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Susceptibility of constructed wetland microbial communities to silver nanoparticles: a microcosm study

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Abstract

Silver nanoparticles (AgNPs) are increasingly used as an antimicrobial agent in various consumer products. Silver release from these products occurs during use, washing and disposal at varying rates, into the wastewater system and eventually into aquatic ecosystems. Constructed wetlands (wetlands designed for water pollution control) represent a unique type of water treatment system which are beginning to receive growing amounts of influent AgNP loadings. In order to examine potential impacts to the health and ongoing utility of constructed wetlands we evaluated the susceptibility and the developed resistence of constructed wetland microbial communities to two types of AgNPs (citrate and polyvinylpyrrolidone (PVP)-coated), and ionic Ag (Ag^+). Microcosms representing an unplanted batch-fed subsurface flow constructed wetland were exposed to an AgNP contaminated (100 µg/l) simulated wastewater for 28 days. Experiments were triplicated and included unexposed controls. Analysis of total Ag in the interstitial water and biofilm matrix via inductively coupled plasma mass-spectrometry (ICP-MS) revealed the majority (> 98%) of AgNPs partitioned from the interstitial water and accumulated in the biofilm and

presumably the sediment matrix. No significant alteration to the microbial community function (assessed via community-level physiological profiling, CLPP) or structure (using denaturing gradient gel electrophoresis, DGGE) was observed following the initial 28 day exposure. Subsequent *ex-situ* dose-response testing over a wide concentration range (0-5 mg/l) provided some evidence for increased resistance to Ag toxicity in the previously exposed microbial communities when compared to the controls. The ex-situ evaluated susceptibility of the microbial communities to AgNPs varied with significant reductions in catabolic activity observed at 0.5 mg/l for ionic Ag, 1 mg/l for citrate AgNPs and 5 mg/l for PVP AgNPs. These findings suggest that wetland microbial communities can tolerate and develop resistance to lower levels of in-situ AgNP exposure in a relatively short time, however ex-situ toxicity is observed past certain threshold concentrations and this threshold varies depending on the original form of the AgNP.

1.0 Introduction

Silver nanoparticles (AgNPs) are increasingly integrated into textiles, food packaging and many household items as an antimicrobial agent (TPEN 2013). The magnitude of production and breadth of applications are growing thereby increasing the likelihood of Ag release to the environment (TPEN 2011). Both ionic Ag and AgNPs are potentially toxic (Morones *et al.* 2005), and recent studies have shown that Ag can leach from AgNP containing consumer products into the wastewater system (Benn and Westerhoff 2008). According to Geranio *et al*. (2009) some garments can release up to 45 % of the total Ag embedded in the textile after a single wash. This released Ag enters wastewater treatment plants and is predicted to then be transferred in unknown amounts to the natural environment (Blaser *et al.* 2008) giving rise to the risk of exposure and accumulation in biota inhabiting aquatic ecosytems (Cleveland *et al.* 2012).

The investigation of AgNPs toxicity on wetland systems can be completed within the context of natural wetlands, or wetlands built for water pollution control (referred to here as constructed wetlands). Existing studies have focused on the behaviour of AgNPs in natural wetlands. Lowry et al. (2012) found that a large percentage of silver dosed to natural wetland mesocosms was transformed into sulfidised (Ag_2S) silver with rapid transfer of silver from the water column into the sediment. Relatively small amounts of Ag were accumulated in different plant species whilst large body burdens were observed in mosquito fish and chironomids (Lowry et al. 2012). Colman et al. (2014a) investigated ecosystem-level effects of AgNPs in natural wetland mesocosms

reporting that exposure led to a series of effects including leaf senescence, declines in phytoplankton biomass, negative impacts to water chemistry, and an overall increase in methane release. Colman et al. (2014) used admittedly high exposure concentrations (2.5 mg/l) more in line with laboratory toxicological studies than likely environmental concentrations, but demonstrated the unique challenges and complexities of such studies, and the ecosystem-level impacts that silver can have. Colman et al. (2014) also observed a relatively clear convergence regarding the effects of different Ag treatments with ionic silver and AgNPs showing similar results, likely due to the rapid transformation of silver once within the mesocosm systems as demonstrated by Lowry et al. (2012).

Although detailed AgNP studies have been completed with natural wetland mesocosm systems, no studies have yet been completed on wetlands designed for water pollution control. Constructed wetlands (CWs) rely on microbiological processes for water treatment (Faulwetter *et al.* 2009). For processes such as organic degradation, nitrification, or denitrification microbial communities housed within biofilm surrounding the CW bed media (usually gravel) play the dominant mechanistic role. With their increasing use as a low-cost, low-maintenance and effective mode of wastewater treatment the constructed wetland represents a system likely to receive Ag released from consumer products. In particular the large numbers of small-scale constructed wetland systems now employed primarily in European countries but also increasingly in North America for the treatment of greywater from individual residences.

Estimated environmental concentrations for AgNPs based on a combination of modelled and analytical results range from 10^{-4} to 102 µg/kg or µg/l with the highest PECs occurring in sediments and wastewater treatment plant effluents (Gottschalk *et al.* 2013). No estimates on current or expected AgNP loadings to CWs have been made in the literature. CWs receiving greywater from a single home represent the system type most at-risk for potentially high AgNP concentrations. Geranio *et al*. (2009) found X-STATIC™ fabric to release approximately 314 ug Ag/g fabric, and AgKilBact™ fabric to release approximately 377 ug Ag/g fabric after a single wash. Assuming a relatively small high efficiency washer (approximately 50L of water) processing 20 sports shirts (approximately 2kg total weight), upward of 15 mg/l could be estimated as a worst case scenario leaving a washing machine and therefore entering CWs. That estimate is based on clothing containing 100% X-STATIC™ or AgKilBact™. This type of silver treated fibre is commonly

incorporated into fabric in the range of 5-20%. Assuming a 10% final concentration in clothing the washer effluent (influent to a single home CW) could reasonably contain 1.5 mg Ag/l. Averaged over an entire day of water release from a single home this estimate would of course become even lower, perhaps on the order of 0.1 mg/l.

Understanding the effect of AgNPs on microbial communities from constructed wetlands is important for the continued operation of wetlands for water pollution control in the face of growing AgNP inlet loadings. If AgNPs have an exceptionally toxic effect, constructed wetlands which are already in use and otherwise operating efficiently for water treatment may not be viable for future wastewater applications, or could require augmentation to ensure continued operation. Several studies have looked at effects in non-CW microbial communities with differing results. Low levels of AgNPs (0.14 mg/kg) were shown to cause reduced enzyme activity in the microbial communities of contaminated soils (Colman *et al.* 2013), whilst concentrations up to 1.0 mg/l reportedly had no effect on the genetic diversity of microbial communities in estuarine sediments (Bradford *et al.* 2009). In contrast, Doiron et al. (2012) reported that exposure concentrations as low as 5 µg/l of polymer-coated AgNPs resulted in decreased bacterial abundance in marine microcosms. Similarly, Fabrega et al. (2009) saw a decrease in the growth of marine biofilm in the presence of AgNPs.

