Cyanobacteria biocomposites for in situ treatment of domestic wastewater

A thesis submitted by

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Abstract

Centralised water treatment infrastructure is increasingly being put under strain due to population growth. In situ treatment (i.e. a distributed treatment network) may minimise or even negate the need for centralised infrastructure. Cyanobacteria grown in open-ponds and photobioreactors are used for wastewater treatment; however these cultivation systems struggle with batch consistency and have a high capital cost. Biocomposites (biomass immobilised on a solid substrate within a semi-porous matrix) theoretically support increased active biomass within a more compact space and prevent cell wash-out, thereby increasing bioremediation efficiency. Wild-type *Synechococcus elongatus* (strains PCC 7942 and CCAP 1479/1A) and a novel engineered strain (SBG363; designed to overproduce and excrete sucrose) were trialled as potential bioremediation biocomposites. Commercial latex-based binders (AURO 320 and 321) were used for biocoatings formulation, and applied to a selection of natural and synthetic textiles to form textile-based biocomposites. Biomass growth was increased by up to 800%, retaining up to 97% of biomass after 72 hours. Sucrose output from *S. elongatus* SGB363 was unaffected. Wild-type immobilised biomass supported up to 80% greater CO_2 sequestration over a 20-day period than its suspension culture control. However, after three and six days of immobilisation there were no significant differences in total protein content, CO_2 removal, or orthophosphate uptake per cell between immobilised and suspension treatments. Whole transcriptome sequencing (RNA-Seq) was attempted to determine the genetic response of *S. elongatus* surviving within a biocoating. However, immobilised samples had very low RNA integrity number equivalents that prevented differential gene expression analysis. This research shows that cyanobacteria textile biocomposites are a promising solution for process intensification in the wastewater treatment industry, and addresses concerns regarding environmental safeguarding. Metabolically active biocomposites can be used for de-centralised wastewater treatment applications thereby alleviating problems associated with Victorian era infrastructure without creating an additional environmental burden.

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Chapter 1. A review: Engineering wastewater treatment systems through biological understanding

1.1 Introduction

1.1.1 Large scale wastewater treatment in the United Kingdom

Ninety six percent of the UK population is served by centralised wastewater treatment plants (WTPs), producing around 11 billion litres of wastewater daily (DEFRA, 2012), and mainly receiving a mixture of domestic and industrial wastewater. Centralised treatment describes a system based on sewer based transportation to large-scale treatment plants (Wilderer and Schreff, 2000), with variation in the time taken to reach the treatment plant depending on location or network (Grievson, 2021) (Figure 1.1). Increasing urbanisation is causing added burden on sewerage infrastructure affecting the quantity and quality of wastewater discharged to the environment (Yates *et al.*, 2019). Most of the UK's water and sewerage infrastructure was built between the late 1800s and early 1900s with limited major upgrading (Lofrano and Brown, 2010).

Privatisation of the UK's water utilities in the mid 1980's led to under-investment, poor maintenance of infrastructure, and a shrinking workforce despite the UK's growing population (Bayliss, 2013). Water losses from centralised systems may be as much as 50%—particularly for underfunded utilities (Bithas, 2008). The number of burst pipes is expected to increase by 32% by 2070, posing a significant threat to infrastructure (Speight, 2015). Current usage levels, the cost of centralised services, and climate change are increasing challenges around water scarcity and contribute to greater inequalities within societies (Bithas, 2008).

Within a WTP, wastewater undergoes up to three stages of treatment depending on the population equivalent (P.E.) of where the treated water is to be discharged (Table 1.1). P.E. is a unit of measure set out in the European Union Urban Wastewater Treatment Directive to assess the polluting potential of wastewater discharges. One P.E. is defined as the organic biodegradable load having a five-day biochemical oxygen demand (BOD) of 60g of oxygen a day. The susceptibility of the receiving body of water to pollution determines the level of treatment to be executed.

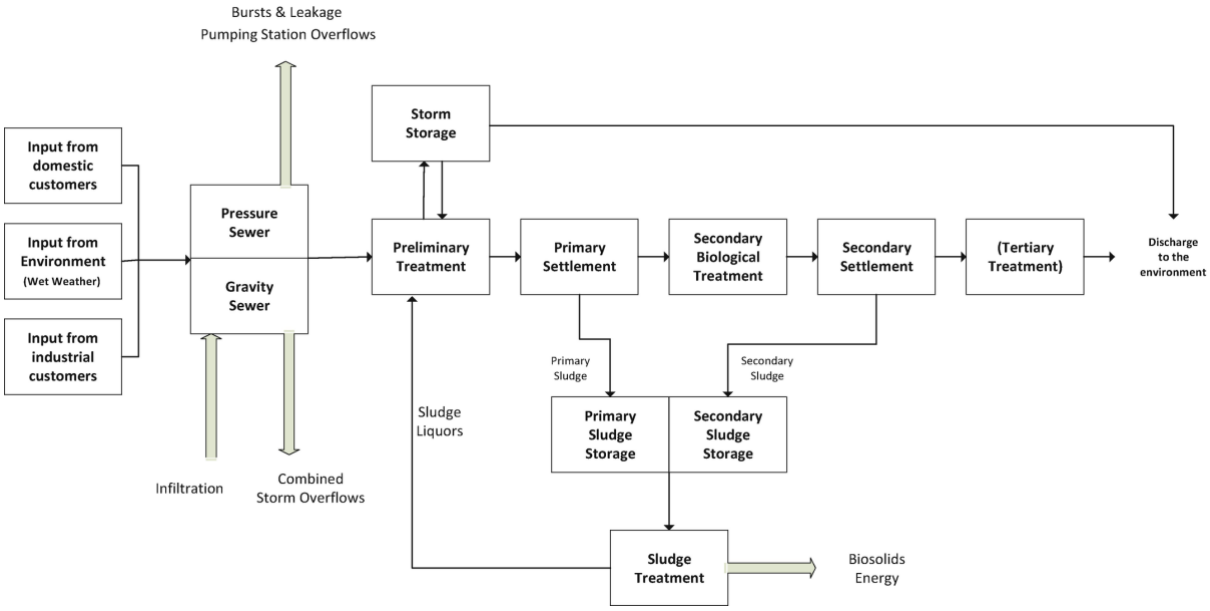

Figure 1.1. A typical process for wastewater treatment in the United Kingdom (Grievson, 2021).

Table 1.1. Treatment level descriptions and discharge locations of treated wastewaters based on UK policy formed by European Union Urban Wastewater Treatment Directive – 91/271/EEC (2012).

Treatment Level	Description	Population Equivalent and Discharge Location
Preliminary	The removal of grit from wastewater by the slowing of water flow causing grit to be deposited into traps. Other large solids are then removed by screening.	Freshwater and estuarine locations with a P.E. of <2000, and coastal waters with a P.E. of <10,000
Primary	The settlement of suspended solids (SS) not removed by primary treatment. There must be a removal of at least 50% of SS and at least a 20% reduction of BOD.	Discharged to areas deemed less sensitive. Estuarine waters with a P.E. between 2000 and 10,000, and coastal waters with a P.E. >10,0000.
Secondary	The biological breakdown of wastewater generally by bacteria. The aim of secondary treatment is to sufficiently reduce the BOD to avoid chronic oxygen depletion in receiving waters. This is done by	Discharged to areas deemed normal. Freshwater and estuarine waters with a P.E. >2000 and coastal waters with a P.E. >10,000.

	aeration with bacterial cultures or trickle filter beads covered with bacterial culture.	
Tertiary	Used to treat a variety of pollutants, thus takes a variety of forms. Includes UV light irradiation and microfiltration for disinfection, and chemical treatment for phosphate and nitrate removal.	Discharged to sensitive areas which would feasibly become eutrophic if discharged wastewater was not tertiary treated. Freshwater, estuarine, and coastal wasters with a P.E. >10,000.

1.1.2 Costs and issues associated with traditional waste management strategies

Despite current policy, treated wastewater that is discharged from large facilities remains a significant factor contributing to the instream concentrations of nitrogen (N) and phosphorous (P), causing eutrophication within freshwater and estuaries (Yates *et al.*, 2019). The persistence of untreated wastewater has knock-on effects on the environment, agriculture, and public health due to excess loading of nutrients, salts, metals and metalloids (Qadir *et al.*, 2015).

By 2015, domestic wastewater treatment was responsible for 3% of global electricity consumption, and 5% of non-CO_2 greenhouse gas (GHG) production (Li *et al.*, 2015). Energy use by water companies has doubled since the 1990s, and is set to double again by 2030 if significant changes are not made; threatening the industry's capacity to meet national GHG emissions targets (CST, 2009). Additionally, policies to reduce CO_2 emissions run counter to the current strategy for wastewater treatment, suggesting major technological innovation is required (Thomas and Ford, 2006a). Previously, water and sewerage companies have modified existing protocols to meet new objectives rather than investing in and developing new processes (Tanner *et al.*, 2018). This may be partially due to continued land usage competition as to prioritise land for water services requires political will, which is unlikely to happen until a crisis point has been reached, such as happened in Singapore after being declared a water sparse country in 2005 (Timm and Deal, 2018). Bioremediation, particularly by photosynthetic microbes, offers specialised treatment of wastewater that can sequester target nutrients and metals from municipal and industrial waste streams (Puyol *et al.*, 2017).

1.1.3 Point-source bioremediation

Utilisation of cyanobacteria in wastewater treatment dates to back to the mid 20th century (Oswald and Gotaas, 1957) and now forms an important part of activated sludge systems in large-scale WTPs (Martins *et al.*, 2011). Cyanobacteria can survive in WTPs due to their broad

abiotic tolerances and metabolic flexibility (Lin *et al.*, 2011; Magana-Arachchi *et al.*, 2011; Beversdorf *et al.*, 2013; Dadheech *et al.*, 2013). The N and P found in wastewater originates in human excretions, detergents, agricultural practices, and industrial chemicals production (Li *et al.*, 2015) and could be viewed as a "free" resource for cyanobacteria cultivation (Cuellar-Bermudez *et al.*, 2017). Provided the biomass is reused, Munoz and Guieysse (2006) argued that this process causes no secondary pollution as no waste products would be generated. Decentralised, or point-source systems, offer an alternative, but must ensure both human and environmental protection, be user friendly, and comply with water regulations through effluent quality monitoring (Oladoja, 2017). Point-source—or start-of-pipe—bioremediation ensures that treatment can be rapid and effective to facilitate safe water supply as end-of-pipe treatments can be more specific to pollutants that are not targeted during conventional bioremediation (Kümmerer *et al.*, 2018). With proper urban planning, wastewater should be viewed as a valuable resource rather than a problem, by encouraging changes in consumer behaviour such as increased energy and water saving, waste recycling, and mixed land use (Grant *et al.*, 2012). There is concern from governmental organisations that perceived failures in distributed wastewater treatment systems may lead to a perception of inappropriate spending of public funds. Farrelly and Brown (2011) advocate that failure should be a valuable knowledge resource for future experimentation, encouraging cultural, policy, and structural reforms for wastewater management. Through this review, the previous successes will be discussed, but ultimately radical change and better biological insight is required to truly transform the wastewater treatment industry using point-source bioremediation.

1.2 Bioremediation

1.2.1 Photosynthetic organisms in the natural world

The first sustained increase in atmospheric oxygen, termed the Great Oxidation event, 2.4 – 2.1 Ga (Lyons *et al.*, 2014) is accredited to the evolution of cyanobacteria (Ettwig *et al.*, 2010). As gram-negative bacteria (Shih *et al.*, 2013), cyanobacteria are the only known prokaryotes able to conduct oxygenic photosynthesis (Hamilton *et al.*, 2016) and are key to carbon, N, and oxygen biogeochemical cycles (Karl *et al.*, 2002; Kulasooriya, 2011). Advances in phylogenetics based on molecular approaches has led to 50 cyanobacteria genera being described since 2000 (Komárek, 2016). Phenotypic diversity observed in the genera has enabled cyanobacteria to tolerate a range of environments, increasing their ability to occupy environmental niches (Hamilton *et al.*, 2016). Their ability to sustain growth in ecosystems of high nutrient levels suggests they would be suitable tools for wastewater remediation (Abed *et al.*, 2014).

Uptake of inorganic N and P contributes to normal cellular function and is regulated by the cells, but can be influenced by pH and dissolved oxygen content of water. Inorganic N can be assimilated as nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) forms through translocation of the plasma membrane where it is reduced to NO_2^- and then NH_4^+. The NH_4^+ can then be used to synthesise amino acids, contributing to the production of genetic materials (DNA and RNA), proteins, and photosynthetic machinery (Barsanti and Gualtieri, 2014; Singh *et al.*, 2019). P is incorporated as hydrogenphosphate or dihydrogenphosphate during phosphorylation of ADP to ATP for photosynthesis and respiration (Cai *et al.*, 2013).

1.2.2 Open pond cultivation

Mass cultivation of micro-photosynthetic organisms began in the mid-20th century in California (Oswald and Gotaas, 1957). Open ponds were utilised due to their low operational cost, regardless of mixing system (Hoh *et al.*, 2016). Open ponds have three main designs; raceway, unstirred, and circular (Figure 1.2). Typically, ponds are constructed and lined with cement, polyethylene, or PVC with a working depth of 0.25 – 1 m (Singh *et al.*, 2016). One of the more effective models of raceway ponds is the High Rate Algal Pond (HRAP). First published in 1963 (Oswald, 1963), HRAPs aim to maximise algal biomass by utilising shallow ponds that are mixed using a paddle wheel with a working depth of 0.3 – 0.5 m (Montemezzani *et al.*, 2015). Compared to other styles of algal pond, HRAPs demand less land, have smaller construction costs, and have reduced evaporative water loss (Young *et al.*, 2017). The energy required to operate HRAPs is low, and the operation and cleaning are simple (Pawar, 2016). The length to width ratio is critical to maximise productivity, with the width sufficiently small to maintain current speed which affects mixing and mass transfer velocity (Chisti, 2007).

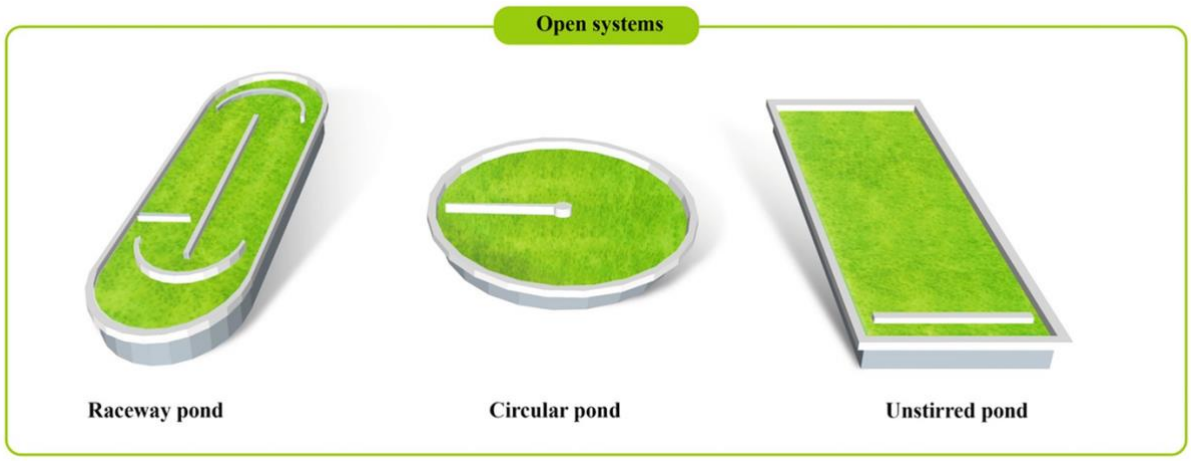

Figure 1.2. The three most common designs for outdoor mass microalgae and cyanobacteria cultivation. Adapted from Zerrouki and Henni (2019).

In a wastewater treatment context, HRAPs can clean to an equivalent of tertiary treatment with biomass productivity of up to 30 tonnes/ha/year, without additional CO_2 supplementation (Park *et al.*, 2011). Gutiérrez *et al.* (2016) treated municipal wastewater by phycoremediation in open HRAPs, reducing ammonia concentration by 97% after four days with a 10% recycling rate with *Chlorella* sp. the dominant alga. Median percentage removal values for N, ammonia, BOD, and P are 61.23, 77.00, 59.00, and 42.73% respectively (Young *et al.*, 2017). Racault and Boutin (2005) demonstrated that a HRAP could treat up to 175g $BOD.m^{-3}\ d^{-1}$ in a 0.2m deep pond under ideal conditions. Cyanobacteria in an open HRAP in the tropics removed up to 96% of NH_4^+, and 87% of P from pig farming wastewater (Olguín *et al.*, 2003). Design of the HRAP was a significant factor in this study, with depth, pH, and temperature all significantly affecting NH_4^+ and P removal. Additionally, strain selection plays a vital role. Renuka *et al.* (2013) showed the ability of filamentous strains (*Phoridium* sp., *Limnothrix* sp., *Anabaena* sp., *Westiellopsis* sp., *Fischerella* sp., and *Spirogyra* sp.) to remove 90% of nitrate-nitrogen (NO_3-N), and 97.8% of phosphate (PO_4^{3-}) from primary treated municipal wastewater after 10 days of treatment. By comparison, a reference unicellular algal strain was only capable of removing 82% of NO_3-N which was significantly lower, but a similar removal rate of PO_4^{3-} was observed.

However, there is poor consistency between open ponds; driven by a combination of abiotic and biotic factors. The 'open' nature of open ponds means that abiotic factors (including light, temperature, and water evaporation) cannot be effectively controlled, which significantly affects algal growth and nutrient removal (Chandra *et al.*, 2019). Light intensity is a key factor affecting the efficiency of algal ponds. In a model developed by Flynn *et al.* (2010), light was deemed the most fundamental parameter influencing microalgal growth, despite many strains being able to acclimate to low light intensities. Arcila and Buitrón (2017) found that a high light intensity treatment of 6213 ± 1186 Wh $m^{-2}\ d^{-1}$ reduced the removal of total nitrogen (TN) (36%) and chemical oxygen demand (COD) (50%) but had the highest removal of P (92%). At lower levels (2741–3799 Wh $m^{-2}\ d^{-1}$) the removal of TN and COD increased to 60% and 89% respectively, whereas P uptake decreased to 28%. Light is so influential that the biomass composition fluctuates significantly within a 24-hour period, e.g. the cyanobacterium *Arthrospira platensis* displayed up to 14% variation in chlorophyll *a* and 21% variation in total phycocyanin concentration in 24h, which Hidasi and Belay (2018) attributed to photoinhibition due to high light intensities and high dissolved oxygen concentrations during the middle of the day. Further, the performance of open ponds fluctuates seasonally. In a temperate full scale pond, biomass was reduced to just 40% of the summer density, resulting in a reduction of NH_4^+ removal to just 47% in the autumn which Sutherland *et al.* (2014) attributed to carbon limitation.

Hydraulic retention time (HRT), i.e. how long cultures are retained within the pond, is another influencing factor. Increased HRT generally increases nutrient removal with around 6-10 days being optimal (Park and Craggs, 2010; Arcila and Buitrón, 2017). Beyond this, there is potential for re-release of PO_4^{3-} due to respiration of the cultures (García *et al.*, 2002; Alemu *et al.*, 2018). HRT is also a significant factor affecting cost of operation - Acién Fernández *et al.* (2018) argued that HRT must be reduced to less than one day at an industrial scale to be economically viable. Furthermore, analysis of 553 datasets using an artificial neural network found that HRT was the critical parameter for microalgal growth in open pond systems (Supriyanto *et al.*, 2019).

The size of the pond is fundamental, and reliance on small-scale experiments may overestimate the productivity of large scale (1 ha or greater) open ponds. Sutherland *et al.* (2020) compared nutrient removal between $5m^2$, $330m^2$, and $10{,}000m^2$ ponds with seasonal variation. NH_4^+ removal was greatest in the $5m^2$ pond regardless of season, despite the maximum rate of photosynthesis not significantly differing between pond size. The $330m^2$ pond is considered a pilot scale pond but was recommended as the optimum size pond by Sutherland *et al.* (2020) as the microalgal productivity was maximised at this size due to increased mixing and higher photosynthetic activity when light was limiting, which was well balanced with capital and operational costs.

Contamination by other organisms is the most significant factor leading to rapid culture crashes and variability in productivity (Mesple *et al.*, 1995). Common biocontaminants include herbivorous zooplankton, bacteria, viruses, fungi, and unwanted non-specific algae species (Yun *et al.*, 2018). The recycling of culture media causes significant increases of bio-contamination, inhibiting productivity of the desired photosynthetic species (Yuan *et al.*, 2019). In the natural environment, these confounding organisms prevent bloom formation with one dominating species, but within the controlled pond system the effects can decimate target species within a few days (Molina *et al.*, 2019).

Zooplankton enter from the environment surrounding the ponds (Canovas *et al.*, 1996), and grazing is responsible for changing the dominant microalgal species, reduction in pond productivity and nutrient uptake, and increased biomass settling (Montemezzani *et al.*, 2016). Dependable and economical treatments for zooplankton require further development to improve the use of open ponds (Montemezzani *et al.*, 2017c). CO_2 asphyxiation is a promising chemical treatment for some zooplankton species (Montemezzani *et al.*, 2017b) and commercially available insecticides have also shown some promise in chemical removal of zooplankton. To minimise potential impacts on the environment, physical controls have been utilised to remove zooplankton from open ponds but are both economically and energetically

expensive with detrimental effects to algal cultures. Temperature has been proposed as a method of zooplankton control in ballast water. Increasing water temperature to 38ºC removed all zooplankton species and most of the phytoplankton (Rigby *et al.*, 1999). However, for use in wastewater treatment it would be detrimental to the productivity of most algal systems and would add a significant operational cost. Filtration is another physical removal method which Montemezzani *et al.* (2017a) demonstrated that larger filtration sizes of 300 and 500µm could completely remove *Moina tenuicornis* populations within four days, while larger filters of 800µm allowed juvenile survival and reproduction. Failure to remove all organisms allow for rapid regrowth and require further treatment (Grob and Pollet, 2016). Filtration is also a relatively economically expensive solution with capital cost of $0.19/tonne of water, with increased costs due to fouling or clogging of the filter (Danquah *et al.*, 2009; Uduman *et al.*, 2010).

Wastewater contains a variety of bacteria, but is dominated by Proteobacteria, Bacteriodes, and Actinobacteria (Unnithan *et al.*, 2014). Phytoplankton-lytic bacteria can inhibit the growth of microorganisms naturally impacting bloom formation and termination using thermostable compounds (Wang *et al.*, 2009). *Bacillus fusiformis* was identified by Mu *et al.* (2007) to decrease chlorophyll *a* concentrations in cultures of *Microcystis aeruginosa, Chlorella,* and *Scenedesmus* suggesting the lytic effect impacts a range of species. These bacteria can also have negative effects on human health. Vardaka *et al.* (2016) conducted 454-pyrosequencing of 31 brands of *Arthospira* (marketed as Spirulina) that had been grown in large open pond cultures, finding 469 operational taxonomic units (OTUs). Making up some of the most prevalent OTUs were *Pseudomonas, Vibrio*, *Bacillus*, and *Enterococcus* – all known for causing human health problems. Furthermore, β-methylamino-l-alanine (BMAA) produced by non-target cyanobacterial species has been found in some dietary supplements of *Arthospira*, *Aphanizomenon*, and *Nostoc.* There has been inconsistency in the amounts of BMAA detected in samples depending on analytical method; regardless, caution should be applied (Manolidi *et al.*, 2019). Concordantly, Roy-Lachapelle *et al.* (2017) found microcystins, anatoxin-a, and BMAA in several samples using the same methods for each sample of commercially available *Arthrospira* and *Aphanizomenon flos-aquae* that had been grown in open ponds. If we consider the desired end-point of open ponds for wastewater treatment – safe, clean water - due consideration must be given to the presence of toxins.

1.2.3 Photobioreactors

To overcome the limited productivity in open pond systems, closed photobioreactors (PBRs) have been designed with an emphasis on maximal productivity whilst remaining economical,

operable, and low maintenance (Singh and Sharma, 2012). PBR designs include flat-plates, columns, annular, tubular and coil reactors (Figure 1.3) (Zerrouki and Henni, 2019). It is often easier to control parameters such as pH, temperature, mixing, gas exchange, evaporation, and biotic influences in PBRs (Ugwu *et al.*, 2008). A significant advantage of PBRs is the reduction in water use, with PBRs using four times less water than open ponds for the same level of biomass production (Yang *et al.*, 2011; Delrue *et al.*, 2012). Posten (2009) defined the four-phases of any given PBR as; 1) the solid phase – cells, 2) the liquid phase – media, 3) the gas phase – air or CO_2 enriched air, 4) the radiation phase – light. Any given PBR can be divided into three distinct zones based on cell growth due to light transfer through the unit. Firstly, as light initially passes through the PBR wall the light intensity exceeds the cell's ability to photosynthesise, having an inhibitory effect. This then reaches a maintenance zone in which cells can balance light and nutrient resources for sustained culture growth. Around the middle of the PBR is a dark zone where there is little light causing a reduced growth rate (Bitog *et al.*, 2011). To ensure maximal biomass production within PBRs the casing material (such as poly vinyl chloride or polyethylene) must be sufficiently transparent whilst remaining mechanically intact. To minimise the impact of poor light penetration, mixing of cultures by aeration, pumping, or mechanical agitation is common (Wang *et al.*, 2012a). Mixing also helps to manage pH, temperature gradients, cell sedimentation, and gas exchange (Carvalho *et al.*, 2006).

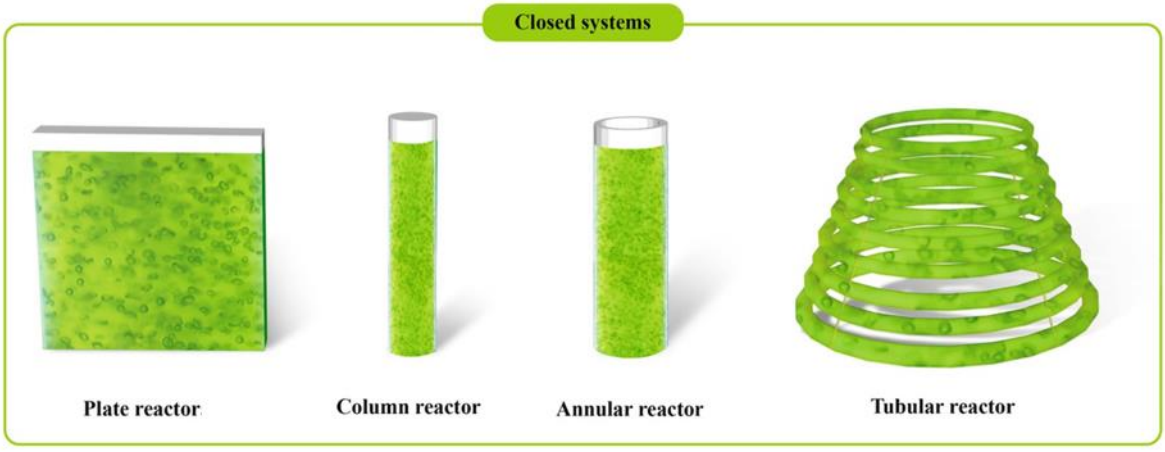

Figure 1.3. Common designs of photobioreactors for microalgae and cyanobacteria cultivation. Adapted from Zerrouki and Henni (2019).

PBRs have had good success in wastewater treatment. Jiang *et al.* (2012) operated a mixed microalgae culture which reduced total phosphorous (TP) by 92%, and NH_4^+ by 99% from municipal wastewater. Likewise, an evolving mixed algal culture removed 75% of TN and 93% of TP from influent wastewater using natural light and wave energy for culture mixing

(Novoveská *et al.*, 2016). Although these results were slightly decreased when compared to Jiang *et al.* (2012) both studies show compelling results for mixed algae cultures in PBRs. Light intensity and duration can significantly influence nutrient recovery. Maximal N and P removal of 7.7 ± 1.6 mg $N \cdot L^{-1} \cdot d^{-1}$ and 1.03 ± 0.21 mg $P \cdot L^{-1} \cdot d^{-1}$ were obtained with continuous illumination of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with no photoinhibition due to good mixing and the central dark zone. However, in a subsequent experiment using the same bioreactor with reduced solar PAR (89 ± 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) N recovery rates were significantly lower when the artificial lighting (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was only used for 12 hours a day at night time, and relied on natural light during day hours (2.2 ± 1.1mg $N \cdot L^{-1} \cdot d^{-1}$) when compared to samples being continuously illuminated with artificial light during the same time period (3.5 ± 1.8 $N \cdot L^{-1} \cdot d^{-1}$) (González-Camejo *et al.*, 2019).

