ASSESSING TOXICITY OF TITANIUM DIOXIDE (TiO_2) NANOPARTICLES ON *PSEUDOMONAS* SPECIES BIOFILMS

by

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Assessing toxicity of titanium dioxide (TiO_2) nanoparticles on *Pseudomonas* species biofilms

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ABSTRACT

Biofilms are essential to the aquatic environment. Recent advances in technology resulted in increased use of nanomaterials (such as titanium dioxide nanoparticles) and their release into aquatic environments with unknown long-term effects. Potential toxicity of titanium dioxide, known for its photocatalytic properties, on *Pseudomonas aeruginosa* (PAO1-gfp) and *Pseudomonas sp.* (CT07-gfp) biofilm formation and proliferation was assessed using flowcells, confocal laser scanning microscopy (CLSM), and total and viable cell release into effluent under different titanium dioxide concentrations (100 ppm, 10 ppm and 1 ppm). COMSTAT software analysis was used to obtain quantitative morphological biofilm data. Results showed that titanium dioxide had a concentration and media-dependent effect on biofilm formation, growth, proliferation and viability. Viable effluent cell counts remained within the same order of magnitude. Biofilm recovery was evident within 24-48 hours after exposure. At environmentally relevant concentration (1 ppm), there was no effect on formation, proliferation or growth of the biofilm.

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ABBREVIATIONS and TERMINOLOGY

CLSM – confocal laser scanning microscopy

CNT – carbon nanotubes

CT07 – *Pseudomonas* sp.

Daphnia magna – small planktonic crustacean (water flea)

DAPI – (4',6-diamidino-2-phenylindole) fluorescent stain

DLS – dynamic light scattering

EPS – exopolymeric substance (extracellular matrix)

ENM – engineered nanomaterial

FCS - fluorescence correlation spectroscopy

GFP – green fluorescent protein

Nano-TiO_2 – titanium dioxide nanoparticle

Nano-Ag – silver nanoparticle

Nano-ZnO – zinc oxide nanoparticle

Nm – nanometer ($1.0x10^{-7}$ cm)

NP – nanoparticle

Oryzias latipes – Japanese rice fish

PAO1 – *Pseudomonas aeruginosa*

Rainbow trout - *Oncorhynchus mykiss*

ROS – reactive oxygen species

SEM - scanning electron microscopy

SSR – simulated solar radiation

TEM – transmission electron microscopy

TiO_2 – titanium dioxide

1. INTRODUCTION

Prokaryotes were the first simple microorganisms that appeared on this planet and up to this day, they represent a large portion of life's genetic diversity (Whitman *et al*., 1998). They are essential to the biogeochemical cycles of the biosphere and produce important components of the earth's atmosphere. In nature, bacteria mostly live in communities called biofilms (Stewart & Franklin, 2008). Biofilms are a staple of any aquatic environment, serving as the bottom of the food web, recycling nutrients and fixing carbon. In recent years, biofilms have been used to treat wastewater, degrade pollutants and help with remediation (Stewart & Franklin, 2008). With advancement of newer technologies in mid-2000s, such as nanotechnology, the effects of nanomaterials on bacterial communities are still unknown. The primary objective of the proposed research is to study the toxicity of titanium dioxide nanoparticles on formation, proliferation, growth and viability of two biofilm-forming bacterial species (separately) – *Pseudomonas aeruginosa* and *Pseudomonas* sp. (related to *P. fluorescens*). These bacterial species have been chosen as model organisms to represent the biofilm communities.

1.1 Brief history of nanotechnology

Richard Adolf Zsigmondy was an Austrian-Hungarian chemist who won a Nobel Prize in chemistry in 1925 for his observation of colloids (Reitstötter, 1966). He was the first person to observe gold nanoparticles in 1912 using the immersion ultramicroscope that he invented (Reitstötter, 1966). K. Eric Drexler popularized the term nanotechnology in 1986 after he listened to the lecture given by Richard Feynman "There's plenty of room at the bottom", after which Drexler wrote a book "Engines of creation: the coming era of nanotechnology".

Nanotechnology refers to manipulation of matter on a near-atomic scale to produce novel structures, materials, and devices. Engineered nanomaterials are materials with at least one external dimension in the size range of approximately 1-100 nanometers and nanoparticles have all three external dimensions at the nanoscale (Klaine, 2008).

1.2 Overview of the nanotechnology uses and its effects on the environment

In the last decade, there has been an exponential growth in nanotechnology field and application of engineered nanomaterials (ENM) in everyday products (Scown *et al.,* 2010). High manufacturing rates and usage could lead to engineered nanomaterial release into the environment during their lifecycle, both deliberately and by accident (Lubick, 2008). As an example, remediation of contaminated groundwater is performed by the use of iron nanoparticles (Zhang *et al.*, 2006), while grinding of nanocomposite materials as a finishing step in engineering of nanomaterials produces powders that could become airborne (Aitken, 2004). A study by Kiser *et al.* (2009) showed that single nanoparticles and spherical aggregates (50 nm to a few hundred nanometers in size) composed of sub-50 nm spheres of titanium and oxygen only were observed in sewage, biosolids and liquid effluent from wastewater treatment plant, as well as commercial products containing titanium dioxide. This shows that nanomaterials can enter the environment through all major pathways: biosolids application and solid waste disposal, wastewater treatment plant effluent and water runoff, and release into the air.

Due to increased use of nanomaterials in recent years, increased levels of nanoparticles might be discharged into the environment through insufficient methods of filtering out nanomaterials in wastewater treatment plants or through unintentional releases such as atmospheric emissions and solid or liquid waste streams from production facilities (Klaine *et al.,* 2008; Gottschalk *et al*., 2009). The main concentration increase would be seen at the wastewater treatment plant (WWTP) discharge zones, which are populated by freshwater organisms such as bacterial communities, invertebrates and vertebrates, and fish (Hoellein *et al.,* 2011).

There are five classes of manufactured nanomaterials: carbon nanotubes (CNT), originated in 1985; metal and metal oxide nanoparticles (such as titanium dioxide or zinc oxide); semiconductor nanocrystals, also known as quantum dots; zero-valent metals; and detrimers (Klaine *et al*., 2008). The small size, high surface area, unique chemical composition and shapes, and surface chemistry are what give engineered nanomaterials their novel properties

(Oberdörster *et al.*, 2005). The focus of this study is on metal oxide nanoparticles, such as titanium dioxide.

Titanium dioxide (TiO_2), silicone dioxide (SiO_2), silver oxide (AgO) and zinc oxide (ZnO) are the most commonly used nanoparticles (NP) as industrial and commercial additives. As an example, they are abundantly present in the natural aquatic ecosystems (Adams *et al.*, 2006). Titanium dioxide is used in common products such as sunscreens as an active ingredient that absorbs UV radiation, or in textiles, cosmetics, personal care, clothing, paints, and food additives as a coloring agent (Klaine *et al.,* 2008; Scown *et al.,* 2010). Due to its known antimicrobial activity, titanium dioxide is used in coatings and glass to promote self-cleaning of the surfaces of the material that have significant exposure to sunlight (Ma *et al.,* 2012). Silver nanoparticles are commonly used in wound dressings, bandages, socks and other textiles due to their antimicrobial properties (Klaine *et al.,* 2008).

Measuring traces of NPs in aquatic systems against the high background of natural colloids poses a difficulty (Klaine *et al.,* 2008). There is a lack of analytical tools capable of characterizing and quantifying particles in their native state in complex environmental settings due to microorganisms, naturally occurring colloids and organic matter (Petosa *et al.,* 2010). Based on the critical review by Klaine *et al.* (2008), it has been agreed that the following properties are an essential requirement when measuring nanoparticles' behaviour in natural waters: chemical composition, mass, particle number and concentration, surface area concentration, size distribution, specific surface area, surface charge/zeta potential, surface contamination, stability and solubility. Similarly, when measuring nanoparticles' behaviour in the marine environment, it is important to consider increased alkalinity, higher ionic strength and wider variety of colloids and natural organic matter (Klaine *et al.*, 2008; Petosa *et al.,* 2010).

Given biofilm essential role in aquatic ecosystems such as nutrient recycling and being the main food source for many microorganisms and invertebrates (Wolfaardt *et al.*, 1994), it is important

to understand potential toxic effects of nanoparticles on biofilm formation, proliferation and function.

Biofilms are important in nitrogen and phosphorus removal in wastewater treatment plant during activated sludge treatment, and so they come into constant contact with NPs. Titanium dioxide is one of the most widespread nanoparticles used by the industries such as textiles, clothing, and beauty product manufacturers. A recent study showed that after a 70-day long exposure of nitrogen removing bacteria to titanium dioxide concentration of 50 mg/L, abundance of nitrifying bacteria (especially ammonia-oxidising bacteria) was greatly reduced, while at 1 mg/L (which is most environmentally relevant), it was unaffected (Zheng *et al.,* 2011). Failure to remove nitrogen from the wastewater prior to its discharge into the water basin may lead to eutrophication, affecting the ecosystem (Scown *et al.,* 2010; Hoellein *et al.,* 2011). Phosphorus remained unaffected at both concentrations. Analysis of biofilm composition showed that nitrogen removing bacteria numbers decreased significantly in the biofilm community compared to phosphorus removing bacteria (Zheng *et al.,* 2011). While concentrations of 50 mg/L are not environmentally-relevant at the moment, concentrations of nanoparticle discharge may increase significantly in the future years due to continual increase in nanomaterial use. Therefore, compromising biofilms' ability to effectively remove and recycle contaminants from the wastewater due to toxic effects may further endanger aquatic habitats near WWTP discharge sites and overall quality of our lake/river water.

A study performed by Peulen and Wilkinson (2011) on *P. fluorescens* concluded that diffusion of nano-silver into the biofilm highly depended on the initial and aggregated size of the particle, as well as porosity and structure of the biofilm. Any particles bigger than 50 nm showed little diffusion into the biofilm, which suggests that biofilms and their composition may hinder the diffusion of NP in the environment that are larger than 50 nm (Peulen and Wilkinson, 2011).

To conclude, assessment of nanomaterial release into the environment should be prioritized in order to facilitate ecological risk assessment. Provided that focus of this thesis is on titanium

dioxide, specific effects of titanium dioxide on biofilms, microorganisms and other biota will be discussed below.

1.3 Regulation of nanotechnology in Canada

Environment Canada and Health Canada jointly hold responsibility for the regulation of chemical substances in Canada. Nanotechnology is regulated using regulatory frameworks designed for chemical substances. According to the National Collaborating Centre for Environmental Health (2011), exposure to nanoparticles is very likely due to the high volume of production of nanomaterials (NCCEH, 2014). Due to the potential differences in how nanoparticles and nanomaterials are generated compared to their macro-sized counterparts, the properties are unique to each substance, which means that it is difficult to predict what effects newly developed nanomaterials will have on human health and the environment. Therefore, there is a requirement for a new field to study nanomaterials and it has been proposed to name it nanotoxicology (Buzea *et al.*, 2007).

1.4 Biofilm as the predominant bacterial communities

Antonie Van Leeuwenhoek discovered bacteria in 1676 on tooth surfaces using his simple microscopes, while Heukelekian and Heller observed that bacteria grew better when there was a surface to which they could attach (Donlan, 2002). The ability of bacteria to respond successfully to environmental stimuli is one of many reasons that made these organisms the most abundant on the planet. The term biofilm is defined as “matrix-enclosed bacterial populations adherent to each other and/or to surfaces and interfaces” (Costerton *et al*., 1987). One of the earliest recorded observations of biofilms date back to 1933 in a paper by Henrici, where the author observed that bacteria prefer to grow upon submerged surfaces (O'Toole *et al.*, 2000). The matrix produced by the bacterial cells in the biofilm is composed of extracellular polymeric substance (EPS), which contains polysaccharides, extracellular DNA, lipids and proteins (Lopez *et al.*, 2010). The matrix protects the cells from biological and chemical antibacterial agents, as well as adverse environmental stresses such as pH changes, UV

radiation, osmotic shock and desiccation (Kokare *et al.*, 2009). Planktonic cells, which are mobile and serve as a biofilm's means of spreading, do not have the protection of an extracellular matrix (O'Toole *et al.*, 2000). It was shown that cells within the biofilm could be 10-1000 times more resistant to effects of antimicrobial agents than their planktonic counterpart (Watnick & Kolter, 2000; Mah & O'Toole, 2001). This biofilm resistance could be partially attributed to the slow growth rate commonly observed in mature biofilms (Mah & O'Toole, 2001).

Bacteria within biofilms were notably more resistant to bacteriophages, amoeboid predators, and to vortex-feeding protozoa (Donlan, 2002; Lopez *et al.*, 2010). A study performed by Desai *et al.* (1998) investigated resistance of planktonic and biofilm cell cultures to antimicrobial agents. It was found that bacteria grown in biofilm cultures were 15 times more resistant to antimicrobial compounds than the equivalent planktonic cultures (Desai *et al.*, 1998). Desai *et al.* (1998) concluded that growth phase and mode of growth had the biggest impact on bacterial resistance to antimicrobial agents. All these factors reinforce the predominance of biofilms in the natural ecosystems as opposed to free-floating planktonic cells.

Biofilms are able to form on all types of surfaces such as plastic, metal, soil particles, glass, wood, medical implants and equipment, and food products (Kokare *et al.*, 2009). In general, hydrophilic surfaces show greater bacterial attachment than hydrophobic surfaces and concentrations of several cations such as sodium or ferric ions in the media aid in attachment of cells to the surfaces (Donlan, 2002; Kokare *et al.*, 2009).

Biofilms occupy the lowest trophic levels and function as efficient catalysts for environmental carbon transformation and mineralization, as well as nutrient recycling and trapping and recycling of cellular components (Costerton *et al.*, 1987; Wolfaardt *et al.*, 1994; Stoodley *et al.*, 2002; Bester *et al.*, 2010). Biofilms are ubiquitous and can have beneficial or detrimental contributions to the environment or host (O'Toole *et al.*, 2000). Biofilm communities also provide an ideal environment to establish a symbiotic relationship between two metabolically

distinct bacteria (Kokare *et al.*, 2009). There are numerous reasons why so many bacterial species form biofilms. They include protection from the environment, ease of nutrient exchange, and potential acquisition of new genetic traits by horizontal gene transfer (Kokare *et al*., 2009).

1.5 Negative impacts of the biofilms

As mentioned above, biofilms are ubiquitous and can have beneficial or detrimental contribution to the environment or host (O'Toole *et al*., 2000). Biofilms are known to populate central venous and urinary catheters in hospitals, prosthetic heart valves, contact lenses or even intrauterine devices, causing infections or biofouling (Kokare *et al.,* 2009). Biofilms are also known to cause otitis media (inflammation of the middle ear), periodontitis (gum infection) and populate individuals with chronic infections such as cystic fibrosis (Kokare *et al.,* 2009). Biofilms cause microbial infections in the food industry, may impact water quality, produce harmful metabolites (e.g. H_2S) and cause clogging and corrosion of water pipe interiors (Costerton *et al.,* 1987; Lopez *et al.,* 2010). On occasion, disinfectant use was shown to enhance the formation of biodegradable substances, which was shown to promote biofilm formation in the distribution system due to microorganisms' ability to use the biodegradable substances as an energy source (Kokare *et al.*, 2009).

1.6 Biofilm benefits and commercial uses

Biofilms serve as the main food source in many aquatic habitats (Costerton *et al*., 1987), and also have an essential role in breaking down sewage and removing nitrogen and phosphorus in wastewater treatment plants (Ashraf *et al*., 2001; Scown *et al.,* 2010). Biofilms can be used in decontaminating polluted sites, and are vital for the health of many aquatic ecosystems (Fuchs *et al*., 1996; Hoellein *et al.,* 2011). EPS, in addition to protecting the cells within the biofilm, has been reported to sequester metal ions, cations and toxins (Kokare *at al.*, 2009).

The study performed by Wolfaardt *et al.* (1994) showed that a biofilm community, cultivated in a continuous-flow cell reactor, was able to accumulate herbicide diclofop methyl, primarily in the exopolymer matrix, and then break it down.

Biofilms' diversity allows researchers to engineer biofilms to remove or break down environmental pollutants (Ashraf *et al*., 2001). The byproducts of turning olives into oil are toxic phenols, which are now removed by using a bed of carbon granules encrusted with biofilms that comprise a range of *Achaea* and *Bacteria* (Bertin *et al.,* 2004). The carbon granules trap the pollutants, which the biofilm degrades (Bertin *et al.,* 2004). If correctly engineered, the biofilm will comprise sufficient genetic diversity to adapt to the specific environment within which it operates, requiring little maintenance (Bertin *et al*., 2004).

While most *P. aeruginosa*-plant interactions are detrimental to the plant, a study has found a *P. aeruginosa* strain that actually supports plant growth (Ashraf *et al*., 2001). *P. aeruginosa* can also degrade polycyclic aromatic hydrocarbons. Conjointly, this suggests the future uses of *P. aeruginosa* for environmental detoxification of synthetic chemicals and pesticides and for industrial purposes and pollution control (Johnson and Olsen, 1997; Ashraf *et al.*, 2001).

1.7 Formation and zoning laws of the biofilms

Bacteria cells forming a sheet on the surface (a biofilm) is considered a steady-state in the bacterial biological cycle as opposed to them swimming freely in the environment (O'Toole *et al*., 2000). Biofilm formation is a bacterial response to environmental stimuli such as nutrient availability (Brown *et al.,* 1977; O'Toole *et al.,* 2000). Alternatively, if the nutrient source is depleted, biofilms revert back to the planktonic state in order to search for other nutrient-dense areas (Kolter *et al*., 1993; Bester *et al.,* 2010). Production of an exopolysaccharide lyase *by P. fluorescens* has been shown under starvation conditions (Allison *et al.,* 1998).

Formation of the biofilm is well documented (Figure 1). Numerous factors favour biofilm formation in the nutrient-dense areas. These factors include substratum effect (roughness of

the surface), conditioning film (chemical modification to the attachment surface by the medium), hydrodynamics, characteristics of the aqueous medium and quorum sensing (cell-to-cell signalling) (Donlan, 2002; Kokare *et al.*, 2009). As a general first step, bacterial attachment is mediated by pilli, fimbriae, flagella and EPS to start the formation or enhancement of microcolonies (Watnick & Kolter, 2000; Kokare *et al.*, 2009). While single-specie biofilms are extremely rare in natural environments, they are used in laboratory conditions to study formation of the biofilms in detail. As an example, in single-species biofilms such as *P. aeruginosa* biofilms, it was found that acyl-homoserine lactones (acyl-HSLs) are important in defining boundaries between bacterial pillars among which motile cells, which maintain their association with the biofilm, swim (Watnick & Kolter, 2000). As Watnick and Kolter (2000) stated in their review, these molecules dictate zoning by-laws in single-species biofilms, while in *P. fluorescens* biofilms acyl-HSLs are responsible for attachment to substratum. Therefore, the exact genetic regulation of biofilm formation and cell attachment differs between bacterial species (O'Toole *et al.,* 2000).

According to Stoodley *et al.* (2002), there are three mechanisms of biofilm formation: redistribution of attached cells by motility (planktonic cells), binary division of attached cells (spreading of the biofilm outwards along the surface) and recruitment of cells from the bulk fluid to the developing biofilm.

The mechanism of redistribution of attached cells by motility starts with attachment of planktonic cells to the substrate (Lopez *et al.,* 2010). The initial attachment is reversible. The next sequential stage is irreversible adhesion by bacterium differentiating into biofilm-associated cells by supressing flagellum synthesis and producing exopolysaccharide glycocalyx polymer (Costerton *et al.*, 1987; Kokare *et al.*, 2009). This process is then followed by cell-to-cell adhesion, maturation (EPS production, binary division and recruitment of cells from the bulk fluid), and finally detachment of planktonic cells to populate new areas (Lopez *et al.,* 2010) (Figure 1). Microelectrode measurements have shown that oxygen concentration and pH fall in

a biofilm as the substratum is approached, suggesting that the environment in a biofilm is not homogenous (Watnick & Kolter, 2000).

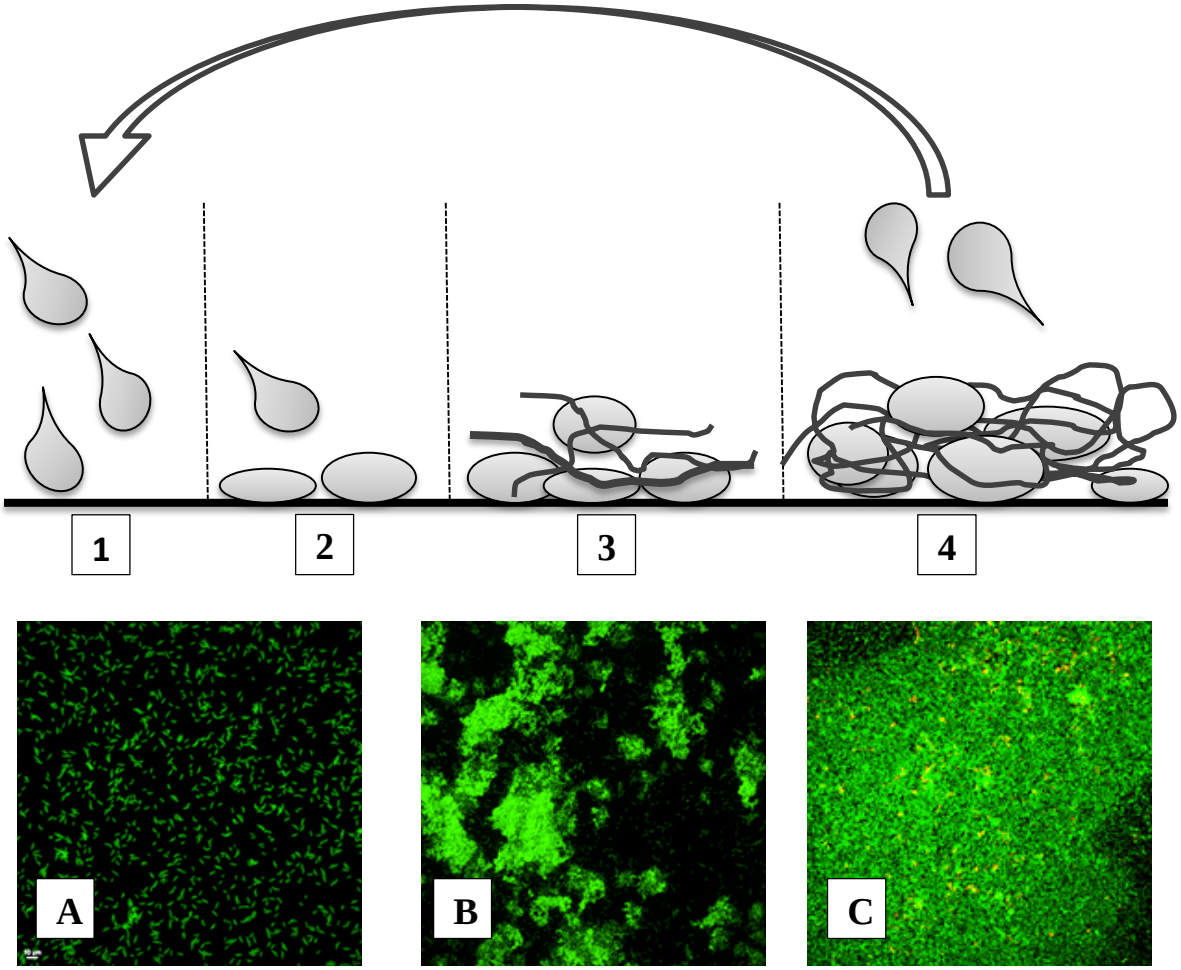


Figure 1: Simplified schematic of widely-accepted biofilm formation. *Step **1** shows planktonic bacterial cells moving towards the surface (substratum), and step **2** shows cells attaching (loosely). Image **A** depicts first 2 steps. Step **3** shows cells that have irreversibly attached and start to produce extracellular matrix and form colonies (image **B**). Step **4** shows established biofilm producing planktonic cells to populate new areas (image **C**).(figured adopted from Annual Review of Microbiology from Biofilms as Complex Differentiated Communities, P. Stoodley et al., Volume **56**, 2002).*

The model of conditioning layer formation, bacterial adhesion and biofilm expansion and then planktonic cell creation is widely accepted. However, certain researchers, for example Bester *et al.* (2005), argue that planktonic cell production happen every step of the way, not only after the maturation of the biofilm, and in fact promote biofilm formation (Bester *et al*., 2009).

Planktonic cells produced by the biofilm have to be viable in order to be able to establish new biofilms elsewhere.

1.8 *Pseudomonas* species

Pseudomonas is a genus of gram-negative, preferably aerobic gammaproteobacteria, belonging to the family *Pseudomonadaceae*. It contains almost 200 species, and among the most studied is *Pseudomonas aeruginosa* (PAO1). It is suggested that the organisms of the *Pseudomonas* genus constitute the most abundant known bacterial species on Earth (Costerton *et al.,* 1995; Palleroni, 2010). *Pseudomonas sp.* (CT07) is a more recent environmental isolate (Bester, 2010). In favourable conditions, these bacterial species form biofilms on practically any surface. Sessile cells have more active reproduction and general metabolism during biofilm formation, which slow down as biofilm matures, while planktonic cells are committed to motility to colonize new areas and thus promote biofilm proliferation (Costerton *et al*., 1987; Lopez *et al*., 2010). Depending on nutrient availability, biofilm structure may differ.

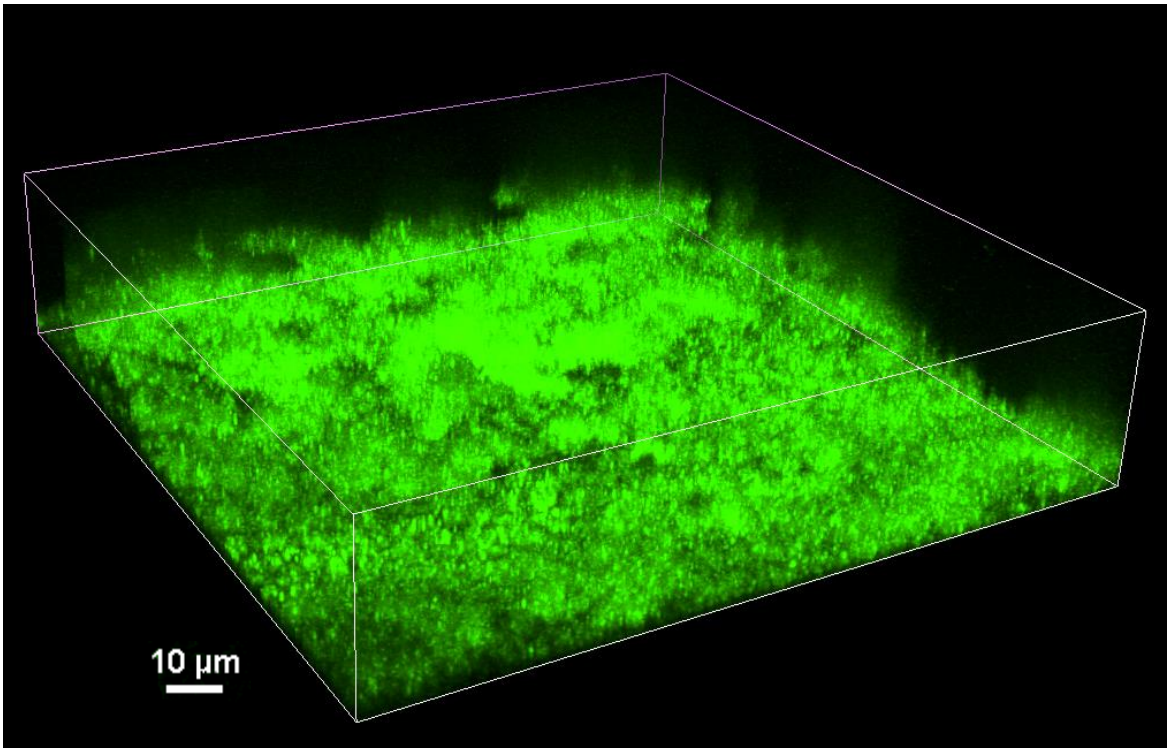


Figure 2: Volume snapshot of 144 hour old CT07-gfp biofilm. *Example of the biofilm's 3-D structure. Image was taken with Nikon 90i-C1 confocal microscope. Resolution is 1024x1024 pixels.*

1.9 *Pseudomonas aeruginosa* (PAO1) and *Pseudomonas* sp. (CT07)

P. aeruginosa is a gram-negative, rod shaped and monoflagellated bacterium able to populate the most diverse habitats on the planet (Peix *et al.,* 2009). *Pseudomonas aeruginosa* (PAO1) was first isolated in 1882 by Carle Gessard from the wound infections of soldiers (Moore and Flaws, 2011). In all oligotrophic aquatic ecosystems, which contain high-dissolved oxygen content but low nutrient content, *P. aeruginosa* is the predominant known inhabitant (Peix *et al*., 2009). Due to its diverse nature, PAO1 inhabits any type of environment, ranging from ponds to plants to human-made devices. It is also an opportunistic human pathogen, meaning it will infect individuals with compromised immune systems (Peix *et al.,* 2009). It is common to find PAO1 isolates in the tissue of cystic fibrosis patients (Costerton *et al.,* 1987). PAO1 is one of the most commonly studied strains of *Pseudomonas* species due to its ubiquity.

Pseudomonas sp. (CT07) is also a gram-negative bacterium, and a recent common environmental isolate from an industrial cooling water tower (Bester, 2010). CT07 is a close relative of *P. fluorescens*, and some researchers consider the two species to be interchangeable.

1.10 Titanium dioxide (TiO_2)

Titanium dioxide (Figure 3) is a metal oxide in a form of a white powder, being odourless and tasteless (Material Safety Data Sheet, n.d.). Photoactivation by UV light occurs at 380 nm wavelength (Kulacki *et al*., 2012). This is the property that makes this NP an effective sterilizing, deodorizing and anti-fouling agent when exposed to sunlight and thus used in the production of windows, paints, cements, tiles and other products. When photoactivated, TiO_2 produces reactive oxygen species (ROSs), which include hydrogen peroxide (H_2O_2), superoxide anion ($O \cdot_2^-$), hydroxyl radicals (·OH), and peroxynitrites (Sharma, 2009). These above-mentioned species contribute to oxidative stress, cell membrane damage, lipid peroxidation, DNA and protein damage, resulting in the damaged cells (Klaine *et al.,* 2008; Ma *et al*., 2012). Ultimately, perforation of the cell membrane occurs and there are potential cytotoxic and genotoxic effects of ROSs on cells (Dalai *et al.,* 2013).

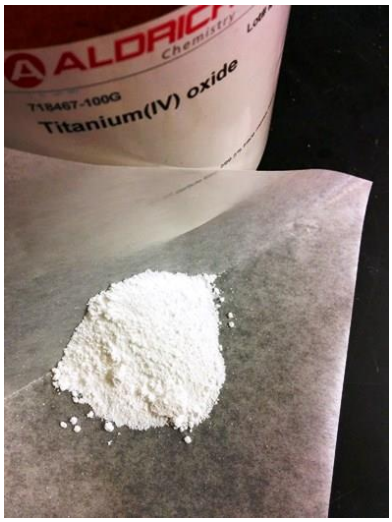


Figure 3: Titanium dioxide nanopowder. *Particle size is roughly 21nm according to the manufacturer.*

1.11 Synthesis and applications of titanium dioxide nanomaterials

Since the start of the twentieth century, titanium dioxide has been widely used as a pigment, as well as additive in sunscreens, paints, plastics, paper, toothpaste, cosmetic products and foodstuffs, and part of electrical ceramics, catalysts, electronics, and electric conductors (Ceresana, 2013). The photocatalytic properties of titanium dioxide were discovered in 1972 by Fujishima and Honda after the phenomenon of photocatalytic splitting of water on a titanium dioxide electrode under ultraviolet (UV) light was observed (Chen and Mao, 2007).

Natural sources of titanium dioxide in the environment consist of the minerals such as ilmenite ($FeTiO_3$) and rutile (TiO_2), which are found in plutonic and metamorphic rocks (Sharma, 2009). There are multiple methods of producing titanium dioxide nanomaterials and nanoparticles out of raw materials, and they include sol-gel method, micelle and inverse micelle method, sol method, hydrothermal method, and solvothermal method (Chen and Mao, 2007). Refer to Chen and Mao (2007) “Titanium Dioxide Nanomaterials: synthesis, properties, modifications and applications” for more detail as the details of the synthesis of titanium dioxide are outside the scope of this study.

Titanium dioxide accounts for roughly 70% of total production volume of pigments worldwide, and roughly 4 million tonnes of it is consumed annually (Shi *et al*., 2013). Titanium dioxide is in top five nanoparticles used worldwide in consumer products.

