

Engineered Solar-Driven Recycling Systems for Sustainable Fertilisation

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Declaration of originality

I hereby certify that the work presented in this thesis is my own and in the instances where this is not the case, the original contributors and sources are acknowledged accordingly.

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“Upon this handful of soil our survival depends. Husband it and it will grow our food, our fuel
and our shelter and surround us with beauty. Abuse it and the soil will collapse and die, taking
humanity with it”

Sanskrit text written in about 1500BC

Abstract

Harvesting food removes essential nutrients for plant growth, such as nitrogen and phosphorous, from the agricultural system. This is currently solved by applying chemical fertilisers, but their overuse contributes to environmental pollution with extensive societal costs. Despite attempts to replace them with nitrogen-fixing cyanobacteria, no global impact has been observed.

In this thesis, I have researched the use of the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 as a provider of biologically available nitrogen for both aqueous and terrestrial biomass production. Model co-culture systems were developed that enabled the transfer of nitrogen from *Anabaena* to a second engineered cyanobacterium, allowing its growth and metabolism. A new protocol for monitoring such co-cultures was developed and verified. Importantly, this protocol enables both singular and filamentous cyanobacteria to be quantified from the same sample. Contaminants were also isolated from an industrial algae pilot plant, with the intention to evaluate if the robustness of engineered biomanufacturing can be influenced by the implementation of co-cultures.

Finally, the same *Anabaena* strain was also tested as a fertiliser of wheat as a model crop plant. Importantly, it was demonstrated that the fertilisation capability of *Anabaena* is enhanced if it is applied alive, suggesting that continued nitrogen fixation may still take place even after

application to soil. Comparisons with a chemical fertiliser indicated that *Anabaena* was similarly efficacious. However, the high cost of algae manufacturing hinders immediate commercial use, necessitating further research for higher cost efficiency.

Overall, this study demonstrates that N_2 -fixing cyanobacteria have the potential to make a difference to both terrestrial and liquid photosynthetic biomanufacturing. The work provides a foundation for continued research into an exciting field of research that has important potential implications for human livelihood and a more sustainable way of life.

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List of Abbreviations

AM	arbuscular mycorrhizal
<i>amt14B</i>	ammonium uptake transporter genes
ANPR	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
APC	Allophycocyanin
ATP	Adenosine triphosphate
BG-11	BG-11 medium
BG-11 ₀	nitrogen-free BG-11 medium
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
DAPI	4'-6-diamidino-2-phenylindole
DW	Dry Weight
EU	European Union
EPA	Eicosapentaenoic Acid
FP-PBR	Flat-Panel Photobioreactor
FSC	Forward Side Scatter
<i>gIfA</i>	glutamine synthetase inactivation factor (IF7A) gene
GS	Glutamine Synthetase
IARI	Indian Agriculture Research Institute
IPTG	Isopropyl-β-D-thiogalactoside
HPLC	High-Performance Liquid Chromatography
Kan	Kanamycin
MHT-PBR	Multilayer Horizontal Tubular Photobioreactor
N	Nitrogen
N+	N-repleted medium (BG-11)
N-	N-depleted medium (BG-11 ₀)
N ₂	Dinitrogen
NH ₃	Ammonia
NH ₄ ⁺	Ammonium
Nm	Neomycin
Nr	Reactive Nitrogen
OD	Optical Density
OD ₇₃₀	Optical Density measured at 730 nm
P	Phosphorus

PC	Phycocyanin
PE	Phycoerythrin
PBR	Photobioreactor
OTU	Operational Taxonomic Unit
Sm	Streptomycin
SN	Supernatant
Sp	Spectinomycin
SSC	Side Scatter
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
WT	Wild Type
Zeo	Zeocin

1

Introduction

When we harvest food, we also remove key nutrients, such as nitrogen (N) and phosphorous (P), from the agricultural system. These are the most frequently limiting macronutrients for plant growth, and therefore have to be replaced in order to sustain food production (Schachtman et al., 1998). This issue is commonly resolved by applying synthetic fertilisers.

1.1 Problems associated with fertilisers production and use

Although di-nitrogen (N_2) is very abundant in air (78% v/v), this molecular form cannot be used by plants. The Haber-Bosch process, invented more than a century ago, allowed N_2 to be

converted into a bioavailable form of N (NH_3) to produce chemical fertilisers. Along with the introduction of newly developed technologies, such as high-yield crop varieties, pesticides, and improved management techniques, the Haber-Bosh process enabled the “Green Revolution” in the second half of the 20th century, in which global agricultural productivity drastically increased. As a result, food prices and hunger, particularly in Asia, were reduced (Pingali, 2012). Without the invention from Haber and Bosch, it is estimated that more than half of the world population would not be fed today (Erisman et al., 2015).

However, this industrial process is unsustainable as it globally consumes 2% of all energy supply and generates 1.6% of the world's total CO_2 emissions, thereby substantially contributing to climate change (Liu, 2014). Additionally, in 2010 the Haber-Bosh process produced twice as much reactive N (Nr) than natural terrestrial sources, generating most of the current anthropogenic Nr (Erisman et al., 2015).

Eighty percent of the NH_3 produced during this industrial process is used as fertilisers for agriculture. Yet only 17% of the N used in food production (including crops, dairy and meat production) is consumed by humans, the rest dissipates into the environment, and ultimately ends up in aquatic habitats (Fowler & Coyle, 2013). This N pollution threatens all ecosystems, but also the atmosphere, causing air pollution, biodiversity loss as well as eutrophication (Erisman et al., 2015). Seventy percent of the N found in the UK rivers come from agricultural land (Muhammed et al., 2018). Only in the European Union, the cost generated by this environmental pollution on human health, ecosystems and climate is estimated to be between €70 billion and €320 billion per year, with 60% of it related to human health (Sutton et al., 2011).

Like N, P is also crucially needed to sustain crop productivity, but the global reserves (mining) are finite and expected to run out in 50-100 years (Cordell et al., 2009). This is why P was added to the EU critical materials list 2014 (Houses of Parliament, 2014). Yet, according to Smil (2000), human activity increases the natural P biogeochemical cycle by:

- Accelerating the erosion, run-off and leaching of P from the fields: forests and grasslands have been increasingly converted into cultivated land, which have higher soil erosion rate,
- Recycling much less organic wastes than in past traditional agricultural systems,
- Discharging human and industrial wastes into natural systems,
- Applying large quantities of inorganic fertilizers: when applying P-based fertilisers, plants will only be able to utilise up to 20%, as the rest will be quickly converted into organic forms that plants cannot assimilate, and will therefore be lost into the environment (Schachtman et al., 1998). This P accounts for 20 to 30% that is found in English rivers, and similarly to N, anthropogenic perturbation of the P cycle leads to eutrophication. As a result, 74% of English lake water bodies fail the EU Water Framework Directive objectives (Houses of Parliament, 2014).

As a result of anthropogenic activities, it is estimated that the N cycle is altered by more than 90% whereas the global C cycle is disturbed by less than 10% (Lillywhite & Rahn, 2005). Furthermore, both global N and P biogeochemical flows are so perturbed that they are recognised to pose a “high risk” of threatening the optimal development of human societies on a global scale, and the principal reason behind this is the high fertiliser application rate in specific regions of North America, Europe and Asia (Steffen et al., 2015).

In contrast, some regions of the world desperately lack N-based fertilisers, like Africa where almost 80% of the continent encounters N-stress problems (Liu et al., 2010). Sub-Saharan African countries for instance apply the lowest rate of fertiliser of any region in the world by far (Gregory & Bumb, 2006), whereas insufficient agricultural productivity, owing to poor soil fertility, is a major contributor of Africa's hunger and poverty (Haggblade & Hazell, 2010; Tittonell et al., 2005). The continent did not benefit from the Green Revolution, which bypassed marginal production environments owing to the lack of infrastructures and low population densities (Pingali, 2012).

A Green Revolution 2.0 is starting to arise, but to be successful, it should not only deliver an increased food production combined with an ecological approach for sustainable agriculture, but also bring stability, resilience and equitability, while targeting small-scale farm holders (Pingali, 2012; Waage, 2013). Indeed, in a vast part of the developing world, more than 80% of the food is produced by smallholders, thus placing them at the forefront of food security. Yet, maintaining less than 2 hectares of land each, they represent the vast majority of the extremely poor and around half of the undernourished people in the world. Mainly living in rural areas where they depend on family agriculture and related activities for their livelihood, they are also specifically vulnerable to climate change (IFPRI, 2005; IFAD & UNEP, 2013).

To conclude, the distribution of plant fertiliser needs to be more equitable, by increasing the supply to regions that presently are lacking nutrient, and to decrease the usage in regions where it is wasted through inefficient practice. In addition, we clearly need to find and implement more sustainable methods for fertilizer production and application given that the global population

continues to increase (48% in the next 100 years), requiring an overall increase of the food production (up to 100% according to Godfray et al. (2012)). This places even further demands on the unsustainable Haber-Bosch process for nitrogen production or on our limited natural resources for phosphate.

Such sustainable fertiliser solutions should also be made available to the underprivileged small farm holders in remote and rural areas, particularly if they are low-cost, eco-friendly, cost-effective, and locally and sustainably produced. Indeed, to significantly combat poverty and ensure long-term food security, especially in a changing climate, empowering the small-scale farmers of these countries to increase their ability to reliably, sustainably and locally produce food is therefore crucial, and will also bring many other environmental, social and economic benefits (IFPRI, 2005; IFAD & UNEP, 2013).

1.2 The potential of photosynthetic microorganisms for bio-fertilisation

Cyanobacteria, also called “blue-green algae”, offer an opportunity with high potential, especially in the framework of “Sustainable Agriculture Intensification” (increasingly recognised as the new paradigm to enhance food security, nutrition, resilience to natural disasters and climate change, as well as reducing poverty). Indeed it aims to “increase food production from existing farmland in ways that have lower environmental impact and which do not undermine our capacity to continue producing food in the future” (Campbell et al., 2014). In other words, as global food

demand increase is positively correlated to the environmental impacts of agricultural expansion (Tilman et al., 2011), sustainable intensification of agriculture consists in de-coupling food production from its environmental negative footprint. For instance, lower greenhouse gas emissions can be achieved by implementing agroforestry strategies or applying precision agriculture (the latter being defined as “a management strategy that uses electronic information and other technologies to gather, process, and analyse spatial and temporal data for the purpose of guiding targeted actions that improve efficiency, productivity, and sustainability of agricultural operations” (Godfray et al., 2012; Lowenberg-DeBoer & Erickson, 2019). Successes in implementing such changes have been reported: Pretty et al. (2006) analysed around 300 agricultural sustainability projects implemented in 12.6 million farms (representing 37 million hectares) in developing countries and found an average of 79% yield boost, combined with water use efficiency improvement and a potential carbon sequestered equivalent to 0.35 t C/ha/y.

Cyanobacteria such as *Spirulina* have traditionally been used as a potential food or nutrient supplement (Lafarga et al., 2020). A complementary possibility is to use algae or cyanobacteria as bio-fertilisers for sustainable plant production. Free-living cyanobacteria are photosynthetic, and some have also the ability to fix atmospheric nitrogen, therefore they would not necessarily compete for energy and carbon with heterotrophic soil microorganisms, as long as they have access to sunlight. Furthermore, the addition of cyanobacteria to agricultural soil has been shown to benefit the growth of many different plant cultivars in multiple ways, including by: accelerating the decades-long recovery process of degraded soils (also in desert regions); enhancing soil fertility (by building up organic matter in soils, a major contributor to soil fertility, fixing

atmospheric di-nitrogen and converting it into a bio-available form for plants – in the case of diazotrophic (i.e. nitrogen-fixing) cyanobacteria, and solubilising insoluble forms of P that plants are not able to utilise) and enhancing resistance to pathogens and diseases (bio-control agent activity) (H. Singh, et al., 2014). Cyanobacteria have also been shown to mitigate heavy metals toxicity to limit their transfer to plants (Al-Sherif et al., 2015), produce plant growth-promoting substances and improving the activities of other profitable microorganisms associated to plant roots (Issa et al., 2014).

1.2.1 Potential for N-fertilisation

As early as the eleventh century, in order to increase rice yields, Vietnamese farmers have been inoculating paddy fields with *Azolla*, a tiny water fern hosting filamentous diazotrophic cyanobacteria, *Anabaena azolla* (H. Singh et al., 2014). Yet scientists discovered only recently the nitrogen-fertilising action of *Anabaena*, and since 1939, technologies for “algalisation”, or cyanobacterial inoculation in fields for fertilising purposes, have been developed, and were made available to Indian rural producers. Indeed, the Indian Agriculture Research Institute (hereafter IARI), New Delhi, deployed this technology amongst farmers: by growing the cyanobacteria and subsequently applying them to their agricultural land. They argued that this technology should allow farmers to sustainably produce bio-fertilisers themselves, and therefore to independently boost their ability to produce food (Kaushik, 2014). India is also now using 4 times more synthetic fertilisers per hectare than the rest of the world, leading to the disastrous environmental pollution consequences previously described, and results in critical soil deterioration (Bhooshan et al., 2018).

Mishra & Pabbi (2004) reported on a technology transfer solution that involves the use of an inoculum composed of *Anabaena*, *Nostoc*, *Aulosira* and *Tolypothrix*, all N-fixing cyanobacteria. The algal mixture was used as a seed for mass production, and 10-12 kg of the produced and sun-dried biomass was applied per hectare of rice paddy. The results showed a 15-20% increase in rice yield, and the inoculated cyanobacteria established themselves in a permanent way after repeated inoculation during 3-4 seasons. Yet, the implementation of such systems amongst farmers was not popular, for the following reasons: (1) owing to the open-air production character of the system, not only did contamination occur very easily, leading to varying final product quality, but also the period of production was constrained to few months in the summer (as the production cannot be pursued during the rainy and winter season), and (2) because of the low density of the culture, large amounts of inoculum per hectare of paddy field were required.

Since then, the technology was considerably improved, and in 2018, a study on 260 households practicing algalisation techniques was reported: on average, these farmers consumed 41.1% less urea when applying cyanobacteria on paddies than “non-algalised” plots, while benefiting from a 1% yield increase and a 3% income increase (Bhooshan et al., 2018).

The technology, much cheaper than synthetic fertilisers in this country, was popularised amongst farmers by IARI, by leading demonstration trials targeted to farmers (Kumar et al., 2015), but also through technology licencing to private companies (Bhooshan et al., 2018). Interestingly, IARI developed in 2015 a system for sharing information and disseminating technologies to the farmers living in even the remotest areas of India via the village post offices (Burman et al., 2015).

The factors leading to the adoption of the technology were also analysed in the study from Bhooshan et al. (2018): results showed that farmers employing the technology had a higher experience and education, and were either owning their land or leasing it.

Transferring such technologies to non-flooded crops would also bring many similar benefits, including diet diversification. If successful, this could help developing countries to lift their net food production, along with enhanced reliable nutrient access and self-sufficiency in a sustainable manner. It has already been demonstrated that some N-fixing cyanobacteria, from the *Anabaena* and *Nostoc* genera, inoculated to nutrient-poor soil collected in Africa, enhanced the soil physico-chemical properties, maize growth and wheat germination (Gheda & Ahmed, 2015; Maqubela, et al., 2009).

Interestingly, cyanobacteria, especially those that are able to fix both carbon and nitrogen, have been reported in several studies to promote the formation of biocrusts that stabilize desert soil and control desertification. For example, in the Qubqi desert of Inner Mongolia, cyanobacterial inoculation accelerated the formation of moss crusts (which appear once the topsoil is stabilised) that enabled the most productive growth of several woody plants after eight years (Lan et al., 2014). Similarly, Li & Hu (2021) found cyanobacteria to be dominant in many, but not all, biocrusts sampled across several thousand kilometres along the Northern China border.

1.2.2 Potential for P-fertilisation

After nitrogen, phosphorus is the second most frequently limiting macronutrient for plant growth. When applying P-based fertilisers, plants will only be able to utilise 20%, as the rest will be quickly converted into organic forms that plants cannot assimilate. However, it has been reported that at least some cyanobacteria have the capacity to solubilise these insoluble forms of phosphate into inorganic P, the only form of P assimilable by plants (Schachtman et al., 1998; Singh et al., 2016). Most cyanobacteria also accumulate polyphosphate at times when it is readily available in the environment, that is then utilised when environmental sources are lacking (Li & Dittrich, 2019). Hence, the accumulation is dynamic, and therefore the optimum concentration of P for fertilisation purposes, is likely to vary greatly according to the conditions in which it has been grown and potentially also harvested and stored.

Not many studies have been carried out in which the ability of cyanobacteria to supply P have been studied. Eukaryotic algae, however, have recently been studied comprehensively as a source of P, and it is not unthinkable that the same principles could apply also for cyanobacteria. Schreiber et al. (2018) applied *Chlorella vulgaris* biomass as a biofertiliser to wheat demonstrating that it was completely able to replace inorganic NPK fertiliser. Notably, they did not separate between the effect of one nutrient relative to another in the algal biomass, hence it is likely that also N and other nutrients contributed towards stimulation of growth. Although studies such as these demonstrate that algal biomass can act effectively as an inorganic fertiliser replacement, they do not explain how the nutrients are transferred from one species to another. Interestingly, very recently they carried out biofertilisation experiments with *C. vulgaris* grown

on a radioactive isotope (^{33}P), and were able to demonstrate rapid transfer of P from the algal biomass (mainly stored as polyphosphate) into all visible parts of the young wheat seedling (Siebers et al., 2018). This provided the first evidence of the transfer of nutrients from algal biomass into plants. It opens up opportunities for follow-on studies that address questions such as which form of each nutrient is optimal for transfer from microbial into plant biomass?

1.2.3 Potential for wastewater treatment

One important opportunity to increase the sustainability impact of algal biofertilisation technologies, is to utilise the fact that microalgae can be cultured using wastewater. However, not all types of wastewaters would be suitable to grow algae, especially if the end product is to be applied on crops. Ali et al. (2021) categorises the different types of wastewater as follows: domestic/municipal (e.g. from households, this category can be further divided into greywater (from kitchen, laundry, ...) and blackwater (urine, faeces)); industrial (e.g. from food production or textile industries); landfill leachates; hospitals and pharmaceuticals; agricultural (pesticides, ...); stormwater and runoff; synthetic wastewater (experimental laboratories). Some of these wastewaters may contain heavy metals and toxic pollutants and therefore will not be suitable to grow algae aimed at fertilising food crops. For example, municipal domestic wastewater is recognised suitable for microalgae cultivation due to its low toxicity and high nutrient content (Suleiman et al., 2020; Wang et al., 2022). Wastewater from certain food industries may also be suitable as demonstrated by Stedt et al. (2022) who cultivated seaweed (macroalgae) using wastewater from salmon farms. In the context of agriculture, algae could be cultured in wastewater in order to (1) remove N and P from the wastewater and (2) recycle that N and P

back to agriculture through application onto the soil. Hence, by removing those nutrients from the wastewater, the algae are effectively also cleaning the water. Recently, *Spirulina*, but also microalgal consortia (isolated from sewage wastewater), that had been grown on wastewater, were used to inoculate soils and shown to enhance respectively plant growth and micronutrient availability (zinc, iron, copper and manganese), in leafy vegetables and wheat (Renuka et al., 2017; Wuang et al., 2016). Addressing micronutrient availability is also highly relevant as the prevalence of micronutrient deficiency is the highest in developing regions like Southeast Asia and Sub-Saharan Africa (Ramakrishnan, 2002).

1.3 Could cyanobacteria-based bio-fertilisers also be used in biotechnological applications?

Another opportunity is to employ a bio-fertiliser approach also in biotechnological processes, since they also require nutrients. Indeed, production of commercially attractive products from photosynthetic microorganisms has gained a lot of attention recently but is, to date, only based on high value products dependent on native metabolic capabilities of extremophile strains.

To improve the viability and expand the scope of algal biotechnology, several challenges still need to be overcome, in particular contamination under less extreme conditions and the overall issues of excessive cost. For instance, when scaling up photo-biotechnological processes, bacterial contamination which does not occur in stringent sterile laboratory conditions is likely to appear (Habtegebriel & Valdramidis, 2023). This is problematic for continuous productivity, resulting in

increased production costs since no product is made when the process is not in operation (e.g. due to cleaning). In addition, particularly when a product is required at high purity, cultivation of microorganisms for biotechnology generally focuses on a single species. Yet such systems are unstable and more prone to perturbation, increasing the opportunity for external contaminating species to settle (Kazamia et al., 2012). On the contrary, in nature, no organism lives in isolation, but rather as part of an ecosystem of varying diversity, and natural microbial communities have been recognized for their high robustness against fluctuations in environmental conditions. However, the mechanisms underlying their stability has long been debated (McNaughton, 1977).

Could the algal bio-fertiliser approach also be used to address some of these outstanding challenges? Mimicking natural systems, e.g. using microbial communities with inter-species exchange of key nutrients, might help both reduce contamination and lower the overall cost, if it is controlled. For example, a controlled consortium, where one organism provides N to another (therefore acting as a bio-fertiliser), could be synthetically built. Indeed, the reciprocal exchange of nutrients between different species, a key concept of ecology, is recognized to enhance the stability of populations (Stachowicz, 2001). It has also been argued that synthetic ecology, by increasing the number of species and therefore reducing the ecological space available for contaminating species, could help to reduce the impact of unavoidable contamination occurring in large-scale algal production (Kazamia et al., 2014). Furthermore, if a nitrogen-fixing bacterium is able to supply sufficient assimilable N to another cyanobacterium or eukaryotic alga that is producing a compound of commercial interest, then it should be possible to omit assimilable N from the culture media. This is a significant benefit, as assimilable nitrogen, a key limiting factor for growth provided typically in the form of nitrate, represents a significant cost for industrial-

scale production. More importantly, if it results in a more stable bioprocess, there are substantial financial benefits from minimising (non-productive) process down-time.

1.4 How could cyanobacteria contribute to recycling nutrients?

Having played a key role in the oxygenation of the Earth's atmosphere around 2.3 billion years ago, cyanobacteria are one of the most ancient forms of life present on the planet (Whitton, 2000). More than 150 genera and 2,000 species are currently described (Singh et al., 2014). They are ubiquitous photosynthetic prokaryotes and display a great morphological diversity. They can be unicellular, and form colonial aggregates (Rippka et al., 1979), or multicellular, forming long filaments of cells called trichomes, which sometimes exhibit branches (when cell division occurs in more than one plane). The vegetative cells of some filamentous species can also differentiate into heterocysts (in which nitrogen fixation takes place), spore-like akinetes (to survive cold and dessication conditions), or form motile short filaments called hormogonia (for dispersion purposes) (Flores & Herrero, 2010).

Cyanobacteria live in aquatic (both marine and fresh water) and terrestrial environments. As they are remarkably tolerant to a large range of temperature (up to 70 °C), pH (lower than 4), water level, salinity and irradiance, cyanobacteria have the capacity to survive in diverse extreme environments, such as polar regions, hyper-saline waters, hot springs or arid deserts (Singh et al., 2014; Singh et al., 2016). In addition, they naturally form symbiotic associations with eukaryotic hosts, like fungi, plants and animals. Most of these symbioses, like the *Azolla-Anabaena* symbiosis, occur thanks to the ability of cyanobacteria to fix atmospheric N₂ and transfer it to the

partner, in exchange for a protected environment from predation and environmental extremes (Whitton, 2000). For example, such symbiosis is estimated to supply up to 60 kg N.ha⁻¹ to rice fields (Vaishampayan et al., 2001).

To understand the complex metabolism of nitrogen fixation and cell differentiation, *Anabaena* sp. PCC 7120, an isolated and sequenced form of *Anabaena azolla*, has been frequently studied as a model organism (Kaneko et al., 2001). Cyanobacteria are able to grow on many different nitrogen sources, either organic or inorganic. When grown in the presence of nitrate, nitrite, urea, cyanate or molecular nitrogen, cyanobacteria convert these first into ammonium, making it the preferred source of nitrogen for the majority of bacteria (Klähn et al., 2015). Uncharged ammonia will also diffuse out of the cell where it is partially trapped due to differences in pH-dependent chemical equilibria (where the positively charged ammonium form is unable to diffuse back in) between the interior and exterior of the cell (Boogerd et al., 2011). Together, these are likely the reasons why most N-fixing cyanobacteria, to minimize the loss of ammonium/a, have specific uptake transporters for ammonium (e.g. *amt1*, *amt4*, and *amtB* in *Anabaena* sp. PCC 7120). The strain *Anabaena* sp. PCC 7120 CSP19, in which the complete *amt14B* cluster has been removed (Paz-Yepes et al., 2008), therefore represents a good initial candidate in order to increase the supply of assimilable N to another partner.

In addition, many cyanobacteria, including *Anabaena* sp., are also capable of over-accumulating inorganic P in the form of polyphosphate, and then slowly release it to support plant growth (Mukherjee et al., 2015). Although this over-accumulation only functions when P is available, it

would at least allow P captured in wastewater to be stored temporarily inside cyanobacteria, and thereafter be provided to the chosen agricultural crop.

To summarise, cyanobacteria could be used as bio-fertilisers to support the growth of plants, but also the growth of another microorganism (in a synthetic community; biotechnological context), in particular with respect to nitrogen and phosphorus. If wastewater is used as a substrate for algae cultivation, it could also reduce the dispersion of both N and P into natural ecosystems, thereby reducing the potential for environmental degradation such as eutrophication. In this PhD study, the focus was placed on defined conditions, wastewater was therefore not utilised.

A second approach is to use the capacity of cyanobacteria to convert the available molecular nitrogen (N_2) into assimilable forms for other organisms (either plants or microorganisms), as already happening naturally in rice paddies (see section 1.2.1). If successful, this approach would allow a reduction in the amount of used chemical fertiliser (for both plants and microorganisms production) and/or the development of microbiological bioprocesses that are more robust to contamination (Kazamia et al., 2012).

The illustration in Figure 1-1 summarises these two bio-fertiliser approaches.

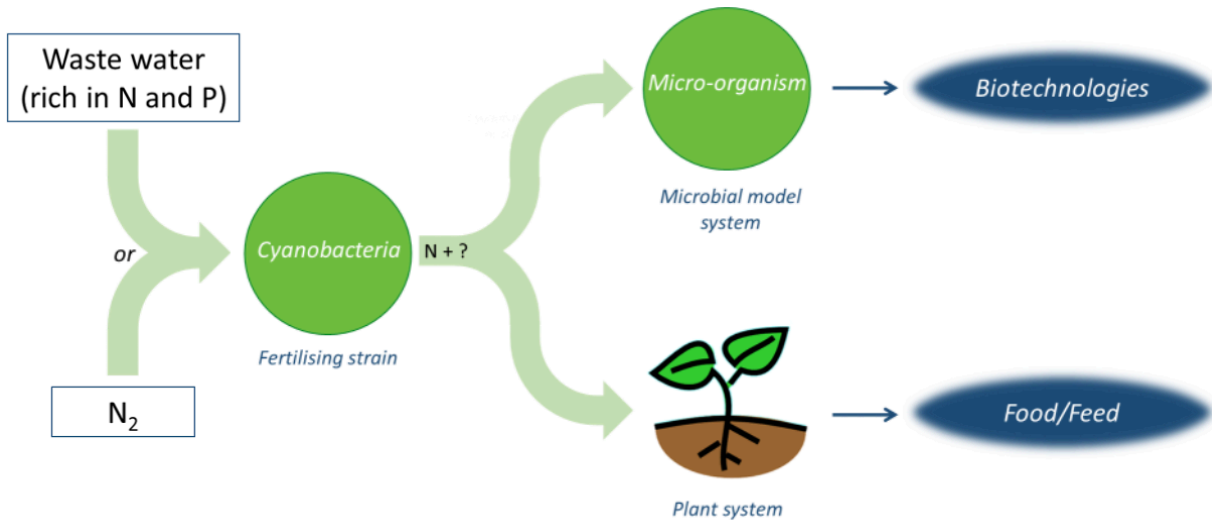


Figure 1-1. Summary of the microbial and plant bio-fertilising systems.

This second approach, in which a nitrogen-fixing cyanobacteria provides nitrogen to support the growth of another organism (microorganism or plant), was the focus of the present research. Ultimately the aim of this PhD study was to contribute towards new solutions that can be used both in developed countries where excessive fertiliser use is a massive problem, and in developing countries, where fertilisers are critically needed.

1.5 Aim and objectives

Viewing biomanufacturing in its broadest potential sense, including both microbes and plants, the overall aim of this applied PhD project was to test the hypothesis that the addition of a second nitrogen-providing species to a bioprocess (either microbial or plant) enhances its sustainability. The word “sustainability” is broad and refers to several aspects in this case. On one hand, it was assumed that less chemical nitrogen fertiliser (or media nutrient) needs to be supplied. As

described above, excess use of chemical nitrogen fertiliser contributes to many global challenges, including environmental pollution and climate change. The second aspect is that the construction of a microbial bioprocess with one (or more) key nutrients absent from the media, which instead is supplied from one species to another, will result in a more stable bioprocess that will suffer less from the impact of other contaminating species, thereby being able to operate more efficiently (i.e. with less resources consumed). Before the sustainability of the constructed bioprocesses can be evaluated however, the initial challenge is to construct a bioprocess where one or more nutrients is shared between one species and another. The main hypothesis of this thesis then became: a free-living nitrogen-fixing cyanobacteria can support all the nitrogen needs of another non-diazotrophic organism (microbial or plant).

To implement a feasible project, the goals of this project were to construct simple model systems that would enable this latter hypothesis to be tested. A nitrogen-fixing cyanobacterium was used consistently as the nutrient donor, with a focus on nitrogen as the exchanged nutrient. The work was further sub-divided into two distinct parts, based on the recipient organism.

In the **first case**, the recipient was another bacterium, engineered to produce a carbon-containing product, ethanol. Here, the aim was to test the hypothesis that engineered nutrient-exchange co-cultures could enhance the stability (i.e. productivity) of biotechnological processes.

In the **second case**, the recipient was a plant. The aim here was to test the hypothesis that nitrogen-fixing bio-fertilisers could replace chemical nitrogen fertiliser. Complementary

experiments were carried out to better understand the cause-effect relationships observed with these model systems.

These aims were achieved via the following planned objectives:

First case - Bacteria-Bacteria co-cultures

1. Development of a method to monitor two different photosynthetic microorganisms simultaneously (Chapter **3**),
2. Construction and evaluation of nutrient-exchanging microbial co-cultures involving two photosynthetic microorganisms, one which was N₂-fixing and the other engineered to biosynthesise a carbon-containing product (ethanol; Chapter **4**),
3. Identification and characterization of bacterial contaminants in an industrial algae cultivation facility (Chapter **6**),
4. Characterization of the impact of bacterial contaminants and/or engineered nutrient-exchange co-culture systems on biomanufacturing with ethanol as the target product.

Second case - Bacteria-Plant co-cultures

5. Development of a scalable cultivation, harvesting and storage protocol for manufacturing of a nitrogen-fixing cyanobacteria to be used as a biofertiliser,

6. Evaluation of biofertiliser at different dosage with different plants and in different soils
(Chapter 5),
7. Characterization of native UK cyanobacteria as an indigenous source of biofertiliser,
8. Evaluation of biofertiliser with end-use customers, i.e. farmers.

Objectives 4 and 8 were not pursued in this project due to the constraints of time and COVID-19 restrictions. Some of the objectives were carried out as a collaboration, esp. objective 3 carried out within the EU FP7 DEMA project (with the overall aim of developing direct photosynthetic ethanol production using cyanobacteria), and objectives 5-7 together with the academic spinout project BIO-F Solutions.

2

Material and Methods

2.1 Chemical & reagents

Chemicals and reagent components, unless otherwise noted, were ordered from Merck (Sigma-Aldrich Ltd., UK), VWR (VWR International Ltd., UK) or Thermo Fisher Scientific (Fisher Scientific Ltd., UK).

2.2 General protocols

2.2.1 Incubation and sterile work

Cyanobacterial cultures, both liquid and on agar plates, were incubated in an AlgaeTron AG 230 light incubator (Photon Systems Instruments, spol. s r.o., Czech Republic) at 30 °C, under continuous illumination by cool white LED panels at $60 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and in a 1-% enriched CO₂ atmosphere (maintained by an Evolution horticultural carbon dioxide controller (Ecotechnics UK Ltd., UK)). Heterotrophic microorganisms (such as contaminants described in Table 2-1) were incubated at 37 °C in either a benchtop Labnet Mini Incubator (ST2185, Appleton Woods Ltd., UK) or a shaker incubator (New Brunswick 44R, Eppendorf, UK) with 200 rpm orbital shaking for 16 h (overnight). Sterile work with both bacteria and cyanobacteria was performed in class I or class II biosafety cabinets under continuous laminar flow.

2.2.2 Laboratory centrifuges

Centrifuge tubes (15 and 50-mL) were centrifuged in a Heraeus Megafuge 16R benchtop centrifuge (Thermo Fisher Scientific, UK). Unless otherwise noted, centrifugation was performed at room temperature.

2.3 Strains

Strains used in this study are outlined in Table 2-1. Isopropyl- β -D-thiogalactoside (IPTG) was added for induction of gene expression of the ICLEE strain, at a concentration of 1 mM at the beginning of the incubation.

Table 2-1. Origin of the strains used in this study.

Strains	Description ^a	Source
<u>Anabaena sp. PCC 7120</u>		
WT	Wild Type	Prof. Enrique Flores ^b
CSP19 (hereafter Δamt)	$\Delta amt::C.K3$; $Amt4^-$, $Amt1^-$, $AmtB^-$, Nm^R	Paz-Yepes et al. (2008)
165 2w4, C2 (hereafter $gifA$ -1)	$P_{petE}-gifA-T_{gifA}::pRSF1010-Sm^R-Sp^R$ in <i>Anabaena</i> WT, expression induced by the presence of copper (3 μ M Cu^{2+})	Malatinszky (2017)
160 20w6, C20 (hereafter WT $gifA$ -2)	$P_{nifHDK}-gifA-T_{gifA}::pRSF1010-Sm^R-Sp^R$ in <i>Anabaena</i> WT, expression under diazotrophic conditions only	Malatinszky (2017)
C20 163 20d5, C20 (hereafter Δamt $gifA$ -2)	$P_{nifHDK}-gifA-T_{gifA}::pRSF1010-Sm^R-Sp^R$ in <i>Anabaena</i> Δamt , expression under diazotrophic conditions only	Malatinszky (2017)
<u>Synechocystis sp. PCC 6803</u>		
UL030	$\Delta psbA2::PpsbA2$ $Zmpdc$ $adhA$, Kan^R $\Delta phaAB::PpsbA2$ $Zmpdc$ $adhA$, Zeo^R	Lopes da Silva et al. (2018)
ICLEE	$\Delta slr0168::PA1lacO1$ $RBSEpdc$ $RBSEadh$, Sp^R , IPTG inducible	Bartasun et al. (2019)
<u>Contaminants^c</u>		
Conta1	iTYA ^d	A4F, Portugal
Conta2	scTYB ^d	A4F, Portugal
Conta3	scTYC ^d	A4F, Portugal
Conta4	bTYD ^d	A4F, Portugal
Conta5	bNAD ^d	A4F, Portugal
Conta6	bTYE ^d	A4F, Portugal
Conta7	nNAG ^d	A4F, Portugal

Conta8	nNAM ^d	A4F, Portugal
Conta9	nNAJ ^d	A4F, Portugal
Conta10	nTYK ^d	A4F, Portugal
Conta11	nNAL ^d	A4F, Portugal
Conta12	ftNAH ^d	A4F, Portugal
Conta13	ftNAI ^d	A4F, Portugal
Conta14	iNAF ^d	A4F, Portugal
Conta15	eNAN ^d	A4F, Portugal
Conta16	eNAO ^d	A4F, Portugal
Conta17	eNAP ^d	A4F, Portugal

^a Abbreviations: Nm: neomycin, Sm: streptomycin, Kan: kanamycin, Zeo: zeocin, Sp: spectinomycin; ^R denotes resistance to the corresponding antibiotic.

^b Professor Enrique Flores is affiliated with IBVF, CSIC and Universidad de Sevilla, Spain.

^c Contaminants isolated from pilot-scale UL030 cultures at A4F, Portugal (a collaborator that specializes in large-scale outdoor cultivation of algae).

^d Isolates classified by A4F according to plating time (i-: inoculation; sc-: start of semi-continuous regime; b-: batch regime; n-: after chemical photobioreactor (PBR) disinfection and neutralisation; ft-: after first heat PBR disinfection; e-end of the test), media (TY (Tryptophan Yeast) or NA (Nutrient Agar)), isolates (letter: A – P).

2.4 Media preparation and antibiotics preparation

2.4.1 Liquid Lysogeny Broth medium

Liquid Lysogeny Broth (hereafter LB) medium was used to grow contaminants (as described in Table 2-1 and section 2.5.4) and was prepared by dissolving 20 g LB Broth (Lennox formulation, L3022, Sigma-Aldrich, UK) to 1 L deionized water in a 1-L Schott screw cap bottle (VWR, UK). The mix was autoclaved at 121 °C for 15 min.

2.4.2 Lysogeny Broth agar medium

Solid Lysogeny Broth medium was used to check whether cyanobacterial cultures (of *Synechocystis* sp. PCC 6803 and/or *Anabaena* sp. PCC 7120, see Table 2-1) were contaminated (as described in 2.5.3) and was prepared by dissolving 6 g LB Broth (Lennox formulation, L3022, Sigma-Aldrich, UK), 4.5 g agar powder (20767.298, VWR, UK) to 300 mL deionized water in 500 mL Schott screw cap bottle (VWR, UK). The mix was autoclaved at 121 °C for 15 min. Solid LB agar was melted by gentle boiling in a laboratory microwave oven and cooled to 60 °C. Twenty millilitres of liquefied LB agar were distributed into 9-cm plastic Petri plates, let solidified in sterile laminar flow and used immediately after solidification.

2.4.3 Liquid BG-11 medium

Liquid BG-11 medium was used to grow cyanobacterial cultures (of *Synechocystis* sp. PCC 6803 and/or *Anabaena* sp. PCC 7120, see Table 2-1) and was prepared according to Rippka et al. (1979). A 100-time concentrated stock solution (100x BG-11) was prepared by dissolving 149.6 g NaNO_3 , 7.49 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 3.6 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.89 g $\text{Na-citrate} \cdot 2 \text{H}_2\text{O}$, 1.12 mL 0.25 M sodium EDTA pH 8.0 and 100 mL 1,000x trace minerals solution. The 1,000-time concentrated trace minerals solution was made as follows: 2.86 g H_3BO_3 , 1.81 g $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 0.22 g $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.39 g $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 0.079 g $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ and 0.0494 g $\text{Co}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$ were dissolved in 1 L deionized water and stored at 4 °C until being used. Standard BG-11 liquid medium was prepared by mixing 10 mL 100x BG-11 stock solution with 1 mL 1,000x ferric ammonium citrate (0.6 g ferric ammonium citrate dissolved in 100 mL deionized water), 1 mL 1,000x Na_2CO_3 (2 g

Na₂CO₃ dissolved in 100 mL deionized water) and 1 mL 1,000x K₂HPO₄ (3.05 g K₂HPO₄ dissolved in 100 mL deionized water), and adjusting the volume to 1 L with deionized water. The mix was autoclaved at 121 °C for 15 min in a 1-L Schott screw cap bottle and kept at room temperature until being used. Antibiotics (Table 2-2), when appropriate, were added at room temperature. The bottle was mixed thoroughly before use to re-dissolve an orange-brown iron precipitation.

2.4.4 BG-11 agar medium

Solid BG-11 agar medium was used to grow cyanobacterial cultures (of *Synechocystis* sp. PCC 6803 and/or *Anabaena* sp. PCC 7120, see Table 2-1) and prepared in 500-mL Schott screw cap bottles in 300-mL batches as follows: 3 mL 100x BG-11 stock solution, 0.3 mL 1,000x ferric ammonium citrate, 0.3 mL 1,000x Na₂CO₃, 0.3 mL 1,000x K₂HPO₄ and 5 mL 1 M TES-NaOH buffer pH 8.2 were mixed with 2.35 g Na₂S₂O₃ · 5 H₂O and 4.5 g Difco Bacto agar (BD, Plymouth, UK) and the volume adjusted to 300 mL with deionized water. One molar TES-NaOH buffer was made by dissolving 57.3 g TES-Na (T0772, Sigma-Aldrich, UK) in 200 mL deionized water, adjusting the pH to 8.2 with 10 N NaOH (16 g NaOH dissolved in 30 mL deionized water and the final volume brought to 40 mL) and making the final volume 40 mL with deionized water. The buffer was filter sterilized using 0.2 µm syringe filters and stored at 4 °C. Bottles of pre-mixed BG-11 agar medium were autoclaved at 121 °C for 15 min and stored at room temperature. Solid BG-11 agar was melted gently by boiling in a laboratory microwave oven and cooled to 60 °C. Antibiotics (Table 2-2), when appropriate, were added to liquefied agar at 60 °C or below. Twenty millilitres of liquefied BG-11 agar were distributed into 9-cm plastic Petri plates, let solidified in sterile laminar flow and used immediately after solidification.

2.4.5 Nitrate-free medium

Nitrate-free media were used to perform diazotrophic cultivation of cyanobacterial strains. Liquid and solid nitrate-free BG-11 (hereafter BG-11₀) was used to grow cyanobacterial cultures (of *Synechocystis* sp. PCC 6803 and/or *Anabaena* sp. PCC 7120, see Table 2-1) and prepared similarly to BG-11 standard medium by leaving out sodium nitrate from the 100x stock solution and making a 100x BG-11₀ stock instead. The rest of the components were added as described for standard BG-11.

2.4.6 Liquid Tryptone Yeast medium

Liquid Tryptone Yeast (hereafter TY) medium was used to grow contaminants (as described in Table 2-1 and section 2.5.4) and was prepared by dissolving 1.8 g Tryptone (T9410, Sigma Aldrich, UK), 0.9 g yeast (70161, Sigma Aldrich, UK), and 0.27 g CaCl₂ · 2 H₂O to 300 mL deionized water in 500-mL Schott screw cap bottle (VWR, UK). The mix was autoclaved at 121 °C for 15 min.

2.4.7 Tryptophan Yeast agar medium

Tryptone Yeast agar medium was used to grow contaminants (as described in Table 2-1 and section 2.5.4) and was prepared by dissolving 1.8 g Tryptone (T9410, Sigma Aldrich, UK), 0.9 g yeast (70161, Sigma Aldrich, UK), 3.6 g agar (20767.298, VWR, UK) and 0.27 g CaCl₂ · 2 H₂O to 300 mL deionized water in 500 mL Schott screw cap bottle (VWR, UK). The mix was autoclaved at 121 °C for 15 min. Solid LB agar was melted by gentle boiling in a laboratory microwave oven. Solid

LB agar was melted by gentle boiling in a laboratory microwave oven. Twenty millilitres of liquefied NA agar were distributed into 9-cm plastic Petri plates, let solidified in sterile laminar flow and used immediately after solidification.

2.4.8 Nutrient Agar medium

Solid Nutrient Agar (hereafter NA) medium was used to grow contaminants (as described in Table 2-1 and section 2.5.4) and was prepared by dissolving 8.4 g Nutrient Agar (70148, Sigma-Aldrich, UK) to 300 mL deionized water in 500-mL Schott screw cap bottle (VWR, UK). The mix was autoclaved at 121 °C for 15 min. Solid LB agar was melted by gentle boiling in a laboratory microwave oven. Twenty millilitres of liquefied NA agar were distributed into 9-cm plastic Petri plates, let solidified in sterile laminar flow and used immediately after solidification.

2.4.9 Antibiotics preparation

To create a stock solution of antibiotic (Table 2-2), antibiotic powder was dissolved in deionized water and filter sterilised with sterile 0.2 µm syringe filter. Stock solutions were stored at -20 °C and thawed at room temperature prior use.

Table 2-2. Antibiotic concentration used to culture cyanobacterial strains.

