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Pseudomonas putida biofilm dynamics following a single pulse of silver nanoparticles

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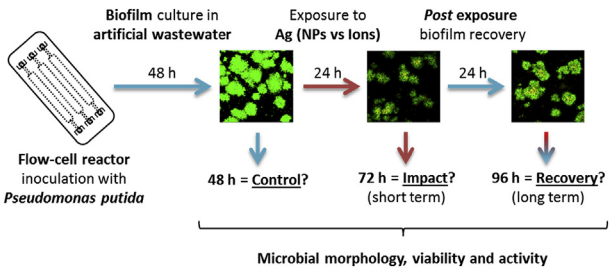
HIGHLIGHTS

- Mature *Pseudomonas putida* mono-species biofilms were cultured under hydrodynamic conditions.
- Short and long term impacts of Ag NPs on biofilm morphology, viability and activity were studied.
- Dose dependent toxicity patterns were reported *post* exposure to 0.01 −100 mg L^{-1}.
- Recovering biofilms were characterised 24 h *post* exposure.
- Dynamic biofilm based models offer enhanced nanosafety assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudomonas putida mono-species biofilms were exposed to silver nanoparticles (Ag NPs) in artificial wastewater (AW) under hydrodynamic conditions. Specifically, 48 h old biofilms received a single pulse of Ag NPs at 0, 0.01, 0.1, 1, 10 and 100 mg L^{-1} for 24 h in confocal laser scanning microscopy (CLSM) compatible flow-cells. The biofilm dynamics (in terms of morphology, viability and activity) were characterised at 48, 72 and 96 h. Consistent patterns were found across flow-cells and experiments at 48 h. Dose dependent impacts of NPs were then shown at 72 h on biofilm morphology (*e.g.* biomass, surface area and roughness) from 0.01 mg L^{-1}. The microbial viability was not altered below 10 mg L^{-1} Ag NPs. The activity (based on the D-glucose utilisation) was impacted by concentrations of Ag NPs equal and superior to 10 mg L^{-1}. Partial recovery of morphology, viability and activity were finally observed at 96 h. Comparatively, exposure to Ag salt resulted in *ca.* one order of magnitude higher toxicity when compared to Ag NPs. Consequently, the use of a continuous culture system and incorporation of a recovery stage extends the value of biofilm assays beyond the standard acute toxicity assessment.

1. Introduction

Current interest in engineered nanoparticles (NPs) is clear given their various attractive physico-chemical properties (Ju-Nam and Lead, 2008; Rai et al., 2014). There are nevertheless legitimate concerns regarding the actual risk associated with the emergence of anthropogenic NPs in the environment (Duester et al., 2014; Eduok et al., 2013). Reported environmental concentrations of the majority of NPs in freshwater systems are in the μg L^{-1} range and likely to increase due to the wide applications of NPs in societal and medical products (Gottschalk et al., 2013; Ju-Nam and Lead, 2008; Rai et al., 2014). Consequently, the potential adverse effects of NPs

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on microorganisms in environmental systems (natural and otherwise) need to be appraised.

Bacteria have already been used intensively in nano(eco)toxicology, especially as planktonic cultures (Holden et al., 2014; Kahru and Ivask, 2013). Biofilms, defined as self-produced matrix enclosed mono or multi-species microbial communities that adhere to biological or non-biological surfaces or interfaces (Stewart and Franklin, 2008), are nonetheless referred as the main living form of bacteria in the environment (Hall-Stoodley et al., 2004). Structurally organized, dynamic and complex ubiquitous biological systems, biofilms have in addition essential beneficial implications (*e.g.* facilitators within the natural environment or in the treatment of wastewaters) (Hall-Stoodley et al., 2004; Stewart and Franklin, 2008). Consequently, biofilm based assays represent a desirable source of information in nano(eco)toxicology.

Despite their relevance, only a handful of nano(eco)toxicological studies has been carried out using biofilms to date. Assays performed under static conditions (*i.e.* here referred to as static biofilms) using microtitre plates or glass slides, coupled with spectrophotometry or confocal laser scanning microscopy (CLSM), have been reported (Choi et al., 2010; Dong and Yang, 2014; Dror-Ehre et al., 2010; Inbakandan et al., 2013; Martinez-Gutierrez et al., 2013; Radzig et al., 2013; Raftery et al., 2014). However, biofilms obtained under hydrodynamic conditions (*i.e.* here referred to as non-static biofilms) are fully hydrated, planktonic free and mature structures compared to the static biofilms (Buckingham-Meyer et al., 2007; Crusz et al., 2012; Weiss Nielsen et al., 2011). Studies based on non-static biofilms are therefore gradually emerging using diverse rotating biological contactor and reactors (Fabrega et al., 2009; Hou et al., 2014; Martinez-Gutierrez et al., 2013; Park et al., 2013). Unlike most reactors, the flow-cell systems present the additional advantages of real time, non-invasive and non-destructive versatile studies (Crusz et al., 2012; Weiss Nielsen et al., 2011). Consequently, a high potential of assay development is associated with the use of flow-cell reactors.

