Bottom-up Construction and Screening of Algae-bacteria Consortia for Volatile Organic Compounds (VOCs) Biodegradation

Author: Zongting Cai

Supervisors: Dr Jagroop Pandhal, Prof. Steve Thornton

Industrial Supervisor: Dr Andrew Goddard

Faculty of Engineering

Department of Chemical and Biological Engineering

The University of Sheffield

November 2023

ABSTRACT

Microbial communities have been exploited in various fields of studies associated with agriculture, the food industry, human health and other related fields. Engineering of microbial communities is an emerging field of research motivated by finding stable and efficient microbial systems as tools to achieve different biological tasks such as bioproduction and bioremediation. Algae and bacteria communities, in particular, are of interest due to the vast metabolic diversity of bacteria, the significant variety of bioproducts derived from algae, as well as the widespread interactions between algae and bacteria in natural systems. However, the successful designing of microbial communities not only underlies a profound understanding of microbial activities but also requires efficient approaches to piece together the known microbial traits to give rise to more complex systems. This project aims to address the lack of efficient methodologies for identifying and engineering algae bacteria consortia with unique functions, while also responding to the requirement of the industrial sponsor, Freeland Horticulture Ltd., which is motivated to combine waste woody and herbaceous biomass combustion with greenhouse CO_2 enrichment to achieve efficient heating, plant growth enhancement, waste reduction, and CO_2 capture. This study demonstrated the bottom-up integration of environmentally isolated phototrophic microalgae and chemotrophic bacteria as artificial consortia to bio-degrade selected representative volatile organic compounds (VOCs) that are of most concern in woody and herbaceous biomass combustion. A high throughput screening method based on 96-well plate format was developed for discovering consortia with bioremediation potential. Screened exemplar consortia were verified for VOCs degradation performance, among which certain robust consortia achieved 90.7%, 92.15 % and near 100% removal (7-day) of benzene, toluene, and phenol, respectively, with initial concentrations of 100 mg/L. VOCs degradation by consortia were mainly attributed to certain bacteria including *Rhodococcus erythropolis*, and *Cupriavidus metallidurans*, and directly contributed to the growth of microalgae *Coelastrella terrestris* ($R= 0.82$, $P<0.001$). This work revealed the potential of converting VOCs waste into algal biomass by algae-bacteria consortia constructed through a bottom-up approach. The screening method enables rapid shortlisting of consortia combinatorial scenarios without prior knowledge

about the individual microorganisms or the need for interpreting complex microbial interactions.

Acknowledgement

First and foremost, I bid my gratitude to God for His timely provision and unwavering guidance, especially during the challenging periods of the 2020 COVID-19 lockdown and the uncertainties of my immigration application in 2023. His strength has been my anchor, allowing me to stand firm in faith and complete my PhD journey.

My profound appreciation goes to my main supervisor, Dr. Jags Pandhal, whose steadfast guidance and mentorship have been indispensable throughout my academic journey. From our initial connection through an online search for PhD opportunities, Dr. Pandhal has been a guiding light, helping me navigate the complexities of scientific research. His commitment to fostering an environment of intellectual freedom while providing the necessary supervision and direction to ensure the success of my work is pivotal. During challenging times beyond work, such as periods of mental strain during the lockdown, my return to my home country for my marriage, and the birth of my daughter, Dr. Pandhal demonstrated understanding and flexibility, ensuring that my well-being was respected. Dr. Pandhal is not only an easy-going and supportive supervisor but also a trusted friend. The opportunities he provided for personal and professional development have been instrumental in shaping my academic journey.

I am deeply thankful to the Grantham Centre for Sustainable Futures and the University of Sheffield, along with Freeland Horticulture Ltd, for generously providing me with a joint scholarship. Also, I want to extend my gratitude to my second supervisor, Prof. Steve Thornton, and technician Andrew Fairburn for their support and access to lab facilities. My thanks also go to my industrial supervisor, Dr. Andrew Goddard, for providing samples and organising field trips related to this project.

My heartfelt thanks go to my family: my wife, Eunice Kayui An Wong, who has been my pillar of strength and brought our daughter into this world; my parents, Mr. Yong Cai and Ms. Ping Li, for their emotional and financial support; and my daughter, Eliana Yoyo Cai Wong, who has brought immense joy and responsibility into my life, and humorously, for being quiet and not crying when I needed to focus on my thesis writing.

I am grateful to my colleague, Elbaraa ElGhazy, for his reliability and assistance with my experiment setups, and to Dr. Esther Karunakaran for her training and knowledge sharing. I also want to thank Ali-Al Haid, Dr. Caroline Evans, Fikayo Adepoju, Hannah Leflay, Helen Wright, James Greenham, Josie McQuillan, Jose Luis, Juliano Bertozzi Silva, Kasia Okurowska, Kirsty L. Franklin, Mengxun Shi and Wan Aizuddin bin Wan Razali for their daily lab help and technical support.

Finally, I would like to thank everyone at the University of Sheffield for contributing to the positive and hopeful atmosphere during my four years and one month of study and in maintaining the spirit of this esteemed institution.

Table of Content

Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (https://www.sheffield.ac.uk/new-students/unfair-means). This work has not been previously presented for an award at this, or any other university.

Publications related to this project:

Cai, Z., Karunakaran, E. and Pandhal, J. (2023) *Bottom-up Construction and Screening of Algae-bacteria Consortia for Bioremediation: A Case Study on Volatile Organic Compounds.* doi:10.21203/RS.3.RS-3204286/V1.(Preprint)

Conferences

1. **Poster event: Microbiology society annual conference 2022**
   04th-07th April 2022 Belfast
   Poster link

2. **Oral Presentation 1: Microbial Cycling of Volatile Organic Compounds: Biogeochemistry to Biotechnology**
   25th-26th May 2022 Norwich
   Presentation slides link
   also available on the society news

3. **Oral Presentation 2: Algae-UK and EBNet webinar, Developments in Algal Gas Scrubbing**
   Online
   recorded available on: youtube

List of Tables

List of Figures

Abbreviations

ARHD	Aromatic-ring-hydroxylating dioxygenase
ASB	Algae-associated bacteria
BTEX	Benzene, toluene, ethylbenzene, xylenes
CCAP	Culture Collection of Algae and Protozoa
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CNC	Cellulose nanocrystals
CNF	Cellulose nanofibrils
COD	Chemical oxygen demand
CoSMO	Synthetic obligatory cooperative system
DCM	Dichloromethane
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EtBr	Ethidium bromide
FISH	Fluorescence in situ Hybridization
FPR	False positive rate
GC	Gas chromatography
GC-FID	Gas Chromatography with Flame Ionization Detector
GCN	Gene copy number
GLA	γ-Linolenic acid
GN	Gram-negative
GP	Gram-positive
GPP	Gross primary production
HAB	Harmful algal bloom
HP-5	Precision-engineered (5%-phenyl)-methylpolysiloxane
HPLC	High-performance liquid chromatography
LB	Luria-Bertani broth

MMP	Maximum power principle
MS	Mass spectrometry
N	Nitrogen
OD	Optical density
P	Phosphorus
PBR	Photobioreactors
PBS	Phosphate-buffered saline
PCC	Pearson correlation coefficients
PCR	Polymerase chain reaction
PE	Photosynthetic efficiency
PGP	Plant growth-promoting
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative Polymerase Chain Reaction
RCD	Ring-cleaving dioxygenase
rDNA	Ribosomal DNA
RMSE	Root-mean-square error
rRNA	Ribosomal RNA
TAE	Tris-acetate-EDTA
TAG	Free fatty acids and triacylglycerol
TAG	Triacylglycerol
THF	Tetrahydrofuran
TN	Total nitrogen
TP	Total phosphorus (TP)
TVC	Total viable count
VOCs	Volatile organic compounds
WGS	Whole-genome sequencing
WWT	Wastewater treatment
3N-BBM	Bold Basal Medium with 3-fold Nitrogen
3N-BBM+V	Bold Basal Medium with 3-fold Nitrogen and Vitamins

Chapter 1: Literature Review

Aims and objectives

- *Brief overview of the industrial context and its association with the objectives of the work.*
- *Introduce the promise of algae bioproducts and their potential applications.*
- *Overview of algae-bacteria interactions, highlighting the current focus, applications, and research approaches in microbial system engineering.*
- *Address limitations and identify research gaps in algae-bacteria consortia engineering studies.*
- *Contextualise algae-bacteria consortia integration approaches*
- *Outline key hypotheses and research questions to guide the study.*
- *Summarise the content and structure of the current work.*

1.1 Introduction

Microalgae and bacteria - two distinct groups of microorganisms, are important components of ecosystems. They are also popular targets in biotechnological research and biomanufacturing applications due to their unique metabolic capabilities. Notably, bacteria are adept at bioremediation, breaking down pollutants and recycling nutrients in various environments. The metabolic diversity of bacteria enables them to colonize diverse niches, from wastewater treatment plants to form symbiotic relationships with plants and algae. Microalgae, on the other hand, known for their efficient photosynthesis, contribute significantly to Gross primary production (GPP). They are versatile solar-driven cell factories that generate an array of bioproducts such as lipids, carbohydrates and pigments with applications spanning from biofuel production to nutraceuticals (Spolaore et al., 2006; Williams and Laurens, 2010).

Algae-bacteria interactions, a result of co-evolution manifests in various forms such as mutualism, competition and commensalism, thereby influencing ecosystem dynamics and bioprocess efficiency (Amin et al., 2015; Croft et al., 2005). Harnessing the synergies between microalgae and bacteria can lead to innovative solutions for sustainable bioproduction and environmental remediation. By strategically combining their strengths, researchers aim to create robust and efficient microbial consortia. This exploration has led to the emergence of algae-bacteria consortia biotechnology, a field that delves into optimizing these interactions for practical applications (Ramanan et al., 2016)

This chapter introduces algae bioproducts and algae-bacteria interactions, highlighting the current focus, applications, and research approaches in microbial system engineering with a special emphasis on the combination of autotrophic microalgae and chemotrophic bacteria. The characteristics of these organisms and the prospects of integrating them are also discussed, given their recognised potential for various biological tasks, emerging as a key research area in microbial system engineering. This chapter also addresses the limitations and identifies research gaps in algae-bacteria consortia engineering studies, providing an overview of similar research including the experimental chapters in this project. In addition, as this project employs the degradation of VOCs concerning the industrial sponsor, Freeland Horticulture Ltd., the general industrial background and the generation, impact and

remediation of common VOCs, particularly focusing on biomass combustion processes, are also briefly reviewed.

1.2 Project background

1.2.1 Industrial context

Global industry relies heavily on the combustion of fossil fuels such as coal, crude oil products and natural gas. These sources of fuels are not only non-renewable but also have a significant contribution to climate change due to heavy CO_2 emissions. A global focus on reducing emissions is influencing almost every industry (IPCC, 2023), driving the adoption of low-carbon, sustainable technologies.

Horticultural cultivation is the controlled growth and production of plants within protective transparent material. It is seen as the advanced form of agriculture to meet the need for higher agriculture production yield in response to the global population increase (Vadiee and Martin, 2012). Greenhouses, especially on large scale, are energy intensive since artificial environmental controls are usually needed for growing plants in all climates. For example, temperature control, particularly when heating is needed, fuels such as natural gas, oil or wood are usually used to deliver hot air into the greenhouses (Nemali, 2022). A major product of combustion processes, CO_2, is essential for photosynthesis, and CO_2 enrichment in greenhouses has long been proved to be beneficial for enhanced plant yield (Mortensen, 1987; Sánchez-Guerrero et al., 2005). In return, greenhouses can serve as carbon capture systems (Wang et al., 2022) and there have been explorations of combining horticulture with energy-intensive sectors as an emission reduction strategy (Marchi et al., 2018).

Combining biomass combustion with horticulture offers a more sustainable approach by integrating heating and CO_2 enrichment with waste reduction (Dion et al., 2011). As the industrial collaborator and one of the sponsors of this project, Freeland Horticulture Ltd. is currently exploring the potential of this approach. As the largest topsoil supplier in the UK, the company is contracted to process 50,000 tonnes of biomass waste annually for waste management companies and local authorities, converting it into bagged horticultural products via composting. However, the company faces challenges in the reduction of oversized waste which is mainly comprised of woody and herbaceous biomass. To address

this challenge, the company aims to adopt biomass combustion while capturing the resulting CO_2 to enhance greenhouse cultivation as a carbon capture strategy.

1.2.2 VOCs types, health impact and mitigation methods

The combustion of biomass causes air pollution in a similar way as that of its fossil fuel counterpart (Chao et al., 2008). Biomass typically comprises complex, large organic molecules and incomplete combustion often yields a considerable amount of pollutants such as particle matter, carbon monoxide (CO), sulfur and nitrogen oxides (SOx, and NOx) as well as VOCs which are primarily aromatic hydrocarbons. Although the components can vary with feedstock type, combustion technologies or efficiency, this project, in collaboration with Freeland Horticulture Ltd. as the industrial sponsor, focuses on the pollutants generated from the combustion of woody and herbaceous biomass. These materials are rich in polymeric substances such as cellulose and lignin, which are believed to result in higher concentrations of volatile organic compounds (VOCs) compared to those produced by fossil fuel combustion (Zhu et al., 2022). Among these, organic acids, aromatic compounds, alcohols and aldehydes are some of the most recognised VOCs derived from woody biomass combustion (Evtyugina et al., 2014).

Despite that the direct toxic effect of these VOCs on plants remains unclear, as demonstrated by laboratory experiments (Cape, 2003), some of these compounds are recognised as major pollutants affecting both indoor and outdoor air quality (Musialik-Piotrowska et al., 2010), and can have a severe impact to human health including eye, skin, respiratory system irritation, poisoning and carcinogenic risk (Collins, 2001). These risks are especially recognised for aldehydes and aromatic hydrocarbons which are usually listed as top mutagenic hazards (Kim and Shim, 2010). For this reason, it is crucial to adopt pre-treatment processes to remove these VOCs from flue gas produced by biomass combustion before it can be fed into the horticultural system. This is also essential for ensuring that the horticultural system complies with requirements such as workplace exposure limits (Health and Safety Executive, 2020) and emission or air quality regulations (Environmental Protection, 2010).

Although the health impact of these VOCs is well recognised, mitigation methods remain largely as physical absorption, combustion strategies optimisation and catalysing processes

(Poddar et al., 1996; Chao et al., 2008; Shi et al., 2021). Considering cost and scalability, it is of particular interest to explore the potential of biological mitigation methods for the efficient remediation of VOCs derived from biomass combustion.

1.3 Bacteria in bioremediation

The biological removal of VOCs as a more sustainable mitigation approach has garnered widespread attention (Guieysse et al., 2008; Ni et al., 2020). These studies emphasize technical innovations and specific testing protocols, while also highlighting the core role of bacterial activities that are responsible for VOC uptake.

Bacteria-driven biological treatment of waste is a well-exploited technology with widespread implementations for mitigating a variety of environmental problems beyond just VOCs. Such technologies have been intensively used in wastewater treatment (WWT), waste gas treatment and soil remediation, where bacteria play a dominant role in breaking down unwanted substrates such as toxic organics compounds, ammonium and ordurous compounds (Antizar-Ladislao, 2010; Numberger et al., 2019; Lu et al., 2012).

For waste gas treatments, where VOCs are usually seen as major pollutants, technologies such as biofilters have been successfully applied on an industrial scale for odour control and toxic chemical removal, with bacteria-led metabolism being the core mechanism. Notably, a considerable amount of known processes occurring in aerobic conditions can be expressed as:

$$Substrate + O_2 \xrightarrow{Bacteria\ metabolism} Biomass + CO_2 + H_2O$$

As key decomposers in ecosystems, the natural diversity of bacteria determines their capability to utilise a variety of substrates. For example, *Pseudomonas putida*, a well-characterised gram-negative bacterium, has been used for the biodegradation of many toxic compounds including formaldehyde (Adroer et al., 1990; Roca et al., 2008), benzene, phenol and toluene (Abuhamed et al., 2004; Reardon et al., 2000a). Other frequently studied

bacteria regarding the biodegradation of these compounds include *Rhodococcus* and *Bacillus*. Many of these bacteria can use hydrocarbons as a source of carbon and energy, converting them into simpler compounds through metabolic processes (Abbasian et al., 2015). A summary of related bacteria species and literature is given in Table 1.1.

Bacteria offers a unique pool of solutions for eliminating specific wide arrays of aromatic pollutants. Also, for certain aromatic compounds, some bacteria share identical metabolic processes as well as the co-existence of intermediate compounds. Although many pathways still require further understanding, this observation suggests the potential to enhance biodegradation efficiency through the co-metabolism of multiple VOCs or the co-culture of different bacterial species that utilise the same intermediates.

Table 1.1 Common bacteria with aromatic compounds degradation abilities

Bacteria	Catabolism pathway /functional genes/enzyme	Target compounds	Reference
Pseudomonas P. putida KT2440	**Beta-Ketoadipate Pathway** • Protocatechuate Branch (pca genes) • Catechol Branch (cat genes) **Homogentisate Pathway** • hmg/fah/mai genes **Phenylacetate Pathway** • pha genes	P-hydroxybenzoate Benzoate Phenylpropanoid compounds Phenylalanine Tyrosine N-phenyl alkanoic acids	Jiménez et al., (2002) Wackett et al., (1988)
Sphingobium *S. paucimobilis SYK-6.* *Sphingobium barthaii KK22* *Sphingomonas xenophaga strain BN6*	**Protocatechuate, catechol branches of the β-ketoadipate pathway** • Protocatechuic catabolic gene clusters	Naphthalenesulfonate polycyclic aromatic hydrocarbons (PAHs)	Pinyakong et al., (2003) Maeda et al., (2020) Higuchi et al., (2022)
Mycobacterium Mycobacterium sp. strain PYR-1	**Novel dioxygenases** (undefined)	Anthracene Phenanthrene	Moody et al., (2001)

Table 1.1 (Continued) Common bacteria with aromatic compounds degradation ability

Bacteria	Catabolism pathway /functional genes/enzyme	Target compounds	Reference
Rhodococcus *Rhodococcus jostii RHA1* *Rhodococcus erythropolis CCM2595* *Rhodococcus sp. AN22*	**Peripheral Pathways** **3-Oxoadipate Pathway:** • Catechol branch (catA, catB, catC gene) • Protocatechuate degradation **Gentisate Pathway** **Modified Ortho-Cleavage Pathway** **Meta-Cleavage Pathway**	Chlorobenzenes Chlorobenzoates Naphthalene Toluene Xylene Aniline	Martínková et al., (2009) Larkin et al., (2005)
Bacillus *Bacillus subtilis strain 168* *Bacillus subtilis*	**Meta cleavage pathway** • yfiE gene (encodes catechol-2,3-dioxygenase) • licBCAH Operon (involves lichenin utilisation) • rbsRKDACB Operon (rbsRKDACB Operon) **β-ketoadipate pathway**	Phenol Catechol	Le et al., (2006) Velupillaimani and Muthaiyan, (2019)

1.4 Algae as microalgae bioproduct and their application

Microalgae are mainly unicellular photosynthetic organisms that are typically found in freshwater and marine environments (Parker et al., 2008) but are also widespread in many environments with a high diversity of more than 200,000 species (Guiry, 2012). With their ecological interactions and biochemical diversity, microalgae have always been a core focus in the study of ecology in aquatic environments (Cembella, 2003). They have also been found in terrestrial environments like soil (Bérard et al., 2005; Abinandan et al., 2019) and rock surfaces (Aburai et al., 2013). Similar to their terrestrial counterparts eukaryotes, plants, microalgae are key photosynthesizers that contribute up to 40% of the marine primary production and play an important role in the earth's ecosystem (Moriarty et al., 1985).

Microalgae are also well known for their high photosynthetic efficiency (PE), which was estimated to be 6-20% over what was observed in most terrestrial plants (1%-2%) (Singh and Ahluwalia, 2012) and as a result, they can produce biomass more rapidly than plants (Brennan and Owende, 2010). This feature relieves the issue of land-use competition, positioning microalgae as a suitable candidate for large-scale cultivation (Naik et al., 2010; Lam and Lee, 2012).

Researchers began to explore the biotechnological applications of microalgae almost three decades ago (Metting, 1996). As a result, microalgal biotechnology is now thriving as a pivotal area in sustainable manufacturing research, given the algae's versatility in producing a range of bioproducts (Fig.1.1). These include lipids (Williams and Laurens, 2010), carbohydrates (Ahmad et al., 2011; Tsukahara and Sawayama, 2005), proteins (Rizwan et al., 2015), vitamins, and pigments (Spolaore et al., 2006), underscoring their potential as sources of renewable energy and valuable bio-product generating factories (also summarised in Table 1.2).

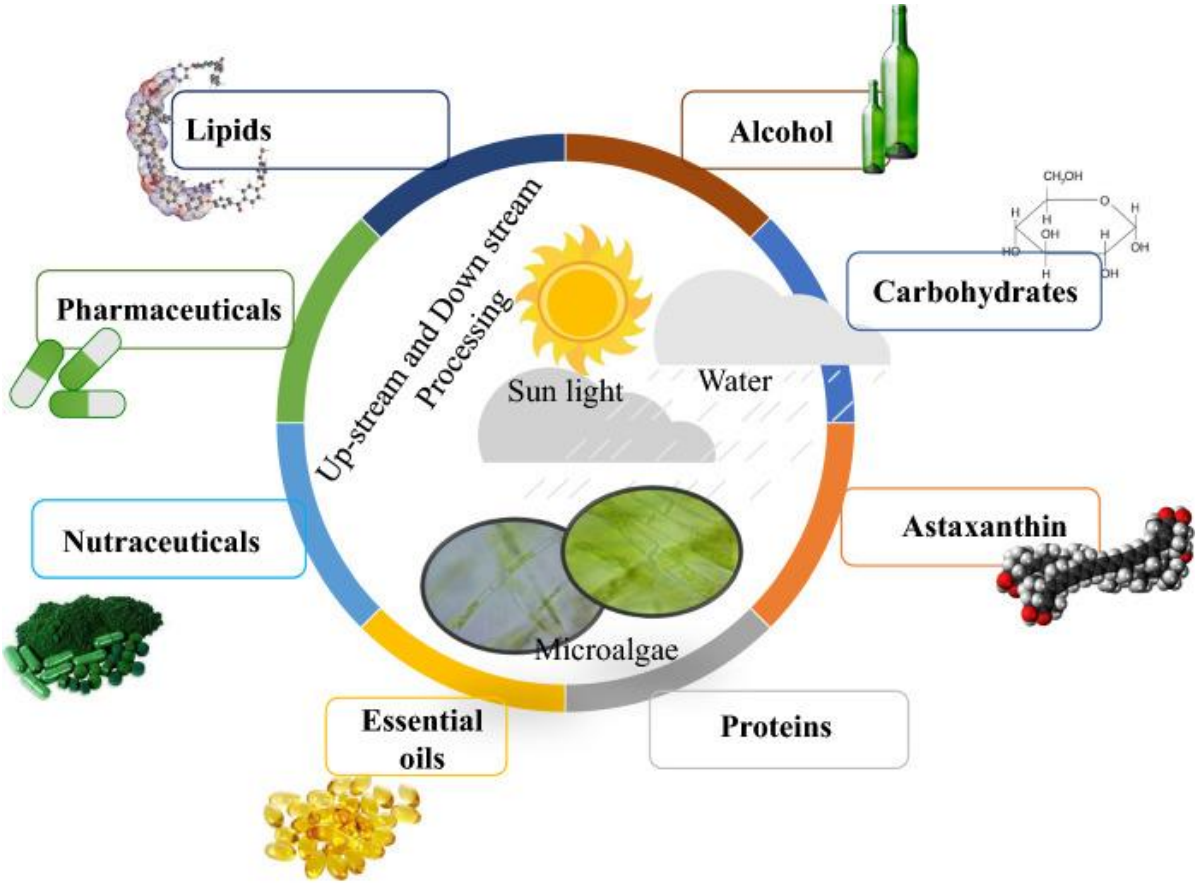


Fig.1.1 Conceptual figure of microalgae bioproducts and their applications, adapted from Udayan et al., (2023).

