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**IMPACTS OF NANOMATERIALS ON SOCIAL
BEHAVIOUR OF MICROORGANISMS**

ANEE MOHANTY

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Impacts of Nanomaterials on Social Behaviour of Microorganisms

Anee Mohanty

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ABSTRACT

Increasing use of nanomaterials (NMs) in a wide range of consumer products has raised concerns about their impacts on natural and engineered ecosystems. NMs released into the ecosystems will potentially influence organisms at all levels of the food chain. Microorganisms are at a low trophic level and play critical roles in maintaining the health of ecosystems. Hence, the impacts of NMs on microorganisms have attracted great interests. However, majority of the toxicological studies of NMs have focused on their bactericidal (killing) and bacteriostatic (growth-inhibiting) effects. Although the concentration of NMs in the environments is expected to be much lower than those used in most nanotoxicity studies, little is known about the influences of NMs at sublethal concentrations on critical functions of microorganisms. The objective of this study was to elucidate the impact of NMs on microbial functions. Specifically, the focus was on the influence of NMs at sublethal levels on bacterial social behaviours including production of communal resources, intercellular communication, and collective antibiotic tolerance.

Cells of *Pseudomonas* species often produce siderophores, a group of organic compounds that facilitate iron uptake for the producers and their neighboring non-producers in microbial communities. Pyoverdine (PVD) is one of the most important siderophores produced by *Pseudomonas aeruginosa* and it is an important communal resource in environmental microbial communities and also a key virulence factor in establishing infections in medical settings. Tellurium nanorods (TeNRs) produced using a metal-reducing bacterium *Shewanella oneidensis* and physicochemically synthesized single-wall carbon nanotubes (SWCNTs) significantly inhibited PVD production in *P. aeruginosa*. Quantitative polymerase chain reaction (qPCR) and RNA-sequencing based transcriptomics analyses revealed that the inhibition was at transcriptional level.

Quorum sensing (QS) is one of the most important signalling systems that bacteria use to coordinate cooperative behaviours. QS signalling has been shown to play a critical role in various natural and engineered bioprocesses. The acylated homoserine lactones (AHLs) is one of the predominant QS signals used by most Gram-negative bacteria. Using *Pseudomonas syringae* and *Pantoea stewartii* as model organisms, the key AHLs

produced by these two organisms were identified and quantified the influences of silver nanoparticles (AgNPs) and single-wall carbon nanotubes (SWCNTs) on the production of different AHLs. *P. syringae* mainly produced 6 short-chain AHLs, while *P. stewartii* produced 11 short- and long-chain AHLs. The presence of AgNPs in *P. syringae* cultures resulted in overall reduction of AHLs. Intriguingly, the production of most AHLs in *P. stewartii* was not significantly affected by either 0.5 µg/ml AgNPs or 20 µg/ml SWCNTs. This study reports the differential effects exhibited by NMs on the production of different signals.

The impact of AgNPs on collective antibiotic tolerance of a sludge wastewater community in terms of live/active community composition was also evaluated. There was no significant change in the community structure in response to the nanomaterial treatment. Moreover, treatment with tetracycline followed by AgNPs treatment did not result in significant change in the antibiotic tolerance profile under the testing conditions.

Taken together, these findings provide a novel insight into environmental impacts of NMs. This study will aid in comprehensive understanding the effect of NMs on various aspects of cell-cell interactions in microbial communities that often play critical roles in maintaining the health of ecosystems in various natural and engineered ecosystems.

LIST OF PUBLICATIONS

JOURNAL PAPERS

Mohanty A, Tan CH, Cao B* (2016) Impacts of nanomaterials on bacterial quorum sensing: Differential effects on different signals. Environmental Science: Nano 3:351-356.

Mohanty A, Wei L, Lu L, Chen Y, Cao B* (2015) Impact of sublethal levels of single-wall carbon nanotubes on pyoverdine production in *Pseudomonas aeruginosa* and its environmental implications. Environmental Science & Technology Letters 2(4): 105-111.

Mohanty A, Liu Y, Yang L, Cao B* (2015) Extracellular biogenic nanomaterials inhibit pyoverdine production in *Pseudomonas aeruginosa*: A novel insight into impacts of metal(loid)s on environmental bacteria. Applied Microbiology and Biotechnology 99(4): 1957- 1966.

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Wu Y, Arumugam K, Tay M, Seshan H, **Mohanty A**, Cao B* (2015) Comparative genome analysis reveals genetic adaptation to versatile environmental conditions and importance of biofilm lifestyle in *Comamonas testosteroni*. Applied Microbiology and Biotechnology 99(8): 3519-3532.

BOOK CHAPTER

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CONFERENCE PRESENTATIONS

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LIST OF ABBREVIATIONS

× g	g-force in centrifuge
μg	Microgram
μL	Microlitre
μM	Micromolar concentration
AgNPs	Silver nanoparticles
AHL	Acyl homoserine lactone
ATCC	American type culture collection
bp	Base-pair
c-di-GMP	Cyclic di-guanylate monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CNTs	Carbon nanotubes
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
ENMs	Engineered nanomaterials
EPS	Extracellular polymeric substances
g	Gram
GFP	Green fluorescent protein
h	Time in hour
ICP-OES	Inductively coupled Plasma Optical Emission Spectroscopy
L	Litre
LB	Luria Bertani medium

M9	M9 minimal media
mg	Milligram
min	Time in minute
mL	Millilitre
mM	Millimolar concentration
MWCNTS	Multi-wall carbon nanotubes
NCBI	National Center for Biotechnology Information
nm	Nanometre
NMs	Nanomaterials
°C	Degree Celcius
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PVD	Pyoverdine
q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic Acids
RPKM	Reads Per Kilobase of transcript per Million mapped reads
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acids
SWCNTs	Single-wall carbon nanotubes

CHAPTER 1. Introduction

1.1 Research background

Nanomaterials (NMs) have a wide range of applications in biology, medicine, agriculture, electronics, environmental monitoring, and consumer goods (Murty, Shankar et al. 2013). The increasing use of products containing NMs over the past decade has posed a great concern of their negative impacts to the environment. NMs can enter the environment through atmospheric emission or as solid or liquid waste from the production facilities, contaminating soils, surface water, and groundwater. Considering the potential release of NMs into the environment, assessing their risks to the health of the ecosystem has been the topic of interest in environmental science and engineering.

The NMs in the environment will potentially impact organisms at all levels of the food chain. Over the past decade, *in vivo* and *in vitro* nanotoxicity studies have been conducted to evaluate the toxicity of various NMs to various living organisms. The impacts of NMs exerted on bacteria are of particular interest because being at the low trophic level they will affect NMs fate, including transfer to higher trophic levels through biomagnification. Furthermore, bacteria play critical roles like nutrient recycling in natural and engineered ecosystems (Peralta-Videa, Zhao et al. 2011). There are plethora of evidences showing many NMs exhibit bactericidal (killing) and bacteriostatic (growth-inhibiting) effects through damaging cell membrane, denaturing proteins and nucleic acids, and generating oxidative stress (Klaine, Alvarez et al. 2008, Neal 2008).

1.2 Research gaps

The toxicity of NMs in terms of bactericidal or bacteriostatic effect has mostly been concentration dependent (Rai, Yadav et al. 2009, Liu, Zeng et al. 2011). However, the discharge concentrations of NMs in the environments is expected to be much lower than those used in majority of nanotoxicity studies and environmentally relevant concentration is expected to be mostly sublethal (see Section 2.2). How NMs at sublethal concentrations affect environmental bacteria in terms of functions is largely unexplored, which has been

recently recognized as a critical gap toward a comprehensive understanding of the impacts of NMs on ecosystems. In various natural and engineered environments, microorganisms are often present as polymicrobial communities in which cells actively interact with each other (Keller and Surette 2006). Microorganisms in communities are functionally linked to each other and any changes in functions of specific bacteria may cause re-structuring of the whole microbial communities and affect the ecosystems (see Section 2.5). One of the most important aspects influencing the function of an ecosystem is the interaction between individual cells, *i.e.*, bacterial social behaviour (Crespi 2001). These social behaviours are partly governed by production of communal resources such as siderophores, quorum sensing signals and other secondary metabolites which are key to cell-cell interaction in a microbial community (West, Griffin et al. 2006, Hibbing, Fuqua et al. 2009) (see Section 2.11). Therefore it is quite plausible to assume that any alteration in the production of these communal resources can possibly alter the structure and function of the whole community in turn affecting the key ecological functions performed by these microbial communities. At sublethal concentrations, NMs may affect the community profile by affecting the production of communal resources and cell-cell interaction. Microbial communities in natural and engineered ecosystems are the drivers of key processes *e.g.*, bioremediation, biogeochemical cycling and waste water treatment (Wagner and Loy 2002, Narihiro and Sekiguchi 2007, Van Der Heijden, Bardgett et al. 2008, Ahmed, Cao et al. 2012). Hence, it becomes very important to study the sublethal effects of different NMs on social behaviour of microorganisms.

1.3 Objectives

This study aims at exploring the sublethal impacts of NMs on the social behaviour of environmental bacteria. The specific tasks include:

(i) Investigate the impacts of NMs on the production of communal resources, *i.e.*, microbial product that is beneficial to the community, by environmental bacteria. Specifically, the impacts of NMs, *i.e.*, Tellurium nanorods (TeNRs) and single-wall carbon nanotubes (SWCNTs), on the production of siderophore by *Pseudomonas aeruginosa* will be explored.

(ii) Explore the impacts of NMs on cell-cell communication through quorum sensing signals. Specifically, the effect of two types of NMs *i.e.*, Silver nanoparticles (AgNPs) and SWCNTs, on the production of quorum sensing signals in two model gram negative bacteria *Pseudomonas syringae* and *Pantoea stewartii* will be tested.

(iii) Determine the effect of NMs on overall structure of microbial community and to study the impact on antibiotic tolerance. AgNPs will be used as model NM and its effect on the antibiotic tolerance of sludge microbial community will be assessed using transcriptomics approach.

1.4 Thesis synopses

Chapter 1 provides a brief background about NMs and their potential toxicity on microorganisms. This chapter emphasizes the need for more comprehensive and in depth understanding of sublethal impact of NMs on social behaviour of bacteria. The objective and scope of the work is described in detail.

Chapter 2 has detailed literature review for the whole thesis. This chapter summarizes and discusses recent research work on the impacts of NMs on microbial communities' structure and function in natural and engineered ecosystems, with an emphasis on their influences on the social behaviour of microorganisms at sublethal concentrations.

Chapter 3 presents the effect of biogenic tellurium nanorods on siderophore production in a model environmental bacteria *P. aeruginosa* both in planktonic as well as biofilm mode of life.

Chapter 4 presents the effect of engineered single-wall carbon nanotubes (SWCNTs) on pyoverdine production in a model environmental bacteria *P. aeruginosa* both in planktonic as well as biofilm mode of life.

Chapter 5 reports the impact of SWCNTs and silver nanoparticles (AgNPs) on quorum sensing signal production in model Gram-negative bacteria *P. syringae* and *P. stewartii*.

Chapter 6 illustrates the impact of AgNP on wastewater treatment community structure and the effect of nanomaterial treatment on the collective antibiotic tolerance of the community.

Chapter 7 provides a summary of the thesis work and proposes some future recommendations

CHAPTER 2. Literature Review

A portion of this chapter has been published as Mohanty A, Wu Y, Cao B (2014) Impacts of engineered nanomaterials on microbial community structure and function in natural and engineered ecosystems. Applied Microbiology and Biotechnology 98: 8457-8468. Permission has been granted by the licensed content publisher "Springer Science" to use the published content as a chapter in this thesis.*

2.1 Nanomaterials (NMs) and applications

Nanomaterials (NMs) are man-made materials with at least one dimension of nanometer size (less than 100 nm). The physiochemical properties of NMs differ markedly from their larger counterparts because of a higher surface to volume ratio, which results in a higher reactivity and unique physicochemical properties (Roduner 2006). The physical, chemical, and mechanical properties manifested by NMs have been exploited for various applications in different industries including electronics, healthcare, water purification, and food packaging (Murty, Shankar et al. 2013). According to a recent survey there are over 1000 NM-enabled consumer products produced by 500 companies ranging over 20 different countries (Rejeski. 2009). The most commonly used NMs in consumer products in the order of their occurrences are silver which is present in highest number of product (259 products) second is carbon based nanomaterials (82), followed by zinc (including zinc oxide) (30), silica (35), titanium (including titanium dioxide) (50), and gold (27) (Weinberg, Galyean et al. 2011). Apart from these commonly used NMs there are many others metals, metal oxide, carbon nanostructures that are present in different consumer products.

Elaborating on the application in different fields, electronics is arguably the field where NMs have brought revolution in miniaturization and increased efficiency of nano electronics system. NMs are far more superior in respect to their optical properties, higher quantum efficiency and more stable to photobleaching. Moreover their optical properties are highly dependent on size. Owing to all these properties an entire gamut of nanosensors are currently being used (Wang 2005). There have been wide ranging application in healthcare industry like diagnosis, therapy and prevention (Lanone and Boczkowski

2006). Because of their small size they can easily be taken up by the cells, but size is one of the many special features of nanoparticles. To list out few applications, they are used as bioconjugates for drug delivery, also used in biodetection of pathogens, fluorescent biological labels, useful in probing DNA and protein structures, used in MRI as contrast enhancers, in tissue engineering as scaffold material, nanomaterial coatings on implants to prevent microbial biofilm formation (Salata 2004, Lanone and Boczkowski 2006). NMs are also contributing in many ways to water purification and environment monitoring as nano-enhanced membranes are used for desalination and water purification (Li, Mahendra et al. 2008, Hoek and Ghosh 2009, Buonomenna 2013, Qu, Alvarez et al. 2013), currently nanomaterial based sensors are being used to monitor water quality against bacteria, heavy metals and toxins (Andreescu, Njagi et al. 2009). Other sectors where NMs find their application includes cosmetics and consumer goods, sunscreens are containing titanium dioxide or zinc oxide particles to prevent skin burn (www.nanotechproject.org/consumerproducts , Mu and Sprando 2010). In food and agriculture industry also NMs are used as nanocomposites for food packaging, antimicrobial nano-emulsions in the decontamination of food equipment, packaging or food processing, also as sensors to monitor pathogens (Weiss, Takhistov et al. 2006, Sozer and Kokini 2009, Duncan 2011), in agriculture they are also used in form of nano-porous zeolites for slow and efficient release of water and fertilisers for plants, NMs are also part of food nutrients and drugs for livestock (Srilatha 2011). From last few years NMs are increasingly being used in construction industry. The various usage includes mixing with reinforcement of concrete to increase the mechanical strength, to produce self-cleaning glass, impregnation of nano-ZnO, -Ag or -TiO₂ particles in wood can prevent their decay as these materials are known to have antibacterial properties (Larsen-Basse, Chong et al. 2006). NMs are also being incorporated into textile to make fabrics which can be stain free, water repellent or attractant and especially to add antibacterial property (Wong, Yuen et al. 2006). Other major application includes making fouling resistant marine paint, in energy sector to make solar cell and hydrogen storage devices.

2.2 NMs as emerging environmental contaminants

NMs can be released into the environment during different stages of the product life cycle, for example, manufacturing, waste disposal, and weathering of the product containing NMs. NMs can enter the environment through point sources such as manufacturing factories or landfills, and non-point sources including the attrition of NMs from products (Wiesner, Lowry et al. 2006) (Figure 2.1). For example, release of silver nanomaterials from washing of textiles (Lorenz, Windler et al. 2012), release of TiO₂ nanomaterials from weathering of paints (Kaegi, Sinnet et al. 2010), and release of NMs from products like spray, sunscreens or cosmetics often occur. Ultimately the NMs released from all the above sources will end up in the air, water and soil through different routes. The above mentioned sources are few direct sources of NMs contamination in environment. Moreover there are several indirect sources of NMs contamination as well. Anthropogenic activities such as mining, smelting, and industrial use have caused serious problems of metal and metal(loid) pollution in nearly every country in the world. The natural and engineered ecosystems are often direct or indirect recipients of toxic metal and metal(loid)s (*e.g.*, Cu, Zn, Cr, Te, Se, and As). A wide range of microorganisms in the ecosystems will potentially come in contact with these pollutants and are capable of transforming metals and metal(loid)s into relatively stable and less toxic NMs, which has been generally considered as one detoxification mechanism (Han and Gu 2010, Ng, Sivakumar et al. 2013). For example, uranyl (UO₂²⁺) can be reduced by certain bacteria to form extracellular uraninite (UO₂) nanoparticles (Suzuki, Kelly et al. 2002, Schofield, Veeramani et al. 2008, Cao, Ahmed et al. 2011). Although the bulk concentration of the biogenic NMs in the environment could be as low as ng per kg of water or sediment samples, local concentration of the biogenic NMs in microenvironments at the micron scale may be significantly higher because of the highly heterogeneous distribution of nanomaterials in biological matrices, particularly in biological systems with a high cell density such as microbial biofilms (Habimana, Steenkeste et al. 2011, Peulen and Wilkinson 2011). The potential influence of these *in situ* generated biogenic NMs in the environment to environmental organisms are largely unexplored.

During the transport of these NMs in the environment, their physicochemical properties might change drastically because of photochemical transformation, oxidation/reduction, adsorption, combustion, precipitation, aggregation, and biotransformation *etc.* (Nowack, Ranville et al. 2012).

Figure 2.1 Flow chart depicting the leaching of nanomaterials to the environment from different sources and their fate in the natural environment. Reprinted with permission from (Wiesner et al., 2006).

To date, there have been no accurate detection and quantification methods for NMs in environmental samples. However, there have been many attempts to model the behaviour and concentration of these NMs in environment. Various models have been proposed to assess the environmental fate of the NMs but all vary to a wide extent in the methodologies, assumption and criteria used (Gottschalk and Nowack 2011). These modeling studies to predict environmental concentration acts as a valuable substitute in absence of actual measurement techniques.

Over the past decade, the toxicity of NMs has been widely studied. Toxicity of NMs have been tested with diverse prokaryotic and eukaryotic cells including microorganisms (Suresh, Pelletier et al. 2013), plants (Lin and Xing 2007, Miralles, Church et al. 2012),

humans (Oberdörster, Maynard et al. 2005, Sharifi, Behzadi et al. 2012). NMs have also shown toxicity to aquatic organisms (Baun, Hartmann et al. 2008, Griffitt, Luo et al. 2008), crustaceans (Heinlaan, Ivask et al. 2008), nematodes (Ma, Bertsch et al. 2009). It is getting increasingly clear that NMs are toxic to different organisms in various levels of food chains. Thus for risk assessment and regulatory purposes, a comprehensive understanding of ecotoxicological effect of NMs is required at several levels *i.e.*, single organisms, communities and higher organisms (Kahru and Dubourguier 2010).

2.3 Toxicity to microorganisms

Microorganisms are ubiquitous in natural and engineered environments and they constitute about one third of earth's total biomass (Ash, Foley et al. 2008). Toxicity of NMs to microorganisms are of special interest as microorganisms play important roles in biogeochemical cycling and are critical in maintaining the health of the ecosystem (Arrigo 2005, Falkowski, Fenchel et al. 2008). Microbial communities are also key to bioprocesses in various engineering applications including wastewater treatment, food processing, and biofuel production (Madigan, Martinko et al. 1997, Antoni, Zverlov et al. 2007). Moreover, microorganisms are comparatively fast and inexpensive to grow which make them attractive model test organisms. Therefore, comprehensive understanding the effect on microorganisms is imperative for ecotoxicity studies of NMs.

In recent years the impacts of NMs on environmental microorganisms have been extensively studied, with an emphasis on antimicrobial activities of NMs at the single cell level, which have been summarized and discussed in several recent research reviews (Li, Mahendra et al. 2008, Hajipour, Fromm et al. 2012, Seil and Webster 2012, Ingle, Duran et al. 2014). Ecotoxicity studies on model microorganisms can focus on survival/viability, cell division, gene mutations and effect on biochemical pathways (*e.g.*, enzyme production). Microbial nanotoxicity studies have mostly focused on the biocidal and biostatic effect of various NMs on different model organisms. The most standard methods include minimal inhibitory concentration (MIC) calculation and enzyme assays.

2.4 Mechanisms of toxicity of NMs to microorganisms

A wide range of NMs exhibit antimicrobial activities (Stoimenov, Klinger et al. 2002, Lyon, Adams et al. 2006, Marambio-Jones and Hoek 2010, Dong, Tang et al. 2012). The small size and high surface area to volume ratio are the unique properties which allow them to closely interact with the microbial cell and the sub-cellular structures (Morones, Elechiguerra et al. 2005, Ng, Sivakumar et al. 2013). Hence, many of them are being widely used in coatings, membrane fabrications, and medical devices (Liu, Qi et al. 2013, Murty, Shankar et al. 2013). Although the exact mechanisms of the antimicrobial effects of NMs may vary for different NMs and different organisms, it has been generally recognized that NMs often have multidimensional effects which include damages caused by direct contact such as damaging the cell membrane and deactivating the membrane bound enzymes as well as indirect damages caused by reactive oxygen species (ROS) inside the cells (Figure 2.2) (Zhang, Jiang et al. 2007, Klaine, Alvarez et al. 2008, Li, Xie et al. 2010, Marambio-Jones and Hoek 2010).

Elaborating on the toxicity mechanism cell membrane is the most accessible site. It is also the site for important cellular functions like material transport, energy transduction and intracellular communication through surface receptors. NMs used in imaging applications target cell membranes for labelling (Dubertret, Skourides et al. 2002). Smaller NMs like quantum dots (less than 5 nm), silver nanoparticles less than 80 nm size are able to enter bacterial cells (Xu, Brownlow et al. 2004, Kloepper, Mielke et al. 2005). Though, larger particles cannot enter the cells some have shown to bind to cell surface and cause damage to the cell membrane *e.g.*, silicon nanoparticles and fullerene derivatives get embedded in the membrane (Jang, Pell et al. 2003) and carboxy fullerene has been shown to cause cell membrane rupturing leading to cell death (Tsao, Kanakamma et al. 1999).