Cyanobacterial strains (described in Table 2-1)	Antibiotics	Stock concentration	Working concentration
<i>Synechocystis</i> sp. PCC 6803 UL030	Kanamycin (Kan)	50 mg/mL	50 µg/mL
	Zeocin (Zeo)	15 mg/mL	15 µg/mL
<i>Synechocystis</i> sp. PCC 6803 ICLEE	Spectinomycin (Sp)	50 mg/mL	50 µg/mL
<i>Anabaena</i> sp. PCC 7120 CSP19 (hereafter Δamt)	Neomycin (Nm)	10 mg/mL	10 µg/mL
<i>Anabaena</i> sp. PCC 7120 165 2w4, C2 (hereafter <i>gifA</i> -1), <i>Anabaena</i> sp. PCC 7120 160 20w6, C20 (hereafter WT <i>gifA</i> -2), <i>Anabaena</i> sp. PCC 7120 C20 163 20d5, C20 (hereafter Δamt <i>gifA</i> -2)	Spectinomycin (Sp)	50 mg/mL	2.5 µg/mL (agar plates); 1.5 µg/mL (liquid medium)
	Streptomycin (Sm)	20 mg/mL	2.5 µg/mL (agar plates); 1.5 µg/mL (liquid medium)

2.5 Microbial culturing conditions

2.5.1 Cultivation of cyanobacteria

Well-plate cultures of cyanobacteria were grown on BG-11 agar plates in the AlgaeTron incubator as described in section 2.2.1. Liquid cultures were inoculated from plates by collecting cells with a plastic inoculation loop and whisking them into 3 mL of fresh BG-11 added in a 6-well plate. After about 7 days 20 mL fresh BG-11 was inoculated with 1 mL of the pre-culture in a 100-mL

Erlenmeyer flask covered with aluminium foil and incubated at 30 °C in the same incubator as for plates, at 180 rpm on a Titramax 1000 vibrational shaker (544-12200-00, Heidolph Instruments GmbH, Germany). For diazotrophic cultivation, BG-11₀ was used lacking sodium nitrate. Solid and liquid media were supplemented with appropriate antibiotics when necessary, at the concentrations described in Table 2-2.

2.5.2 Cryopreservation of cyanobacteria

The cyanobacterial culture to cryopreserve was grown in 100-mL Erlenmeyer flasks until late exponential phase ($0.7 < OD_{730} \leq 1$ measured in a 1-cm cuvette) in 20 mL BG-11 liquid medium supplemented with the appropriate antibiotics (Table 2-2), under standard growth conditions. 750 µL of culture were transferred to a 2-mL Nalgene cryogenic vial (Thermo Scientific, UK) and gently mixed with 250 µL of 80% (w/v) sterile glycerol (autoclaved) by inverting the tube a couple of times. The cryotube was immediately placed in a cooling block (pre-cooled at -20 °C for 1 h) and transferred to -80 °C as quickly as possible. Cryotubes were moved to labelled plastic boxes at -80 °C after 3 hours in the cooling block.

2.5.3 Contamination check

At the end of each culture, or when cryopreserving, liquid cultures of cyanobacteria were checked for bacterial contamination by spreading a loop previously whisked in the cyanobacterial culture across an LB agar plate. The plate was then incubated for 7 days at 37 °C in the Mini Incubator (described in section 2.2.1) to verify if any bacterial growth occurred. These conditions

will not allow all contaminants to be detected, however, it is particularly useful given that the surrounding laboratory mostly work with *Escherichia coli*.

2.5.4 Bacterial cultivation

Contaminants received from A4F, Portugal, were grown and maintained on agar plates (using either Tryptophan Yeast for conta1, conta3, conta4, conta6 and conta10, or Nutrient Agar for conta5, conta7-9, and conta11-17) at 37 °C in a benchtop Labnet Mini Incubator (ST2185, Appleton Woods Ltd., UK). Liquid cultures were inoculated from plates by transferring individual colonies with a plastic inoculation loop into 5 mL liquid LB (for bNAD, nNAG, conta13, conta16 and conta17) or liquid TY (for bTYD) medium in 50-mL centrifuge tubes. Cultures were incubated at 37 °C with 200 rpm orbital shaking for 16 h (overnight) in a shaker incubator (New Brunswick 44R, Eppendorf, UK). To enable proper aeration of the culture during incubation, tube caps were loosened.

2.5.5 Cryopreservation of bacteria (contaminants)

Overnight cultures of contaminants grown at 37 °C in 50-mL centrifuge tubes in 5 mL liquid TY or NA, depending on the strain (see 2.5.4). Seven hundred and fifty microlitres of culture were transferred to a 2-mL Nalgene cryogenic vial (Thermo Scientific, UK) and gently mixed with 250 µL of 80% (w/v) sterile glycerol (autoclaved) by inverting the tube a couple of times. The cryotube was immediately placed in a cooling block (pre-cooled at -20 °C for 1 h) and transferred to -80 °C

as quickly as possible. Cryotubes were moved to labelled plastic boxes at -80 °C after 3 hours in the cooling block.

2.5.6 Co-cultivation of cyanobacteria

Axenic cultures of *Synechocystis* sp. PCC 6803 (either ICLEE or UL030) and *Anabaena* sp. PCC 7120 (either wild-type, CSP19 (hereafter Δamt), WT *gifA*-1/2 and Δamt *gifA*-2) were grown in 100-mL Erlenmeyer flasks in 20 mL BG-11 liquid medium supplemented with the appropriate antibiotics (Table 2-2) as well as IPTG if relevant, under standard growth conditions (described in 2.5.1). When mid-exponential phase ($0.5 < OD_{730} \leq 0.7$ measured in a 1-cm cuvette) was reached, contamination of each of these pre-cultures was checked for contamination on LB plates as described in section 2.5.3 above, and cultures washed 3 times in sterile deionized water (centrifugations were performed for 5 min for *Anabaena* and 15 min for *Synechocystis*, both at 5,000 rpm). Thereafter, the cyanobacterial cultures were combined in 100-mL Erlenmeyer flasks (either in BG-11 or BG-11₀), all diluted to a final OD_{730} of 0.1 for both *Synechocystis* and for *Anabaena* strains, unless otherwise stated, in a final volume of 20 mL. With ICLEE, IPTG was also added to a final concentration of 1 mM. The following combinations were performed in replicates, both in BG-11 (N-repleted conditions) and BG-11₀ (N-depleted conditions):

- *Synechocystis* (either ICLEE or UL030) alone,
- *Synechocystis* (either ICLEE or UL030) with the different strains of *Anabaena* (WT, Δamt , WT *gifA*-1/2 and Δamt *gifA*-2).

2.5.7 Co-cultivation of contaminants with *Synechocystis*

Cultures of *Synechocystis* sp. PCC 6803 ICLEE or UL030 were grown in 100-mL Erlenmeyer flasks in 20 mL BG-11 liquid medium supplemented with the appropriate antibiotics (Table 2-2) as well as IPTG if relevant, under standard growth conditions. Contaminants received from A4F were cultivated as described in section 2.5.4. When cultures reached mid-exponential phase ($0.5 < OD \leq 0.7$ measured in a 1-cm cuvette; OD_{730} and OD_{600} respectively for *Synechocystis* and contaminants), *Synechocystis* pre-cultures were checked for contamination on LB plates as described in section 2.5.3 above, and cells (both *Synechocystis* and contaminants) washed 3 times in sterile deionized water (centrifugations were performed for 5 min for the contaminants and 15 min for *Synechocystis*, both at 5,000 rpm). Thereafter, the cyanobacterial and contaminants cultures were combined in 100-mL Erlenmeyer flasks (either in BG-11 or BG-11₀), diluted to a final OD_{730} of 0.02 for *Synechocystis* and 0.04 for contaminants, in a final volume of 20 mL (unless otherwise stated). IPTG was also added to a final concentration of 1 mM if necessary.

2.6 Wheat fertilisation experiments

2.6.1 Cyanobacteria cultivation

Fresh BG-11 (1.5 L) was inoculated with 200 mL of a preculture of *Anabaena* sp. PCC 7120 WT (grown in the conditions described in section 2.5.1) in two 5-L Erlenmeyer flasks closed with a foam stopper, and incubated at 30 °C, at 150 rpm and under white fluorescent light at 120

$\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a growth chamber. Air was bubbled into the culture. The set-up is shown in Figure 2-1.



Figure 2-1. Set-up used to produce biomass for the wheat growth experiments.

Once a week, 1 L cyanobacterial biomass was harvested at the exponential phase and 1 L of fresh BG-11 media added to each flask for semi-continuous culture. The biomass harvested was centrifuged at 10,000 g for 10 min. Thereafter, the supernatant (hereafter SN) was set aside for future experiments, and the wet biomass (precipitate) was separated for further independent treatments: untreated (i.e. fresh alive cyanobacterial biomass (either washed with deionized water (10,000 g, 10 m) or unwashed)); dead cyanobacterial biomass (unwashed, oven-dried (80 °C, 24 hours)) and freeze-dried biomass (10 mL of biomass in falcon tubes was covered with parafilm with holes and placed in a Heto Dry winner freeze-drier for 48-72 hours). The supernatant and biomass were all stored at 4 °C, until soil inoculation with wheat.

2.6.2 Experimental set-up

Triticum aestivum (spring wheat, Limagrain, UK) seeds were bleach sterilized by shaking them in a 50% sodium hypochlorite solution and rinsing them in deionized water 8 times. The seeds were then placed in a petri dish (391-2018, VWR) with a filter paper soaked in deionized water. These petri dishes were covered with aluminium foil and placed in a growth room chamber, set at 20 °C in day/night cycles of 50%:55% relative humidity under a 16h:8h photoperiod at $120 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity. After 4-6 days, germinated seeds were transplanted to soil as described below. Wheat fertilisation experiments were carried out, either in a growth room chamber or in an outside greenhouse.

2.6.2.1 Plant growth room experiment

A pot experiment was conducted in a growth room chamber at Imperial College London for 39 days with wheat (*Triticum aestivum*), in vermiculite (Medium grade, VER, Fargro, UK (characteristics can be found in the Appendice II)). Sterilised and germinated seeds were transplanted to vermiculite (dispensed in 5 x 5 cm plastic pots), and *Anabaena* sp. PCC 7120 WT biomass, grown as described in 2.6.1, was applied onto the vermiculite surface right after transplanting.

Anabaena biomass (0.08 g, or its equivalent before drying) per pot was applied directly on top of the vermiculite (either pipetted (liquid applications) or weighed and applied (solid applications)). The treatments were as follows: fresh alive cyanobacterial biomass (either washed with

deionized water or unwashed), dead cyanobacterial biomass (unwashed, oven-dried (80 °C, 24 hours)), supernatant and untreated (negative control). Each of the plant pots were separated in weighing boats to prevent cross-contamination.

The plants were maintained at 20 °C in day/night cycles of 50%:55% relative humidity under a 16h:8h photoperiod at 120 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity. All plant pots were maintained wet by regular spraying with tap water and incubated for 39 days.

2.6.2.2 Outdoor Silwood Park greenhouse

A pot experiment was conducted in an outdoor greenhouse at Silwood Park (Imperial College London) for 42 days with wheat (*Triticum aestivum*), in soil collected from an annually ploughed field at Silwood Park (Imperial College London). Prior to starting the experiment, the soil was air dried, sieved (2 mm), and homogenised. Seedlings were transplanted to Silwood Park soil (dispensed in plastic pots of 5.1 cm diameter) and either *Anabaena* sp. PCC 7120 WT or urea were applied to the soil surface, right after transplanting at the following application rates: 0, 20, 40, 70, 120, 150 and 210 kg N/ha, using the area of the pots. Urea was weighed and applied onto the soil surface, whilst *Anabaena* (grown as described in 2.7.1) was pipetted as a liquid onto the soil surface (the volume applied was based on its nitrogen content (11.6%), as per Table 5-1). Each of the plant pots were separated in saucers to prevent cross-contamination.

The potted plants were placed in the outdoor greenhouse at Silwood Park, and maintained in day/night cycles of 20°C: 15°C, 50%:55% relative humidity under a 16h:8h photoperiod at 120-

130 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity. All plant pots were maintained wet by regular spraying of tap water and incubated for 42 days.

2.6.3 Data collection

At the end of each experiment, growth parameters of the wheat seedlings were recorded: chlorophyll *a* quantification (see section 2.7.6), number of leaves, dry weights of roots and shoots (oven-drying at 80 °C for 48 hours), height (defined as the longest part of the plant by measuring from the surface of the vermiculite next to the stem to the longest part of the plant when hold up next to the measuring tape) and standing height (defined as the highest point of the plant by measuring from the surface of the vermiculite to the highest standing leaf, not being hold).

2.7 Analytical methods

2.7.1 Optical Density measurements

Cyanobacterial growth was observed by Optical Density (OD) measurements using a Tecan Infinite M200 Pro plate reader (Tecan AG, Switzerland). Cell culture dilutions were added either to a 1-mL cuvette or a 96 flat-bottom well-plate, and OD read at an absorbance wavelength of 600 nm (heterotrophic bacterial cultures (i.e. contaminants)) or 730 nm (cyanobacterial cultures).

2.7.2 Microscopic analysis of cyanobacterial cultures

Liquid cultures of cyanobacteria were sampled for 200 μL homogenous samples into 1.5-mL Eppendorf microcentrifuge tubes under sterile conditions, and the culture was returned to incubation. The sample was serially diluted if necessary, and 10 μL of each dilution step was transferred on a glass microscope slide. Sample droplets were covered with a glass cover slip and the edges of the slip were painted with transparent nail polish to avoid quick drying of the culture. A fluorescent microscope (Axioplan 2, Zeiss, Germany) was used in bright field mode to study samples. Hundred-times magnification was used for overviewing the sample and 400x (with a drop of immersion oil between the objective and the cover slip) for cell counting analysis (described in the next section).

2.7.3 Cell counting

Synechocystis sp. PCC 6803 cell numbers were determined by cell counting using an improved Neubauer counting chamber (Brand, Germany). Between 30 and 300 cells per sample were counted from a known volume of culture, under a bright-field light microscope and a Student's t-test (as described in 2.8.1) performed.

2.7.4 Ethanol quantification

Two millilitres of each culture were centrifuged (4,000 g, 10 min) and the supernatant was used for metabolite analyses. Ethanol was quantified with the following two methods:

- High-Performance Liquid Chromatography (HPLC) using a 1200 series Agilent HPLC (Agilent Technologies, Germany). Samples were chromatographed under isocratic conditions using a prepacked HPLC carbohydrate analysis AMINEX HPX-87H column (300 x 7.8 mm, hydrogen form, 9 µm particle size, 8% cross linkage) with industrial grade guard cartridge (30 x 4.6 mm, hydrogen form) (Bio-Rad, USA) was heated up to 60 °C. The mobile phase was 5 mM sulphuric acid in water (HPLC grade) with a flow rate of 0.6 mL/min. The signals were acquired with a refractive index detector,
- With an enzymatic procedure using the Ethanol kit (R-Biopharm AG, Darmstadt, Germany) according to manufacturer's instructions. This assay was proportionally scaled-down to be carried out in a 96-microtiter plate (with a sample volume of 6.67 µL, and a final volume of 210 µL).

Standards and blanks were included in every analytical run. A series of samples with serial dilutions of absolute ethanol (VWR) were analysed and used to create a standard curve.

2.7.5 Ammonia quantification by the Willis method

The protocol for measurement of ammonia/ammonium was originally described by Willis et al. (1996). All dilutions except where stated were dissolved in deionized water. The method was modified to be carried out in a 96-well microtiter plate. The reaction buffer contained sodium salicylate (1.28 g), $\text{Na}_3\text{PO}_4 \times 12 \text{ H}_2\text{O}$ (1.6 g) and sodium nitroprusside (0.02 g) dissolved in 40 mL water prepared in a 50-mL centrifuge tube in a fume hood, covered with aluminium foil and stored at 4 °C. A hypochlorite solution (40x) was prepared fresh (1 mL bleach (10–15% active

chlorine) in 39 mL water). Standards were prepared by diluting a 1 M ammonium chloride stock (0.535 g NH_4Cl in 30 mL water) in water: 0.01, 0.02, 0.04, 0.05, 0.08, 0.1, 0.15, 0.2, 0.5, 0.8, 1, 5 and 10 mM. Each sample was filtered through 10-kDa molecular weight cut-off filter (516-0227, VWR) to remove protein. The reactions were assembled in a 96-well clear bottom polystyrene plate (734-0954, VWR). Standards (including a blank) and samples were first aliquoted into each respective well. Two hundred microlitres of the reaction buffer were then added to each well containing a sample using a multichannel pipette. To start the assay, fifty microlitres of the Hypochlorite solution was added immediately thereafter and mixed with the pipette. The plate was covered with a lid and incubated in a plate shaker (PHMP, Grant Instruments (15 min, room temperature, 900 rpm)). The absorbance was read at 685 nm using a Tecan Infinite M200 Pro multimode reader. The concentration of ammonia was calculated by fitting a linear curve using the standards.

2.7.6 Chlorophyll *a* quantification

For cyanobacteria, chlorophyll *a* was quantified according to the protocol described by Zavřel et al. (2015). Chlorophyll *a* extractions were performed under modest irradiance (up to $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ of white light or $10 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ of green light) in order to prevent degradation of extracted pigments. Each cyanobacterial culture was concentrated by centrifugation (3,000 g, room temperature, 10 minutes), washed in 20 mL of fresh BG-11 medium and then resuspended in 500 μL of fresh BG-11 medium. One millilitre of methanol (VWR), precooled to 4 °C, was added and mixed by gentle pipetting up and down. Samples were then covered with aluminium foil, incubated at 4 °C for 20 minutes to extract the pigments from the cells. The methanol extract

was centrifuged (15,000g, 4 °C, 7 min) and the chlorophyll *a* concentration determined by measuring the absorbance at both 665 and 720 nm in a 1-cm cuvette. The amount of pigment was calculated with the following equation:

$$\text{Chlorophyll } a \text{ } [\mu\text{g/mL}] = (\text{absorbance at } 665 \text{ nm} - \text{absorbance at } 720 \text{ nm}) * 12.95$$

Standards and blanks were included in every analytical run. A series of samples with serial dilutions of absolute ethanol (VWR) were analysed and used to create a linear standard curve.

For wheat seedlings, chlorophyll *a* was quantified according to the protocol described by Bojović et al. (2005). Samples of leaves from 8 replicates were collected and finely chopped for immediate analysis. The homogenised fresh material (0.5 g) was placed into a 1.5 mL Eppendorf tube containing 1.5 mL of 80% acetone (VWR). The preparation was crushed using a pipette tip, vortexed for 20 seconds and centrifuged (15,000g, room temperature, 15 minutes). The supernatant was then placed into a new Eppendorf tube containing 0.5 mL of 80% acetone and the crush, vortex and centrifugation above repeated. The supernatant was transferred into an Eppendorf tube containing the previous supernatant. Two hundred microlitres of the supernatant was then transferred to a 96-well plate. Standards and blanks were included in every analytical run. A series of samples with serial dilutions of absolute ethanol (VWR) were analysed and used to create a standard curve. The chlorophyll *a* concentration was determined by measuring the absorbance at both 647 nm and 664 nm, and calculated with the following equation:

$$\text{Chlorophyll } a \text{ } [\mu\text{g/mL}] = 17.8 * (\text{absorbance at } 647 \text{ nm}) + 7.3 * (\text{absorbance at } 663 \text{ nm})$$

2.7.7 DNA extraction and 16S rRNA sequencing of bacterial contaminants

DNA was extracted from cultures with a simple freeze-thaw procedure, in which 250 µL of bacterial suspension were centrifuged (10,000 rpm, 3min), resuspended in 50 µL of deionized water, and subsequently incubated at 99 °C for 5 minutes and placed in ice for 5 minutes. 2 µL of the supernatant obtained following centrifugation (10,000 rpm, 3min) was used to amplify 16S rRNA genes by PCR using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primer set (Weisburg et al., 1991). The PCR products were stained with 10 µL of 6X Purple Gel Loading Dye (New England Biolabs, United States) and subsequently run through a 1% Tris-acetate-EDTA (TAE) agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, United States) at 85 volts for 40 minutes. The target band was extracted and purified from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen, Netherlands), following the protocol provided by the manufacturer. Finally, purified PCR products were sent to Source BioScience (United Kingdom) for sequencing with their Sanger Sequencing services using the primers specified above. The sequences of each bacterial isolate were submitted to NCBI and subjected to BLAST searches to identify the closest hit in the NCBI 16S ribosomal RNA sequences database.

2.7.8 Flow cytometry

Samples were acquired using a 14-colour BD LSRFortessa flow cytometer equipped with a platereader, for Class II work (BD LSRFortessa II, BD Biosciences, UK), with the laser and filter configuration described in the table below.

Table 2-3. Lasers and filters of the LSRFortessa II flow cytometer used in this study.

Laser wavelength	Filters	Emission from
405 nm (violet)	450/50	DAPI*, used for beads gating
488 nm (blue)	Forward Scatter (FSC)	
	488/10	Side Scatter (SSC)
	530/30	Phycoerythrin (PE)
	695/40	Chlorophyll <i>a</i>
561 nm (yellow-green)	586/15	Used for beads gating
640 nm (red)	610/20	Used for beads gating
	670/14	Phycocyanin (PC), APC**

*4'-6-diamidino-2-phenylindole.

**Allophycocyanin

Falcon 5-mL Round Bottom Polystyrene Test Tubes (with Cell Strainer Snap Cap) or 96-well U-bottom plates (respectively 352235 and 10520832, Thermo Fisher Scientific, UK) were used to load samples on the cytometer, with 1 mL or 200 μ L of cultures (diluted to an OD₇₃₀ of 0.03), respectively. 50 μ L of counting beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific, UK) were added to each tube, or 30 μ L for wells. A minimum of 8,000 beads were recorded for each sample. All settings were kept constant throughout the analysis. The loader settings were as follow for the 96-well plate measurements: sample flow rate: 2 μ L/s; sample volume: 180 μ L; mixing volume: 100 μ L; mixing speed: 250 μ L/s; number of mixes: 5 mixes; wash volume: 800 μ L. Forward scatter (FSC), side scatter (SSC) and fluorescence signals were plotted on a logarithmic scale and analyses were conducted on the FlowJoTM V10 software (BD Biosciences). The gating strategy for analysing *Synechocystis* and *Anabaena* was as follows:

background noise exclusion: histogram vs. chlorophyll *a*; *Synechocystis* 6803 gate: PE vs. PC, APC (height) vs. APC (area) (doublets removal); *Anabaena*: PE vs. chlorophyll *a*.

Staining for cell viability was performed by the addition of DAPI (0.3% (v/v), 4'-6-diamidino-2-phenylindole, Fisher Scientific Ltd., UK) for 5 minutes. Positive control aliquots were resuspended in 4% (v/v) formaldehyde for 20 minutes and rinsed twice in Phosphate-Buffered Saline (PBS).

For imaging flow cytometry, cultures were resuspended in 4% (v/v) formaldehyde for 20 minutes (fixation) and rinsed twice in PBS. Data were acquired using a Luminex ImageStream MK2 (Luminex Corporation, USA), whose laser configuration is described in the table below.

Table 2-4. Lasers of the Luminex ImageStream MK2 flow cytometer used in this study are summarised below.

Laser wavelength	Emission from
488 nm (blue)	Phycoerythrin
	Chlorophyll <i>a</i>
642 nm (red)	Phycocyanin

2.7.9 High-throughput 16S rRNA gene amplicon sequencing, processing and analysis

To determine the taxonomic profile of the algae communities, high-throughput 16S rRNA gene amplicon sequencing was performed as described by Celis et al. (2018). In brief, the primers Pro341F (5'-CCTACGGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3')

(Takahashi et al., 2014) were used to amplify the hypervariable regions V3 and V4 of the 16S rRNA gene in Bacteria and Archaea as well as of the 16S rRNA in chloroplasts of single-celled algae strains. After a PCR approach, resulting amplicons were pooled in equimolar amounts for subsequent Illumina MiSeq sequencing (2 x 300 bp) (Illumina, San Diego, CA, USA), applying the paired-end protocol.

After sequencing, adapter and primer trimming were performed through an inhouse pipeline (Wibberg et al., 2016). For amplicon processing and analysis, a pipeline including FLASH v.1.2.11 (Magoc et al., 2011), USEARCH v.11.0.667 (Edgar, 2010), and the Ribosomal Database Project (RDP) classifier v.2.9 (Wang et al., 2007) was used as described recently (Maus et al., 2016; Liebe et al., 2016; Klassen et al., 2017).

2.8 Statistical analysis

2.8.1 Student's t-test

Assuming a normal distribution (Fay & Gerow, 2013), a two-sided Student's t-test was used to determine statistically significant changes across independent samples, with asterisks indicating significance (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.005$). All experimental data presented in this study is shown as average $\pm$ SD from three biological replicates, unless stated otherwise.

2.8.2 ANOVA

The means of the replicates $\pm$ standard deviation are presented. Statistical analyses were conducted using the OriginPro 2018b software (Academic version). The treatments were compared against each other using one or two-way ANOVA. Differences were considered significant at a probability level of $P < 0.05$.

3

Establishing a method for high-throughput analysis of a photosynthetic consortium including filamentous species

3.1 Introduction

The species selected for the model nitrogen-sharing co-culture study (see Chapter 4) were *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) and the diazotroph (i.e. nitrogen fixer) and filamentous *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), both photosynthetic cyanobacteria. These two species were selected as (1) they are both well-established laboratory strains with

well-defined metabolism (i.e. published metabolic models), (2) they can both be cultured with the same media (BG-11) and (3) *Synechocystis* was the target biocatalyst of the DEMA consortium

To quantify the impact of the tested variables in the experimental system, total biomass is the first parameter to be monitored by regularly measuring the Optical Density (OD) at 730 nm. At this specific wavelength, no cyanobacterial pigments are absorbing, therefore OD measurements only represent light scattering from the cells (Murton et al., 2017). However, these OD measurements do not allow to distinguish the growth of *Synechocystis* from the growth of *Anabaena* when co-cultivated.

To monitor the growth of each species selectively, direct microscopic cell counting using a hemocytometer can be performed easily with *Synechocystis* single cells (and was carried out for the first co-culture experiment, as described in the next chapter, section 4.3.1). However, this would not discriminate between healthy and alive from unhealthy or dead cells. Moreover, counting *Anabaena* (either the number of filaments or the number of cells per filaments) is challenging using direct microscopy as long filaments (up to 2,000 μm) tend to entangle (Jin et al., 2018).

Alternatively, flow cytometry can be used. It is a rapid, precise and automated cell counting method giving the possibility to simultaneously record multiple parameters per cell, whilst only requiring a small sample volume (Poniedziałek et al., 2017). Flow cytometry is already well established in medical, veterinary and environmental studies (Poniedziałek et al., 2017), and has been widely used in marine ecology since the 1980s, and more recently applied to freshwater

picophytoplankton studies for phylogenetic, physiological and ecophysiological purposes (Andersen, 2005). For example, flow cytometry is used to characterise microbial community dynamics at the single-cell level in marine, freshwater and wastewater environments, assess and monitor water drinking water quality (Coggins et al., 2020). Notably, it enabled the determination of new species, such as *Prochlorococcus*, a 0.6 µm cyanobacterium displaying an uncommon pigmentation, and likely the most abundant photosynthetic organism on the planet (van Dijk et al., 2010).

Flow cytometers work as follows: a cell suspension is injected into the cytometer's sheath fluid, which acts as an entrainment fluid to carry and align hydrodynamically the particles (cells, debris and media noise) with the direction of the flow as they pass by the instrument's lasers (laser beam), as shown on Figure 3-1. Cells therefore flow in a single file and are analysed one by one by laser interrogation (at rates of up to 10,000 cells per second) (Andersen, 2005). As summarised in Table 2-3, the Fortessa II flow cytometer used in this study is equipped with 4 lasers (violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (640 nm)).

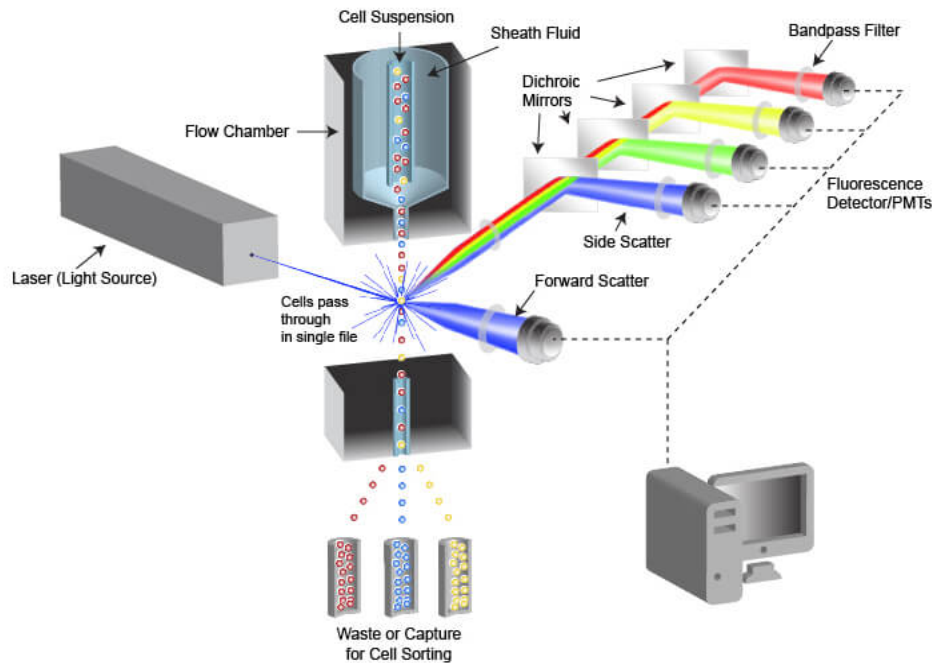


Figure 3-1. Schematic overview of a flow cytometer coupled with a cell sorter.

Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com) (Appendix I). Note that cell sorting was not performed in this study (the Fortessa II does not include a cell sorter).

The instrument then detects and records both scattered laser light and fluorescent emission signals. The former, scattered laser light, is quantified in two directions by detectors placed:

- in front of the laser beam to quantify the Forward Scatter (FSC), proportional to relative cell size,
- at a 90° angle from the laser beam (Side Scatter SSC), proportional to internal cell complexity or granularity.

As a particle crosses the laser beam and scatters light or fluoresces (Figure 3-2), a pulse (peak) of photon emission is created over time and converted into a voltage peak (known as an “event”).

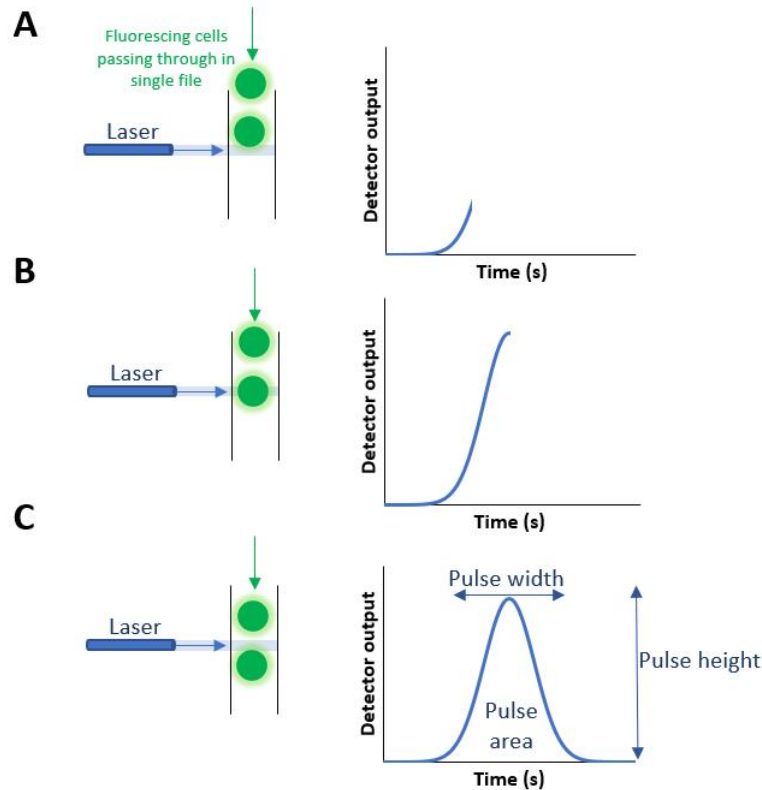


Figure 3-2. Creation of a voltage pulse as a particle (cell) crosses the flow cytometer laser beam. As a particle moves through the laser beam (panels A to C), a pulse is recorded.

The flow cytometer then records the following measurements:

- pulse height: pulse maximum value (typically when the particle is at the centre of the laser beam),
- pulse width: time of the signal duration as the particle passes through the laser beam,
- and pulse area: total signal emitted as the particle moved through the laser beam (integral of the height over width (time)) (Hoffman, 2009).

Importantly, the pulse width is linked to the length of the particle as it passes through the laser beam, and therefore can be used to discriminate between filamentous cyanobacteria and single cell cyanobacteria (van Dijk et al., 2010).

As a result, cells with different features can be counted and characterised based on their differences in size, internal complexity and fluorescence properties (McKinnon, 2019).

Additionally, samples can be treated (with fluorescent dyes for instance) to quantify:

- other intrinsic features such as the chemical composition of the biomass (lipid, carbohydrates, proteins, nucleic acids and pigment content),
- metabolic activity (cellular enzyme activity, autofluorescence of native pigments and cell viability) of each cell within a heterogeneous population (Hyka et al., 2013),
- and detect contaminating bacteria or viruses (Andersen, 2005).

Finally, coupling a flow cytometer with a cell sorter allows single cells from a heterogeneous cell population to be isolated and collected for further analysis (e.g. obtain cells overproducing a compound of interest or axenic cultures) (Hyka et al., 2013).

Flow cytometry can therefore be used as a tool for the development of efficient biotechnological processes with microalgae (Hyka et al., 2013), and is well suited to monitor cell growth and metabolic activity of the different partners of a photosynthetic microbial co-culture (Dunker et al., 2017; Dunker et al., 2013; Smith et al., 2016; Spiegelman et al., 2005).

In particular, flow cytometry is adapted to discriminate sub-populations within heterogeneous samples, as microalgae and cyanobacteria possess fluorescent pigments such as chlorophyll *a*, phycocyanin and phycoerythrin (Andersen, 2005). When excited by blue light (488 nm), chlorophyll *a* and phycoerythrin emit respectively red (maximum emission wavelength ~670 nm) and yellow-orange fluorescence (maximum emission wavelength ~575 nm), whilst phycocyanin

emits red fluorescence (maximum emission wavelength ~660 nm) when excited by red light (>640 nm) (van Dijk et al., 2010). Two of the lasers available with the Fortessa II flow cytometer are particularly useful for detecting photosynthetic pigments (see Table 2-3):

- a 488 nm laser detecting fluorescence in the 695 ± 40 nm channel (chlorophyll *a*) and 530/30 nm (phycoerythrin) channels,
- and a red laser (640 nm) detecting fluorescence in the 670/14 nm channel (phycocyanin).

It is important to note that, although filament-forming cyanobacteria are widespread in eutrophic systems, flow cytometry has not been frequently used to study this class of organisms. This may be explained by the fact that they are organised in long filaments whose individual cells cannot be distinguished as they flow past the lasers, unless the filaments are disrupted and single cells generated (Azevedo, 2012). However, if single cells analysis is not necessary, filaments on their own can be recorded. For example, intact filaments of up to 1,000 μm have been detected by flow cytometry and even sorted without damaging them, using voltage pulse width as a selective parameter (van Dijk et al., 2010). Furthermore, the recent development of imaging flow cytometry could potentially allow to monitor not only the number of cells per filament but also the length of filaments without disrupting them. Indeed, imaging flow cytometry is a platform which combines the features of fluorescent microscopy and flow cytometry, and links each events of the cytogram to a microscopic image, therefore giving information such as size distribution, cell life cycle, morphology, etc., opening a new window of opportunities for biological research (Dunker, 2019). Imaging flow cytometry has therefore the potential to provide information on the community composition at rates of throughput that are otherwise impossible

to attain using conventional monitoring methods. In this study, entire filaments were counted by flow cytometry, without disrupting them.

3.2 Chapter overview

The importance of developing methods to effectively study filamentous cyanobacteria has been recently highlighted (Jin et al., 2018). As far as I am aware, traditional flow cytometry has not previously been used to monitor such filamentous species in culture whilst maintaining their integrity, i.e. when keeping the filaments intact, except for two methodology publications (van Dijk et al., 2010; Barker et al., 2012). This chapter therefore aimed to test the hypothesis that traditional flow cytometry can be used to monitor a co-culture that includes a filamentous cyanobacterium (*Anabaena*) in a robust and reliable fashion.

This over-arching hypothesis was tested by breaking the task down into testing three separate hypotheses addressed one after the other:

1. **Hypothesis 1:** *Synechocystis* cells and *Anabaena* filaments can be effectively differentiated by flow cytometry,
2. **Hypothesis 2:** *Anabaena* filaments can be reliably counted using flow cytometry,
3. **Hypothesis 3:** The experimental workflow can be improved with higher throughput.

The aim of this chapter was also to establish the analytical methodology to support follow-on experiments in Chapter 4.

3.3 Results

3.3.1 Hypothesis 1: *Synechocystis* cells and *Anabaena* filaments can be effectively differentiated by flow cytometry

To reliably monitor the growth of both *Synechocystis* and *Anabaena* during co-cultivation, a clear separation without overlap would ideally be achieved on the cytograms. In order to evaluate how axenic samples of *Synechocystis* and *Anabaena* behave on the flow cytometer, the two strains (1) *Anabaena* sp. PCC 7120 WT (hereafter *Anabaena* WT) and (2) *Synechocystis* engineered to produce ethanol (*Synechocystis* sp. PCC 6803 ICLEE, hereafter ICLEE) were subjected to flow cytometry (as described in 2.7.8). Briefly, liquid pre-cultures were used to inoculate 20 mL fresh BG-11 media (section 2.5.1) in a 100 mL Erlenmeyer flask to a starting OD₇₃₀ of 0.1 (as measured in a Tecan M200 plate reader) and incubated in a PSI Instruments AlgaeTron 230 at 30 °C, 180 rpm, 1% (v/v) CO₂ and 60 µE.m⁻².s⁻¹. After seven days of cultivation, each culture was checked for contamination by plating on LB agar and over-night incubation at 37 °C with cultures deemed axenic when there was no sign of colony-formation (as described in 2.6.3). These conditions will not allow all contaminants to be detected, however, it is particularly useful given that the surrounding laboratory mostly work with *Escherichia coli*.

Falcon 5-mL Round Bottom Polystyrene Test Tubes (with Cell Strainer Snap Cap) (352235, Thermo Fisher Scientific, UK) were next used to load samples on the cytometer, with 1 mL or 200 µL of each culture (diluted to an OD₇₃₀ of 0.03, as described in 2.7.8). 50 µL of counting beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific, UK) were added to each tube,

and a minimum of 8,000 beads were recorded for each sample. Each axenic culture was analysed separately with the flow cytometer as detailed in 2.7.8, and the corresponding cytograms combined *in silico* thereafter. As *Synechocystis* and *Anabaena* are respectively unicellular (~2 µm in diameter (Boone & Castenholz, 2012)) and filamentous (up to 2,000 µm long (Jin et al., 2018)), their difference in shape should enable a distinct separation of the 2 species. The following parameters, commonly used for phytoplankton, were used:

- Forward scatter (FSC), proportional to cell size, and FSC-W is related to the length of the particle crossing the laser beam, and therefore can be used to discriminate filamentous from non-filament cyanobacteria,
- Side Scatter (SSC), proportional to internal cell complexity,
- Chlorophyll *a* fluorescence (488 nm excitation, 695/40 nm emission),
- Phycoerythrin (hereafter PE) fluorescence (488 nm excitation, 530/30 nm emission),
- Phycocyanin (hereafter PC) fluorescence (640 nm excitation, 670/15 nm emission).

The cytograms obtained with axenic cultures are depicted in Figure 3-3.

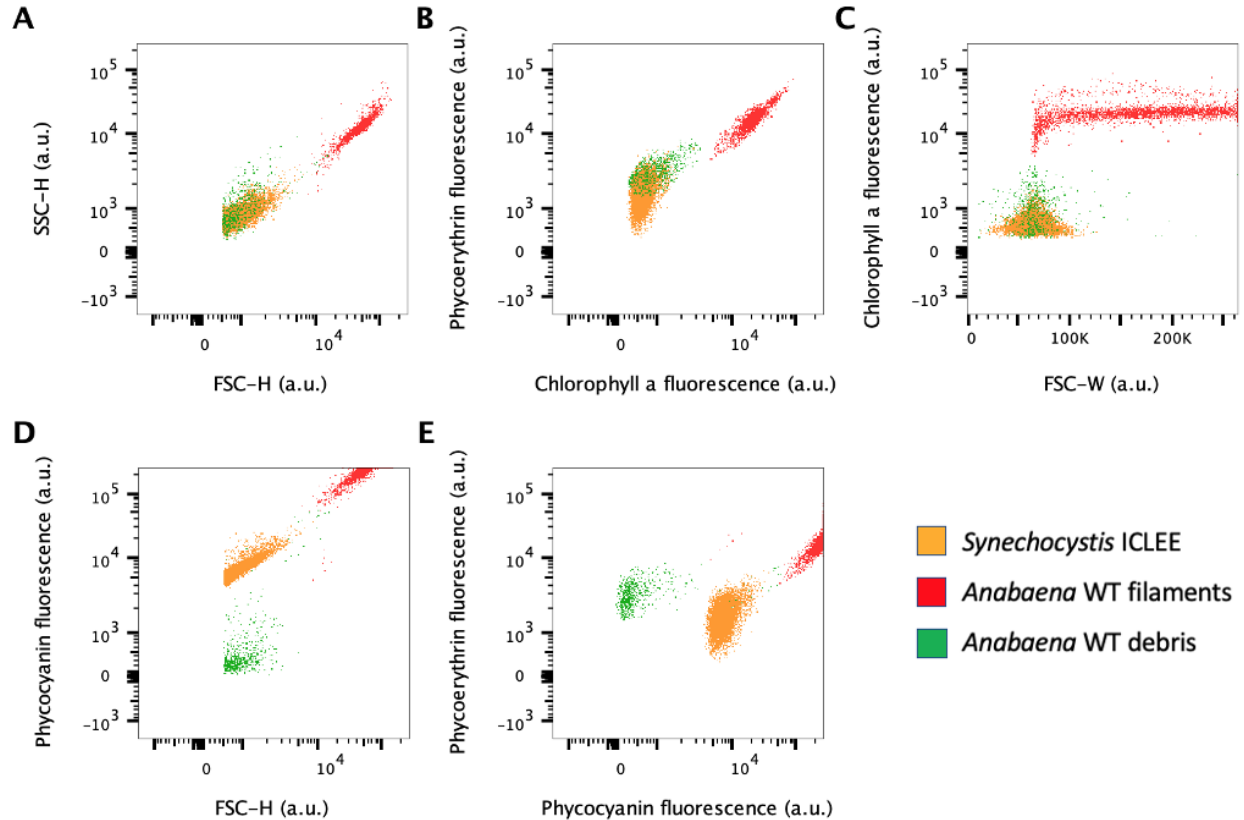


Figure 3-3. Flow cytometric cluster analysis of axenic cultures of *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 ICLEE engineered to produce ethanol, combined in silico.

Axenic cultures of *Anabaena* sp. PCC 7120 WT and *Synechocystis* sp. PCC 6803 engineered to produce ethanol (ICLEE) were separately analysed by flow cytometry (using BD LSRFortessa II, as detailed in 2.7.8) and the corresponding populations combined *in silico* and plotted against different filters (all pulse heights except where otherwise stated): SSC-H: Side Scatter pulse height; FSC-H: Forward Scatter pulse Height; Phycoerythrin fluorescence pulse Height (488 nm excitation, 530/30 nm emission); Chlorophyll *a* fluorescence pulse Height (488 nm excitation, 695/40 nm emission); FSC-W: Forward Scatter pulse Width; Phycocyanin fluorescence pulse Height (640 nm excitation, 670/14 nm emission). Individual panels represent: (A) internal cell complexity vs. cell size, (B) phycoerythrin fluorescence vs. chlorophyll *a* fluorescence, (C) chlorophyll *a* fluorescence vs. the length of the particle crossing the laser beam (FSC-W), (D) phycocyanin fluorescence vs. cell size and (E) phycoerythrin fluorescence vs. phycocyanin fluorescence. The colouring scheme is as follows: orange: ICLEE; green: *Anabaena* WT filaments debris; red: *Anabaena* WT filaments.

As expected, the uniformly sized *Synechocystis* cells (orange cluster in Figure 3-3) form a very well-defined cluster on all commonly used parameters. On the contrary, *Anabaena* cells form 2 clusters (in red and green on Figure 3-3), with one (green population) sometimes overlapping with *Synechocystis* population. Note that media noise was gated out (as described in 2.7.8). As

discussed in detail below, it was first assumed that the green population represented debris of *Anabaena* filaments that had been dislodged from the filaments.