1.4.1 Algae lipid

Lipids are one of the main bioproducts associated with microalgae. Microalgae typically have a high content of lipids (20-50%), depending on the strain (Udayan et al., 2023). Some algae genera, such as *Chlorella* and *Nannochloropsis*, can have up to 53% or even 77% lipid content (Deshmukh et al., 2019; Ferreira et al., 2019) and these lipids are usually composed of free fatty acids and triacylglycerols (TAGs). Research has also been carried out to enhance lipid productivity and biomass by optimising culturing strategies, such as nitrogen starvation and salt stress to boost lipid accumulation. This not only results in the accumulation of lipids but also alters the lipid composition, making them more tailored and suitable for different purposes (Mirizadeh et al., 2020).

With algae's capability to efficiently convert CO_2 into biomass through photosynthesis, algae lipid production can aligned with biofuel research (Ullah et al., 2014). Large-scale cultivation of algae, especially with species or strains being intentionally selected for high lipid content,

is already being implemented for biodiesel production using open pond cultivation systems (Hallenbeck et al., 2016). Additionally, some early studies explored the integration of algae cultivation with industrial-scale carbon capture in facilities such as power plants, serving as a biological emission control process (Iglina et al., 2022). Lipid production remains a prominent topic in these studies, as the combination of carbon capturing and algae cultivation enhances sustainability by addressing carbon emissions (Fabris et al., 2020).

Apart from the topic of sustainable biofuel, algae lipids also contain a high amount of polyunsaturated fatty acids (PUFAs), a vital component of the human diet (Udayan et al., 2023) Algae lipid extracts also see application as food additives for human nutrition, especially docosahexaenoic acid (DHA), which is already commercially available from algae (Spolaore et al., 2006).

1.4.2 Carbohydrates and polymers

Carbohydrates are another primary component of algae biomass and can be converted into ethanol and biogas (Chandrasekhar et al., 2023). The carbohydrate content in microalgae is converted into fermentable sugars through the hydrolysis of microalgal biomass using acid, alkaline, or enzymatic pretreatments, which are usually used for ethanol production (Müller et al., 2023). Similar to lipids, the carbohydrate content in algae can be increased through cultivation strategies such as nitrogen starvation. Although the use of algal biomass for ethanol production is relatively uncommon, as many studies prioritise lipid production for algae diesel and algal carbohydrates, it is sometimes viewed as a secondary objective or a byproduct (Brennan and Owende, 2010). Nonetheless, there are still works focusing on intentionally enhancing starch production in *Chlorella* (Cheng et al., 2017) and *Chlorella zofingiensis* (Zhu et al., 2014) for bioethanol production purposes.

Fibrous polymers are a major content of algae carbohydrates. Recently, algae-derived cellulose has become an attractive research focus in the context of biodegradable plastic materials from renewable sources (Zanchetta et al., 2021). Some microalgae, especially under genus *Nannochloropsis*, have been estimated to contain up to 78% cellulose (dry biomass) under optimal conditions (Jeong et al., 2017). These species are currently at the

forefront of algae-derived cellulose research, yielding a variety of crude materials for cellulose products such as cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF). These products exhibit unique chemical and physical properties and have promise in the textile industry, food industry and biomedical applications (Zanchetta et al., 2021).

1.4.3 Algae as a source of pigments

Microalgae are renowned for their ability to produce a variety of chromatic compounds related to photosynthesis, making them a significant source of natural pigments. While chlorophyll is the most abundant pigment in the world and has been documented for health benefits and wide application in the food industry and for medicinal purposes (Solymosi and Mysliwa-Kurdziel, 2016), microalgae like *Chlorella sp*., *Chlamydomonas sp*., and *Scenedesmus sp.*, have become primary commercial sources of chlorophyll, with successful large-scale production (da Silva Ferreira and Sant'Anna, 2017). Another type of essential pigment, carotenoids, plays a pivotal role in photosynthesis by capturing light and transferring energy to chlorophyll via singlet–singlet excitation transfer (Maoka, 2020). Their potential health benefits and antioxidant properties have also been well-studied, with β-carotene, astaxanthin, lutein and fucoxanthin being some of the more research attractive carotenoids (Sluijs et al., 2015). Microalgae especially *Dunaliella* and *Chlamydomonas reinhardtii*, are some of the up-scaled natural sources of these carotenoids (Jacob-Lopes et al., 2019) and have gained prominence in the nutraceutical, and cosmetic industries and in food fortification. Notably, astaxanthin is currently recognized as the most potent anti-oxidative carotenoid (Pogorzelska et al., 2018). The global astaxanthin market was estimated at 1 billion in 2019. While nearly 95% of this was synthetic, the primary natural source is microalgae, especially *Haematococcus pluvialis* (Stachowiak and Szulc, 2021).

1.4.4 Other bioproducts from algae

Beyond these products mentioned above, microalgae can also be the source of a variety of other bio-functional compounds that are suitable for human consumption and animal feed as nutrients and therapeutic supplements such as vitamins (Becker, 2013). According to Arora and Philippidis, (2023), previous studies have covered the production of 10 types of key vitamins from over 25 genera of microalgae. Additionally, protein constitutes a significant portion of algal biomass, making up more than half of the total content in certain genera such as *Chlorella vulgaris* (58%) and cyanobacteria *Spirulina* (60%) (Spolaore et al., 2006). However, the utilization of microalgae protein in the food industry is often constrained by its limited digestibility and bioavailability, as well as concern about its safety in consumption (Wang et al., 2021).

Except for cell products, microalgae as living biomass can be incorporated into the soil, as a bio-fertiliser (Mahapatra et al., 2018). These algal additions mediate both biological and chemical properties of the soil, such as increasing the N-P ratio, thereby enhancing soil fertility and ultimately boosting crop production. *Chlorella vulgaris* and cyanobacteria *Spirulina platensis* are some of the most commonly used species for soil fertility enhancement and it has been reported that they can increase rice production (Ammar et al., 2022).

In summary, microalgae are versatile organisms that serve as a rich source of lipids for biofuel production, carbohydrates for ethanol and biogas, and fibrous polymers for biodegradable plastics. They can also provide high-value products like pigments and vitamins. Given these benefits, microalgae have been seen as solar-driven cell factories.

Table 1.2 Algae-associated bioproducts

Bioproduct	Microorganism names	Function/Application	Reference
Lipids			
Free fatty acids Triacylglycerol (TAG) Polyunsaturated fatty acids (PUFAs) • Docosahexaenoic acid (DHA) • Eicosapentaenoic acid (EPA) • γ-Linolenic acid (GLA)	*Chlorella spp.* *Nannochloropsis spp*. *Scenedesmus spp.* *Botryococcusbraunii* *Stichococcus bacillaris*	Biodiesel Food supplements	Olivieri et al., (2011) Sivakumar et al., (2014) Deshmukh et al., (2019) Ferreira et al., (2019) Udayan et al., (2023)
Carbohydrates Polysaccharides (pectin, agar, alginate) Starch Cellulose and hemicellulose	*Chlorella* vulgaris CCAP 211/11B *Chlamydomonas reinhardtii* UTEX 90 *Scenedesmus obliquus CNW-N* *Nannochloropsis sp* *Spirogyra peipingensis*	Bioethanol Biogas Biopolymer	Hirano et al., (1997) Illman et al., (2000) Dragone et al., (2011) Chen et al., (2013a)
Protein	*Synechococcus spp.* *Scenedesmus obliquus* *Chlorella vulgaris* *Aphanizomenon flos-aquae*	Animal feed Human nutrition Cosmetics Bio-functional proteins/peptide	Becker, (2007) Chronakis and Madsen, (2011) Samarakoon and Jeon, (2012)

Table 1.2 (Continued) Microalgae-associated bioproducts

Bioproduct	Microorganism names	Function/Application	Reference
Pigments Chlorophyll Carotenoids • Beta-carotene • Astaxanthin • Lutein	*Chlorella sp.* *Scenedesmus sp.* *Chlamydomonas sp.* *Dunaliella sp.* *Haematococcus pluvial*	Food additives. Antioxidants Nutraceuticals Pharmaceutical	Pogorzelska et al., (2018) Jacob-Lopes et al., (2019) Solymosi and Mysliwa-Kurdziel, (2016) Sluijs et al., (2015) da Silva Ferreira and Sant'Anna, 2017) Stachowiak and Szulc, (2021).
Vitamins Vitamin A Group B Vitamin Vitamin C (ascorbic acid) Vitamin E (α-tacopherol) Vitamin K1 Vitamin D3	*Tetraselmis suecica* *Dunaliella tertiolecta* *Chlorella sp.* *Nannochloropsis sp.*	Animal feed Food industry. cosmeceutical Pharmaceutical	Becker, (2013) Arora and Philippidis, (2023)
Bio-stimulants and Bio-fertilizers	*Chlorella vulgaris* *Sargassum sp* *Gracilaria verrucosa*	Agriculture	Mahapatra et al., (2018) Ammar et al., (2022).

1.5 Algae-bacteria interaction

While bacteria play a primary role in pollutant bioremediation, the integration of microalgae brings several benefits. Algae, as solar-powered cells not only capture CO_2 and supply O_2 to aid bacterial respiration but also contribute to resource recovery by offering a variety of valuable bio-products as discussed in Session 1.4. Such integration has long been explored under the field termed 'algae-bacteria consortia biotechnology' and its wide applications are illustrated in Fig.1.2.

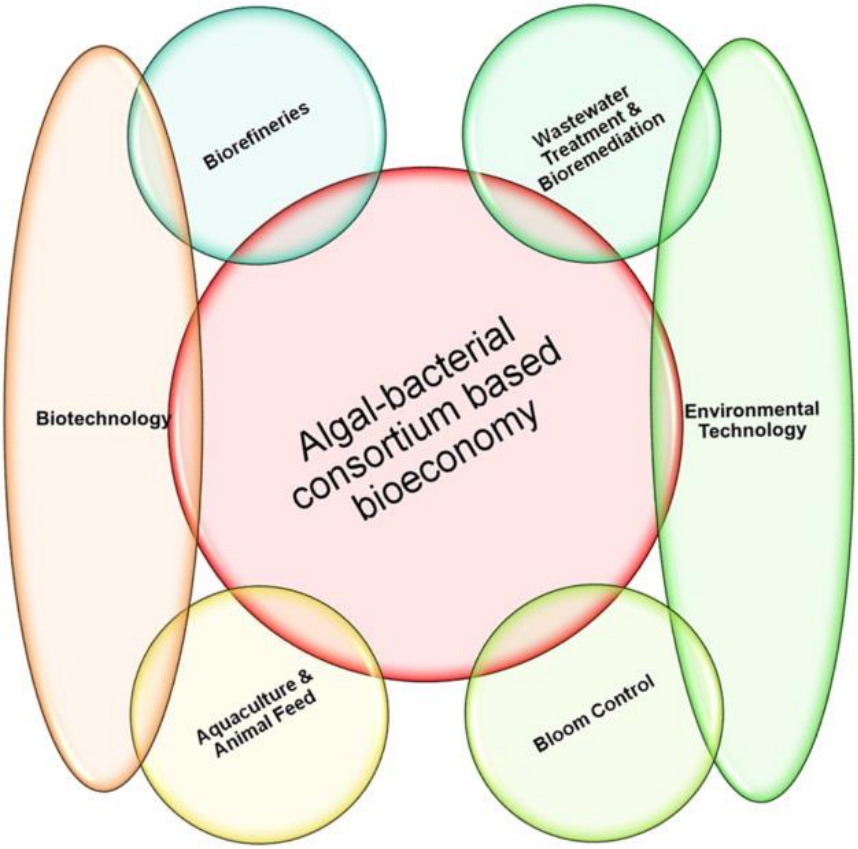


Fig.1.2 Applications of algal–bacterial interactions for biotechnology and environmental sectors, adapted from (Ramanan et al., 2016).

1.5.1 Co-existing of algae in nature environment

Algae-bacteria communities are some of the most prevalent and fundamentally mixed microbial systems in natural environments. Research has shown that algae and bacteria are closely linked evolutionarily (Ramanan et al., 2016). It is believed that nearly every microalgae species is associated with specific bacteria, which makes it challenging to obtain axenic algae cultures (Amin et al., 2015).

The most well-known interaction between algae and bacteria is mutualism with nutrient exchange as one of the foundations underpinning their coexistence. A classic example of micronutrient exchange is the sharing of Vitamin B12 between algae and bacteria, as exemplified by the algae *Chlamydomonas reinhardtii* and the bacterium *Sinorhizobium meliloti* (Croft et al., 2005; Xie et al., 2013). Further research has highlighted the evolutionary significance of this mutualism, emphasising that Vitamin B12 exchange can be widespread between broader algae and bacteria species. (Kazamia et al., 2012).

Mutualism is not limited to micronutrient exchange but can involve a broader range of substances. In aquatic environments for example, algae are responsible for providing a large amount of dissolved organic carbon (DOC) that can work as the major carbon source for the survival of heterotrophic bacteria (Weinberger et al., 1994; Takemura et al., 2014). The mutualistic relationship between algae and bacteria plays a crucial role in cycling a wider array of elements, including nitrogen, sulfur, phosphorus and iron (Amin et al., 2009; Ask et al., 2009; Cho et al., 2015) For instance, some nitrogen-fixing bacteria, such as *Mesorhizobium*, *Azospirillum*, and *Rhizobium*, can promote the growth of algae like *Chlorella*, which lack nitrogen-fixing abilities. These bacteria supplement the algae, especially in nutrient-poor environments where they exchange nutrients with algae for other algae-originated organic carbon for survival (He et al., 2009; Kim et al., 2014).

1.5.2 Diverse Interactions in agae-bacteria Communities

Beyond mutualism, early observations of toxin-mediated inhibition between microalgae and bacteria suggest that metabolites play a significant role in affecting the growth of competitors (Arzul *et al.*, 1999). Subsequently, more detailed studies have revealed how the

dynamic functioning of these metabolites triggers and influences complex behaviours, such as chemical defence, among participants in each interaction (Pohnert, 2004). Beginning with the introduction of the term 'psychosphere' (Bell and Mitchell, 1972) recent studies have explored these interactions from ecological perspectives.

Previous studies have not only uncovered the ubiquity of cross-kingdom chemical communication between algae and bacteria but also highlighted its importance in the co-evolution of microorganisms (Cirri and Pohnert, 2019). As a result of long-term co-evolution, many metabolites serve as signalling chemicals and play a crucial role in algae-bacteria interactions (details in Table 1.3). The exchange of these metabolites can lead to either beneficial effects such as growth promotion and nutrition, or detrimental behaviours, such as pathogenicity, inhibition and antibiotic resistance (Zhang *et al.*, 2012; Cirri and Pohnert, 2019). For instance, some well-known 'phycosphere' bacteria, such as *Pseudomonas*, *Bacillus*, *Alteromonas*, *Cytophaga*, and *Micrococcus*, can produce algicidal agents that induce morphogenesis in algae (Lovejoy et al., 1998; Zhang et al., 2020). These bacteria have been closely linked to algal blooms and their algicidal effects are often species-specific, which greatly inspired the study of bacterial elimination of harmful algae (Jin et al., 2017).

1.5.3 Efforts to harness algae-bacteria interactions

Though many known algae-bacteria interactions can be beneficial to algae, the co-existence of bacteria with algae has often been viewed as a contamination issue before these interactions are harnessed, particularly in phycology studies (Dittami et al., 2014) and algae-refinery systems, including both open ponds and closed photobioreactors (PBRs). As mentioned in the previous section, most algae are highly dependent on certain types of bacteria in their life span (Dittami et al., 2014), Eliminating these algae-associated bacteria (ASB) is extremely challenging and sometimes impossible on a large scale without costly sterilisation processes (Lee, 2001). Conversely, bacteria-driven processes, such as WWTs, sometimes identify certain algae as problematic colonizers and the removal or prevention of these algae has been a primary focus of research (Azarian et al., 2007; Joh et al., 2011).

Exploring the interactions between individual algae and bacteria reveals the potential applications of algae-bacteria consortia. This inspired the attempts to intentionally select microbes for co-culture with algae (Table 1.3). An early study by Mouget et al., (1995) suggested that two *Pseudomonas* isolated from laboratory algal cultures, *Pseudomonas diminuta* and *Pseudomonas vesiculuris*, could stimulate the growth of co-cultured green microalgae *Scenedesmus bicellularis* and *Chlorella sp.,* simply by providing CO_2 and consuming oxygen to maintain a preferable condition for photosynthesis.

As the knowledge surrounding the algae-associated microbiome (or phycosphere) becomes more available, the intentional selection of microbial partners for specific algae species has emerged as a viable option for the construction of consortia with specific biological tasks. For instance, a study by Dong and Zhao, (2004) obtained 12.92 mg/L of astaxanthin production in a mixed culture of algae *Haematococcus pluvialis* and a yeast *Phaffia rhodozyma*, which was 3.5- and 11-fold higher than those in axenic cultures of the two species, respectively. Wirth et al., (2015) mixed microalgae, *Chlamydomonas sp.* and *Scenedesmus sp*., with bacteria *Rhizobium sp.* and increased algal biomass yields by up to 24% as the result of O_2-CO_2 exchange. Due to the same mechanism, Papone et al., (2012) obtained enhanced lipid production in *Chlorella sp. KKUS2* by choosing fungi *Toluraspore YU5/2* and *Toluraspore Y30* for co-culturing. In addition to function enhancement and productivity promotion (Xie et al., 2013; Amin et al., 2015; Segev et al., 2016), the adverse interaction between certain algae and bacteria can also be utilised to address certain environmental challenges. For example, harmful algal bloom (HAB) control can be achieved by bacteria that release algicidal molecules including 1-acetyl-β-carboline(Kim et al., 2015), Orfamide A (Aiyar et al., 2017) and urocanic acid (Kim et al., 2018; Zhuang et al., 2018).

Table 1.3 Applications of algae/bacteria co-culture and microbe selection criteria.

Purpose (Enhance)	Co-cultured Microorganism		Microbe selection criteria	Reference
	Algae	Partner microbe		
Lipid production	*Chlorella vulgaris TISTR 8261* *Chlorella sp. KKUS2*	*Trichosporonoides spathulata JU4-57* *Toluraspore YU5/2, Toluraspore Y30*	• Both microalgae and yeast strains have high lipid production rates; • O_2 and CO_2 exchange enhances algal photosynthesis and fungal aerobic growth. • O_2 and CO_2 exchange enhances algal photosynthesis and fungal aerobic growth.	(Kitcha and Cheirsilp, 2014) (Papone et al., 2012)
Astaxanthin production	*Haematococcus pluvialis*	*Phaffia rhodozyma*	• Both *H.pluvialis and P.rhodozyma* are astaxanthin producers; • CO_2 produced by *P.rhodozyma enhances algae photosynthesis;* • O_2 released by algae is a key substrate for β-carotene ketolase to catalyse astaxanthin synthesis in *P.rhodozyma.*	(Dong and Zhao, 2004)
Biomass	*Isochrysis galbana 8701*	*Ambrosiozyma cicatricosa*	• Mutualism and O_2 and CO_2 exchange enhance	(Cai et al., 2007)
Exopolysaccharides (EPS)	*Weissella confusa 11GU-1*	*Propionibacterium freudenreichii JS15*	• EPS functionality.	(Villacorte et al., 2015)
2-keto-l-gulonic acid[1]	*Gluconobacter oxydans*	*Ketogulonicigenium vulgare*	• Recognized metabolic cooperation between *G. oxydans* and *K. vulgare* • Predicted metabolic behaviours of G*. oxydans* after genetic modification.	(Wang et al., 2016)

[1] the precursor of vitamin C

1.6 Approaches to algae-bacteria consortia engineering

As discussed in the previous session, although the co-existence of algae and bacteria is a well-recognised natural phenomenon, many earlier phycology studies viewed bacteria as contaminants, and systems primarily treated algae as undesirable. However, more recent studies have attempted to deliberately select bacterial partners for algae co-culture, which is now recognised as a strategy to enhance the functionality of microbial systems. This shift is driven by the deeper understanding of the algae-associated microbiome, particularly through studies of classical ecological models, such as the mutualism between algae and bacteria. These models have been highlighted for their evolutionary and ecological significance, enriching the 'phycosphere' concept.

Despite these achievements, initiatives to harness algae-bacteria interactions are often motivated by either: 1) recognising new and intriguing microbial behaviours and exploring their potential applications, or 2) directly using documented microorganisms as models. Although these interactions have been exploited for various biological tasks, including waste treatment, bioproduct production, and harmful algae control, there are no clear guidelines for actively seeking and identifying broader algae-bacteria interactions. Also, there is a lack of efficient methodologies to progress from simply identifying interesting interactions towards engineering algae-bacteria consortia with desirable traits and functions.

1.6.1 Top-down approaches

An easy and straightforward approach to utilise algae-bacteria interactions is the top-down route, which involves deliberately introducing mixed communities containing algae and bacteria. The principle behind such combinations often centres on the division of labour (Min et al., 2011), or the occupation of different niches. An early study by Humenik and Hanna, 1971 demonstrated that combining the green algae *Chlorella* with bacteria in activated sludge led to a sufficient supply of algal-originated O_2 for bacteria and increased algal protein production within a WWT system. This finding inspired numerous later studies exploring advantages over bacteria-only systems, such as self-supporting systems and enhanced nutrient removal (Min et al., 2011; Inoue and Uchida, 2013).

