DEPARTMENT OF MOLECULAR SCIENCES

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Investigating the impact of emerging human pollutants on marine *Synechococcus*

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Disclaimer

I certify that all the work presented in this thesis have not been published in any others work and this is solely my own work. I have cited all the materials where credit is due. My work has got the research ethics approval with biosafety number 5201600490.

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Abstract

Pollution of the marine environment as a result of human activity is an issue of growing concern. Detection of pharmaceuticals and personal care products (PPCPs) in marine ecosystems is increasingly being reported and may adversely impact marine organisms. Toxicity testing of PPCP-related substances has focussed largely on model organisms with limited ecological relevance. I examined the impact of emerging pollutants on representative strains of *Synechococcus,* a highly abundant key marine primary producer. *Synechococcus* growth was tracked over 72 hrs using three independent techniques: optical density at 750nm, *in vivo* chlorophyll a fluorescence and autofluorescent cell counts by flow cytometry, to compare common cyanobacterial growth monitoring methods. Silver and zinc oxide nanoparticles significantly inhibited growth of all tested strains at concentrations above 5 mg/L and 0.01 mg/L respectively. For triclosan, concentrations above 0.1 mg/L were inhibitory to growth. Oxybenzone and ibuprofen had no significant impact on growth at concentrations up to 10 mg/L and 5 mg/L respectively. Oxygen production was significantly reduced in *Synechococcus* CC9311 on exposure to triclosan and silver nanoparticles, indicating these substances can impact photosynthesis. *Synechococcus* species showed higher sensitivity to zinc nanoparticles and lower sensitivity to the other tested compounds than reported for most previously tested marine organisms, indicating the need to extend ecotoxicological testing of non-model organisms, particularly photosynthetic prokaryotes.

1. Introduction

The ocean plays an indispensable role by supporting various life forms on earth. It is inhabited by numerous organisms ranging from the smallest bacteria to the biggest mammals [1-4]. Our environment is increasingly subject to pollution from a wide variety of human-derived contaminants. The sources of those anthropogenic pollutants include the agricultural release of fertilizers, pesticides, antibiotics from the rearing of farm animals, the release of organic compounds and heavy metals from industry and discharge of sewage [5, 6]. Other activities contributing to marine pollution include maritime transport, oil extraction, aquaculture and an increase of tourism and recreational activities near coastal areas [4, 7]. The comprehensive review by Halpern *et al*., [8] into ocean pollution in 2008 estimated that an area of around two million square kilometres is considered under high impact from anthropogenic pollutants, while a further region comprising more than 41% of the world's ocean was projected to be under medium to high impact [8]. The only regions thought to be subject to lower impact are near-polar regions, hardly visited by humans [9].

Pharmaceuticals and personal care products (PPCPs) are an emerging class of marine pollutant [10-12]. Pharmaceutical products have been extensively produced and used for many decades. Pharmaceutical products are not only prescribed for human use, but are heavily relied upon by the livestock industry [7]. Personal care products range from soaps, toothpaste to all laundry, bathing products and other cosmetics that are in everyday use [13]. Such products are discharged, often at high levels, into marine water bodies without further modification [5, 11]. Industrialisation and high population density near coastal areas increase the amount of waste entering the coastal environment [8, 14]. The global detection of these compounds in fresh and marine surface waters at a low but biologically relevant levels indicates a potential risk to the aquatic ecosystem [12]. Evidence is mounting that anthropogenic pollution is a burden to all marine organisms regardless of size and habitat.

Marine picocyanobacteria represent highly abundant phytoplankton communities found in virtually every area of the ocean surface, dominating tropical to subtropical waters [15]. The genera *Synechococcus* and *Prochlorococcus* are the most abundant picocyanobacteria in the ocean and they are estimated to contribute to up to one third of the total primary production on Earth [16, 17]. They also provide energy and organic sources to other organisms in the ocean [15, 18]. *Synechococcus* is found to be distributed ubiquitously in freshwater, brackish water and in all the marine environments from coastal to open ocean [19, 20]. Their population is greater in nutrient rich regions compared to oligotrophic areas and they contribute to the regulation of global biogeochemical cycles and nutrient cycling in marine ecosystems [18, 21] .

Several ecotoxicological studies have been conducted to look at the impact of PPCPs on marine and estuarine algae, fish, mammals and other invertebrates [12, 22-25]. As standard ecotoxicology assays

are not easily applied to environmental bacteria, little is known about the impact of these compounds on marine bacteria. Given the importance of marine bacteria in our ocean, investigation of the impact of anthropogenic pollutants on marine picocyanobacteria, such as *Synechococcus* represents an important addition to current ecotoxicology research.

1.1 Cyanobacteria

Photosynthetic organisms present in aquatic ecosystems are reported to contribute to 45% of the photosynthesis on Earth [26]. They supply organic matter required for sustaining other life in aquatic ecosystems like open ocean, rivers, lakes and estuaries. Thus, photosynthetic organisms determine the overall productivity and biological flow of energy [27]. Cyanobacteria represent an ancient, diverse group of organisms. They are oxygenic photosynthetic prokaryotes which can adapt to an astonishing array of changing environmental conditions [28]. They can be seen in all surface environments in the unicellular or filamentous form. The photosynthetic apparatus in cyanobacteria have photosystem reaction centres bound to thylakoid membrane with the main photosynthetic pigment being chlorophyll a. A distinctive feature of most cyanobacteria is their accessory light-harvesting complex called phycobilisomes, illustrated in Figure 1 [29]. These complexes contain the phycobiliproteins, phycocyanin and allophycocyanin, while some cyanobacteria produce an additional phycobiliprotein called phycoerythrin (PE) [30, 31]. This accessory light-harvesting complex is located on the outer surface of the thylakoid membrane. It absorbs and transfers light energy to the photosystem reaction centres [32].

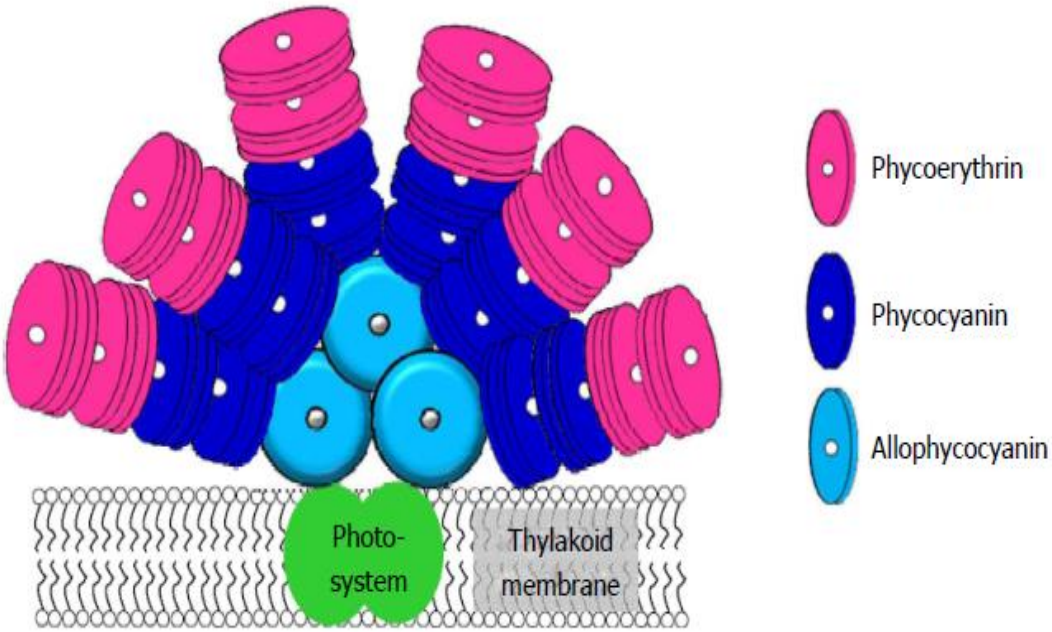

Figure 1: The structure of phycobilisomes and associated pigments in cyanobacteria (taken from Sonani *et al.,* 2016 [33]

1.2 Marine picocyanobacteria

Marine picocyanobacteria are important contributors to global carbon fixation in certain regions of the ocean [16]. *Synechococcus* is found to be distributed ubiquitously in freshwater, brackish water and in all marine environments from coastal to open ocean [19, 20] with concentrations of up to 1.5x 10^6 cells per mL in some regions [17]. Their population is greater in nutrient rich, mixed and coastal regions compared to oligotrophic areas [27]. They have been reported to be present at higher numbers in areas with low salinities or low temperatures. They contribute to the regulation of global biogeochemical cycle and nutrient cycling in the marine ecosystem [18, 34]. Marine *Synechococcus* contain chlorophyll a as a primary light harvesting pigment along with other accessory pigments. The particular composition of accessory pigments determines the colour of every individual strain of *Synechococcus* [35]. Cyanobacteria without phycoerythrin pigment appear blue-green colour [31]. Those cyanobacteria with olive-green to reddish orange colour contain phycoerythrin with different ratios of phycocyanin to phycoerythrin. *Synechococcus* strains isolated from the open ocean are generally reddish-orange due to the prevalence of phycoerythrin pigment in them [36].

1.3 Phylogenetic classification and distribution

A large degree of genetic variation amongst the marine and estuarine strains of *Synechococcus* has been revealed by molecular phylogeny analysis [37] and based on 16S rDNA sequence variation, *Synechococcus* lineages have been divided into three sub-clusters 5.1, 5.2 and 5.3 [18, 28]. Out of the three, sub-cluster, 5.1 is the largest containing 10 distinct genetically different clades. *Synechococcus* from each clade possess distinguishing physiologies that allow them to adapt and distribute to a wide range of environment. Each clade has distinct strains which differ in motility or accessory light harvesting protein complexes and their ability to carry out chromatic adaptation [36, 38, 39]. For example, WH8102 strain from clade III is motile while strains from other clades are not. Quantitative molecular assays have confirmed that each clade occupies a different niche and are ecologically distinct lineages or ecotypes [40]. The distribution of representative *Synechococcus* strains from each clade of subcluster 5.1 and their biogeography across marine regions are presented in Table 1 [41].

The complete genome sequences of many marine *Synechococcus* strains have been determined and are available in the NCBI genome database. Coutinho *et al*., 2016 [19] summarised the detailed characteristics of the 24 *Synechococcus* genomes available in 2013. The following representatives of the main ecotypes (clades I-IV) are among the most well studied isolates: *Synechococcus* CC9311 strain (clade I) isolated from California current (coastal), CC9605 (clade II) isolated from California current (oligotrophic), WH8102 (clade III) isolated from Sargasso Sea (oligotrophic) and BL107 (clade IV) isolated from Blanes Bay, Mediterranean Sea and were chosen for testing in this work.

Synechococcus trains CC9311 and WH8102 are perhaps the most well studied, being amongst the first genome sequenced and have been used in a number of comparative genomic and stress response studies [42-44]. These strains have been used as representative coastal (CC9311) and oligotrophic (WH8102) habitat isolates and examined to determine their physiological and transcriptomic response to a range of stressors, often showing differences in their responses. A study by Varkey *et al*., 2016 [45] , on the effect of low temperature on two different strains of *Synechococcus* showed a decrease in the growth and photosynthesis of strain WH8102 while strain BL107 was unaffected, with indications this was due to increasing the abundance of proteins involved in photosynthesis. Work by Tetu *et al*., 2013 [46] on the effect of DNA damaging agents mitomycin C and ethidium bromide on CC9311 and WH8102 indicated that CC9311 was able to tolerate higher levels of these toxicants. Another study on copper toxicity looking at *Synechococcus* strains WH8102, CC9311 and CC9605 demonstrated increased tolerance to copper in coastal strains compared to the open ocean strain [44]. This work indicates that different strains may respond quite differently to other stressors, such as exposure to environmental pollutants.

Table 1: Distribution of representative *Synechococcus* strains from each clade of subcluster 5.1 and their biogeography across many marine regions (adapted from Huang *et al*., 2012 and Coutinho *et al.*, 2016 [19, 41]).

Synechococcus			
Sub cluster 5.1	**Representative isolates**	**Biogeography**	**Isolate reference**
I	CC9311, WH8020	Temperate to polar waters, co-occurrence with clade IV	[47, 48]
II	CC9605, WH8012	Tropical/ subtropical waters	[38, 48]
III	WH8102, WH8103	Oligotrophic waters	[48]
IV	BL107, CC9902	Temperate to polar waters, co-occurrence with clade I	[47, 48]
V-VII	WH7803, WH7805, WH8018, UW92, RS9920	Oceanic waters	[48]
VIII	RS9917, WH8101	Hypersaline waters	[49]
IX	RS9916	Rarely detected	[48]
XI-XIV		Gulf of Aqaba subsurface water (predominant)	[27]
XV/XVI	UW69, UW106, UW105, UW140	Sargasso Sea (isolates and environmental sequences)	[36]
CRD1/CRD2	MITS9220, UW179	Costa Rica upwelling dome (predominant)	[50]
WPC1/WPC2	KORDI-56	East China Sea and East Sea (isolates and environmental sequences)	[51]
CB1-CB3	CB0201, KORDI-53	Chesapeake Bay, summer	[52, 53]

1.4 Pharmaceutical products

Pharmaceutical products include a broad category of substances that are used to treat diseases or injury. The presence of pharmaceutically active compounds in the terrestrial and aquatic environment

has been acknowledged and their impact on other non-target organisms is of growing concern [54]. In the European Union alone about 3000 pharmaceutical products are used, including analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others [55]. The type and concentration of pharmaceuticals differ among countries depending on prescription and population size.

Despite this, there are currently limited assessments of the impact of pharmaceuticals in the marine environment in many geographical regions. Gaw *et al*., 2014 [56] reviewed the available information and reported on forty-nine studies which have looked into the presence of pharmaceuticals in the coastal environment. Of these, twenty studies were conducted in Europe, twenty-one in Asia, six in North America and one each in South America and Oceania. Prichard *et al*., 2016 [12] have reviewed the data on levels of 113 pharmaceuticals and their metabolites in coastal waters, reporting concentrations from the nanogram to microgram scale.

1.5 Personal care products

Personal care products (PCPs) include a wide variety of products that we use on everyday basis starting from soaps, toothpaste, shower products, fragrances, insect repellents to sunscreen [13]. Compared to pharmaceuticals, less data is available on the occurrence and toxicity of PCPs in the marine environment [57]. However, several studies have reported the presence and bioaccumulation of personal care products in the aquatic environment. The major classes of PCPs detected in the marine environment includes disinfectants, UV filters, musks and other substances like silver nanoparticles [4, 58].

1.6 Pharmaceuticals and personal care products as emerging pollutants

The utilisation of pharmaceuticals and personal care products (PPCPs) is rapidly increasing worldwide. PPCPs covers the broad categories of all types of human and veterinary prescribed pharmaceutical products and personal care products [13]. The presence and bioaccumulation of PPCPs in fresh as well as marine waters has been reviewed by many authors [7, 13]. Although their concentration is generally fairy low (ng/L-µg/L), studies have reported impacts of such anthropogenic agents on both freshwater and marine organisms [54]. Pharmaceutical products are intended to act on a target with a specific mode of action. When such products are released into the environment, they may exert a negative effect on non-target populations [59]. Therefore, their uncontrolled release needs to be monitored. Unlike pharmaceuticals, personal care products are designed for external use and enter the environment without chemical modification [54]. After their use PPCPs enter the sewage system. Sewage treatment plants separate the compounds into two phases. The hydrophobic parts get eliminated in the solid sludge while hydrophilic ones are discharged through effluents [57]. Current

sewage treatment is insufficient to remove such compounds thereby these compounds often end up in the environment. Wastewater effluents, landfill waste, submarine outfalls, runoff via rivers and streams, aquaculture, agriculture, recreational activities are all potential sources of PPCPs which enter into the marine system [4, 58].

1.7 Sources of Pharmaceuticals and personal care products

The main sources of pharmaceuticals and personal care products are the effluent water from sewage [56].The wastewater generated from households, hospitals as well as manufacturing industries continue to discharge pharmaceuticals and other personal care products. The removal of such compounds from sewage depends on the physico-chemical properties of the compound and effectiveness of removal ranges from 10 to 100% [56]. Inefficiently treated waste water is finally discharged into water bodies contaminating the freshwater and marine water bodies. The PPCPs present in solid waste discharge and activated sludge contaminate the soil and finally reach water bodies [60]. In the marine environment, the coastal and ocean outfall contains discharged sewage from waste water treatment plants. In coastal areas, groundwater sewage can also be a source of PPCPs. Waste disposal in coastal areas including the leachate from landfills, seafills are another source of PPCPs entering the coastal environment. Rodriguez *et al.*, 2013 [61] report concentrations of pharmaceuticals up to 0.027 mg/L from landfill leachate on the island of Mallorca. Personal care products can enter the environment through recreational activities and tourism in coastal areas, which is increasing as these areas around the world continue to attract more tourists [55, 62]. The direct washing of sunscreen by swimmers and surfers is a main source of contamination of UV filters in coastal areas.

Here, I review four categories of PCPPs chosen as the focus of this study, which include substances of high environmental relevance and for which limited ecotoxicology information is currently available.

