Soil Biology & Biochemistry 69 (2014) 141–146

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Substrate and nutrient limitation of ammonia-oxidizing bacteria and archaea in temperate forest soil

J.S. Norman*, J.E. Barrett

Department of Biological Sciences (MC 0406), Virginia Tech, 2125 Derring Hall, Blacksburg, VA 24061, USA

ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form
31 October 2013
Accepted 3 November 2013
Available online 18 November 2013

Keywords:
Ammonia
Oxidizing
amoA
Potassium
Phosphorus
Bacteria
Archaea
qPCR
Coweeta
Forest

ABSTRACT

Ammonia-oxidizing microbes control the rate-limiting step of nitrification, a critical ecosystem process, which affects retention and mobility of nitrogen in soil ecosystems. This study investigated substrate (NH_4^+) and nutrient (K and P) limitation of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in temperate forest soils at Coweeta Hydrologic Laboratory, a long-term ecological research site in western North Carolina, USA. We investigated substrate and nutrient limitation by amending soils with either ammonium or a nutrient solution containing P and K, then assessing the growth of these organisms during *in situ* soil incubations. We found substantial growth of both AOA and AOB during all incubations including unamended control incubations. Our results demonstrate that substrate availability limits nitrification by AOB and that high levels of substrate addition inhibit the growth of AOA in these soils. We found no evidence for nutrient limitation of AOB, though nutrient addition indirectly stimulated nitrification by AOB through increased nitrogen mineralization. Our data did suggest nutrient limitation by AOA, though it is unclear whether AOA significantly contribute to ammonia oxidation in this system. Furthermore, we show that AOB are responsible for the majority of ammonia oxidation in high substrate, high nutrient conditions.

Published by Elsevier Ltd.

1. Introduction

Photoautotrophic organisms, such as plants, respond to nutrient addition by increasing rates of carbon fixation through photosynthesis, often resulting in increased growth rates (Chapin et al., 1987). The effect of fertilization on rates of carbon fixation by chemoautotrophic organisms is harder to predict however, since these organisms acquire energy for carbon fixation by the oxidation of reduced inorganic compounds. For example, ammonia-oxidizing archaea (AOA) and bacteria (AOB), which perform the rate-limiting step of nitrification, primarily use ammonium as the substrate for energy acquisition in support of chemoautotrophic growth. This energy-based demand on the inorganic nitrogen (N) pool may affect the rate at which AOA and AOB acquire other major soil nutrients, such as phosphorous (P) and potassium (K).

AOB were first discovered in the 19th century and thrive under high nutrient conditions in pure culture (Martens-Habbena et al., 2009). AOA were first isolated in pure culture in 2005 (Könneke et al., 2005) and exist in oligotrophic environments such as those found in the open ocean, where they may be responsible for the majority of ammonia oxidation (Martens-Habbena et al., 2009). AOA and AOB also exhibit significantly different ammonia oxidation kinetics in pure culture (Martens-Habbena et al., 2009). Since these organisms control the rate-limiting step of nitrification, a critical process that regulates the mobility of N in soil, understanding the independent effects of substrate and nutrient availability on AOA and AOB activity is key to understanding controls over the nitrification in any environment.

Here we test whether AOA and AOB are substrate-limited by ammonium availability or nutrient-limited by P and K in temperate forest soils. To investigate substrate and nutrient limitation of AOA and AOB, we amended forest soil with either ammonium (NH_4^+) or a nutrient solution containing both P and K. We then measured the growth response of both AOA and AOB to these additions during net nitrification incubations. Growth was assessed by estimating changes in copy number of domain-specific ammonia monooxygenase subunit A (*amoA*) genes. In this paper, we use the term nutrient to refer to elements such as P and K, which ammonia-oxidizing microbes (AOM) only use to meet assimilatory demand; we assume that assimilatory demand for NH_4^+ is low, relative to substrate requirements of these organisms, and therefore consider NH_4^+ only as a substrate for chemoautotrophic growth by AOM. We

* Corresponding author. Tel.: +1 540 231 6679; fax: +1 540 231 9307.
E-mail addresses: jenorma1@vt.edu (J.S. Norman), jebarre@vt.edu (J.E. Barrett).

0038-0717/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.soilbio.2013.11.003

predicted that AOB would exhibit increased growth in response to NH_4^+ additions, while high levels of NH_4^+would inhibit the growth of AOA, as has been shown in culture based studies (e.g. Haztenpichler et al., 2008; Martens-Habbena et al., 2009). We also predicted that ammonia oxidation would exhibit a positive, saturating response to NH_4^+ addition reflective of Michaelis–Menten enzyme kinetics. Finally, we predicted that nutrient amendment would not directly affect ammonia-oxidizing microbes since these organisms should require excess NH_4^+, relative to P and K, to fill both assimilatory and energetic requirements.

2. Materials and methods

2.1. Site description and incubation conditions

This experiment was performed at Coweeta Hydrologic Laboratory, a United States Forest Service research facility and National Science Foundation Long-Term Ecological Research site, in Otto, North Carolina, USA. During the summer of 2011, we excavated approximately 5 kg of soil from a forested reference watershed (Coweeta watershed 18), which has remained undisturbed since 1927 and contains a mix of hardwood tree species (Swank and Vose, 1997). Soil was passed through an ethanol-sterilized 4 mm sieve, to remove small rocks and fine roots, and then mixed to homogenize. The sieved soil was divided into five 1-kg sub-samples, which we separately amended with two levels of ammonium chloride (NH_4Cl) (high and low substrate treatment), two levels of monobasic potassium phosphate (KH_2PO_4) (high and low nutrient treatment), or with distilled water (control treatment).