1.12 Chemical and physical properties of titanium dioxide

In the laboratory, TiO_2 was shown to aggregate and precipitate from the water column relatively fast (within 24 hours), going from 27 nm to 700-1000 nm, with an average floc size ranging from 160-250 nm (Adams et al., 2006; Kulacki *et al*., 2012), while in the environment, sedimentation and aggregation of these nanoparticles is more difficult to deduce due to water currents and turbulence, contact with microorganisms and colloids (Sharma, 2009). Therefore, it is currently unclear whether the materials in aggregates retain the same toxic potential and bioavailability to biota as their nanosize counterparts (Klaine *et al*., 2008). An experimental study done by Romanello and Fidalgo de Cortalezzi (2013) confirmed that average aggregate size of titanium dioxide particles was around 200-300 nm, and attempts to break the aggregates by ultrasonication were unsuccessful. The study also showed that pH closest to the point of zero charge of the nanoparticle (which is pH of 4 for slurry) caused a faster rate of aggregation and presence of divalent cations in the solution caused aggregation at a wider range of pHs (Romanello & Fidalgo de Cortalezzi, 2013).

Additionally, many experiments with nanoparticles in the laboratory settings use concentrations of TiO_2 that are much higher than those occurring in the environment, and general conditions in the laboratory are not the same as those of aquatic environments, which poses another difficulty when considering experimental result relevance in environmental applications (Nowack and Bucheli, 2007; Klaine *et al.,* 2008).

Due to these unique properties of nano-titanium dioxide in aqueous solution, chemical properties of TiO_2 are highly dependent on both its chemical and physical characteristics such as size, crystal structure, specific surface area, particle shape and charge, purity, solubility, agglomeration rate, and any substance that it is in contact with (especially if NPs are coming

from WWTP discharge) (Skocaj *et al*., 2011). Therefore, development of reliable and standardized means of nanoparticle characterization both in the laboratory setting and in the environment is a project to be undertaken.

A study conducted by Gottschalk *et al.* (2009) used the life-cycle perspective of engineered nanomaterial (ENM)-containing products to calculate predicted environmental concentrations of multiple nanoparticles (nano-TiO_2, nano-ZnO, nano-Ag, CNT, and Fullorene) in the US, Europe and Switzerland. The study concluded that for sewage treatment effluents, there could be a current risk to aquatic organisms from nano-TiO_2, nano-ZnO and nano-Ag due to annual increase in ENM use (Gottschalk *et al.,* 2009). While a number of studies have been conducted to assess potential nanoparticle toxicity on bacterial biofilms, algal communities, as well as invertebrates and vertebrates, no conclusive evidence is available to date on the extent of titanium dioxide potential toxicity on biota. However, a few studies discussed below have shown that titanium dioxide may interfere with the aquatic food chain on the level of the grazers.

A study done by Gurr *et al.* (2005) reported that ultrafine anatase-sized titanium dioxide particles (10-20 nm) induced oxidative DNA damage, lipid peroxidation and increased hydrogen peroxide production in BEAS-2B cells (human bronchial epithelial cells) in the absence of photoactivation. The study also found that rutile-size 200 nm particles induced hydrogen peroxide damage without photoactivation (Gurr *et al.*, 2005). A different study conducted by Park *et al.* (2011) found that cytotoxicity and oxidative stress of TiO_2 nanoparticles highly depended on physio-chemical properties, such as structure (rutile, anatase, or mixture) and photocatalytic potency. Conclusively, the study found that certain TiO_2 particles are cytotoxic to keratinocyte cells even without photoactivation (Park *et al*., 2011).

1.13 Titanium dioxide "doping"

All of the experiments mentioned above used UV lamps to irradiate titanium dioxide to cause reactive oxygen species production. Given information available in the literature, visible

spectrum (such as room lighting) does not have the capacity to fully photoactivate titanium dioxide. No study has given exact amount of photoactivation of titanium dioxide in the visible spectrum available from the room lighting, but it is presumed to be low. In order to increase the photoactivation of TiO_2 by visible light (termed band gap narrowing), numerous studies have recently developed "doping" of titanium dioxide with nitrogen, carbon, sulfur, iodine, or other ions (Asahi *et al.,* 2001; Ohno *et al.,* 2004). Ohno and colleagues (2004) substituted S (S^{4+}) for some of the titanium dioxide atoms in the lettuce, synthesizing chemically modified titanium dioxide photocatalysts. The team found that "doped" titanium dioxide solution showed strong absorption of visible light and photoactivation by wavelengths longer than 440 nm (Ohno *et al*., 2004).

1.14 Known toxicity of titanium dioxide on bacterial biofilms

Matsunaga *et al.* (1985) conducted one of the earliest photocatalytic/microbial studies. In the study, powdered titanium dioxide was suspended in an aqueous solution of planktonic cells (10^3 cells/ml) and photocatalytic killing of planktonic cells was observed with greater than 2-log reduction in viable cells under metal-halide lamp (Matsunaga *et al.*, 1985). The review done by Sharma (2009) stated that 72% reduction in growth of *E. coli* occurred at titanium dioxide concentration of 5000 mg/L, which is well above the current environmental levels. The study also concluded that toxicity of titanium dioxide nanoparticles is dependent on the size, form and concentration of the nanoparticles in the solution (keeping in mind that TiO_2 particles have high aggregation) (Sharma, 2009). Different microorganisms reacted differently to the same toxicological conditions (Sharma, 2009). Additionally, pH, ionic composition and strength of aqueous suspension on aggregation of NP, chemical composition of NP and any other unknown effects of organic matter on NP structure have a high impact on diffusion into the biofilm (Sharma, 2009; Peulen and Wilkinson, 2011).

There is now a growing body of literature documenting photocatalytic killing of planktonic cells using TiO_2 nanopowders (Gage *et al.*, 2005). Some researchers report oxidation of coenzyme A and decreased respiration as the main reason for cell death, while others report oxidative

damage to cell membrane and leakage of cell contents. Gage *et al.* (2005) looked at photocatalytic versus non-photocatalytic treatment of *Pseudomonas aeruginosa* (using UV-A light). Overall findings were that photocatalytic treatment of planktonic PAO1 cultures with titanium dioxide provided a 4-log reduction in the number of viable cells compared to a 1-log reduction by UV alone (Gage *et al.*, 2005). However, biofilm bacteria showed no susceptibility to photocatalytic treatment, although UV treatment alone caused a 2- to 3-log reduction in viable cells (Gage *et al.*, 2005). It is a well known fact that UV-A light alone can cause damage to bacterial cells either via oxidative damage to cell membrane and respiration, or by direct damage to membrane-bound enzymes (Fernandez & Pizarro, 1996).

A more recent study by Polo *et al.* (2011) looked at effects of photocatalytic titanium dioxide on populations of planktonic cells of *P. aeruginosa, P. stutzeri* and *B. cereus* and on biofilm population of *P. aeruginosa* exclusively. Just like in the study by Gage *et al.* (2005) there was a reduction in planktonic cell populations (1-log reduction for *P. aeruginosa* and *Bacillius* sp, and 2-log reduction for *P. strutzeri*). However, the photocatalytic treatment had no effect on the *P. aeruginosa* biofilms (Polo *et al.*, 2011). Gage *et al.* (2005) proposed a number of explanations as to why titanium dioxide had no effect. One of the explanation was that reactive oxygen species produced by titanium dioxide were potentially being scavenged by media components such as phosphates or phosphates could have been blocking active sites on the catalyst surface and therefore preventing photoactivation of TiO_2 (Gage *et al*., 2005). Alternatively, attached cells could have evoked a response that increased their resistance to ROSs or other effects of titanium dioxide (Gage *et al.*, 2005). It was also observed by multiple authors that Gram-negative bacteria are more susceptible to photocatalytic effects of titanium dioxide due to breakage of the outer lipopolysaccharide membrane (Polo *et al.,* 2011).

1.15 Known toxicity of titanium dioxide on other microorganisms, invertebrate and vertebrates

A number of studies have looked at accumulation of NPs in biofilms, their toxic effects, and passage of accumulated nanoparticles up the food chain. Ma *et al.* (2012) worked with *Daphnia*

magna and *Oryzias latipes* (Japanese medaka), testing phototoxicity of titanium dioxide on both organisms. As expected, larger concentrations of TiO_2 in suspension caused faster aggregation of NPs and their sedimentation (Sharma, 2009; Ma *et al.,* 2012). The results showed that NP concentration under 100 mg/L in regular lighting showed no effect, while concentrations between 150-1500 mg/L caused mortality ranging from 15-55% (Ma *et al*., 2012). In simulated solar radiation (SSR) conditions, which are equal to realistic environmental conditions, all TiO_2 concentrations caused a significant increase in *Daphnia* mortality of up to 4 orders of magnitude compared to regular laboratory conditions (Ma *et al.,* 2012). Japanese medaka were less sensitive than *Daphnia*, but SSR conditions increased TiO_2 toxicity 100 fold, causing concentration-dependent mortality (Ma *et al.*, 2012). The article by Ma *et al.* (2012) provides numerous references to studies performed on toxicity of titanium dioxide on microorganisms and invertebrates in its discussion section.

Campos *et al.* (2013) have done a recent study on effects of TiO_2 NPs on the *Daphnia* food chain, and found that NP aggregates bind algae and sediment in concentrations higher than 10 mg/L, effectively making *Daphnia's* food source unavailable. A review by Stocaj *et al.* (2011) has found that irradiated 25 nm TiO_2 nanoparticles proved to be toxic to certain freshwater algae species, and Kulacki *et al.* (2012) showed that toxic effects of TiO_2 on algal species became negligible at levels of 1 ppm (this concentration is environmentally relevant). However, there was an accumulation of TiO_2 NP in the biofilm, which was later shown to be transferred up the food chain and accumulated in the herbivorous snail *Physa acuta*, which was grazing on the dried biomass (Kulacki *et al.,* 2012). Measurements were performed using inductively coupled plasma atomic emission spectrometry (Kulacki *et al.*, 2012).

A recent study by Dalai *et al.* (2013) focused on cytotoxicity of titanium dioxide nanoparticles at low concentrations (1 mg/L or less) on freshwater microalgae *Scenedesmus obliquus*. Dalai *et al.* (2013) concluded that there was damage to algal cells at concentrations of 1 mg/L and 0.5 mg/L under irradiated conditions due to ROS formation (irradiation simulating sunlight conditions). However, the team also found damage to algae in the dark conditions at the same

concentrations, which they attributed to adsorption of titanium dioxide nanoparticles to the cell surfaces, which ultimately led to internalization of NPs by the algae and distortion of intracellular components (Dalai *et al*., 2013). From this alone, it can be concluded that concentration of TiO_2 in the solution and irradiation play a role in its toxicity. Most laboratory concentrations surpass those currently present in the environment. However, due to nanoparticle sedimentation, they are slowly accumulating in the benthic ecosystems and may be re-suspended into the water column, increasing concentrations.

Irradiated TiO_2 NPs showed low to moderate toxicity to invertebrates and vertebrates (Skocaj *et al*., 2011), and Zhu *et al*. (2010) were the first to provide evidence of TiO_2 NP accumulation transfer between *Daphnia* and *Danio rerio* (zebrafish) by dietary exposure. *Oncorhynchus mykiss* (rainbow trout), when exposed to 10 or 100 mg/kg TiO_2 nanoparticle diets for 8 weeks (with a 2 week recovery) showed only some mild oxidative stress and subtle chemical disturbances in the brain (Ramsden *et al.,* 2009). Sandworms, on the other hand, showed sub-lethal effects with NPs being visualized in the gut lumen using TEM (Skocaj *et al*., 2011). Lastly, increased levels of cadmium and arsenic were found in carp's brain tissue, after fish were subjected to irradiated TiO_2 NPs, which suggests that NPs might be vectors for other toxicants and potentially nutrients (Skocaj *et al.,* 2011).

The Canadian Centre for Occupational Health and Safety released a summary in March of 2013 based on research conducted by the International Agency for Research on Cancer (IARC). The summary stated that titanium dioxide, a compound that is naturally occurring in the environment in the form of ilmenite and rutile, has been classified as a potential human carcinogen (Canadian Centre for Occupational Health and Safety, 2013). Currently no studies have been done addressing toxicity of macro-molecules of titanium dioxide in the environment.

A more detailed and extensive toxicological overview of titanium dioxide was compiled by Shi *et al*. (2013). Table 1 shown below depicts the summary of sections 1.14 and 1.15 on the effects

of titanium dioxide nanoparticles (under irradiated and non-irradiated conditions) on different species across multiple trophic levels.

Organism	Effect	Source
E. coli	72% reduction in growth at TiO_2 concentration of 5000 mg/L	Sharma (2009)
***Pseudomonas aeruginosa* (PAO1)**	4-log reduction planktonic cells in TiO_2 + UV, and 2-log reduction in planktonic cells just under UV Biofilm – no susceptibility to TiO_2+UV	Gage *et al*. (2005)
Multiple algae species	Irradiated 25 nm TiO_2 particles – toxic to algae Effects of 1 ppm of TiO_2 had negligible effect	Stocaj *et al*. (2011) Kulaki *et al.* (2012)
***Scenedesmus obliquus* (freshwater microalgae)**	Damage to cells under irradiated and non-irradiated conditions at concentrations of 1 mg/L and 0.5 mg/L.	Dalai *et al*. (2013)
Daphnia magna	TiO_2 only: 150-1500 mg/L caused mortality ranging from 15-55% TiO_2 + UV: 4 order of magnitude increase in mortality	Ma *et al*. (2012)
Daphnia magna	At concentrations higher than 10 mg/L, TiO_2 binds algae and sediments out of the solution, causing starvation	Campos *et al.* (2013)
Oryzias latipes	Simulated solar radiation caused 100 fold increase in concentration-dependent mortality	Me *et al*. (2012)
***Physa acuta* (snail)**	TiO_2 bioaccumulation in snail feeding on biofilms exposed to TiO_2	Kulaki *et al.* (2012)
***Danio rerio* (zebrafish)**	TiO_2 accumulation transfer between *Daphnia* and zebrafish	Zhu *et al.* (2010)
***Oncorhynchus mykiss* (rainbow trout)**	Showed mild oxidative stress when exposed to 10 or 100 mg/kg TiO_2 nanoparticle diets for 8 weeks	Zhu *et al*. (2010)

Table 1. Summary table of effects of titanium dioxide nanoparticles (under irradiated and non-irradiated conditions) on different species across multiple trophic levels.

2. HYPOTHESIS

The study of potential impact of manufactured nanomaterials on aquatic organisms is a growing field (Campos *et al.,* 2013). In order to make the best risk assessment of nanoparticle toxicity on biofilms, more information is required on nanoparticle interaction with the biofilms both in light and dark environments (presence or absence of visible light). Biofilms are a staple of any aquatic environment, participating in nutrient cycling, waste digestion and serving as part of the food chain. Additionally, biofilms are important in wastewater treatment plant activated sludge treatment, and so they come into constant contact with nanoparticles among other contaminants. While biofilms are diverse communities that encompass many bacterial species, *Pseudomonas aeruginosa* (PAO1) and *Pseudomonas sp.* CT07 are selected to represent the biofilm communities in the environment as model organisms.

The null hypothesis for the study is that titanium dioxide (TiO_2) nanoparticles have no effect on the formation, growth, proliferation and viability of *Pseudomonas sp.* (CT07) and *Pseudomonas aeruginosa* (PAO1) biofilms at specific concentrations.

3. STUDY OBJECTIVES

The first objective of the proposed research is to study the potential toxicity of titanium dioxide nanoparticles on proliferation, growth and viability of a biofilm-forming bacterial species *Pseudomonas* sp after the biofilm has been established. The intention is to observe at what concentrations (if any) titanium dioxide nanoparticles affect growth and viability of the biofilm.

The second objective is to study the potential toxicity of titanium dioxide on formation of the two biofilm-forming bacteria species (separately) – *Pseudomonas aeruginosa* (PAO1) and *Pseudomonas* sp. CT07. Additionally, changes in biofilm structure, proliferation, growth and viability will also be observed.

Impacts will be measured using parameters such as formation, proliferation, viability and growth of the biofilm. Due to UV unable to penetrate deep into the layers of the water, the

exact mechanism of titanium dioxide toxicity will not be studied. However, the study plans to observe if there is an effect in room light versus no room light (dark) conditions to observe if any damage is done to the biofilm in the absence of light. Lastly, the study will be conducted using flowcell systems to simulate the natural environmental with turbulence and directional flow.

4. MATERIALS and METHODS

The flow-cell protocol used in this experiment has been adopted from Bester *et al*., (2005) paper. A detailed materials and methods section of flow-cell design can be found in Dr. Bester's doctoral dissertation (2010) titled "Biofilm-derived planktonic cell yield: a mechanism for bacterial proliferation". Introduction of high TiO_2 concentrations into the system, test for green fluorescent protein (GFP) leakage and 10 versus 20 z-stacks check were novel procedures. Summary of the protocol can be found in Appendix A.

Flow-cell reactor provides continuous flow of nutrients and contaminants into the system. The continuous flow simulates current and prevents planktonic cells from multiplying within the reactor.

Figure 4 below depicts general flowchart of the experimental design, with a more detailed methods flowchart available in in Figure 7.

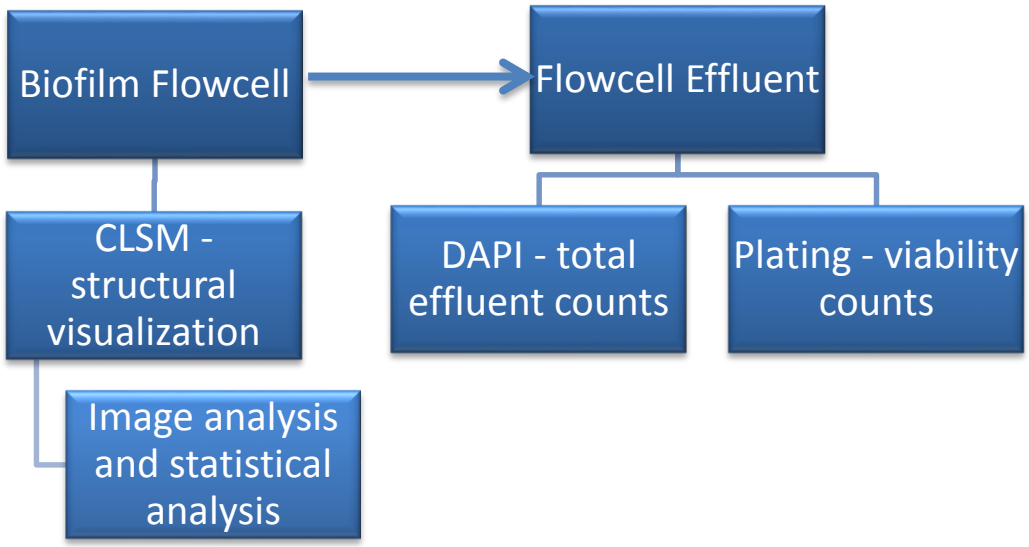


Figure 4: General flowchart of the experimental design. *Biofilm flowcell was subjected to CLSM imaging, while effluent was used for total effluent counts and viable planktonic cell counts.*

4.1 Bacterial strains used

The Gram negative, motile rod-shaped *Pseudomonas* sp. CT07 isolated by Bester *et al*. (2005) from water in an industrial cooling system was used as the test organism. *Pseudomonas aeruginosa* (PAO1) was the second strain used in a single experiment for comparison purposes. Both species were tagged with green fluorescent protein (GFP) (provided by Dr. Wolfaardt's lab).

4.2 Media used for cultivation and experiments

All but two of the experiments were performed using modified AB defined medium (final concentration of 1.51 mM $(NH_4)_2SO_4$, 3.37 mM Na_2HPO_4, 2.20 mM KH_2PO_4, 137 mM NaCl, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $CaCl_2 \cdot 2H_2O$, and 0.001 mM $FeCl_3$) (Clark and Maaloe, 1967) with 1 mM Na-Citrate$\cdot 2H_2O$ as the carbon source and distilled water. The other two experiments were performed using 0.3 g/L Tryptic Soy Broth (TSB) media with original 0.14 mM glucose concentration and increased 1 mM glucose concentration.

CT07-gfp and PAO1-gfp cultures have been propagated on 3 g/L Tryptic Soy Broth (TSB) agar plates on bi-weekly basis.

Overnight culture used as inoculum for biofilm studies was incubated at 24°C ± 2°C with shaking at 300 rpm in a modified AB medium described above with 10 mM Na-Citrate$\cdot 2H_2O$ as the carbon source.

4.3 Titanium dioxide concentrations used

Titanium (IV) oxide nanopowder (Sigma-Aldrich, ~21 nm particle size) was added directly into the media flask prior to autoclaving. Concentrations of 100 parts per million (ppm), 10 ppm and 1 ppm were used. Concentration of 1 ppm is closest to the estimated environmentally relevant concentration, while 100 ppm was considered to be significantly higher than current environmental exposure.

4.4 System design and sterilization procedure

A peristaltic pump (Gilson, Minipuls 3) was used to pump the AB modified media and all of its contents through a flowcell at a volumetric flow rate of 10-12 mL/h. The flowcell contained 4 channels. Dimensions of each channel were 5 mm by 4.3 cm by 6 mm, giving it a total volume of approximately 1.3 cm^3. The total dilution rate was calculated to be 9.2/hr, which suggests roughly 9 reactor volumes pass through every hour and wash away planktonic cells before they have time to multiply. Silicone tubing (inner diameter of 0.157 cm) was used for influent to allow for gas exchange as *Pseudomonas* bacteria require oxygen for optimal metabolism. A new cover slip with dimensions of 5.2 cm by 7 cm was glued onto a flowcell before each experiment with silicone glue. Bubble traps were inserted in the system about 25 cm prior to the flowcell entry point (Figure 5).

The whole system was sterilized with 10% (v/v) commercial bleach before each experiment for 2-3 hours at a rate of 8 mL/h. Sterile distilled water was pumped through the system for at least 12 hours at a rate of 10 mL/h to wash out the remains of the bleach. Before inoculation, the system was be flushed with sterile defined medium (Figure 6).

Titanium dioxide was kept in suspension with magnetic stirring.

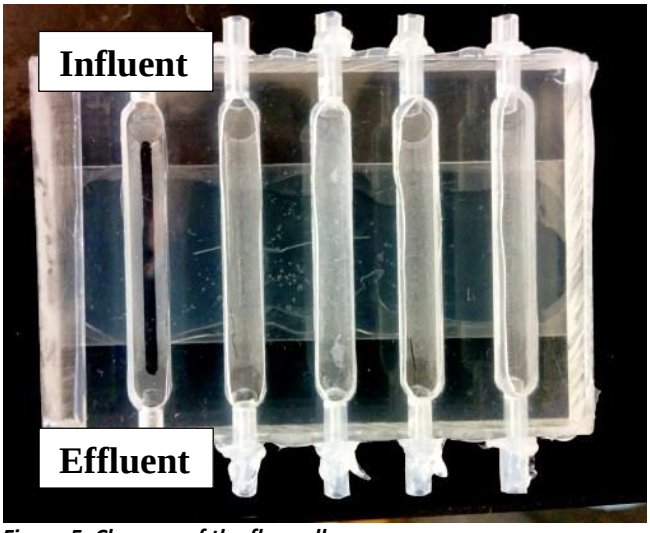


Figure 5: Close-up of the flowcell.

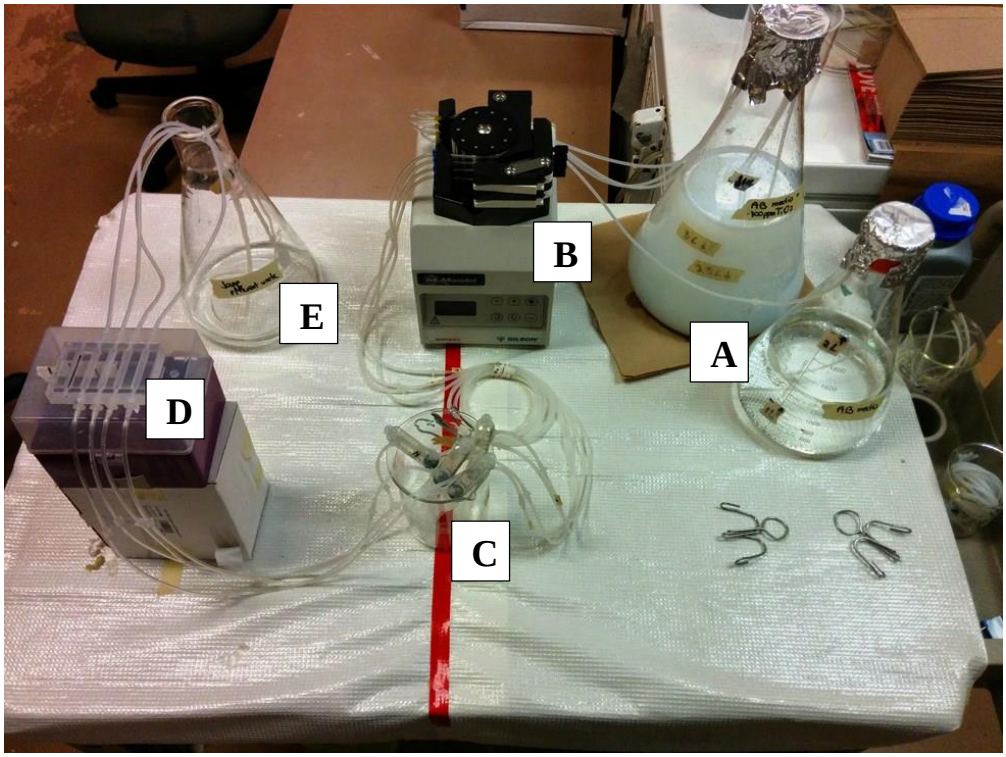


Figure 6: Flowcell system set-up*. The image depicts common system set-up.* ***A)*** *media flasks (cloudy media flask contains titanium dioxide);* ***B)*** *peristaltic pump;* ***C)*** *bubbletraps;* ***D)*** *flowcell; and* ***E)*** *effluent container.*

4.5 Characterization of TiO_2 aggregates using particle size analyser

Based on literature, the size of titanium dioxide nanoparticles greatly affected their toxicity to biofilms. Therefore, to characterize the size of titanium dioxide aggregates in defined AB media, a Brookhaven Particle Sizer BIC20 was used together with the proprietary software of Brookhaven called BIC Particle Size. The system was set up as described above (without culture inoculant) and flowcell influent and effluent samples were collected. Average titanium dioxide aggregate size was recorded for influent and effluent samples.

In order to identify medium component causing high aggregation of titanium dioxide nanoparticles, a set of flasks was set up with different AB defined medium components excluded. Additionally, MilliQ water with only titanium dioxide was set up as a control. Samples were tested for aggregate size at 30 minutes, 60 minutes and 18 hours. After the initial 60

minutes, samples were sonicated for 30 seconds to test for sonication effect on the aggregate size.

The software generated the following results for each sample: effective diameter (mean value), and standard error. Results available in Appendix B.

4.6 Biofilm inoculation

A cell culture was grown in an AB defined medium overnight. Optical density (OD at 600 nm) readings were performed on an Eppendorf BioPhotometer. In order to standardize the inoculate amount of cells at around 10^9 CFU/mL, 0.1 OD at 600 nm was used as an approximation of numbers to inject 100 µL of overnight culture into each flow cell channel through the influent tubing using a sterile 25G x $1\ ^1/_2$ needle and a sterile 1 mL syringe. The cells were given 20-30 minutes to attach to the flow cell walls with the pump turned off and upstream tubing clamped to prevent cells from swimming upstream. Normal flow rate of 10-12 mL/h was resumed afterwards.

4.7 10 versus 20 z-stacks – obtaining representative data

Based on Korber *at al.* (1992), the field of view of *Pseudomonas fluorescens* biofilm with a minimum of $1x10^5$ μm^2 was sufficient to obtain representative data. Each image taken of *Pseudomonas* sp. in the following experiments had a resolution of 1024 by 1024 pixels, and each pixel had dimensions of 0.3108 by 0.3108 µm (data provided by ICS file from EZ-C1 software). Therefore, the total area scanned per z-stack would be 101288.91 μm^2. At 10 z-stacks per channel, the total area imaged would add to up to roughly $1x10^6 \mu m^2$. To establish that 10 z-stacks are in fact sufficient to obtain representative data, 20 z-stacks per channel were taken. A biofilm was grown and imaged at 72 hours after inoculation. No bubble traps were used and no clamping of the tubing during imaging was done. Data available in Appendix C.

4.8 Step-by-step flowchart of the experiment methods

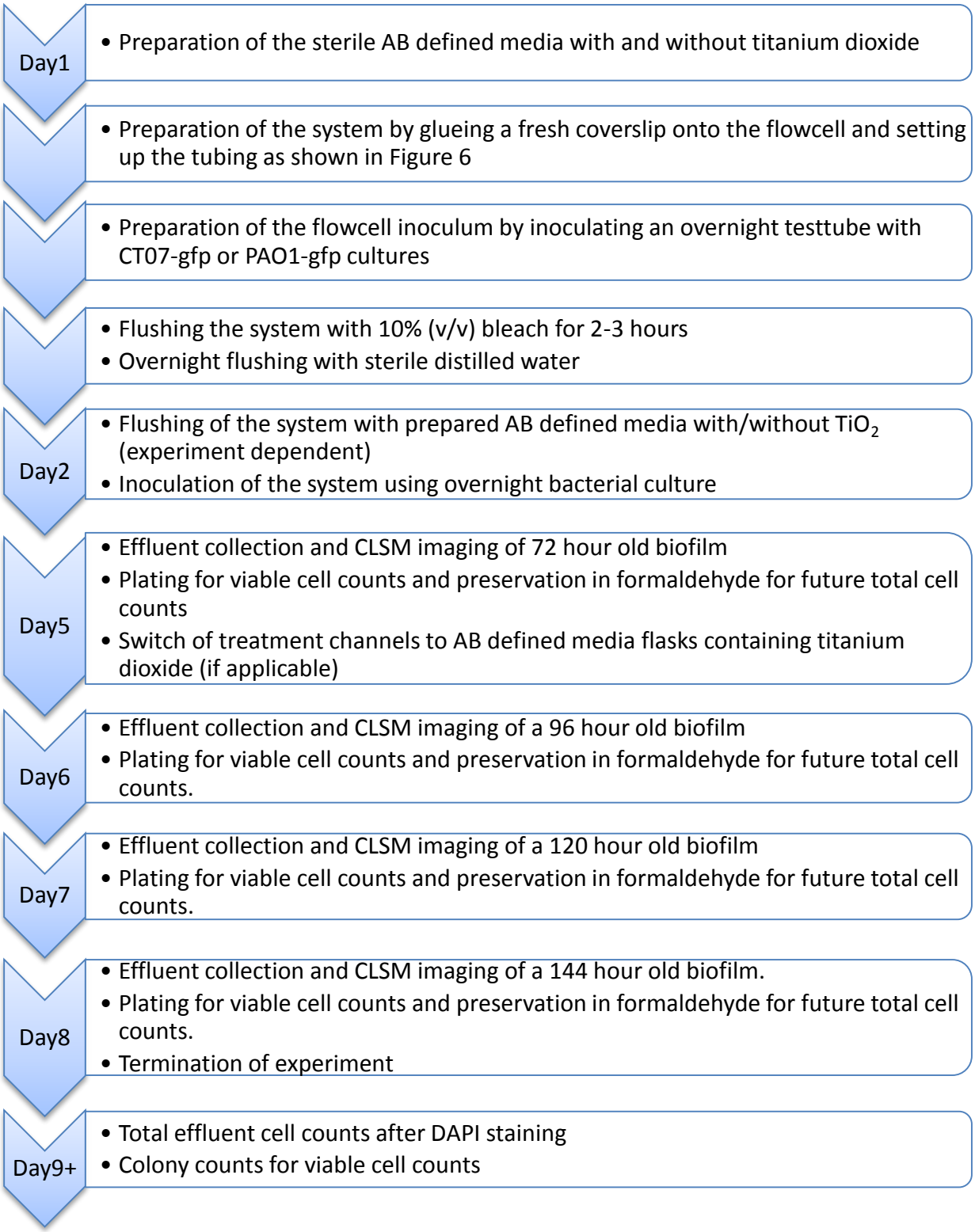


Figure 7: Detailed day-by-day flowchart of the experiment methods

4.9 Assessment of titanium dioxide toxicity using CLSM

Toxicity of TiO_2 on a) developed biofilm (introduction of TiO_2 at 72 hours), and b) developing biofilm (co-inoculation – introduction of TiO_2 at 0 hours) was assessed in single-culture biofilms of *Pseudomonas* sp. gfp (CT07) and *Pseudomonas aeruginosa* gfp (PAO1) grown in a flowcell using a modified AB media. Developed biofilm would have full substratum coverage compared to partial substratum coverage of the developing biofilm. Four channels were established: a) 1 reference and 3 treatment channels for the developed biofilm, and b) 2 treatment and 2 reference channels for co-inoculation. For a fully developed biofilm, one treatment channel was sacrificed every 24 hours of exposure by introduction of double stranded DNA basepair intercalating stain, so for a 72 hour exposure time 3 channels were required. Only one reference was established due to the 4-channel limit on the flowcell.