Despite the success of PBRs in treating wastewater, few are operating at commercial scale (Bertucco *et al.*, 2014). Primarily, this is due to the high operating costs of PBRs, which is estimated to be 4 – 9 times higher than open pond systems regardless of the design (Dogaris *et al.*, 2015). When comparing the cost for lipid production, Richardson *et al.* (2012) found the price per gallon was \$12.73 and \$31.61 for open ponds and PBRs respectively using a Monte Carlo financial feasibility model. Slade and Bauen (2013) also completed a cost model to compare production of microalgae in open ponds and PBRs. The projected case cost of microalgal production was 0.4 € kg^{-1} in an open pond, compared to ~3.8 € kg^{-1} in a tubular PBR. Interestingly, the majority of the cost in open ponds were due to operation, unlike the PBR where the capital cost was the most expensive factor. Huang *et al.* (2017) attributed these high operating costs to high capital investment, short lifespan, cost of cleaning, and temperature control of the PBR. The capital investment for 1000L batch PBRs can be up to \$33,600, with more modern prototypes estimated at \$3709 (Erbland *et al.*, 2020). Furthermore, the design will significantly influence the desired minimum biomass selling price (MBSP). For a horizontal tubular reactor the MBSP is \$0.78 kg^{-1}, but for a helical reactor the price increases to \$1.91 kg^{-1} due to the initial capital costs (Clippinger and Davis, 2019). These are low in comparison to the estimated cost of production of flat panel bioreactors which range between \$2.89- \$9.56 kg^{-1} (Banerjee and Ramaswamy, 2019). It is important to note that all the studies previously mentioned use different methods to estimate the cost of biomass production, and therefore these values should be used as estimates only, rather than definitive values. Additionally, biomass must be separated from the effluent. Common methods are centrifugation, filtration, sedimentation, dissolved air flotation, chemical flocculation, or electric field separation. All

have significant cost implications and may result in the biomass being unusable for future treatments (Hoh *et al.*, 2016).

1.2.4 Process intensification

Despite PBRs increasing the productivity of suspension cultures, scale-up inefficiencies continue to limit the economic feasibility of liquid cultures. Process intensification (PI) was conceptualised in the mid-1990's, and is often used to reduce the physical size of operations while achieving a production objective (Dautzenberg and Mukherjee, 2001). PI is a well-studied area of chemical engineering as it relies on novel engineering strategies (Van Gerven and Stankiewicz, 2009). As well as size reductions, PI has a specific emphasis on sustainability, with a focus on reduction of energy usage and waste production (Moulijn *et al.*, 2008), with an increase in safety (Laird, 2013). However, seven areas that have previously hindered PI technologies have been identified (Lutze *et al.*, 2010): 1) reliance on current technology; 2) risk of start-up; 3) expense of new plant facilities; 4) concern regarding health and safety; 5) decisions regarding how and where to apply PI; 6) few current PI technologies in place; and, 7) a lack of criteria to evaluate PI technology.

This does not mean we cannot apply PI to wastewater management. PI offers novel technology to an industry that is very much behind the times (Nikačević *et al.*, 2012). There are questions that must be answered before and during PI regarding cost, operation, and trials (Tian *et al.*, 2018). PI has been successfully implemented into the oil and gas, pharmaceutical, victual, and chemical industries, but water management has been considerably overlooked (Coward *et al.*, 2018). Based on their review of potential PI technologies, one of the recommendations was that distributed water treatment facilities could help meet PI and sustainability goals. For biotechnology, PI has already had influence by producing "quantum leaps" in performance (Prado-Rubio *et al.*, 2016). PI technology should be applicable to a range of photosynthetic microorganisms at a large scale (Joshi and Gogate, 2018).

1.2.5 Biocomposites as process intensification

Moving to a water-minimal system for microalgae or cyanobacterial growth is a potential method of PI for bioremediation. In this system, organisms typically grown in suspension open-ponds or PBRs would be immobilised onto a solid substrate with a binding system for retention. The characteristics of these biocomposites include one or more discontinuous phases that provide solid support, and a continuous phase creating a matrix around the discontinuous (Chandramohan and Marimuthu, 2011). Biocomposites using natural fibres are a widely used option due to their high tensile strength, low weight, and resistance to degradation (Gurunathan *et al.*, 2015). Natural fibres encompass plant, animal, and mineral materials which can be further

subdivided. Metabolically active cells can be combined to this structure to provide functional tools for a range of applications (Holzmeister *et al.*, 2018). It is important to note that the definition of a biocomposite is separate from biofilm reactors. Although there has been notable success in the development and treatment of domestic wastewaters utilising biofilms (Gou *et al.*, 2020) it does not meet the requirements of PI, and requires the user to have substantial knowledge of algae culturing techniques to maintain the biofilm (Hamano *et al.*, 2017).

To be truly functional as a biocomposite, biomass must be retained whilst allowing the exchange of molecules across a semi-permeable membrane (de-Bashan and Bashan, 2010). The matrix in which cells are held should also act as protection from contaminating species whilst providing room for cellular maintenance and growth (Perullini *et al.*, 2007). Biocomposites containing photosynthetic microorganisms theoretically support greater amounts of biomass within a smaller surface area with greater cellular retention (Flickinger *et al.*, 2007). Due to this increased retention and biomass there is greater flexibility in bioreactor design leading to increased bioremediation when compared to suspension or biofilm counterparts (Eroglu *et al.*, 2015). It is well documented that the behaviour of cells in 3D matrices significantly differ to those grown on 2D surfaces in biomedical applications (Ozbolat *et al.*, 2016; Jensen and Teng, 2020). 3D scaffolds support sustained cell proliferation and metabolic activity than their suspension counterparts making them more suitable for bioremediation of environmental pollutants (Zhao *et al.*, 2019). The ability to develop 3D structures containing metabolically active cells permits the development of customisable culture systems with minimal costs (Mehrotra *et al.*, 2020).

1.2.6 Previous successes of immobilised microbes

Many immobilised bioreactor designs initially focussed on entrapment within gel-like matrices such as alginate, chitosan, or gelatine (Desmet *et al.*, 2014; Pannier *et al.*, 2014; Lode *et al.*, 2015; Blocki *et al.*, 2017). Despite all the mentioned studies having some success in maintaining cellular metabolic activity there are significant problems with gel immobilised samples. Most notably is cell leaching due to matrix breakdown over time, and the effect of encapsulation on long-term viability due to interaction between the immobilisation matrix and the cells. The use of gel-hardeners such as $CaCl_2$ resulted in less efficient bioremediation of phosphate and NH_4^+ by alginate immobilised *Synechococcus elongatus* and cell leakage was observed from day six onwards (Castro-Ceseña *et al.*, 2016). Furthermore, increasing the thickness of a chitosan or alginate matrix to prevent cell leaching will only reduce mass transfer of target nutrients such as N, P, and CO_2 (Cortez *et al.*, 2017). The use of cross-linkers in hydrogel systems can cause intracellular damage to microalgae and cyanobacteria; most likely

due to the presence of glutaraldehyde (Moreno-Garrido, 2008). Furthermore, as gels are often up to 90% water, they are prone to rapid desiccation with poor mechanical properties when rehydrated due to damage to their 3D structure (Malik *et al.*, 2020).

To bypass the issues of gel immobilisation, Cortez *et al.* (2017) advocates for latex-based immobilisation to porous scaffolds. When immobilised to paper substrates with latex, the cyanobacterial biocomposites displayed up 7-10x higher O_2 production and CO_2 fixation over an operational period of 20 days compared to suspension cultures (Bernal *et al.*, 2014). This is not to say that latex based immobilisation is without issues – film formation and therefore subsequent efficacy of the biocomposites can be affected due to the coating formulation, drying, substrate choice, and biomass loading (Flickinger *et al.*, 2017). There are, however, methodologies available to mitigate these stressors. For example, Gosse *et al.* (2012) developed an immobilisation system that utilised wet latex that did not require drying to adhere to paper surfaces, reducing osmotic stress on all strains of microalgae and cyanobacteria used. Alternatively, Bernal *et al.* (2017a) reported on the use of dielectrophoresis to improve chain formation of *S. elongatus* PCC 7002 resulting in a maximal coverage of monolayers of the organism. This resulted in reduced self-shading without any reduction in cell viability. More recently, In-na *et al.* (2020) demonstrated that immobilised microalgae and cyanobacteria can significantly outperform their suspension counterparts for CO_2 sequestration over multi-week operations when immobilised within loofa biocomposites.

1.2.7 Affecting the success of biocomposites

As mentioned before, latex offers an alternative to gel based immobilisation systems as the reduce issues with mass transfer limitations. Latex films form in three stages; 1) consolidation – the evaporation of water to form a packed latex particle network, 2) compaction – particle deformation begins to eliminate void space between latex particles, and 3) coalescence – polymer diffusion is initiated between particles in contact with one another to form the complete film (Figure 1.4) (Price *et al.*, 2017).

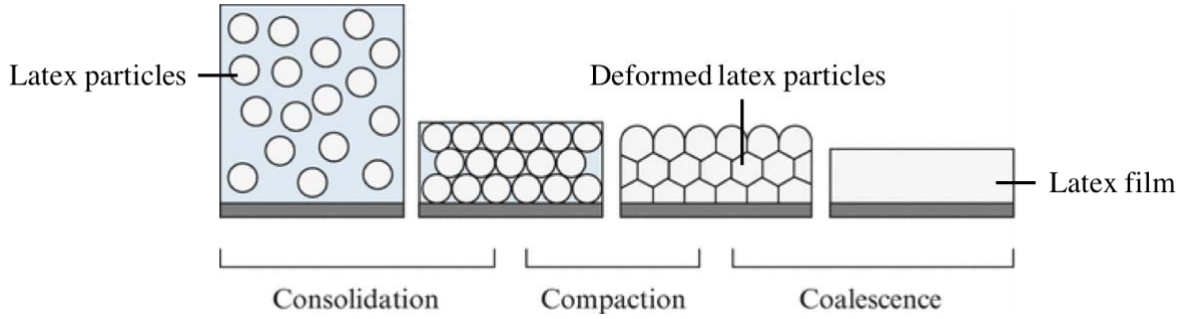

Figure 1.4. Schematic of the formation of latex films on a surface. Adapted from Price *et al.* (2017).

The formation of universal films is affected by several factors including glass transition temperature, particle size and distribution, particle morphology, humidity, and pigments (Wicks *et al.*, 1993; Reyes *et al.*, 2007; Chen *et al.*, 2011b; Churinthorn *et al.*, 2015; Ugur and Sunay, 2016; Limousin *et al.*, 2019). The drying temperature can have significant effects on latex coalescence on porous substrates, where there absorption of the aqueous phase of the latex is rapidly absorbed into the substrate which may result in cracking of the latex (Mesic *et al.*, 2019). However, the optimal drying temperature for an evenly dried film in this study was 40^{O}C which would not be suitable for the immobilisation of biomass as it would cause significant damage to cells. There is also concern regarding gas and liquid mass transfer to immobilised microbes which may limit cellular productivity. There is an inverse relationship between film thickness and mass transfer, therefore film thickness should be minimised whilst ensuring microbes remain immobilised yet metabolically active (Schulte *et al.*, 2016). The mass transfer coefficient is increased from the suspension culture when 6.5mg of *Clostridium ljungdahlii* OTA1 were immobilised from 26 $k_La_{apparent}$ (h^{-1}) to 56 $k_La_{apparent}$ (h^{-1}) respectively, with a linear increase when the amount of biomass was increased. However, Bernal *et al.* (2017b), found that there was a drop in hydrogen production by immobilised *Rhodopseudomonas palustris* as the latex film increased in thickness and cell retention which they attributed to mass transfer limitations as a result of poor moisture diffusion coefficients and gas permeability. This is compounded by In-na *et al.* (2020) finding that CO_2 mass transfer was limited by latex coatings by calculating the CO_2 diffusivity of the latex when 26 μm thick (10^{-15} – 10^{-13} m^2 s^{-1}) compared to the diffusivity of microporous solids (~ 10^{-8} m^2 s^{-1}), liquids (~ 10^{-9} m^2 s^{-1}), and polymer films (~ 10^{-13} m^2 s^{-1}). Therefore, a balance must be struck between latex thickness which will impact cell retention versus cells remaining metabolically active with mass transfer of CO_2 and nutrients via the growth media not being limited.

Textiles offer an alternative scaffold system as the textile industry is in a prime position to shift to a circular economy from a linear (take-make-dispose) one (Koszewska, 2018). The

production of textiles is set to increase to 130 million tonnes per annum by 2025 (Yang Qin, 2014), with more than 60% of textiles produced being derived from synthetic oil-based materials (Koszewska, 2018). In particular, polyester-cotton blend fabrics have the largest environmental impact during their production as it is difficult to recycle blended materials (Zhang *et al.*, 2018), with textiles making upto 5% of landfill waste in 2014 (Weber *et al.*, 2017). When selecting textiles it is important to consider the rugosity as the increased number of microstructures at the surface increases the hydrophobicity which may affect the formation of a uniform biocoating (Melki *et al.*, 2019). When utilising woven fabrics, the diameter and spacing of the weave affects the size of the interyarn pores which can increase the swelling capacity of the textile (Sarkar *et al.*, 2009) which may lead to imperfections of the biocoatings. Additionally, highly porous fabrics such as cotton, have high levels of pore-collapse due to structural fragility leading to alterations in the number and distribution of pores (Dhiman and Chattopadhyay, 2020). Conversely, non-woven fabrics pore size is affected by the fibre density, with less dense fibres having larger pores (Alassod and Xu, 2020) which may lead to poorer retention of immobilised microbes. This porosity ultimately impacts the homogenous formation of latex binders. The more porous structures result in faster wicking which results in non-uniform film formation across the substrate (Khosravi *et al.*, 2014).

1.3 Understanding the metabolic response to immobilisation

1.3.1 The need for greater biological understanding

Despite the application and evident successes of PI to bioremediation there is still a significant gap in understanding the organisms' response to these engineered environments. To progress the technology from research to commercial application, an increase in system productivity and reproducibility is required, which Osanai *et al.* (2017) argued can be achieved through understanding molecular biology and biochemistry of the target organisms. In many engineering projects utilising microalgae and cyanobacteria, a trial and error approach has been adopted resulting in delayed progress (Lü *et al.*, 2011). Although discussing systems biology in the context of biofuels, Klanchui *et al.* (2017) advocated that a better understanding of cellular metabolism is essential to optimising engineering processes — a concept that should also be applied to bioremediation. By approaching engineered systems with a multi-omics (genomics, proteomics, and transcriptomics) toolbox a more universal understanding of the biological agent in the system can be achieved compared to the traditional approach of measuring abiotic factors (Pinu *et al.*, 2019).

Previously used physiological responses to immobilisation include; chlorophyll fluorescence (Rooke *et al.*, 2008), oxygen production (Léonard *et al.*, 2010), metal uptake (De Philippis *et*

al., 2003; Chakraborty *et al.*, 2011), carbon-14 assimilation (Rooke *et al.*, 2011), N and P uptake (Lau *et al.*, 1997; Fierro *et al.*, 2008; Castro-Ceseña *et al.*, 2016), hydrogen production (Das *et al.*, 2015; Swe Cheng *et al.*, 2017), and CO_2 sequestration (Ekins-Coward *et al.*, 2019; Umar *et al.*, 2019). Although all are insightful and have helped to progress their respective fields, Flickinger *et al.* (2017) argued that indirect methods of understanding the impact of immobilisation does not provide enough information about the biology of the system and provides little insight to the gene regulation of the organisms.

1.3.2 Transcriptomics

Dickson *et al.* (2012) was one of the earliest studies to examine gene expression of immobilised *Synechocystis*. Using microarrays and qRT-PCR it was determined that encapsulation in gels significantly altered the expression of up to 1143 genes compared to liquid media. Advances in whole genome sequencing and annotation have provided a wealth of information regarding cyanobacterial genomes and provide a basis for transcriptomic experiments (D'Agostino *et al.*, 2016). As of 2018, there are more than 200 cyanobacteria genomes sequenced and publicly accessible (Hagemann and Hess, 2018). Cyanobacteria lend themselves to biotechnological applications due to their metabolic plasticity; however, this plasticity is why it is imperative to study this metabolic flux to ensure control and sustain functioning in biotechnology (Angermayr *et al.*, 2009). Although proteomics made some significant advances in this area, studies were often limited due to non-representative results as 2D gel electrophoresis techniques exclude a number of protein types (Burja *et al.*, 2003) and often only reveal around 20% of the organism's proteome (Gao *et al.*, 2015). Therefore, the combination of transcriptomics and high-throughput sequencing, commonly referred to as RNA-sequencing (or RNA-Seq) has been the preferred method for gene expression studies since 2015 (Tachibana, 2015). A comprehensive review of transcriptomic methodologies is outside the scope of this thesis, however the reader should refer to Lowe *et al.* (2017). In short, the RNA-Seq methodology is as follows - total RNA is extracted from the organism, and converted to cDNA by reverse transcription. During this process, ribosomal RNA can be depleted. Short fragments of cDNA, usually around 100 base pairs (bp) are sequenced by fragmentation, followed by amplification of randomly primed cDNA, and ligation. These short sequences are then amplified by PCR and then aligned to a reference genome. The accuracy of RNA-Seq is improved by the number of reads of a sample as overlap of the fragments and detection of low-abundance transcripts increases (Kukurba and Montgomery, 2015; Lowe *et al.*, 2017) (Figure 1.5).

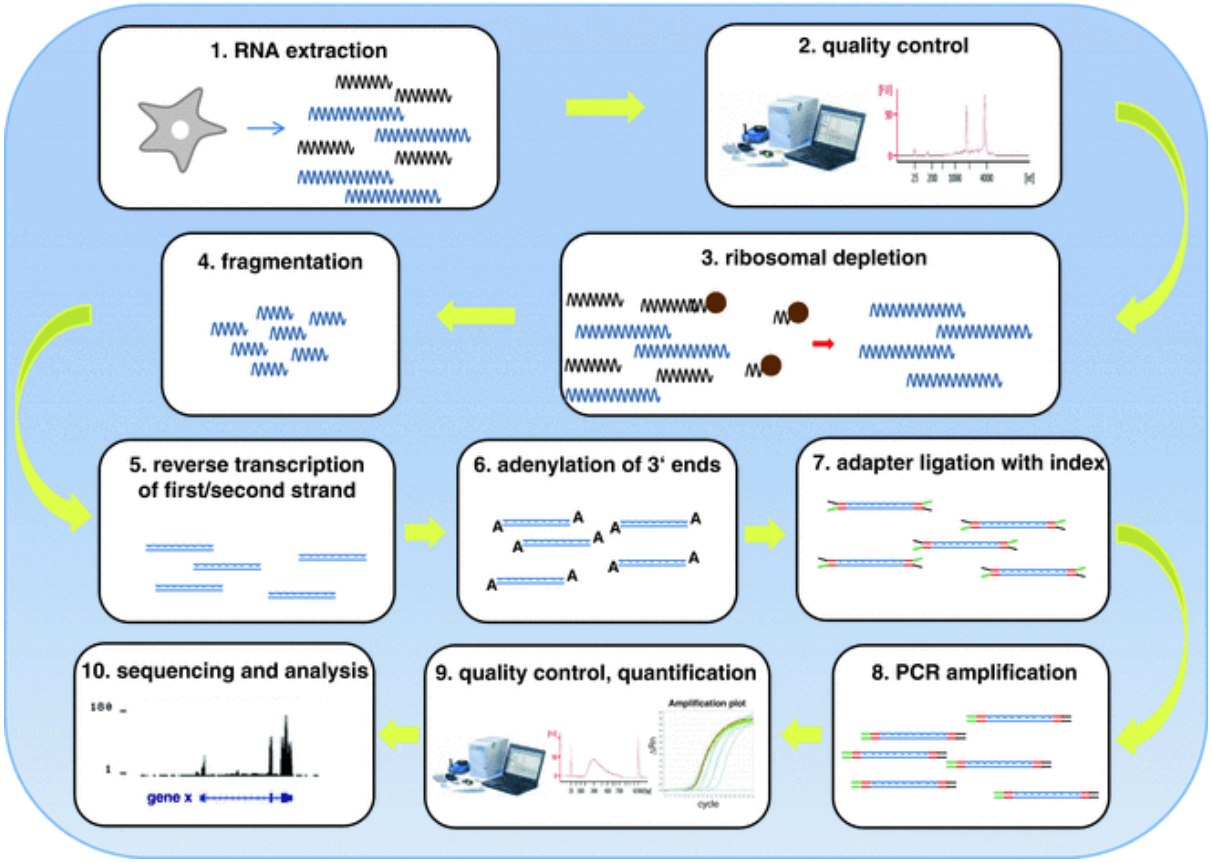

Figure 1.5. A simplified process for RNA-Seq library preparation prior to computational analysis (Goeman and Fanciulli, 2016).

After sequencing, computational analysis is required to determine differential gene expression. With the increased affordability of RNA-Seq the number of analytical packages to process the vast amounts of data has increased, resulting in significant divergence in analysis (Conesa *et al.*, 2016). Analysis pipelines generally comprise four stages; 1) quality control and mapping to a reference genome, 2) quantification of reads with genes, 3) normalisation of gene expression data, and 4) modelling of sample to produce statistical values (Stark *et al.*, 2019) (Figure 1.6). At each of these stages there are a number of approaches which can be combined in the analysis pipeline affecting the conclusions of studies (Seyednasrollah *et al.*, 2015). This was exemplified by Sahraeian *et al.* (2017) using 39 analysis packages, resulting in 490 analyses. When looking for differentially expressed genes (DEG), DESeq2 had higher detection when compared to edgeR, but accuracy was significantly affected by alignment methods, with alignment-free techniques delivering higher quality results. Although the molecular toolbox for cyanobacteria is lacking compared to *E. coli* and *S. cerevisiae*, the cost and ease of transcriptomic studies is improving for select strains of cyanobacteria (Sun *et al.*, 2018). It is important to note that some of the most popular packages for DEG analysis, such as DESeq2, edgeR, and Limma (Dillies *et al.*, 2013) assume that genes are invariant which is not appropriate

for prokaryotic DEG analysis as most levels of gene expression are altered under stress conditions (Berghoff *et al.*, 2017). To correct this, Mahmud *et al.* (2020) have developed a prokaryotic-specific pipeline for differential gene expression and pathway analysis – ProkSeq. ProkSeq utilises shrunken log2 fold changes (LFC) developed by Zhu *et al.* (2019) to normalise the nucleotide base count during differential gene expression analysis to ensure data are comparable between samples. LFC utilises information from all genes to reduce the higher levels of variation observed in genes with low expression. In ProkSeq this is performed by default prior to DESeq2 and saved as a separate output.

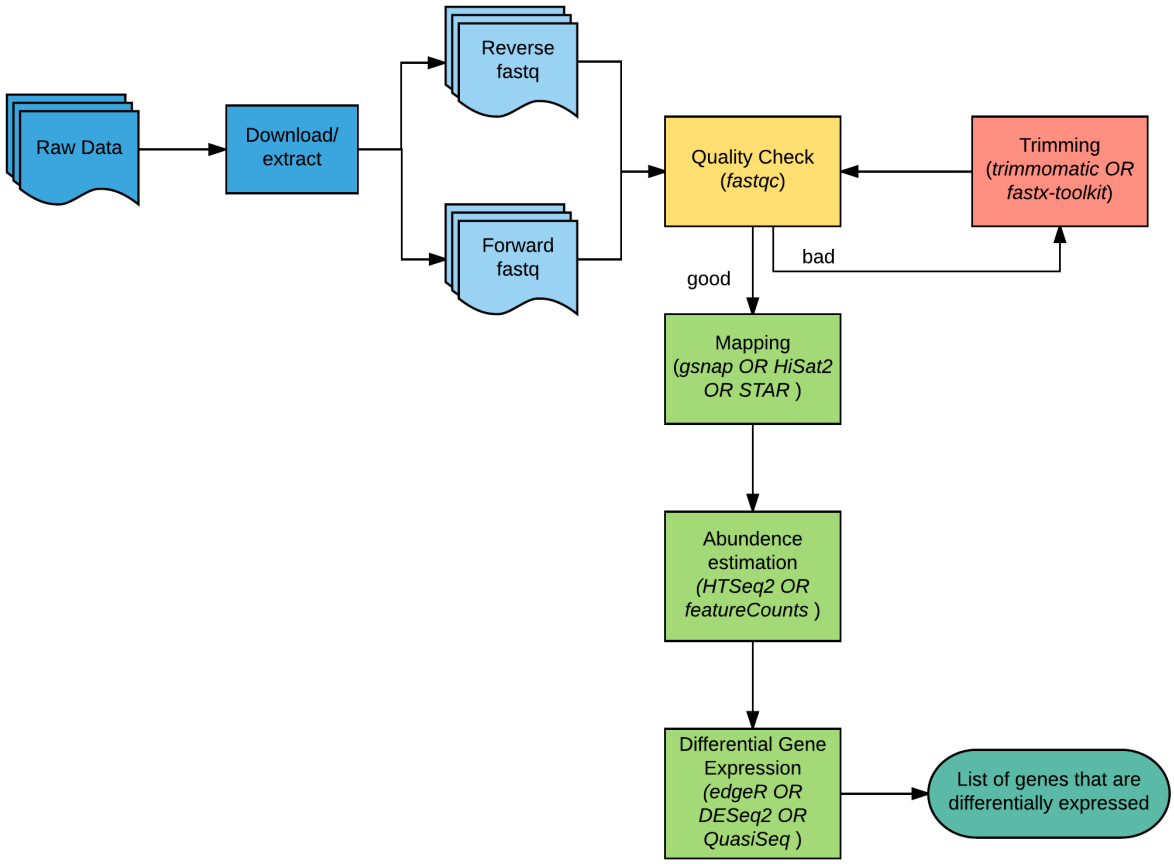

Figure 1.6. An overview of RNA-Seq analysis pipeline with suggested work packages (Chudalayandi, 2020)

There are a wealth of papers utilising RNA-Seq –Scopus returned over 28,000 papers returned that include 'RNA-Seq' in their title, abstract, or key words. In contrast, there are only 53 results for 'RNA-Seq' and 'cyanobacteria', and none for 'RNA-Seq', 'cyanobacteria', and 'immobilise/immobilize' (27.10.2020). Dickson *et al.* (2012) provide, as far as the author has found, the only study researching the global transcriptomic response of immobilised cyanobacteria. Dickson *et al.* (2012) utilised microarrays, but found that up to 1143 genes were

differentially expressed (>1.5 fold change, $P < 0.01$) by *Synechocystis* sp. PCC 6803 after immobilisation in silica gel. In contrast, there were 254 documents returned when searching for 'cyanobacteria' and 'immobilisation', and 'cyanobacteria' and 'bioremediation' returned 601 results. This suggests there is a large gap in the literature combining the efficacy of immobilised cyanobacteria as bioremediators, and our biological understanding of this process as discussed by Dickson and Ely (2013). Particular attention should be paid to the photosynthetic machinery as exemplified by Dickson *et al.* (2012). Fernández-González *et al.* (2020) focussed on the *rbc*L and *psb*A genes associated with RuBisCO and photosystem II respectively. Both increased in expression with increased N supply. Concordantly, P limitation caused 823 genes to be differentially expressed after P limitation, while genes involved in both photosystems were significantly downregulated (Teikari *et al.*, 2015). Additionally, the physical confinement of cyanobacteria into biocomposites may affect the photosynthetic machinery as demonstrated by (Moore *et al.*, 2020). Physical restriction in 2.5% agarose led to disassociation of phycobilisomes from the photosynthetic reaction centres, which the authors attributed as a failsafe mechanism when space is limited, despite nutrients and light availability not limiting. Although it is hypothesised that there will be some mass transfer limitation of nutrient, lack of available space may also have a significant effect on gene expression related to photosynthesis. There is, however, a significant gap in the literature examining the genetic response of cyanobacteria in wastewater even when in suspension ponds or PBRs. We attribute this to a lack of collaborative projects between engineering and molecular biology which would add significant value to the data being collected by both disciplines. There are several stressors exerted on cyanobacteria during mass cultivation in wastewater, the genomic response to which is relevant to industrial biotechnology (Rachedi *et al.*, 2020).