Figure 2.2 Mechanisms of toxicity of NMs: (A) toxicity of metal and metal oxide NMs, (B) toxicity of carbon NMs (i) Physical damages to cell membrane through insertion, extraction and “nano-knives” (ii) ROS- mediated oxidative damages to redox chains in cell membrane and intracellular components such as proteins and DNA (iii) Wrapping of NMs onto bacterial cell surface blocks nutrient access. Although the cells in this illustration are Gram-negative bacteria, the toxicity mechanisms shown here are also applicable to Gram-positive bacteria. Panel (B) was reprinted with permission from (Hegab, ElMekawy et al. 2016).

Toxicity of NMs also results due to protein destabilization and oxidation. Nanoparticle-protein conjugates are now being developed for variety of applications, but it has been

shown that direct physical interaction with NMs can alter the structure and activity of the protein *e.g.*, gold nanoparticle or SWCNTs containing electrodes could partially denature the enzyme glucose oxidase (Liu, Paddon-Row et al. 2004). NMs can also damage nucleic acids, fullerenes have been shown to cause deformation of DNA strand (Takenaka, Yamashita et al. 1999, Zhao, Striolo et al. 2005) and quantum dots can cause breakage of supercoiled DNA strand (Green and Howman 2005).

A large number of research focusing on the mechanism of NM toxicity, have attributed the toxicity to generation of Reactive Oxygen Species (ROS). ROS can damage every component of cell including the membranes, proteins, nucleic acid. In membranes ROS can oxidize the double bonds on membrane phospholipids resulting in lipid peroxidation. This will be followed by increase in membrane fluidity and increased membrane permeability (Cabiscol, Tamarit et al. 2010). These ROS generated by NMs can also damage iron-sulphur clusters in the protein cofactors, formation of disulphide bonds between the sulphur containing amino acids. DNA damage due to ROS results due to strand breaks, cross-linking and adducts of nitrogen bases and sugars. Measurement of ROS species by different assay methods can sometime give false readings due to interference of NMs with the assay reagents (Wörle-Knirsch, Pulskamp et al. 2006, Lyon, Brunet et al. 2008).

NMs exert their toxic effect by interfering with the energy transduction and electron transfer chain. These processes are indirectly affected by the membrane damage occurring due to NMs. They are also affected directly when they come in contact with any redox active NMs which can withdraw electrons from the electron transport chain. Fullerenes have been shown to inhibit respiration in *E.coli* (Mashino, Usui et al. 2003). Cerium dioxide nanoparticles cause the oxidation of membrane components involved in electron transport chain (Thill, Zeyons et al. 2006).

Another phenomenon which is widely linked to the toxicity of NMs, is release of toxic materials like ions or heavy metals from them. Silver ions has been widely implicated in the toxicity mechanism of silver nanoparticles (Kittler, Greulich et al. 2010). These metals

or ions can accumulate inside the cells and exert toxic effects. Silver ions can interact with DNA, thiol groups of enzymes/proteins, thus inactivating them (Feng, Wu et al. 2000).

2.5 Impact of NMs on microbial community

To study the impact of NMs on microorganisms it is important to understand the microbial life style in natural environment. Over the past few decades, it has been increasingly clear that our understanding of microorganisms from laboratory-based pure culture studies is not true representation of their *in situ* lifestyle (Costerton, Cheng et al. 1987). Microorganisms in most natural, engineered, and medical settings are found to be growing as cooperative communities with complex physiology and behaviour (O'Toole, Kaplan et al. 2000, Hall-Stoodley, Costerton et al. 2004, Kjelleberg and Givskov 2007). Microorganisms often present in these microbial communities drive various biological processes such as biogeochemical cycling, nutrient removal in wastewater treatment, and anaerobic digestion (Wagner and Loy 2002, Narihito and Sekiguchi 2007, Van Der Heijden, Bardgett et al. 2008, Ahmed, Cao et al. 2012). Although antimicrobial activities of NMs have been extensively studied at the single cell and population level, elucidating the influence of NMs on microbial communities is a critical aspect in the comprehensive understanding of nanotoxicity. In the past several years, the influence of NMs on microbial communities in various natural and engineered ecosystems has been explored and some representative studies are summarized in Table 2.1. Recent studies on the influence of NMs on natural and engineered ecosystems focused on effects of NMs on structure and function of microbial communities in soil, river, marine and wastewater treatment facilities.

Table 2.1 Summary of recent research work (2008 onwards) on influence of NMs on microbial communities.

NMs (concentration, size, incubation period)	Microbial community	Approaches	Key findings	References
CARBON BASED NANOMATERIALS				
C ₆₀ (1 µg/g soil in aqueous suspension or 1000 µg/g soil in granular form; up to 180 days)	Soil microbial community	Phospholipid fatty acid (PLFA) analysis and community profiling (PCR-DGGE); soil enzyme activity assay (β -glucosidase, acidphosphatase, dehydrogenase, and urease)	No significant changes in microbial community structure. Organic matter and salts control availability of C ₆₀ in soil, which will ultimately control the exposure level and toxicity of C ₆₀ .	(Tong, Bischoff et al. 2007)
C ₆₀ (agglomerates of 50 nm to µm at 0, 5, 25, and 50 mg/kg dry soil; up to 14 days)	Soil microbial community	Substrate-induced respiration (SIR) assay; CFU count; PCR-DGGE	No effect on total respiration; 3-4 fold reduction of fast-growing bacteria immediately after addition of C ₆₀ . Protozoan community is less sensitive. Change in community profile is not in a concentration-dependent manner.	(Johansen, Pedersen et al. 2008)

C ₆₀ (50 mg/g of dry biomass)	Biosolids from anaerobic wastewater treatment sludge	Monitoring production of CO ₂ and CH ₄ ; PCR-DGGE	No change in microbial community structure and function in methanogenesis.	(Nyberg, Turco et al. 2008)
Graphene oxide (up to 3000 mg/L; up to 5 h)	Microbial community in activated sludge	Bacterial metabolic activity and viability assay; Scanning electron microscope (SEM); ROS and oxidative stress measurement	Bacterial metabolic activity, bacterial viability, and biological removal of nutrients, such as organics, nitrogen and phosphorus were significantly impacted by the presence of GO in the activated sludge.	(Ahmed and Rodrigues 2013)
Single-walled carbon nanotubes (SWCNTs) (up to 1000 µg/g of soil; up to 32 days)	Soil microbial community	Microbial biomass C and N analysed for total organic carbon and total organic nitrogen contents using a TOC-VCPH/CPN analyzer; enzyme activity assay	SWCNTs of concentrations at 300–1000 µg/g soil significantly lowered activities of most enzymes and microbial biomass. SWCNTs exhibited more severe impacts on enzyme activity and biomass than MWCNTs.	(Jin, Son et al. 2013)
SWCNTs (0.03 to 1 mg/g soil; 25 days)	Soil microbial community	PLFA	Biomass of major microbial groups including Gram-positive and Gram-	(Jin, Son et al. 2014)

					negative bacteria and fungi decreased. Gram-positive bacteria could adapt to soil disturbance by SWCNTs via changing lipid composition.	
SWCNTs (up to 1000 mg/L)	Anaerobic granular sludge microbial community	Confocal laser scanning microscopy (CLSM); 16S rRNA cloning library construction and sequencing	No significant effect on the methane yield. On the contrary they induced faster substrate utilization and methane production rates.	(Li, Tong et al. 2015)		
SWCNTs (0 to 3.5 g/L; up to 60 days)	Phenol wastewater treatment microbial community	16S rRNA amplicon sequencing; SEM;	SWCNTs protected microbes from inactivation rather than cytotoxic effects, possibly by inducing the production of bound extracellular polymeric substances (EPS).	(Qu, Ma et al. 2015)		
			Bacterial community structure significantly shifted after SWCNTs addition and did not recover later.			
Multi-walled carbon nanotubes (MWCNTs) (up to 5000 µg/g soil)	Soil microbial community	Microbial biomass C, N, and C:N ratio	Enzymatic activities as well as microbial biomass C and N decreased significantly.	(Chung, Son et al. 2011)		

MWCNTs (inner and outer diameter of 10 nm and 25 nm, respectively; 50 and 200 µg/mL)	Soil microbial community	PCR-DGGE pyrosequencing analysis	and	Relative abundances of Bacteroidetes and Firmicutes were found to increase, whereas Proteobacteria and Verrucomicorbia decreased with increasing concentration of CNTs. Dominant phylotypes maintained with treatments.	(Khodakovskaya, Kim et al. 2013)
MWCNTs (10-10,000 mg/kg of soil)	Soil microbial community	Pyrosequencing fatty acid profiling	and	No effects on soil respiration, enzymatic activities, and microbial community structure at 10, 100 and 1000 mg/kg. Increased fungal fatty acid methyl ester markers at the highest treatment. Decreased abundance of some bacterial genera like <i>Derxia</i> , <i>Holophaga</i> , <i>Opitutus</i> and <i>Waddlia</i> . Increased abundance of <i>Rhodococcus</i> , <i>Cellulomonas</i> , <i>Nocardioides</i> and <i>Pseudomonas</i> .	(Shrestha, Acosta-Martinez et al. 2013)
SWNTs, MWNTs, C ₆₀ , and colloidal graphite	River water and wastewater microbial community	Fluorescence-based, nucleic acid assay		Inactivation of microorganisms in river water and wastewater treatment plant (WWTP) samples.	(Kang, Mauter et al. 2009)

METAL NANOPARTICLES

Carboxy-functionalized Silver Nanoparticle (Ag NPs) (0.01–1 mg/L; up to 5 days)	Natural water microbial community	PCR-DGGE; restriction length polymorphism (T-RFLP)	Terminal fragment	Significantly changed the bacterial community structure, affecting individual cells and reduced bacterial metabolic activity. Communities diverged by as much as 54–56%. Compared to the untreated samples.	(Das, Williams et al. 2012)
Ag-NPs (Gum Arabic (GA) and citrate stabilized) (pulse and continuous additions of 0.2 and 2 ppm)	Wastewater microorganisms from batch reactors (SBRs)	T-RFLP		COD and ammonia removal decreased by as much as 30% or greater directly after spikes, SBRs were able to recover within 24 h and resume removal near 95%. GA AgNPs lowered diversity more than citrate AgNPs. The citrate and GA AgNPs treated SBRs never reached the same diversity they had prior to Ag addition.	(Alito and Gunsch 2013)
Ag-NPs (1 mg/L; up to 24 h)	Activated sludge microbial communities	Heterotrophic counts (HPCs); DGGE	plate PCR–	Certain microbial species in the intact activated sludge were highly sensitive. Can impact the cell culturability depending on the physical structure of the activated sludge flocs.	(Sun, Sheng et al. 2013)

Ag-NPs (25 or 1000 µg/L; 30 days)	Estuarine sediment microbial community	Inductively coupled plasma-optical emission spectrometry (ICP-OES); PCR-DGGE	Negligible differences in bacterial diversity in treated samples. Chloride ions in estuary water can cause AgCl complexes formation and aggregation of Ag-NPs which might lead to removal of Ag-NPs from aqueous phase.	(Bradford, Handy et al. 2009)
Ag-NPs (21 nm size; 0, 1, and 10 mg/kg; up to 250 days)	Microbial community of Landfill bioreactors	qPCR (methanogenic of population calculation).	AgNPs at the concentration of 1 mg/kg solids have minimal impact on landfill anaerobic digestion, but a concentration at 10 mg/kg or higher inhibit methanogenesis and biogas production.	(Yang, Xu et al. 2012)
Ag-NPs (PVP capped) (up to 2.5 mg/L; 10 days)	Wastewater treatment microbial communities	Pyrosequencing	Shift in niche populations in both aerobic and anaerobic sludge.	(Doolette, McLaughlin et al. 2013)
Ag-NPs (PVA capped) (size of 21 nm; up to 40 mg/L)	Microbial community of anaerobic sludge digester in wastewater	qPCR; Environmental scanning electron microscopy- dispersive spectroscopy	<i>Methanosaeta</i> and <i>Methanomicrobiales</i> were the dominant methanogens, and the methanogenic diversity and population remained largely unchanged after nanosilver exposure and anaerobic digestion. No significant difference in	(Yang, Chen et al. 2012)

	treatment plants	EDS)		biogas and methane production between the treated and control.	
Ag-NPs (Size less than 15 nm; 1, 50, or 200 mg/L; up to 24 h)	Wastewater biofilms	HPC enumeration); DGGE	(Bacterial PCR-	Biofilms were highly tolerant to Ag-NPs.	(Sheng and Liu 2011)
Ag-NPs (size 65 ± 30 nm; 0–2000 μ g/L; up to 24 h)	Marine biofilms	T-RFLP; CLSM		Ag-NPs significantly affected the community structure. Even at the highest concentrations studied the major taxonomic groups are not displaced. Treatment reduced normal biofilm development and succession in the samples.	(Fabrega, Zhang et al. 2011)
Ag-NPs (~29 nm; 1 mg/L; up to 25 days)	Activated sludge microbial community	T-RFLP; Respirometric assays	ICP-OES;	Prolonged period of nitrification inhibition and increase of ammonia/nitrite concentration in wastewater effluent. Nanosilver exposure did not affect the growth of heterotrophs responsible for organic matter removal. Population of ammonium and nitrite-oxidizing bacteria, <i>Nitrospira</i> decreased, while <i>Nitrobacter</i>	(Liang, Das et al. 2010)

Ag-NPs (50 nm uncoated and 15 nm PVP coated; 0-300 mg/L for uncoated and 1 and 10 mg/L for coated; up to 24 h)	Surface soils (top 10–30 cm) of Toccoa sandy loam	Nitrate analysis	was washed out after the shock loading.
Ag-NPs (original size 13.0 ± 3.3 nm; after aggregation, 63.5 ± 15.2 to 79.2 ± 18.1 nm; 0.1 to 50 mg/L; 50 days)	Microbial communities in sequential batch reactors	Pyrosequencing 16S rRNA	PVP coated Ag-NPs suppressed the soil nitrification process more effectively. Ag-NPs exhibited a greater toxicity to nitrifying bacteria than Ag ⁺ in soils. (Masrahi, VandeVoort et al. 2014) Rate constants of both biological nitrification and organic oxidation decreased exponentially with an increase in Ag-NP concentration, but nitrification was more severely inhibited than organic oxidation even at low Ag NP concentrations (<1 mg Ag L ⁻¹). Heterotrophic bacteria exhibited a higher tolerance to Ag-NPs than nitrifying bacteria. Diversity of microbial community and treatment efficiency decreased. (Jeong, Im et al. 2014)
Ag-NPs (5 nm at 0.05 ppm, and 35 nm at	Activated sludge from waste water	Pyrosequencing; qPCR; SEM	AgNP treated samples had less microbial diversity than control. The effect was more with 35 nm and decreased the (Yang, Quensen et al. 2014)

40 ppm; 7 days)	treatment plant			abundance of <i>Chloroflexi</i> , <i>Firmicutes</i> and <i>WS3</i> , but increased the population of <i>Acidobacteria</i> . Biological nitrogen removal was also hampered by reducing the abundance of <i>Nitrosomonas</i> , and the absence of <i>Nitrosococcus</i> . qPCR showed a four-fold decrease in copy number of the ammonia monooxygenase subunit A gene (<i>amoA</i>), a functional gene marker for ammonia-oxidizing bacteria. All the above effect was not observed in 5nm AgNP or AgNO ₃ treated samples.
Ag-NPs (6.0 ± 2.0 nm; influent concentration of 0.10 mg/L; 61 days)	Membrane bioreactor activated sludge system	Atomic Absorption Spectroscopy (AAS); T-RFLP; qPCR	No significant change in the nitrifying bacterial community structures after treatment with Ag-NPs.	(Zhang, Liang et al. 2014)
			There was no significant changes in gene copy numbers of <i>amoA</i> gene, the 16S rRNA genes of <i>Nitrospira</i> spp. And <i>Nitrobacter</i> spp. after long-term treatment with Ag-NPs.	
			EPS concentration increased significantly	

					after long-term Ag-NPs dosing.	
Au and Ag nanoparticles (10-20 nm; 50 µg/L - 2 mg/L; 45 days)	AOB enrichment cultures from river sediments estuary wetlands	PCR-DGGE; Pyrosequencing			Ag-NPs significantly inhibited bacterial ammonia oxidation in aquatic environment in a concentration dependent manner; inhibition was resulted primarily from a decrease in AOB biodiversity and abundance reduction. AOB biodiversity and abundance as well as activity were not inhibited by Au-NPs.	(Luo, Chen et al. 2014)
Copper nanoparticles (Cu NPs) (220 ± 25 nm; 0.1–10 mg/L)	Wastewater treatment microbial communities	qPCR			Increased the number of denitrifiers (especially N ₂ O reducing denitrifiers) but decreased nitrite accumulation. Total nitrogen (TN) removal was enhanced and N ₂ O generation was reduced. Ammonia and phosphorus removals were not affected.	(Chen, Wang et al. 2012)
Zero-valent iron nanoparticles (nZVI)	Soil microbial community	PLFA; multiple induced (MSIR)	Functional substrate-respiration		Microbial community structures in sandy soils were the most, and clay soils the least, vulnerable to nZVI (impact of nZVI on soil microbial communities is dependent on organic matter content and	(Pawlett, Ritz et al. 2013)

				soil mineral type). Reduction of arbuscular mycorrhizal fungi.
Nano zero valent iron (nFe ⁰)	Groundwater and aquifer sediment	Pyrosequencing; chromatography	Gas	No sulphate reduction was observed in (Kumar, presence of nFe ⁰ and in presence of Omoregie et al. 2014) aquifer material, 1% nFe ⁰ was shown to inhibit the sulphate reducing bacteria (SRB) activity almost completely.
Cobalt NPs (Co NPs) (2–60 nm with an average of 28 nm); Zero valent iron NPs (Fe NPs) (2–58 nm with an average of 25 nm); Nickel NPs (Ni NPs) (~20 nm); Ag NPs (2–50 nm with an average of 35 nm). 550 mg of NPs sprinkled on soil surface (23.6 cm × 26.7 cm); 50 days	Soil microbial community	Pyrosequencing		Different NPs migrated through soil (Vishal, Daniel et al. 2014) matrix at different rates. No significant decline in microbial richness.
Ag-NPs (52 ± 12 nm); nanoCeO ₂ (33 ± 12 nm).	Nitrifying sequencing batch	Ion chromatography; qPCR; Pyrosequencing		Ag-NPs and Ag ⁺ dosing treatment showed (Ma, Metch et al. a decrease in microbial diversity,

20 mg/L; 42 days.	reactor	and TEM-EDS	especially in case of Ag ⁺ .	2015)
			Relative abundance of <i>amoA</i> genes decreased significantly in Ag ⁺ treatment compared to Ag-NPs and controls.	
			<i>Bacteroidetes</i> became more dominant in Ag ⁺ treatment, while <i>Proteobacteria</i> became more dominant in Ag-NPs treated reactors.	