Recent examples are still commonly found in WWT processes, where algae cultivation is intentionally integrated with sewage treatment. This integration facilitates high nitrogen and phosphorus removal rates and the unique capability of heavy metal uptake, with bacteria serving as primary biological nutrient removers and algae working as supplementary elements (He and Xue, 2010; Liu et al., 2012; Kesaano and Sims, 2014). Some studies even view bacteria contamination as beneficial for algae-bacteria co-cultures. For instance, Zhang et al. (2012) found that bacterial contamination in the heterotrophic cultivation of *Chlorella pyrenoidosa* enhanced the removal of total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) in wastewater.

While significant progress has been made, the initiation of consortia and the principles shaping community structures are often overlooked. Many studies examine the overall community structure or combine known microorganisms with desired traits, explaining observed results without delving into interactions at the community or cellular level. To uncover fundamental interaction mechanisms and design rules for combining algae and bacteria for specific applications, efficient methods integrating a top-down approach with functional insights are needed.

1.6.2 Bottom-up approach

With ever-increasing knowledge surrounding algae-bacteria interactions (Section 1.1) and the fast-expanding scope of biological engineering to better harness the capability of microbial consortia, researchers emphasize the importance of understanding individual microbes. This is because the behaviour of microbial consortia is significantly influenced by the characteristics of their microbial members (Bhatia et al., 2018; Jiang et al., 2017). Many microorganisms are well-documented, with readily accessible data on their biological characteristics, metabolic properties, and specific traits. Such information facilitates a bottom-up approach to microbial consortia research as referencing this data for microbe selection is essential for the successful integration of individual microbe into synthetic consortia (Fig.1.3).

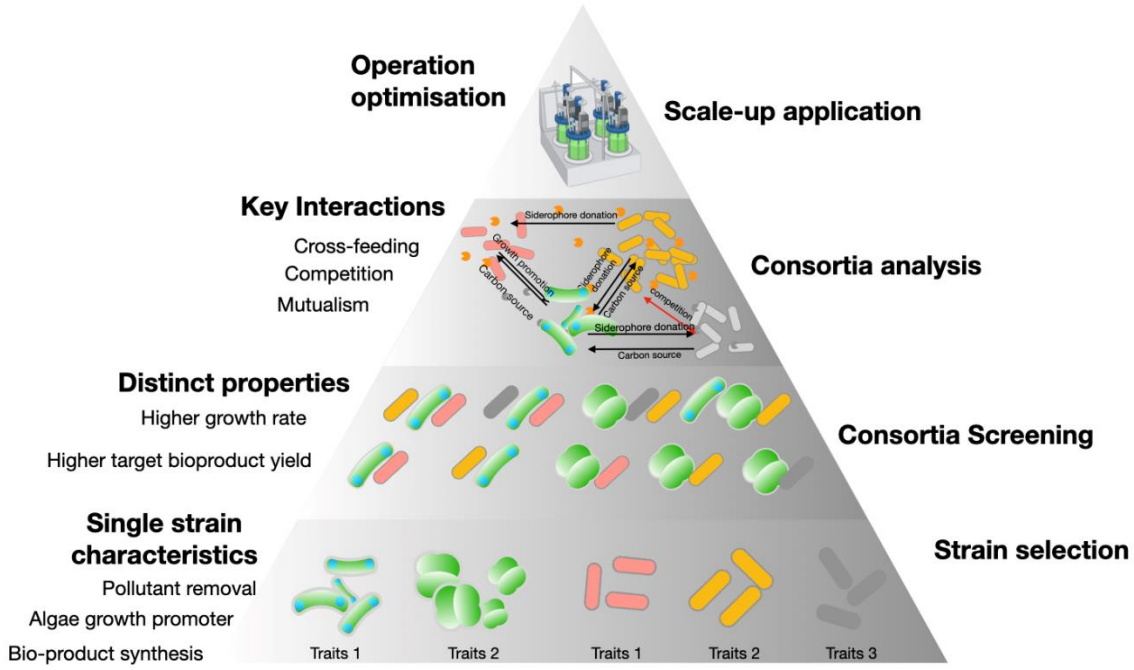


Fig.1.3 Conceptual demonstration of bottom-up approach for algae-bacteria consortia construction. This involves selecting individual microbial candidate based on prior knowledge about their characteristics and traits, and then integrating them to create synthetic consortia. Certain objects in this figure were created on *Biorender.com*.

The advancement of meta-omics studies has enhanced the feasibility of microbe selection based on the known functions and dynamics of specific biological molecules within each consortium member (Bedia et al., 2018). Metabolomics, particularly in environmental studies involving microorganisms, has shed light on algae-bacteria interactions (Daly et al., 2022). Many previously identified algae-associated bacteria have single or multiple metabolite connections with their algae counterparts and this knowledge is already well-available in different fields including phytoplankton communities, and marine ecosystem studies (Table 1.4). These *in-situ* findings underlying typical algae-bacteria interactions can be important references for laboratory interpretation of the same mechanisms for diverse purposes in algae biotechnology.

Understanding the role of microbial metabolites also opens opportunities for using genetic tools in consortia engineering, as metabolic pathways are determined and mediated by associated genes. The synthetic obligatory cooperative system (CoSMO) designed by Shou et al., (2007) serves as a classic example, wherein two gene-knockout yeast strains survive through metabolites cross-feeding. Similarly, Therien et al., (2014) used glycogen synthase knockout mutant strains of *Synechococcus sp. 7002* to provide acetate supporting *Chlamydomonas reinhardtii* growth for lipid production.

Table 1.4 Examples of metabolites molecules associated with algae-bacteria interactions.

Algae	**Associated bacteria**	**Metabolite molecule(s)**	**Function/Effect description**	**Effect type**	**Reference**
Chlamydomonas reinhardtii	*Pseudomonas protegens*	Orfamide A	Deflagellation and killing by compromising Ca^{2+} homeostasis in *C. reinhardtii*	– –	Aiyar *et al.*, (2017)
Gymnodinium catenatum, Chattonella marina, Heterosigma akashiwo	*Pseudoalteromonas*	1-acetyl-β-carboline	Active algicidal molecules produced by *Pseudoalteromonas*, cause algae cell-lysis, responsible for the natural elapse of some marine harmful algal bloom (HAB)	– –	Lovejoy, Bowman and Hallegraeff, (1998); Kim, Son and Jeong, (2015)
Emiliania huxleyi	*Phaeobacter gallaeciensis*	Roseobacticidesz	A family of rare bacterial terpenoids produced by *P. gallaeciensis*, having an algicidal effect, inhibit the growth of *E. huxleyi.*	– –	Seyedsayamdost et al., (2011)
Phaeocystis globose,	*Bacillus sp. strain B1*	*Urocanic acid, N-acetylhistamine, L-histidine*	Single and combined algicidal effect by increasing algal intracellular reactive oxygen species (ROS) level.	– –	Zhuang et al., (2018)

Table 1.4 (Continued) Examples of metabolites molecules associated with algae-bacteria interactions.

Algae	Associated bacteria	Metabolite molecule(s)	Function/Effect description	Effect type	Reference
Phaeodactylum tricornutum	*Pseudomonas chlororaphis JK12,*	*Allantoic acid, Urocanic acid, Cytidine 2′,3′-cyclic phosphate, Uridine 2′,3′-cyclic phosphate, Chlorinated tryptophan*	Single and combined algicidal effect mediated by dissolved oxygen (DO) availability.	**– –**	Kim et al., (2018)
Chlamydomonas reinhardtii	*Sinorhizobium meliloti,*	Vitamin B12 (Colalamin)	Produced by cobalamin-producing bacteria, promotes algae growth, and enhances algae thermal resistance.	**+ +**	Xie *et al.*, (2013)
Pseudo-nitzschia multiseries, Emiliania huxleyi	*Sulfitobacter sp, Phaeobacter inhibens*	*Auxins*	Plant hormones, growth-promoting for plants and algae, Can be produced by some bacteria	**+ +**	Segev et al., (2016) , Amin et al., (2015)

1.6.3 Challenges for algae-bacteria consortia construction

The specific focus and choice of target regarding the algae-bacteria interaction presents a major challenge for top-down approaches. As discussed above, top-down approaches often fail to reveal the interactions within the community, let alone the fundamental mechanisms that could guide the future design and refinement of the consortia. This is primarily due to the overwhelming number of possible interactions. To bridge this gap, previous studies have attempted to uncover how algal-bacteria consortia adapt to specific bioremediation applications using omics tools and from an evolutionary perspective (Okurowska et al., 2021; Qu et al., 2021; Si et al., 2022). However, to understand the fundamental interaction mechanisms and to establish design rules for combining specific algae and bacteria, more efficient methods for integrating top-down data collection with functional insights need to be developed.

The biggest challenge facing the bottom-up approach is predictability. Firstly, the biology of microbiomes is under the effect of a variety of ecological rules (Prosser et al., 2007; Shade et al., 2012). Many classical ecological principles for example, competition (Griffin et al., 2004; Hibbing et al., 2009), ecosystem succession (Fierer et al., 2010; Jiménez et al., 2017), mass cycling and trophic interactions (Gralka et al., 2020), have been well-recognised in the context of microbiology and were found as important shaping forces of microbial community assembly. Despite the growing knowledge of microbial ecology empowered computational tools that provide important insight toward consortia structure and stability (Coyte et al., 2015; Daly et al., 2022) as well as the enhanced ability of prediction (Zomorrodi and Segrè, 2016; Marsland et al., 2019), to simulate microbial consortia behaviour, experimental validation is still needed before these theoretical models can be reliably applied in practical applications.

Furthermore, in microbial systems with multiple species, extra complexity is expected due to the diversity of both interacting components and interacting networks or pathways. This can lead to more complicated system-scale behaviours beyond the function of individual components as a result of emerging properties i.e., effects of inner interactions being synthetically combined (Gilbert and Henry, 2015), which makes the microbial behaviours more difficult to predict. As pointed out by Olson et al., (2012), it is almost impossible to

design or optimise complex microbial systems without acquiring a large quantity of data about the whole system under many factors. Due to this reason, most modelled synthetic algae-bacteria communities are constrained to two-member systems (Mujtaba et al., 2017; Contreras-Angulo et al., 2019) and the selection for algae-bacteria pairs (Le Chevanton et al., 2013), whilst studies involving communities with multiple microbial members remain rare.

1.7 Conclusion

Global industries are evolving to shift from fossil fuel combustion for energy generation to more sustainable technologies in response to climate change and updating policies. Horticulture, as one of the emitters due to fossil fuel use for temperature control, has explored the integration of combustion for heating with CO_2 enrichment which serves as a sustainable carbon capture measure. Freeland Horticulture Ltd., the industrial collaborator and one of the sponsors for this project, aims at a novel approach by combining waste biomass combustion with greenhouse CO_2 enrichment to achieve efficient heating, enhance plant growth, and reduce waste while capturing CO_2. However, the specific waste concerning the company is mainly woody and herbaceous and produces VOCs which are a major concern for human health and air quality, and potentially detrimental to plant growth. Current mitigation methods for these VOCs are largely based on physical absorption, combustion techniques optimisation, and catalytic processes. There is an increasing need to develop sustainable solutions that are both feasible to the problems concerning the industrial sponsor and suitable for applications in broader fields.

Biological methods offer promising alternatives for remediating VOCs. Whilst bacteria seem to dominate the field of broader pollutant bioremediation, the inclusion of microalgae in the process presents attractive benefits. Microalgae, known for their adaptability, play a significant role in ecosystems. These photosynthetic organisms are not only pivotal in aquatic ecosystems but also able to produce valuable bioproducts like lipids for biofuel, carbohydrates for ethanol and biogas, fibrous polymers for biodegradable plastics, and pigments for various industries. Their ability for efficient photosynthesis makes them solar-driven cell factories and an attractive focus for biotechnology research.

Algae-bacteria communities, prevalent in natural environments, have evolved intricate interactions that include mutualism, competition, and nutrient exchange. The integration of algae and bacteria under the context of 'algae-bacteria consortia biotechnology', relies on these interactions to enhance bioproduction or bioremediation processes. Efforts to harness such interactions have been approached through both top-down and bottom-up strategies. The top-down approach which focuses on introducing mixed communities, has wide applications in wastewater treatment sectors for pollutant removal. On the other hand, the bottom-up approach relies on the meticulous selection and genetic engineering of strains to construct synthetic consortia tailored for specific tasks. However, challenges persist in both approaches. The top-down approach often struggles with specificity as a result of the overwhelming possibility of interactions, while the bottom-up approach faces predictability issues due to the complexity of multi-species systems and the need for extensive data. Despite these challenges, understanding and utilising algae-bacteria interactions continues to be a promising avenue for sustainable biotechnological and addressing environmental challenges.

Given the industrial context and the promising potential of algae-bacteria interaction, this project aims to develop an effective methodology for identifying and optimising algae-bacteria consortia capable of bioremediating volatile organic compounds (VOCs) associated with biomass combustion. To address the challenges highlighted in this review from the associated field of study, this project aims to resolve specificity and predictability issues in the engineering of algae-bacteria consortia, particularly in multi-species systems, through robust experiment design and data collection as well as the precise modelling of the relevant microbial systems. Additionally, this project tests and validates the functionality of the algae-bacteria consortia of interest, assessing their potential for upscaling to real industrial-scale operations. The project has also attempted to analyse the selected consortia to deepen the understanding of algae-bacteria dynamics, providing insights into microbial interactions and offering guidance for future work.

1.8 Project objectives

In response to the industrial context and the challenges identified in the literature review, the specific aims and objectives of this project are set as follows:

1. Microorganism acquisition: Obtain phototrophic microalgae and chemotrophic bacteria with novel metabolic capabilities to degrade target VOCs concerning biomass combustion.
2. Consortia construction: Integrate characterised algae and bacteria to establish artificial algae-bacteria consortia.
3. Consortia screening: Develop a screening method to assess the performance of the consortia and rapidly shortlist promising candidates.
4. Performance verification: Confirm the VOCs biodegradation abilities of the screened consortia and evaluate the accuracy of the screening method.
5. Community structure analysis: Analyse the microbial community structure within the consortia to set clear objectives for future in-depth studies, such as proteomics research focusing on interactions or functional roles.

1.9 Core hypothesises.

To achieve the above-stated objectives, the following hypotheses will serve as the core research guidelines for this project:

- Hypothesis 1: Areas polluted by VOCs are more likely to harbour microorganism with beneficial traits due to prolonged natural selection. The isolated microorganisms from these areas are expected to have pre-existing interaction pathways or connections.
- Hypothesis 2: An active consortium, along with its culturing environment, can be viewed as an ecosystem wherein microalgae, functioning as the ecosystem's 'flora',

serve as the primary providers of energy and carbon. The efficiency of algae photosynthesis plays a crucial role in regulating the energy influx into the system.

- Hypothesis 3: Random combinations of candidate microbes can stochastically recreate pre-existing interactions and also give rise to new emergent properties in vitro. Selective stress from VOCs can aid in screening and identifying consortia of interest for this project.
- Hypothesis 4: In an established consortium, certain microbial members may be pivotal in determining the overall performance of the consortium. These core microbes can be identified by comparing the performance of consortia in their presence/absence.

1.10 Thesis outline

The thesis is organized into six chapters. Chapter 1 introduces the topic and reviews relevant literature. Chapters 2 to 5 outline the four key experiments conducted, the steps of which are also illustrated as a graphic abstract (Fig.1.4). Chapter 6 provides a brief summary of the work, discusses future research, and explores potential applications of the methods developed in the project.

Chapter 1 provides an overview of algae bioproducts and relevant research about algae-bacteria interactions, highlighting the current focus, applications, and research approaches for combining algae and bacteria for various biological tasks. It discusses the characteristics and potential for the integration of photosynthetic microalgae and chemotrophic bacteria and points out research gaps in algae-bacteria consortia engineering. This chapter also briefly introduces the context of the industrial background, covering the project's industrial sponsor’s case study.

Chapter 2 focuses on the acquisition and characterisation of microalgae and bacteria candidates for creating consortia capable of biodegrading specific VOCs, i.e., benzene, toluene, phenol and THF. The chapter outlines the methodologies and assays used in microbe isolation, selective culturing, characterisation, and identification. It also discusses

the possible behaviours or interactions in co-culturing microbial systems as a potential aid in the engineering of robust consortia for efficient VOCs biodegradation.

Chapter 3 introduces the bottom-up approach to integrating individual algae and bacteria isolates to form algae-bacteria consortia. By measuring chlorophyll abundance as a proxy of consortia performance, a high-throughput screening method based on 96-well microplates was developed which facilitated rapid shortlisting of algae-bacteria combinations. The experimental design includes three scoring indexes to assess the algae growth-promoting potential and functional stability of the consortia without the need for interpreting complex interaction networks.

Chapter 4 presents a case study on selected consortia with distinctive performance as identified in Chapter 3, serving as a validation of the screening method. The VOCs biodegradation ability was verified using Gas Chromatography with Flame Ionization Detector (GC-FID), and algae biomass was examined for correlation with VOCs degradation. The results were compared with screening outcomes to assess the validity and predictive accuracy of the screening method.

Chapter 5 shows a subsequent analysis of the bacterial community structure of the exemplar algae-bacteria consortia examined in Chapter 4 using dye-based qPCR. The chapter quantifies the bacterial population in the consortia and profiles the bacterial community structure. The findings reveal core bacteria within the consortia and provide insights for future in-depth studies, such as proteomics studies focusing on interactions or functional roles.

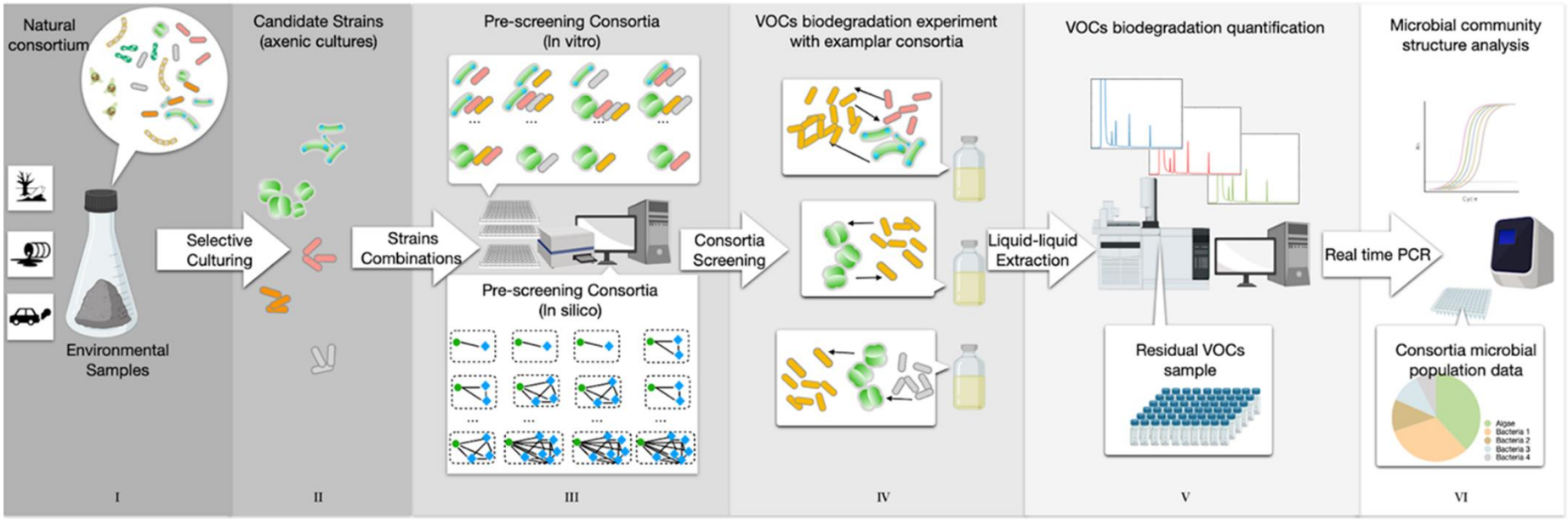


Fig.1.4 Graphic abstract of experiments conducted. **I.** Environmental microbe isolation and selective culturing. **II.** Identification and characterisation of isolated microbes. **III.** and **IV.** Consortia construction, screening, and assessing in 96-well plates. **V.** GC-FID Analysis on VOCs degradation performance of exemplar consortia. **VI.** Exemplar consortia microbial community structure analysis using qPCR. Note: Certain objects in this diagram were created using *Biorender.com.*

Chapter 2: Microbial Candidates Acquisition and Characterisation

Aims and objectives

- *Isolation of microalgae and bacteria candidates for their potential in the biodegradation of specific VOCs.*
- *Identification and characterisation of obtained microorganisms.*
- *Evaluation of co-culturing potential based on published literature*

2.1 introduction

Microbial communities have been used as important biological tools for a variety of purposes associated with agriculture, the food industry and human health. Artificial engineering of microbial communities is an emerging field of research driven by the discovery of stable and efficient microbial systems. The selection of microbial members is fundamental to the successful design of microbial communities due to its direct association with the functions of the microbial system and the efficiency of target tasks.

In-depth characterisation of microorganisms, such as those listed in the literature review (Table 1.3 and Table 1.4), facilitates the selection of suitable microbial candidates for algae-bacteria consortia engineering. Existing knowledge has been used to categorise these modelled microbes based on growth dynamics, interactions, and metabolic pathways, which have wider applications in enhancing productivity in a more controlled manner. Also, molecular tools developed based on these microorganisms enhance the feasibility of modification or manipulating the consortia towards achieving higher performance. Despite the wide availability of modelled microbes with promising functions and traits which appear to be beneficial for a specific test, randomly selected microbial members could however lead to the failure of the entire microbial systems due to the risk of ecological compatibility (metabolic conflict) and potential pathogenicity. These risks could be even more pronounced under the context of this study where microorganisms of entirely different trophic classifications are to be combined.

Natural environments present an attractive source of microorganisms due to several benefits. Firstly, natural habitats like water, soil and rocks are inhabited by a variety of microalgae (Bérard et al., 2005; Aburai et al., 2013) and bacteria (Torsvik et al., 1996). Microbes isolated from these environments are usually adapted to conditions where the microbiome usually possesses unique traits for surviving extreme conditions or degrading specific compounds (Dash et al., 2013). These metabolic abilities provide inherent advantages in various applications such as the production of biopolymers (El-Saied et al., 2007; Vishnuvardhan Reddy et al., 2008), biosurfactants (Elazzazy et al., 2015), or enhanced fermentation processes based on effective feedstock breakdown (Taylor et al., 2012). Also, microorganisms usually exist in microbial communities where interactions are established.

These pre-existing interactions with their naturally evolved synergies, can benefit the study and engineering of laboratory-assembled consortia.