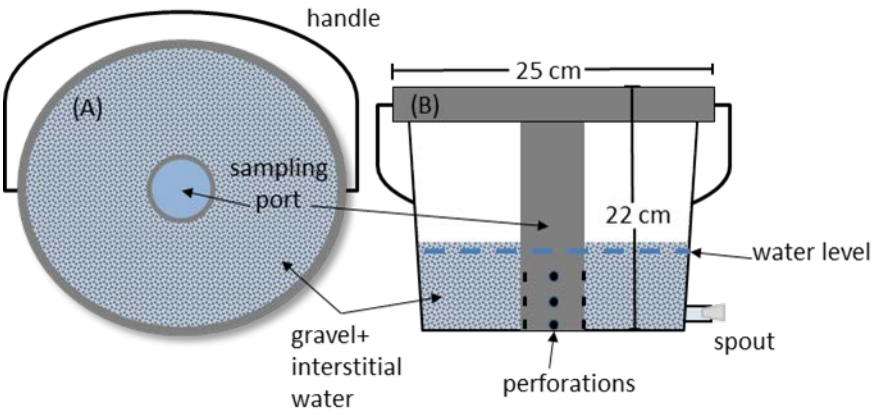
Genetic fingerprinting methods are frequently used to study microbial communities in environmental samples (Boon *et al.* 2002). In denaturing gradient gel electrophoresis (DGGE), the number and relative abundance of the dominant ribotypes in a sample can be determined based on the number and intensity of the bands. Community-level physiological profiling (CLPP) can be used to assess the catabolic function of microbial communities based on substrate utilisation (Weber and Legge 2010a). CLPP is a rapid and sensitive method to measure differences in catabolic activity, richness, diversity, and has been successfully applied to the assessment of microbial assemblages in constructed wetlands (Weber and Legge 2013; Weber *et al.* 2011; Zhang *et al.* 2010). In addition, the first known *ex-situ* method of microbial community functional (based on the catabolic capabilities) toxicity testing was recently developed and described using the concepts underlying the CLPP method and applied to constructed wetland samples exposed to AuNPs (Weber *et al.* 2014).

This study set out to evaluate the impacts of different types of AgNPs (citrate and polyvinylpyrrolidone coated) in addition to ionic Ag on constructed wetland microbial communities associated with both the biofilm and interstitial waters. Changes in microbial community function and structure were monitored in wetland microcosms over a period of 28 days following exposure to 100 µg/l AgNPs. The distribution and toxicity of each type of AgNP and potential for development of microbial community resistance was further assessed via *ex-situ* community based functional toxicity testing over a wider exposure range.

2.0 Materials and methods

2.1 Experimental design

CW microcosms (Figure 1) were built in triplicate for each type of AgNP (citrate, and polyvinylpyrrolidone coated), ionic Ag, and an unexposed control, using 5 L polypropylene containers giving a total of 12 experimental systems. The microcosms were filled with 2 kg of coarse granitic gravel (Ø 10-15 mm) and inoculated by homogenously mixing 0.5 kg of similar biofilm-containing gravel obtained from the surface of a local horizontal subsurface flow constructed wetland, treating tertiary domestic wastewater (De Pinte, Belgium). The microcosms were fitted with a 32-mm-diameter perforated central sampling well and a small outlet spout made of silicone tubing with a plastic stopper for draining. The microcosms were fed with 0.5 l of a simulated wastewater (pH 7.4) based on that described by Weber *et al*. (2011), consisting of 1 g/l molasses, 0.049 g/l urea, 0.0185 g/l $NH_4H_2PO_4$, (Merck, Darmstadt, Germany). After filling the water level was just below the surface of the gravel. The microcosms were operated in batch mode with weekly draining equating to a hydraulic retention time (HRT) of 7 days. Solids were retained in the microcosms for the duration of the experiment. The microcosms were allowed to acclimatize for 19 days (longest time permitted due to project constraints) before the simulated wastewater was spiked to a final Ag concentration of 100 µg/l. The exposure concentration is higher than some predicted environmental concentrations e.g. (Blaser *et al.* 2008), but falls within a reasonable worst-case scenario for CWs (as described earlier). The chosen concentration also provides a balance between environmental relevance, analytical feasibility and the aim of investigating the development of Ag resistance in the exposed microbial community over the relatively short term of the experiment. After the acclimatization period, the microcosms were operated for 28 days.



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159 **Figure 1**: Diagram of a constructed wetland microcosm showing (A) aerial view (B) cross-sectional view.
160 Diagram is not to scale.

161

162 **2.2 Nanoparticle stock solutions**

163 Citrate AgNPs with a manufacturer stated average particle size of 10 nm and at a concentration of
164 100 mg/l were obtained from PlasmaChem GmbH (Berlin, Germany) as a colloidal dispersion. PVP
165 AgNPs in powder form (20-30 nm, SkySpring Nanomaterials, Inc., Houston, TX, USA) were
166 dispersed using Milli-Q water (EMD Millipore Corp., Billerica, MA, USA) followed by 15 minutes in
167 an ultrasonic bath (Sonorex Super RK103H, Bandelin electronic GmbH, Berlin, Germany) to give a
168 final concentration of 100 mg/l. The ionic Ag standard solution (Plasma HIQU, 1000 ± 2 μg Ag/ml
169 in 2 – 5 % HNO_3) was purchased from ChemLab NV (Zedelgem, Belgium). All AgNP suspensions
170 were stored in the dark at 4-8 °C. The concentrations of the Ag stock dispersions were verified by
171 ICP-MS analysis (Section 2.3) prior to their use in spiking the simulated wastewater.

172

173 **2.3 Nanoparticle characterization**

174 Characterisation of the PVP and citrate AgNPs used in this study was performed on representative
175 stock solutions and the nanoparticle spiked simulated wastewater. Scanning/Transmission
176 Electron Microscope images (S/TEM) (Quanta FEG 250, FEI, Oregon, USA) were collected to
177 qualitatively assess the particle morphology and provide size distribution data. The elemental
178 identity of the imaged particles was confirmed using energy dispersive X-ray spectroscopy (EDS)
179 (EDAX, USA). A representative EDS spectra is included as supplementary information (SI Figure 1).
180 A droplet of each sample was placed onto a carbon/formvar coated copper TEM grid and allowed
181 to dry. Images were collected at 30 Kv, spot size 1 using a dark field (DF) S/TEM detector. The

nanoparticle size distribution was measured for single particles or particle agglomerations in a representative image. Particle diameter was derived from the area measured using the analyze particles function in the free software program ImageJ. The mean particle diameter, based on number distribution, and the zeta potential was determined using a Dynamic light scattering instrument (DLS) (Brookhaven 90 Plus/Zeta, Brookhaven Instruments Corp., New York, US). All sample measurements were performed in triplicate at 22 °C using a 659 nm laser positioned at a measuring angle of 90 °. The results of this characterization are shown in Figure 2. Total Ag content in the different AgNPs stock solutions was quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Vista-MPX CCD Simultaneous ICP-OES, Varian, Agilent Technologies, Santa Clara, CA, USA). Sample aliquots of 2.5 mL were combined with 3.0 mL 65 % HNO_3 (Chem-Lab, Zedelgem, Belgium) and subjected to open vessel microwave digestion (MARS, CEM Corp., Matthews, NC, USA) at 100 °C and 600 W for 1 h. The concentration of all stock solutions was verified before spiking the wastewater to be fed into the wetland microcosms.