METAL OXIDE NANOPARTICLES

TiO ₂ (size 15-20 nm; 0-2.0 mg/g soil) and ZnO (size 20-30 nm; 0-0.5 mg/g soil); up to 60 days	Soil bacterial communities	Respirometric assays; T-RFLP	Both nano-TiO ₂ and nano-ZnO reduced microbial biomass. Both NMs altered the composition of the soil bacterial community; ZnO showed stronger effects.	(Ge, Schimel et al. 2011)
TiO ₂ NPs (70-90 nm; 50 mg/L; up to 70 days)	Bacterial community in activated sludge	PCR-DGGE; SEM	FISH; Significantly decrease total nitrogen (TN) removal efficiency from 80.3% to 24.4%. Biological phosphorus removal was unaffected. Reduced the diversity of microbial. Abundance of nitrifying bacteria, especially ammonia-oxidizing	(Zheng, Chen et al. 2011)

				bacteria, was highly decreased.	
TiO ₂ NPs (under natural levels of UV radiation)	Planktonic and biofilm communities of surface water	Polarization microscopy; Raman spectroscopy; Membrane integrity assay	Cell integrity was compromised.	(Battin, Kammer et al. 2009)	
TiO ₂ NPs (primary size of 20–30 nm that agglomerated in deionized water to 194 nm; 20 mg/g; up to 288 days)	Soil bacterial communities	T-RFLP	Nano-TiO ₂ altered the bacterial community composition and reduced diversity.	(Jin, Son et al. 2013)	
TiO ₂ NPs (size less than 25 nm; 2–200 mg/L)	Bacterial community in activated sludge	PCR-DGGE; TEM	SEM; 200 mg/L of TiO ₂ NPs significantly reduced microbial diversity in the activated sludge. Abundance of denitrifying bacteria was significantly decreased.	(Li, Cui et al. 2013)	
TiO ₂ NPs: PW6 (100% anatase; ~81.5 nm) and P25 (84% anatase and 16% rutile; ~22.9 nm); 0-	Freshwater microbial community	Cytotoxic assay using Live/Dead BacLight Bacterial viability kit, staining and direct	Under simulated solar illumination PW6 and P25 reduced the abundance of viable <i>Bacillus subtilis</i> and <i>Aeromonas hydrophila</i> , while stimulated growth of	(Binh, Tong et al. 2014)	

25 mg/L; up to 2 h			counting microscopy	using	<i>Arthrobacter</i> sp. and <i>Klebsiella</i> sp.	
TiO ₂ NPs (80% anatase) and 20% rutile); 21 nm; concentration 1 mg/kg and 500 mg/kg of soil; up to 90 days	Soil communities	microbial	qPCR; chromatography	Gas	In most soils of the present study, TiO ₂ NPs had no significant effect on either C-mineralization potential activity or on bacterial abundance at both the concentration tested up to 90 days.	(Simonin, Guyonnet et al. 2015)
NPs of organic polymers, Mo/NaO, TiO ₂ , TiSiO ₄ , CdSe/ZnS quantum dots, Au, Fe/Co (up to 30 days)	Soil community	bacterial	SEM; PCR- DGGE		Altered bacterial community. Inorganic NMs had less effect on the microbial community except TiO ₂ and the Au nanoparticles.	(Nogueira, Lopes et al. 2012)
TiO ₂ NPs (Anatase; 5–10 nm); TiO ₂ NPs (Rutile; ~55 nm); Zero valent Cu NPs (2–60 nm with an average of 25 nm); Ag NPs (~35 nm); ZnO NPs (~35 nm); 10 ppm or 0.0625 mg/kg soil; up to 120 days	Soil community	microbial	Tag-encoded amplicon pyrosequencing (TEFAP)	FLX	ZnO and Cu NPs were not toxic to soil bacterial community. Ag NPs and TiO ₂ changed bacterial richness and composition in wavering pattern as a function of time. TiO ₂ in Rutile phase has more prolonged influence than Anatase phase.	(Shah, Jones et al. 2014)

Nano-Al ₂ O ₃ , nano-TiO ₂ , nano-SiO ₂ , and nano-Fe ₂ O ₃	<i>E. coli</i> and <i>Salmonella</i> co-culture	Conjugative Transfer of the RP4 Plasmid	Nanoalumina can promote the conjugative transfer of the RP4 plasmid from <i>Escherichia coli</i> to <i>Salmonella</i> spp. by up to 200-fold	(Qiu, Yu et al. 2012)
Copper oxide (CuO) and magnetite (Fe ₃ O ₄) NPs (< 50 nm; 1% and 0.1%)	Microbial communities in a sandy loam (Bet-Dagan) and a sandy clay loam (Yatir)	PCR-DGGE; 454 high throughput sequencing and qPCR	In the Yatir soil, 1% CuO exposure cause a significant decrease in oxidative potential and changes to community composition. Fe ₃ O ₄ changed the hydrolytic activity and bacterial community composition in Bet-Dagan soil but did not affect the Yatir soil bacterial community. In Bet-Dagan soil, abundance of bacteria from the Bacilli class decreased after addition of 0.1% CuO but increased with 1% CuO, while in Yatir soil their abundance was reduced with 1% CuO. Other important soil bacterial groups, including <i>Rhizobiales</i> and <i>Sphingobacteriaceae</i> , were negatively affected.	(Frenk, Ben-Moshe et al. 2013)
ZnO NPs	Microbial	PCR-DGGE	Nitrogen and phosphorus removal	(Puay, Qiu et al.

(66.25 ± 36.61 nm; 1 mg/L; up to 63 days)	community in sequencing batch reactor		efficiencies were also severely affected (2015) along with poor settling properties of the activated sludge. Changes in the bacterial community structure and a reduction in the bacteria diversity was observed.
CuO and ZnO NPs (25 nm; 10 and 20 µg/ g soil; 30 days)	Soil microbial community	Most Probable Number (MPN) method and serial dilution plating method	Both metal oxide NPs inhibited CH ₄ oxidation activity of soil. (Mohanty, Rajput et al. 2014)
γ-Al ₂ O ₃ and γ-Al ₂ O ₃ coated with humic acid (20–50 nm; 100 g/L; up to 400 h)	Anaerobic microbial community in granular sludge	Gas chromatography and SEM	Lower methane production (Alvarez and Cervantes 2012)
CeO ₂ , Fe ₃ O ₄ , SnO ₂ (0, 10 and 100 mg/kg of dry soil; 7 or 60 days)	Soil microbial biomass	Microbial C/N ratio	The microbial C/N ratio increased with (Vittori Antisari, Fe ₃ O ₄ and SnO ₂ NPs probably due to the Carbone et al. predominance of microbial communities 2013) such as ectomycorrhizae. Changes the bacterial/fungal biomass ratio.

Silica, palladium, gold and copper NPs (0.013% w/w or 0.066% w/w; 15 days)	Soil microbial community	Fatty Acid Methyl Ester (FAME) Profile analysis	Statistically insignificant influence of the nanoparticles on the soil microbial community profile.	(Shah and Belozerova 2009)
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2.6 Influence on microbial community structure

Microbial communities in soils play an essential role in biogeochemical cycling, agricultural production, and plant diversity (Jean-Marc, Tawna et al. 1994, Zak, Holmes et al. 2003, Falkowski, Fenchel et al. 2008). Studies have shown that of NMs, such as surface charge, size, aggregation, could be altered in environmental media (Lowry, Gregory et al. 2012, Dobias and Bernier-Latmani 2013, Liu, Jin et al. 2014). Different NMs migrate at different rates in the soil matrix thus exhibit different effects on the soil microbial community (Vishal, Daniel et al. 2014). Soil type also affects the influence of NMs. Microbial communities in sandy and clay soils have been shown to respond differently to CuO and magnetite nanomaterials. Beta-dragon soil which contains lower amount of organic matter and soil clay portion was shown to have significant shift in microbial composition. Both magnetite and CuO NMs showed an increase in Bacilli class members. Further the relative abundance of Actinobacteria increased significantly in response to treatment with 0.1% CuO treatment whereas relative abundance of Betaproteobacteria was increased after treatment with 1% CuO. Yatir soil which has higher organic and clay content, after exposure to 1% CuO, the relative abundance of Bacilli class was reduced while the total population did not change significantly (Frenk, Ben-Moshe et al. 2013). Similar results were obtained with zero-valent iron nanomaterials where microorganisms in sandy soils were more susceptible than those in clay soils and was shown to have bactericidal effect on Gram negative bacteria and it also caused reduction in arbuscular mycorrhizal fungi (Pawlett, Ritz et al. 2013). The influence of NMs on soil microbial communities was also affected by organic matter and salt contents in the soil (Tong, Bischoff et al. 2007). Aquatic microbial communities were also examined for the effect of NMs. Reports on the impacts of NMs on microorganisms in both planktonic and biofilm modes of growth are available for freshwater and marine systems. Carbon based NMs were shown to inactivate cells in monocultures as well as in river water microbial communities (Kang, Mauter et al. 2009). Similarly, Ag nanoparticles changed the community profile of fresh water microbial communities (Das, Williams et al. 2012). In addition to planktonic cultures, aquatic biofilm communities as impacted by NMs have also been reported. Biofilms are surface-associated microbial communities with

cells embedded in a polymeric matrix (Hall-Stoodley, Costerton et al. 2004, Flemming and Wingender 2010). Although biofilm communities often exhibit a higher tolerance to toxic contaminants or antimicrobial agents than their planktonic counterparts (Hall-Stoodley, Costerton et al. 2004, Harrison, Ceri et al. 2007, Cao, Majors et al. 2012, Ding, Peng et al. 2014), the exposure to AgNPs changed community structure of marine biofilms, affecting biofilm development and succession; however, major taxonomic groups were not displaced (Fabrega, Zhang et al. 2011). Cell integrity in biofilm communities could also be compromised by NMs, *e.g.*, TiO₂ NPs (Battin, Kammer et al. 2009).

Although most studies have reported a reduction in microbial diversity upon NMs exposure, there were also reports of NMs not having significant effects on community structures (Tong, Bischoff et al. 2007, Nyberg, Turco et al. 2008, Bradford, Handy et al. 2009, Shah and Belozeroova 2009).

For the effects of NMs on engineered ecosystems, microbial communities in waste or wastewater treatment bioprocesses are often used as model systems and most studies focused on the community composition and community function in terms of efficiency of the bioprocesses. The impacts of NMs on both anaerobic and aerobic treatment processes have been studied and the most commonly studied bioprocesses include methanogenesis and nitrogen and phosphorous removal (Table 2.1). Intriguingly, although many previous reports demonstrated that different types of NMs such as AgNPs and graphene oxide had negative impact on ammonium-oxidizing bacteria and nitrite-oxidizing bacteria (Nyberg, Turco et al. 2008, Liang, Das et al. 2010, Ahmed and Rodrigues 2013), there were also reports showing that anaerobic sludge digester microbial communities were not affected by these NMs (Nyberg, Turco et al. 2008, Yang, Chen et al. 2012). The discrepancies could be because of different physicochemical properties of the NMs and their complex interactions with the organic and inorganic matters in the microbial communities.

2.7 Influence on microbial community function

Whether the exposure to NMs affects microbial community functions is another important aspect that has been relatively less explored. Several recent studies examined the impacts

of NMs on community function. Most commonly studied functions were methanogenesis and nutrient removal in waste water treatment plants. For example, methanogenesis was found to be inhibited by NMs such as Al_2O_3 and the toxicity of Al_2O_3 was reduced when coated with humic acids (Alvarez and Cervantes 2012). AgNPs were shown to inhibit methanogenesis in landfill bioreactor in concentration dependent manner where 1 mg/kg AgNPs didn't show any impact on biogas production but 10 mg/kg AgNPs reduced biogas production (Yang, Xu et al. 2012). However, another study showed that no effect on methanogenesis could be observed when biosolids from anaerobic sludge were exposed to fullerene (C60) for few months (Nyberg, Turco et al. 2008).

Nitrogen removal process was also reported to be inhibited by NMs like TiO_2 (Zheng, Chen et al. 2011, Li, Cui et al. 2013) and AgNPs (Liang, Das et al. 2010, Masrahi, VandeVoort et al. 2014). Graphene oxide was shown to negatively impact the waste water treatment process specially it affected the nutrient removal (phosphorous and nitrogen) in a concentration dependent manner. It also increased the water turbidity and reduced the sludge dewaterability thus deteriorating the effluent quality (Ahmed and Rodrigues 2013). AgNPs were shown to inhibit nitrification process in soil microbial community as well as in waste water treatment plant (Jeong, Im et al. 2014, Masrahi, VandeVoort et al. 2014). In activated sludge treatment process, titanium dioxide was shown to reduce nitrogen and phosphorous removal efficiency (Zheng, Chen et al. 2011). In contrast, CuO NPs was reported to increase the abundance of denitrifiers (especially N_2O reducing denitrifies) and enhanced total nitrogen removal (Chen, Wang et al. 2012).

To explain the influence of NMs on community structure and function, most studies suggested an organism-specific toxicity of NMs, *i.e.*, different microorganisms in the community have different susceptibility to the NMs. For example, some bacteria are more tolerant to AgNPs than others (Sun, Sheng et al. 2013). Heterotrophic microorganisms were found more tolerant to AgNPs than nitrifying bacteria (Jeong, Im et al. 2014). It has also been shown that MWCNTs selectively reduced the population of a few phyla (*e.g.*, Proteobacteria and Verrucomicrobia) and increased the cell count of several other phyla (*e.g.*, Bacteroidetes and Firmicutes) (Khodakovskaya, Kim et al. 2013). MWCNTs were shown to increase the population of few bacteria genera like *Rhodococcus*, *Cellulomonas*,

*Nocardioide*s and *Pseudomonas*, which are well known degraders of recalcitrant aromatic compounds (Khodakovskaya, Kim et al. 2013, Shrestha, Acosta-Martinez et al. 2013). In another study, Gram-positive bacteria were found capable of adapting to SWCNTs by changing their membrane lipid composition (Jin, Son et al. 2014). The mechanisms of organism-specific effects of NMs remain largely unknown and need to be further explored.

2.8 Commonly studied NMs for impacts on microbial community

The most commonly used NMs in these studies include carbon based and of metal and metal oxides because these NMs are widely used in various consumer products and thus more likely to end up in the environments as emerging contaminants. Ag, ZnO, TiO₂ nanomaterials and carbon based nanomaterials such as fullerene, single-wall carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) are the mostly used NMs for elucidating impacts of NMs on microbial communities in natural and engineered ecosystems. Many studies used more than one type of NMs to examine material-specific effects on microbial communities (Ge, Schimel et al. 2011, Shah, Jones et al. 2014). Interestingly, even for the same type of NMs, the influences on microbial communities could vary significantly. For example, Johansen et al. (2008) reported that C₆₀ significantly affected the soil microbial community and reduced the number of actively growing bacteria by 3-4 folds, while several other studies showed no significant effects on the microbial community profiles (Tong, Bischoff et al. 2007, Nyberg, Turco et al. 2008). The shape, size, and concentration of the NMs varied among previous studies, which could attribute to the inconsistencies reported in different studies. For example, rutile and anatase are the two mineral phases of TiO₂ nanoparticles and the rutile phase has been shown to exhibit a longer effect than the anatase phase (Shah, Jones et al. 2014). In addition, surface modification of NMs also has profound effects on their influences on microbial communities. For example AgNPs coated with polyvinylpyrrolidone (PVP) were shown to be more toxic to nitrifying bacteria (Masrahi, VandeVoort et al. 2014). On the contrary, coating of humic acid on Al₂O₃ NPs reduced their toxicity to methanogenic activity (Alvarez and Cervantes 2012). In another study, compared with AgNPs stabilized

with citrate, AgNPs stabilized with gum Arabic (GA) showed a stronger impact on microbial diversity (Alito and Gunsch 2013).

2.9 Commonly used techniques to study impacts NMs on microbial community

Most previous studies elucidating the impacts of NMs on microbial communities focused on the changes of microbial composition in the communities. Commonly used techniques to profile microbial communities in previous studies include PCR-DGGE, T-RFLP, quantitative real-time PCR, phospholipid fatty acid (PLFA) analysis, and functional multiple substrate-induced respiration (MSIR) profiles analysis, and fluorescent in situ hybridization (FISH). One of the powerful and widely used technique is use of microscopy like confocal laser scanning microscopy, electron microscopy (SEM and TEM) which are combined with different staining techniques and fluorescent probes to visualize the changes in response to NMs treatment. PCR-DGGE and T-RFLP are the most commonly and earliest community fingerprinting techniques used for studying the impact of NMs on microbial composition in environmental samples. The methods analyses total DNA extracted from all the microbes present in the sample. A particular gene or DNA region which is unique to each bacterial species, is then amplified by PCR. Advantages of these methods are they can be performed quickly and relatively cheaply, and the analyses can accommodate a large number of samples simultaneously. Although these methods remains to be the most widely used tool for community analysis, the trend of increasingly applying sequencing technologies such as pyrosequencing to investigate the impacts of NMs is clear (Zhang, Shao et al. 2011, Ye, Zhang et al. 2012).

As most of the microorganisms remain unculturable till now, culture independent techniques to study microbial community structure have gained popularity. Starting with low-throughput 16S rRNA pyrosequencing to high throughput genome-wide sequencing approaches, the field has made significant progress in last decade. Genome-wide sequencing such as metagenomics and metatranscriptomics have revolutionized the study of complex microbial community. 'Meta'omic' approach combines DNA/RNA sequencing with study of proteins and metabolites and is a powerful tool for collective analysis of

biochemical function, systems-level interactions and quantification of complex microbial community.

Especially metagenomics and metatranscriptomic are most widely used tools for assessing the genomic composition and diversity in microbial community. While DNA-based metagenomics can identify the structure and metabolic potential of the whole community (Eisen 2007), metatranscriptomic can give more precise information about function and activity environmental community. Though metatranscriptomic is comparatively a new technical development but has been successfully used for characterization of structure and function within a variety of microbial communities. The major bottleneck in metatranscriptomics study remains the isolation of good quality RNA as it is prone to degradation and complex matrix of environmental samples makes it difficult to isolate pure and quality RNA required for sequencing. The Illumina platform is currently preferred for sequencing, and widely used in microbial community analysis for rRNA gene surveys (Bartram, Lynch et al. 2011, Caporaso, Lauber et al. 2012).

However, sequencing based approaches often generate large quantities of data and require reference genomes for the annotation of the sequencing data and great efforts on bioinformatics analysis, which remain major challenges of this approach. While next generation sequencing methods are gaining popularity because of the above stated challenges there are very few studies available so far which used these high throughput sequencing methods to study effect of NMs on microbial communities. Following are the most recent studies in this field and all of them have used amplicon sequencing method. To study the effect engineered nanomaterials on freshwater wetlands microbial communities, separate mesocosms were treated with Ag^0 , Ag_2S , CuO , or CuS NPs. 16S amplicon sequencing was used using Illumina MiSeq platform to study the composition of the community. Specifically, researchers suggest that *Cyanobacteria* was vulnerable to Ag^0 but not Ag_2S NPs and they also observed a sharp decrease in *α -proteobacteria* common across the CuO and CuS NPs and Cu^{2+} treatments but overall the environmental community was evidently resilient despite a worst case scenario of high concentration (Moore, Stegemeier et al. 2016). Another study used the same 16S rRNA amplicon sequencing to study the effect of SWCNTs on the sludge microbes of phenol wastewater

treatment system, the sequencing data showed that bacterial community structure significantly shifted after SWCNTs addition and did not recover afterward. *Zoogloea* increased significantly upon SWCNTs addition. Towards the final stage, *Rudaea* and *Mobilicoccus* increased, while *Burkholderia*, *Singulisphaera*, *Labrys* and *Mucilaginibacter* decreased notably (Qu, Ma et al. 2015). One more study uses the same technique of amplicon sequencing to study the effect of sulfidised-silver nanoparticles (Ag₂S-NPs) on soil microbial community, they combined quantitative PCR and species sensitivity distribution (SSD) methods along with the sequencing to calculate the threshold toxicity concentration for silver based nanoparticle (Doolette, Gupta et al. 2016).

2.10 Mechanisms of toxicity to microbial community

Although extensive studies have been carried out to understand the impacts of NMs on microbial communities, most focused on the overall changes in community profiles and community function caused by organism-specific toxicity of NMs. To understand the exact mechanism behind the change in community structure and function, it will be interesting and important to explore the influence of NMs on intercellular interactions in defined multi-species cultures and in complex microbial communities in natural and engineered ecosystems. Intercellular interaction and communication between members of community in a given ecological niche is the key to fascinating group behaviours and many more critical functions (Jacob, Becker et al. 2004)

2.11 Social behaviour: the key to community structure and function

Communication and cooperation are long considered the crux of sociality. Increasing volume of research shows that unicellular microorganisms manifest complex and fascinating social lives (West, Griffin et al. 2006, West, Diggle et al. 2007). The social behaviour is reflected in many ways starting from cooperation to protect themselves from predators and adverse environmental condition, to secure food, to reproduction or dispersal to a new locality (Crespi 2001). The social lives of microbes have a significant impact on various members of ecosystem. Following are the major areas where cooperative behaviours of microbes play key roles (i) in the ability of microbes to cause

infection in humans, animals, and plants (Williams, Camara et al. 2000, André and Godelle 2005) and (ii) waste treatment and nutrient recycling (Valle, Bailey et al. 2004) (iii) promoting plant growth (Kiers, West et al. 2002).

Bacteria are known to have a myriad of ways to exhibit cell-cell interactions in community level. Following are few well studied systems involved in interactive behaviour.

2.11.1 Production of communal resources

Public goods are defined as metabolites secreted by certain members of the community but are beneficial to others occupying the same niche (West, Griffin et al. 2006). Some of the common examples of microbial public goods are siderophores (iron scavenging molecule), antibiotics, rhamnolipids, biosurfactants, exopolysaccharides and proteases (West, Diggle et al. 2007). These molecules are central to the cooperation and complex multicellular behaviour elucidated by microbial community in nature (Dunny, Brickman et al. 2008). Their importance lies in the myriad of cellular functions these molecules are involved in starting from nutrient acquisition to protection against predators. Hence, any alteration on the production of the public goods can alter the structure and function of the whole community and in turn affect the key ecological function performed by these microbial communities.

One primary example of public goods in environmental microbial communities is siderophore, a group of organic compounds produced by bacteria to facilitate the uptake of iron. Iron is an essential element for growth of all microorganisms but its bioavailability is low under circumneutral pH. One mechanism to acquire iron is the production and secretion of siderophore to scavenge iron (Wandersman and Delepelaire 2004). Siderophores have very high affinity for Fe^{3+} with the affinity constant constant $>10^{30} \text{ M}^{-1}$ (Drechsel and Jung 1998, Haas, Eisendle et al. 2008). Apart from iron sequestering, siderophores are also known to enhance virulence (Lamont, Beare et al. 2002), enhance plant-microbe interactions (Ahmed and Holmström 2014), and drive microbial evolution (Keller and Surette 2006, Cordero, Ventouras et al. 2012). Siderophores also promote the growth of uncultured bacteria (D'Onofrio, Crawford et al. 2010). In addition, researchers

have shown that siderophore deficient mutants lacks the ability to form biofilm in *Pseudomonas aeruginosa* (Harrison and Buckling 2009). The above wide ranging functions of siderophores suggest that any alteration in the production of these bioactive compounds can have ever lasting impact on bacterial community. There are over 500 types of siderophores known so far and mostly these are produced wide variety of bacteria and few of them are well studied (Figure 2.3 A). Siderophores are produced by several gene clusters and are assembled by nonribosomal cytoplasmic peptide synthetases (Neilands 1995). The production as well as uptake of siderophore-iron complex from the environment is a multi-step process (Figure 2.3 B and C).

Figure 2.3 (A) Structures of different types of siderophores. Schematic representation of siderophore-mediated iron uptake in (B) Gram-negative and (C) Gram-positive bacteria. Reprinted with permission from (Saha, Saha et al. 2013) and from (Andrews, Robinson et al. 2003) for panel (A) and (B, C) respectively.