1.8 Non-steroidal anti-inflammatory drugs

While antibiotics are the pharmaceutical agents most often screened for in environmental surveys, non-steroidal anti-inflammatory drugs are also pharmaceuticals of high environmental relevance and generally less well researched in terms of environmental impact. Non-steroidal anti-inflammatory drugs, which include compounds such as ibuprofen, diclofenac, ketoprofen and naproxen, are used to treat many symptoms including fever, inflammation and rheumatic arthritis [4]. Such compounds and their metabolites are often detected in sewage treatment plants, surface water and even in the marine environment in nanogram to microgram per litre concentrations [63]. Ibuprofen is the non-steroidal anti-inflammatory drugs most frequently detected and investigated in seawater. In surface water, ibuprofen has been detected in concentrations in the rage of a few micrograms, with the

average concentration of 0.03 μg/L in rivers of Korea [64], 22 μg/L in sewage treatment plant effluent and 84 μg/L in sewage treatment plant influents. This is an indication that sewage treatment plants are the major source of ibuprofen contamination in marine environment, with reports that levels can be as high as 1000ng/L [7]. Moreover, the presence of ibuprofen in seawater has been reported in the range of 0.01 to 2730 ng/L [55, 65, 66]has also been detected in marine sediments [67].

1.9 Disinfectants

Disinfectants are used in households on an everyday basis and there is a high potential to release them in the aquatic environment. Some of the examples of compounds detected are 4-methyl phenol, triclosan, triclocarban, phenol, biphenylol [6]. Among them, triclosan and triclocarban were the most detected compound in terms of frequency and concentration in surface water and waste treatment plans[68]. Triclosan and triclocarban are used as an antimicrobial in soaps, creams, fragrances. Several studies on surface water and waste treatment plants [57, 69, 70] reported the presence of triclosan, and its derivative methyl-triclosan, with the highest concentration of 74 ng/L (surface water) to 650ng/L (waste treatment plans) [71]. Studies on monitoring 95 different chemical compounds by USGS in the United States found that 56.8% of surface water samples contained triclosan at a concentration of 50 ng/L or higher [13, 72]. The methyl derivatives of triclosan are lipophilic and they are more likely to be accumulated in aquatic organisms. Studies on fish showed the detection of methyl triclosan with 2100 ng/g of fish lipids [72]. The ability to accumulate triclosan and its methyl derivatives vary among different species [73]. In algae, triclosan accumulates to a greater degree than methyl triclosan, but in aquatic plants triclosan fails to accumulate [74, 75]. Triclosan also reaches marine environments, particularly estuaries and coastal regions, from sewage treatment plants. Studies by Wu *et al*., 2007 [76] detected triclosan in a concentration of 99.3ng/l across the Victoria Harbour in China.

1.10 UV filters

Sunscreen is widely used to protect our skin from harmful radiation from the sun. UV filters are an ingredient of sunscreen and other cosmetics that can absorb photons from sunlight and rapidly return them to ground state thereby minimising the damaging effect of those photons [77]. Previously, sunscreen products incorporated minerals like zinc oxide and titanium oxide. They act as inorganic UV blockers (both UVA and UVB). Addition of these minerals provides broad spectrum protection, less penetration in the skin and lack of skin irritation. New sunscreen products have replaced these minerals with their nano size particles to reduce the opaqueness of the sunscreen. Thus, zinc oxide nanoparticles (ZnO NPs) , titanium oxide nanoparticles ($nTiO_2$) (with diameter less than 100 nm) are used more and more frequently in the manufacture of sunscreens [78] and they can comprise up to

25% of some sunscreen products. Combinations of up to ten different UV filter compounds can be found in sunscreen and cosmetics [79]. The major source of UV filter contamination is from direct release by swimmers in the coastal areas and wastewater treatment plants. Twenty-seven different compounds are currently certified to be used in cosmetics, plastics and much more [55]. Examples of commonly applied UV filters includes ZnO NPs, $nTiO_2$, 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), 4-methyl-benzilidine-camphor (4MBC), benzophenone-3 (BP3), octocrylene (OC).

Among the UV filters, BP-3 and OC have been detected at the highest concentrations in seawater, with 2013 ng/l and 1409 ng/l reported in studies from Folly Beach, South Carolina [80]. Benzophenone-3 is an active ingredient in many personal care products and has been declared an emerging anthropogenic pollutant by the U.S environment protection agency [77]. Boating, swimming and residential waste contribute to major dumping of BP-3. Levels of BP-3 have been detected on coral reefs at 0.4 to 3.8 part per trillion. The half-life of BP-3 is short but they can be renewed continuously and act as a pseudo-persistent pollutant. Bioaccumulation of UV filters was reviewed by Arpin point *et al.,* 2016 [7] who report that UV filters accumulate in marine mollusks [81-83].

Studies on the quantification of zinc oxide nanoparticles are very few and there is a lack of analytical instruments capable of characterising nanoparticles from seawater or sediments [84, 85]. Expected environmental concentrations have been calculated based on the market penetration of nanoparticles using mathematical model based methods. The estimated concentrations vary among the models used with concentrations in marine surface water estimated to range from 0.1-76000 ng/L and 1.6-3194 μg/L in marine sediments [86, 87].

1.11 Silver nanoparticles

Silver nanoparticles (Ag-NPs) are widely used in various types of consumer products ranging from medicines, food products to electronics and cosmetics [88, 89]. Advancements in the field of nanotechnology and antimicrobial activity of Ag-NPs led to the incorporation of Ag-NPs in many personal care products including toys, kitchen items, paints, wound dressings to name a few [90]. Such widespread use of Ag-NPs containing products means this substance often reaches marine environment through waste disposal. The predicted environment concentration of silver nanoparticles in aquatic ecosystem ranges from few ng/l to mg/kg and thus the investigation of Ag-NPs toxicity in the marine environment is very important.

1.12 Impact of PPCPs

Studies available on the impacts of PPCPs on marine organisms are limited. Currently available data reviewed by Prichard and colleagues *et al.,* 2016 [12] showed the impacts of such emerging

pollutants on marine organisms from different tropic levels. Some of the notable impacts are given below.

1.12.1Non-steroidal anti-inflammatory drugs

Studies on the impact of non-steroidal anti-inflammatory drugs on marine organisms are very limited. In vertebrates and invertebrates, non-steroidal anti-inflammatory drugs like ibuprofen have been known to influence the cyclooxygenase pathway which is concerned with the synthesis of eicosanoids [91, 92]. Eicosanoids are important regulators of reproduction and therefore they are expected to affect the reproduction in marine organisms. Previous studies have reported negative effects of ibuprofen on marine organisms including sea mussels [93, 94] fish and microalgae [24, 95] and *Ruditapes philippinarium* [93]. Zanuri *et al.,* 2017 studied the effect of ibuprofen on marine invertebrates and exposure to 20 minutes of ibuprofen at a concentration of ≥ 1μg/L was shown to affect the gametes of *P. miliaris* and the success of fertilisation in *A. rubens* [96]. Moreover, ibuprofen exposure on marine blue mussels *Mytilus edulis trossulus* for two weeks demonstrated a significantly reduction in growth rate and impairment of the mussels' reproductive fitness at a concentration above 1mg/L [97].

1.12.2 Disinfectants

Although disinfectants are detected in concentrations at the nanogram to microgram scale, studies have demonstrated they can affect freshwater and marine organisms at this low level. In comparison to other disinfectants, triclosan was found to be more toxic to organisms from different trophic groups [13]. Algae and invertebrates were more sensitive to triclosan than fish and vascular plants. Brausch *et al.*, 2010 [13] proposed that the reason for higher sensitivity of algae to triclosan is likely due to disruption of fatty acid synthesis and the enoyl acyl carrier reductase pathway [98, 99], membrane destabilisation [100] and uncoupling of oxidative phosphorylation [101]. Studies on the impact of triclosan on marine organisms showed evident hyperpolarization of marine microalga *Tetraselmis suecica* [102] and decrease hemocyte metabolism and viability in *Haliotis tuberculata* [103].

1.12.3 UV filters

Studies into the toxicological effects of UV filters on marine organisms are limited. In mammals, BP-3 can induce activation of estrogen receptor proteins and has anti-androgenic activity [104]. Similar effects were also observed in studies of fish. BP-3 causes endocrine disruption, decreased egg production and egg hatching rates [105]. Downs *et al.,* 2009 [106] studied the effects of BP-3 on coral planulae and showed an increase in coral bleaching and DNA lesions, thus demonstrating genotoxicant effects on corals. BP-3 at a concentration of 10 to 1.6 pg/cell induces depolarization of the microalgae *Tetraselmis suecica* [7]. The impact of BP-3 on marine organisms belonging to

different trophic levels was also studied by Paredes *et al*., 2014 [107], reporting a significant reduction in the growth of all tested organisms (*Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*) [107].

Compared to other UV filters, the toxic effects of zinc oxide nanoparticles have been extensively studied in marine organisms, as reviewed by Yung *et al*., 2014 [108]. Studies on marine phytoplankton, crustaceans, fish have demonstrated that the impact of ZnO NPs is more pronounced in phytoplankton than crustaceans and fish [109]. Miller *et al.,* 2010 [110] report a significant difference in growth rate after 96 hours of exposure to ZnO NPs for four species of phytoplankton, *Thalassiosira pseudonana*, *Skeletonema marinoi, Dunaliella tertiolecta* and *Isochrysis galbana* with IC50 values of 4.56, 2.3, 0.85 and 1.19 mg/L respectively. In another study on marine eukaryotic algae *D. tertiolecta*, growth was affected with 50 % effective concentration (EC50) of 1.95 mg/L [78]. Similarly, ZnO NPs negatively affected another two marine phytoplankton, *Phaeodactylum tricornutum* and *Alexandrium minutum,* at a concentration of 10 mg/L [111].

1.12.4 Silver nanoparticles

Most of the available data on Ag-NPs toxicity is focused on freshwater organisms including algae, crustaceans and fish. At present, there is a limited amount of data on marine organisms. Studies on freshwater algae have shown a reduction of biomass and biofilm volume and photosynthesis [89]. Work by Gambardella [112] *et al.*, 2015 has looked at the impact of Ag-NPs on marine organisms belonging to different tropic levels, including primary producers and consumers, and showed that Ag-NPs can cause a change in mobility (swimming behaviour of crustaceans, sperm movement in sea urchin) and mortality in a dose-dependent fashion [112]. In this work algae (*Dunaliella tertiolecta*, *Skeletonema costatum*) were impacted with an IC50 of 0.9 and 3.1 mg/L respectively, cnidaria (*Aurelia aurita*- jellyfish) were reported to have an EC50 of < 0.1 mg/L, for crustaceans (*Amphibalanus amphitrite* and *Artemia salina*) the EC50 was < 0.1 mg/L while for echinoderms (*Para- centrotus lividus*) an EC50 of 0.55 mg/L was reported. Such work indicated that jellyfish, sea urchins and green algae are more sensitive to Ag-NPs than diatoms [113]. Work has also been carried out on corals, where a concentration of 0.05 mg/L was reported to alter behaviour and inhibit swimming at the larval stage 114].

1.13 Project objectives

Increasing population density, inhabitation of coastal zones and general increases in the use of pharmaceuticals and personal care products all increase the likelihood of detecting higher concentrations of PPCPs in the ocean in the future. As indicated by the above review of available literature on the impact of PPCPs on marine organisms, this is a notable environmental issue but further ecotoxicology assessment is needed. To date, no such studies have been conducted on marine bacteria which occupy an important place in all ocean ecosystems. Marine *Synechococcus* represents a genetically diverse, important group of phytoplankton in the ocean. This phytoplankton community provides organic nutrients to other organisms in higher trophic levels and plays an important role in biogeochemical cycling of carbon. As *Synechococcus* is distributed ubiquitously in the marine environment including brackish, coastal and open ocean to polar regions, studying the impact of PPCPs on *Synechococcus* is important in increasing our understanding of how marine ecosystems will be affected by such emerging human pollutants. Thus, this project represents an important, innovative approach to monitoring the impact of emerging anthropogenic pollutants on marine bacteria which are at the base of the food chain.

1.14 Specific questions addressed

In my project, I aim to answer the question of how a range of different PCPPs, which have been observed at relatively high concentrations in seawater, impact marine *Synechococcus.* The following substances were selected as representatives of major classes of pharmaceuticals and personal care products which are found in the marine environment: zinc oxide nanoparticles, silver nanoparticles, BP-3 from cosmetics, triclosan as a disinfectant and ibuprofen as a non-steroidal anti-inflammatory drug. This work describes the development of assays to monitor the impact of potential toxicants on the growth of a panel of representative marine *Synechococcus* strains. In this work, I also developed assays to track the impact of toxicants on photosynthetic oxygen production in marine *Synechococcus,* which is put forward as a useful indicator of how toxic compounds impact the physiology and photosynthetic capacity of primary producers.

2. Materials and methods:

2.1 *Synechococcus* cultures and growth conditions

Representative strains of *Synechococcus* from the major clades CC9311 (I), CC9605 (II), WH8102 (III), and BL107 (IV) were selected for this study. The strains were cultured on artificial seawater (ASW) [116] at a pH of 8.0 and temperature of 22 °C under constant illumination with 35 μmol photons m^{-2} s^{-1} and agitation at 200 r.p.m. in an Infors HT multitron standard incubator. The cultures were first acclimated to the incubator conditions for three successive generations and then actively growing cells in early logarithmic phase were used to carry out the experiments.

2.2 Growth curve study

Marine *Synechococcus* are relatively slow growing bacteria which are highly sensitive to variations in environmental conditions and media composition. Growth curves were performed for all strains to determine growth characteristics under experimental conditions to ensure appropriate sampling regimes were applied to each strain. The growth curve of all the strains was first studied in three different types of culture vessels; Glass Erlenmeyer flasks, cell culture flasks (high grade polystyrene, greiner bio-one CELLSTAR, cell culture flask) and 24 well cell culture plate (high grade polystyrene, greiner bio-one). The glassware used was cleaned thoroughly, including an overnight acid wash in 10% HCl, and sterilised by autoclaving. Plasticware used was all single-use, optically clear and sterile prior to set up. Cultures were established in all culture vessels using initial cell densities of around 5 x 10^4 - 10^5 cells/ml (O.D ~0.03) with final volumes of 25 mL in 125mL glass flasks, 25 mL in 50mL cell culture flask and 2 ml in 24 well cell culture plates. Growth of cultures was monitored every 24 hours over 72 hours by measuring optical density at 750 nm (described below), and tracking changes in *in vivo* chlorophyll a autofluorecence, measured in arbitrary fluorescence units (reported here as relative fluorescence units RFU) on excitation at 440 nm emission at 580 nm (BMG-PHERAstar, described below). The technique of using *in vivo* measurement of primary photosynthetic pigments as a proxy for changes in biomass and therefore population growth is commonly applied to marine picocyanobacteria including marine *Synechococcus* strains [117-119]. All cultures were set up in triplicate.

2.3 Optical Density measurements

Population growth of all *Synechococcus* cultures was indirectly monitored by measuring the optical density at 750 nm (Beckman DU 640 spectrophotometers, Beckman Instruments, Inc., Fullerton, CA, USA). This was performed using 1ml of culture transferred to Sterna cuvettes and absorbance was measured at 750 nm after performing blank measurement with sterile ASW.

2.4 *In vivo* measurements of fluorescent pigments

Marine *Synechococcus* contain a large diversity of pigments. All strains used in this study contain both chlorophyll a and phycoerythrin, which is their major light harvesting protein. The *in vivo* fluorescence emission spectra for each of the cultures was measured for each *Synechococcus* strain scanning from 300 to 750 nm with excitation at 400 nm in luminescence spectrometer (Perkin Elmer, LS 55). The identified wavelength was used to choose the correct optic mode for the BMG-PHERAStar plate reader.

Assays were performed to optimise measurement of *in vivo* relative fluorescence of both chlorophyll a and phycoerythrin for our test strains using a BMG-PHERAStar plate reader. Measurements were made with the F1 optic mode (440 600 580) which allows excitation with 440 nm and emission at 600 nm (chlorophyll) and 580 nm (phycoerythrin). All measurements were performed using 384 well non-binding, black plates with a sample volume of 25 µl (Interpath: 781906). The optimal gain adjustment setting for each strain was determined using late exponential phase (densely pigmented) cultures (Table 2) and used in all experiments.

Table 2: Gain settings and focal height for each strain

Strain	Gain A	Gain B	Focal height
CC9311	1119	722	6.4
CC9605	1003	637	6.4
BL107	1085	644	6.5
WH8102	1055	694	6.3

2.5 Flow cytometry

Flow cytometry has been extensively used in phytoplankton research to count cells and standard protocols make use of the distinct autofluroescence of *Synechococcus* cells (<2 m)[120]. Population growth was measured directly where possible by flow cytometric analysis of *Synechococcus* cultures to generate cell counts.