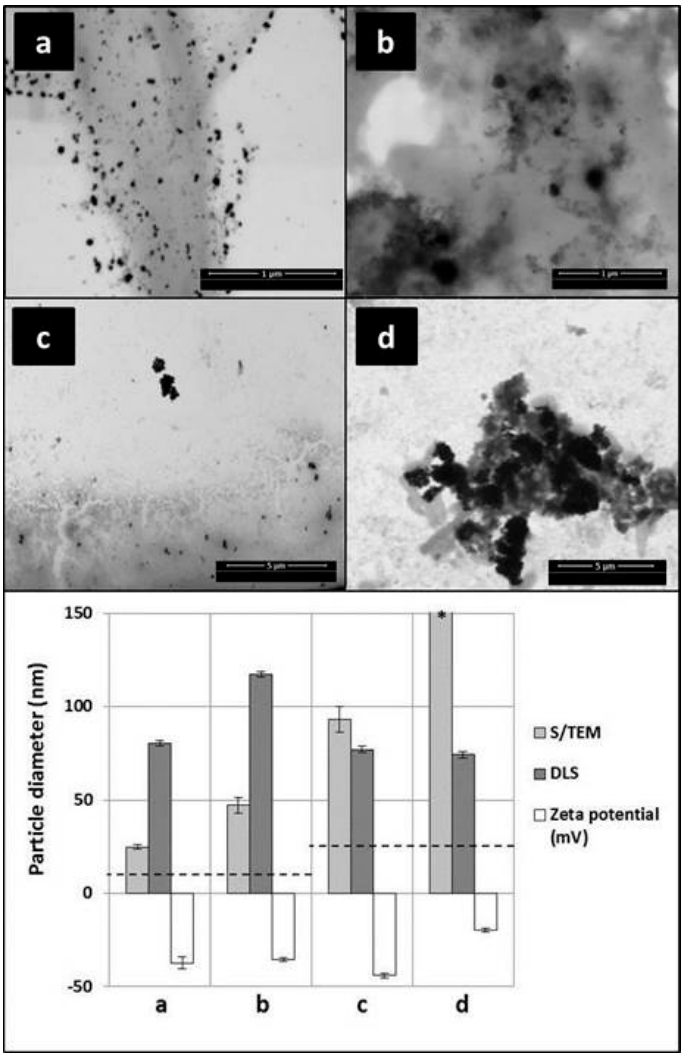


Figure 2: S/TEM images for citrate coated AgNPs in the original stock solution (a) and in the simulated wastewater (b), PVP coated AgNPs in the original stock solution (c) and in the simulated wastewater (d). Bar chart shows the mean particle diameter calculated using S/TEM images and DLS. Error bars show 1 std. error for S/TEM and Zeta potential data, and 1 geometric standard deviation (GSD) for DLS data. The horizontal dashed line indicates the particle size quoted by the supplier.

The percentage of ionic Ag in AgNP stock solutions (defined here as <10 kDa molecular weight cut-off (MWCO)) was determined using centrifugal ultrafiltration (UF) devices (Amicon Ultra-4, EMD Millipore Corp., Billerica, MA, USA; MWCO of 10kDa). For this, 4.0 ml was pipetted from the stocks/samples into UF devices and subjected to centrifugation (Megafuge 1.0, Heraeus, Hanau, Germany) at 3735 g for 15 min. Afterwards, the filtrates were diluted ten times with internal standard solution (IS) (10 μg Rh/l (Chem-Lab, Zedelgem, Belgium) in 2 % HNO_3), and analysed directly for total Ag content by means of inductively coupled plasma-mass spectrometry (ICP-MS)

(Elan DRC-e, PerkinElmer, Inc., Waltham, MA, USA). Both citrate and PVP coated AgNPs were > 99% particulate. External calibration standards were used for ICP-MS analyses, and recalibrations were performed every 20 samples. Blank samples and reference standards were included at the beginning and the end of each intra-analysis batch of 20 samples for quality control purposes. All measurements were performed in triplicate unless stated otherwise. The detection limit of the ICP-MS was 0.016 μg Ag/l. Additional details can be found in SI Table 1.

2.4 Water chemistry measurements

Water quality was characterized weekly at the end of the 7 day HRT in the sampling well for pH (Model 520A pH meter, Orion Research Inc., Boston, MA, USA), dissolved oxygen (DO) (Portable D.O. meter, HI 9143, Hanna Instruments, Woonsocket, RI, USA), and total organic carbon (TOC). TOC in the wastewater was measured using a TOC-Vcpn analyser (Shimadzu, Kyoto, Japan) within 3 days of sample collection during which time the samples were stored in a refrigerator at 4^{o}C.

2.5 Microbial community analysis

2.5.1 Community-level physiological profiling

Community-level physiological profiling (CLPP) was used to study impacts to microbial community function in the microcosms. The profiles were created by gathering substrate utilization data from 96 well (31 carbon sources and 1 blank in triplicate) Biolog® Ecoplates (Biolog, Hayward, CA, USA). Interstitial waters were collected after 28 days from the sampling well of each microcosm using a long glass 50 mL pipette (following a 3 times well volume flush) into 50 mL autoclaved glass bottles. Biofilm samples were collected after 28 days by shaking 75 g of gravel from each microcosm in 750 mL phosphate buffer saline (10 mM Na_2HPO_4 (Chem-Lab, Zedelgem, Belgium), 8.5 g/l NaCl (Chem-Lab, Zedelgem, Belgium), pH 7.4) for 3 h (approximately 200 rpm) and in the dark according to Weber and Legge (Weber and Legge 2010b). The 75g of gravel was comprised of 25g from replicated system giving a composite sample. Each well of the Biolog® Ecoplate was inoculated with 100 μL of sample using an 8 channel pipette. Direct inoculation without pre-treatment of the samples was possible as both the interstitial water and biofilm extracts were sufficiently clear. The plates were incubated in the dark at room temperature and were read using an absorbance microplate reader at 590 nm (Infinite® 200PRO, Tecan Group Ltd., Männedorf, Switzerland and PowerWave$_X$340, BioTek Instruments, Inc., Winooski, VT, USA) at 0, 14, 24, 43,

48, 65, 72 and 115 hours post inoculation. Data from the time point of 65 h was selected for further analysis based on the suggestions of Weber and Legge (2010a).