1.4 Synthetic Biology

1.4.1 Cyanobacteria and synthetic biology

With systems biology providing greater insight into cellular processes and metabolic activity during bioremediation, this detailed knowledge can be exploited by metabolic engineers to enhance bioremediation through the introduction of new or repeated metabolic pathways (Dangi *et al.*, 2019). Synthetic biology combines genetic engineering, systems biology, and computational modelling to design biological parts and systems, or redesign existing systems for greater productivity; tailoring the organism to work as effectively as possible (Huang *et al.*, 2010; Sharma *et al.*, 2018). Much of the effort of cyanobacterial synthetic biology has been geared towards greater production of fuels and fine chemicals via improving photosynthesis by enhancing light harvesting efficiency and CO_2 fixation (Wang *et al.*, 2012b).

Cyanobacteria—coined as the green *E. coli* (Ruffing and Kallas, 2016)—are good candidates for synthetic biology due to their naturally efficient production pathways for proteins and carbohydrates. Modification of the cyanobacterial genome can enhance the productivity and use of cyanobacteria in biotechnology (Ng *et al.*, 2020). Cyanobacteria offer advantages over the traditional models of *E. coli* and *S. cerevisiae* due to their low nutrient requirements and their metabolic plasticity, enabling them to respond to environmental fluctuations (Xiong *et al.*, 2017). They are also advantageous over microalgae due to their higher photosynthetic levels and growth rate with minimal nutrient requirements (Quintana *et al.*, 2011). The potential of cyanobacteria has been unlocked through advances of omics technologies and the increasing capacity to manipulate genetic sequences (Santos-Merino *et al.*, 2019). Compared to eukaryotic microalgae and plants, the cyanobacterial genome is relatively amenable to engineering (Ducat *et al.*, 2011; Lau *et al.*, 2015). The novel metabolic pathways are the result of combining enzymatic activities of various metabolic networks within a host cell or "chassis" (Hagemann and Hess, 2018). Generally, manipulation of the genome is for either improving strain tolerance or improving carbon flux to the desired product (Sengupta *et al.*, 2018; Singh *et al.*, 2018). Sun *et al.* (2018) detailed the progress of cyanobacterial synthetic biology which has rapidly advanced with the advent of CRISPR/Cas9 systems, but is still lacking compared the metabolic toolboxes available for *E. coli* and *S. cerevisiae*. Cyanobacteria have multiple chromosomes which means that ensuring a homozygous mutant is produced can be difficult as often selection must be done over multiple generations increasing the time and resources required for a single modification (Ramey *et al.*, 2015). CRISPR/Cas9 systems have been utilised to overcome this polyploidy as it affects all copies of the chromosome simultaneously as first demonstrated by the disruption of the *nblA* gene of *S. elongatus* UTEX 2973 (Wendt *et al.*, 2016). The success of Wendt *et al.* (2016) modification using CRISPR/Cas9 was that the Cas9 must be expressed transiently using the *nblA*-targeting pCRISPomyces-2 plasmid, as high levels of the Cas9 protein resulted in cell death.

1.4.2 Synthetic biology and wastewater bioremediation

Much of the literature regarding cyanobacteria and synthetic biology is focussed on increasing production of desired chemicals, with the potential for using wastewater as a growth medium due to its high nutrient density (Bhuvaneshwari *et al.*, 2019). It could be argued that wastewater treatment could benefit from synthetic biology. Pathak *et al.* (2018) noted that genetic engineering can aid culture maintenance, biomass production, and be combined with the production of high-value metabolites. Wastewater treatment already relies, to some degree, on photosynthetic microbes for bioremediation; this could be intensified by enhancing the robustness of target synthetic microbes, simultaneously increasing CO_2 capture (Gerotto *et al.*,

2020). Enhanced biosorption and biotransformation of nutrients or pollutants from wastewater will lead to higher treatment efficiency, but this is still within its infancy and limited to laboratory scale experiments. Li *et al.* (2018) genetically engineered *S. elongatus* UTEX 2973 with a reduced doubling time, thus increasing the growth rate by introducing the *pilN* gene encoding the Tfp pilus assembly protein. Furthermore, strains could be modified to increase uptake of target pollutants (Nzila *et al.*, 2016), as first demonstrated by Chakrabarty (1985) with an engineered strain of *Pseudomonas* to enhance oil degradation. More recently, *Synechocystis* sp. PCC 6803 expressed *mrlA* encoding microcystinase from *Sphingopyxis* sp. USTB-05, increasing microcystin degradation three-fold when compared to the native strain (Dexter *et al.*, 2018). Although microcystins and oil are not significant problems for domestic waste, the ability of recombinant strains to target specific compounds is promising to tailor synthetic strains for domestic waste streams and help society reach the desired zero-waste targets (Garlapati *et al.*, 2019). However, there are concerns that strains developed in tightly controlled lab environments will not be sufficiently robust to survive in a mixed-waste stream (with variable pH, temperature, and nutrient availability), with the overexpression of novel genes or pathways causing significant burden on the overall metabolic activity of the cell (Zhang *et al.*, 2020).

1.4.3 Concerns regarding synthetic biology

There are concerns about the use of synthetic microbes (Dana *et al.*, 2012). Accidental release of synthetic strains of photosynthetic microbes could have significant, permanent effects on ecosystems (Gressel *et al.*, 2013). With large-scale cultivation, the escape of synthetic strains is inevitable, thus care should be taken to ensure the strain could not establish outside of a controlled environment (Gressel *et al.*, 2014). Containment within PBRs provides less risk as reactor design could feature mitigation measures such as sterilisation prior to release of wastewater, but open-ponds must have enhanced containment such as catchment areas, filters, or UV/gamma irradiation, and monitoring strategies when containing synthetic strains (Glass, 2015). Physical controls can be extremely costly, so biocontainment is recommended by engineering strains to have specific requirements for survival, or gene expression that would not be naturally found. Clark *et al.* (2018) developed a *Synechococcus sp.* 7002 mutant that required 5% CO_2 supplementation for growth and lactate production. The *ccmK2k1LMN* operon was deleted meaning the carbon concentrating mechanism was disrupted. Due to the deletion of the operon, concerns about genetic drift were alleviated as the high levels of CO_2 required cannot be found environmentally. Alternatively, a synthetic strain of *S. elongatus* PCC 7942 has been developed to be dependent on phosphite (PO_3^{-3}) rather than any other form of P. Phosphite is uncommon in the natural environment meaning if any escape were to occur the

strain would not be viable long-term (Motomura *et al.*, 2018). Although this strategy was effective for biocontainment, it would not be suitable for bioremediation applications as the strain would not be effective for PO_4^{-3} removal. Alternatively, control of expression of the target genes can be achieved by the insertion of the *lacI* gene upstream of the target gene, which represses expression unless isopropyl β-D-1-thiogalactopyranoside (IPTG) is present (Ducat *et al.*, 2012; Lin *et al.*, 2020; Qiao *et al.*, 2020).

Despite science attempting to prevent accidental release and utilising genetic controls, synthetic strains are subject to legislative regulations, frameworks, and risk assessments. The development of risk assessments is complex as the fitness of the synthetic strain, all native species of photosynthetic microbes, and all environmental perturbations must be accounted for if models for risk assessment are to have any real meaning (Gressel *et al.*, 2014). Additionally, Marchant *et al.* (2011) argued that legislation has not been updated or advanced as rapidly as the science and technology. Furthermore, communication within biological science, particularly synthetic biologists and ecologists is often hindered by differences in terminology and research techniques (Kuiken *et al.*, 2014). Across research disciplines the definition of "risk" regarding synthetic biology varies. This variation influences behaviour associated with synthetic biology as simple questions of "what is the risk?" becomes multifaceted, and thus more complicated (Wolfe *et al.*, 2016). To help disciplines effectively communicate, the concept of "trading zones" was coined by Gorman (2002). "Trading zones" encourage communication between disciplines to solve existing problems and generate novel scientific ideas. It is important that these trading zones are holistic and welcome the input of a range of disciplines not limited to scientists, sociologists, and policy makers (Gorman, 2010). The argument can also be made to involve the public to prevent animosity towards, and improve understanding of synthetic organisms (Tilman *et al.*, 2009). The immobilisation of synthetic strains potentially alleviates some of the concerns about the accidental release of synthetic organisms by acting as another physical control. When immobilised onto a loofah scaffold there was just 0.61% release of *S. elongatus* PCC 7942 after 72 hours of immobilisation (In-na *et al.*, 2020). If both physical and genetic controls were applied to synthetic species, the risk of release to environment could significantly reduced and would pave the way to process intensification of wastewater treatment.

1.5 Conclusion

By 2050, it is predicted that 26% of the global population will be affected by organic water pollution due to the rapid rise in urbanisation, with people in developing nations disproportionately affected (Wen *et al.*, 2017). Environmental risk differs from economic,

social, and health risk, but should be considered when policy is developed regarding wastewater management (Hanjra *et al.*, 2012). Despite these risks, proper utilisation and treatment of wastewater may prove an invaluable resource when one considers the shortage of fresh water and increasing volumes of wastewater produced (Saad *et al.*, 2017). The wastewater treatment industry is rapidly approaching failure to meet the demands, and without radical change will lead to water insecurity and scarcity. Process intensification using immobilised and synthetic photosynthetic microbes may offer an alternative to centralised systems, acting as start-of-pipe, or point-source, bioremediators. This has been exemplified by the Horizon 2020 project, Living Architecture (LIAR). LIAR aimed to develop a selectively programmable PBR "wall" that could be installed into human dwellings, through a convergence of engineering, computing, and synthetic biology. Although LIAR was successful in all of these, it was noted that performance was ultimately limited by both wild-type and synthetic *Synechococcus elongatus* being used in suspension in the PBR and microbial fuel cell portions of the wall. Ultimately, process intensification of the bio-components of the wall is needed to maximise productivity which we propose can happen with the immobilisation of both the wild-type and synthetic strains being immobilised into our iteration of a biocomposite. The immobilisation of synthetic strains alleviates some of the concerns of accidental release to the environment, whilst increasing the productivity of the whole system.

2 Textile-based cyanobacteria biocomposites for potential environmental remediation applications

2.1 Introduction

The growing global human population is placing increasing demands on water resources—demands which are unlikely to be met with current centralised treatment systems and practices (Lofrano and Brown, 2010; Verstraete and Vlaeminck, 2011). If the water industry is to keep pace, major technological innovations rather than incremental and piecemeal shifts in treatment practices are required (Thomas and Ford, 2006b; Tanner *et al.*, 2018). The urgency for technology and process transition is heightened when viewed from a climate change context, with municipal wastewater treatment accounting for approximately 3% of global electricity consumption and 5% of non-CO_2 greenhouse gas emissions (Li *et al.*, 2015).

Decentralised (or point-source) systems present an alternative option, but to be effective they must be affordable, ensure both human and environmental safety, be user friendly (effectively zero maintenance), be fully compliant with effluent discharge regulations, and (ideally) should make a tangible contribution to the circular economy (Munoz and Guieysse, 2006; Grant *et al.*, 2012; Li *et al.*, 2015; Cuellar-Bermudez *et al.*, 2017; Oladoja, 2017). Phycoremediation (the use of algae or cyanobacteria for environmental clean-up) is one approach that could deliver many of these requirements, with the capacity to combine wastewater and atmospheric remediation within a single treatment option, all the while generating biomass for bioprocessing (Olguín, 2003; Rawat *et al.*, 2011; Kumar *et al.*, 2018; Ansari *et al.*, 2019).

Due to their broad abiotic tolerances and metabolic flexibility, the use of cyanobacteria in wastewater treatment is well established (Oswald and Gotaas, 1957), forming an important part of mixed community activated sludge systems (Martins *et al.*, 2011). However, targeted treatment processes based primarily on cyanobacteria are still under development. The very nature of suspension-based cultivation (generally conducted in high rate algae pond systems) remains one of the main challenges facing the roll-out of microalgae and cyanobacteria treatment processes, with operational issues including poor batch-to-batch consistency, high economic cost of biomass separation, and contamination by non-target species (Christenson and Sims, 2011); notwithstanding the substantial land requirement. Closed photobioreactors (PBRs) have been employed to mitigate abiotic variation and contamination (Mata *et al.*, 2010; Tredici *et al.*, 2015); however, capital and operational costs are prohibitive (Huang *et al.*, 2017; Acién *et al.*, 2018).

The need for suspension-based systems is being subverted with the advent of a range of biofilm reactors (Zhuang *et al.*, 2018; Peng *et al.*, 2020a; Waqas *et al.*, 2020; Yang *et al.*, 2020), treating domestic (Gou *et al.*, 2020), industrial (Johnson *et al.*, 2018; Hillman and Sims, 2020) and even marine wastewaters (Peng *et al.*, 2020b). However, these systems still require sufficient operator competency to culture and maintain a metabolically active biofilm (David *et al.*, 2015; Hamano *et al.*, 2017). Iterations of these systems that reduce, or ideally eliminate, the need for regular culture maintenance is desirable in terms of process cost, accessibility and acceptability. Attempts in this direction include trialling phototrophic granular biomass (photogranules) (Kumar and Venugopalan, 2015; Trebuch *et al.*, 2020); which, while promising in the context of a wastewater treatment plant, would not necessarily be suitable if the ambition for decentralisation extended as far as treatment systems for individual properties. Given such a scenario, a more radical vision of a biofilm reactor is needed.

Engineered biofilms, also known as living biocomposites, are gaining traction in atmospheric and low flow wastewater bioremediation applications (Estrada *et al.*, 2015; In-na *et al.*, 2020). These biocomposites comprise living microbes immobilised within a semi-porous matrix (binder), forming a biocoating, which is then applied to a structural scaffold (de-Bashan and Bashan, 2010); theoretically supporting greater biomass loading per unit area with minimal cell loss (Flickinger *et al.*, 2007). Unlike natural biofilm systems, the use of immobilised biomass allows greater flexibility in reactor design, enabling increased reaction rates due to improved robustness, with greater reuse of biomass (Eroglu *et al.*, 2015). Successful immobilisation has been demonstrated to improve O_2 production (an indicator of photosynthetic activity) between 7-10x compared with suspension cultures (Bernal *et al.*, 2014). However, the efficacy of biocomposites can be affected by many factors during preparation; including, but not limited to the coating formulation, substrate properties, and the extent of biomass loading (Flickinger *et al.*, 2017); although novel fabrication methods show promise (Fidaleo *et al.*, 2014; Bernal *et al.*, 2017a; Chen *et al.*, 2020).

The current study drew inspiration from the EU H2020 Living Architecture project, which sought to develop highly distributed autonomous wastewater treatment systems for individual properties (Armstrong *et al.*, 2017). We have extended the scope of the Living Architecture brief by evaluating some of the key technical constraints to immobilising cyanobacteria as biocomposites. Using two strains of *Synechococcus elongatus* (PCC 7942 and CCAP 1479/1A), we screened a range of binders formulated from latex, petroleum, shellac or chitosan, with four affordable and easily accessible textile scaffolds (a woven 80/20 polyester-cotton blend, a woven 100% bamboo, a non-woven 40/60 wool-polyester blend, and a non-woven 100%

polyester). Binders were screened for toxicity and adhesive properties, with biocomposite viability determined by measuring net photosynthetic CO_2 uptake over a 20-day experimental period.

2.2 Methods

2.2.1 Cell cultivation

Synechococcus elongatus PCC 7942 was grown in Blue-Green Medium (BG11), and *S. elongatus* CCAP 1479/1A in Jaworski's Medium (JM) without cyanocobalamin, thiamine HCl, and biotin, in 10 L polycarbonate (Nalgene) carboys with constant air supply at 18 °C ± 2 ºC, and a 16L:8D photoperiod (mean luminance of 35 µmol m^2 s^{-1}) using 30 W daylight-type fluorescent tubes (Sylvania Luxline Plus, n = 6).

2.2.2 Binder adjustments

A selection of binders were screened for suitability for cyanobacteria immobilisation. Ten synthetic acrylic, styrene and polyurethane latex binders were selected from a library of proprietary and commercial binders, gifted by Prof. Michael Flickinger, North Carolina State University, USA, having previously been assessed for microbial immobilisation. Six plant-based binders (AURO Paint Company, UK and Germany) were similarly selected (Bernal *et al.*, 2014; In-na *et al.*, 2020). The initial percentage solids content of the liquid binders was determined by oven drying to a constant mass at 100 °C. The binders were adjusted to pH 7 using 0.1 M or 0.5 M acetic acid, or 0.1 M or 0.5 M ammonium hydroxide (Table 2.1). Shellac (orange, pure, flake, Fisher Scientific, CAS-9000-59-3, MDL no. 148309) was ground to a fine powder by mortar and pestle and dissolved in 90% ethanol at 50 ºC. Chitosan (99.9%, ACROS Organics, CAS-9012-76-4, MDL no. MFCD00161512) was dissolved at 18 ºC in 0.3 M acetic acid.

Table 2.1. Name, coding and available compositional details of the binders used in this study. Also shown are the binder adjustments required to make 1 mL of neutral pH binder. AA = acetic acid, AH = ammonium hydroxide, N/A = not available.

Binder	Code	Main components	Initial % solids content	Binder volume (mL)	dH_2O to dilute to 5% (mL)	Neutraliser	Final volume (mL)
AURO 160 (silk gloss woodstain, clear)	160	Castor oil, cellulose, colophony glycerol ester, drying agents, metal soaps, linseed oil, mineral pigments, silicic acid, surfactants made from rapeseed oil, water, xanthane	13.68	0.364	0.564	0.5M AA	0.073
AURO 251 (clear lacquer, glossy)	251	Castor oil, castor oil as amino soap, cellulose, colophony glycerol ester, drying agents, metal soaps, linseed oil, mineral fillers, sunflower oil, surfactants made of rapeseed and castor oils, water	29.10	0.172	0.802	0.5M AA	0.026
AURO 261 (clear lacquer, matt silk)	261	Castor oil, castor oil as amino soap, cellulose, colophony glycerol ester, drying agents, metal soaps, fatty acids, linseed oil, mineral fillers, silicic acid, sunflower oil, surfactants made of rapeseed oil, water	19.52	0.257	0.718	0.5M AA	0.026
AURO 320 (emulsion paint, white)*	320	Mineral fillers, water, Replebin®, titanium dioxide, cellulose, surfactants made of rapeseed-, castor oil, ammonia and thiazoles	52.57	0.095	0.811	0.5M AA	0.093
AURO 321 (emulsion paint, white)	321	Mineral fillers, water, Replebin®, titanium dioxide, cellulose, surfactants made of rapeseed-, castor oil, ammonia and thiazoles	52.85	0.094	0.890	0.1M AA	0.016
AURO 379 (colour wash binder)	379	Water, shellac (as ammonia soap), alcohol, xanthane, walnut oil, rosemary oil, lavender oil	9.01	0.545	0.273	0.1M AA	0.182
Rhoplex SG-10M	1	Butyl acrylate/Methyl methacrylate	51.30	0.098	0.891	0.1M AA	0.011
Interkem A510	2	Acrylic polymer	25.08	0.201	0.781	0.1M AA	0.018
Baycusan C-1000	3	Polyester polyurethane	52.60	0.096	0.903	0.1M AA	0.001
Baycusan C-1004	4	Polyester polyurethane	42.68	0.119	0.858	0.5M AH	0.024
Nuplex Setaqua 6776	5	Acrylic polymer	42.77	0.118	0.835	0.1M AA	0.047
Rhoplex SF-012	6	Acrylic polymer	42.74	0.117	0.883	-	0.000
Rhoplex SF-3122	7	Acrylic polymer	64.36	0.079	0.909	0.1M AA	0.012
JP 912	8	Acrylic polymer	51.12	0.098	0.882	0.1M AH	0.020
PD-0413	9	N/A	80.37	0.063	0.931	0.1M AA	0.006
HB 3691-M	10	Polyester polyurethane	48.42	0.105	0.848	0.1M AA	0.047

2.2.3 Toxicity testing

The binders were initially screened for cell growth impacts. One millilitre of binder was added to 1 mL of 7-10 days old culture (n=3) in a multiwell plate. In separate wells, 1 mL of binder and 1 mL of growth medium were combined to screen for interactions. One millilitre of cells diluted with 1 mL of sterile de-ionised water (dH_2O) was run as a baseline for cell growth. Each treatment replicate was mixed daily by forward and reverse pipetting for seven days, when cell density was determined using an improved Neubauer haemocytometer with a Leica DMi 8 microscope with bright field contrast at 400x magnification and viewed using LasX software. The most suitable binder for each strain was carried forward for subsequent adhesion testing, wherein the range of percentage solids contents was expanded to 2.5, 7.5, and 10% (>10% solids inhibits gas exchange (Umar, 2018)).

2.2.4 Adhesion testing

The textile substrates were: 1) a woven 80/20 polyester-cotton blend (cotton) purchased from Aow RungRuang Co. Ltd, Bangkok, Thailand; 2) a woven 100% bamboo (bamboo) was purchased from WBL Fabrics, UK; 3) a non-woven 40/60 wool-polyester blend (wool blend); and 4) a non-woven 100% polyester (polyester) were purchased from the Wool Felt Company, UK. The polyester-cotton blend was selected as it exhibited high levels of cyanobacteria attachment in a separate study (In-na et al. unpublished). The textiles were autoclaved, then dried at 105 ºC for 3 h, and stored in a silica gel desiccator until use. When required, the textiles were cut into 0.5 x 0.5 cm^2 pieces. Cyanobacteria were centrifuged at 1720 relative centrifugal force (RCF) (Sigma Laboratory Centrifuges, 3K18 C) for 30 min at 20 ºC. If sufficient biomass wasn't recovered, the supernatant was removed and additional culture was added to the pellet and the centrifugation step was repeated. The cyanobacteria and binders were combined in sterile Eppendorf tubes, first by gentle pipetting, and then by vortex mixing. A range of cell-binder formulations (biocoatings) were produced at 1, 2.5, 5, and 10% (v/v) cell content to understand the binder's robustness. Once combined, 100 μL of each biocoating was pipetted onto the textiles to form biocomposites. In a separate Eppendorf tube, the equivalent volume of culture was added to the same volume of dH_2O and counted using a Neubauer haemocytometer to estimate biocoating cell density and hence biocomposite cell loading. The biocomposites were dried in darkness at 20 ± 2 ºC for 24 h, then added to 1 mL of growth medium in well plates wrapped in aluminium foil to prevent photosynthesis. The plates were placed on a two-dimensional orbital shaker (Techlifer orbital rotator shaker lab UPC 789458170564), set to 80 rpm. Biocomposites were moved to a new well of fresh medium after 1, 24, and 48 h with the

total time spent in the medium being 72 h, allowing for cumulative cell release to be calculated using a Neubauer haemocytometer.

2.2.5 Scanning electron microscopy

The biocomposites were imaged using scanning electron microscopy following adhesion testing. Samples were dried at 105 °C for 3 h, stored in a desiccator before being attached to 12 mm diameter pin stubs using carbon tape, and imaged using a Hitachi TM 3000 SEM with a backscattered electrons system. All biocomposites were observed in two or more randomised locations using a 5 or 15 kV accelerating voltage.

2.2.6 CO_2 uptake

Informed by the toxicity and adhesion tests, biocomposites were taken forward for CO_2 uptake tests. Samples were prepared as described for adhesion testing, but the textiles were cut into 1 x 5 cm strips. Formulations were pipetted 1 cm from the top of the strip so the growth medium could only reach cells by capillary action. Five millilitres of sterile growth medium was pipetted into a 50 mL sterile, clear Wheaton glass serum bottle. Biocomposite samples were then placed into the bottle and suspended using 0.15 mm sterile nylon thread to prevent the cells from being submerged. The bottles were sealed using a rubber butyl stopper and a crimped aluminium cap. Each bottle was flushed with 45 mL of 5% CO_2-enriched air using a hypodermic needle to pierce the rubber stopper without breaking the seal. Samples containing the equivalent number of cells in suspension were placed in sealed bottles as controls. Every two days a sample of the headspace was withdrawn using a hypodermic needle and an air-tight syringe and the percentage CO_2 content was analysed using a G100 GEOTech CO_2 meter. Bottles were then re-flushed with 5% CO_2-enriched air. The moles of CO_2 fixed by the cells was calculated using equation 1. This was repeated for 20 days and a cumulative total of fixed CO_2 was calculated.

$$\text{fixed } CO_2 \text{ (mol)} = \frac{[(5.00\% - \%CO_2 \text{ recorded}) \times 45] \times 10^{-3} \text{ (L)} \times \text{system pressure (atm)}}{0.082 \text{ (L atm mol}^{-1}\text{K}^{-1}) \times \text{system temperature (K)}} \quad \text{[Eq 1]}$$

Biocomposites comprising 5% solids binder with 2.5% cell content, 10% solids binder with 2.5% cell content, and 5% solids with 10% cell content were tested following initial screening trials (data not shown). Additionally, a 5% solid binder with 2.5% cell content cotton biocomposite was tested with an artificial urine rather than a defined growth medium to better reflect a wastewater scenario (Brooks and Keevil, 1997).

2.2.7 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8. Data were tested for normal distribution (Shapiro-Wilk test). For toxicity data, equality of variance was determined using the Brown-Forsythe test and one-way ANOVA with Tukey *post-hoc* testing. For adhesion data,

two-way ANOVA was used with Tukey *post-hoc* testing. Sphericity was assumed due to measurements as the experimental design was based on matching, not repeated measures (Maxwell *et al.*, 2017). For CO_2 uptake, repeated measures two-way ANOVA with Tukey *post-hoc* testing was conducted. For the adhesion and CO_2 data, sphericity was violated, therefore a Geisser-Greenhouse correction was performed (Greenhouse and Geisser, 1959).

2.3 Results

2.3.1 Binder toxicity

The toxicity data for both *S. elongatus* strains were normally distributed (Shapiro-Wilk test, $P > 0.05$), with significant differences between control cultures and 5% solids AURO binders (PCC 7942: ANOVA, $F = 483.3$, df = 6, 17, $P < 0.001$; CCAP 1479/1A: ANOVA, $F = 70.67$, df = 6, 14, $P < 0.001$), synthetic binders (PCC 7942: ANOVA, $F = 10.3$, df = 10, 35, $P < 0.001$; CCAP 1479/1A: ANOVA, $F = 82.09$, df = 10, 22, $P < 0.001$), and natural binders (shellac and chitosan) (PCC 7942: ANOVA, $F = 128.7$, df = 2, 9, $P < 0.001$; CCAP 1479/1A: ANOVA, $F = 224.6$, df = 2, 6, $P < 0.001$).

Of the AURO binders, only AURO 320 and 321 supported significantly improved growth, whereas AURO 160, 251, 261 and 379 returned cell densities significantly lower than controls ($P < 0.05$); therefore in this context they were classed as toxic (Figure 2.1). On this basis, only AURO 320 and 321 were considered for follow-on CO_2 fixation trials. None of the synthetic binders supported improved growth relative to controls with a singular exception of binder-9 with *S. elongatus* CCAP 1479/1A; however, this was not deemed to be a sufficiently large growth improvement to justify automatic inclusion in subsequent CO_2 trials. The shellac and chitosan treatments had significantly decreased cell densities for both strains, with shellac killing the cultures.

