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Ciliate dependent production of microbial anthranilic acid occurring within aspen litter

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ABSTRACT

Terrestrial protozoa and their role in the soil microbial loop are intricately linked to the functioning of forest soils. Yet, in spite of their recognition as vital components of soil ecosystems, protozoa remain understudied when compared to other soil microorganisms. In addition to directly stimulating soil nutrient levels by releasing bacterial nutrients, soil protozoa may affect plant growth indirectly by promoting bacteria that produce plant auxins. We conducted a four week incubation study using defaunated trembling aspen leaves that were selectively inoculated with ciliates. Ciliates modified microbial community structure, as assessed with phospholipid fatty acid (PLFA) analysis. Using High Performance Liquid Chromatography (HPLC), we found that the presence of ciliates did not favor bacteria that produce the plant auxin, indole-3-acetic acid (IAA). Instead, ciliates were associated with the production of anthranilic acid, which indicates that they were responsible for maintaining populations of *r*-selected bacteria within a relatively stable and nutrient poor environment. Additionally, while ciliates were observed using phase contrast microscopy, the commonly used PLFA indicator for soil protozoa (20:4) was not detected therefore it was shown to be a poor and potentially invalid biomarker.

1. Introduction

In forest ecosystems, there is an abundant protozoan community that runs the gamut of soil ecological niches; they are grazers of bacteria, fungi and other protozoa; they are saprotrophs involved in the primary and secondary stages of litter decomposition; and they are detrivores, consuming the products of litter decomposition (Adl and Gupta, 2006). In particular, protozoa such as soil ciliates are nearly ubiquitously distributed worldwide (Foissner, 2006), have high species diversity (Foissner et al., 2008) and are able to quickly colonize soils *via* aerosol dispersal (Altenburger et al., 2010). When active, ciliates have a disproportionally large spatial influence relative to their size due to their high mobility within the soil matrix (Adl, 2007). Despite their influence and prevalence, the role of ciliates and other protozoa in soil biogeochemical processes is often overlooked or only briefly touched upon in studies of forest ecosystems.

As early as the onset of the 20th century, soil protozoa were recognized as important drivers of the soil nitrogen cycle (Waksman, 1916). Studies from the later part of the last century showed that protozoans stimulate nitrogen mineralization in soils (Griffiths, 1986), promote nitrification (Griffiths et al., 1999), and that their activities lead to higher nitrogen contents in plant tissues (Clarholm, 1985; Kuikman and Van Veen, 1989; Kuikman et al., 1991). The culmination of research conducted throughout the 20th century led to the conceptualization of the "soil microbial loop". In brief, soil protozoa participate in the "soil microbial loop" by increasing nutrient availability in the soil environment as their nutritional needs are less than the bacteria they consume (Coleman, 1994).

Selective grazing of bacteria by protozoa can influence the composition of microbial communities (Rønn et al., 2002b; Griffiths et al., 1999), which can have far-reaching ecological effects. In particular, protozoa-induced compositional changes to the soil microbial community can increase the activity of auxin-producing bacteria, an important plant growth hormone (Bonkowski and Brandt, 2002). Protozoa have been shown to produce root systems that are larger with more lateral branching and therefore able to access moisture and nutrients from a larger volume of soil (Bonkowski and Brandt, 2002), have longer fine roots and more root tips (Bonkowski et al., 2001). These changes to plant root architecture have led to the expansion of the "soil microbial loop" concept to include non-nutritional influences in addition to grazing-related nutritional effects (Bonkowski, 2004).

Given their diverse roles in ecosystem function, protozoan communities are excellent candidates for describing the status of

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soil ecosystems as well as for monitoring effects of human impact on soil health. Soil ciliates, in particular, are ideal as they are widely distributed (Foissner, 2006). However, at present their use as indicators is hindered by the fact that a great number of species have not been described, and their enumeration and identification are both time consuming and require a high level of expertise (Foissner, 1999a). Molecular methods appear to be a potential workaround for many of the methodological hurdles; however, applying these methods to soil ciliates is still in its infancy and requires extensive fundamental groundwork (Lara and Acosta-Mercado, 2012). One possible currently available option to detect the presence of soil ciliates is the use of microbial phospholipid fatty acids (PLFAs). Currently, the PLFA 20:4 is a general biomarker for protozoa commonly used in the soil ecology literature (Frostegård et al., 1997; Sampedro et al., 2006; Thoms et al., 2010). Its use as a potential protozoan biomarker appears to link back to the work of Lechevalier and Lechevalier (1988), who summarized pure culture studies profiling the lipid signatures of amoeba, flagellates and ciliates. However, the use of 20:4 as a general indicator of soil protozoa may not be appropriate as fatty acids greater than 20 carbon are also found in higher plants and eukaryotic cells (Zelles, 1997, 1999). Additionally Rønn et al. (2002a), using mixed cultures of flagellates and amoebae, found that 20:4 was not detectable or was present in unpredictable quantities even when the presence of flagellates and amoebae was confirmed through traditional MPN techniques. Given these issues, the use of 20:4 as a protozoan biomarker may be inconsistent with the current evidence available in the literature and requires further testing against traditional enumeration methods of mixed cultures.