2.5.2 DNA extraction and purification

Total DNA was extracted from the interstitial water and biofilm samples (previously detached from gravel using the above protocol for prepping CLPP solutions) by filtering 50 mL of each through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) using a sterile vacuum filtration system and the filters stored at -20 °C. For DNA extraction 200 mg of glass beads and 1000 μL of lysis buffer (100 mM Tris/ EDTA/ NaCl, 1% PVP40, 2% SDS, pH 7) (Sigma Aldrich/ Chem-Lab, Belgium) were added to the tube containing the rolled filter paper. The tube was then shaken vigorously twice with a FastPrep® automated homogenizer (MP Biomedicals, Santa Ana, CA, USA) for 30 s at 1600 rpm and then centrifuged for 5 min at maximum speed (16220 rpm). Thereafter, the supernatant was mixed with 500 μL of phenol:chlorophorm:isoamilic alcohol (Sigma Aldrich, Belgium), after which it was centrifuged for 1 min at maximum speed (16220 rpm). Then, the supernatant was mixed with 700 μL of chloroform (Sigma Aldrich, Belgium) and centrifuged for 1 min at maximum speed (16220 rpm). 45 μL of 3 M sodium acetate and 500 μL of ice-cold isopropyl alcohol (both Sigma Aldrich, Belgium) were then added to the supernatant. The tubes were mixed and kept at -20 °C at least for 1 h, after which they were centrifuged for 30 min at 4 °C at maximum speed (16220 rpm). Finally, the pellet was left to dry and thereafter resuspended in 50 μL of Tris-EDTA buffer (Sigma Aldrich, Belgium). The extracted DNA samples were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration of DNA in the samples was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) and the quality of the DNA was verified using agarose gel electrophoresis. The samples were stored at -20 °C until further processing.

2.5.3 PCR amplification

The V3 region of 16S rRNA genes from the microbial community was amplified by PCR using Universal primers (338F-ACTCCTACGGGAGGCAGCAG with a GC-clamp and 518R-ATTACCGCGGCTGCTGG) based on the protocol described by Øvreås et al. (1997). The master mix contained the following components: 0.2 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10x Taq Reaction Buffer ($MgCl_2$-free), 1.25 U/50 µL of Taq DNA

Polymerase, 400 ng/µL 31 of bovine serum albumin, and DNase and RNase free filter sterilised water (Thermo Scientific, Waltham, MA, USA). 1 μL of extracted DNA was added to 24 μL of mastermix. The PCR was carried out in a thermal cycler T100™ (Bio-Rad, Hercules, CA, USA) as follows: an initial denaturation step for 5 min at 94 °C; then 30 cycles of 1 min at 95 °C, 1 min annealing at 53 °C, and 2 min DNA synthesis at 72 °C; concluding with a final elongation step of 10 min at 72 °C.

2.5.4 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was used to assess the structure of the microbial community in the wetland microcosms. Triplicated samples were pooled and run as a single sample. DGGE was performed for PCR products using an INGENY phor-U 2x2 (Ingeny International BV, The Netherlands) according to the manufacturer's instructions. The PCR products were loaded on a polyacrylamide gel (8 % wt/vol) (acrylamide/bisacrylamide solution (37.5:1), Bio-Rad, Hercules, CA, USA) in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The gradient ranged from 45 % to 60 %. The 100 % denaturant contained 7 M urea (Bio-Rad, Hercules, CA, USA) and 40 % (v/v) formamide (Bio-Rad, Hercules, CA, USA).

Samples were run for 18 h at 100 V and 60 °C. After the run, the gel was stained with SYBR Green solution (50 μl/l in 1x TAE-buffer; Applied Biosystems, Grand Island, NY, USA) for 20 min, after which the gel was imaged on a UV trans illumination table (OptiGo, Isogen Life Science, De Meern, The Netherlands). The images were imported into an analysis software (GelCompar II, Applied Maths, Belgium) for determination of the number and intensity of bands. See SI Figure 2 for DGGE gel image.

2.5.5 Ex-situ dose-response testing of AgNPs

Ex-situ experiments were conducted to test the effects of AgNPs on the catabolic capabilities of biofilm microbial communities within the wetland microcosms and adaptation of the community to Ag toxicity. Biofilm microbial communities were detached from the gravel of microcosms that had previously been exposed to 100 µg/l for 28 days and from the control with no Ag exposure as described in Section 2.5.1 and detailed in Weber *et al.* (2014). An aliquot (19.6 ml) microbial community solution was placed in a sterile 50 ml C-tube and 0.4 ml of AgNP stock solution added to give final exposure concentrations of 0.5, 1, 2, 5 mg Ag/l. The sample was thoroughly mixed by

30 second hand shaking and then each well of a Biolog® Ecoplate was inoculated with 100 µl. The plate was then incubated for 5 days and absorbance readings collected as described above.

2.5.6 Data analysis

CLPP data were analyzed according to Weber and Legge (2010a) based on average well color development (AWCD), Richness and Diversity. Richness is a measure of the number of different carbon sources utilised by a microbial population, and is calculated here as the number of wells with a corrected absorbance greater than 0.25 AU. Diversity is expressed here in terms of the Shannon index applied to the utilisation of all 31 individual substrates contained on the Biolog® Ecoplate. Carbon source utilisation patterns (CSUPs) from CLPPs and banding patterns from DGGE were subjected to principal component analysis (PCA). Normality of the data was assessed and, if necessary, corrected according to Weber *et al.* (2007). The mean CSUP of the triplicated treatments was used in PCA analysis. The statistical significance of differences ($p<0.05$) in results was assessed using a factorial ANOVA followed by a post-hoc Tukey HSD for differences between each treatment and group and a one way ANOVA followed by a 2-sided Dunnett's test comparing each dose concentration against the control. Differences are not significant in the absence of a reported p-value. Statistical analyses were performed in STATISTICA version 12, StatSoft® and XLSTAT 2013, Addinsoft©.

3.0 Results and Discussion

3.1 Water quality

Water quality within the CW microcosms was comparable for all treatments and did not fluctuate significantly over time. Over the course of the 28 day exposure period water pH in all systems was in the neutral range (7.3 – 8.2), TOC was 2.4 – 4.5 mg/L and DO was typically low but showed an increase from less than 1mg/L after 7 days of operation to between 2 and 5 mg/L for the remaining duration of the experiment. There was no indication of an impact of Ag exposure on water quality based on these parameters (SI Table 2).

3.2 Nanoparticle characterization

Characterisation results for the investigated AgNPs are shown in Figure 2. The mean particle diameter measured in the stock solutions using DLS were 80.3 and 77.1 nm for Citrate and PVP coated AgNPs respectively. Analysis of S/TEM images differed with values of 24.8 and 93.3 nm.