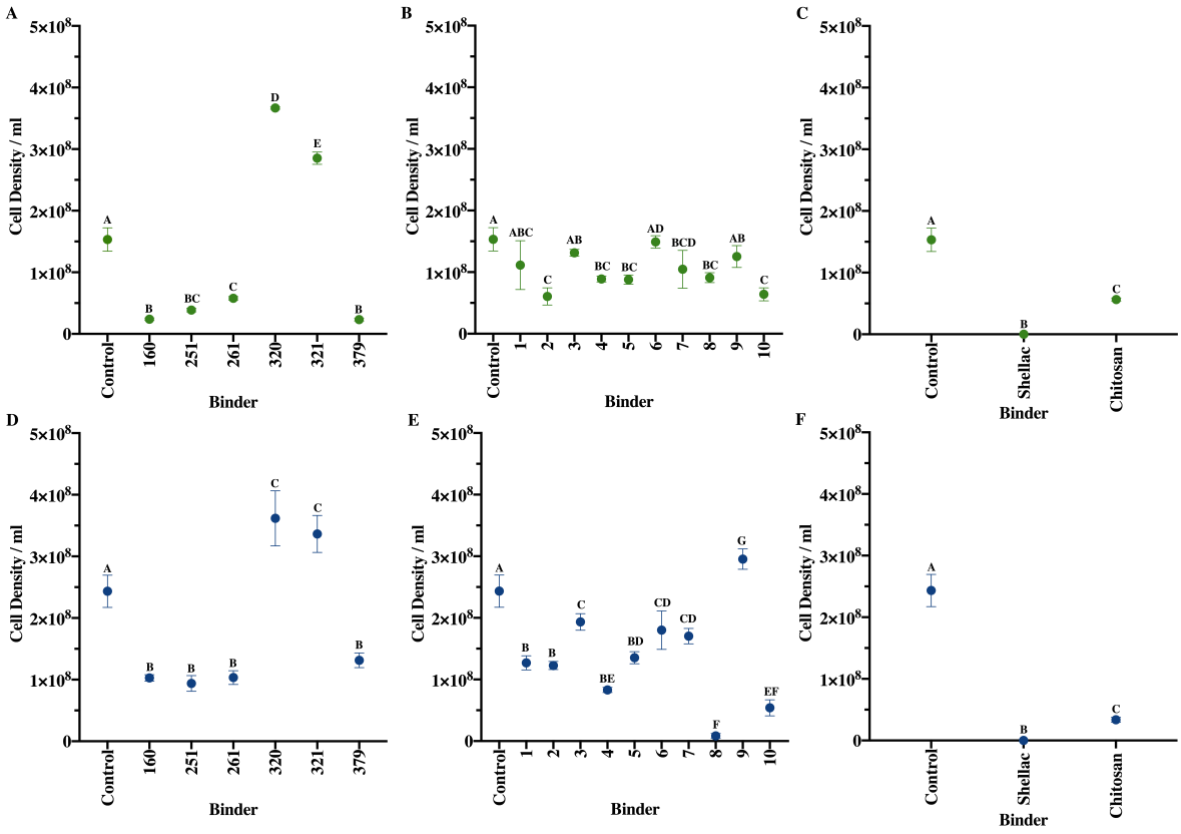

Figure 2.1. Cell density (cells per mL, mean ± SD) of *Synechococcus elongatus* PCC 7942 (panels A-C) and *S. elongatus* CCAP 1479/1A (panels D-F) after a seven-day contact toxicity test with binders of 5% solids content. Significant differences are indicated on individual graphs by different letters. Refer to Table 1 for binder details.

There was a significant increase in growth with respect to controls for *S. elongatus* PCC 7942 with AURO 320 (ANOVA, $F = 23.06$, df = 4, 13, $P < 0.001$), with the best growth support by the 2.5% solids treatment ($P < 0.05$). Similarly for *S. elongatus* CCAP 1479/1A with AURO 321 (ANOVA, $F = 5.182$, df = 4, 10, $P = 0.016$) (Figure 2.2), although in this case the best growth was with the 7.5% solids treatment ($P < 0.05$).

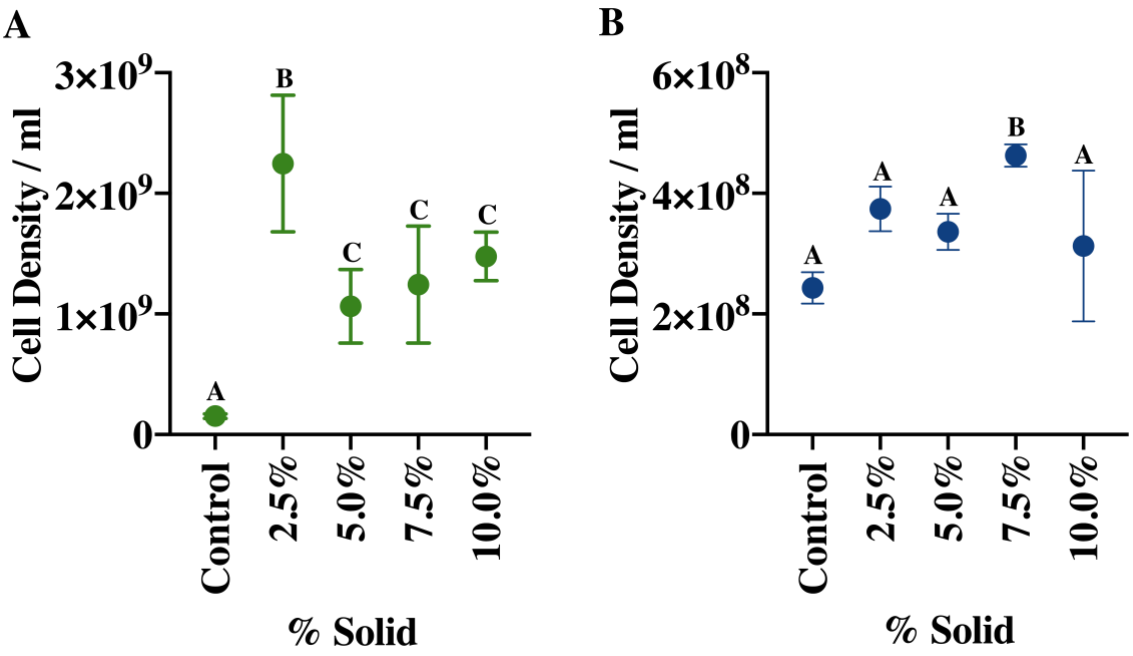

Figure 2.2. Cell density (cells per mL, mean ± SD) of A) *Synechococcus elongatus* PCC 7942 with AURO 320 and B) *S. elongatus* CCAP 1479/1A with AURO 321, after a seven day contact toxicity test with varying percentage binder solids content. Significant differences are indicated on individual graphs by different letters.

2.3.2 Adhesion

Scanning electron micrographs of representative *S. elongatus* PCC 7942 biocomposites are presented in Figure 2.3, showing variation in biocoating integrity. Figure 2.3A illustrates the densely packed nature of the biocoating, but also shows that the biocoatings cannot be regarded as monolayers. Equally, poorly coated areas of the fibres are seen (Figure 2.3B&C), as well as a more complex 3D architecture where the pore space between fibres have allowed the biocoating to penetrate further into the textile structure (Figure 2.3D).

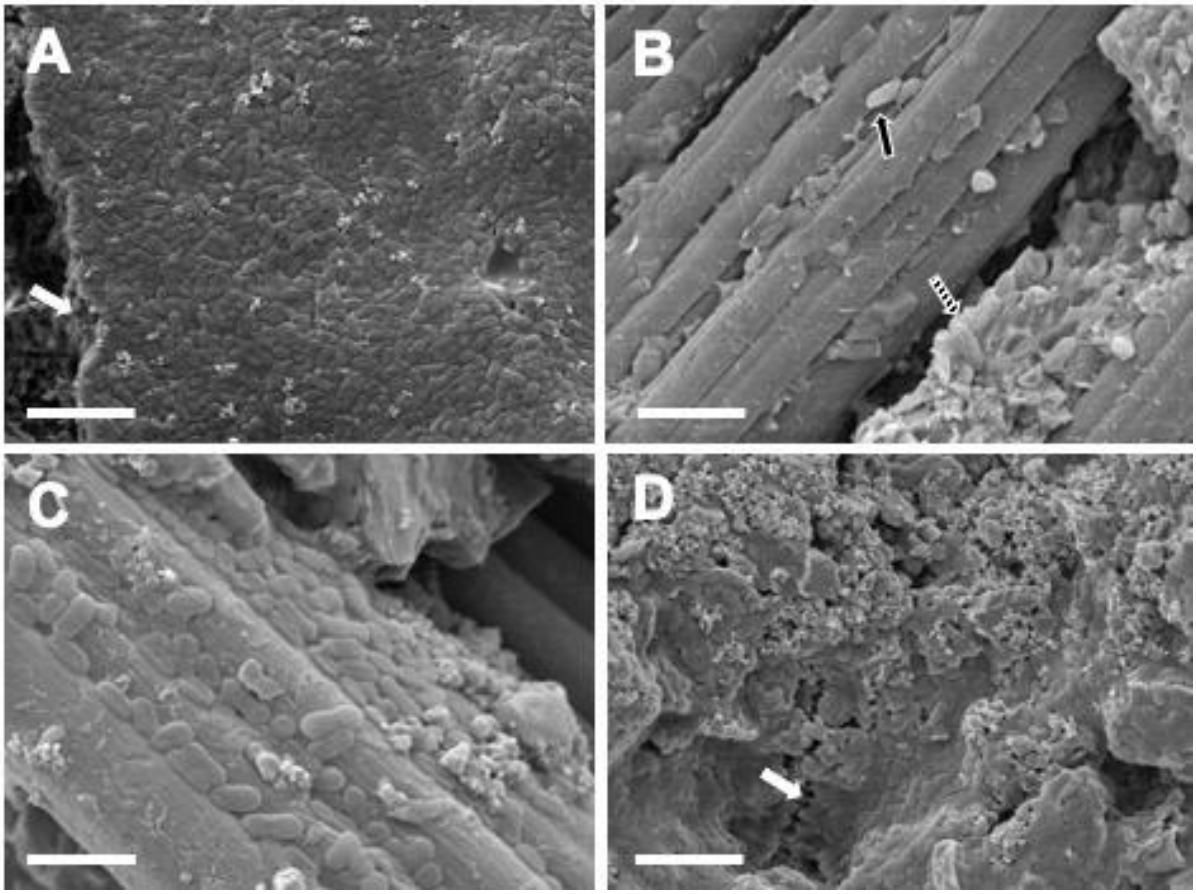

Figure 2.3. Scanning electron micrographs of *Synechococcus elongatus* PCC 7942 biocomposites made with 5% solids content and 2.5% cell loading: A) cotton, arrow indicates that the biocoating was several cells thick, scale bar = 10 μm; B) bamboo, black arrow indicates a cell attached to the bamboo fibre, the hatched arrow indicates a section of biocoating (binder with cells), white arrow indicates a point of cell loss from the biocoating, scale bar = 5 μm; C) wool, scale bar = 10 μm, and D) polyester, arrow indicates a fracture in the biocoating, scale bar = 10 μm.

Significant interactions between textile type and binder solids content affected cell retention when a 1% cell loading was used (two-way ANOVA: $F = 4.267$, df = 12, $P = < 0.001$), with textile type (two-way ANOVA: $F = 15.93$, df = 3, $P < 0.001$) and solids content (two-way ANOVA: $F = 31.38$, df = 4, $P < 0.001$) having significant effects. Cells retention was not significantly different for the bamboo at any binder solids content but the other textiles experienced significant losses (Figure 2.4), particularly for higher solids contents, with wool and polyester having the poorest cell retention properties.

When a 2.5% cell loading was used there was no significant interaction between textile and binder solids content (two-way ANOVA: $F = 1.732$, df = 12, $P = 0.096$). Cell retention was

significantly reduced for all textiles at any binder solids content relative to the control (Figure 2.4). For cotton and bamboo, there was a general trend of improved cell retention with increasing solids content, but this was not evident for either the wool blend or the polyester.

There was a significant interaction between textile and binder (two-way ANOVA: $F = 4.365$, df = 12, $P = < 0.001$) when 5% cell loading was used, with textile type (two-way ANOVA: $F = 11.40$, df = 3, $P < 0.001$) and binder solids content (two-way ANOVA: $F = 16.16$, df = 4, $P < 0.001$) having significant effects. There was no significant release for either cotton or bamboo at any solids content (Figure 2.4), however the wool and polyester textiles experienced significant cell loss at 7.5 and 10% solids.

There was also a significant interaction between textile and binder at the highest cell loading (10%) (two-way ANOVA: $F = 5.052$, df = 12, $P < 0.001$), with textile (two-way ANOVA: $F = 14.26$, df = 3, $P < 0.001$) and binder solids (two-way ANOVA: $F = 27.12$, df = 4, $P < 0.001$) both significant factors. The bamboo had no significant cell loss and the cotton only had significant loss at 2.5% solids, whereas the wool blend had significant release at 7.5 and 10% solids content and the polyester at 2.5, 7.5, and 10% solids content.

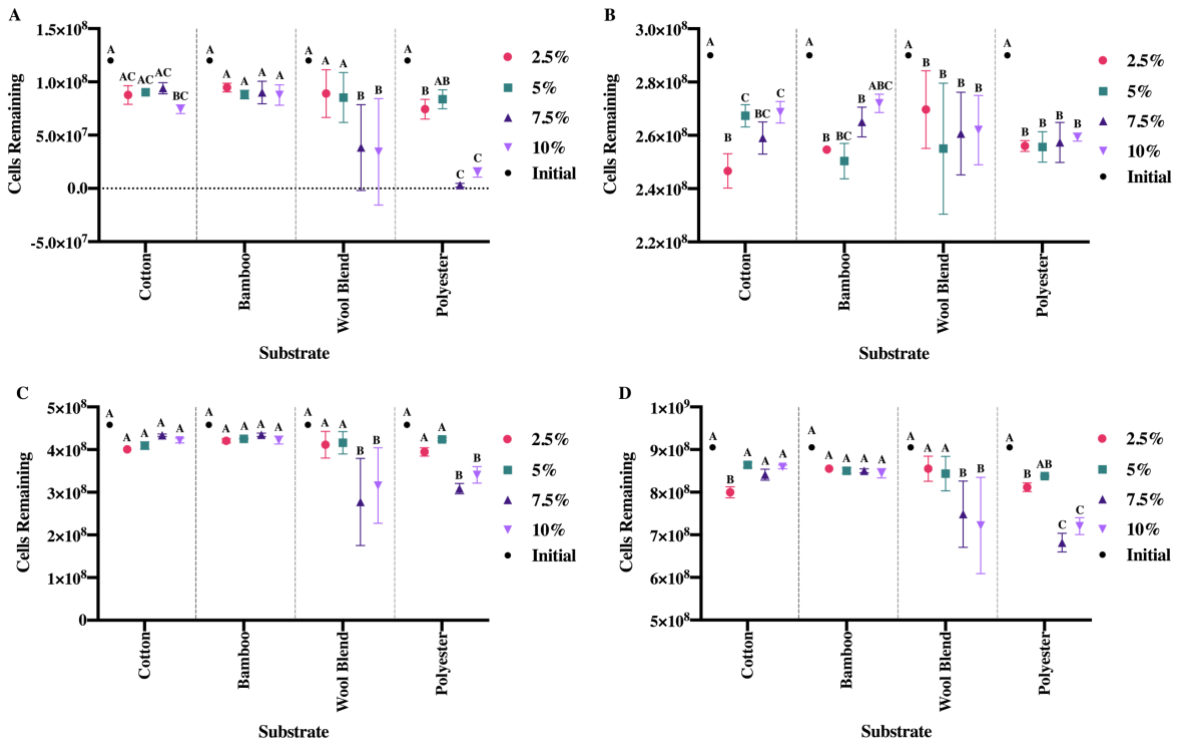

Figure 2.4. Cell retention (mean ± SD) of *Synechococcus elongatus* PCC 7942 immobilised within AURO 320 on woven 80/20 polyester-cotton blend (cotton), woven 100% bamboo (bamboo), non-woven 40/60 wool-polyester blend (wool blend), and non-woven 100% polyester (polyester) biocomposites after 72 h. Different cell loading levels were trialled: (A)

1%, (B) 2.5%, (C) 5%, and (D) 10%. Different letters indicate significant differences within textiles for each cell loading level.

In the equivalent adhesion tests with *S. elongatus* CCAP 1479/1A, there was also a significant interaction between textile and binder solids content at a cell loading of 1% (two-way ANOVA: $F = 14.3$-, df = 12, $P < 0.001$). Textile (two-way ANOVA: $F = 48.55$, df = 3, $P < 0.001$) and binder solids content (two-way ANOVA: $F = 151.7$, df = 4, $P < 0.001$) were significant factors. All textiles experienced some cell loss although the bamboo was unaffected by the binder solids content. The other textiles experienced increased cell loss at high binder solids content (either 7.5 or 10% solids) (Figure 2.5). The wool blend and the polyester treatments performed very poorly at higher solids content, in some instances (polyester 7.5 and 10%) causing all cells to be released. A markedly different pattern was observed at a cell loading of 2.5%. There was no significant interaction between textile and binder solids (two-way ANOVA: $F = 1.533$, df = 12, $P = 0.153$). Whereas all treatments did release cells, this was either not worsened at higher solids content or, in the case of cotton, cell retention tended to improve.

The 5 and 10% cell loading treatments returned very similar patterns in terms of cell release. In both cases there were significant interactions between textile and binder solids (5% cell loading - two-way ANOVA: $F = 3.307$, df = 12, $P = 0.002$; 10% cell loading - two-way ANOVA: $F = 5.708$, df = 12, $P < 0.001$). Textile was a significant factor (5% cell loading – two-way ANOVA: $F = 9.607$, df = 3, $P < 0.001$; 10% cell loading – two-way ANOVA: $F = 13.99$, df = 3, $P < 0.001$) as was binder solids content (5% cell loading – two-way ANOVA: $F = 20.99$, df = 4, $P < 0.001$; 10% cell loading - two-way ANOVA: $F = 21.74$, df = 4, $P < 0.001$). The cotton and bamboo textiles had no significant cell losses at any binder solids content at either cell loading levels. The performance of the wool blend was almost identical between the cell loading rates, with only the 7.5 and 10% binder solids losing cells. Whereas the polyester textile did tend to lose cells at higher binder solids, this loss was not quite as high at the 7.5% cell loading level.

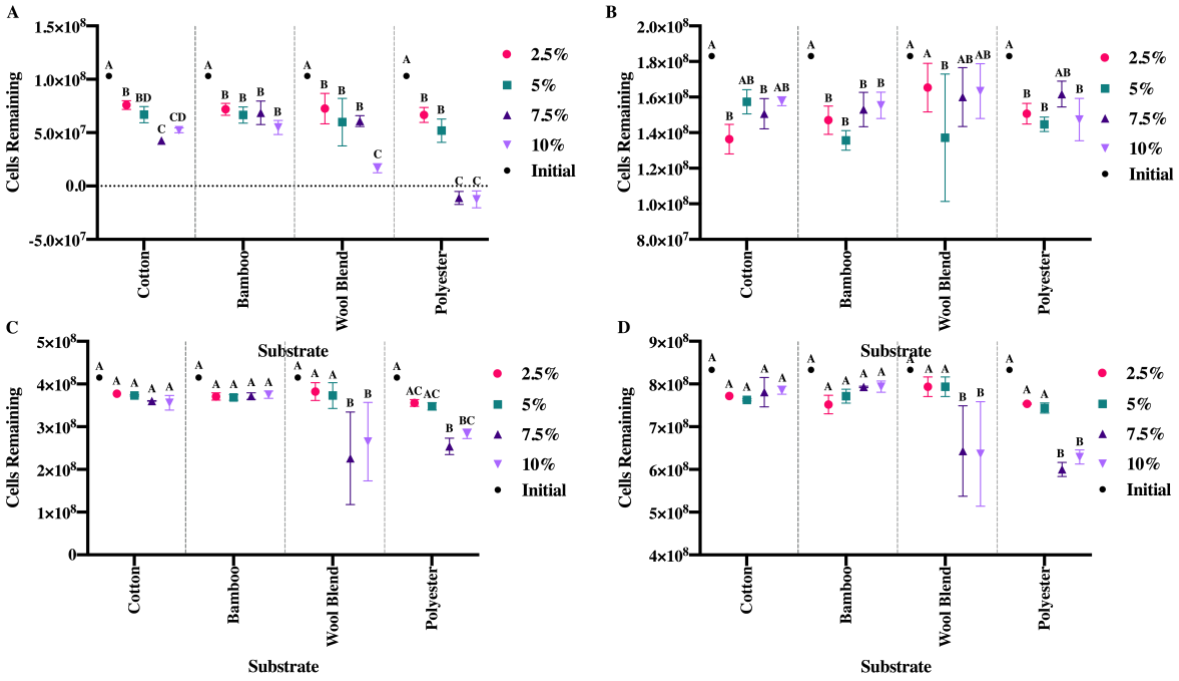

Figure 2.5. Cell retention (mean ± SD) of *Synechococcus elongatus* CCAP 1479/1A immobilised within AURO 320 on woven 80/20 polyester-cotton blend (cotton), woven 100% bamboo (bamboo), non-woven 40/60 wool-polyester blend (wool blend), and non-woven 100% polyester (polyester) biocomposites after 72 h. Different cell loading levels were trialled: (A) 1%, (B) 2.5%, (C) 5%, and (D) 10%. Different letters indicate significant differences within textiles for each cell loading level.

2.3.3 CO_2 uptake for S. elongatus PCC 7942 biocomposites

For biocomposites with 5% solids content and 2.5% cell loading, there was a significant interaction between textile and time (days) in terms of CO_2 consumption (two-way ANOVA: $F = 6.871$, df = 40, $P < .0001$), with textile (two-way ANOVA: $F = 9.192$, df = 4, $P < 0.001$) and time (two-way ANOVA: $F = 909.4$, df = 10, $P = 0.016$) significant factors for *S. elongatus* PCC 7942 (Figure 2.6A). Cotton and polyester had significantly more CO_2 uptake per cell than the suspension control from days 2 and 6 respectively. However, there was no significant difference in CO_2 uptake per cell between textiles within each time point.

There was a significant interaction between textile and time in terms of CO_2 uptake for *S. elongatus* PCC 7942 with 10% solids content AURO 320 (two-way ANOVA: $F = 6.871$, df = 40, $P < 0.001$), with textile (two-way ANOVA: $F = 9.192$, df = 4, $P = 0.002$) and time (two-way ANOVA: $F = 909.4$, df = 10, $P < 0.001$) significant factors (Figure 2.6B). The CO_2 uptake

of the suspension control was significantly higher that the biocomposites at each time point. The bamboo was the poorest performing biocomposites.

For *S. elongatus* PCC 7942 biocomposites with 5% solids content and 10% cell loading, textile and time had significant interactions (two-way ANOVA: $F = 14.68$, df = 40, $P < 0.001$), with both time (two-way ANOVA: $F = 631.8$, df = 10, $P < 0.001$) and textile (two-way ANOVA: $F = 17.98$, df = 4, $P = 0.001$) significant factors (Figure 2.6C). From day eight onwards, cells immobilised to cotton or bamboo had significantly lower cumulative CO_2 uptake than the suspension control, whereas the wool and polyester were not significantly different to the control. The CO_2 uptake of all treatments (including the controls) was markedly reduced compared with preceding trials.

For the *S. elongatus* PCC 7942 biocomposites fabricated with 5% solids content and 2.5% cell loading, there were significant interactions between textile, time and growth media (two-way ANOVA: $F = 49.88$, df = 30, $P < 0.001$), with time (two-way ANOVA: $F = 1053.00$, df = 10, $P < 0.001$), textile and media (two-way ANOVA: $F = 77.98$, df = 3, $P < 0.001$) all significant factors (Figure 2.6D). From day four onwards, BG11 treatments had greater cumulative CO_2 uptake than their respective suspension and cotton controls. With artificial urine, cumulative CO_2 uptake was not significantly different either in suspension or immobilised. From day six, cumulative CO_2 uptake with BG11 was significantly increased with respect to all other treatments regardless of immobilisation state. Suspension cultures failed to significantly increase cumulative CO_2 after day four in artificial urine, and day six in BG11, in contrast to immobilised samples which had significant increases in cumulative CO_2 at all time points until day 16.

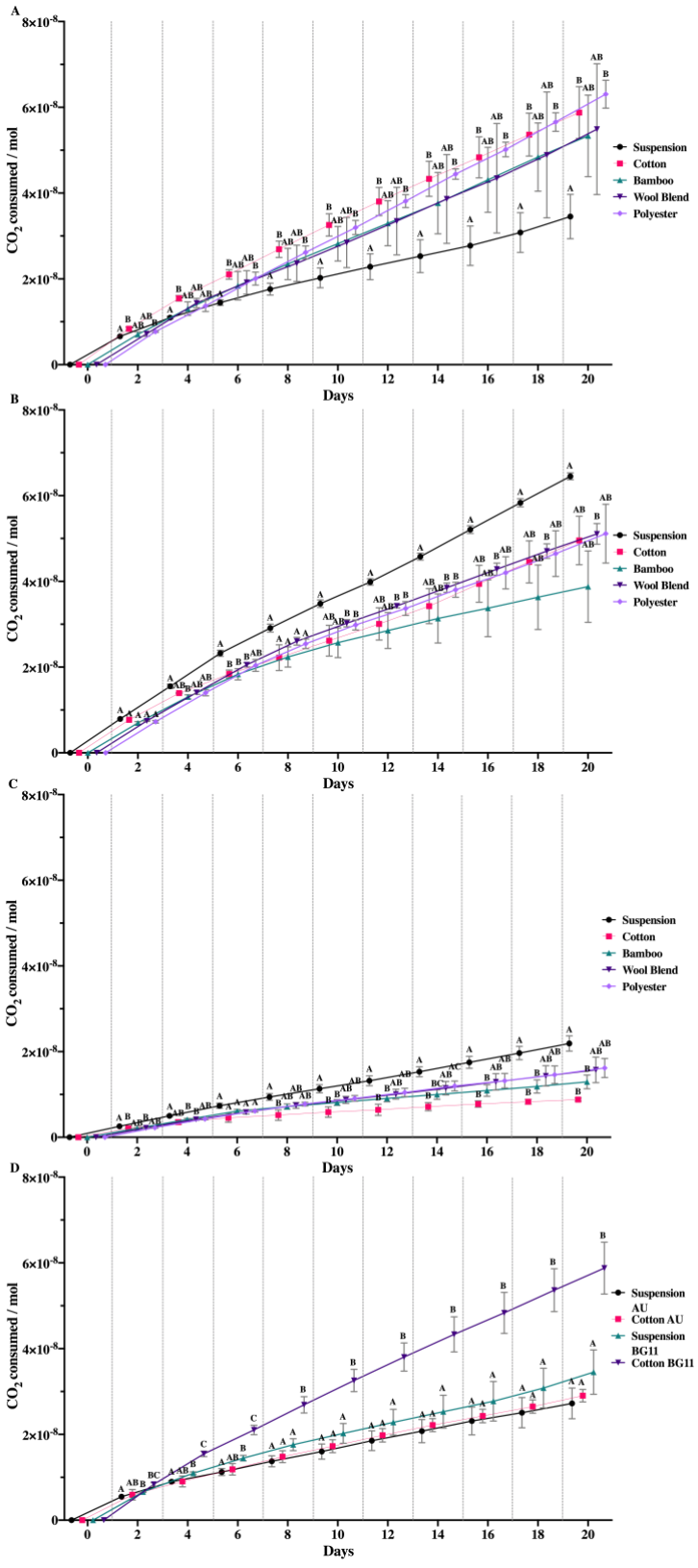

Figure 2.6. Cumulative uptake (mean ± SD) of CO_2 per cell for *Synechococcus elongatus* PCC 7942 immobilised within AURO 320 over a 20 day period on woven 80/20 polyester-cotton blend (cotton), woven 100% bamboo (bamboo), non-woven 40/60 wool-polyester blend (wool blend), and non-woven 100% polyester (polyester) biocomposites with varying binder solids content, cell loading, and growth media; A) 5% solids with 2.5% cell loading in BG11, B) 10%

solids with 2.5% cell loading in BG11, C) 5% solids with 10% cell loading in BG11, and D) 5% solids and 2.5% cell loading with either artificial urine or BG11. Different letters indicate significant differences within time points on individual graphs.