One potential issue for the subsequent co-culture studies was that the *Anabaena* debris cluster overlapped with that of *Synechocystis* in the three initial cytograms obtained (Fig. 3-3, panels A-C). To obtain a clear discrimination between the *Anabaena* green cluster and the *Synechocystis* (orange cluster) without overlap, the cytograms showing PC fluorescence (Figure 3-3, panels D and E) were used, in which the different populations were well separated. On these cytograms, *Anabaena* filaments in the red cluster are partially beyond the chart edges. However, this is not an issue as this population can be correctly gated with the SSC vs. FSC or phycocyanin fluorescence vs. chlorophyll *a* fluorescence plots. As a result, these cytograms were systematically drawn as part of the gating strategy when analysing samples (as described in section 2.7.8).

Surprisingly, two distinct populations were observed in the cytograms when analysing the axenic *Anabaena* culture. Panels A, B and C of Figure 3-3 show the overlap in the green (*Anabaena*) and orange (*Synechocystis*) clusters, suggesting that these *Anabaena* cells (green population), at the tested voltage, are of similar size, internal cell complexity as well as display a similar chlorophyll *a* and phycoerythrin fluorescence as *Synechocystis* cells (~2 µm in diameter (Boone & Castenholz, 2012)). On the contrary, the red cluster (also *Anabaena*) contains cells with significantly higher values for the aforementioned parameters. This red cluster also appears as a horizontally stretched cluster on the pulse width channel (related to the length of the particle crossing the laser beam, panel C), suggesting filaments of varying lengths. This is in accordance with the

results obtained by Van Dijk et al. (2020) who made similar observations. The red cluster can therefore be argued to correspond to the *Anabaena* filaments (which can be up to 2,000 μm long (Jin et al., 2018)), whereas the green cluster contains debris derived from the filaments. To verify this hypothesis, two additional experiments were carried out as described below.

To test the hypothesis that the green cluster in cytograms obtained with axenic culture corresponds to cellular debris, four separate aliquots of the same axenic *Anabaena* culture grown in BG-11 media (as described in 2.5.1) were tested for viability using DAPI, a nucleic acid stain at a 0.3% (v/v) concentration. This viability dye is not able to permeate live cells and can thereby be used to discriminate between intact and membrane-damaged cells (Hyka et al., 2013). As a control, half of the DAPI-stained aliquots were also fixed with 4% (v/v) formaldehyde for 5 minutes (as detailed in section 2.5.8), and used as a positive control to separate on the cytograms between dead and alive cells as formaldehyde permeates membranes without damaging the cells themselves (Hyka et al., 2013). To verify that no *Synechocystis* cells overlap with *Anabaena* clusters on the cytograms, a separate aliquot of *Synechocystis* was prepared (without DAPI staining). These aliquots were all separated analysed using BD LSRFortessa II (as detailed in section 2.7.8). The obtained cytograms are depicted in Figure 3-4.

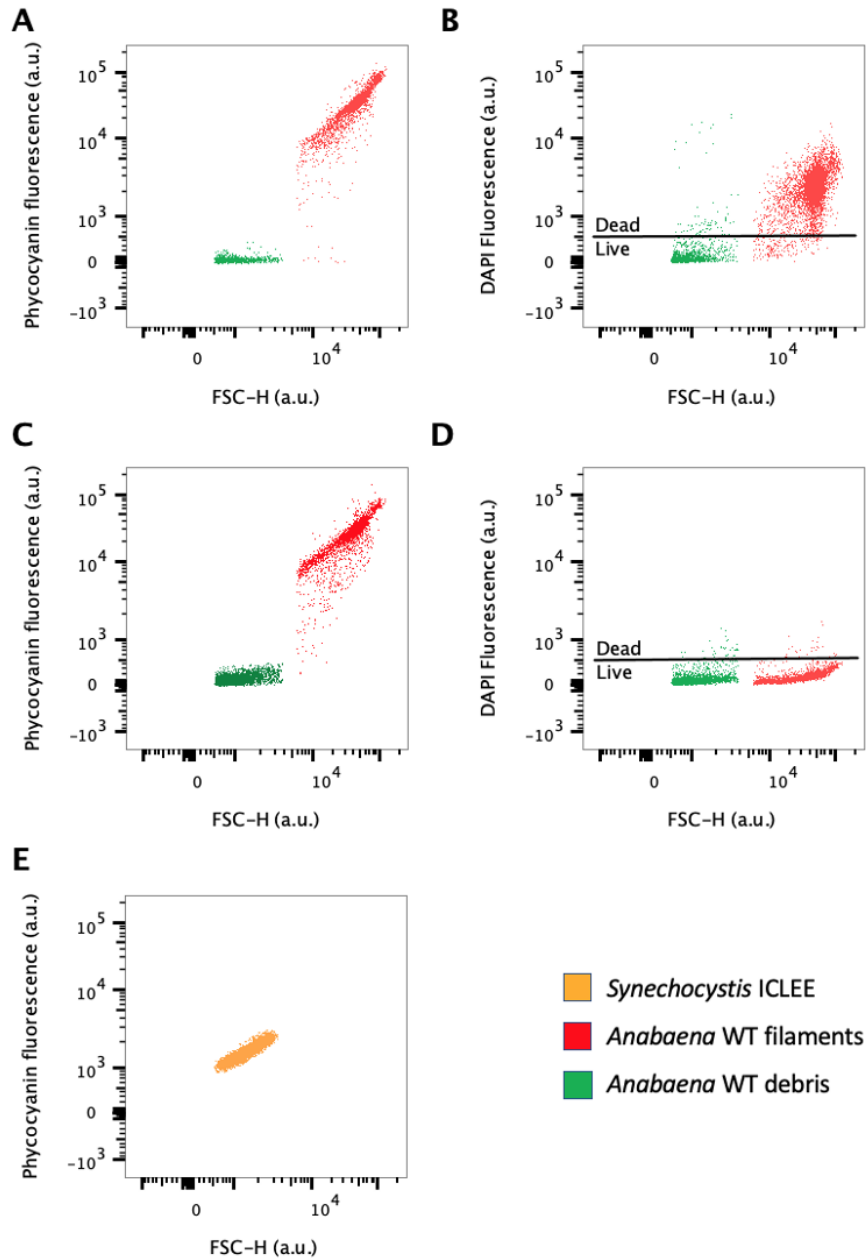


Figure 3-4. Flow cytometric analysis of axenic *Anabaena* sp. PCC 7120 WT cultures cell viability.

Equal amounts (by OD₇₃₀) of axenic cultures of *Anabaena* sp. PCC 7120 WT were subjected to flow cytometry (using BD LSRFortessa II, as described in 2.5.7), stained with DAPI (A-D). As a positive control, cells were fixed with 4% (v/v) formaldehyde for 5 minutes (A-B) prior to DAPI staining (0.3% (v/v)). The black line on (B) and (D) distinguished between live and membrane-compromised cells: panel (B) corresponds to the positive control (cells were compromised by cell fixation (with formaldehyde) prior to the flow cytometry analysis), whilst panel (D) corresponds to the sample of *Anabaena* to be tested for cell viability. (E) *Synechocystis* sp. PCC 6803 ICLEE (unstained) as a control to prove *Anabaena* is not contaminated. *Anabaena* sp. PCC 7120 WT and *Synechocystis* sp. PCC 6803 ICLEE engineered to produce ethanol were separately analysed by flow cytometry, gated according to the method described in section 2.5.7 and the corresponding populations plotted against different filters (all pulse heights): FSC-H: Forward Scatter pulse height; Phycocyanin fluorescence pulse Height (640 nm excitation, 670/14 nm

emission); DAPI fluorescence pulse Height (405 nm excitation, 450/50 nm emission). Individual panels represent: (A, C, D) phycocyanin fluorescence vs. cell size and (B, D) DAPI fluorescence vs. cell size. Colouring scheme is as follows: orange: ICLEE; green: *Anabaena* WT filaments debris; red: *Anabaena* WT filaments.

Panels A and C on Figure 3-4 show the 2 clusters of *Anabaena* as described previously (Figure 3-3). The *Synechocystis* cluster, shown in panel E (Fig. 3-4), is absent from panels A and C, demonstrating that the *Anabaena* culture used for this viability experiment was not contaminated by the unicellular cyanobacterium *Synechocystis*. Aliquots stained with DAPI are shown in panels B and D, with panel B corresponding to the dead cells that were fixed with formaldehyde prior to the flow cytometry analysis, i.e. it would be expected that all filaments should be stained with DAPI (hence displaying a higher fluorescence). Interestingly, the red cluster displayed a higher fluorescence on panel B, but only a few events for the green cluster can be seen with a higher fluorescence (i.e. above the black line), suggesting there is possibly no nucleic acids to be stained in the green cluster, which would be the case if the green cluster is composed of cell debris.

Therefore, axenic cultures of both *Synechocystis* WT and *Anabaena* WT were analysed with an imaging flow cytometer (as detailed in section 2.7.8) to visually ascertain what the green cluster described above corresponds to, and compared with the traditional flow cytometry cytograms (Figure 3-3). The obtained results are shown in Figure 3-5 below.

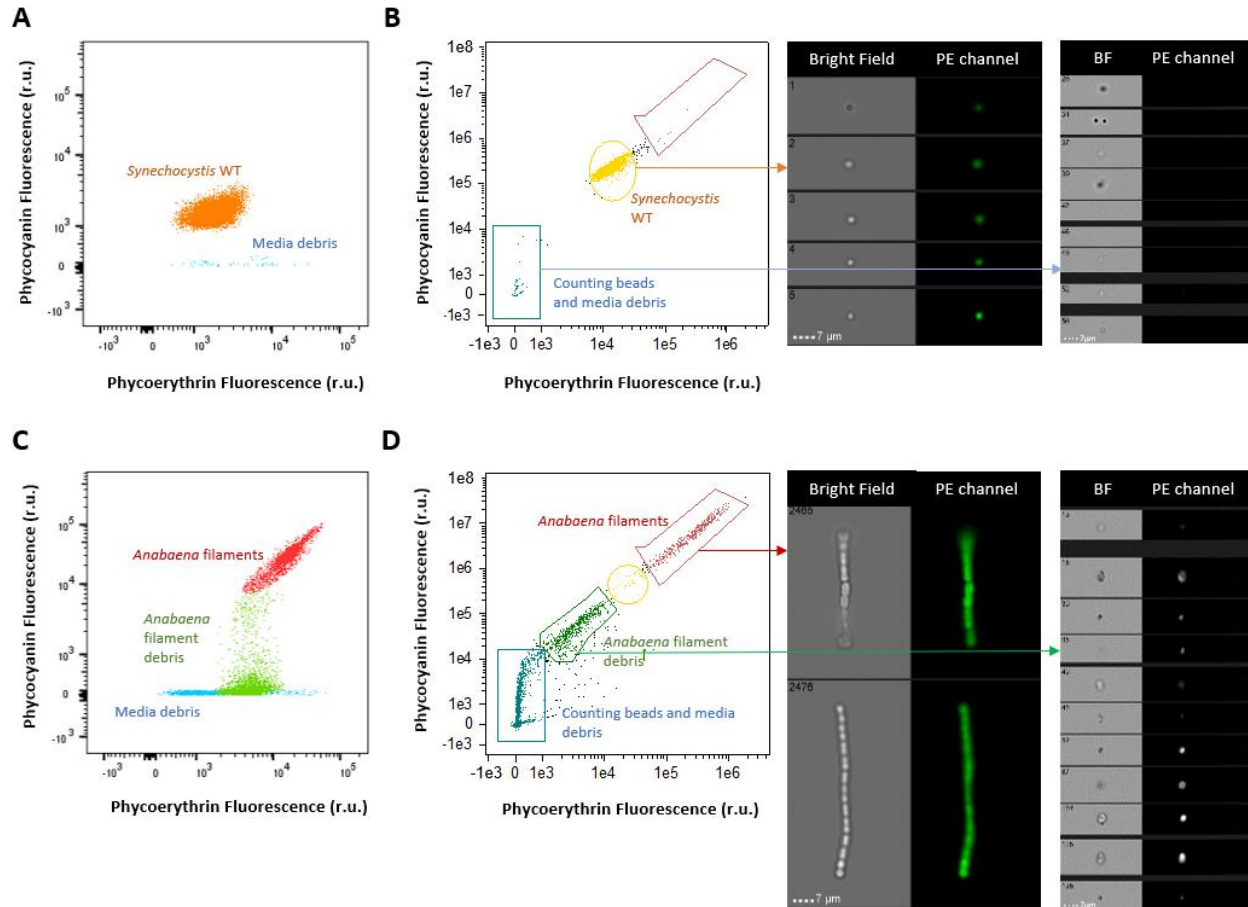


Figure 3-5. Comparison of cytograms obtained by both flow cytometry and imaging flow cytometry, with axenic cultures of *Synechocystis* sp. PCC 6803 WT (A and B) and *Anabaena* sp. PCC 7120 WT (C and D).

Axenic cultures of *Synechocystis* WT (panels A-B) and *Anabaena* WT (panels C-D) were separately analysed with both a flow cytometer (BD LSRFortessa II); panels A and C) and an imaging flow cytometer (Luminex ImageStream MK2), panels B and D), gated according to the method described in section 2.7.8 and the corresponding populations plotted against different filters (all pulse heights): phycocyanin fluorescence pulse Height (640 nm excitation, 670/14 nm emission); phycoerythrin fluorescence pulse Height (488 nm excitation, 695/40 nm emission). Individual panels represent phycocyanin fluorescence vs. phycoerythrin fluorescence. Note that the populations are not expected to fall exactly within the same gates on the flow cytometer and imaging flow cytometer, given they are different instruments. Panels B and D include microphotographs examples of individual *Synechocystis* cells (panel B), and *Anabaena* filaments and debris (panel D) obtained with the help of Dr. Maggio Roberta, using the bright field (BF) and phycoerythrin (PE) channels.

The flow cytometric analysis of axenic cyanobacterial cultures, acquired on both a traditional flow cytometer (BD LSRFortessa II, BD Biosciences, UK) and an imaging flow cytometer (Luminex ImageStream MK2, Luminex Corporation, USA), resulted in similar cytograms, with a well-defined cluster for *Synechocystis* (orange and yellow populations, respectively on panels A and B, Fig. 3-5), and two separate clusters for *Anabaena* (in green and red on panels C and D, Figure 3-5). Note that the populations are not expected to fall exactly within the same gates on the flow cytometer (panels A and C of Fig. 3-5) and imaging flow cytometer cytograms (panels B and D of Fig. 3-5), given they are different instruments. As each event of the imaging flow cytometry is linked to a microscopic image, each population on cytograms can be visually ascertain. The *Synechocystis* population (Fig. 3-5, panel B) showed unicellular round cells fluorescing on the PE channel, as expected, whilst media debris were not. Regarding *Anabaena* (Fig. 3-5, panel D), the events linked to the red cluster appear fluorescing on the PE channel of the corresponding microscopic images, whilst media debris (blue colour, Fig. 3-5, panels B and D) are not. This confirm that the red cluster (Fig. 3-5, panels C and D) corresponds to the filaments of *Anabaena*, whilst the green clusters in the same cytograms correspond to the debris from filaments as shown in panel D, Fig. 3-5. This is also in line with conclusions by van Dijk et al. (2010) who found that the filaments of *PseudoAnabaena* appeared on the cytogram as a stretched cluster well separated from the culture debris. Consequently, the green populations shown in both Figure 3-3 and panels C and D of Fig. 3-5, corresponding to the filament debris, were not taken into account when monitoring the *Anabaena* population.

Interestingly, imaging flow cytometry could also be used to quantify the number of cells per filaments or the length of filaments, as shown in the panel C of Figure 3-6 below. Axenic cultures

of *Synechocystis* WT and *Anabaena* WT (diluted to an OD₇₃₀ of 0.03) were separately analysed with the imaging flow cytometer (Luminex ImageStream MK2) and combined in silico. The length of *Anabaena* filaments (shown in panel C, Fig. 3-6) and diameter of *Synechocystis* cells (shown in panel E, Fig. 3-6), can be determined by creating a masking strategy (Fig. 3-6, panel A), using the object mask followed by the skeleton mask. The object mask segments the starting mask to closely identify the area corresponding to the cells, whilst the skeleton mask provides the barebone structure of the cell. The gating strategy is as follows: gating unicellular *Synechocystis* and *Anabaena* filaments by plotting the chlorophyll *a* fluorescence vs. the number of pixels in the starting mask (panel A); sub-gating unicellular for singlets using the aspect ratio vs. the area of the particle (panel D, unfeasible with *Anabaena* filaments); calculating the length of each particle (respectively *Anabaena* filaments (panel C) and diameter of *Synechocystis* cells (panel D)).

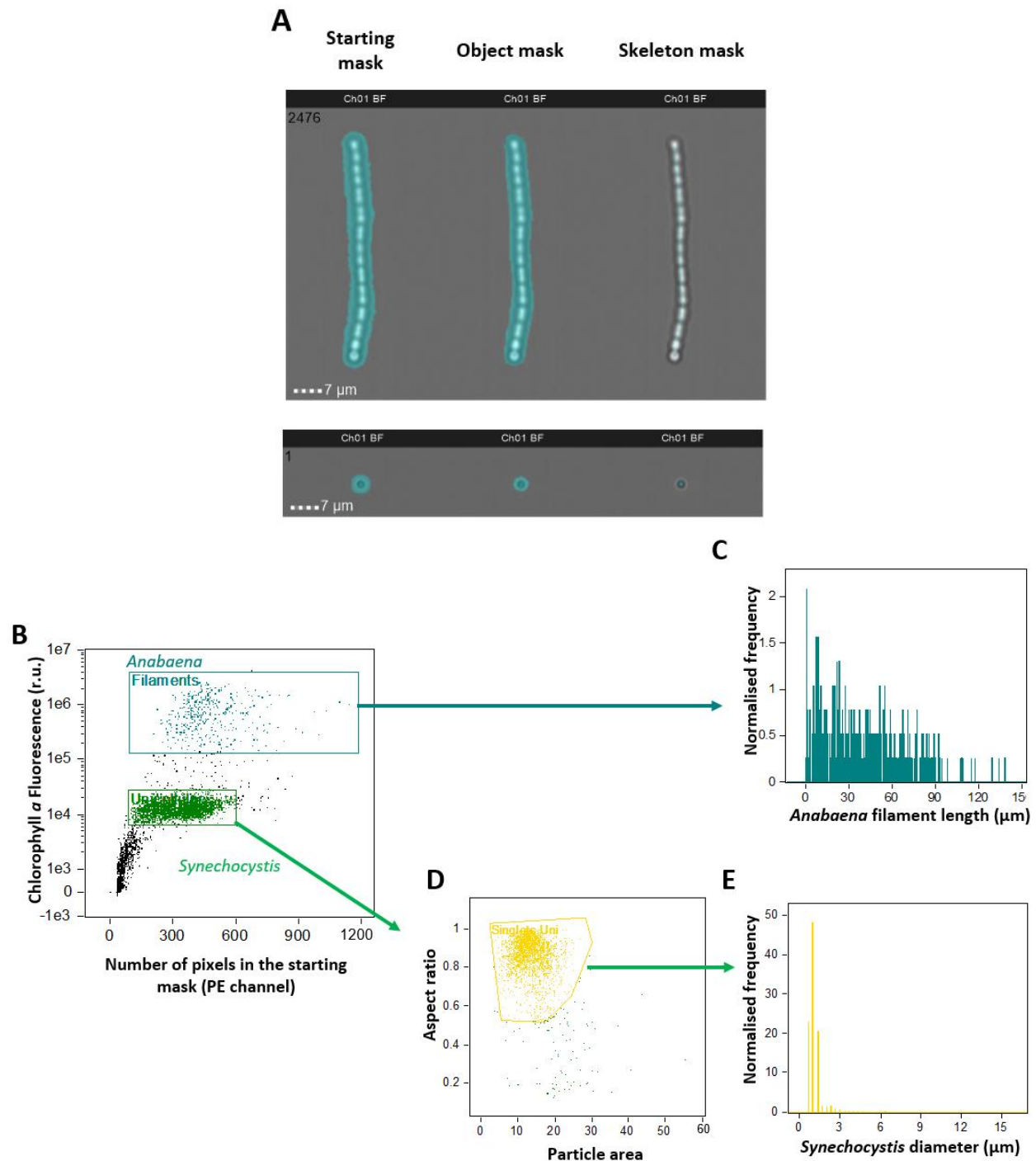


Figure 3-6. The length of *Anabaena* filaments.

Axenic cultures of *Synechocystis* WT and *Anabaena* WT (diluted to an OD_{730} of 0.03) were separately analysed with the imaging flow cytometer (Luminex ImageStream MK2) and combined *in silico*. The length of *Anabaena* filaments (C), and diameter of *Synechocystis* cells (E), can be determined by creating a masking strategy (panel A), using the object mask followed by the skeleton mask. The object mask segments the starting mask to closely identify the area corresponding to the cells, whilst the skeleton mask provides the barebone structure of the cell. Gating strategy is as follows: (A) gating unicellular *Synechocystis* and *Anabaena* filaments by plotting the chlorophyll *a* fluorescence vs. the number of pixels

in the starting mask; (D) sub-gating unicellular for singlets using the aspect ratio vs. the area of the particle (unfeasible with *Anabaena* filaments); (C) and (E) calculating the length of each particle (respectively *Anabaena* filaments and diameter of *Synechocystis* cells). Aspect ratio: describes how round or oblong an object (minor axis divided by the major axis of the mask); Area of the particle: number of pixels in the mask. This figure was obtained with Dr. Prabhjoat Chana's help.

Figure 3-6 also allowed to determine the length of the *Anabaena* filaments (from a few μm to up to 140 μm , panel C) and the diameter of *Synechocystis* (around 1 μm).

In summary, the work carried out in section 3.3.1 tested hypothesis 1 using two different flow cytometers. In both cases, similar cytograms were obtained with the appropriate gating. Although axenic *Anabaena* cultures grown in nitrogen-repleted media resulted in separate populations (i.e. "debris" and filaments), it was clearly distinct from the single population obtained with axenic *Synechocystis* when combined *in silico*. Hence, it was concluded that hypothesis 1 was confirmed although mixed cultures (Fig. 3-8 below) still had to be tested.

3.3.2 Hypothesis 2: *Anabaena* filaments can be reliably counted using flow cytometry

Given that there are only two publications using flow cytometry to quantify the number of cyanobacterial filaments (van Dijk et al., 2010; Barker et al., 2012), two further experiments, described in the next two sections, were carried out to (1) validate the use of flow cytometry against more traditionally use methods, such as direct cell counting and (2) validate the use of flow cytometry to monitor co-cultures.

3.3.2.1 Comparison with direct cell counting

In addition to counting cells and filaments by flow cytometry (as described in 2.5.8), aliquots of axenic cultures of *Synechocystis* WT and *Anabaena* WT (diluted to an OD₇₃₀ of 0.03) were also subjected to cell counting using a hemocytometer (as described in 2.7.3). The number of cells and filaments counted by the two different methods is shown in Figure 3-7. For both *Synechocystis* cells and *Anabaena* filaments, no statistically significant difference ($P > 0.05$ with Student's t-test (described in section 2.8.1)) in both cell and filaments numbers was found between the two methods suggesting that flow cytometry is a reliable method to record cells and filaments, at least at this cell density (OD₇₃₀ of 0.03) and growth phase. This dilution rate was therefore used for all subsequent experiments. Note that the error bars are smaller in the case of the flow cytometry measurements, as automated cell counting is more robust than human cell counting (Andersen, 2005).

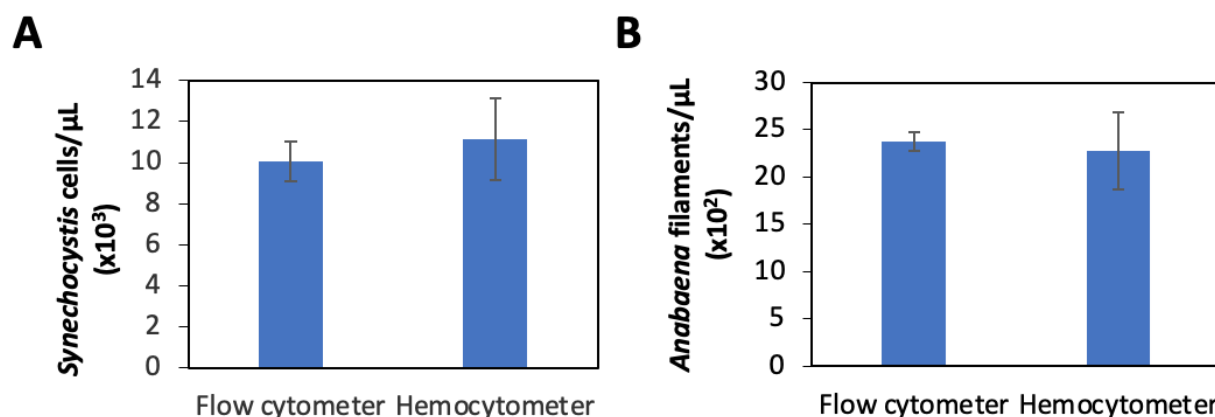


Figure 3-7. Flow cytometry is a reliable method to record the number of (A) *Synechocystis* single cells and (B) *Anabaena* filaments in a mixed population.

Aliquots of axenic cultures of *Synechocystis* WT and *Anabaena* WT (diluted to an OD₇₃₀ of 0.03) were separately analysed with both a flow cytometer (BD LSRFortessa II) or a Neubauer hemocytometer to compare total (A) *Synechocystis* single cells and (B) *Anabaena* filaments densities (respectively cells or filaments/μL). No statistically significant difference ($P > 0.05$) was found when performing a Student's t-

test (described in section 2.8.1). Error bars display standard deviation of the mean from (A) 3 and (B) 4 replicates.

3.3.2.2 Flow cytometry analysis of two partners of the co-culture, mixed at different ratios

All the analyses above were carried out with axenic cultures or in silico combination of axenic cultures. Next, it was important to next verify whether the flow cytometer enables monitoring of the two species that are grown together in a single culture. Known concentrations of *Synechocystis* and *Anabaena* cells were analysed by flow cytometry, either axenically or mixed together at different ratios (*Synechocystis*:*Anabaena* ratios of 25:75, 50:50, 75:25), all diluted to an OD₇₃₀ of 0.03 (as described in 2.7.8). The number of cells and filaments that were counted in both the axenic and mixed samples were found to correlate (Figure 3-8), suggesting that both filaments and individual cells from the two species can be reliably monitored by flow cytometry when they are mixed in a co-culture when diluted to an OD₇₃₀ of 0.03, thereby confirming hypothesis 2.

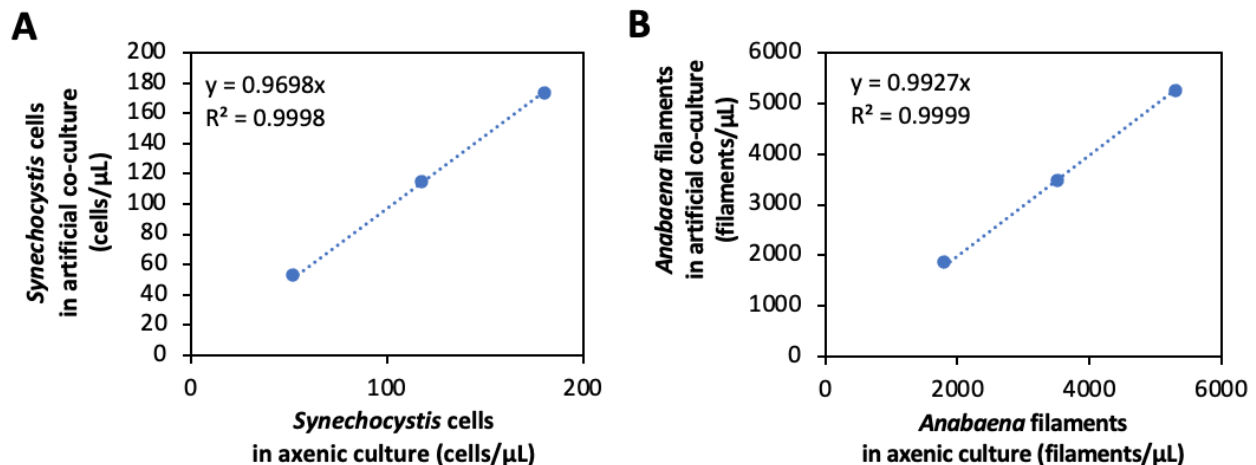


Figure 3-8. The number of cells and filaments in co-cultures of (A) *Synechocystis* and (B) *Anabaena* can be reliably monitored with flow cytometry.

Known concentrations of (A) *Synechocystis* and (B) *Anabaena* filaments were analysed by flow cytometry (BD LSRFortessa II), either axenically or mixed together at different ratios (*Synechocystis*:*Anabaena* ratios of 25:75, 50:50, 75:25), all diluted to an OD₇₃₀ of 0.03. The dotted lines represent the linear correlation cells or filaments when comparing numbers obtained with the co-culture and axenic cultures.

3.3.3 Hypothesis 3: Cyanobacteria can be studied by high-throughput flow cytometry

The Fortessa II flow cytometer can acquire samples from both single tubes and a microtiter plate, the latter allowing a fully automated sample acquisition of a large number of samples.

Yet, a switch to microtiter plates could potentially raise issues. The cells, as well as the counting beads (used for absolute counting of the cells) could settle to the bottom of the wells, resulting in a misleading count, especially for the last wells of the microtiter plate given the time required for the sampler to go through the plate in total. To avoid this, parameters such as mixing volume, speed and number of mixes by the sample probe in each individual well before sampling can be adjusted to obtain the most homogeneous sample for acquisition (described in section 2.7.8).

Given that the cyanobacteria used in this study tend to settle by gravity without sufficient mixing, the effect of the sample holder was tested. Aliquots of the same culture of *Synechocystis* was transferred to both tubes and 96-well plate, for comparison (as described in 2.7.8). No statistically significant difference ($P > 0.05$) in cell numbers was found between the two methods with the parameters tested (panel A, Figure 3-9), suggesting that microtiter plates can reliably be used for high throughput acquisition of *Synechocystis*. Importantly, there was also no carry over by the sampling probe between wells as cell counts were similar between wells for both species (panel B, Figure 3-9).

Additionally, a microplate with all 96 wells filled with the same culture was analysed to check whether the cell concentration in wells located at the end of the microtiter plate would suffer from a discrepancy compared to the first wells of the microtiter plate (for both *Synechocystis* and *Anabaena*, respectively panels C and D, Figure 3-9). No statistically significant difference ($P > 0.05$) was found between the first and last samples, allowing to conclude that hypothesis 3 is correct.

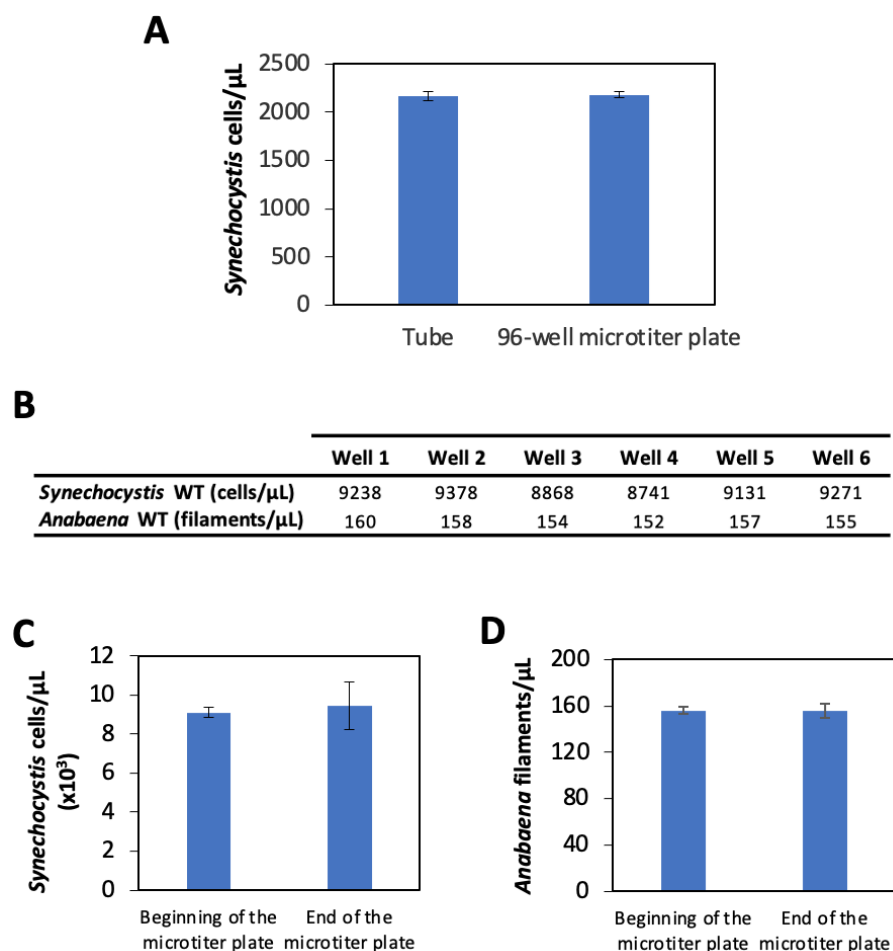


Figure 3-9. Microtiter plates can be reliably used for high-throughput absolute counting of unicellular (*Synechocystis* sp. PCC 6803 WT) and filamentous cyanobacteria such as *Anabaena* sp. PCC 7120 WT.

(A) Comparison of cell concentrations of the same *Synechocystis* WT culture acquired from both a tube (1 mL working volume) and a 96-microtiter plate (200 μL working volume). No statistically significant difference ($P > 0.05$) was found when performing a Student's t-test (described in section 2.8.1)

(B-D) In another experiment, axenic cultures of both *Synechocystis* WT and *Anabaena* WT were transferred into all wells of two 96-well microtiter plates. Data from the first and last 6 wells of the microtiter plate are shown. No statistically significant difference ($P > 0.05$) was found when performing a Student's t-test (described in section 2.8.1).

Error bars display standard deviation of the mean from (A) 4 and (C-D) 6 replicates.

3.4 Discussion

As far as I am aware, flow cytometry with both single cell and filamentous cyanobacteria has not been reported previously. Van Dijk et al. (2011) was the first to develop a method to analyse

filamentous cyanobacteria in flow cytometry and described the development of the method as challenging. Several other studies have followed including Azevodo et al. (2012) and Doppler et al. (2022), but their focus was solely on axenic cultures. High-throughput analysis of mixed cultures is of applied interest for several reasons. Firstly, environmental samples are likely to be mixed. Studies on mixed cultures containing both single-cell and filamentous species is relevant for the food industry. For example, *Spirulina* sp. is a popular food supplement but if contaminated with filamentous cyanobacteria, it could become a serious issue given that many filamentous cyanobacteria are capable of synthesising cyanotoxins (Christensen et al., 2020).

The ultimate objective with the establishment of the flow cytometry methodology in this study was to monitor the growth of the non-diazotroph *Synechocystis*, in order to understand if the diazotrophic *Anabaena* could support its growth. Guided by the previous work of Van Dijk et al. (2011) and advice from the Flow Cytometry Facility at Imperial College London, a method was established using axenic samples of each strain type. Analysis of the obtained data, including both cytograms and individual microscopic images, suggested that clear differentiation of clusters was possible, as also reported by Van Dijk (2011) and other flow cytometry studies using varying biological samples (Kraft et al., 2021). Follow-up experiments were carried out to ensure that the method was robust, for example, with respect to the placement of samples within a microtiter plate (Figure 3-9, panels C and D). Even though cyanobacteria tend to settle over time, this did not influence the flow cytometry analysis, most likely thanks to the mixing by the sample probe before sampling. The suitability of the general method was also validated by comparison with direct cell counting under a microscope using a hemocytometer (Fig. 3-7).

Finally, the key experiment was to analyse varying mixtures of the two cell types (Fig. 3-8), which confirmed for the first time, as far as I am aware, that flow cytometry is a suitable method also for the analysis of mixtures of filamentous and single-cell cyanobacteria. Due to the lack of previous publications on this topic, it is not possible to compare directly with other studies. However, Graham et al. (2018) analysed environmental samples containing both unicellular and filamentous *Anabaena* species, using both flow cytometry (FlowCam) and light microscopy. They concluded that there was a positive correlation in cell numbers between the two methods of analyses, supporting the conclusion.

For subsequent experiments in this thesis, the most important aspect was to be able to measure the number of *Synechocystis* cells in presence of a filamentous cyanobacteria (*Anabaena*), as the main hypothesis of this study was: a free-living nitrogen-fixing cyanobacteria (*Anabaena*) can support all the nitrogen needs of another non-diazotrophic organism (i.e. *Synechocystis*). Hence, the ability to also count *Anabaena* filaments was a bonus.

Interestingly, filaments reaching up to 140 μm in size were observed (Fig. 3-6, panel C) in this present study. In contrast, far longer filaments were measured previously as filament length in *PseudoAnabaena* strain CCY 9704 and *Planktothrix rubescens* exceeded 1,000 μm (Van Dijk et al., 2011), *Anabaena* species reached 1,000 μm (Komárek & Johansen, 2015) and 1,135 μm (Doppler et al., 2022). Could this have been due to filaments breaking up during flow cytometry? It is possible but unlikely given (1) that filaments are noted to be extremely difficult to break on purpose (for example, requiring sonication (Azevedo et al., 2012)), and (2) the comparison with

direct filament counting (Figure 3-9) showed no statistical difference in filaments counts between the two methods.

A more likely explanation is that *Anabaena* cultured in our laboratory had smaller filaments because it was cultured under nitrogen-repleted conditions whilst the abovementioned publications used environmental samples or defined nitrogen-depleted media (Doppler et al., 2022), but it remains to be tested in future studies by comparing the effect of media.

3.5 Chapter conclusion

This chapter aimed to answer the following question: can traditional flow cytometry be used to monitor a co-culture that includes a filamentous cyanobacterium (here *Anabaena*) in a robust and reliable fashion? The experiments that were carried out, framed around three hypotheses, confirmed that *Synechocystis* cells and *Anabaena* cells can be effectively (1) differentiated (Figure 3-3) and (2) reliably counted on flow cytometry (Figure 3-8), thereby answering this question positively. The developed methodology will for the first time allow filamentous species to be monitored in a mixed population (Fig. 3-8), whilst maintaining their integrity. Furthermore, the pre-requisite experiments have been carried out to improve the experimental workflow with higher throughput sampling (Figure 3-9). The method was therefore established and ready to be used to study the nitrogen-sharing co-cultures (Chapter 4).

The use of the imaging flow cytometry protocol may also be useful to study further the effect of the environment (e.g. nitrogen presence or absence in the media) on the length of filaments and

heterocyst numbers. Unfortunately, the difficult access to the imaging flow cytometer as well as the COVID-19 pandemic interfered, so these experiments could not be performed.

Note that the growth dynamics of the two cyanobacteria was studied in the next chapter.

4

Microbial fertilisation: developing a microbial synthetic consortium dependent on atmospheric nitrogen

4.1 Introduction

Nitrogen is a key nutrient that is shared amongst microbial communities in natural ecosystems. For example, nitrogen fixed via biological nitrogen fixation represents the principal external source of bioavailable nitrogen in the oceans: up to 50% of the nitrogen fixed by diazotrophs is

released to the extracellular environment and consumed by the surrounding planktonic community, in the form of either ammonium or dissolved organic nitrogen (Berthelot et al., 2016). For mass cultivation of photoautotrophic microorganisms, the supply of nitrogen in media represents a high cost hampering its overall sustainability (Grizeau et al., 2016). Large-scale cultures are also commonly contaminated by opportunistic microorganisms and limiting the availability of nutrients for contaminants is a key strategy to minimize their influence (Kazamia et al., 2012).

Hence, the development of artificial symbioses that can utilise nitrogen-fixing organisms to provide the necessary nitrogen to a photoautotrophic microorganism is of commercial interest. Recently, several artificial symbioses have been described in the literature, involving different diazotrophs including:

- *Azotobacter vinelandii* species (either wild-type or engineered) were able to support the growth of both cyanobacteria and eukaryotic algae such as *Chlamydomonas* sp. (Preininger et al., 2006), *Neochloris* sp. and *Scenedesmus* sp. (Villa et al., 2014), *Synechococcus* sp. (Smith & Francis, 2016), as well as *Chlorella* sp. and *Pseudokirchneriella* sp. (Ortiz-Marquez et al., 2012). Remarkably, two tri-partite symbiosis involving *Azotobacter vinelandii* have been demonstrated: one with the bacteria *Bacillus licheniformis* and *Paenibacillus curdlanolyticus* (Kim et al., 2008), and another long-living one with the green algae *Chlamydomonas* sp. and the fungus *Alternaria* in carbon- and nitrogen free-medium (Lorincz et al., 2010),
- *Bacillus* sp. supported the growth of *Chlorella* sp. (Hernandez et al., 2009),

- *Azospirillum brasilens* supported the growth of *Chlorella sorokiniana* (de-Bashan et al., 2016),
- *Mesorhizobium sangaii*, was shown to enhance both biomass and lipid production of *Chlorella vulgaris* (Wei et al., 2020).

Note that some of these artificial consortia also support the production of molecules of commercial interest such as lipids (Ortiz-Marquez et al., 2012; Wei et al., 2020), polyhydroxybutyrate and alginate (Smith & Francis, 2016). Notably, none of these previous studies employed a photosynthetic diazotroph as the nitrogen-fixing species.

One goal with this thesis was to develop a model multi-species nitrogen-exchange system using the photosynthetic and nitrogen-fixing model organism *Anabaena* as a donor of nitrogen to terrestrial plants, with the aim to eventually reduce the need for nitrogen fertiliser. Instead of terrestrial plants, a second cyanobacterium that is not capable of nitrogen-fixation was used in this chapter as the initial target recipient organism with the intention to establish a simpler first model system. A second aim, once such a two-species co-culture had been developed, was to test the hypothesis developed by our partners in the DEMA consortium, that the introduction of additional species into a fixed ecological space limits the ability of unwanted species to influence the primary target of that biotechnological process (Kazamia et al. (2018), see also Chapter 6). Although there is a substantial difference between cyanobacteria and terrestrial plants, it can be argued that a bacteria-only system is simpler and quicker to work with.

Hence, the practical aim of the present chapter was to build a fully photosynthetic consortium involving nitrogen fixation by a free-living diazotroph (*Anabaena* sp. PCC 7120), which would then provide fixed nitrogen to a photosynthetic cyanobacteria producing a compound of commercial interest (*Synechocystis* sp. PCC 6803). These two species were selected as (1) they are both well-established laboratory strains with well-defined metabolism (i.e. published metabolic models), (2) they can both be cultured with the same media (BG-11) and (3) *Synechocystis* was the target biocatalyst of the DEMA consortium.

Both *Synechocystis* and *Anabaena* are cyanobacteria. The total biomass of the culture can be monitored by measuring the Optical Density (OD) at 730 nm. As no cyanobacterial pigments absorb at this specific wavelength, measurements at this wavelength therefore only represent light scattering from the cells (Murton et al., 2017). However, to specifically monitor the growth of *Synechocystis*, an ethanol-producing strain of *Synechocystis* was used at first. The quantifiable excreted product (ethanol) thereby acts as an indicator of the metabolic state (or health) of the nitrogen-receiving host, and the success of the biotechnological process. The production of ethanol by cyanobacteria was the applied biotechnological target of the DEMA consortium, but here it was primarily considered an indicator for metabolic activity in the nitrogen-receiving strain.

In addition to ethanol quantification, direct cell counting with a hemocytometer was carried out for the development of the proof-of-concept (section 4.3.1). As manual cell counting is particularly challenging and not accurate for filamentous microorganisms, the flow cytometry method described in Chapter 3 was developed.

In summary, the two selected partners for the co-culture were:

- *Synechocystis* sp. PCC 6803, engineered to produce ethanol (strains ICLEE and UL030), a non-diazotrophic cyanobacterium (hereafter referred to as “*Synechocystis*”),
- *Anabaena* sp. PCC 7120, a filamentous and heterocyst-forming diazotroph (hereafter referred to as “*Anabaena*”).

Figure 4-1 describes the general model system, with *Anabaena* acting as a bio-nitrogen fertiliser for the biotechnologically relevant *Synechocystis* strain that is compromised by contamination by an unknown third partner.