Biofilms in the treatment channels were exposed to TiO_2 concentrations of 100 ppm, 10 ppm and 1 ppm. The overall biofilm structure and integrity were assessed with confocal laser scanning microscopy (CLSM) Nikon Eclipse 90i-C1 microscope and camera, a 40x objective and 485 nm laser and appropriate emission filter (515/530) to visualize green fluorescent protein (GFP) pigment of intact cells. The effect of titanium dioxide nanoparticles on the integrity of the cell membranes was assessed by Propidium Iodide staining (Invitrogen, concentration 30 μM) and CLSM, using 685 nm laser and an appropriate emission filter (650LP). Propidium Iodide intercalates into double-stranded DNA or double-stranded RNA in a stoichiometric manner and therefore only labels cells with compromised membranes (most likely dead cells) since PI cannot pass through an intact cell membrane. A set of images of planes at various depths within the sample with same x and y coordinates is known as the z-stack. 10 Z-stacks were taken per channel for statistical analysis using EZ-C1 software (developed by Nikon Corporation for confocal microscope image acquisition). Step size for z-stack was set to 0.80 nm. The control experiment to assess the potential loss of GFP from cells due to compromised cell membranes will be discussed later.

Introduction of titanium dioxide to a 72 hour old biofilm was used to test for effects on structure of an already developed biofilm, while introduction of titanium dioxide at inoculation (0 hour old biofilm) was used to primarily test for effects of titanium dioxide on formation of the biofilm and any structural differences during its development.

4.10 Effect of visible light on titanium dioxide toxicity

Visible light has wavelengths ranging typically from 390 nm to 700 nm, while titanium dioxide photoactivates best at 380 nm. Therefore, the effect of visible light (room light in the laboratory) on toxicity of titanium dioxide due to photoactivation was tested. The above described procedure was followed for the experimental set up. In order to eliminate the potential photoactivation effect by fluorescent lighting, the flask with titanium dioxide was covered in tin foil to prevent light from penetrating into the liquid and the experiment was kept away from direct lighting.

4.11 Diagnosis of GFP leakage from CT07-gfp cells

Potential damage from TiO_2 to cell membranes could have caused GFP leakage, and therefore fluorescence loss. To ensure that no valuable data were lost during CLSM imaging, an evaluation of GFP leakage from bacteria cells due to potential damage from TiO_2 was assessed using FM1-43 lipid stain (Invitrogen) and overall design described above. The FM1-43 stain's primary targets are lipids and cell membranes, but it does not penetrate the membrane.
One reference and one treatment channel were set up using the same protocol described above. Biofilms were given 72 hours to develop in identical conditions after which the treatment channel was subjected to 100 ppm of titanium dioxide in the presence of visible light. CLSM was performed at 0, 24, 48 and 72 hours of exposure without the use of PI, since it is not a vital stain. However, after the initial scan at 72 hours of exposure, both channels were injected with FM1-43 lipid stain to visualize the whole biomass since FM1-43 binds to lipids and cell membranes. Results available in Appendix D.

4.12 Viable cell counts

A series of dilutions (up to 60 times) were done in sterile 0.9% saline solution using the effluent collected. The dilutions were plated in duplicate on 3 g/L TSB agar plates with an Eppendorf Xplorer pipette using a drop plate method (Herigstad *et al.,* 2001). Plates were allowed to grow for a minimum of 48 hours at room temperature in order to perform colony counts with the naked eye.

4.13 Total effluent cell counts

The total number of cells produced by the biofilm was assessed by combining 450 μL of the effluent with 80 μL of formaldehyde and stored at -4°C for a maximum of 1 week. Of the stored effluent, 100-150 μL were stained with DAPI (final concentration 250 μg/mL) for 20 minutes in the dark and mixed with 1 mL of 0.9% saline. The stained cells were funneled onto a membrane filter with 0.4 µm pore size. The filter was placed on a slide, and a small drop of antifadent mounting medium was added before the cover slip. Cells were visualized under a Nikon Eclipse 90i-C1 microscope using UV illumination and a DAPI filter cube with a 60x/1.4 oil-immersion objective or a 20x/0.75 Plan Fluor objective. The cells were counted in 30 fields if 60 x/1.4 oil-immersion objective was used, or in 10 fields if 20x/0.75 Plan Fluor objective was used. The fields were randomly chosen and counts were done using ImageJ software. A Sony CCD color camera was used as the detector for image acquisition.

Total counts were used to back-calculate average number of cells in the effluent per mL. An excel sheet designed to calculate CFU/mL from images taken with 60x/1.4 oil-immersion objective was used. The Excel sheet was designed by Dr. Kroukamp and provided by Dr. Bester.

4.14 Confocal Laser Microscopy Image Analysis

Image analysis was performed with NIS-Elements, IrfanView and COMSTAT (using a MATLAB package) software. NIS-Elements, developed by Nikon Corporation, was used for extraction of images from z-stacks and IrfanView was used for grayscaling the images. COMSTAT (Heydorn *et al.*, 2000) was used for quantitative analysis of the biofilm's structure.

4.15 Quantification of biofilm structure by COMSTAT

The novel computer program COMSTAT, designed by Heydorn and colleagues from the Technical University of Denmark, allowed for quantitative characterization of three-dimensional biofilm images to produce quantitative data. The following parameters were used to obtain a quantitative structure of the biofilm: biofilm biomass ($\mu m^3/ \mu m^2$), average thickness (μm), roughness coefficient, surface to volume ratio ($\mu m^2/ \mu m^3$) and maximum thickness (μm) (Heydorn *et al*., 2000).

Overall bio-volume, defined as the number of biomass pixels in all images of a stack, provides an estimate of the biomass of the biofilm. In biofilm literature, mean biofilm thickness is more commonly used. Maximum thickness ignores pores and voids inside the biofilm and simply provides a value for any given location. Biofilm roughness is an indicator of the biofilm heterogeneity and surface to volume ratio provides information on how much of the biofilm's surface is open to the surrounding environment from which nutrients and other components may diffuse to the bacteria within the micro-colonies.

4.16 Analysis of results and statistical analysis

Microsoft Excel was used to calculate averages and standard deviations for 10 values within each z-stack per parameter mentioned above obtained from CLSM images using the COMSTAT program.

For statistical analysis of all CLSM results, such as differences between biomass and average thickness values, a student t-test was performed with equal variance, 2 tails and a confidence interval of 95% (alpha level of 0.05). When p-values were close to 0.05, the exact value would be stated. For p-values smaller than 0.001, $p < 0.001$ will be stated. Microsoft Excel was used for t-test analysis.

For total effluent counts, ImageJ software was used to count the cells within each image taken.

5. RESULTS and DISCUSSION

5.1 Planktonic cell population of the tubing upstream of the flowcell

In one of the subsequent experiments, a decrease in reference channel biomass and average thickness at 144 hours (72 hours of exposure) was puzzling at first (example: Figure 18). Therefore, an experiment was designed to grow CT07-gfp biofilm in the two channels (duplicates) for the duration of 168 hours (7 days) and observe if a similar decrease would occur (data not included). At 144 hours after inoculation it was noted that the thickness of the biofilm started to decrease based on the number of slices in each z-stack (no COMSTAT analysis was performed). After careful inspection of the flowcell and the tubing it was noted that biofilm has grown upstream of the flowcell and populated the silicone tubing (Figure 8). Such outgrowth could have potentially happened in the 10 ppm exposure experiment. In both cases the pump was stopped during the imaging process, but the influent tubing was not clamped, allowing planktonic cells to swim upstream of the flowcell and establish a new biofilm.

Within the next 24 hours (total time of 168 hours) the biofilm extended further up the tubing towards the nutrient-rich media, in the process starving the biofilm located downstream in the flowcell. This phenomenon may explain the potential drop in the biomass and average thickness in the reference channel after 120 hours since inoculation.

Therefore, for all future experiments the tubing was clamped just upstream of the flowcell to minimize the chance of planktonic cells establishing biofilm in the silicone tubing. Since the CLSM imaging can only be performed on a flowcell, biofilm established upstream would starve the biofilm growing in the flowcell and no usable data would be obtained.

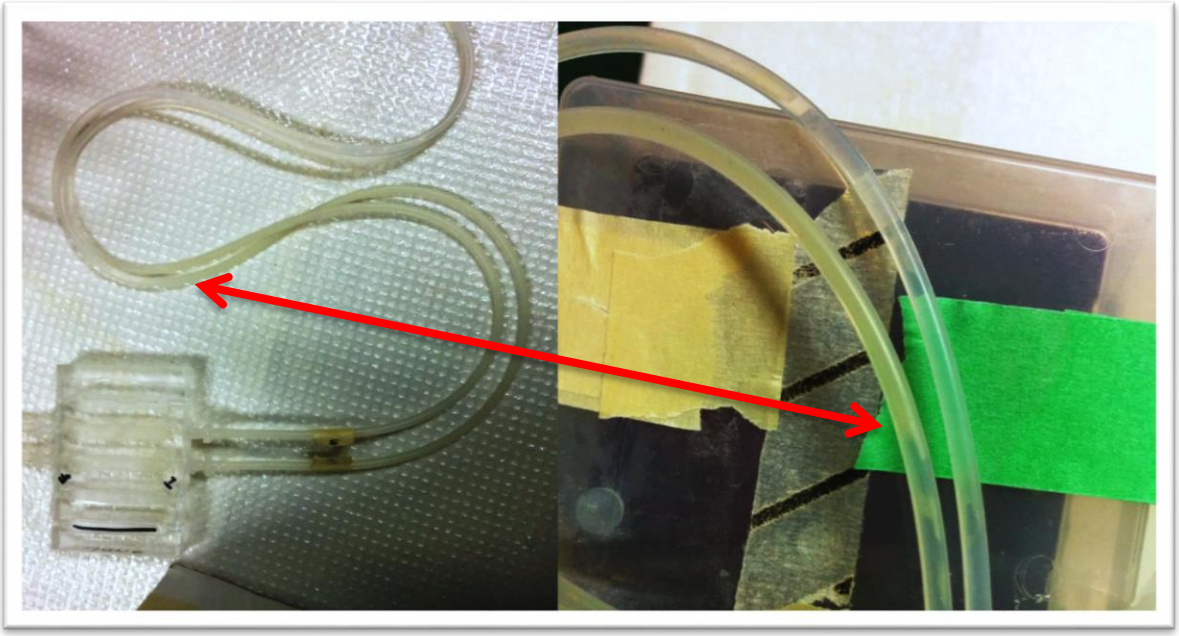


Figure 8: Picture of the tubing upstream of the flowcell at 144 hours after the inoculation with CT07-gfp cells. *Arrows point to interphase between biofilm-populated silicone tubing and un-populated tubing. CT07-gfp biofilm gives off a yellow color. Picture was taken with Nexus 5 phone.*

5.2 Effects of Titanium Dioxide nanopowder on biofilms

For the following set of experiments, biofilms were grown in AB defined medium for 72 hours in the presence of visible light. One reference and three treatment channels were prepared for each experiment. For the first three experiments, three treatment channels were subjected to different concentrations of titanium dioxide (100 ppm, 10 ppm and 1 ppm) in the presence of visible light. For the fourth experiment, three treatment channels were subjected to 100 ppm of TiO_2 in the absence of visible light. The effect of titanium dioxide concentrations on proliferation, growth and viability of biofilms was assessed.

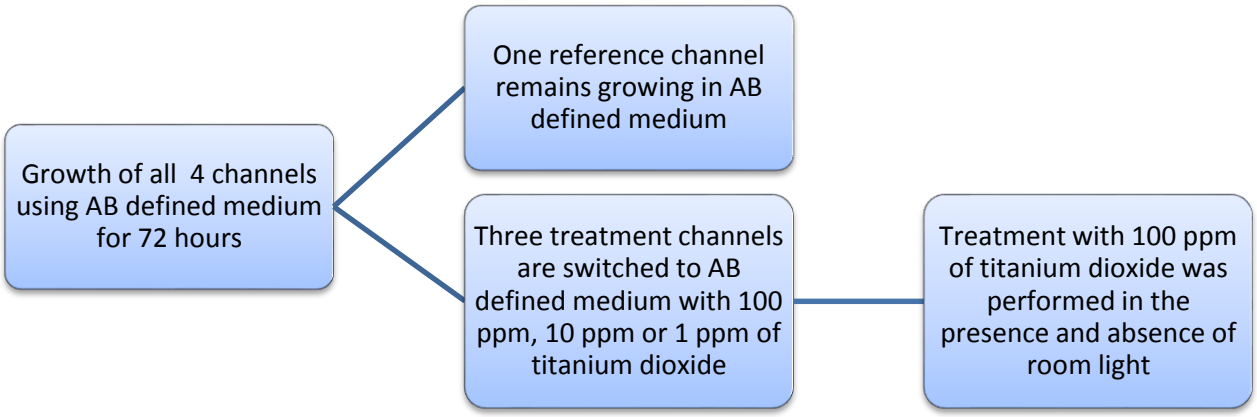


Figure 9: Diagram above shows a flowchart of the experiments performed in section 5.2.

5.2.1 Effects of 100 ppm of titanium dioxide on 72 hour old biofilm in visible light

After deciding on the media composition and number of z-stacks to be taken, and the fact that GFP would not leak from the compromised cells, the effects of titanium dioxide concentration of 100 ppm on mature biofilm (72 hours old) was tested. The experiment was conducted under regular laboratory fluorescent lighting during the day, and lights shut off at night. Bubble traps were used to prevent bubble accumulation in the flowcell channel and the tubing was clamped during imaging to prevent planktonic cells from swimming upstream and establishing a biofilm.

The effect of 100 parts per million (ppm) concentration of titanium dioxide on 72 hour old CT07-gfp biofilm (considered fully formed) was assessed using confocal laser scanning microscopy (CLSM), effluent viable cell counts and total cell counts. Confocal laser scanning microscopy images and effluent samples were taken at 24 hour intervals for up to 72 hours after the introduction of the contaminant (TiO_2).

Based on COMSTAT and statistical analysis it is evident that 100 ppm concentration of TiO_2 had a noticeable impact on biomass production between treatment and reference biofilms during the first 48 hours of exposure ($p < 0.001$ at 24 and 48 hours) and on average thickness ($p < 0.05$ at 24 hours, and $p < 0.001$ at 48 hours). After 72 hours, while the biofilm biomass did not reach the same levels as the reference channel, there was no evident significant difference between the treatment and the reference channel, which suggests that the biofilm was able to proliferate (Figure 10).

Given information available in the literature, the visible light spectrum (such as fluorescent lighting and daylight) poorly photoactivates titanium dioxide, since maximum activation occurs at UV-A (370-380 nm). No study has given exact photoactivation parameters of titanium dioxide in the visible spectrum available from the fluorescent lighting. However, the studies suggest that the percentage of the photoactivation by visible light is low. In order to increase the effectiveness of TiO_2 photoactivation by visible light (band gap narrowing), numerous studies have looked into "doping" of titanium dioxide with nitrogen, carbon, sulfur, iodine, or other ions (Asahi *et al*., 2001; Ohno *et al.*, 2004). Additionally, as mentioned in the introduction, Gurr *et al.* (2005) and Park *et al*. (2011) showed that physio-chemical properties highly effect titanium dioxide photoactivation.

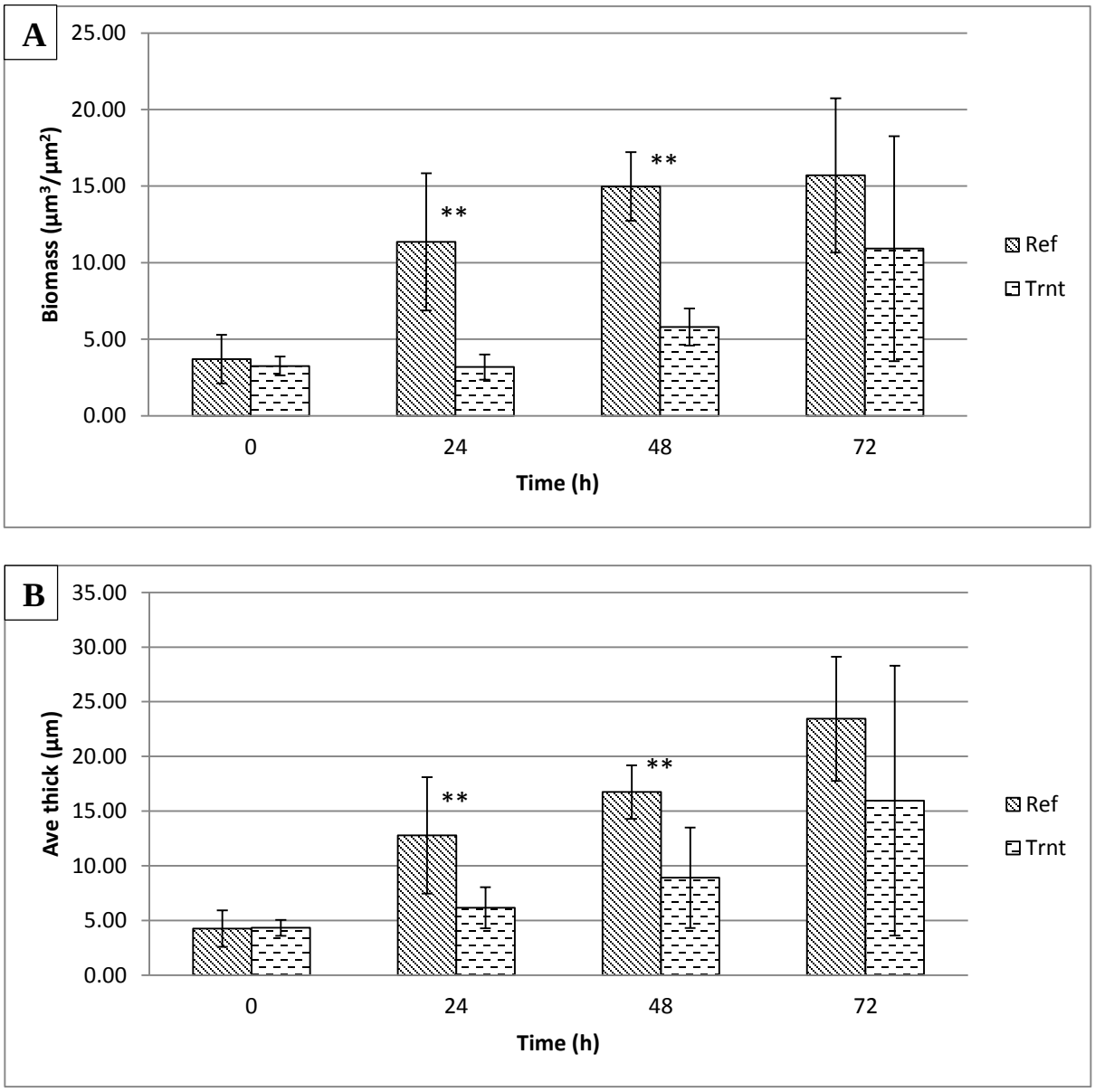
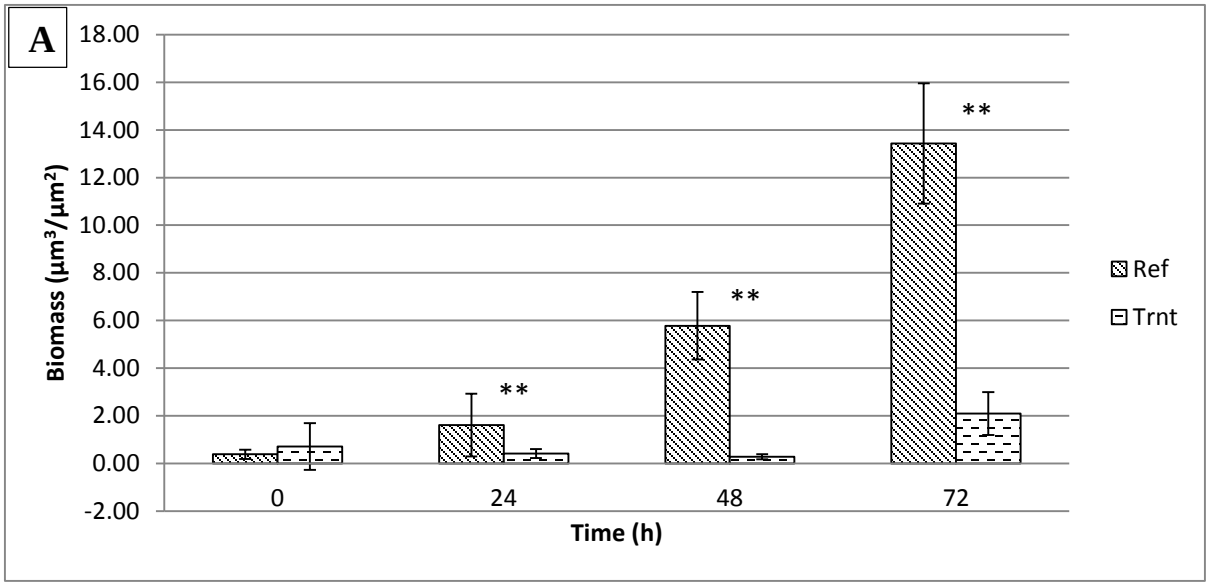


***Figure 10**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of reference (ref) and treatment (trnt) channels at 0 hours exposure (72 hour mark of biofilm growth), 24, 48 and 72 hour exposure to titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 100 ppm of titanium dioxide. At time zero, biofilms in reference and treatment channels are 72 hours old, grown in identical conditions. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*



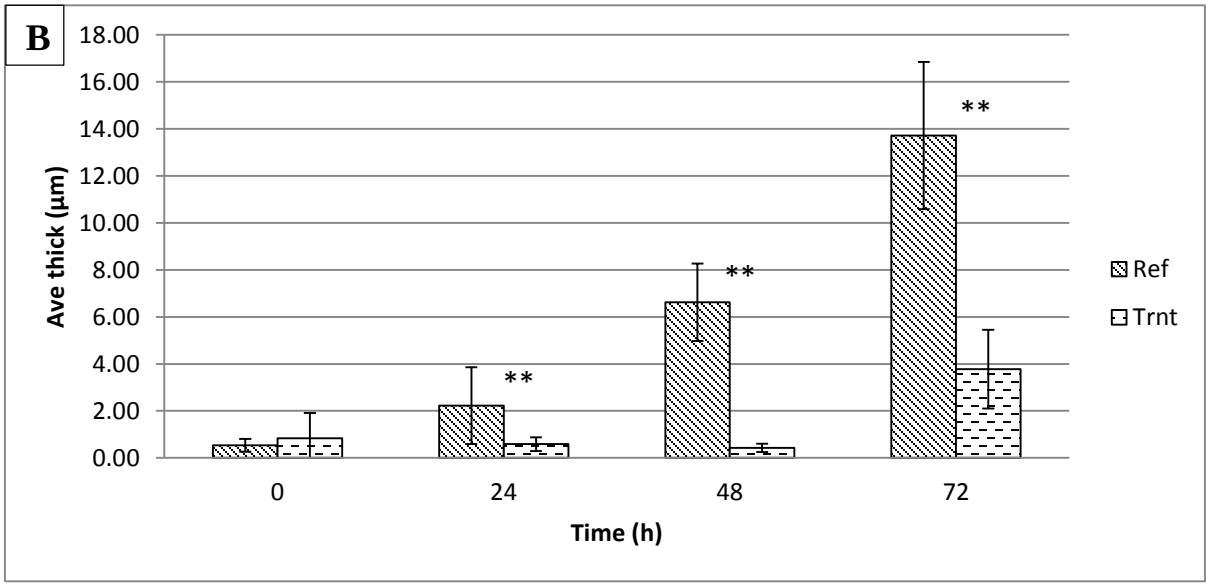


***Figure 11**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (μm) values of reference (ref) and treatment (trnt) channels at 0 hours exposure (72 hour mark of biofilm growth), 24, 48 and 72 hour exposure to titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in μm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 100 ppm of titanium dioxide. At time zero, biofilms in reference and treatment channels are 72 hours old, grown in identical conditions. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

Each biofilm is a diverse ecosystem and therefore there is an inherent difference in proliferation and growth rate. There are multiple explanations for the biofilm behaviour observed in Figure 10. One potential explanation for stagnation in biofilm growth at 24 hours of exposure is damage from reactive oxygen species produced by titanium dioxide. Two studies mentioned showed that titanium dioxide produces hydrogen peroxide without being photoactivated (Gurr *et al.,* 2005; Park *et al*., 2011). The second possibility is adsorption of titanium dioxide nanoparticles to the cell surface, followed by internalization of nanoparticles by bacterial cells and distortion of intracellular components. This pathway was suggested by Dalai *et al.* (2013) after the experiment where titanium dioxide exhibited damage to algae in the dark conditions. The third explanation is simply the inherent difference in proliferation of the biofilm, though unlikely based on results shown in Figure 11.

Figure 11 shows biomass and average thickness values for a CT07-gfp biofilm, which was exposed to 100 ppm of titanium dioxide under fluorescent lighting at time 0 hours. These results came from experiment where cells were checked for GFP leakage (Appendix D). Unlike 24 hour stagnation in biofilm growth in Figure 10, the period of stagnation in Figure 11 is twice the time (48 hours), which is significant for bacterial growth (provided *Pseudomonas* has been reported to have generation time of 0.58 hours). At 72 hours, the biofilm in the treatment channel proceeded to grow and proliferate, while the reference channel biofilm has been proliferating throughout the exposure time. It is highly unlikely that the biofilm in the treatment channel would arrest its proliferation in the presence of nutrients. Therefore, it is logical to conclude that 100 ppm of titanium dioxide had an effect on biofilm growth and proliferation and initiated a stress response from CT07-gfp cells.

Polo *et al.* (2011) found that PAO1 sessile cells of the biofilm grown on slides in ABT minimal medium supplemented with 0.5% (wt/vol) glucose developed resistance to reactive oxygen species produced by photoactivated titanium dioxide (titanium dioxide concentration of 3 g/L, or 3000 ppm). Additionally, Mah and O'Toole (2001) reported that biofilms are resistant to hydrogen peroxide penetration due to catalase-mediated degradation. Therefore, one potential

explanation for biofilm recovery from the stagnant state after 72 hours of exposure is the development of the resistance to products of titanium dioxide (such as hydrogen peroxide). Although photoactivation was not measured and therefore it cannot be specified if titanium dioxide was photoactivated, there is potential for production of reactive oxygen species such as hydrogen peroxide in absence of light (Gurr *et al.,* 2005; Park *et al*., 2011). As mentioned by Polo *et al.* (2011) and Mah and O'Toole (2001), bacterial cells have mechanisms for combating hydrogen peroxide.

Alternatively, EPS produced by the biofilm severely hinders the passage of any particle bigger than 50 nm (Peulen and Wilkinson, 2011). Therefore, if an alternate mechanism was at work where titanium dioxide particles were internalized by bacterial cells, two explanations can be offered for eventual recovery of the biofilm: 1) the majority of titanium dioxide aggregates were too large to penetrate deep into the biofilm matrix and therefore only a handful of smaller aggregates could cause the initial damage, and 2) the cell blocked the absorption of titanium dioxide. It was not possible to assess though whether titanium dioxide nanoparticles were trapped within the biofilm or not.

The viable cell counts presented in Figure 12 show that the exposure to titanium dioxide did not reduce or prevent biofilm from producing viable planktonic cells. With the rate of production remaining relatively similar compared to the untreated channel. The review by Stewart and Franklin (2008) argues that diversification and genetic changes of the bacteria within the biofilm aids in survival against antimicrobial agents and constant environmental changes, and Desai *et al.* (1998) found that antimicrobial resistance increased in planktonic cultures as they approached stationary phase. Therefore, one of the possible reasons that explain the similarity in viable cell production is that planktonic cells produced by the established biofilm under stress conditions may have altered the genetic expression and developed a higher resistance to the effects of titanium dioxide.

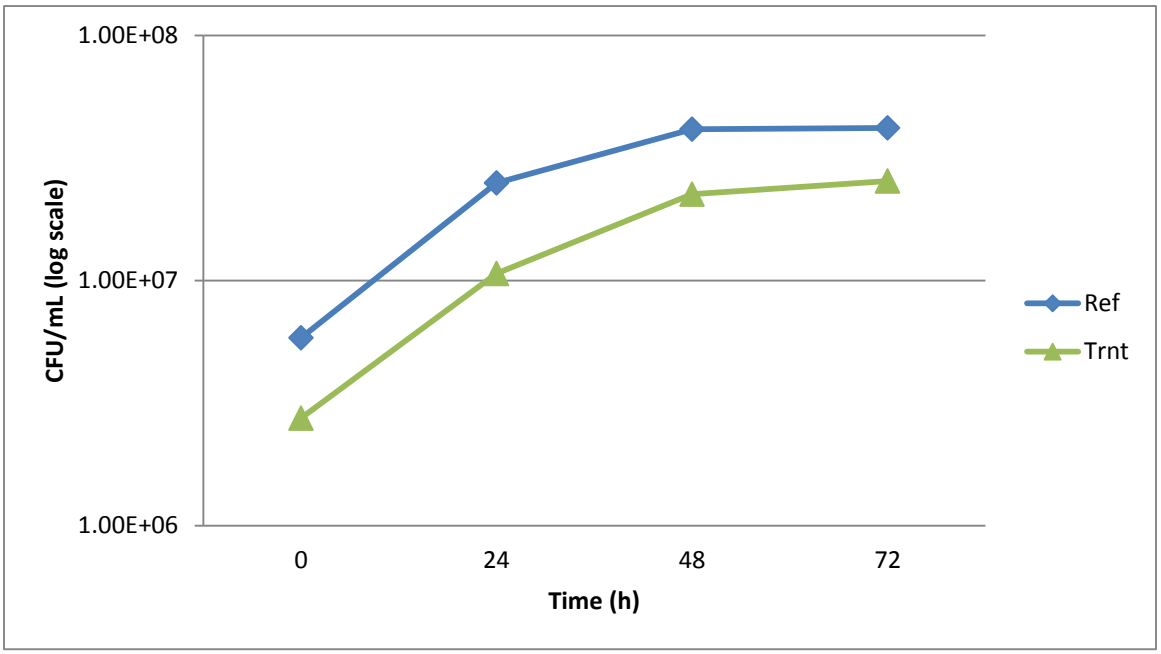


***Figure 12**: **Viable cell counts at 24 hour intervals during treatment with 100 ppm of TiO_2.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm in regular room lighting conditions and CT07-gfp biofilm in treatment (trnt) channel and reference (ref) channel. In the graph, x-axis shows time of treatment and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Figure 13 shows that values were within the same order of magnitude in total effluent cells from treatment and reference channels. This may suggest that titanium dioxide does not promote biofilm shedding and that slower biomass increase in the treatment channel was not due to biofilm losing its biomass via effluent cell, but perhaps due to stress response and cell damage that interfered with cell division.

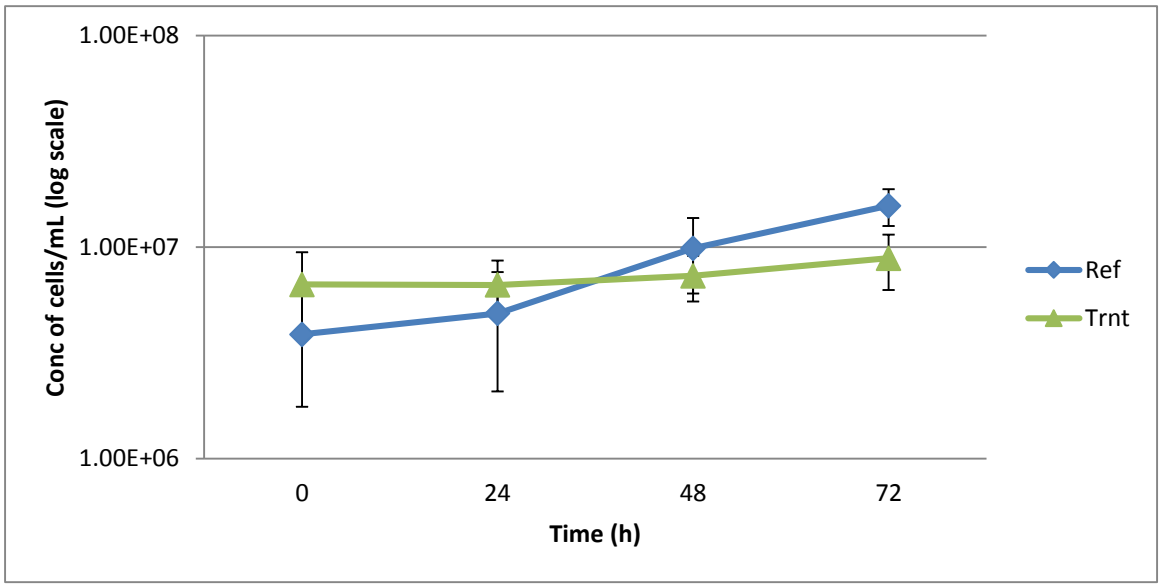


Figure 13: Total effluent cells counts at 24 hour intervals during treatment with 100 ppm of TiO_2. *Average concentration of cells per mL values obtained in the tests with titanium dioxide concentration of 100 ppm in regular room lighting conditions and CT07-gfp biofilm in treatment (trnt) channel and reference (ref) channel. In the graph, x-axis shows time of treatment and y-axis shows log (conc. of cells) values at each time point for both treatment and reference channels.*

Lastly, the percentage of compromised biomass, as indicated by Propidium iodide staining in Figure 14, suggests that the initial effect of titanium dioxide (effect within 24 hours) had a higher damaging impact on the integrity of the cell membranes within the biofilm than longer term exposure (up to 72 hours). Propidium iodide binds to the DNA within the cell, and cannot cross cell membrane that is fully intact (eBioscience, 2010).

