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# Application of bacteriophages to selectively remove *Pseudomonas aeruginosa* in water and wastewater filtration systems

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ABSTRACT

Water and wastewater filtration systems often house pathogenic bacteria, which must be removed to ensure clean, safe water. Here, we determine the persistence of the model bacterium *Pseudomonas aeruginosa* in two types of filtration systems, and use *P. aeruginosa* bacteriophages to determine their ability to selectively remove *P. aeruginosa*. These systems used beds of either anthracite or granular activated carbon (GAC), which were operated at an empty bed contact time (EBCT) of 45 min. The clean bed filtration systems were loaded with an instantaneous dose of *P. aeruginosa* at a total cell number of 2.3 (±0.1 [standard deviation]) × $10^7$ cells. An immediate dose of *P. aeruginosa* phages (1 mL of phage stock at the concentration of 2.7 × $10^7$ PFU (Plaque Forming Units)/mL) resulted in a reduction of 50% (±9%) and >99.9% in the effluent *P. aeruginosa* concentrations in the clean anthracite and GAC filters, respectively. To further evaluate the effects of *P. aeruginosa* phages, synthetic stormwater was run through anthracite and GAC biofilters where mixed-culture biofilms were present. Eighty five days after an instantaneous dose of *P. aeruginosa* (2.3 × $10^7$ cells per filter) on day 1, 7.5 (±2.8) × $10^7$ and 1.1 (±0.5) × $10^7$ *P. aeruginosa* cells/g filter media were detected in the top layer (close to the influent port) of the anthracite and GAC biofilters, respectively, demonstrating the growth and persistence of pathogenic bacteria in the biofilters. A subsequent 1-h dose of phages, at the concentration of 5.1 × $10^6$ PFU/mL and flow rate of 1.6 mL/min, removed the *P. aeruginosa* inside the GAC biofilters and the anthracite biofilters by 70% (±5%) and 56% (±1%), respectively, with no *P. aeruginosa* detected in the effluent, while not affecting ammonia oxidation or the ammonia-oxidizing bacterial community inside the biofilters. These results suggest that phage treatment can selectively remove pathogenic bacteria with minimal impact on beneficial organisms from attached growth systems for effluent quality improvement.

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## 1. Introduction

Biofiltration, a method that combines adsorption and biodegradation processes in a filtration system, has been used for many years in water and wastewater treatment facilities to improve water quality (Chien et al., 2008; Feng et al., 2012; Le Coustumer et al., 2012; Li et al., 2012; Yang et al., 2011). Commonly used biofiltration systems, such as the anthracite

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and granular activated carbon (GAC) biofilters, can remove organic matter, ammonia, micropollutants, disinfection byproducts, and odor-causing compounds (Donner et al., 2002; Hargesheimer and Watson, 1996; Ho et al., 2011; Wahman et al., 2011). Of these biofilters, GAC biofilters have higher sorption capacity to remove organic compounds than anthracite biofilters. This is because of GAC's porous structure and higher specific surface area, despite the fact that anthracite has the same chemical composition as GAC. On the other hand, because anthracite is a compact or dense mineral coal, its use results in longer filter runs and less head loss than GAC biofilters. Regardless of which biofilter is used, however, both provide an excellent environment for the growth of pathogenic bacteria, which colonize the filter media and form biofilms (Camper et al., 1985; September et al., 2007; Stewart et al., 1990; Vaerewijck et al., 2005). If the biofilm detaches, or if the biofilter media releases particles, these pathogens, along with other bacteria, can spread into the biofilter effluent. Recent studies show that GAC filters used to treat swimming pool water are contaminated by *Pseudomonas aeruginosa* after 4 months of operation (Uhl and Hartmann, 2005). *P. aeruginosa* is an opportunistic pathogen known to easily form biofilms resistant to antibiotics and antiseptics (Drenkard and Ausubel, 2002; Dunne, 2002; Mah et al., 2003; Whiteley et al., 2001), and is often found in the drinking water supply (Anaissie et al., 2002). This contamination is difficult to remove, even by backwashing at free chlorine concentrations of >2 mg/L. Moreover, the efficiency of chlorine disinfection decreases over time. Therefore, more effective methods for *selectively* removing bacterial pathogens in biofiltration systems are urgently needed in order to maintain beneficial bacteria and to minimize cost.