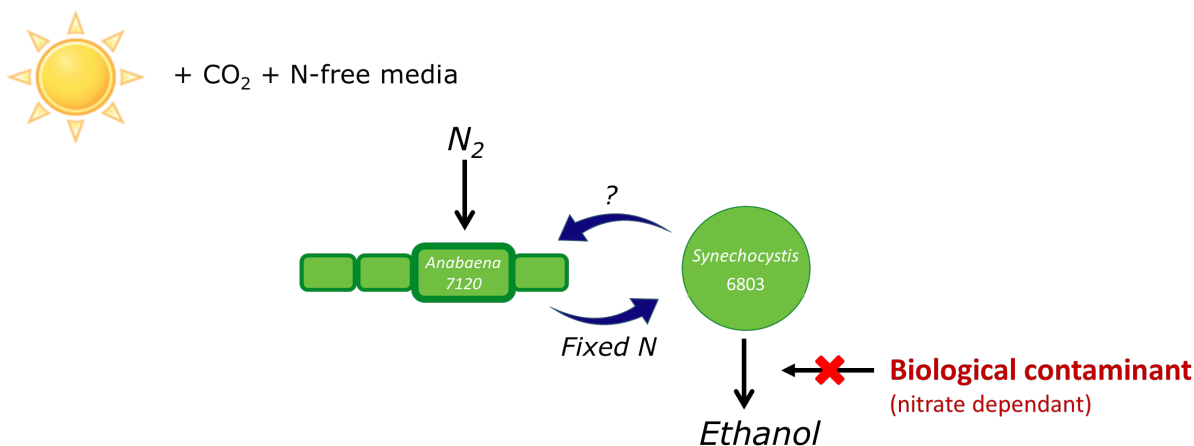


Figure 4-1. Model system including a diazotroph providing fixed nitrogen to the ethanol-producing bacteria *Synechocystis* in nitrogen-deprived media.

In this microbial photosynthetic system, a N-fixer organism, *Anabaena* 7120, supplies a non-diazotroph engineered to produce ethanol (*Synechocystis* 6803) with fixed N. Ethanol serves as a proxy for active metabolism. As the available ecological niche would be more occupied by having multiple species in the culture, we hypothesised that the space for contaminants would be minimised.

4.2 Chapter overview

The hypotheses that were aimed to be tested with the experimental system shown in Figure 4-1 were the following:

1. **Hypothesis 1:** The free-living nitrogen-fixing *Anabaena* 7120 supports the growth and ethanol production of the non-diazotrophic *Synechocystis* in N-depleted media. If, under N-depleted conditions, more *Synechocystis* cells were counted and more ethanol was measured in co-cultures compared to the axenic *Synechocystis* only control, the N fixed by the diazotrophic cyanobacteria could be assumed to provide N to the non-diazotroph to sustain its growth and metabolic activity,
2. **Hypothesis 2:** The increase in community complexity in the co-culture makes it more robust towards contamination. In order to test this hypothesis, the initial plan was to charge the axenic control cultures and 2-partner co-cultures with contaminants identified in Chapter 6 and then quantify the impact on growth of the target biocatalyst and ethanol accumulation. However, the COVID-19 pandemic interfered, so this hypothesis could not be tested.

A series of additional hypotheses were developed during this study to solve technical challenges that arose whilst establishing the experimental system aimed to test the above hypotheses:

- **Hypothesis 3:** The experimental system for sharing nitrogen between the diazotrophic and the non-diazotrophic cyanobacteria can be robust and reproducible,
- **Hypothesis 4:** Ethanol production (i.e. metabolic activity) is limited by the quantity of nitrogen provided by *Anabaena*,

- **Hypothesis 5:** Combinations of environmental conditions and/or strains exist that allow *Anabaena* to better support the growth of *Synechocystis* under nitrogen-depleted conditions,
- **Hypothesis 6:** Flow cytometry can be used to monitor the growth of two different cyanobacteria species in a co-culture'
- **Hypothesis 7:** Pre-culturing *Anabaena* in N-depleted media allows it to share nitrogen more quickly with *Synechocystis*.

The different experiments aiming to test these hypotheses are described in the following sections.

4.3 Results

4.3.1 Hypothesis 1: The free-living nitrogen-fixing *Anabaena* 7120 supports the growth and ethanol production of the non-diazotrophic *Synechocystis* in N-depleted media

4.3.1.1 Co-cultures with *Anabaena* and one ethanol-producing *Synechocystis* strain (ICLEE)

Initial experiments with the ethanol-producing *Synechocystis* ICLEE strain were performed to establish the experimental system. The ICLEE strain acquired from Paulina Bartasun (Bartasun et al., 2019) and the wild-type and Δamt *Anabaena* strains were all maintained in liquid BG-11 media (2.4.3) under standard growth conditions (2.5.1) with regular re-inoculation into fresh

media. Individual axenic control cultures or combinations of each of the two strains (depending on the chosen experimental treatment) were used to inoculate 30-mL main cultures of media to an equivalent starting OD₇₃₀ of 0.1 for *Synechocystis* ICLEE and 0.2 for *Anabaena* followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.5.1).

The *Anabaena* Δamt strain lacks the complete *amt* cluster which is believed to encode the *Amt* transporter responsible for the uptake of ammonium (NH₄⁺) and ammonia (NH₃) in the media (Perin et al., 2021). Intracellular NH₃ freely diffuses through the membrane and is lost unless it is recovered by active uptake transport catalysed by *Amt* (Boogerd et al., 2011). It was therefore assumed that cultures of the Δamt mutant would accumulate a higher concentration the NH₃/NH₄⁺ in the media and would support better the growth of *Synechocystis*.

Two treatments were tested, with all cultures containing ICLEE:

- Presence or absence of assimilable nitrogen: culture media were either BG-11 (N-repleted conditions (N+), i.e. media including nitrogen) or BG-11₀ (N-depleted conditions (N-), i.e. media without nitrogen),
- Presence (*Anabaena* 7120 WT) or absence (*Anabaena* 7120 Δamt) of the Δamt transporter.

As a control, the growth and ethanol production of axenic ICLEE was also included. OD measurements at 730 nm were taken at day 0, 7, 14 and 25 (as described in 2.7.1), the number of *Synechocystis* cells counted after 7 and 25 days of cultivation with an hemocytometer (as

described in 2.7.3), and the ethanol produced by ICLEE measured by HPLC after 7, 14 and 25 days of cultivation (as described in 2.7.4). The results obtained are shown in Figure 4-2.

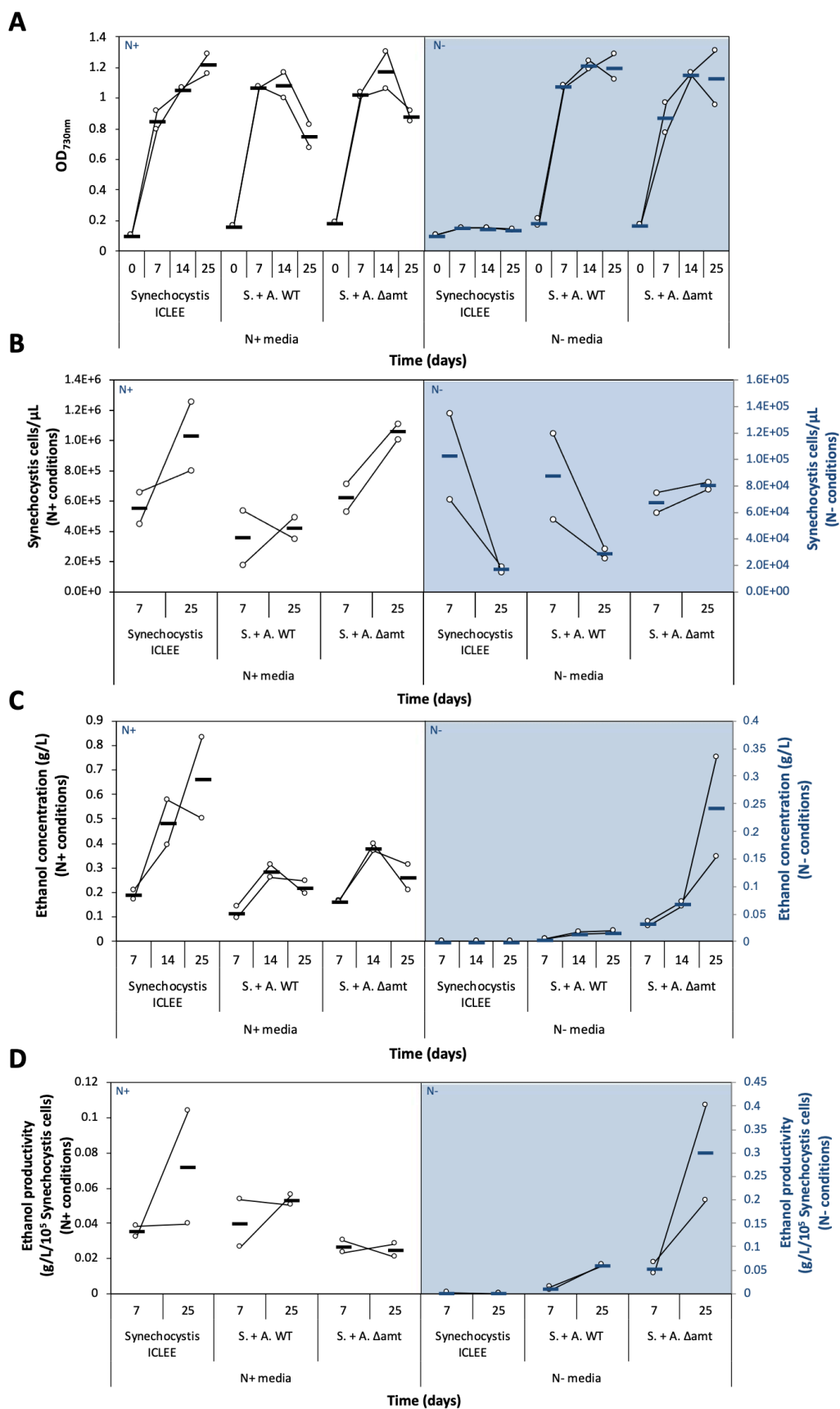


Figure 4-2. A synthetic N₂-dependent nitrate-free co-culture employing N₂-fixing 7120 and ethanol-producing *Synechocystis* ICLEE.

Individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT or Δamt) were used to inoculate 20-mL main cultures of media to an equivalent starting OD₇₃₀ of 0.1 for *Synechocystis* and 0.2 for *Anabaena*, followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.5.1).

(A) Total cyanobacterial biomass measured by OD at 730 nm, over time of the different cultures (axenic ICLEE; ICLEE in co-cultivation with *Anabaena* WT; and ICLEE in co-cultivation with *Anabaena* Δamt) in both N-repleted (N+) and N-depleted (N-) conditions.

(B) Variation over time of the number of *Synechocystis* cells counted with an haemocytometer under light microscopy in the different conditions.

(C) Ethanol productivity of the engineered *Synechocystis* ICLEE, in the different conditions, measured by HPLC at day 7, 14 and 25.

(D) Ethanol productivity relative to cell count at day 7 and 25, for 10⁵ *Synechocystis* ICLEE cells.

For (B-D), the scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

White and coloured backgrounds respectively represent media with (N+, i.e. BG-11) and without nitrogen (N-, i.e. BG-11₀). S.: *Synechocystis* ICLEE; A. WT: *Anabaena* WT; A. Δamt : *Anabaena* Δamt .

Circles and bars represent individual values and means, respectively (n=2).

As positive and negative controls, ICLEE was grown axenically in BG-11 (complete growth medium containing assimilable N) and BG-11₀ (identical to BG-11 except lacking assimilable N), respectively. As expected, ICLEE grew in the optimal conditions (i.e. in BG-11, N-repleted conditions) and no growth could be detected under N-depleted conditions (panel A, Figure 4-2). This was also supported by the cell count (panel B, Figure 4-2).

ICLEE was also co-cultivated in N-repleted conditions together with either *Anabaena* WT or Δamt mutant strains to verify if both cyanobacterial species could be grown together and one partner is not toxic for the other, as some cyanobacteria (such as *Microcystis aeruginosa*) are known to produce toxins in the presence of a competitor (Dunker et al., 2017). When co-cultivated with *Anabaena* (irrespective of the strain), ICLEE grew and produced ethanol in a similar range as when grown axenically after 7 days, but thereafter declined except for the growth of *Synechocystis* with the Δamt mutant (Fig. 4-2, panels B and C). Observations under the

microscope showed that ICLEE became dominant in both co-cultures in N-repleted media, as few *Anabaena* filaments were noticed, suggesting that ICLEE grows faster than *Anabaena* under these conditions.

In nitrogen-deprived media (diazotrophic conditions), the total cyanobacterial biomass in cultures of ICLEE co-cultivated with either *Anabaena* WT or the Δamt mutant, followed a typical microbial growth curve (panel A, Fig. 4-2). However, the number of *Synechocystis* cells in both co-cultures were much lower in N-depleted conditions than in N-repleted media on day 7 (panel B, Fig. 4-2), meaning that the *Anabaena* filaments were becoming dominant in the diazotrophic co-cultures, which is in line with the microscopic observations.

Additionally, when co-cultivated diazotrophically with *Anabaena* WT, the number of *Synechocystis* cells decreased from day 7 to day 25. It remained similar in the co-culture with the *Anabaena* Δamt mutant: at day 25, almost 3 times more *Synechocystis* cells were counted when co-cultivated with the *Anabaena* Δamt mutant than when co-cultivated with *Anabaena* WT.

As cultures with ICLEE accumulated more ethanol when ICLEE was co-cultured with *Anabaena* Δamt (0.24 g/L), than when cultured axenically in N-depleted media (0 g/L, negative control) after 25 days of cultivation (Fig. 4-2, panel C), these results tentatively support the hypothesis that the free-living nitrogen-fixing *Anabaena* 7120 can support the nitrogen needs of *Synechocystis*. Unfortunately, a statistical analysis was not possible given this experiment was only performed with duplicates, hence all the results shown in Fig. 4-2 are only indicative.

It is interesting to note that both growth (panel B, Fig. 4-2) and ethanol production (panel C, Fig. 4-2) in co-cultures under diazotrophic conditions were greater with *Anabaena* Δamt compared to *Anabaena* WT. On day 25, the accumulation of ethanol was 14 times greater with the Δamt mutant than with the WT. Hence, while the Δamt mutant was able to stimulate a 3-fold increase in cellular growth, the impact on ethanol was much greater. This supported the decision to also monitor metabolic activity, not just growth. Although, co-cultures of ICLEE and the *Anabaena* Δamt mutant only accumulated a third of the ethanol compared to the axenic ICLEE culture in nitrogen-repleted media suggesting the availability of nitrogen was still limiting ICLEE. Interestingly, the results suggest that ICLEE produced more than 4 times more ethanol per cell when co-cultivated diazotrophically with *Anabaena* Δamt rather than in any N-repleted cultures (panel D, Fig. 4-2).

4.3.1.2 Replacement of the ethanol-producing strain ICLEE with UL030

Partners in the DEMA consortium reported far higher ethanol titres with another engineered *Synechocystis* strain called UL030. Preliminary tests in our laboratory confirmed that cultures of UL030 accumulated up to seven times higher ethanol concentration than ICLEE under optimal conditions, i.e. N-repleted media (Figure 4-3) (Lopes da Silva et al., 2018).

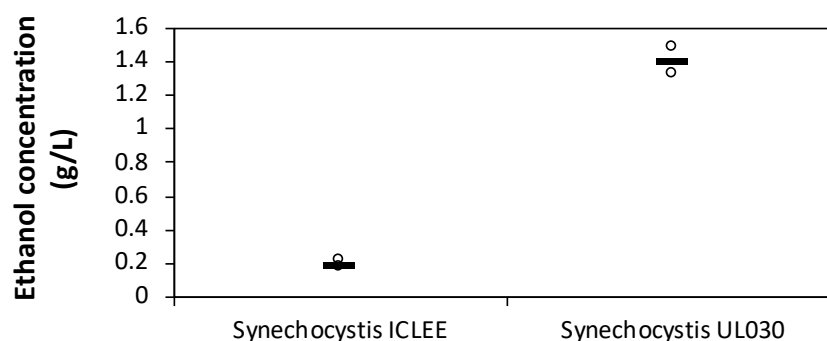


Figure 4-3. Comparison of the ethanol produced by two mutants of *Synechocystis*.

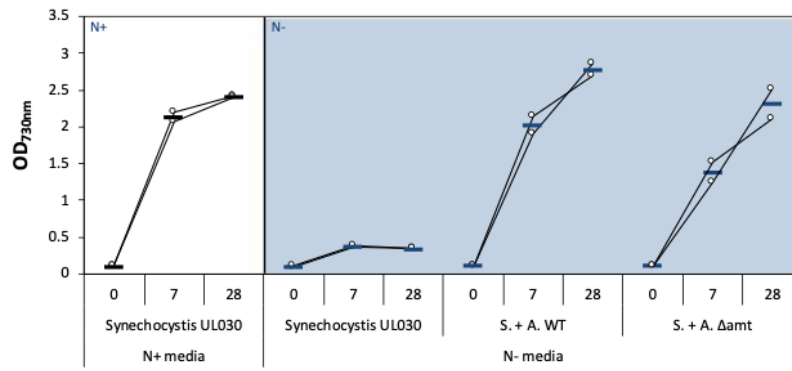
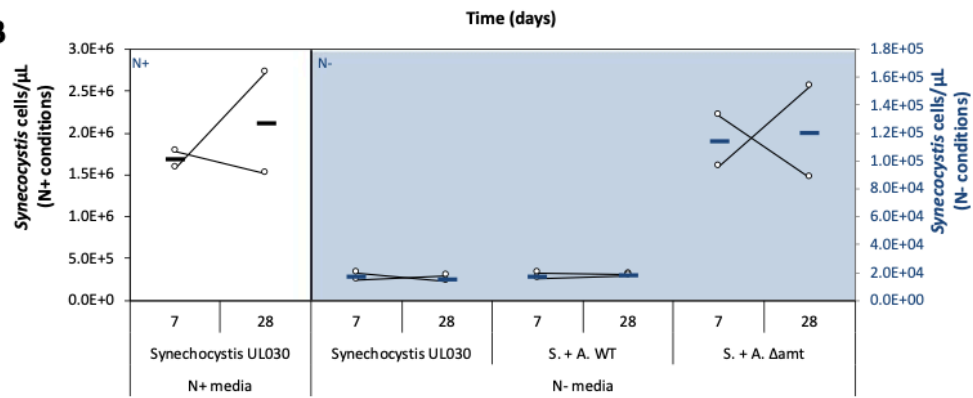
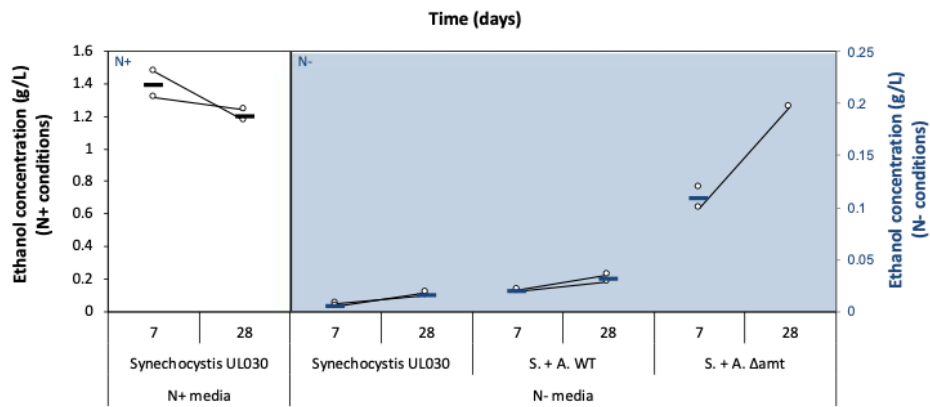
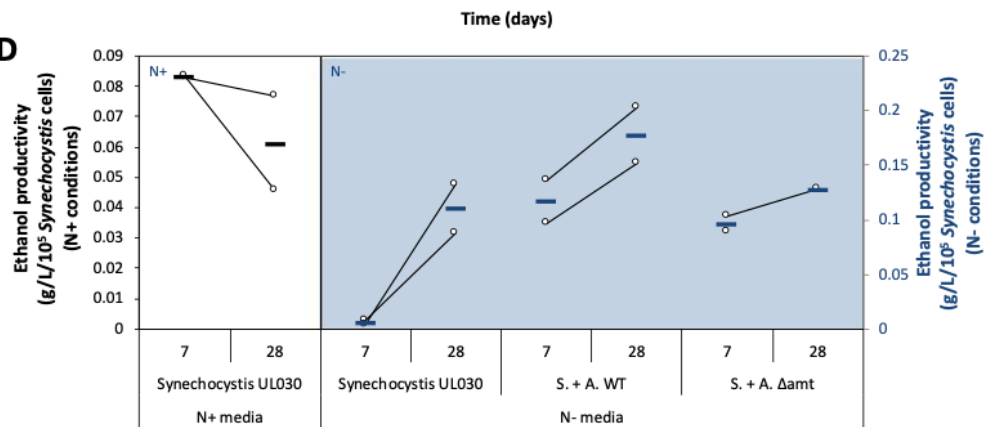
Synechocystis ICLEE and UL030 were cultivated in BG-11 (N-repleted media) and the ethanol production was quantified by HPCL after 7 days of cultivation.

Circles and bars represent individual values and means, respectively (n=2).

With the hope that changing to UL030 may reduce the number of cultures in which the accumulated ethanol was below the limit of detection, the co-cultivation experiment shown in Fig. 4-2 was repeated using UL030 instead of ICLEE, with the same experimental design. Once again, axenic UL030 was used as a control for growth and ethanol production in both BG-11 (N-repleted media (N+), positive control) and BG-11₀ (N-depleted media (N-)).

Individual axenic control cultures or combinations of *Synechocystis* UL030 and *Anabaena* (either WT or Δamt) were used to inoculate 20-mL main cultures of media to an equivalent starting OD₇₃₀ of 0.1 for ICLEE and 0.2 for *Anabaena* strains. Cultures were incubated under standard growth conditions (as described in 2.5.1). OD measurements at 730 nm were taken at day 0, 7 and 28 (as described in 2.7.1), the number of *Synechocystis* cells counted after 7 and 28 days of cultivation with an hemocytometer (as described in 2.7.3), and the ethanol produced by ICLEE measured by HPLC after 7 and 28 days of cultivation (as described in 2.7.4).

The results are summarised in Fig. 4-4.

A**B****C****D**

Time (days)

Figure 4-4. A synthetic N₂-dependent nitrate-free co-culture employing N₂-fixing *Anabaena* and another ethanol-producing *Synechocystis* UL030.

Individual axenic control cultures or combinations of *Synechocystis* UL030 and *Anabaena* (either WT or Δamt) were used to inoculate 20-mL main cultures of media to an equivalent starting OD₇₃₀ of 0.1 for *Synechocystis* and 0.2 for *Anabaena*. Cultures were incubated under standard growth conditions (as described in 2.6.1).

(A) Total cyanobacterial biomass measured by OD at 730 nm, over time, of the different cultures (axenic UL030; UL030 in co-cultivation with *Anabaena* 7120 WT; and UL030 in co-cultivation with *Anabaena* Δamt) in both N-repleted (N+; white background) and N-depleted conditions (N-; coloured background).

(B) Variation over time of the number of *Synechocystis* cells counted with a haemocytometer under light microscopy in the different conditions.

(C) Ethanol productivity of the engineered *Synechocystis* UL030, in the different conditions, measured by HPLC at day 7 and 28.

(D) Ethanol productivity relative to cell count at day 7 and 28, for 10⁵ *Synechocystis* UL030 cells.

For (B-D), the scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

White and coloured backgrounds respectively represent N-repleted conditions (N+, i.e. BG-11) and N-depleted (N-, i.e. BG-11₀). S.: *Synechocystis* UL030; A. WT: *Anabaena* WT; A. Δamt : *Anabaena* Δamt . Circles and bars represent individual values and means, respectively (n=2), except for the ethanol production from the co-culture UL030 + 7120 Δamt , at day 28 when a problem with the HPLC was encountered.

As expected, in N-repleted media, UL030 grew and produced ethanol in nitrogen-repleted media (positive control), while no growth and ethanol could be detected in N-depleted conditions (negative control). This also correlated with the cell count depicted in panel B, Fig. 4-4.

Importantly, in support of the hypothesis that the free-living nitrogen-fixing *Anabaena* 7120 can support the nitrogen needs of another non-diazotrophic organism, cultures with ICLEE accumulated more ethanol when co-cultured with *Anabaena* Δamt (0.2 g/L) than when cultured axenically in N-depleted media (0.02 g/L, negative control) after 28 days of cultivation (panel C, Fig. 4-4). Note that the 0.2 g/L value (co-culture ICLEE and *Anabaena* Δamt) is only from one replicate as the HPLC stopped working whilst measuring the duplicate. Unfortunately, a statistical analysis was not possible given this experiment was only performed with duplicates.

Additionally, in support of the hypothesis that *Anabaena* Δamt supports better the growth of *Synechocystis* than *Anabaena* WT in diazotrophic conditions, cultures with ICLEE accumulated more ethanol when ICLEE was co-cultured with *Anabaena* Δamt , than when co-cultured with *Anabaena* WT (panel C, Fig. 4-4). On day 28, the accumulation of ethanol was 6 times greater with the Δamt mutant than with the WT.

In general, although statistical analysis was not possible given the experiment was only performed with duplicates, the results were similar to those obtained in the first experiment with ICLEE. In media devoid of added nitrogen, the *Anabaena* Δamt mutant, supported the growth and ethanol production of both the engineered *Synechocystis* strains.

Surprisingly, co-cultures with the *Anabaena* Δamt mutant and UL030 only produced around 0.2 g/L of ethanol, i.e. 16% of what it produced axenically in nitrogen-repleted media, even though more cells were observed (approximately 50% more comparing panels B in Fig. 4-2 and Fig. 4-4). In contrast, even though axenic ICLEE accumulated seven times less ethanol under N-repleted conditions (Fig. 4-3), it accumulated a similar amount of ethanol in diazotrophic co-cultures (0.24 g/L, panel C, Fig. 4-2) as UL030 in the same conditions (0.2 g/L, panel C, Fig. 4-4). Although these results were only preliminary given the shortage of replicates, they suggested that something else limited ethanol accumulation. The UL030 strain was prepared in a different wild-type background than ICLEE, and comparisons between such "sub-strain" variants have shown differences at the genome sequence level (Trautmann et al., 2012). Unfortunately, while the experiment shown in Fig. 4-3 with UL030 was only performed in duplicates, the HPLC stopped

working when processing the last sample of the run, i.e. data from the second replicate of the co-culture UL030 with 7120 Δamt was missing.

The aim of the next experiments was therefore to repeat this experiment and find an alternative method to ethanol quantification given that the HPLC was repeatedly stopping in the middle of sequence runs. It is possible, but not verified, that the cultures accumulated an unknown molecular species that blocked the HPLC used for ethanol quantification. They were thought to be constituents of the cyanobacterial slime coat. This was solved by switching to an enzymatic assay for the following experiments (Ethanol kit (R-Biopharm AG, Darmstadt, Germany)).

Additionally, after repeated attempts to verify the ethanol productivity of axenic cultures of UL030 in N-repleted media, we realised that the strain was genetically unstable (i.e. produced no measurable ethanol). Similar observations were also made by the DEMA collaborator who provided the strain. Subsequent experiments were therefore carried out using ICLEE as a partner of the synthetic consortium.

4.3.2 Hypothesis 3: The experimental system for sharing nitrogen between the diazotrophic and the non-diazotrophic cyanobacteria can be robust and reproducible

To optimise the experimental co-culture system with the aim to obtain more robust and reproducible results, two sub-hypotheses, described below, were formulated and subsequently tested:

- Hypothesis 4: Ethanol production (i.e. metabolic activity) is limited by the quantity of nitrogen provided by *Anabaena*. The experiments so far were performed with an optical density ratio of one *Synechocystis* to two *Anabaena* (i.e. a ratio of ICLEE:7120 = 1:2). Increasing the initial concentration of *Anabaena* in the co-culture was assumed to increase the amount of nitrogen available to ICLEE, and therefore increase the ethanol concentration in the media.
- Hypothesis 5: Combinations of environmental conditions and/or strains exist that allow *Anabaena* to better support the growth of *Synechocystis* under nitrogen-depleted conditions.

4.3.2.1 Hypothesis 4: Ethanol production (i.e. the metabolic activity of *Synechocystis*) is limited by the quantity of nitrogen provided by *Anabaena*

Varying the inoculum of the two different strains was assumed to affect the quantity of *Anabaena* relative to *Synechocystis*. If true, this should increase the amount of nitrogen available per *Synechocystis* cell and thereby allow testing the hypothesis that ethanol production is limited by the quantity of nitrogen provided by *Anabaena*.

So far, in previous above experiments, co-cultures were inoculated with a ratio of *Synechocystis* cells to *Anabaena* cells of 1:2 (i.e. the ratio of ICLEE:7120 = 1:2), so this ratio was kept as a control for the experiment described here. Controls were therefore as follows:

- Axenic ICLEE in N-repleted conditions (BG-11 (N+), positive control for growth and ethanol production),

- ICLEE in co-cultivation with either *Anabaena* 7120 WT or Δamt , in BG-11₀ (N-depleted media, N-), inoculated to a ratio 1:2 (control with regards to the first experiment (described in section 4.3.1.1)).

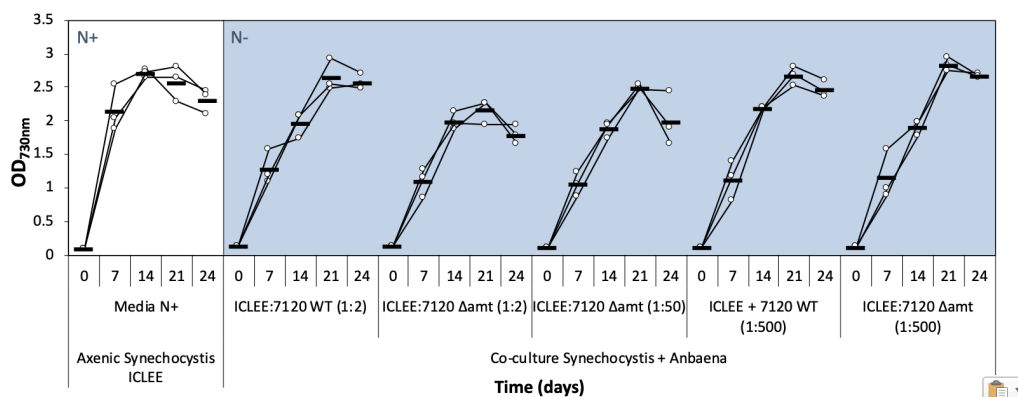
The treatments tested the initial ratio (ICLEE:7120) between the two partners of the consortium cultured in BG-11₀ (N-depleted media, N-):

- 1:500, with both 7120 WT and 7120 Δamt mutant,
- 1:50, with 7120 Δamt mutant.

The experiment was started by inoculating 20 mL of media (either BG-11 or BG-11₀) with individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT or Δamt) to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* (and OD₇₃₀ for *Anabaena* corresponding to the following ICLEE:7120 ratios: 1:2, 1:50 and 1:500), followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.5.1). OD measurements at 730 nm were taken at day 0, 7, 14, 21 and 24 (as described in 2.7.1), and the ethanol produced by ICLEE measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany) after 7, 14, 21 and 24 days of cultivation (as described in 2.7.4).

The results of this experiment are shown in Fig. 4-5.

A



B

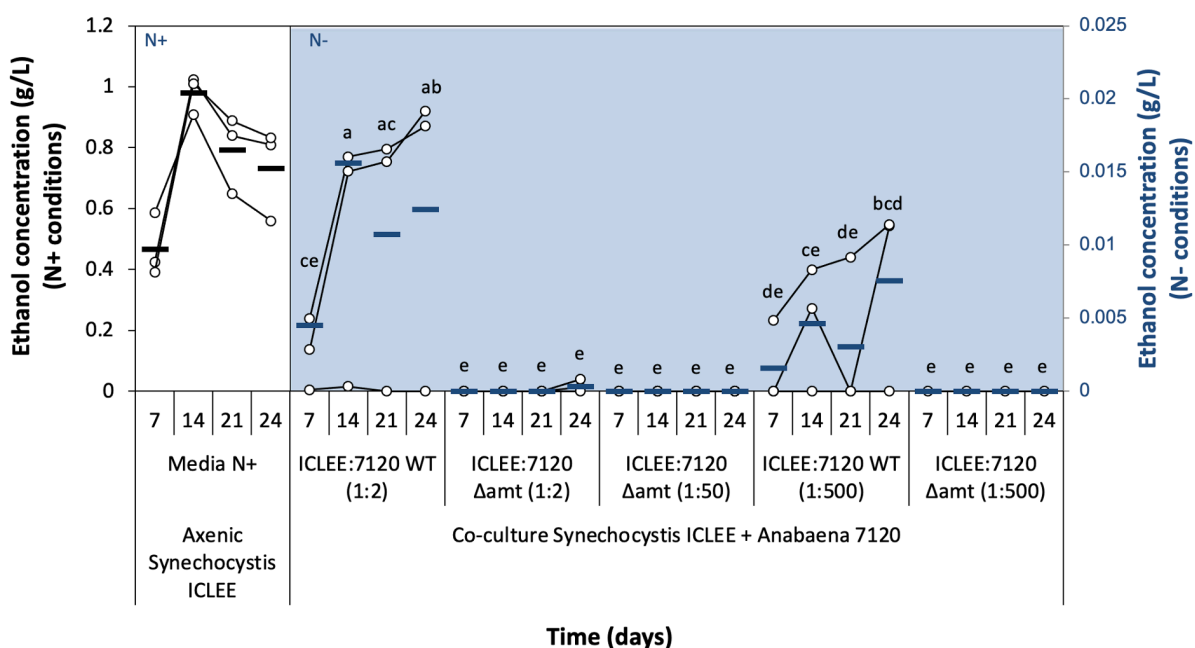


Figure 4-5. Testing whether varying initial ratios between the two consortium partners could lead to higher ethanol production from *Synechocystis* ICLEE.

Individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT or Δamt) were used to inoculate 20-mL main cultures of media to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* (and OD₇₃₀ for *Anabaena* corresponding to the following ICLEE:7120 ratios: 1:2, 1:50 and 1:500) followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.5.7).

(A) Total cyanobacterial biomass measured by OD at 730 nm, over time, of the different cultures (axenic ICLEE in N-repleted-conditions (N+); ICLEE in co-cultivation with *Anabaena* 7120 WT or Δamt , with varying ratios (1:2; 1:50 and 1:500) in N-depleted conditions (N-)).

(B) Ethanol productivity of the engineered *Synechocystis* ICLEE, in the different conditions, measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany)) at day 7, 14, 21 and 24. The scale for co-cultures (under diazotrophic conditions) is shown on the right, whilst the scale for axenic culture (N-repleted) is shown on the left. Means which do not share the same letter e.g. a, b, etc., are significantly different (P < 0.05, two-way ANOVA).

White and coloured backgrounds respectively represent N-repleted (N+, i.e. BG-11) and N-depleted conditions (N-, i.e. BG-11₀). ICLEE: *Synechocystis* ICLEE; 7120 WT: *Anabaena* WT; 7120 Δamt : *Anabaena* Δamt .

Circles and bars represent individual values and means, respectively (n=3).

As expected, in the optimal conditions (i.e. in N-repleted media), ICLEE grew and produced ethanol, and all the cultures grew well (panel A, Fig. 4-5).

However, under diazotrophic conditions, only three out of 15 cultures accumulated any ethanol even though all showed photosynthetic growth. Less than 0.02 g/L accumulated when ICLEE was co-cultivated with either the *Anabaena* WT and Δamt mutant (panel B, Fig. 4-5), whereas in the first experiment (described in section 4.3.1.1, panel C, Figure 4-2), this value was 0.02 g/L and 0.24 g/L (when co-cultivated with *Anabaena* WT and Δamt mutant respectively) after 25 days of cultivation. This complicated further analysis with regards to the possible impact of initial ratios of strains on the ethanol production. Contaminating species were found (as per the protocol described in 2.5.3) in the flasks containing the consortium ICLEE/7120 Δamt after 21 days of cultivation, whereas the axenic ICLEE in N-repleted conditions was not contaminated.

*4.3.2.2 Hypothesis 5: Combinations of environmental conditions and/or strains exist that allow *Anabaena* to better support the growth of *Synechocystis* under nitrogen-depleted conditions*

With the continued aim to establish a robust nitrogen-sharing co-culture system, and to replicate results obtained in earlier experiments (e.g. Fig. 4-2), two new treatments were introduced into

previous experimental designs: (1) alternative modifications to nitrogen-metabolism, and (2) different initial culture pH.

Two new strains (constructed by a previous PhD-student, David Malatinszky (2017)) that over-expressed *gifA* in the *Anabaena* WT (A. WT *gifA*-2) and the Δamt mutant (A. Δamt *gifA*-2) background (described in 2.3) were used in this study. GifA is a regulatory protein (inactivation factor) that inhibits the activity of the glutamine synthetase (hereafter GS) by direct protein-protein interaction (Galmozzi et al., 2010). As the glutamine synthetase is the primary assimilator of ammonia in cyanobacteria (Luque & Forchhammer, 2008), it was hypothesised that strains overexpressing *gifA* should allow a build-up of fixed N in the extracellular environment.

All experiments described until now were carried out in media buffered at pH 8. Since the media pH will influence the equilibrium between ammonia and ammonium (Boogerd et al., 2011), and therefore consequently also the loss of N by *Anabaena*, it was also decided to evaluate a higher initial pH (pH 10). Both cyanobacteria species are known to grow at such a high pH.

Combining treatments tested previously with these two new treatments (*gifA*/pH), one large experiment was designed incorporating the following treatments in co-cultures with ICLEE:

- Initial culture media pH of 8 vs. 10,
- Nitrogen-repleted (N+) vs. -depleted (N-) media,
- *Anabaena* 7120 WT vs. Δamt ,
- Over-expression of *gifA* vs. no over-expression.

The following controls were also included:

- Axenic ICLEE in nitrogen-repleted media (positive control, should synthesise ethanol),
- Axenic *Anabaena* 7120 wild-type over-expressing *gifA* (A. WT *gifA*-2; to ensure that it grows).

All co-cultures were inoculated with ICLEE and one of the different *Anabaena* strains listed above, at a 1:2 ratio of ICLEE:7120 (described in 4.3.1.1), in 20 mL media (either BG-11/BG-11₀ adjusted to pH 8, as described in 2.4.3) or BG-11/BG-11₀ at pH 10 (using 3-(cyclohexylamino)-1-propanesulfonic acid, thereafter CAPS) adjusted to pH 10 with 10 N NaOH) with individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT, Δamt , WT *gifA*-2 or Δamt *gifA*-2) to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* and 0.02 for *Anabaena*, followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.6.1). OD measurements at 730 nm were taken at day 0, 4, 14 and 25 (as described in 2.7.1), the ethanol produced by ICLEE was measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany) after 4, 14 and 25 days of cultivation (as described in 2.7.4), and the ammonium concentration in the supernatant measured with the modified, micro-scale Willis method (Willis et al. (1996), described in 2.7.5) after 4 and 25 days of cultivation. The results of this experiment are shown in Fig. 4-6.

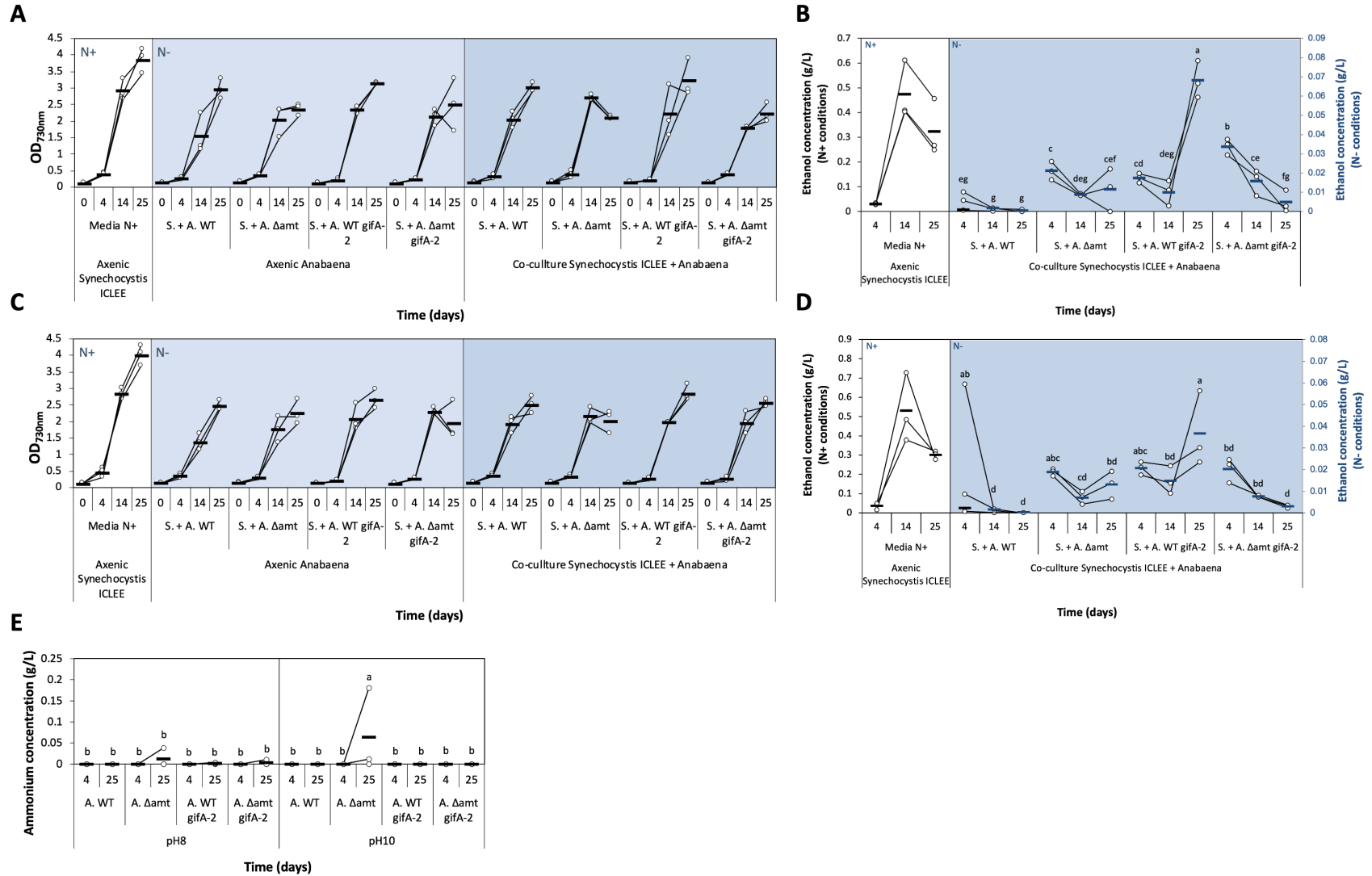


Figure 4-6. Testing whether different *Anabaena* mutants can sustain the ethanol production of a non-diazotroph producing ethanol, ICLEE, at pH 8 (A-B) and 10 (C-D).

Individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT, Δamt , WT *gifA*-2 or Δamt *gifA*-2) were used to inoculate 20-mL main cultures of media (either BG-11/BG-11₀ adjusted to pH 8) or BG-11/BG-11₀ at pH 10 (using CAPS) adjusted to pH 10 with 10 N NaOH) to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* and 0.02 for *Anabaena*, followed by the addition of 1 mM IPTG. Cultures were incubated under standard growth conditions (as described in 2.5.1).

(A, C) Total cyanobacterial biomass measured by OD at 730 nm, over time, of the different cultures (axenic ICLEE in N repleted-conditions (N+); ICLEE in co-cultivation with *Anabaena* either WT or Δamt , overexpressing *gifA* or not, in diazotrophic conditions (N-)).

(B, D) Ethanol productivity of the engineered *Synechocystis* ICLEE, in the different conditions, measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt)) at day 4, 14 and 25. The scale for co-cultures (under diazotrophic conditions) is shown on the right, whilst the scale for axenic culture (N-repleted) is shown on the left.

(E) Ammonia accumulation in axenic cultures of different *Anabaena* strains under diazotrophic conditions (N-) at day 4 and 25, at both pH8 and 10. Ammonium was measured colorimetrically by the Willis micro-scale assay (described in 2.7.5) from cell-free supernatants.