2.3.4 CO_2 uptake for S. elongatus CCAP 1479/1A biocomposites

There was a significant interaction between textile and time for biocomposites made with 5% solids content and 2.5% cell loading (two-way ANOVA: $F = 5.325$, df = 40, $P < 0.001$), with time (two-way ANOVA: $F = 646.7$, df = 10, $P < 0.001$) and textile (two-way ANOVA: $F = 4.987$, df = 4, $P = 0.018$) significant factors. From days 4–18 there were no significant differences in cumulative CO_2 uptake between suspended and immobilised cultures, with the exception of the wool blend on day 20 (Figure 2.7A). Over time, only the wool and polyester made significant increases in cumulative CO_2 uptake.

For biocomposites fabricated with 10% solids and a 2.% cell loading (Figure 2.7B), there was a significant interaction between textile and time (two-way ANOVA: $F = 6.249$, df = 40, $P < 0.001$), with time (two-way ANOVA: $F = 1426.00$, df = 10, $P < 0.001$) and textile (two-way ANOVA: $F = 8.404$, df = 4, $P = 0.003$) significant factors. There was no significant difference in cumulative CO_2 uptake between textiles. From days 8 and 10 onwards the bamboo and polyester had significantly lower cumulative CO_2 uptake than the suspension control.

There was a significant interaction between textile and time (two-way ANOVA: $F = 1.564$, df = 40, $P = 0.038$) with time (two-way ANOVA: $F = 536.0$, df = 10, $P < 0.001$) being significant, but not textile (two-way ANOVA: $F = 1.564$, df = 40, $P = 0.158$) for biocomposites made with 5% solids content and 10% cell loading (Figure 2.7C). Only the cotton biocomposite did not have significantly different cumulative CO_2 uptake compared to the suspension culture. However, from day eight onwards there was no significant difference in cumulative CO_2 uptake between the suspension culture or textiles.

In the final experiment, with biocomposites made with 5% solids content and 2.5% cell loading, there were significant interactions between textile, time and the growth media (two-way ANOVA: $F = 8.613$, df = 30, $P < 0.001$), with time (two-way ANOVA: $F = 326.1$, df = 10, $P < 0.001$) and textile and media (two-way ANOVA: $F = 13.68$, df = 3, $P = 0.016$) significant factors (Figure 2.7D). The use of artificial urine with the immobilised biocomposites did not cause a significant change in cumulative CO_2 uptake compared to the equivalent JM treatment until day 18. The impact of artificial urine on the suspension or immobilised samples was less clear over time. The suspended samples had significantly greater cumulative CO_2 uptake on

days 4 and 8. Biocomposites exposed to either artificial urine or JM had significant increases at each subsequent measurement over the full experimental period, but the suspension treatment did not make significant increases on after days 2 and 10 respectively.

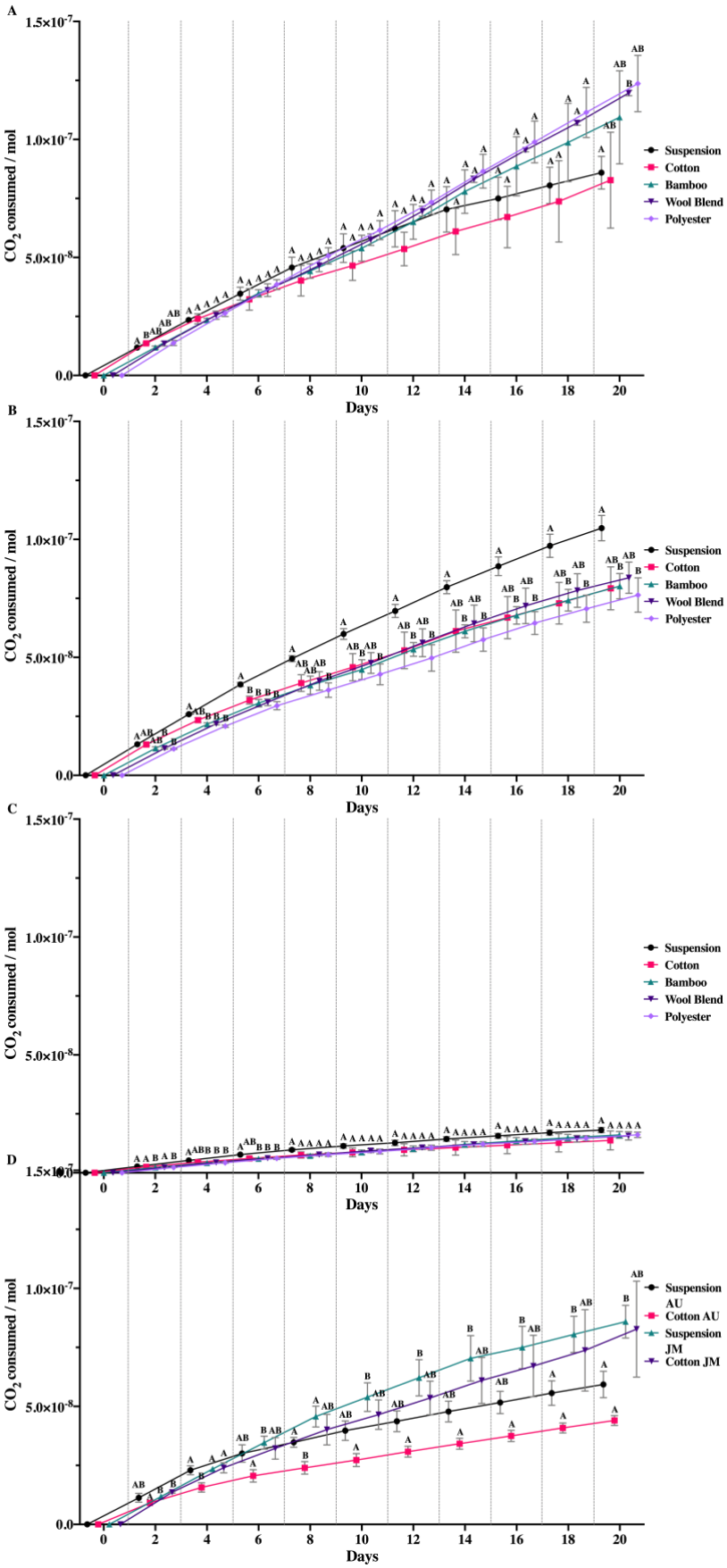

Figure 2.7. Cumulative uptake (mean ± SD) of CO_2 per cell for *Synechococcus elongatus* CCAP 1479/1A immobilised within AURO 321 over a 20 day period on woven 80/20 polyester-cotton

blend (cotton), woven 100% bamboo (bamboo), non-woven 40/60 wool-polyester blend (wool blend), and non-woven 100% polyester (polyester) biocomposites with varying binder solids content, cell loading, and growth media; A) 5% solids with 2.5% cell loading in BG11, B) 10% solids with 2.5% cell loading in BG11, C) 5% solids with 10% cell loading in BG11, and D) 5% solids and 2.5% cell loading with either artificial urine or BG11. Different letters indicate significant differences within time points on individual graphs.

2.4 Discussion

The objective of this study was to investigate some of the main technical steps required to develop robust, low cost, low maintenance living cyanobacterial biocomposite systems that could be deployed for a range of phycoremediation applications. As a system, the biocomposites are inherently simple, comprising three core elements; a scaffold to provide the physical means of supporting the structure (in this study this role was fulfilled using a range of commercially sourced textiles), a binder (ideally porous) to secure the cells to the scaffold while allowing for the diffusion of water and gases across the thin polymer film, and an appropriate microorganism. Textiles were chosen as they are cheap, easily obtained and could extend the technical life of the textile product if discarded fabrics are repurposed as biocomposites. Textiles also have the added benefit of being highly porous scaffolds with a large surface area for biocoating adhesion. Their physical flexibility would also allow textile biocomposites to be deployed in a range of physical spaces, and could deliver remediation using both dead-end and tangential flow (in addition to the capillary action and wicking demonstrated here), although the latter would be favoured.

We chose to test a range of textiles, ranging from 100% natural fibres (bamboo) to fully synthetic (100% polyester), and incorporating two textile blends (a 40/60 wool-polyester blend and an 80/20 polyester-cotton blend); however other scaffolds have been tested (Akhtar *et al.*, 2004; Bernal *et al.*, 2014; Eroglu *et al.*, 2015). Polyester was chosen as it is ubiquitous throughout the textiles industry and biocomposites could offer an additional recycled product. Further, we have separately demonstrated that polyester is amenable to conversion to cyanobacterial biocomposites for carbon capture applications, particularly the 80/20 blend used here (In-na et al. unpublished); although we acknowledge the need to drastically reduce plastics use and subsequent release into the environment (Barnes *et al.*, 2009). The wool blend was chosen against the backdrop of a collapse in the wool price (currently trading at a six year low, August 18th, 2020, www.tradingeconomics.com), with many UK wool producers having to destroy their fleeces. At the opposite end of the scale we chose bamboo, and despite it being an

expensive material, the growing demand for bamboo clothing is driving increased production that should, in time, reduce wholesale prices.

Binder choice was guided by prior experience. A range of synthetic binders were screened, and although we previously documented toxicity issues when used in combination with eukaryote microalgae (In-na *et al.*, 2020), they had not been assayed against cyanobacteria. However, the outcomes were very similar, with all binders proving toxic with the exception of PD-0413 (binder 9). Nonetheless, we elected not to promote this binder for further testing due to its unknown properties and focused more effort on the AURO latex coatings, which are more accessible.

AURO coatings are formulated from exclusively natural ingredients and are hypoallergenic; they were chosen on the premise that they should have lower toxicity to the cyanobacteria. This assumption was not entirely justified as four of the six AURO binders returned cell densities below the controls. Without a detailed reverse engineering of the formulations it is not possible to identify where the issues lie; however, comparing the published constituents reveals that binders 160, 251, and 261 all contain metal soaps (to promote coating drying) which are water-insoluble compounds comprising alkaline earth or heavy metals (the manufacturer does not declare which metals are involved) contained within carboxylic acids (Robinet and Corbeil-a, 2003; Noble, 2019). We also cannot discount the possibility that the impact on cell growth was not due to the polymer film being impermeable, thereby impeding gas and water exchange. Binder 379, which has shellac as a constituent of the formulation, also supported poor growth. Given that the shellac-only treatment killed all of the cyanobacteria, it is not unreasonable to deduce that the shellac had a role in the poor performance of this binder.

The two AURO emulsion coatings (320 and 321) successfully supported strong cyanobacteria growth (with some variation between the strains). These observations corroborate our previous findings with these coatings when used in combination with a loofah sponge scaffold (In-na *et al.*, 2020), consistently outperforming suspension controls, often by orders of magnitude. Other than not containing metal soaps or shellac, the reasons for such consistent growth promotion remain speculative. Both binders list ammonia as part of the formulation, which potentially could be exploited by *S. elongatus* as an additional nitrogen source (Ludwig and Bryant, 2012), although nitrogen limitation is unlikely to have been an issue in the short (72 hour) toxicity tests. The other notable differences are the inclusion of Replebin® (a plant alcohol ester with organic acids) as a proprietary ingredient, and titanium dioxide as a pigment. We have no further details on the chemical composition of Replebin® and speculation would be unhelpful; however, a potential role—albeit paradoxical—for TiO_2 cannot be discounted. TiO_2 is

increasingly used in photochemistry based water treatment to kill cyanobacteria and destroy their cyanotoxins (He *et al.*, 2020; Pestana *et al.*, 2020); yet a recent study has found that colloidal TiO_2 and cyanobacteria extracellular polymeric substances (EPS) interact, promoting colloidal stability, reducing photochemical damage (Xu *et al.*, 2020), and potentially enhancing the adsorption of organic molecules, particularly those with phosphate and nitrogen moieties. Further investigation into the functional aspects of the AURO 320 and 321 binders is clearly warranted.

Despite previous studies successfully immobilising microalgae and cyanobacteria in chitosan (Aguilar-May *et al.*, 2007; Aguilar-May and del Pilar Sánchez-Saavedra, 2009; Eroglu *et al.*, 2015), there was a significant reduction in growth during toxicity testing; potentially due to suboptimal pH conditions during the curing process (Kuan *et al.*, 2015). A more structure approach to developing chitosan within a biocomposite architecture would probably address these issues.

The second determinant of a successful binder is its capacity to retain the cells once the biocoating is formed. SEM imaging revealed variation in the coverage of the textile fibres by AURO 320 and 321, and that the manual application approach used here does not deliver a cellular monolayer with the topographically complex textiles. The polyester-cotton and bamboo textiles were both woven; the diameter and spacing of the weave affects the inter-yarn pore size, subsequently affecting the swelling ability of the textile as the liquid flows between the pores, with tighter weaves allowing less swelling (Gibson *et al.*, 1999)—hence reducing mechanical stress on the binder, particularly during drying (Jons *et al.*, 1999). Additionally, the hydrophobicity of woven fabrics is influenced by its roughness, structure, and geometry, with rougher surfaces being more hydrophobic (Melki *et al.*, 2019). Wettability is also influenced by pore structure. The non-woven textiles (the 40/60 wool-polyester blend and the 100% polyester) had larger pores, which should result in a rougher surface structure with greater wettability than the woven textiles, thereby increasing interactions between the surface and the growth medium (Zhu *et al.*, 2006), and potentially affecting latex film formation (Khosravi *et al.*, 2014). When coating latex onto porous substrates the water can leave through evaporation and wicking, with the extent of wicking affected by the textile's hydrophobicity. We would therefore expect that the biocoatings would have better coverage and deeper penetration with the non-woven textiles. Increasing the solids content of the binder would have ameliorated this effect and helped create more even films with improved adhesive properties.

Interestingly, the ammonia in the AURO binders may have physically affected the cotton and bamboo. Ammonium is utilised in the cotton industry to penetrate the cellulose by breaking

hydrogen bonds, with the resulting fibres having improved stability and abrasion resistance (Dornyi *et al.*, 2008). Additionally, the increased cell density in the adhesion test may also have affected film formation by increasing viscosity and prevented levelling of the film (Desjumaux *et al.*, 2000).

The viability of the biocomposites was determined by net CO_2 uptake over a 20 day semi-batch trial, with reference to the equivalent cell density in suspension culture. The biocomposites with 5% solids content and 2.5% cell loading performed well, easily surpassing the controls, although over time there was some slippage of the biocoating from the wool blend and polyester biocomposites into the liquid media pool. Latex films can begin to disintegrate after just 10 days, primarily due to photodegradation but also from microbial activity (Lambert *et al.*, 2013). Furthermore, there is potential for swelling of rubber latex of up to 20% under exposure to continuous moisture which could contribute to biocoating failure and subsequent release of cells (Cesar *et al.*, 2020).

When the solids content was increased to 10% to prevent slippage, the CO_2 uptake was reduced compared to the suspension controls. The increased thickness of the binder and the reduced number of pores will have reduced light penetration, gas and nutrient exchange through mass transfer limitation (Pires *et al.*, 2013; Miranda *et al.*, 2017), which we confirmed in our previous work (In-na *et al.*, 2020). Future iterations of these biocomposites should focus on maximising pore number and size (i.e. permeability) without compromising the structural integrity of the biocoating or by exceeding the cell size of the immobilised organism. A number of options exist to achieve this, including incorporating water soluble fillers within the binder (Lyngberg *et al.*, 2001) and, at a more technical level, including halloysite nanoclays (Chen *et al.*, 2020).

To increase CO_2 uptake more biomass was loaded into the system; however, the CO_2 uptake per cell was significantly reduced when compared with the 2.5% biocoatings. Increasing cell loading in immobilised systems is not necessarily a panacea for poor performance, as demonstrated in alginate immobilised systems (Chevalier and de la Noüe, 1985; Lau *et al.*, 1997; Hameed, 2007). In scenarios where resource limitation will become an issue (such as the single addition of nutrients in the current study), by increasing cell loading you only exacerbate resource competition. Whereas we did not specifically measure nutrient levels, it is reasonable to assume a degree of limitation given the 20 day duration without nutrient renewal. This would have reduced pigment synthesis, compromised photosynthetic efficiency, and therefore reduced CO_2 fixation (Ruan *et al.*, 2018). In the context of wastewater remediation this may be less of an issue as nutrient deficiency is less likely outside of static systems as tested here.

To better reflect the performance of the biocomposites when exposed to effluent, the defined growth media were replaced with a basic artificial urine. Both *S. elongatus* strains had reduced CO_2 uptake with the artificial urine. Most cyanobacteria have one or more genes that transport and catabolise urea to ammonia and CO_2 which can then be used for metabolism (Veaudor *et al.*, 2019). Li and co-workers compared the proteomic response of *Synechococcus* sp. WH8102 when grown on either nitrate or urea, with the urea treatments having greater RuBisCO activity but reduced carbonic anhydrase activity, suggesting a higher carboxylation efficiency which reduces demand for CO_2 (Li *et al.*, 2019). The artificial urine had sufficient phosphate, therefore deviation from stochiometric balance does not explain the observations. Once reliable biocomposites are developed there is need for further optimisation based on real effluent.

Aside from the materials components of the biocomposites, careful selection (and potentially adaptation) of the immobilised organism is also needed. We have observed substantive differences in performance between two strains of the same species, with *S. elongatus* PCC 7942 better equipped for immobilised existence. We documented similar variation (albeit with *S. elongatus* CCAP 1479/1A delivering the better results) using loofah sponge-based biocomposites for carbon capture. For biocomposites to reach their potential there is an imperative to incorporate species with established phycoremedation pedigree in addition to convenient laboratory strains.

2.5 Conclusion

We set out to explore the technical constraints to fabricating textile-based cyanobacteria biocomposites that could be deployed for a range of environmental remediation applications. In particular, these approaches (pending further optimisation) could become an effective treatment option for highly distributed wastewater treatment infrastructure, potentially within the context of individual properties. This study drew inspiration from the EU H2020 project Living Architecture (Armstrong *et al.*, 2017), which had the goal of developing similar highly distributed wastewater treatment infrastructure; however, the Living Architecture technology is deeply embedded within the mindset of suspension-based treatment systems. We sought to circumvent this approach by developing engineered immobilised biocomposites that would accelerate the maturation of the Living Architecture vision and lead to the miniaturisation (i.e. intensification) of the Living Architecture infrastructure; which will be vital if it is to deliver on its promise.

3 Genetic response of cyanobacteria to immobilisation

3.1 Introduction

An optimised biocomposite formulation has been developed following a robust biocomposite development programme. However, an important and yet mostly unanswered question remains— how do the cells survive in an environment that is vastly different from their natural planktonic (suspension) lifestyle, i.e. forcefully constrained to a benthic existence within an artificial version of an exopolymeric matrix?

Cortez *et al.* (2017) argue that the thick matrix of chitosan and alginate results in mass transfer limitations which may be mitigated by using thin nanoporous coatings such as latex. Evidence in support of this hypothesis was provided by Bernal and co-workers who immobilised cyanobacteria to paper substrates with latex, recording up to 10 times higher O_2 production than suspension controls (Bernal *et al.*, 2014). However, In-na *et al.* (2020) provided evidence that latex biocoatings restrict CO_2 mass transfer.

Immobilised biomass has the potential to remove nutrients from wastewater at an equivalent or increased rate relative to their suspension counterparts. The central objective of this thesis is to develop biocomposites that may be deployed for wastewater treatment applications; however, it is uncertain whether the additional physical barrier presented by a binder (particularly relatively untested binders, e.g. latex) will limit the nutrient scrubbing performance of these systems. Evidence suggests that chitosan and alginate binders may perform well; for example, growth and nitrate removal was not significantly different for a shrimp farm isolate of *Synechococcus elongatus* immobilised in chitosan, yet chlorophyll *a* and protein concentrations were significantly greater (Aguilar-May and del Pilar Sánchez-Saavedra, 2009). Also, there was significantly more chlorophyll *a* when *S. elongatus* was immobilised in alginate beads when compared with suspension treatments (Ruiz-Güereca and del Pilar Sánchez-Saavedra, 2016). However, alginate coatings are susceptible to cell leakage, reducing their operational duration. Furthermore, it is not as simple as increasing the hardness of the alginate shell as when the alginate and hardener ($CaCl_2$) concentration was increased, the phosphate and ammonium removal was significantly reduced (Castro-Ceseña *et al.*, 2016).

Nielsen *et al.* (2010) argue that the efficacy of wastewater bioremediation relies on sufficient understanding of the systems biology of organisms in the treatment system. Being photoautotrophs, CO_2 and nutrient availability are fundamental factors that affect cyanobacterial metabolism, with reduced access potentially measurable through the production of proteins and transcription and translation from gene regulation. Although there are papers

studying the influence of immobilisation on CO_2 or nutrient uptake, and indirect methods of measuring viability such as staining, fluorescence microscopy, and enzyme activity (Flickinger *et al.*, 2017), there is very little known about gene regulation of microbes in biocoatings at any stage of the process (Dickson and Ely, 2013; Flickinger *et al.*, 2017).

Proteomic studies can verify transcriptional patterns, helping to assess molecular responses to nutrient or CO_2 limitation (Harke and Gobler, 2013). Protein can account for up to 60% of dry weight in cyanobacteria, but requires high amounts of nitrogen and phosphorous to produce it (Silva-Benavides and Torzillo, 2012). Nutrient supply can influence resource allocation to various macromolecular pools (e.g. proteins, nucleic acids, or metabolites) (Moore *et al.*, 2013), potentially influencing the success of the biocomposites. In particular, proteins associated with photosynthesis, RuBisCO (*rbc*L) and photosystem II (psbA) are decreased during nutrient limitation (Fernández-González *et al.*, 2020). CO_2 uptake can affect protein synthesis, thereby affecting cellular metabolic integrity (Mou *et al.*, 2017) and growth rate (Klumpp *et al.*, 2009). Protein abundance can therefore provide insight into the variation of cellular functions in response to environmental fluctuations (Wegener *et al.*, 2010).

High-throughput sequencing provides a route to a much greater understanding of the specific roles and processes that microbial consortia play in wastewater bioremediation (Perera *et al.*, 2019). Understanding the transcriptomes can aid productivity in cyanobacterial systems (Krishnan *et al.*, 2018). Allocation of resources that affect cell survival can be influenced by environmental stressors, particularly those affecting the photosynthetic apparatus and carbon metabolism as demonstrated by Beck *et al.* (2017). *S. elongatus* PCC 7942 has a fully sequenced genome and metabolome (NCBI accession PRJNA10645), giving deep insight into gene function. RNA-Seq experiments are useful for differential gene analysis. The advantage of RNA-Seq is that it allows the investigator to look at the regulation of every gene within an organism's genome. This contrasts with targeted gene studies (e.g. using q-PCR), as these risk overlooking wider gene network responses. For example, if only select genes relating to CO_2 and phosphorous metabolism were monitored we may miss significant changes elsewhere relating to storage molecules, cellular repair, cellular signalling, etc.

RNA-Seq has also helped elucidate the response of *S. elongatus* PCC 7942 to nitrogen starvation, with 545 genes differentially expressed compared to a non-nitrogen starved cultures. Genes encoding for PSII were downregulated by a log fold-change (logFC) of 4.2, whilst nitrate-nitrite uptake genes were up-regulated by 1.1 logFC. Interestingly, *cbbLS,* which encodes for RuBisCo, was not differentially expressed during nitrogen starvation (Choi *et al.*,

2016). The use of RNA-Seq in this study allowed Choi *et al.* (2016) to identify the rearrangement of cellular transport of the carbon and nitrogen pathways.

Genes related to protein translation can also vary with stress conditions, including CO_2 limitation, light intensity, and nutrient depletion (Billis *et al.*, 2014). Global supplies of phosphorous are dwindling, so recovery of lost phosphorous from wastewater is integral to sustainable management (Rittmann *et al.*, 2011). Phosphorous, which is utilised in nucleic acids, phospholipid structures, proteins, and metabolite production, is essential for cell metabolism (Blank, 2012). Phosphate uptake is dependent on a periplasmic binding protein, PstS, and an associated ABC transporter, PstCAB (Scanlan *et al.*, 2009) and theoretically increases with cell size or density of the phosphate uptake system (Krumhardt *et al.*, 2013). One of the most widely studied operons for phosphorous uptake in cyanobacteria is the Pho operon, which is controlled by the *PhoU* gene which encodes a negative regulator of transport with high phosphorous affinity and polyphosphate-degrading enzymes (Solovchenko *et al.*, 2019).

Changes to light regimes can also influence resource synthesis, with *S. elongatus* PCC 7942 doubling its RuBisCO content whilst a five-fold decrease of phycobilisome synthesis occurred when moved from a light environment of 50 μmol m^2 s^{-1} to 500 μmol m^{-2} s^{-1} (MacKenzie *et al.*, 2005). Increased expression of RuBisCO can improve cell growth, chlorophyll *a* concentration, and oxygen evolution in a genetically engineered strain of *Synechocystis* (Liang and Lindblad, 2017). Theoretically, a wild-type strain naturally overexpressing RuBisCO would have similar increases in these physiological parameters.

This study aimed to understand the genetic response of *S. elongatus* to immobilisation. The physiological parameters of CO_2 uptake, phosphate uptake, total protein, and fraction of the protein that is RuBisCo, were determined at three and six days after immobilisation on a woven 80/20 polyester cotton blend. RNA extraction and transcriptomic study was attempted for *S. elongatus* PCC 7942 after three days of immobilisation, but could not be completed for *S. elongatus* CCAP 1479/1A as an annotated genome is not accessible.

3.2 Methods

3.2.1 Cell cultivation

Synechococcus elongatus PCC 7942 was grown in Blue-Green Medium (BG11), and *S. elongatus* CCAP 1479/1A in Jaworski's Medium (JM) without cyanocobalamin, thiamine HCl, and biotin, in 10 L polycarbonate (Nalgene) carboys with constant air supply at 18 °C ± 2 ºC,

and a 16L:8D photoperiod (mean luminance of 35 µmol m^2 s^{-1}) using 30 W daylight-type fluorescent tubes (Sylvania Luxline Plus, n = 6).

3.2.2 Biocomposite formation

Biocomposites were prepared in triplicate following the methodology described in Chapter 2. In short, autoclaved and dried woven 80/20 polyester-cotton blend (cotton) purchased from Aow RungRuang Co. Ltd, Bangkok, Thailand were cut into 1cm x 5cm strips. AURO 320 and 321 was adjusted to 5% solid for *S. elongatus* PCC and *S. elongatus* CCAP respectively. A wet cell paste (WCP) of 5% solid was used for each strain. The binder and WCP was combined by vortex mixing, and a separate Eppendorf of 5% WCP and dH_2O was prepared to estimate the number of cells that were pipetted onto each substrate. Formulations were pipetted 1cm from the top of the strip, so the growth media could only reach cells by capillary action. Five mL of sterile growth media were pipetted into 50mL sterile, clear Wheaton glass serum bottles. Samples were placed into the bottles and suspended using 0.15mm sterile nylon thread to prevent the cells being submerged due to substrate collapse. The bottles were sealed using a rubber butyl stopper and crimped aluminium seal. Samples were flushed with 45mL of 5% CO_2 enriched air using a hypodermic needle to pierce the rubber stopper without breaking the seal. Samples containing the equivalent number of cells in suspension, and samples of the 5% solid binder without cells were placed in sealed bottles as controls.

3.2.3 CO_2 uptake

After three days, the gas environment was sampled using a hypodermic needle and syringe and the percentage CO_2 content was determined using a G100 GEOTech CO_2 meter. Samples that were going to undergo analysis on day 6 were replenished with CO_2. The moles of CO_2 absorbed by the cells was calculated using the following equation:

$$\text{fixed } CO_2 \text{ (mol)} = \frac{[(5.00\% - \%CO_2 \text{ recorded}) \times 45] \times 10^{-3} \text{ (L)} \times \text{system pressure (atm)}}{0.082 \text{ (L atm mol}^{-1}\text{K}^{-1}) \times \text{system temperature (K)}} \quad \text{[Eq 1]}$$

3.2.4 Nutrient uptake

The growth media were sampled at days three and six and centrifuged at 5000 RCF for 30 minutes to remove any biomass or debris from the substrates. Hach Lange Phosphorous total/phosphate ortho (LCK 348) test kits were used to determine the orthophosphate concentration following manufacturer's instructions, and measured using a Hach Lange spectrophotometer (DR2800). Briefly, ammonium molybdate and antimony potassium tartrate form an antimony-phospho-molybdate complex in an acidic environment with phosphorous-containing solutions. Ascorbic acid is used to reduce the complex and form a blue-colour which is measured at 650nm. The intensity of the blue colour is dependent on the concentration of

orthophosphate. The initial starting concentration of BG11 and JM were determined using the same method.

