Image caption: Biofilm reactors at a wastewater treatment facility.

Investigating Fate of Engineered Nanoparticles in Wastewater Biofilms

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Abstract: Engineered nanoparticles incorporated into consumer products have shown to negatively impact vital ecosystems once released into the environment. As wastewater reuse practices become increasingly necessary in areas of water scarcity, innovative wastewater treatment applications will be required. Attached growth (i.e. biofilm) processes for wastewater treatment generate less waste and are easier to operate compared to activated sludge. This study examines the interaction between silver nanoparticles (Ag-NPs) and wastewater biofilms. Two bench scale reactors were used to examine the impact of Ag-NPs on model biofilm, as well as the attachment of Ag-NPs to biofilm. The insights provided offer a basis for understanding the removal capabilities of Ag-NPs from wastewater through biofilm processes.

Key Points:

- Silver nanoparticles can attach to model wastewater biofilm without significantly impacting biofilm biomass.
- Wastewater biofilm can become stressed under exposure to 1 mgL^{-1} of silver nanoparticles.
- By applying a mass balance, model biofilm Comamonas testosteroni was observed to accumulate 0.172 ng mm^{-2} of silver nanoparticles.

Introduction

The application of silver nanoparticles (Ag-NPs) has expanded exponentially within manufactured products such as food packaging, cosmetics, and textiles (Boxall et al., 2009). Reuse of treated wastewater for various purposes such as drinking water, irrigation water, and/or cooling water is now a reality and will continue to increase as traditional freshwater sources become progressively stressed. Although Ag-NPs have previously been referred to as emerging contaminants, their presence is now a long-term issue that might have damaged vital microbiological ecosystems (de Faria et al., 2014). By modeling the fate and transport of Ag-NPs, environmentally relevant quantities will vary depending on location type. These concentrations are predicted generally in the range of 0.003 – 100 ngL^{-1} (Mitrano et al., 2012). Wastewater treatment plants, an important barrier between consumers and their surroundings, are not designed specifically for the removal of Ag-NPs (Walden and Zhang, 2016). As wastewater influent complexity increases, treatment plants should be re-evaluated for their processing efficiency. Likewise, as competing demands increase upon limited freshwater resources, reuse practices of treated wastewater will increase across the United States, including Arkansas. Consequently, there is a pressing need for economical yet effective regionalized wastewater treatment. Biofilm systems (Figure 1) are easy to maintain and convenient for small communities. Here, we investigated the role of wastewater biofilms in the removal of Ag-NPs from waste streams. The goal of this proposal investigated the following hypotheses: (1) ENPs within wastewater can attach to biofilms without significantly altering nutrient reduction capacity; and (2) under certain steady-state parameters, biofilms can become an environmental sink for ENP to accumulate within the extracellular polymeric substances (EPS). Ag-NPs were exposed to model wastewater bacteria Comamonas testosteroni in two differently sized bench scale reactors for Ag-NP impact on biomass and removal from suspension. Ongoing work will explore dual and mixed species combinations with additional bacteria Acinetobacter calcoaceticus and Delftia acidovorans (Andersson et al., 2008).

Methods

Experimental design

The three species were first tested for biofilm forming capacity. A biofilm formation assay was conducted in a clear 96 well plate with 2% crystal violet as previously described (Djordjevic et al., 2002; O'Toole, 2011). A control experiment was conducted for 28 days to observe the time for a mature biofilm to form within the CDC biofilm reactor (BioSurface Technologies, Bozeman, MT), and to monitor biological reduction capacity in the absence of Ag-NPs. A non-limiting synthetic wastewater inoculated with D. acidovorans was fed and recycled through the CBR as nitrate, phosphate, sulfate, chlorides, COD, and pH were monitored. Shorter experiments with C. testosteroni used as a feed into the CBR and the custom flow cell were also performed for 48 hours. For the shorter experiments, the feed was switched to sterile synthetic wastewater to remove planktonic cells from the system. Then, biofilm was exposed to a spike of about 1 mg L^{-1} Ag-NPs (CBR) and 2 mgL^{-1} (flow cell) for 30 minutes.