Based on visual analysis between percentages of compromised biomass of treatment channels, there is visible difference between the percentage of compromised biomass between 24 and 48 hours of exposure. However, there is little difference between 48 and 72 hours of exposure. When comparing reference channel and treatment channel at 72 hours of exposure, there is a significant difference in the statistical values ($p < 0.001$). However, each biofilm is unique and cannot be reproduced even under controlled laboratory conditions. Therefore, there would be natural variance in the percentage of compromised biomass within each individual biofilm considering that cells die at different rates within each biofilm.

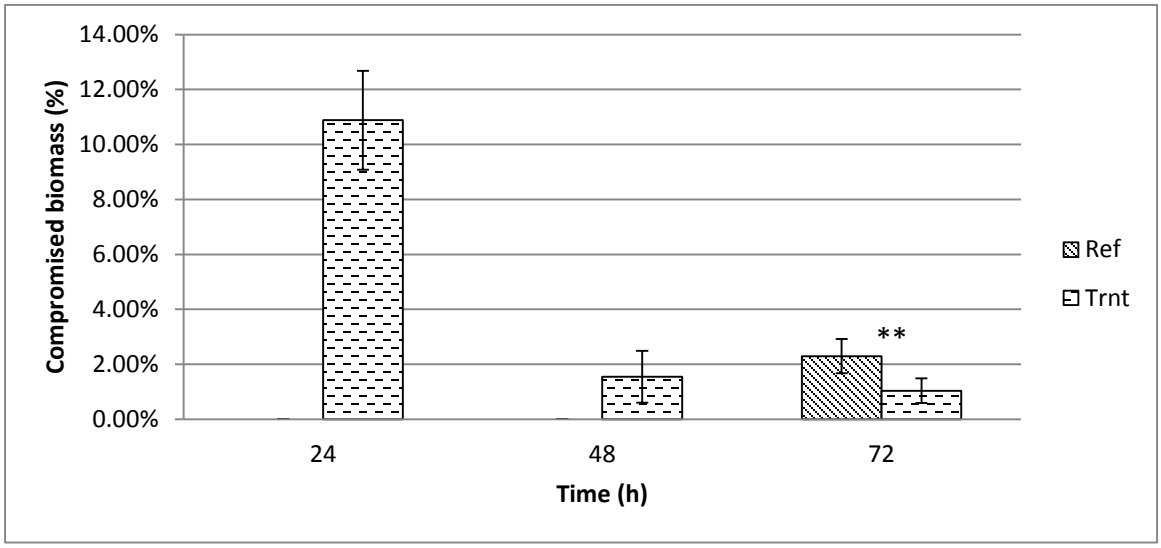


***Figure 14*: Percent of compromised biomass in treatment and reference channels during treatment with 100 ppm of TiO_2.** *The bar graph shows compromised biomass (%) of treatment (trnt) channels at 24, 48 and 72 hours of exposure and reference (ref) channel biomass at 72 hours (72 hours of treatment exposure, or 144 hours of total growth). Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

It is difficult to say whether or not the percentage of compromised cells is a reliable means of assessing the extent of the titanium dioxide damage to the biofilm. Cells within the biofilm get damaged and naturally die. Propidium iodide does not distinguish between viable cells with compromised membranes and dead cells, although it is a common stain used to identify dead cells (eBioscience, 2010). However, considering these results in conjunction with results from Figure 10, the initial higher value of compromised biomass coincides with insignificantly lower biomass production in the first 24 hours (0.08 $\mu m^3/\mu m^2$ decrease in the first 24 hours). The subsequent decrease in compromised biomass coincides with total biomass increase in the treatment channel at 48 hours of exposure to titanium dioxide (a 2.62 $\mu m^3/\mu m^2$ increase). Despite the fact that the percentage of compromised biomass was significantly higher in the reference channel than the treatment channel at 72 hours of exposure, there was still an increase in overall biomass production in both channels. Therefore, the percentage of compromised biomass is a very general indicator of biofilm growth and proliferation, and in no way correlates to the viable cell production.

A study performed by Gage *et al.* (2005) looked at effects of UV light and titanium dioxide coated slides on biofilm viable cells and on planktonic cells *of Pseudomonas aeruginosa* (PAO1). The results showed a 1- to 2-log reduction in viable cells on UV illuminated slides with and without titanium dioxide coating. The reduction occurred within the first 8-24 hours, after which viable cell counts increased above the initial counts. This study concluded that reduction of the biofilm cells was primarily due to UV irradiation and TiO_2 photocatalytic activity was insignificant. Provided UV irradiation was not used in this experiment, no visible effect on planktonic cell production is in accord with the study by Gage *et al.* (2005).

Considering all available data from the experiment where a 72 hour old biofilm was exposed to 100 ppm of titanium dioxide (Figures 10-14), it is possible to conclude that titanium dioxide at concentrations of 100 ppm had an effect on biofilm's growth rate, in some cases more severe than others (difference in data between Figures 10 and 11). Based on similarity in effluent cell counts and viability counts it can be assumed that titanium dioxide toxicity does not act by killing off newly formed cells, otherwise total effluent counts would be greater than reference and viable planktonic counts would be lower. Therefore, it is possible that CT07-gfp cells potentially supress their division in the presence of titanium dioxide in order to divert the resources to either damage control or towards developing resistance to titanium dioxide or reactive oxygen species created by titanium dioxide. Gage *et al.* (2005) have suggested this mechanism as one of the potential means of bacterial biofilms surviving titanium dioxide exposure. The exact mechanism remains unknown.

As an alternative explanation for biofilm proliferation after 48 hours, hydrogen peroxide and other free radicals have a half-life that can vary greatly on the composition of the media they are in. According to the MSDS, the half-life of hydrogen peroxide may very between 8 hours and 20 days. A study done by Häkkinen *et al.* (2004) on boreal lakes with high dissolved organic carbon and increased metal concentrations found that the half-life of hydrogen peroxide varies between 1.4 hours and 58.2 hours. Hydrogen peroxide is considered the most stable molecule among reactive oxygen species with the longest half-life (Cruz de Carvalho, 2008). Hydroxyl

radicals, on the other hand, are highly reactive with half-life of only about 10^{-9} s, regardless of the solution they are in. The short half-life of hydrogen peroxide and other free radicals in the defined AB medium and lack of production of new reactive oxygen species may be one of the reasons why a biofilm subjected to titanium dioxide is able to proliferate after 48 hours.

To conclude, after the initial halt in biofilm biomass increase during the first 24-48 hours of exposure to 100 ppm of TiO_2 in the visible light spectrum, the CT07-gfp biofilm was able to proliferate. It is visible in biomass and average thickness increases (Figures 10 and 11), as well as decrease in percentage of compromised cells (Figure 14). Despite the initial 24 hour halt in biomass increase, viable planktonic cell production was not affected (Figure 12) and total planktonic cell counts were in the same order of magnitude (Figure 13). From these results it can be concluded that titanium dioxide at concentration of 100 ppm had a significant impact on the biofilm growth and proliferation within the first 48 hours, but not on its ability to produce viable planktonic cells. Currently, the concentration of titanium dioxide in the environment is estimated to be less than 1 ppm, including man-made and natural sources. Therefore, in this context, concentration of 100 ppm is well above environmentally relevant levels.

Summary:

- Titanium dioxide concentration of 100 ppm has significantly impaired increase of biomass and average thickness of the biofilm within the first 48 hours after exposure.
- CT07-gfp biofilm was able to proliferate after 48 hours of exposure.
- Concentration of 100 ppm of titanium dioxide had no observable effect on viable cell production.
- Total effluent counts were within the same order of magnitude for reference and treatment.
- Method adjustment: bubble traps were used and tubing was clamped upstream of the flowcell.

5.2.2 Effects of 100 ppm of titanium dioxide on a 72 hour old biofilm in the absence of light

The next experiment in the series of subjecting developed biofilm (72 hours old) to titanium dioxide was exposure to 100 ppm of TiO_2 in the absence of room light. The flask containing titanium dioxide has been wrapped in tin foil to prevent light from penetrating. The majority of the tubing and the flowcell were covered with tinfoil as well to prevent light exposure, but loosely enough to allow for gas exchange. The goal of the experiment was to see if room lighting (visible light) had an impact on effects of titanium dioxide on the biofilm. One hundred ppm of TiO_2 was used since it had the biggest impact in previous experiments. Bubble traps were not used in this experiment because previous experiments did not encounter any problems with bubbles in the flowcell. However, there was bubble formation observed primarily in the reference channel.

Confocal laser scanning microscopy images and effluent samples were taken at 24 hour intervals for up to 72 hours after the introduction of TiO_2. The upstream tubing was clamped during imaging to prevent planktonic cells from swimming upstream and establishing a biofilm. Based on COMSTAT analysis (Figure 15), the biomass and average thickness of the reference channel are unusually fluctuating, decreasing at 24 and 72 hours (additional data in Appendix I). Provided that it is a reference channel, possible explanations are either contamination in the channel (which was not observed visually), or passing of the bubbles distorting and dislodging parts of the biofilm off the substratum. Potential changes in the ambient temperature could have caused bubble formation. Therefore, comparison between biomass and average thicknesses of reference and treatment channels will not be done.

The biomass of the treatment channel decreased significantly in the first 24 hours of exposure compared to the starting points at time 0 hours ($p < 0.001$), and then increased at 48 and 72 hours. The average thickness decreased significantly in the first 24 hours of exposure ($p < 0.001$) and then increased significantly in the next 24 hours ($p < 0.001$) and continued to increase up to 72 hours. Looking at the biomass and average thickness changes in the treatment channel in total, the trend resembles that of the biomass and average thickness

changes in experiment performed in visible light with concentration of 100 ppm of TiO_2 (Figure 10).

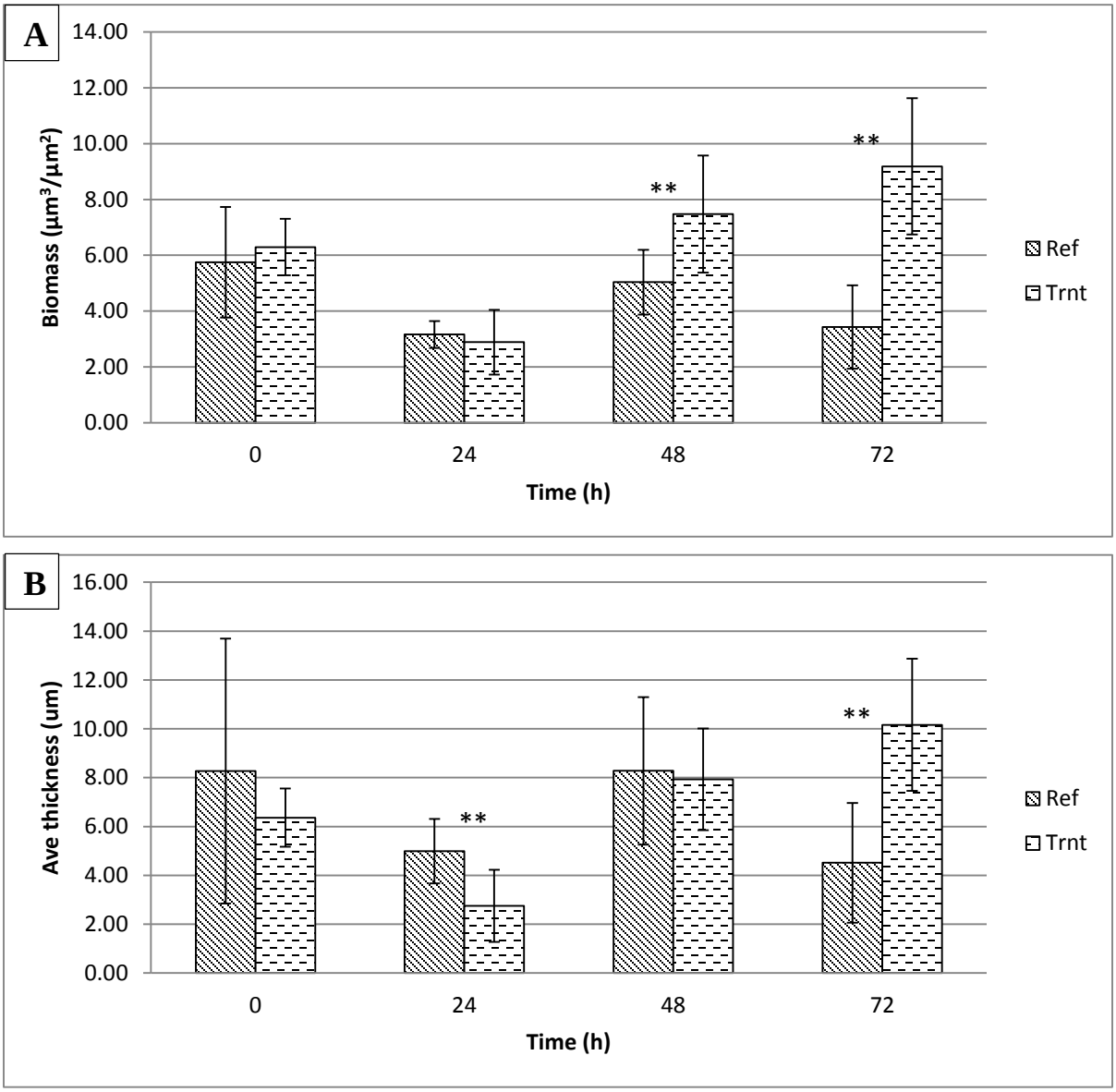


***Figure 15**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2 in absence of visible light**. Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of reference (ref) and treatment (trnt) channels at 0 hours exposure (72 hour mark of biofilm growth), 24, 48 and 72 hour exposure to titanium dioxide in absence of room light. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 100 ppm of titanium dioxide. At time zero, biofilms in reference and treatment channels are 72 hours old, grown in identical conditions with room lights. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

The percentage of compromised biomass (Figure 16) shows that at 24 hours, roughly 40.5% of the biomass was compromised in the treatment channel. The value dropped to 16% and remained there for 48 and 72 hours of exposure. There was little difference between the values at 24, 48 and 72 hours of treatment channel and here was no significant difference between treatment and reference channels at 72 hours of exposure. Combining these results together with COMSTAT analysis from Figure 15, it can be argued that titanium dioxide had a damaging effect on the CT07-gfp biofilm within the first 24 hours, after which the biofilm was able to minimize the damage and resume growth in the next 48 hours.

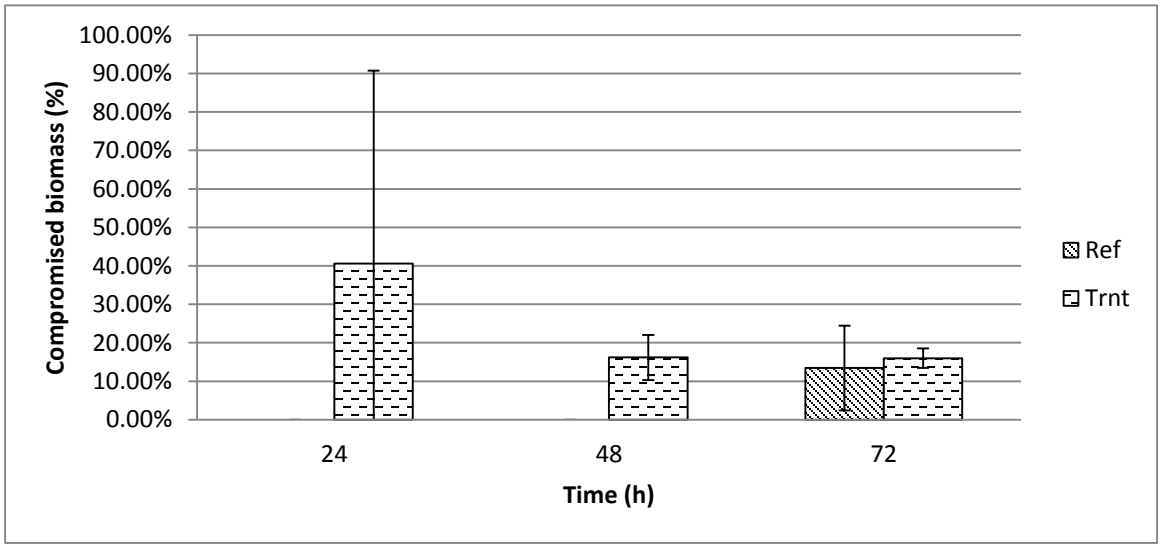


***Figure 16**: **Percent of compromised biomass in treatment and reference channels during treatment with 100 ppm of TiO_2 in absence of visible light**.. The bar graph shows compromised biomass (%) of treatment (trnt) channels at 24, 48 and 72 hours of exposure and reference (ref) channel biomass at 72 hours (72 hours of treatment exposure, or 144 hours of total growth). Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

Recalling that studies have shown that titanium dioxide is capable of producing hydrogen peroxide in the absence of photoactivation, damage to the biofilm cells in the first 24 hours may be attributed to lipid peroxidation and oxidative DNA damage due to H_2O_2. After 48 hours of exposure, cells could have adapted and mounted a defensive response. Titanium dioxide could have also caused damage by cell adhesion and internalization.

Similarly to the previous experiments, concentration of 100 ppm of titanium dioxide in the absence of visible light did not have an effect on the viability of the planktonic cells produced by the biofilm (Figure 17).

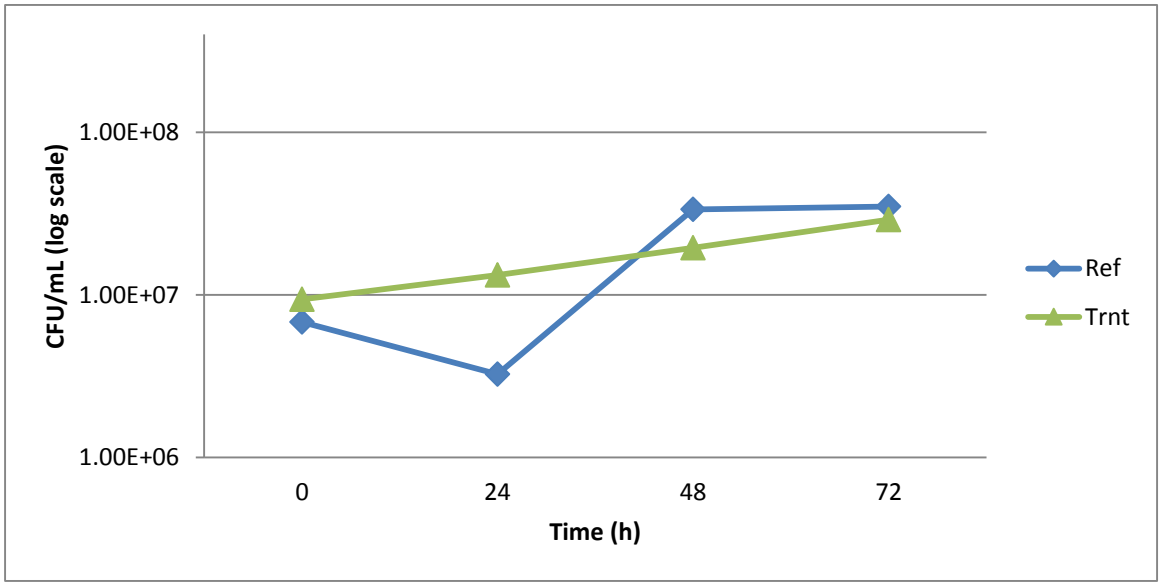


***Figure 17*: Viable cell counts at 24 hour intervals during treatment with 100 ppm of TiO_2 in absence of light**. *CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm in the absence of regular room lighting and CT07-gfp biofilm in treatment (trnt) channel, and reference (ref) channel. In the graph, x-axis shows time of treatment and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Therefore, even in the absence of room lighting titanium dioxide at concentration of 100 ppm had an inhibiting effect on CT07-gfp biofilm within the first 24 hours of exposure. Comparing results of 100 ppm titanium dioxide concentration in the presence of room lighting and 100 ppm in the absence of room lighting it can be concluded that titanium dioxide possesses a mechanism of cell damage that is independent of photoactivation by UV-A or fluorescent lighting. Adams *et al.* (2006) reached a similar conclusion during the experiment they conducted where *E. coli* and *B. subtilis* bacterial cultures in petri dishes containing MD media were subjected to a range of concentrations (from 10 ppm to 5000 ppm) of titanium dioxide, zinc oxide and silicone dioxide nanoparticles in both light and dark conditions. Light conditions were simulated by leaving petri dishes on the windowsill in direct sunlight for 6 hours between 9 am and 3 pm, while petri dishes were wrapped in tin foil to simulate dark conditions. The team did not suggest an alternate mechanism of titanium dioxide toxicity.

However, the fact that in the presence of visible light titanium dioxide at concentration of 100 ppm had a longer delaying effect on biofilm recovery (24 hours in the dark versus 48 hours in the light), it is possible that visible light does assist in titanium dioxide photoactivation and therefore production of reactive oxygen species such as hydrogen peroxide. The goal of this experiment was to observe effect in the absence of light, and not exact mechanism of toxicity.

Summary

- Titanium dioxide concentration of 100 ppm in the dark has decreased biomass and average thickness of the biofilm within the first 24 hours after exposure.
- Biofilm resumed proliferation after 24 hours of exposure.
- There was no effect on the viability of planktonic cells.
- The percentage of compromised biomass decreased from 40.5% at 24 hours to 16% at 48 and 72 hours of exposure.
- Due to bubbles potentially affecting biofilm biomass and average thickness in the reference channel, bubble traps were introduced in subsequent experiments.

5.2.3 Effects of 10 ppm of titanium dioxide on 72 hour old biofilm in visible light

Similarly to the previous experiment with 100 ppm of titanium dioxide in the visible light, a 72 hour old CT07-gfp biofilm was subjected to a lower concentration of titanium dioxide (10 ppm) in the flowcell. Confocal laser scanning microscopy images and effluent samples were taken at 24 hour intervals for up to 72 hours after the introduction of the contaminant (TiO_2). The experiment was performed without bubble traps and without clamping of the influent tubing during imaging. The pump was stopped during imaging.

COMSTAT data obtained and statistical analysis performed show that there was a significant difference in biomass and average thickness values between reference and treatment channels after 24 hours of exposure ($p < 0.001$) as indicated in Figure 18. At the 48 hour mark of exposure to 10 ppm of TiO_2, there was no significant difference in biomass values obtained between reference and treatment channels. The average thicknesses of the reference channel

was significantly higher than the treatment channels at 48 hours ($p < 0.05$), but significantly lower at 72 hours ($p < 0.001$). At 72 hours of exposure, the treatment channel's biomass was greater than the reference.

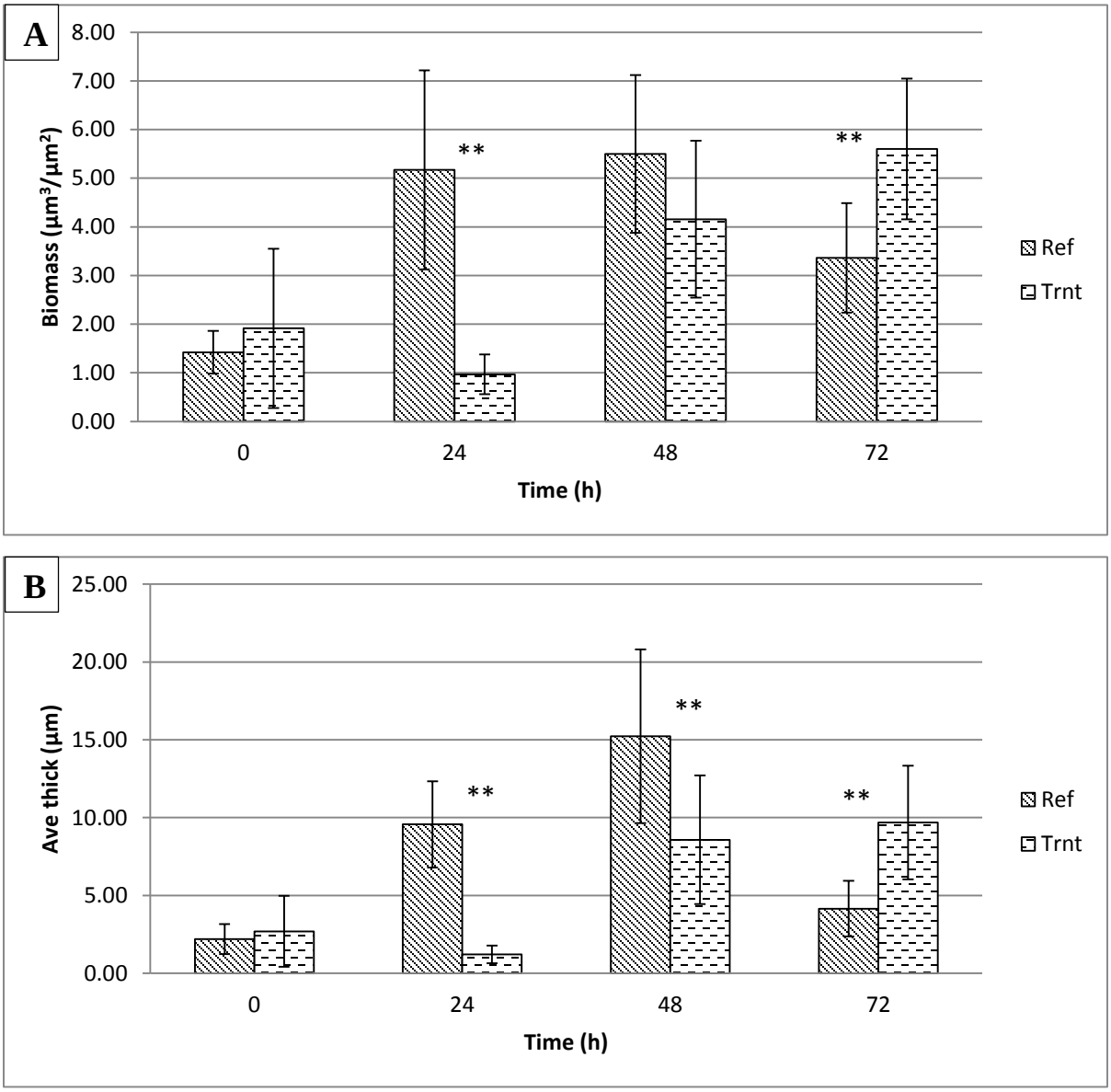


***Figure 18**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 10 ppm of TiO_2.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (μm) values of reference (ref) and treatment (trnt) channels at 0 hours exposure (72 hour mark of biofilm growth), 24, 48 and 72 hour exposure to titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in μm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 10 ppm of titanium dioxide. At time zero, biofilms in reference and treatment channels are 72 hours old, grown in identical conditions. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

The potential reason for the decrease in the reference biomass and average thickness at 72 hours will be discussed below. However, there is a trend in treatment channel biomass and average thickness change similar to the 100 ppm experiment: an initial decrease in the treatment biomass in the first 24 hours followed by a steady increase as seen at 48 and 72 hours. This suggests that a biofilm subjected to 10 ppm of TiO_2 was able to resume its proliferation after 24 hours, seemingly at a greater rate than the biofilm exposed to 100 ppm.

The percentage of the compromised biomass in Figure 19 shows that there is a difference between the percent of compromised biomass after 24 and 48 hours of exposure and that there is little difference between 48 and 72 hours of exposure to titanium dioxide. The percent of compromised biomass in the reference channel was significantly lower than in the treatment channel after 72 hours of exposure ($p < 0.001$). Comparing the percent of compromised biomass between 100 ppm and 10 ppm treatments, it can be pointed out that at a TiO_2 concentration of 10 ppm, the biofilm sustained 5 times more damage in the initial 24 hours than at 100 ppm. These results may be explained by looking at the value for surface to volume ratio for biofilms exposed to 100 ppm and 10 ppm of TiO_2. At 0 hours of exposure, surface to volume ratio of the treatment channel that was to be exposed to 100 ppm was 3.17 $\mu m^2/\mu m^3$, while the biofilm that was to be exposed to 10 ppm had a surface to volume ratio of 3.69 $\mu m^2/\mu m^3$. At 24 hours of exposure, 100 ppm exposed biofilm ratio was 1.98 $\mu m^2/\mu m^3$ versus 2.55 $\mu m^2/\mu m^3$ for 10 ppm exposed biofilm (values are available in Appendices F and G).

Higher surface to volume ratio indicates that more of the biofilm's surface is exposed to the surrounding environment. If titanium dioxide could impact on a higher surface area, potentially more damage could be done. Additionally, recalling the fact that titanium dioxide is known to form large aggregates, it is possible that an increased concentration (100 ppm) stimulated larger aggregate formation, which in turn decreased titanium dioxide's ability to penetrate into the biofilm matrix. At a concentration of 10 ppm, perhaps titanium dioxide particles were able to penetrate deeper into biofilm matrix and cause more damage during the initial stages due to

higher surface area exposed. The type of damage done by titanium dioxide to the biofilm was not assessed.

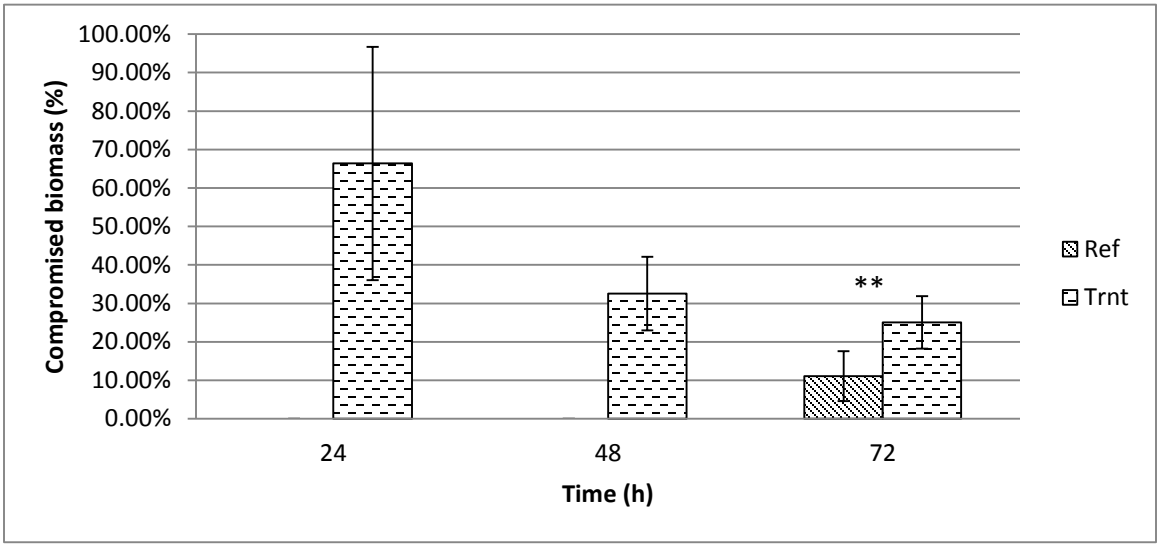


***Figure 19**: **Percent of compromised biomass in treatment and reference channels during treatment with 10 ppm of TiO_2.** The bar graph shows compromised biomass (%) of treatment (trnt) channels at 24, 48 and 72 hours of exposure and reference (ref) channel biomass at 72 hours (72 hours of treatment exposure, or 144 hours of total growth). Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

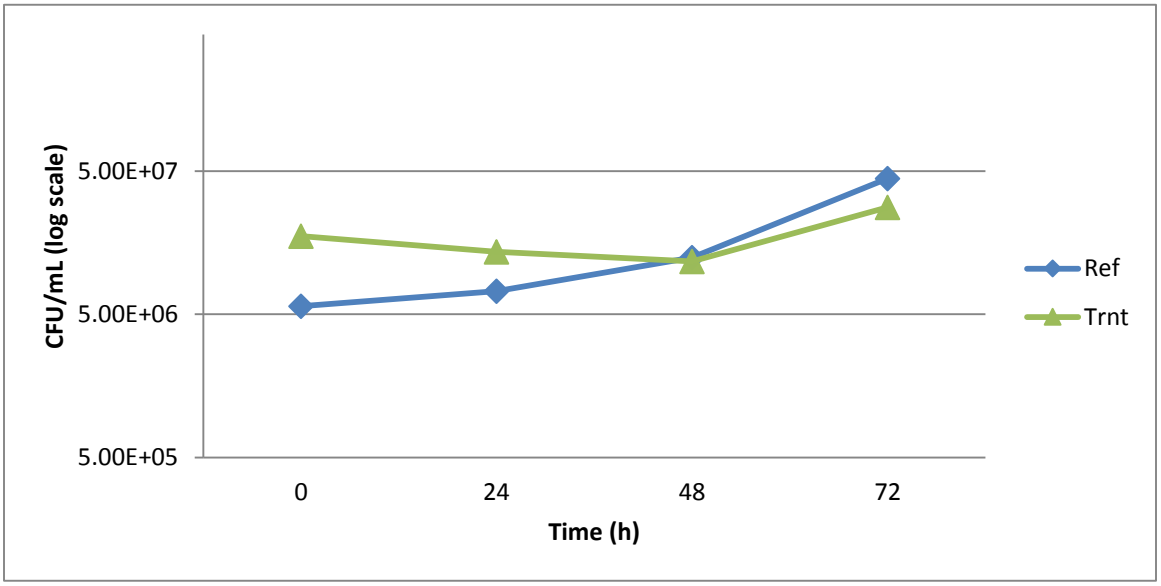


***Figure 20**: **Viable cell counts at 24 hour intervals during treatment with 10 ppm of TiO_2.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm in regular room lighting conditions and CT07-gfp biofilm in treatment (trnt) channel, and reference (ref) channel. In the graph, x-axis shows time of treatment and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

As seen from Figure 20, the viable cell counts remained in the same order of magnitude for reference and treatment channels, which suggest that titanium dioxide at a concentration of 10 ppm had no effect on viable planktonic cells of CT07 biofilm. This finding is in agreement with the data from Figure 12, which show that higher concentration of 100 ppm did not have any effect on viable counts.