3.2.5 Proteins

After three and six days, samples were removed from the sealed systems. Suspension samples were agitated by vortex and then centrifuged at 5000 RCF for 30 minutes at 4 ºC. A pre-cycle was run without samples to ensure the centrifuge temperature was appropriate. Once samples had been centrifuged the supernatant was discarded. For biocomposites, the 1cm^2 area over which the cell/binder was pipetted was cut from the surrounding fabric. Total protein was extracted using a modified version of the Pattanayak *et al.* (2015) method. Three hundred microliters of lysis buffer (8M urea + 20mM HEPES) was added to the cell pellet or biocomposite along with 0.05g of 0.1mm acid washed glass beads. Samples were vortexed for 30 seconds, then iced for one minute for a total of five rounds. Samples were then centrifuged at 4000 RCF for three minutes to separate the beads and cellular debris. Total protein concentration was determined by the Bradford Assay, for which 30µL of sample was added to a cuvette, and one mL of Bradford reagent was added. Samples were incubated at room temperature for five minutes and absorbance measured using a UV-Vis spectrophotometer (CARY 100) at 595nm.

SDS-Page gels were run to analyse individual protein changes between suspension and biocomposites samples. Samples were prepared by adding 3µL of sample buffer comprising 1:9 beta-mercaptoethanol:4x Laemmli buffer (Bio-Rad) to 9µL total protein sample. Samples were heated to 95 ºC for five minutes to ensure complete digestion of disulphide bonds. Ten microlitres of sample was loaded into each well of a precast polyacrylamide gel (12% Mini-PROTEAN® TGX™ Precast Protein Gels, 12-well, Bio-Rad). A Tris/Glycine/SDS running buffer was prepared following manufacturer's instructions (10x, Bio-Rad). A standard protein ladder (Precision Plus Protein™ Dual Xtra Prestained Protein Standards, Bio-Rad) was added to the first and last well of each gel. The current was set to 200 V and run for 31-40 minutes to allow full separation of protein bands. Gels were removed from the cassettes and washed with 200mL of dH_2O. They were then stained in Coomassie stain for one hour with agitation, then destained using DI water before visualisation. Gels were visualised using a Gel Doc EZ Imaging System (Bio-Rad) and analysed using Image Lab software (Bio-Rad). For each lane of the gel, bands were detected with high sensitivity and the molecular weights determined using the two standard protein ladders. The relative band percentage was then determined, and bands of a molecular weight (kDa) of 55 and 13 with 10% variation were determined as the large and small RuBisCO subunits.

3.2.6 RNA extraction and sequencing

RNA extraction was conducted after three days of immobilisation or suspension treatment of *S. elongatus* PCC 7942 only, with five samples for each treatment. *S. elongatus* CCAP 1479/1A was not sequenced due to no annotated genome being available for alignment. Extractions were completed using the Zymo Quick-RNA Fungal/Bacterial Miniprep kit following the manufacturer's instructions. A centrifuge pre-cycle was run without samples to ensure the centrifuge was at temperature. Suspension samples were agitated and then centrifuged at 5000 RCF for 30 minutes at 4 ºC. The supernatant was discarded. For biocomposites, the 1cm^2 area to which the cell/binder was pipetted was cut from the surrounding fabric. The power bead beating step was conducted for 60 seconds to ensure complete removal from the biocomposites and lysis of the cells. A gDNA removal step was conducted between steps 6 and 7 using DNAse1 on-column removal. Following extraction, the quantity and quality of the RNA for each sample was recorded three times using a NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer. Samples contained at least 50mg/mL of RNA with a 260/280 value of ~2.0 and a 260/230 value of 1.8-2.2. Samples were sequenced at the Centre for Genetic Medicine (CGM), Newcastle University. QC checks of samples and ribosomal RNA depletion was conducted by the CGM. Forty million 75-base pair single reads were conducted per sample using Illumina Next Seq. Single reads were suitable for this experiment as only count data was required for mapping to a transcript after alignment (Stark *et al.*, 2019). RNA Integrity Number (RIN) for each sample was determined using the RNA ScreenTape Analysis assay in the Agilent TapeStation. The output RIN was adapted for prokaryotic samples using the Agilent 2100 Bioanalyser Expert software.

3.2.7 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8. Data were tested for normal distribution (Shapiro-Wilk test). Repeated measures two-way ANOVA with Tukey *post-hoc* testing was conducted with Geisser-Greenhouse correction to correct for sphericity for all samples (Greenhouse and Geisser, 1959). To conduct statistical analysis for the RuBisCO fractions, an arcsine transformation was performed on percentage data prior to statistical analysis.

Analysis of the transcriptome data would have followed the methodology detailed by Xu and Miao (2020). The annotated reference genome of *S. elongatus* PCC 7942 was accessed from the GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/NC_007604.1). The quality of reads would have been assessed using FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads would have been trimmed

to high-quality only and mapped to the reference genome via Bowtie2 (Langmead and Salzberg, 2012). Gene expression would have been quantified using the method presented by HTSeq as fragments per kilobase of exon per million reads mapped (FPKM) (Anders *et al.*, 2015). Differentially expressed genes would have been determined using the Bioconductor package DESeq2 which uses a model based on the negative binomial distribution (Love *et al.*, 2014). Genes must have a *p*-value < 0.05 and log fold change values ≥ 2 to be considered as differentially expressed.

3.3 Results

3.3.1 S. elongatus PCC 7942

There were no significant interactions between immobilisation state and time for CO_2 uptake, orthophosphate uptake, and extracted protein concentration over the six day experimental period (repeated measures two-way ANOVA; CO_2 - F = 6.397, df = 1, P = 0.0647; Orthophosphate - F = 0.0901, df = 1, P = 0.779; Protein - F = 4.463, df = 1, P = 0.102) (Figure 3.1).

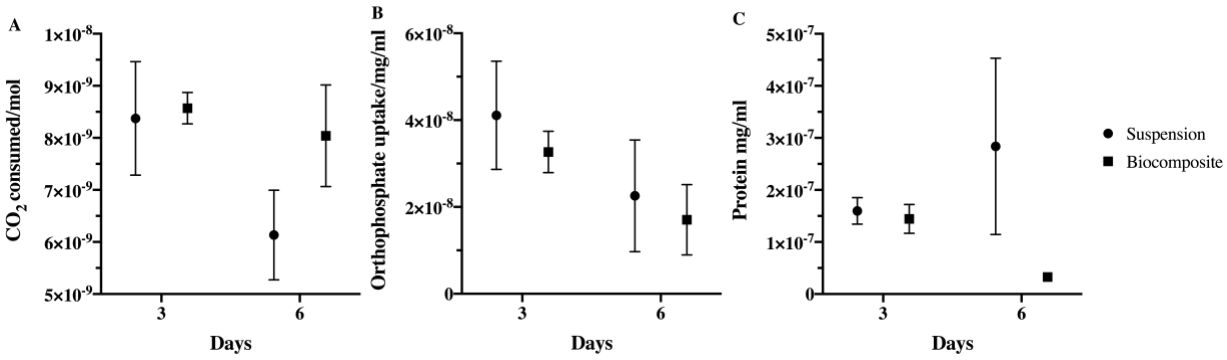

Figure 3.1. Mean (± standard deviation) of A) CO_2 uptake (mol), B) orthophosphate (mg/mL) uptake, and C) extracted protein (mg/mL) by *Synechococcus elongatus* PCC after three and six days in a 5% CO_2 environment.

The fraction of the total soluble protein that was the large or small RuBisCO subunit did not significantly vary between immobilised and suspension samples after three or six days (repeated measures two-way ANOVA; large RuBisCO subunit - F = 0.7585, df = 1, P = 0.4329, small RuBisCO subunit - F = 2.353, df = 1, P = 0.1998) (Figure 3.2).

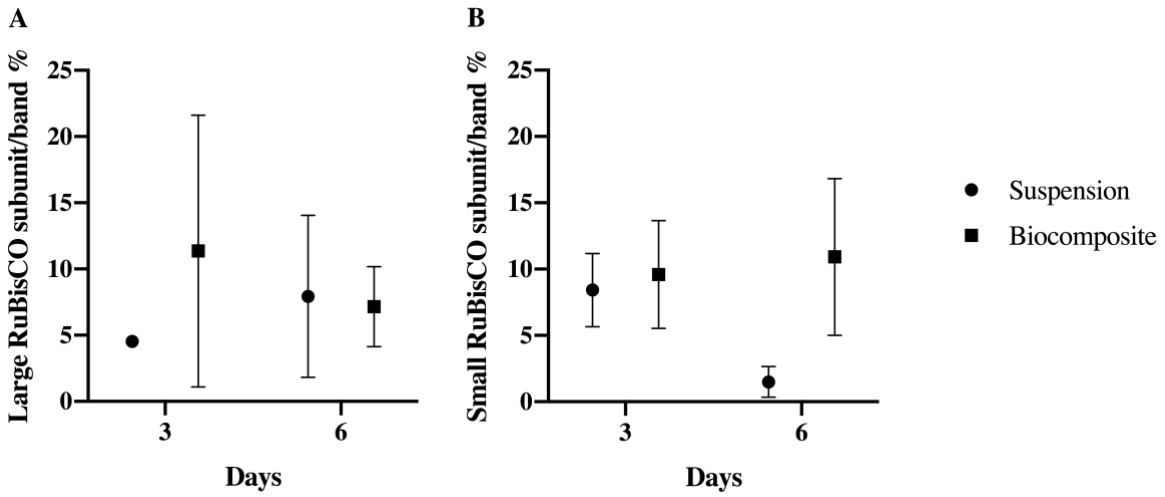

Figure 3.2. Mean band percentage (± standard deviation) of the total soluble protein that was the large (A) or small (B) RuBisCO subunit *Synechococcus elongatus* PCC after three and six days in a 5% CO_2 environment.

3.3.2 S. elongatus CCAP 1479/1A

Despite there being no significant difference in orthophosphate uptake, or total protein uptake, there was a significant difference in CO_2 uptake due to time and immobilisation (repeated measures two-way ANOVA; CO_2 - $F = 88.66$, df = 1, $P = 0.0007$; Orthophosphate - $F = 0.5603$, df = 1, $P = 0.4958$; Protein - $F = 3.693$, df = 1, $P = 0.1309$) (Figure 3.3). Time was also a significant factor in CO_2 uptake ($F = 22.72$, df = 1, $P = 0.0089$) but only the suspension samples had significantly greater CO_2 uptake on day 6 (*post-hoc* Bonferroni's multiple comparison test, $P < 0.05$). Immobilisation alone, however, did not cause significant variation in CO_2 uptake ($F = 4.9$, df = 1, $P = 0.0913$) but the biocomposites did have significantly greater CO_2 uptake on day three (*post-hoc* Bonferroni's multiple comparison test, $P < 0.05$).

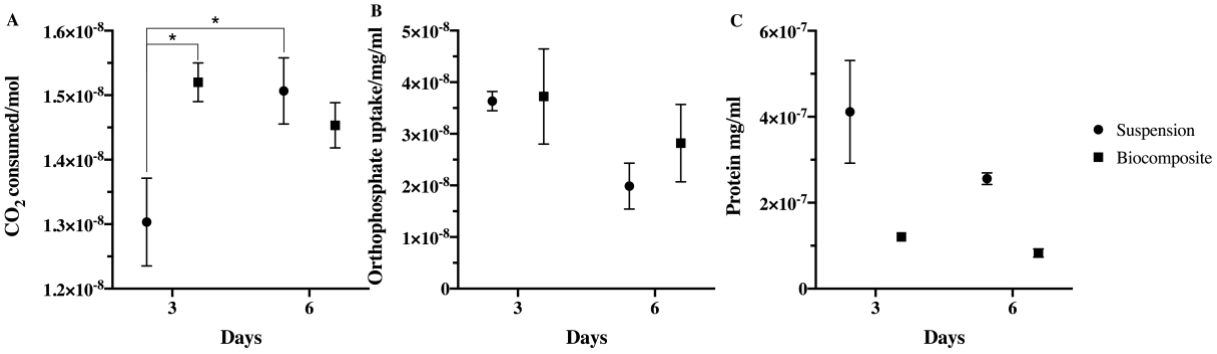

Figure 3.3. Mean (± standard deviation) of A) CO_2 uptake (mol), B) orthophosphate (mg/mL) uptake, and C) extracted protein (mg/mL) by *Synechococcus elongatus* CCAP after three and

six days in a 5% CO_2 environment. Asterisks indicate significant difference between samples at each time point on each graph (post-hoc Bonferroni's multiple comparisons test, $P < 0.05$).

Interestingly, the fraction of the total soluble protein that was the large RuBisCO subunit expressed by *S. elongatus* CCAP was not significantly influenced by the interaction between immobilisation and time ($F = 0.6874$, df = 1, $P = 0.4537$) but did significantly affect the expression of the small RuBisCO subunit ($F = 14.11$, df = 1, $P = 0.0198$). Both time ($F = 12.16$, df = 1, $P = 0.0252$) and immobilisation ($F = 11.24$, df = 1, $P = 0.0.085$) significantly influenced the expression of the small subunit. The suspension samples had a significantly higher proportion of the small subunit compared to the immobilised samples on day three, and to the suspension samples on day six. The biocomposite samples were not significantly different between days 3 to 6 (*post-hoc* Bonferroni's multiple comparison test, $P < 0.05$) (Figure 3.4).

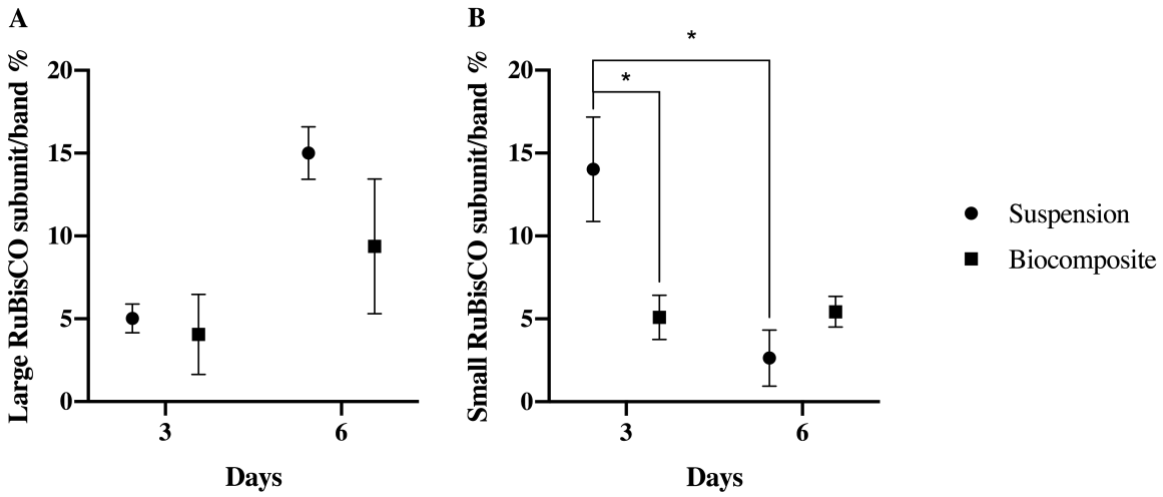

Figure 3.4. Mean band percentage (± standard deviation) of the total soluble protein that was the large (A) or small (B) RuBisCO subunit *Synechococcus elongatus* CCAP after three and six days in a 5% CO_2 environment. Significant differences are indicated by asterisks on individual graphs (post-hoc Bonferroni's multiple comparisons test, $P < 0.05$).

3.3.3 RNA-Seq: Quantities and qualities of samples

The extracted concentration of RNA was higher for the suspension samples, but the extracted concentration of RNA from the biocomposites had surpassed the required concentration from the sequencing facility (50 µg/mL) (Table 3.1). For both sets of samples, the A260/A280 were all within the acceptable range, indicating high purity RNA but the A260/A230 values were lower than the ideal value of two (Table 3.1).

Table 3.1. Mean values of RNA concentration (mg/ml), A260/A280, and A260/A230 values recorded in triplicate using a NanoDrop™ One/OneC Microvolume UV-Vis spectrophotometer. Suspension samples are indicated with an S, and biocomposites with a B.

Sample	RNA Concentration (µg/mL)	A260/A280	A260/A230
S1	179.8	2.00	1.78
S2	259.0	1.97	1.79
S3	126.1	2.04	0.66
S4	364.7	2.04	1.73
S5	167.9	1.86	1.34
B1	81.2	1.98	1.54
B2	88.6	2.05	0.57
B3	70.4	2.03	1.06
B4	92.0	2.03	1.78
B5	52.3	2.00	1.53

3.3.4 RNA-Seq: RIN^e values

For each sample, the RIN^e value and observations of each sample were determined. The biocomposite samples were notably more degraded that the suspension samples (Table 3.2). With the exception of sample B1, the lower ribosomal fragment was missing from all biocomposite samples. The gel electrophoresis output (Figure 3.5) from the TapeStation Assay demonstrated the reason for the reduced 23S/16S area in Table 3.2.

Table 3.2. RNA Integrity number, size of area, and observations made from RNA ScreenTape Analysis (TapeStation, Agilent). Suspension samples are indicated with an S, and biocomposites with a B.

Sample	RNA Integrity Number Equivalent	23S/16S (Area)	Observations
S1	8.1	0.6	-
S2	6.2	0.7	-
S3	6.9	0.5	-
S4	6.8	0.6	-
S5	7.6	0.5	-
B1	2.3	-	Upper ribosomal fragment degraded
B2	1.8	-	Lower ribosomal fragment missing
B3	1.8	-	Lower ribosomal fragment missing
B4	1.7	-	Lower ribosomal fragment missing
B5	1.6	-	Lower ribosomal fragment missing

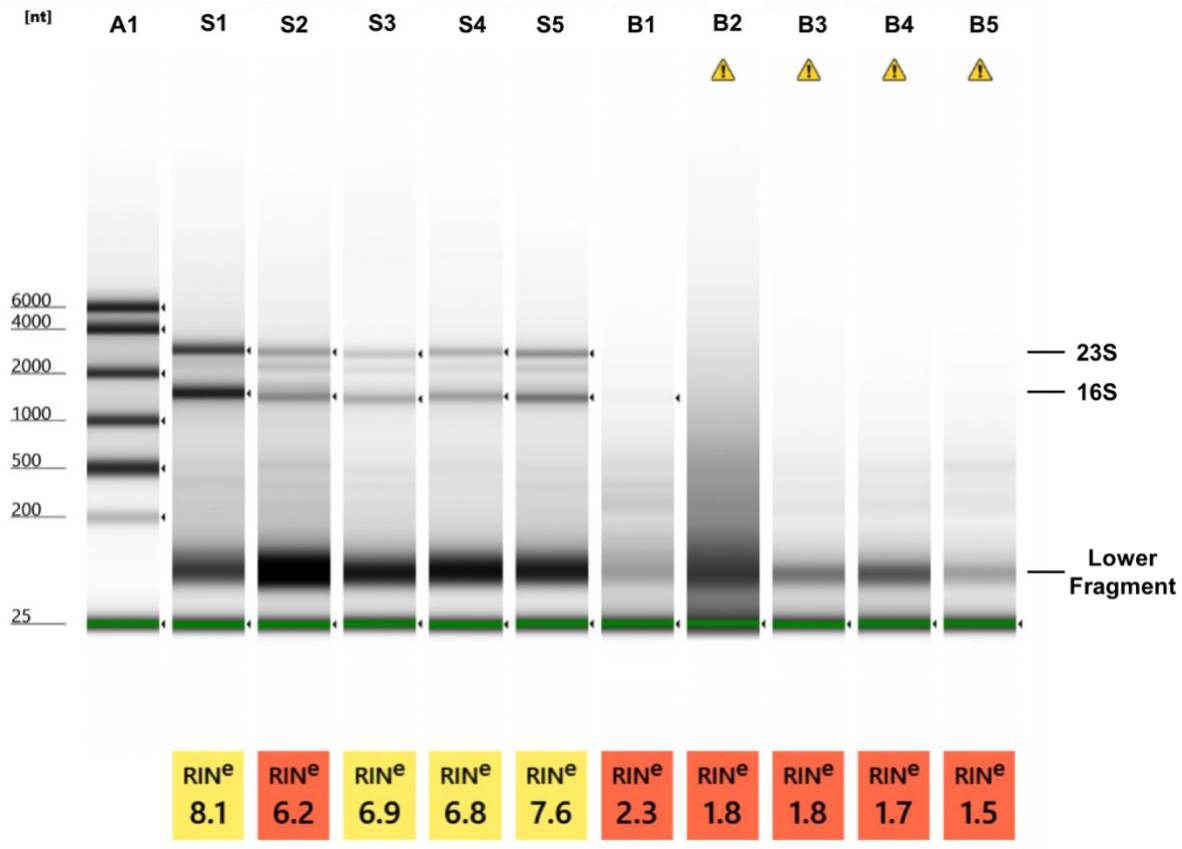

Figure 3.5. Gel electrophoresis output from RNA ScreenTape Analysis (TapeStation, Agilent). The control ladder in lane A1 allows for identification of the 23S, 16S, and lower fragments. The RNA Integrity Number equivalent (RIN^e) is presented for each lane. Lane A1 is the standards ladder. Suspension samples are indicated with an S, and biocomposites with a B.

3.4 Discussion

3.4.1 Physiological data and RuBisCO concentrations

The physiological parameters for *S. elongatus* PCC 7942 were not significantly different, suggesting that the immobilised cells were able to survive within the biocoating. *S. elongatus* CCAP 1479/1A, however, did have significant differences in CO_2 uptake after three days and a reduction in the small RuBisCO subunit. This indicates that the cells in the biocomposite were taking up more CO_2 but did not require as much RuBisCO for fixation. Depending on light intensity, the carbon fixation rate by *S. elongatus* ranges between 1.3–7.8 $\mu mol \cdot min^{-1} \cdot mL^{-1}$ with increased irradiance increasing carboxysome synthesis, thus increasing RuBisCO and improving carbon fixation (Sun *et al.*, 2016). However, cells contained in the biocomposite likely experience light limitation if not on the surface of the coating, potentially explaining the lowered RuBisCO small subunit expression. Therefore the increased CO_2 uptake by the biocomposite is paradoxical. In a given RuBisCO complex there are eight large and small subunits (Bracher *et al.*, 2017). These are arranged as anti-parallel tetramers with the small

subunits capping the large ones due to the presence of the chaperone proteins GroEL and GroES that promote folding of the RuBisCO proteins (Hayer-Hartl *et al.*, 2016). Perera *et al.* (2019) argued the importance of proteomics to understand cell metabolisms, with SDS-polyacrylamide gel electrophoresis allowing for comparative studies. It is, however, important to note that gel electrophoresis can only reveal around a maximum of 20% of an organism's proteome (Mishra *et al.*, 2019). Therefore, it is more likely that there was an issue with protein separation in the SDS-Page gel, resulting in a misrepresentation of band percentage of the RuBisCO small subunit of the suspension samples due to another protein around the same 13 kDa size of the small subunit resulting in the significant increase observed.

3.4.2 RNA quality and transcriptomics

Depending on the threshold used, RIN/RIN^e values must be above 3.95 (Weis *et al.*, 2007) or a high as 8 (Imbeaud *et al.*, 2005), but more commonly above 6 (Gallego Romero *et al.*, 2014). The poor RIN^e values may have been associated with the low A260/A230 values. These may have been low due to contamination by salts, carbohydrates, proteins, or aromatic compounds (Gallagher and Desjardins, 2007) but this is unclear in this case. Due to the low quality RIN^e of the biocomposite samples, differential gene expression (DGE) analysis was not possible due to poor quality RNA leading to unequal gene coverage and increased false positive rates in DGE (Wang *et al.*, 2016). Schuierer *et al.* (2017) argue that the quality and number of samples are critical to the proper understanding of DGE which would not be possible in this case due to the highly degraded biocomposite samples. Despite being unable to complete DGE analysis on these samples, previous literature serves as a good indicator to what may have been observed if abiotic stressors were exerted on the biocomposites.

Cyanobacteria respond to multiple stressors by having plastic central metabolisms, including the glycolysis pathway, Calvin-Bensham-Bassham cycle, oxidative pentose phosphate pathway, and tricarboxylic acid pathway (Cui *et al.*, 2020). Although the only results with significant difference were CO_2 consumption per *S. elongatus* CCAP cell and the small RuBisCO subunit band %. The environment in the biocoating is markedly different to the usual suspension lifestyle of *S. elongatus*, therefore changes to gene regulation would be expected to ensure cell survival. The physiological parameters of CO_2 uptake, phosphate uptake, and protein concentration may be reflected in differential gene expression.

Under phosphate limitation, RNA synthesis was reduced to one-quarter of the original value, with net protein synthesis completely stopped between 30-36 hours in a *Synechococcus* strain (Grillo and Gibson, 1979). Under stress conditions it is expected that cyanobacteria redirect metabolism to focus on carbohydrate production rather than proteins, often undergoing

proteolysis (Duarte and Costa, 2017). Proteins containing phosphorous are involved in photosynthesis and two-component signalling pathways (Yang *et al.*, 2013), indicating that phosphate limitation may cause severe reduction in cell metabolism. This phenomenon is well documented, with phosphorous limitation significantly affecting nucleic acid synthesis and the Calvin-Benson-Bassham cycle, with the former affecting photosynthetic efficiency by decreasing the amount of photosynthesis associated reactants (Barsanti and Gualtieri, 2014). Specifically, proteins involved in the dark (*rbc*L) and light (*psb*A) reactions decreased in quantity during nutrient limitation in *Synechococcus* sp. (Fernández-González *et al.*, 2020). Additionally, Teikari *et al.* (2015) found that *Anabaena* sp. responded to phosphorous depletion by a reduction in expression of genes relating to central metabolism, photosynthesis, and transcription and translation. Overall, 823 genes were differentially expressed after four days of phosphorous limitation. Concordantly, *Synechococcus* sp. WH8102 had differential gene expression of 1266 genes under phosphorous limitation, with 24 of these strongly upregulated. A third of these 24 are associated with transport, with six being phosphorous-specific ABC transporters, possibly to sequester larger amounts of phosphorous (Tetu *et al.*, 2009).

Some cells in the biocomposite may have received a lower photon flux density due to cell shading, which is beneficial to the PSII reaction centre as the cell is able to replace D1 proteins (Ohnishi *et al.*, 2005), those cells at the surface of the biocomposite were not able to migrate to a region of lower light intensity. The excess light at the surface of the biocoating may induce ROS production, which inhibits synthesis of proteins, including those relating to photosystems (Takahashi and Murata, 2008). To survive higher photon densities, *Synechococcus* utilises a generalist strategy and maximises gene expression of ROS detoxification enzymes (*katG*, *sodB*), DNA repair (*recA)*, and dissipation of excess energy (*crtR*, *ocp*) (Mella-Flores *et al.*, 2012). Additionally, drying of the biocomposites may have influenced protein expression. Potts (1986) found that immobilisation and subsequent rewetting of *Nostoc commune* UTEX 584 caused a reduction in proteins between 18–97 kDa after drying, despite continued protein synthesis. Upon rewetting, no heat-shock or water-stress regulons were induced suggesting that immobilisation does not affect cells in predictable ways. It is, however, important to note that protein expression and concentration of cells fluctuates with the age of the culture, with older cultures having significantly reduced protein concentrations, potentially due to nutrient limitation (Elsalhin *et al.*, 2016).

