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Effect of tetracycline on ammonia and carbon removal by the facultative bacteria in the anaerobic digester of a sewage treatment plant

Richard Grabert, Raj Boopathy*, Rajkumar Nathaniel, Gary LaFleur

Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA

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ABSTRACT

This study was conducted to see the effect of tetracycline on nitrogen assimilation and carbon removal in an anaerobic digester of a sewage plant. Samples of sewage were collected from an anaerobic digester sludge. Consortium of nitrogen assimilating bacteria were isolated from the sample and its ability to assimilate ammonia at different concentrations of tetracycline was measured along with carbon removal. The results indicate that while high concentrations of tetracycline of more than 100 mg/L delayed the growth of the bacteria, the resistant bacteria grew after a lag period and the removal of nitrogen and carbon was unaffected even at the highest tetracycline concentration of 250 mg/L tested in this study.

1. Introduction

The use of antibiotics became widespread in the 1950s, but this soon led to new problems. Bacteria with a resistance to antibiotics started to appear soon after antibiotic use. The bacteria were able to acquire antibiotic resistance genes through mutations and through gene transfer. The main method of antibiotic gene transfer appears to be through horizontal gene transfer (Salyers et al., 2004). In this method, bacteria acquire the genes from other bacteria. Depending on the antibiotic, different genes may exist that allow the bacteria to survive the antibiotic. For example, 38 different tetracycline resistant genes have been found and described in bacteria (Roberts, 2005).

Tetracycline was first patented in 1953 and became available for commercial use in the year 1978. Tetracycline is a commonly prescribed antibiotic used to treat numerous infections, including acne, cholera, and syphilis. Tetracycline is also widely used in the cattle and poultry industries. It is also included in the World Health Organization's List of Essential Medicines. Tetracycline belongs to a large family of antibiotics, known as the tetracyclines. These were first discovered as natural products in members of the *Streptomyces* genus (Jukes, 1985). Tetracycline works by binding to the 30S ribosomal subunit and preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor and thus prevents protein synthesis in bacteria. The antibiotic passively diffuses through hydrophilic pores and then uses active transport to penetrate the inner-cell membrane.

Nitrogen is an important part of life as it is one of the key building blocks and is one of the main molecular components in DNA, RNA, and amino acids. Nitrogen is cycled from one form to another by various organisms through different processes including nitrogen fixation, ammonification, nitrification, denitrification, and assimilation. Nitrogen fixation is the process by which atmospheric nitrogen is converted into ammonia. Since atmospheric nitrogen is mostly unreactive, nitrogen fixation is one of the few ways to make nitrogen available for biological processes. Nitrogen fixation often happens in soil with the nitrifying bacteria around the roots of plants, like legumes and rice (Postgate, 1998). Ammonification is the degradation of dead and decaying material and conversion of protein to ammonia by heterotrophic bacteria in the environment. Nitrification is an aerobic process carried out by a group of bacteria and archaea. The bacteria that carry out ammonia oxidation are known as ammonia-oxidizing bacteria (AOB) and the archaea are known as ammonia-oxidizing archaea (AOA). The most studied genera of AOBs are *Nitrosomonas* and *Nitrosococcus* (Kowalchuk and Stephen, 2001). The nitrite oxidation step, which converts nitrite to nitrate, is carried out by bacteria in the genera *Nitrobacter* and *Nitrospira* (Ward, 1996). These bacteria are chemoautotrophs and use carbon dioxide as their carbon source (Marsh et al., 2005).

Denitrification is the process by which nitrate is reduced to molecular nitrogen and other gaseous nitrous oxide intermediates. Denitrification is an anaerobic bacterial process carried out by bacteria called denitrifiers. The bacteria use a reduction pathway that transforms nitrate to nitrite to nitric oxide to nitrous oxide to dinitrogen. Denitrification is often carried out by bacteria in the genus *Pseudomonas,* such as *P. denitrificans* (Carlson and Ingraham, 1983).

* Corresponding author.
E-mail address: Ramaraj.Boopathy@nicholls.edu (R. Boopathy).

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Assimilation is the process of converting nitrate and ammonia into organic compounds, such as amino acids and proteins. Most microorganisms that assimilate nitrogen do so by utilizing ammonia or by converting other forms enzymatically into ammonia (Johansson and Gest, 1976). The enzyme glutamine synthatase is required for the initial uptake of ammonia, with other enzymes responsible for catalyzing the utilization of ammonia (Magasanik, 1982). Glutamines, glutamates, and aspartates are the main amino donors in the subsequent synthesis reactions and all microorganisms that use ammonia as a source of nitrogen.

In sewage treatment plants, nitrification, denitrification, and assimilation play a key role in removing the excess ammonia from sewage. If this ammonia is left untreated, it can have detrimental effects on the environment and lead to eutrophication. The process of sewage treatment has multiple steps. The sewage treatment plant in Thibodaux, Louisiana, USA uses activated sludge and trickling filter as the aerobic process and anaerobic digester as the anaerobic process to remove carbon, nitrogen, phosphorous, and suspended solids in the wastewater.

The primary solids from primary settling tank and the secondary solids from the secondary settling tank travels separately to the anaerobic digester, which allows the sludge to further settle out and also provides an opportunity for the denitrifiers to turn the nitrate in the sludge into nitrogen gas and any residual carbon to methane and CO_2. The sludge from the bottom is pumped into sludge drying beds periodically.