White and coloured backgrounds respectively represent media with (N+ (BG-11)) and without nitrogen (N- (BG-11₀)). S.: *Synechocystis* ICLEE; A. WT: *Anabaena* WT; A. *amt*: *Anabaena* Δamt .

Circles and bars represent individual values and means, respectively (n=3). For (B), (D) and (E), means which do not share the same letter e.g. a, b, etc., are significantly different ($P < 0.05$, two-way ANOVA).

As expected, in the optimal conditions (i.e. in N-repleted media), ICLEE grew and produced ethanol, and all the cultures grew well (panel A, Fig. 4-6). Negative controls were not included since earlier experiments showed clearly that there was negligible growth of ICLEE and metabolism (0.0004 and 0.0021 g/L ethanol after 7 days, and 0 g/L after 25 days, as shown in 4.3.1.1) under nitrogen-depleted conditions.

Although more ethanol was observed in these co-cultures (Fig. 4-6) than in the previous experiment (Fig. 4-5), there was still little ethanol (up to 0.08 g/L) produced by ICLEE when co-cultivated in diazotrophic conditions at either pH, irrespective of the time of sampling (panel B, Fig. 4-6). Unfortunately, contaminating species were once again found (as per the protocol described in section 2.5.3) in all flasks of this experiment.

At pH 8 (after 25 days), ICLEE co-cultured with *Anabaena* WT strains overexpressing *gifA-2* produced more ethanol (0.07 g/L, Fig. 4-6, panel B) than any of the other *Anabaena* strains (statistically significant difference on day 25, $P < 0.05$, two-way ANOVA), reaching 21% of what it would produce axenically in optimal conditions (i.e. in nitrogen-repleted media).

At pH 10 (after 25 days), ICLEE co-cultured with *Anabaena* WT strains overexpressing *gifA-2* also produced more ethanol (0.04 g/L, Fig. 4-6, panel D) than any of the other *Anabaena* strains (statistically significant difference on day 25, $P < 0.05$, two-way ANOVA).

Surprisingly, no ammonium was detected in any of the axenic *Anabaena* supernatants except for the *Anabaena* Δamt mutant at pH 10 after 25 days, statistically higher than any other *Anabaena* strains ($P < 0.05$, two-way ANOVA, Fig. 4-6, panel E).

4.3.3 Hypothesis 6: Flow cytometry can be used to monitor the growth of two different cyanobacteria species in a co-culture

Following the challenges with ethanol quantification and the lack of easy monitoring of *Synechocystis*, we decided to test the hypothesis that the flow-cytometry based method developed in Chapter 3 could be used to monitor the microbial co-culture systems. Flow cytometry can be used to monitor the growth of two different cyanobacteria species in a co-culture.

The hypothesis tested in 4.3.2.2 ("Combinations of environmental conditions and/or strains exist that allow *Anabaena* to better support the growth of *Synechocystis* under nitrogen-depleted

conditions") was also still relevant. Hence, we decided to test more *Anabaena* mutants, and a new sub-hypothesis was added to test the possibility that slow heterocyst-formation prevents *Anabaena* from supporting *Synechocystis* well. The testable hypothesis (7, below) was based around the idea that *Anabaena* pre-cultured in nitrogen-depleted media would be able to fix N₂ more quickly after it was added to (i.e. used to inoculate) the co-cultures. In other words, if the vegetative cells had already differentiated into heterocysts during the pre-culturing period, there would be no need for delay due to heterocyst differentiation, thereby increasing chances that the growth of *Synechocystis* could be supported more quickly due to the absence of a lag period (before the N-fixation in the co-culture starts).

Hypothesis 7: *Pre-culturing in N-depleted media allows Anabaena to more quickly share nitrogen with Synechocystis.*

The combined experiment used the following *Anabaena* strains: WT, Δamt , *gifA*-1 (P_{petE} -*gifA*- T_{gifA} copper inducible, thereafter A. WT *gifA*-1) and A. WT *gifA*-2 (P_{nifHDK} -*gifA*- T_{gifA}) in a WT background (described in Table 2-1).

Prior to inoculating the main co-cultures, the *Anabaena* strains were grown either in BG-11 or BG-11₀. The results were divided between *Anabaena* pre-grown under N-repleted (N+) or N-depleted (N-) media (Figures 4-7 and 4-8, respectively).

In summary, the following treatments were included in the combined experiment, some aimed at testing the two hypotheses whilst others with the intention to replicate previous results:

- *Anabaena* pre-cultured in N-repleted (N+) or N-depleted (N-) media,

- *Anabaena* strains with varying modifications to nitrogen-metabolism (WT, Δamt , A. WT *gifA*-1, A. WT *gifA*-2).

These controls were also included:

- Axenic ICLEE in both N-repleted (positive control for growth and ethanol production) and N-depleted (negative control for growth and ethanol production) media,
- ICLEE in co-cultivation with *Anabaena* 7120 WT, in N-depleted media (ratio ICLEE:7120 being 1:2, control with regards to the experiment described in 4.3.1.1),
- ICLEE in co-cultivation with *Anabaena* 7120 Δamt , in N-depleted media (ratio ICLEE:7120 being 1:2, control with regards to the experiment described in 4.3.1.1),
- ICLEE in co-cultivation with *Anabaena* WT overexpressing *gifA* (A. WT *gifA*-1), in N-depleted media (ratio ICLEE:7120 being 1:2),
- ICLEE in co-cultivation with *Anabaena* Δamt overexpressing *gifA* (A. WT *gifA*-2), in N-depleted media (ratio ICLEE:7120 being 1:2).

The experiment was started by inoculating 20 mL media (either BG-11 or BG-11₀) with individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT, Δamt , WT *gifA*-2 or Δamt *gifA*-2) to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* and 0.02 for *Anabaena*, followed by the addition of 1 mM IPTG (for all flasks) and 3 μ M copper (Cu²⁺) (for the flasks including *Anabaena* WT *gifA*-1). Cultures were incubated under standard growth conditions (as described in 2.5.1). OD measurements at 730 nm were taken at day 0, 4, 14 and 25 (2.7.1), the number of *Synechocystis* cells and *Anabaena* filaments counted at day 0, 7, 14 and 21 days of cultivation by flow cytometry (as described in 2.7.8) and the ethanol produced by ICLEE

measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany) after 4, 14 and 25 days of cultivation (as described in 2.7.4).

The results are summarized in Figure 4-7 and Figure 4-8 (*Anabaena* pre-cultured in N-repleted media (N+) and N-depleted (N-) media, respectively).

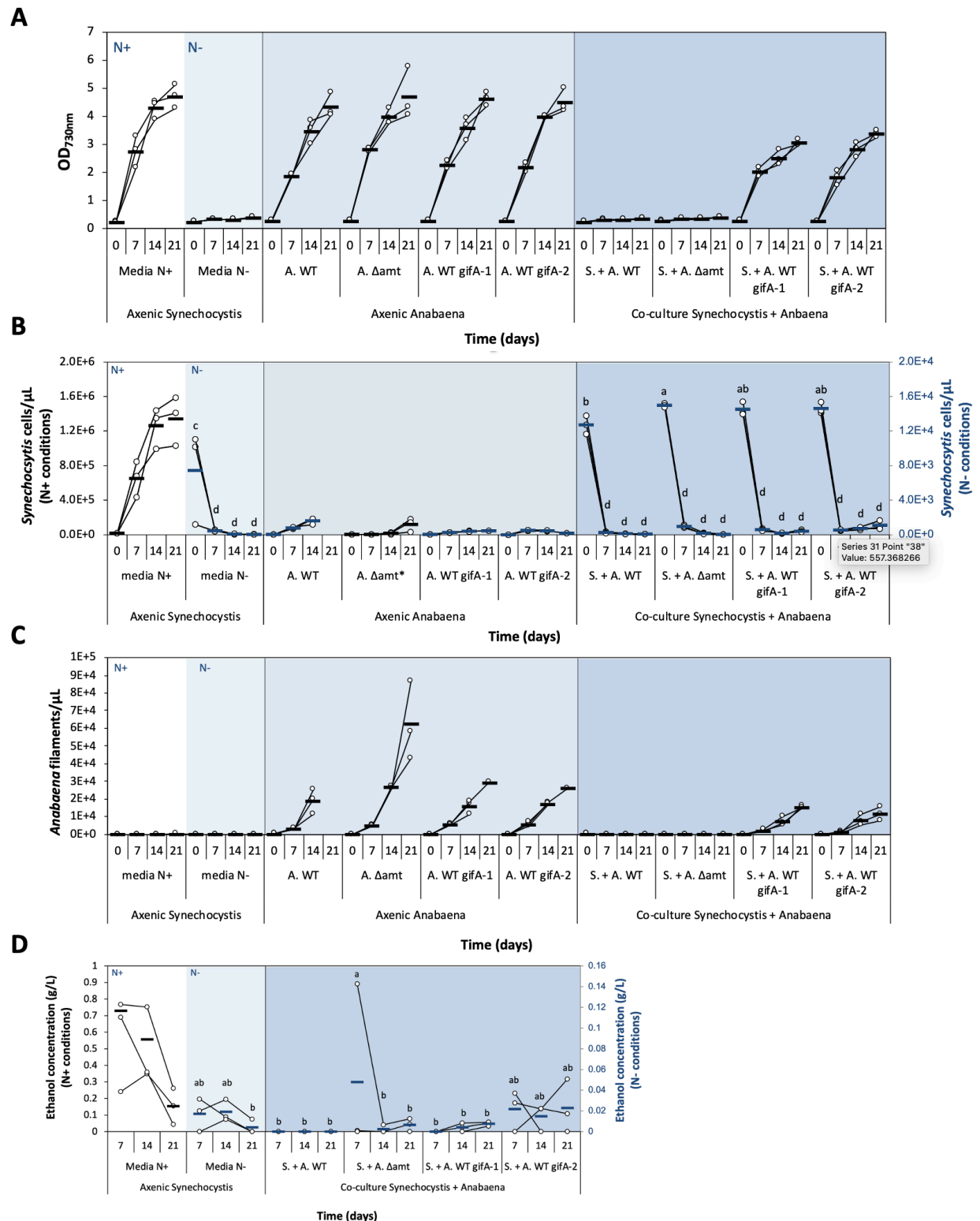


Figure 4-7 Testing whether different *Anabaena* mutants (pre-cultured in N-repleted conditions) can sustain the ethanol production of a non-diazotroph producing ethanol, monitored by flow cytometry. Individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT, Δamt , WT *gifA*-1 or WT *gifA*-2) were used to inoculate 20-mL main cultures of media (either BG-11/BG-11₀) to

an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* and 0.02 for *Anabaena*, followed by the addition of 1 mM IPTG (for all cultures), and 3 μ M Cu²⁺ (for cultures including WT *gifA*-1). Cultures were incubated under standard growth conditions (as described in 2.5.1).

(A) Total cyanobacterial biomass measured by OD at 730 nm, over time, of the different cultures in both N-repleted (N+; white background) and N-depleted (N-; coloured background) conditions.

(B) Variation over time of the number of *Synechocystis* ICLEE cells counted by flow cytometry. The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right (except for the axenic *Anabaena* Δ amt culture whose scale is shown on the left (*)).

(C) Variation over time of the number of *Anabaena* filaments counted by flow cytometry.

(D) Ethanol productivity of the engineered *Synechocystis* ICLEE, in the different conditions, measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany)) at day 7, 14 and 21. The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

White and coloured backgrounds respectively represent media with (N+, i.e. BG-11) and without nitrogen (N-, i.e. BG-11₀). S.: *Synechocystis* ICLEE; A. WT: *Anabaena* WT; A. Δ amt: *Anabaena* Δ amt.

Circles and bars represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc., are significantly different (P < 0.05, two-way ANOVA).

As expected, under optimal conditions (i.e. in N-repleted media), ICLEE grew and produced ethanol, and all the cultures grew well (panel A, Figure 4-7). Surprisingly, ethanol production decreased on day 21 compared to the previous experiments.

Unfortunately, regardless of *Anabaena* pre-culture conditions (i.e. N-repleted or depleted), all co-cultures with *Anabaena* WT and the Δ amt mutant died (panels A, Figure 4-7 and Figure 4-8), as confirmed by the yellow appearance of the cultures. As no contaminating strains (usually heterotrophic, hence not fluorescing) were found whilst analysing these cultures on flow cytometry, the reasons for these cultures' death remain unknown, especially as this was never an issue previously.

When *Anabaena* was pre-cultured under either non-diazotrophic or diazotrophic conditions (panels D of Fig. 4-7 and 4-8, respectively), there was no statistically significant difference between the ethanol produced under diazotrophic conditions, either by ICLEE axenically or in co-

culture ($P > 0.05$, two-way ANOVA described in 2.8.2). After a sharp decrease from day 0 to day 7, the number of *Synechocystis* cells in co-culture were as low as in the negative control (axenic *Synechocystis* in nitrogen-depleted media) for the duration of both experiment (no statistically significant difference, $P > 0.05$, two-way ANOVA, panel B of Figures 4-7 and 4-8).

Comparisons in ethanol titre between ICLEE co-cultures with *Anabaena* WT *gifA*-2 strains having pre-cultures grown under non-diazotrophic (Fig. 4-7, panel D) vs. diazotrophic conditions (Fig. 4-8, panel D) revealed no statistically significant difference on day 21 ($P > 0.05$, two-way ANOVA), suggesting that the method of pre-culturing did not make a difference to co-culture outcomes.

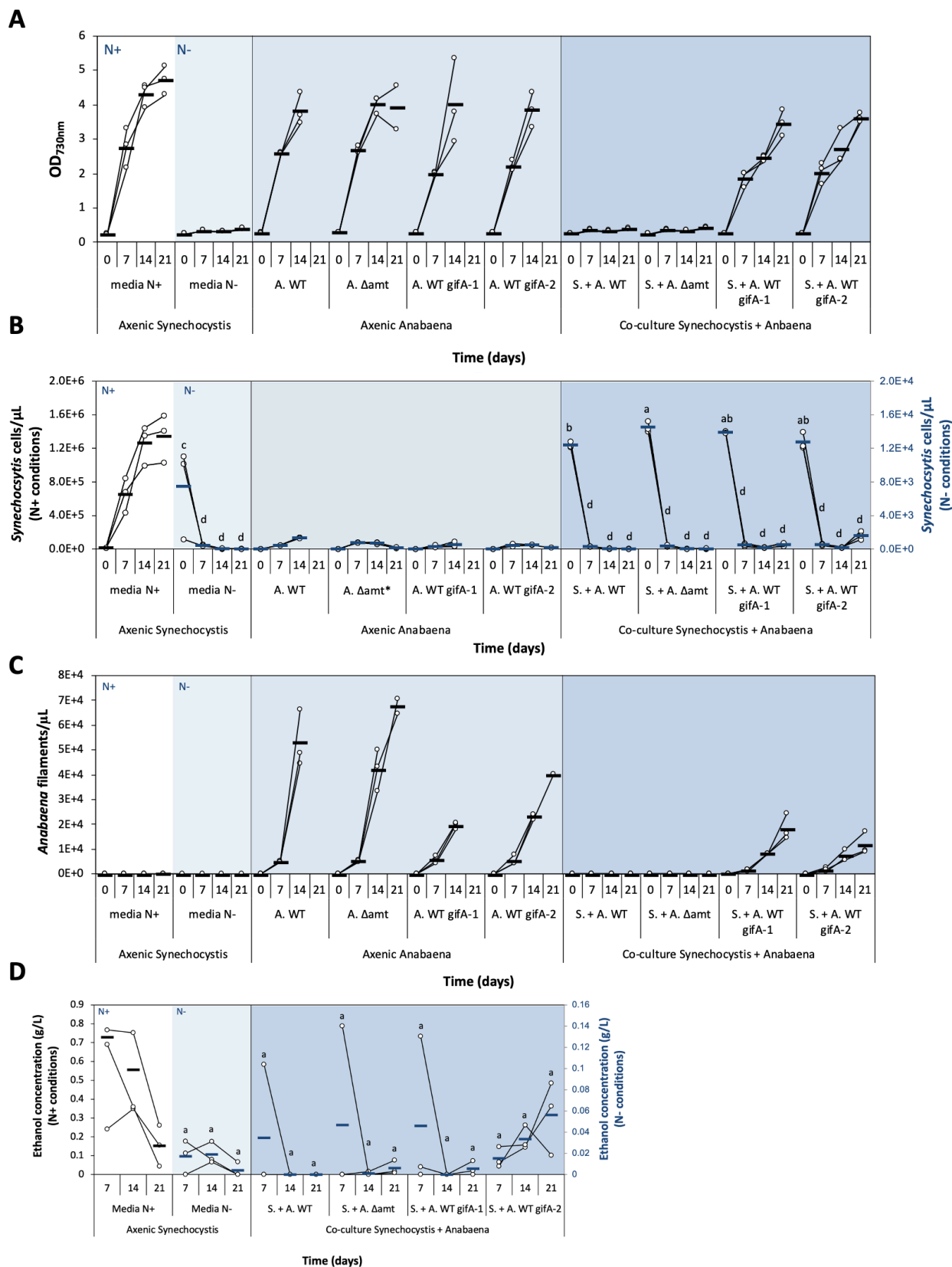


Figure 4-8. Testing whether different *Anabaena* mutants (pre-cultured in diazotrophic conditions) can sustain the ethanol production of a non-diazotroph producing ethanol, monitored by flow cytometry.

Individual axenic control cultures or combinations of *Synechocystis* ICLEE and *Anabaena* (either WT, Δamt , WT *gifA*-1 or WT *gifA*-2) were used to inoculate 20-mL main cultures of media (either BG-11/BG-11₀) to an equivalent starting OD₇₃₀ of 0.01 for *Synechocystis* and 0.02 for *Anabaena*, followed by the addition of 1 mM IPTG (for all cultures), and 3 μ M Cu²⁺ (for cultures containing A. WT *gifA*-1). Cultures were incubated under standard growth conditions (as described in 2.5.1).

(A) Total cyanobacterial biomass measured by OD at 730 nm, over time, of the different cultures in both N-repleted (N+) and N-depleted (N-) conditions.

(B) Variation over time of the number of *Synechocystis* ICLEE cells counted by flow cytometry. The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

(C) Variation over time of the number of *Anabaena* filaments counted by flow cytometry.

(D) Ethanol productivity of the engineered *Synechocystis* ICLEE, in the different conditions, measured with an enzymatic assay (Ethanol kit (R-Biopharm AG, Darmstadt, Germany)) at day 7, 14 and 21. The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

White and coloured backgrounds respectively represent media with (N+, i.e. BG-11) and without nitrogen (N-, i.e. BG-11₀). S.: *Synechocystis* ICLEE; A. WT: *Anabaena* WT; A. *amt*: *Anabaena* Δamt .

Circles and bars represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc., are significantly different (P < 0.05, two-way ANOVA).

Interestingly, flow cytometry data suggested that *Synechocystis* cells were still present in the co-cultures on day 21, i.e. at the end of the experiment (Figure 4-9) but that they had not been included in the count shown in Fig. 4-8, panel B. This was due to these cells not falling within the gate defined by the positive control (axenic ICLEE in N-repleted media, blue cluster shown in Fig. 4-9), as the cells had lost some of their fluorescence (fluorescence is part of the gating strategy, as described in 2.7.8), though not as much as the negative control (axenic ICLEE in N-repleted media, Fig. 4-9). On the cytograms (Fig. 4-9), this is reflected by a second (red) cluster being concentrated on the very left on the PC fluorescence channel. The red cluster was therefore defined on the cytograms as the *Synechocystis* cells which do not fall into the (blue) positive control gate (as shown in Fig. 4-9). Axenic ICLEE cells in N depleted media (negative control) die

quickly, which is reflected by the low OD₇₃₀ (e.g. panel A, Fig. 4-8) over the course of the cultivation. These cultures also rapidly turn yellow (in a few days).

Additionally, Figure 4-9 illustrates that ICLEE cells in co-culture with both *Anabaena* WT *gifA*-1 and WT *gifA*-2 are maintained but not dead, i.e. they are still fluorescent, only at a level between the positive and negative controls. This not surprising given that they are likely to be lacking sufficient N. Yet, are they still metabolically active? The fact that co-cultures with ICLEE display a higher fluorescence than the negative control (Fig. 4-9) suggest so. If such cells are still metabolically active, it would be important to capture their presence by adjusting the gate parameters.

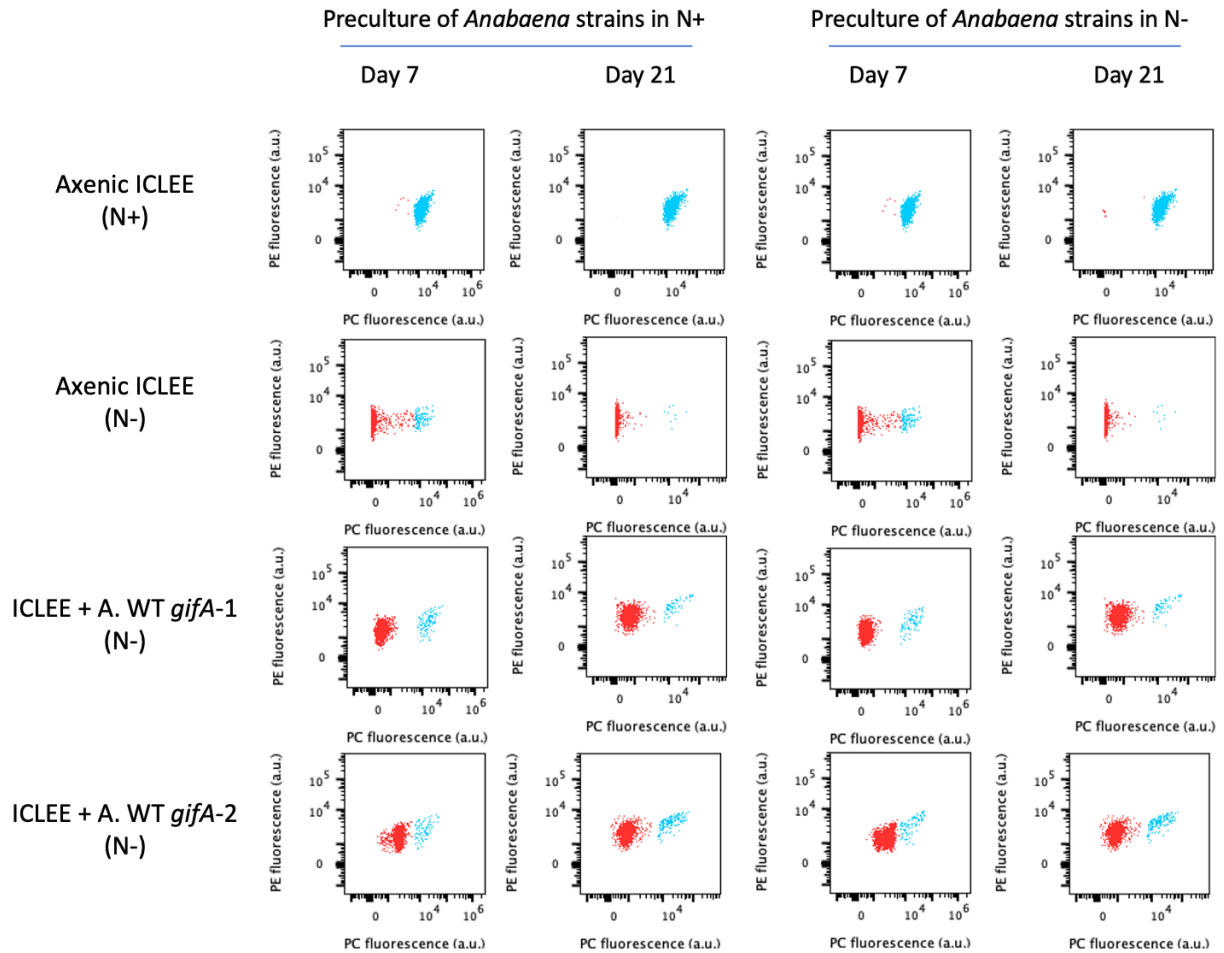


Figure 4-9. Cytograms showing the two *Synechocystis* populations, grown axenically and in co-cultures. Blue cluster was gated thanks to the positive control (axenic ICLEE in N-repleted media), whilst the red cluster represents ICLEE cells which do not fall in the positive control gate and display a lower fluorescence.

To include this red cluster of “surviving” *Synechocystis* cells, the gating strategy was adjusted, by expanding on the cytograms the gate of the positive control to include all the *Synechocystis* cells, but without including cells which fall where the negative control falls (i.e. at the very left as shown on Fig. 4-9). As each dot (or event) on the cytogram corresponds to a cell (as described in 3.1), any population in a gate/cluster can be quantified. Results, shown in Figure 4-10, suggest that *Synechocystis* did grow in co-cultures with *gifA*-2, at a rate that was approximately 5-fold slower

than under N-repleted conditions, but at a higher rate than the negative control (statistically significant difference, $P < 0.05$, two-way ANOVA), regardless of the pre-culture media used.

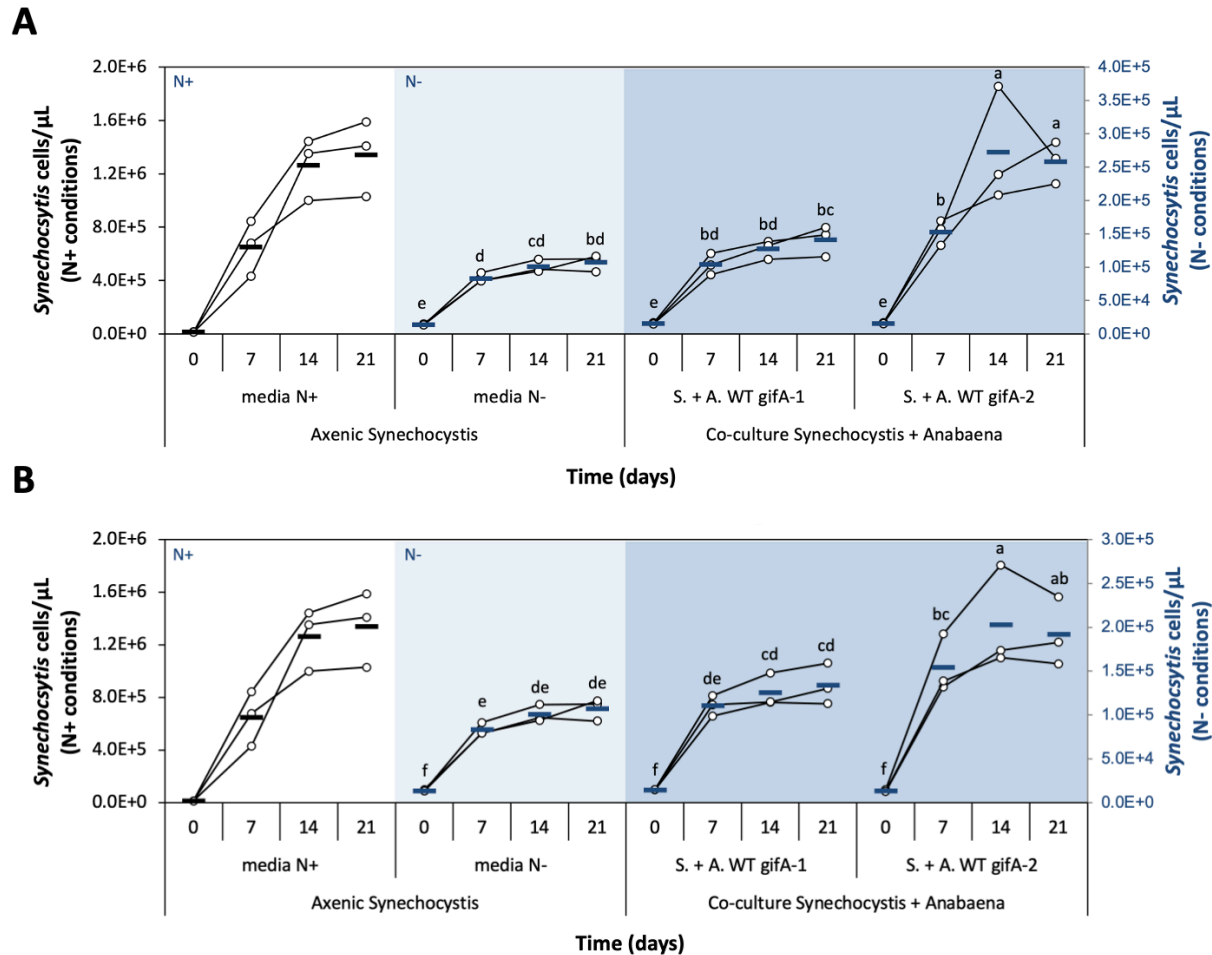


Figure 4-10. Variation over time of the number of *Synechocystis* cells displaying different fluorescence, counted by flow cytometry.

Values in the white background (N+) corresponds to the positive control (i.e. axenic ICLEE in N-repleted media, blue cluster in Fig. 4-9), while values in the coloured background (N-, irrespectively of the shade) correspond to the diazotrophic cultures.

(A) Preculture of *Anabaena* in N-repleted media.

(B) Preculture of *Anabaena* in N-depleted media.

The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right. S.: *Synechocystis* ICLEE; A. WT: *Anabaena* WT.

Circles and bars represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc., are significantly different ($P < 0.05$, two-way ANOVA).

Interestingly, when the same re-evaluation of the flow cytometry data (i.e. gate adjustment described just above) was also done for the axenic culture of the *Anabaena* Δamt mutant that displayed a surprisingly high number of *Synechocystis* cells (panel B, Figure 4-7), a high number of *Synechocystis* cells with a lower fluorescence than the positive control, but higher than the negative control (statistically significant difference, $P < 0.05$, two-way ANOVA), were found. Hence, it is likely that this culture was contaminated with unicellular cyanobacteria (likely *Synechocystis*, due to operator mistake) which were present all throughout the experiment but displayed a lower fluorescence (Figure 4-11). Unfortunately, no measurement for ethanol quantification was carried out with these control cultures (axenic *Anabaena*).

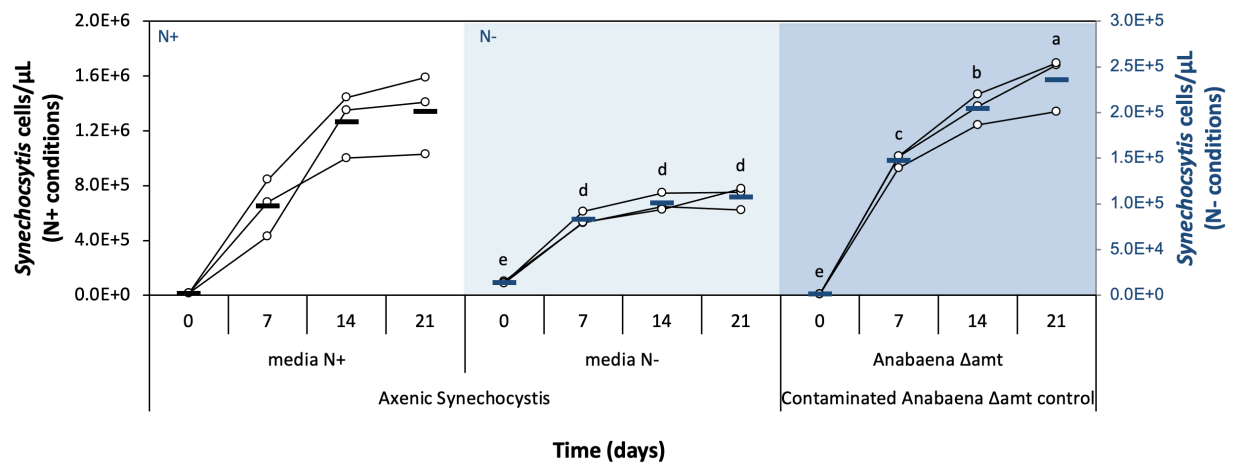


Figure 4-11 *Anabaena* culture supposedly axenic was contaminated by *Synechocystis* ICLEE cells and grew over time.

Values in the white background (N+) corresponds to the positive control (i.e. axenic ICLEE in N-repleted media, blue cluster in Fig. 4-9), while values in the coloured background (N-, irrespectively of the shade) correspond to the diazotrophic cultures.

The scale for the N-repleted conditions is shown on the left, while the scale for the N-depleted conditions is shown on the right.

Circles and bars represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc., are significantly different ($P < 0.05$, two-way ANOVA).

4.4 Discussion

Previous studies with cyanobacteria engineered to synthesise ethanol have obtained titres (i.e. concentrations) of more than five grams per litre (Gao et al., 2012). The present study started within the EU consortium DEMA project and therefore used strains available in that project, even if their productivities were lower than those reported in the literature.

The initial aims of this project were to establish a co-culture dependent on nitrogen-exchange between *Anabaena* and *Synechocystis*, but due to the number of challenges and attempts to solve those bottlenecks, additional hypotheses were formulated and tested.

Ethanol produced by engineered *Synechocystis* was used as an indicator of metabolic activity. The only previous example of a similar consortium, though not involving any nitrogen-fixer, was with two engineered strains of *Synechocystis* 6803 (hence requiring nitrogen in the media), displaying an improved ethanol production (Velmurugan & Incharoensakdi, 2020). Hence, this is a relatively new field and as this chapter shows, it is not simple to establish such a model co-culture system even with only two species.

Due to the complexity of this chapter, each of the six hypotheses (1, 3-7 (hypothesis 2 could not be tested)) is examined one by one.

Hypothesis 1: The free-living nitrogen-fixing *Anabaena* 7120 supports the growth and ethanol production of the non-diazotrophic *Synechocystis* in N-depleted media. The growth of ICLEE and UL030 in diazotrophic co-cultures was supported by the *Anabaena* Δamt strain, but not by

Anabaena WT, in two experiments (Fig. 4-2B, 4-4B), though there were an insufficient number of replicates for statistical analyses. This preliminary conclusion was supported in a subsequent experiment where the *Anabaena* WT *gifA*-2 strain supported the growth of ICLEE (Fig. 4-10), in which the difference was statistically significant. Similarly, the Δamt mutant supported ethanol production in early experiments (Fig. 4-2C and 4-4C), while the WT and *gifA*-2 strains resulted in ethanol accumulation in later experiments (Fig. 4-5B, 4-6B and 4-6D). Several of these were statistically significant in the third week of culturing. Although there was a curious change in co-cultures with the Δamt strain between the early and late experiments, we concluded that the available data support this hypothesis, both in the case of growth and ethanol, but it does depend on which *Anabaena* strain is used to inoculate the co-culture. This conclusion assumes that the only reason for the growth and ethanol synthesis of *Synechocystis* under diazotrophic conditions is because some unknown molecular form(s) of nitrogen are shared between *Synechocystis* and *Anabaena*. We have been unable to find any similar literature although nitrogen-fixing bacteria have previously been shown to support the growth of non-diazotrophic bacteria (Nascimento et al., 2015) and *Rhizobium* species are well-known for sharing nitrogen with leguminous plants.

Hypothesis 3: The experimental system for sharing nitrogen between the diazotrophic and the non-diazotrophic cyanobacteria can be robust and reproducible. This hypothesis was formulated after several experiments failed to be reproduced and contamination was frequently observed with the Δamt strains, e.g. Fig. 4-5, 4-7 and 4-8. Following the shift to *gifA*-2 over-expressing strains with pre-culturing under diazotrophic conditions, the flow cytometry method developed in Chapter 3 allowed to verify that *Anabaena* allowed the growth of *Synechocystis*

(Figure 4-10), in addition to what was observed with Δamt in earlier experiments. Despite this, there were also several unexpected outcomes (i.e. shifting outcomes with Δamt). Hence, we cannot rigorously conclude that this hypothesis holds true at this point, although sufficient well-functioning experiments could be completed to progress our understanding on several other hypotheses. Further experiments are still needed in future studies to establish more robust and reproducible experimental systems.

Hypothesis 4: Ethanol production (i.e. metabolic activity) is limited by the quantity of nitrogen provided by *Anabaena*. This hypothesis links to the first one. Two specific tests are relevant. Firstly, attempts were made to vary the initial ratio between the N-donor and N-recipient at the time when the co-culture was inoculated. Unfortunately, this experiment (Fig. 4-5) largely failed, as culture contaminations were observed at the end of the experiment. Secondly, all the experiments that supported the first hypothesis regarding ethanol do suggest that nitrogen-availability influences the ethanol-production of *Synechocystis*. However, it is still unknown what the molecular form(s) of that nitrogen are, especially as ammonium was only detected in any of the axenic *Anabaena* supernatants except for the *Anabaena* Δamt mutant at pH 10 after 25 days, statistically higher than any other *Anabaena* strains (Fig. 4-6, panel E). However, this higher ammonium concentration did not correlate to a higher ethanol production (Fig. 4-6, panel D). A separate study in the same research laboratory also concluded that there was no measurable ammonia in any of the Δamt cultures (Perin et al., 2021). The deletion of *amt* in *Rhodobacter capsulatus*, a heterotrophic nitrogen-fixing bacterium, did result in substantial accumulation of

ammonia in the culture (Yakunin et al., 2002). One possibility is that *Anabaena* 7120 has complementing ammonia-uptake transporters that have yet to be discovered.

Although there was no statistically significant difference in the ethanol concentration between co-cultures with *Anabaena* WT and Δamt strains in later experiments (Fig. 4-6 onwards), the co-cultures with *gifA-2* did accumulate more ethanol in some experiments (Fig. 4-6). Nevertheless, there was also no measurable ammonium in *gifA-2* over-expressing cultures. Curiously, the effect of *gifA-2* over-expression on ethanol accumulation was only observed in the WT background but not in the Δamt background. Hence, we were not able to make any clear conclusion regarding this third hypothesis and further experiments are needed. In hindsight, it would have been interesting to also test the manual addition of commercial sources of inorganic or organic nitrogen to axenic diazotrophic ICLEE cultures at different times.

Hypothesis 5: Combinations of environmental conditions and/or strains exist that allow Anabaena to better support the growth of Synechocystis under nitrogen-depleted conditions.

As described above, we first evaluated the effect of *amt* deletion on the ability of *Anabaena* to support the growth of *Synechocystis* under diazotrophic conditions. When in later experiments it was not possible to reproduce this, we formulated this hypothesis which also relates to hypothesis 2. To answer this new hypothesis new experiments were carried out where we added new *Anabaena* strains over-expressing GifA. In support of this hypothesis, similar results as was previously observed with Δamt were then observed with the *gifA-2* over-expression strain, as discussed above. We also tested the effect of culture pH (Fig. 4-6 panels A-B (pH 8.0) vs. C-D (pH

10.0)), but found no statistically significant differences. We conclude that the hypothesis can be confirmed but believe that further optimisation of the experimental system is still possible.

Hypothesis 6: Flow cytometry can be used to monitor the growth of two different cyanobacteria

species in a co-culture. This hypothesis was formulated as a precursor to shifting the method of monitoring *Synechocystis* given challenges with ethanol-measurements using HPLC. Flow cytometry also allowed estimated cell counts to be obtained from experiments shown on Figure 4.7 and onwards. In many cases, the *Synechocystis* cell counts correlated with measurements of ethanol in the third week of culturing, as expected, but not OD₇₃₀. Importantly, flow cytometry gave unexpected new insights, including the finding of two distinct sub-populations of *Synechocystis* in both axenic and co-cultures (Fig. 4-9) and the discovery of contaminating *Synechocystis* in supposedly axenic *Anabaena* cultures (Fig. 4-11).

Hypothesis 7: Pre-culturing Anabaena in N-depleted media allows it to share nitrogen more

quickly with Synechocystis. The final hypothesis relates to efforts to optimize the experimental system in hypothesis 4. Experiments testing this hypothesis were conclusive. There was not a statistically significant improvement in ethanol accumulation on day 21 in co-cultures with *Anabaena* over-expressing *glnA-2* when comparing *Anabaena* pre-cultured under diazotrophic conditions (Fig. 4-8D) with that under non-diazotrophic conditions (Fig. 4-7D). Also, the number of *Synechocystis* cells in co-cultures was not statistically significantly affected by pre-culturing conditions (Fig. 4-8B vs. 4-7B). The method of pre-culturing did therefore not make a difference to co-culture outcomes.

Given the novelty of this experimental system there are few comparisons with the literature that are possible. Agawin et al. (2007) carried out competition experiments between the N-fixing cyanobacteria *Cyanothece* and the microalgae *Chlorella*, in media containing a very low N content. They found that the population densities at first increased for both species. After ten days, *Cyanothece* became dominant in the co-culture, and *Chlorella* only reached ~4% of its steady-state population density typically observed in a monoculture, meaning that *Cyanothece* does not provide enough N to *Chlorella* to sustain its growth. Interestingly, when they changed the N-recipient to a cyanobacterium, *Synechococcus bacillaris*, the nitrogen excreted by *Cyanothece* allowed continued co-existence of both species.

Finally, on the nature of the nitrogen-containing molecules exchanged by the *gifA-2* over-expressing strains, most previous studies have focused almost exclusively on the molecular biology. In one study, however, Klähn et al. (2018) observed measurable differences in external accumulation of glutamate in *Anabaena gifB* mutants, a related gene that also plays a role in the regulation of nitrogen-metabolism. In future studies it would be of great interest to follow up on this observation.

4.5 Chapter conclusion

Is the free-living nitrogen-fixing *Anabaena* 7120 able to support the growth and ethanol production of the non-diazotrophic *Synechocystis* in N-deprived media? From a series of different experiments, it must be concluded that the answer is at least a conditional yes, confirming that hypothesis 1 is likely true. Artificial photosynthetic communities between two cyanobacteria,

displaying ethanol production in the absence of assimilable nitrogen, was demonstrated repeatedly.

To the best of our knowledge, this work describes the first artificial “live” fully photosynthetic consortium involving nitrogen fixation by a free-living diazotroph (*Anabaena* sp. PCC 7120), which provides fixed nitrogen to another photosynthetic but non-diazotrophic cyanobacteria producing a compound of commercial interest. In addition, it is the first time that the effect of a co-culture on an engineered metabolism is studied.

The addition of flow cytometry analysis significantly helped to understand the outcome from the co-cultures. In the final experiment (Fig. 4-9), the flow cytometry data provided powerful insight into the two distinct populations, and also suggested that phenotypic changes were taking place (e.g. changes in fluorescence by one of the species) that otherwise would have been difficult to quantify by other means.

Going forward, the flow cytometry analysis platform will undoubtedly be valuable and there are a range of options possible. In particular, it would be relevant to also evaluate other N-fixing cyanobacteria such as *Cyanothece* species (Agawin et al., 2007), and to compare these with N-fixing heterotrophic microorganisms.

The fact that ammonium production could be detected in some strains, but not others, suggests that there are more ways than one in which assimilable N can be shared with co-culture partners. The mechanisms behind the *gifA*-1/2 strains ability to support *Synechocystis* should be studied further.

It would also be of great interest to evaluate the impact of contaminants on the metabolic productivity of either axenic or N-sharing co-cultures (hypothesis 2). Although the ethanol productivities of those two systems were quite different, at least the relative impact would be of interest to measure. This was not done because of COVID-19 restrictions.

5

Plant fertilisation: developing a cyanobacterial wheat fertiliser

5.1 Introduction

The results in the previous chapter suggested that *Anabaena*, and particularly the Δamt and/or *gifA* mutants, were able to supply nitrogen to another organism not capable of fixing nitrogen itself. Instead of supporting other bacteria, could this concept also be transferred to terrestrial plants? N-fixing cyanobacteria have indeed already been used to inoculate rice paddies, yet other crops have so far received little attention (Hussain & Hasnain, 2011). In this chapter, the aim was

to investigate whether *Anabaena* is able to support the growth of terrestrial plants under nutrient-limiting conditions. Although *Anabaena* biomass contains a wide range of elements in addition to nitrogen, this study focused on the latter. The selected crop plant was wheat as it is the most important cereal crop in the UK, and the second globally (DEFRA, 2016; Velu et al., 2016).

One of the reasons why cyanobacteria and eukaryotic algae are appealing as a N biofertiliser is that they have a very high protein content, in many cases exceeding 50% of total dry weight matter. For example, cyanobacteria and algae contains on average 35-55% protein per dry weight (López et al., 2010) and measurements with selected *Anabaena* and *Nostoc* strains range between 40-50% protein per dry weight (Vargas et al., 2002). Whether or not all the proteins become available to the plants, either directly or via microbial turnover, remains uncertain. However, fertiliser trials with pure protein suggest that N contained within protein is readily available to plants (Adamczyk et al., 2010).