Reactor descriptions and setup

The CBR is a 1 liter glass beaker with a polyethylene lid which holds 8 polyethylene rods, each with three removable polyethylene coupons serving as an attachment site for biofilm growth. The working volume is about 350 mL. The custom flow cell holds three removable polyethylene coupons, and has a working volume of about 2 mL. The synthetic wastewater consisted of nutrient broth (300 mgL^{-1}), KH_2PO_4 (44 mgL^{-1}), NaOH (16.7 mgL^{-1}), $CaCl_2{\cdot}2H_2O$ (132.4 mgL^{-1}), $MgSO_4{\cdot}7H_2O$ (100 mgL^{-1}), $C_6H_{12}O_6$ (140 mgL^{-1}), KNO_3 (3 mg L^{-1}), $NaHCO_3$ (175 mgL^{-1}), $MnSO_4{\cdot}7H_2O$ (12.8 mgL^{-1}), $(NH_4)2SO_4$ (118 mgL^{-1}), and $FeCl_3{\cdot}6H_2O$ (5 mgL^{-1}). The CDC biofilm reactor (CBR), flow cell, connectors/tubing, and synthetic wastewater solution were autoclaved at 121°C for 30 minutes prior to each experiment (Model 522LS Gravity Steam Sterilizer, Getinge, New York). The experimental setup (Figure 2) included the CBR or flow cell connected to a peristaltic pump set at 10 and 1 mL min-1 flow rate, respectively.

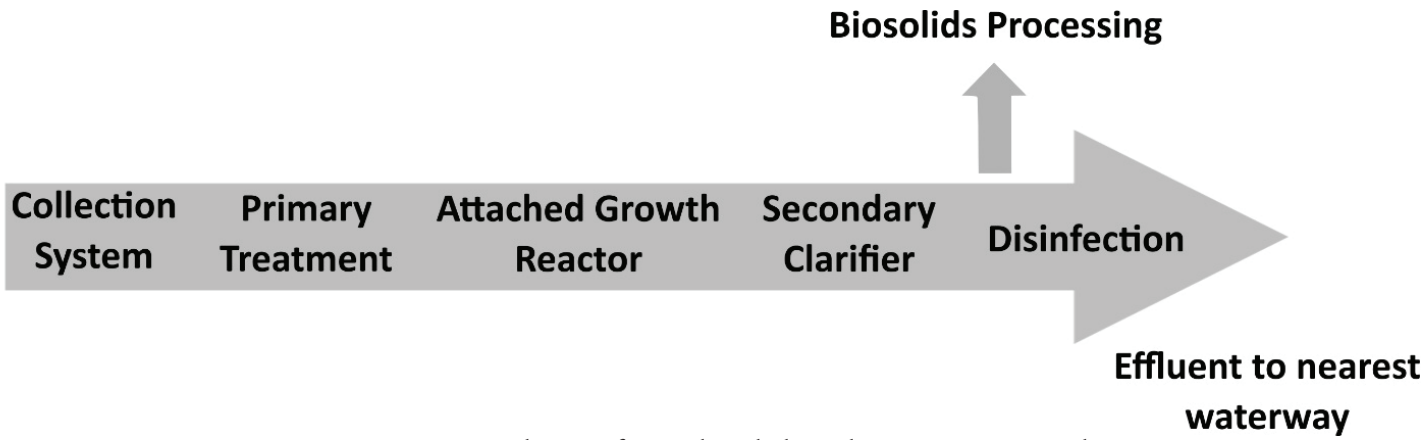

Figure 1. Representative schematic of a typical attached growth wastewater treatment plant.

Figure 2. (left) The experimental setup included a peristaltic pump and autoclavable tubing to circulate synthetic wastewater through the CDC biofilm reactor (CBR). (right) A close up shows the detachable polyethylene sampling coupons suspended in the CBR for biofilm testing.

Biofilm analysis with CBR

Biofilm amount was determined from Hoescht 33342 cell stain with an upright confocal fluorescence microscope (Nikon Eclipse Ni-E upright microscope, Nikon Instruments, Melville, New York). For biofilm stress, a modified dichlorofluorescein (DCF) assay was used as previously described in black-sided clear bottomed 96-well plates (Corning 3603, Corning, MA) and analyzed on a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., VT) (Wang and Joseph, 1999).