These values are higher than the manufacturer stated sizes and suggest agglomeration had occurred to some extent in the stock solutions prior to addition to the simulated wastewater. Agglomeration was also evident in the simulated wastewater by both DLS and S/TEM imaging, and was most evident for PVP coated AgNPs in the simulated wastewater (Figure 2d). This was also reflected in the Zeta potential of the stock and wastewater solution whereby PVP coated AgNPs in the wastewater solution showed a Zeta potential of lower magnitude (-20 mV) and therefore higher propensity for agglomeration. Citrate coated AgNPs in both the stock and wastewater solutions (and the PVP stock) had comparatively high negative Zeta potential suggesting greater particle stability. From these results and despite the relative stability suggested based on Zeta potential results it is clear that agglomeration of particles was occurring to some extent prior to addition to the wetland microcosms. EDS spectra (SI Figure 1) show no significant Ag sulfidisation or chlorination to occur directly after adding Ag to the original simulated wastewater solution, however this does not discount it from happening while in the microcosms over longer time periods. Agglomeration is an important parameter to monitor as the decreased surface area and increased mass of particle agglomerates will alter behaviour including rates of dissolution and partitioning behaviour.

The mass of AgNPs added to the systems in the spiked simulated wastewater was reduced by approximately 98 % in the water column after 7 days (the maximum period between batch loading of wastewater) for all systems. This amounted to the removal of 98 ppb, leaving a concentration of 2 ppb. All of this remaining silver was verified to be AgNPs (via 10kDa MWCO filters) with no ionic silver detected (even in the system dosed with ionic silver). Due to these low concentrations characterization of the remaining particles via DLS or TEM was not possible.

The distribution of Ag in the microcosms was determined at the end of the 28 day exposure period (Figure 3) and the majority was found to reside in the biofilm with levels approximately ten-fold higher than in the interstitial water. This distribution was comparable for all treatments with the exception of citrate coated AgNPs where the mass was approximately half the amount in the biofilm compared to other Ag types after 28 days of operation. This was not explained by concomitant higher levels in the interstitial water and may be due to increased loss during the weekly drain and fill cycle, or due to partitioning into the accumulated organic matter and sediment rather than the biofilm. The overall recovery of Ag based on the sum of biofilm and

interstitial water Ag was approximately 55%. In a study by Weber et al. (2014), evidence was given for the microbiological degradation of the citrate coatings surrounding AuNPs. Although not accounted for in this study it is possible that degradation of the citrate coatings occurred here as well. This could affect partitioning behaviour through increased formation of AgS compounds leading to deposition in the sediment and lower availability to the biofilm dwelling microbes. Minimal ionic Ag was detected in the interstitial water of any of the microcosms including those dosed with ionic Ag, which again suggests rapid particle formation/association. Rapid decreases in the concentration of Ag from the interstitial water (water column) have been reported elsewhere (Colman *et al.* 2014; Lowry *et al.* 2012). In a comprehensive study by Colman *et al.* (2014) using natural wetland mesocosms, Ag concentrations in the interstitial water fell by up to 67% within 24 hours of dosing. The decrease was attributed to release of large quantities of chloride ions (Cl^-) from submerged and floating macrophytes which led to the formation of AgCl(s). In the absence of plants in the present study the observed low levels of Ag in the interstitial water could be partially due to some AgCl(s) formation via small amounts of added Cl- in the simulated wastewater feed, however more likely due to the formation of Ag_2S(s), via larger amounts of added sulphur in the simulated wastewater feed, which is known to occur readily under low oxygen conditions or through reaction with organic matter (Levard *et al.* 2012; Levard *et al.* 2011). This would explain the lack of ionic Ag after 28 days exposure with the low levels of DO (1-3 mg/l) observed here. As stated the entire mass of Ag added to the systems is not accounted for in Figure 3. As this study was focused on the microbial community the biofilm was sampled in such a way that mainly gravel-adhered biofilm was collected and did not include significant amounts of accumulated solids from the sediment layer at the base of the microcosms. It is suspected that unaccounted Ag had settled into the sediment layer or been removed during the drain-fill cycle. Lowry et al. (2012) investigated the long-term fate of AgNPs in a simulated natural wetland reporting that AgNPs added to the water column rapidly settled into aquatic sediments and that a large fraction of dosed Ag was sulfidised to Ag_2S(s). Water chemistry data collected over the same period was similar in all systems throughout the period, further suggesting that the type of AgNP or ionic silver dosing did not affect the systems in different ways (see SI Table 2).

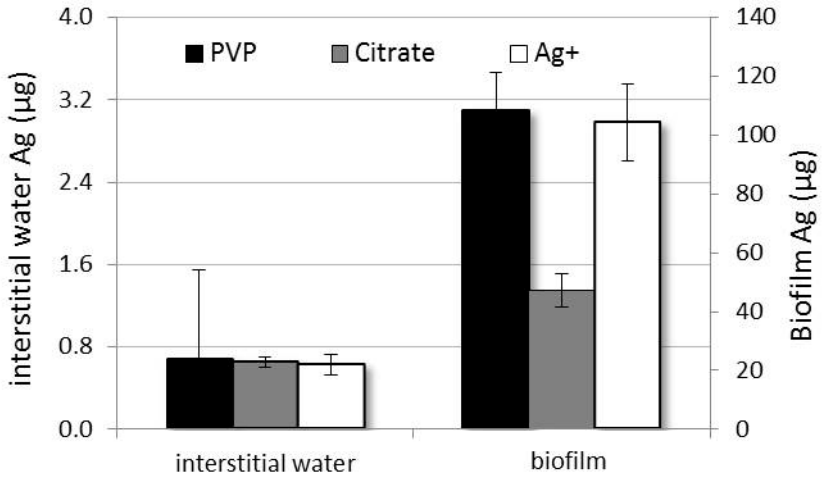
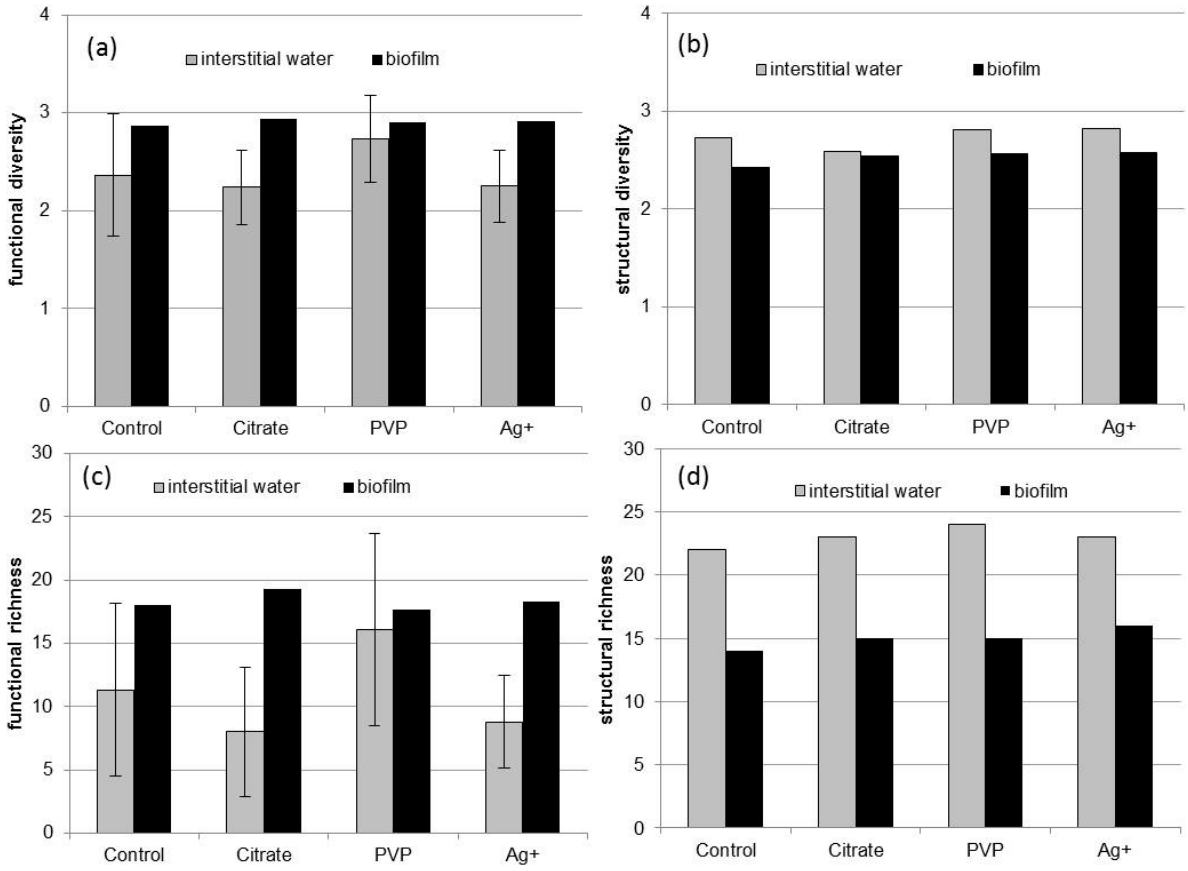