Based on COMSTAT data shown in Figure 18 and percent of compromised cells shown in Figure 19, it may be concluded that while titanium dioxide at a concentration of 10 ppm caused a decrease in biomass and average thickness of the CT07 biofilm within the first 24 hours (similar to 100 ppm experiment), the biofilm was able to resume proliferation at some point between 24 and 48 hours. The viable cell counts were not affected by the titanium dioxide concentration as compared to the reference channel.

As mentioned in section 5.1, establishment of biofilm upstream of the flowcell affected biomass and average thickness of the reference channel at 144 hours since inoculation. One hypothesis as to why the biomass and average thickness did not decrease in the treatment channels is that titanium dioxide prevented effective biofilm formation in the tubing upstream of the treatment channel between 72 and 144 hours since inoculation. Therefore, the biofilm in the flowcell was receiving enough nutrients and was able to proliferate after 24 hours of TiO_2 exposure despite presence of titanium dioxide in the channel. The ability of planktonic cells to establish biofilms in the presence of titanium dioxide was tested in subsequent experiments.

Summary:

- Titanium dioxide concentration of 10 ppm decreased biomass and average thickness of the biofilm within the first 24 hours.
- Average thickness of the treated channel was significantly lower than reference channel at 48 hours, while biomass was not significantly smaller.
- CT07-gfp biofilm was able to proliferate after 24 hours of exposure.
- There was no observable effect on the viable cell production.

- The unexpected drop in reference biofilm biomass and average thickness at 72 hours (144 hours total) could be attributed to the biofilm extending into the tubing upstream of the flowcell and therefore limiting nutrient availability to the flowcell biofilm. Clamping of the influent tubing was employed in subsequent experiments.

5.2.4 Effects of 1 ppm of titanium dioxide on 72 hour old biofilm

The effect of 1 part per million (ppm) concentration of titanium dioxide on a 72 hour old CT07-gfp biofilm was assessed using confocal laser scanning microscopy and viable cell counts. As a reminder, 1 ppm of titanium dioxide is currently considered to be close to an environmentally relevant concentration. Confocal laser scanning microscopy images and effluent samples were taken at 24 hour intervals for up to 72 hours after the introduction of the contaminant (TiO_2).

Additionally, the upstream tubing was clamped during imaging when the pump was stopped to prevent planktonic cells from swimming upstream and establishing a biofilm and bubble traps were used to prevent bubble formation in the flowcell.

Based on the COMSTAT data obtained (Figure 21), at time 0 hours there was a statistically significant difference between biomass values of reference and treatment channels ($p = 0.001$). However, given that each biofilm is unique, such differences are acceptable during biofilm formation and initial growth (within 72 hours). The average thicknesses of reference and treatment channels at 0 hours did no differ significantly. At 24, 48 and 72 hours of exposure the biomass and average thickness values of treatment and reference channels did not differ significantly, which suggests that there was no effect on the biofilm growth and proliferation from the 1 ppm concentration of titanium dioxide.

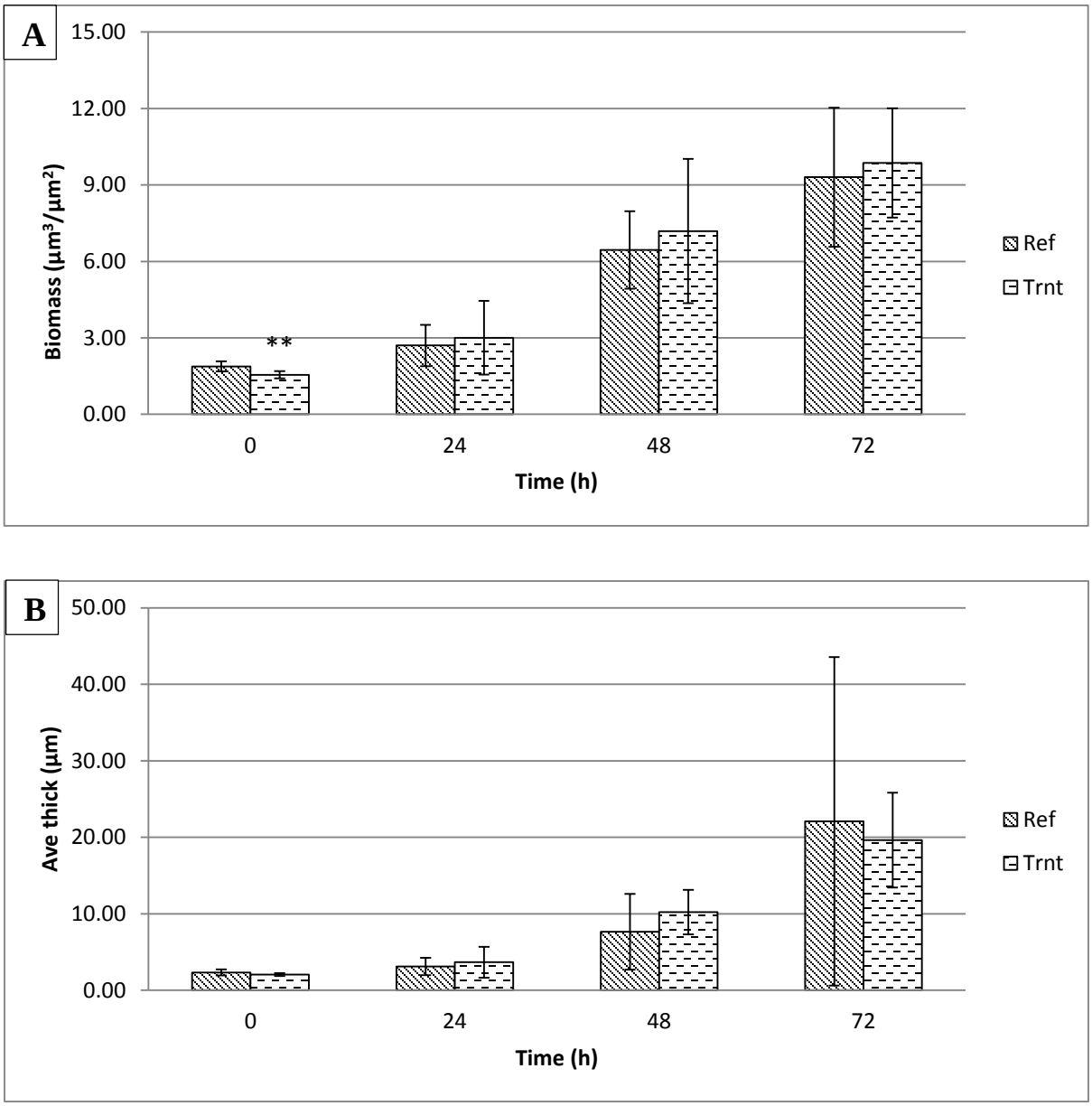


***Figure 21**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 1 ppm of TiO_2.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of reference (ref) and treatment (trnt) channels at 0 hours exposure (72 hour mark of biofilm growth), 24, 48 and 72 hour exposure to titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 1 ppm of titanium dioxide. At time zero, biofilms in reference and treatment channels are 72 hours old, grown in identical conditions. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

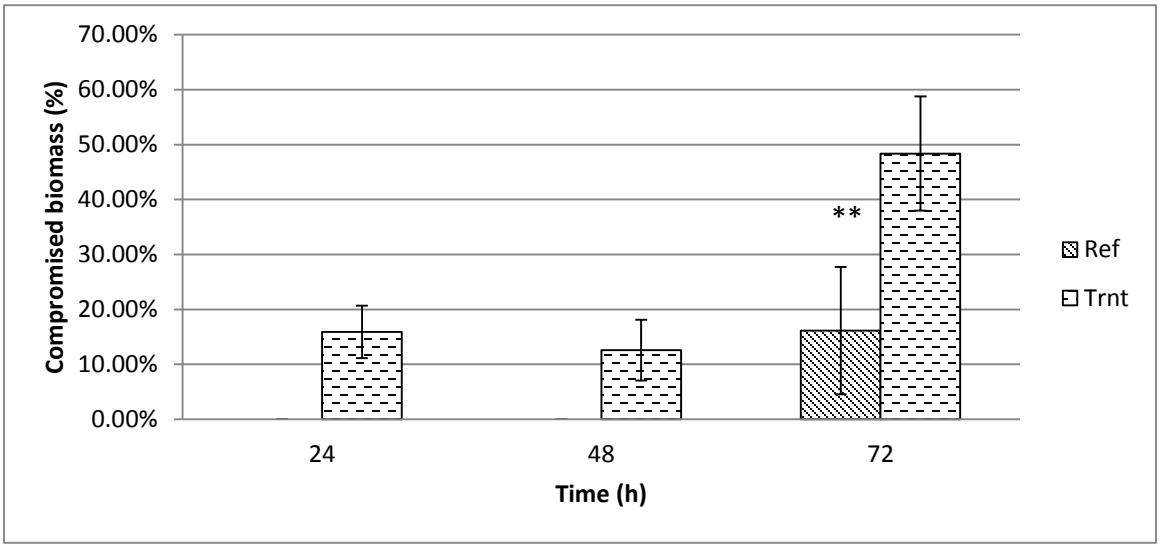


***Figure 22*: Percent of compromised biomass in treatment and reference channels during treatment with 1 ppm of TiO_2.** *The bar graph shows compromised biomass (%) of treatment (trnt) channels at 24, 48 and 72 hours of exposure and reference (ref) channel biomass at 72 hours (72 hours of treatment exposure, or 144 hours of total growth). Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

However, the percent of compromised biomass (Figure 22) suggests that at 72 hours of exposure there was increased damage to the biofilm cells in comparison with 24 and 48 hours. The data also shows that there was a significant difference between percent of compromised cells in reference and treatment channels at 72 hours ($p < 0.001$).

These data, coupled together with data from Figure 21, suggest that low concentrations of titanium dioxide (1 ppm) may take longer to compromise the membrane of the cells (based on PI staining), which may explain increase in percentage of compromised biomass at 72 hours. In comparison, a study by Zheng *et al*. (2011) exposed nitrogen removing bacteria to 50 mg/L of titanium dioxide (50 ppm) for 70 days and found that there was a significant reduction in the numbers of viable bacteria, while exposure for the same amount of time to 1 mg/L (1 ppm) showed no effect. Perhaps a longer time frame than 72 hours of exposure to 1ppm of titanium dioxide is required to better assess whether the increase in percent of compromised cells at 72 hours of exposure has a significant implication. Titanium dioxide concentration of 1 ppm does not prevent biofilm growth and proliferation for the first 72 hours of exposure. This may be

simply due to low concentration and not enough reactive oxygen species accumulation in the media and due to lower adhesion rate of titanium dioxide particles to cells.

Lastly, looking at Figure 23, at 48 hours there is almost a full 1-log reduction in viable cell production in the treatment channel compared to the reference channel. However, at 72 hours the production of viable cells from the treatment channel reached almost the same level as the reference. If titanium dioxide did have an impact on the viability of planktonic cells, the duration was for 24 hours with recovery at 72 hours. However, the temporary decrease in viable cell production could also be a coincidence and a natural developmental stage of that particular biofilm (full data available in Appendix H).

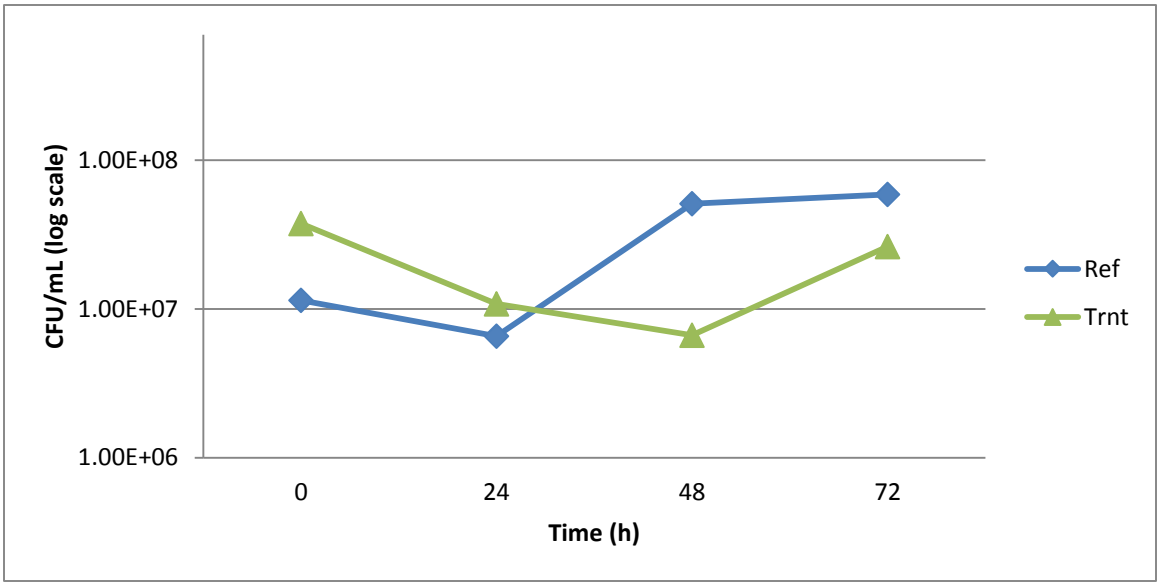


***Figure 23**: Viable cell counts at 24 hour intervals during treatment with 1 ppm of TiO_2. CFU/mL values obtained in the tests with titanium dioxide concentration of 1 ppm in regular room lighting conditions and CT07-gfp biofilm in treatment (trnt) channel, and reference (ref) channel. In the graph, x-axis shows time of treatment and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Summary:

- Titanium dioxide concentration of 1 ppm did not have any impact on biofilm's growth and proliferation.
- There was a 1-log reduction in viable planktonic cells at 48 hours, followed by an increase to same order of magnitude at 72 hours.

- Based on percent of compromised biomass a longer than 72 hour exposure experiment would be suggested.
- The method was adjusted – tubing upstream of the flowcell was clamped during the imagine when the pump was turned off.

In conclusion, Figure 24 below summarises the biomass values between the following treatments: 100 ppm in full room light, 100 ppm in the dark, 10 ppm in room light and 1 ppm in room light.

Based on the observations from Figure 24, there is a visible effect of titanium dioxide on growth and proliferation of CT07-gfp biofilm in both light and dark conditions, though with varying magnitude. There is also an effect on biofilm growth and proliferation at 100 ppm and 10 ppm of titanium dioxide, and no visible effect at 1 ppm.

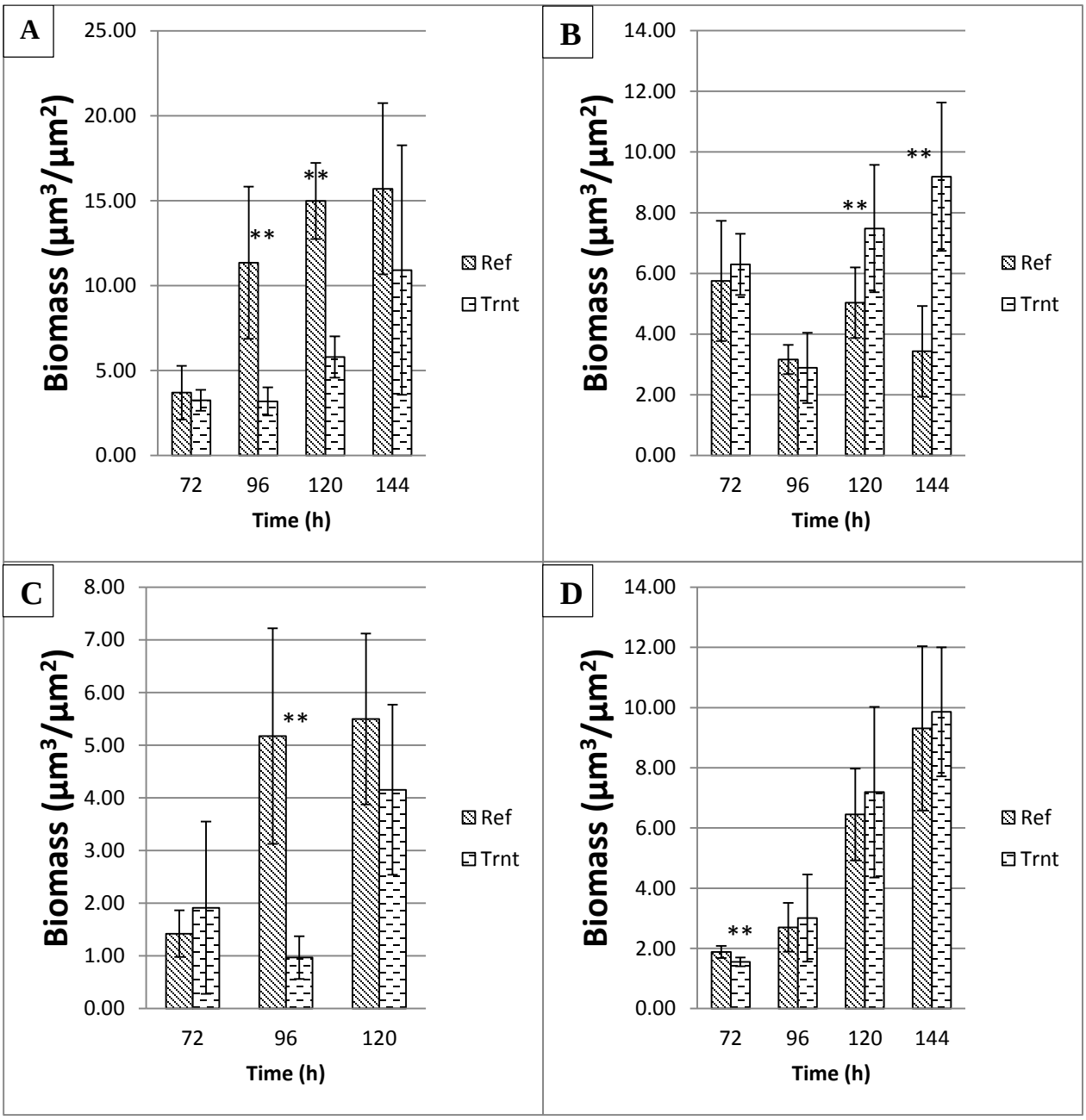


Figure 24: Biomass change over time in CT07-gfp biofilm treatment and reference channels during exposure of treatment channel to a) 100 ppm in full light, b) 100 ppm in the dark, c) 10 ppm in the light, and d) 1 ppm in the light, of TiO_2. *Time (hours) corresponds to time elapsed since inoculation and 72 hours mark the start of biofilm exposure to titanium dioxide. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference (P > 0.05).*

5.3 Effects of titanium dioxide nanopowder on the formation of the biofilm (co-inoculation)

The study by Gage *et al.* (2005) showed that TiO_2-coated slides exposed to UV illumination can kill PAO1 planktonic cell suspensions within a few hours. There is a growing body of literature that examines if titanium dioxide does in fact have the capacity to prevent biofilm formation, and *Pseudomonas aeruginosa* (PAO1) is a commonly used model organism. *Pseudomonas* sp. CT07 is a recent environmental isolate, and therefore little work has been done with this specific strain. This section will address the results obtained by inoculating a flowcell in the presence of titanium dioxide concentration of 100 ppm with CT07-gfp cells as well as PAO1-gfp. All experiments were performed in regular room lighting, but using two different media. A light spectrum was not measured. For reference and treatment, duplicate channels were set up for redundancy.

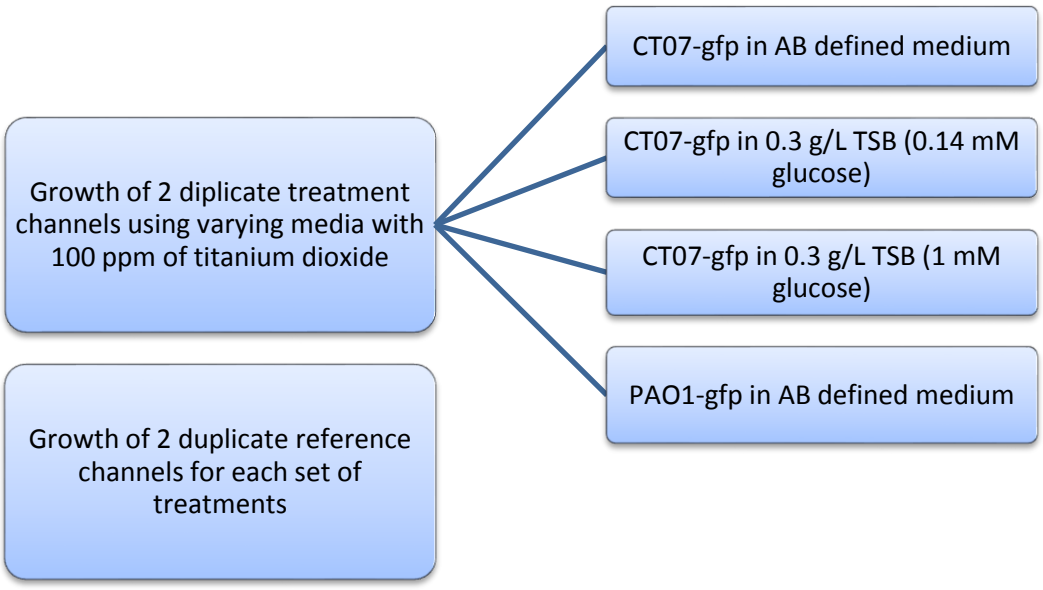


Figure 25: Diagram above shows a flowchart of the experiments performed in section 5.3.

5.3.1 Effects of 100 ppm of titanium dioxide on formation, growth, viability and structure of the CT07-gfp biofilm in AB defined media with 1 mM citrate

The flowcell system was set up similarly to the experiments in section 5.2, with bubble traps to prevent bubbles (which could form in the tubing upstream of the flowcell) from disrupting the biofilm formation. The key difference was that the inoculation of CT07-gfp tagged cell culture occurred into a channel that contained defined AB media with 100 ppm of titanium dioxide concentration (co-inoculation). Therefore, the focus of the experiment was to see if CT07-gfp planktonic cells would be able to form a biofilm in the presence of titanium dioxide concentration of 100 ppm.

Confocal laser scanning microscopy images and effluent samples were taken every 24 hours between 72 and 144 hours after the inoculation. The upstream tubing was clamped during imaging when the pump was stopped to prevent planktonic cells from swimming upstream and establishing a biofilm. Figure 26 displays biomass and average thickness of treatment and reference channels. Based on statistical analysis (t-test), all 4 biomass sets differ significantly between treatment and reference channel (p-values < 0.05, data available in Appendix J). The average thickness differed significantly only at 120 and 144 hours after inoculation.

Based on numerous studies available that suggest that there is a toxic effect on planktonic cells from titanium dioxide, it was expected that biofilm would not be able to form in the presence of titanium dioxide due to damage to planktonic cells. The unexpected part was that not only did the biofilm in the presence of titanium dioxide form, it was proliferating and increasing its biomass and average thickness significantly faster than the reference counterpart after 120 hours. Unfortunately, due to time constraints, only 5 z-stacks were obtained for the second reference and treatment channels for redundancy purposes. Therefore not enough of the biofilm was imaged to have a representative data. However, effluent was collected from all 4 channels and therefore data on viable cell counts is available below.

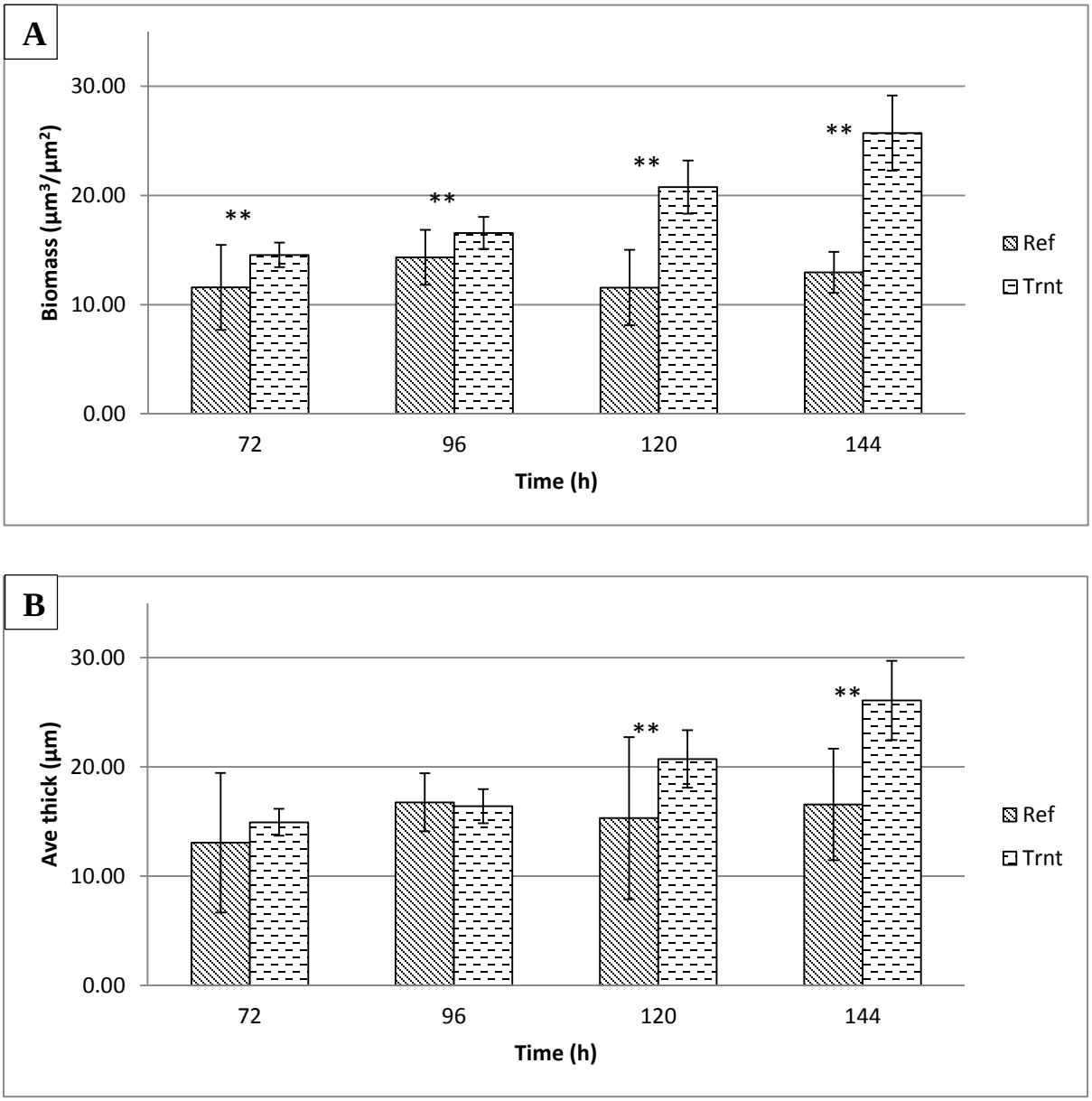


***Figure 26**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2 in AB defined medium.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of one reference (ref) and one treatment (trnt) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

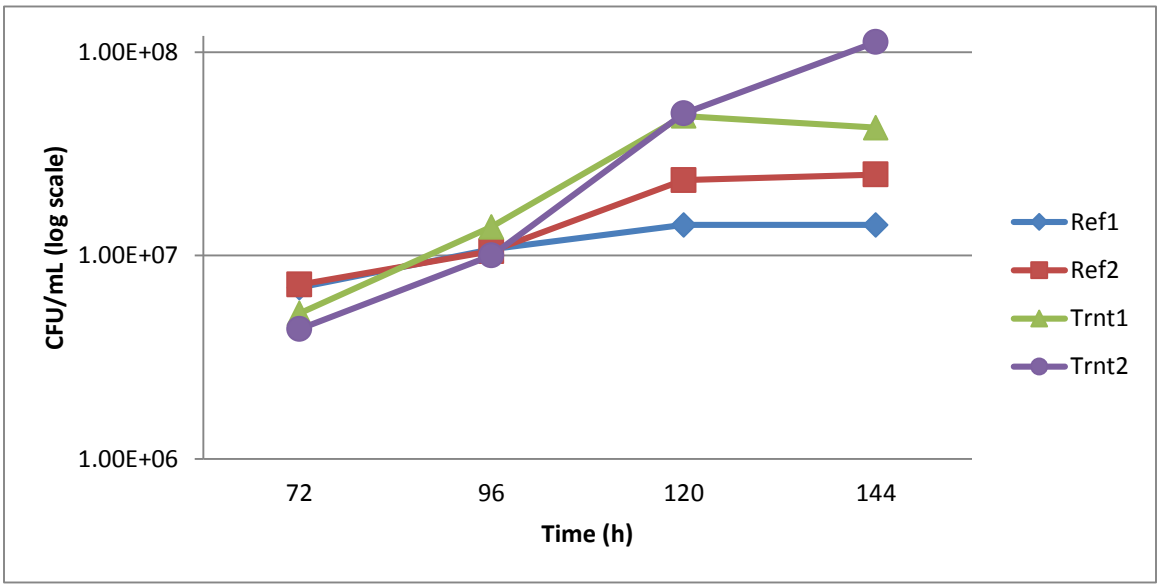


***Figure 27**: **Viable cell counts during treatment with 100 ppm of TiO_2 in AB defined medium.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm at co-inoculation, defined AB media and CT07-gfp biofilm in treatment (trnt1 and trnt2) channel, and reference (ref) channel. In the graph, x-axis shows total time since inoculation and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Figure 27 presents viable cell counts from 2 treatment and 2 reference channels. Between reference channel #1 (COMSTAT data available in Figure 26) and treatment channel #2 (COMSTAT data not available), there is almost a 1-log difference, while reference #1 and treatment #1 channels both produced similar viable cell numbers. Combining these findings together with COMSTAT data in Figure 26 it may be concluded that in the presence of 100 ppm of titanium dioxide, CT07-gfp cells are able to form biofilm and continue to produce viable planktonic cell numbers. Additionally, the increase in the biomass and average thickness of the biofilm subjected to titanium dioxide is greater than the reference channel, and viable cell counts are slightly higher than those produced by the reference channel. Perhaps due to early exposure to titanium dioxide in planktonic state, the cells were able to develop resistance to the potential bactericidal effects of TiO_2. The developing biofilm would have inherent resistance and any further planktonic cells produced by the biofilm would be more resistant to titanium dioxide toxicity and therefore proliferate at the same rate as the biofilm that was not exposed to titanium dioxide.