Importantly, different processing of the cyanobacterial biomass is likely to impact its biofertilisation efficacy. Indeed, processing which kills the cyanobacteria will result in a fertiliser which only provides nutrients upon its degradation and breakdown by other microorganisms of the rhizosphere. On the contrary, a processing which maintains cell viability will result in a fertiliser containing alive N-fixing cyanobacteria which may also provide many additional benefits to the crops, through other chemical or physical interactions with both plants and other organisms present in the rhizosphere. Cyanobacteria have indeed been recognised as “ecosystem engineers”, thanks to their ability to excrete large amounts of extracellular polysaccharides which

work as a cement creating a favourable environment for other microorganisms to grow (Rossi et al., 2017). Of particular interest is the possibility that cyanobacteria which remain alive in the soil continue to fix new N_2 in the soil and thereby continue their contribution to the soil N budget beyond what it contains within its biomass at the time of application. This is the first hypothesis tested in this chapter. However, if *Anabaena* penetrates deep enough inside the soil it will not receive any light. Then, there are two possibilities: (1) N-fixation stops because the heterocyst runs out of ATP (adenosine triphosphate), or (2) *Anabaena* is able to sustain its metabolism in the absence of light through the consumption of reduced carbon compounds. The latter may either be stored intracellularly (e.g. polysaccharides) or be supplied by plants or the degradation of other microorganisms in the rhizosphere (Smercina et al., 2019). From a limited number of studies, no substrate which enables complete chemo-organoheterotrophic growth of *Anabaena* sp. PCC 7120 has yet been described (Stebegg et al., 2012), although unpublished studies from our group (Laura Bonaldo, Pers. Commun.) suggest that fructose can be utilised in total darkness, albeit growth is slow.

Although plant biofertiliser trials with alive cyanobacteria have previously been conducted, so far no one appears to have tested the above hypothesis, i.e. whether or not it matters that N-fixing cyanobacteria are alive when it is applied as a fertiliser. Previous trials with nitrogen-fixing cyanobacteria (Do Nascimento et al., 2015; Maqubela et al., 2009), cyanobacteria that are not capable of nitrogen fixation (Wuang et al., 2016) and eukaryotic algae (Schreiber et al., 2018) demonstrate the potential of algal biomass to be used as a partial or even complete replacement of inorganic NPK fertiliser. Whether an additional bonus from N-fixation can take place *in situ*,

however, still remains to be proven. The answer to this question would have implications for processing, storage and distribution of the biofertiliser.

Unfortunately, it is expensive to produce algal biomass (Tredici et al., 2016). Hence, the quantities required to fertilise cereal crops in successful trials done so far are unlikely to be economically viable, as acknowledged by some authors (Nascimento et al., 2019; Schreiber et al., 2018). It is therefore surprising that very few articles have examined the relationship between biomass dosage and fertilisation efficacy, especially in comparison with chemical inorganic fertilisers. Hence, it would also be interesting to understand how the efficacy of *Anabaena* biofertilisation varies according to dosage, in comparison with an inorganic fertiliser, when compared on a total N-content basis. Thus, the second hypothesis is that increasing dosage of *Anabaena* results in improved plant growth when nitrogen is limiting. The outcome of such a test will depend on many factors including the existing nutritional status of the soil and crop plant, as well as other abiotic and biotic factors. But, to begin with, the aim here is to test these three hypotheses with experimental systems that are as simple and defined as possible.

5.2 Chapter overview

This chapter aims to test the following two hypotheses addressed one after the other:

1. **Hypothesis 1:** *Anabaena* which remains alive in the soil continues to fix new N_2 in the soil and contributes to the soil N budget beyond what it contains within its biomass at the time of application,

2. **Hypothesis 2:** Increasing dosage of *Anabaena* results in improved plant growth when nitrogen is limiting.

Urea was used as a positive nitrogen control whilst the negative control contained no added nutrients.

The laboratory work testing the first hypothesis (section 5.3.2), was performed by Ms. Helen Yang (FYP student, Microbial Metabolic Engineering Group, Imperial College London, UK), supervised by myself, whilst the laboratory work testing the second hypothesis (section 5.3.3) was performed by both myself and Dr. Emanga Alobwede (Technician, Microbial Metabolic Engineering Group, Imperial College London, UK). The information generated by all experiments were interpreted and evaluated by myself.

5.3 Results

5.3.1 Biomass elemental characterisation of *Anabaena* 7120

Prior to any experiments with plants, *Anabaena* sp. PCC 7120 WT was cultured in N-repleted media (BG-11) in two 5-L Erlenmeyer flasks closed with a foam stopper, and incubated at 30 °C, at 150 rpm and under white fluorescent light at 120 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a growth chamber (PSI Instruments), and air was bubbled into the culture, as described in 2.6.1. Biomass was harvested and freeze-dried (as described in 2.6.1), and twenty grams of biomass (in triplicates) were sent

to NRM Laboratories for analysis of its nutrient elemental composition. The obtained results are shown in Table 5-1.

Table 5-1. Nutritional content of *Anabaena* sp. PCC 7120 WT

	Unit	Nutritional content
Total Nitrogen DUMAS*	% w/w	11.6 ± 0.8
Total Phosphorus	% w/w	1.0 ± 0.1
Total Potassium	% w/w	0.4 ± 0.2
Total Calcium	% w/w	0.3 ± 0.0
Total Magnesium	% w/w	0.3 ± 0.0
Total Sulphur	mg/kg	10338.0 ± 1443.9
Total Carbon	%	46.0 ± 0.7
Carbon:Nitrogen ratio	:1	4.0 ± 2.0
Total Manganese	mg/kg	495.0 ± 217.5
Total Zinc	mg/kg	76.6 ± 12.8
Total Iron	mg/kg	2171.0 ± 374.5
Total Boron	mg/kg	22.9 ± 3.9
Selenium	mg/kg	<0.02

*Total nitrogen DUMAS consists of both available and non-available nitrogen in the sample. Performed by NRM Laboratories, UK. Mean ± standard error (n=3).

Interestingly, the total N content was far higher than earlier publications had suggested (Vargas et al., 1998) since 11.6% (w/v) N per dry weight is the equivalent of 72% (w/v) protein per dry weight, assuming that the average nitrogen content of protein is 16% (w/v). However, *Anabaena* also accumulates storage compounds which contain N such as cyanophycin (Burnat et. Al, 2014).

5.3.2 Hypothesis 1: *Anabaena* which remains alive in the soil continues to fix new N₂ in the soil and contributes to the soil N budget beyond what it contains within its biomass at the time of application

N-fixing cyanobacteria from the *Nostoc* and *Anabaena* genera have already been demonstrated to improve soil physico-chemical properties and plant growth (Gheda & Ahmed, 2015; Maqubela et al., 2019). Yet, the mechanism or molecules by which N-fixing cyanobacteria fertilise plants is yet to be described (Perin et al., 2019). Two sub-hypotheses were therefore formulated: (a) any protein contained within the *Anabaena* biomass that is applied to the soil eventually transforms into plant-available nitrogen, or (b) *Anabaena* continues to fix new nitrogen even after application to the soil. The latter could still be driven by sunlight if the algae are situated at the top of the soil (until it is washed away by the rain), or the cells may derive sufficient energy required for nitrogen-fixation from reduced carbon compounds secreted by the plant. To separate between pre-existing nitrogen and newly fixed nitrogen, the ideal treatments would be to compare wild-type *Anabaena* with a deletion mutant of *Anabaena* that was unable to fix nitrogen. Unfortunately, no such strain was available. Therefore, methods to inactivate its metabolic activity without compromising the N content (N being the focus of this study) were considered: oven-drying and autoclaving of the biomass were therefore evaluated as they are simple methods and the equipment available.

Anabaena sp. PCC 7120 WT biomass was cultivated as described in Chapter 2 (section 2.6.1), centrifuged (10,000 g for 10 min) to separate the cells from the aqueous supernatant and then

subjected to the following treatments: (1) no treatment, i.e. fresh *Anabaena* (positive control), (2) autoclaving (121 °C, 20 min) or (3) oven-drying (80 °C, 24 hours). Two milligrams of each preparation (or its equivalent OD₇₃₀ for fresh biomass) was subjected to elemental CN-analysis using a Flash 2000 Elemental Analyser to obtain C and N contents. The results are shown in Figure 5-1.

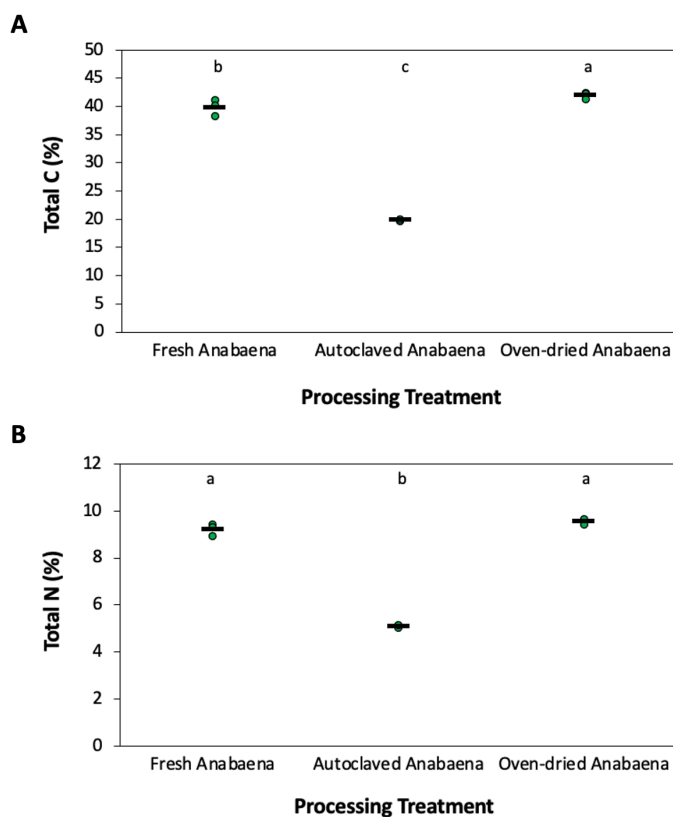


Figure 5-1. The C and N content of *Anabaena* sp. PCC 7120 WT is influenced by its processing method. Two milligrams of *Anabaena* biomass grown in BG-11 (as described in 2.7.1), either fresh (OD₇₃₀ equivalent of 2 mg), autoclaved (121 °C, 20 min) or oven-dried (80 °C, 24 hours) was subjected to a Flash 2000 Elemental analyser (as described in 2.6.1) to obtain (A) total C (%) and (B) total N (%). Circles and bards represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc. are significantly different (P < 0.05, one-way ANOVA).

Figure 5-1 shows that autoclaving resulted in a substantial drop in both total C and total N contents (approximately half compared to no treatment, i.e. fresh *Anabaena*), whilst oven-drying only marginally affected the total C: the C contents were 40% and 42% (fresh and oven-dried respectively, statistically significant difference) and the N contents were 9.2% and 9.5% (fresh and oven-dried respectively, not statistically significant difference). Oven-drying was therefore the selected method taken forward. Note that the NRM analysis (Table 5-1) and the Flash Elemental analysis (Fig. 5-1) led to a slightly different total C (46% vs. 40%, respectively) and total N (11.6% vs. 9.2%, respectively), likely due to (1) the difference in methods (e.g. Total DUMAS for the NRM analysis vs. Flash Elemental analyser) and (2) the difference in biomass processing (freeze-dried biomass for the NRM analysis vs. fresh *Anabaena*).

Additionally, prior to the fertilisation experiment with plants, the viability of both fresh and oven-dried biomass was tested by liquid culture growth. Briefly, the dried biomass was first resuspended in BG-11, then 20 mL of BG-11 were inoculated with either fresh or oven-dried biomass (starting at an OD₇₃₀ of 0.1), and the growth was followed by monitoring OD₇₃₀ regularly. The obtained results are shown in Figure 5-2, and confirmed that the oven-dried biomass is indeed dead. Note that between days 4 and 7, the AlgaeTron ran out of CO₂, and was replaced from day 7 onwards, hence the drop in OD₇₃₀. However, this does not impact the overall outcome of this experiment (i.e. oven-drying *Anabaena* at 80 °C for 24h results in dead biomass).

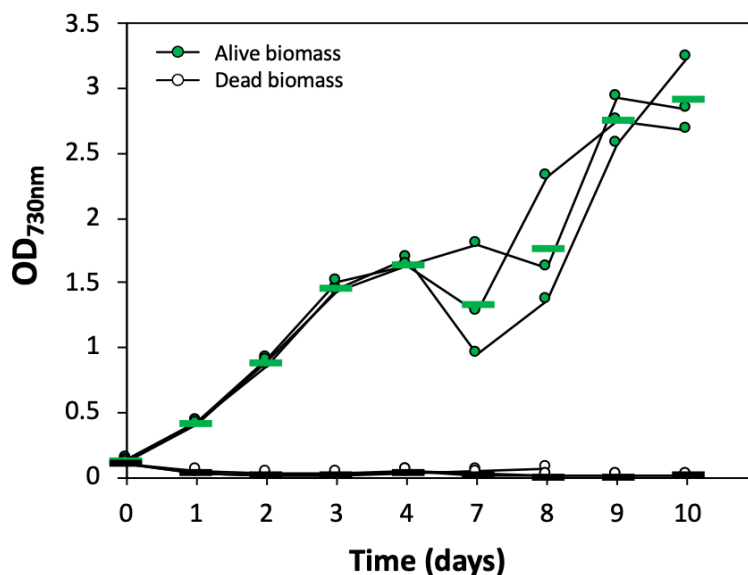


Figure 5-2. Impact of processing *Anabaena* sp. PCC 7120 WT on cell viability.

Anabaena biomass, either in liquid culture (maintained as described in section 2.5.1, green circles) or oven-dried (80 °C, 24 hours, black circles) was inoculated in BG-11 and OD₇₃₀ was monitored over time. Bars display the mean from three replicates. Green = liquid *Anabaena* (green) vs. oven-dried (black).

The two *Anabaena* WT preparations, alive (i.e. fresh) and dead (i.e. oven-dried), were thereafter used as treatment on small pots with planted wheat seedlings. Briefly, the pot experiment was conducted in a growth room chamber at Imperial College London for 39 days with wheat (*Triticum aestivum*) seedlings planted in vermiculite (as described in 2.6.2.1). Vermiculite is a relatively well-defined growth medium with a very low concentration of nutrients (Appendix II), and importantly it is allowed to be used inside Imperial College London's indoor greenhouse facility. As described in 2.6.2, sterilised and germinated seeds were transplanted to vermiculite (dispensed in 5 x 5 cm plastic pots). The *Anabaena* sp. PCC 7120 WT biomass was thereafter applied onto the vermiculite surface right after transplanting the wheat seedlings.

Anabaena application rates were based on the industry standard for nitrogen fertiliser application in the UK, 160 kg N/ha (DEFRA, 2010). This equates to an addition of 0.08 g of dry

weight cyanobacteria (or its OD₇₃₀ equivalent before drying) per pot. The preparations were applied directly on top of the vermiculite (either by pipette (liquid applications) or weighed and scattered on top of the soil (solid applications)). The experimental design utilised the following treatments:

- **Alive** cyanobacterial biomass, either:
 - a. **Washed** with deionized water,
 - b. **Unwashed** cyanobacterial biomass (i.e. fresh biomass),
- **Dead** cyanobacterial biomass (unwashed, oven-dried (80 °C, 24 hours)),
- **Supernatant** at the stage of cyanobacterial biomass harvesting (negative control for both the dead and alive unwashed biomass treatments),
- **Untreated** (negative control for the alive washed biomass treatment).

Both washed and unwashed liquid culture were tested to verify whether the residual nutrients coming from the culture medium could impact the results.

The plants were maintained at 20 °C in day/night cycles of 50%:55% relative humidity under a 16h:8h photoperiod at 120 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity. All plant pots were maintained wet by regular spraying with tap water and incubated for 39 days. After 39 days, the plants were harvested and the following growth parameters recorded (as described in 2.6.3): the number of leaves, dry weights (of both shoots and roots, after drying at 80 °C for 48 hours), chlorophyll *a* concentration (as an indicator for nitrogen accumulation ((Evans, 1989), as described in 2.7.6), height (defined as the longest part of the plant by measuring from the surface of the vermiculite next to the stem to the longest part of the plant when hold up next to the measuring tape) and

standing height (defined as the highest point of the plant by measuring from the surface of the vermiculite to the highest standing leaf, not being hold). The obtained results are shown in Figure 5-3, while Figure 5-3 shows 3 replicates of each treatment after 39 days of growth.

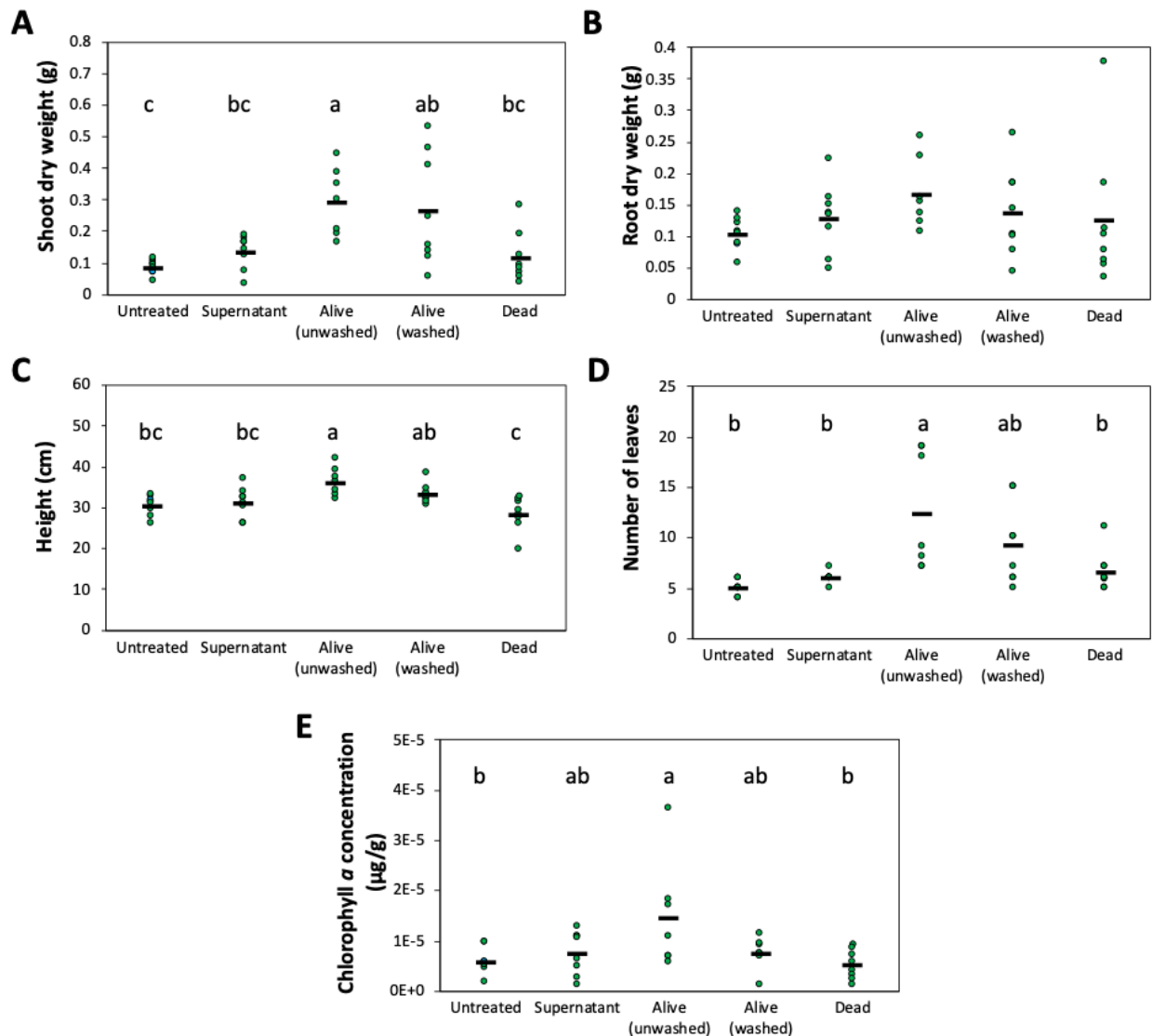


Figure 5-3. Effect of alive and dead cyanobacterial fertilisation on wheat growth parameters.

Wheat seedlings were fertilised following transplantation in vermiculite soil with 0.08 g of alive (washed and unwashed), dead, or supernatant (same volume as washed treatment) of *Anabaena* sp. PCC 7120 biomass. The following parameters were measured after 39 days: (A) Shoot dry weight, (B) root dry weight, (C) height, (D) number of leaves and (E) chlorophyll *a* concentration in shoots. Circles and bars represent individual values and means, respectively (n=8). Means which do not share the same letter e.g. a, b, etc., are significantly different ($P < 0.05$, one-way ANOVA).

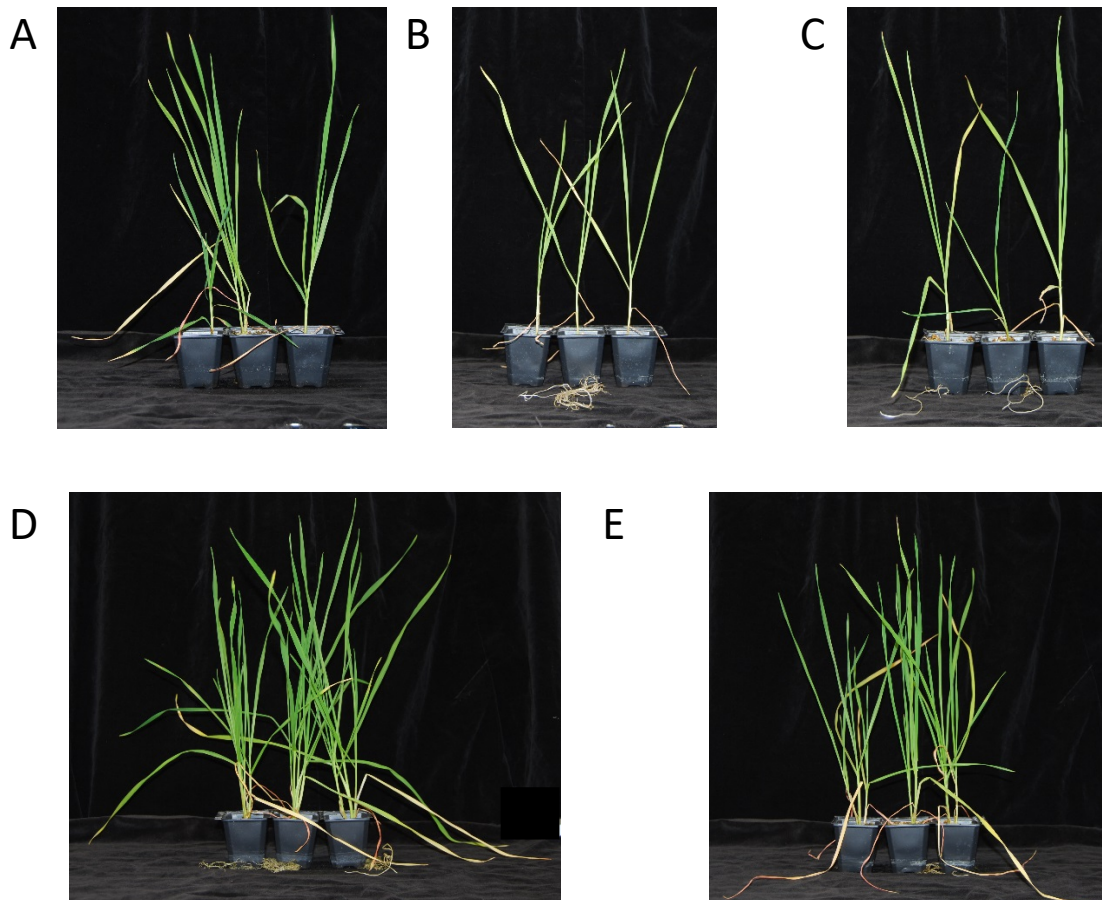


Figure 5-4. Pictures representing 3 replicates of each treatment after 39 days.

Wheat seedlings were fertilised following transplantation in vermiculite soil with 0.08 g of alive (washed and unwashed), dead, or supernatant (same volume as washed treatment) of *Anabaena* sp. PCC 7120 biomass. Pictures were taken 39 days after transplantation. Treatments are as follows: (A) Dead *Anabaena* biomass; (B) Untreated; (C) Cyanobacterial supernatant; (D) Alive unwashed *Anabaena*; (E) Alive washed *Anabaena*.

It should be noted that this experiment lacks a positive control, as an initial goal of this first experiment was to evaluate whether *Anabaena* can promote any growth at all in this experimental system.

For all measured parameters, there was no statistically significant difference between supernatant, untreated and dead *Anabaena* treatments (Fig. 5-3). However, the pictures shown on Figure 5-4 suggest that the pots which received dead *Anabaena* (A) grew better than the

untreated (B) and supernatant (C) treatments, but not as well as the ones which received alive biomass.

Applying alive *Anabaena* 7120 (both washed and unwashed) led to the highest shoot dry weights: alive washed *Anabaena* increased the shoot dry weight by 3.1-fold compared to the untreated control, and alive unwashed *Anabaena* 7120 increased it by 2.2-fold compared to the supernatant treatment (panel A, Fig. 5-3). This is line with visual observations (Fig. 5-4): wheat seedlings which were fertilised with alive *Anabaena* (both washed and unwashed) appear to be visually healthier than with the other treatments. There was no significant difference between the washed and unwashed alive biomass on any of the wheat plant growth parameters. Applying unwashed alive *Anabaena* significantly improved all measured parameters compared to the supernatant treatment, except for the root dry weight and chlorophyll *a* concentration, whilst applying washed alive *Anabaena* only improved shoot dry weight compared to the negative control (i.e. untreated) (Fig. 5-3).

Given that unwashed alive *Anabaena* significantly improved all measured parameters compared to the supernatant treatment (except for the root dry weight and chlorophyll *a* concentration), the second hypothesis was tested using unwashed alive biomass, as it simplified the experimental process.

5.3.3 Hypothesis 2: Increasing dosage of *Anabaena* results in improved plant growth when nitrogen is limiting

After having identified the optimal method of processing *Anabaena* biomass prior to its application, the second hypothesis was then tested: increasing dosage of *Anabaena* results in improved plant growth when nitrogen is limiting. This will also help determine the optimal dosage of application which is important as producing cyanobacteria biomass is costly and consumes plentiful resources, including land area (Tredici et al., 2016). In the following experiment, urea was used as an inorganic N source as it is readily available in a life sciences laboratory and alternatives such as ammonium nitrate are difficult to source given their access is highly regulated (i.e. they can be used to generate explosives).

Note that this experiment differed from the previous one in three important ways: (1) a different soil medium was used, (2) different dosages of biomass applications were tried, and (3) the biomass treatment was compared with an inorganic nitrogen source (urea, positive control).

The pot experiment was conducted in an outdoor greenhouse at Silwood Park (Imperial College London) for 42 days with wheat (*Triticum aestivum*), as described in 2.6.2.2, in soil collected from an annually ploughed field at Silwood Park (Imperial College London). Due to the non-sterile nature of the soil, this experiment was not allowed to be carried out in the South Kensington Greenhouse. Prior to starting the experiment, the soil was air dried, sieved (2 mm), and homogenised. Unfortunately, the physico-chemical properties of the Silwood Park soil were not evaluated.

Seeds were sterilised and pre-germinated 5 days prior to the start of the experiment (as described in 2.6.2). Seedlings were transplanted to Silwood Park soil (dispensed in plastic pots of 5.1 cm diameter) and either *Anabaena* sp. PCC 7120 WT or urea were applied to the soil surface, right after transplanting. The following application rates were selected, derived from the R209 Fertiliser Manual (DEFRA, 2010): 0, 20, 40, 70, 120, 150 and 210 kg N/ha, using the area of the pots. Urea was weighed and applied onto the soil surface as a dry powder, whilst *Anabaena* (grown as described in 2.6.1) was pipetted as a liquid onto the soil surface (the volume applied was based on its nitrogen content (11.6%), as per Table 5-1).

The potted plants were placed in an outdoor greenhouse at Silwood Park (Imperial College London), and maintained in day/night cycles of 20°C: 15°C, 50%:55% relative humidity under a 16h:8h photoperiod at 120-130 $\mu\text{E} \cdot \text{m}^2 \cdot \text{s}^{-1}$ light intensity. All pots were maintained wet by regular spraying of tap water. After 42 days, the plants were harvested and the shoot and root dry weights as well as shoot heights were recorded. The obtained results are described in Figure 5-5.

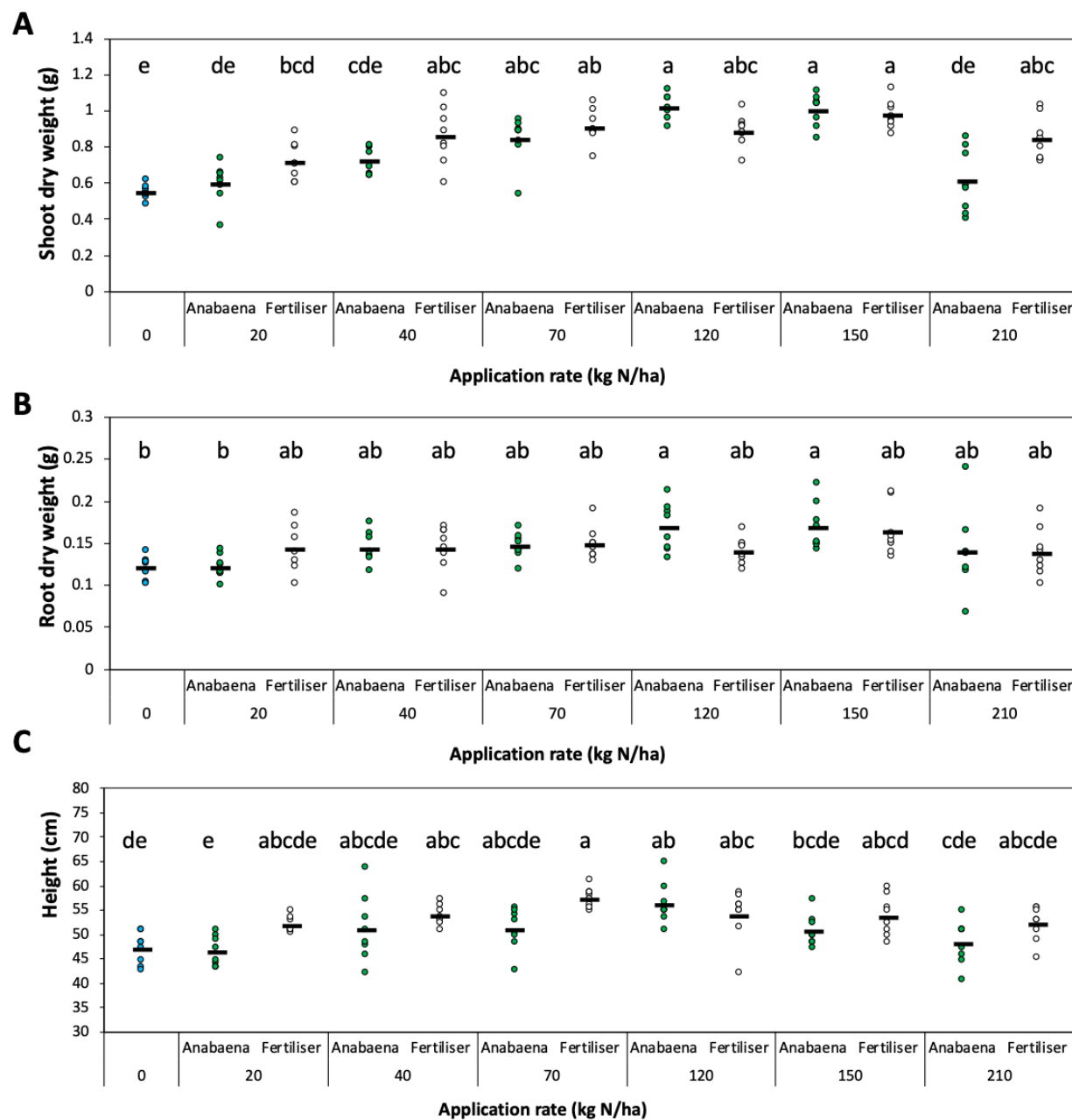


Figure 5-5. Wheat response to different dosages of cyanobacterial fertiliser, in comparison to urea.

Wheat plants were supplemented 5 days after germination with 0, 20, 40, 70, 120, 150 and 210 kg N/ha of either *Anabaena* sp. PCC 7120 biomass or urea (fertiliser). (A) Shoots height, (B) shoots dry weights and (C) roots dry weights were measured after 42 days.

Circles and bars represent individual values and means, respectively (n=8). Means which do not share the same letter e.g. a, b, etc., are significantly different ($P < 0.05$, two-way ANOVA).

There was no statistically significant difference between the cyanobacterial and urea treatments on shoot dry weights, for all application rates except for the highest one (210 kg N/ha) (panel A, Fig. 5-5).

However, urea significantly improved wheat shoot biomass from 20 kg N/ha onwards, whilst *Anabaena* only improved wheat shoot biomass from an application rate of 70 kg N/ha and onwards, all relative to the untreated control.

The optimum dosage for *Anabaena* was 120 kg N/ha, whilst for urea the optimum was 150 kg N/ha. At the highest application rate (120 kg N/ha), *Anabaena* did not stimulate growth and there was no statistically significant difference between the fertilisation with *Anabaena* and the negative control. Yet, urea applied at this rate led to a significant difference with the negative control.

Root dry weights were overall not significantly different compared with the negative control, except for plants fertilised with *Anabaena* applied at 120 and 150 kg N/ha (panel B, Fig. 5-5).

Anabaena only improved wheat height at 120 kg N/ha, whilst urea improved it at 40, 70, 120 and 150 kg N/ha (panel C, Fig. 5-5), all relative to the untreated control. There was also no statistically significant difference between the cyanobacterial and urea treatments on wheat height at all application rates.

5.4 Discussion

When crops are harvested and transported elsewhere, there is a loss of nutrients from the farming system that needs to be replenished. Although N_2 can be made available through chemical reactions catalysed by light and fixed by free-living microorganisms in the soil, it is usually insufficient to maintain high-yielding crop production. Hence, farmers add inorganic nitrogen fertiliser (as well as other elements) typically as a salt that includes urea, ammonium and/or nitrate. However, only approximately 50% of the nitrogen applied as fertiliser gets taken up by the crop plant (Bijay-Singh & Craswell, 2021).

The different methods of preparing *Anabaena* biomass had a major impact on the efficiency of the fertilisation. Comparing wheat seedlings treated with dead or alive *Anabaena* biomass (Fig. 5-3), it was clear that alive biomass was more effective as a biofertiliser. This therefore confirms **hypothesis 1** (*Anabaena* which remains alive in the soil continues to fix new N_2 in the soil and contributes to the soil N budget beyond what it contains within its biomass at the time of application) as tentatively being true. Caution is used as it remains unknown *how* stimulation of plant growth is achieved. In particular, it is not known whether the effect observed in Fig. 5-3 is due to continued N_2 -fixation or not. Another possibility is that *Anabaena* secretes either enzymes and/or metabolites that help make inorganic nutrients (e.g. P) more bioavailable (Timofeeva et al., 2022), or stimulates growth (i.e. acting as hormones), and that these functions also require the cyanobacterial cells to be alive. Other effects may not require *Anabaena* cells to remain alive, such as the effect of extracellular polysaccharides on the water-holding capacity of soil (Costa et al., 2018). Further experiments are still clearly needed to understand the mechanism behind the

whole-plant observations. Aside from constructing and testing *Anabaena* mutants that are incapable of N₂-fixation, another possibility is to utilise stable isotope label (i.e. ¹⁵N) to culture *Anabaena* and then to track its movement into both soil and plant.

In addition, it is known that arbuscular mycorrhizal fungi (AM) can help crops to assimilate nutrients. For example, AM fungi significantly enhanced the growth of wheat under low nitrogen and low C:N ratio conditions (Ingraffia et al., 2020). However, a meta-analysis of farm-based trials concluded that AM fungi had minimal impact on the growth of crops (Ryan & Graham, 2018). It cannot be excluded that fungi were present in the non-sterile soil used in our experiments and influenced the outcome, however, we assume that all treatments would have been similarly affected. Future studies could examine the impact of AM fungi in combination with *Anabaena*, and soil sterilization are also worth considering.

To the best of my knowledge, these results can be compared with two previous studies inoculating wheat with cyanobacteria in nutrient-poor soil, even though these studies were carried out during a very short time and did not use the exact same species of cyanobacteria:

- This study: whilst 0.08 g/pot was applied, experiments lasted 39 days, and got a 3.1-fold increase in shoot DW, but no increase in root dry weight,
- Gheda & Ahmed (2015) applied 10 g of freshly harvested *Anabaena cylindrica* on potted Egyptian degraded soil and reported, after only 14 days, a >1.4-fold increase of roots dry weight yet no significant different was found in the shoot dry weight,

- Similarly, El-Shinnawil et al. (2016) applied 4 mL of a 15-day old *Anabaena variabilis* culture to wheat grown in agar BG-11₀ media, and after 15 days noticed a 2.7-fold increase in root dry weight, with only a 1.2-fold increase of in shoots dry weight.

As the establishment of the plants is one of the most critical stages of plant life, a significant increase in root growth is likely to be important for crop yield (Gheda & Ahmed, 2015), but it is unfortunately not possible to directly compare with their data as they did not provide any information regarding the quantity of biomass (in terms of dry weight or culture density) that was applied. Nor did they apply any statistical analysis on most of their data. In contrast, in the experimental results shown in Fig. 5-2 in the present study, the greatest response in measured parameters to alive biomass application was in the dry shoot weight. Although eight replicates were used in this present study, the variation was substantial, and the experiment would benefit from being repeated with a larger number of replicates and in different kinds of soil.

With respect to biomass processing, applying dead algae to wheat improved none of the measured parameters (compared to both the untreated and supernatant treatments, Figure 5-3). Furthermore, the pictures in Figure 5-4 show that the plants which received dead algae grew better than the untreated and supernatant treated ones, but not as well as the ones which received alive biomass. This could be explained by the dead biomass starting to break down in the soils, allowing nutrients to be released and shared with the roots, but in a less efficient way than the alive algae. Whether this relates to the speed of release and/or loss of nutrients through volatilization or drainage, remains unclear. The outcome could also have been affected by the

low water retention capacity of vermiculite, and/or the relatively short duration of the experiment.

Comparing with other published data, a study performed for a much longer period (34 weeks) using dead flakes of cyanobacterial biomass reported a 36% increase in dry weight of wheat stubble, along with an 45% increase in grain yield, following the inoculation of organically poor soil with 0.6 g of cyanobacterial consortia biomass including *Anabaena doliolum* (Nisha et al., 2007). Furthermore, the authors of this study only dried the biomass at 60 °C, whereas 80 °C have been used in this present study. There were a number of important differences relative to this present study, including the method of drying, which could have impacted the measured parameters.

However, it is important to note that storage issues may be encountered with alive biomass: when stored a few months in the 4 °C fridge, the biomass started to turn blue, as cyanobacterial pigments were released presumably as a result of cellular death. Storing cyanobacteria in a powder form may alleviate this issue, as the powder did not reveal any colour difference. Yet, applying dead biomass to wheat did not improve the plant growth as well as applying alive biomass, but a longer experiment could have led to different result, as reported by Nisha et al. (2007). The drying method could also potentially impact the quality of the bio-fertiliser. In a very thorough experiment, Schreiber et al. (2018) compared wet (i.e. untreated) and spray-dried *Chlorella vulgaris* (a eukaryotic alga) treatments on wheat and found that the latter was statistically significantly more efficient than the former. Furthermore, algalisation practices for

rice paddies in India occurs with sun-dried cyanobacteria, and IARI recommends farmers to store the powder up to 3 years, at room temperature (Sahoo & Seckbach, 2015).

It is not clear yet if N becomes available by exudation or by microbial degradation after the cell death (J. S. Singh et al., 2016). Future experiments could consider applying un-targeted Liquid Chromatography-Mass Spectrometry analysis of the supernatant of *Anabaena* stored or treated under varying conditions to shed light on the molecular nature of the shared nutrient(s). In conclusion, if these recommendations are combined with what was learned in our experiment, the ideal storage method may involve drying that preserves cellular viability. Such methods are available in the brewing yeast industry and deserve further attention and testing in order to reach the best product, i.e. it becomes important to compromise between efficacy of the product and the cost and ease of use related to processing, storage and shelf-life.

For the second hypothesis, varying doses of both *Anabaena* and an inorganic N control (urea) were evaluated (see 5.3.3, Fig. 5-5). Interestingly, there was no statistically significant difference between the cyanobacterial and urea treatments on shoot dry weights, for all application rates except for the highest one (210 kg N/ha) (panel A, Fig. 5-5), suggesting that *Anabaena* can fully replace the chemical fertiliser.

However, urea significantly improved wheat shoot biomass from 20 kg N/ha onwards, whilst *Anabaena* only improved wheat shoot biomass from an application rate of 70 kg N/ha and onwards, all relative to the untreated control. This surprisingly large difference may relate to the fact that urea is immediately available to the plant whilst *Anabaena* cells may need to lyse before

any nitrogen (mainly protein) becomes available. On the other hand, urea may more easily be lost if it rains during application. Hence, the timing of application likely plays an important role in determining how effective any fertiliser treatment is.

The optimum dosage for *Anabaena* was 120 kg N/ha, whilst for urea the optimum was 150 kg N/ha. Surprisingly, at the highest application rate (210 kg N/ha), *Anabaena* did not stimulate growth and there was no statistically significant difference between the fertilisation with *Anabaena* and the negative control. Yet, urea applied at this rate led to a significant difference with the negative control. One possibility is that the high dosage affected the *Anabaena* treatment through aggregation whilst urea was still easily water-soluble. Further experiments could consider breaking filaments to reduce aggregation.

Root dry weights were overall not significantly different compared with the negative control, except for plants fertilised with *Anabaena* applied at 120 and 150 kg N/ha (panel B, Fig. 5-5). This may relate, at least in part, to the fact that wheat roots may grow longer when nutrients are lacking (Guo et al., 2019).

Overall, it can be argued that the data support **hypothesis 2** (increasing dosage of *Anabaena* results in improved plant growth when nitrogen is limiting) as confirmed to be true, but the cyanobacteria treatment did not perform as well as the inorganic treatment.

Searching the literature, few comparable experiments in the literature have been found and all studies have used eukaryotic algae instead of cyanobacteria. In most cases, these studies have been carried out with either only a single or one relevant dosage tried (Schreiber et al., 2018;

Nascimento et al., 2019). For example, Nascimento et al. (2019) only tested 150 and 450 kg N/ha, with the higher dosage being beyond normal practice, in the European Union at least. The most relevant study is that of Rupawalla et al. (2021) who compared different dosages of mixed eukaryotic algae with mixed inorganic nitrogen fertiliser on the growth of spinach. Whilst the inorganic fertiliser was more effective, on a molar nitrogen basis, than the algae fertiliser under the tested conditions, there was an optimum maximum dose for both treatments followed by sharp negative impact.

It is important to note that there is a general lack of rigorous studies on this applied topic (applied use of cyanobacteria to stimulate crop growth) in the literature.

It is unfortunate that there have not been further studies done with respect to dosage, and that the experiment described in section 5.3.3 suggested that a relatively high cyanobacterial application rate would likely be needed to stimulate wheat growth to its fullest potential. One hundred and twenty kg N/ha is the equivalent of ~1 ton *Anabaena* dry weight per ha, which if produced in the manner proposed by Tredici et al. (2016) would cost approximately €12,600/ha, clearly surpassing the typical value of wheat harvested in the EU: 6 tons of wheat per ha is sold at €230 per ton (EC Commission, 2021) and would generate approximately €1,380 per ha, one tenth of the fertiliser cost. This conclusion is similar to that of Schreiber et al. (2018). In many cases the EU CAP subsidies may constitute the main farm income anyway.

Despite these unfavourable financial estimations, there is still a chance that cyanobacteria-based biofertilisers may also be used for higher value crops, and the effect of repeated applications

over multiple seasons has not yet been evaluated either. If the cyanobacteria remain alive within the soil, it would be interesting to understand if they continue to contribute to nitrogen inputs over time? Such experiments would be very interesting to undertake though it would require long-term research.

5.5 Chapter conclusion

This chapter aimed to test the two following hypotheses which can also be summarised as two questions:

- Does *Anabaena* need to be alive for an efficient fertilisation?
- How does *Anabaena* compare with synthetic fertilisers (urea)?