The acylated homoserine lactones (AHLs) are QS signals used by most Gram-negative bacteria (Fuqua, Parsek et al. 2001). The AHL-type QS systems have been identified in a large number of Gram-negative bacterial species belonging to the α , β , and γ subclasses of proteobacteria (Eberl 1999) and have been shown to be highly conserved across different species. These include pathogens such as *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Serratia liquefaciens*, plant symbionts such as *Rhizobium spp.*, environmental bacteria such as *Nitrosomonas spp.* and *Geobacter uraniumreducens* (Case, Labbate et al. 2008). In these bacteria, the AHL-type QS systems have been shown to regulate a wide variety of bacterial physiological processes including bioluminescence (Nealson and Hastings 1979), biofilm formation (Parsek and Greenberg 2005), host pathogenesis (de Kievit and Iglewski 2000, Miller, Skorupski et al. 2002), plasmid transfer, motility, secondary metabolite production (Latifi, Winson et al. 1995), and sporulation (Camilli and Bassler 2006). The AHL-type QS has also been demonstrated to play a critical role in engineering processes such as membrane biofouling (Dobretsov, Teplitski et al. 2009) and granulation in biological wastewater treatment (Shrout and Nerenberg 2012, Tan, Koh et al. 2014). The ubiquitous presence of the AHL-type QS systems in environmental bacteria and their capability in regulating a broad range of bacterial functions suggest a pivotal role of the AHL-type QS in defining and shaping complex community structures and functions for ecological services. Environmental factors such as nutrient availability (Bollinger, Hassett et al. 2001), temperature, pH (Yates, Philipp et al. 2002) and environmental stresses (Lazazzera 2000) may contribute to different quorum sensing responses. For example, it has been shown that a short-term starvation may enhance quorum sensing signal production in *Myxococcus xanthus* (Shimkets 1999).

2.11.3 Collective antibiotic tolerance

In the past few decades, antibiotic resistance has emerged as a global crisis because of indiscriminate usage of antibiotics. The antibiotic resistance is widespread in most of the natural and engineered environment and spreading among environmental bacteria at an alarming rate (Ventola 2015). There have been a lot of efforts for detailed understanding of the evolution of antibiotic resistance in terms of origin and dissemination (Davies 1994, Martínez 2008, Davies and Davies 2010). But antibiotic resistance and different

mechanisms associated with it has mainly been investigated at the single level and only recently focus has shifted to the community level studies. It is increasingly clear that bacteria at the community level can survive treatment even if they are not genetically resistant. “Inoculum effect” highlights the fact that cells at a higher density are more tolerant to drug treatment compared to culture with low cell density (Tan, Smith et al. 2012). Moreover, it is apparent now that population dynamics and social interactions play a very important role in development of resistance. Hence, there is effort to understand the underlying communal interactions and population level processes. This will help us answer the question of “how a population of bacteria survive antibiotics treatment when a single bacteria cannot”. Such collective tolerance can be attributed to the various processes, *e.g.*, QS, biofilm formation, cooperative production of antibiotic inactivating enzymes, antibiotic titration and mutualistic interactions. (Vega and Gore 2014, Meredith, Srimani et al. 2015).

Biofilms are more tolerant to antimicrobials than their planktonic counterparts (Lewis 2001). One main reasons for this phenomenon is attributed to the physical structure of biofilms which creates a diffusion barrier by secreting extracellular polysaccharide, DNA and proteins (Anderson and O'toole 2008). The cells are also in close contact with each other in biofilms which promotes plasmid transfer and as most of the environmental biofilms are multispecies communities they help in increasing the host range of the mobile genetic elements (Madsen, Burmølle et al. 2012).

2.12 Summary

Because of an increasing use of NMs in a wide range of industries, the presence of NMs in the environment is unavoidable. Most of the earlier studies to assess the toxicity of NMs focused on the mechanisms of bactericidal effects (Figure 2.2) on single species laboratory cultures at higher concentrations, which might not be a true representation of the environmentally relevant concentration. Further, extensive studies were carried out to understand the impacts of NMs on microbial communities (Table 2.1), most focused on the overall changes in community profiles and community function caused by organism-specific toxicity of NMs. In addition to bactericidal activities, sub-lethal impact of NMs

on microbial physiology and functions is a novel and critical aspect of the NMs-microorganism interactions, in particular, the impact of NMs on various social behaviours such as public goods production and intercellular communication via signaling molecules, which are key to fascinating structures and functions of microbial communities in ecosystem. Thus to comprehensively understand the impacts of NMs on the environment and the health of the ecosystem, it is imperative to elucidate the impacts of NMs on social behaviour of microorganisms.

CHAPTER 3. Biogenic Tellurium Nanorods Inhibit Pyoverdine Production in *Pseudomonas aeruginosa*

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3.1 Introduction

Various microorganisms such as bacteria, fungi, yeast and plant extracts containing redox active enzymes and are capable of reducing metals and metal(oids) to produce NMs (Mohanpuria, Rana et al. 2008, Kumar and Yadav 2009, Durán, Marcato et al. 2011). Although both cells and biofilm matrix of environmental microorganisms are capable of transforming toxic metal(loid) contaminants into nanomaterials, the potential impacts of the biogenic nanomaterials (bio-NMs) on the ecosystems are largely unexplored. In addition, most econanotoxicity studies were conducted using engineered nanomaterials while the impacts of bio-NMs that may be generated in the environment have never been explored.

Tellurium (Te) is a rare metalloid element that has been widely used as an alloy component for products such as solar cells (Singh, Rangari et al. 2004), optical modulators (Staupendahl and Schindler 1982) and gas sensors (Tsiulyanu, Marian et al. 2001). The increasing usage of Te has led to environmental contamination by the highly soluble and toxic oxyanion TeO_3^{2-} (Dopp, Hartmann et al. 2004). Various bacteria have been reported capable of reducing soluble TeO_3^{2-} to insoluble $\text{Te}(0)$ nanomaterials, as a result of either detoxification, redox maintenance, or respiration (Yurkov, Jappé et al. 1996, Moscoso, Saavedra et al. 1998, Trutko, Akimenko et al. 2000, Klonowska, Heulin et al. 2005). Some

environmental bacteria such as *Shewanella oneidensis* are capable of producing bio-NMs extracellularly (Narayanan and Sakthivel 2010, Kalathil, Lee et al. 2011, Sintubin, Verstraete et al. 2012).

P. aeruginosa is an environmental bacterium and an opportunistic pathogen that can establish infections in immunocompromised patients. The infection caused by *P. aeruginosa* is very resistant to antimicrobials because of several reasons and the major one being the ability of this organism to form biofilms (Wagner and Iglewski 2008, Rybtke, Jensen et al. 2011).

Herein, it is described for the first time a biogenic nanomaterial that can significantly inhibit the production of PVD in *P. aeruginosa*. Specifically, tellurium nanorods (TeNRs) were obtained through bioreduction of tellurite by a metal-reducing bacterium *Shewanella oneidensis* MR-1 and demonstrated that the biogenic TeNRs could effectively inhibit the production of PVD. Although a variety of NMs have been demonstrated to exhibit excellent bactericidal activities (Klaine, Alvarez et al. 2008, Marambio-Jones and Hoek 2010), NMs that can effectively attenuate bacterial public goods production at sublethal concentrations have never been reported.

3.2 Materials and methods

3.2.1 Biosynthesis of TeNRs by *S. oneidensis*

Stock cultures of *S. oneidensis* MR-1 were maintained in LB medium with 20% glycerol at -80°C. The bacterial cells were grown in LB medium containing 100 µg/ml potassium tellurite. The cultures were bubbled with nitrogen gas for 5 min and the headspace in the culture was less than 3% of the total volume. After 72 h, cells were removed by centrifugation at 5,000g for 20 min. The supernatant was then concentrated using Millipore Amicon centrifugal filter units (MWCO ~3 kDa). Te nanorods (TeNRs) were harvested by using ultracentrifugation at 100,000g for 30 min, washed three times with MilliQ water and dried at 50°C under vacuum.

After an overnight exposure to TeO_3^{2-} , *S. oneidensis* cell suspension was placed on a glass microscope slide and dried in an oven at 70°C. The dried sample was then stained using 4'-

6- diamidino-2-phenylindole (DAPI). Briefly, 5 μ l of DAPI solution (1 mg/mL) was dropped onto the sample and incubated at room temperature, in the dark, for 45 min. The glass slide was then rinsed with ultrapure water to remove excess DAPI. The stained samples were examined using an inverted epifluorescence microscope (Microscope Axio Observer.Z1, Carl Zeiss). Sample for Transmission electron microscopy (TEM) images and energy dispersive X-ray spectroscopy (EDX) were prepared by drying a drop of TeNRs solution on a carbon-coated copper grid (Shahjamali, Bosman et al. 2012).

3.2.2 Inductively coupled plasma mass spectrometry

To determine the dissolution of TeO_3^{2-} from the biogenic TeNRs, the suspension of 100 μ g/ml TeNRs in ABTGC medium was filtered through a centrifugal filter unit (molecular weight cutoff 3 kDa; Amicon Ultra-4 3K). The total concentration was quantified using an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-e, Perkin Elmer; Waltham, MA) as described previously (Klonowska, Heulin et al. 2005, Kim, Kanaly et al. 2012).

3.2.3 Quantification of pyoverdine in *P. aeruginosa* cultures

P. aeruginosa PAO1 wild type and mutant strains were cultivated at 37°C in ABT minimal medium (15 mM $(\text{NH}_4)_2\text{SO}_4$, 40 mM Na_2HPO_4 , 20 mM KH_2PO_4 , 50 mM NaCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 and 0.01 mM FeCl_3) supplemented with 30 mM glucose and 5 g/l casamino acids (ABTGC) (Chua, Tan et al. 2013) in the absence or presence of TeNRs at different concentrations. Pyoverdine fluorescence (Ex 398 nm, Em 460 nm) (Greenwald, Hoegy et al. 2007) and optical density at 600 nm (OD_{600}) were recorded using a TECAN infinite M200PRO plate reader. All the fluorescence measurements were carried out together with cell-free controls to rule out the abiotic effects of TeNRs. In addition, conditioned media (cell-free supernatant of overnight cultures) supplemented with TeNRs were used as another set of controls to check for the interference of TeNRs on the fluorescence measurements.

3.2.4 PvdA-gfp assay

P. aeruginosa strain containing *p_{pvdA-gfp}* reporter (Kaneko, Thoendel et al. 2007, Yang, Nilsson et al. 2009) was cultivated in ABTGC medium at 37°C in the absence or presence of TeNRs at different concentrations. GFP fluorescence (Ex 395 nm, Em 509 nm) and OD₆₀₀ were measured using a TECAN infinite M200PRO plate reader.

3.2.5 Quantitative Polymerase Chain Reaction (qPCR)

P. aeruginosa PAO1 was cultivated in ABTGC medium at 37°C in the absence or presence of TeNRs (50 µg/ml). After 9 h, 0.5 ml of the culture was mixed with 1 ml of RNA protect (Qiagen mini RNA prep) and centrifuged at 6,000g for 10 min. Total RNA content was extracted using a commercially available kit (Qiagen mini RNA prep) following the instructions from the manufacturer. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, DE, USA). Two µg of the total RNA was used for first-strand cDNA synthesis by using a commercially available kit (Fermentas Life Sciences, EU). The resultant cDNA was used in qPCR reactions on ABI StepOnePlus system (Life Technologies, CA). Primers (efficiency >90% for all primers) were obtained from Sigma Life Sciences and their sequences are listed in Table 3.1. PCR was carried out with 1 µl of first-strand cDNA in a total volume of 20 µl containing 0.2 mM of each primer, 10 µl of PCR KAPA SYBR® FAST qPCR Master Mix ABI (2X) Prism™, 0.4 µl ROX high. Amplification parameters used were: initial activation 3 min at 95°C, then 40 cycles of 3 s at 95°C and 30 s at respective appropriate annealing temperature. Experiments were performed in triplicates for all the genes. The amount of each target gene was normalized to a reference gene *tsf* and compared to control samples (Xie, He et al. 2011). Data analysis was performed using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{untreated sample})$, $\Delta C_T = C_T(\text{target gene}) - C_T(\text{tsf})$, and C_T is the threshold cycle value for the amplified gene (Livak and Schmittgen 2001).

Table 3.1 PCR primer details.

Gene name	Forward primer	Reverse primer	Product length	Annealing temperature (°C)
<i>tsf</i>	CCTTCAACGAGAAG CTGACC	CTTTCAGGTTGAC CACGACA	180 bp	54.2
<i>pvdA</i>	GGGCGCCCTGGAA GTGCTGTT	GCTCTGCCCCGCCG CCGATAATG	575 bp	64.0
<i>fpvA</i>	ACACCGGCGAAAC CAAGAGCAACT	TAGACCGCGCCGA CGTAGGGAATG	388 bp	57.0
<i>pvdE</i>	CACCGCCCCCGTCG CTATCG	GGTCTTGTCCGGG GCCTCCTGTT	369 bp	58.3
<i>pvdN</i>	CGAGCGACGCCGG CAATAAATGG	GTTGCACGGCGAA GTAGCGACCTG	240 bp	57.6
<i>pvdS</i>	TCGGGGCCCGGAGG AAGAAGG	CGGCGGGCGCTGA GATGG	296 bp	62.7
<i>pvdT</i>	AACCGCATCGCCAT CCCCTACTCC	TCACGCCGATCCC GCCTACCAG	277 bp	62.7

3.2.6 iTRAQ-based proteomic analysis

Proteomic analysis was conducted using an iTRAQ-coupled two-dimensional LC-MS/MS technique as described previously (Zhou and Chen 2011). Briefly, cells of *P. aeruginosa* PAO1 grown in ABTGC medium in the absence or presence of 50 µg/ml TeNRs at late exponential growth phases were harvested by centrifugation at 3000g for 20 min at 4°C and lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris-HCl, pH 8.0) by intermittent sonication. Unbroken cells were removed by centrifugation at 3000 × g for 10 min at 4 °C. The supernatants containing the cytoplasmic proteins were collected by centrifugation at 120,000g for 60 min at 4°C. Protein concentration was determined by Bradford assay using γ-globulin as a control. Proteins from each sample were precipitated by cold acetone at −20 °C. The proteins (~100 µg) were reduced, cysteine blocked, digested and labeled with respective isobaric tags using iTRAQ reagent Multiplex kit (Applied Biosystems Inc., CA, USA) according to manufacturer's protocol. The analysis of iTRAQ-labeled peptide mixtures were performed on a combination of an Agilent 1200 nanoflow LC system (Agilent Technologies Inc., USA) and a 6530 Q-TOF mass spectrometer (Agilent Technologies Inc., USA). The identification and quantification of the proteins were performed using Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Software Revision A.03.03.084 SR4). Each MS/MS spectrum was searched for species *P. aeruginosa* against the UniProt protein database.

The following criteria were required to consider a protein for further statistical analysis: two or more distinct peptides had to be identified and the fold change had to be greater than 2 or less than 0.5.

3.2.7 Transposon mutagenesis library and mutant screening

The *P. aeruginosa* Δ *motB* and Δ *fliF* mutants were identified from a transposon mutagenesis library screen, which was constructed by using the Mariner transposon vector pBT20, as previously described (Wang, Chua et al. 2013). Transconjugants carrying transposon insertion were picked from the selective plates and inoculated into microtiter plate wells containing LB medium. Mutants that produced PVD at a comparable level in the presence and absence of 50 μ g/ml TeNRs were selected and saved for further analysis. For identification of the transposon insertion site, the sequence flanking the Mariner transposon in selected mutants was identified by arbitrary polymerase chain reaction (PCR), as previously described (Friedman and Kolter 2004).

3.2.8 Cytotoxicity assay of biogenic TeNRs

Human bronchial epithelial cells (BEAS-2B; ATCC, Manassas, VA, USA) and murine macrophages (RAW264.7; ATCC, Manassan, VA, USA) were used to test cytotoxicity of the biogenic TeNRs. Both cell types were cultured in complete DMEM (high glucose Dulbecco's Modified Eagle's Medium; PAA Laboratories Inc., MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% Antibiotics/Antimycotics (PAA Laboratories) at standard culture conditions (37°C, 5% CO₂) and sub-cultured in the ratio of 1:2 to 1:4 at 90% confluency. All cells were passaged at least 3 times before using for the cytotoxicity experiments. Cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8/WST-8; Dojindo Molecular Laboratories Inc., Japan) following the protocols provided by the manufacturer. Cells were seeded in 96-well plates at a seeding density of 10⁴ cells per well. After 24 h incubation, the culture medium was replaced by freshly prepared TeNRs suspension in the cell culture medium and incubated for another 22 h. 10 μ l of CCK-8 was then added to each well and the cells were further incubated for 2 h after which the absorbance at 450 nm was measured. Wells containing cells and TeNRs suspension without CCK-8 was used as background.

3.2.9 Flow cell biofilms

Biofilm formation under hydrodynamic conditions was evaluated using multi-channel flow cells (BioCentrum-DTU, Denmark). The dimensions and assembly of the flow cell systems were described elsewhere (Crusz, Popat et al. 2012). Each channel of the flow cells was inoculated using 0.4 ml diluted overnight cultures in modified ABTG medium ($OD_{600} \sim 0.15$). After inoculation, the medium flow was stopped for initial attachment (~1 h) onto the glass slide used in the flow cells. Then air-saturated modified ABTG medium was continuously supplied with a flow rate of 6 ml/h for biofilm growth in each channel.

3.2.10 Confocal Laser Scanning Microscopy (CLSM) imaging

All CLSM image acquisitions were performed using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring of green fluorescence (488 nm) and cyan fluorescence (458 nm). Three-dimensional images and sections were generated using the IMARIS software package (Biplane AG, Zürich, Switzerland). 40X oil immersion lens were used for visualizing the biofilm morphology at 24 h, 72 h, 120 h, 132 h and 144 h. GFP fluorescence of the cells were observed with the excitation at 488 nm and emission at 509 nm. PVD fluorescence was observed with the excitation and emission at 405 nm and 461 nm, respectively. After 120 h, TeNRs were introduced to the biofilms along with the media at a concentration of 50 $\mu\text{g/ml}$. Confocal images were taken after 12 h and 24 h of TeNRs addition.

3.2.11 Analysis of CLSM images.

The CLSM images were analysed using IMARIS software (version 7.6.4; Bitplane, Zurich, Switzerland). The structure of macrocolonies was reconstructed from Z-series images.

3.2.12 RNA extraction from biofilms

Silicon tubing (Masterflex L/S 16, *i.e.*, 3.2 mm) of 20 cm long was used to grow biofilms for RNA extraction. Biofilms were grown for 120 h and then media was changed to ABTG supplemented with TeNRs at a concentration of 50 $\mu\text{g/ml}$. Biofilms were harvested

after 24 h exposure to TeNRs. Biofilm biomass obtained after 144 h growth in ABTG medium without TeNRs was used as the control. RNA was extracted from all the samples using commercially available Qiagen mini RNA prep kits following manufacturer's instructions (RNeasy Mini Kit, Qiagen). Experiments were conducted in triplicates.

3.2.13 RNA sequencing

Quality of the RNA samples was determined using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on a Bioanalyzer RNA 6000 Nano Chip (Agilent). Next-generation sequencing library preparation was performed by following TruSeq RNA Sample Preparation v2 protocol (Illumina) with modifications: The mRNA purification step was omitted and instead, 200 ng of total RNA was directly added to the elute-fragment-prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely tagged with one of Illumina's TruSeq LT RNA barcodes to allow library pooling for sequencing. Library quantitation was performed using Invitrogen's Picogreen assay and the average library size was determined by running the libraries on a Bioanalyzer DNA 1000 chip (Agilent). Library concentration was normalized to 2 nM and the concentration was validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol and Illumina's PhiX control library as a standard. Libraries were then pooled at equal volumes and sequenced in two lanes of an Illumina HiSeq2500 rapid run at a final concentration of 7.2 pM and a read-length of 101 bp paired-end.

3.2.14 Computational analysis

The Illumina RNA sequencing raw data were deposited in the NCBI Sequence Read Archive database (<http://www.ncbi.nlm.nih.gov/sra>) with an accession number SRP045514. The sequence reads were assembled and analysed in RNA-Seq and expression analysis application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark).

The

P.

aeruginosa

PA01

genome

(<http://www.ncbi.nlm.nih.gov/nuccore/110645304>) was utilized as the reference genome for the assembly. The following criteria were used to filter the unique sequence reads: minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. Data were normalized by calculating the reads per kilobase per million mapped reads (RPKM) for each gene (Mortazavi, Williams et al. 2008). Results were annotated using databases PseudoCAP (<http://www.geneontology.org/GO.current.annotations.shtml>) and www.pseudomonas.com. Statistical analyses ANOVA and t-test were performed. The fold change ratio (R) of each gene expression was calculated as the ratio of RPKM in treated sample to that of the control.

3.3 Results and discussion

3.3.1 Biogenic TeNRs from TeO_3^{2-} reduction by *S. oneidensis*

TeNRs have been successfully prepared via physicochemical routes including chemical reduction and photolytic preparation (Liu, Hu et al. 2004, Zhu, Wang et al. 2004, Gao, Lu et al. 2008, Webber and Brutchey 2009). The unique helical chain conformation of Te crystals typically leads to a highly anisotropic growth to form one-dimensional (1D) structures such as nanorods (Webber and Brutchey 2009). TeNRs can also be produced by bacteria grown on Te oxyanions such as tellurite (TeO_3^{2-}), which offers a “green” approach for the synthesis of Te nanomaterials under facile conditions in the absence of organic solvents. For example, although TeO_3^{2-} is highly toxic to most microorganisms and has been used as an antimicrobial agent for decades (Turner, Borghese et al. 2012), *S. oneidensis* has been reported to be able to reductively transform TeO_3^{2-} to Te (0) in the form of rod-like nanostructures, *i.e.*, TeNRs, intracellularly and at the cell exterior (Klonowska, Heulin et al. 2005, Kim, Kanaly et al. 2012).

