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Emergent microbial food webs in ecological treatment systems for wastewater: Insight from stable carbon isotopes

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ABSTRACT

The overall significance of microbial predation to the natural disinfection process of bacterial fecal pathogens in ecological treatment systems (ETS) is not well understood, due to the complex interactions and difficulties associated with quantifying components of the microbial food web in situ. A ^{13}C-labeled biological tracer was developed via the growth of K-12 *Escherichia coli* on D-(+)-glucose derived from *Zea mays* which is naturally enriched in ^{13}C due to the C_4 photosynthetic pathway. A microcosm experiment was conducted utilizing partially treated wastewater collected from a functioning ETS at the Darrow School in New Lebanon, New York. A filter-feeding ciliate *Tetrahymena pyriformis* and cyclopoid copepod *Cyclops* sp., were introduced to the microcosms to simulate an emergent pelagic food web in an ETS. The ^{13}C-labeled *E. coli* tracer successfully produced a δ^{13}C signature that was distinct from alternative carbon sources in the simulated food web. Predation of *E. coli* by *T. pyriformis* was observed when the ^{13}C-labeled *E. coli* tracer was the sole carbon source, however predation of *E. coli* by *T. pyriformis* in the presence of alternative carbon sources was negligible; the ciliate exhibited a δ^{13}C value most similar to the total dissolved solids in the ETS water. Minor predation of *T. pyriformis* by *Cyclops* sp. was observed elucidating a potential link to higher trophic webs. Sensitivities of the developed tracer and its feasibility for in situ analysis of emergent food webs are discussed.

1. Introduction

Ecological engineering research has advanced our understanding of nutrient removal, retention and transformation in constructed ecosystems and ecological technologies for wastewater treatment such as treatment wetlands (Kadlec and Wallace, 2009; Verhoeven and Meuleman, 1999) and living machines (Todd and Josephson, 1996). However, there is still limited information on the ability of such systems to naturally remove microbiological contaminants (Kadlec and Wallace, 2009; Morsy et al., 2007).

Numerous mechanisms have been identified in the literature as being responsible for the natural disinfection of bacterial pathogens of fecal origin. These processes include filtration, sedimentation, adsorption to biofilms, solar UV inactivation, microbial predation, oxidation, bacterial and viral lysis, as well as natural death and competition with other microbes (Decamp and Warren, 1998; García et al., 2008; Molleda et al., 2008; Morsy et al., 2007; Redder et al., 2010; Reinoso et al., 2008; Yang et al., 2000). The role of microbial predation in wastewater treatment is often considered among the various factors affecting disinfection but is rarely quantified in situ due to the complexity of the ecology associated with these types of organisms (Kuppardt et al., 2010).

Bacterivory in aquatic ecosystems has been attributed to phagotrophic (particle ingesting) protists such as heterotrophic flagellates, ciliates and dinoflagellates (Sherr and Sherr, 2002; Weisse, 2002), with predatory ciliates playing a significant role in eutrophic freshwater ecosystems (Sherr and Sherr, 2002). The role of ciliated protozoa in the predation of pathogenic bacteria has been emphasized in research pertaining to both conventional and ecological wastewater treatment (Curds, 1992; Decamp and Warren, 1998; Decamp et al., 1999). Chabaud et al. (2006) found that removal rates of pathogen indicator bacteria on biofilm slides treated with cycloheximide are 10 times less than on biofilms with an active protist community and that bacterivory by protozoa could

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account for 60% of the removal efficiency of pathogen indicator bacteria related to biofilms. Decamp and Warren (1998) observed feeding rates of various species of ciliated protozoa isolated from treatment wetlands on *E. coli* cells, and suggest that predatory ciliates may be capable of all reported *E. coli* removal rates from wastewater treated with horizontal subsurface flow constructed wetlands utilizing the root zone method. However, Decamp and Warren (1998) also suggest that ciliates would most likely be unable to maintain maximum feeding rates in situ due to numerous ecological factors. These factors include complex relationships associated with the microbial food web such as intra- and interspecies predation, predation by organisms from higher trophic levels, as well as the availability and utilization of multiple food sources (Little et al., 2008; Sanders and Wickham, 1993; Sherr and Sherr, 2002; Weisse, 2002).

The link between the microbial food web and higher trophic levels can be significant in both freshwater and marine ecosystems producing indirect cascading effects on both bacterial population size and composition, a relationship often referred to as a trophic cascade. As a common metazoan predator in both marine and freshwaters, with selective feeding habits, copepods have been known to utilize protozoa as an important food source (Sanders and Wickham, 1993). A mesocosm experiment conducted by Zöllner et al. (2003) reported a significant increase in bacterial production along a gradient of increasing copepod density, which supports the indirect trophic cascade effect on bacterial populations as a result of the decreased grazing pressures on bacteria due to the predation of protozoa by copepods.

The analysis of stable carbon isotopes (^{12}C and ^{13}C) is an accepted method for determining food sources in both aquatic and terrestrial food webs (Gannes et al., 1998; Kwak and Zedler, 1997; Peterson and Fry, 1987; Sulzman, 2007). The relative abundance of these two isotopes of carbon, denoted by $\delta^{13}C$, can also be used to examine the transfer of carbon through food webs as only minimal fractionation (+1.0‰) occurs between successive trophic transfers (Peterson and Fry, 1987). As different metabolic processes associated with the various photosynthetic pathways in the transformation of carbon dioxide to organic carbon result in distinct $\delta^{13}C$ values, carbon sources at the base of food webs can be determined by detecting differences in the $\delta^{13}C$ values of organisms (Gannes et al., 1998).