Figure 3: The total mass of Ag measured in the interstitial water and biofilm of the experimental microcosms after 28 days of operation. Values were determined using total Ag data derived from microwave assisted acid digested samples with ICP-MS analysis. Error bars represent 1 standard deviation (n=3). No Ag was detected in the control microcosms.

3.3 Microcosm microbial community impacts

The richness and diversity of the microbial community in terms of both function and structure was examined at the end of the 28 day exposure period (Figure 4). Functional diversity and richness, Figure 3 A and C respectively, was higher for the biofilm microbial community in comparison to the interstitial water for all treatments. PVP AgNPs appeared to have a slightly positive impact on the interstitial water community function with richness and diversity being slightly higher than the control, whereas all other exposures resulted in functional richness below that of the control. Structural richness and diversity, Figure 4 B and D respectively, was higher in the interstitial water compared to the biofilm. The dose of 100 µg/l used in this study was selected to provide some degree of relevance to concentrations that might be encountered in constructed wetlands. Overall, no distinct impacts either negative or positive were seen in the functional or structural richness or diversity of the CW microcosm microbial communities.



419

420 **Figure 4** – Functional and structural diversity (a and b) and richness (c and d) of microbial communities in
421 wetland microcosms after 28 days exposure to 100 µg/l of AgNPs or ionic Ag in simulated waste water.
422 Error bars for interstitial water samples are based on 1 standard deviation (n = 3). Biofilm samples were
423 combined and run as a single sample. Richness is calculated as the number of carbon sources utilised via
424 CLPP for function, or number of identifiable bands in a DGGE sample for structure. Diversity is calculated
425 using the Shannon index. For DGGE analysis triplicated samples were pooled and run as a single sample.

426

427 Silver nanoparticle toxicity effects have been recorded for microbial communities at
428 concentrations lower than that used here (100 µg/L). For example, Kroll et. al (2016) found
429 significant effects to stream microbial communities (periphyton) after exposure to 20 µg/L PVP
430 coated AgNPs or ionic silver. Specifically they found a decrease in diversity and population density.
431 Additionally, when examining the biofilm (extracellular polymeric substances contained in the
432 biofilm) the ratio of polysaccharides to proteins changed (assumed from C/N ratio), and the
433 biofilm mass was reduced by about 50%. Although the results found here do not report any
434 significant effects, they are consistent with other studies looking at microbial communities and

435 biofilms associated with water treatment (as opposed to environmental microbial communities
436 such as periphyton). Zhang et al. (2014) investigated the exposure of membrane bioreactor
437 biofilm, treating synthetic wastewater, to 60 days of AgNPs at a concentration of 100 µg/L. They
438 found no significant impact to the microbial communities or water treatment performance as the
439 AgNPs (or ionic silver) were adsorbed or precipitated quickly in the system. Zhang et al. (2014)
440 also found a developed silver resistance in the biofilms, as measured through the abundance of
441 the silE gene (silver resistance gene).
442
443 Some of the resilience of biofilms can be attributed to the ability for the extracellular polymeric
444 substance (EPS) matrix to itself act as a barrier, limiting the contact of silver (ionic or particle form)
445 with the microbial communities themselves. Joshi et al. (2012) showed the production of EPS by
446 engineered *E. Coli* provided protection from AgNPs most likely due to an enhanced aggregation
447 effect in the EPS matrix. Joshi et al. (2012) also showed the addition of an artificial EPS analogue
448 (xanthan) to provide a similar protection. Kang et al. (2014) also showed that the EPS produced by
449 *E.Coli* in suspension hindered the intracellular penetration of ionic silver, and that during the
450 penetration of ionic silver through the biofilm matrix ionic silver was reduced into nanoparticles
451 (10-30 nm in diameter) and immobilized in the EPS matrix before they could reach the cell. It is
452 likely that the general resilience of the CW biofilm microbial communities recorded here is in part
453 due to the EPS matrix acting as a barrier. With respect to the interstitial communities it is possible
454 that the interstitial microbial communities were also able to produce an EPS exudate, as shown
455 with suspended *E. Coli* by Kang et al. (2014), however the lack of significant effects are also likely
456 due to the rapid removal of the silver from the water column.
457
458 It is also noted that there is significant potential for the ongoing accumulation of Ag within the
459 aquatic sediment and, although not evaluated here, could lead to eventual re-release of Ag in
460 significant concentrations to the interstitial water and biofilm. Two recent studies more
461 representative of a sudden mobilisation event of AgNPs from aquatic sediment investigated the
462 impacts of elevated levels (2 mg/l) of Ag and found negative, cascading, ecosystem level impacts
463 to water chemistry and plants in natural wetland mesocosms (Colman *et al.* 2014) and significantly
464 increased mortality in *Fundulus heteroclitus* larvae and embryo in microcosms (Bone *et al.* 2015).
465 However, in the latter study, Bone et al. (2015) found that the results of microcosm exposures

were not replicable at the meso-scale and attributed the disparity in part to decreased levels of UV light in laboratory microcosm experiment.