This chapter introduces the acquisition of microalgae and bacteria candidates to make up consortia in a bottom-up way. Environmentally isolated bacteria and microalgae were characterised for their metabolic capabilities for the biodegradation of specific VOCs. Microbial species or strains with known traits reported in previous studies were also examined. This chapter presents the experimental methodologies and assays used in microbial isolation, selective culturing, identification, and characterisation based on the checklist (Table 2.1) of desired traits for the construction of consortia. From the results of the microbial identification and characterisation, this chapter also presents other research findings on the microbial candidate, where available. It also discusses their potential behaviour or interactions in co-culturing microbial systems, which can potentially aid in the engineering of robust consortia which can efficiently biodegrade VOCs.

Table 2.1 Checklist for desired traits for microalgae selection

Process	**Desired traits**
Environmental sampling	• Resistant to gasoline and diesel pollutants: containing a variety of monoaromatics, diaromatics to polyaromatic compounds (Sharma et al., 2013). • Pre-adapted *in situ* and coevolved environmental communities, • High diversity to provide adequate microbial candidates
Adaptive culturing	• Rapid growth • VOC resistant
Preliminary screening	• VOC resistant • Culturable *in vitro*
Microbial identification and characterisation	• The ability of VOC degradation • Suitable for scale-up • Potentially synergistic interactions

2.2 Methodology

2.2.1 Synthetic VOCs feeds

As presented in Chapter 1, aromatic compounds, furans and aldehydes are the main VOCs of concern associated with biomass combustion. In this research, benzene, toluene and phenol were selected as aromatic pollutants as these compounds are also typical target substrates in bacterial biodegradation experiments (Reardon et al., 2000; Hamed *et al.*, 2003; Abuhamed *et al.*, 2004). Tetrahydrofuran and formaldehyde were selected as representative VOC pollutants as these compounds themselves or their homologues are abundant in biomass combustion waste streams. All the selected chemicals have some extend of solubility in water (Table 2.2) at room temperature which allows the preparation of aquatic VOCs media.

Table 2.2 Solubility of VOCs compound in water and their stock concentration.

Compound	**Solubility in water g/L**	**Condition**
Benzene	1.8	25 °C
Toluene	0.535	25 °C
Phenol	84	20 °C
Tetrahydrofuran	freely soluble	N/A
Formaldehyde	400	25 °C

Solubility data source: International Programme on Chemical Safety (IPCS)

2.2.2 Microbial candiates acquisition

2.2.2.1 Laboratory collections

Microorganisms characterised in previous published studies were also considered in this project. *Chlorella vulgaris* CCAP 211/211B is one of the most studied microalgal strains in biochemistry (Chen et al., 2011; Safafar et al., 2016) and has been placed on the list of algae candidates due to its availability in our laboratory.

Also, bacteria *Pseudomonas putida F1, Pseudomonas oleovorans, Pseudomonas sp. OX1, Pseudomonas aeruginosa, Rhodococcus sp EH831,* and *Bacillus subtilis DM-04* were put on the bacteria candidates list due to their robust ability to degrade VOCs pollutants. However, only *Pseudomonas putida KT2440* was acquired from the Department of Animal and Plant Science, University of Sheffield.

2.2.2.2 Environmental isolation

Ideally, the industrial combustion systems associated with this project would be the best source for isolating microorganisms that are adapted to the VOCs of interest, the field work plan was delayed and eventually cancelled due to COVID-19 disruptions. Instead, sites exposed to crude oil-associated compounds were considered viable options for sourcing microorganisms. Petroleum-derived compounds, especially BTEX compounds, are chemically similar to the VOCs selected for this study (Montagnolli et al., 2015). Microorganisms isolated from such areas, like pitch lake (Schulze-Makuch et al., 2011) or petroleum-contaminated soils (Obayori et al., 2008; Xia et al., 2017), have been shown to possess genes associated with the degradation of hydrocarbons or have demonstrated the ability to degrade these compounds. Guided by these studies, a site under prolonged exposure to exhaust and crude oil products was chosen for the increased possibility of isolation and enrichment of bacteria and microalgal candidates that are either tolerant or even able to degrade the VOCs of interest. Soil samples were taken from road verges with apparent oil spillage from the closest diesel pump located at 53 °23 ’11.88” N, 1°28 ’46.02” W (Fig 2.1 a). After removing the plant debris and surface soil (3-5 cm in depth), 30 mL of soil sample was collected using a sterilised steel teaspoon and 50 mL falcon tube (Fig.2.1 b). Twenty surface samples were collected from road curbs and a wall with signs of photosynthetic organism

growth (Fig.2.1 c) using sterile swabs moisturized by PBS buffer and which were placed in two falcon tubes (Fig.2.1 d). All samples were taken in winter, December 2019 and stored in the laboratory fridge at 4 °C before processing.

Fig.2.1 Environmental sampling. a) Fuel station, b) Soil sample, c) wall surface, d) swab samples.

2.2.2.3 Sample processing

Ten grams of soil sample was suspended in sterile PBS solution to a final volume of 30 ml. The soil suspension was then split into 6×5 mL aliquots and transferred to heterotrophic and autotrophic flasks. Swab tips of surface samples were mixed with 30 mL of PBS solutions followed by a 3 min vortex at the highest speed to release the adherent microorganisms. The resulting suspension was aliquoted into 6×5 mL volumes before being transferred into heterotrophic and autotrophic flasks. Instead of an environmental study, this experiment is to isolate and enrich candidate microbes for subsequent experiments. Therefore, all the flasks were prepared in duplicate, and no statistical analysis was performed for each sample and replicas. Culturing flasks were filled with 45 mL of different media or growth substrates aimed at promoting a different mode of microorganism growth. Each flask had a final culture volume of 50 mL with the VOCs concentration upon experiment initiation as detailed in Table 2.3.

Table 2.3 Media and VOCs concentration of different culture flasks

Flasks configuration	Media	Light scheme	VOCs Concentration (mg/L)				
			B	T	P	THF	FA
Heterotrophic	PBS media + LB broth	Dark (24 h)	10	10	10	10	/
Autotrophic	3N-BBM+V media	Light (24 h)	10	10	10	10	/

LB broth final concentration 2 g/L; B: Benzene, T: Toluene, P: Phenol, THF: Tetrahydrofuran, FA: Formaldehyde (FA was not added due to unavailability during the initial culturing).

2.2.2.4 Adaptive culturing and microorganism isolation

Heterotrophic flasks were designed for selective isolation of bacteria and growth inhibition of autotrophic microorganisms. LB broth was added as a carbon source to support growth. Selective stress was introduced using a low concentration (10 mg/L) of each VOC. The flasks were sealed with breathable cotton films and placed in a 30 × 30 × 15 cm plastic box with pored cap which was designed to slow down the VOCs escaping in the culture while allowing air exchange. Cultures flasks were incubated at room temperature 20-22°C for 4 weeks. Growth status was visually observed daily. To maintain the nutrient availability and VOCs concentrations in the cultures, partial media refreshment was conducted every 2 days by adding 5 mL of heterotrophic media containing the same concentration of VOCs. However, to avoid disturbance of cell densities in the culture, media refreshing was only performed when evident signs of microbial growth such as increased turbidity and colour changes of cultures were observed.

To isolate microalgae, autotrophic cultures were stored and incubated using the same method under identical conditions as those in the heterotrophic flasks, except for additional illuminance. Growth status was monitored daily. Upon observing evident signs of

microorganism growth such as cultural turbidity and colour changes, partial media refreshment was performed following the same 2-day schedule.

After four weeks of adaptive culturing, all flasks were vortexed to mix thoroughly before 5 mL culture suspensions were taken. After centrifuging (2000 g, 5 min), acquired pellets were washed using corresponding fresh autotrophic/heterotrophic media and subsequently transferred into another batch of flasks and incubated for another 4 weeks. This serves a subculture of the initial adaptive culturing and the incubation condition remained the same.

At the end of the subculturing, 5 mL culture suspensions were taken from each flask and centrifuged (2000 g, 5 min) to separate biomass from the medium. Biomass pellets were re-suspended in 5 mL normal PBS buffer followed by 3-7 series of 10-fold dilutions before being spread out on Petri dishes embedded with corresponding media agar. Inoculated Petri dishes were incubated for one week at room temperature with or without illuminance (24 h, 7 days) and growth status was monitored daily. Any isolated bacteria and microalgae colonies were presumptively classified according to colony morphologies and the total viable count (TVC) test results and microscope check. Media and classification criteria in this step are detailed in Table 2.4.

Table 2.4 Culturing media and preliminary classification criteria of isolates

Microorganism	**Media**	**Colony Morphology**				**Cell Morphology**		
		Colour	Surface	Shape	Margin	Shape	Colour	Size
Bacteria	LB agar*	×	×	×	×			
Microalgae	3N-BBM+V agar*					×	×	×

*Agar was prepared in 1.5% w/v concentration in corresponding media; Symbol × refers to the related criteria that have been considered; Microalgae were classified via microscopic observation on individual cell morphology due to their identical colony morphology, but larger cell size compared to bacteria.

2.2.2.5 Enrichment and axenic culture

Classified bacteria and microalgae were assigned with a unique ID number. The associated colonies were selected using sterile inoculation loops for sub-culture. A series of sub-

cultures was performed using 96-well plates limiting dilution specified by Lo Surdo and Bauer, (2012) with a culture volume of 200 µL in each well combined with the streaking plate method for further purification.

For bacteria subcultures, uniform colony morphology was considered a sign of axenic bacteria growth. For microalgae, 180 µl of culture was taken from each batch of dilution and was subsequently pipetted (20 µl per drop) on Petri dishes containing LB agar. After 72 h incubation in the dark at room temperature, plates with no bacteria colonies formed were considered bacteria-free.

2.2.2.6 Microorganism growth and VOCs resistance experiment

The average growth rates of each microalgae candidate were calculated using the following equation (Eq. 2.1)

$$r = \frac{N_t}{N_{t-1}} \times 100\% \qquad \text{Eq.2.1}$$

r *:* average growth rate*;*

N_t*:* Quantity of microorganisms at time t;

N_{t-1}*:* The number of microorganisms at the preceding time step, t

t*:* The specific point in time or the time interval being considered.

which serves as one of the key criteria for algae candidate selection in this project. Also, to validate the VOCs resistance and determine their resistance threshold, all microalgae and bacteria isolates along with the laboratory collections, i.e., algae *C. vulgaris* CCAP 211/211B and bacteria *P. putida KT2440*, were cultured with elevated VOC concentrations. Consequently, to assess their ability to utilise VOCs as carbon sources, all bacteria isolates were cultured in a carbon-deprived medium, i.e., PBS solution containing the same concentration of VOCs as the only carbon sources. Media selections and VOCs concentration are detailed in Table 2.5. The choice of VOCs concentration was guided by prevalent BTEX degradation studies (Kim et al., 2008; Jiang et al., 2015).

Table 2.5 Culturing condition, media, and VOCs concentration for VOCs resistant experiment.

Microorganism	Media	VOCs concentration (mg/L)				Illuminance	Shaking
		B	T	P	THF		
Bacteria	LB broth	100	100	100	100	Dark	120 rpm
	PBS solution	100	100	100	100	Dark	120 rpm
Microalgae	3N-BBM+V	40	40	40	40	24 h	120 rpm
		100	100	100	100	24 h	120 rpm
		200	200	200	200	24 h	120 rpm

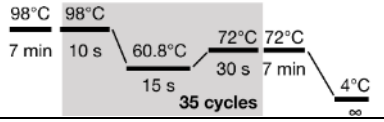
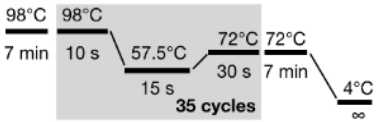
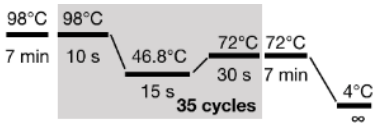
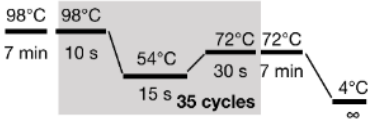
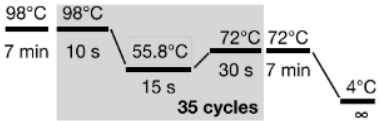
BTP: benzene (B) and toluene (T) and phenol (P). Tetrahydrofuran (THF);

2.2.2.7 Microorganism identification

Characterization of the bacteria and algae isolates was achieved by DNA sequencing of the 16S rRNA gene (bacteria) and 18S rRNA (microalgae). Individual colonies (48 h culture, LB agar) of different bacteria isolates were picked using sterilised tooth sticks and suspended in 20 µl of nuclease-free water (QIAGEN) in 0.2 mL PCR tubes. Template DNA samples were obtained by a modified heat-lysis protocol (Englen and Kelley, 2000) at 98°C for 5 min using a thermocytometer (ABI 9700). Partial 16S rDNA PCR was performed using Phusion High-Fidelity PCR Kit (Thermo Scientific™) according to the kit manual and recommended PCR program settings. Two different sets of forward and reverse primer pairs which bind different regions on the bacterial 16S rRNA gene were used however, this chapter presents results based on primer set 535(F) and CD(R) which achieved successful amplification (Table 2.6).

Microalgal template DNA was extracted from fresh individual colonies (120 h culture) through 10 min incubation at room temperature in 20 µl of dilution buffer provided in Phire Plant Direct PCR Kit (Thermo Scientific™). Partial 18S rDNA PCRs were performed following kit protocols using four pairs of forward and reverse primers and recommended PCR program settings (Table 2.6).

Table 2.6 Primer selection and PCR program setting

Template DNA	Primer		Amp. length	Program settings	Reaction Volume	Reference
	Name	**Sequence (5'-3')**				
Bacteria 16s rDNA	515 (F)	GTGCCAGCMGCCGCGGTAA	440 bp		50 µl	Rudi et al., (1997); TURNER et al., (1999)
	CD (R)	CTTGTGCGGGCCCCCGTCAATTC				
Algae 18s rDNA	18s-SS3 (F)	GGTGATCCTGCCAGTAGTCATATGCTTG	1800 bp		50 µl	Khaw et al., (2020); Matsumoto et al., (2010)
	18s-SS5(R)	GATCCTTCCGCAGGTTCACCTACGGAAACC				
	ITS_5.8 (F)	GAAGTCGTAACAAGGTTTCC	800 bp		50 µl	Timmins et al., (2009)
	ITS_5.8 (R)	TCCTGGTTAGTTTCTTTTCC				
	PA (F)	AACCTGGTTGATCCTGCCAG	500 bp		50 µl	Alemzadeh et al., (2014)
	SSU-inR1 (R)	CACCAGACTTGCCCTCCA				
	P45 (F)	ACCTGGTTGATCCTGCCAGT	550 bp		50 µl	Duong et al., (2012)
	P47(R)	TCTCAGGCTCCCTCTCCGGA				

The PCR products were verified by gel electrophoresis on 1% ultrapure agarose gel (Invitrogen™) prepared using Tris-acetate-EDTA (TAE) buffer containing 0.5 µg/mL of ethidium bromide (EtBr) as fluorescent stain. Amplification product bands corresponding to target lengths were collected and further purified using the QIAquick Gel Extraction Kit (QIAGEN) following the kit protocol. The extracted DNA concentrations were measured using a NanoDrop 2000/2000 Spectrophotometer (Thermo Scientific™). Bacteria DNA samples were submitted to the Sheffield Microarray/Genomics Core Facility (The University of Sheffield, UK) and sequenced using a 3730 DNA analyser (Applied Biosystems®). Microalgae DNA sample was sequenced by GENEWIZ® (Takeley Sanger Sequencing Laboratory, UK) using Illumina® Platforms. All sample sequencing analysis was bidirectional and searched for their species or genus identities using NCBI-BLAST.

2.2.2.8 Preliminary algae-bacteria co-culture

To determine if the isolated algae and bacteria are co-culturable and investigate potential algae growth-promoting effects, each bacteria isolate was pairwisely co-cultured with two chosen algae candidates, respectively in 96-well plates. For the microalgae inoculant, cultures at the stationary phase were centrifuged and the resulting pellets were washed twice using the 3N-BBM+V. 10 µl of algae cells were subsequently distributed into each well. Bacteria inoculants were prepared using single colony suspensions in the same medium. Each well contained 200 µL of 3N-BBM media with 100 mg/L concentrations of each VOC compound and approximately 10^3 algae cells as counted using a hemocytometer. The inoculated 96-well plates were incubated at room temperature under continuous illuminance for 7 days and their growth patterns were visually monitored.

2.2.3 Material and equipment

2.2.3.1 Media and VOCs solutions.

In the above-mentioned experiments, all media was prepared as concentrated sterile stocks. The final media was prepared by mixing sterile stocks with deionised water to reduce media preparation time, minimise VOCs losses and facilitate calculation, especially when handling ppm concentration chemicals. All media dilution was performed aseptically in a vertical laminar airflow hood to ensure sterility.

3N-BBM+V media were used as culturing media and processing buffer was prepared using the recipe as provided on the Culture Collection of Algae and Protozoa (CCAP) website and detailed in Table 2.7

Table 2.7 3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified) stocks

Component	**Amount**
Mineral (Per Litre)	
$NaNO_3$	75 g
$CaCl_2.2H_2O$	2.5 g
$MgSO_4.7H_2O$	7.5 g
KH_2PO_4	17.5 g
$K_2HPO_4.3H_2O$	7.5 g
NaCl	2.5 g
Trace Elements (Per Litre)	
Na_2EDTA	0.75 g
$FeCl_3.6H_2O$	0.097 g
$MnCl_2.4H_2O$	0.041 g
$ZnCl_2.6H_2O$	0.005 g
$CoCl_2.6H_2O$	0.002 g
$Na_2MoO_4.2H_2O$	0.004 g
Vitamins (Per 100 mL)	
Vitamin B1 (Thiamine hydrochloride)	0.12 g
Vitamin B12 (Cyanocobalamin)	0.1 g

To prevent precipitation, minerals and vitamins were prepared separately as stock1 ($NaNO_3$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, NaCl), stock2 (KH_2PO_4, $K2HPO_4.3H_2O$), stock3 (all trace elements) and stock 4 (Vitamins); Make up to 1litre media with dH_2O and 10 mL stock1 and stock2, 6 mL stock3, 1 mL stock4.

LB broth (Miller) for bacteria culturing was prepared in ×5 stocks. PBS solution used for sample processing, microbial culturing, washing, and dilution was prepared according to the recipe in Table 2.8.

Table 2.8 PBS (Phosphate Buffered Saline) (1X, pH 7.4)

Component	**Amount**
KCl	8 g
Na_2HPO_4	200 mg
KH_2PO_4	1.44 g
KH_2PO_4	245 mg

For VOCs solutions, all the compounds were prepared as 5× stocks (Table 2.9) in PBS buffer (1×) and 3N-BBM+V media (1×) by injecting anhydrous liquid-form chemicals (Sigma-Aldrich) using a 1 mL gas-tight syringe (ThermoScientific™) to ensure accuracy.

Table 2.9 VOCs solutions (5X)

Component	**Concentration (3N-BBM+V)**	**Concentration (PBS buffer)**
Benzene	500 mg/L	500 mg/L
Toluene	500 mg/L	500 mg/L
Phenol	500 mg/L	500 mg/L
Tetrahydrofuran	500 mg/L	500 mg/L

Due to the low solubility of nonpolar chemicals in water, the stocks were prepared using a water bath (50 °C).

TAE buffer for agarose DNA electrophoresis was prepared following the recipe in Table 2.10.

Table 2.10 TAE buffer (Tris-acetate-EDTA) (10X)

Component	**Amount**
0.5M EDTA	20 mL
Glacial acetic acid	11.4 mL
Tris	48.5 g

Components were added following the sequence specified in the table.

2.2.3.2 Optical density (OD) measurements and microscope imagery

Chlorophyll a and chlorophyll b which exhibit maximum absorbance at wavelengths around 480 nm and 680-685 nm (Datt, 1998) respectively, were measured to verify the growth statuses of algae isolate in plastic cuvettes with a light path of 1 cm (Fisher Scientific™) using Spectronic 200E spectrophotometer (ThermoScientific™).

An Olympus BX51 microscope (company, place, country) was used for colony classification, cell morphology checking and hemocytometer cell counting. High-resolution microscopy images were captured by a camera coupled with a PC installed with ProgRes CapturePro 2.6 software. All microscopic image processing was conducted using MATLAB 2020.

2.3 Result

2.3.1 Microorganism isolate

Following the adaptive culturing, 26 bacteria isolates were obtained from heterotrophic-incubated environmental samples. Along with the *P.putida KT2440* strain sourced from the Animal and Plant Science, University of Sheffield, a total of 27 bacterial collections were procured. VOCs resistance experiment suggested that out of these colletions, 26 demonstrated VOCs resistance as evidenced by their observed growths in LB media which contained up to 100 mg/L concentrations for each VOC. These VOCs-resistant bacterial isolates were included in the candidate bacteria collections (Fig.2.2). Hypha-forming microorganisms from environmental samples were excluded as they fall outside the scope of this project.

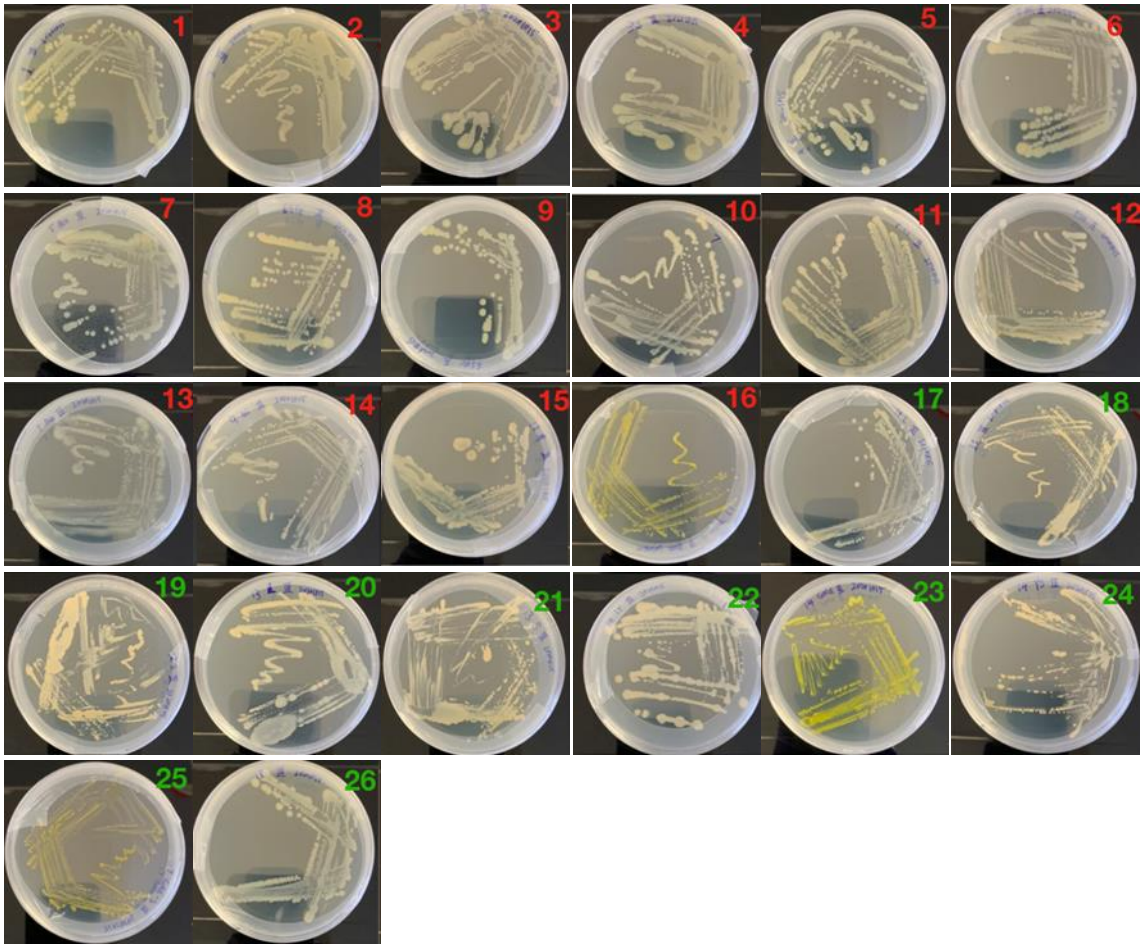


Fig.2.2: VOC-resistant bacterial isolates. Red numbers indicate bacteria isolated from mixed colonies, while green represents isolates from a single colony.