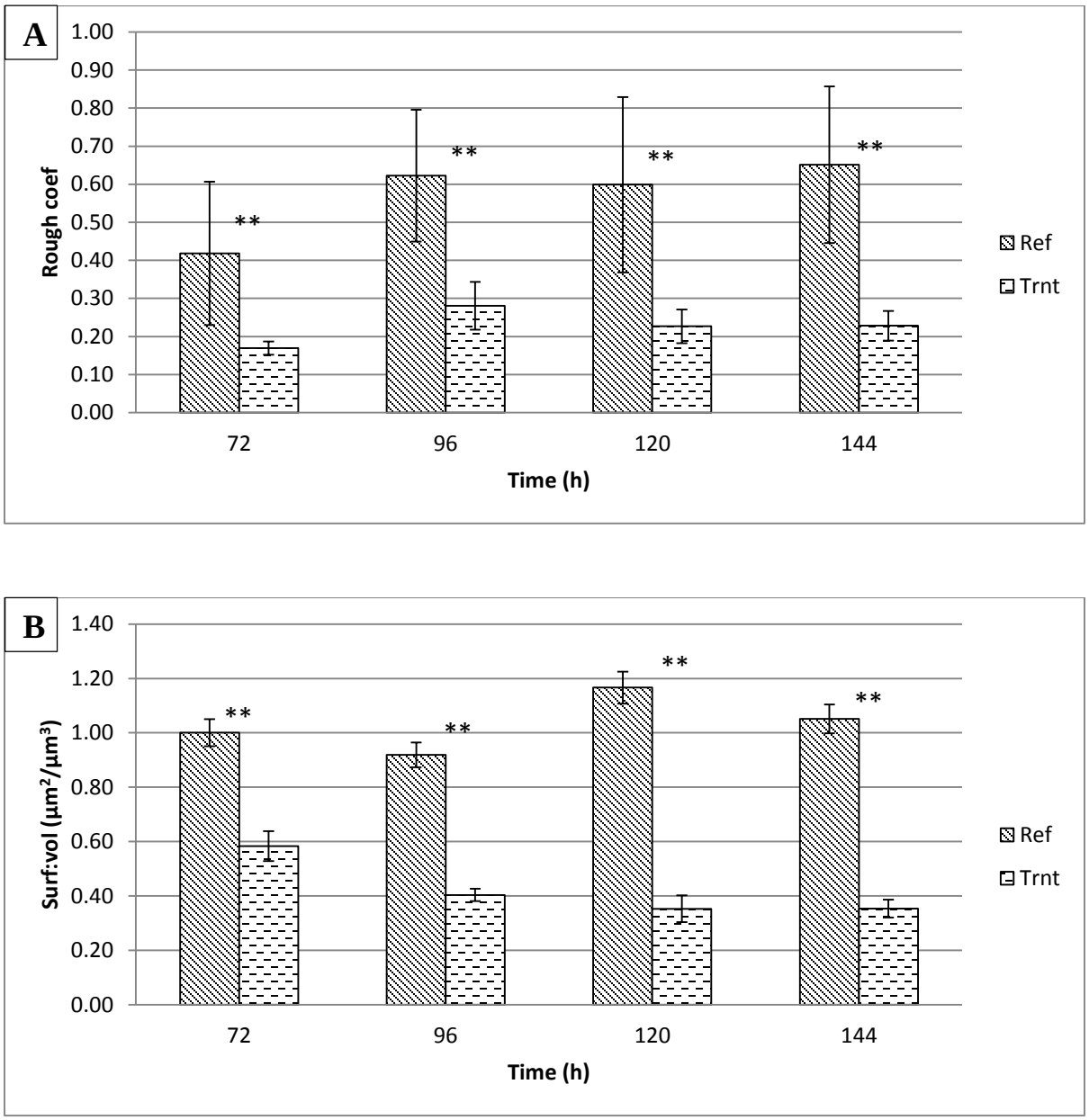


***Figure 28**: **Roughness coefficient and surface to volume ratio change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2 in AB defined medium**. Averages of **a)** roughness coefficient (no units) and **b)** surface to volume ratio (µm²/µm²) of one reference (ref) and one treatment (trnt) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** roughness coefficient of the biofilm and **b)** surface to volume ratio of the biofilm in µm²/µm². Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

During the scanning of the treatment and reference channels with CLSM it was noted that biofilm structure in general in the reference channel differed from that of the treatment. Therefore, further COMSTAT analysis looked into roughness coefficient and surface to volume ratio difference between treatment and reference channels. As a reminder from the materials and methods section, biofilm roughness is an indicator of the biofilm heterogeneity and surface to volume ratio provides information on how much of the biofilm's surface is open to the surrounding environment from which nutrients and other components may diffuse to the bacteria within the micro-colonies. The results shown in Figure 28 clearly indicate that the roughness coefficient and area to volume ratio of the reference (untreated) biofilm is significantly higher than the treatment biofilm at all of the time points ($p < 0.05$, data available in Appendix J).

It can be seen from one of the z-stacks from both reference and treatment channels in Figure 29 and the data from Figure 28 that the architecture of the biofilm differs in the treatment channel and the reference channel. This can be potentially attributed to titanium dioxide exposure in the treatment channel. It seems that the biofilm exposed to 100 ppm of titanium dioxide has a more flat and compact architecture as opposed to the reference channel. Such architecture may limit biofilm's exposure to titanium dioxide. Although extensive characterization of the biofilm's architecture is outside the scope of this study, looking at general parameters that help describe the 3-D shape of the biofilm in further experiments with co-inoculation provides a better insight into how CT07-gfp biofilm is able to potentially tolerate a concentration of 100 ppm of titanium dioxide and thrive in that environment at least for the first 144 hours.

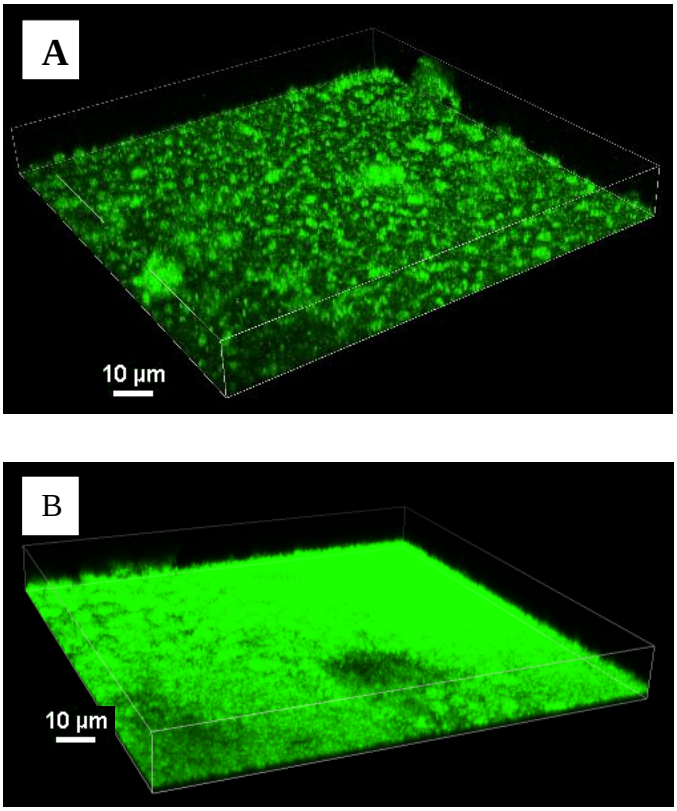


Figure 29: Volume snapshot of 144 hour old CT07-gfp biofilm in a) *reference channel,* ***and b)*** *treatment channel with 100 ppm of TiO_2. Image was taken with Nikon 90i-C1 confocal microscope. Resolution is 1024x1024 pixels. Image was prepared in NIS-Elements.*

Summary:

- CT07-gfp bacterial cells are capable of establishing a biofilm in the presence of 100 ppm of titanium dioxide in regular light conditions.
- Titanium dioxide did not hinder or stop the growth and proliferation of the biofilm.
- The levels of viable cells produced by the established biofilm under the treatment conditions were in the same order of magnitude as cells produced by the reference channel.
- The architecture of the treatment channel's biofilm differed from the reference – a more flat, compact structure.

5.3.2 Effects of 100 ppm of titanium dioxide on formation, growth, viability and structure of the CT07-gfp biofilm in 0.3 g/L Tryptic Soy Broth (TSB)

It was established by the previous experiment that CT07-gfp cells are capable of forming biofilm in the presence of 100 ppm of titanium dioxide in defined AB media (section 5.3.1). The biofilm was continuously increasing its biomass and average thickness, and the viable effluent counts were in the same magnitude as the reference channel. Therefore, looking at biofilm proliferation results from section 5.3.1, it can be hypothesized that CT07-gfp cells will be able to form biofilm in the presence of titanium dioxide concentrations lower than 100 ppm.

An experiment similar to the one described in 5.3.1 was performed in Dr. Wolfaardt's lab by Natasha Wierre in the summer of 2012. The preliminary results suggested that CT07-gfp cells were not capable to form biofilm in the presence of 100 ppm of titanium dioxide (complete result set is not available). Upon looking into the methods section, it was noted that the set-up of the flowcell system, bacterial stain used and concentration of titanium dioxide were the same. However, the medium used was not a defined AB medium but rather a 0.3 g/L TSB medium.

Therefore, an experiment was performed using the same conditions as the experiment from section 5.3.1, except 0.3 g/L of TSB medium was used. While both treatment channels were intact after 72 hours, one of the reference channels had a leak and therefore was full of air after first 72 hours. Because of this, data from only 1 reference channel are available. The other thing noted was that titanium dioxide would settle out of the AB defined media if left undisturbed and therefore required magnetic bar stirring to keep it in suspension. When the flask with 0.3 g/L TSB and concentration of 100 ppm of titanium dioxide was left undisturbed for a few hours, the majority of titanium dioxide stayed suspended in the media. Unfortunately, no samples were analyzed to determine particle size. However, knowing the concepts of settling velocity it could be concluded that aggregate size was smaller. The magnet bar was still used to stir the media to keep the methods consistent across the experiments.

During first CLSM imaging 72 hours after inoculation, it was visually noted that no biofilm was forming in the treatment channels. However, CT07-gfp cells were visible near substratum. Upon close inspection using time lapse of 10-20 seconds at a time (data not presented), it was observed that while certain cells remained stationary at the glass surface, others would move slightly about their own axes or even shift from their current location. Based on the model of biofilm development (Figure 1) this observation suggested that cells were in the process of adhering themselves to the glass surface of the cover slip. On the contrary, there was visible colony formation in the reference channel (Figure 30).

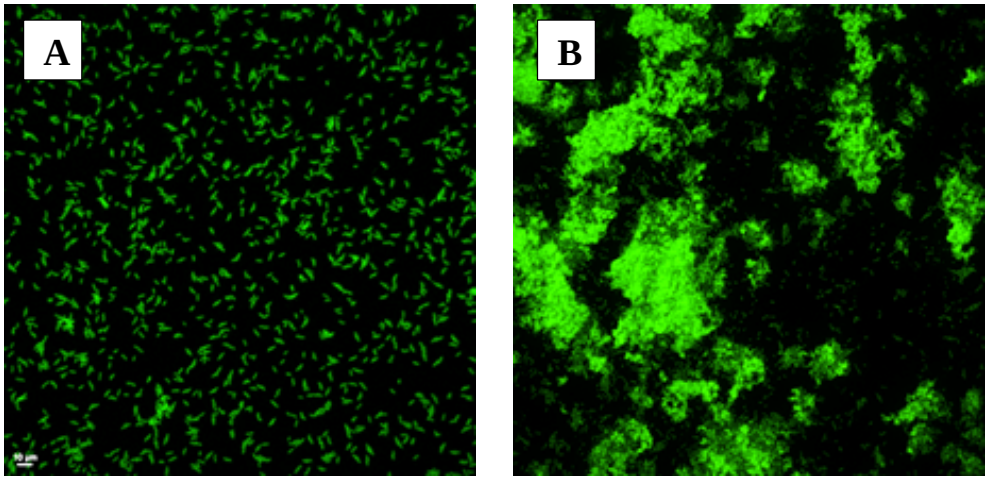


Figure 30: Area snapshot of 72 hour old biofilm from treatment and reference channels. *Treatment is shown in image* ***a)*** *with 0.3 g/L TSB media containing 100 ppm of TiO_2, and reference is shown in image* ***b)****. Images were taken with Nikon 90i-C1 confocal microscope and 40x objective. Resolution is 1024x1024 pixels. Images prepared in NIS-Elements software.*

Figure 31 shows biomass and average thickness data from a reference and 2 treatment channels. As described above, at 72 hour only single cells were visible on the substratum, while there were few microcolonies forming in the reference channel. By 96 hours, visibly small microcolonies began to form in both treatment channels.

At 72 hours, the means of the average thicknesses of the biofilms in the treatment channels were significantly lower than the mean of the average thickness of the reference (actual values provided in Appendix K). Between 96 and 144 hours, formation and growth of the biofilm was observed in both treatment channels and Figure 31 supports this finding as there is a significant

increase in the biomass and average thickness. However, the values of biomass and average thickness in the treatment channels are significantly lower than those of the reference, which suggests that a concentration of 100 ppm of titanium dioxide had an impact on the growth of the biofilm.

From these findings it can be estimated that formation of the biofilm in the treatment channel took at least 24 hours longer than in the reference channel. However, provided each biofilm is its own unique organism and community, this 24 hour discrepancy may be the result of natural causes. The fact that biomass and average thickness increase in the reference channel is more than double compared to both treatment channels cannot be dismissed as a coincidence. It can be concluded then that 100 ppm concentration of titanium dioxide does have a hindering effect on biofilm growth and formation in 0.3 g/L TSB media. However, CT07-gfp cells are capable of forming biofilm in these conditions. Thinking back to section 5.3.1, titanium dioxide concentration of 100 ppm had no effect on planktonic cell production. Therefore, there is a possibility that inherently CT07-gfp planktonic cells may not be affected by titanium dioxide through an unknown mechanism.

Looking further at the general architecture of the biofilm, Figure 32 shows that while there is a statistically significant difference in all of roughness coefficient values between treatments and reference ($p < 0.05$), and difference between surface to volume ratio at 72, 96 and 144 hours ($p < 0.05$), the architecture of both treatment channel biofilms and reference biofilm is very similar (Figure 33). Therefore, no definite conclusions can be drawn in regards to titanium dioxide effect on structure of the biofilm under these experimental conditions.

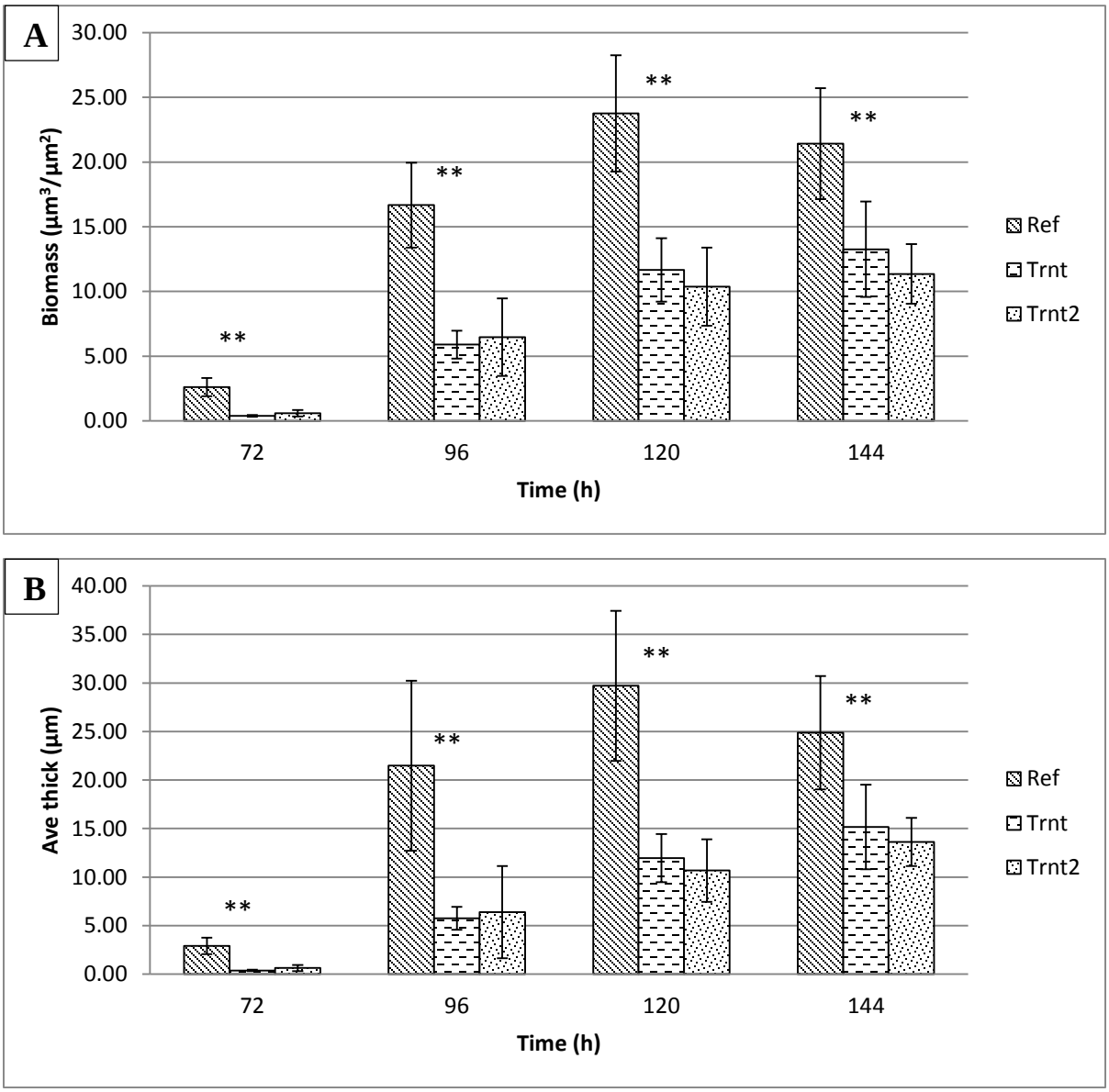


***Figure 31**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels after co-inoculation with 100 ppm of TiO_2 in 0.3 g/L TSB.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (μm) values of one reference (ref) and two treatment (trnt 1 and trnt2) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in μm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote statistically significant difference between the average values of treatment#1 and reference ($P < 0.05$).*

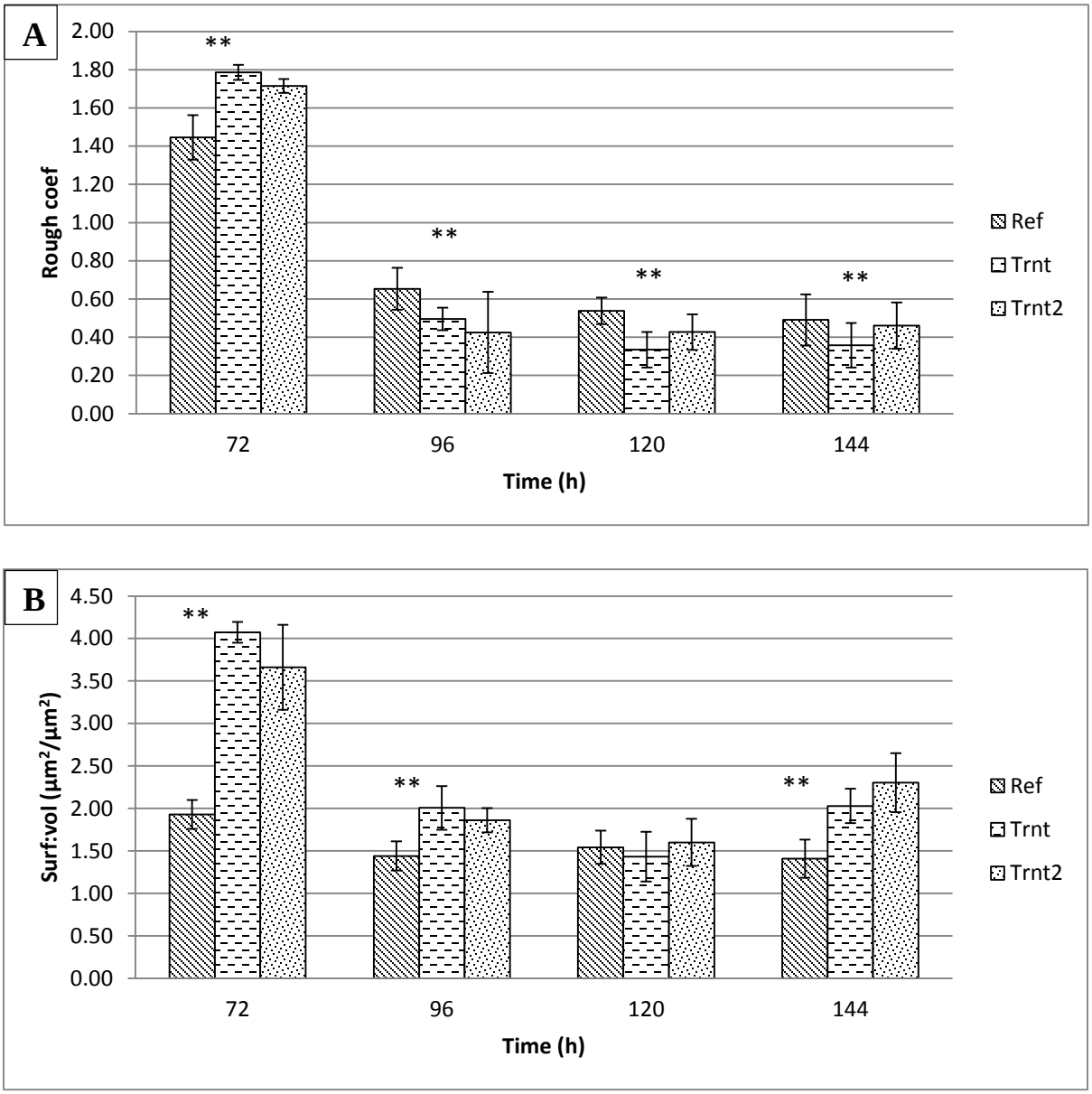


***Figure 32**: **Roughness coefficient and surface to volume ratio change over time in CT07-gfp biofilm reference and treatment channels after co-inoculation with 100 ppm of TiO_2 in 0.3 g/L TSB.** Averages of **a)** roughness coefficient (no units) and **b)** surface to volume ratio ($\mu m^2/\mu m^2$) of one reference (ref) and two treatment (trnt1 and trnt2) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** roughness coefficient of the biofilm and **b)** surface to volume ratio of the biofilm in $\mu m^2/\mu m^2$. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment#1 and reference ($P < 0.05$).*

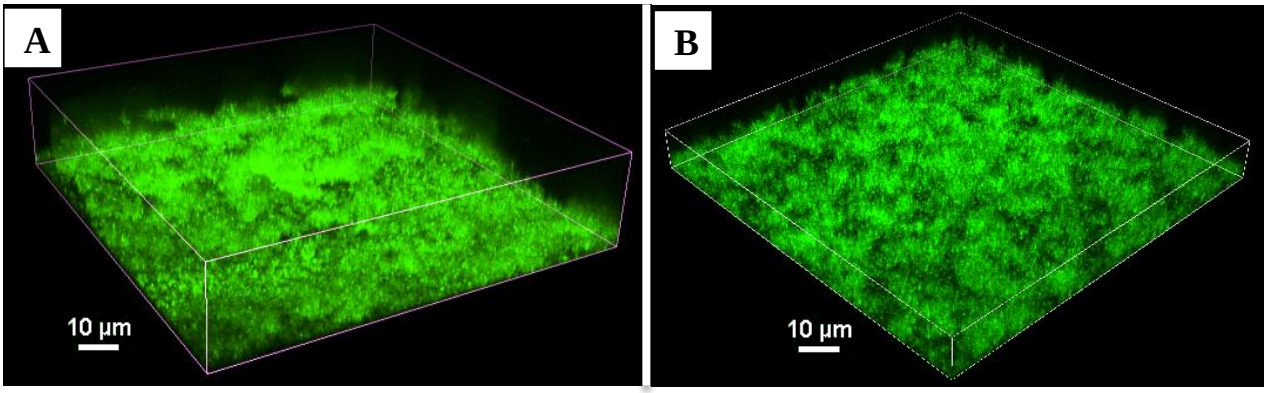


Figure 33: Volume snapshot of 144 hour old biofilm from treatment and reference channels. *Treatment is shown in image* ***a)*** *with 0.3 g/L TSB media containing 100 ppm of TiO_2, and reference is shown in image* ***b)****. Images were taken with Nikon 90i-C1 confocal microscope and 40x objective. Resolution is 1024x1024 pixels. Images prepared in NIS-Elements software.*

Lastly, the titanium dioxide concentration of 100 ppm had no noticeable effect on viability of planktonic cell production (Figure 34). All cell counts remained roughly within the same magnitude.

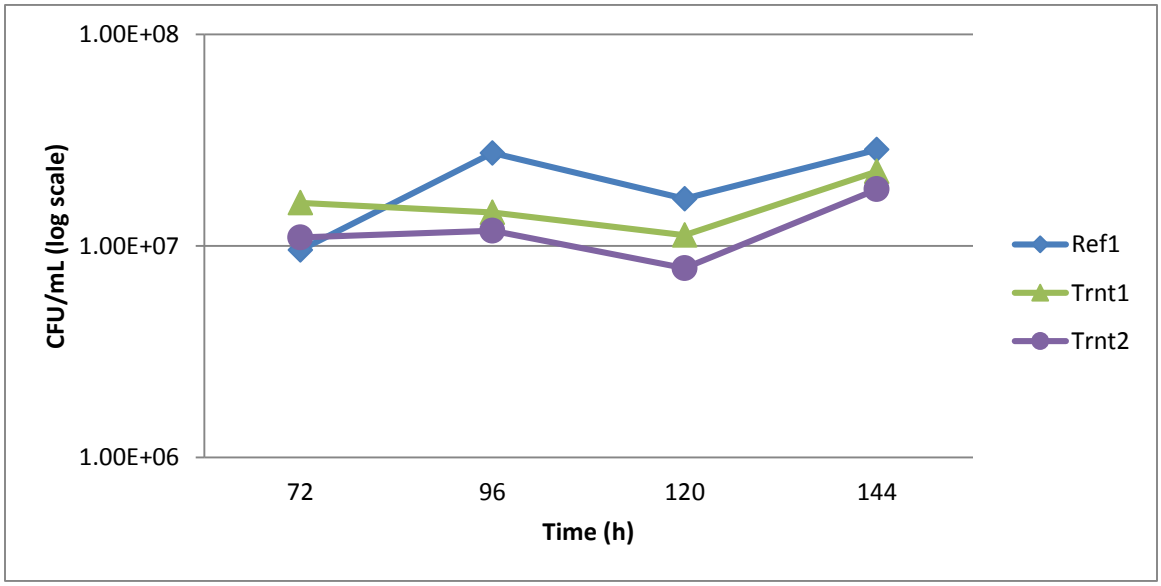


Figure 34*: **Viable cell counts during treatment with 100 ppm of TiO_2 in 0.3 g/L TSB.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm at co-inoculation, 0.3 g/L TSB media and CT07-gfp biofilm in treatment (trnt1 and trnt2) channels, and reference (ref) channel. In the graph, x-axis shows total time since inoculation and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

The experiment performed in section 5.3.1 had identical conditions to the experiments described in this section – the same bacterial strain, same light and temperature conditions, and same titanium dioxide concentration. The only component of the experiment that differed was the medium used – 0.3 g/L of TSB instead of AB defined medium. To recall, AB defined medium had 1mM citrate and caused high aggregation of titanium dioxide while 0.3 g/L TSB medium had a concentration of 0.14 mM glucose (rough manufacturer's estimate) and, as mentioned above, low sedimentation of titanium dioxide was observed. There was no noticeable effect from titanium dioxide on viable planktonic cell production. Combination of components in TSB media caused lower aggregation of titanium dioxide, which could in turn increase the toxicity to the biofilm, increase penetration depth by titanium dioxide aggregates and reactive oxygen species production, stimulating slower growth. Lower carbon source (0.14 mM glucose versus 1 mM citrate) could have given the biofilm less resources to combat the damage dealt by the TiO_2 toxicity mechanism. Additionally, the concentration of the carbon source and the different type of carbon source could have also affected biofilm's capacity to endure toxicity of titanium dioxide.

Summary:

- CT07-gfp bacterial cells are capable of establishing a biofilm in the presence of 100 ppm of titanium dioxide in regular light conditions in 0.3 g/L TSB medium.
- The growth rate of the treatment channel's biofilm was about one third (1/3) of the reference's.
- Titanium dioxide concentration of 100 ppm had no noticeable effect on viability of planktonic cell production.
- The architecture of the treatment channel's biofilm and reference biofilms were comparable.

5.3.3 Effects of 100 ppm of titanium dioxide on formation, growth, viability and structure of the CT07-gfp biofilm in 0.3 g/L Tryptic Soy Broth (TSB) with 1 mM glucose

In order to compare if the low carbon availability had an impact on rate of biofilm formation or if in fact the lower aggregate size of titanium dioxide had an impact on rate of formation, an experiment was designed to increase glucose concentration in 0.3 g/L of TSB to 1 mM. This was done by adding glucose to the 0.3 g/L media to match the 1 mM citrate levels on defined AB media. Otherwise, the experiment was run using the same set-up as described for section 5.3.2 with a single reference and two treatment channels. Even with increased glucose concentration, titanium dioxide was still suspended in the media flask when left undisturbed for a few hours.

Figure 35 shows that at 72 hours, reference biofilm biomass value was about 9 times greater than biofilm biomass in treatment channel #1 (trnt1). These results are in line with results from Figure 31, where at 72 hours there was formation of microcolonies in the reference channel, and only single cells loosely attached in the treatment channels. Similarly to the biomass, average thickness of the reference channel at 72 hours was about 10 times greater (Figure 35). Visually it was observed that at 72 hours, single cells were covering the substratum of the treatment channels, loosely attached. The same effect was observed as in experiment 5.3.2 – certain cells were stationary at the surface, while others would slowly move about or turn around their own axes. At the same time, reference channel had solid coverage of the substratum and small microcolonies forming. Between 96 and 144 hours, the biomasses and average thicknesses of treatment channels' biofilms steadily increased, but were still significantly smaller than the reference.

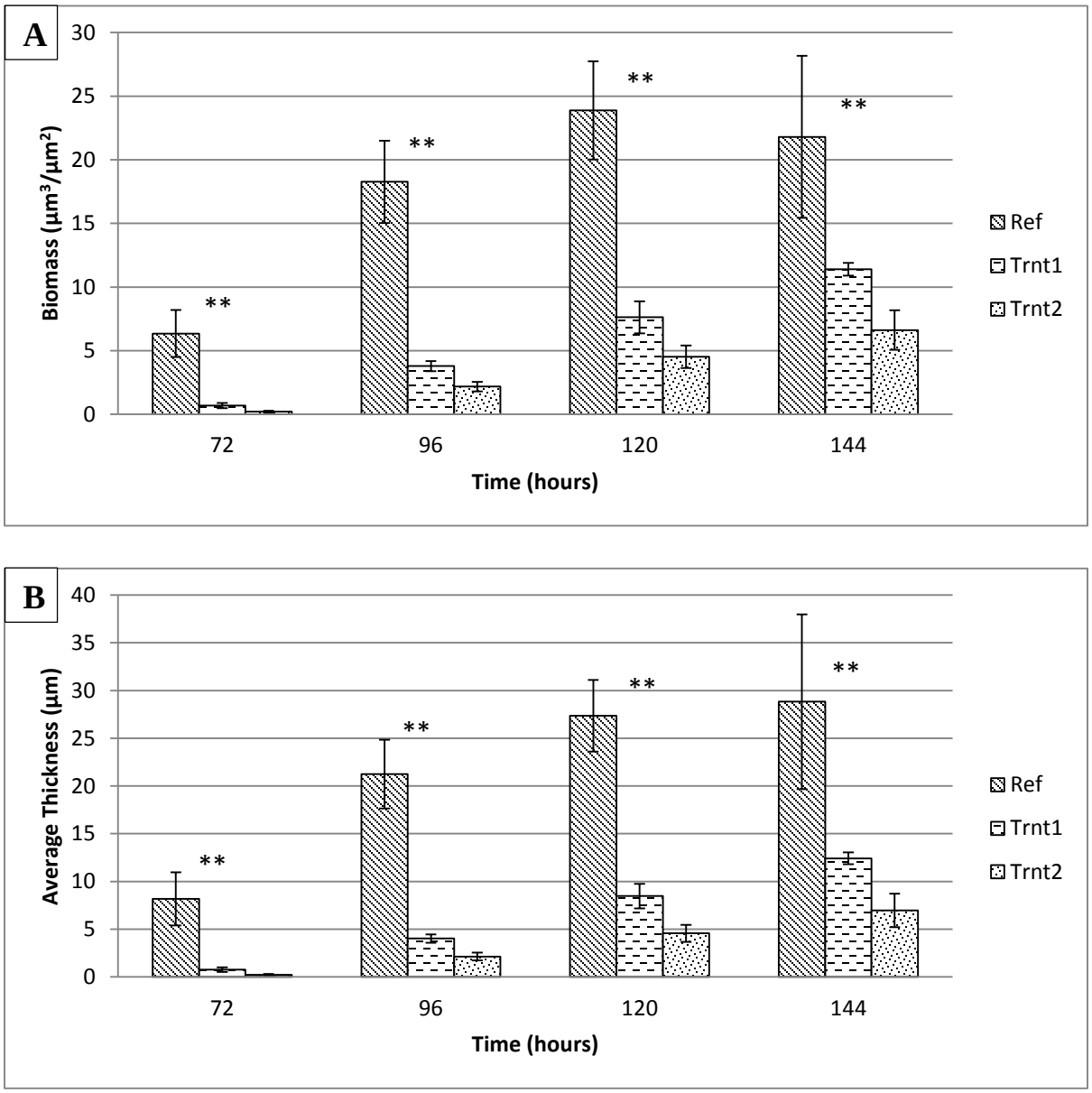


***Figure 35**: **Biomass and average thickness change over time in CT07-gfp biofilm reference and treatment channels after co-inoculation with 100 ppm of TiO_2 in media with 1 mM glucose**. Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of one reference (ref) and two treatment (trnt 1 and trnt2) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote statistically significant difference between the average values of treatment#1 and reference ($P < 0.05$).*

Looking at the roughness coefficient and area to volume ratios in Figure 36, data suggest that reference channel had a higher homogeneity at 72 hours than treatment channels, although

the treatment channels' biofilms roughness coefficient decreased as the time went on. Surface to volume ratio among all 3 suggests that biofilms were equally exposed to the surrounding media.