Applications of flow-cell reactors were reported in (eco)toxicology for the testing of silver sulphadiazine and solvent styrene on *Pseudomonas* spp. biofilms (Bjarnsholt et al., 2007; Halan et al., 2011). Examples in nano(eco)toxicology are currently particularly scarce with the sole contribution being the study by Fabrega et al. (2009) where the interactions between Ag NPs and *Pseudomonas putida* biofilms were investigated (*e.g.* accumulation and uptake of NPs). These authors especially stressed the need of complementary studies dedicated to the assessment of long term effects (*i.e.* including recovery) of NPs on complex systems such as biofilms. This was further emphasised in recent literature (Handy et al., 2012) as an area not being considered in most of the nano(eco) toxicological studies published so far.

The present study builds on these pioneer examples (Bjarnsholt et al., 2007; Fabrega et al., 2009; Halan et al., 2011) and aims to assess the temporal impact following a single pulse of NPs on non-static mono-species biofilm morphology, viability and activity using flow-cell reactors. Silver (Ag) is prioritised given that it is a well-known bactericidal agent and one of the most widely used NPs in a large range of applications (Morones et al., 2005; Rai et al., 2014). *P. putida* based biofilms are considered since they are used with flow-cell reactors and are commonly proposed as an environmental bacterial model (Bjarnsholt et al., 2007; Fabrega et al., 2009; Halan et al., 2011). Consequently, the dynamics (considering the impact in the short term as well as the potential recovery in the long term) of mature *P. putida* biofilms (considering morphology, viability and activity) in response to a single pulse of Ag NPs and salts are here reported and discussed.

2. Material and methods

2.1. Material

The biofilm reactor consisted of inverted Perspex flow-cells (CLSM compatible) and bubble traps purchased from DTU Systems Biology (Lyngby, Denmark) used in combination with 24 × 50 mm glass coverslips (1.5 mm thick) from SLS UK Ltd and silicone (Versilic) and Marprene (Watson Marlow UK Ltd) tubings as reported previously (Crusz et al., 2012; Weiss Nielsen et al., 2011). Representative Ag NPs (*i.e.* JRCNM03000a also named Ag NM-300K NPs, which are negatively charged nanoparticles with a primary size *ca.* 15 nm delivered in suspension at 10% (w/v) in 4% (v/v) each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat) were obtained from the European Commission's Joint Research Centre (Ispra, Italy) and characterised previously (Klein et al., 2011; Mallevre et al., 2014). The Filmtracer Live/Dead® Biofilm Viability Kit was purchased from Life Technologies UK Ltd. D-glucose, silver nitrate ($AgNO_3$), phenol and sulphuric acid were from Fisher Scientific UK Ltd. Rely^{+}On Virkon® disinfectant was from DuPont. A silver single element standard was purchased from Perkin Elmer UK Inc.

2.2. Methods

2.2.1. Culture of *P. putida* mono-species biofilms in a flow-cell reactor

Biofilms were cultured under hydrodynamic (laminar) conditions using parallelised flow-cells as schematised in Fig. 1. Wastewater isolated *Pseudomonas putida* BS566::*luxCDABE* (hereafter referred to as *P. putida BS566*) (Wiles et al., 2003) was used as a model bacterium for establishing mono-species biofilms. All experiments were performed in artificial wastewater (AW), the composition of which is reported elsewhere (Mallevre et al., 2014) using D-glucose at 0.5% (w/v) as sole carbon source.

The set up reactor was cleaned with Virkon® 1% (w/v) then extensively washed with sterile deionised water at 15 mL channel^{-1} h^{-1} using a 205U multi-channel cassette pump (Watson Marlow UK Ltd). The channels were then filled with sterile AW and left at minimal flow rate overnight. Prior to the inoculation, the bacterium was pre-cultured overnight at 28 ± 2 °C under shaking conditions (140 rpm) in AW then diluted in order to reach a final concentration *ca.* 10^7 CFU mL^{-1} (corresponding to a dilution about 1:100). Each channel was then independently inoculated with 200 µL of freshly prepared cell suspension by: clamping off the tubing upstream of each flow-cell (Fig. 1, position A), disconnecting the tubing downstream of the flow-cells (Fig. 1, position B) and injecting the bacterial suspension within the channels (Fig. 1, position C). After inoculation, the tubings were re-connected and unclamped; the flow-cells were then incubated 1 h (*i.e.* flow off, glass coverslip on bottom). The biofilms were cultured (*i.e.* glass coverslip on top) for 48 h in AW with a consistent flow rate of 3 mL channel^{-1} h^{-1}.

2.2.2. Experimental scenario of culture, exposure and recovery

Stock suspensions of Ag NPs at 100 mg L^{-1} were freshly prepared in AW prior to each experiment, sonicated (2 × 8 min in a Kerry ultrasonic water bath at 38 ± 10 KHz), then serially diluted to give final concentrations of 0, 0.01, 0.1, 1, 10 and 100 mg L^{-1} applied for 24 h at 3 mL channel^{-1} h^{-1} on 48 h old biofilms. Ag ions (applied as $AgNO_3$) were similarly tested at final concentrations of 0, 0.001, 0.01, 0.1, 1 and 10 mg L^{-1}. Virkon® 1% (w/v) was tested as a toxicant positive control. After exposure, upstream tubings were purged and the system filled with fresh AW (*i.e.* free from any toxicant) for an additional 24 h of culture at 3 mL channel^{-1} h^{-1}. Three time points