This was performed using a Beckman Coulter Cytoflex S equipped with three lasers 488 nm, 405 nm and 561 nm and volumetric counting hardware. Measurements were carried out by both standard filter set up as well as the violet side scatter configuration as per the manufacturer's instructions which improved population resolution for these small cells. The data were analysed using

CytExpert 2.0 software. 50 µl aliquots of cultures were collected every 24 hours and were fixed with 50 µl of 10% paraformaldehyde then made up to a final volume of 500 µl with filtered ASW media. All samples were stored in covered boxes at 4 °C and analysed within a week of collection. Prior to the experiments, the instrument was calibrated by using flow check fluorophores (Beckman Coulter lot no. AJ201; ~3 µm fluorospheres with a fluorescence emission of 410 nm to 800 nm when excited at 405 nm, 488 nm or 635 nm) to ensure consistency of instrument performance. Populations of *Synechococcus* were identified based on both scattering characteristics and fluorescence (pigmentation). Well-clustered *Synechococcus* populations were observed in 585/42(PE) and 690/50 (chlorophyll a) dot-plots and populations were gated based on this plot. Cell counts were obtained by volumetric counting of the gated populations which showed both PE and chlorophyll a florescence, excluding non-fluorescent cells from the analysis. The samples were loaded onto a 96 well clear plate and run in plate mode with 10,000 events recorded for 60 seconds at a flow rate of 40µl /min.

2.6 Solvent sensitivity test

To determine an appropriate solvent to use for compounds with low water solubility, a solvent sensitivity test was performed with the following solvents: methanol, ethanol, acetone, dimethyl sulfoxide (DMSO), and N, N dimethyl-formamide. 0.01% of all solvents were selected for this experiment as the final concentration of solvent in the toxicity test can be made lower than 0.01%. Control (no solvent) cultures and 0.01% final volume solvent addition cultures were set up for each *Synechococcus* culture and growth data collected every 24 hours over a 72-hour period.

2.7 Test Substances

The substances selected for initial testing cover the major classes of pharmaceuticals and personal care products and have been reported in seawater at more than one location around the world. The tested concentration range and solvent used for each compound are given in Table 3. All tested substances and reagents used in the study were purchased from Sigma Aldrich (Australia) and were of molecular grade purity.

Table 3: Selected compounds with desired solvent and their range to be tested.

Substance	Solvent	Range(mg/l)
Silver nanoparticles (nano-powder; <100nm size)	Water	0.001-10
Zinc oxide nanoparticles (dispersion; <100nm size)	Water	0.01-1
Triclosan	DMSO	0.001-1
Oxybenzone	DMSO	0.1-10
Ibuprofen	DMSO	0.1-5

Silver nano powder (Cat. No. 576832, <100 nm particle size, contains PVP as dispersant, 99.5% trace metals basis) and zinc oxide dispersion (cat. No. 721077**,** <100 nm particle size (TEM), ≤40 nm avg. part. size (APS), 20 wt. % in H_2O) were purchased from Sigma Aldrich. Stock solutions of nanoparticle products were prepared by suspending powdered nanoparticles in sterile milli-Q water followed by vortexing and sonication of 20 minutes using an ultrasonics sonicator. A stock solution of 1g/L was prepared in ASW media and then diluted to required concentrations using the ASW media. All the solutions were prepared on the day of the experiment.

Substances with low water solubility required a solvent carrier to dissolve the compounds. Triclosan (Cat.no. PHR 1338; Sigma Aldrich) was dissolved in DMSO to a final concentration of 300 g/L. This stock solution was then diluted with ASW to prepare a working stock of 0.08 g/L. Triclosan was tested across a concentration range of 0.001 to 1 mg/L, with each concentration prepared from the working stock. All the test solutions and solvent control contained the equivalent % of DMSO as low as 0.001%. Oxybenzone (Cat.no.H36206; 2-Hydroxy-4-methoxybenzophenone; Sigma Aldrich) and Ibuprofen (Cat. No. 14883; Sigma Aldrich) were both prepared similarly, with each compound dissolved in DMSO to generate a concentrated stock, then subsequently diluted in ASW to generate a working stock. For these compounds, the tested concentration range was 0.1 to 10 mg/L. All working solutions were prepared on the day of the experiment from the working stock.

2.8 Toxicity assays

Standard guidelines exist for testing the impact of chemicals on the growth of freshwater alga and cyanobacteria [121]. These guidelines were followed where possible with some minor adaptations specific for growth of marine *Synechococcus*. Assays were conducted with the cultures within the early to mid-exponential growth phase corresponding to a starting optical density (750 nm) of 0.03. All experiments were carried out in triplicate. Cultures with no added compound were used as a control for water soluble compounds. A negative (test medium) control without solvent and a solvent control at a concentration equivalent to the test solutions was used as a control for poorly water-soluble compounds. Measurements were taken every 24 hours for 72 hours. The concentrations reported for each substance are nominal concentrations. For each substrate tests were conducted to determine if the substrate contributed to background fluorescence or O.D, where this was the case (zinc oxide dispersion) substrate only dilution series were measured and subtracted from all the test measurements. The pH of the test solution was measured at the start and at the end of each and was unchanged throughout the experiment (pH 8.0).

2.9 Growth rate

The growth of *Synechococcus* cultures were monitored using three independent methods: optical density measurement at 750 nm, relative fluorescence of chlorophyll a pigment and counting the fixed autofluorescent cells in Beckman Coulter Cytoflex S flow cytometer. The average growth rate of the *Synechococcus* cultures was calculated by using the formula described in OECD (201) guidelines for testing of chemicals in fresh water algae and cyanobacteria [121].

$$\mu_{i\text{-}j} = \frac{\mathrm{lnXj} - \mathrm{lnXi}}{\mathrm{tj} - \mathrm{ti}} (\mathrm{day}^{-1})$$

where: $\mu_{i\text{-}j}$ is the average specific growth rate from time i to j; X_i is the biomass at time *I;* X_j is the biomass at time *j*.

The percent inhibition of growth rate for each treatment replicate was calculated using the equation

$$\%\mathrm{I_r} = \frac{\mu_C - \mu_T}{\mu_C} \text{ ^ } 100$$

where: $\%\ \mathrm{I_r}$: percent inhibition in average specific growth rate; μ_C mean value for average specific growth rate (μ) in the control group; μ_T average specific growth rate for the treatment replicate.

2.10 Photosynthetic oxygen evolution measurements

Experiments monitoring oxygen evolution in *Synechococcus* CC9311 were conducted using substances that clearly affected growth. Silver nanoparticles at concentrations 0.5, 1, 5 and 10 mg/L and triclosan at concentrations 0.001,0.01 and 0.1 mg/L were selected for this study. Oxygen

measurement of ZnO NPs were not performed due to limitation in time. Oxygen evolution was measured using respiration vials with integrated optical oxygen sensors (REDFLASH® indicator) equipped with an adapter ring to attach optical fibres with a multi-channel optical oxygen meter (Firesting, Pyro Science, Germany). The measurements were recorded using the Pyroscience software 'Pyro Oxygen Logger'. The respiration vials were calibrated before each use using a 2-point calibration to set stable 100% and 0% oxygen calibration values for all four channels. The 100% air saturation solution was prepared in a 200 ml culture flask filled with 150 ml of ASW media, while a 0% air saturation solution was generated by addition of 1.05 g/L of sodium hydrosulfite to ASW media. Both solutions were placed flat inside a shaker incubator for 30 min at 22 °C with 100 rpm mixing prior to use in calibration. Oxygen measurement experiments were set up using standard growth conditions but with 40 ml of culture in 200 ml acid-washed glass conical flasks with triplicate biological samples. Respiration vials were completely filled with test culture without any air bubbles inside. Upon excitation with a red light on the sensor, a luminescence was emitted at near infra-red range (NIR). Presence of oxygen produces a quenching effect on the emission of NIR allowing oxygen production to be calculated (inversely proportional to NIR emission). All standard growth measurements were taken in parallel with oxygen measurements.

A dipping probe temperature sensor maintained the temperature at 22 °C during the measurement with a circulating thermostat-regulated water bath. The light source during the experiment was provided by white light equivalent to that used for standard incubator growth.

2.11 Statistical analysis

Prior to the statistical analysis, the normal distribution of population was tested by a Shapiro Wilk normality test. The data were statistically analysed by one way analysis of variance (ANOVA) using Graph Pad prism 7. Dunnett's tests and Tukey's post hoc comparisons were made when significant differences were detected. The level of significance was set at $p < 0.05$ for all testing conditions. All errors quoted in this study are ±95% confidence intervals with the numbers of replicate presented in brackets (n).

3. Results:

3.1 Growth curve study

Marine *Synechococcus* strains CC9311, CC9605, WH8102 and BL107 were grown from early to mid-exponential stage over three days in three different culture vessels to determine if growth was consistent across each. Growth was followed using optical density measurements and *in vivo* relative fluorescence of chlorophyll a. Analysis of growth curve data (Figure 2) indicated that there was no significant difference between glass Erlenmeyer flasks, high-grade polystyrene cell culture flasks and high-grade polystyrene 24 well plates (ANOVA test followed by Tukey's multiple comparisons; $p > 0.05$). Subsequent standard growth experiments were conducted in 24 well plates to enable the testing of multiple strains and conditions concurrently.

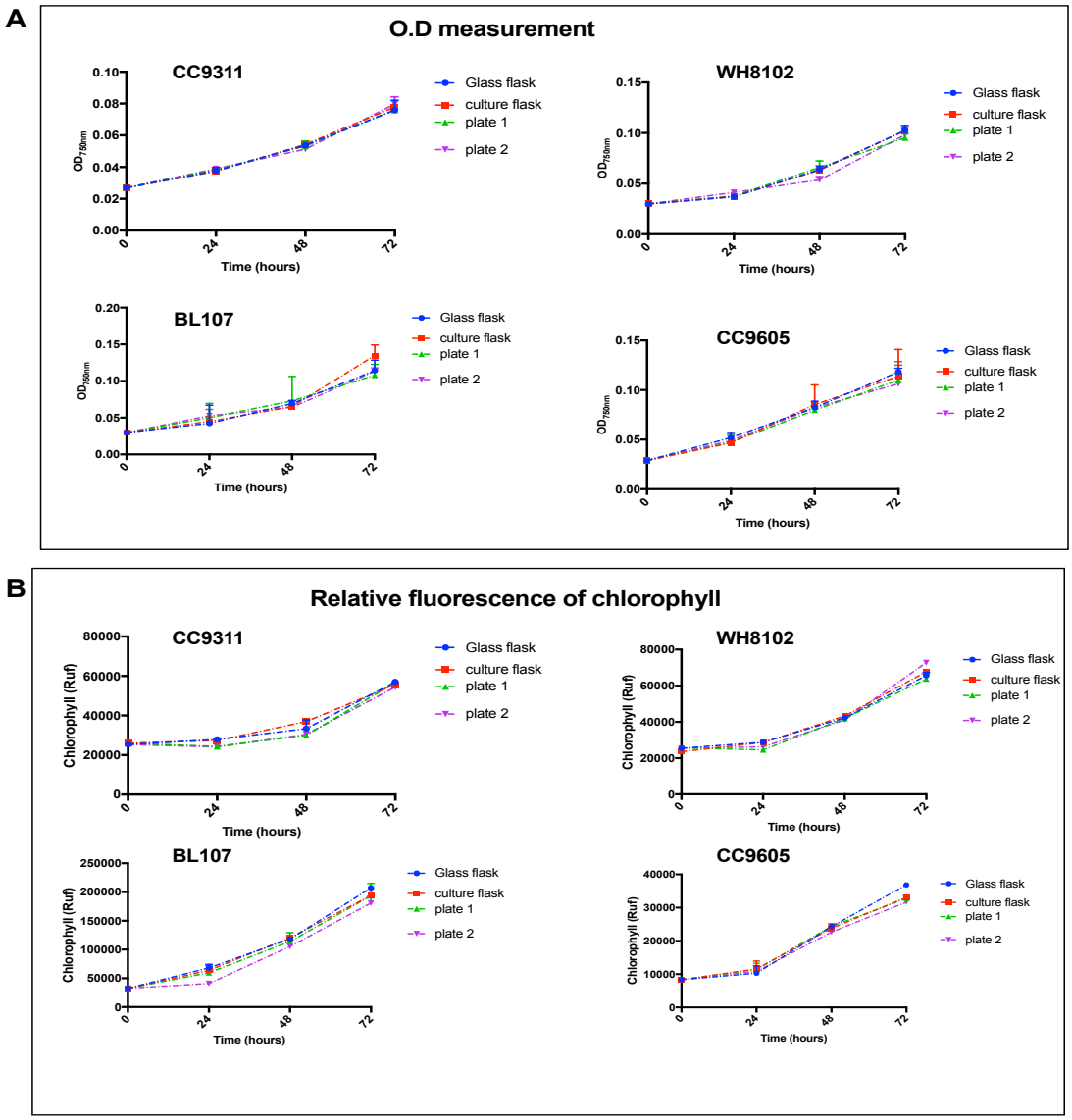

Figure 2: Growth curves of four different strains of *Synechococcus* in three different types of culture vessels. Growth data is derived from (A) optical density (B) *In-vivo* relative fluorescence of chlorophyll a, as described in the Materials and Methods. The error bars indicate S.D (n=3).

3.2 Solvent sensitivity test

Solvent sensitivity tests were conducted on three different *Synechococcus* strains using 0.01% solutions of methanol, ethanol, acetone, DMSO, and dimethyl formamide in ASW media. The growth curve data for each solvent and the control (ASW with no added solvent) over 72 hours were recorded (Figure 3). For all four strains, DMSO and dimethyl formamide were found to have no significant impact on growth (p >0.05; Dunnett's multiple comparisons test) while the other three solvents; methanol, ethanol, and acetone showed a clear significant difference (p < 0.001). Thus, DMSO was selected as a solvent to carry the poorly water-soluble substances in further growth experiments.

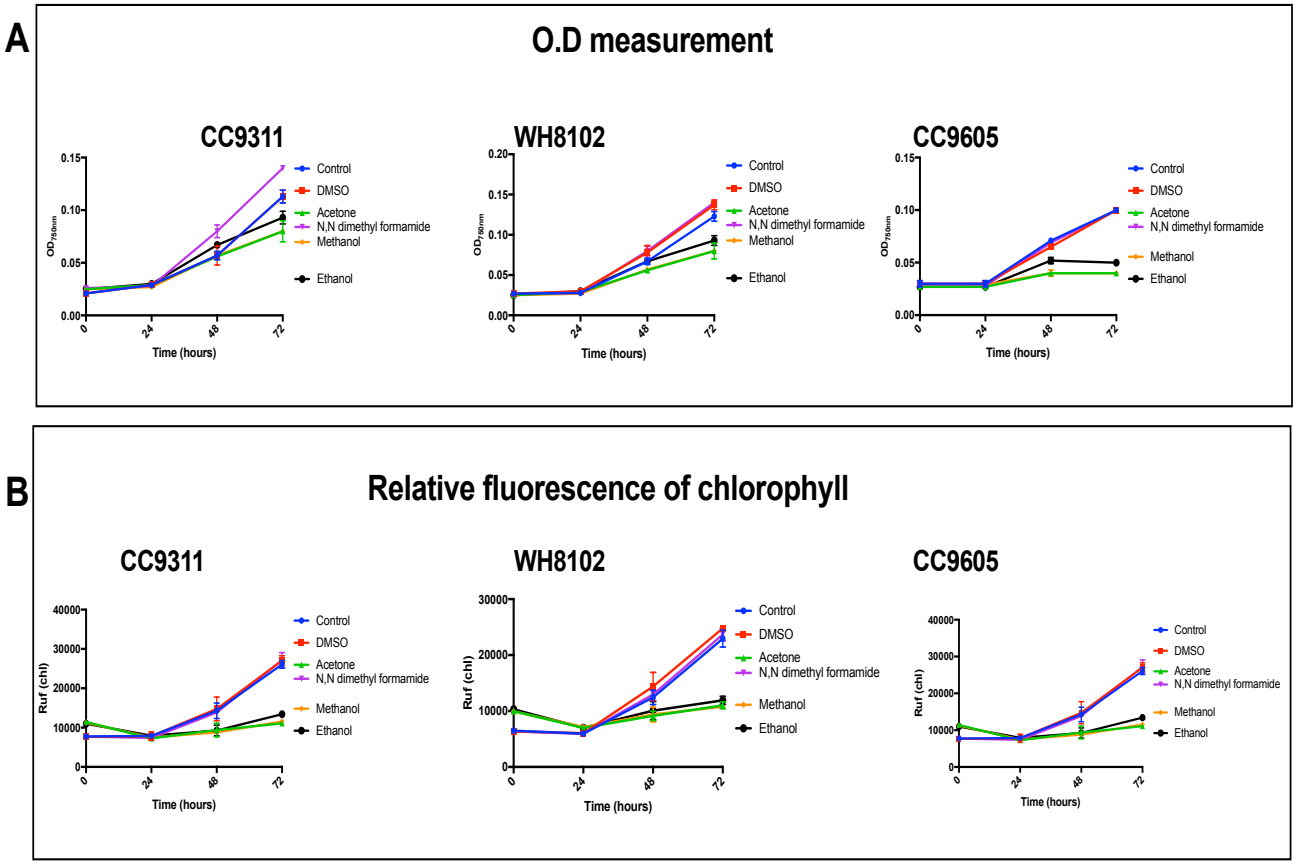

Figure 3: Growth curves of three different strains of *Synechococcus* in the presence of five different types of solvents. Growth data is derived from (A) optical density and (B) *In-vivo* relative fluorescence of chlorophyll a, as described in the Materials and Methods. The error bars indicate S.D (n=3).