In summary, it must be concluded that *Anabaena* can effectively replace urea as a fertiliser under the tested conditions, if the dosage is chosen appropriately. In addition, it would appear that the *Anabaena* fertilisation effect is more effective when its cells are kept alive.

The effect of *Anabaena* 7120 on the growth performance of wheat was studied in vermiculite (as a proxy for nutrient-poor soil) as well as real soil, with both alive and dead biomass. Results showed that applying alive cyanobacterial biomass significantly improved dry weight of the shoots, a very important parameter for farmers to increase yield. And importantly, the cyanobacterial biofertiliser compares well with urea, a chemical fertiliser. This result could be due to the improved soil N levels in soils, as reported by Gheda & Ahmed (2015). Yet a key question remains unanswered: is *Anabaena* 7120 continuously feeding the plants with N by being

alive or is most or all of the N input due to the degradation of cells and subsequent uptake by the plants, either directly or through turnover in the rhizosphere, or can the effect also be attributed to other factors?

Further complicating the interpretation of these and other published experiments is the fact that *Anabaena* contains not only nitrogen but also phosphorus, carbon, and many micronutrients (as shown on Table 5-1). This issue is particularly apparent in the work of Schreiber et al. (2018) which only considers the phosphorus content of the algal biomass used. Instead, as described here, the focus on a particular nutrient is only used in order to normalize the input.

Understanding whether the biofertiliser effect of *Anabaena* on wheat depends on “live” N-fixation or not will also be important, since it has considerable impact on the choice of methods used for harvesting, processing and storage. To fully understand the cause-and-effect relationships on this matter is not simple but worthwhile to pursue from a translational perspective. Notably, cyanobacteria are only able to accumulate carbon and nitrogen, but they may also influence the availability of phosphorus and possibly also other micronutrients through enzymatic activities. All such activities would depend on both (i) viability and (ii) continued metabolic activity.

Another question of interest is understanding if other factors also will influence the outcome? For example, a global meta-analysis of 171 studies on biofertilisers shows that N-fixers are generally the best inoculants if inoculated along with P solubilisers, as plant growth and biological N fixation are driven by P availability (Schütz et al., 2018).

Following these experiments, field trials at Silwood Park were also attempted to evaluate *Anabaena* fertilisation under more realistic conditions of commercial use, but on two occasions, birds ate the seeds once applied despite the mitigation measures put in place, preventing completion of the intended experiment.

Looking ahead, the financial estimates suggest that the current method of fertilisation with *Anabaena* will struggle to compete with current chemical fertiliser options. Hence, further innovation and optimisation is needed to commercialize such a technology. However, as only one cyanobacterium strain has been tested with one plant crop, under two environmental conditions, a number of other options still remain to be tested.

6

Analysis of contamination in pilot-scale cultures of cyanobacteria

Notes from the author:

Part of this work was done as a collaboration with researchers from A4F (Catarina Cabanas, Diana Gomes and Dr. Tiago Guerra), the University of Cambridge (Dr. Matthew Davey and Prof. Alison Smith), and the Scottish Association of Marine Science (Dr. David Green). The work reported in Figure 6-8, Figure 6-9 and Figure 6-10 was carried out by these collaborators. All the remaining results were generated by myself.

6.1 Introduction

In nature, few if any species live in isolation. Most organisms belong to and interact with other members of evolved and diverse local communities. The development of next-generation sequencing has resulted in a considerable understanding of the microbial ecology in many natural locations (Boughner & Singh, 2016). However, such studies applied to anthropogenic microalgal production systems are scarce, especially at larger scale, and are mostly published in the context of wastewater treatment, but the high nutrient concentration of these systems makes them very different from microalgae production systems (Lian et al., 2018). In contrast, industrial biotechnology assumes or at least aims to be based on single species monocultures. However, no cost-efficient large-scale culture is likely to be axenic and that is particularly true for algal biotechnology. Furthermore, when algae are genetically engineered, their fitness is likely to be reduced, further increasing the opportunity for invasive species to overtake the culture (Sebesta et al., 2022). The presence of additional unintentional species in industrial-scale microalgae cultures, especially those open to the environment, is regarded as a major problem that impacts productivity and adds further to the already high cost of algal biotechnology (McBride et al., 2014). These species are typically labelled “contaminants”, but they have also in some instances been found to benefit algal growth, improve salinity and thermal tolerance, provide both micro- and macro-nutrients, as well as facilitate downstream processing (Lian et al., 2018). It is fair to conclude that there is an overall lack of understanding and appreciation of the impact of microbial co-inhabitants and parasites on the industrial growth of algae (Carney & Lane, 2014), particularly when the strain is engineered for a biotechnological

purpose. Yet, the benefit could be immense. For example, Sapphire Energy, Inc. identified the fungal pest responsible for open ponds culture crashes which allowed them to develop a procedure to identify, track and manage this unwanted contaminant (McBride et al., 2014). Ideally, we could learn from such studies and even adopt unintended neighbours for increased culture robustness. This so-called “synthetic ecology” approach has been hypothesised (Kazamia et al., 2012) but, as far as I know, there is no example where this has been empirically tested at industrial scale. For instance, a 100-L closed photobioreactor, inoculated with *Chlorella vulgaris*, selected from the Great Lakes region, and therefore inherently contaminated (not axenic), was continuously operated for 1 year, and the ratio of algae to bacteria remained stable during the whole period, even when changes in operations were introduced (Cutshaw et al., 2020).

In the past, contaminants found in cultures of wild-type algae have been characterised, but only from small-scale laboratory photobioreactors, and for the first time in 2018, from closed photobioreactors of different volumes, up to 200 L (Fulbright et al., 2018). However, these contaminants were not independently tested directly with the species of interest to determine their impact on growth and production of the compound of commercial interest, except for of a small number of studies from long-term small-scale algal cultures (Cho et al., 2015; Park et al., 2008; Watanabe et al., 2005; Yun et al., 2017).

More recently however, bacteria, isolated from a 25,000-L outdoor raceway pond, in which *Nannochloropsis oceanica*, a high-yielding microalga rich in eicosapentaenoic acid (EPA), was continuously grown for 3 months, were shown to improve both biomass and EPA content by >2-fold when independently co-cultivated with *N. oceanica* at laboratory scale (B. Liu et al., 2020).

The next step forward for algal biotechnology is to employ genetically engineered strains as it will open opportunities to widen commercial opportunities for potentially more sustainable solutions to our resource-hungry world that do not clash with agriculture (Perin & Jones, 2018). Only one publication that studied bioreactors with genetically engineered strains was found, reporting on the analyses of contaminants in an outdoor 6-L culture of *Synechocystis* sp. PCC 6803 engineered to produce ethanol (Zhu et al., 2017). Their analysis informed them how to modify the culture (increase pH) to rescue bioproduction, impaired by an ethanol-scavenging proteobacterium.

In this chapter, one of the first systematic analysis of the ecology of pilot-scale algal cultures (50- and 1,200-Litres) using cyanobacteria engineered to produce a commodity product (ethanol) is reported. The engineered strain was cultured at pilot-scale by A4F, Portugal, an algal biotechnology company partner in the DEMA consortium. Even though A4F has extensive expertise with eukaryotic algae, attempts to culture these engineering cyanobacteria resulted in repeated “crash” (defined as sudden end to growth and/or loss of photosynthetic pigments) or sudden decrease in alcohol concentration. This chapter analysed the contaminants isolated from such cultures and provides an initial understanding of the causes responsible for these cultures and productivity crashes and will allow mitigating measures to be developed in the future.

6.2 Chapter overview

This chapter aimed to understand what caused the culture crashes and/or disappearance of ethanol in the cyanobacteria cultures at A4F. The work was based on the following hypotheses:

1. **Hypothesis 1:** Cultures of *Synechocystis* sp. PCC 6803 UL030 that experience a “crash” or loss of ethanol contain other non-target microbial species,
2. **Hypothesis 2:** The culture “crash” and loss of ethanol are both caused by unwanted microbial contaminants.

The first hypothesis was tested by sampling and characterising microorganisms isolated from the pilot-scale cultures with 16S rRNA sequencing. Subsequent follow-up experiments by our collaborators Dr. Matthew Davey and Dr. David Green utilised 16S rRNA meta-barcoding which we compared with the 16S rRNA sequence data we obtained.

The second hypothesis was answered adding individual isolated contaminants back into axenic cultures of a cyanobacteria engineered to produced ethanol (*Synechocystis* sp. PCC 6803) (section 6.3.2). These cultures were then monitored for photosynthetic growth and ethanol concentration.

6.3 Results

6.3.1 Hypothesis 1: Cultures of *Synechocystis* sp. PCC 6803 UL030 that experience a “crash” or loss of ethanol contain other non-target microbial species

The ethanol-producing *Synechocystis* sp. PCC 6803 UL030 strain (hereafter UL030, described in Lopes da Silva et al., 2018) was cultured three times, inside a contained greenhouse, in A4F’s pilot

units in Lisbon, Portugal. The photobioreactors used were a flat-panel photobioreactor (FP-PBR) with a 50-L working volume (for Tests A and B) and a multilayer horizontal tubular PBR (MHT-PBR) with a 1,200-L working volume (for Test C), as shown in Figure 6-1. Several rounds of successively larger volume pre-cultures were prepared prior to inoculation of the main 50- or 1,200-L cultures (note that details from A4F are not available). The cultivation medium used for all tests was A4F's proprietary undisclosed industrial MMF medium. The pH values were kept between 7 and 8 by automated CO₂ additions, which also function as a carbon source. The maximum temperature set-point was set to 25 °C, however the culture temperatures could temporarily be lower than that setup according to the outside temperature. Homogenization of the cultures was achieved through aeration in Tests A and B, and with a centrifugal pump for Test C. These cultures faced crashes or decrease in ethanol production (described hereafter), and the analysis of contaminants isolated from such cultures could help in explaining what is causing the culture to crash and allow the development of mitigating measures.

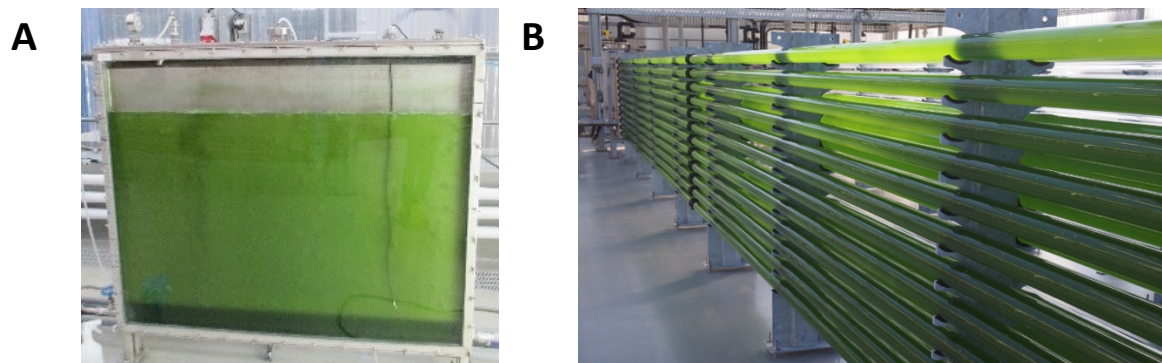


Figure 6-1. Cultivation of the engineered ethanol-producing *Synechocystis* sp. PCC 6803 UL030 at A4F, Portugal.

(A) FP-PBR: Flat-Panel Photobioreactor, 50-Litre working volume. (B) MHT-PBR: Multilayer Horizontal Tubular Photobioreactor, 1,200-Litre working volume.

Whole-culture samples were taken regularly (summarised in Table 6-1) from both photobioreactors pictured in Figure 6-1 during UL030 cultures to monitor biomass production (as dry weight per litre (g/L)), ethanol production and invasive contaminants, all described in section 6.3.3. The samples were collected from a sample valve wiped with ethanol and using sterile equipment (syringe, 50 mL Falcon tubes). All handling was carried out in a laminar flow hood. To obtain a representative picture of the photobioreactor (hereafter PBR) contamination and the maximum number of different culturable strains, each sample taken from the PBRs was streaked out onto two different growth media: NA (Nutrient agar) and TY (Tryptone yeast extract) and incubated at 37 °C for 1-2 days (as described in 2.5.4). Colonies formed on each plate were counted to assess total bacterial counts (CFU/mL) and representative samples from individual colonies were subsequently isolated and sent to Imperial College London for continued analysis (incl. 16S rRNA sequencing). Seventeen isolates were classified according to the following nomenclature:

- plating time (inoculation (i), start of semi-continuous regime (sc), batch regime (b), after neutralisation (n), first heat treatment (ft), end of test (e)),
- media (NA, TY),
- individual isolates (A-P).

Three strains were isolated from the first two Flat-Panel cultures whilst 11 different strains were isolated from the large tubular reactor.

Individual uniform colonies isolated from each culture were transferred into liquid cultures, cryo-preserved and subjected to 16S rRNA sequencing (described respectively in 2.5.5 and 2.7.7). Briefly, DNA was extracted from each culture and subjected to PCR using primers adhering to the conserved but variable region of the 16S rRNA gene (V4). Gel-purified PCR amplicons were cleaned using the QIAquick Gel Extraction Kit (Qiagen) and submitted for sequencing by Source BioScience (UK).

Table 6-1 summarises the analysis of each of the individual isolated contaminants, as well as the observations made by A4F at the time of isolation. Species identification through 16S rRNA sequencing was possible with 13 out of 17 isolates (i.e. with >80% BLAST query hit). Despite unique morphologies and colours, those 13 isolates were identified as 7 or 8 species (genus level, with >80% BLAST query hit), representing only 3 phyla (*Proteobacteria*, *Bacteroidetes* and *Actinacteria*).

A4F Test#	Photo-bioreactor	Contaminant code	A4F contaminant code ¹	Blast hit (>80% query cover)	Cultivation day	A4F observation on the day of contaminant isolation
Test A	Flat Panel PBR	Conta1	iTYA	<i>Porphyrobacter</i> sp.	0	Colony isolated from PBR inoculation day
		Conta2	scTYB	<i>Sphingomonas</i> sp. or <i>Blastomonas</i> sp. ²	22	-
		Conta3	scTYC		30	After this day, the ethanol concentration started to decrease until no ethanol was detected
Test B		Conta4	bTYD	<i>Flavobacterium</i> sp.	13	Colonies isolated the day after the maximum ethanol concentration was reached in the reactor. Afterwards, the ethanol concentration started to decrease until no ethanol was detected in the culture
		Conta5	bNAD			
		Conta6	bTYE	No hit		
Test C	Tubular Multilayer Horizontal Multilayer PBR	Conta7	nNAG	<i>Pseudoxanthomonas</i> sp.	0	Colonies isolated after chemical disinfection of PBR
		Conta8	nNAM	No hit		
		Conta9	nNAJ	<i>Pseudomonas</i> sp.		
		Conta10	nTYK			
		Conta11	nNAL	0	Colonies isolated after heat disinfection of PBR	
		Conta12	ftNAH			
		Conta13	ftNAI	0	Colony isolated immediately after PBR inoculation	
		Conta14	iNAF			No hit
		Conta15	eNAN	<i>Microbacterium</i> sp.	22	Colonies isolated at the end of the cultivation
		Conta16	eNAO			
Conta17	eNAP	<i>Ochrobactrum</i> sp.				

Table 6-1. Contaminants identified by 16S rRNA sequencing at ICL in the different ethanol-producing pilot-scale cultures at A4F, at different time points of the cultivations.
Contaminants in bold were selected for further study.

¹Isolates classified by A4F according to plating time (i-: inoculation; sc-: start of semi-continuous regime; b-: batch regime; n-: after chemical PBR disinfection and neutralisation; ft-: after first heat PBR disinfection; e-end of the test), media (TY or NA), isolates (letter: A – P).

²Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

6.3.2 Hypothesis 2: The culture “crash” and loss of ethanol are both caused by unwanted microbial contaminants

Large-scale cultures are difficult, if not impossible, to replicate and the resources required to operate each test run means that it is not possible to include replicates or controls in parallel. Hence, to better understand the impact of the microbial contaminants isolated from the PBRs on the ethanol productivity of the biotechnological system, representative cultures at the laboratory scale level were reconstituted, by spiking UL030 shake flask cultures with selected contaminants.

Six heterotrophic bacteria (highlighted in bold in Table 6-1, isolated from Tests B and C) were selected and independently co-cultivated in BG-11 media with the ethanol-producer UL030 under controlled laboratory conditions (as described in 2.5.7). Note that A4F were not able to share their MMF media (confidentiality). The aim was to evaluate their impact on cyanobacterial growth and ethanol production. As a control, UL030 was also grown axenically. All cultures were carried out in triplicate, with the same starting density of *Synechocystis* and contaminant at the beginning (with a *Synechocystis*:contaminant ratio of 1:1; OD₇₃₀ = 0.01). All pre-cultures of *Synechocystis* were regularly confirmed to be lacking contaminants by plating on LB agar plates incubated for 7 days at 37 °C.

During each culture, the total biomass was followed with OD measurements at 730 nm, as shown in Figure 6-2. Most of the co-cultures with the contaminants accumulated biomass (conta4, conta5, conta7 and conta16), indicating that there was no major interaction between the ‘contaminant’ and the cyanobacterium. The exception were co-cultures with conta13 (*Pseudomonas* sp., bleaching of the culture) and conta17 (*Ochrobactrum* sp., after 5 days the culture was visually affected).

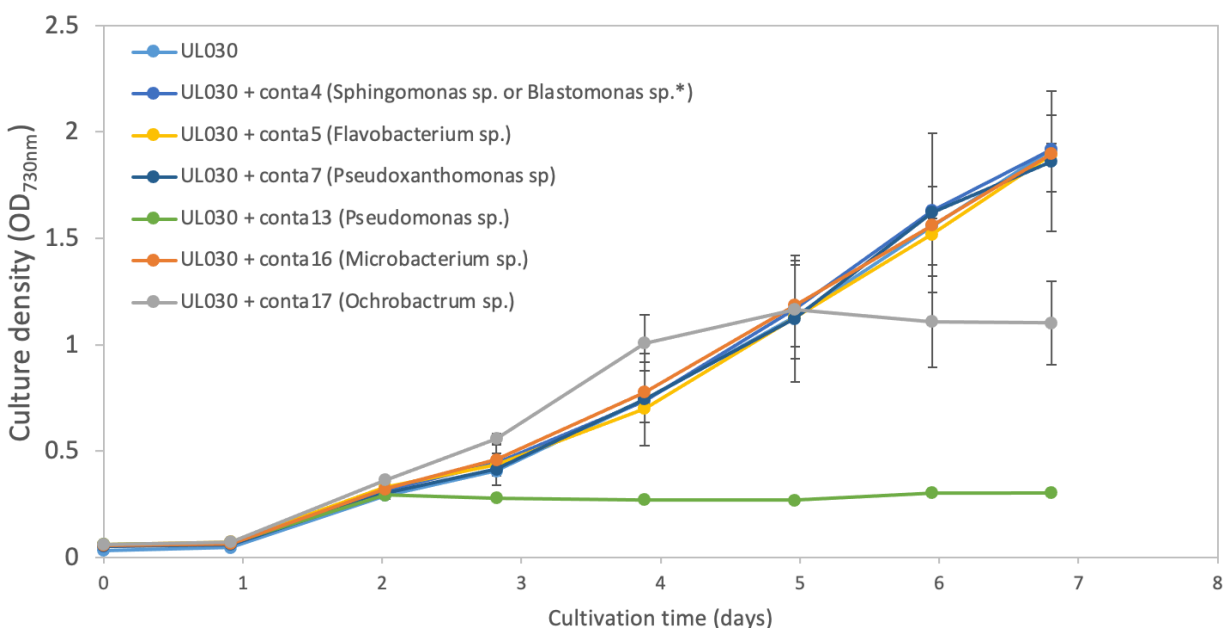


Figure 6-2. Evolution of the total bacterial biomass over time.

Total bacterial biomass measured by OD at 730 nm over time, of the different co-cultures (axenic UL030 and UL030 in co-cultivation with conta4 (*Sphingomonas* sp. or *Blastomonas* sp.*), conta5 (*Flavobacterium* sp.), conta7 (*Pseudoxanthomonas* sp.), conta13 (*Pseudomonas* sp.), conta16 (*Microbacterium* sp.) and conta17 (*Ochrobactrum* sp.), grown in BG-11 and inoculated with a 1:1 ratio (*Synechocystis*:contaminant). Error bars display standard deviation of the mean from two replicates.

*Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

To specifically determine the impact of the contaminants on the growth of UL030, daily chlorophyll *a* extractions were performed in order to follow the growth of the cyanobacterium

UL030 (as described in 2.7.6). A linear correlation was found between the chlorophyll *a* content and the concentration of UL030 (Figure 6-3). The experiment was done in both water and in suspension together with a contaminant, in order to verify that the contaminant's presence did not interfere with chlorophyll *a* extraction. The result suggests that the quantity of extracted chlorophyll *a* could be used as a direct indicator of the growth of UL030 even when co-cultured together with heterotrophic bacteria.

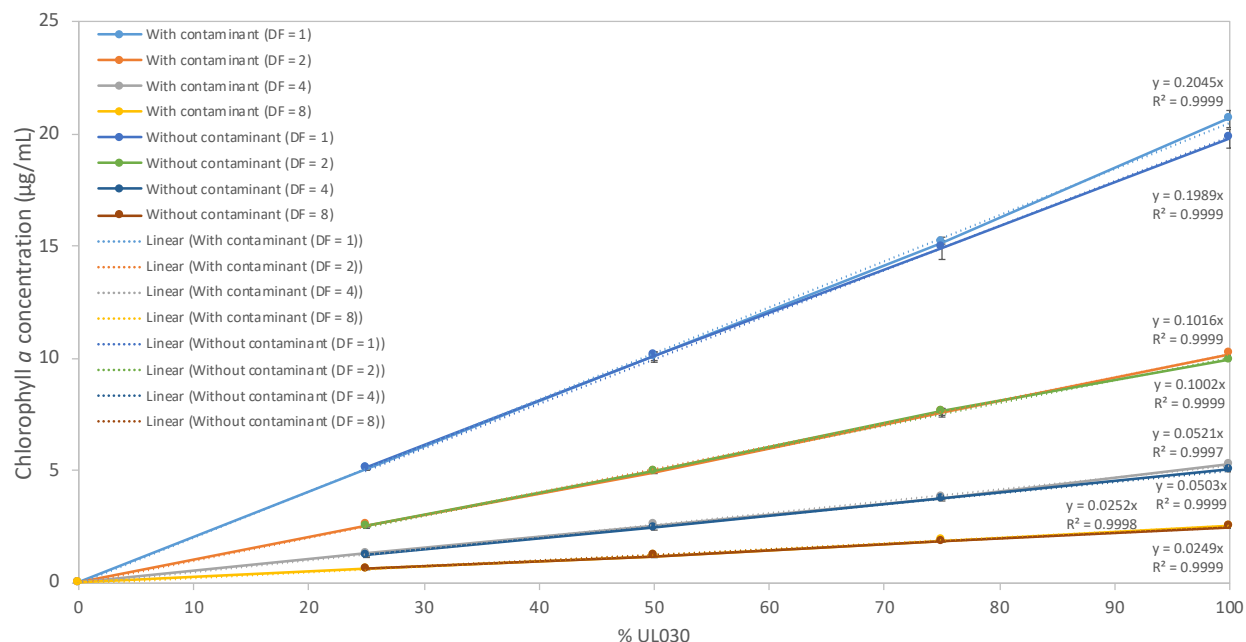


Figure 6-3. Chlorophyll *a* content can be used as an indicator of *Synechocystis* UL030 growth when co-cultivated with an heterotrophic bacterium.

Synechocystis UL030 was mixed with either a contaminant (conta17 (*Ochrobactrum* sp.) or water at different proportion (0-100%), at various dilution factors (DF), and the chlorophyll *a* content of the different suspension extracted and quantified. The dotted lines represent the linear correlation between the Chlorophyll *a* content and UL030 concentration in suspension.

Error bars display standard deviation of the mean from two replicates.

The chlorophyll *a* measurements plotted in Figure 6-4 confirmed that the growth of UL030 is not impaired by some of the contaminants (conta4, conta5, conta7 and conta16). However, conta13

(*Pseudomonas* sp.) and, to a lesser extent, conta17 (*Ochrobactrum* sp.), directly affected the growth of UL030, explaining the lower total biomass measured at an optical density of 730 nm.

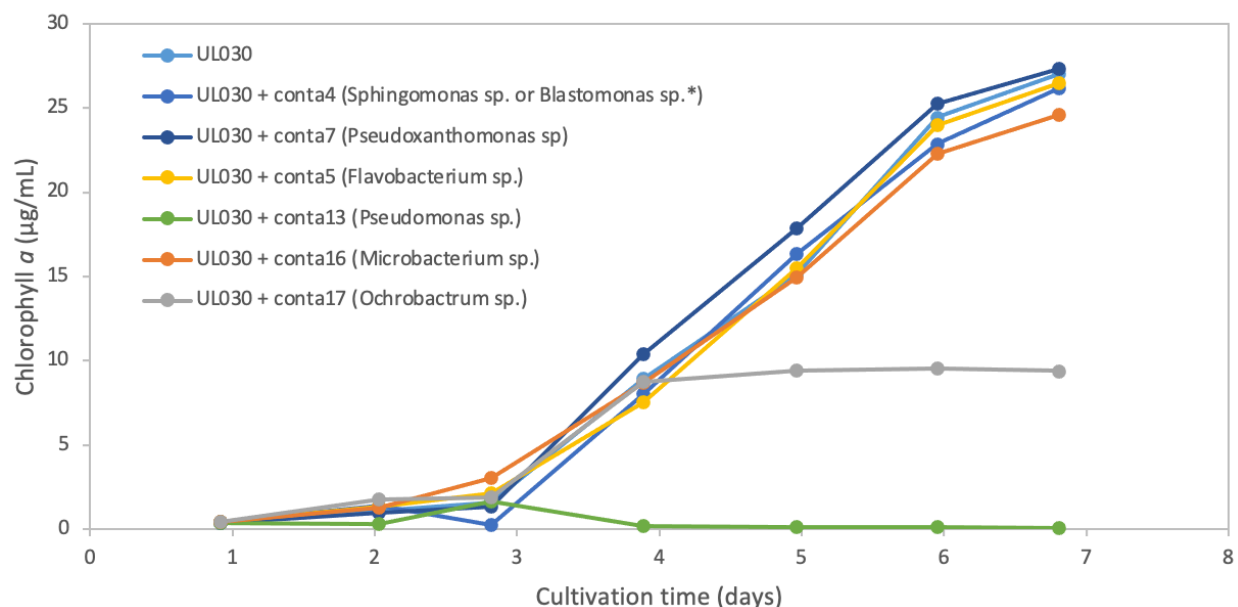


Figure 6-4. Impact of contaminants on *Synechocystis* UL030 growth.

Growth of the cyanobacteria UL030, followed by the chlorophyll *a* concentration, over time, of the different co-cultures (axenic UL030 and UL030 in co-cultivation with conta4 (*Sphingomonas* sp. or *Blastomonas* sp.*), conta5 (*Flavobacterium* sp.), conta7 (*Pseudoxanthomonas* sp.), conta13 (*Pseudomonas* sp.), conta16 (*Microbacterium* sp.) and conta17 (*Ochrobactrum* sp.)) in BG-11 conditions. Standard deviation (SD) were minimal, thus the non-visible error bars. Error bars: $\pm$ SD, $n = 3$.

*Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

Given algal productivity was shown to be dependant on the initial concentration of bacterial inoculation (B. Liu et al., 2020), two of these contaminants were tested with varying initial ratios of UL030 relative to the contaminant itself (from 2 to 4 times more cyanobacteria than contaminant). Conta13 (*Pseudomonas* sp.) and conta5 (*Flavobacterium* sp.) were selected as they have opposite effects on the growth of UL030: the first preventing it, and the latter not. Results, plotted on Figure 6-5, show that these varying initial concentrations do not change the previous outcome. However, it would be interesting to replicate this experiment with smaller or greater

ratios: in the phycosphere, there are 100 to 1,000 more bacteria than microalgae, and synthetic co-cultivation experiments have been tried with bacteria to microalgae ratios varying from 0.3 to 100 (B. Liu et al., 2020).

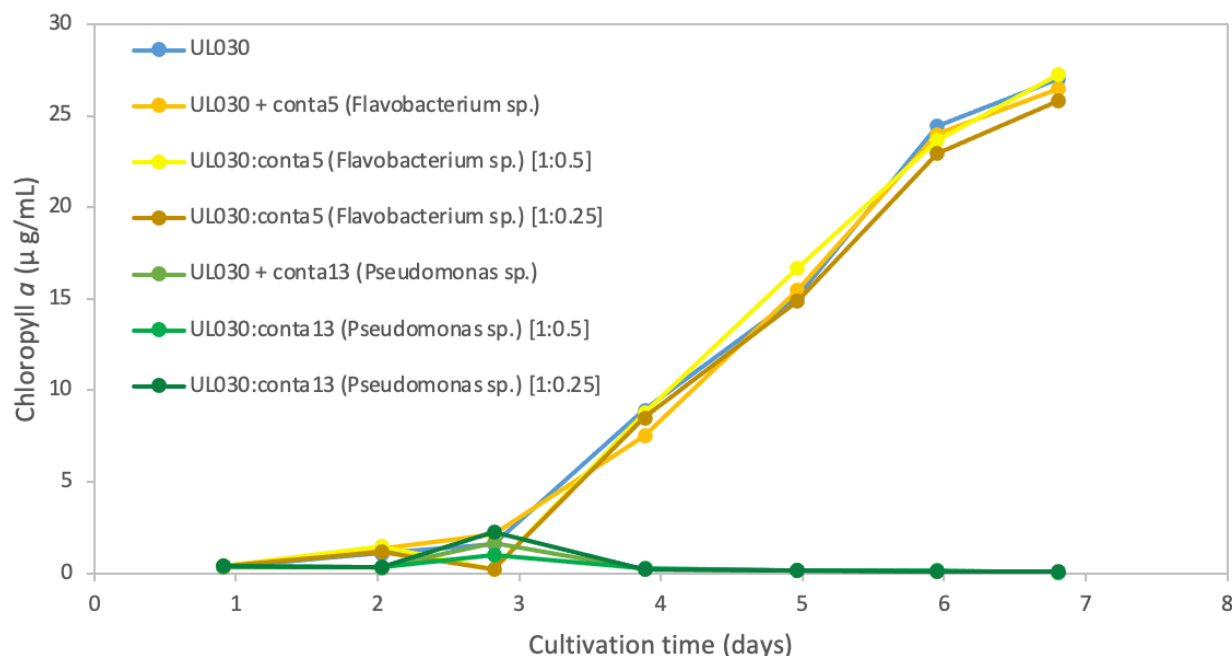


Figure 6-5. Impact of contaminants on *Synechocystis* UL030 growth, with varying initial ratios.

Growth of the cyanobacteria UL030 in shake flasks, indicated by the chlorophyll *a* concentration, over time, of the different co-cultures (axenic UL030 and UL030 in co-cultivation with conta5 (*Flavobacterium* sp.) and conta13 (*Pseudomonas* sp.) in BG-11 conditions. Different initial ratios were tested, varying from 2 to 4 times more cyanobacteria than contaminant.

Standard deviation (SD) were minimal, thus the non-visible error bars. Error bars: $\pm$ SD, $n = 3$.

In parallel, each contaminant was grown axenically to verify its viability after the several washings done prior to the co-cultivation implementation (necessary to remove the rich media of the contaminants preculture, as the media used in the co-culture experiment (BG-11, containing no carbon source) is different). Each contaminant was inoculated in fresh rich media at the beginning of the co-culture experiments. Their growth was monitored (OD at 600 nm) for 35 hours. All

selected contaminants grew (Figure 6-6), though conta4 and conta7 had a longer lag phase compared to the other contaminants.

Importantly, all of these contaminants display a quicker growth rate than UL030 (Figure 6-4), meaning that they can potentially outgrow the cyanobacteria when present in the culture.

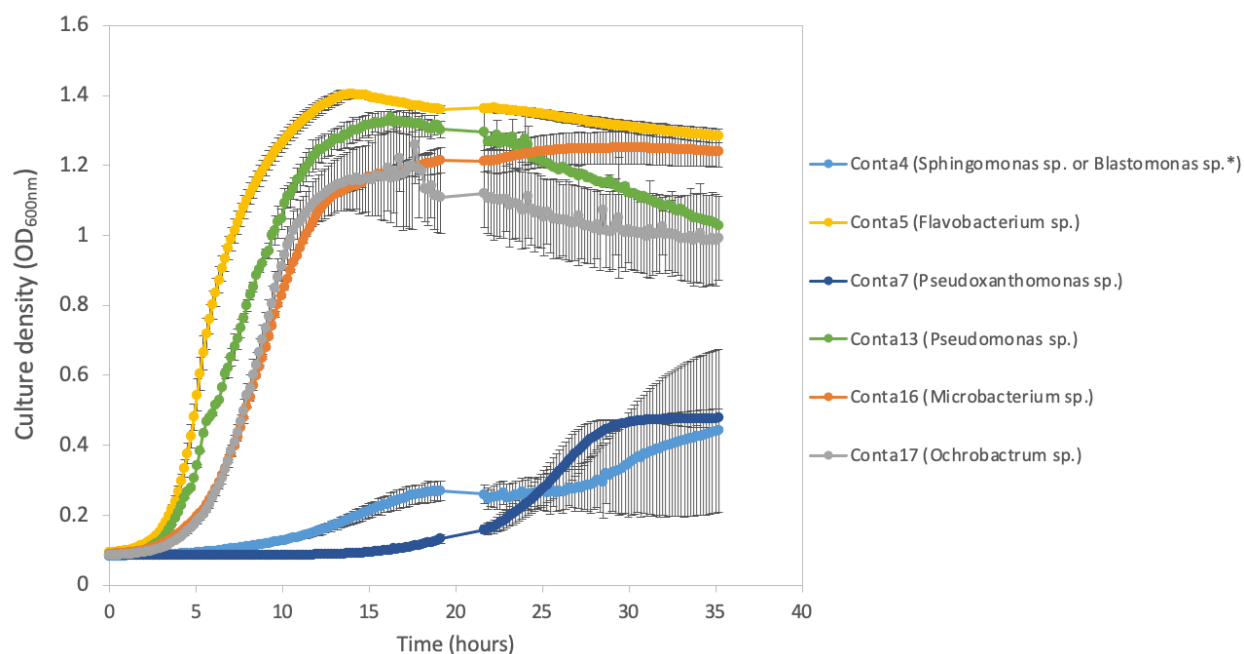


Figure 6-6. Growth of each contaminant in rich media, over 36 hours microtiter plate reader.

Growth (37 °C, 200 rpm) was carried out in a Tecan M200 microtiter plate reader. Each contaminant was also grown separately in the relevant rich media (LB or Tryptone Yeast) to make sure their growth was not impacted by the preparatory washings.

Error bars display standard deviation of the mean from two replicates.

*Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

After 7 days of cultivation, the ethanol titre in samples of the media was quantified by HPLC, as described in 2.7.4. results are shown on Figure 6-7. Results indicate that the accumulation of ethanol was significantly (up to 30x) lower or completely impaired when UL030 is cultivated together with all the contaminants, at all the tested ratios.

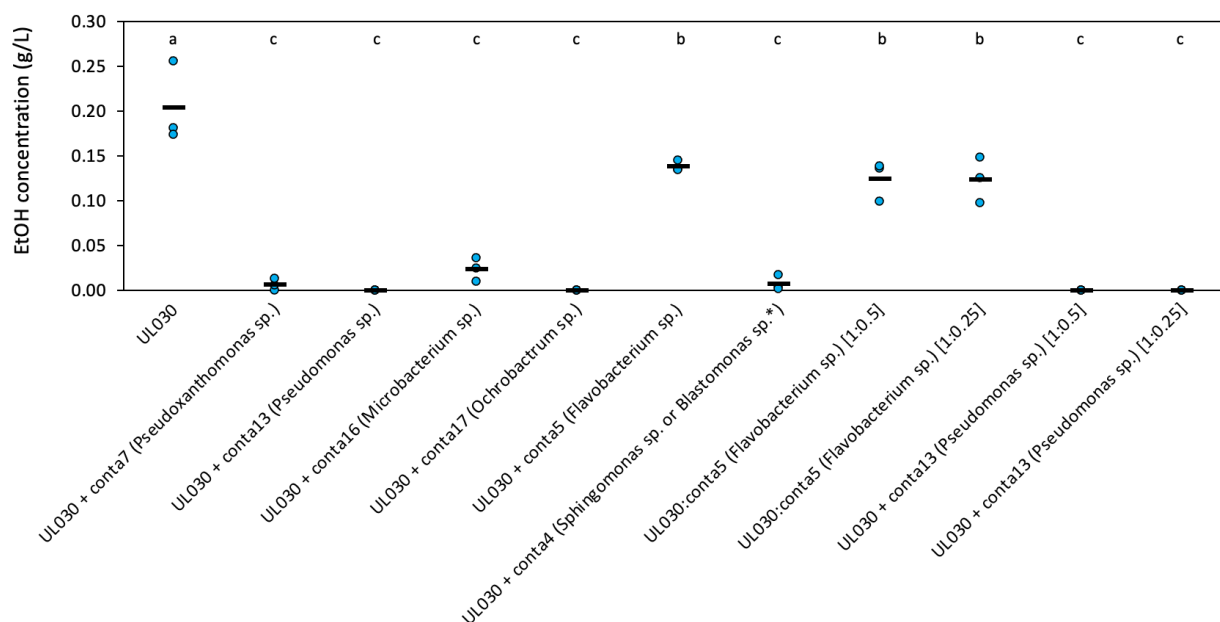


Figure 6-7. Ethanol concentration measured in the media after 7 days of cultivation.

Synechocystis UL030 was grown axenically or in co-culture with a contaminant at a *Synechosystis*:contaminant ratio of 1:1 (conta4 (*Sphingomonas* sp. or *Blastomonas* sp.*), conta5 (*Flavobacterium* sp.), conta7 (*Pseudoxanthomonas* sp.), conta13 (*Pseudomonas* sp.), conta16 (*Microbacterium* sp.) and conta17 (*Ochrobactrum* sp.)) or different initial ratios (with conta5 (*Flavobacterium* sp.) and conta13 (*Pseudomonas* sp.)). The ethanol concentration quantified after 7 days by HPLC.

Circles and bards represent individual values and means, respectively (n=3). Means which do not share the same letter e.g. a, b, etc. are significantly different ($P < 0.05$, one-way ANOVA).

*Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

Table 6-2 summarizes the outcome of the reconstitution experiments, distinguishing between 3 categories of contaminants:

- Contaminants with no impact on UL030 growth but negatively impacting the ethanol produced (conta4, conta7 and conta16). The most likely explanation why these three isolates influenced the ethanol titre without affecting the growth of UL030, is that they utilised it as a substrate,
- Contaminants with impact on both UL030 growth and ethanol production (conta13 and conta17). Hence it is possible that both affected the growth of UL030 and/or that they

consumed the ethanol, by outgrowing UL030, competing for nutrients or are pathogenic, provoking UL030 cell lysis. Note that decreasing the ratio of UL030:conta13 to 100:25 (%OD₇₃₀) did not change this outcome in terms of growth and ethanol production of UL030 (Figure 6-5).

Table 6-2. Summary of the contaminants isolated from A4F's pilot-scale photobioreactor cultures and their impacts on both the growth and ethanol production of UL030.

Contaminant code	Blast hit (>80% query cover)	Impact on UL030 growth	Impact on UL030 ethanol production
Conta4	<i>Sphingomonas</i> sp. or <i>Blastomonas</i> sp.*	●	●
Conta5	<i>Flavobacterium</i> sp.	●	●
Conta7	<i>Pseudoxanthomonas</i> sp.	●	●
Conta13	<i>Pseudomonas</i> sp.	●	●
Conta16	<i>Microbacterium</i> sp.	●	●
Conta17	<i>Ochrobactrum</i> sp.	●	●

The red colour means that the contaminant has a negative impact on either the growth or ethanol production of UL030, contrarily to the green colour.

*Genus could not be determined, this contaminant belong to the family *Sphingomonadaceae*.

6.3.3 Comparison with the pilot-scale cultures

The experiments described in above sections aimed to understand the impact of each contaminant on UL030 growth and ethanol production. Although only a single contaminant was tested in each trial, the results help explain the reasons behind the issues observed when A4F attempted to culture ULO030 at pilot-scale. In the light of these results, each of the 3 pilot-scale cultures performed at A4F was re-examined. Indeed, these pilot-scale cultures, whose growth

and ethanol production were monitored all along by A4F, suffered from either culture crashes and/or a decrease in ethanol accumulation.

To determine cell density and ethanol concentration of the pilot-scale cultures, several samples were taken at regular intervals throughout cultivation by A4F, Lisbon, Portugal. Biomass growth was observed in all cultures with similar productivities ($3 \text{ g}_{\text{DW}}/\text{m}^2/\text{day}$). Notably, ethanol accumulation was only observed in the first two 50-L flat-panel cultures (Tests A and B), with a maximum productivity of 240 mg/L (total ethanol accumulated in the supernatant at the time of sampling) obtained in Test B, whilst no ethanol accumulated in the 1,200-L tubular reactor culture (Test C). In all tests, the decrease in ethanol concentration in the culture occurred simultaneous with an increase in bacterial load.

Additionally, 16S rRNA meta-barcoding was carried out with some samples from A4F pilot-scale cultures, in order to obtain an understanding of the culture composition and determine the key species responsible for reduced ethanol accumulation (as described in 2.7.9). The 16S rRNA meta-barcoding was carried out by Dr. David Green (Scottish Association of Marine Science, United Kingdom), Dr. Matthew Davey and Prof. Alison Smith, Department of Plant Sciences, University of Cambridge, United Kingdom.

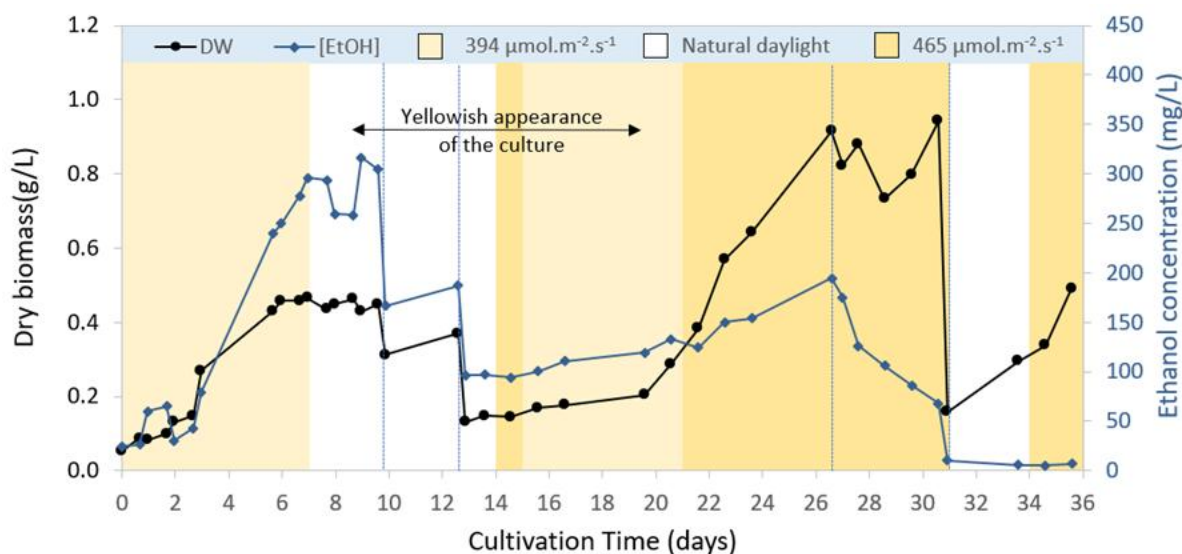
The below paragraphs, analysing each pilot-scale culture subsequently, were compiled using the results described in the previous section, as well as data and observations from A4F, Dr. David Green and Dr. Matthew Davey, which have been re-evaluated by the author of this thesis.