Biofilm analysis with flow cell

The flow cell system has the advantage of a smaller working volume than the CBR, allowing for quick biofilm formation and simple mass balance measurements. C. testosteroni was recycled through the flow cell for 48 hours to establish a mature biofilm. Then, sterile synthetic wastewater was pumped through for 10 minutes to eliminate any planktonic cells. 2 mg L^{-1} Ag-NPs were aseptically injected into the cell. After 30 minutes, sterile wastewater was used to flush the flow cell of any unattached Ag-NPs for 10 minutes. All effluent was retained and analyzed for total volume and total silver concentrations. All effluent was collected in sterile centrifuge tubes for mass balance measurements. To remove biofilm from the coupon for ICP-MS, each coupon was aseptically removed from the flow cell and inserted into a sterile tube with 5 mL of DDI water. The tubes were vortexed for 5 minutes. The coupon was removed, and the total volume was brought up to 10 mL and acidified with 2.5% nitric acid for ICP-MS. The concentration of silver ion was measured by centrifugal filtration and ICP-MS.

Silver synthesis

Silver nanoparticles were formed using sodium borohydride to reduce silver nitrate with sodium citrate as a capping agent (Mulfinger et al., 2007). All glassware was washed with phosphorus free detergent, rinsed three times with tap water, then rinsed three times with deionized water (Elga Process Water System (18.2 $M\Omega \cdot cm^{-1}$) Purelab flex, Veolia, Ireland). The reduction of silver nitrate occurred as follows:

$$AgNO_3 + NaBH_4 \rightarrow Ag + 0.5H_2 + 0.5B_2H_6 + NaNO_3$$

$$4Ag+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ago + C_6H_5O_7H_3 + 3Na+ + H+ + O_2$$

The formation of Ag-NPs was confirmed by scanning the absorbance from 300 – 700 nm with a UV-vis spectrophotometer (Beckman Coulter, CA, USA.). The concentration of Ag-NPs was measured with ICP-MS. Particle size was verified with TEM (Jeol, USA) and DelsaNano (Beckman Coulter, Life Sciences, USA).

Statistical analysis

All statistics and plots were generated in SigmaPlot (Systat Software, Inc., version 12.5) where statistic p values less than 0.05 were considered significant.

Results

Biofilm formation assay. The capability to form biofilm was investigated for the bacteria combinations discussed using a crystal violet microtiter 96-well plate assay. For all single and multiple combinations with these species a strong biofilm was formed. Of the three single assays, A. calcoaceticus forms a significantly stronger biofilm than C. testosteroni or Delftia acidovorans (Figure 3, $p < 0.05$). There was no significant difference

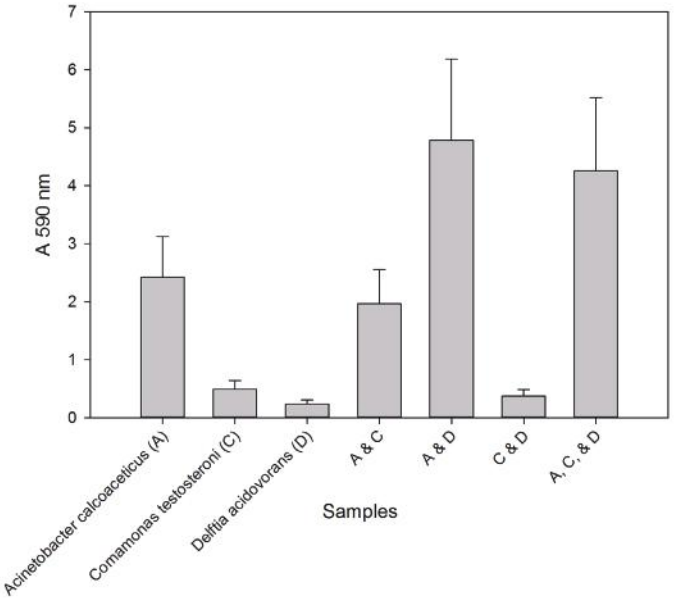

Figure 3. Biofilm formation assay results from crystal violet staining with standard error (n=3) for each species single, duel, and mixed. A greater absorbance reflects increased ability to form biofilm.

between the assay of all three mixed and the assay of A. calcoaceticus & D. acidovorans. (Stepanovic et al., 2000; O'Toole, 2011).