Two microalgae isolates obtained from the soil sample and one from plant leaves were isolated after autotrophic adaptive culturing. The isolates were preliminarily identified as three different species according to their distinct cell morphologies (Fig.2.3) with temporary labels as *ST*, *SA* and *LA*, respectively. Although three microalgae candidates exhibited resistance to 10 mg/L VOCs concentrations in the adaptive culturing period, microalage *LA* , isolated from plant leaves surfaces, was later excluded due to slow growth and strong adhesion on the glassware surface. ST and SA exhibited good mixing and suspension properties when cultivated in liquid culture.

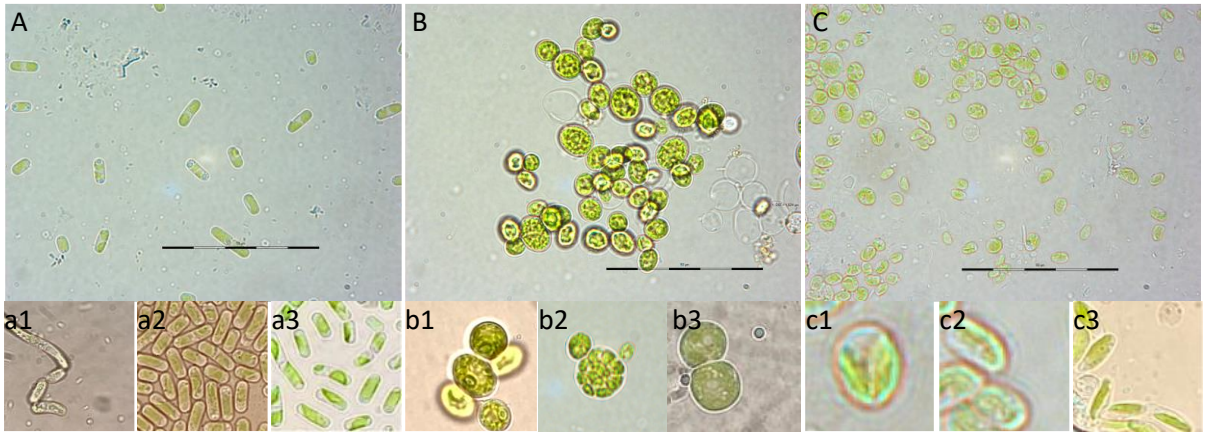


Fig.2.3 Microscopic images of microalgae isolates A (*ST*), B (*SA*), and C (*LA*) and their morphology variations during different growth stages: individual cells (a2, b1,c2,c3), cell division (a1,a3;b2;c1).

2.3.2 Characteristics of isolates

Carbon-deprived culturing in PBS solution revealed that at least 6 bacteria isolates were able to utilise VOCs as growth substrates (Fig.2.4) suggesting their promising capability for VOCs degradation. Also, two bacteria isolates (ID:1 and 2) exhibited strong fluorescence under UV light (Fig.2.5) which was accidentally discovered during UV sterilisation of the laminar airflow hood.

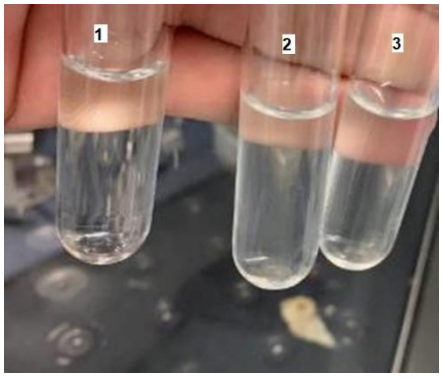


Fig.2.4 Bacteria isolates grew in PBS solution, using VOCs as only growth substrates. 1: Blank medium, 2 and 3 Bacteria growth resulted in the turbidity of the media

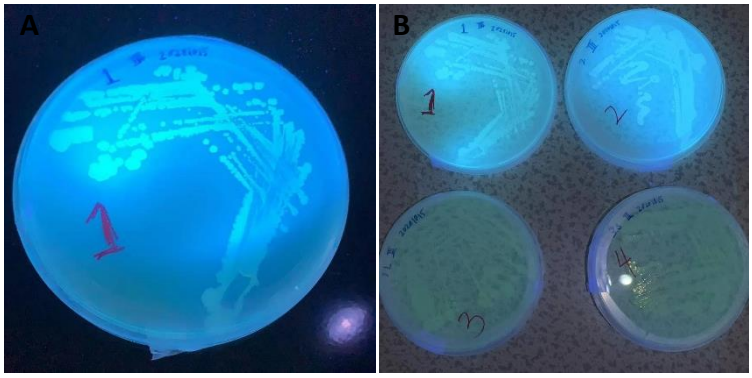


Fig.2.5 A**:** Bacteria isolates exhibit fluorescence; B**:** comparison between fluorescent and non-fluorescent cultures; culture media: LB agar.

Characterization of growth in microalgae isolates (Fig.2.6) indicated that over an 8-day cultivation period in normal 3N-BBM+V media, ST achieved a growth of 1000×10^4 cells/mL, while *SA* reached a lower cell density of 45×10^4 cells/mL. *ST* exhibited a lower maximum growth rate of 51.5% (observed on day 7) and had a shorter lag phase (1 day) compared to SA which had a maximum growth rate of 64.9% but a prolonged lag phase (≥ 4 days).

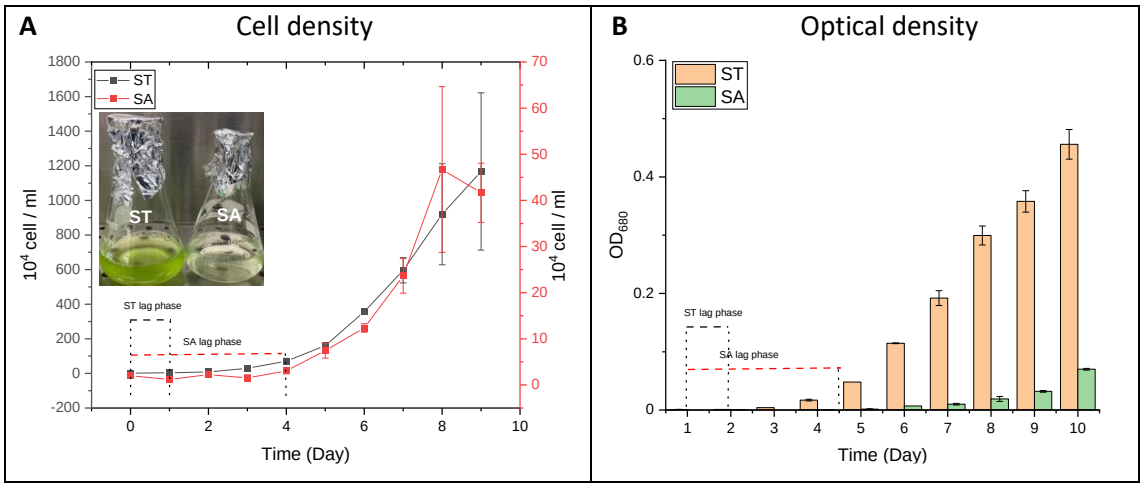


Fig.2.6 Growth curves and lag phases of microalgae isolate ST and SA in VOCs-absent culture condition. Visualised in both cell density data (A) and optical density at 680 nm (B).

It is worth noting that *SA* exhibited pigment accumulation (Fig.2.7) after prolonged culturing (≥14 days). The orange pigment was later identified as a carotenoid characterised by its absorbance peak lying between 450 and 470 nm according to spectrophotometer wavelength scan results (data not shown).

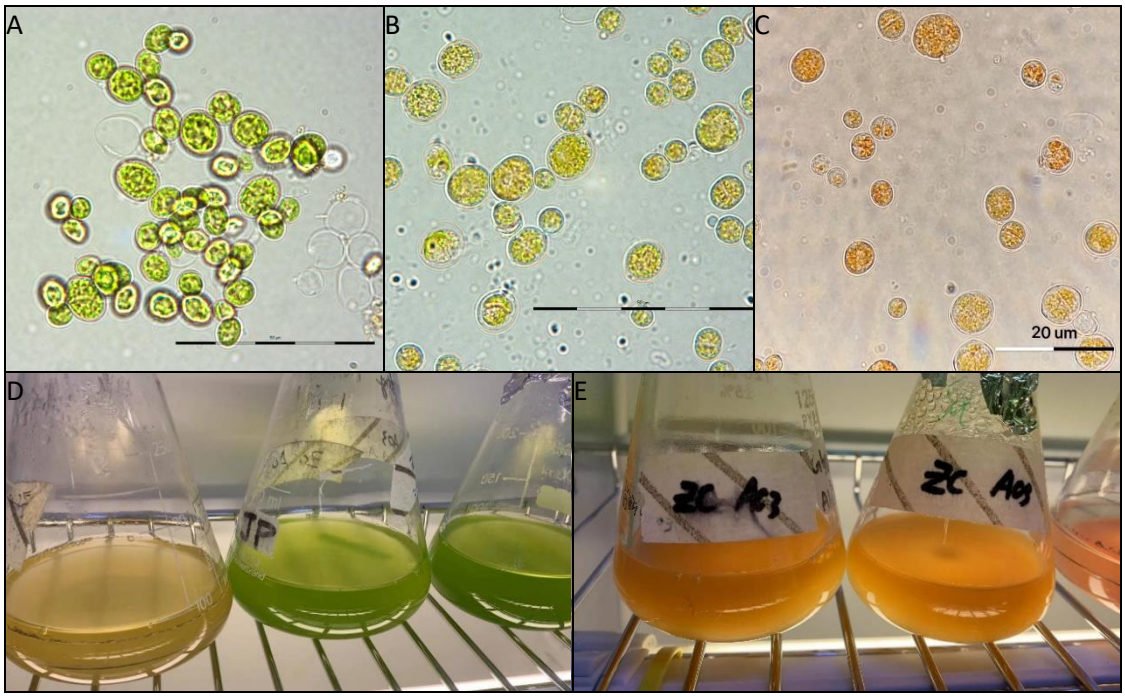


Fig.2.7 Microscopy image of microalgae isolate *SA* and colour change over time, A: 0-14 days, B:≥14 days C: preservation stock ≥ 30 days, magnification: 10 ×100; D:14 days; E: ≥ 30 days.

The VOC resistance experiment showed that both algae demonstrated some degree of resistance to VOCs, as evidenced by their growth even under increased VOCs concentrations (Fig.2.8). Notably, SA exhibited stronger resistance to VOCs, as suggested by its survival in media where each of the four VOCs were present at a concentration of 200 mg/L. Moreover, the growth of SA was consistently higher than that of ST cells under these conditions. This suggests that the SA is a more suitable algae candidate for consortia for VOCs biodegradation. However, the laboratory collection *C. vulgaris* CCAP 211/211B was not able to grow in the presence of 40 mg/L of VOCs (data not shown) and was subsequently excluded from the candidate list.

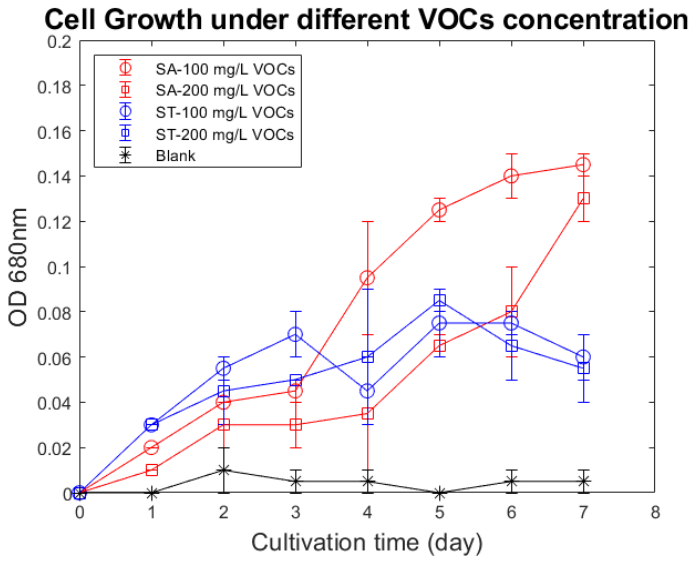


Fig.2.8 Optical densities at 680 nm of algae isolate ST and SA grown in media containing different concentrations of VOCs

2.3.3 PCR results

PCR successfully amplified the partial 16S rDNA of bacteria in all 26 samples. The amplification product exhibited a uniform length of about 440 bp as presented in gel electrophoresis results (Fig.2.9)

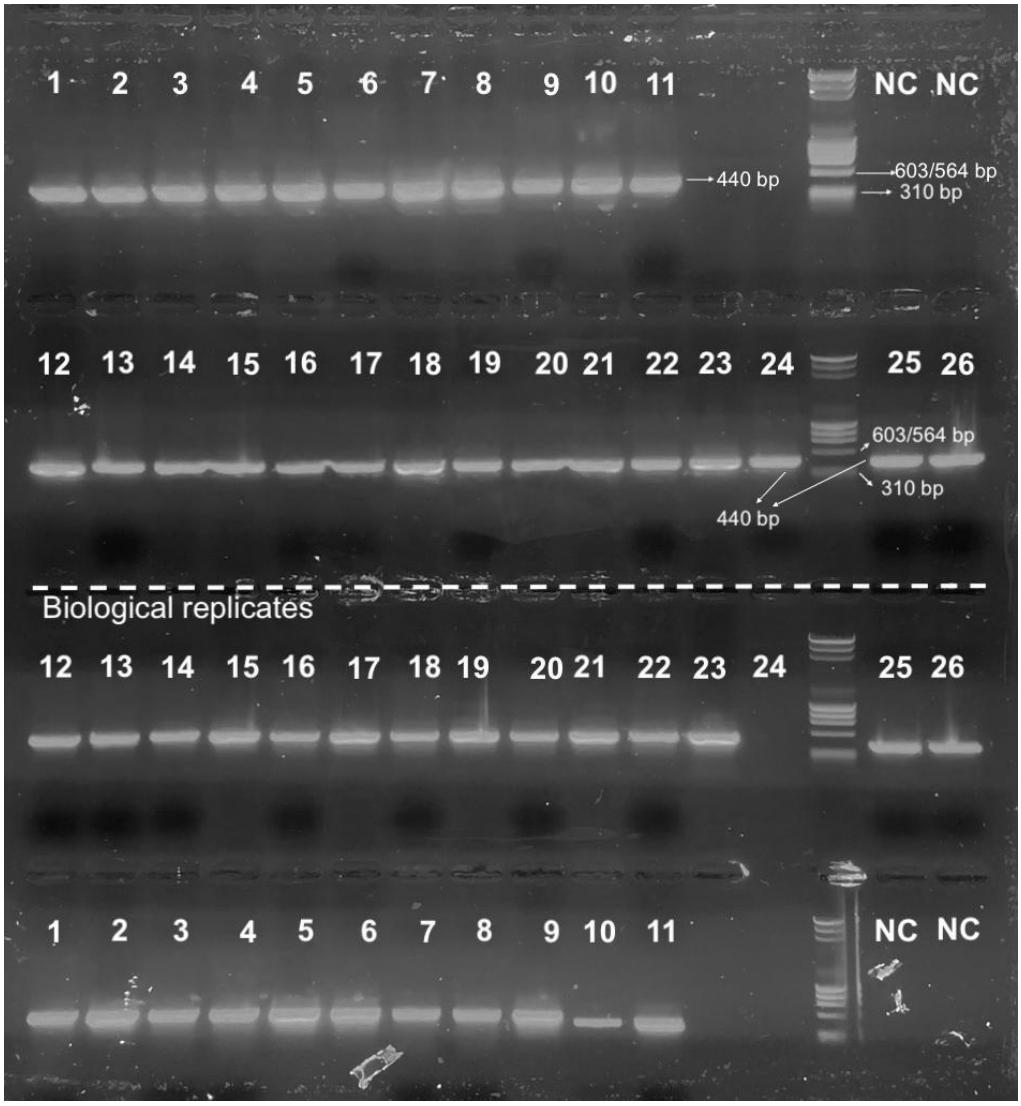


Fig.2.9 Bacteria partial 16S rDNA PCR gel electrophoresis. Primer sets selection: 515 (F) and CD (R) with a target amplification length of 440 bp. DNA ladder: Quick-Load® DNA Marker, Broad Range (NEB #N0303). *Bacteria 24 had negative amplification in its biological replicate indicated by the absence of a fluorescent band.

For microalgae 18S rDNA PCR, multiple primer sets were used but only successful amplification was presented (Fig.2.10). Notably, primer sets ITS-5.8 F, and ITS-5.8 R which were expected to yield DNA fragments of 700 bp (Timmins et al., 2009), produced amplification products of ~1200 bp in algae ST. Despite the abnormal amplifications, all DNA fragments resulted in single bands, indicating specific PCR amplifications. As a result, all of these bands were gel-purified and submitted for sequencing.

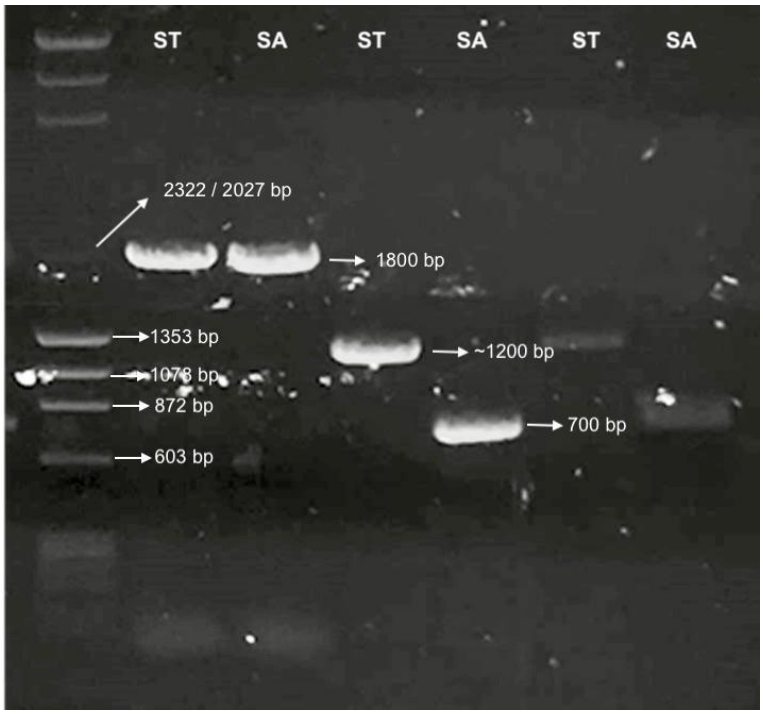


Fig.2.10 Microalgae 18S rDNA PCR gel electrophoresis, primer sets 18s-SS3 and 18s-SS5 (lane 1-2); ITS-5.8 F, ITS-5.8 R (lane 3,4,5,6).

2.3.4 Microorganism identification

DNA sequences were NCBI BLAST-analysed using Nucleotide Collections (Standard databases) and the best match results suggested that the 25 bacteria isolates belong to 10 genera while the two sequenced microalgae were identified as *Deuterostichococcus epilithicus* and *Coelastrella terrestris*, respectively (Table 2.11).

Table 2.11 Microorganism identification results

Bateria/microalgae label	**NCBI BLAST results**	**Match**
1	*Pseudomonas fluorescens*	100%
2	*Pseudomonas sp.*	100%
3	*Pseudomonas syringae*	100%
4	*Stenotrophomonas sp. L18Cg*	100%
5	*Stenotrophomonas sp.*	100%
6	*Pseudomonas laurentiana*	100%
7	*Stenotrophomonas sp. L18Cg*	100%
8	*Stenotrophomonas rhizophila*	100%
9	*Stenotrophomonas sp. B3_22*	100%
10	*Achromobacter sp. strain Bbqt9*	97.92%
11	*Pseudomonas sp. strain SeaQual_P_B845W*	100%
12	*Pseudomonas laurentiana*	100%
13	*Delftia tsuruhatensis*	100%
14	*Delftia tsuruhatensis*	100%
15	*Cupriavidus metallidurans*	100%
16	*Plantibacter flavus*	100%
17	*Rhodococcus sp.*	100%
18	*Rhodococcus sp.*	100%
19	*Rhodococcus erythropolis*	100%
20	*Rhodococcus sp.*	100%
21	*Rhodococcus sp.*	100%
22	*Ochrobactrum anthropi*	100%
23	*Agromyces atrinae strain P27 (T)*	100%
24	*Rhodococcus sp.*	100%
25	*Plantibacter sp.* (not same as 16)	100%
26	*Pseudomonas putida KT2440*	N/A*
ST	*Deuterostichococcus epilithicus (Stichococcus sp.)*	99%
SA	*Coelastrella terrestris*	99.8%

*Note: Bacteria 26 is a known strain of *Pseudomonas putida KT2440* obtained from laboratory collection and was not sequenced.