3.3 Effect of zinc oxide nanoparticles on marine *Synechococcus*

The four different strains of *Synechococcus* were exposed to different concentrations of zinc oxide nanoparticles (ZnO NPs) for 72 hrs and changes in the population were monitored by O.D measurement, the relative fluorescence of chlorophyll a and cell counting using flow cytometery.

All four strains of *Synechococcus* were found to have reduced growth at 72 hrs following exposure to ZnO NPs at concentrations of 0.01, 0.1, 0.5 and 1 mg/L compared to the control (Figure 4). This general trend was consistent for each of the methods used to track changes in the population over time. The overall reduction in growth on exposure to each test concentration compared to the control was found to be significant for all strains and for all growth measurement methods ($p < 0.001$; ANOVA test followed by Dunnett's test). The impact of exposure to different ZnO NP concentrations on each strain at 72 hrs was compared (Figure 4, Table 4). For strains WH8102 and CC9605 concentrations of 0.01 mg/L and above resulted in equivalently strong reductions in growth (no significant difference in growth impacts between the different levels of toxicant). For CC9311 0.01 mg/L exposure had an intermediate degree of impact, and slowed growth to a lesser degree than higher levels of toxicant (growth changes were significantly different between 0.01 mg/L and higher concentrations, Table 4). For BL107, growth as monitored by optical density measurement was also different between 0.01 mg/L and higher concentrations, but was not significantly different based on the other methods of growth measurement. For all strains ZnO NP concentrations above 0.1 mg/L resulted in similar responses ($p > 0.05$).

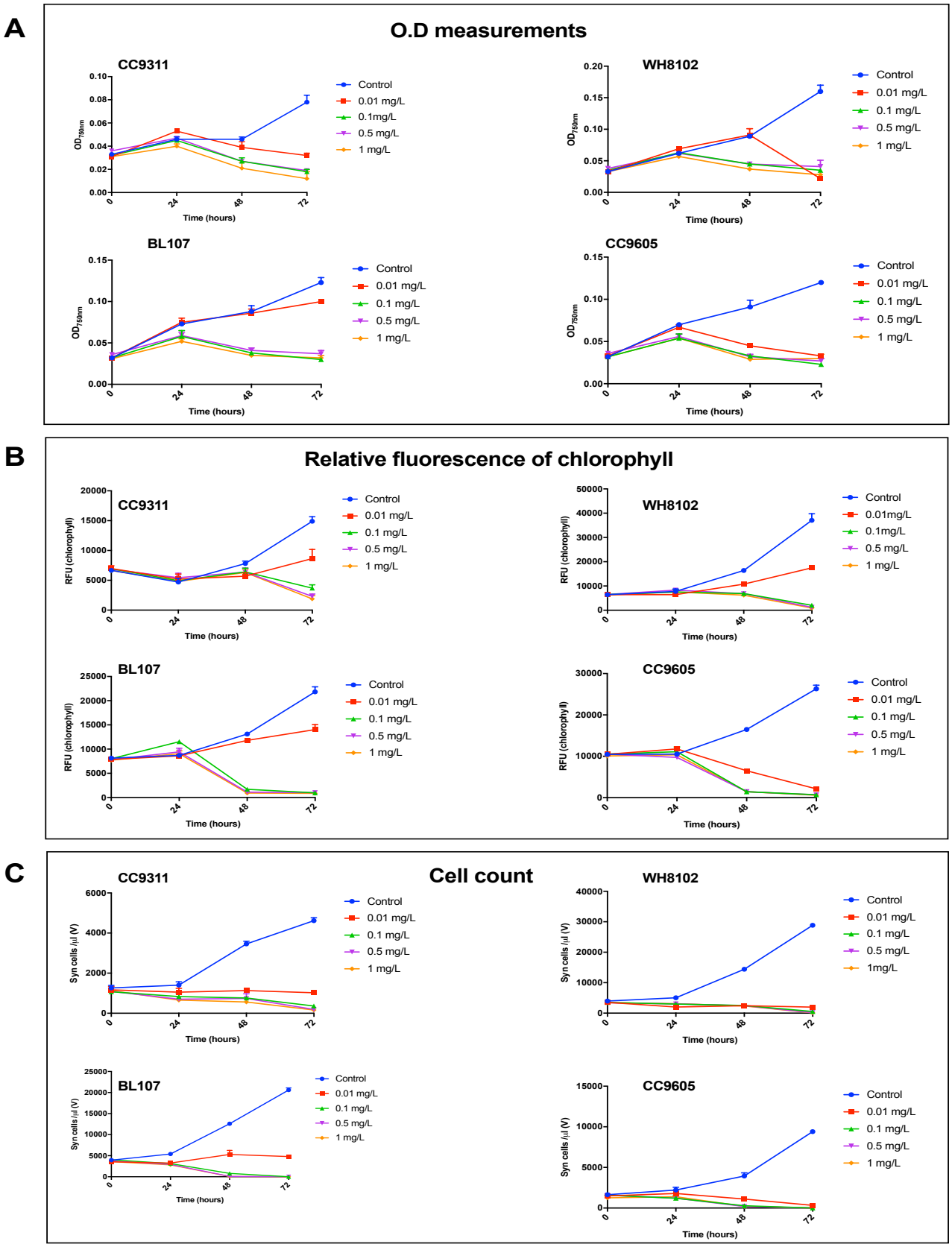

Figure 4: Growth curves of four different strains of *Synechococcus* in the presence of 0.01, 0.1, 0.5 and 1 mg/L of ZnO NPs. Growth data is derived from (A) optical density (B) *In-vivo* relative fluorescence of chlorophyll a and (C) flow cytometric cell count measurements, as described in the Materials and Methods. The error bars indicate S.D (n=3).

All *Synechococcus* strain control cultures were calculated to have to a mean growth rate of ~0.02 day^{-1} over 72 hrs. For cultures exposed to ZnO NPs, a negative growth rate was observed from nominal concentrations of 0.1 mg/L and above in all the three growth monitoring methods (Figure 5).

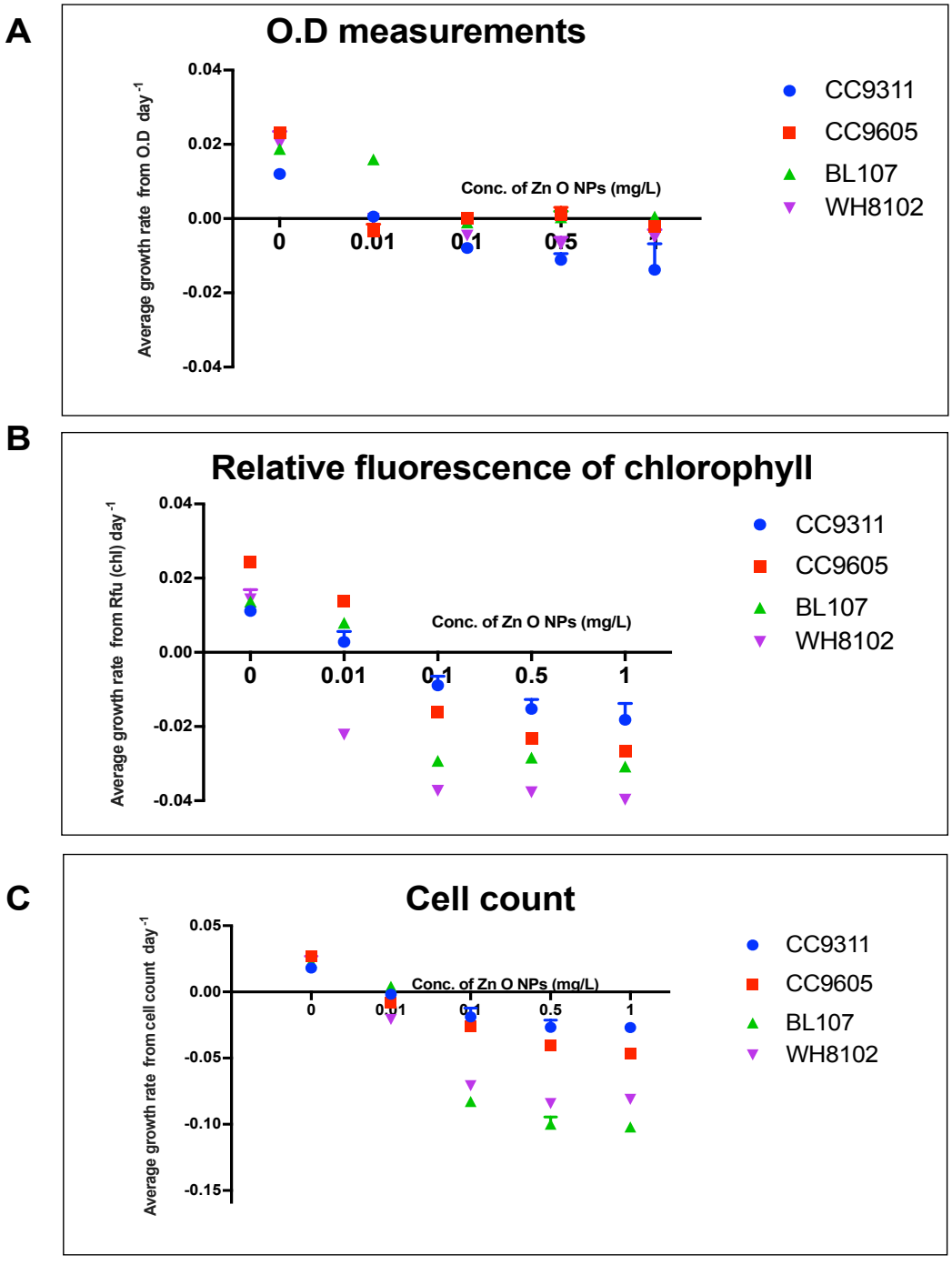

Figure 5: Average growth rate of four strains of marine *Synechococcus* in the presence of different concentrations of ZnO NPs as measured by (A) O.D measurement at 750nm (B) *In vivo* relative fluorescence of chlorophyll a and (C) flow cytometric cell count as described in the Materials and Methods. The error bars indicate S.D (n=3).

Table 4: Comparison of growth impacts from different concentrations of ZnO NPs at 72 hrs.

	0.01 mg/L				0.1 mg/L				0.5 mg/L				1 mg/L			
	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4
C	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
0.01 mg/L					*	ns	**	ns	*	ns	**	ns	**	ns	**	ns
					**	ns	ns	ns	**	ns	ns	ns	**	ns	ns	ns
					**	ns	ns	ns	**	ns	ns	ns	**	ns	ns	ns
0.1 mg/L									ns	ns	ns	ns	ns	ns	ns	ns
									ns	ns	ns	ns	*	ns	ns	ns
									ns	ns	ns	ns	ns	ns	ns	ns
0.5 mg/L													ns	ns	ns	ns
													ns	ns	ns	ns
													ns	ns	ns	ns

Results generated by ANOVA followed by Tukey's multiple comparison tests for four strains of *Synechococcus* represented by S_1(CC9311), S_2 (WH8102), S_3(BL107), S_4(CC9605). Asterisks indicate significance at different P value cut-offs with p <0.05 (*), p <0.001(**), ns indicates results were not significant ($p \geq 0.05$). Green shading indicates analysis of OD measurement data, salmon shading indicates chlorophyll a relative fluorescence data, yellow shading indicates flow cytometry cell counts.

To determine if ZnO NPs exposure impacts the ratio of key photosynthetic pigments the *in vivo* PE to chlorophyll a relative fluorescence ratio was determined for all strains over the course of the experiment (Figure 6). In all four strains, the ratio of PE/chlorophyll a was significantly different from the control at 72 hrs time (ANOVA test followed by Dunnett's test; $p < 0.001$). This ratio was found to increase on exposure to all ZnO NPs concentrations above 0.01 mg/L after 24-48 hrs.

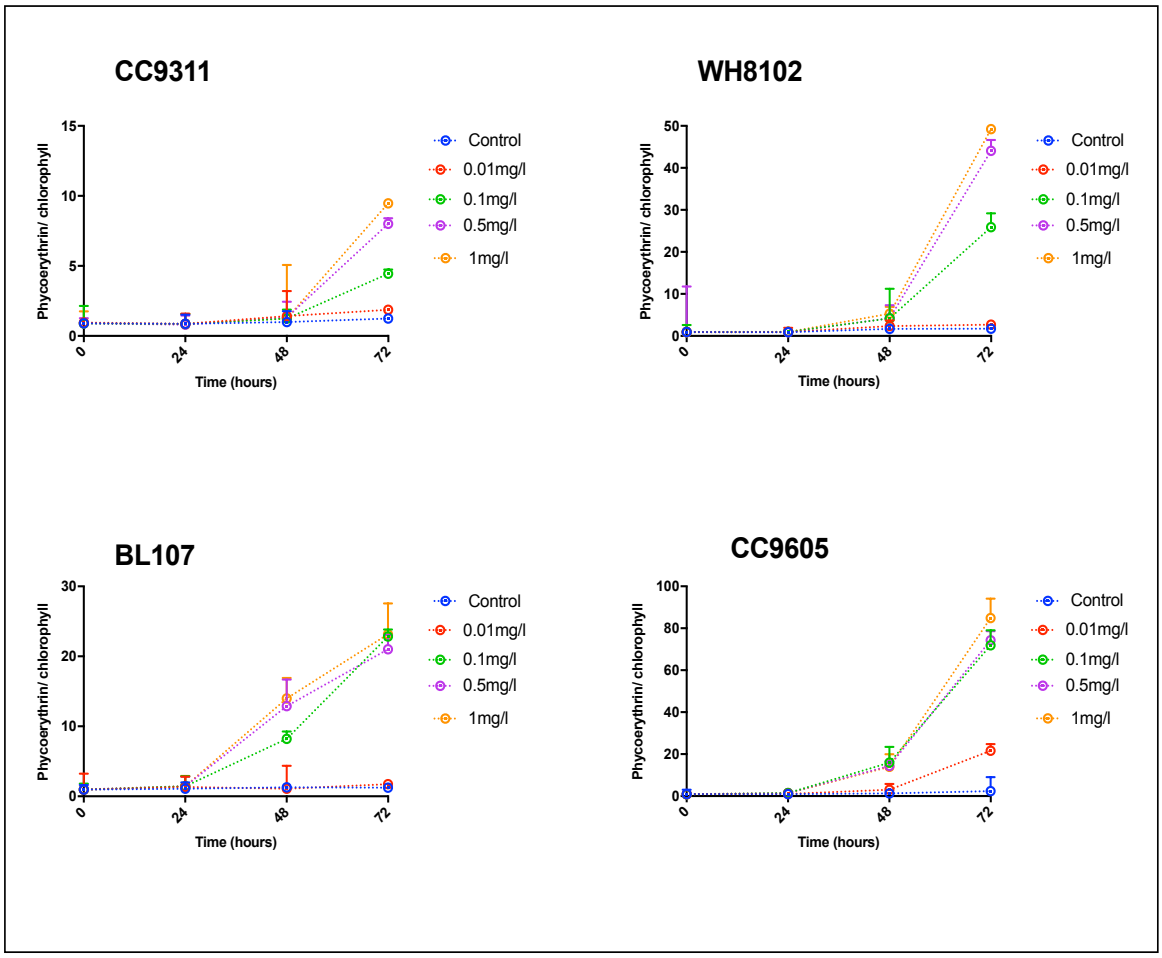

Figure 6: *In vivo* Phycoerythrin/chlorophyll a ratio of four different *Synechococcus* strains after exposure to different concentrations of ZnO NPs, measured as relative fluorescence at excitation with 440 nm and emission at 580 nm (phycoerythrin) and 600 nm (chlorophyll a). Measurements were made every 24 hours over a 72-hour period using the PHERAstar instrument. The error bars indicate S.D (n=3).

3.4 Effect of silver nanoparticles on marine *Synechococcus*

The four different strains of *Synechococcus* were exposed to different concentrations of silver nanoparticles (Ag NPs**)** (0.001, 0.01, 0.1, 1 and 10 mg/L respectively) for 72 hrs and changes in the population were monitored by O.D and relative fluorescence of chlorophyll a measurement.

Analysis of growth data at 72 hrs from both the methods showed that concentrations of Ag NPs up to 1 mg/L did not appear to affect the growth of tested *Synechococcus* strains. At 10 mg/L, growth of all strains was clearly inhibited (Figure 7). Statistical analysis showed a significant difference ($p < 0.001$) between the control and 10 mg/L and also between the lower concentrations and 10 mg/L after 72 hrs exposure (Table 5).