3.5 Conclusion

Due to the poor quality of the extracted RNA from the biocomposite samples it was not possible to complete the main objective of this Chapter. Future work should focus on optimisation of

RNA extraction, with snap-freezing of samples in liquid nitrogen potentially improving yields and integrity. There were, however, some promising results – immobilised biomass did not have significant changes in CO_2 uptake, orthophosphate uptake, or protein synthesis. Cells could survive and complete biological functions at comparable levels to suspension cultures. This is encouraging for utilising biocomposites for bioremediation applications. A more detailed genomic picture would provide greater insight into the genetic regulation of biocoatings.

4 Immobilising metabolically engineered *S. elongatus* for wastewater treatment: A proof of concept for intensified wastewater treatment

4.1 Introduction

Photosynthetic microalgae and cyanobacteria are important platforms for the synthesis of novel products and processes (de Farias Silva and Bertucco, 2016). Their amenability to genetic transformation (Kim *et al.*, 2017; Patel *et al.*, 2019) opens the possibility for improvements in numerous applications, including; bioenergy (Baebprasert *et al.*, 2011; Zhu *et al.*, 2014), food additives (Poliner *et al.*, 2018), and industrial enzyme and chemicals production (Brasil *et al.*, 2017; Gudmundsson *et al.*, 2017; Lin *et al.*, 2017; Betterle and Melis, 2018). In addition to fuels and biochemicals, native microalgae and cyanobacteria have growing importance in the delivery of a range of ecosystem services (Berdalet *et al.*, 2016; Calahan *et al.*, 2018), including the bioremediation (phycoremediation) of municipal, industrial, and agricultural wastewaters (Dubey *et al.*, 2011; Idi *et al.*, 2015; Gonçalves *et al.*, 2016; Lyu *et al.*, 2016; He *et al.*, 2017). Cyanobacteria are particularly suited for bioremediation due to their metabolic flexibility and broad environmental tolerances (Whitton and Potts, 2012). The scale of the wastewater treatment challenge (Sato *et al.*, 2013), particularly given an estimated 80% of wastewaters are discharged untreated (WWAP, 2017), makes the water industry a prime candidate for process intensification, i.e. the use of novel processes to achieve improvements in process performance alongside orders of magnitude reduction in infrastructure size and cost (Coward *et al.*, 2018).

Phycoremediation has a role in achieving these objectives; however, dependence on native strains may limit process effectiveness. Further, reliance on conventional microalgae cultivation methods—which are predominantly suspension systems using open ponds and raceways, or closed system PBRs (Chen *et al.*, 2011a; Chiaramonti *et al.*, 2013; Slegers *et al.*, 2013)—introduces other complications such as limitations in microalgae cell density (commonly 0.5-3 g L^{-1} (Raeesossadati *et al.*, 2014)) and the potential for biomass loss (i.e. process dilution) once the treated waters are discharged. Alternative approaches based on biofilm reactors are being trialled (Zhou *et al.*, 2018; Wu *et al.*, 2019; Chaiwong *et al.*, 2020; Gou *et al.*, 2020) which ameliorate many of the deficiencies of suspension based cultivation systems, i.e. higher biomass loading per unit area and improved retention of metabolically active biomass within the treatment system. However, these systems are still limited by species and strain choice, although incremental improvements have been made using adaptive evolution (Xu *et al.*, 2018; Cheng *et al.*, 2019). Harnessing metabolic engineering to improve phycoremediation performance, for example by developing tailored services to remove or degrade chemicals that otherwise bypass conventional treatment processes, could be a

disruptive innovation. If delivered in an intensified form, e.g. as engineered biofilms, significant advancement towards wastewater treatment intensification would be made (Kümmerer *et al.*, 2018; Wang *et al.*, 2018).

There are understandable environmental concerns about growing engineered strains in open ponds (Flynn *et al.*, 2010); however, biofilms—if engineered to prevent cell release—could alleviate public concerns over the in situ use of metabolically engineered organisms. This trinity of challenges (metabolic engineering, biomass concentration, and biomass retention) describe the main conditions needed to deliver flexible and future-proofed process intensification for wastewater treatment. The current study forms part of a wider project (EU Horizon 2020: Living Architecture) aiming to demonstrate the potential for metabolic engineering for wastewater management, in which a phototrophic cyanobacterium (*Synechococcus elongatus*) and two heterotrophic bacteria (*Escherichia coli* and *Pseudomonas putida*) operate as an engineered suspension-based microbial consortium optimised for phosphate removal (García-Jiménez *et al.*, 2018; Uluşeker *et al.*, 2019; Nogales *et al.*, 2020). The consortium was engineered to operate in obligate positive feedback: *S. elongatus* 363 secretes sucrose for uptake by *E. coli* SBG610, which secretes fructose for *P. putida* SBG714, which in turn secretes vitamin B_{12} for *S. elongatus*. In this study, we extend the brief of the overall project by demonstrating proof-of-concept using the engineered strain of *S. elongatus* immobilised as a living biocomposite (a combination of metabolically active cells bound to an inert structural scaffold). The objective was to successfully and securely immobilise the organism without compromising its metabolic function and, crucially, without compromising the rate of secretion and export of sucrose.

4.2 Methods

4.2.1 Cell modification and cultivation

Synechococcus elongatus PCC7942 was genetically modified to produce *S. elongatus* 363 (classed as hazard group 1), which was designed to overproduce and secrete sucrose following the stable insertion of six genes into the neutral sites of the chromosomes (Table 4.1). It was grown in modified BG-11 medium (Stanier *et al.*, 1971) with additional 10 mM HEPES to buffer pH. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) was added to induce sucrose production. Cultures of sucrose induced and non-induced cells were grown in 500 mL flasks, containing 300 mL of media, with no external air supply. Flasks were incubated at 28-30 ºC on an orbital shaker at 190 RPM, under a 16:8 light:dark photoperiod using fluorescent tube lighting.

Table 4.1. Genetic modification of *Synechococcus elongatus* PCC7942 to make *S. elongatus* 363. The shorthand, full name, and role of the gene are presented with the corresponding plasmid used to integrate into neutral sites 1 or 2 of all copies of *S. elongatus* chromosomes.

Gene	Full Name	Role	Plasmid	Insertion Site
pgi	glucose-6-phosphate isomerase	Converts fructose-6-phosphate to glucose-6-phosphate	pMSM230	1
pgmt	phosphoglucomutase	Converts glucose-6-phosphate to glucose-1-phosphate	pMSM230	1
galU	UTP-glucose-1-phophate uridylytransferase	Converts glucose-1-phosphate to UDP-glucose	pMSM230	1
sps	sucrose phosphate synthase	Combines UDP-glucose and fructose-6-phosphate to sucrose	pMSM249	2
cscB	sucrose permease	Allows export of sucrose from the cell	pMSM249	2
lacI	Lac repressor	Inducible repressor of sps and cscB genes	pMSM249	2

4.2.2 Biocomposite development: toxicity and adhesion testing

The biocomposites were formed following the methodology in Chapter 2. The substrates used were the 80:20 cotton-polyester blend, a 40:60 wool-polyester blend, and a 100% non-woven polyester. Binder choice (commercial latex-based coatings: AURO 320 and AURO 321; AURO Paint Company, UK) and binder solids content (5%) was guided by the prior research on cyanobacteria biocomposites (Umar *et al.*, 2019) and data Chapter 2. As the engineered *S. elongatus* had not previously been evaluated for tolerance to immobilisation in this form, a series of toxicity and adhesion assays were conducted. Briefly, binder pH was adjusted to between 6.5 and 7.5 to prevent cell death, of which 1 mL was added to 1 mL of seven to ten days old cultures in triplicate in 24-multiwell plates. The pH adjusted binder and growth medium were combined to screen for interactions. One millilitre of cell culture was diluted with 1 mL of autoclaved dH_2O to provide a baseline for cell growth. Each replicate was mixed daily by forward and reverse pipetting for seven days, after which, relative percentage growth was

determined using an improved Neubauer haemocytometer with a Leica DMi 8 microscope (x200 magnification) running LasX software.

For adhesion testing, the textiles were autoclaved, dried at 105 °C for 3 h, and stored in a desiccator until use. Cells were centrifuged at 1720 relative centrifugal force (RCF) for 30 min at 20 °C. Cells and binders were combined in Eppendorf tubes to create a 2.5% biocoating which was pipetted onto the textiles to saturation (summarised in Figure 4.1). In a separate Eppendorf tube, the same volume of cell culture was added to 1 mL of dH_2O and counted to calculate the number of cells in the biocoating. The resulting biocomposites were dried in darkness at 20±2 °C for 24 h. The dried biocomposites were then added to 1 mL of growth medium in well plates, wrapped in aluminium foil to prevent cell division, and agitated on an orbital shaker at 80 RPM. Biocomposites were moved to a new well of fresh medium after 1, 24, and 48 h with the total time spent in media being 72 h. Cell release from the biocomposite into the growth medium was determined by cell counts at these time intervals.

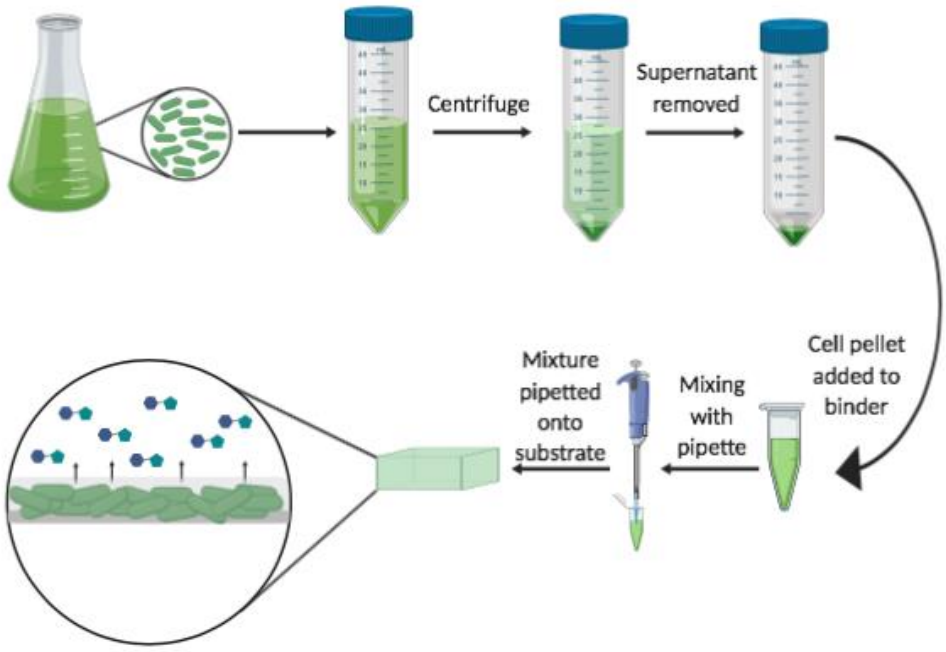

Figure 4.1. Schematic describing the process of biocomposite fabrication using *Synechococcus elongatus* 363 producing sucrose.

4.2.3 Sucrose analysis

Sucrose produced in response to IPTG was determined using a sucrose/D-glucose enzymatic assay (item number K-SUCGL, Megazyme). The enzymatic reactions are described in equations 1-3:

$$\text{sucrose} + H_2O \rightarrow \text{D-glucose} + \text{D-fructose (enzyme 1 – invertase)} \quad [\text{Eq. 1}]$$

D-glucose + H_2O + O_2 → D-gluconate + H_2O_2 (enzyme 2 – glucose oxidase) [Eq. 2]

$2H_2O_2$ + p-hydroxybenzoic acid + 4-aminoantipyrine → quinoneimine dye + $4H_2O$ (enzyme 3 – peroxidase) [Eq. 3]

The quantity of quinoneimine dye is equivalent to the quantity of D-glucose in the sample. Absorbance was measured spectrophotometrically at 510 nm, and converted to sucrose equivalents (mg/mL) using equation 4 if the absorbance was between 0 – 0.5:

$$= (abs/2.1184) * (342/180) \quad [Eq. 4]$$

If the initial absorbance was greater than 0.5, the samples were diluted with dH_2O to within the working range. The value of 2.1184 was determined from a calibration curve from linear regression of standards absorbance. The sucrose concentration was then normalised to the number of cells within the culture (mg/mL per cell).

4.2.4 Statistical analysis

Statistical analysis was conducted using IBM® SPSS® Statistics (Version 22). Data were tested for normal distribution (Kolmogorov-Smirnov test) and equal variance (Levene's test). Kruskal-Wallis and post-hoc Mann Whitney *U*-tests were employed for non-normal or unequal variance data. To ensure fair comparisons were made between induced and non-induced cultures, if either dataset had non-normal or unequal variance, Kruskal-Wallis and Mann-Whitney *U*-tests were used for both treatments. To determine whether there was any variation in growth between induced and non-induced cyanobacteria, cell counts were converted to arcsine transformed percentages to control for differing cell loadings and subjected to statistical testing.

4.3 Results

4.3.1 Toxicity testing

Both AURO binders were non-toxic to the IPTG induced *S. elongatus*, with cell density increasing significantly compared with the controls (Kruskal-Wallis test, K = 5.6, df = 2, $P < 0.05$; Figure 4.2A), although there was no significant difference between the binders (Mann-Whitney *U*-test, $P < 0.05$). Cells continued to produce and secrete sucrose with no significant change versus the control and no significant difference between binders (Kruskal-Wallis test, K = 1.882, df = 2 $P > 0.05$; Figure 4.2B). It was noticeable that the range in sucrose production values corresponded closely to the cell density range, although not necessarily with the median values. Of the binders, AURO 321 appeared to offer the more stable performance.

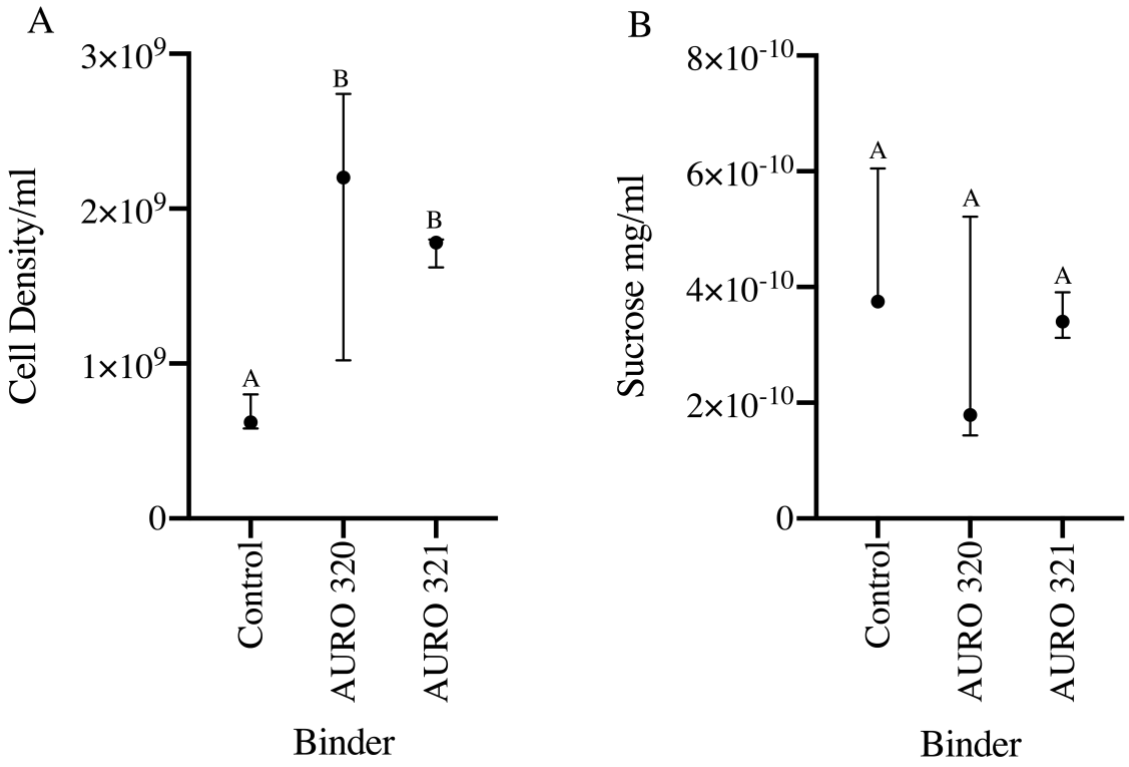

Figure 4.2. Cell density (A), and sucrose production (mg/mL) per cell (B), for IPTG induced *Synechococcus elongatus* 363 following a seven-day contact toxicity test with AURO 320 and 321 binders. Data presented as median and range. Shared letters indicate no significant difference between treatments.

The non-IPTG induced cultures also experienced significantly better growth compared to the controls (Kruskal-Wallis test, K = 5.6, df = 2, $P < 0.05$; Figure 4.3A), with AURO 320 supporting substantially higher growth that AURO 321 (Mann-Whitney *U*-test, $P < 0.05$). The culture, being non-induced, did not produce or secrete sucrose in any treatment (Figure 21B). There was no significant difference in growth between induced and non-induced cultures (Kruskal-Wallis test, K= 7.6, df = 3, $P > 0.05$).

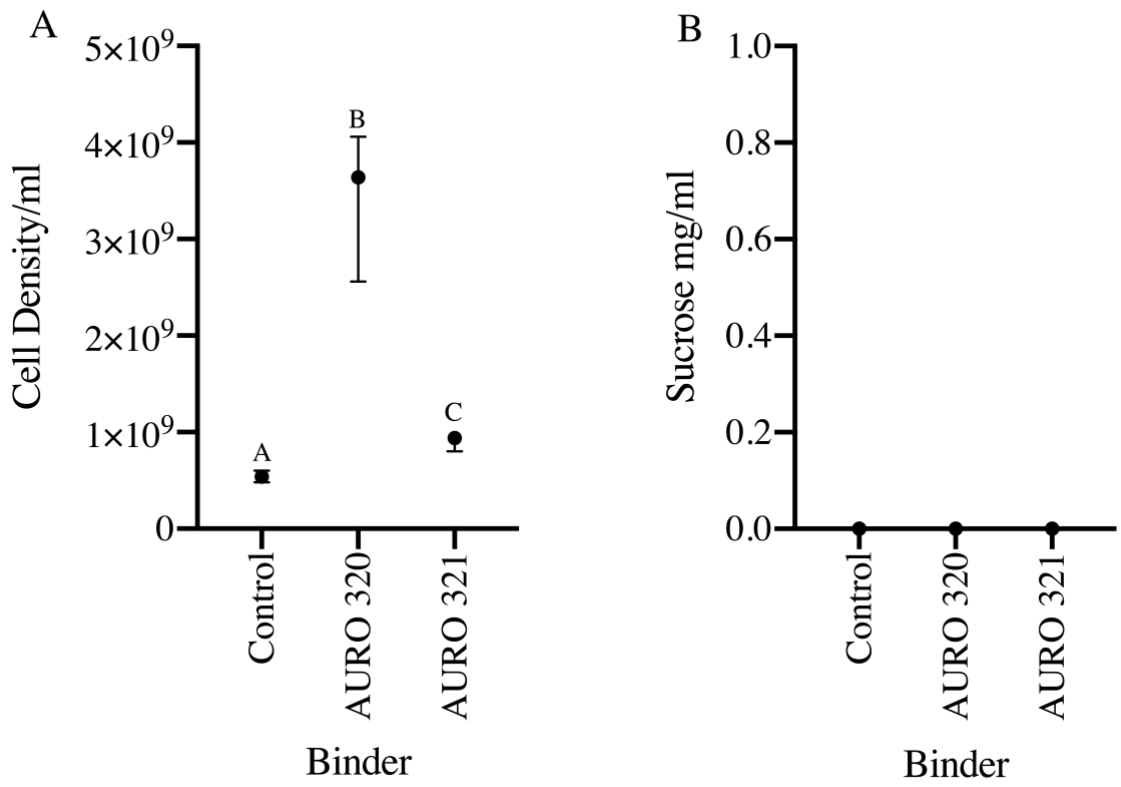

Figure 4.3. Cell density (A), and sucrose production (mg/mL) per cell (B), for non-induced *Synechococcus elongatus* 363 following a seven-day contact toxicity test with AURO 320 and 321 binders. Data presented as median and range. Shared letters indicate no significant difference between treatments.

4.3.2 Biocomposite performance: Adhesion and sucrose release

Cells were lost from all biocomposites (Kruskal-Wallis test, K = 18.198, df = 6, $P < 0.05$; Figure 4.4A), with the wool-AURO 320 combination showing the greatest median loss (1.57×10^8 cells), retaining 81.81% of cells within the biocomposite. Wool-321, polyester-320 and polyester-321 fared significantly better, retaining 88.07, 87.60 and 92.24% of cells respectively. The best performing biocomposites were cotton-320 (97.45%) and cotton-321 (97.80%). There was a difference in cell loss between induced and non-induced cells, with more cells lost from the induced wool-320 and polyester-321 (Kruskal-Wallis test, K = 32.2, df = 11, $P < 0.05$; Mann-Whitney *U*-test, $P < 0.05$). Cumulative sucrose secretion in BG11 after 72 hours of adhesion testing did not conform to normal distribution (Kolmogorov-Smirnov, $P < 0.05$). There was a significant difference in normalised sucrose secretion in biocomposites containing induced *S. elongatus* 363 (Kruskal-Wallis test, K = 16.534, df = 6, $P < 0.05$; Figure 4.4B). Curiously, more sucrose was leached per cell from biocomposites that supported the poorest cell retention, i.e. both wool biocomposites and polyester-320. There was no significant

difference between binders when used with matching scaffolds, except for polyester with cells immobilised with AURO 321 producing less sucrose than cells immobilised with AURO 320 (Mann-Whitney *U*-test, $P > 0.05$).

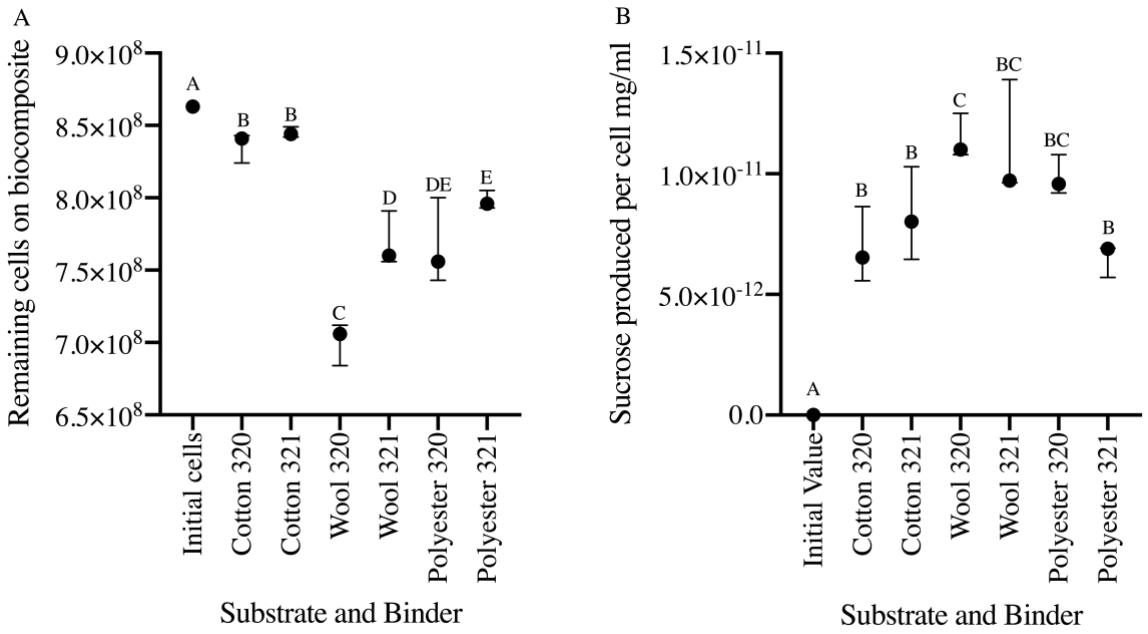

Figure 4.4. Cell retention (A) of IPTG induced *Synechococcus elongatus* 363 from fabric (cotton, wool or polyester) biocomposites with AURO 320 and 321 binders; (B) sucrose production (mg/mL per cell). Data presented as median and range. Shared letters indicate no significant difference between treatments.

As with the induced cells, similar proportions of non-induced cells were lost from all biocomposites: cotton-320 = 97.92%, cotton-321 = 98.05%, wool-320 = 87.78%, wool-321 = 91.55%, polyester-320 = 88.82%, and polyester-321 = 95.84% (Kruskal-Wallis test, K = 19.286, df = 6, $P < 0.05$; Figure 4.5A). Non-induced S. elongatus 363 did not produce or secrete sucrose when immobilised with either binder (Figure 4.5B).

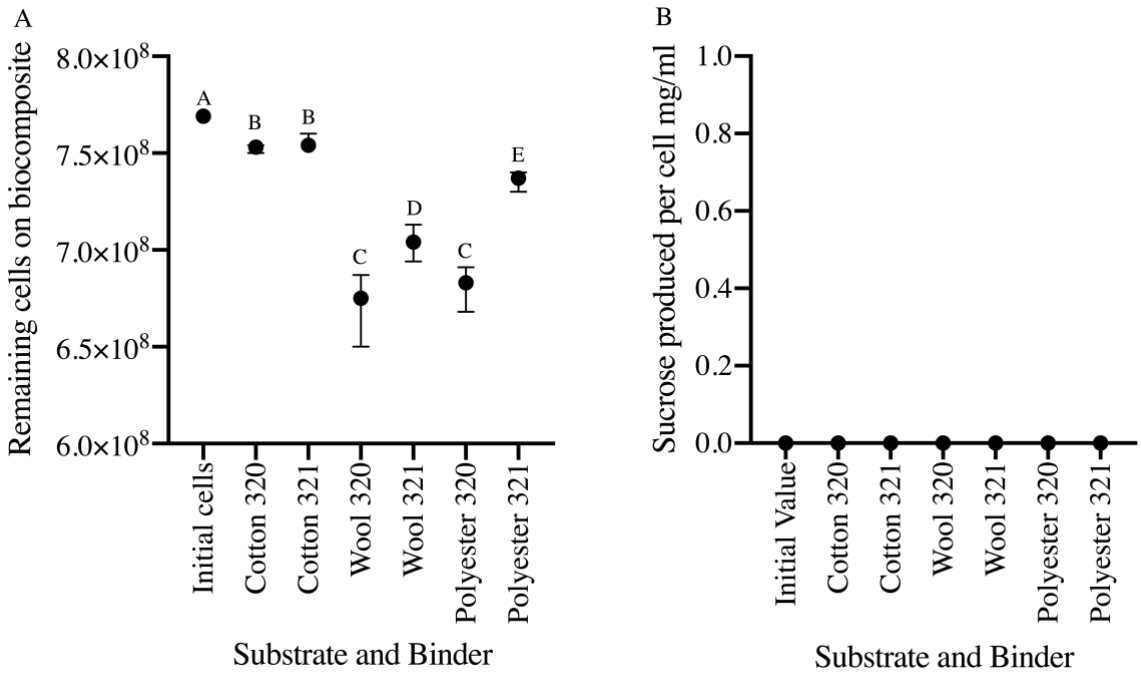

Figure 4.5. Cell retention (A) of non-induced *Synechococcus elongatus* 363 from fabric (cotton, wool or polyester) biocomposites with AURO 320 and 321 binders; (B) sucrose production (mg/mL per cell). Data presented as median and range. Shared letters indicate no significant difference between treatments.

4.4 Discussion

In this study, we successfully demonstrate proof-of-concept that metabolically engineered cyanobacteria can be immobilised within a functional biocomposite that could be deployed as part of a targeted wastewater remediation strategy. We utilised engineered *Synechococcus elongatus* as a chassis and successfully incorporated it into a prototype textile-based biocomposite, demonstrating cell survival, the maintenance of metabolic performance and, a high rate of retention of cells within the biocomposite structure.