Previous samples we have taken from Coweeta had a maximum NH_4^+ concentration of 10.54 μg NH_4^+–N/g wet weight of soil. In the low substrate addition treatment, we assumed that the soil contained roughly the same background NH_4^+ concentration, corrected for moisture, and added enough NH_4Cl to double this amount. Similarly, in the high substrate addition treatment we added enough NH_4Cl to increase the ambient NH_4^+ to 10 times the background value. We designed our low nutrient treatment and high nutrient treatment to increase inorganic soil P and K molar concentrations by 1/10th of the amounts that we increased inorganic N concentrations in our low substrate treatment and high substrate treatment soils, respectively. All additions were made as solutions dissolved in 40 mL of distilled water in order to avoid increasing the water content of the soil by more than 10%, based on previous data. Solutions were applied with a spray bottle while soil was mixed by hand. Control treatments were amended with 40 mL of distilled water to control for changes in soil moisture associated with substrate and nutrient amendment.

Following the amendment procedure, we used soil from each treatment to conduct 28-day buried-bag incubations. Buried-bag assays exclude plant roots from affecting soil inorganic N pools thereby allowing NH_4^+ and nitrate (NO_3^-) to accumulate over the course of incubation (Eno, 1960). The rates at which total inorganic nitrogen (TIN) and NO_3^- accumulate during buried-bag incubations were used to estimate respective mineralization and nitrification rates in each bag. To conduct buried-bag assays, we filled Whirl-pak (Nasco, CA, USA) bags from each treatment with approximately 100 g of soil each. Whirl-pak bags are made of polyethylene, which allows for gas exchange, but not water exchange during incubation. Bags were sealed as recommended by the manufacturer. We stored three bags of each treatment at 4 deg. C (day 0 bags) until analysis and incubated 3 replicate bags in the ground, which were excavated for analysis after 28 days (day 28 bags). Incubations were conducted in the same location from which soil was initially collected, and bags from each treatment were randomly distributed in the ground during incubation.

While estimates of net nitrification could be affected by denitrification occurring during buried-bag incubations, this possibility was minimized by both increased aeration when the soil was sieved and the gas permeability of the bags we used. Furthermore, by adding substrate and nutrient solutions with spray bottles while soil was mixed by hand, we assured that there were no large saturated zones, which would serve as hotspots of denitrification. We therefore assume that the magnitude of this flux was small and consistent across treatments and that nitrification represents the major control on the nitrate pools at the end of buried-bag incubations.

2.2. Soil chemical analyses

Soil pH of day 0 samples was estimated by measuring the pH of a 1:2 soil:water slurry using an Orion 3-star benchtop pH meter (Thermo Fisher Scientific, MA, USA) (McLean, 1982). Soil moisture content was estimated by mass loss of a ~10-g subsample of day 0 and day 28 bags after overnight drying at 105 °C. Inorganic N was extracted from day 0 and day 28 samples by suspending 5-g of soil in 50 mL of 2 M KCl, and agitating for 30 min at 250 RPM on an orbital shaker table (Bundy and Meisinger, 1994). Bulk extracts were filtered through pre-leached 11-micron filter paper (Whatman International Ltd, Kent, UK) and then filtered through 0.7-μM glass fiber syringe filters (Tisch Scientific, OH, USA) prior to storage at −20 °C until further analysis. NH_4^+ and NO_3^- concentrations in KCl extractions were measured using a Lachat flow-injection autoanalyzer (Hach company, Loveland, CO, USA), and values were reported as μg N–NH_4^+/g dry weight of soil and μg N–NO_3^-/g dry weight of soil, respectively. TIN was defined as (μg N–NH_4^+ + μg N–NO_3^-)/g dry weight of soil. Net nitrification was calculated for each incubation by subtracting average day 0 NO_3^- for that treatment from day 28 NO_3^- in each bag (Eno, 1960). Similarly, net mineralization was calculated for each incubation by subtracting average day 0 TIN for that treatment from day 28 TIN in each bag (Eno, 1960).

2.3. Available ammonia (NH_3) estimations

Ammonia (NH_3) rather than ammonium (NH_4^+) is thought to be the substrate oxidized by AOB (Suzuki et al., 1974). NH_3 concentrations depend not only on the amount of NH_4^+ in a given environment but also on the pH of that environment. We therefore estimated the amount of NH_3 available in each day 0 bag after addition using day 0 NH_4^+ concentration and day 0 pH data by Equation (1).

$$[NH_3] = \left[NH_4^+\right]\left(10^{(\text{day 0 pH}-9.25)}\right) \quad (1)$$

Equation (1) is based on the Henderson–Hasselbalch equation, and assumes that the p*K*a of NH_3/NH_4^+ is 9.25.

2.4. Soil DNA extraction and quantitative PCR

DNA was extracted from ~0.25 g of soil from Day 0 and Day 28 soil samples, using MOBIO powersoil® DNA isolation kits. The manufacturer's instructions were followed except that DNA was eluted in 100 μL of solution C6 warmed to 55 deg. C to maximize elution efficiency, and an extra ethanol wash step was employed as recommended for soils with high humic content. Quantitative polymerase chain reaction (qPCR) was used in conjunction with AOA and AOB specific primers to estimate AOA and AOB abundance by quantifying gene copy number of ammonia monooxygenase subunit A (*amoA*) genes characteristic for each group. All qPCR reactions were performed in triplicate using a Biorad CFX96 quantitative thermocycler set to read SYBR green fluorescence.