Antibiotics are known to be present throughout the sewage treatment process and these antibiotics and antibiotic resistance genes (ARGs) may have an effect on sewage treatment. Different studies have shown the presence of antibiotics and ARGs in sewage treatment. The human body does not metabolize antibiotics, so they are expelled into the sewage (Zhang et al., 2009). As these antibiotics concentrate in the sewage systems, bacteria in the sewage treatment process are accidentally being exposed and selected for antibiotic resistance. These antibiotics are creating antibiotic resistant bacteria and have been found in many sewage treatment plants (Ghosh et al., 2009; Zhang et al., 2009). Antibiotic resistant bacteria have been found in the Thibodaux Sewage Treatment Plant (Everage et al., 2014). The genes needed for antibiotic resistance have been found throughout the entire sewage treatment plant process, with two studies done at the Thibodaux Sewage Treatment Plant showing the presence of sul1, tet(A), and tet(X) genes in the treated effluent of the sewage plant (Naquin et al., 2015, 2017) showing resistance to sulfonamide and tetracycline respectively. These antibiotics and ARGs could be having significant impacts on the bacteria that perform the sewage treatment process in removing carbon and nitrogen in the wastewater through nitrogen assimilation process. One study examined the occurrence of antibiotics at sewage treatment plants in Japan and showed that antibiotics below concentrations of 0.05 mg/L had no effect on these bacteria (Ghosh et al., 2009). Effect of higher concentrations of antibiotics on the anaerobic digestion process was not extensively studied. Therefore, this study was conducted to test whether carbon and nitrogen removal by bacteria is affected by the presence of tetracycline at high concentrations.

2. Materials and methods

2.1. Sample collection

The sludge sample was collected from the anaerobic digester of Thibodaux sewage treatment plant in a 1 L sterilized Nalgene collection bottle. The sample was transported to the lab and kept in a refrigerator before use.

2.2. Antibiotic analysis

Samples were analyzed for the presence and concentration of various antibiotics in the sludge. The antibiotics in the water sample were analyzed using HPLC equipped with two Model 210 solvent pumps, a Model 320 programmable multi-wavelength ultraviolet (UV) detector set at 260 nm, a Model 410 system autosampler (Varian, Walnut Creek, CA), and an LC-CN 4.6 mm-i.d × 25 cm HPLC column (C-18 Supleco column 15 cm × 4.6 mm ID) with a particle size of 5–6 μm. The sample was run in an isocratic mode. The mobile phase was 25 mMKH_2PO_4: acetonitrile (40:60) at a flow rate of 1.0 ml/min with an injection volume of 15 μL. The run time was 20 min. The pressure was < 950 psi. Standard antibiotics were used to quantify the antibiotics in the sample.

2.3. Development of bacterial consortium

A 10 ml sample of the sludge sample was transferred to a 200 ml basic mineral salt medium (BMS) with ammonia as nitrogen source. The carbon to nitrogen ratio in the medium was 15:1. BMS was made with 3.5 g of KH_2PO_4, 1.5 g of K_2HPO_4, 0.1 g of $MnSO_4$, 0.1 g of NaCl, 0.1 g of $(NH_4)_2SO_4$, 1.5 g of glucose, and 0.5 g of yeast extract in 1 L of deionized water. The medium was incubated aerobically at the ambient temperature of 22 °C until the optical density reached 0.400 at 600 nm wavelength. The bacteria that are grown on facultative as it came from the anaerobic digester, but was grown under aerobic condition. This developed consortium served as the inoculum for further studies.

2.4. Effect of tetracycline on nitrogen and carbon removal

The tetracycline was obtained from Sigma Aldrich Chemical Company (St. Louis, MO). The effect of various concentrations of tetracycline on carbon and nitrogen removal was studied in culture bottles with 100 ml BMS. The concentrations used include 5, 10, 25, 50, 100, and 250 mg/L tetracycline with a control culture that did not have any antibiotics. A 5% inoculum was used to start the experiment. Triplicate cultures were maintained in each concentration. Samples were periodically taken for bacterial growth, ammonia, and carbon analysis.

2.5. Analytical methods

The bacterial growth was monitored by optical density using a spectrophotometer set at 600 nm wavelength. The organic carbon in the sample was monitored using chemical oxygen demand (COD) analysis. The amount of chemically oxidizable carbon (COD) was measured using colorimetric Reactor Digestion Method using HACH LR COD following the method given by HACH (1999). Ammonia was analyzed using the colorimetric Nessler Method (HACH, 1999).

2.6. Tetracycline resistance genes

Kirby-Bauer Antibiotic Resistance Disc assays were performed on the consortium used in this experiment. A bacterial lawn of consortium was spread with aseptic technique onto a petri dish containing 10 ml of Mueller-Hinton Agar. After spreading, four antibiotic discs were applied to each plate. The four antibiotic discs were tetracycline, oxytetracycline, ampicillin/sulbactum, and streptomycin.

The methods for isolating tetracycline resistance genes was adapted from Naquin et al. (2015). One mL from each culture was inoculated into a 50 ml centrifuge tube containing 20 ml of the necessary Basic Mineral Salt Medium and incubated at 37 °C for 24 h. The tubes were then centrifuged in a IEC HN-S2 centrifuge for 3000 revolutions per minute for 15 min. The DNA was extracted from the pellet using a MO BIO Laboratories, Inc. PowerSoil® Genomic DNA Isolation Kit.

Genes were amplified by polymerase chain reaction (PCR). PCR was performed using tetracycline resistance gene primers obtained from Sigma Aldrich Co. (Table 1). The PCR mix in each tube contained 32.2 μL of DI water, 5 μL of Deoxyribonucleotide triphosphate (DNTP), 5 μL of PCR buffer, 0.5 μL of taq polymerase, 5 μL of the extracted DNA,