Bacteriophage (phage) treatment, which target specific bacteria, is a promising alternative to current disinfection strategies, which target all bacteria, rather than just the pathogenic bacteria of interest. A phage can attach to the host bacterial cells by recognizing specific receptors, and then replicate within the bacteria by injection of its genome into the bacteria's cytoplasm. Phage progeny are synthesized inside the cell, and lytic phages are released for a new cycle of infection. In particular, phage treatment has been shown to effectively remove *P. aeruginosa in vitro* (Hall et al., 2012) and in its biofilms (Fu et al., 2010; Zhang and Hu, 2013), but has not yet been investigated in applied environments.

The effectiveness of phage treatment in selectively removing pathogenic bacteria in a mixed-species biofilm is largely unknown. To our knowledge, only a few studies have shown that phage can control dual-species biofilms (Kay et al., 2011; Sillankorva et al., 2010), not to mention more complicated biofilm systems. The transport dynamics of phage and bacteria in the filtration systems makes the efficiency of phage treatment even more uncertain due to the non-uniform distributions of bacteria and phages in the porous media (Kay et al., 2011; Långmark et al., 2005). Furthermore, it is necessary to determine how *P. aeruginosa* phages could affect the growth of beneficial bacteria, such as nitrifiers, in the biofiltration systems because they are essential (but sensitive) organisms involved in ammonia oxidation. Although phages infect specific bacterial hosts, a side effect is that lytic phage infection of host cells also produces enzymes that degrade extracellular polymeric substances (EPS), which promote biofilm formation and protect biofilm bacteria (Donlan, 2009; Glonti et al., 2010; Hanlon et al., 2001). Therefore, the objectives of this study were to investigate the persistence of *P. aeruginosa* bacteria in anthracite and GAC biofilters, to evaluate their removal by phages, and to further assess the impact of *P. aeruginosa* phage treatment on nitrification and nitrifying communities in order to determine their selectivity.

## 2. Materials and methods

### 2.1. P. aeruginosa *and* P. aeruginosa *phage stocks*

*P. aeruginosa* (ATCC 39018) was incubated in a Luria Broth medium overnight on a shaker (200 rpm) at room temperature until the stock cultures contained an average of $2.3 \times 10^8$ CFU/mL (colony-forming units per milliliter). A mixture of *P. aeruginosa* phages was isolated from wastewater by the double-layer agar method (Miller, 2001) following the literature procedure (Knezevic and Petrovic, 2008). After two rounds of enrichment and 1× phosphate buffered saline (PBS) soaking, a phage stock (>$10^9$ PFU/mL, plaque-forming units per milliliter) was obtained. Nucleic acid (RNA and DNA) analysis indicated the isolated phages were RNA phages (Zhang and Hu, 2013), and the morphology of the phages was determined by Transmission Electron Microscopy (TEM) using detailed procedures described elsewhere (Zhang and Hu, 2013) (see also Fig. S1). After RNA extraction and gel electrophoresis, two clear RNA bands with different sizes were found, indicating more than one type of bacteriophage was isolated and enriched.

### 2.2. *Anthracite and GAC filtration systems*

Anthracite (average particle size 0.9–1.0 mm) was collected from the filter beds at the Columbia Water Treatment Plant in Columbia, MO, USA. A commercially available GAC (DARCO, Sigma, St. Louis, average particle size 0.8–1.7 mm) was selected for comparison. The physical parameters of these filter media are listed in Table S1. Duplicate glass columns (25 mm ID and 15 cm bed depth) were packed with sterilized anthracite (62.3 g/column) or GAC media (27.1 g/column) to serve initially as the clean bed filters, and later as the biofilters, in which mixed-culture biofilms were allowed to form. While the total bed volume in either the anthracite or GAC filters was 70.5 mL, the difference in the porosities of the filter media resulted in differences in the pore volumes of anthracite and GAC biofilters: 31 mL and 46 mL, respectively. All the column experiments were conducted in duplicate at room temperature (24 ± 2 °C). In biofiltration experiments, however, one biofilter column was subjected to phage treatment after day 125 while the other one remained untreated to serve as a control.

### 2.3. *Fate of phage and bacteria in the clean anthracite or GAC filter*

The clean bed filters were fed with a 0.85% sodium chloride solution at a flow rate of 1.6 mL/min, corresponding to an

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