TeNRs used in this study were obtained through microbial reduction of TeO_3^{2-} by *S. oneidensis* using lactate as an electron donor. It was observed that black precipitates, presumably aggregates of TeNRs, at the cell exterior upon the exposure of *S. oneidensis* cells to TeO_3^{2-} (Figure 3.1A). TEM-EDX showed that the biogenic TeNRs obtained here were about 10-20 nm wide, up to several hundreds of nm long, and were comprised

entirely of Te (Figure 3.1B and C). In this study, focus was on the novel antivirulence activity of the biogenic TeNRs and further detailed material characterization of the TeNRs was not included. In fact, the reduction of TeO_3^{2-} by *S. oneidensis* has been reported in literature and the Te(0) nanorods obtained from bacterial reduction of TeO_3^{2-} under similar experimental conditions have been extensively characterized (Klonowska, Heulin et al. 2005, Kim, Kanaly et al. 2012)

Figure 3.1 (A) Extracellular precipitates of TeNRs (black) in a suspension of cells (blue) visualized by overlay of bright-field and fluorescence imaging. Cells were stained with DAPI. (B) TEM image and (C) EDX spectrum of the biogenic TeNRs.

3.3.2 Inhibition of PVD biosynthesis by biogenic TeNRs

In an effort to elucidate impacts of the biogenic TeNRs on microorganisms, *P. aeruginosa* was grown in the presence of TeNRs. Interestingly, TeNRs (up to 100 $\mu\text{g/ml}$) have no negative impact on bacterial growth, as evidenced by comparable or even slightly higher specific growth rate for all treated cultures, compared to the controls (Table 3.2), suggesting that the biogenic TeNRs do not exhibit bactericidal activity against *P. aeruginosa*.

Table 3.2 Influence of TeNRs on growth of *P. aeruginosa* PAO1 in ABTGC media at 37°C. Specific growth rate was determined from $\mu = d \ln (OD_{600})/dt$, where OD_{600} was the optical density at a wavelength of 600 nm. Values are means $\pm$ standard deviation (n=3).

Concentration of TeNRs ($\mu\text{g/ml}$)	Specific growth rate (h^{-1})
0	0.42 ± 0.04
25	0.49 ± 0.09
50	0.49 ± 0.04
100	0.50 ± 0.05

Chemically synthesized Te nanomaterials with different structures such as nanorice, nanopencil and nanowire have been reported to exhibit excellent antibacterial activity because of the oxidative dissolution of TeO_3^{2-} from the Te nanomaterials (Lin, Lee et al. 2012).

Here, the biogenic TeNRs did not show bactericidal activity, possibly due to a high stability and resistance to molecular oxygen of the biogenic TeNRs. To confirm this, ICP-MS was used to quantify the concentration of TeO_3^{2-} in the ABTGC medium with the presence of 100 $\mu\text{g/ml}$ TeNRs but no TeO_3^{2-} could be detected after 24 h. The resistance to oxidative dissolution and the lack of bactericidal activity of the biogenic TeNRs open a new window of novel applications other than being used as a bactericidal agent. Although the biogenic TeNRs did not exhibit bactericidal activity against *P. aeruginosa*, the fluorescence of pyoverdine (PVD) was significantly lower in the cultures with TeNRs compared to those without TeNRs at both exponential and stationary phases of growth, and the attenuation of PVD fluorescence by TeNRs occurred in a concentration dependant manner (Figure 3.2).

Figure 3.2 Influence of TeNRs (0, 25, 50, 100 µg/ml) on PVD fluorescence (normalized by cell density) in *P. aeruginosa* PAO1 cultures growing in ABTGC media in the exponential (6 h) and stationary phase of growth (12 h). Data shown are mean $\pm$ standard deviation (n=3)

The attenuation of PVD fluorescence by TeNRs suggests a potential for using TeNRs as a novel antivirulence agent by targeting PVD production. To further investigate whether the attenuation of PVD fluorescence by TeNRs was because of the inhibition of the production of PVD in the cultures, a *pvdA::gfp* reporter strain was employed which allowed the biosynthesis of PVD to be monitored based on GFP fluorescence. In the presence of TeNRs, the GFP fluorescence in the cultures of the reporter strain, in both exponential and stationary phases of growth, was significantly lower than for cultures without TeNRs (Figure 3.3 a), suggesting that the TeNRs inhibited the expression of *pvdA* gene and, hence, PVD biosynthesis. Quantitative polymerase chain reaction (qPCR) analysis revealed that the expression levels of several other key PVD biosynthesis genes in *P. aeruginosa* exposed to 50 µg/ml of TeNRs were also significantly lower than the no-TeNRs controls (Figure 3.3 b), confirming the inhibitory effect of TeNRs on the biosynthesis of PVD in *P. aeruginosa*.

Figure 3.3 (a) Influence of TeNRs (0, 25, 50, 100 µg/ml) on GFP fluorescence (normalized by cell density) in *P. aeruginosa* reporter strain *pvdA-gfp* growing in ABTGC media at the exponential phase (6 h) and stationary phase (12 h) of growth. (b) Influence of TeNRs (50 µg/ml) on the expression level of key genes involved in PVD production and uptake. Values were normalized to the housekeeping gene *tsf*. PvdS: a transcriptional regulator; PvdE: an export ABC transporter to transport PVD precursors across the inner membrane; PvdN and PvdP: periplasmic enzymes involved in chromophore formation; PvdQ: a periplasmic enzyme for removal of fatty acid chains from PVD precursors; PvdT: a subunit of the ATP-dependent efflux pump for PVD secretion; FpvA: an outer membrane transporter for import of ferri-pyoverdine. Data shown are mean $\pm$ standard deviation (n=3).

The inhibition of PVD biosynthesis by biogenic TeNRs at the transcriptional level was further validated at the protein level through a proteomics approach. Among the proteins

differently expressed in *P. aeruginosa* in the presence or absence of TeNRs were siderophore-related proteins PvdA, FpvA, FptA and PchB (Table 3.3).

Table 3.3 Down-regulation of proteins involved in siderophore production and uptake in *P. aeruginosa* PAO1 in the presence of biogenic TeNRs (50 µg/ml). ^aThe percentage of matching amino acids from identified peptides divided by the total number of amino acids in the sequence; ^bThe ratio of protein production level in biogenic TeNRs-incubated cells to control cells without TeNRs.

Locus tag	Protein	%Cov ^a	Ratio ^b
PA2386	L-ornithine 5-monooxygenase, PvdA	40	0.50
PA2398	Ferri-pyoverdine receptor, FpvA	17	0.32
PA4221	Ferri-pyochelin receptor, FptA	8	0.45
PA4230	Salicylate biosynthesis protein, PchB	33	0.4

Consistent with the qPCR data, both PvdA and FpvA, the key proteins involved in PVD biosynthesis and ferri-PVD uptake, respectively, were down-regulated in the presence of biogenic TeNRs. Interestingly, PchB and FptA, the proteins that play an important role in pyochelin biosynthesis and ferri-pyochelin uptake, were also found down-regulated, suggesting that the biogenic TeNRs also inhibit the production of pyochelin in *P. aeruginosa*. In addition to PVD, pyochelin is another major siderophore that *P. aeruginosa* produces to meet its need for iron. Although PVD appears to be more essential than pyochelin in iron depleted conditions, both PVD and pyochelin are considered to be virulence factors of *P. aeruginosa*. The inhibitory effects of the biogenic TeNRs on the production of both PVD and pyochelin render them a promising antivirulence agent against *P. aeruginosa*.

3.3.3 Polysaccharides, amyloids, and flagellar motility in cell-TeNRs interaction

The inhibitory effect of TeNRs on PVD biosynthesis led us to address how TeNRs at the cell exterior inhibit intracellular gene expression. In *P. aeruginosa*, two major extracellular polysaccharides Pel and Psl as well as functional amyloids Fap have been demonstrated to play important roles protecting bacteria from various unfavourable conditions (Dueholm, Petersen et al. 2010, Colvin, Gordon et al. 2011, Mann and Wozniak 2012, Billings, Millan et al. 2013). In addition, the polysaccharide Psl has been

reported to be able to act as a signal to stimulate biofilm formation of *P. aeruginosa* (Irie, Borlee et al. 2012). To test whether the extracellular polysaccharides and amyloids are involved in the interaction between TeNRs and *P. aeruginosa*, the production of PVD was monitored in mutant strains lacking extracellular polysaccharides ($\Delta pelA$, $\Delta pslBC$, $\Delta pelA\Delta pslBCD$) or amyloids ($\Delta fapC$) in the presence and absence of TeNRs. The results showed that the lack of extracellular polysaccharides Pel and Psl or amyloids did not abolish the inhibitory effect of TeNRs on PVD production (Figure 3.4), suggesting that the inhibition of PVD production by TeNRs is not mediated by extracellular polysaccharides and amyloids.

Figure 3.4 PVD fluorescence (normalized by cell density) in 12 h cultures of *P. aeruginosa* PAO1 wild type and mutants lacking polysaccharides or amyloids in ABTGC media with (50 µg/ml) or without TeNRs. Data shown are mean $\pm$ standard deviation (n=3).

Further the interaction of TeNRs with the mutants in a transposon mutagenesis library was tested. Interestingly, inhibition of PVD production could not be observed for mutant strains $\Delta motB$ and $\Delta fliF$ (Figure 3.5). MotB and FliF are proteins involved in flagella mediated motility and it has been reported that the mutants lacking MotB or FliF has a significantly compromised motility (Grünenfelder, Gehrig et al. 2003, Doyle, Hawkins et al. 2004, Chevance and Hughes 2008). As an important appendage, bacterial flagella not

only generate motility but can also sense physical properties of the local environment. For example, flagella have been reported to be used by *Vibrio parahaemolyticus* to sense surface contact, which triggers a programme of gene expression promoting colonization and virulence (Gode-Potratz, Kustusch et al. 2011). Here, the abolishment of the inhibitory effect of TeNRs on PVD production in the motility-deficient mutants implies an involvement of flagellar movement in the cell-TeNRs interaction. The molecular mechanism by which the interaction between biogenic TeNRs and flagellar movement negatively influences PVD biosynthesis, however, remains to be identified.

Figure 3.5 PVD fluorescence (normalized by cell density) in 12 h cultures of *P. aeruginosa* PAO1 wild type and motility-deficient mutants $\Delta motB$ and $\Delta fliF$ in ABTGC media with (50 $\mu\text{g/ml}$) or without TeNRs. Data shown are mean $\pm$ standard deviation (n=3).

3.3.4 Cytotoxicity of biogenic TeNRs

It was demonstrated that the production of PVD in *P. aeruginosa* could be inhibited by biogenic TeNRs. To further demonstrate the clinical potential of the biogenic TeNRs, CCK-8 viability assay was carried out to evaluate their cytotoxicity by using human bronchial epithelial cells BEAS-2B and murine macrophages RAW264.7. The presence of TeNRs (up to 100 $\mu\text{g/ml}$) did not influence the metabolic rate of BEAS-2B and RAW264.7 cells, suggesting a lack of cytotoxicity by the TeNRs against mammalian cells

(Figure 3.6). Thus, the biogenic TeNRs hold a promise as a novel no-biocidal antivirulence agent against *P. aeruginosa* and possibly other bacterial pathogens.

Figure 3.6 CCK-8 viability assay for RAW cells and BEAS-2B cells in the presence of TeNRs at different concentrations: 0, 25, 50, 100 µg/ml. Metabolic rate is shown as the dehydrogenase activity which reduces WST-8 salt to formazon dye. Untreated cells were used as controls. Data shown are mean $\pm$ standard deviation (n=3).

3.3.5 TeNRs reduce PVD production in *P. aeruginosa* biofilms

Biofilm mode of growth is predominant in environmental microorganisms. To study the impacts of TeNRs on siderophore production in biofilms, the GFP-tagged *P. aeruginosa* strain PAO1-*gfp* was grown in flow cell biofilm reactors and monitored the spatiotemporal production of PVD in biofilms. The production of PVD and the biofilm development in the flow cells examined using CLSM are shown in Figure 3.7.

Figure 3.7 Distribution of PVD in *P. aeruginosa* biofilms. Biofilms were grown using GFP-labeled *P. aeruginosa* for 5 days in modified ABTG medium. CLSM images contain top-down views (x-y planes) and side views (x-z, y-z planes). Scale bar 20 μm . Results are representative of three separate experiments.

At the initial stage of biofilm growth (24 h), scattered cells attached to the bottom of the flow cells, while nearly no PVD fluorescence could be detected. After 72 h, surface coverage of $\sim 44\%$ was achieved with an average biofilm thickness of $\sim 5 \mu\text{m}$ and cell aggregates or microcolonies (up to $20 \mu\text{m}$ in diameter) could be observed. At this stage, although most cells co-localized with PVD, the PVD fluorescence was highly intensified in microcolonies than that of scattered cells. In the 5-day old (120 h) biofilms of *P. aeruginosa*, mushroom-shaped multicellular structures (up to $80\text{--}100 \mu\text{m}$ in diameter and $40\text{--}50 \mu\text{m}$ in height) could be observed, which has been reported as the feature of mature biofilms for *P. aeruginosa* (Stoodley, Sauer et al. 2002). In mature *P. aeruginosa* biofilms,

it was found that PVD fluorescence could only be observed in the thin “lawn” of the biofilm and the stalk surface of the mushroom-shaped structures, which is in agreement with previous reports on the spatially resolved expression of *pvdA*, a key gene in PVD biosynthesis (Kaneko, Thoendel et al. 2007, Yang, Nilsson et al. 2009).

Highly heterogeneous 3-dimensional (3-D) structures of mature biofilms often pose a challenge to diffusion of chemicals in biofilms, limiting the efficacy of conventional biocides such as antibiotics (Costerton, Lewandowski et al. 1995, Stewart and William Costerton 2001, Drenkard 2003, Stewart 2003). Diffusion of dispersed NMs in biofilms is even more problematic (Peulen and Wilkinson 2011). Here it was shown that PVD in mature *P. aeruginosa* biofilms mainly present in the thin bacterial “lawn” of the biofilms and the stalk surface of 3-D mushroom structures, suggesting that diffusion may not limit the interaction of NMs with the cells that are active in PVD production.

Because the 3-D mushroom-shaped structures, a key feature of a mature biofilms for *P. aeruginosa*, were observed in 5-day old biofilms under the above experimental conditions, TeNRs were introduced to the biofilms at 120 h to investigate the interaction between TeNRs and mature biofilms. Figure 3.8 shows the influence of biogenic TeNRs on PVD production in mature *P. aeruginosa* biofilms. As shown in Figure 3.8, PVD in the control biofilms was mainly observed in the thin bacterial “lawn” and the stalk surface of 3-D mushroom structures.

Figure 3.8 Biogenic TeNRs reduce PVD production in mature *P. aeruginosa* biofilms. a–b CLSM images of 5-day-old GFP-labeled *P. aeruginosa* biofilms upon exposure to TeNRs (50 µg/ml) for 12 h (A) and 24 h (B). The control biofilms were grown in parallel with the TeNR-treated biofilms in different flow cell channels. Controls in a and b were biofilms grown in the absence of TeNRs for 144 h (120 h + 12 h) and 154 h (120 h + 24 h), respectively. C–D Biovolume ratio of PVD-producing biomass to total biofilm biomass, indicating the amount of PVD in unit biovolume of the biofilms. CLSM images contain top-down views (x-y planes) and side views (x-z, y-z planes). Scale bar 20 µm. Results are representative of three separate experiments.

After exposing to TeNRs for 12 h the biofilm showed a markedly lower PVD fluorescence, while little or no PVD fluorescence could be detected in the biofilm with a 24 h exposure to TeNRs. Quantitative image analysis further revealed that the PVD-

producing biovolume fraction in mature biofilms decreased from 67% to 33% and from 95% to 3% after 12 h and 24 h treatment, respectively (Figure 3.8 C and D).

Intriguingly, although PVD production in mature biofilms was significantly inhibited by TeNRs, biofilm structures were still maintained, suggesting that TeNRs do not trigger massive cell detachment. To confirm this, cell detachment rate were quantified for the control and treated biofilms (Figure 3.9 A). Cell detachment rate for both the control and treated biofilms was about 10^8 - 10^9 CFU $\cdot$ h $^{-1}$ $\cdot$ mm $^{-2}$. Although the fluorescence intensity of PVD in the effluent of the biofilms exposed to TeNRs was found significantly lower (Figure 3.9 B), there was no significant difference in cell detachment from the control and treated biofilms ($p = 0.145$). This result implies that, when exposed to the TeNRs, cells in biofilms decrease PVD production and most likely remain in the biofilms, creating local microenvironments lacking PVD, which will have a great impact on the structure and function of the microbial communities in the microenvironments.

Figure 3.9 (A) Cell detachment rate from 5-day-old *P. aeruginosa* biofilms during 24-h further growth in modified ABTG medium with or without TeNRs (50 μ g/ml). Experiments were carried out with at least three replicates for each condition. Paired Student's t test, $p = 0.145$. (B) PVD fluorescence in effluents from 5-day-old *P. aeruginosa* biofilms during 24-h further growth in modified ABTG medium with or without TeNRs (50 μ g/ml). Experiments were carried out in triplicate.

3.3.6 Transcriptomic changes

The results show that TeNRs significantly inhibit PVD production in mature biofilms of *P. aeruginosa* while do not disrupt biofilm structures. To further investigate the

mechanism of action, the effect of TeNRs was examined on gene expression profiles of *P. aeruginosa* cells in mature biofilms through a RNA-seq based transcriptomic approach. Genes involved in biosynthesis and transport of siderophores were of particular interest. The exposure to TeNRs resulted in a significant decrease in the expression of genes involved in siderophore production (Table 3.4).

Table 3.4 Siderophore related genes that are down regulated in treated samples compared to control.

Annotation	Gene	Gene Product	Log ₂ (R)
PA0781	-	Outer membrane receptor proteins, mostly Fe transport	-5.7
PA4837	-	Outer membrane receptor proteins, mostly Fe transport	-5.14
PA1922	-	Outer membrane receptor for ferrienterochelin and colicins	-4.41
PA4224	<i>pchG</i>	Pyochelin biosynthetic protein PchG	-2.93
PA2386	<i>pvdA</i>	L-ornithine N5-oxygenase	-2.66
PA2424	<i>pvdL</i>	PvdL	-2.48
PA2413	<i>pvdH</i>	PvdH	-2.37
PA4230	<i>pchB</i>	Salicylate biosynthesis protein PchB	-2.28
PA4218	<i>ampP</i>	Siderophore transporter activity	-2.25
PA4225	<i>pchF</i>	Pyochelin synthetase	-2.2
PA4231	<i>pchA</i>	Salicylate biosynthesis isochorismate synthase	-2.17
PA4226	<i>pchE</i>	Dihydroaeruginosic acid synthetase	-2.12
PA2400	<i>pvdJ</i>	PvdJ	-2.02
PA2399	<i>pvdD</i>	Pyoverdine synthetase D	-1.83
PA4288	<i>pchD</i>	Pyochelin biosynthesis protein PchD	-1.82
PA2395	<i>pvdO</i>	Pyoverdine biosynthetic process	-1.82
PA4229	<i>pchC</i>	Pyochelin biosynthetic protein PchC	-1.76
PA2392	<i>pvdP</i>	PvdP	-1.64
PA2385	<i>pvdQ</i>	PvdQ	-1.52
PA2425	<i>pvdG</i>	PvdG	-1.43

PA2390	<i>pvdT</i>	ATP-binding cassette (ABC) transporter complex	-1.41
PA4221	<i>fptA</i>	Fe(III)-pyochelin outer membrane receptor precursor	-1.22
PA2397	<i>pvdE</i>	Pyoverdine biosynthesis protein PvdE	-0.99
PA2396	<i>pvdF</i>	Pyoverdine synthetase F	-0.86
PA2398	<i>fpvA</i>	Ferripyoverdine receptor	-0.75

The production of PVD in *P. aeruginosa* involves biosynthesis of PVD precursors in the cytoplasm (Schalk and Guillon 2013), the transport of the precursors into the periplasmic space where the precursors are enzymatically modified to PVD (Yeterian, Martin et al. 2010, Schalk and Guillon 2013), and the export of PVD through an efflux (Hannauer, Yeterian et al. 2010) system. Genes responsible for the biosynthesis of PVD precursors (*pvdL*, *pvdJ*, *pvdD*, *pvdH*, and *pvdA*), transferring PVD precursors from cytoplasm to the periplasmic space (*pvdE*), maturation of PVD precursors in the periplasmic space (*pvdQ*, *pvdO*, and *pvdN*), and exporting mature PVD out of the cells (*pvdT*) were found down-regulated in the biofilm cells treated with TeNRs. Interestingly, it was found that, in addition to PVD related genes, several genes involved in the synthesis of pyochelin (*pchA*, *pchB*, *pchC*, *pchD*, *pchE*, and *pchF*), another important siderophore in *P. aeruginosa* for iron uptake, were also significantly down-regulated. In addition, the expression of two genes encoding siderophore-binding proteins (*fptA* and *fpvA*) also decreased in the biofilm cells treated with TeNRs.

The decreased expression of the siderophore genes in the biofilms suggests that TeNRs inhibited the production of iron-chelating siderophores PVD and pyochelin in mature biofilms at the transcriptional level. The molecular mechanism by which the TeNRs negatively influences the transcription of siderophore genes in the biofilms, however, remains elusive and merits further investigations.