Nutrient reduction capacity

The CBR setup as a closed system with recycle was inoculated with D. acidovorans; nitrate, phosphate, sulfate, chlorides, COD, and pH were monitored to test for nutrient changes without Ag-NPs present. Minimal or no change was observed for nitrate, phosphate, sulfate, chlorides and pH. COD was reduced to approximately 18.8 mgL^{-1} from above detection limit after 10 days. We concluded that the quantity of biofilm formed within this reactor type with single species D. acidovorans is not sufficient for nutrient reduction testing.

Silver nanoparticle formation

The Ag-NPs exhibited the expected UV-vis peak at 395-400 nm for nano-sized silver. The average particle size from photon correlation spectroscopy was 7.9 nm, and confirmed with TEM (Figure 4). ICP- MS measured a stock solution concentration of 76 mgL^{-1}, with less than 10% ionic silver present. This stock was stored in the dark and verified as unchanged with UV-vis at each use.

CBR experiment

In the CBR system, C. testosteroni exhibited insignificant change in biomass after Ag-NP exposure (p=0.1323). This is consistent with previous conclusions that wastewater biofilms are tolerant to toxic loadings. However, reactive oxygen species present reflected significant cell stress after the 30-minute treatment (Figure 5, p = 0.0132). The CBR experiment addresses the first hypothesis that Ag-NPs can attach without significantly altering biomass.

Flow cell experiment

The amounts of Ag-NPs per coupon (Table 1) were all less than 0.1 ng mm^{-2}. The total silver recovered from biofilms was 0.172 ng mm^{-2}. This is a first step toward proving the second hypothesis that biofilms can become a sink for Ag-NPs.

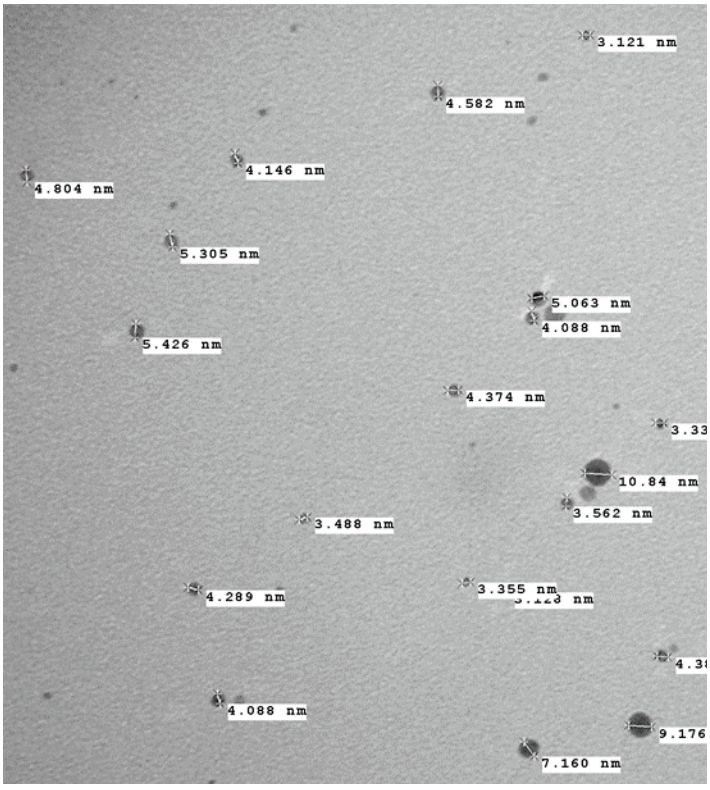

Figure 4. Transmission electron microscope image of silver nanoparticles (Ag-NPs) verifying the formation of nano-sized particles. Embedded within the image are diameters of randomly selected particles.