6.3.3.1 A4F Test A

Figure 6-8 describes the evolution of the first pilot-scale culture carried out by A4F in the Flat-Panel Photobioreactor for 36 days (Test A shown in Table 6-1). Note that the cultivation was performed by A4F, and the graph on panel A (Fig. 6-8) was created by A4F as no raw data were shared. Biomass growth was linear over time, except from day 7 to 20 during which the culture had a yellowish appearance (panel A, Figure 6-8). The dry weight of the biomass reached the highest value in all three cultivations at day 30 (~1 g/L (total ethanol accumulated in the supernatant at the time of sampling)), whilst the ethanol concentration increased during the first 12 days (with a maximum of ~300 mg/L at day 9), and at lower rate, until day 26, when it dropped to finally becoming negligible from day 30 onwards, despite the increasing biomass dry weight. Contaminants were observed by microscopic observation from cultivation day 6 and increased in number until the end of the experiment. The bioreactor culture was sampled for plating of contaminants at cultivation days 0, 22 and 30, and 3 invasive species were isolated and subsequently identified by 16S rRNA sequencing (Table 6-1):

- Conta1 at day 0: *Porphyrobacter* sp.,
- Conta2 and conta2, at days 22 and 30: *Sphingomonas* sp. or *Blastomonas* sp. (the genus could not be determined, however this contaminant belongs to the family *Sphingomonadaceae*).

A



B

		Cultivation Day						
Phylum	Genus	6	10	22*	30*	30*	36	36
Cyanobacteria	<i>Synechocystis</i> PCC 6803	728154 (99.3)	349488 (47.1)	610795 (77.6)	88916 (13.7)	78960 (12.1)	100724 (12.1)	149336 (24.6)
Bacteroidetes	<i>Chryseobacterium</i>	1137 (0.2)	478 (0.1)	411 (0.1)	51 (0.0)	1430 (0.2)	580275 (69.8)	59 (0.0)
Proteobacteria	<i>ANPR**</i>	202 (0.0)	54 (0.0)	71 (0.0)	4 (0.0)	433 (0.1)	79547 (9.6)	24 (0.0)
Proteobacteria	<i>Blastomonas</i>	39 (0.0)	82 (0.0)	352 (0.0)	432429 (66.6)	457070 (66.9)	724 (0.1)	376962 (62.1)
Proteobacteria	<i>Porphyrobacter</i>	16 (0.0)	387547 (52.2)	170797 (21.7)	124022 (19.1)	111304 (17.0)	11 (0.0)	76665 (12.6)

Figure 6-8. A4F Test A - Evolution of biomass, ethanol concentration and bacterial diversity as function of cultivation time for *Synechocystis* sp. PCC 6803 UL030 growing in A4F 50-L Flat-Panel Photobioreactor for 36 days.

(A) Evolution of UL030 biomass dry weight (DW) and ethanol concentration as function of cultivation time. The background colours represent the light intensity received by the photobioreactor (white full spectrum LED modules were used to supplement natural light). The vertical blue dotted lines show the manual renewal of the media decided by A4F. Culture was sampled for plating of contaminants at cultivation days 0, 22 and 30. Contaminants were observed by microscopic observation from cultivation day 6 and increased in number until the end of the experiment. The graph provided by A4F, Portugal.

(B) Number of taxonomic assignments (reads) and percentage contributions (in parenthesis) for the main five genus-level assigned OTUs (Operational Taxonomic Units) for 16S SSU rRNA. Percentage contribution values are the mean relative abundance of the taxon in percentage of total sequences. One measurement

was carried from each day of sampling and All values are ranked in descending order of the first sample day values for each test. Dominant species for each sampling day are highlighted in bold. *Same sample day as the 16S study from this thesis (Table 6-1); ** = Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium. Data obtained from Dr. Matthew Davey, University of Cambridge, and Dr. David Green, Scottish Association of Marine Sciences.

16S rRNA meta-barcoding was carried out on days 6, 10, 22, 30 and 36 (panel B, Figure 6-8).

Synechocystis *UL030* was the dominant genus detected on both days 6 and 22 (over 77% of total reads), however *Porphyrobacter*, *Blastomonas* and *Chryseobacterium* OTUs (Operational Taxonomic Units) were dominant (over 52%) on days 10, 30 and 36 (note that technical replicates of day 36 differ). This correlates with A4F observations: UL030 biomass dry weight had indeed been steadily increasing until day 6, whilst the culture displayed a yellowish appearance at day 10, but seemed to be recovering from day 22 (increase of both biomass dry weight and ethanol concentration, though limited for the latter). Days 30 and 36 were also marked by very low ethanol concentration (<0.1 g/L).

On day 10 of the cultivation, the culture had a yellowish appearance, possibly because *Porphyrobacter* was the dominant genus in the culture (52% of the total reads, also isolated on agar plate at the start of the cultivation). However, this contaminant was also significantly present on both days 22 and 30 (~19% of total reads), during which UL030 biomass dry weight was increasing, suggesting that at low concentration (<22% of the total reads), the contaminant has no impact, whereas at high concentration, this contaminant bacteria has the potential to severely impact the culture. Note that *Porphyrobacter* species, isolated from a 25,000-L outdoor raceway pond with the target species *N. oceanica*, was shown to improve the growth of the algae by 2.53 in laboratory co-culture compared to the axenic control (B. Liu et al., 2020).

Blastomonas was present at a negligible level on day 22 (352 reads, accounting for 0% of total reads), but became the dominant genus (>62%) on both days 30 and (possibly) 36. As a contaminant from either the *Sphingomonas* or *Blastomonas* was isolated on agar plate (conta2 and conta3) both on days 22 and 30 (Table 6-1), the meta-barcoding result suggests that the contaminant isolated on agar plate at days 22 and 30 is therefore likely to belong to the *Blastomonas* genus.

Days 22 and 30 are both characterised by either a constant level or a decrease in ethanol concentration, whereas the biomass production continued to grow. When inoculated with UL030, conta4 (*Blastomonas* sp.) affected UL030's ethanol production without impacting its growth (Table 6-2), suggesting that the reason for the decrease in ethanol concentration in the A4F pilot-scale reactor could be that this strain becomes dominant in the later stages of the culture. Another possibility is that *Chryseobacterium* sp. (as identified with the 16S rRNA meta-barcoding), or that others not successfully characterised contaminants are also able to consume ethanol but do not greatly influence the growth of UL030.

6.3.3.2 A4F Test B

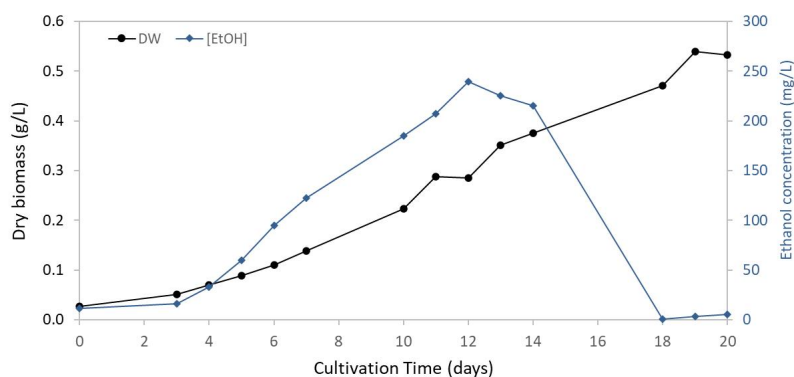
Figure 6-9 describes the evolution of the second pilot-scale carried out by A4F in the Flat-Panel Photobioreactor (Test B shown in Table 6-1). Note that the cultivation was performed by A4F, and the graph on panel A (Fig. 6-9) was created by A4F as no raw data were shared. Biomass growth was linear with time, but ethanol concentration suddenly dropped at day 12 (panel A,

Figure 6-9). Contaminants were observed by microscopic observation from cultivation day 12 and increased in number until the end of the experiment.

After reaching the maximum ethanol concentration in the culture, three invasive species were isolated (at day 13), and 2 were identified by 16S rRNA sequencing (Table 6-1):

- Conta4: *Sphingomonas* sp. or *Blastomonas* sp. (the genus could not be determined, however this contaminant belongs to the family *Sphingomonadaceae*),
- Conta5: *Flavobacterium* sp.

A



B

		Day	
Phylum	Genus	3	20
Proteobacteria	<i>Blastomonas</i>	222551 (56.1)	447649 (56.1)
Cyanobacteria	<i>Synechocystis</i> PCC 6803	124993 (31.5)	14170 (1.8)
Proteobacteria	<i>Porphyrobacter</i>	46639 (11.8)	54 (0.0)
Unclassified	Unclassified	2181 (0.6)	4845 (0.6)
Bacteroidetes	<i>Flavobacterium</i>	3 (0.0)	294335 (36.9)
Proteobacteria	<i>Stenotrophomonas</i>	0 (0.0)	34598 (4.3)

Figure 6-9. A4F Test B - Evolution of biomass, ethanol concentration and bacterial diversity as function of cultivation time for *Synechocystis* sp. PCC 6803 UL030 growing in A4F 50-L Flat-Panel Photobioreactor for 20 days.

(A) Evolution of UL030 biomass dry weight (DW) and ethanol concentration as function of cultivation time. Culture was sampled for plating of contaminants at cultivation day 13. Contaminants were observed by microscopic observation from cultivation day 12 and increased in number until the end of the experiment. The graph was provided by A4F, Portugal.

(B) Number of taxonomic assignments (reads) and percentage contributions (in parenthesis) for the main five Genus-level assigned OTUs for 16S SSU rRNA. Percentage contribution values are the mean relative abundance of the taxon in percentage of total sequences. One measurement was carried from each day of sampling. All values are ranked in descending order of the first sample day values for each test. Dominant species for each sampling day are highlighted in bold. Data obtained from Dr. Matthew Davey, University of Cambridge.

16S rRNA meta-barcoding was carried out on days 3 and 20 (Figure 6-9, panel B). Mainly bacterial species (*Porphyrobacter*, *Blastomonas* and *Flavobacterium*) were observed on both days (>87% of total reads), and there were few reads corresponding to cyanobacteria (less than 32%). This

coincides with A4F microscope observations, suggesting many bacteria in the later time point samples.

Porphyrobacter bacteria accounted for 12% of the total reads on day 3, and became negligible on day 20 (54 reads, accounting for <1% of total reads), confirming the hypothesis drawn from A4F's first pilot scale culture (A4F Test A), in which a low concentration (<22%) of this contaminant did not impact the general health of the culture. As in A4F Test A, this contaminant was identified when ethanol accumulated in the culture, suggesting that this contaminant is unlikely to be responsible for the drop of ethanol in the media.

Flavobacterium sp. was also present at day 3, yet its presence was negligible (3 reads, accounting for 0% of total reads), but on day 20 it accounted for 37%. This contaminant was also isolated on day 13 (contaminant 5) on agar plate, suggesting its presence all throughout the cultivation. In laboratory co-cultivation experiments, *Flavobacterium* sp. was found to consume ethanol (Table 6-2).

The dominant genus was *Blastomonas* (>56%) on both days 3 and 20. As a contaminant from either the *Sphingomonas* or *Blastomonas* genus (belonging to the family *Sphingomonadaceae*) was isolated on agar plate at day 13 (Table 6-1), the meta-barcoding outcome suggests again that the contaminant isolated on agar plate at day 13 is likely to belong to the *Blastomonas* genus.

These latter two bacteria (*Flavobacterium* sp. and *Blastomonas* sp.), co-cultivated under controlled laboratory conditions with UL030, did not affect cyanobacterial growth (Figure 6-4), and both showed a clear impact on the cyanobacterial ethanol production (Table 6-2). Even though *Blastomonas* was the dominant Genus (>56%) on both days 3 and 20 (Figure 6-9, panel

B), the ethanol concentration was steadily increasing until day 12, and dropped suddenly. This contaminant is therefore unlikely to be the reason behind the drop of ethanol in the pilot-scale cultivation, though it might have prevented a higher ethanol concentration during the first 12 days of the cultivation. Another possible explanation for the sudden drop of ethanol in the photobioreactor could be the increase in the concentration of the *Stenotrophomonas* genus (from 0% to 4.6% of total reads between days 3 and 20), whose presence even at low concentration could be sufficient to scavenge ethanol in the media (assuming it grows on ethanol).

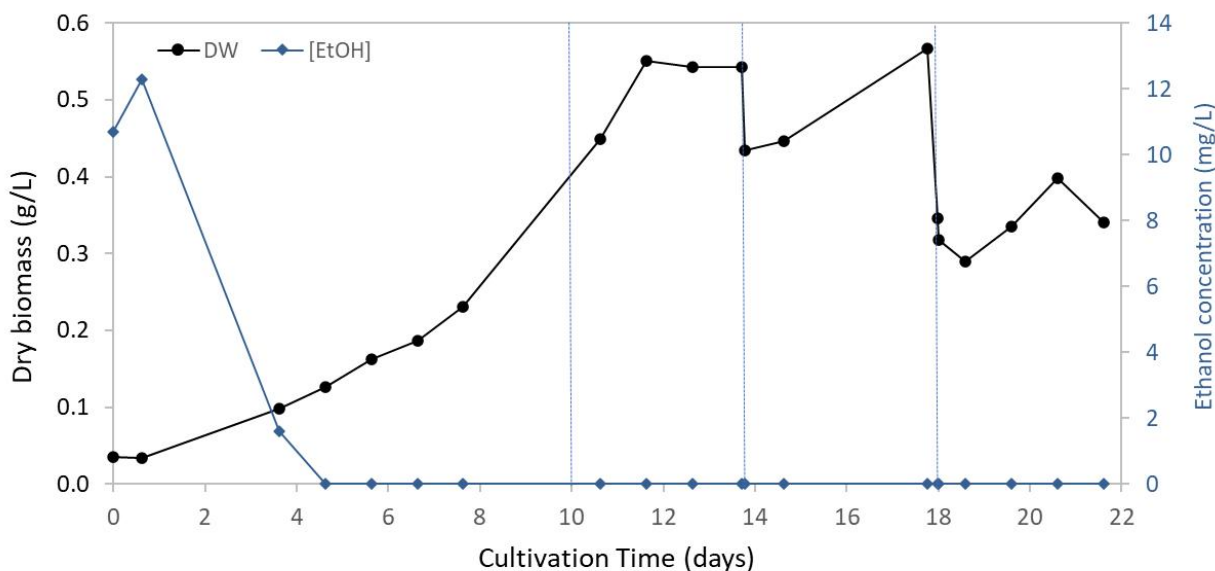
6.3.3.3 A4F Test C

Figure 6-10 describes the evolution of the first pilot-scale carried out by A4F in the 1,200-L Multilayer Horizontal Tubular Photobioreactor (Test C shown in Table 6-1). Note that the cultivation was performed by A4F, and the graph on panel A (Fig. 6-10) was created by A4F as no raw data were shared. Compared to Test B (in Flat-Panel Photobioreactor), the dry weight biomass of UL030 reached similar levels (~0.5 g/L) in ~12 days (Figure 6-9, panel A), despite a plateau between day 12 and 14. From day 21, the culture started to display a yellowish appearance, suggesting an imminent collapse of the culture. Importantly, almost no ethanol was detected. The maximum ethanol concentration was 12 mg/L on day 1, thereafter reaching undetectable levels from day 5 and onward. In contrast, an ethanol concentration of 304 mg/L and 239 g/L was observed in Tests A and B, respectively. Contaminants were observed by microscopic observation since the beginning of cultivation and increased in number until the end of the experiment. The media was filtered on day 13, in an attempt to get rid of the contaminants.

Samples for plating of contaminants were taken on the first day of cultivation (after different sterilisation methods, as well as immediately after the PBR inoculation) and at the end of the cultivation (day 22). In total, 11 invasive species were isolated and 9 were identified by 16S rRNA sequencing (Table 6-1):

- Conta7 at day 0: *Pseudoxanthomonas* sp., isolated after the chemical disinfection of the PBR (day 0),
- Conta9 to conta13 at day 0: *Pseudomonas* sp. (conta9 to conta11 were isolated after the chemical disinfection of the PBR, whilst conta12 and conta13 were isolated after heat sterilisation of the PBR),
- Conta15 and conta16 at the end of the cultivation: *Microbacterium* sp.,
- Conta17 at the end of the cultivation: *Ochrobactrum* sp.

A



B

		Cultivation day		
Phylum	Genus	14	15	22*
Bacteroidetes	<i>Chryseobacterium</i>	196525 (56.4)	144 (0.0)	62647 (7.9)
Cyanobacteria	<i>Synechocystis</i> PCC 6803	65532 (18.8)	584432 (99.3)	45747 (5.7)
Proteobacteria	<i>ANPR**</i>	45542 (13.1)	31 (0.0)	30202 (3.8)
Proteobacteria	<i>Methyloversatilis</i>	11630 (3.3)	3 (0.0)	86977 (10.9)
Proteobacteria	<i>Tistrella</i>	1970 (0.6)	126 (0.0)	428219 (53.7)

Figure 6-10. A4F Test C - Evolution of biomass, ethanol concentration and bacterial diversity as function of cultivation time for *Synechocystis* sp. PCC 6803 UL030 growing for 22 days in A4F Multilayer Horizontal Tubular Photobioreactor.

(A) Evolution of UL030 biomass dry weight (DW) and ethanol concentration as function of cultivation time. Culture was sampled for plating of contaminants at cultivation day 0 and 22. The vertical blue dotted lines show the addition of nutritive medium. The second one was combined with a filtration of the medium. Contaminants were observed by microscopic observation since the beginning of cultivation and increased in number until the end of the experiment. From day 21, the culture started to display a yellow appearance. The graph was provided by A4F, Portugal.

(B) Number of taxonomic assignments (reads) and percentage contributions (in parenthesis) for the main five Genus-level assigned OTUs for 16S SSU rRNA. Percentage contribution values are the mean relative abundance of the taxon in percentage of total sequences. Dominant species for each sampling day are highlighted in bold. One measurement was carried from each day of sampling. All values are ranked in descending order of the first sample day values for each test. *Same sample day as the 16S study from this thesis (Table 6-1); ** = *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*. Data obtained from

Dr. Matthew Davey, University of Cambridge, and Dr. David Green, Scottish Association of Marine Sciences.

16S rRNA meta-barcoding was carried out on days 14, 15 and 22 (Figure 6-10, panel B). Mainly bacterial species (*Chryseobacterium* and *Tisterlla*, >53% of total reads) were observed on both days 14 and 22, whilst *Synechocystis* was only dominant on day 15 (99% of total reads). Interestingly, none of these bacterial species were identified by the agar plate streak out method. *Tisterlla*, identified only in this larger volume pilot scale culture, was dominant towards the end of the cultivation (53% of total reads), and could be the reason behind the crash of the culture at the end. *Chryseobacterium* could also be responsible for the lack of ethanol in the media, given this contaminant was possibly also present in A4F Test A in high concentration (70% of total reads) at the end of the cultivation when no ethanol was present.

None of the contaminants isolated and identified with 16S rRNA sequencing (*Pseudoxanthomonas* sp., *Pseudomonas* sp., *Microbacterium* sp. and *Ochrobactrum* sp.) were identified with meta-barcoding at any stage of the cultivation. One explanation for this may lie in the unsuitability of meta-barcoding for untargeted analysis of ecosystems (Cowart et al., 2015).

From the 4 contaminants isolated by the agar plate method, 2 of them negatively impacted cyanobacterial growth (conta13 (*Pseudomonas* sp.) and conta17 (*Ochrobactrum* sp.)), whilst the other 2 had no impact (conta7 (*Pseudoxanthomonas* sp.) and conta16 (*Microbacterium* sp.)) in reconstitution experiments (Figure 6-4). Yet, they all affected the accumulation of ethanol (Figure 6-5). No ethanol was detected during this large-scale cultivation experiment, whilst some biomass accumulation was observed, though it remained low and variable after day 12, possibly

because of the contaminants preventing UL030 growth or consuming the produced ethanol. Given that most of the bacteria identified by the agar plate method were able to prevent ethanol accumulation, most likely through consumption as a reduced carbon source, it is not unreasonable that also other species such as *Chryseobacterium* and *Tisterlla* also would do the same.

To understand the impact of isolated bacteria on the growth of UL030, the relative timing at which they were found should be considered. For example, *Pseudoxanthomonas* sp. (conta7) was isolated right after chemical sterilisation of the MHT-PBR. From the start of the inoculation, UL030 biomass was continuously growing, meaning this contaminant did not significantly hinder UL030, which correlate with the result found in laboratory-controlled experiments.

Similarly, *Pseudomonas* sp. (conta13) was isolated right after heat sterilisation of the MHT-PBR and impacted the growth of UL030 in the laboratory-controlled experiments. Yet, from the start of the inoculation, UL030 biomass did accumulate. One possibility is that conta13 was present at very low concentration from the start, and thereafter started to dominate the culture, finally causing biomass accumulation to plateau from day 10 onwards. The filtration intervention trialled on day 13 seems to have been sufficient enough to recover the culture as the biomass started to grow again, meaning that it could have been successful in removing this and/or other unwanted contaminant(s). Another explanation could be that a more virulent species of *Pseudomonas* contaminated the culture. For example, *Pseudomonas* sp. could also be the reason behind the bleaching of the culture at day 22.

At the end of the culture, which saw a decrease of cyanobacterial biomass concentration, two other different contaminants were isolated, *Microbacterium* sp. (conta16) and *Ochrobactrum* sp. (conta17). Whilst the first one did not affect the growth of UL030 in the reconstitution experiments, the second one did, meaning that in the pilot-scale cultivation, *Ochrobactrum* sp. could be at least partially to blame for the culture starting to crash (yellowish appearance at day 21).

6.4 Discussion

The scale-up of any microbial biotechnological process is well-known to be costly and time-consuming, due to a number of challenges including contamination and a lack of performance due to changes in the operating environment (e.g. gas-exchange, mixing) (Crater & Lievense, 2018). The issue of contamination is particularly challenging for phototrophic microorganisms as they grow slower than heterotrophs, and operators are advised to *manage* contamination rather than *prevent* it (Borowitzka & Vonshak, 2017). At the beginning of this collaborative study, the experience of several partners was that the cultures of *Synechocystis* would become contaminated during the scale-up process. Based on the collective insight of the DEMA consortium, we formulated the following hypotheses:

Hypothesis 1: Cultures of Synechocystis sp. PCC 6803 UL030 that experience a “crash” or loss of ethanol contain other non-target microbial species. Seventeen different individual isolates (Table 6-1) were isolated from large-scale algae bioreactors by streaking out PBR samples from 3 different cultures onto two different growth media (NA and TY). Subsequent 16S rRNA

sequencing was successful with thirteen out of seventeen isolates (four isolates could not be sequenced successfully, i.e. <80% BLAST query hit). Subsequent 16S rRNA sequencing was successful with thirteen out of seventeen isolates. Analyses of the sequencing data grouped these thirteen members into three different phylogenetic groups (Proteobacteria, Bacteroidetes and Actinacteria), all of them prokaryotic bacteria (see section 6.3.1).

It is likely that the limited range of culturing methods (i.e. NA and TY) constrained the selection of microorganisms that could be recovered. For example, the isolation of fungi or grazing eukaryotes would require alternative methods. Observations by the A4F bioreactor operators supported the conclusion that bioreactors were or became contaminated. For example:

- Test A (Fig. 6-8), the operator reported "yellowish appearance" and after 14 days there was no further increase in ethanol and none was measured after 28 days,
- Test B (Fig. 6-9), no ethanol was measured after 14 days,
- Test C (Fig. 6-10), no ethanol was measured after 2 days.

Although it was not known what kind of contamination (i.e. bacteria, fungi, etc) was responsible for these observations, high-throughput 16S gene amplicon sequencing with Illumina MiSeq by Cambridge confirmed the presence of a range of different bacteria (often with good match to the 16S rRNA sequencing, e.g. Test B). In some samples there was even a larger number of reads for non-target species than with the target species, *Synechocystis*.

In conclusion, bacteria were recovered in every A4F photobioreactor culture, hence, the hypothesis was confirmed. This doesn't exclude the possibility that bacteria will always be found

in any large-scale bioreactor (Borowitzka & Vonshak, 2017) and that they are not necessarily the cause of the undesirable culture changes that were observed (crash, product consumption).

Hypothesis 2: The culture “crash” and loss of ethanol are both caused by unwanted microbial contaminants. To test hypothesis 2, individual contaminants were re-introduced to an axenic laboratory culture of the engineered ethanol-producing UL030 *Synechocystis* strain and the growth of the cyanobacterium as well as the accumulation of ethanol were monitored over a seven-day period (see 6.3.2).

Only two out of six contaminants influenced the growth of *Synechocystis* (Fig. 6-4). Repeated experiments with varying ratios of *Synechocystis*:contaminant did not result in any different outcomes (Fig. 6-5). Interestingly, the effect of each contamination on ethanol accumulation diverged from that of growth. As summarised in Table 6-2, five of the six tested contaminants influenced ethanol production whilst only two affected the growth of *Synechocystis*, at the tested ratios.

This means that three of those contaminants most likely consumed the ethanol produced by *Synechocystis*, whilst the other two contaminants may have affected ethanol accumulation indirectly, by their effect on *Synechocystis* growth.

In conclusion, the results clearly show that all six tested contaminants were capable of affecting either the growth of *Synechocystis* and/or the accumulation of ethanol, most likely through consumption as a reduced carbon source. We hypothesised that it may be challenging to manufacture any chemical product with a slow-growing phototroph if that chemical also can act

as a substrate for contaminating species. One method of testing this hypothesis in future studies would be to compare scale-up with a wild-type strain that is not engineered to secrete ethanol or other chemicals. An alternative strategy is to implement a procedure to rapidly remove the product once it has been secreted, however, the lower the titre, the higher the energetic cost will be to separate it from water (Pothakos et al., 2018).

A set of contaminants are now ready to be used in experiments that aim to test such strategies, such as the nitrogen-exchange co-culture systems developed in Chapter 4. Such experiments should be considered for future studies as we ran out of time in the present study to complete this initial aim.

Comparisons with the literature

Previously, Watanabe and colleagues (2008) found that the presence of *Sphingomonas spp.*, isolated from a long-term xenic laboratory culture of *Chlorella sorokiniana* had no effect on the growth of the latter. In contrast, the same species found in the phycosphere of *Chlorella vulgaris* in wastewater stimulated the growth of *Chlorella vulgaris* (Cho et al., 2015).

From the latter study, *Flavobacterium* sp. that was also isolated from a *C. vulgaris* phycosphere improved the growth of *Chlorella*, whilst our isolate of the same species (conta5) did not influence the growth, but negatively impacted the ethanol production of UL030, at the tested ratio.

Finally, *Microbacterium* sp., isolated from a long-term xenic laboratory culture of *Chlorella sorokiniana* increased the growth of the latter according to Watanebe et al. (2008), whereas the same species isolated from the soil along with *Chlorella vulgaris* had no effect on the growth of the latter (Thi Vu et al., 2010).

In this present study, four contaminants were isolated and found in reconstitution experiments to have no deleterious impact on the growth of UL030 (belonging to the *Blastomonas*, *Flavobacterium*, *Pseudoxanthomonas* and *Microbacterium* genera, as shown on Fig. 6-4 and 6-5), but all consumed ethanol (Fig. 6-7).

Blastomonas sp., which did not impair the growth of UL030 but did impair ethanol production in the co-cultivation experiments (Table 6-2), was isolated from the two cultivations in the Flat-Panel Photobioreactor (Tests A and B) which both suffered from a sharp decrease of ethanol accumulation (panel A of Fig. 6-8 and 6-9 respectively). Yet, this contaminant was present in both cultures from the start, and even dominant from day 3 in the case of A4F Test B (56% of total reads (panel B, Fig. 6-9)), which saw a maximum of ethanol accumulated at day 12 (panel A, Figure 6-9), suggesting it might not be the one responsible for the loss of ethanol in the cultivation.

However, *Pseudoxanthomonas* sp. and *Microbacterium* sp., respectively isolated before the start and at the end of the cultivation in the Multilayer Horizontal Tubular Photobioreactor, which did not accumulate ethanol (A4F Test C, as shown on panel A, Fig. 6-10), were shown to impact ethanol production in the co-cultivation experiment, without impairing UL030 growth (Table 6-

2). Thus, these 2 contaminants, in particular *Pseudoxanthomonas* sp. isolated before the start of the inoculation, could be using ethanol as a substrate and preventing its accumulation in the photobioreactor. It is also possible that the heat sterilization, carried out after the isolation of *Pseudoxanthomonas* sp., was effective in getting rid of this specific contaminant. None of these two contaminants were identified with the 16S rRNA meta-barcoding. This may be due to limitations with the 16S rRNA meta-barcoding method (Rizal et al., 2020; Lamb et al., 2019).

In this same pilot-scale culture, *Pseudomonas* sp. and *Ochrobactrum* sp., respectively isolated before the start and at the end of the cultivation, were shown to impact both UL030 and ethanol production in the co-cultivation experiments (Table 6-2), suggesting that these contaminant species can simply outgrow UL030 (faster growth rate), compete for nutrients or are pathogenic, provoking UL030 cell lysis. Yet, *Pseudomonas* sp. was likely not present at high concentration (not identified in the 16S rRNA meta-barcoding), given UL030 grew during this test, whereas *Ochrobactrum* sp. could be responsible for the culture crash (yellowish appearance, panel A, Fig. 6-10).

Other species from different genera with a notable presence in the cultivation (>10% of total reads at some points) were identified thanks to the 16S rRNA meta-barcoding:

- *Chryseobacterium*: identified in Test C and possible Test A. In both tests, this contaminant was dominant when there was no ethanol,
- *Porphyrobacter* (conta1): identified in A4F Tests A and B. Significantly present from day 3 in Test B (11.8% of total reads) when ethanol was being accumulated in the culture, it's unlikely to be the contaminant responsible for the culture crash of Test A and the sudden

drop of ethanol from day 13 in Test B, unless it became dominant at this stage and has a strong negative effect,

- *ANPR* species: identified in both A4F Tests A and C, represented lower than 13% of the total reads, but were also present when there was no ethanol in the culture,
- *Methyloversatilis* and *Tisterlla*: both identified in A4F Test C, with the latter becoming dominant towards the end of the cultivation (panel B of Figure 6-9), could be responsible for the lack of ethanol accumulation in this cultivation. *Tisterlla* also completely overcame the culture (54% of the total reads, whilst *Synechocystis* sp. PCC 6803 reads account for less than 6%), which could explain the collapse of the culture (yellowish appearance) at day 22.

Note that none of the isolated contaminants displayed a growth promoting effect, at the tested initial ratios (1:1, 1:0.5 and 1:0.25, in favour of UL030). Nevertheless, it would be important to test these contaminants with different initial ratios. The initial microalgae to contaminating bacteria ratio was indeed shown to impact the overall algae production of *N. oceanica* in co-culture experiments: a low starting concentration of the contaminating bacteria (ratio 1:6 in favour to the bacteria) can have a negative, a neutral or slight positive impact on the growth of the algae *N. oceanica*, whereas a high concentration (ratio 1:60 in favour of the bacteria) drastically enhanced the algal growth, and vice-versa (B. Liu et al., 2020). The same author also mentions the phycosphere in which the ratio microalgae to bacteria can be as high as 1:1000. Ratios of the predator bacteria to cyanobacterial prey have also shown to be a key factor in the biological control of cyanobacterial bloom (at laboratory scale) (Ndlela et al., 2018).

Interestingly, the 16S rRNA meta-barcoding data showed greater abundance of other bacterial reads (non-cyanobacterial) at most of the time points. The phycosphere of UL030 in the A4F pilot-scale tests was dominated by *Proteobacteria* (such as *Porphyrobacter* and *Blastomonas*) and *Bacteroidetes* (*Flavobacterium*, *Chryseobacterium*). This result coincides with previous studies: these phyla were indeed found to be the most abundant in natural marine environment (Fulbright et al., 2018), in cyanobacterial blooms (Ndlela et al., 2018), but also in a 100-L closed PBR culture continuously operated for 1 year with the algae *Chlorella sorokiniana* (Cutshaw et al., 2020), in enclosed PBR cultures of the algae *Nannochloropsis salina* ranging from 5 mL to 200 L (Fulbright et al., 2018), as well as in 25,000-L outdoor raceway pond cultures of *Nannochloropsis oceanica* (B. Liu et al., 2020). *Proteobacteria* are also the most studied biological control agents of cyanobacterial bloom, but only implemented at laboratory scale so far (Ndlela et al., 2018).

Also, *Pannonibacter phragmitetus*, a proteobacterium which has been previously reported to jeopardise the growth of an ethanol-producing *Synechocystis* sp. PCC 6803 in outdoor non-sterilised 6-L photobioreactors as well as completely scavenge the ethanol accumulated in the media (Zhu et al., 2017), was not identified in this study.

Broader implications

Importantly, only bacteria were identified with 16S rRNA sequencing in this study. However, algae, virus, fungi and zooplankton also have the potential to contaminate cultures and provoke undesirable consequences (Zhu et al., 2020). For instance, the microalga *Poterioochromonas* has been reported to feed on *Synechocystis* (Touloupakis et al., 2016).

Characterization of contaminating bacteria in pilot-scale production could allow to prevent their negative impact, as did Sapphire Energy, Inc. with a fungal pest (McBride et al., 2014), or even develop appropriate strategies preventing their apparition. For instance, contaminants without any negative impact could be used as ‘probiotics’, as they are occupying the available ecological niche: bacterial communities obtained from crashed microalgae cultures were found to protect microalgae from rotifer grazing at laboratory scale (Fisher et al., 2019). The benefit of supplementing photosynthetic cultures with such probiotics can also go beyond the contamination management strategy. It could also improve both biomass production and target product productivity. Indeed, bacteria, isolated from a 25,000-L outdoor raceway pond, in which *N. oceanica*, a high-yielding microalga rich in eicosapentaenoic acid (EPA), was continuously grown for 3 months, were shown to improve both biomass and EPA content by >2-fold when independently co-cultivated with *N. oceanica*. However this strategy was only implemented at laboratory scale (B. Liu et al., 2020).

In this study, no bacteria with such probiotic effect were isolated.

Another strategy for improved culture robustness is to modify culture operation. For instance, the ethanol accumulation in an engineered-*Synechocystis* cultivation in outdoor non-sterilised photobioreactors was successfully rescued by increasing the pH, as the ethanol-scavenging proteobacterium was shown to be sensitive to high pH (Zhu et al., 2017), and in this present study, *Proteobacteria* were found the most abundant phylum during the whole cultivation or towards the end of the cultures. Additionally, this high pH strategy was shown to prevent

collapses of *Synechocystis* mass cultures due to a contaminating microalga (Touloupakis et al., 2016).

Another attractive strategy is to utilise a so-called xenobiotic nutrient strategy already demonstrated with terrestrial plants (López-Arredondo & Herrera-Estrella, 2012), eukaryotic algae (Loera-Quezada et al., 2016), heterotrophic bacteria and yeast (Shaw et al., 2016) and cyanobacteria (Motomura et al., 2018). The focus so far has been on using the rare-in-nature molecule phosphite (PO_3^{2-}) as a source of phosphorus (Figueroa and Coates, 2017; Casida, 1960; Metcalf and Wolfe, 1998), instead of the more commonly used phosphate (PO_4^{3-}). *Synechocystis* was previously engineered to grow on phosphite (Polyviou et al., 2015), as well as *Synechococcus*, which was successfully cultured outdoor in non-sterile media and 100-L reactors and 1,000-L raceway ponds (González-Morales et al., 2020).

6.5 Chapter conclusion

Next-Generation Sequencing allowed considerable progress in microbial ecology research, but there is a lack of knowledge regarding the microbiome and its role in anthropogenic microalgae production, especially at larger scale than laboratory production (Lian et al., 2018).

In this study, heterotrophic bacteria co-existing with the species of interest in pilot-scale bioreactor cultures were characterised with 16S rRNA sequencing and in lab-scale reconstitution co-cultures. This led to the identification of contaminants which either consume the biofuel or which negatively influence the growth of the biofuel producing strain, and provided insights as

to why both biomass and ethanol production were highly variable in A4F pilot-scale cultures of UL030.

In the future, this analysis would greatly benefit from the following experiments:

- Obtain the species identified with the 16S rRNA meta-barcoding which are likely to have an impact on the UL030 growth and ethanol production (such as *Chryseobacterium* sp.) from culture collection and test them (as well as conta1 (*Porphyrobacter* sp.)) independently with UL030,
- Test different ratios cyanobacteria:bacteria,
- Test the strategies described above (increase of pH, *Synechocystis* engineered to utilise phosphite as a substrate) in a UL030 culture charged with the deleterious contaminant,
- Charge the co-cultures described in Chapter 4 (once re-established) with the contaminants affecting the growth and the ethanol production: they will serve as the perfect agents to examine the hypothesis that more complex ecological systems have greater robustness.

7

Conclusion

Nitrogen still remains a major problem for global agriculture. In higher-income countries, excessive and inefficient use of nitrogen results in pollution causing both medical and environmental problems, costing tax-payers in the EU more than €100 billion annually (Sutton et al., 2011). Meanwhile, inequitable and insufficient availability of nitrogen in lower-income regions affects the livelihood and opportunities for small-hold farmers. More effort is needed to solve these two interrelated but clearly different challenges.

Few species are naturally capable of biological N_2 -fixation, some cyanobacteria are among them. Although the use of such N_2 -fixing cyanobacteria to stimulate agricultural productivity have been

studied and applied (particularly in rice paddies) for decades, the initial premise of this PhD study was that opportunities to exploit this naturally existing capability should be pursued further.

It can be argued that this topic, at least from a biotechnological perspective, has not been studied exhaustively and that further work is still needed. For example, if we could understand the mechanism(s) by which cyanobacteria stimulate the growth of plants, it would help to select for or even engineer improved traits. This thesis presents one such extension of current knowledge, after which many more can still follow.

In Chapter 3, I developed a Flow Cytometry based method for quantification of a mixture of cyanobacteria species, one unicellular and the other filamentous. Highly effective separation between the two species was possible, enhancing the available information that can be gathered from co-cultures involving filamentous cyanobacteria. The method may also provide an opportunity to accurately quantify the integrity and heterogeneity of filamentous cyanobacteria populations.

In Chapter 4, I developed cyanobacteria-only co-cultures involving *Synechocystis* and *Anabaena*. The former species was engineered to produce ethanol as a proxy indicator for metabolic activity, whilst the second species had the duty of fixing N₂ for both species to survive under N-depleted diazotrophic conditions. Although this series of experiments was plagued by inconsistency and technical challenges, possibly caused by the operator, several developments were achieved. Firstly, some of the *Anabaena* strains were able to support the growth and metabolism of its cognate partner, as could be witnessed through the accumulation of ethanol. This was achieved

either by a mutant lacking ammonia-uptake, or by another mutant with modified regulation of glutamine synthetase. In both cases, this most likely resulted in the N-fixing strain exporting ammonia that was quickly consumed by the partner, as no statistically significant ammonia accumulation was measured. It is also possible that other N-species were released into the culture for *Synechocystis* to consume and future studies would benefit from identifying the molecular nature of N exchange. Secondly, the flow cytometry method proved useful and provided interesting insight into some of the *Synechocystis* populations, as their fluorescence properties appear to have shifted. Overall, the co-culture studies suggest that *Anabaena* can be used to provide nitrogen to other species, be it in bioreactors or for agriculture.

In Chapter 5, the study moved to also include plants. As it had already been observed previously with similar species (Do Nascimento et al., 2015), *Anabaena* was also highly capable of fertilising wheat. Most importantly, the work in this thesis demonstrated for the first time, as far as I am aware, that alive N₂-fixing cyanobacteria are better able to stimulate the growth of a plant, than that which is dead, suggesting but not proving that N₂-fixation may continue even once applied to the soil. Clearly, it is early days yet, and a wide range of other permutations of experimental design and treatments are possible, but the results are promising and deserve to be studied further.

In Chapter 6, I isolated and characterized contaminants from an industrial pilot facility that was culturing similar engineered ethanol-producing *Synechocystis* strains, as was used in Chapter 4. When reconstituted in the laboratory, different bacterial contaminants had different effects on the intended biotechnological application. Some affected the growth of *Synechocystis* whilst

others only consumed the product. Only one contaminant was found that did not interfere substantially with ethanol bioproduction. The aim with the contaminant study was to identify candidates that subsequently could be used to test the hypothesis that co-cultures may exhibit higher robustness. Unfortunately, due to a lack of time and the COVID-19 pandemic, these experiments could not be performed. The study still provided interesting insight and lessons into challenges for the scale-up of engineered cyanobacteria, especially when the product of the intended process is food for many different bacteria.

Overall, this study demonstrates that N_2 -fixing cyanobacteria have the potential to make a difference to both terrestrial and liquid photosynthetic biomanufacturing. Several interesting new and potentially publishable insights were made but clearly there is still much more work to be done. This work provides a foundation for continued research into an exciting field of research that has important potential implications for applications which are both crucial for human livelihood and have the potential to enable a more sustainable way of life.

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Appendices

Appendix I. Reproduction approval for Figure 3-1.

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----- Original Message -----

From: Valton, Marine A M [m.valton@imperial.ac.uk]

Sent: 3/18/2021 9:46 AM

To: customerservice.eu@cellsignal.com; support@cellsignal.com; sales@cellsignal.com; info@ozyme.fr

Subject: Permission to reprint a diagram from your website

Dear Cell Signaling Technology Team,

I am completing my PhD thesis at Imperial College London entitled 'Engineered Solar-Driven Recycling Systems for Sustainable Fertilisation'.

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Marine Valton

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Appendix II. Characteristics of vermiculite, used for the development of the Proof-of-Concept, realised by NRM laboratories.

Determinand	Unit	SOIL
Dry Matter (Fresh)	% w/w	92.8
Hot Water Soluble Boron	mg/l	0.1
Total Copper	mg/kg	3.2
Total Zinc	mg/kg	31.3
Total Lead	mg/kg	<5
Total Arsenic	mg/kg	5.5
Total Cadmium	mg/kg	<0.1
Total Nickel	mg/kg	<10
Total Chromium	mg/kg	16.0
Total Mercury	mg/kg	<0.02
Total Selenium	mg/kg	<0.09
TPH [C5-C44] (Fresh)	mg/kg	<20
Aliphatic TPH >C5-C6 (Fresh)	mg/kg	<0.2
Aliphatic TPH >C6-C8 (Fresh)	mg/kg	<0.4
Aliphatic TPH >C8-C10 (Fresh)	mg/kg	<4
Aliphatic TPH >C10-C12 (Fresh)	mg/kg	<4
Aliphatic TPH >C12-C16 (Fresh)	mg/kg	<4
Aliphatic TPH >C16-C21 (Fresh)	mg/kg	<4
Aliphatic TPH >C21-C35 (Fresh)	mg/kg	<9
Aliphatic TPH >C35-C44 (Fresh)	mg/kg	<12
Aromatic TPH >C5-C7 (Fresh)	mg/kg	<0.02
Aromatic TPH >C7-C8 (Fresh)	mg/kg	<0.02
Aromatic TPH >C8-C10 (Fresh)	mg/kg	<0.02
Aromatic TPH >C10-C12 (Fresh)	mg/kg	<4
Aromatic TPH >C12-C16 (Fresh)	mg/kg	<4

Determinand	Unit	SOIL
Aromatic TPH >C16-C21 (Fresh)	mg/kg	<4
Aromatic TPH >C21-C35 (Fresh)	mg/kg	<9
Aromatic TPH >C35-C44 (Fresh)	mg/kg	<0.2
Benzene (Fresh)	mg/kg	<0.02
Toluene (Fresh)	mg/kg	<0.2
Ethyl Benzene (Fresh)	mg/kg	<0.04
m&p-Xylene (Fresh)	mg/kg	<0.2
o-Xylene (Fresh)	mg/kg	<0.1
PAH EPA16 (Fresh)	mg/kg	<1
Naphthalene (Fresh)	mg/kg	<0.05
Acenaphthylene (Fresh)	mg/kg	<0.05
Acenaphthene (Fresh)	mg/kg	<0.05
Fluorene (Fresh)	mg/kg	<0.05
Phenanthrene (Fresh)	mg/kg	<0.1
Anthracene (Fresh)	mg/kg	<0.05
Fluoranthene (Fresh)	mg/kg	<0.1
Pyrene (Fresh)	mg/kg	<0.1
Benzo[a]anthracene (Fresh)	mg/kg	<0.1
Chrysene (Fresh)	mg/kg	<0.1
Benzo[b]fluoranthene (Fresh)	mg/kg	<0.1
Benzo[k]fluoranthene (Fresh)	mg/kg	<0.1
Benzo[a]pyrene (Fresh)	mg/kg	<0.1
Indeno[1,2,3-cd]pyrene (Fresh)	mg/kg	<0.1
Dibenzo[a,h]anthracene (Fresh)	mg/kg	<0.1
Benzo[g,h,i]perylene (Fresh)	mg/kg	<0.1