2.3.5 Preliminary algae-bacteria co-culture

The preliminary co-culture experiment generated 24 algae-bacteria pairs for each algae candidate. The layout of co-cultures in 96-well plates and the corresponding growth images are shown in Fig.2.11. For algae ST (*D. epilithicus*), it was surprising to note that all pairs as well as the axenic control wells, displayed almost identical low growth patterns characterised by no visible colour changes. In contrast, algae SA (*C. terrestris*) exhibited more distinct growth characterised by visually discernible colour changes. Particularly, strong growth-promoting effects on *C. terrestris* were observed in wells containing two *Pseudomonas* (no.1 and no.2) and five *Rhodococcus* (no.18-21 and no.24). The *Rhodococcus* species demonstrated a notably potent algae growth-promoting effect compared to the bacteria *Pseudomonas* as evidenced by deepened colour in their corresponding culture.

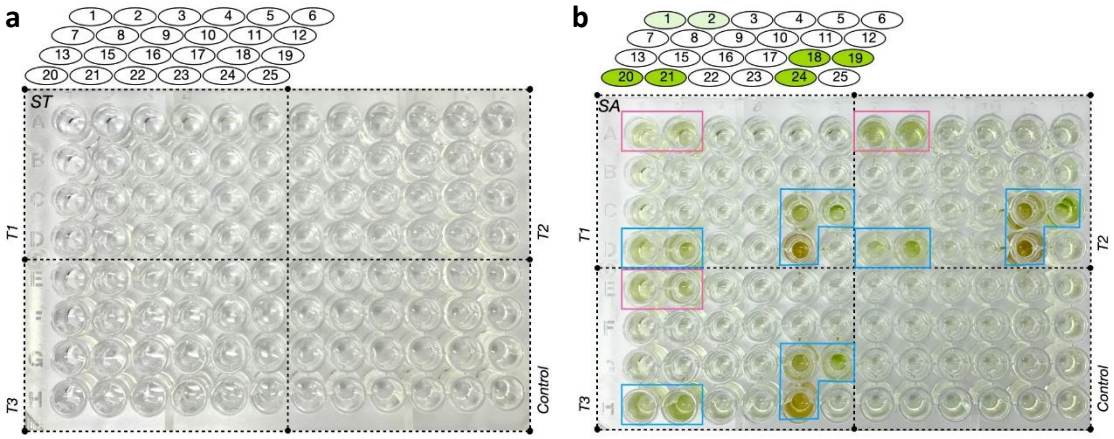


Fig.2.11 Pairwise algae-bacteria co-culture. a: growth pattern on day 0; b: growth pattern on day 7 Triplicate test group T1, T2, T3: microalgae + 24 bacteria isolates (no.15 and no.25 were not included due to poor reviving status caused by prolonged stock preservation) and control group: axenic microalgae.

2.4 Discussion

In this study, all microbial isolates obtained from environmental samples were found to be VOC resistant. Furthermore, certain bacteria isolates demonstrate the trait of utilizing VOCs as growth substrates. This increased the potential for the successful construction of algae-bacteria consortia with VOCs biodegradation capabilities which can either be attributed to synergistic microbial interactions or a direct result of VOCs degrading microorganisms metabolism. This section consolidates the experimental findings in this chapter and presents a comprehensive overview of the identified microorganisms, integrating known knowledge from existing literature. Based on this information, potential microbial interaction mechanisms are also anticipated to serve as groundwork for subsequent experiments.

2.4.1 VOCs degraders

Carbon-free culture suggests that at least 6 isolates can grow with VOCs as carbon sources, which provides 'guaranteed' candidates for degrading one or more types of VOC compounds of interest. The 16s rDNA sequencing result further verified these findings, where gram-negative (GN) bacteria *Pseudomonas* and gram-positive (GP) *Rhodococcus* were found to be dominant genera. These two genera of bacteria have been well known for their aromatic compound degrading capability and utilising them as a growth substrate (Cébron et al., 2008; Kauppi et al., 1998). Two isolates were identified as *Delftia*, a GN bacterium known to be able to degrade low molecular weight phenolic compounds (Juárez-Jiménez et al., 2010) and even aniline as sole carbon and nitrogen sources (Sheludchenko et al., 2005). Although no growth was observed for this bacteria using VOCs as solitary carbon sources, it is worth labelling it as a potential VOC degrader.

Pseudomonas, *Rhodococcus* and *Delftia* species possess multiple enzyme systems to break down aromatic compounds (Shigematsu et al., 2003; Kauppi et al., 1998; Patrauchan et al., 2008). According to full-length genome analysis by McLeod et al., (2006), a model strain *Rhodococcus jostii RHA1* for example, has been predicted to possess at least 200 oxygenases and 30 pathways associated with aromatic compounds catabolism. To obtain more efficient

VOCs degrading consortia, it is worth combining *Pseudomonas*, *Rhodococcus* and *Delftia tsuruhatensis* which possess similar VOCs degrading enzyme systems.

In most aromatic compound degrading bacteria, the initial step in degradation pathways involves the incorporation of two oxygen atoms onto the aromatic rings via catalysis by aromatic-ring-hydroxylating dioxygenases (ARHDs) (Kimura and Kamagata, 2009). ARHDs comprise a family of enzymes that are functional and structurally similar which commonly have a Rieske iron-sulfur centre [Fe2S2], a conserved region responsible for electron transfer (Kauppi et al., 1998; Jouanneau et al., 2011). The catechol products ARHDs catalyse are essential substrates of ring-cleaving dioxygenases (RCDs) which causes further oxygenolytic cleavage of the aromatic nucleus. Studies have classified RCDs into two groups (intradiol and extradiol) according to their attacking point on the aromatic rings. Yet both types utilise non-heme Fe(III) or non-heme Fe(II) to cleave the aromatic nucleus ortho to the hydroxyl substituents (Vaillancourt et al., 2006). Regardless of the diversity of ARHDs and RCDs, the universality of iron ions in these enzymes made it reasonable to believe that iron availability plays an important role in VOCs degrading capability of engineered consortia concerning this project.

2.4.2 VOCs resistant bacteria

Although the remaining bacterial isolates did not show direct evidence of VOCs degradation, it is imperative to consider their potential significance firstly, for their VOCs resistant abilities as confirmed in this experiment and secondly, their other distinct attributes as identified in previous research. A detailed discussion of these traits and their broader implications will be presented in the subsequent section of this chapter.

2.4.3 Microalgae

Alga ST, *D. epilithicus,* is a green algae species that belongs to the family of *Stichococcaceae.* As a relatively newly reported chlorophyte, studies concerning *D. epilithicus* only emerged in 2020 and are primarily focused on its phylogenetic differentiation from closely related taxa (Yoon et al., 2020; Pröschold and Darienko, 2020). While information about its characteristics is limited to cell morphologies, several other microalgae from its sister genus

Stichococcus, can serve as useful references for predicting the behaviour of *D. epilithicus* due to their similar characteristics and high genetic proximity. For example, *Stichococcus bacillaris,* a soil-inhabiting microalga, has drawn research attention due to its lipid-rich trait and high biomass production potential (up to 3.7 g/L in 6 days) (Sivakumar et al., 2014), high tolerance against up to 15% CO_2 (Olivieri et al., 2013) and an estimated 1.5% photosynthetic efficiency (Olivieri et al., 2011). Given these traits and the observed rapid growth in this study, *D. epilithicus* has exhibited some potential to be a robust algae candidate. However, its behaviour under higher VOC concentrations, together with the absence of evident growth in the preliminary co-culturing experiment suggests that it may not be the most suitable candidate for consortia which are designed to function in high VOC concentrations.

Another identified microalga, *C. terrestris* exhibits more interesting traits. As its name suggests, *C. terrestris* is a terrestrial microalga like many species in the same genera that have been isolated from a variety of terrestrial environments such as soil, rock surface and tree bark (Hodač et al., 2016). Most *Coelastrella* have traits such as rapid growth, lipid-rich and resistance to stress. Surprisingly, one strain in this genus, *Coelastrella sp. F50* has been reported to be extremely thermotolerant i.e., can withstand 50 °C for 8 hours according to Hu et al., (2013) while a similar trait was also observed in *C. terrestris* isolate from one of our parallel projects using a photobioreactor under high-temperature culturing condition (36 °C) (data not shown). Moreover, the accumulation of carotenoids in *C. terrestris* observed in this study was also documented in other research on the same species (Saxena et al., 2020) as well as other same-genera species (Abe *et al*., 2007; Aburai *et al.*, 2013). This has been widely identified as a protection strategy against photoinhibition, UV radiation and oxidative stress. Rauytanapanit et al., (2019) revealed that the carotenoid synthesis in *C. terrestris* cells is a result of nutrient scarcity after prolonged culturing and is associated with astaxanthin derivatives, yet detailed mechanisms remain to be examined. Artificial triggering and shortening of carotenoid synthesis via intensive light exposure and salinity stress (1.5 g/L NaCl) have been proposed as an effective approach for making *Coelastrella* potential sources of natural pigment (Hu et al., 2013). Based on this information, the observed alterations in pigment content within *C. terrestris* have further heightened interest in its potential role in microbial consortia as well as the prospective value of their bioproducts.

2.4.4 Algae growth promoting

The preliminary co-culturing experiment of algae and bacteria isolates has shown highly similar patterns amongst the biological replicates, suggesting consistency of the co-culture methodology. For the alga, *C. terrestris*, a distinct growth-promotion effect was observed with *Pseudomonas* and *Rhodococcus*. This finding aligned with their abilities to grow in PBS buffer containing VOCs, indicating a potential association between VOCs degrading capability and algae growth promotion. Furthermore, *Rhodococcus* seems to exhibit a more obvious algae growth-promotion effect on *C. terrestris* which might be attributed to its more efficient VOCs catabolising and detoxifying ability, or to another mutualistic interaction that has yet to be identified. Notably, the unique yellowish pigment accumulation induced by bacteria No.24 (Well D5, D11 and H5) suggested that bacteria No.24 is of a different strain or species to the other four *Rhodococcus* candidates. Also, as pigment changes in *C. terrestris* have been reported to be a response to a variety of adverse factors such as oxidative stress or nutrient limitation, it is, therefore, reasonable to see *C. terrestris* pigment development as a stress indicator as profiled in the algae-bacteria interactions. Since the pigment changes in *C. terrestris* is a response to various stressors such as oxidative or nutrient limitations (Rauytanapanit et al., 2019), it can be hypothesized that bacteria No.24 is a fast-growing *Rhodococcus* that may rapidly consume available nutrients in the media and thereby induce stress in *C. terrestris.*

2.4.5 Microbial Interaction potential

Apart from the observed algae growth promotion, other types of interactions are anticipated to occur based on information on identified microorganisms in the literature. In the algae-bacteria co-culturing system, nutrient exchange is expected to be the most dominant interaction. Algae can provide carbon sources for heterotrophic bacteria which in return, supply CO_2 via degradation of organic carbon to facilitate algae photosynthesis. Another noteworthy interaction is the potentially enhanced biodegradation of VOCs in the presence of photosynthetic oxygen. This aligns with the findings of Mukherjee and Bordoloi, (2012) whose study revealed the enhanced bacterial biodegradation of BTX (i.e. benzene, toluene, and xylene) after the addition of hydrogen peroxide (H_2O_2) as an oxygen source.

In addition, many identified bacteria in this study are known for their plant-associated or even plant growth-promoting (PGP) effects. *Cupriavidus metallidurans* is a plant-associated bacterium and is also well known for its heavy metal resistance (Estrada-de los Santos et al., 2011). The three *Plantibacter* isolates also have the potential to induce PGP due to the close association of this genera with plants. Particularly, *Plantibacter flavus* is a well-studied bacteria that possesses multiple genes encoding the synthesizing of plant growth-regulating compounds including auxin, cytokinin biosynthesis and 1-aminocyclopropane-1-carboxylate deaminase (Mayer *et al.,* 2019). A similar trait has also been found in *Achromobacter* according to Jiménez-Vázquez *et al.*, (2020) who observed changes in plant root architecture caused by *Achromobacter sp. 5B1* via auxin mediations, which subsequently enhances plant growth. Finally, *Ochrobactrum anthropic* has also been reported for its PGP effect and endophytic relationship with certain plants, as well as novel applications in agriculture (Chakraborty et al., 2009; Meng et al., 2014). Although these bacteria have no previous study of their association with algae in literature nor any significant growth promotion effect on *D. epilithicus* and *C. terrestris* observed in the primary co-culture experiment, the evolutionary proximity and metabolic similarities between plants and microalgae suggest the need for further research, especially with a focus on their potential algae growth-promoting effects.

Siderophore-mediated cooperation and competition is another type of plausible microbial interaction to occur in a microbial consortium. Siderophores are iron-chelating compounds produced by certain microorganisms to uptake essential iron elements from the surrounding environment and have been identified as essential survival strategies in certain bacteria (Johnstone and Nolan, 2015). Siderophores are metabolically expensive to individual producers but benefit the overall microbial community (Griffin et al., 2004; Harrison et al., 2008) and can induce either cooperative or competitive behaviour depending on whether it is shared or privatised among different species (Niehus et al., 2017). The fluorescent colonies of two isolates (bacteria no.1 and 2) observed in this study are believed to be the result of fluorescent pyoverdine (PVD), a common siderophore produced by *Pseudomonas* species (Lamont et al., 2006). Apart from this, one *Delftia* strain (*D. tsuruhatensis MTQ3)* has been reported to be siderophore-producing (Guo et al., 2016) despite the lack of observational evidence in this study. Given these findings, a hypothesis can be proposed that

the fluorescent *Pseudomonas* (No.1 and 2) and the two *Delftia* isolates might either have a competitive advantage for iron uptake or act as a cooperative siderophore donor depending on the type of siderophore receptors in other microorganisms in the same consortium. Manipulation on iron availability thus presents an attractive option to mediate the cooperation/competition behaviour of this strain in a co-culturing system.

In summary, the experimental results and literature information about these microbial isolates indicate a range of potential interactions, some of which remain unidentified. These interactions in a co-culturing system may result in combined benefits within the consortia, such as reducing oxidative stress, decreasing VOCs toxicity and enhancing stability against external threats and disturbances These potential interactions along with those previously discussed, are conceptually summarized in Table 2.12

Table 2.12 Traits of identified algae and bacteria and their possible role in consortia and interaction (in conceptual diagrams)

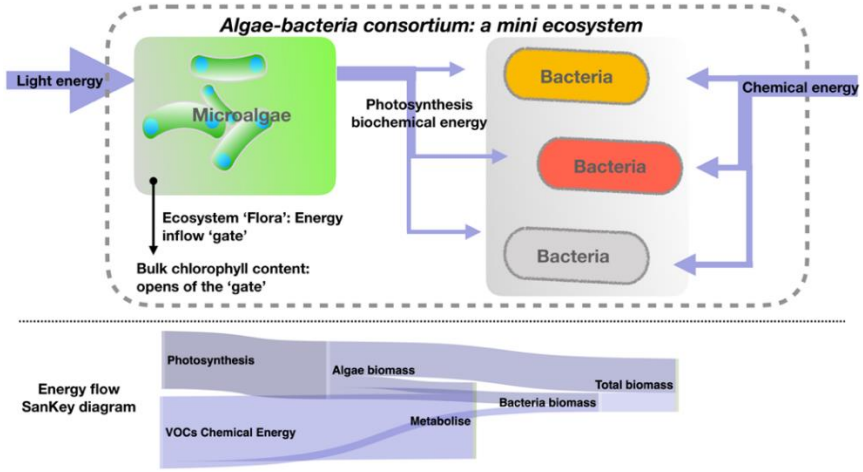
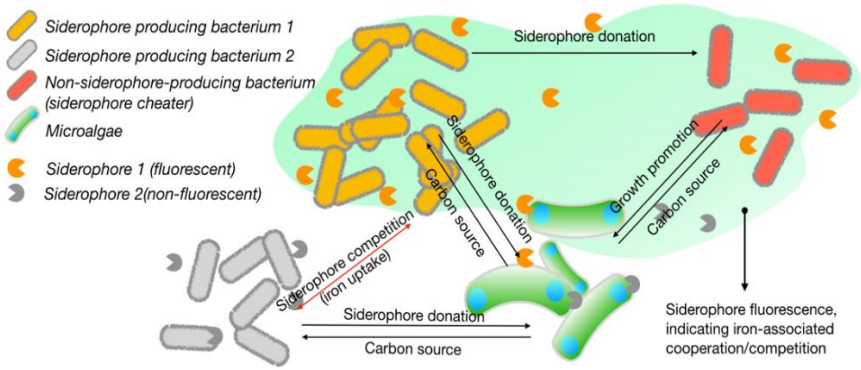
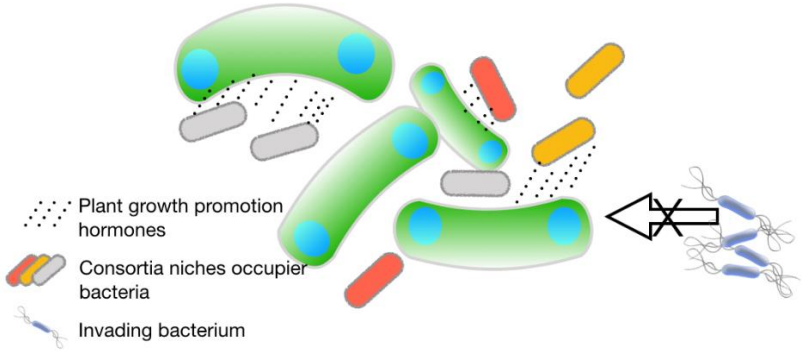
Microorganism	Traits	Role in consortia	Other possible interaction
Microalgae			
Deuterostichococcus epilithicus	• Lipid-rich • High biomass production • Fast-growing • CO_2 tolerance (15%) • VOCs resistant	• CO_2 fixation • Oxygen source • Consortia productivity indicator	
Coelastrella terrestris	• High biomass production • Carotenoid production • Thermo-resistance • VOCs resistant • High biomass accumulation • Carotenoid producing	• Carbon fixer • Oxygen source • Consortia productivity indicator • Oxidative stress indicator	
Confirmed VOCs-degrading bacteria			
Pseudomonas spp.	• VOCs resistant • VOCs-dependent growth • Fast-growing • Providing producing	• VOCs degrader • Iron-mediated competition/cooperation indicator	
Rhodochoccus spp.	• VOCs resistant • VOCs-dependent growth • Fast-growing	• VOCs degrader	

Table 2.12 (Continued) Traits of identified algae and bacteria and their possible role in consortia and interaction (in conceptual graphs)

Microorganism	Traits	Role in consortia	Other possible interaction
VOCs resistant bacteria			
Plantibacter spp.	• VOCs resistant • Plant growth-promoting	• Potential algae growth promoter • Oxidative stress buffering microorganisms • Toxicity buffering microorganisms • Ecological nitches occupiers	
Cupriavidus metallidurans	• VOCs resistant • heavy metal resistant • highly plant-associated		
Ochrobactrum anthropi	• VOCs resistant • Plant growth-promoting		
Achromobacter sp	• VOCs resistant • Plant growth-promoting		
Stenotrophomonas sp.	• VOCs resistant • Nitrogen-fixing		

2.5 Conclusions

This chapter presented acquisition and selection of candidate microorganism as an initial step toward successful VOC-degrading algae-bacteria consortia construction. A collection of environmental microorganisms that are resistant to VOCs including benzene, toluene, phenol and tetrahydrofuran, were successfully isolated and enriched. Two microalgae species and 26 bacteria belonging to 9 different genera were identified using 18s and 16s rDNA sequencing. Both microalgae species, *Deuterostichococcus epilithicus* and *Coelastrella terrestris* have shown desirable growth rates and capability for biomass accumulation while *C. terrestris* has higher VOCs tolerance limits despite their longer lag phase in axenic culturing. Also, *C. terrestris* has been observed to be carotenoid-producing which is the response of adverse factors and has garnered interest for further applications such as high-value pigment harvesting as well as to serve as an indicator in monitoring algae-bacteria interactions. Two *Pseudomonas* and 4 *Rhodococcus* isolates were able to grow in nutrient-free media using VOCs as the sole carbon source suggesting their VOCs-degrading capability thus aligning with the findings of their aromatic hydrocarbon catabolic capability in other studies.

The preliminary algae-bacteria co-culturing conducted in 96-well plates has confirmed growth promotion for algae *C. terrestris* by *Pseudomonas* and *Rhodococcus.* This finding aligns with the VOCs degrading capabilities observed in these bacteria. While other identified bacteria showed no significant effect on *C. terrestris* growth, most of these bacteria have well-documented plant growth-promoting (PGP) potentials in literature, which presents new perspectives in anticipating their potential interactions with algae.

This study is to the best of our knowledge, the first research aimed at establishing algae-bacteria consortia from a bottom-up approach. This work yielded sufficient microbial candidates and a basic understanding of their characteristics, which serves as a benchmark for further study regarding more complex algae-bacteria consortia engineering.

Chapter 3: Algae-bacteria Consortia Screening—the Design of a High Throughput Method

Aims and objectives

- *Present the design of a screening method for identifying high-performance algal-bacterial consortia regarding VOC biodegradation.*
- *Explain and validate the monitoring of chlorophyll fluorescence as a proxy for VOC biodegradation.*
- *Detail the adoption of a bottom-up approach within the framework of the experimental design.*
- *Define the scoring indexes and explain how consortia combination structures were exploited to uncover consortia performance.*
- *Interpret the screening results for assessing individual microorganisms and identifying high-performance consortia regarding VOC biodegradation.*

3.1 Introduction

As addressed in the literature review (Chapter 1), while microorganisms from natural environments have evolved to thrive in their native habitats by means of a variety of metabolic capabilities, artificial consortia composed of microorganisms tailored for specific compound breakdown, present a promising strategy to harness microbial potential with enhanced precision and heightened specificity for targeted biological tasks. However, the successful construction of such consortia with ideal capabilities and maintaining their functional stabilities remain major biological and engineering challenges facing algae-bacteria co-culturing. Although knowledge surrounding the algae-associated microbiome is improving (Okurowska et al., 2021; Qu et al., 2021; Si et al., 2022), studies on intentionally selected bacterial for co-culturing with specific algae species remain difficult due to the complex nature of microbial communities.

Firstly, microbial communities are subject to the rules of internal factors such as inter and intra-species interaction (Comolli, 2014), as well as system behaviour due to greater diversity of biochemical characteristics and an increased number of interactive components (Gilbert and Henry, 2015). Secondly, external factors such as physical and chemical conditions pose a significant influence on the behaviour of microbial communities. External factors usually cross-interact with internal factors, thus leading to the loss and gain of specific functions as a result of microbial adaptation (Fernandez et al., 2014), which leads to extra complexity. Thirdly, almost all microbiomes are subject to a variety of ecological rules (Prosser et al., 2007; Shade and Handelsman, 2012). Many classical ecological principles for example, competition(Griffin et al., 2004; Hibbing et al., 2009), ecosystem succession(Fierer et al., 2010; Jiménez et al., 2017), mass cycling and trophic interactions (Gralka et al., 2020) have been well-studied in the context of microbiology and provides useful overviews about the major shaping forces of microbial community assembly. This however highlights the lack of comprehensive knowledge and data for the controllability and optimisation of complex microbial communities and the lack of efficient tools and methodologies to process these data.