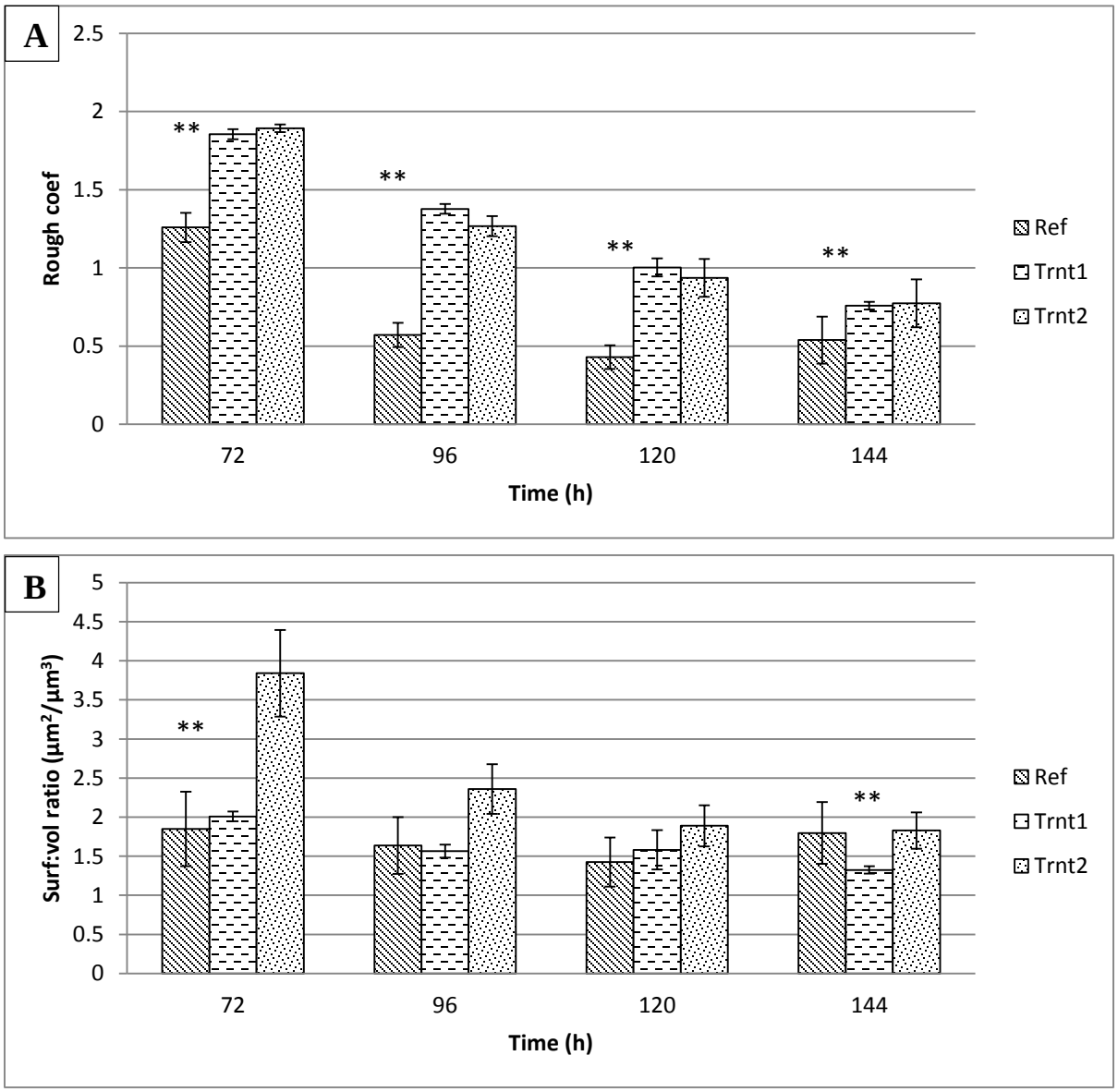


***Figure 36**: **Roughness coefficient and surface to volume ratio change over time in CT07-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2 in 1 mM glucose.** Averages of **a)** roughness coefficient (no units) and **b)** surface to volume ratio ($\mu m^2/\mu m^2$) of one reference (ref) and two treatment (trnt1 and trnt2) channels at 72, 96, 120 and 144 hours after inoculation. Treatment channel media contains 100 ppm of titanium dioxide. Y-axis shows **a)** roughness coefficient of the biofilm and **b)** surface to volume ratio of the biofilm in $\mu m^2/\mu m^2$. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment#1 and reference ($P < 0.05$).*

Lastly, looking at the amount of viable planktonic cells produced, there was a 1-log difference between both treatment channels and a reference at 72 and 96 hours after co-inoculation (Figure 37). However, at about 120 hours, the viable cell levels of both treatment channels reached roughly the same order of magnitude as the reference channel. This is the only scenario where viable cell counts were affected by exposure of biofilm to titanium dioxide concentration of 100 ppm.

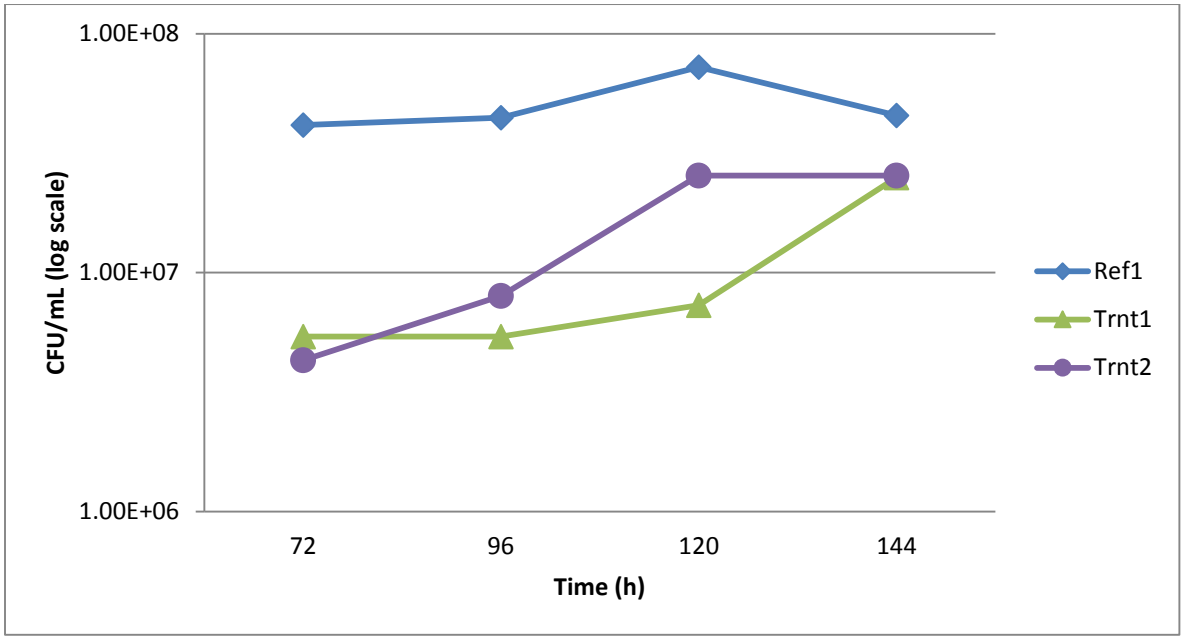


***Figure 37**: **Viable cell counts during treatment with 100 ppm of TiO_2 in 1 mM glucose.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm at co-inoculation, 0.3 g/L TSB media with 1 mM glucose and CT07-gfp biofilm in treatment (trnt 1 and trnt2) channels, and reference (ref) channel. In the graph, x-axis shows total time since inoculation and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Connecting all the data together, it seems that 100 ppm of titanium dioxide had a roughly 24-hour delaying effect on formation of the CT07-gfp biofilm in 0.3 g/L TSB media (regardless of the final glucose concentration) compared to the AB defined media. The rate of biofilm proliferation in 0.3 g/L TSB media (regardless of glucose concentration) was also slower for both treatment and reference channel compared to 1 mM citrate. This is evident when looking at biomass values of reference channels at 72 hours: average biomass value is 11.58 $\mu m^3/\mu m^2$ for AB defined medium reference and 6.35 $\mu m^3/\mu m^2$ for 0.3 g/L TSB 1 mM glucose (additional

values available in Appendix L). Reference channel in AB defined medium developed almost double the biomass than reference channel in 0.3 g/L TSB 1 mM glucose media, which carries a practical significance. However, the treatment and reference biofilms that developed in 0.3 g/L TSB media had more similar architecture than reference and treatment that developed in the AB defined media. Lastly, only this experiment with 1 mM glucose carbon source had a 1-log difference in viable planktonic cells production between treatments and reference channel, while no distinguishable difference was noted in the other experiments. These results are inconsistent with Figure 34, which shows no effect on viable cell counts in 0.3 g/L TSB media. In the grand scheme of things, 1-log reduction did not reduce biofilm's proliferation in both treatment channels. Thus, this may be attributed to variability in biofilm growth.

It took CT07-gfp cells about 24 hours longer to establish first microcolonies as compared to the reference channels regardless of the concentration of glucose in the solution. Treatment biofilms grown in 0.3 g/L TSB media (regardless of the concentration) had significantly lower biomass and average thickness values compared to the reference at any time point. On the contrary, CT07-gfp cells grown in the AB defined media with 100 ppm of titanium dioxide were able to form microcolonies and establish a biofilm at the same time as the reference (no 24 hour delay). The growth rate was comparable between the reference and treatment biofilms in AB defined media. Therefore, media composition plays a role in CT07-gfp biofilm's susceptibility to effects of titanium dioxide.

It was established that glucose availability had no impact on growth of the biofilm subjected to 100 ppm of titanium dioxide (comparing Figures 31 and 35). Looking back at the observation where titanium dioxide, when undisturbed, did not settle out in the flask containing 0.3 g/L TSB media it was hypothesized that aggregate size is small. There was a more profound impact on biofilm formation time and growth rate when biofilm was grown in 0.3 g/L TSB media with 100 ppm of titanium dioxide than in AB defined media with 100 ppm of titanium dioxide. Therefore, it may be concluded that decreased aggregate size had a negative impact on formation and growth of the CT07-gfp biofilm. Similarly, Sawai *et al.* (1996) found that decreasing particle size

had higher toxicity, although the team did not work with titanium dioxide. The opinions on the matter of particle size affecting toxicity of titanium dioxide are divided to this day.

Summary:

- CT07-gfp bacterial cells are capable of establishing a biofilm in the presence of 100ppm of titanium dioxide in regular light conditions in 0.3 g/L TSB media with 1 mM glucose.
- The growth rate of the treatment channel's biofilm was about a quarter (1/4) of the reference's.
- Titanium dioxide concentration of 100 ppm had 1-log reduction in viable cell production at 72 and 96 hours followed by an increase to same order of magnitude as the reference.
- The architecture of the treatment channel's biofilm and reference biofilms were comparable.
- Final glucose concentration (0.14 mM versus 1 mM) made no difference in the formation time or growth rate of the biofilm in the presence of 100 ppm of titanium dioxide.
- Potential reduction in size of titanium dioxide aggregates in 0.3 g/L TSB media could be responsible for the 24 hour delay in biofilm formation and overall reduced growth for the duration of the experiment.

5.3.4. Effects of 100 ppm of titanium dioxide on formation, growth, and viability of the PAO1-gfp biofilm in AB defined media with 1 mM citrate

Pseudomonas aeruginosa (PAO1) has been one of the model biofilms for a few decades. PAO1 is a well-studied bacterium, both in its biofilm and planktonic forms. There is currently a lot of literature available on effects of titanium dioxide on sessile cells and planktonic cells viability and resistance development (Mah and O'Tool, 2001; Gage *et al.*, 2005; Polo *et al*., 2011) . Therefore, an experiment was performed on PAO1 to compare and contrast how titanium dioxide would affect formation and proliferation of PAO1-gfp biofilm and planktonic cells. Experimental set up was identical to experiment 5.3.1: bubble traps were set up, tubing was clamped and 2 reference and 2 treatment channels were inoculated with overnight cultures.

Treatment channel contained media with 100 ppm of titanium dioxide. The carbon source was AB defined medium with 1 mM citrate and experiment was performed in regular room lighting.

Figure 38 shows biomass and average thickness of 2 reference and 2 treatment channels. At 72 hours after co-inoculation, reference channel #2 and treatment channel #2 had similar biomass and average thickness values. Data for reference #1 were not available for 72 hours. At 96 and 120 hours, average thickness and biomass of reference channels were roughly double that of the treatment channels. At 144 hours, the gap between treatment and reference channels' biomasses and average thicknesses decreased. These results suggest that PAO1-gfp cells were capable of establishing a biofilm during exposure to 100 ppm of titanium dioxide without time lag (unlike CT07-gfp cells). However, growth rate and proliferation of PAO1-gfp biofilm at initial stages was slower in the presence of titanium dioxide compared to the reference channel.

A number of studies mentioned above suggested that PAO1-gfp sessile cells are capable of tolerating hydrogen peroxide. Two recent studies performed by Polo *et al.* (2011) and Gage *et al.*, (2005) both reported photocatalytic titanium dioxide killing of PAO1 planktonic cell cultures. However, Gage and colleagues reported that UV-A played the key role in cell damage, since TiO_2-coated slides were not toxic to the bacteria. The level of photoactivation is not known in this experiment, however few studies mentioned previous suggested that titanium dioxide produces reactive oxygen species without being photoactivated by UV-light.

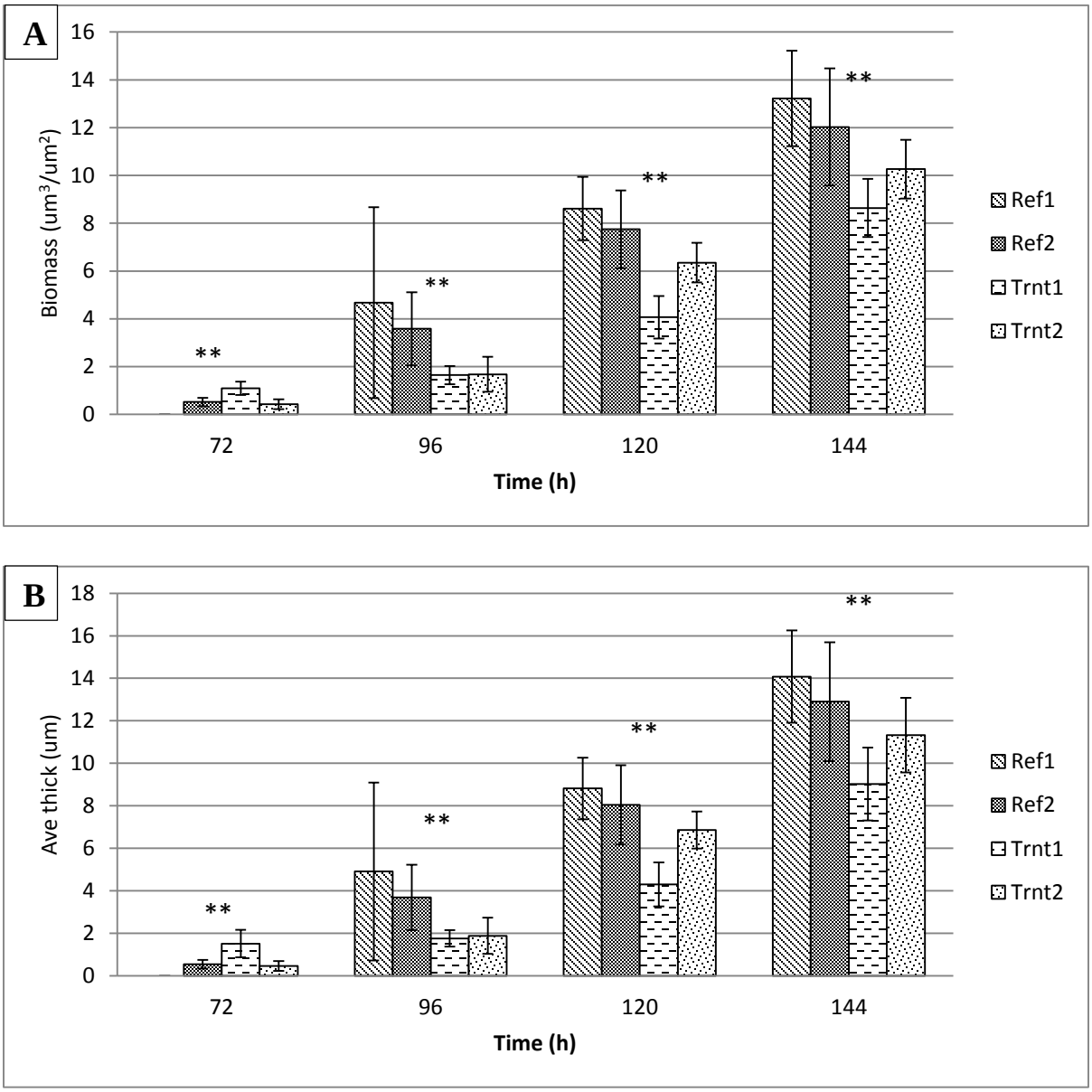


***Figure 38**: **Biomass and average thickness change over time in PAO1-gfp biofilm reference and treatment channels during exposure of treatment channel to 100 ppm of TiO_2.** Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (μm) values of two reference (ref1 and ref2) and two treatment (trnt1 and trnt2) channels at 72, 96, 120 and 144 hours after inoculation are shown. Treatment channel media contained 100 ppm of titanium dioxide. Y-axis shows **a)** biomass ($\mu m^3/\mu m^2$) of the biofilm and **b)** average thickness (μm). Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the total time elapsed since inoculation. Error bars show standard deviation. ** over a set of bars per time denote significant difference between the average values of treatment1 and reference2 ($P < 0.05$). Data for ref1 at 72 hours is not available.*

Looking at the viable planktonic cells produced, there is no evident significant difference between the treatment and reference channels (Figure 39). The cells remain within the same

order of magnitude. Therefore, it can be concluded that 100 ppm of titanium dioxide did not have a significant impact on viability of the PAO1 biofilm.

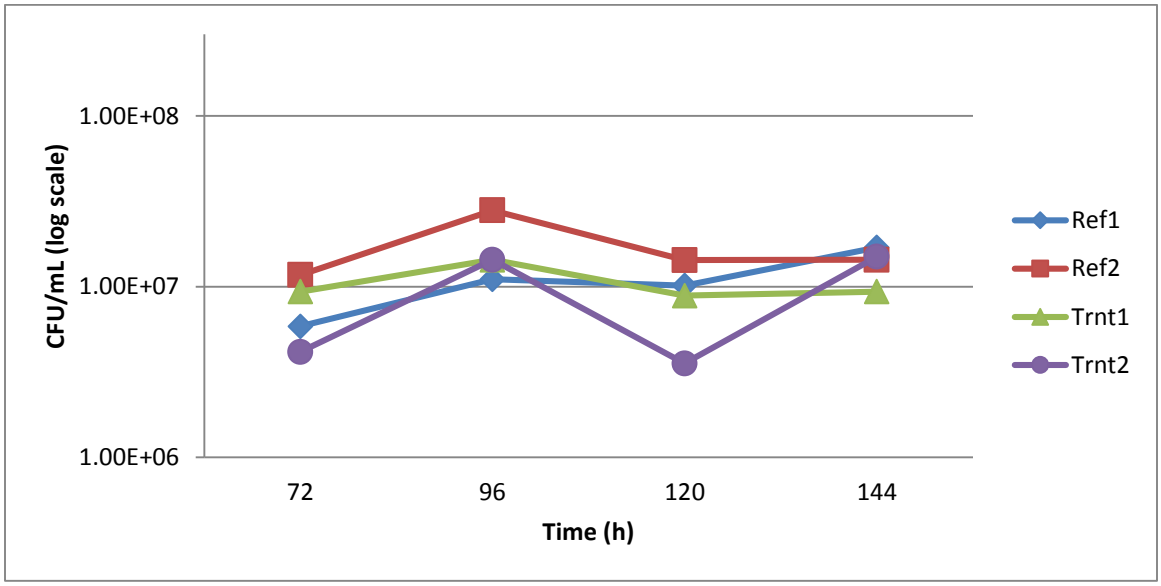


***Figure 39**: **Viable cell counts during co-inoculation treatment of PAO1 biofilm with 100 ppm of TiO_2.** CFU/mL values obtained in the tests with titanium dioxide concentration of 100 ppm at co-inoculation, AB defined media and PAO1-gfp biofilm in treatment (trnt1 and trnt2) channels, and reference (ref1 and ref2) channels. In the graph, x-axis shows total time since inoculation and y-axis shows log (CFU/mL) values at each time point for both treatment and reference channels.*

Roughness coefficient in both treatment channels was slightly higher than in reference channels at 72 and 96 hours (data available in Appendix M). However, by 120 hours, biofilms in reference and treatment channels were showing the same heterogeneity. Similarly, surface to volume ratio of treatment channels was higher at 72 and 96 hours of exposure, and then dropped down and evened out with the reference, suggesting that biofilms in reference and treatment channels had similar exposure to the surrounding media. These results suggest that 100 ppm of titanium dioxide in AB media had no consequence on biofilm's architectural design.

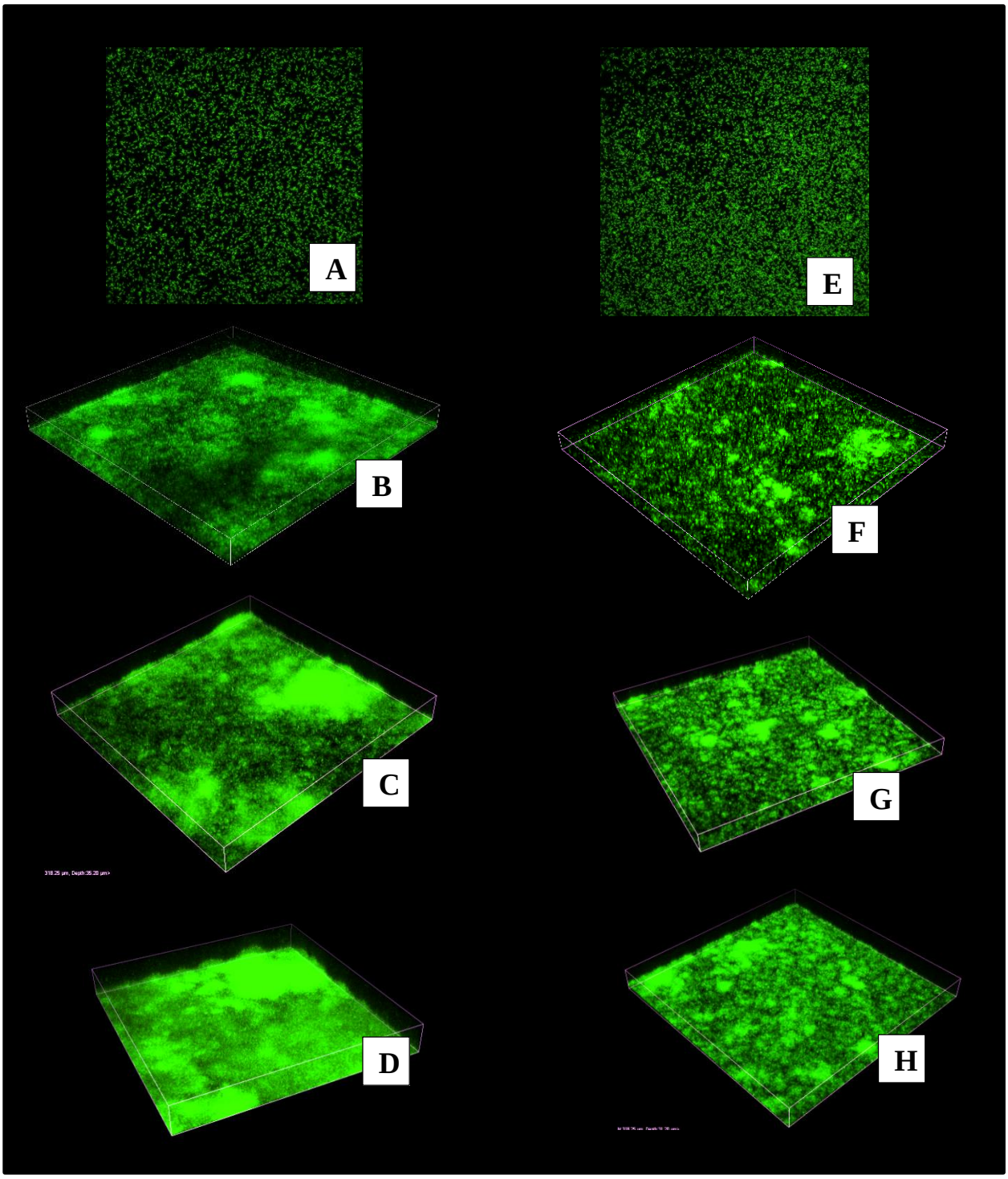


Figure 40: Time series from 72-144 hours of PAO1-gfp biofilm development in reference channel and treatment channel with 100 ppm of TiO_2. A-D *depict snapshots of reference channel at* ***a)*** *72 hours since inoculation (2-D),* ***b)*** *96 hours (3-D),* ***c)*** *120 hours (3-D), and* ***d)*** *144 hours (3-D).* ***E-H*** *depict snapshots of treatment channel with 100 ppm of TiO_2 at* ***e)*** *72 hours since inoculation (2-D),* ***f)*** *96 hours (3-D),* ***g)*** *120 hours (3-D), and* ***h)*** *144 hours (3-D).*

Summary:

- PAO1-gfp bacterial cells are capable of establishing a biofilm in the presence of 100 ppm of titanium dioxide in regular light conditions in AB defined media.
- The growth rate of the treatment channel's biofilm is about half of the reference at 96 and 120 hours after inoculation, but is then increased at 144 hours.
- Titanium dioxide concentration of 100 ppm had 1-log reduction in viable cell production at 120 hours in treatment channels.
- Architecture of the biofilm was similar between treatment and reference channels.

6. CONCLUSIONS

Literature suggests that titanium dioxide requires UV-A light for effective photoactivation and reactive oxygen species production. However, production of reactive oxygen species such as hydrogen peroxide has been documented without photoactivation of titanium dioxide. Reactive oxygen species is the most widely-accepted pathway by which titanium dioxide damages cells.

Titanium dioxide (at any concentration) did not prevent or permanently block biofilm formation, proliferation and growth, and did not have a significant observable effect on viable cell production. At a higher concentration of 100 ppm on developed biofilm there was a halt to biomass production and there was an increased percentage of compromised biomass in the first 24 hours. Also, during co-inoculation experiments, 100 ppm of titanium dioxide in 0.3 g/L TSB media (both 0.14 mM glucose concentration and 1 mM glucose concentration) caused a 24 hour delay in formation of the biofilm. Therefore, it can be concluded that there is an alternative mechanism by which titanium dioxide exerted damage on CT07 biofilm. The novel pathway has been suggested where titanium dioxide adhesion to cells causes cells to internalize the nanoparticle and thus cause content disruption. The focus of the study was to observe if titanium dioxide exhibited any toxic effect on the biofilm. The study of the mechanism of toxic effect was not part of the scope, therefore UV light was not a mandatory variable.

Other mechanisms could have contributed to biofilm's ability to recover from titanium dioxide damage. Low levels of reactive oxygen species in continuous flow reactor could have been insufficient in long term to damage cells, especially since half-life of certain free radicals is extremely short. Additionally, change in gene expression to combat free radicals could have caused bacterial cells to develop resistance to titanium dioxide nanoparticles.

In conclusion, titanium dioxide has a concentration and media-dependent effect on biofilm formation, growth, proliferation and viability. Conclusively, titanium dioxide showed no observable effect on viable cell production of the biofilm. At close to environmentally relevant concentration (1 ppm), there was no effect on formation, proliferation or growth.

7. RECOMMENDATIONS and PROTOCOL IMPROVEMENT

Sonication of titanium dioxide in the media was not possible without significantly increasing chance of contamination of the media and the system. However, sonication of the titanium dioxide aggregates within the flask and tubing may greatly reduce their size and therefore produce more accurate results in regards to "true" nanoparticle effect on biofilm formation. However, provided that titanium dioxide naturally aggregates in the environment due to colloids and ionic background, the results obtained in the laboratory are potentially environmentally relevant.

Although AB defined media was adjusted to decrease the size of the titanium dioxide aggregates, it did not completely eliminate the problem. Development of the defined media that would prevent or severely decrease titanium dioxide aggregation is one of the suggestions for future studies. However, development of new media requires considerable work and time, and was outside the scope of this project.

Decreasing the tubing length between the flask containing titanium dioxide and the flowcell with biofilm could potentially decrease the distance travelled by reactive oxygen species produced by photoactivated titanium dioxide, since ROS have a short half-life. This may

increase the impact of the same concentrations of titanium dioxide on the biofilm's formation and growth. The use of "doped" titanium dioxide may increase photoactivation strength in visible light conditions and therefore increase effects of TiO_2 on the biofilm. The use of doped-TiO_2 is a recent development. Additionally, measurement of free radicals in the effluent would provide more insight into ROS formation potential of titanium dioxide.

Synergistic or antagonistic toxicity effect of titanium dioxide with other metals and chemicals was not considered in this study, however it is recommended for the future research.

A protocol design involving field work is suggested for future experiments to increase environmental relevance of the experiments. Additionally, the use of epifluorescent nanoparticles to track their location in the biofilm is suggested, with future visualization of titanium dioxide nanoparticles within the biofilms. This may provide insight into what exactly titanium dioxide binds to, if it does, and how deep it can penetrate into the biofilm.

Lastly, although not directly related to the set of these experiments, it would be interesting to observe the change in planktonic and sessile cells' protein expression profile between exposed and non-exposed biofilms. Perhaps that would give insight into how cells are coping with potential toxicity exerted by titanium dioxide.

APPENDICES

Appendix A

Summary of the experimental design

As mentioned in the Materials and Methods section of the thesis, the experimental set-up and protocol has been adopted from Dr. Bester's doctoral dissertation (2010). Overall set-up is described in section 4.4 and shown in Figures 5 and 6. Protocol is summarized in Figure 7.

More detailed account for protocol is as follows. All work was done in sterile conditions.

Flowcell design

Each channel has the following dimensions within the flowcell block – it has to be at least 7 mm from the side of the flowcell block, and 5 mm from the top and bottom of the flowcell block. Distance between channels has to be at least 5 mm. The drill edge has to be 5 mm in diameter with a flat edge to make a flat bottom of the channel. Depth of the channel was 6 mm. Side holes were drilled 3 mm away from the top edge using 1/8 drill size.

Flowcell should never been cleaned with alcohol, otherwise it runs a risk of cracking; soap and water only.

Medium preparation

Distilled water with A-10 solution (and appropriate titanium dioxide concentration when needed) was autoclaved together. Other medium components such as FB-New, $FeCl_3$ and Sodium Citrate had to be autoclaved and added post-fact, due to precipitation of medium ingredients if autoclaved all together.

System set-up and inoculation

Figure 6 depicts the system set-up. Flasks with media (and titanium dioxide) would be pumped with peristaltic pump through bubble traps into the flowcell and then effluent container. Flow

cell would be angled with influent point being higher than effluent to prevent titanium dioxide build up on the bottom of the channels.

The system would be sterilized with 10% (v/v) commercial bleach for 2-3 hours, after which autoclaved distilled water would be pumped through for about 12 hours (overnight). In the morning, media would be swapped for distilled water to saturate the system.

Overnight culture would be growth in about 5 mL of media (with 10 mM citrate concentration) and injected into the flowcell with pump stopped and tubing clamped very slowly. Needle entry point would be covered by silicone glue to prevent leakage. After injection, the system would be left alone for about 20 minutes for cells to attach, after which clamps would be taken off and flow resumed. The inoculation would generally happen on Friday to allow for 72 hour growth over the weekend. 0.1 OD reading (OD at 600 nm) would mean about 100 μL of culture would be inoculated into the channel.

Effluent collection and distribution

Effluent collection would happen every day right before CLSM. In turn, effluent tubing of each channel would be disconnected and effluent would be collected into a 1.5 mL eppendorf tube. From this effluent after 30 seconds of vortex (to break up any cell clumps), 100 μL would be used for viable cell plating and 2x450 μL would be mixed with 2x80 μL of formaldehyde (and kept in the fridge) for total cell counts.

Propidium Iodide (PI) staining

At final concentration of 30 μM, 500 μL of stain were injected per channel with pump stopped and tubing clamped. Stain would be left in the dark for 20 minutes, after which it would be washed out for about 10 minutes.

Confocal Laser Scanning Microscopy

For GFP visualization, 485 nm laser was used. If PI was present in the channel, 635 nm laser was

used together with 485 nm. First z-stack would be always at the influent end of the channel, and each following z-stack would be taken about 1-2 mm down the channel. This way 10 z-stacks would allow for imaging of the first half of the channel. Images would always be taken at the center of the channel.