The binders were non-toxic and actually promoted growth in both induced and non-induced treatments, particularly AURO 320. Similar growth promoting patterns were previously seen with the same binders using wild type strains of *S. elongatus* (CCAP14791/A was increased by AURO 320 and PCC7942 was increased by both AURO 320 and 321(In-na *et al.*, 2020)). The binders are commercial latex-based emulsion paints marketed for application to internal surfaces; they are therefore not formulated to support microbial growth or to retain microbes within the coating structure. However, this serendipitous trait makes them amenable for biocomposites development. None of the biocomposites were fully efficacious when it came cell retention, although the cotton biocomposites lost fewer than 3% of the loaded cells. These

figures are broadly in line with prior work on wild type strains immobilised within loofah sponge-based biocomposites (In-na *et al.*, 2020). These findings engender confidence that optimised latex-based binders can be formulated that will successfully retain all cells within the structure over extended operational periods.

S. elongatus naturally utilises sucrose as an osmoprotectant. There was no significant difference in sucrose production compared with control cultures. When comparing between induced and non-induced *S. elongatus* 363, sucrose secretion had no significant effect on adhesion. Induced cells continued secreting sucrose during the adhesion test, despite being kept in darkness. The mechanism for this is unclear although carbon fixation enzymes have been found to accumulate in salt-stressed cells indicating a redirection of energy away from primary metabolism (Pandhal *et al.*, 2009). Under low stress conditions glycogen production and storage accounts for up to 20% of fixed carbon (Hendry *et al.*, 2017), with glycogen and sucrose metabolic pathways competing for carbon (Lin *et al.*, 2020). Both glycogen and sucrose production utilise fructose-6-phosphate (F6P) from the Calvin-Benson-Bassham cycle. To produce glycogen, F6P is converted to glucose-1-phosphate (G1P) with an intermediary glucose-6-phosphate, causing glycogen and sucrose production to compete; G1P is either processed to glycogen by glucose-1-phosphate adenylyltransferase and glycogen synthase enzymes or converted to uridine diphosphate glucose by UDP-glucose pyrophosphorylase and combined with F6P by sucrose-phosphate synthase to the intermediary sucrose-6-phosphate which is catalysed by sucrose-phosphate phosphatase to intercellular sucrose. The activation of this pathway redirects a significant proportion of fixed CO_2 away from biomass accumulation. *Synechococcus* sp. cannot export sucrose, halting production due to limited intracellular space and reducing photosynthetic activity as the cells are unable to balance the carbon flux (Qiao *et al.*, 2018). Strains without the insertion of a transporter are unable to secrete sucrose, potentially limiting the quantity produced. To increase production, *S. elongatus* PCC 7942 was transformed by inserting the sucrose permease transporter *cscB* from *E. coli* with an IPTG inducible promoter into the neutral site 3 vector (Ducat *et al.*, 2012). The recombinant strain channelled up to 80% of fixed CO_2 into sucrose and saw a 10-fold increase in sucrose export compared to wild-type *S. elongatus* PCC 7942 (Duan *et al.*, 2016).

Our findings differ from sucrose production studies conducted in suspension. Many studies observed that as sucrose production increased, cell growth decreased or ceased entirely. Reduced growth rates may be caused by an increased expression of *rpaB* (Moronta-Barrios *et al.*, 2013). Moronta-Barrios and co-workers demonstrated that overexpression of *rpaB* prevented cell elongation, causing cell growth to cease. *rpaB* works with the environmentally

responsive sensor kinase *Nb1S*, creating the essential *Nb1S-RpaB* pathway that can regulate environmental stressors and influence photosynthesis (Wilde and Hihara, 2016). The production and secretion of sucrose increased proportionally with an increase in the quantum yield of photosystem II and oxidation levels of the electron transport chain (Abramson *et al.*, 2016). The activity of the Calvin-Benson-Bassham cycle is not reduced within hours of osmotic stress and cells produce sucrose to mitigate osmotic stress, to the detriment of cell growth. The overexpression of *RpaB* ceases cell growth but increases sucrose production two-fold (Abramson *et al.*, 2016). During this, photosystem II showed a 5.2% increase in activity allowing the inference that energy is redirected into sucrose production rather than growth. Similar decreases in growth of cultures that were overexpressing *sps* and *spp* genes with negligible amounts of glycogen production were observed despite expression of glycogen synthesis genes showing no significant expression variation (Lin *et al.*, 2020).

Many studies focus on gene regulation but neglect enzyme V_{max}. Under carbon limited conditions the sucrose production pathway would ordinarily be overexpressed compared to primary metabolism (Abernathy *et al.*, 2017). It was hypothesised that sucrose-phosphate synthase may be the rate limiting enzyme in sucrose production due to modifications in *sps* expression having the greatest effect on sucrose production; excess carbon may be available if *sps* expression is maximised (Du *et al.*, 2013). This excess carbon could be redirected as glycogen for growth stored by the cells prior to induction with IPTG (Guerra *et al.*, 2013). When induced, metabolism would have redirected fixed carbon into sucrose metabolism rather than glycogen. As the export of sucrose is creating a continuous sink, both growth and sucrose production can continue. AURO binders are likely responsible for this excess energy as all cultures with AURO binders, regardless of type of binder or sucrose induction, showed significant growth compared to cultures without binders.

Traditionally, cultivation of photosynthetic microorganisms has occurred in open ponds and PBRs in suspension systems. The accidental escape of microorganisms from these systems is inevitable (Gressel *et al.*, 2014), although an engineered algae was found to not outcompete natural strains from local lakes in mixing experiments, but dispersion was rapid and it cannot be assumed that every engineered strain would not outcompete natural populations (Szyjka *et al.*, 2017). The stochastic nature of the natural environment and "the paradox of plankton" (Hutchinson, 1961) mean that modelling the potential of an engineered spill from laboratory data would not meaningfully contribute to robust risk assessments. Therefore, it is recommended to culture engineered strains with selective pressures, also known as biocontainment. Biocontainment can be abiotic factors such as salinity, pH, temperature, and

high CO_2 genetic modifications (Clark *et al.*, 2018). In the case of *S. elongatus* 363, *lacI*—a lac repressor—was added, meaning the modification would not be expressed without the presence of IPTG as the genes are under the control of the lac repressor.

4.5 Conclusion

Biocomposites show promise to support and intensify wastewater treatment by outperforming suspension-based processes. Further work is needed to refine biocomposite composition, for instance to improve biocoating porosity and thereby increase mass transfer rates (Chen *et al.*, 2020); although this must be achieved without compromising the biocoating's capacity to securely retain cells within the biocomposite structure. Aside from alleviating concerns over the escape of transgenic organisms (Rosenberg *et al.*, 2008), biocomposites negate biomass loss from cell washout (as per suspension based algae culture systems), occupy smaller areas, can be reused for multiple treatments, and will lower the operational cost of wastewater treatment (Mallick, 2002; Eroglu *et al.*, 2015), therefore meeting the requirements for process intensification.

5 Synthesis Chapter

5.1 Summary of thesis

This thesis has highlighted the need for process intensification of the wastewater treatment industry by utilising higher amounts of biomass that is less susceptible to wash-out from the systems. Immobilised cyanobacteria show promise as a target organism due to their metabolic flexibility, well sequenced genomes, and genetic manipulability.

Through the literature review a key gap in process intensification research was identified. Too often interdisciplinary work is neglected which may slow the rate of progress of innovative processes and technology. Living Architecture (LIAR) is a prime example of the successes that can be achieved when multidisciplinary teams work together. Collaboration between researchers with expertise in metabolic and synthetic biology, unconventional computing, robotics, algaeponics, bioenergy, and architectural design resulted in the production of a next-generation selectively-programmable bioreactor that was prototyped in Bristol in just three years. The exemplary work of LIAR sets a precedent for multidisciplinary projects that take novel ideas from method development to advanced biology with in-situ testing.

Chapter 2 focussed on refinement of immobilising cyanobacteria to textile substrates using non-toxic binders. Following the methodologies developed In-na *et al.* (2020) a strong emphasis was placed on ensuring biomass remained attached to the chosen substrates whilst ensuring the biocoating remained metabolically active. Up to 95% biomass was retained on substrates using the AURO binders. The uptake of CO_2 was a simple parameter to determine metabolic activity of *S. elongatus* during this refinement period. The data demonstrated that under certain conditions, biocoatings could outperform their suspension counterparts regardless of substrate used. However, when the thickness of the latex was increased, or more biomass was incorporated into the biocoating, CO_2 uptake was reduced necessitating further research into the optimal concentrations of both factors.

As noted in Chapter 2, a range of factors can affect the success of the biocoating. Collecting CO_2 uptake data alone, however, does not elucidate the causal reasons for success or failure. In a scaled back experiment, we sought to answer a simple research question – does immobilisation of *S. elongatus* PCC 7942 significantly alter the expression of genes compared to a suspension counterpart? Due to mass transfer limitations caused by the latex binder, it was hypothesised we would see significant changes of expression levels of genes related to the central metabolism of *S. elongatus* as access to fundamental resources would be reduced. Unfortunately, during the RNA extraction process the quality and quantity of RNA was

disrupted for all immobilised samples. Optimisation of extraction protocols should be determined by utilising different extraction kits or methodologies, or a methodology for clean-up of the RNA post extraction should be developed to ensure good RNA integrity. Additionally, RNA-Seq experiments with a greater number of factors including growth in artificial urine, longer and multiple time periods, and different substrates and binders should be conducted. This should also be continued if experiments are scaled up as operational size may affect gene expression. In Chapter 2, CO_2 uptake was significantly reduced when an artificial urine medium was used rather than a standard growth media. Careful consideration should therefore be applied as one purpose of this thesis is to utilise biocomposites for treatment of domestic wastewater. The mechanism responsible for this reduced uptake remains unclear as many cyanobacteria have genes responsible for the uptake of nitrogen in urea (Veaudor *et al.*, 2019) but was unfortunately outside the scope of this thesis.

The work thus far had focussed on utilising model wild-type strains of *S. elongatus.* As part of the LIAR project, a genetically recombinant strain of *S. elongatus* was developed (*S. elongatus* 363) to over-express and export sucrose as a feed source for *E. coli* in a novel microbial fuel cell. As noted in the literature review, LIAR was a phenomenal success in technology development but productivity was ultimately limited by the organisms' planktonic lifestyle. As a proof of concept, *S. elongatus* 363 was immobilised into a biocoating with almost 98% of the biomass remaining, outperforming the wild-type strains in Chapter 2. Although the sucrose output per cell was not significantly different when binders were used, its promising that sucrose was still leached from the biocoatings. It would be useful to perform a similar RNA-seq experiment to the one detailed in Chapter 3 on the impact of immobilisation on *S. elongatus* 363 sucrose production. As a proof of concept, we are happy with the results of this Chapter as the first known example of synthetic cyanobacteria biomass being immobilised, but a more robust experiment including CO_2 uptake, nutrient uptake, and proteomics is recommended.

Although we have highlighted where future research should be directed, this thesis provides a strong basis for the utilisation of biocomposites as a method for process intensification for bioremediation. When compared to large suspension cultures either in open ponds or photobioreactors, we predict that biocomposites ameliorate problems associated with suspension cultures (Table 5.1). More biomass can fit in the same footprint, less biomass is lost to washout, and concerns regarding synthetic strains are reduced.

Table 5.1. Comparison of key factors affecting biomass production in open ponds, photobioreactors, and biocomposites.

	Open Pond	Photobioreactor	Biocomposite
Productivity	Variable	High	High
Capital cost	Low	High	Low
Maintenance cost	Low	High	Low
Footprint	High	High	Low
Contamination	High	Low	Low
Cell loss	High	High	Low
Energy consumption	Low	High	Low
Reuse of biomass	Low	Low	High
Biosecurity	Low	High	High

5.2 Future applications of immobilised photosynthetic microbes

Much of this thesis has been written within the context of wastewater treatment in the UK which is in urgent need of radical new processes. The vision of LIAR could be executed utilising the technology presented in this thesis as the minimal-water to support biomass approach would allow for greater volumes of wastewater to be treated. The results of this thesis present a novel alternative for bioremediation of wastewater that can be applied in a range of settings. Across the globe the human population is rapidly expanding with the United Nations estimating it will hit 9.7 billion by 2050 (UN, 2019). The current linear economy that utilises resources in a take-make-dispose fashion is unsustainable and will not be able to support the rapidly growing population of the future. A shift to circular economies is required to eliminate waste through creating closed loop processes (Sariatli, 2017). Circular economies necessitate restorative and regenerative design disrupting many aspects of chain production and consumption (Esposito *et al.*, 2018). Wastewater fits well in this model, where we can utilise it as a valuable resource for production of fine chemicals. When water treatment is coupled with the production of bio-products the environmental impact of wastewater clean-up is reduced in terms of water usage, power consumption, and waste generation (Arashiro *et al.*, 2018; Sutherland and Ralph, 2020).

Immobilised biomass, as presented in this thesis, acts as a chassis for increasing bio-production as more biomass can be utilised in the same area coupled with free growth media.

Immobilised biomass could act as a delivery system to parts of the planet where transport of liquid cultures is impractical. As cyanobacteria and microalgae are nutrient dense for key nutraceuticals such as phycobiliproteins, β-carotene, and astaxanthin, immobilised biomass could act as a simple delivery mechanism that preserves these. Alternatively, there is evidence that protein from photosynthetic microbes is an eco-friendly alternative to livestock protein, with *Chlorella* and *Arthospira* having well-balanced amino-acid profiles required for human health according to the World Health Organisation (Caporgno and Mathys, 2018). Condensing the required amounts of biomass onto 3D biocomposites may help prevent non-communicable diseases induced by poor nutritional balance as delivery and maintenance will be reduced. This could also be applied to synthetic strains of microalgae or cyanobacteria producing and secreting pharmaceuticals or use as edible vaccination strategies which currently rely on liquid cultures and lyophilisation (Kurup and Thomas, 2020).

More significantly, however, this work fits into a vision of a green future. We need to focus on designing homes that are sustainable and utilise green technologies. We advocate for utilisation of LIAR-like technologies in these buildings for wastewater management but suggest extending the technological applications of immobilised biomass.

Currently, 92% of the global population is being exposed to unsafe levels of air pollution, with 3 million deaths linked to outdoor air exposure in 2012 (WHO, 2016). Carbon capture is another key technology that has previously utilised suspension biomass. Humans exhale between 4 – 5% CO_2 which could be sequestered by immobilised biomass in the home. Previously, Dittmeyer *et al.* (2019) detailed a process that utilised air conditioning units to remove CO_2 from dwellings, but this process relied on the captured carbon being stored on the seabed. Instead, why not utilise a green technology? This technology need not be cumbersome or obvious in the home but could take the form of something as simple as wallpaper or curtains. The immobilised biomass could utilise CO_2 and moisture in the air, acting as in situ air purifiers. Previously, the industrial designer Adam Miklosi designed the *Chlorella* pavilion (Figure 5.1) where people would be able to visit and benefit from the oxygen produced in symbioses with the microalgae in photobioreactors (Miklosi, 2013). Miklosi detailed the benefit of symbioses with microalgae so why are we not incorporating these into our living spaces? Similar sentiments were utilised in the 2015 design project – Living Things by Frier and Douenias (2015). *Spirulina* was utilised to produce oxygen in photobioreactors disguised as light fittings in a home setting with a control panel situated in another part of the home (Figure 5.2). Frier

and Douenias (2015) emphasised the importance of living in symbioses with microorganisms in our homes as these living structures improve air quality, and utilise our excess light and heat energy. Our process-intensified vision allows for humans to reap the benefits of symbioses in our homes.

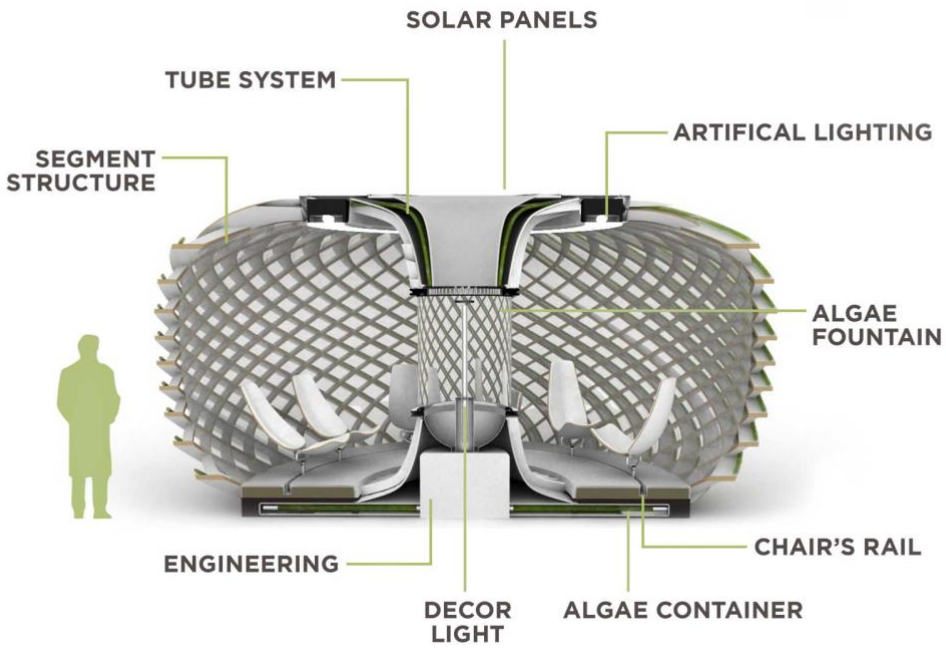

Figure 5.1. The *Chlorella* pavilion designed by Miklosi, 2013 where people can visit to reap the benefits of breathing oxygen produced by the microalgae.

Figure 5.2. *Spirulina* bioreactors disguised as light fittings as part of the Living Things project (Frier and Douenias, 2015).

On a much larger scale, whole building façades could be turned into intensified photobioreactors as the next-generation step to the BIQ project which utilises natural sunlight for microalgal growth and heat production for the attached building (Wurm and Pauli, 2016). With the estimate that 60% of the global population will soon live in cities (WHO, 2016) there is scope to develop eco-cities powered by photosynthetic biomass. Much like the BIQ house, the (EcoLogicStudio) is an architectural group focussed on building integrated nature and environmental design. Their view of cities as "agri-urban" ecosystems has led to the design of BioCities that utilises bioenergy to function. Previous designs include the Algae Canopy, Urban Algal Folly, BioBombola, and BioTechHUT (Figure 5.2). The aims of these designs include CO_2 sequestration, oxygen production, light generation, and food production.

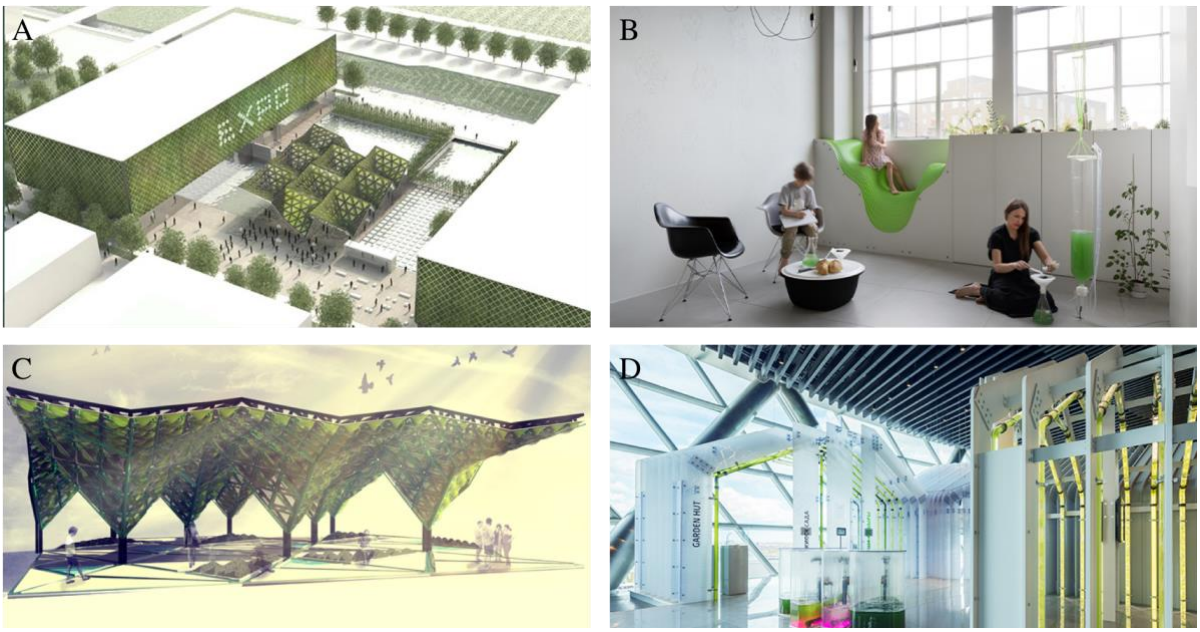

Figure 5.3. Designs from the ecoLogicStudio; A) Urban Algal Folly, B) BioBombola, C) the Algae Canopy, and D) BioTechHUT (EcoLogicStudio).

As we begin to envisage a future with immobilised photosynthetic microbes, the possibilities are vast. However, to produce sufficient amounts of immobilised biomass we must have significant amounts of textiles to work with. The fashion industry produces up to 92 million tonnes of waste textile per year with biodegradable materials making up a much less substantial proportion since the mid-1990's (Niinimäki *et al.*, 2020). Although drastic change is needed in this industry to vastly reduce the amount of waste produced, some of the waste could be repurposed for biocomposite production. Although we have utilised the biocomposites for static treatments, there is scope for wearable algae-infused clothes. Blond and Bieber (2014) have developed Algaemy – textiles utilising microalgae as a pigment in their garments production as an eco-friendly alternative to traditional dyes. As the garments age the textiles change colour leading to an ever-evolving piece of fashion. A more extreme version of this is the Algaculture symbiosis suit used to perform The Algae Opera (Figure 5.4) developed by Burton and Nitta (2012). As a wearable piece of art and fashion, CO_2 exhaled by the opera singer was used to grow the microalgae which was then intended to be used as a food source.

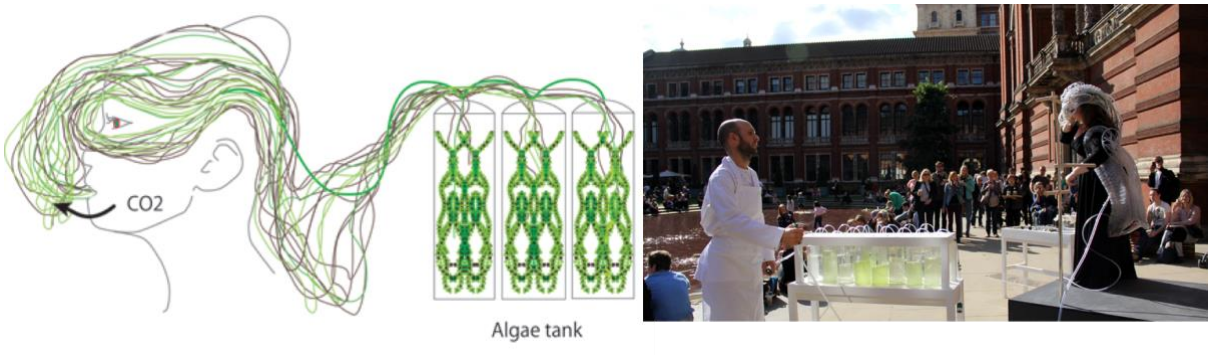

Figure 5.4. The Algaculture symbiosis suit design (Left) and opera singer wearing the Algaculture symbiosis suit to exhale CO_2 to grow the microalgae (Right).

5.3 Extra-terrestrial intensification

Although an art project in 2012, there is scientific merit to the work done by Burton and Nitta. As humans plan to colonise space there are significant concerns about how oxygen will be produced, water will be recycled, shielding from radiation, and how food will be established on the Martian surface. Previously, *Chlorella vulgaris* has been successfully cultured on the International Space Station for 180 days as part of a trial for the Environmental Control and Life Support System (Niederwieser *et al.*, 2018). Perhaps even more significantly, as part of the BIOlogy and Mars EXperiment (BIOMEX) a strain of cyanobacterium, *Chroococcidiopsis* sp. CCMEE 029 was embedded on artificial martian and lunar mineral environments and exposed to the space environment. After 1.5 years of exposure to space with temperatures ranging between -4°F to 116°F, *Chroococcidiopsis* sp. CCMEE 029 survived on the artificial Martian surface with low level of DNA damage and regained metabolic activity (de Vera *et al.*, 2019). Detailed by Aronowsky (2017), taking full stores of physicochemical systems (water, oxygen, and food) on long missions would not be feasible due to the significant amount of weight it would add, necessitating bioregenerative systems powered by microalgae and cyanobacteria. Synthetic biology is set to aid long-duration space missions and formation of lunar and Martian bases. Menezes *et al.* (2015) identifies six challenges for long-term survival in space; resource utilisation, manufacturing, life support, medicine and human health, cybernetics, and terraforming. Synthetic biology may provide cost-effective and bioregenerative resources for long term space colonisation. Utilising our system of immobilised biomass would add an extra cost-saving mechanism that is less prone to leakage than a suspension based system.

Ultimately, long term survival in space relies on being able to build structures quickly and reliably on the Martian surface. NASA have designed Myco-Architecture which will grow at destination with the aim that the structures will be able to support long-term human survival as a self-repairing material. Growth of myco-architecture does, however, rely upon the presence of oxygen which is absent on Mars. The cyanobacterial species, *Anabaena variabilis* 7120, has been chosen as a test strain due to its diazotrophic and photosynthetic capabilities. Oxygen and sugars produced by *Anabaena* will be used as a feedstock for *Mycelium* and *Bacillus subtilis* to grow and fill the plastic bladder that was seeded on Earth (Figure 5.5) (Rothschild *et al.*, 2019). In preliminary tests, however, the mycelium failed to grow as the cultures of *A. variabilis* were too low to produce sufficient volumes of oxygen. Utilising our system of increased biomass loading, and therefore greater oxygen production, may aid efforts to sustain a Martian colony. Additionally, in the current model proposed by NASA, the cyanobacteria will then be grown in suspension bioreactors to provide a continuous oxygen supply to settlers with the expectation that Martian water sources will be available for cultures. Employing an immobilised, water-minimal system as proposed in this thesis could aid long-term survival as we have shown that immobilised cyanobacteria can out-perform suspension cultures for CO_2 uptake.

Figure 5.5. Growth of Myco-Architecture on the Martian surface where water, CO_2, and heat are delivered by robots to promote algal activity which will feed mycelium and *Bacillus subtilis* to produce inhabitable structures (Rothschild et al., 2019).

Moreover, this thesis has demonstrated that bio-products produced by immobilised cyanobacteria are able to leach from the biocoatings. The applications of immobilised

microalgae and cyanobacteria in space extend to many aspects of extra-terrestrial survival. Synthetic strains could be engineered to produce essential food supplements to ensure inhabitants health such as essential amino acids, vitamins, antioxidants, and pharmaceuticals as proposed on Earth. Additionally, surrounding of Martian structures in photosynthetic biomass may protect inhabitants from the much higher levels of radiation on Mars compared to Earth (Matula and Nabity, 2016). Much like the LIAR design, photosynthetic microbes could also be used to power microbial fuel cells supplying Martian bases with a source of bio-energy alongside solar panels. Although this technology will aid colonisation it does raise ethical concerns. We currently still do not know if there is life on Mars, and colonisation of Mars by humans will ultimately also introduce trillions of other species carried in the human microbiome risking the search for native life on Mars and the rights that these potential "Martians" have (Cowley, 2019).

Conclusion

Immobilised cyanobacteria and microalgae is a prime candidate to aid the green revolution to a circular economy. As resources on Earth become more limited, inequalities within society will continue to grow with a linear economy. Innovative utilisation of renewable resources and recycling of existing materials will help form a circular economy, ultimately reducing inequalities in society and benefitting the health of Earth. As humans turn their attention to space, there are of course ethical concerns regarding colonising another planet. The wellbeing of those colonisers is of prime concern. Deploying intensified photosynthetic biotechnology should reduce risk to those early colonisers as they can provide several services with minimal human maintenance. Designed symbioses with photosynthetic microbes could reduce the environmental burden human population growth is putting on the Earth, whilst aiding human ambitions to colonise other planets. Attention to fixing the problems humans have created on Earth should be prioritised before colonising another planet that ultimately we have no right to.

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