In this study, we inoculated defaunated aspen leaf litter with ciliates and other microorganisms isolated from an aspen forest floor in order to test how ciliates participate in specific aspects of the soil microbial loop. In particular we wanted to test i) if ciliate activity changes the structural composition of the microbial community and promotes auxin-producing bacteria and ii) if ciliates enhance the release of nitrogen from leaf litter. Additionally, we assessed the effectiveness of PLFA analysis as a tool to detect and potentially quantify ciliates by measuring and analyzing microbial phospholipids against direct counts of ciliates.

2. Materials and methods

2.1. Experimental design

The experiment was laid out as a 2-factor completely randomized design (2 treatments and 3 time periods) with three replications per treatment for a total of 18 experimental units. The treatments applied to samples included two different combinations of microbial inoculants so that half of the samples were inoculated with only forest floor bacteria ('bacteria-only' treatment) while the other half were inoculated with forest floor bacteria and ciliates ('bacteria + ciliate' treatment). After the inoculations were applied, the samples were randomly assigned to three different time or incubation periods (week-1, week-2 and week-3) at which point they were destructively sampled for analysis. A replicated set of samples was also taken immediately after inoculation to establish the conditions after the fumigation treatment.

2.2. Mesocosm preparation

The leaf litter used in the study was collected in August of 2009 from trembling aspen (*Populus tremuloides* Michx.) stems grown at the University of Alberta's Ellerslie Research Farm, Edmonton, Alberta, Canada. Stems were from aspen seedlings germinated from local open pollinated seeds that were planted in 2004 and subject to climactic conditions of the Edmonton region. Upon collection all leaves were air-dried and stored. For this experiment, the leaf litter was coarsely ground, weighed and soaked in distilled water for 2 h and then allowed to drain until water ceased to pool underneath the material. Protozoa were removed by fumigating the litter with chloroform in two 48-h cycles (Bonkowski et al., 2001). After fumigation the litter was rinsed with sterilized distilled water in a sterile laminar flow hood, drained and weighed. The moisture content at field capacity, calculated by using the initial dry weight of the litter and the final wet weight, was estimated to roughly 300%.

Reusable filter holders (Nalgene reusable filter holders with receiver, Thermo Fisher Scientific, Waltham) were used as the incubation mesocosms. The mesocosms were autoclaved at 120 °C for 20 min prior to addition of the leaf litter. Further, the mesocosms were kept sterile from atmospheric contamination by plugging all points of access found on the lid and on the receiver of the mesocosms with silicon plugs or sterilized cotton fiber. Each mesocosm contained 16 g (3.88 g dry weight) of the wet leaf litter placed evenly in the upper chamber on top of a 2.5 cm sand bed supported by a layer of glass wool. All mesocosms were kept within a sterile airtight acrylic glove box with inlet and outlet valves during the entire duration of the study. Gas exchange within the glove box was maintained with compressed building air passed through a 0.25 μm air filter. Sample moisture was conserved by bi-weekly monitoring of the mesocosm weights and adding water when required. Sterilized water and the implements used for maintaining water content were kept within the glove box.

2.3. Inoculant preparation

Microbial inoculants used to create the bacteria-only and bacteria + ciliate treatments were prepared from the forest floor of a mature site of trembling aspen collected from the Fort McMurray region of Alberta in the summer of 2010. Fresh surface litter was removed on site before the underlying decomposition layers (F and H horizons based on the Canadian system of soil classification) of the forest floor were sampled. The forest floor sample was kept cold during transport back to the laboratory, and then hand sorted to remove coarse woody fragments and roots, thoroughly homogenized to a depth of 10–15 cm and air-dried. The material was rewetted to field capacity using a soak and drain method (Puustjarvi, 1973) one month prior to the preparation of the inoculations. After draining, the forest floor was weighed and transferred to a large plastic tub, spread to a depth of 3 cm (to prevent anaerobic conditions) and covered. The moisture content was maintained by daily weighing to monitor moisture loss, with rewetting as necessary.

Three inoculants were prepared for this study, the first, the ciliate inoculant was made by creating a 1:20 solution (by weight) of moistened forest floor with 10% standard soil solution (SSS) plus wheat grass (Adl et al., 2008) and incubating for two days at 25 °C. The culture viability as a source of soil protozoa was ensured by inspecting it with an inverted phase contrast microscope at 160×. A sample of this protozoa culture, taken from the water column to avoid contamination by amoebae, was then added to a solution of 10% SSS plus wheat grass. This solution was incubated for five days to ensure that all flagellates were consumed to extinction by the ciliates. This was verified by inspecting the solution for flagellates with a hemocytometer under a phase contrast microscope at 400× before the solution was used as an inoculant for the bacteria + ciliate treatment.

The second inoculant, the bacteria inoculant #1 was prepared from forest floor bacteria mixing distilled water to the moistened aspen forest floor (3:1 water:forest floor). The solution was stirred