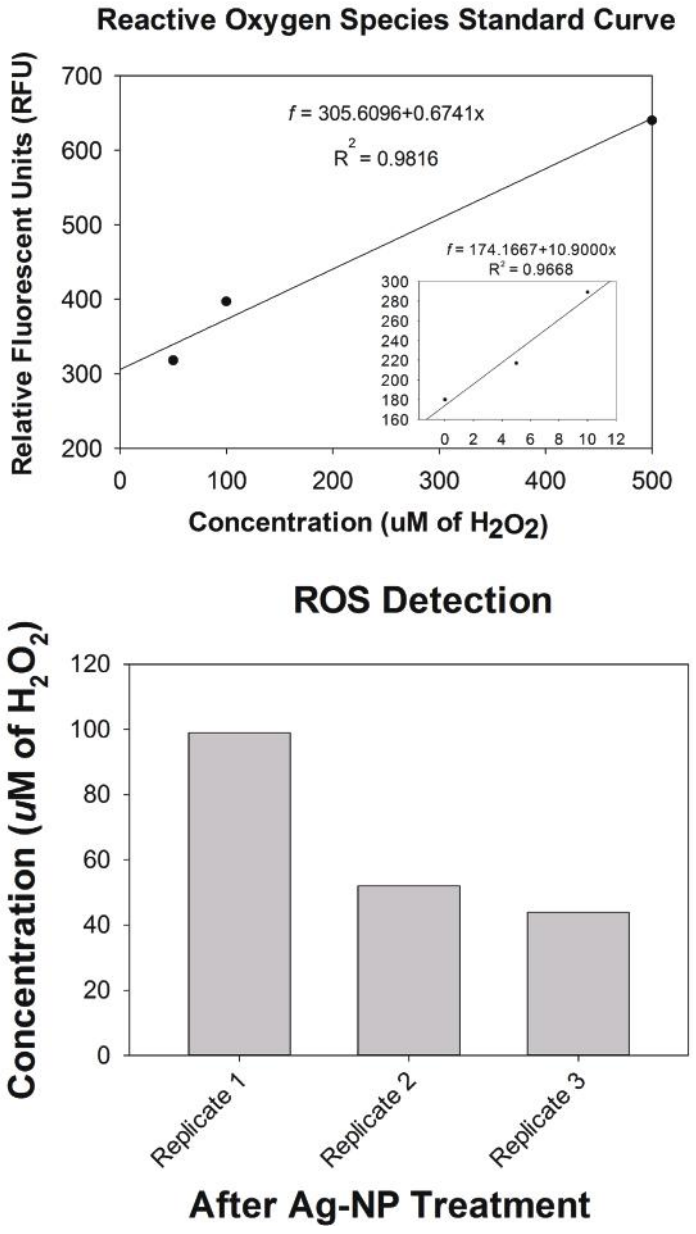

Figure 5. (a) RFU measurements were converted to concentration from the standard curve. (b) Reactive oxygen species measurements after a 30-minute exposure to 1 mg L-1 Ag-NPs.

	Ag-NPs per coupon (ng/mm^2)	Ag-NPs per coupon (ng/mm^2)	Ag-NPs per coupon (ng/mm^2)	Total Silver in Biofilms (ng)	Percent Recovered	Total Silver Accumulation (ng/mm2)
Control (No Biofilm)	-	-	-		104	-
Comamonas testosteroni	0.091	0.053	0.028	21.8	91.7	0.172

Table 1. Silver mass balance: the amount of silver retained on each coupon removed from the flow cell measured by ICP-MS.

Conclusions, Recommendations and Benefits

Model wastewater biofilm shows potential to resist acute exposure to environmentally relevant quantities of Ag-NPs. Further, this model biofilm can accumulate Ag-NPs into its biofilm structure. This fundamental look at the Ag-NP – biofilm interactions shows minimal potential for Ag-NP accumulation. However, the resistance to detachment in the presence of Ag-NPs shows the capability of even a single wastewater type species to tolerate toxic loadings. We recommend continuing this work with other model species and a more complex biofilm community.

Although ENPs have been commonly referred to as 'emerging' contaminants, the presence of ENPs is now a persistent and long term issue that may have already damaged vital microbiological ecosystems. The goal is to explore realistic environmental conditions in wastewater biofilm systems that control the removal and release of potentially toxic ENPs (silver nanoparticles, Ag-NPs), thereby establishing the fundamental groundwork that will enable innovative use of biofilm processes in wastewater treatment for water reuse and recycling in areas of water scarcity. By investigating water supply and quality problems, this research directly addresses the goals of the AWRC. Likewise, by exploring issues that are of immediate concern in arid and semi-arid climates, this research furthers the U.S. Geological Survey's national water mission to increase knowledge of water quality and quantity. The United States Environmental Protection Agency (EPA) published many examples of current water reuse practice in Region 9 district (serving Arizona, California, Hawaii, Nevada, Pacific Islands and Tribal Nations), and reuse will continue to increase as traditional fresh water sources become increasingly stressed (Fachvereinigung Betriebs- und Regenwassernutzung e, 2005).

Acknowledgements

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