Although the advancement of computational tools provides deeper insight into consortia structure, stability (Coyte et al., 2015) and enhanced ability of prediction (Zomorrodi and Segrè, 2016; Marsland et al., 2019), most explicitly modelled synthetic algae-bacteria communities are constrained to two-member systems (Mujtaba et al., 2017; Contreras-Angulo et al., 2019) and the search for pairwise co-cultures (Le Chevanton et al., 2013), while multi-microbe studies are rare. Most studies involving naturally occurring algae-bacteria consortia (Min et al., 2011; Zhang et al., 2012; Inoue and Uchida, 2013) rely on examining the overall community structures but fail to provide deeper information about microbial interactions. These interactions however, although having been recognised as keys to the stable and efficient operation or accurate modelling of the algae-bacteria biosystems (Del Rio-Chanona et al., 2019; Jiang et al., 2021), still leave a large gap toward practicality implementation due to their complexity.

This chapter presents the design of a screening method which enables the identification of algal-bacterial consortia that are capable of bioremediation of VOCs of interest, i.e. benzene, toluene, phenol and THF. This study is motivated by the lack of knowledge on how to construct stable synthetic algae-bacteria consortia and the absence of efficient tools or methodologies accessible in most research laboratories. A bottom-up route was the core approach to integrate environmentally isolated microalgae and chemotrophic bacteria (detailed in Chapter 2) in the formation of VOCs-degrading consortia.

By implementing a microplate-scale screening method and measuring chlorophyll abundance in this closed system, a high throughput data generation approach was developed which facilitated rapid shortlisting of algae-bacteria combinations. In addition to algae growth data, this experimental design takes advantage of the consortia combination structure to give rise to two scoring indexes which uncovered the algae growth-promoting potential and functional stability of consortia without the need for interpreting complex interaction networks.

3.2 Methodology

3.2.1 Consortia designing and microbial candidate configuration.

Chapter 2 described the characterisation of 26 bacteria belonging to 9 genera as well as two microalgae *Deuterostichococcus epilithicus* and *Coelastrella terrestris*. Given the vast number of potential combinations, which is theoretically $2^{(26+2)}$ (i.e.,268,435,456), it becomes highly infeasible to explore all these combinations in culture-based *in vitro* culture studies. To test as many consortia combinations as possible while avoiding the generation of a large number of combination scenarios, bacteria identified in Chapter 2 were first shortlisted according to functionalities 1) the ability to degrade VOCs as a carbon source for growth; and 2) whether repeated microbes are included, i.e., microbes of the same genera and having similar colony morphologies.

The shortlisted microbes were grouped to form consortia, each of which consisted of one alga, one VOC-degrading bacterium (degrader) and at least one bacterium without any obvious VOC degradation traits (non-degrader). This generated a collection of 6 VOC-degrading bacteria (degraders) and 7 non-VOC-degrading bacteria (non-degrader) and *Coelastrella terrestris* (Table 3.1) as the "building bricks" for the algae-bacteria consortia.

Table 3.1. Microbial candidates involved in constructing algae-bacteria consortia

Microbe type	Microbe label	Identification
Bacteria: VOC Degraders	A	*Pseudomonas fluorescens*
	B	*Rhodococcus erythropolis*
	C	*Pseudomonas sp.*
	D	*Delftia sp.*
	E	*Rhodococcus sp$_1$.*
	F	*Rhodococcus sp$_2$.*
Bacteria: VOC Non-Degraders	1	*Pseudomonas syringae*
	2	*Agromyces atrinae*
	3	*Cupriavidus metallidurans*
	4	*Ochrobactrum anthropi*
	5	*Plantibacter flavus*
	6	*Plantibacter sp.*
	7	*Rhodococcus sp$_3$.*
Microalga	Ag	*Coelastrella terrestris*

Microalgae inoculant was distributed into 96-well plates with even cell densities controlled at approximately 1000 cells per well except for blanks. Microplate wells that were only inoculated with algae were marked as controls. Bacteria overnight culture in LB broth were pipetted (1 µL per bacterial candidate) separately into the corresponding wells according to the predetermined combination scenarios. After inoculation, each well was topped up to a 200 µL final culture volume using 3N-BBM+V medium containing benzene, toluene, phenol and tetrahydrofuran, 100 mg/L per compound. Inoculated 96-well plates were applied with optical sealing films (SealPlate Sealing Film, sterile, Elkay) which securely sealed individual wells while leaving approximately 200 µL headspace. All these plates were then incubated at 25 °C with even and continuous luminance for 7 days. Although cultivation parameters such as growth media, temperature, pH, vessels, and starter ratios can impact microbial interactions, these were not within the scope of this research. Details of microplate layouts are presented in Table S1, Supplementary Material 1.

3.2.2 Data acquisition

Instead of examining any bacterial growth pattern, algae growth was determined to be the sole data source reflecting the overall VOCs degradation within the entire consortia. Since the screening experiment was performed as a closed system without external gaseous exchange, the nature of consortia configuration mimicked closed ecosystems as hypothetically illustrated in the conceptual diagram (Fig.3.1). In a consortium, microalgae are expected to have limited or no growth due to their autotrophic nature and the lack of access to atmospheric CO_2 as carbon sources. Instead, bacterial degradation of VOCs thus determines the availability of CO_2 as a carbon source, which directly limits the maximum potential growth of algae. Based on this hypothesis, algae growth is expected to serve as an indicator of overall VOCs degradation in the entire consortium.

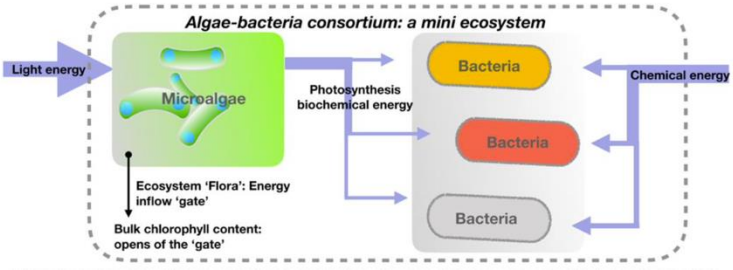


Fig.3.1 An ecosystem model of the algae-bacteria consortium in the high throughput screen. An algae-bacteria consortium and its containing environments is an ecosystem where the majority of carbon sources for the growth of the entire microbial community are from VOCs degradation by bacteria with algal photosynthesis as the main energy input and algae biomass as the final VOC-originated carbon sinks.

To quantify the growth of algae, bulk chlorophyll concentration was measured in terms of fluorescence using a Spark® 10M multimode microplate reader (Tecan). The plates first underwent a linear shaking process at 810 rpm speed and 2 mm amplitude for 10 seconds to remove bubbles without suspending the cells. The fluorescence was measured as emission at a wavelength of 690 nm and an excitation wavelength of 390 nm. Since the algae cells tend to grow at the bottom of the plate, the instrument measurement optical setting was selected as 'bottom', and each well was exposed to 30 flashes to balance accuracy and processing time.

To check the correlation of algae cell density with the chlorophyll fluorescence signals, a trial measurement was performed using axenic *C. terrestris* culture, an algae-bacteria mix culture of *C. terrestris* and 7 bacteria, as well as axenic culture of a previously characterised non-candidate alga *D. epilithicus* as external control. All three samples were pre-grown for 7 days and underwent two-fold dilutions to form algae cell densities gradient in the 96-well plate which were later fitted with their fluorescence data for measurement validation.

3.2.3 Screening data processing

To facilitate calculations, fluorescence data generated by the plate reader were first reshaped from their 96x1 microplate format to 1x8 vectors that represent daily growth data from day 0 to day 7, corresponding to the matching wells on the 96-well plate. Then the reshaped fluorescence data vectors were normalised by subtracting the average data of blanks, followed by standardisation via rescaling to a (0, 1) range using Eq.3.1.

$$F_{rescale} = a + \left[\frac{F - F_{min}}{F_{max} - F_{min}}\right](b - a) \qquad \text{Eq.3.1}$$

F refers to the fluorescence data that are rescaled to an arbitrary interval [*a b*].

The quality of fluorescence data and the consistency of datasets between biological replicates were subjected to a Pearson correlation test as well as root-mean-square error (RMSE) given by:

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(F1_i - F2_i)^2}$$

And/or RMSE percentage:

$$RMSE\% = \frac{RMSE}{\max(F1 \cup F2) - \min(F1 \cup F2)}$$

Where $\boldsymbol{n}$ is the total number of data point within a single replicate, $\boldsymbol{F1_i}$ is the $\boldsymbol{i}^{\text{th}}$ fluorescence data point in replicate $\boldsymbol{F1}$, and $\boldsymbol{F2_i}$ is the $\boldsymbol{i}^{\text{th}}$ fluorescence data point in replicate $\boldsymbol{F2}$.

Instead of looking at the endpoint growth in each well on day 7, individual consortium was assessed for its performance according to the value of its growth score (***GS***), defined as the

difference in its standardised fluorescence data versus the average fluorescence data of axenic algae controls, using Eq.3.2.

$$GS = \sum_{0}^{n=7} (F_{Day\,n} - C_{Day\,n}) \qquad \text{Eq.3.2}$$

GS: The additional (positive) or reduced (negative) value fluorescence data $F_{Day\,n}$ in a consortium versus that in the control group $C_{Day\,n}$ at a specific sampling time (*Day 0* to *Day 7*).

Besides, any consortium with bacterial candidate numbers >2 is categorized as a **super-consortium** which consists of **sub-consortia** that have different contributions of ***GS*** to its **super-consortia** as demonstrated in the example (Fig.3.2).

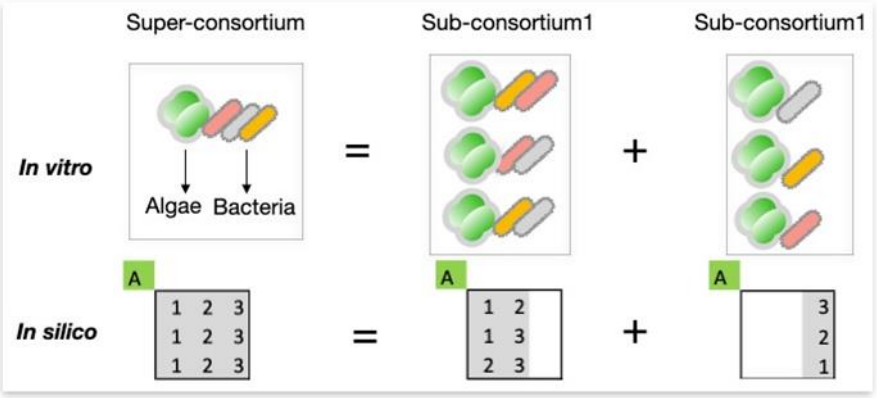


Fig.3.2 Example of a 3-bacteria super consortium and its sub-consortia. The consortium can be seen as a super-consortium and split into 3 different pairs of sub-consortia, with corresponding *in silico* expression as matrix manipulation. Each row of the matrix represents an option for breaking down the original consortium demonstrated in the *in vitro* diagram. Letter 'A' refers to algae and numbers 1, 2, and 3 represent co-cultured bacteria.

This combinational feature of consortia design gives rise to two additional parameters, growth contribution index (***CI***) and functional stability (***FS***), which reflects the strength and stability of the algae growth-promoting effect of different bacteria combinations (Fig.3.3).

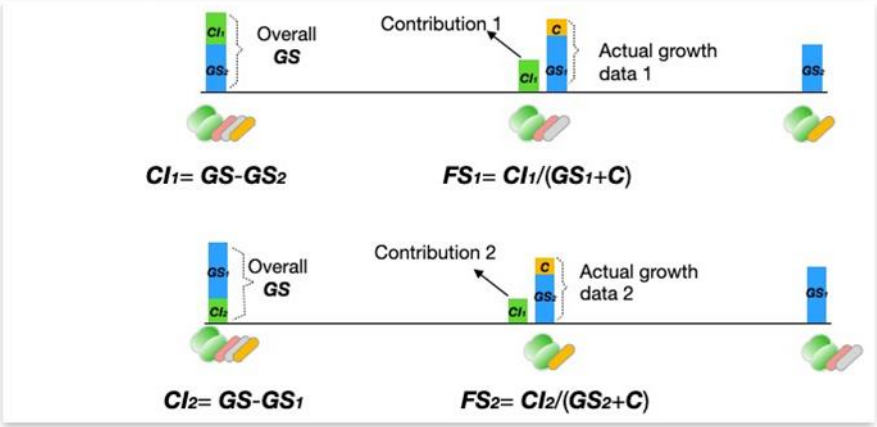


Fig.3.3. Conceptual illustration for the definition of screen parameters ***CI*** and ***FS***.

The ***CI*** is defined as the difference between the ***GS*** of the **super consortium** and the ***GS*** of its counterpart or pairing **sub-consortium** (Eq.3). Likewise, ***FS*** of a given **sub-consortium** with a specific **super-consortium** is defined as the ratio between its ***CI*** to the super-consortium and its own ***GS*** (Eq.3.3).

$$\begin{cases} Cons = Sub1 \cup Sub2 \\ CS_1 = GS - GS_2 \\ CS_2 = GS - GS_1 \\ FS_1 = \dfrac{CI_1 + C}{GS_1 + C} \\ FS_2 = \dfrac{CI_2 + C}{GS_2 + C} \end{cases} \qquad \text{Eq.3.3}$$

$Cons, Sub1\ and\ Sub2$ are the superset and its two subsets which represent the sup. $CI_1, CI_1\ and\ FS_1, FS_2$ are the contribution and Functional Stability indexes of $Sub1\ and\ Sub2, repectively.$ GS, GS_2, GS_1 refers to the actual growth scores of $Cons, Sub1\ and\ Sub2.\ C$ is the sum of 7-day fluorescence data of axenic algae control.

The combinational feature of the consortia in this study allows a **single consortium** to have multiple choices of **super-consortia**, which mathematically exhibit hierarchical structures that include multiple ***CI*** (Fig.3.4), as well as ***FS*** index under different super-consortia.

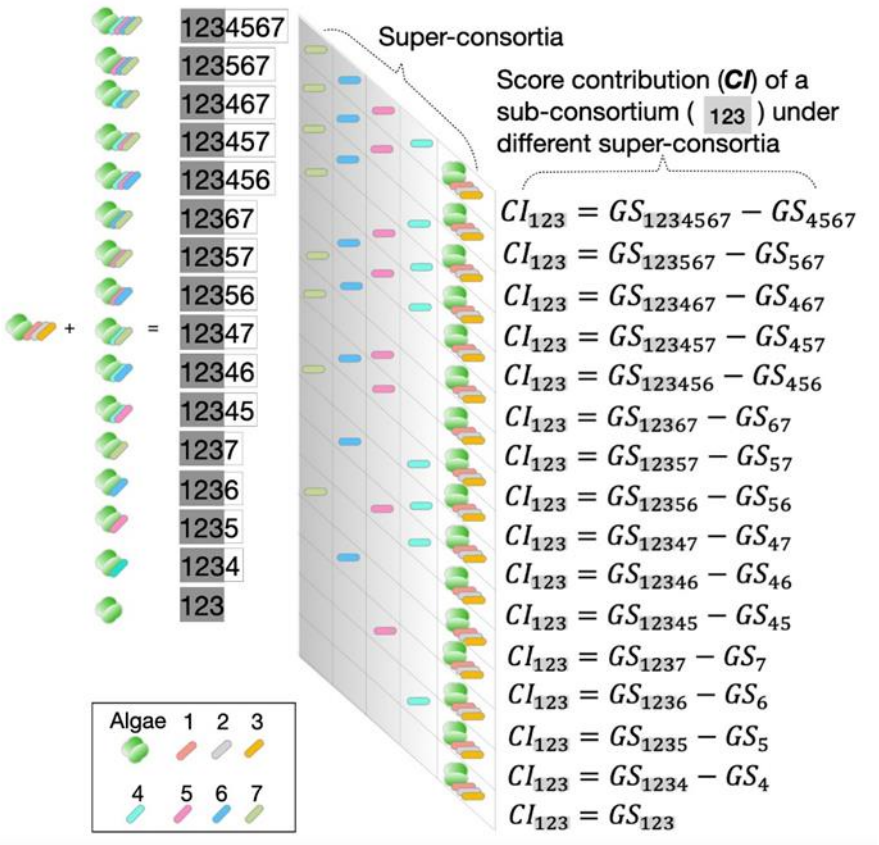


Fig.3.4, Hierarchical structures of multiple ***CI*** of the exemplar consortium ***'123'*** under different super-consortia.

To present the overall growth contribution and functional stability of the given consortium, ***CI*** and ***FS*** calculated under multiple super-consortia, were processed using Eq.3.4.

$$\begin{cases} CI = \sum_{n=0}^{7-s} \sum_{i=1}^{\binom{7-s}{n}} CI_i \\ FS = \sum_{n=0}^{7-s} \sum_{i=1}^{\binom{7-s}{n}} FS_i \end{cases} \qquad \text{Eq.3.4}$$

$\boldsymbol{CI}$ and $\boldsymbol{FS}$ are the overall growth contribution index and Functional Stability index of a given consortium, i.e. average value of $\boldsymbol{CIi}$ and $\boldsymbol{FSi}$ which are the single contribution and Functional Stability indexes obtained under different super-consortia; Consortium size $\boldsymbol{s}$ refers to the number of non-degrader bacteria.

3.2.4 Combination significance and single microbe effect test

To analyse how bacteria combinations affect consortia behaviours, Student t-test was performed against ***GS***, ***CI*** and ***FS*** values. For any combination of certain bacteria, the 768 consortia automatically fall into two collections, 1) that either contain the same bacteria member or 2) those without these bacteria. The two collections served as two sample sets for double-tailed t-tests (non-paired) with a p-value threshold set as 0.05 for determining significant ($p \leq 0.05$) and non-significant ($p > 0.05$) combinations. The p-values were then adjusted via Benjamini-Hochberg correction to minimise the false positive rate (FPR). These adjusted p-values are known as q-values (Benjamini and Hochberg, 1995) and are calculated according to the following steps: 1) for a given set of p-values:

$$P = \{p_1, p_2, p_2, \cdots, p_m\}$$

2) sort the P to get:

$$P' = \{p'_1, p'_2, p'_3, \cdots, p'_m\}$$

so that

$$p'_1 \leq p'_2 \leq p'_3, \cdots \leq p'_m$$

Where $\boldsymbol{m}$ is the total number of the number of tests, $\boldsymbol{p_i}$ is the p-value of individual tests and $\boldsymbol{p'_j}$ are the sorted p-values in ascending order. The sorted p-values were assigned ranks $\boldsymbol{r}$, which correspond to their positions in ascending order. For example, the $\boldsymbol{k}$-th p-value in P' is defined by:

$$r(p'_k) = k$$

Then a given p-values $\boldsymbol{p'_k}$ with rank of $\boldsymbol{k}$, the FPR-adjusted p-values $\boldsymbol{q_k}$ can be calculated using Eq.3.5:

$$q_k = p'_k \times \left[\frac{m}{r(p'_k)}\right] \qquad \text{Eq.3.5}$$

Eq.5 Calculation of FPR-adjusted p-values (also known as q-values) via Benjamini-Hochberg correction.

The effects of single bacterial member were analysed by comparing consortia with and without that bacterium. ***GS***, ***CI*** and ***FS*** values subject to the presence and absence of each bacterial member were normalised by rescaling within the range of -1 to 1 using the same method specified in Eq.3.1. In addition, the statistic correlations among the three parameters, together with their correlation between growth data of flask cultures, were evaluated via Pearson correlation coefficients (R-value) with p=0.05 as the significant threshold.

3.2.5 Computational tools and software

All data processing, screening parameters calculations and statistical calculations in this study were performed using MATLAB 2023 (MathWorks Inc., United States).

3.3.Result

3.3.1 Consortia growth and assay consistency

Using candidate microalgae *C. terrestris* and a shortlisted collection of 6 degraders and 7 non-degraders bacteria, a total number of $6\times 2^7 = 768$ unique consortia were generated. As a result, *C. terrestris* growth was measured in a total number of 1920 samples, including biological duplicates for each consortium ($768\times2 = 1536$) in twenty 96-well plates. The left-over wells were assigned to 90 controls, 102 blanks and 32×6 consortia containing only algae and degraders. The algae growth observed in these combinations exhibited distinct patterns, while these patterns among replicates exhibited noticeable consistency, suggesting that *C. terrestris* growth was influenced by specific bacterial combinations rather than occurring randomly (Fig.3.5). Details of microplate layouts are presented in the Table S1, Supplementary Material 1.

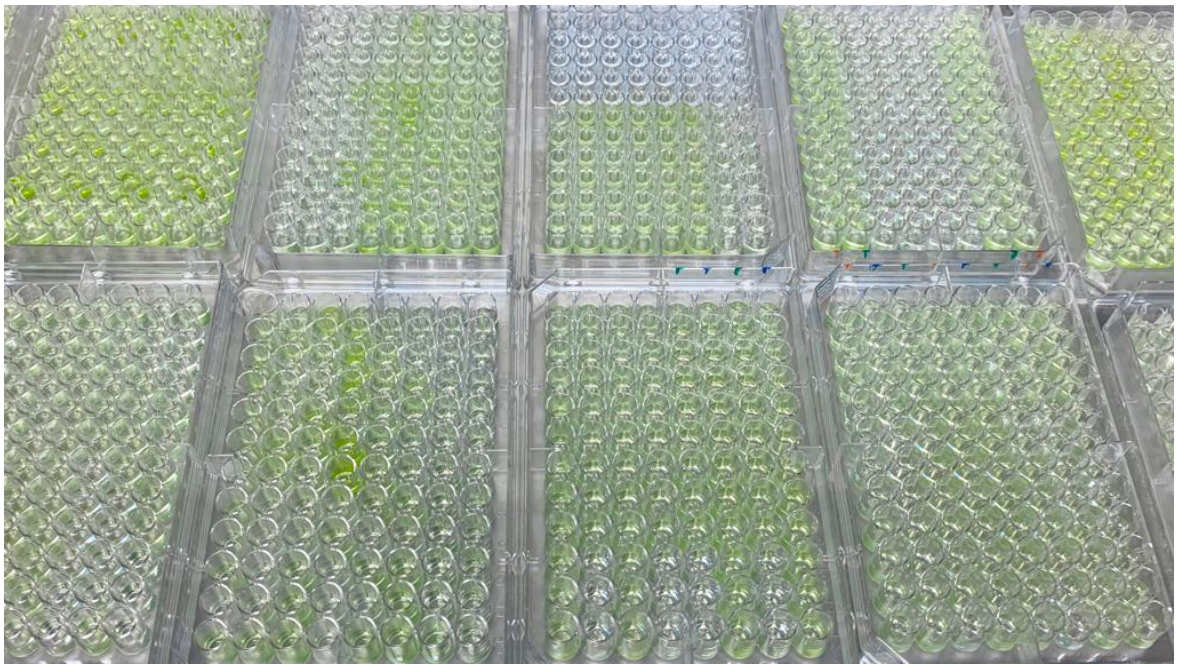


Fig.3.5 Partial image of growth patterns observed in 96 well plates whilst screening consortia.