3.4 Conclusions

Using *P. aeruginosa* as a model environmental bacterium, it was shown that TeNRs generated from TeO_3^{2-} inhibits the production of PVD, a siderophore plays an important role in shaping environmental microbial communities, promoting plant growth, and causing infections. This study reports for the first time that TeNRs generated from metal(loid)s can alter function of environmental bacteria, providing a novel insight into the environmental impacts of NMs. In addition, most ecotoxicity studies were conducted using engineered NMs while the impacts of TeNRs that may be generated in the environment have never been explored. The results also provide a novel aspect of the environmental impacts of NMs.

CHAPTER 4. Impact of Sublethal levels of Single-Wall Carbon Nanotubes on *P. aeruginosa* and its Environmental Implications

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4.1 Introduction

In Chapters 3, it was shown the inhibition of PVD production in *P. aeruginosa* planktonic cultures and mature biofilms by biogenic TeNRs. Since the biogenic TeNRs used in Chapters 3 were produced by bacteria, whether commonly used engineered nanomaterials (ENMs) can cause similar impacts on bacterial functions still remains. This chapter focuses on the sublethal impacts of one of the commonly used ENMs on *P. aeruginosa* as a model environmental microorganism.

Carbon nanotubes (CNTs) are long, hollow cylindrical nanostructures wrapped up by graphene sheets (Iijima 1991). Based on the number of graphene sheets present, CNTs can be catalogued as single-wall CNTs (SWCNTs) and multi-wall CNTs (MWCNTs). CNTs have many potential applications in various fields due to their unique physical, electrical and mechanical properties. For example, CNTs can be used in environmental application as sensors, sorbents, filters for removing pathogens and viral particles (Brady-Estévez, Kang et al. 2008, Mauter and Elimelech 2008, Arias and Yang 2009), in biomedical applications such as detection, imaging and drug delivery (Kam, O'Connell et al. 2005, Liu, Tabakman et al. 2009, Lu, Gu et al. 2009), in high strength composites, energy storage devices (Baughman, Zakhidov et al. 2002). SWCNTs have been demonstrated to exhibit stronger antimicrobial activities than MWCNTs (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009, Vecitis, Zodrow et al. 2010, Tiraferri, Vecitis et al. 2011).

The potential use of SWCNTs in commercial and industrial settings along with their strong antibacterial activities has raised serious concerns about their impacts on human health and the environment. Previous studies on the toxicity of SWCNTs mainly focused on human health and very recently some important work on their environment impacts have also been carried out (Helland, Wick et al. 2008). The SWCNTs in the environment will potentially impact organisms at all levels of the food chain. The impacts exerted on bacteria are of particular interest because these organisms are at low trophic level and play critical roles in natural and engineered ecosystems.(Peralta-Videa, Zhao et al. 2011) Most studies working on bacteria-SWCNT interactions focused on the toxicity to individual bacteria (Kang, Pinault et al. 2007, Liu, Wei et al. 2009). However, SWCNTs in the environment are expected to be present in low concentrations that may not significantly affect bacterial viability or growth (Petersen, Zhang et al. 2011). Little is known about the sublethal impacts of SWCNTs on bacterial functions, which is a critical gap toward a comprehensive understanding of the impacts of SWCNTs on ecosystems.

The objective of this study was to explore sublethal impacts of SWCNTs on environmental bacteria. Specifically, using *P. aeruginosa* as a model environmental bacterium, the impacts of SWCNTs was examined on the production of PVD, a “public goods” that has been implicated to play a critical role in environmental microbial communities.

4.2 Materials and methods

4.2.1 Synthesis of SWCNTs

SWCNTs were synthesized by arc-discharge method (AP-SWNT, tube diameter ~1.4 nm, Carbon Solutions, Inc. US) and purified by a centrifugation-based method (Wei, Li et al. 2010). Surface functional groups were added by refluxing purified nanotubes in concentrated nitric acid (68%, Merck) for 3 h. The functionalized nanotubes were recovered by filtration and washed with 0.2 M sodium hydroxide solution (Merck) to remove small carbon debris generated during acid treatment (Wang, Shirley et al. 2009). The solid was then washed with ~500 ml distilled (DI) water to remove any adsorbed ions and dried in a vacuum oven overnight. Suspension of 1 mg/ml nanotubes was prepared by

tip sonication performed at 20W for 1 h in an ice-water bath (Sonics, VCX-130). Zeta-potential of the nanotube suspension was measured using a ZetaPALS particle size analyzer (Brooks Instrument, US). At pH 6.3 the zeta potential was -39.30 ± 1.02 mV.

4.2.2 Cell viability assay

Bacterial viability was tested by drop-plate method (Chen, Nace et al. 2003, Ding, Peng et al. 2014, Wu, Ding et al. 2014). *P. aeruginosa* PAO1 (ATCC 15692) cultures were grown in presence of SWCNTs (0, 40, 80 $\mu\text{g/ml}$) at 37°C for 24 h in a shaking incubator 200 rpm) (initial OD₆₀₀ ~0.08). For viability assay, samples were withdrawn in regular intervals and diluted for 10 to 10⁸ times and 10 μl of each dilution was dropped onto LB agar plates. Eight replicates for each dilution were used. After 24 h incubation, colonies were enumerated as colony forming unit (CFU) (Chen, Nace et al. 2003).

4.2.3 Quantification of PVD in *P. aeruginosa* cultures

PVD fluorescence (Greenwald, Hoegy et al. 2007) and optical density at 600 nm (OD₆₀₀) were recorded using a TECAN infinite M200PRO plate reader (please refer to section 3.2.3 for detailed method). Cell density was determined by a drop-plate based CFU count method (Ding, Peng et al. 2014). All the fluorescence measurements were carried out together with cell-free controls to rule out the abiotic effects of SWCNTs. In addition, conditioned media (cell-free supernatant of overnight cultures) supplemented with SWCNTs (40, 80 $\mu\text{g/ml}$) were used as another set of controls to check for the interference of SWCNTs on the fluorescence measurements.

4.2.4 Transcriptional assay for *pvdA*

The assay was performed according to the protocol mentioned in Section 3.2.4 of Chapter 3. The reporter strain was cultivated in ABTGC medium at 37°C with and without SWCNTs at different concentrations (0, 40, 80 $\mu\text{g/ml}$). TECAN infinite M200PRO plate reader was used to monitor GFP fluorescence (Ex 485 nm, Em 535 nm) and OD₆₀₀.

4.2.5 Flow cell biofilms

The influence of SWCNTs on biofilms was evaluated using multi-channel flow cells (BioCentrum-DTU, Denmark). The dimensions and assembly of the flow cell systems have been described elsewhere (Crusz, Popat et al. 2012, Zhang, Ng et al. 2014). Each channel of the flow cells was inoculated using 0.4 ml diluted overnight cultures of *P. aeruginosa* PAO1 in M9 minimal medium (48 mM Na₂HPO₄; 22 mM KH₂PO₄; 9 mM NaCl; 19 mM NH₄Cl; 2 mM MgSO₄; 0.1 mM CaCl₂ supplemented with 0.4% glucose) (OD₆₀₀ ~0.15). After inoculation, medium flow was stopped to allow the cells attach (~1 h) onto the glass coverslips used in the flow cells. Then air-saturated medium was continuously supplied with a flow rate of 8 ml/h for biofilm growth in each channel. After 96 h, SWCNTs were introduced to the biofilms along with the media at a concentration of 20 µg/ml.

4.2.6 Confocal Laser Scanning Microscopy (CLSM) imaging

Biofilms grown in the flow cell systems were imaged (20X lens) using a confocal laser scanning microscopy (CLSM) (Carl Zeiss Microscopy LSM 780) equipped with detectors and filter sets using the method described in Section 3.2.10, Chapter 3.

4.2.7 Quantification of cell detachment

Detachment of cells from the biofilms was quantified using a drop-plate method (Chen, Nace et al. 2003). Briefly, the effluents from the flow cells were collected in regular intervals and were serially diluted. Then, 6 replicates of 10 µl from each of selected dilutions were plated onto an LB agar medium. Colony forming units (CFU) were enumerated after overnight incubation at 37°C.

4.2.8 Inhibition of PVD biosynthesis by SWCNTs in *P. fluorescence*

P. fluorescence strain OE28.3 (Demot and Vanderleyden 1991) was grown in ABTGC medium at 30°C in presence of varying concentration of SWCNTs. PVD fluorescence (Ex 398 nm, Em 460 nm) (Greenwald, Hoegy et al. 2007) and optical density at 600 nm (OD₆₀₀) were recorded using a TECAN infinite M200PRO plate reader.

4.2.9 Quantitative Polymerase Chain Reaction (qPCR)

P. aeruginosa PAO1 was grown in ABTGC medium at 37°C in the absence or presence of SWCNTs (40 µg/ml). After 9 h, cells were harvested and total RNA was extracted using a commercially available kit (Qiagen mini RNA prep) following the instructions from the manufacturer. qPCR was performed using the method described in Section 3.2.5.

4.2.10 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

Cells were grown both in presence and absence of 40 µg/l SWCNTs in ABTGC media for 24 h and harvested by centrifugation at 6000g for 15 min. The cell pellets were lysed at 4°C in lysis buffer (0.1% SDS, 0.5 M TEAB, 50 mM protease inhibitor tablet) with intermittent vortexing and 15-20 min sonication on an MSE-Soniprep-150 Sonicator (Sanyo Gallenkamp, Leicestershire, UK) for 5 s with a 10 s cooling interval between each pulse. The lysate was filtered through a 0.2 µm syringe filter. The iron concentration was quantified using an ICP-OES (PerkinElmer Optima DV2000). To determine the leaching of iron from SWCNTs, iron in the suspension of 40 µg/l SWCNTs in the ABTGC medium was also measured.

4.2.11 Statistical analysis

The IBM SPSS (www.ibm.com/software/sg/analytics/spss/) was used for statistical analyses, including two-way ANOVA. Details of the statistical analyses can be found in respective result sections.

4.3 Results and discussion

4.3.1 SWCNTs inhibit PVD production

The fluorescence of PVD is often used to reflect PVD production in *P. aeruginosa* cultures because PVD is a water-soluble fluorescent siderophore ³¹. In the presence of SWCNTs, the signal of PVD fluorescence (normalized by growth as indicated by cell density CFU/ml) in *P. aeruginosa* cultures was attenuated significantly in a SWCNT-concentration dependent manner (Figure 4.1). PVD fluorescence was quantified for the conditioned media (cell-free culture supernatant) before and after supplemented with

SWCNTs and found no interference of SWCNTs on PVD fluorescence measurement. At a concentration of 40 or 80 $\mu\text{g/ml}$, SWCNTs caused a 51-70% or 85-96% decrease, respectively, in PVD production capability of *P. aeruginosa* cells in exponential (6-12 h) and stationary (15-24 h) growth stages. A two-way ANOVA was performed to investigate the effect of time and concentration. No significant effect from time or the time-concentration interaction was found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

Figure 4.1 Influence of SWCNTs on PVD fluorescence (normalized by cell density) in *P. aeruginosa* cultures growing in ABTGC medium. Data shown are means $\pm$ the standard deviation ($n = 3$). A two-way ANOVA shows that no significant effect from time or time-concentration interaction can be found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

A reduced growth of *P. aeruginosa* in the presence of SWCNTs was observed (Table 4.1), which is consistent with previous reports demonstrating an antibacterial activity of SWCNTs against *P. aeruginosa* (Kang, Pinault et al. 2007, Brady-Estévez, Kang et al. 2008). The minimal inhibitory concentration (MIC) of SWCNTs under the above experimental conditions was $\sim 200 \mu\text{g/ml}$. No cell growth could be observed at a dose of lethal levels ($>200 \mu\text{g/ml}$). In the presence of 40 or 80 $\mu\text{g/ml}$ SWCNTs, cell growth was

still observed, resulting in a final cell density of $1.25 \pm 0.33 \times 10^9$ and $1.18 \pm 0.24 \times 10^9$, respectively. Hence, a question arises: are there any sublethal impacts of SWCNTs on the cells? In particular, in this study whether SWCNTs affect the PVD production of viable cells was examined. To address this question, a biomarker strain that allows the monitoring of the expression of a key gene involved in PVD biosynthesis was employed.

Table 4.1 Density of viable cells in *P. aeruginosa* cultures with or without SWCNTs. Data shown are mean $\pm$ standard deviation (n=8).

SWCNTs ($\mu\text{g/ml}$)	Cell density (CFU/ml)		
	0 h	24 h	Ratio (24 h vs. 0 h)
0	$1.30 \pm 0.19 \times 10^7$	$1.15 \pm 0.26 \times 10^{11}$	8800
40	$1.77 \pm 0.27 \times 10^7$	$1.25 \pm 0.33 \times 10^9$	71
80	$1.80 \pm 0.21 \times 10^7$	$1.18 \pm 0.24 \times 10^9$	65

Gene *pvdA* codes an enzyme L-ornithine N5-oxygenase responsible for the hydroxylation of L-ornithine, which represents an early step in the biosynthesis of PVD in *P. aeruginosa* (Visca, Ciervo et al. 1994). To monitor PVD biosynthesis in viable cells, a reporter strain *P_{pvdA}-gfp* in which a gene encoding green fluorescent protein (GFP) was under control of the promoter of gene *pvdA* was employed and the GFP fluorescence was used as an indicator to monitor the biosynthesis of PVD. Figure 4.2 shows the GFP fluorescence (per CFU/ml) of the biomarker strain *P_{pvdA}-gfp* in planktonic cultures with or without SWCNTs. From a two-way ANOVA using the factors time and concentration, no significant effect from time or the time-concentration interaction was found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

Figure 4.2 Influence of SWCNTs on GFP fluorescence in *P. aeruginosa* reporter strain *pvdA-gfp* growing in ABTGC medium. Data shown are means $\pm$ the standard deviation ($n = 3$). A two-way ANOVA shows that no significant effect from time or the time–concentration interaction can be found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

In the presence of SWCNTs, GFP fluorescence was markedly lower than that of the cultures without SWCNTs, which suggests that the expression of gene *pvdA*, and hence, PVD biosynthesis, was significantly inhibited by SWCNTs. To confirm this, the expression of several key genes involved in PVD biosynthesis was quantified by using qPCR. Relative gene expression levels as impacted by SWCNTs at a concentration of 40 µg/ml are shown in Figure 4.3.

Genes responsible for the biosynthesis of PVD precursors (*pvdA*), transferring PVD precursors from cytoplasm to the periplasmic space (*pvdE*), maturation of PVD precursors in the periplasmic space (*pvdN*), and exporting mature PVD out of the cells (*pvdT*) were found down-regulated in the cells treated with SWCNTs (Figure 4.3). In addition, the expression of two genes encoding a transcriptional regulator (*pvdS*) and a siderophore-binding protein (*fpvA*) also decreased in the cells treated with SWCNTs (Figure 4.3).

Figure 4.3 Influence of SWCNTs (40 $\mu\text{g/ml}$) on the expression level of key genes involved in PVD production at 9 h in the midexponential growth phase. Values were normalized to the housekeeping gene *tsf*. The C_T values for *tsf* remained unchanged for the control and the treated cells (24.68 ± 0.92 and 23.84 ± 0.30 , respectively), suggesting that the expression of *tsf* is not affected by SWCNTs. Abbreviations: PvdS, transcriptional regulator; PvdE, export ABC transporter to transport PVD precursors across the inner membrane; PvdN, periplasmic enzymes involved in chromophore formation; PvdT, subunit of the ATP-dependent efflux pump for PVD secretion; FpvA, outer membrane transporter to import ferri-pyoverdine; PvdA, lysine/ornithine *N*-monooxygenase. Data shown are means $\pm$ the standard deviation ($n=3$). Statistical significance determined by a paired Student's *t* test ($p < 0.001$).

In *P. aeruginosa*, there are three well-characterized quorum sensing systems namely *las*, *rhl*, and *pqs* (Wilder, Diggle et al. 2011) which interact with each other and coordinate the expression of a number of genes including those responsible for PVD production (Stintzi, Evans et al. 1998, Hentzer, Wu et al. 2003, Williams and Cámara 2009). To further examined whether the inhibitory effect of SWCNTs on PVD production was because of an influence on bacterial quorum sensing signalling, by using qPCR the expression of the key quorum sensing genes *lasA*, *rhlA* and *pqsA* in the bacterial cultures were quantified with and without SWCNTs treatment. The results show that the presence of SWCNTs did not inhibit the expression of these quorum sensing genes (Figure 4.4). Compared with the cultures with no SWCNTs, the quorum sensing systems in the treated cultures were

slightly enhanced, suggesting that the influence of SWCNTs on quorum sensing is not the primary cause for the decreased PVD production.

Figure 4.4 The expression level of key quorum sensing genes *pqsA*, *rhlA* and *lasA* in the absence or presence of SWCNTs (40 $\mu\text{g/ml}$) as revealed by qPCR analysis. Values were normalized to the housekeeping gene *rpoD*. Data shown are mean $\pm$ standard deviation ($n=3$).

An intriguing question one would ask is how SWCNTs at cell exterior affect gene expression inside the cells. In previous studies, Dimpka et al. reported that CuO nanoparticles inhibited PVD production in *P. chlororaphis* O6, where Cu ion release has been implicated to play a key role (Dimpka, Mclean et al. 2012, Dimpka, McLean et al. 2012). In this study, it was shown that SWCNTs inhibit PVD production at the gene transcriptional level. Could it be because that SWCNTs release ions, in particular, iron to the growth media? Iron level in growth media is known to be a key factor influencing PVD biosynthesis. A high iron level of inhibits PVD production. In this study, the synthesis of SWCNTs did not involve iron and the absence of iron in the SWCNTs was also confirmed by elemental mapping using Transmission Electron Microscopy- Energy-dispersive X-ray spectroscopy (TEM-EDX) (data not shown). Further ICP-OES was employed to quantify iron concentrations in the media with or without SWCNTs and found that the presence of SWCNTs slightly decreased the iron levels in the media (0.89 ± 0.01 vs. 1.00 ± 0.02 μM , $p < 0.05$), which could be due to the adsorption of iron onto

the SWCNTs. Hence, the possibility of an additional iron supply from SWCNTs to the cultures can be ruled out. Interestingly, although SWCNTs inhibit PVD production, the iron level in the cells grown in the presence of SWCNTs was found slightly higher than that of the control cells (350 ± 1 vs. 322 ± 5 pmol/mg protein, $p < 0.05$). These results suggest that, in addition to PVD and hence, PVD-mediated iron uptake system, SWCNTs may also affect other iron uptake systems (Andrews, Robinson et al. 2003, Cornelis and Dingemans 2013).

Based on the data obtained in this study and previous work, the hypothesis is that certain extracellular appendages of the bacteria such as surface proteins, exopolysaccharides, pili, and flagella might be interacting with the SWCNTs and transmit the signals across cell membranes. However, the exact mechanism for PVD inhibition by SWCNTs requires further investigations.

4.3.2 SWCNTs reduce PVD production in *P. aeruginosa* biofilms

In natural environments, microorganisms are often found as surface- or interface-associated assemblies encased in a self-produced polymeric matrix known as biofilms (Hall-Stoodley, Costerton et al. 2004, Cao, Majors et al. 2012, Ding, Peng et al. 2014). In addition, it has been shown that PVD is an important factor influencing biofilm formation of *P. aeruginosa* (Banin, Vasil et al. 2005). Hence, the influence of SWCNTs on *P. aeruginosa* biofilms using a GFP-tagged strain in flow cell biofilm reactors was further examined. After 96 h, mushroom-shaped multicellular towers, typical 3D structures of mature *P. aeruginosa* biofilms, could be observed (Figure 4.5).

Figure 4.5 Mature biofilm structure showing PVD expression. Biofilms were grown using GFP-labeled *P. aeruginosa* for 4 days in M9 medium. CLSM images contain top-down views (x-y planes) and side views (x-z, y-z planes). Scale bar 10 μm . Results are representative of three separate experiment.

SWCNTs were then introduced to the pre-grown mature biofilms and PVD production in the biofilms was evaluated based on PVD fluorescence obtained from CLSM image analysis. Quantitative image analysis revealed that the PVD fluorescence (blue) in the mature biofilms (green) in the SWCNTs-treated (up to 48 h) and the control biofilms was comparable (Figure 4.6), suggesting no significant influence of SWCNTs on PVD production in mature *P. aeruginosa* biofilms (all $p > 0.05$). Intriguingly, in the presence of SWCNTs, the rate of cell detachment from the biofilms was higher and PVD fluorescence in the effluents from the SWCNTs-treated biofilms was lower than that of the control (Figure 4.7). Taken together, the results show that the exposure to SWCNTs for up to 48 h did not significantly influence PVD production in cells encased in mature biofilms; however, it promoted cell detachment from the biofilms and decreased PVD production of the detached cells.

Figure 4.6 CLSM images of mature *P. aeruginosa* biofilms upon exposure to SWCNTs (20 $\mu\text{g/ml}$) for (A) 12, (B) 24, and (C) 48 h. The control biofilms were grown in parallel with the SWCNT-treated biofilms. Controls in panels A–C were biofilms grown in the absence of SWCNTs for 108 h (96 h + 12 h), 120 h (96 h + 24 h), and 144 h (96 h + 48 h), respectively. (D) Biovolume ratio of PVD-producing biomass to total biofilm biomass, indicating the amount of PVD in the unit biovolume of the biofilms. CLSM images contain top-down views (x – y planes) and side views (x – z and y – z planes). The scale bar is 10 μm . Results are representative of three separate experiments.

Figure 4.7 (A) Cell detachment and (B) PVD fluorescence in effluents from mature *P. aeruginosa* biofilms in the absence or presence of SWCNTs (20 µg/ml). Experiments were conducted in triplicate.