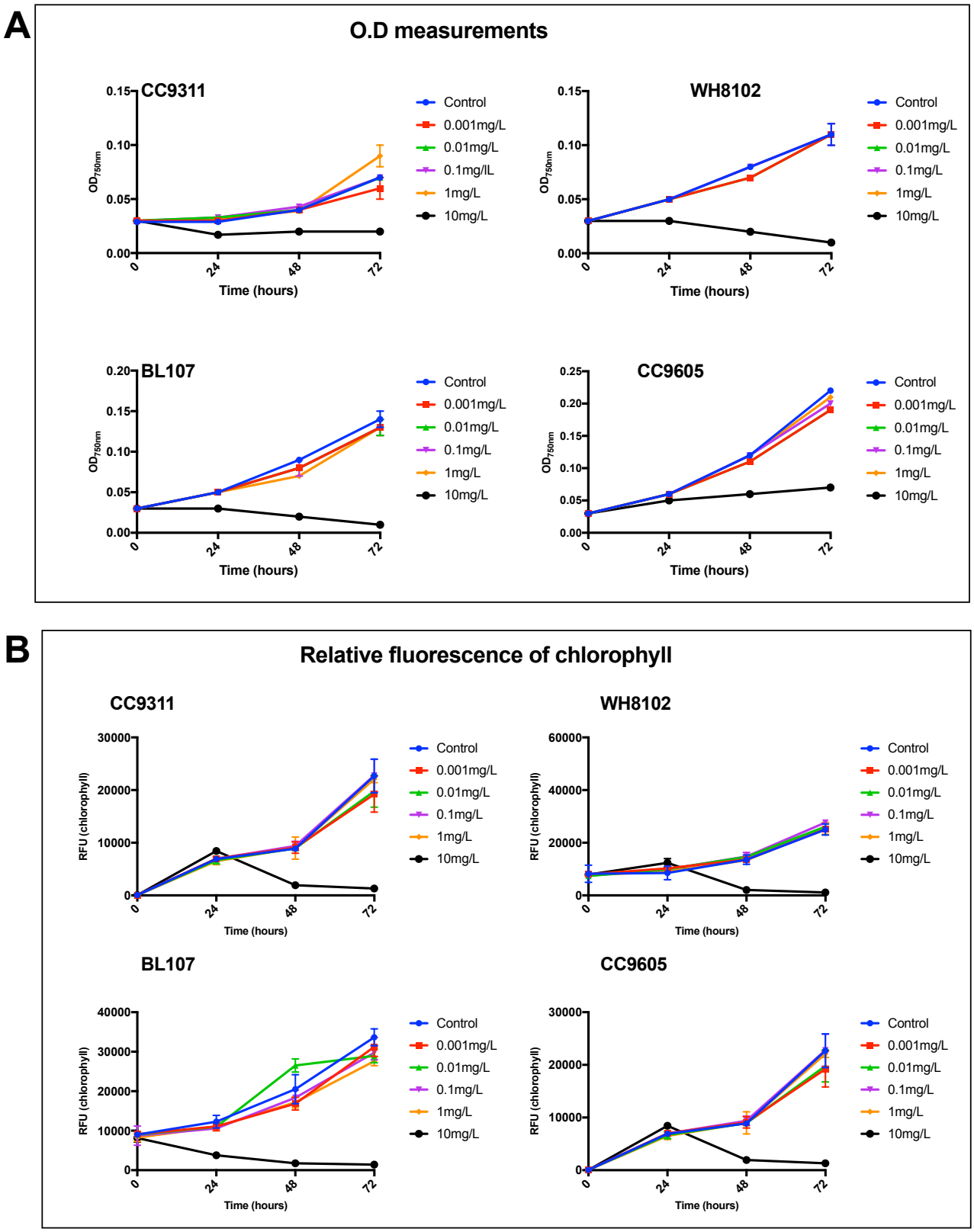

Figure 7: Growth curves of four different strains of *Synechococcus* in the presence of 0.001, 0.01, 0.1,1 and 10 mg/L of silver nanoparticles Growth data is derived from (A) optical density and (B) *In vivo* relative fluorescence of chlorophyll a, as described in the Materials and Methods. The error bars indicate S.D (n=3).

All *Synechococcus* strain control cultures were calculated to have to a mean growth rate of ~0.02 day^{-1} over 72 hrs. For cultures exposed to Ag NPs at concentrations of 1 mg/L and below the growth rate was not significantly different from the control. However, exposure to a nominal concentration of 10 mg/L Ag NPs resulted in a negative growth rate in all four strains (Figure 8).

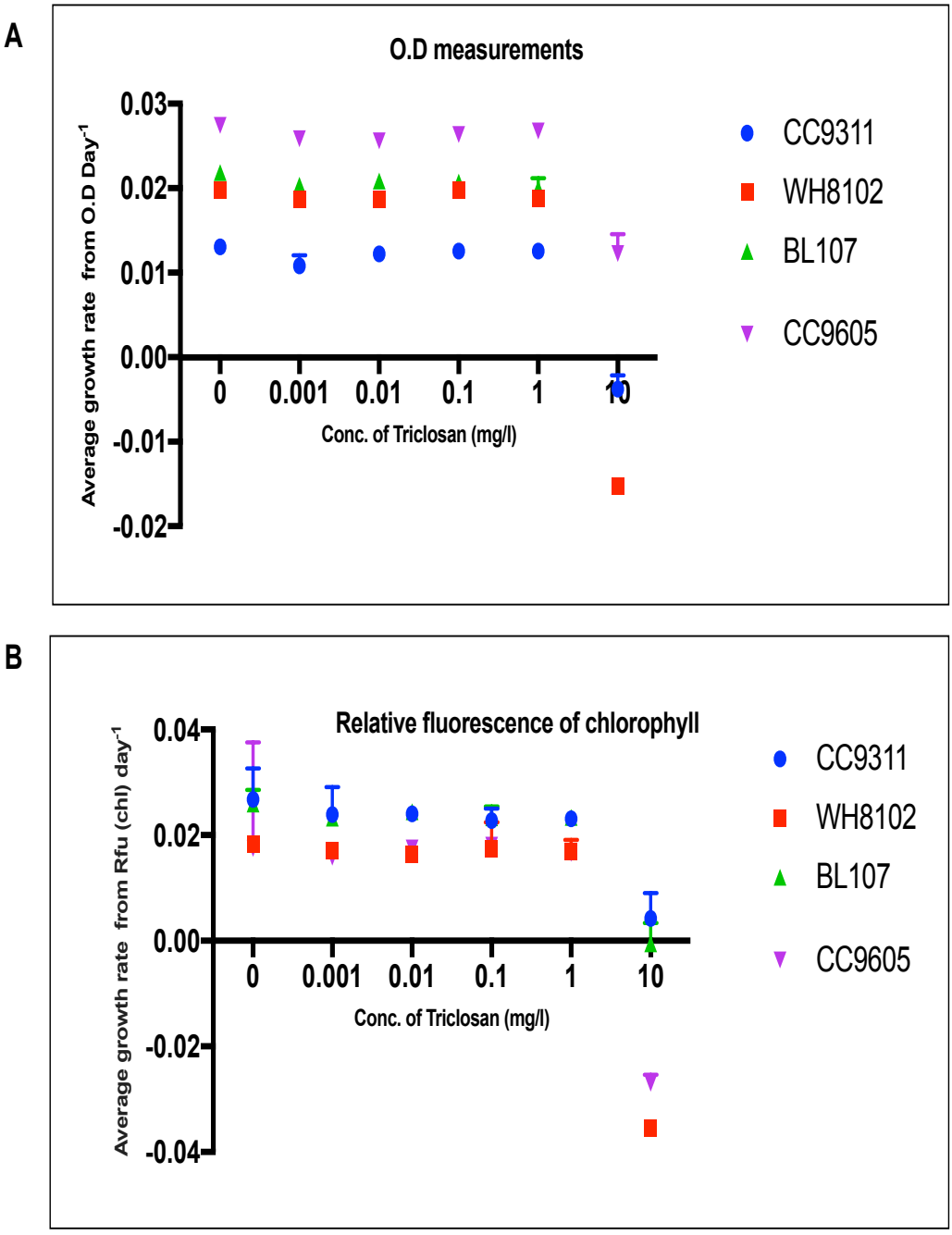

Figure 8: Average growth rate of four strains of marine *Synechococcus* in the presence of different concentrations of silver nanoparticles as measured by (A) O.D measurement at 750nm and (B) *In vivo* relative fluorescence of chlorophyll a as described in the Materials and Methods. The error bars indicate S.D (n=3).

Table 5: Comparison of growth impacts from different concentrations of Ag NPs after 72 hr exposure.

	0.001 mg/L				0.01 mg/L				0.1 mg/L				1mg/L				10mg/L			
	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4
C	ns	ns	ns	ns	n	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	**
	ns	ns	ns	ns	n	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	**	**	**	**
0.001 mg/L					n	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	**
					n	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	**
0.01 mg/L									ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	**
									ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	**
0.01 mg/L													ns	ns	ns	ns	**	**	**	**
													ns	ns	ns	ns	**	**	**	**
1 mg/L																	**	**	**	**
																	**	**	**	**

Results generated by ANOVA followed by Tukey's multiple comparison tests for four strains of *Synechococcus* represented by S_1(CC9311), S_2 (WH8102), S_3(BL107), S_4(CC9605). Asterisks indicate significance at different P value cut-offs with p <0.05 (*), p <0.001(**), ns indicates results were not significant ($p \geq 0.05$). Green shading indicates analysis of OD measurement data, salmon shading indicates chlorophyll a relative fluorescence data.

The ratio of *in vivo* PE to chlorophyll a relative fluorescence was determined for all strains over the course of the experiment. For concentrations of 1 mg/L and lower concentrations were found to be close to 1, while for 10 mg/L the ratio was much higher for all four strains (At 72 hrs ratios were as follows: 1.7 for CC9311, 1.8 for WH8102, 1.1 for BL107 and 1.3 for CC9605).

3.5 Effect of triclosan on marine *Synechococcus*

The four different strains of *Synechococcus* were exposed to different concentrations of triclosan (0.001, 0.01, 0.1 and 1 mg/L respectively) for 72 hrs and changes in the growth of each population were monitored as for ZnO NPs. A solvent control (0.001% DMSO in ASW media) was run in addition to the ASW only control and was found to be not statistically different ($p > 0.05$) from the control in all test conditions, as shown in Table 6.

Table 6: Comparison of growth impacts from different concentrations of triclosan at 72 hrs.

	C+S				0.001 mg/L				0.01 mg/L				0.1 mg/L				1mg/L			
	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4
C	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	**	**	**	ns	**	**	**	**
	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	*	**	**	ns	*	**	**	**
	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*	**	**	**	**	**	**
C+S					ns	ns	ns	ns	ns	*	ns	ns	**	**	**	ns	**	**	**	**
					ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	ns	**	**	**	**
					ns	ns	ns	ns	ns	ns	ns	ns	**	**	**	*	**	**	**	**
0.001 mg/L									ns	*	ns	ns	**	**	**	ns	**	**	**	**
									ns	ns	ns	ns	**	**	**	ns	*	**	**	**
									ns	ns	ns	ns	**	**	**	**	**	**	**	**
0.01 mg/L													**	**	**	ns	**	**	**	**
													**	**	**	ns	*	**	**	**
													**	*	**	*	**	*	**	**
0.1 mg/L																	ns	ns	ns	**
																	ns	ns	*	**
																	ns	ns	ns	ns

Results generated by ANOVA followed by Tukey's multiple comparison tests for four strains of *Synechococcus* represented by S_1(CC9311), S_2 (WH8102), S_3(BL107), S_4(CC9605). Asterisks indicate significance at different P value cut-offs with p <0.05 (*), p <0.001(**), ns indicates results were not significant ($p \geq 0.05$). Green shading indicates analysis of OD measurement data, salmon shading indicates chlorophyll a relative fluorescence data, yellow shading indicates flow cytometry cell counts.

From O.D measurement data, 0.1 and 1 mg/L of triclosan showed an impact on the growth of CC9311, WH8102 and BL107 strains (Figure 9) and at 72 hrs a significant difference was observed between the control and these two concentrations ($p < 0.001$). However, no significant difference was observed ($p > 0.05$) between the means of 0.1 and 1 mg/L indicating these concentrations elicited a comparable response in these three strains of *Synechococcus*. This general trend was consistent for each of the methods used to track changes in the population over time (Table 6).

There was some indication that growth of WH8102 was also negatively impacted by exposure to 0.01 mg/L of triclosan, as there was a significant difference ($p < 0.05$) in mean optical density between this concentration and both controls at 72 hrs, however no impacts were observed for this concentration based on the other growth measurements (Table 6).

In CC9605, a significant growth impact was observed only at 1 mg/L concentration and the response to 0.1mg/L triclosan exposure differed to that of the other tested strains (Figure 9). Statistical comparisons of strain responses showed that growth of CC9605 after 72 hr exposure to

0.1 mg/L triclosan was not significantly different to that of control from O.D and chlorophyll a fluorescence measurements, while for other strains, this was significantly different ($p < 0.001$) from all three methods. It suggests that CC9605 is the least sensitive to triclosan of the tested strains.

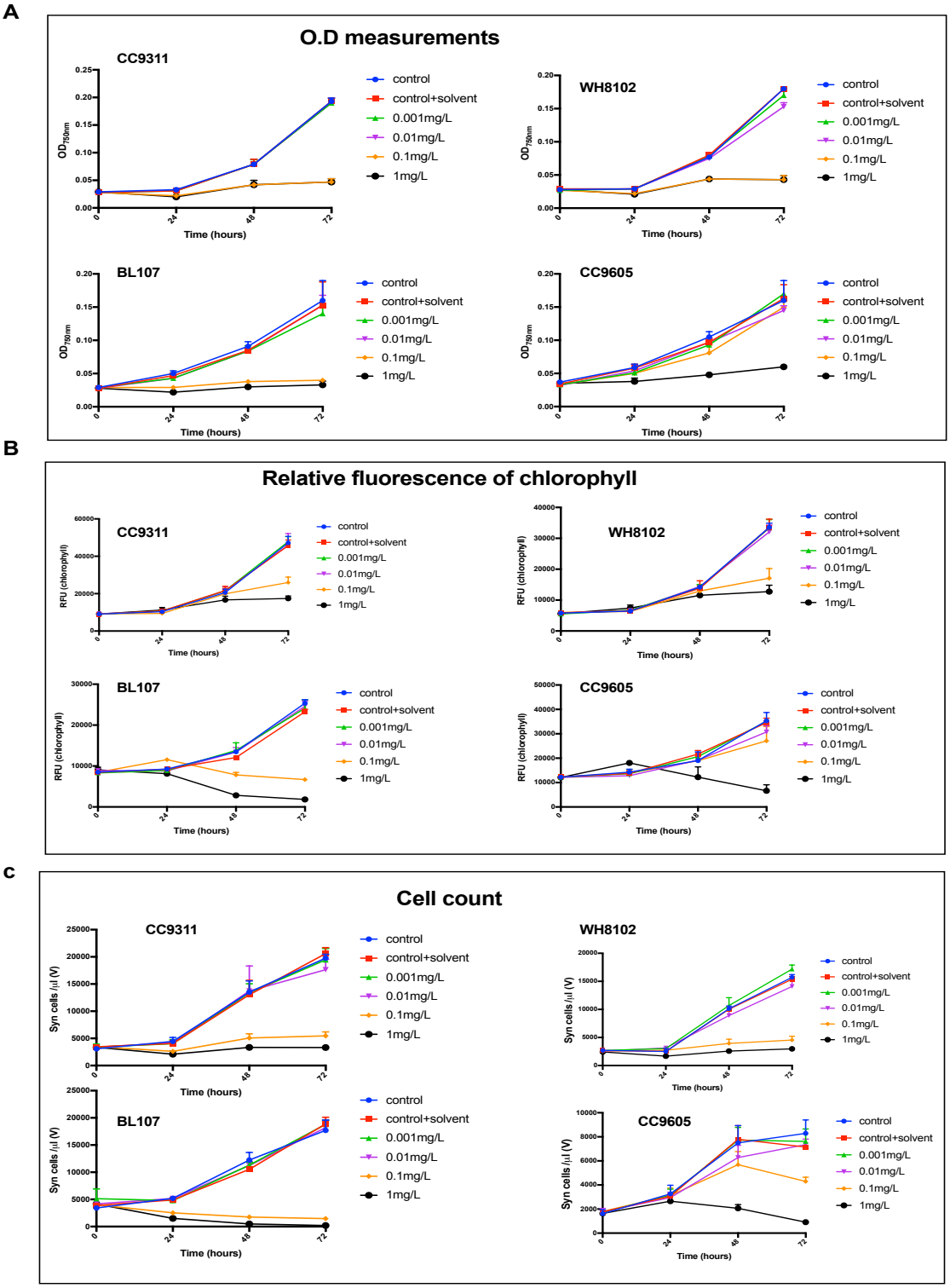

Figure 9: Growth curves of four different strains of *Synechococcus* in the presence of 0.001, 0.01, 0.1 and 1 mg/L of triclosan. Growth data is derived from (A) optical density (B) *In vivo* relative

fluorescence of chlorophyll a and (C) flow cytometric cell count measurements, as described in the Materials and Methods. The error bars indicate S.D (n=3).

All *Synechococcus* strain control cultures were calculated to have to a mean growth rate of ~0.02 day^{-1} over 72 hrs. For cultures exposed to triclosan at concentrations of 0.01 mg/L and below the growth rate was not notably different from the control. In strains CC9311, WH8102 and BL107 exposure to nominal concentrations of 0.1 mg/L and above resulted in a strong reduction in growth rate (Figure 10). While for CC9605 strains the reduction in growth rate was notable at 1 mg/L.

