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Bottle effects alter taxonomic composition of wetland soil bacterial communities during the denitrification enzyme activity assay

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ABSTRACT

The denitrification enzyme activity assay (DEA) and other *ex situ*, incubation-based methods are widely used for measuring biogeochemical transformations. DEA does not provide direct measurements of denitrification rates *in situ*, but rather denitrification *potential* under laboratory conditions over the course of an incubation with resource limitations removed. Despite recognized limitations, DEA has several advantages. In particular, it is a relatively simple and low-cost method considered reliable for comparing relative differences in denitrification, for example between areas subjected to different management treatments. However, a critical but, to our knowledge, untested assumption of DEA is that bacterial composition remains static during the assay, i.e., the microbial community being assayed is equivalent to the original community collected from the field. If this assumption is violated, it could result in flawed estimates of relative differences between samples. We tested the static-community assumption using high-throughput sequencing to measure differences in bacterial community composition between samples from two different wetland vegetation types over the course of standard DEA incubations. We also compared samples that were or were not treated with a standard amendment solution to differentiate amendment effects from "bottle effects" of time spent under storage and laboratory conditions. We found that initially distinct bacterial communities became less similar to each other during short-term cold storage, but then became more similar during incubation, with a net result of samples converging in composition. Surprisingly, there were no effects of amendment solution; bottle effects alone accounted for these changes. This raises concern that potentially important differences in wetland denitrification, or other processes measured using *ex situ* methods, could be obscured by changes in bacterial community composition that arise as an artifact of laboratory incubations.

1. Introduction

Alteration of the global nitrogen cycle is one of the strongest drivers of ecosystem change (Vitousek et al., 1997b; Galloway et al., 2003; Galloway et al., 2004; Gruber and Galloway, 2008). Anthropogenic changes to the nitrogen cycle have increased biologically available nitrogen, contributing to species invasions, biotic homogenization, environmental acidification, and negative human health impacts (Vitousek et al., 1997a; Galloway et al., 2003). Denitrification is a key component of the global nitrogen cycle that converts nitrogen from a biologically available form (nitrate) to gaseous products (dinitrogen and nitrous oxide) that are inaccessible to most organisms. Thus, it is an important ecosystem service that can help offset nitrogen pollution. Denitrification is an anaerobic respiratory process that is catalyzed primarily by bacteria, and the ability to denitrify is widely distributed across the bacterial phylogenetic tree, with over 50 bacterial genera known to be capable of denitrification (Shapleigh, 2006).

There is great interest in understanding the rates at which different ecosystems perform denitrification. Unfortunately, measuring denitrification rates has proven difficult. There are

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several approaches commonly used to quantify denitrification, but they all have recognized limitations (see Davidson and Seitzinger, 2006; Groffman et al., 2006). Some methods are difficult to implement in the lab or field, others require equipment not present in most laboratories, and many are expensive. The number of samples required to capture a spatially and temporally heterogeneous process like denitrification compounds these issues (McClain et al., 2003; Davidson and Seitzinger, 2006; Groffman et al., 2009a, 2009b).

The denitrification enzyme activity assay (DEA) is the most frequently used method for quantifying denitrification potential (Groffman et al., 2006). DEA is a laboratory incubation of field-collected soil (Groffman et al., 1999, 2006). Soil and an amendment solution containing glucose, nitrate, and chloramphenicol (to remove carbon and nitrogen limitations and stop bacterial enzyme production, respectively) are placed into a jar that is then sealed. The jar is flushed with helium to create an anoxic environment and acetylene is added to inhibit enzymatic conversion of N_2O to N_2. Gas samples are collected from the headspace of the jar at multiple time points and analyzed for N_2O concentration; these data are then used to calculate potential denitrification rate.

DEA does not provide a direct measurement of field denitrification rates. Rather, it is a measure of *potential* denitrification under ideal conditions (Groffman et al., 2006). DEA has appeal because it is relatively inexpensive, easily reproducible, and thought to provide a measure of denitrification that can reliably compare relative differences between treatments (Groffman et al., 2006). Like other methods for measuring denitrification, it has shortcomings. For example, denitrification could be underestimated due to acetylene inhibition of nitrification (Walter et al., 1979; Seitzinger et al., 1993).