Recent technological advancements in microbial ecology have provided novel insights into the microscopic world, and the integration of the fields of microbial ecology with engineering is highly encouraged in order to utilize this knowledge for the optimization or implementation of new technologies for wastewater treatment (Daims et al., 2006). Numerous studies have used stable isotope probing (SIP) techniques via ^{13}C-labeled biomarkers such as DNA or *r*RNA coupled with density gradient centrifugation to track microbial predation (Boschker and Middelburg, 2002; Kuppardt et al., 2010). However, Boschker and Middelburg (2002) have noted that labeling intensity must be very high for these techniques in order to observe an effective signal via density gradient centrifugation. There is also caution associated with the use of biomarkers, as they are not always representative of bulk diets due to fractionation during the synthesis of specific biochemical compounds (Boschker and Middelburg, 2002). Further complications include effects of isotopic routing, in which various components of an organism's diet are isotopically heterogeneous, and can be used in the synthesis of specific tissues or components of tissues; isotopic routing makes assumptions about the representation of bulk diets by specific biomarkers difficult to validate (Gannes et al., 1997). Thus, the analysis of bulk stable carbon isotopes may provide a better representation of the highly heterogeneous and complex diets of aquatic microorganisms.

The purpose of this study was to develop a ^{13}C-labeled biological tracer in order to examine the incorporation of a bacterial fecal pathogen as a significant carbon source in a simulated microbial food web. This study focused on analyzing the effectiveness of the tracer to produce a unique isotopic signature in the presence of multiple trophic levels and alternative carbon sources. This research represents the initial steps required to determine the feasibility of bulk stable carbon isotope analysis as an in situ method for examining the role of bacterial fecal pathogens in the microbial food web, as well as determining linkages to higher trophic levels in constructed ecosystems for wastewater treatment.

2. Methods

2.1. Tracer development

A ^{13}C-labeled biological tracer was developed by cultivating a strain of *Escherichia coli* on a minimal D-(+)-glucose broth media utilizing D-(+)-glucose ($\delta^{13}C$ value = -10.4 ± 0.1‰; $n = 5$) derived from *Zea mays*. Since *Z. mays* is a grass that utilizes the C_4 mode of photosynthesis its tissues tend to be more enriched in ^{13}C ($\delta^{13}C$ ranging from -20 to -10‰) compared to tissues derived from C_3 photosynthesis ($\delta^{13}C$ ranging from -34 to -22‰) (Gannes et al., 1998). D-(+)-glucose (purity ≥99.5%; Sigma–Aldrich Inc.) was used as the main carbon source in a minimal D-(+)-glucose broth medium. A 0.5% D-(+)-glucose by mass solution was mixed as a control broth medium containing 2.5 g D-(+)-glucose reagent, 5.0 g tryptone (vegetable derived; Sigma–Aldrich Inc.) microbiological reagent, and 2.5 g reagent grade NaCl (Sigma–Aldrich Inc.) added to 500 ml of Milli-Q filter sterilized (0.2 μm) water. An additional 1.0 and 1.2% D-(+)-glucose solutions were also mixed by increasing the mass of D-(+)-glucose reagent at the expense of tryptone with increasing concentrations of D-(+)-glucose by dry weight of 50 and 75% respectively, compared with a dry weight concentration of D-(+)-glucose of 25% for the control solution. This was done in order to determine the optimal mixture that would minimize other carbon sources introduced by the tryptone reagent without limiting other nutrients required for successful cell growth. Five replicates of each solution were measured out in 4 ml aliquots in culture tubes (totaling 15 culture tubes) and sterilized via autoclave (121 °C, 15 psi for 30 min) in preparation for inoculation with *E. coli*.

A K-12 strain of *E. coli* was obtained from the Microbiology Laboratory at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) in Syracuse, New York. *E. coli* stock cultures were maintained in 4 ml of sterile 0.5% D-(+)-glucose broth described previously and incubated in the dark for 24 h at 37 °C on a shaker table set to 50 strokes per min and then stored at 4 °C. In order to maintain a healthy stock culture, 100 μl of the stock culture was transferred to new broth using a sterile pipette and the above procedure repeated every 2 weeks. 100 μl of the stock culture was introduced to each of the 0.5, 1.0, and 1.2% D-(+)-glucose broth mixtures and their replicates described above, and incubated for 48 h at 37 °C and 50 strokes per min. A preliminary trial indicated that an incubation time of 24 h did not provide enough biomass for a successful stable isotope analysis. A new culture was grown directly before initiating the experiment.

After 48 h 1 ml was extracted from each of the culture tubes with a sterile pipette and placed in sterile culture tubes for cell enumeration where they were then fixed with 111 μl of 10% by volume glutaraldehyde solution and stored in the dark at 4 °C for 24 h before being prepared for cell enumeration via epifluorescence microscopy. After extraction for cell enumeration the remaining 3 ml in each culture flask was concentrated on a 0.7 μm (25 mm diameter) glass fiber filter under gentle vacuum, rinsed with 30 ml