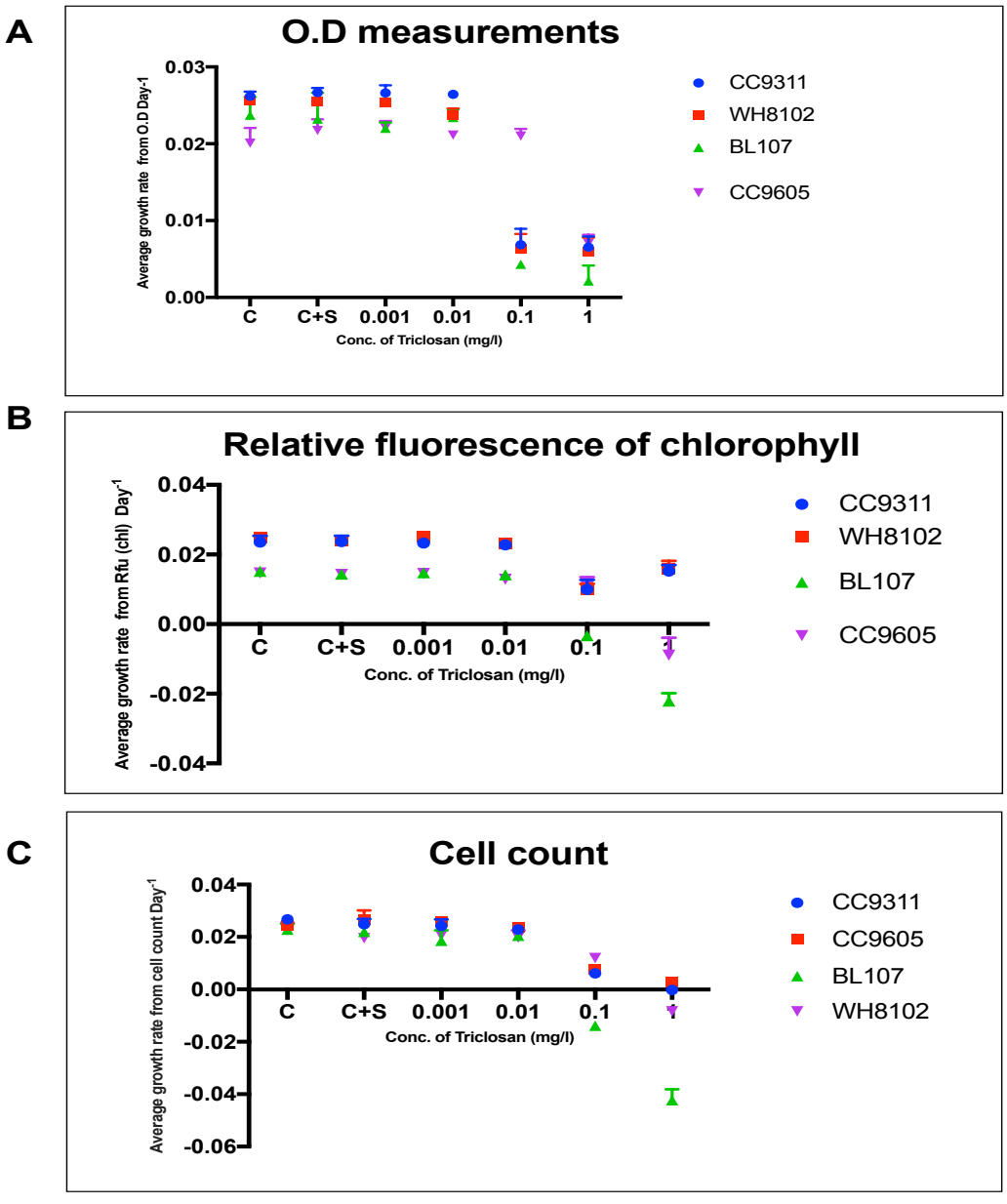

Figure 10: Average growth rate of four strains of marine *Synechococcus* in the presence of different concentrations of triclosan as measured by (A) O.D measurement at 750nm (B) *In vivo* relative fluorescence of chlorophyll a and (C) flow cytometric cell count as described in the Materials and Methods. The error bars indicate S.D (n=3).

The ratio of the *in vivo* relative fluorescence of PE to chlorophyll a for all strains over the course of the experiment showed no significant difference from the control culture without triclosan (data not shown).

3.6 Effect of oxybenzone on marine *Synechococcus*

The four different strains of *Synechococcus* were exposed to different concentrations of oxybenzone (BP-3) (0.1, 1, 5 and 10 mg/L respectively) for 72 hrs and changes in the population were monitored as for other test substances. A solvent control was also run, as described for triclosan, and was not statistically different ($p > 0.05$) from the control for any of the strains as assessed by any of the population growth measurements. All the tested concentration showed equivalent growth in all the three growth monitoring methods (Figure 11).

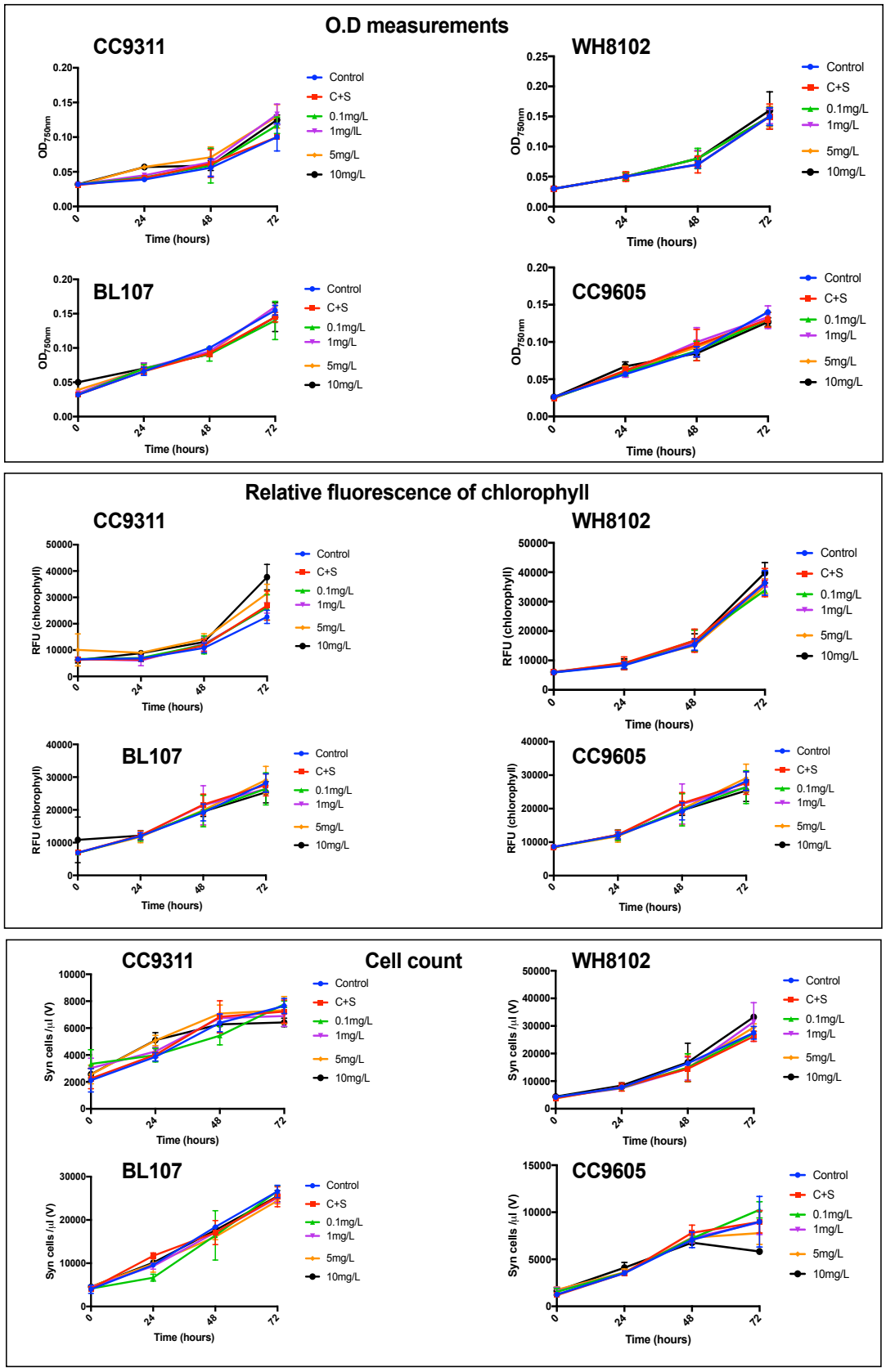

Figure 11: Growth curves of four different strains of *Synechococcus* in the presence of 0.1,1, 5 and 10 mg/L of BP- 3. Growth data is derived from (A) optical density (B) *In-vivo* relative fluorescence of chlorophyll a and (C) flow cytometric cell count measurements, as described in the Materials and Methods. The error bars indicate S.D (n=3).

Statistical analysis of culture growth at 72 hrs, as assessed by each of the three population growth measurements, showed there was no significant impact ($p > 0.05$) on growth of any strain for any of the tested BP-3 concentrations (Table 7).

Table 7: Comparison of growth impacts from different concentrations of BP-3 at 72 hrs.

	C+S				0.001 mg/L				0.01 mg/L				0.1 mg/L				1mg/L			
	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4
C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
C+S					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
0.001 mg/L									ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
									ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
									ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
0.01 mg/L													ns	ns	ns	ns	ns	ns	ns	ns
													ns	ns	ns	ns	ns	ns	ns	ns
													ns	ns	ns	ns	ns	ns	ns	ns
0.1 mg/L																	ns	ns	ns	ns
																	ns	ns	ns	ns
																	ns	ns	ns	ns

Results generated by ANOVA followed by Tukey's multiple comparison tests for four strains of *Synechococcus* represented by S_1(CC9311), S_2 (WH8102), S_3(BL107), S_4(CC9605). Asterisks indicate significance at different P value cut-offs with $p < 0.05$ (*), $p < 0.001$(**), ns indicates results were not significant ($p \geq 0.05$). Green shading indicates analysis of OD measurement data, salmon shading indicates chlorophyll a relative fluorescence data, yellow shading indicates flow cytometry cell counts.

3.7 Effect of ibuprofen on marine *Synechococcus*

The four different strains of *Synechococcus* were exposed to different concentrations of ibuprofen (0.01,0.1, 1 and 5 mg/L respectively) for 72 hrs and changes in the population were monitored by O.D measurement and relative fluorescence of chlorophyll a measurement.

Analysis of growth data from both the methods indicated that 0.01, 0.1, 1 and 10 mg/L of ibuprofen did not induce any noticeable impacts on any of the tested strains of *Synechococcus* (Figure 12). As in the BP-3 experiment, the solvent control was not statistically different ($p > 0.05$) from the

control for any of the strains and statistical analysis of culture growth at 72 hrs, as assessed by each of the three population growth measurements, showed no significant impact ($p > 0.05$) on growth of any strain for any of the ibuprofen concentrations.

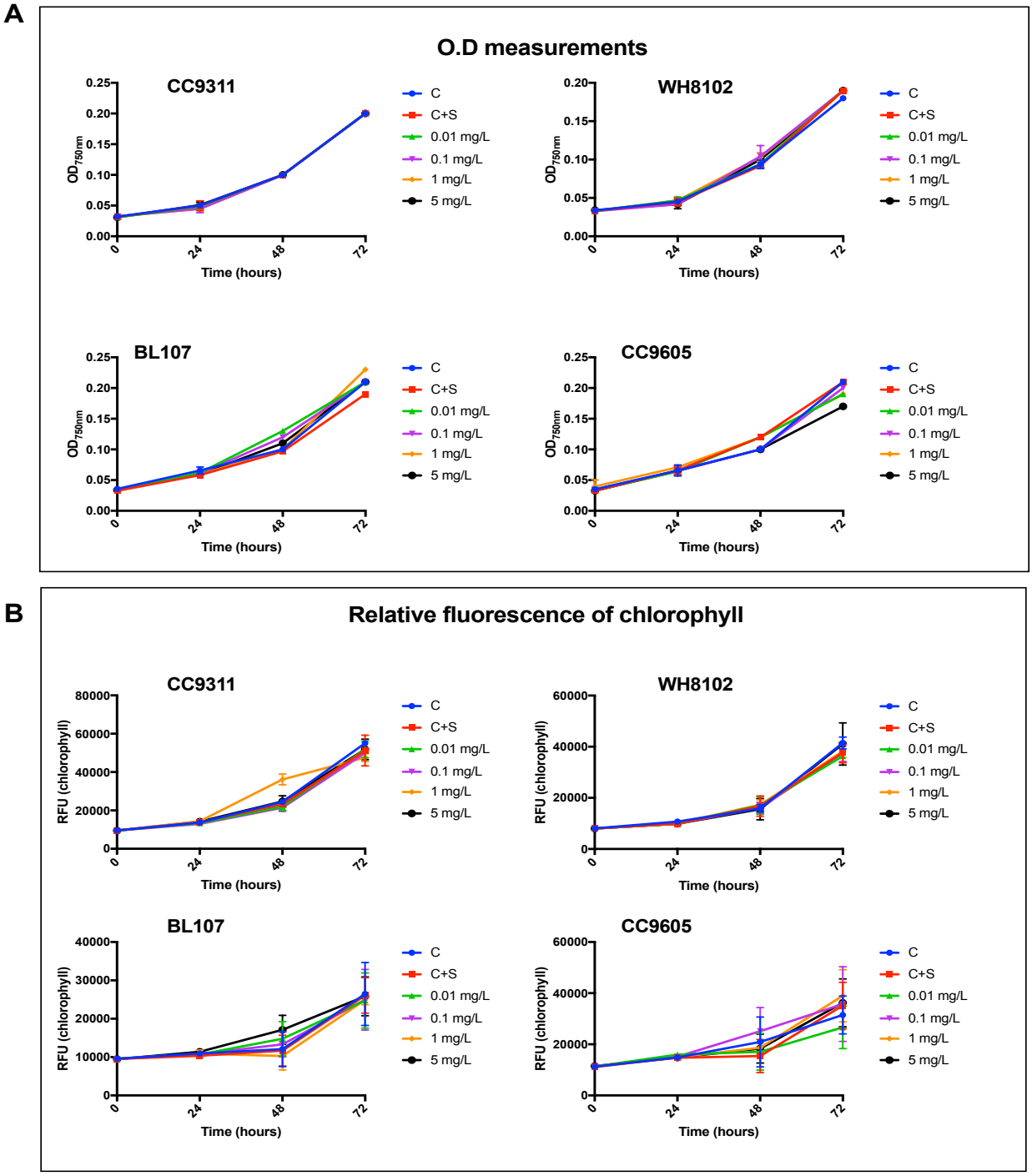

Figure 12: Growth curves of four different strains of *Synechococcus* in the presence of 0.01,0.1, 1 and 5 mg/L of ibuprofen. Growth data is derived from (A) optical density and (B) *In-vivo* relative fluorescence of chlorophyll a, as described in the Materials and Methods. The error bars indicate S.D (n=3).

3.8 Oxygen production

3.8.1 Effect of Ag NPs on oxygen evolution

Initial growth tracking experiments determined that population growth of all four strains of *Synechococcus* was negatively impacted by exposure to 10 mg/L Ag NPs, while concentrations of 1 mg/L and below showed no significant impact on growth over 72 hrs (Table 5). Oxygen production was measured over 72 hrs for *Synechococcus* CC9311 cultures exposed to 0.5, 1, 5 and 10 mg/L of silver nanoparticles to determine whether exposure resulted in physiological impacts beyond changes in population growth. Oxygen evolution measurements were taken in parallel with population growth measurements at 24, 48 and 72 hrs after experimental set up. To normalise oxygen evolution to culture biomass, oxygen evolution per cells (µl) was calculated.

The oxygen evolution of the lowest concentration (0.5 mg/L) of Ag NPs was slightly higher than the control while exposure to 1 mg/L Ag NPs resulted in equivalent oxygen evolution with control at all time points. There was no detectable oxygen evolution in cultures exposed to 5 and 10 mg/L Ag NPs in all the evaluated time points (Figure 13, A). At 72 hrs, a significant difference was observed between the means of control and all the tested concentrations as shown in Figure 13 (B).