4.3.3 SWCNTs inhibit PVD production in other *Pseudomonas* species

Production of siderophores is a highly conserved characteristic in many environmental bacteria, especially *Pseudomonas* species (Meyer, Geoffroy et al. 2002). For example, *P. fluorescence* is a plant root coloniser commonly found in soils and it protects plant roots from fungal infection by scavenging iron with the help of siderophores (Leong 1986, Preston, Bertrand et al. 2001). SWCNTs could also inhibit siderophore production in *P. fluorescence* in a concentration dependent manner (Figure 4.8), suggesting that the inhibitory effect of SWCNTs on siderophore production is not limited to the model

organism, *i.e.*, *P. aeruginosa*, used in this study and similar effects may be observed for many other environmental bacteria.

Figure 4.8 Concentration-dependent inhibitory effect of SWCNTs on PVD production in *P. fluorescence*. Relative fluorescence intensity (normalized by OD₆₀₀) indicates the amount of PVD produced in the cultures.

4.4 Conclusions

Environmental bacteria in natural settings communicate, cooperate or compete with each other. These interactions are often mediated by extracellular metabolites such as siderophores. Siderophores are produced by certain environmental bacteria and can be effectively shared among closely related species and can give competitive advantage to some species over others in any ecological niche. This study reports for the first time that SWCNTs at sublethal concentrations inhibit siderophore production in an environmental bacterium. Siderophore secreted by certain environmental bacteria that can be exploited by local community members with cognate receptors and has been implicated to be key components in establishing microbial communities. The results reveals an important sublethal impact of SWCNTs on siderophore-enabled cell-cell interactions in microbial communities, which often exist and play critical roles in maintaining health of ecosystems in various natural and engineered environments.

CHAPTER 5. Impacts of Nanomaterials on Bacterial Quorum Sensing: Differential Effects on Different Signals

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5.1 Introduction

The most common approach to supply information and synchronize social activity in bacteria is the production of certain chemicals as signalling molecules. Contaminants in the environments may interact with environmental bacteria and interfere with their quorum sensing (QS) signalling systems, altering various bacteria driven processes, *e.g.*, biogeochemical cycles, nutrient uptake by plants, wastewater treatment, and pathogenesis. One group of contaminants that are of the emerging concern is NMs, *i.e.*, materials with a size $\leq 100\text{nm}$ in at least one dimension (Cao 2004, Buzea, Pacheco et al. 2007). Owing to their unique physicochemical properties, NMs have been increasingly used over the past decade across various industries.(Lines 2008) This leads to the possibility of NMs release into the environments at different stages of the nanomaterial-containing product life cycle. However, to date, very limited studies exploring the impacts of NMs on bacterial QS signalling have been reported and conclusions in most studies have been drawn based on nanomaterial-induced changes in certain QS-regulated functions (Wagh, Patil et al. 2013, Naik and Kowshik 2014) rather than direct quantification of QS signals. Since each bacterial species usually produces a number of different QS signals, our knowledge on how these QS signals responds differently to NMs is lacking but required for a comprehensive understanding of the effects of NMs on bacterial QS systems.

The objective of this study was to investigate influences of NMs on different AHL-type QS signals. Gram-negative bacteria *Pseudomonas syringae* and *Pantoea stewartii* were used as model organisms and both have been reported to produce AHL-type QS signals

(Quiñones, Dulla et al. 2005, Koutsoudis, Tsaltas et al. 2006, Tan, Koh et al. 2015). The NMs used in this study were silver nanoparticle (AgNPs) and single-wall carbon nanotubes (SWCNTs). The influences of AgNPs and SWCNTs on the production of several key AHLs in *P. syringae* and *P. stewartii* were quantitatively assessed. This study reports for the first time on the effects of NMs on the production of individual QS signals.

5.2 Materials and methods

5.2.1 Bacterial strains and nanomaterials

Stock cultures of *P. syringae* pv. tomato DC3000 (DC3000) (Buell, Joardar et al. 2003) and *P. stewartii* (NCBI accession no. KC252900) (Tan, Koh et al. 2015) were maintained in lysogeny broth (LB) medium with 25% glycerol at -80°C. Cultures were grown aerobically at 30°C in LB medium. The SWCNTs were synthesized using the arc-discharge method (AP-SWNT, tube diameter of ~1.4 nm, Carbon Solutions, Inc.) and purified through a centrifugation-based method as previously described (Section 4.2.1). AgNPs with sodium citrate as stabilizer with a size of 20 nm (TEM-based) were purchased from Sigma-Aldrich (Singapore).

5.2.2 Cell viability assay

P. stewartii and *P. syringae* were grown in LB in the presence of SWCNTs (0-500 µg/ml) or AgNPs (0-2 µg/ml) at different concentrations. The optical density at 600 nm (OD₆₀₀) was used to monitor bacterial growth. Since NMs may interfere with OD₆₀₀ readings, cell growth was further quantified using a Colony Forming Unit (CFU) method (Chen, Nace et al. 2003, Ding, Peng et al. 2014, Wu, Ding et al. 2014).

5.2.3 Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

Cells were grown in presence and absence of AgNPs at 0.5 µg/ml for 24 h. The culture supernatant was harvested by centrifugation at 6000g for 15min. The cell free supernatant was filtered through a 0.2 µm syringe filter. The silver ion concentration was quantified using an ICP-MS instrument (ELAN DRC-e, Perkin Elmer; Waltham, MA) as previously described. (Section 4.2.10)

5.2.4 Extraction of AHLs from bacterial cultures

P. stewartii and *P. syringae* were grown in LB in the presence and absence of 20 µg/ml SWCNTs or 0.5 µg/ml AgNPs at 30°C in a shaking incubator (200 rpm). Samples taken at 0 h and 24 h were used to quantify cell growth following the CFU method described above (Chen, Nace et al. 2003). Extraction of AHLs from each bacterial culture at the early stationary phase (24 h) were conducted as previously described (Shaw, Ping et al. 1997). Briefly, 100 ml of dichloromethane was mixed with 50 ml of the culture supernatant. The dichloromethane extract was dried in a nitrogen gas stream and then resuspended in methanol/water (1:1 v/v) for further analysis by HPLC-MS/MS (Shimadzu, Singapore).

5.2.5 Quantification of AHL signals

The parameters for LC-MS/MS were set according to a previously reported procedure for AHL analysis with slight modifications (Tan, Koh et al. 2014). The XR-ODS C18 column and a flow rate of 0.3 ml/min were used for all samples. The mobile phase consisted of a linear gradient (40–95%) of solvent B (methanol with 0.1% formic acid) and solvent A (25 mM ammonium formate with 0.1% formic acid). Following LC separation, samples were then ionized by electrospray ionization under positive mode and detected using the multiple reaction monitoring (MRM) approach (Morin et al., 2003; Decho et al., 2009). Each standard MRM profile, including specific LC retention time, appearance of precursor ion *m/z* and two transition ions, as well as relative intensity of the two transition ions, was used as a reference. A full scan, ranging from *m/z* 100 to 350 was used to identify the putative AHLs, coupled with precursor ion scan mode was conducted in comparison with the standards. The following synthetic AHLs (>97%) purchased from Sigma-Aldrich (Singapore) were used as AHL standards: *N*-butyryl-DL-homoserine lactone (C4-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL), *N*-(3-oxohexanoyl)-DL-homoserine lactone (3OC6-HSL), *N*-heptanoyl-DL-homoserine lactone (C7-HSL), *N*-octanoyl-DL-homoserine lactone (C8-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3OC8-HSL), *N*-decanoyl-DL-homoserine lactone (C10-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3OC10-HSL), *N*-dodecanoyl-DL-homoserine lactone (C12-HSL), *N*-(3-oxododecanoyl)-

L-homoserine lactone (3OC12-HSL), *N*-(3 hydroxydodecanoyl)-DL-homoserine lactone (3OHC12-HSL), *N*-tetradecanoyl-DL-homoserine lactone (C14-HSL) and *N*-(3-oxotetradecanoyl)-Lhomoserine lactone (3OC14-HSL). The standard curves were constructed with a concentration ranging from 0.5 to 200 µg/ml for absolute quantification of signals in real samples. Every AHL had two transition ions which were used to identify the AHL and the transition ion with the highest intensity was employed to construct the standard curves. Post run analysis such as analyte peak areas integration was done using LabSolutions (Shimadzu). The limits of detection and quantification for each AHL were calculated with a signal-to-noise ratio of 3.3 and 10, respectively. Triplicate blank injections were performed between sample injections to avoid sample carry over.

5.2.6 Statistical analysis

All statistical analyses were conducted using Prism 6 Version 6.04 (GraphPad, U.S.A.) or PRIMER 6 Version 6.1.15 & PERMANOVA+ Version 1.0.5 (PRIMER-E, United Kingdom). Method of multivariate analysis community diversity commonly used in microbial ecological studies, were adopted to determine the overall changes of AHL profiles between different treatments. The AHL profiles were clustered based on Euclidean distance with logarithm (X+1) transformation and visualized using nMDS plot.

5.3 Results and discussion

5.3.1 Determination of sub-inhibitory concentrations for AgNPs and SWCNTs

The AgNPs and SWCNTs inhibited growth of *P. syringae* and *P. stewartii* in a concentration dependent manner, which is consistent with previous studies on bactericidal activities of NMs (Kang, Pinault et al. 2007, Kim, Kuk et al. 2007). The minimal inhibitory concentration (MIC) of AgNPs and SWCNTs under the above experimental conditions was ~1 µg/ml and ~200 µg/ml respectively. No growth inhibition was observed for AgNPs and SWCNTs at a concentration of 0.5 µg/ml and 20 µg/ml, respectively, against both organisms after 24 h of cell growth. This was further confirmed by the CFU count method (Table 5.1). Hence, the sub-inhibitory concentrations of 0.5 µg/ml and 20 µg/ml were used for AgNPs and SWCNTs, respectively, to examine their effects on QS signalling in *P. syringae* and *P. stewartii*.

Table 5.1 Growth of *P. syringae* and *P. stewartii* cells in LB medium in the absence (control) and presence of 0.5 µg/ml AgNPs or 20 µg/ml SWCNTs. Data shown are mean ± standard deviation (n=5).

Growth Condition		CFU (cells/mL)	
		0 h	24 h
<i>P. syringae</i>	Control	$1.12 \pm 0.13 \times 10^7$	$4.20 \pm 0.83 \times 10^9$
	AgNPs	$1.14 \pm 0.26 \times 10^7$	$4.60 \pm 0.54 \times 10^9$
	SWCNTs	$1.30 \pm 0.36 \times 10^7$	$9.80 \pm 0.14 \times 10^9$
<i>P. stewartii</i>	Control	$1.18 \pm 0.13 \times 10^7$	$2.02 \pm 0.04 \times 10^{10}$
	AgNPs	$1.14 \pm 0.16 \times 10^7$	$1.96 \pm 0.08 \times 10^{10}$
	SWCNTs	$1.16 \pm 0.11 \times 10^7$	$2.04 \pm 0.16 \times 10^{10}$

5.3.2 Identification of key AHLs produced by *P. syringae* and *P. stewartii*

The AHLs produced by the two bacteria were identified using LC-MS/MS based on standard curves and the mass spectra of 13 synthetic AHLs as quantitative references (Figure 5.1 A). Different AHLs profiles were obtained for these two organisms. *P. syringae* produced mostly 6 short-chain AHLs namely C4-HSL, C6-HSL, 3OC6-HSL, C7-HSL, C8-HSL, and 3OC8-HSL. Among which C6-HSL and 3OC6-HSL (~2.23 mM each) were the most abundant QS signals (accounted for ~98% of total signals) (Figure 5.1B). Whereas *P. stewartii* produced 11 different types of AHLs including both short- and long-chain AHLs: C4-HSL, C6-HSL, 3OC6-HSL, C7-HSL, C8-HSL, 3OC8-HSL, 3OC10-HSL, C12-HSL, 3OC12-HSL, C14-HSL, and 3OC14-HSL (Figure 5.1C). The most abundant AHL signal in *P. stewartii* was 3OC6-HSL (accounted for ~81% of total signals).

Figure 5.1 (A) Total ion chromatogram of a mixture of 13 standard AHLs (spiked into the sample matrix). (B) Profile of AHLs produced by *P. syringae*. (C) Profile of AHLs produced by *P. stewartii*. For a better clarity, the peaks for certain AHL signals with low abundance are shown with a 10× (*), 100× (**), or 1000× (***) amplification.

5.3.3 Influence of AgNPs and SWCNTs on production of different AHLs

For *P. syringae*, in presence of 0.5 $\mu\text{g/ml}$ AgNPs, the production of all the 6 AHLs except 3OC8-HSL was significantly lower compared with control cultures without AgNPs (Figure 5.2). In particular, the presence of AgNPs in *P. syringae* cultures resulted in >50% reduction of C4-HSL, ~24% reduction of C6-HSL, and ~31% reduction of 3OC6-HSL. The concentration of Ag^+ in the culture media containing 0.5 $\mu\text{g/ml}$ AgNPs after 24 h of

incubation was determined to be 12.5 ± 0.4 $\mu\text{g/L}$ using ICP-MS. Then the impact of AgNO_3 at a concentration of ~ 12.5 $\mu\text{g/L}$ was examined on the model organisms and no inhibitory effect on QS signalling could be observed. Hence, the effects reported here can be attributed to the nanoparticulate nature of AgNPs. In contrast, in the presence of 20 $\mu\text{g/ml}$ SWCNTs, the level of most AHLs remained unchanged except for C4-HSL and C8-HSL, which exhibited a reduction of $\sim 13\%$ and $\sim 21\%$, respectively. Intriguingly, the production of most AHLs in *P. stewartii* was not significantly affected by either 0.5 $\mu\text{g/ml}$ AgNPs or 20 $\mu\text{g/ml}$ SWCNTs. The only exception was C4-HSL, which was observed a $\sim 21\%$ reduction in the presence of AgNPs (Figure 5.3).

Figure 5.2 Influence of AgNPs (0.5 $\mu\text{g/ml}$) and SWCNTs (20 $\mu\text{g/ml}$) on the production of different AHLs by *P. syringae*. The identity and quantity of individual AHLs present in each sample were compared with the multiple reaction monitoring profiles of 13 standard AHLs. A total of 6 AHLs are shown (the remaining 7 AHLs were below the detection limit). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Error bars are defined as standard error of the mean ($n = 6$). the dotted line represents the limit of detection.

Figure 5.3 Influence of AgNPs (0.5 $\mu\text{g/ml}$) and SWCNTs (20 $\mu\text{g/ml}$) on the production of different AHLs by *P. stewartii*. The identity and quantity of individual AHLs present in each sample were compared with the multiple reaction monitoring profiles of 13 standard AHLs. A total of 11 AHLs are shown (the remaining 2 AHLs were below the detection limit). (** $p < 0.01$) Error bars are defined as standard error of the mean ($n = 6$).

A nonmetric Multidimensional Scaling (nMDS) analysis of the AHLs produced by both *P. syringae* and *P. stewartii* as impacted by AgNPs and SWCNTs is shown in Figure 5.4. In *P. syringae*, samples from the AgNP-treated cultures formed a distinct cluster and the tight clustering was influenced mostly by C4-HSL.

Figure 5.4 Overall AHL profiles in (A) *P. syringae* and (B) *P. stewartii* by nonmetric Multidimensional Scaling (nMDS) in the nanomaterial-treated (0.5 μ g/ml AgNPs or 20 μ g/ml SWCNTs) and no-treatment control conditions. Experiments were conducted in 6 replicates.

These results suggested a significant shift in the AHL profiles, in particular, C4-HSL, when *P. syringae* was grown in the presence of AgNPs. The samples from SWCNT-treated cultures closely resembled the control without SWCNTs and were clustered together, indicating no overall changes in the AHL profiles. In contrast, all the samples from *P. stewartii* cultures with and without AgNPs or SWCNTs were largely scattered and no discrete clusters could be found, suggesting no overall changes in the AHL profiles produced by *P. stewartii*. Taken together, the results show that the effects of NMs on bacterial QS signalling are highly dependent on the bacterial species, and more importantly, the production of different QS signals may respond differently to the contaminant exposure. The production of AHLs is a complex biological process and the exact mechanism on how NMs interfere with different AHL synthesis pathways needs further investigation.

The differential effects of different NMs on AHLs production in different species might be attributed to a combination of nanomaterial physicochemical property and bacterial response mechanism to environmental stressors. The physio-chemical properties such as sizes, shapes, compositions of both the materials used are very different which might lead to different biological effects. Previous reports have suggested different modes of action for different NMs. For example, AgNPs are known to attach to the bacterial cell membrane and alter their permeability and respiration. They also can penetrate into the cells and interact with biomolecules such as proteins (Matsumura, Yoshikata et al. 2003) and nucleic acids (Feng, Wu et al. 2000), while SWCNTs mostly act through direct physical contact or exert oxidative stress which compromises the cell membrane integrity (Kang, Pinault et al. 2007). Moreover the degree of aggregation for both the NMs, in presence of the organic matter in the culture media might be different that may in turn affect their bioavailability. These are some of the plausible reasons why the effect was seen with only AgNPs and not SWCNTs.

Furthermore, the effect of AgNPs is also different for different bacterial strains. This host specificity could be attributed to variety of bacterial processes but one plausible explanation could be, extracellular polymeric substance (EPS) might play a very crucial role in the penetration of NMs in bacterial culture media. Both the bacterial strains used in

this study might vary in terms of their EPS composition (Nimtz, Mort et al. 1996, Keith, Keith et al. 2003) and thus might interact very differently with NMs.

In previous studies, the effects of NMs on bacterial QS signalling have been reported mostly based on the assay of QS-regulated activities such as violacein production in *Chromobacter violaceum* (Wagh, Patil et al. 2013, Naik and Kowshik 2014). However, a number of different QS signals are often produced concurrently and each may involve in regulating different bacterial activities. For example, violacein production has been shown to be induced by short-chain AHLs (C4-C8) and inhibited by AHLs with *N*-acyl side chains from C10 to C14 (McClean, Winson et al. 1997). The results reveal that the production of different AHL-type QS signals may respond differently to NMs at sub-inhibitory concentrations. Both *P. syringae* and *P. stewartii* are soil bacteria that are usually associated with plant roots (Cha, Gao et al. 1998, Dumenyo, Mukherjee et al. 1998) and the AHLs produced by these organisms have been shown to regulate various interactions between bacteria and their plant hosts (von Bodman, Bauer et al. 2003). The understanding of differential effects of NMs on the production of different AHLs would allow us to better assess their impacts on specific bacteria-host interactions. In addition, QS represents both intra- and inter-species signalling and QS-enabled cooperation is beneficial to the local population or community as a whole (Waters and Bassler 2005). Hence, differential effects exhibited by NMs on different QS signals in certain QS signal producers may result in a complex impact on the overall function of the whole community.

5.4 Conclusions

Taken together, the results reveal that the effects of NMs on bacterial QS signalling are highly dependent on bacterial species, and more importantly, the production of different QS signals in one species may respond differently. This study significantly advances the current knowledge toward a comprehensive understanding of the sublethal effects of NMs on bacterial QS signalling.

CHAPTER 6. Effect of Silver Nanoparticles on Collective Antibiotic Tolerance of Wastewater Sludge Microbial Community

6.1 Introduction

The increased resistance of microbial biofilm communities has been widely studied and it is increasingly clear that the communal interactions and social dynamics hold the key. This type of cooperative behaviour is interesting in the context of antibiotics treatment, known as collective antibiotic tolerance. It is widely reported that resistant to antibiotics in bacteria can be intrinsic or they can acquire it as a result of genetic mutation or horizontal gene transfer via plasmids, transposons and integrons (Blair, Webber et al. 2015). Along with widespread usage of antibiotics, other toxic environmental pollutants, *e.g.*, heavy metals (Baker-Austin, Wright et al. 2006, Seiler and Berendonk 2012), triclosan (Carey and McNamara 2015), disinfection by products (Lv, Jiang et al. 2014) also contributes to increased antibiotics resistance. This type of co-selection or cross-protection is seen in case of shared regulatory response for antibiotics and anthropogenic pollutants. Mechanisms of antibiotic tolerance at community level are clearly more complex than single cell level. Therefore, there is an increased thrust towards a full understanding of the environmental factors that lead to evolution and proliferation of antibiotics resistance in natural environment.

NMs are one of the emerging contaminants and their ever increasing usage across different industry will lead to their leaching into environment. The most commonly used NM in consumer products is silver, which is present in highest number of product (259 products) (Weinberg, Galyean et al. 2011). Interestingly, there have been some studies showing NMs can promote antibiotics resistance by enhancing horizontal gene transfer. Nano alumina was shown to promote conjugative transfer of the RP4 plasmid transfer across genera due to the membrane damage caused by oxidative stress (Qiu, Yu et al. 2012). However, this study was limited to laboratory based pure cultures. Very recently another study reported the effect of silver nanoparticles on antibiotic gene profile in

wastewater bacterial community (Ma, Metch et al. 2016). Using metagenomics based approach the study finds that the silver nanoparticle treatment increases the abundance of antibiotic resistance genes (ARGs) compared to untreated control and silver ion treated reactors. But the major limitation of metagenomics information is, it is based on DNA from both live and dead cells and the increased copy number of certain genes may or may not get reflected in the respective gene expression.

Moreover, apart from the above mentioned studies there has not been much information on how NMs can affect the collective antibiotic tolerance of a complex microbial community. The objective of this study was to evaluate the effect of silver nanoparticles (AgNPs) on the antibiotics tolerance of wastewater microbial community using metatranscriptomics to study the microbial population in terms of the change in live/active population. AgNPs were used as the model NM for its universal presence and tetracycline was used as the model antibiotic because tetracycline class of antibiotics is one of the most widely used for treatment of human and live stocks (Chopra and Roberts 2001).

