impact in this respect are protists. Protists are abundant in soils of coniferous forest ecosystems and

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http://dx.doi.org/10.1016/j.ejsobi.2015.05.004
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according to food web models, play a major role in carbon and nitrogen cycling in these systems [21]. Within coniferous forest ecosystems, protozoa and bacteria are particularly abundant in ant nests [15,19,22], and a mechanism that could be crucial for nutrient liberation are predator–prey interactions between protists and bacteria as protists are important microbiota grazers [21,23,24]. This interaction is generally known as a microbial loop and is considered important for the release of mineral nitrogen from consumed microbial biomass, since protozoa excrete much of the ingested nitrogen as ammonium [25]. Overgrazing can decrease microbial activity, but more often grazing effects result in enhanced microbial turnover [26,27]. Protists have been found to boost the decomposing activity of bacteria, because bacterial populations that are grazed are metabolically more active [24,28,2]. Moreover, protozoan grazing can change composition of bacterial community because gram-positive (G+) bacteria are generally much harder to digest by protists than gram-negative (G−) bacteria [30–32]. Bearing this in mind, ant nests are useful model systems to study microbial mechanisms involved in carbon and nutrient dynamics of organic hotspots in the forest floor.

In this study we focused on different roles of bacteria and protists and especially on their interaction affecting nitrogen turnover and nitrogen flow in nutrient-rich (ant nest) and nutrient-poor (forest floor) materials. We carried out an experiment using microcosms with sterilized ant nest or forest floor material, either inoculated with litter bacteria alone or with bacteria and protists together. We subsequently sampled leaching water from the microcosms and analysed the leachates for mineral forms of nitrogen at regular intervals. Also, respiration of the microcosms was measured during the whole incubation period. Our hypotheses were that 1) higher microbial activity (i.e. respiration) will occur in the ant nest material, especially when inoculated with bacteria and protists together, 2) higher amounts of mineral nitrogen will be leached from the ant nest material, 3) even more nitrogen will be leached in presence of protists, and 4) bacterial community composition will be shifted towards G+ bacteria in presence of protists.

2. Materials and methods

2.1. Sampling and preparation of the materials

Litter materials were sampled in a forest stand dominated by Norway spruce (*Picea abies* (L.) H. Karst.) with rare beech (*Fagus* sp.) and oak (*Quercus* sp.) situated at 600 m above m.s.l. on the southern slope of the Klet Mountain in South Bohemia (Czech Republic) in August 2012. Approximately 200 cm^3 of ant nest material was sampled from the centre of five randomly selected ant (*Formica aquilonia* Yarrow) nests of a similar size. The centre of the nests was sampled as there is the highest microbial activity [33,34]. For comparison, the same volume of litter material was sampled from the forest floor at a depth of 0–5 cm where microbial activity is highest [35]. The forest floor material (L horizon), mainly represented by conifer needles, was sampled in the nests' surroundings, approximately five meters from each nest. Materials were then transferred to the laboratory and thoroughly mixed. Ants were separated from the ant nest material using pincers. Fresh ant nest and forest floor material was then put in thin layers into plastic bags and γ-ray sterilized (isotope Co^{60}, total dose 40 kGy) [36]. Afterwards, the materials were kept at 4 °C until the incubation started. Total carbon and nitrogen content was determined in dried and crushed materials on a CN analyser. Material pH was measured in a 1:10 material:distilled water solution using a glass electrode. Ammonia and nitrate were extracted from the materials by $KAl(SO_4)_2$ solution and determined using the Kjeldahl method [37]. Properties of the sterile materials are summarized in Table 1.

2.2. Preparation of the inocula

To prepare bacterial inocula, ca. 30 g of fresh ant nest or forest floor material were mixed with 200 ml of sterile water and incubated for two hours. Bacteria were isolated from the litter materials by a sequence of filtering steps according to Rosenberg et al. [32]. First, the suspension was filtered through a paper filter to separate the organic materials. Two subsequent filtering steps with 3 and 1.2 μm filters (Cellulose Acetat Filters (Minisart®), Sartorius) under sterile conditions resulted in a filtrate with a mixed diverse bacterial community. The filtrates were incubated for three days at room temperature in Neffs Modified Amoebae Saline (NMAS) medium [38] and then checked for contaminations by protists under the microscope. The protozoa-free bacterial inocula from ant nest and litter material were pooled and thoroughly mixed together for subsequent litter inoculation.

To prepare protozoan inocula, app. 5 g of fresh ant nest or forest floor material were mixed with 20 ml of NMAS medium supplemented with 1:9 v:v sterile nutrient broth (NB-NMAS, Oxoid) in a Petri dish [38]. The suspension was incubated for seven days at room temperature. Then, protists (amoebae, flagellates, ciliates) were identified at 100× magnification using an inverted microscope (Nikon TE2000) only to be sure that all the groups were present in the inoculum. Ten ml of inoculum were transferred using a Pasteur pipette into a new Petri dish with 20 ml of NB-NMAS medium and further incubated for two days at room temperature. The inocula from ant nest and litter material were pooled and thoroughly mixed together for subsequent litter inoculation.

During the incubation period microbial and protozoan communities adapted to the available resources in the respective litter treatments could establish.

2.3. Microcosm experiment

The design of the microcosms corresponded to those used by Bonkowski et al. [29]. Microcosm chambers consisted of perspex tubes (150 mm height, 60 mm diam) placed on ceramic lysimeter plates. The chambers could be drained under natural conditions of soil matric potential by lowering the atmospheric pressure in a box below the plates. Removable, air-tight lids on top of the chambers allowed watering of microcosms. Small vials attached to the lids contained 5 ml 1 N KOH to trap CO_2 for respiration measurements. All microcosm chambers had been sterilized by soaking in 70% ethanol.

Into each of the microcosms, 3 g of either ant nest or forest floor material was put and the materials were consecutively watered with 3 ml of sterile distilled water. All microcosms were inoculated with 1 ml of bacterial inoculum. Moreover, one half of the microcosms was inoculated with 0.5 ml of the protozoan inoculum (BP treatment). To the other bacteria-treated half, 0.5 ml of NB-NMAS medium was added to get the same moisture and nutrient conditions (B treatment). We had 12 replicates for each treatment plus 12 blanks (empty microcosms) for respiration measurements, in total 60 microcosms. The incubation started at the beginning of September 2012 and continued for 21 days at room temperature under sterile conditions in a sterile bench.

Measurements of microcosm respiration started on the third day of incubation to avoid the first burst of respiration after inoculation and then were done regularly, daily at the beginning and at an interval of two days after six days of incubation. Respiration was determined by titration of 1 N KOH with 0.01 M hydrochloric acid, after precipitation by barium chloride according to Schinner et al. [37]. Microcosms were leached weekly with 20 ml of sterile

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