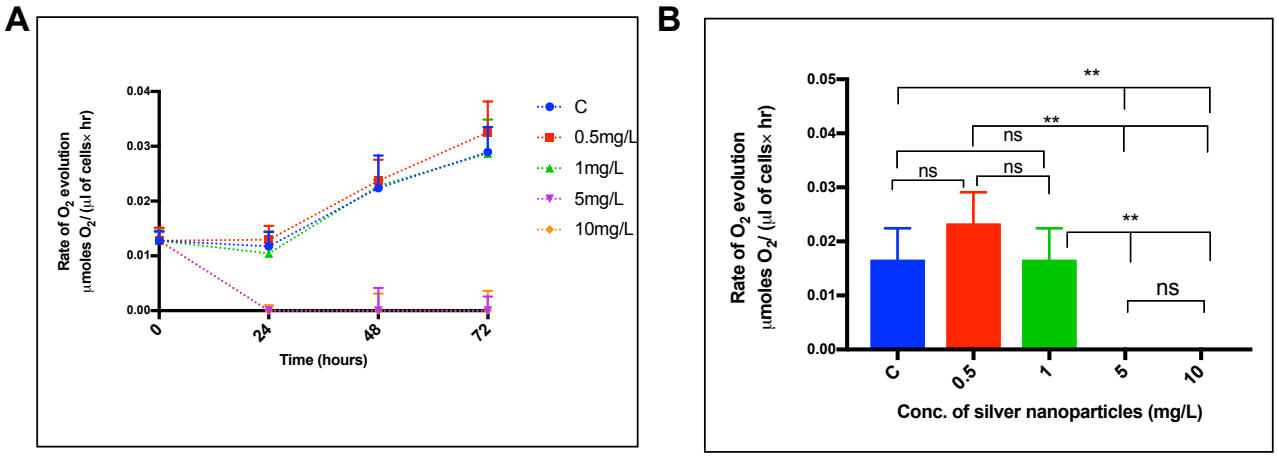

Figure13: Rate of oxygen evolution (normalised to cells per µl) for *Synechococcus* CC9311 after exposure to different concentrations of silver nanoparticles. (A) measured at 24, 48 and 72 hrs (B) bar graph showing the difference in oxygen evolution at 72hrs, C represents control. ANOVA followed by Tukey's test. Asterisks indicate significance at different P value cut-offs with $p < 0.05$ (*), $p < 0.001$(**), ns indicates results were not significant ($p \geq 0.05$).

The relative fluorescence of phycoerythrin and chlorophyll a measurement at 72 hrs showed a significant reduction in chlorophyll a at 5 and 10 mg/L of silver nanoparticles while 0.5 mg/L was found to be higher than control and 1 mg/L showed equivalent chlorophyll a fluorescence to control as shown in figure 14.

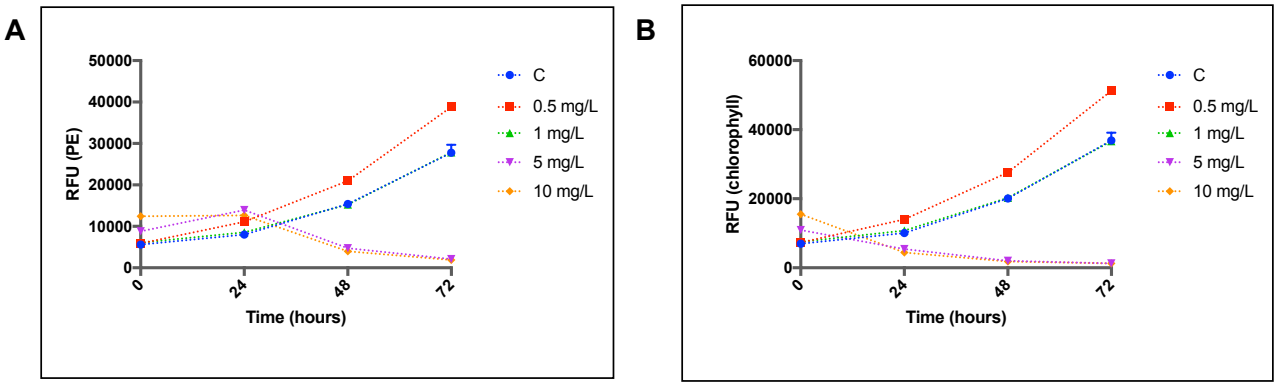

Figure 14: The *in vivo* relative fluorescence of (A) phycoerythrin and (B) chlorophyll a measured for *Synechococcus* CC9311 cultures exposed to 0.5, 1, 5 and 10mg/L concentration of silver nanoparticles for 0-72 hrs. The error bars indicate S.D (n=3).

The population of autofluorescent CC9311 cells as measured by flow cytometry was analysed for changes in mean photosynthetic pigments. This showed a similar pattern of reduction in the relative mean fluorescence of chlorophyll a and phycoerythrin levels within the autofluorescent population for populations exposed to high concentrations (5 and 10 mg/L) of Ag NPs (Figure 15).

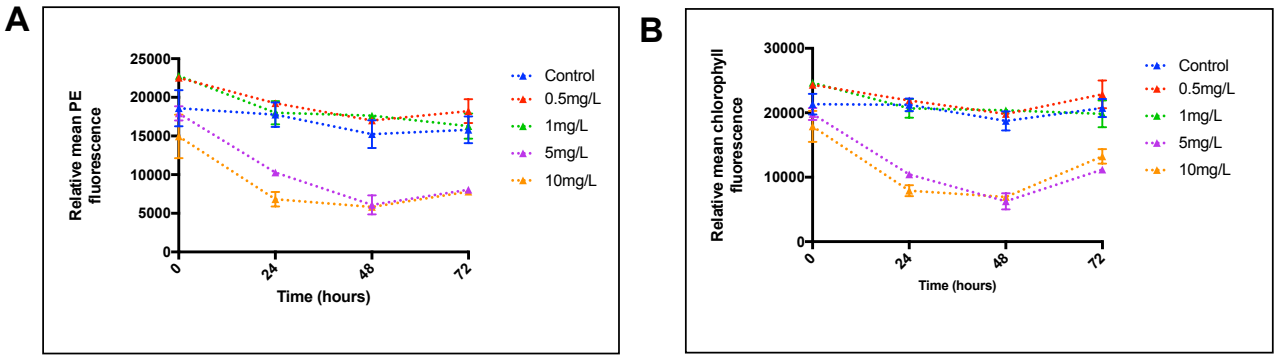

Figure 15: The relative mean fluorescence of (A) phycoerythrin and (B) chlorophyll a data obtained from flow cytometery data (Cytoflex S) for *Synechococcus* CC9311 cultures exposed to 0.5, 1, 5 and 10mg/L concentrations of silver nanoparticles for 0-72 hrs. The error bars indicate S.D (n=3).

3.8.2 Effect of triclosan on oxygen evolution

Initial growth tracking experiments determined that population growth of all four strains of *Synechococcus* were negatively impacted by exposure to 0.1 mg/L triclosan, while concentrations of 0.01 mg/L and below showed no significant impact on growth over 72 hrs (Table 6).

The oxygen evolution on *Synechococcus* CC9311 strain exposed to 0.001, 0.05 and 0.1 mg/L of triclosan was measured in parallel with population growth measurements at 24, 48 and 72 hrs after experimental set up. To normalise oxygen evolution to culture biomass, oxygen evolution per cells (µl) was calculated.

The results of the present study demonstrated that at 72 hrs, a significant (p<0.001) decrease in oxygen evolution was observed at 0.1 mg/L of triclosan when compared to the control as shown in figure 16. A slight decrease in oxygen evolution was observed in 0.001 and 0.05 but they were not significantly different (p > 0.05) to the control.

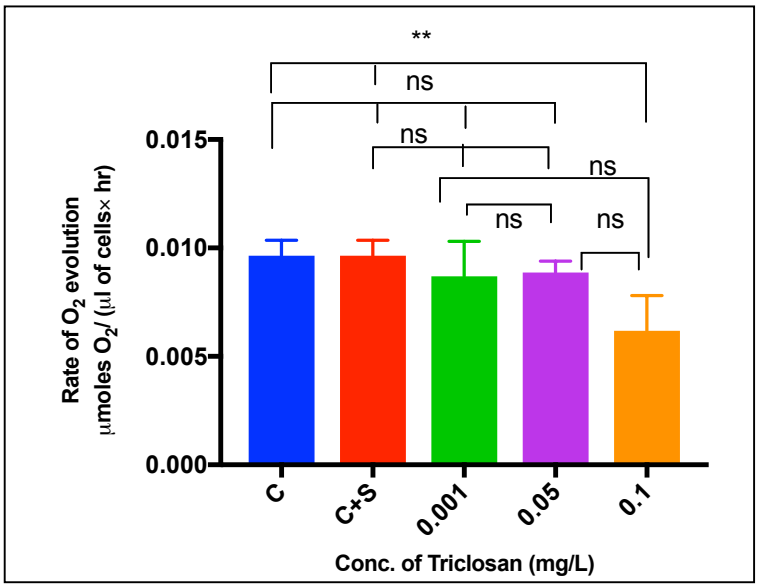

Figure 16: Rate of oxygen evolution (normalised to cells per µl) for *Synechococcus* CC9311 after exposure to different concentrations of triclosan showing the difference in oxygen evolution at 72hrs, C represents control and C+S represents solvent control containing 0.001% DMSO ANOVA followed by Tukey's test. Asterisks indicate significance at different P value cut-offs with p <0.001(**), ns indicates results were not significant (p ≥ 0.05).

The relative fluorescence of phycoerythrin and chlorophyll a measurements showed a significant decrease (p <0.001) in both photosynthetic pigments at 0.1 mg/L concentration of triclosan (Figure 17). Photosynthetic pigment analysis from the flow cytometry data also showed a decrease in relative mean fluorescence of chlorophyll a at 0.1 mg/L (Figure 18 B) while phycoerythrin showed different

pattern in all the evaluated time points (Figure 18 A).

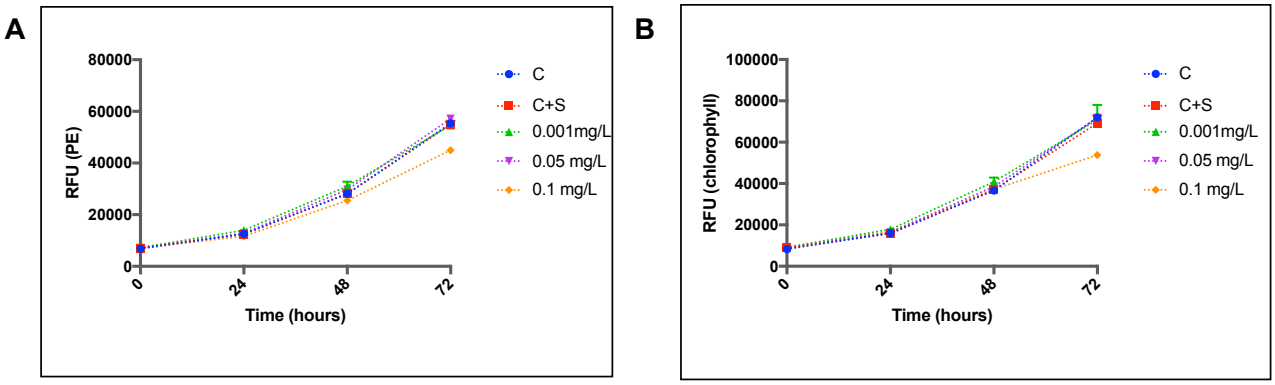

Figure 17: The *in vivo* relative fluorescence of (A) phycoerythrin (PE) and (B) chlorophyll a measured for *Synechococcus* CC9311 cultures exposed to 0.001, 0.05 and 0.1 mg/L concentrations of triclosan for 0-72 hrs. The error bars indicate S.D (n=3).

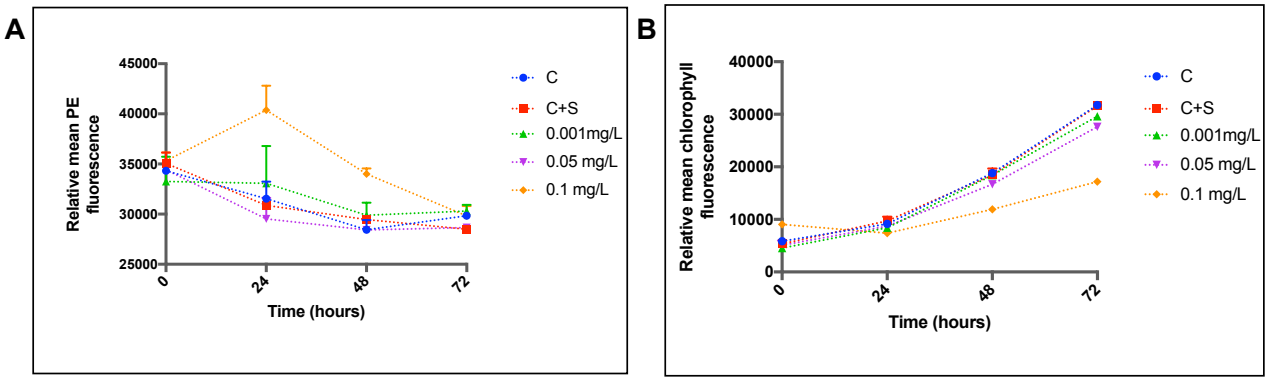

Figure 18: The relative mean fluorescence of phycoerythrin (PE) and chlorophyll a data obtained from flow cytometer (Cytoflex S) for *Synechococcus* CC9311 cultures exposed to 0.001, 0.05 and 0.1 mg/L concentration of triclosan for 0-72 hrs. The error bars indicate S.D (n=3).

4. Discussion

4.1 Impact of ZnO NPs on marine *Synechococcus*

The growth of *Synechococcus* was negatively affected by the presence of zinc oxide nanoparticles over the concentration range tested. Past studies have been carried out on the effect of ZnO NPs on marine organisms belonging to a number of different taxonomic groups and provide some information on the mechanisms of ZnO nanoparticle toxicity [109, 122-124]. However, to the best of our knowledge, this is the first study to report the impacts of ZnO nanoparticles on marine *Synechococcus* species, an abundant and important group of ocean primary producers. Our preliminary screening assay showed that ZnO nanoparticles at a nominal concentration of 0.01 mg/L and above strongly reduced the population growth rate (Figure 4) as measured by optical density, relative chlorophyll a fluorescence, and autofluorescent cell counts.

The observed effect of ZnO nanoparticles in our study suggests that marine *Synechococcus* may be more sensitive than other marine phytoplankton species and algae. Miller *et al.,* 2010 [110] reported impacts of ZnO nanoparticles on four species of phytoplankton, *Thalassiosira pseudonana*, and *Skeletonema marinoi, Dunaliella tertiolecta* and *Isochrysis galbana*. This work showed a significant difference in growth rate at concentrations of 0.5 mg/L or above. In another study on the growth of marine eukaryotic algae *Dunaliella tertiolecta*, concentrations of 1 mg/L or higher were required to significantly reduce growth [89].

All the tested concentrations of ZnO NPs above 0.1 mg/L showed similar response in our study. The pH of the media influences the bioavailability of the nanoparticles and Wong *et al.,* 2013 [109] described the alteration of the surface charge of the nanoparticles when they were exposed to increased pH from neutral to pH 8.0 and proposed that the changes in the surface charge affected the attachment of nanoparticles onto cells surface and altered the toxicity. Thus, similar impacts above a range of concentrations observed in our study might be due to the aggregation of nanoparticles and the bioavailability of nanoparticles to the cultures could be equivalent in those concentrations.

Changes in the ratio of photosynthetic pigments in PE rich freshwater *Synechococcus* under stress conditions have been demonstrated in past studies [125]. In our study, under exposure to ZnO NPs, the ratio of PE to chlorophyll a increased significantly at 72 hrs in all four strains in a dose dependent manner. The study of Newby *et al.,* 2017 on *Synechococcus* sp. IU 625 [126] demonstrated an increased ratio of allophycocyanin to chlorophyll a under the stress of zinc chloride. While Postius *et al.,* 1997 described the reduction of the PE/ chlorophyll ratio in *Synechococcus* spp. Strain BO 8808 under the stress of elevated light intensity [44]. A change in composition and function of phycobiliproteins attached to the stromal surface of thylakoid membrane has been reported in response to stress conditions and can result in pigmentation changes in cyanobacteria [127]. Similar changes may have occurred in the experiments performed here and might have contributed to the

difference in the ratio of fluorescence intensity from PE and chlorophyll a observed. Further examination of changes in overall pigment content and the ratio of different pigments could be conducted using pigment extraction techniques [125] to gain a more direct understanding of how ZnO NPs impact the photosynthetic process in these organisms.

The growth analyses carried out in our study showed that the CC9311 strain may be more sensitive to ZnO NPs than WH8102, CC9605 and BL107. This is perhaps surprising given that past studies on copper stress found CC9311 to be more tolerant than strain WH8102 [44]. Comparison of transporters from different *Synechococcus* strains using TransportDB 2.0 [128] showed that CC9311 strain has more (five) zinc-iron Permease (ZIP) family transporter proteins than CC9605 (one) and WH8102 (zero). The ZIP family of proteins are responsible for transporting different types of cations including zinc inside the cells. Thus, increased sensitivity of *Synechococcus* CC9311 to ZnNPs may be related to the presence of more zinc transporters which may facilitate more rapid movement of zinc into the cell. Studies by Filippis *et al*., 1981 reported the inhibition of enzymes required in photosynthesis by the presence of excess of metal ions [129]. In *Euglena gracilis*, the NADPH-reductase enzyme was found to be inhibited by metal ions including zinc and cadmium ions [130]. Another study also demonstrated the replacement of magnesium ions by zinc ions in the chlorophyll molecules that attached to the light harvesting complex II, which can result in inhibition of the PSII reaction centres in green alga [131]. Further experimentation looking at the impact of a range of metals on these *Synechococcus* strains would be of interest to determine the sensitivities of different strains and what the genetic basis of such differences might be.

Our preliminary study did not cover a sufficient concentration range to determine the EC50 for ZnO NPs on marine *Synechococcus* but provides a start point for such work. Further work using an extended range of concentrations and measuring the exact concentrations of ZnO NPs in each dilution would be required to provide data on these toxicology endpoints. Characterisation of the stress response at the genetic level, using techniques such as transcriptomics, would also be useful in understanding the mechanism of ZnO NPs toxicity on marine *Synechococcus*.