6.2 Materials and Methods

6.2.1 Sludge collection

The sludge was collected from a laboratory scale column-type enhanced biological phosphorus removal (EBPR) reactor with alternate anaerobic and aerobic cycle operating at 30° C after ~60 days of operation. The MLVSS of the sludge taken from reactor was adjusted to 1 g/L and were grown in 250 mL flaks with a working volume of 50 mL, under aerobic condition at 30° C with 200 rpm shaking. MLSS and MLVSS analyses were carried using methods described previously (Eatson, Clesceri et al. 2005). The sludge microorganisms were grown in the same mineral medium used in the EBPR reactor containing 400 mg COD/L, the ammonia and phosphate concentrations were 20 mg N-NH₄⁺/L and 20 mg P-PO₄³⁻/L respectively. Every 24 h, the sludge was allowed to settle for 1 h and half the volume was replaced with fresh synthetic mineral medium mentioned above, to replenish the nutrients.

6.2.2 Nanomaterial and antibiotic treatment

AgNPs with sodium citrate as stabilizer with a size of 10 nm (TEM-based) were purchased from Sigma-Aldrich (Singapore). AgNPs and Ag⁺ (AgNO₃, Merck, Singapore) with concentration 0.1 µg/ml, along with the mineral media were added to the sludge every 24 h till 144 h.

The stock solution of tetracycline antibiotic (Sigma, Singapore; purity of > 98%) was prepared in 70% ethanol. After 144 h, all the three groups (control, AgNP treated and Ag⁺ treated) were exposed to tetracycline treatment at 1000 µg/ml for 24 h. Equal volume of 70% ethanol was added to the untreated samples to account for the background.

6.2.3 Viability measurement

The viability of the sludge microbial community was assessed by using the Live/Dead staining (LIVE/DEAD[®], BacLight[™] Cell Viability Assays, Thermo Fisher Scientific Inc.). Briefly, 100 µL of sludge was evenly mixed with 10 µL of SYTO9/propidium iodide working solution (final concentration 6.68 µM and 40 µM, respectively), after thorough mixing samples were kept in dark at room temperature for at least 30 min. Stained samples were imaged at 488 nm excitation (SYTO9) as well as 561 nm excitation (propidium iodide) using confocal microscopy. Quantitative image analysis was done using IMARIS (Version 8.2.0; Bitplane, Zurich, Switzerland). The ratios of live and dead cells (L/D ratio) were calculated from the confocal images.

6.2.4 Phosphate measurement using ion chromatography

The IC system (Shimadzu Prominence) with a Conductivity detector (CDD) was used for phosphate measurement. Shodex IC-SI-90 4E 250mm x 4.0mm column and a flow rate of 1.1 ml/min was used. The mobile phase used consisted of 12 mM Sodium Hydrogen Carbonate and 0.6 mM Sodium Carbonate. The column oven temperature was maintained at 40⁰C. Run time was 23.8 min/sample.

6.2.5 RNA extraction

The sludge samples were collected by centrifugation at 8,000g at 4⁰C for 8 min, fixed in liquid nitrogen for 30 min and immediately stored at -80⁰C for subsequent RNA extraction. Total RNA was extracted from sludge using ZR Soil/Fecal RNA MicroPrep™ (Zymo Research, USA) according to the manufacturer's guidelines and DNA was removed using the TURBO DNA-free kit (Applied Biosystems, Singapore). RNA quantity and purity was determined using NanoDrop spectrophotometer (Thermo Scientific, DE, USA) and Qubit® RNA HS Assay Kit by Qubit® 2.0 Fluorometer (Life Technologies) according to the manufacturer's protocol. The integrity of RNA was measured on a 2200TapeStation system (Agilent Technologies) with R6K ScreenTape.

6.2.6 RNA sequencing

Next-generation sequencing library preparation was performed by following TruSeq RNA Sample Preparation v2 protocol (Illumina) with modifications: 200 ng of total RNA was directly added to the elute-fragment-prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely tagged with one of Illumina's TruSeq LT RNA barcodes to allow library pooling for sequencing. Library quantitation was performed using Invitrogen's Picogreen assay and the average library size was determined by running the libraries on a Bioanalyzer DNA 7500 chip (Agilent). Library concentration was normalized to 4 nM and the concentration was validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using KAPA Library Quantification Kit Illumina® platforms. Libraries were then pooled at equal volumes and sequenced in one lane of an Illumina HiSeq2500 rapid run at a final concentration of 11 pM and a read-length of 101 bp paired-end. All raw RNA sequencing data used in this study are publicly available from NCBI under BioProjectID: SRP077608.

6.2.7 Sequence analysis using RiboTagger

The raw reads were quality filtered and adapter removed, were processed for rRNA profiling using RiboTagger (available at <https://github.com/xiechaos/ribotagger>). Briefly, a universal primer was used to scan for the 16S rRNA hypervariable region (V4) in order to detect 33 nt sequences immediately next to the V4 region of 16S rRNA, using a position specific scoring matrix (PSSM). Short reads not able to cover the full 33 nt region were discarded. This PCR free approach has the advantage of minimizing amplification bias (Logares, Sunagawa et al. 2014). The taxonomy of individual V4 tag was classified based on the GreenGenes and Silva 16S rRNA databases. Each different V4 tag is used as a signature sequence to represent one operational taxonomic unit.

6.2.8 Statistical analysis

All statistical analyses were performed using R (www.r-project.org), Prism 6 Version 6.04 (GraphPad, U.S.A.) or PRIMER 6 Version 6.1.15 & PERMANOVA+ Version 1.0.5 (PRIMER-E, United Kingdom). For read normalization, sample reads were randomly subsampled with a replacement method to an even depth using QIIME (v.1.8). Community diversity indexes including Simpson and Shannon indexes were determined for each sample. ANOVAs and Sidak's post-tests were conducted to examine differences between samples. Adjusted p-values were reported. Read count matrixes were constructed from the evenly sampled read data, which was further normalized using $\log(X+1)$ transformation. The relative abundance of each tag/OTU was estimated and expressed as a portion of the total reads in each sample. The read count matrixes were further resembled using Bray-Curtis similarity measure prior to visualization using nMDS plots.

6.3 Result and Discussion

6.3.1 Effect of AgNPs on viability

The three groups: control, AgNP and Ag⁺ were seeded with the same inoculum in triplicates. Cell viability was monitored every 24 h using ATP measurement and Live/Dead staining. With time the cell viability decreases across all groups irrespective of treatment (Figure 6.1). At 48 h and 96 h the % live cells are comparable across all the

three groups. But at 144 h the ratio decreases slightly. Ag⁺ or AgNPs treatment didn't contribute to loss of viability at any time point as values were not statistically different across all the groups. This gradual decrease in viability across all the groups might be due to nutrient limitation and toxic metabolite accumulation with time.

Figure 6.1 Viability of sludge biomass over time was assessed through Live/Dead staining. The total biovolume of viable (live) and non-viable (dead) communities were calculated from CLSM images. Ratio of live cells biovolume to total biovolume represents the % live cells. Error bars are defined as standard error of mean ($n=8$).

6.3.2 Effect of AgNPs on community function

The activity of the microbial community was also assessed during the treatment period. As the sludge was collected from the lab scale EBPR reactor, it was expected to have phosphate (P) release and uptake activity by phosphate accumulating organisms (PAOs). For EBPR system, cyclic anaerobic/aerobic phases are maintained which encourages P release and uptake by PAOs. During anaerobic phase internal phosphate is hydrolysed and orthophosphate is released into the media. The experimental conditions were different from the original reactor as the sludge was maintained under aerobic conditions but every 24 h during changing the spent media with fresh media, the sludge was taken out of shaker and was allowed to settle down for 1 h creating a transient anaerobic phase. After sludge settlement the spent media was withdrawn and phosphate levels were measured in the effluent. An apparent P release activity was observed and a decreasing trend is observed

over time and didn't differ between the groups (Figure 6.2). This trend can be correlated with the cell viability which also decreases over time. Another possible reason for the decrease in P release might also be due to shift in community profile over time.

Figure 6.2 Phosphate concentration was measured in the spent media at every 24 h interval. A decreasing trend is observed across all the group over time. Error bars are defined as standard error of mean (n=3, biological replicates).

6.3.3 Effect of AgNPs on overall community composition

Analysis of the total rRNA sequences was done to investigate bacterial diversity and richness using Shannon and Simpson diversity indices. In this study, Shannon and Simpson indices showed a similar pattern, *i.e.*, the community diversity significantly increased from seed culture (0 h) to 144 h samples (Figure 6.3 A and B). The increase in diversity after 144 h is regardless of the treatment type and there was no significant difference ($p > 0.6$) between control, Ag⁺ and AgNP groups. As diversity also considers OTU abundances, the results show more evenly distributed abundance profiles after few days.

Figure 6.3 (A) Shannon index (B) Simpson index. Mean value of diversity for multiple OTUs were compared for seed culture and different groups (Ctrl, Ag⁺ and AgNP) after 144 h. Significant differences are indicated as follows: *** $p < 0.001$, **** $p < 0.0001$. Error bars defined as standard error of mean ($n=3$, biological replicates).

The three different groups after 144 h were also compared with the seed culture using non-metric multidimensional scaling (nMDS) based on the mean relative abundance of OTUs (Figure 6.4) revealed tight clustering of all the three groups and was distinctly separated from the seed culture. This further suggests no difference between AgNPs and Ag⁺ treatment with control after 144 h.

Figure 6.4 Nonmetric multidimensional scaling (nMDS) based on rRNA sequences compares the seed culture and different groups (Ctrl, Ag+ and AgNP) after 144 h. The seed culture form a separate cluster compared to the rest of the samples.

Furthermore, the relative abundances of top 100 OTUs, which represent ~ 90% of the total sequences, were compared across different samples. The relative abundance of different tags in seed samples was significantly different than samples of three different groups after 144 h (Figure 6.5). A closer observation suggests, the major change was in family Piscirickettsiaceae (Tag 2) whose relative abundance in seed culture was ~44% but it decreased significantly to 7-12% across all the groups. There is not much literature available linking it to the EBPR activity, most members of this family are aerobes found in water (Zahou, Mlika et al. 2015). The other major trend was the decrease in abundance of *Candidatus Accumulibacter* (hereafter referred to as Accumulibacter) represented by the Tag 6, 13 and 19. Accumulibacter is a well-known PAO and has been reported in many laboratory reactors and full scale EBPR plants (Crocetti, Hugenholtz et al. 2000, He, Gall et al. 2007). This is not very surprising as all the cultures were grown under constant shaking and it has already been reported that excessive aeration suppresses the growth of PAOs (Carvalho, Oehmen et al. 2014). This may also be directly linked to decrease in P levels with time in all the three groups (Figure 6.2). The relative abundance of other tags

in top 20 showed overall increase across all the three groups compared to seed culture, the most significant being the genus *Zoogloea* (Tag 1) and *Thauera* (Tag 3) where ~ 10 fold increases was observed. Both the above genus belonging to the class of betaproteobacteria are abundant in industrial waste water plants and known to be the important denitrifiers (Juretschko, Loy et al. 2002, Wagner, Loy et al. 2002, Thomsen, Kong et al. 2007). Excessive growth of *Zoogloea* genus has been known to negatively affect the EBPR process by causing bulking due to excessive production of extracellular polymeric substances (EPS) (Montoya, Borrás et al. 2008).

Figure 6.5 Bar chart displaying the community composition of each sample, including the 100 most abundant taxa in all samples.

Combining all the above analysis shows that there was no significant difference in terms of active and live population composition in response to AgNPs or Ag⁺ treatment. More than 50% Tags were unclassified, which highlights an inherent problem of accurately annotating short read-length tag sequences from hypervariable region of 16S rRNA (Wang, Garrity et al. 2007, Liu, DeSantis et al. 2008).

6.3.4 Effect of AgNPs on the collective antibiotic tolerance

To test the effect of AgNPs on collective antibiotic tolerance, sludge was treated with AgNPs and Ag⁺ for 144 h before subjecting all the three groups to tetracycline antibiotic treatment. All the further comparisons were done among three conditions: all the three groups (ctrl, Ag⁺ and AgNP) at 144 h (referred to as “prior treatment” from here on), treatment with 70% EtOH (the final concentration of ethanol in samples was calculated to be 3.5%) and, treatment with tetracycline (EtOH.Tet). The effect of treatment on viability of sludge microbial community was assessed by Live/Dead staining (Figure 6.6). After 24 h of tetracycline treatment reduction (~25-30%) in % live cells was observed for all the three groups: ctrl, Ag⁺ and AgNP. There was no significant reduction in viability with EtOH treatment compared to prior treatment samples.

Figure 6.6 Viability of sludge biomass after tetracycline treatment was assessed through Live/Dead staining. The total biovolume of viable (live) and non-viable (dead) communities were calculated from CLSM images. Ratio of live cells biovolume to total biovolume represents the % live cells. Error bars are defined as standard error of mean ($n=8$).

There was a reduction of viability in all the three groups but there still was a major fraction of live cells after 24 h of antibiotic treatment. However, this observation is not very surprising as wastewater treatment plants have been shown to be the hotspots for spread of ARGs and antibiotic resistance bacteria (Rizzo, Manaia et al. 2013). Because of its extensive usage the tetracycline resistance genes are frequently found in various natural (Chee-Sanford, Aminov et al. 2001, Mackie, Koike et al. 2006) and engineered environments like waste water treatment plants (Auerbach, Seyfried et al. 2007).

Further, analysis of total rRNA reads using non-metric multidimensional scaling (nMDS) based on the mean relative abundance of OTUs for all the samples showed three distinct clusters: prior treatment samples, samples with ethanol treatment and treatment with tetracycline. The three different groups: control, Ag⁺ and AgNP, didn't show much variation within each treatment category and all clustered together (Figure 6.7).

Figure 6.7 Nonmetric multidimensional scaling (nMDS) based on rRNA sequences compares the three groups (Ctrl, Ag⁺ and AgNP) before and after tetracycline treatment. Three distinct clusters are: pretreatment samples, the ethanol and tetracycline treated samples.

Further, Shannon and Simpson diversity indices were calculated for each group within the three different categories (Figure 6.8). There was no significant difference observed within

groups post antibiotics treatment but interestingly both Shannon and Simpson index shows a significant difference post ethanol treatment, for the Ag^+ and AgNP samples the diversity indices were lower than control (p -value < 0.05).

Figure 6.8 (A) Shannon index (B) Simpson index. Mean value of diversity of multiple OTUs were compared for prior treatment, ethanol treatment and tetracycline treatment for all the three groups (Ctrl, Ag^+ and AgNP). Significant differences are indicated as follows: * $p < 0.05$, ** $p < 0.01$. Error bars are defined as standard error of mean ($n=3$, biological replicates).

To further analyse the impact of AgNPs on antibiotic tetracycline tolerance, top 10 most abundant Tags which represent ~55% of the total reads were plotted (Figure 6.9). The heatmap shows the abundance of each Tag varies with the different treatments. But

variation between control, Ag⁺ and AgNP were not observed for most of them except for Tag 1 (Zoogloea) and Tag 3 (Thauera). In response to ethanol treatment the AgNP group enriched for Zoogloea species more compared to control ($p < 0.0001$). In case of Tag 3 (Thauera) both Ag⁺ and AgNP groups have higher relative abundance compared to control ($p < 0.0001$) after ethanol treatment. Tetracycline treatment also showed, Tag 3 (Thauera) being enriched in Ag⁺ group compared to control but this effect was not observed in AgNP group.

Figure 6.9 Heatmap of top 10 most abundant taxa. Each column in the heat-map represents one sample, while each row represents a taxa. The color intensity of the panel is abundance as percentage of the total community.

Overall it was seen that tetracycline treatment enriched the tags which were less abundant before treatment (Tag 6 and 7) and proportionately lowered the earlier abundant species (Tag 1, 5 and 8). But there were no significant difference among control and AgNPs treated samples.

6.4 Conclusions

NMs are being considered as emerging contaminants owing to their usage across different industries. Silver nanomaterials are among the most commonly used ones and are mainly known for their antibacterial properties. Along with that, earlier studies pointed towards a possible effect of NMs on antibiotics resistance which is a major global concern. For the first time, this study uses the RNA sequencing approach to assess the live population composition of a wastewater sludge bacterial community in response to AgNPs and further to study effect on NMs treatment on subsequent antibiotic exposure. The concentration of AgNPs used in this study represents the environmentally relevant concentration with increasing dosage over few days. This study highlights that lower concentration of NMs might not show immediate acute effect on the composition and collective antibiotic tolerance of wastewater community but it will be important to look at the long term impact of NMs exposure on environmental microbial communities.

CHAPTER 7. Conclusions and Recommendations

7.1 Overall conclusions

The presence of NMs in the environment is unavoidable because of an increasing use of NMs in a wide range of industries. Bacteria are important targets to consider while evaluating nanoecotoxicity, as NMs will invariably come in contact with them in water, sewage, soils, and sediments with potential consequences on overall health of the ecosystem. As most bacteria in nature exist in multi-species communities, interspecies interactions among bacteria of different species are important. Cell-cell interactions affect the overall structure and function of microbial communities. To comprehensively understand the impacts of NMs on the environment and the health of the ecosystem, it is imperative to elucidate the impacts of NMs on social behaviour of microorganisms. Impacts of NMs on different aspect of social behaviour *i.e.*, public goods production (Chapters 3 and 4), cell-cell interaction through quorum sensing (Chapter 5), and protective interactions in the context of antibiotic tolerance (Chapter 6) were reported in this thesis.

Firstly, the impacts of NMs on pyoverdine production in a model organism *P. aeruginosa* were investigated. Tellurium nanorods (TeNRs) were found significantly inhibit pyoverdine (PVD) production in *P. aeruginosa* in both planktonic cultures and mature biofilms. Similarly, single-walled carbon nanotubes (SWCNTs) could also inhibit PVD production in *P. aeruginosa*. Interestingly, although SWCNTs has a bactericidal activity against *P. aeruginosa*, the cells that survived the treatment of SWCNTs exhibit a decrease in PVD biosynthesis. PVD plays an important role in growth of *P. aeruginosa* as it facilitates the uptake of iron into the bacterial cell and it has also been implicated in regulating virulence factors required for establishing infection.

Secondly, the impact of NMs on quorum sensing signal was evaluated using two model microorganisms, *i.e.*, *Pseudomonas syringae* and *Pantoea stewartii*. The effect of SWCNTs and AgNPs were tested on the above two model organisms and interestingly, a differential effect was observed. The presence of AgNPs in *P. syringae* cultures resulted in

overall reduction of AHLs signals. In contrast, in the presence of SWCNTs, the level of most AHLs remained unchanged except. Intriguingly, the production of most AHLs in *P. stewartii* was not significantly affected by either AgNPs or SWCNTs. Different AHLs signals are produced concurrently and are involved in regulating different bacteria activities. NMs treatment showed a differential profile compared to untreated control which might have implication in the overall activity of the organism and community as a whole.

Finally the effect of NMs were evaluated on collective antibiotic tolerance of a microbial community. AgNPs was used as the model NM and the effect was tested on sludge microbial community from an enriched EBPR reactor. It was observed that there was no significant difference in the live/active population composition in response to AgNPs treatment. Furthermore, the effect of AgNP didn't show any significant effect on collective antibiotic tolerance in terms of population composition.

Taken together, this study demonstrates sublethal impacts of NMs on bacterial functions that are critical to their social behaviours and provides novel insights into ecotoxicity of NMs.

7.2 Recommendations for future research

In this thesis, it was shown that NMs can influence different behaviours by interfering with production of PVD, AHLs signals, and antibiotic tolerance. The exact molecular mechanisms of the interference remains elusive. Interspecies interactions among bacteria of different species are important as most bacteria in nature exist in multi-species communities. Cell-cell interactions affect the overall structure and function of microbial communities. Future efforts should focus on studying the impacts of NMs on cell-cell interactions in microbial communities. The studies will be conducted using “defined multispecies communities” and “enriched microbial communities”.

7.2.1 Impacts of NMs on production of communal resources

For the PVD inhibition by TeNRs and SWCNTs, it was shown that the effect was at transcriptional level as key genes involved in PVD synthesis, maturation and uptake were

downregulated in the treated samples. Thus it is imperative to know how the signal is transmitted from outside environment to the cell and regulate gene expression. Random mutagenesis library need to be constructed and then screened for specific genes involved in this signal transduction pathway.

In this thesis, the impacts of NMs on the production of siderophores by single species planktonic culture and biofilms were evaluated. This work can be extended to examine total siderophore production by enriched microbial communities from natural and engineered ecosystems, for example, sediments from the urban waterways and sludge from Wastewater Treatment Plants. It will be interesting to see whether NMs treated producer cell and untreated cells behave differently.

7.2.2 Impacts of NMs on cell-to-cell communication

With respect to effect of NMs on QS signals, it has to be further explored whether the effect in *P. syringae* is at the genetic level. For that, qPCR can be used to quantify the key genes involved in the production of different AHLs. It will also be interesting to look at the other key functions like c-di-GMP level and biofilm formation ability when QS signals are downregulated.

Moreover, the effect of NMs on QS signals at the community level using will be another important step towards the comprehensive understanding of the ecotoxicity of NMs. These studies can be conducted using defined or enriched microbial communities. Specifically, the next step in studying the impact of NMs on QS will be examining the phenotypic changes occurring due to change in QS signals.

7.2.3 Impacts of NMs on collective antibiotic tolerance

In the last chapter, the effect of AgNP on the antibiotic tolerance of sludge microbial community using RNA sequencing was examined. By looking at the rRNA the live/active community structure of the sludge can be evaluated. In future, it would be interesting to analyse the mRNA sequences to gain an understanding into the changes in gene expression levels in response to NMs treatment. The concentration of NMs may not be very high to cause killing of the cells but may induce subtle changes at the level of gene

expression and thus changing the function and structure of the whole community in long run.

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