Principal component analysis (PCA) was used to further investigate impacts to the microbial community function and structure after 28 days of exposure. Analysis of the carbon source utilisation patterns (CSUPs) and DGGE banding patterns revealed that the functional potential of the biofilm microbial community differed from that of the interstitial water, but within the biofilm there were no differences observed between treatments and the control (Figure 5 A). In the case of interstitial water samples, the microbial community treated with ionic Ag had developed differently from the other treatments and the control. According to the PCA analysis using DGGE data, the interstitial water and biofilm samples are populated by different microbial communities and again less variation was observed in the biofilm communities, with the ionic Ag exposed interstitial waters developing differently than the other interstitial communities (Figure 5 B) suggesting a slight impact for this type of Ag exposure when compared to AgNPs. Although no statistical analysis could be completed for the structural data this small subtle effect is discussed here as it is not surprising for ionic silver to have an effect of some magnitude.

Differing toxicities for ionic Ag and AgNPs have been described but the reported data are not consistent. Kwok et al. (2012) demonstrated that both citrate and PVP AgNPs were less toxic to early life stage *O. latipes* than gum arabic (GA) AgNPs, but also noted that all NPs were three to ten times less toxic than ionic Ag ($AgNO_3$). Conversely Bone *et al.* (2015) found that AgNPs were more toxic than ionic Ag to *F. heteroclitus* embryos in mesocosm exposures. The microcosm microbial community impacts noted here, however, are more pertinent to subtle changes in function and structure rather than blatantly negative effects. Microbial community analysis as employed can be advantageous when trying to elucidate more subtle shifts in ecosystem function for which the potential ramifications are less clear. Microbial community function is known to vary depending on location within a constructed wetland. Interstitial water communities typically demonstrate a lower functional richness and different physiological profile compared to gravel or rhizospheric associated microbial communities (Weber and Legge 2013). The combined observations in this study of lower functional richness in interstitial water communities compared to the biofilm (Figure 4A and C) and different carbon source utilisation patterns for each community (Figure 5A) conform to this existing understanding of microbial functionality in

constructed wetlands. It is interesting then to observe the opposite trend for community structure as determined by DGGE (Figure 4 B and D) with higher structural richness and diversity in the interstitial water compared to the biofilm. Biofilm community structural profiles were also distinct from interstitial water communities based on the occurrence and relative intensity of DGGE bands (Figure 5B). A possible explanation for this is that the biofilm develops competitively to maximise function and may therefore be composed of a smaller number of species that are better suited to the specific experimental conditions, such as exposure to Ag, leading to higher function but with reduced species richness. With this respect a more in depth analysis of the biofilm communities was completed with a specific goal to investigate a potential developed resistance to silver.

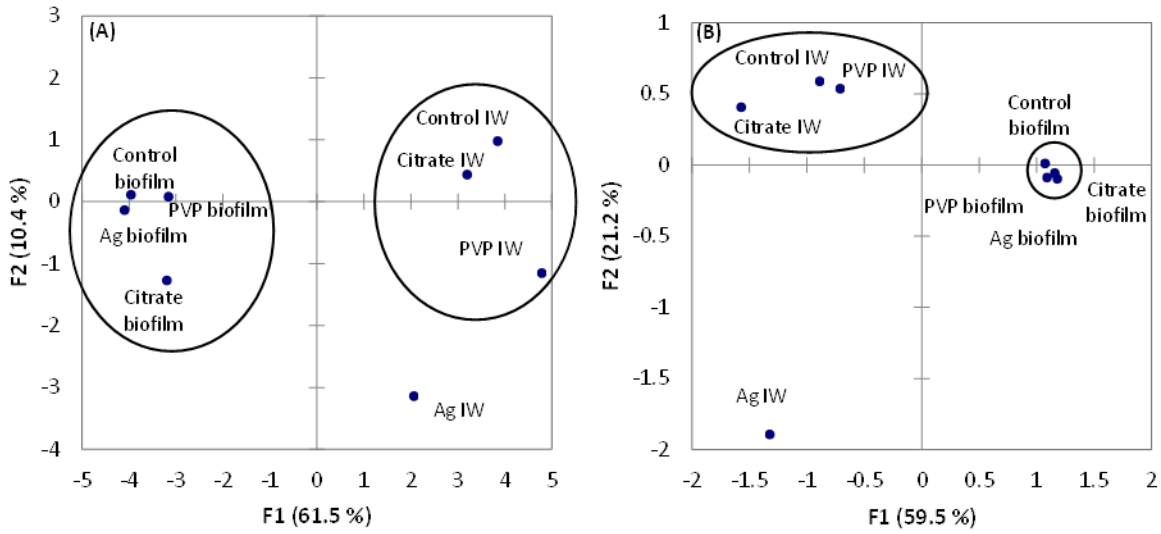
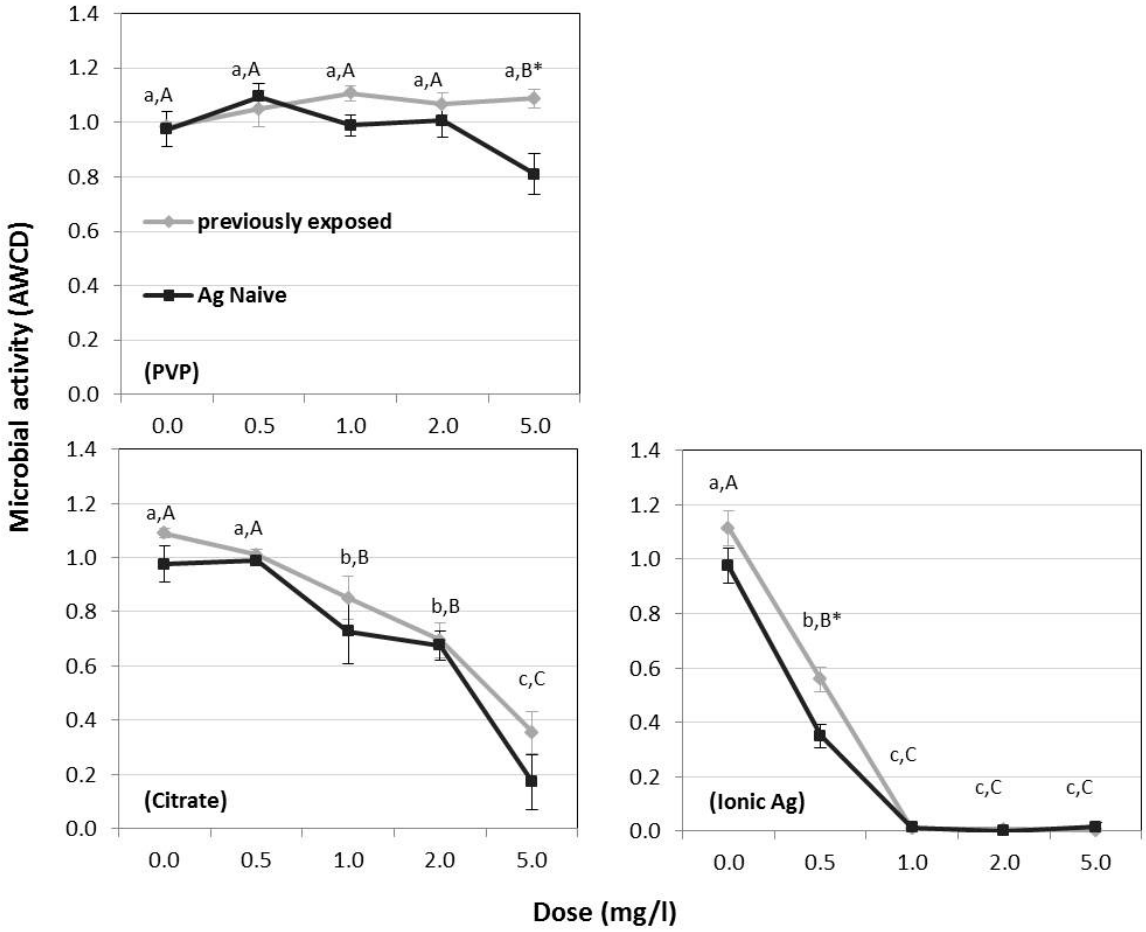