More generally, DEA is an *ex situ*, incubation-based method. It is intended to measure the function of denitrifying bacteria present at the time of sampling, and a key assumption of DEA is that bacterial community composition remains static during the course of the incubation, so that the bacteria being assayed match those found in the original field-collected samples (Groffman et al., 1999). To prevent change in composition, chloramphenicol, an antibiotic that blocks bacterial protein synthesis, is added as part of an amendment solution to prevent reproduction that could alter relative abundances of different taxa (Groffman et al., 1999). However, this may not be sufficient for avoiding "bottle effects," whereby confinement in an artificial environment drives shifts in bacterial composition and dominance (Vollenweider and Nauwerck, 1961; Paerl, 1982). A recent study demonstrated significant changes to marine-sediment microbial communities over the course of the acetylene reduction assay (an *ex situ* incubation for measuring nitrogen fixation) (Fulweiler et al., 2015). In a study using tropical forest soils, cold storage and laboratory incubation caused changes in nitrogen mineralization, immobilization, and nitrification rates relative to *in situ* measures (Arnold et al., 2008). To our knowledge, whether bacterial community composition remains static over the course of DEA incubation has not been tested. If composition is not static, this would call into question the method's reliability for making relative comparisons between treatments, as differences could be under- or over-estimated depending on patterns of microbial community turnover under laboratory conditions.

This study was performed to evaluate whether bacterial community composition changes during DEA incubation. In comparing DEA rates for two hypothetical bacterial communities (e.g., from different study sites or treatments) that initially have distinct microbial communities, there are four potential scenarios for how pairwise differences in community composition could change over the course of the DEA incubation (Fig. 1). If community composition of samples remained relatively static during the incubation, then a core assumption of the DEA method would be validated (Fig. 1a), which would enable differences in denitrification potential to be attributed to treatments of interest.

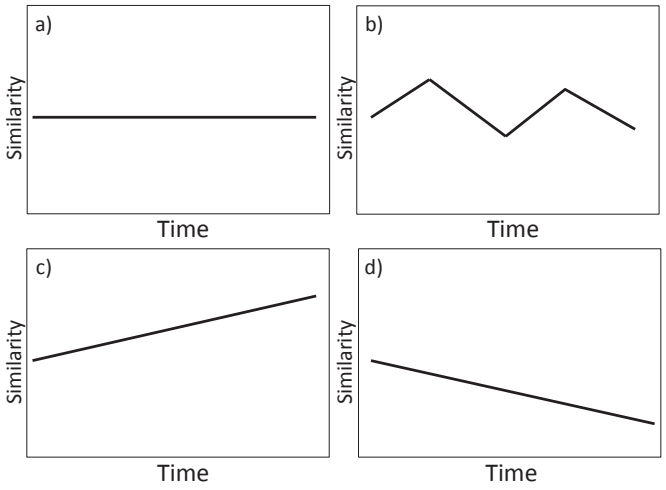

Fig. 1. Four scenarios of how pairwise similarity in bacterial community composition between treatments could change over time: a) *No change*. This would be ideal, as the incubation itself would not confound relative differences between treatments of interest. Alternatively, if the incubation process does alter pairwise similarity, it could do so in a manner that b) is *idiosyncratic*, c) leads to *convergence* in composition that would underestimate treatment differences, or d) leads to *divergence* that would overestimate differences.

Under the alternative scenarios, potential treatment effects are confounded by changes in bacterial communities associated with the incubation itself, either due to time spent in an artificial environment and/or amendment additions. These outcomes pose problems for relative comparisons of treatments. In the second scenario (Fig. 1b), the bacterial communities change with the incubation, but in an idiosyncratic manner. This pattern would make treatment comparisons unreliable because one treatment might exhibit increased rates of denitrification while the other showed decreased rates simply because of stochastic turnover in community composition during the incubation; this would make comparisons highly sensitive to the temporal window sampled. In the third scenario (Fig. 1c), bacterial communities converge in composition due to selective filtering during the incubation. This could lead to underestimation of differences in denitrification potential, making interpretation overly conservative. The final scenario is divergence of communities (Fig. 1d). This would occur if the incubation filtered out different subsets of bacteria in each treatment, potentially leading to overestimation of differences in denitrification potential and making interpretation overly liberal.

We experimentally investigated the temporal dynamics of bacterial community composition during DEA incubation to determine which of these trajectories occurred. We performed incubations on soil samples collected from two distinct plant communities found within a single wetland, systems that are well known to support denitrification due to the presence of anoxic soils (Groffman and Hanson, 1997). DEA would be a reliable method for measuring relative differences in denitrification if initially distinct bacterial communities remained static and similarity did not change throughout the incubation (Fig. 1a). We hypothesized that, instead, amendment additions and incubation time would act as selective pressures on microbial communities, resulting in convergence in community composition (Fig. 1c), i.e., selection toward increasingly artificial "laboratory communities."