4.2 Impact of Ag NPs on marine *Synechococcus*

Previous literature has reported toxicity of silver nanoparticles in a range of marine organisms belonging to different trophic levels, showing dose dependent toxicity across a number of selected species [112, 132, 133]. However, to the best of our knowledge such work has not previously been carried out on marine *Synechococcus* species, an abundant and important group of ocean primary producers. The findings from this study suggest that Ag NPs at concentrations up to 1 mg/L have no discernible impact on the growth of marine *Synechococcus*, while 10 mg/L does negatively impact growth.

Previous silver nanoparticle toxicity studies [112] on marine organisms belonging from different trophic levels demonstrated that algae, diatoms, echinoderms, cnidarians and crustaceans were impacted by Ag NPs at concentrations of 0.9, 1.6, 0.02, 0.1 and 0.4 mg/L respectively. Comparing to these observations, *Synechococcus* appears to be less sensitive. One possible difference in sensitivity could be the used of nano powder instead of suspension in our study, as previous laboratory freshwater toxicity studies on Ag NPs, have shown powder nanoparticles prepared in water by sonication gave reduced toxicity compared to those in suspension [134-137]. Asghari *et al.,* 2012 [115] propose a possible explanation for this could be the aggregation of most of the nano powders even after sonication and agitation. Park and Choi *et al*., [138] reported an improved method in nano powder preparation involving 13 hrs of sonication followed by stirring for seven days and filtering through a 100 nm membrane. This process was reported to enhance the released of silver ions thus increasing the toxicity and the lethal dose of Ag NPs in *Daphnia* was found to be 0.001-0.002 mg/L. In our study, nano powders were sonicated for 20 min which may have been insufficient to create a suspension and therefore result in lower apparent toxicity. Another reason might be the size of the nanoparticles used (< 100 nm), with previous literature also indicating that toxicity of Ag NPs is depended on the size of the nanoparticles [132]. Lodeiro *et al.,* 2017 [139] recently reported that exposure to Ag NPs NM 300K at 1.1 mg/L reduced the biomass and photosynthetic activity to coastal diatom *Chaetoceros curvisetus.* In this work nanoparticles of < 20 nm size were reported to be more toxic than 20-80 nm size particles.

A higher ratio of PE/chlorophyll a was observed in *Synechococcus* cultures exposed to high Ag NPs concentrations (10 mg/L) indicating impacts beyond growth rate reduction after the exposure to silver nanoparticles. This observation was similar to what was seen with ZnO NPs exposure, as described above, indicting the increase in PE/chlorophyll a ratio in *Synechococcus* cultures might be common in cultures exposed to different metal nanoparticles.

To look beyond the impacts on growth and alteration in photosynthetic pigments, we also looked at whether Ag NPs exposure impacts oxygen evolution in marine *Synechococcus*. Previous studies have looked at a range of biochemical parameters related to photosynthesis, such as oxygen evolution, carbon dioxide fixation, ATP production or chlorophyll content, as indicators of a stress response in many algal species [129].

Results from this work showed that in *Synechococcus* CC9311, exposure to Ag NPs at concentrations of 5 mg/L and above resulted in no oxygen evolution in all the evaluated time points indicating Ag NPs can impact this aspect of photosynthesis. In parallel to this observation, the *in vivo* relative fluorescence of chlorophyll a and phycoerythrin measured by both BMG-PHERAStar and flow cytometry decreased compared to the control. This is similar to the observation from recent studies on the marine diatom *C. curvisetus* by Lodeiro *et a*l., [139], which also demonstrated the

photosynthetic impairment and loss of photosynthetic pigment chlorophyll a after exposure to Ag NPs. However, exposure to Ag NPs at concentrations of 0.5 mg/L resulted in a slight increase in oxygen evolution and an increase in photosynthetic pigments, chlorophyll a and phycoerythrin. This could be a physiological response which may enable these cells to better cope with the stress caused by Ag NPs. To gain more insight into the changes in pigment content, further work such as extraction and analysis of pigment could be performed. The decrease in chlorophyll a pigment in the presence of Ag NPs at concentrations of 5 mg/L and above, combined with the decrease in oxygen production per µl of cells, suggests that exposure to high levels of silver nanoparticles is likely to affect the capacity of these cultures to undergo oxygenic photosynthesis.

The range of Ag NPs tested, preparation method and size of the nanoparticles in our preliminary study would need to be modified in further experiments to gain a better understanding of the potential toxicity of Ag NPs and to enable the calculation of EC50 values. As little is known about the mechanism of toxicity of Ag NPs on marine phytoplankton, future work to look at the mode of action would be worthwhile in future studies. Characterisation of the stress response at the genetic level, using techniques such as transcriptomics, may be useful in understanding the mechanism of Ag NPs toxicity on marine *Synechococcus*.

4.3 Impact of triclosan on marine *Synechococcus*

Triclosan toxicity studies on marine organisms are very few and limited to certain species [103, 112, 140]. To the best of our knowledge, this is the first study to report the impacts of triclosan on marine *Synechococcus* species. Our preliminary screening assay showed that triclosan at a nominal concentration of 0.1 mg/L and above strongly reduced the population growth rate as measured by optical density, relative chlorophyll a fluorescence and autofluorescent cell counts (Figure 9). Past studies have reported varied levels of sensitivity in different marine species after exposure to triclosan. Compared to fish, marine algae and invertebrates were found to be more sensitive to triclosan from past studies [13]. Studies on marine phytoplankton species *Dunaliella tertiolecta* [25] showed a significant reduction in growth at concentrations of 3.55 µg/L and above. Another study by Seoane *et al.,* 2017 [102] showed a significant decrease in growth on marine microalga *Tetraselmis suecica* after exposure to triclosan concentrations of 0.2 mg/L and above. Triclosan has been demonstrated to inhibit lipid biosynthesis by inhibiting the enoyl-acyl carrier protein reductase in bacteria [98] and plants [141]. As both algae and cyanobacteria have enoyl-acyl carrier protein reductase enzyme, Eriksson *et al*., 2015 [140] proposed this might be an important part of the triclosan mechanism of action in microalgae and bacteria.

The sensitivity to triclosan varied among different strains of *Synechococcus*. Strain WH8102 appeared to be most sensitive, followed by BL107 and CC9311 while CC9605 appeared to be the

least sensitive. Past studies have indicated that the coastal strain of *Synechococcus* CC9311 may have a greater ability to adapt and respond to the change in its environment compared to the open ocean strain WH8102 due to the presence of more regulatory systems, histidine kinase sensors and response regulators encoded in CC9311 genome than in WH8102 [42]. The only previous study looking at the impact of chemical toxicants on *Synechococcus* strains [46] reported WH8102 was more sensitive to DNA damaging agents, ethidium bromide and mitomycin C than the coastal strain CC9311, which is comparable with what we observed for triclosan toxicity.

The impact of triclosan exposure on oxygen production was examined, as this is a physiological change which is likely to impact on *Synechococcus* primary productivity and therefore may potentially also impact organisms at the higher trophic levels in the food chain. From our study, triclosan negatively impacted on photosynthesis, as inferred by reduction in the rate of oxygen evolution at a nominal concentration of 0.1 mg/L concentration or higher. This was the concentration found to have a negative impact on the growth rates. Past studies have reported the toxic effect of triclosan on photosynthesis of marine periphyton at a concentration (EC50) of 0.8 mg/L, as determined via pulse amplitude modulation (PAM) fluorescence measurements of fluorescence yield, ϕ_{IImax}, and nonphotochemical quenching of photosystem II [140]. Similarly Franz *et al*., 2008 [142] in studies on marine diatom *Nitzschia palea* showed an inhibition of photosynthetic yield after exposure to triclosan at a concentration (EC50) of 0.9 mg/L. While a specific mode of action for triclosan toxicity on photosynthetic processes has not been reported, Franz *et al*. commented that uncoupling of oxidative phosphorylation fitted well with the observed effects on photosynthesis of marine diatom, *Nitzschia palea.*

The *in vivo* relative fluorescence of chlorophyll a and mean chlorophyll a fluorescence from flow cytometry data also showed a clear reduction in this pigment. Similar to our study, decrease in chlorophyll a after the exposure to triclosan was observed in studies by Backhaus *et al*., 2011 [143] and Johansson *et al*., 2014 [144] on marine periphyton. Unlike exposure to ZnO NPs and silver nanoparticles, the ratio of PE/chlorophyll a remained unchanged following triclosan exposure.

This study provides a starting point in determining the impact of triclosan on marine *Synechoccoccus* strains. It would be of interest to further investigate the impact of triclosan at the genetic level using techniques such as transcriptomics, which may help in understanding the mechanism of triclosan toxicity on marine *Synechococcus* and the basis for the different sensitivity of tested strains.

4.4 Impact of BP-3 on marine *Synechococcus*

The coastal areas around the world continue to be subject to high population growth and human use. Many chemical UV filters are incorporated in the formulation of sunscreen and among the UV filters,

BP-3 was detected at the highest concentration in seawater. Our study aimed to determine the impact of BP-3 on marine *Synechococcus* and no significant impact was observed after exposure to BP-3 (0.1, 1, 5 and 10 mg/L) for 72 hrs. In contrast to our results, recent studies on marine algae *T. suecica* [102] reported a stimulatory effect on growth and an increase in fluorescent pigment to algae after exposure to concentrations of 0.2 mg/L and above for BP-3. Studies by Paredes *et al.,* 2014 [107] reported the release of organic and inorganic nutrients after the dissolution of sunscreen in seawater which could potentially fuel the algal growth. Thus, observation on *T. suecica* could be related to this finding. While previous studies on the impact of BP-3 on marine organisms *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata* showed a significant reduction in the growth of all organisms at 4-5 μg/L [107]. Among the tested marine organisms tested, *Isochrysis galbana* was found to be most sensitive to BP-3. BP-3 was reported to interfere with estrogenic activity, resulting in developmental and reproductive toxicity on freshwater fish, *Pimephales promelas* [104]. However, the mechanism of BP-3 toxicity on marine organisms remains to be elucidated. The absence of hormonal regulation in *Synechococcus* and other bacteria might be the reason for their being no observed impact from BP-3 on marine *Synechococcus*.

4.5 Impact of ibuprofen on marine *Synechococcus*

Ibuprofen is a non-steroidal anti-inflammatory drug detected commonly detected in seawater. Currently studies on the impact of ibuprofen on marine organisms are very limited. In this study, the impact of ibuprofen on marine *Synechococcus* was investigated, finding that nominal concentrations of ibuprofen up to 5mg/L did not appear to induce any significant impact on growth over 72 hrs exposure. In contrast to our study, a recent study on marine invertebrates echinoderms, *Asterias rubens* and *Psammechinus miliaris*, and the polychaete worm *Arenicola marina* showed that exposure to ≥ 1μg/L of ibuprofen for 20 min affects the gametes of *P. miliaris* and the success of fertilisation in *A. rubens* [96] . Another study on the impact of ibuprofen on marine blue mussels *Mytilus edulis trossulus* for two weeks demonstrated a significantly reduction in growth rate and impairment of the mussels' reproductive fitness at a concentration above 1mg/L. Ibuprofen was suspected to have an influence on the sex steroid hormone through the steroidogenic pathway [95] and also impact the cyclooxygenase pathway which is concerned with the synthesis of eicosanoids [91, 92]. Eicosanoids are important regulators of reproduction. Based on these studies and other work the mechanism of ibuprofen toxicity is therefore thought to be largely due to effects on reproductive processes.

The absence of an impact of ibuprofen on marine *Synechococcus* in our study may be due to the absence of complex sex steroid hormone mechanism in *Synechococcus* and other bacteria. Ibuprofen at 0.01 mg/L concentration was found to have a stimulatory effect on the freshwater

cyanobacteria *Synechocystis* [145], however the cause for this stimulation was not determined. Thus, while ibuprofen induced a varied response among the marine invertebrates and vertebrates, the concentrations tested in this study did not appear to impact growth of marine *Synechococcus* either positively or negatively.

4.6 Comparison of techniques for monitoring cyanobacterial growth

In our study three independent techniques: optical density measurement at 750nm, *in vivo* chlorophyll a fluorescence and autofluorescent cell counts by flow cytometry were used to monitor the growth of the *Synechococcus* cultures. These are all commonly used techniques for monitoring growth of cynaobacteria and fit with the OECD guidelines for monitoring the reduction of cyanobacterial growth which suggest a range of possible growth monitoring techniques can be used. All three techniques showed mostly comparable results in regard to growth impacts following exposure to tested substances. One notable exception was in the reported growth rate for both zinc oxide nanoparticles and triclosan, where higher concentrations did not negatively impact growth rate as measured by spectrophotometry, while other techniques indicated clearly negative growth rates. This observation may be due to the concurrent accumulation of dead cells in these cultures which would contribute to turbidity and therefore the recorded optical density, while the other two techniques measure properties of live cells (fluorescence) and thus were more suitable for detecting population decline in response to chemical exposure. This is a known limitation of using optical density measurements for monitoring population growth of microbial cultures.

5. Conclusions and future directions

This study was conducted to investigate the impact of emerging pollutants -pharmaceuticals and personal care products on marine *Synechococcus*, an important primary producer of the ocean. From this broad category of products, four personal care products (silver nanoparticles, zinc oxide nanoparticles, triclosan, BP-3) and one pharmaceutical product (ibuprofen) which are detected in marine environments, often at high levels, were selected and their impacts on four strains of marine *Synechococcus,* covering its major clades were studied.

From this study*, Synechococcus* was found to be impacted by zinc oxide nanoparticles, silver nanoparticles, and triclosan. Zinc oxide nanoparticles were found to have the greatest impact on growth of marine *Synechococcus* among the all tested substance. A varied response in growth measured by O.D, the relative fluorescence of chlorophyll a and cell count by flow cytometer was observed among the strains on exposure to zinc oxide nanoparticles. CC9311 was found to be the most sensitive to zinc oxide nanoparticles (≥ 0.01 mg/L) followed by WH8102, CC9605 and BL107 (≥ 0.1 mg/L). We found marine *Synechococcus* to be impacted by relatively low concentrations of zinc oxide nanoparticles compared to other previously studied marine organisms, indicating that these organisms may be particularly sensitive to this toxicant.

The *Synechococcous* strains tested in this study were also found to be sensitive to triclosan, with variations in response observed among the strains. The findings here indicate oligotrophic open ocean strain WH8102 may be the most sensitive (≥ 0.01 mg/L) followed by CC9311 and BL107 (≥ 0.1mg/L). The strain CC9605 was the least sensitive to triclosan (≥ 1mg/L).

Exposure to relatively high concentrations (10 mg/L) of silver nanoparticles did elicit a clear growth response in all four strains, however further work with different nanoparticle preparation may be required to gain a better insight into the true extent of silver nanoparticle toxicity. Two other substances, BP-3 and ibuprofen did not show any significant impact on marine *Synechococcus* growth in the assays performed here.

The project was also designed to investigate whether concentrations of PPCPs which negatively impacted *Synechococcus* growth also resulted in other measureable physiological changes. The evidence from this study suggests that photosynthetic oxygen production in *Synechococcus* strain CC9311 was impacted by silver nanoparticles and triclosan and that this might be linked to reductions in photosynthetic pigments under toxicant exposure.

While the toxicity assay performed in this study provide important insights into the potential impacts of all the selected pharmaceuticals and personal care products, there is an ample scope for further investigation. To follow up on this study, the concentration of all the tested substances need to be extended that span the concentrations that would enable calculations of EC50 values. Measuring

the exact concentrations of each substance in each dilution is also needed to provide data on toxicology endpoints.

To gain greater insight into the exact nature of the impact for each compound the viability of the cells could be determined by staining with viability stains (example -SYTOX green) prior to cell counting using flow cytometry. Determination of whether metal nanoparticles accumulate within cells in each of the cultures, for example using inductively coupled plasma–mass spectrometry analysis of cellular and supernatant fractions, would further help in gaining an understanding of the mechanism of toxicity of nanoparticles. It would also be of interest to look for structural and morphological changes or membrane irregularities in the cell, as visualised by transmission and/or scanning electron microscopy. Characterisation of the stress response at the genetic level, using techniques such as transcriptomics, would also be useful in gaining an understanding of the mechanism of toxicity for zinc oxide nanoparticles, silver nanoparticles and triclosan on marine *Synechococcus.*

In this work, we report the impacts of *in vitro* toxicant exposure on environmentally relevant marine *Synechococcus* species, which provide a starting point for understanding environmental impacts of a set of emerging human pollutants. However, in the marine environment these organisms will exist as part of complex communities, and impacts will differ in this context [146]. There is a growing focus in the emerging field of microbial ecotoxicology to elucidate the impact of pollutants of whole communities, rather than single species [147].

Gaining an insight into the likely environmental impacts of emerging pollutants is complicated by a number factors. In the environment, it is likely that a range of biotic and abiotic factors may affect the bioavailability of pollutants. It is also important to consider that multiple stressors may be present in the environment, and examining the impacts of pollutants in mixtures, as well as the combined impact of environmental stressors and toxicant exposure is critical to gaining information on likely outcomes of anthropogenic impacts on the marine environments.

6. References

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