Figure 5: Principal component analysis of A) community function based on CSUPs of the Biolog® Ecoplates and B) community structure based on the DGGE banding patterns after 28 days exposure in laboratory microcosms. IW = interstitial water, Ag = ionic Ag, citrate = citrate coated AgNPs, PVP = polyvinylpyrrolidone coated AgNPs.

3.4 Microbial community Ag resistance

The effect of AgNPs and ionic Ag on the development of community resistance was assessed *ex-situ* via the quantification of catabolic capabilities after 28 days exposure to Ag (previously exposed), and a control group with no exposure (denoted as Ag naïve). A significant ($p<0.05$) decrease in the catabolic capability with increasing dose was observed for all Ag types except for

PVP AgNP exposed communities with prior exposure (Figure 6). The minimum community level catabolic effect concentration (MCLCEC), as described by Weber *et al.* (2014) varied between treatments. In most cases the dose response trend for previously exposed and naïve communities was similar with the exception of one instance. The MCLCEC of 1 mg/l for citrate NPs was higher than for ionic Ag (0.5 mg/l). For the communities treated with PVP AgNPs, an MCLCEC was only observed in the Ag naïve community at a concentration of 5 mg/l. The exposure to higher concentrations of ionic Ag ceased the catabolic activity of the microbial community completely (at 1mg/L), which was not observed for PVP and citrate NPs. Previous exposure to NPs or ionic Ag appeared in some cases to increase resistance to Ag toxicity with significant differences ($p<0.05$) in effect between the previously exposed and naïve communities at 5 mg/l for PVP AgNPs and 0.5 mg/l for ionic Ag. These results are consistent when analyzing raw data (as shown) or normalizing to the original no-dose response. p-values for comparisons between naïve and previously exposed communities for ionic silver at 0.5 mg/l are 0.005 and 0.015 for non-normalized and normalized data respectively. p-values for comparisons between naïve and previously exposed communities for PVP AgNPs at 5 mg/l are 0.011 and 0.013 for non-normalized and normalized data respectively. Data has been displayed here as non-normalized to better assist in future study comparisons between different authors. These concentrations are higher than any PECs for surface waters or sediments (Gottschalk *et al.* 2013), however are within the realm of possibility for CWs. These results highlight the possibility for variability in the development of Ag resistance depending on the mode of exposure. Current understanding of microbial resistance to Ag is based mainly on clinical studies with exposure to ionic Ag (Silver *et al.* 2006). A better understanding of Ag resistance in the environment will be possible when the mode of toxicity is clearly understood, which is not yet the case (Choi *et al.* 2010). It has been suggested that the mode of toxicity for AgNPs is similar to that of Ag ions (Sintubin *et al.* 2011) acting through the inhibition of essential enzyme activity. The *ex-situ* dose response experiments performed in this study (Figure 6) showed significant differences in the toxicity trends and resistance development for each type of AgNP and ionic Ag.



548

549 **Figure 6** - Dose-response curves for the effect of different types of AgNPs on biofilm microbial
550 communities. Exposures were carried out ex-situ using Biolog® Ecoplates. Previously exposed refers to
551 communities having undergone 28 days exposure to 100 µg/l Ag. Naïve refers to the control communities
552 maintained under identical conditions but without previous exposure to Ag. Error bars are 1 standard
553 deviation (n = 3). Lower case letters (a,b,c) = previously exposed, upper case (A,B,C) = naïve. Significant
554 difference ($p<0.05$) between treatments indicated by different letters above the data point. *= significant
555 difference ($p<0.05$) between previously exposed and naïve groups.

556

557 The mechanisms by which biofilm-bound microbial communities resistance to Ag toxicity may
558 increase over time conceivably involves a combination of increasing numbers of persistent cells
559 (Keren *et al.* 2004), gene expression responses (Lenz *et al.* 2008), efflux systems (Silver and Phung
560 1996) or horizontal gene transfer (Gunawan *et al.* 2013). Although not yet mentioned in the
561 literature for CW microbial communities, one of the simplest and more easily understood
562 mechanisms of resistance may be that biofilm dwelling microbes are inherently provided physical
563 protection by extracellular polymeric substances as shown in conventional wastewater treatment

systems (Sheng and Liu 2011). Resistance may therefore be due at least in part to a microbial communities ability to build and maintain viable biofilms. Many factors will influence this process including development time prior to exposure and environmental factors such as shear stress, oxygenation and nutrient availability. Along the lines of Blanck et al. (1988), this study shows there may be some potential for creating pollution induced tolerance in CW microbial communities. The period of exposure in this experiment was relatively short and therefore, longer-term studies to elucidate the potential for the development of Ag resistance and its physiological basis in wetland biofilms would be useful. Additional experiments using sulfidised AgNPs would also be informative. As observed by Lowry et al. (2012) AgNP transformation can occur fairly rapidly. Better understanding sulfidisation kinetics in the relatively short distribution systems preceding CWs would also be helpful to fully understand what particle types require priority investigation moving forward.

4.0 Conclusions

This is the first study to look at the effects of different AgNPs on both the function and structure of microbial communities in constructed wetlands. Low doses of Ag did not exert significant toxic effects in the short term whether ionic or in nanoparticle form but did lead to subtle changes in both functional and structural microbial community profiles. Higher doses of AgNPs (>500 µg/l) significantly reduced microbial community function in *ex-situ* tests in the case of citrate AgNPs and ionic Ag. PVP coated AgNPs were shown to have limited toxicity in this study. Some evidence of the development of resistance to toxicity was observed in previously exposed microbial communities for all types of Ag (particulate and ionic). Longer term and larger scale studies to elucidate the potential effects of Ag accumulation in constructed wetlands are needed to answer important remaining questions on the potential environmental impacts of continued accumulation and eventual release of Ag over time.

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