Using EZ-C1 software, 40x objective would be selected as objective in use. For GFP, 485 nm laser and 515/530 emission filter would be selected. For PI, 635 nm laser and 650LP filter would be selected. Step size for z-stack would be set at 0.8 nm, and image size would be 1024 by 1024 pixels. Substratum would always be selected as reference point, and 'bottom' of the biofilm would be when no more fluorescence could be detected. If there would be too much noise from the PI stain or GFP, average of 2 or 3 would be selected in the software.

Viable cell plating and DAPI staining

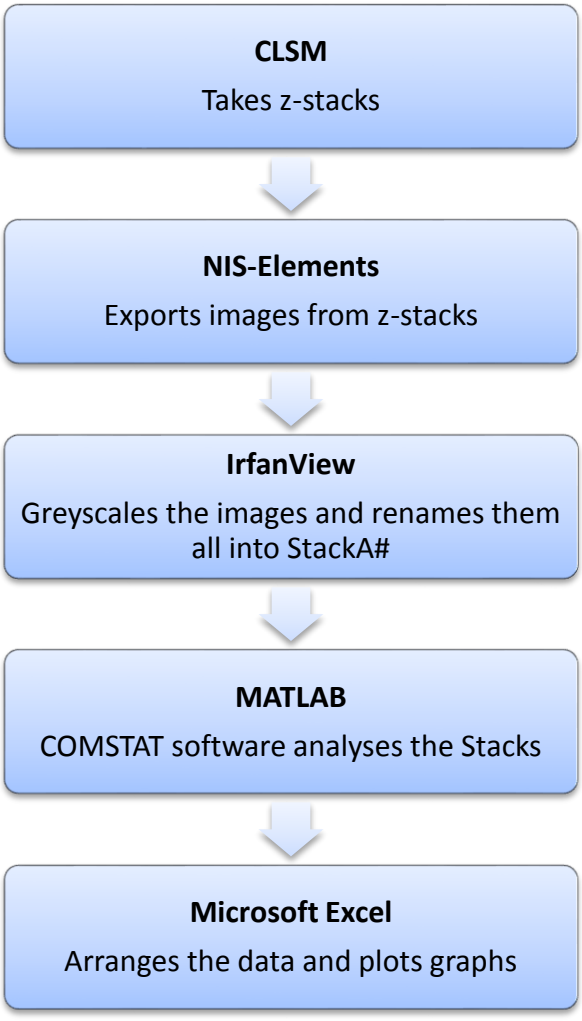
Six dilutions would be performed (100 μL of cells into 900 μL of 0.9% saline), the initial 100 μL coming from the eppendorf tube with effluent collected. Last 3 dilutions would be plated in duplicates on 3 g/L agar plates after thorough vortex to allow cells to disperse evenly. Ten drops of 10 μL each would be dispensed by Eppendorf Xplorer pipette. Plates would be left right side up for 15-20 minutes to let the liquid get absorbed by the agar, after which plates would be flipped agar side up and left for at least 48 hours before counting colonies. Between 30 and 300 colonies/sample was considered acceptable.

Cells preserved in formaldehyde would be retrieved from the fridge and gently vortexed. 100-150 μL of cells would be removed into a new eppendorf tube and mixed with 50 μL of DAPI (final concentration 250 μg/mL). After 20 minutes of staining in the dark, sample would be funnelled onto a membrane filter and placed on a slide with antifadent mounting medium and then coverslip. Original volume of cells stained would be adjusted if too many cells would be in a single field of view on the filter.

Epifluorescent microscopy would be used to visualize cells on the filter. If using 60x oil objective, 30 images would be taken in random fields of the filter. If using 20x objective, 10 images would be taken in random fields of the filter. ImageJ cell counter would be used as software to count cells and excel sheet provided by Dr. Otini Kroukamp would be used to back-calculate average number of cells in the effluent.

Image Analysis of CLSM images

The following 2 folders would be created within each channel folder containing 10 z-stacks per channel: exports and greyscale. Schematic below depicts the analysis process



NIS-Elements

NIS-Elements software would be used to export all images from each z-stacks/channel into the Exports folder. If only 485 nm laser was used, all channels would be merged into one ('invert overlays' option selected). However, if both 488 nm and 635 nm lasers were used, then 'invert overlays' options would be deselected to allow for both channels to be exported.

IrfanView

This program would be used to batch-convert images to grayscale. Images would also be renamed into "StackA#, StackB#, etc" and placed into "Greyscale" folder.

Info files

Within each greyscale folder, an info file would be created for each stack with a matching name. Info files carry information such as range (# of slices in the stack), pixel size of the objective resolution (x and y), and step size (z). So, for StackA#, StackA.info file would be created.

COMSTAT in MATLAB

After designating a path towards the greyscale folder within MATLAB program, the following commands were used to analyse the files:

- checkall – the check the folder for any errors in files or any files that are missing or do not belong according to information presented in the Info files
- look (look at 1 single image) or lookall (to look at the whole stack) to set threshold value that COMSTAT will use to analyse the images
- comstat – command that allows the user to run analysis on the stack

Text files created by the program with results of the analysis were saved in appropriate greyscale folders. Microsoft Excel is then used to arrange the data and produce graphs.

Appendix B

Verifying the actual size of titanium dioxide aggregates in AB defined media

Table A shows samples originally taken from the flowcell system in AB defined media without bacterial inoculant. The aggregates sizes are above nanoscale, and therefore would not be considered nanoparticles. Additionally, effluent size is increased compared to the influent size.

Sample origin	Eff. Diameter (nm)	St. error (±)
Influent 1	1295.8	52.4
Effluent 1	2255.9	639.1
Influent 2	1447.9	104.2
Effluent 2	1730.6	80.7

Table A. Aggregate size of titanium dioxide nanoparticles at 100 ppm concentration. *Sample origin means where from the samples was taken (before the flowcell or after). Eff. Diameter means effective diameter of the aggregate in nanometers and St. Error means standard error of the sample. Samples are 24 hours old.*

In order to identify what caused such high aggregation of titanium dioxide nanoparticles, a set of flasks was set up with different AB defined medium components excluded. Based on results shown in Table B it can be concluded that compounds in the A-10 component of the media, which originally included 1.51 mM $(NH_4)_2SO_4$, 3.37 mM Na_2HPO_4, 2.20 mM KH_2PO_4, 179 mM NaCl, have caused the highest aggregation. The possibility of glucose as an alternative carbon source was also considered and included in this test. However, results showed that glucose caused higher aggregation of titanium dioxide nanoparticles than citrate.

Missing media component	Eff. diameter (nm)	St. error (±)
A-10	799	22
FB-New	1769	132.4
$FeCl_3$	2106	137
Glucose (contains citrate)	1945.6	190.8
Citrate (contains glucose)	2204.9	263.3

Table B. Aggregate size of titanium dioxide nanoparticles at 100 ppm concentration with exclusion of different components of AB defined media. *Content of media components: A-10 (1.51 mM $(NH_4)_2SO_4$, 3.37 mM Na_2HPO_4, 2.20 mM KH_2PO_4, 179 mM NaCl), FB-New (0.1 mM $MgCl_2{\cdot}6H_2O$, 0.01 mM $CaCl_2{\cdot}2H_2O$), $FeCl_3$ (0.001 mM $FeCl_3$). Eff. Diameter means effective diameter of the aggregate in nanometers and St. Error means standard error of the sample. Samples are 3 hours old.*

The time it would take for titanium dioxide nanopowder (original particle size ~21nm) to aggregate in absence of specific AB defined medium components was investigated next and results are provided in Table C.

	Missing media component	Carbon source	Eff. diameter (nm)	St. error (±)
3 hours	A-10	Citrate	799	22
	FB-New		1769	132.4
	$FeCl_3$		2106	137
	Glucose		1945.6	190.8
	Citrate	Glucose	2204.9	263.3
21 hours	A-10	citrate	633.4	28.5
	FB-New		1585.9	67.8
	$FeCl_3$		1356	89.9
	Glucose		1721.5	131.8
	Citrate	glucose	2006	112.3

Table C. Changes in 100 ppm titanium dioxide aggregate size over time with different media components excluded. *Content of media components: A-10 (1.51 mM $(NH_4)_2SO_4$, 3.37 mM Na_2HPO_4, 2.20 mM KH_2PO_4, 179 mM NaCl), FB-New (0.1 mM $MgCl_2{\cdot}6H_2O$, 0.01 mM $CaCl_2{\cdot}2H_2O$), $FeCl_3$ (0.001 mM $FeCl_3$). Eff. Diameter means effective diameter of the aggregate in nanometers and St. Error means standard error of the sample. Samples are 3 and 21 hours old.*

Finally, since titanium dioxide may aggregate independent of any other compound present, an experiment was set up with MilliQ water and 100 ppm of titanium dioxide.

Time elapsed	Effective size (nm)	St. error (±)
30 min	670.8	17.1
60 min	583.9	41.3
Sonicated for 30 sec after 60 minutes	541.8	23.3
18 hours	630	44.1

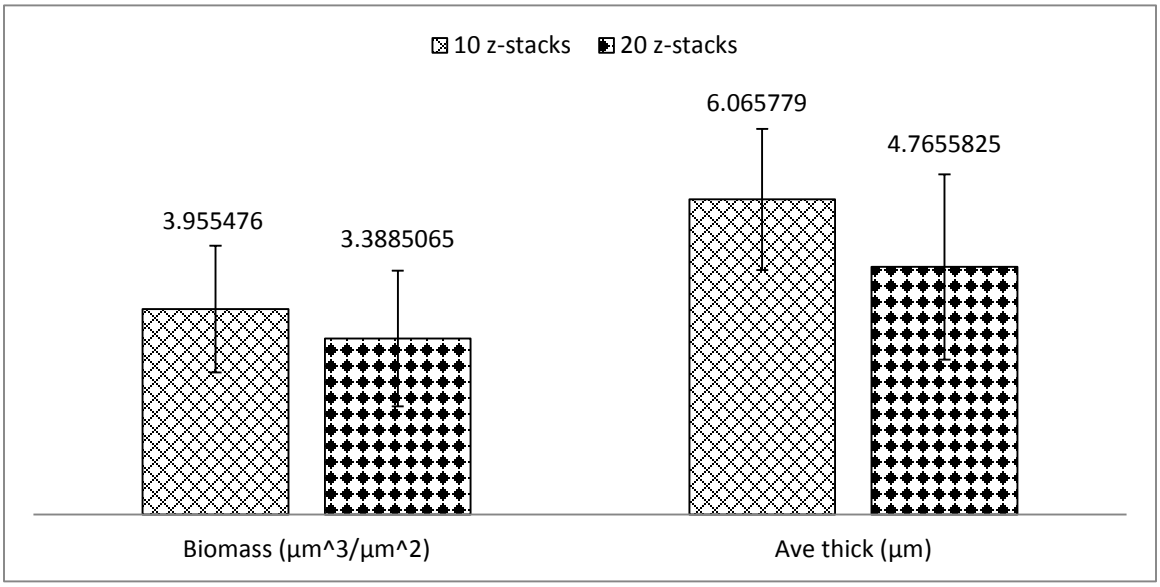
Table D. Aggregate size of 100 ppm of titanium dioxide in MilliQ water. *Eff. Diameter means effective diameter of the aggregate in nanometers and St. Error means standard error of the sample.*

The average aggregate size from Table C in the absence of A-10 component and the average aggregate size at 18 hours from Table D are similar, which suggests that other media components do not contribute significantly towards the aggregation.

Salts and buffers in A-10 components of AB defined media facilitated titanium dioxide nanoparticles to aggregate into roughly 1500 nm particles. Therefore, as a potential solution to decrease aggregate size the concentration of NaCl in the media has been decreased from 179 mM to 139 mM. That was the final concentration in AB defined media used for all further experiments. Sonication was not considered due to an increased chance of media contamination. Additionally, titanium dioxide does naturally aggregate in the environment, therefore making these concentrations environmentally relevant.

Appendix C

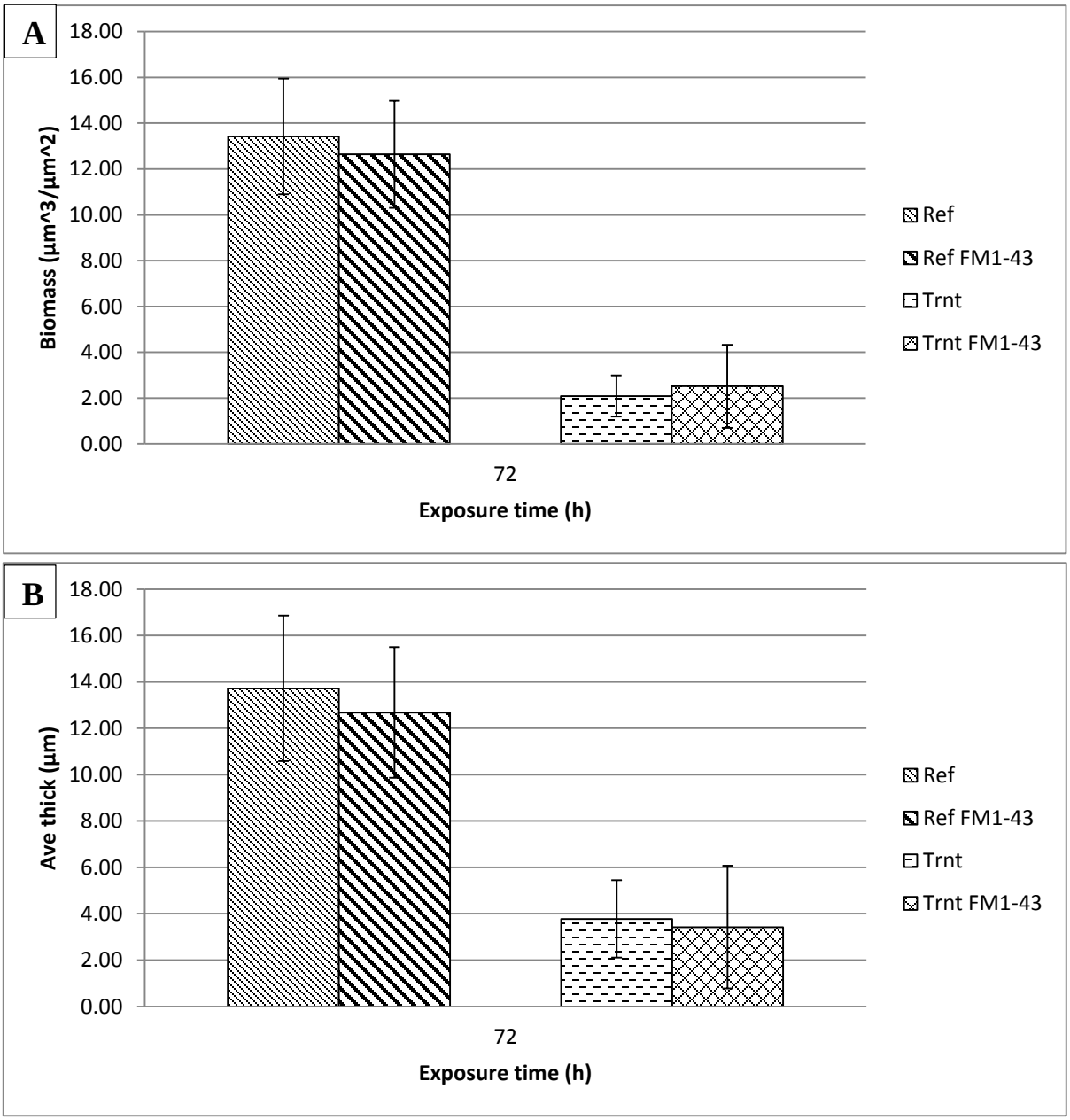
Figure below shows that there was no statistical difference between biomass and average thickness values through analysis of 10 and 20 z-stacks. Ten z-stacks per image were thus considered sufficient for all biofilm morphology analysis during this project.



Statistical difference between 10 and 20 z-stacks per same channel. *Averages of biomass ($\mu m^3/\mu m^2$) and average thickness (µm) of the CT07-gfp biofilm. Each value above the column corresponds to the average value of 10 or 20 z-stacks from COMSTAT analysis. The biofilm is 72 hours old. Error bars show standard deviation.*

Appendix D

Checking for green fluorescent protein (GFP) leakage from CT07-gfp cells due to exposure to TiO_2

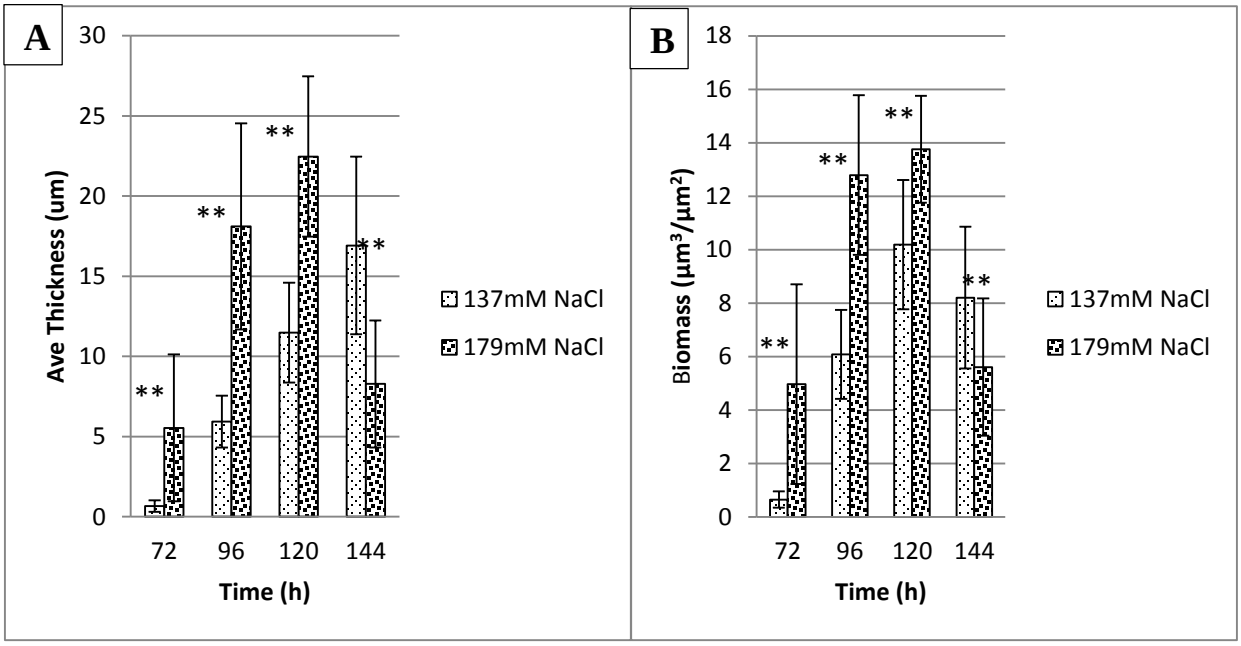


Comparison of biomass and average thickness values between GFP and FM1-43 stain emissions after 72 hour exposure to 100 ppm of TiO_2. *Averages of **a)** biomass ($\mu m^3/\mu m^2$) and **b)** average thickness (µm) values of reference (ref) and treatment (trnt) channels after 72 hour of exposure to titanium dioxide Ref and Trnt by itself means that only emission of GFP was obtained. Ref- and Trnt FM1-43 mean that channels were injected with FM1-43 lipid stain and then imaged. Y-axis shows **a)** biomass of the biofilm in $\mu m^3/\mu m^2$ units and **b)** average thickness of the biofilm in µm. Each value corresponds to the average value of 10 z-stacks from COMSTAT analysis. Time corresponds to the exposure time of treatment channel to the 100 ppm of titanium dioxide. Error bars show standard deviation.*

Appendix E

Effect of different salt concentrations on biofilm growth

Higher salt concentration potentially caused higher aggregate size in titanium dioxide nanoparticles so the sodium chloride concentration was decreased from 179 mM to 137 mM. However, the effect of decreased sodium chloride concentration on growth and proliferation of the biofilm was assessed to see if the combination of sodium chloride concentration, aggregate size and biofilm growth rate was optimal.



***Difference in biomass values between two sodium chloride concentrations**. Averages of **a)** biomass (µm³/µm²) and **b)** average thickness (µm) values of biofilms grown in 137 mM sodium chloride media and 179 mM sodium chloride media. ** over a set of bars per time denote significant difference between the average values of treatment and reference ($P < 0.05$).*

Based on the data shown in figure above, the results suggest that the biofilm grows at about twice the rate in higher salt concentration for at least the first 120 hours, which is a significant difference. However, for the purposes of the experiments performed in this study, the growth rate of biofilm in media containing 137 mM sodium citrate is sufficient.

All tables in appendices below show Average (Ave), standard deviation (St. Dev), reference channels (REF#), treatment channels (TRNT#) and time of exposure (h)

Appendix F

Averages and standard deviations of 72 hour old CT07-gfp biofilm subjected to 100 ppm of titanium dioxide under fluorescent lighting.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^3/\mu m^2$)	Max thick (µm)
0h					
REF					
Ave	3.70	4.25	0.67	3.60	21.84
St. Dev	1.58	1.66	0.20	0.76	5.68
TRNT					
Ave	3.26	4.33	1.03	3.17	30.08
St. Dev	0.61	0.73	0.14	0.21	5.32
24h					
REF					
Ave	11.35	12.78	0.34	1.98	30.32
St. Dev	4.48	5.33	0.11	0.41	6.27
TRNT					
Ave	3.18	6.17	1.07	4.49	51.12
St. Dev	0.83	1.89	0.16	0.37	21.20
48h					
REF					
Ave	14.98	16.74	0.23	1.84	35.52
St. Dev	2.24	2.45	0.03	0.19	3.31
TRNT					
Ave	5.80	8.90	0.72	3.56	45.52
St. Dev	1.21	4.59	0.20	0.77	19.76
72h					
REF					
Ave	15.70	23.44	0.24	3.60	54.00
St. Dev	5.04	5.68	0.08	0.65	11.41
TRNT					
Ave	10.92	15.95	0.88	2.92	65.84
St. Dev	7.34	12.34	0.34	0.85	43.32

Appendix G

Averages and standard deviations of 72 hour old CT07-gfp biofilm subjected to 10 ppm of titanium dioxide under fluorescent lighting.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
0h					
REF					
Ave	1.42	2.20	1.37	3.62	36.80
St. Dev	0.44	0.96	0.14	0.32	9.11
TRNT					
Ave	1.91	2.70	1.17	3.69	27.76
St. Dev	1.64	2.28	0.43	0.82	9.99
24h					
REF					
Ave	5.17	9.57	0.97	2.55	69.52
St. Dev	2.05	2.77	0.16	0.63	23.56
TRNT					
Ave	0.97	1.21	1.47	3.95	21.76
St. Dev	0.41	0.57	0.16	0.58	6.11
48h					
REF					
Ave	5.50	15.24	1.08	2.53	84.64
St. Dev	1.62	5.58	0.21	0.49	23.51
TRNT					
Ave	4.16	8.57	1.08	3.07	81.12
St. Dev	1.61	4.13	0.21	0.53	36.79
72h					
REF					
Ave	3.36	4.15	0.86	2.78	41.20
St. Dev	1.12	1.79	0.16	0.42	22.54
TRNT					
Ave	5.60	9.69	0.83	2.64	53.84
St. Dev	1.45	3.65	0.17	0.59	31.90

Appendix H

Averages and standard deviations of 72 hour old CT07-gfp biofilm subjected to 1 ppm of titanium dioxide under fluorescent lighting.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
0h					
REF					
Ave	1.88	2.32	1.10	3.14	21.68
St. Dev	0.20	0.39	0.05	0.10	4.02
TRNT					
Ave	1.55	2.03	1.26	3.31	28.72
St. Dev	0.15	0.18	0.07	0.09	10.41
24h					
REF					
Ave	2.70	3.09	0.96	2.70	36.88
St. Dev	0.81	1.13	0.16	0.30	15.49
TRNT					
Ave	3.00	3.66	0.97	2.63	30.48
St. Dev	1.45	2.02	0.17	0.43	14.52
48h					
REF					
Ave	6.45	7.65	0.53	1.54	39.52
St. Dev	1.52	4.95	0.13	0.40	19.85
TRNT					
Ave	7.19	10.22	0.78	2.04	66.48
St. Dev	2.83	2.91	0.20	0.68	15.03
72h					
REF					
Ave	9.31	22.08	0.74	2.35	99.28
St. Dev	2.73	21.48	0.20	0.61	45.50
TRNT					
Ave	9.86	19.63	0.72	2.00	98.24
St. Dev	2.15	6.20	0.09	0.33	32.45

Appendix I

Averages and standard deviations of 72 hour old CT07-gfp biofilm subjected to 100 ppm of titanium dioxide under dark conditions.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
0h					
REF					
Ave	5.75	8.27	0.69	2.32	45.84
St. Dev	1.98	5.42	0.15	0.21	16.64
TRNT					
Ave	6.29	6.36	0.57	1.48	35.52
St. Dev	1.01	1.19	0.12	0.29	8.19
24h					
REF					
Ave	3.16	4.99	0.93	3.34	60.48
St. Dev	0.48	1.32	0.20	0.35	27.74
TRNT					
Ave	2.89	2.75	0.62	2.60	32.24
St. Dev	1.16	1.48	0.17	0.81	16.26
48h					
REF					
Ave	5.04	8.28	0.79	2.87	63.36
St. Dev	1.16	3.02	0.24	0.68	21.68
TRNT					
Ave	7.48	7.93	0.39	1.43	36.08
St. Dev	2.10	2.08	0.13	0.45	10.98
72h					
REF					
Ave	3.43	4.52	0.83	2.90	53.28
St. Dev	1.50	2.45	0.27	0.73	17.30
TRNT					
Ave	9.19	10.16	0.46	1.23	50.00
St. Dev	2.44	2.71	0.08	0.28	13.84

Appendix J

Co-inoculation averages and standard deviations of CT07-gfp biofilm subjected to 100 ppm of titanium dioxide in AB defined medium.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
72h					
REF					
Ave	11.58	13.05	0.42	1.00	52.80
St. Dev	3.89	6.40	0.19	0.11	29.12
TRNT					
Ave	14.56	14.92	0.17	0.58	33.04
St. Dev	1.13	1.23	0.02	0.06	5.57
96h					
REF					
Ave	14.35	16.76	0.62	0.92	86.80
St. Dev	2.52	2.66	0.17	0.16	22.30
TRNT					
Ave	16.56	16.39	0.28	0.40	51.92
St. Dev	1.47	1.57	0.06	0.02	8.44
120h					
REF					
Ave	11.56	15.31	0.60	1.17	92.48
St. Dev	3.46	7.43	0.23	0.13	42.09
TRNT					
Ave	20.77	20.73	0.23	0.35	51.84
St. Dev	2.44	2.63	0.04	0.05	3.99
144h					
REF					
Ave	12.96	16.56	0.65	1.05	96.72
St. Dev	1.87	5.11	0.21	0.17	44.94
TRNT					
Ave	25.72	26.09	0.23	0.35	62.72
St. Dev	3.45	3.62	0.04	0.03	10.45

Appendix K

Co-inoculation averages and standard deviations of CT07-gfp biofilm subjected to 100 ppm of titanium dioxide in 0.3 g/L TSB medium.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
72h					
REF					
Ave	2.60	2.90	1.45	1.93	46.16
St. Dev	0.70	0.84	0.12	0.17	18.87
TRNT1					
Ave	0.38	0.38	1.79	4.07	8.24
St. Dev	0.07	0.08	0.04	0.12	1.92
TRNT2					
Ave	0.60	0.63	1.72	3.66	11.60
St. Dev	0.25	0.32	0.04	0.50	9.76
96h					
REF					
Ave	16.67	21.47	0.65	1.44	75.68
St. Dev	3.28	8.77	0.11	0.17	33.28
TRNT1					
Ave	5.90	5.76	0.50	2.01	17.60
St. Dev	1.08	1.18	0.06	0.26	3.29
TRNT2					
Ave	6.47	6.39	0.43	1.86	18.40
St. Dev	3.00	4.76	0.21	0.14	19.11
120h					
REF					
Ave	23.75	29.71	0.54	1.54	74.24
St. Dev	4.49	7.73	0.07	0.20	16.88
TRNT1					
Ave	11.66	11.95	0.34	1.43	26.72
St. Dev	2.45	2.47	0.09	0.29	2.24
TRNT2					
Ave	10.37	10.67	0.43	1.60	31.04
St. Dev	3.01	3.23	0.09	0.28	10.70
144h					
REF					
Ave	21.41	24.88	0.49	1.41	66.48
St. Dev	4.29	5.84	0.13	0.22	16.16

TRNT1					
Ave	13.26	15.17	0.36	2.03	33.76
St. Dev	3.69	4.35	0.12	0.20	5.20
TRNT2					
Ave	11.36	13.62	0.46	2.30	42.32
St. Dev	2.30	2.47	0.12	0.35	16.34

Appendix L

Co-inoculation averages and standard deviations of CT07-gfp biofilm subjected to 100 ppm of titanium dioxide in 0.3 g/L TSB 1 mM glucose medium.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (µm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (µm)
72h					
REF					
Ave	6.35	8.17	1.26	1.85	65.28
St. Dev	1.85	2.79	0.09	0.48	18.88
TRNT1					
Ave	0.69	0.77	1.85	2.01	25.84
St. Dev	0.20	0.25	0.03	0.06	3.16
TRNT2					
Ave	0.21	0.21	1.89	3.84	15.52
St. Dev	0.07	0.09	0.02	0.55	5.82
96h					
REF					
Ave	18.27	21.24	0.57	1.64	81.76
St. Dev	3.23	3.59	0.08	0.36	24.56
TRNT1					
Ave	3.79	4.03	1.38	1.57	38.24
St. Dev	0.39	0.44	0.03	0.08	3.81
TRNT2					
Ave	2.18	2.14	1.27	2.36	24.72
St. Dev	0.38	0.40	0.06	0.32	3.76
120h					
REF					
Ave	23.88	27.36	0.43	1.43	74.72
St. Dev	3.85	3.76	0.08	0.32	12.75
TRNT1					
Ave	7.62	8.47	1.00	1.58	45.04
St. Dev	1.27	1.29	0.06	0.25	4.17
TRNT2					
Ave	4.52	4.57	0.94	1.89	31.84
St. Dev	0.89	0.89	0.12	0.26	5.17
144h					
REF					
Ave	21.80	28.83	0.54	1.80	86.64
St. Dev	6.36	9.16	0.15	0.39	31.67

TRNT1					
Ave	11.40	12.42	0.76	1.32	47.04
St. Dev	0.51	0.61	0.02	0.05	2.41
TRNT2					
Ave	6.62	6.97	0.77	1.83	36.40
St. Dev	1.55	1.75	0.15	0.23	8.18

Appendix M

Co-inoculation averages and standard deviations of PAO1-gfp biofilm subjected to 100 ppm of titanium dioxide in AB defined medium.

	Biomass ($\mu m^3/\mu m^2$)	Ave thick (μm)	Rough coef	Sur: vol ($\mu m^2/\mu m^3$)	Max thick (μm)
72h					
REF1					
Ave	N/A	N/A	N/A	N/A	N/A
St. Dev	N/A	N/A	N/A	N/A	N/A
REF2					
Ave	0.52	0.54	1.66	4.76	8.88
St. Dev	0.18	0.20	0.10	0.12	0.59
TRNT1					
Ave	1.09	1.51	1.24	4.33	10.00
St. Dev	0.28	0.65	0.19	0.14	1.26
TRNT2					
Ave	0.43	0.46	1.74	5.31	9.84
St. Dev	0.21	0.23	0.12	0.43	1.41
96h					
REF1					
Ave	4.67	4.91	0.94	2.87	20.48
St. Dev	3.99	4.18	0.62	1.13	8.49
REF2					
Ave	3.58	3.68	0.83	2.80	20.48
St. Dev	1.53	1.54	0.25	0.52	4.53
TRNT1					
Ave	1.65	1.76	1.26	3.64	17.12
St. Dev	0.38	0.39	0.12	0.32	3.05
TRNT2					
Ave	1.68	1.88	1.29	4.04	16.00
St. Dev	0.73	0.85	0.20	0.80	2.56
120h					
REF1					
Ave	8.61	8.81	0.43	1.80	30.88
St. Dev	1.33	1.46	0.09	0.24	4.58
REF2					
Ave	7.75	8.04	0.32	2.12	27.04
St. Dev	1.63	1.87	0.06	0.30	5.30
TRNT1					
Ave	4.07	4.30	0.57	3.28	17.60
St. Dev	0.89	1.03	0.09	0.34	3.44

TRNT2					
Ave	6.35	6.86	0.41	2.21	22.88
St. Dev	0.83	0.88	0.05	0.38	2.27
144h					
REF1					
Ave	13.22	14.08	0.28	1.51	35.92
St. Dev	2.00	2.17	0.04	0.24	4.87
REF2					
Ave	12.03	12.90	0.24	1.80	34.32
St. Dev	2.45	2.80	0.02	0.25	6.36
TRNT1					
Ave	8.64	9.02	0.28	1.92	24.96
St. Dev	1.21	1.72	0.03	0.21	3.30
TRNT2					
Ave	10.26	11.32	0.26	2.15	32.08
St. Dev	1.23	1.76	0.03	0.16	4.97

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