The correlation analysis between the chlorophyll fluorescence datasets resulted in an R-value of 0.816, indicating a strong positive linear correlation between the two biological replicates as visualised in Fig.3.6a. Also, the extremely low p-values of 8.68×10^{-276} evidence the data consistency between the consortia and their replicates against randomness. The RMSE of the two datasets was determined to be 5.6153, or 13.03% as presented in percentage, showing the magnitude of the differences between the two replicates, as illustrated in Fig.3.6b.

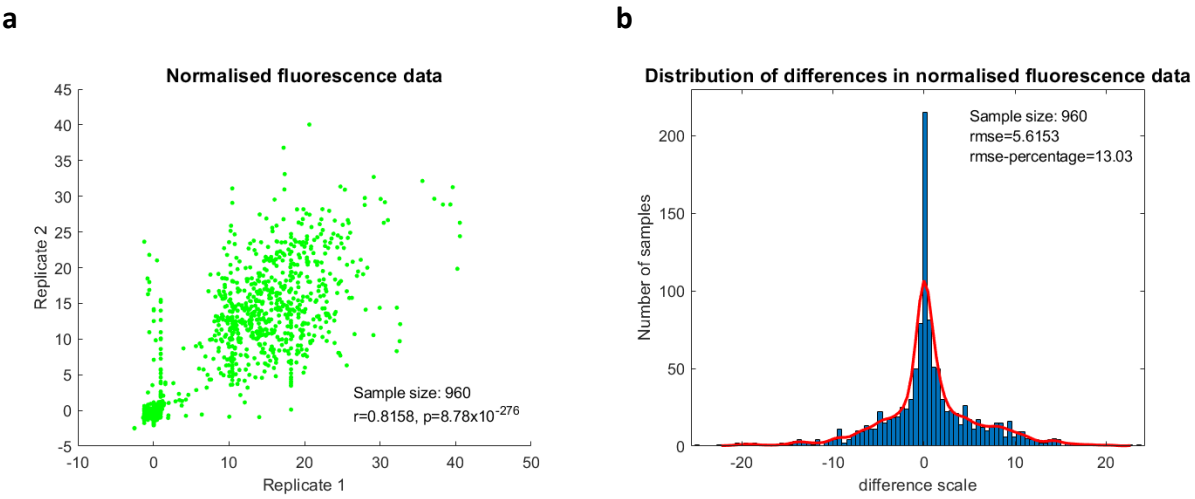


Fig.3.6 Fluorescence data consistency test between replicates. a) Correlation of normalised fluorescence data between two biological replicates. b) Distribution and magnitude of difference be two biological replicates

3.3.2 Fluorescent data validation

Chlorophyll fluorescence (Ex 390 nm, Em 690) data collected by the plate reader showed strong linear correlations to dilution factors in the three calibrating samples (Fig.3.7). While the y-intercepts of the three samples differed due to variations in initial cell densities and chlorophyll content in different algae species, the gradients (1-degree polynomial) of three sets of data were close to 0.693, the natural logarithm of 2, which closely aligned with the feature of two-fold dilutions. This evidenced the high accuracy in the quantification of algae biomass by directly measuring chlorophyll fluorescence, despite the presence of bacteria which could potentially interfere with fluorescence signals.

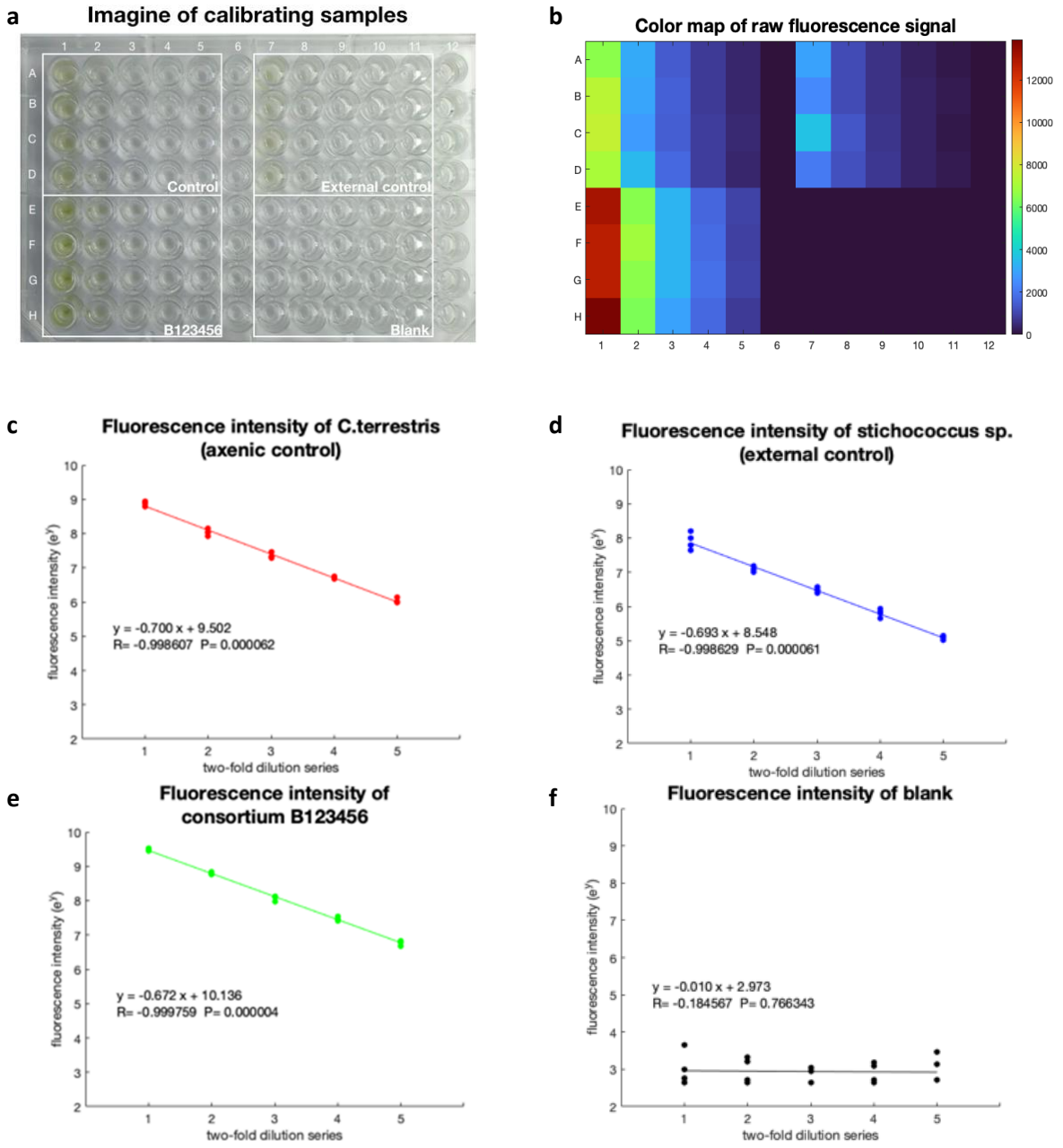


Fig.3.7 Linear fitting of the fluorescence signal (natural logarithm) and algae cell density (two-fold dilution series) in axenic control (a), external control (b), exemplar algae-bacteria consortium (c) and blank (d).

3.3.3 Comparison of screening results

The distributions of the three consortia assessment parameters across the 768 unique consortia are detailed below, together with a visualisation of the results. Among the 768, 679 had positive ***GSs*** values while only 87 were ***GS***-negative (Fig.3.8 a, b), exhibiting a bimodal distribution (Fig.3.8 d) skewed towards the positive side. Upon examining the

relationship or correlation between the ***GSs*** distribution and the consortia bacteria combination (full data in Supplementary Material 2), it was found that all bacteria seemed to hit evenly in ***GS***-positive consortia but non-degrader *3* had a stronger association with high ***GSs*** and was present in 62.8% (241/384) of consortia with ***GS*** value higher than the median value 5.59, while other non-degraders were present in 50% or less. Furthermore, non-degrader *3* presented in 28 out of 38 consortia with top-scored ***GS***, among which 22 also contained degrader *F*. ***GS***-negative consortia are distinctly characterised by the absence of degraders *B*, *E* and *F* and non-degrader *3*. Notably, consortia containing degrader *F* exhibited some of the highest ***GS*** values as visualised in Fig.3.8 c.

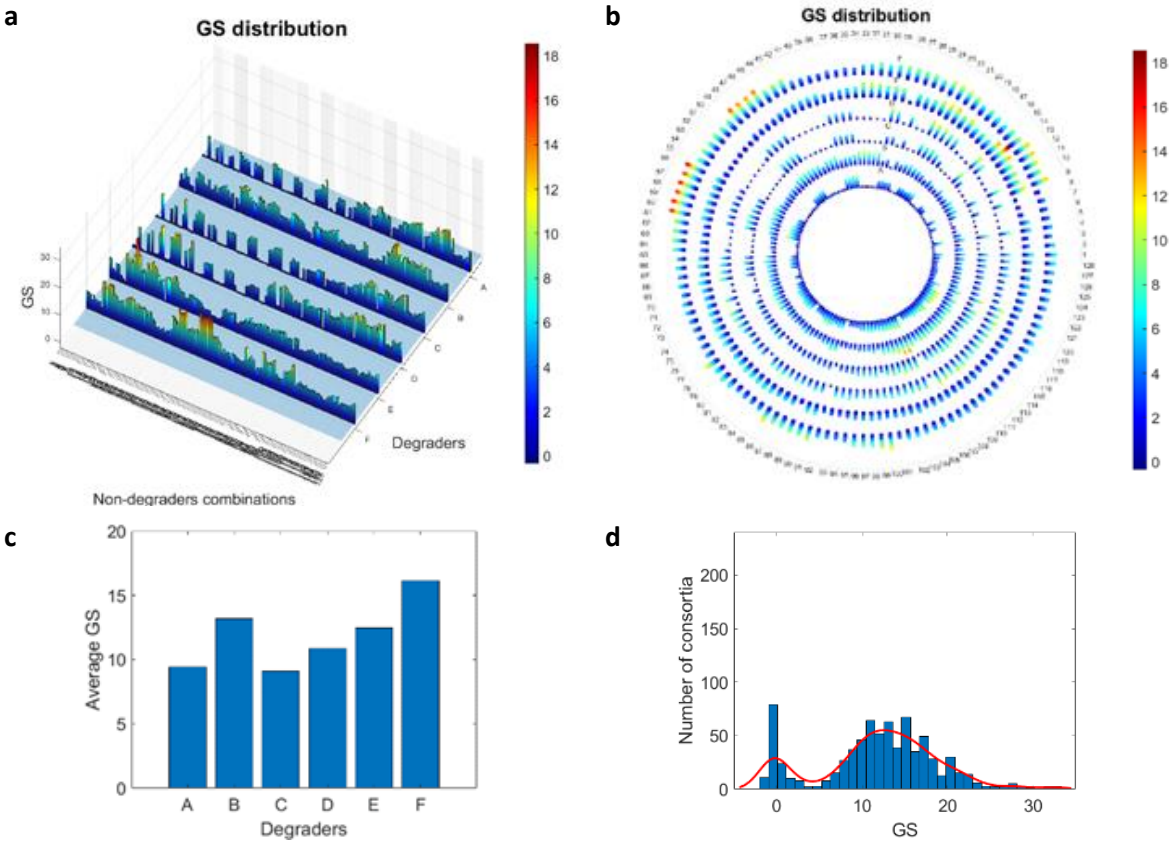


Fig.3.8 Growth score (***GS***) of screened consortia, visualized in bar chart (a), polar plot (b), average values under 6 different degraders (c) and histogram demonstration of ***GS*** value distribution (d). Details of non-degrader combinations are given in Table S2, Supplementary Material 1.

Unlike ***GS***, ***CI*** had a broader distribution across both negative and positive ranges but was also skewed towards the positive domain as 587 consortia showed positive ***CI*** values (Fig.3.9 d). Interestingly, positive ***CI*** values were found evenly in consortia containing degraders *A*, *B*, *C* and *D*, while high ***CI*** values ($\geq$ 1.97, median) seem to be subject to the presence of non-degrader *3*. In contrast, ***CI*** values were significantly lower in consortia containing degrader *E* and *F* among which half (138 /256) were found negative.

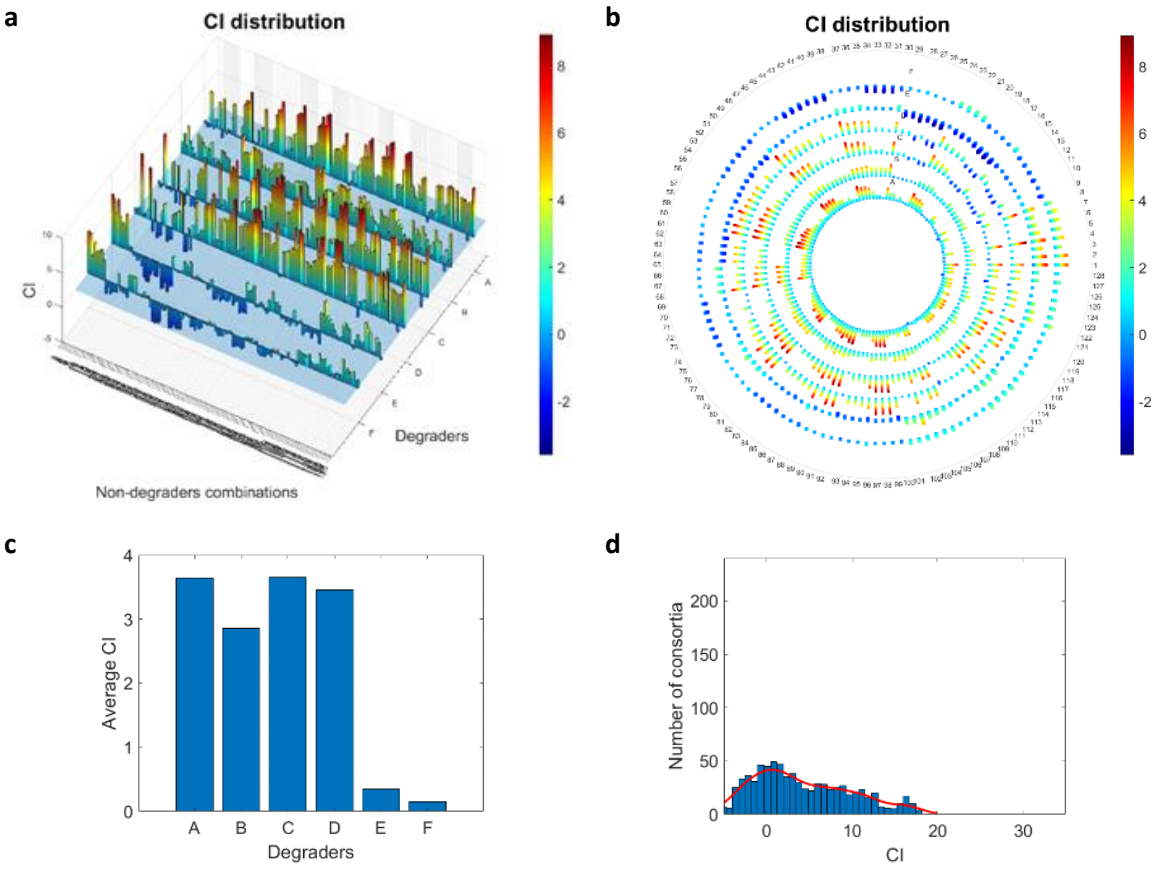


Fig.3.9 Contribution index (***CI***) of screened consortia, visualised in bar chart (a), polar plot (b), average values under 6 different degraders (c) and histogram demonstration of ***CI*** value distribution (d). Details of bacteria combinations are given in Table S2, Supplementary Material 1.

Out of the 768 consortia, the majority (517) had ***FS*** values lower than 1 featured by a peak (Fig.3.10 d) that lies between the range of 0 and 0.6. The remaining 249 consortia had an ***FS*** value greater than 1 and were closely associated with degrader *A*, *C* and *D*. However, this association only occurred either when the non-degrader was absent or the non-degrader was present. ***FS*** values that were close to 1 were only found in a few 2-bacteria consortia based on degrader *E* and *F*, and a couple of 5-bacteria consortia based on degrader *A*, *C* and *D*.

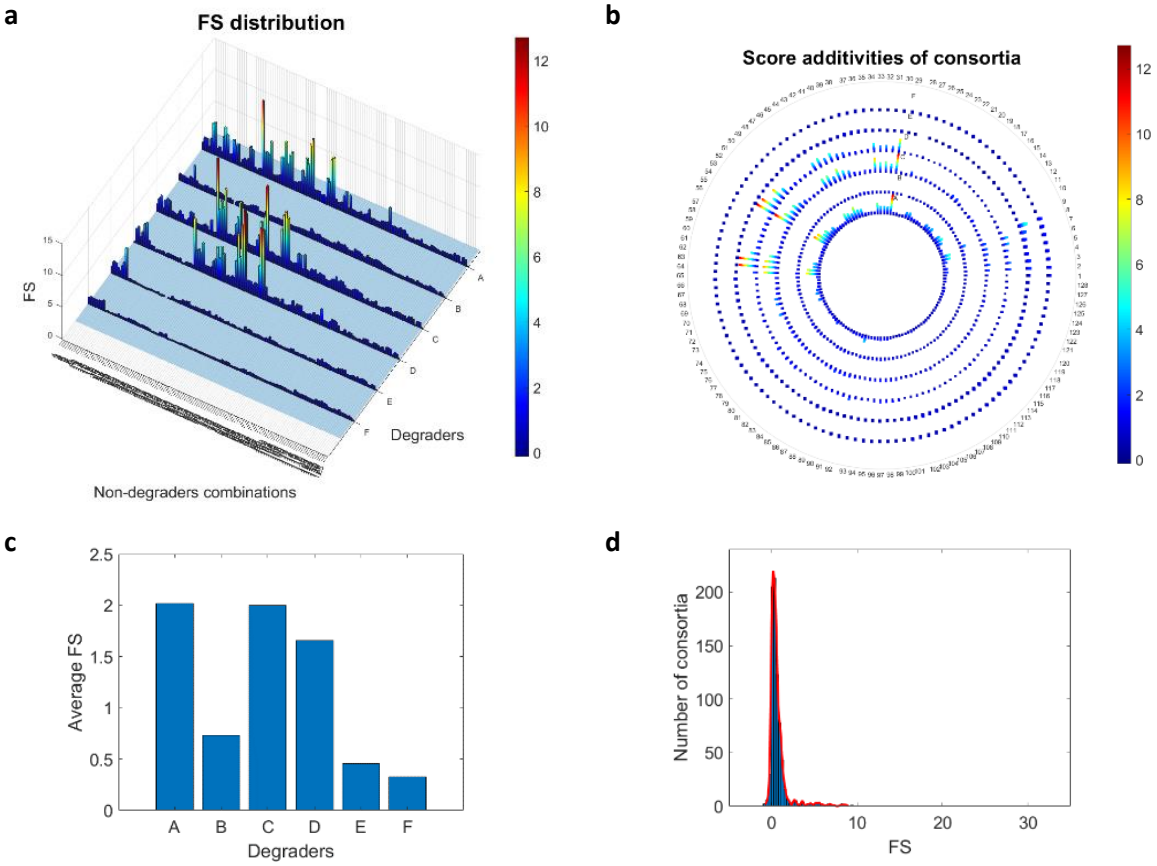


Fig.3.10 Functional stability (***FS***) of screened consortia, visualised in bar chart (a), polar plot (b), average values under 6 different degraders (c) and histogram demonstration of ***FS*** value distribution (d). Details of bacteria combinations are given in Table S2, Supplementary Material 1.

3.3.4 Combination significance and single microbe effect test

Significance tests on the screening results revealed how the three parameters vary as the bacteria combination changes. After false discovery rate (FDR) correction, ***GS*** was found most affected ($p<0.05$) by 63 consortia (Fig.3.11) which are associated with the presence/absence of degrader *B, F*, and non-degrader *3* (Fig.3.11 c, insert). ***CI*** values vary significantly under the presence and absence of 273 consortia wherein degraders *A*, *C* and *D*, and non-degraders *3* and *7* appear to be the primary bacteria that led to ***CI*** variation. A larger number (475) of consortia were found to significantly affect ***FS*** values. These consortia were featured by the absence of Degrader *B*. Non-degraders, *3* and *7*.

a

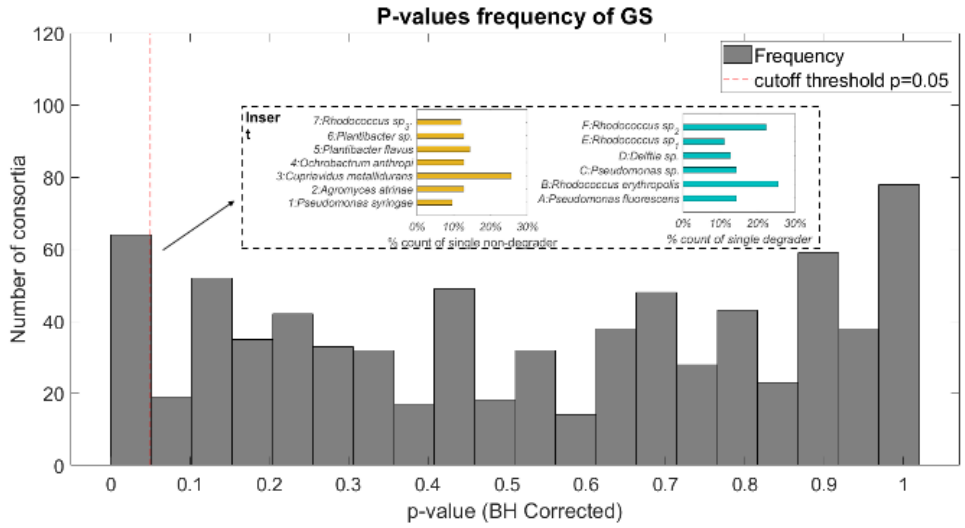
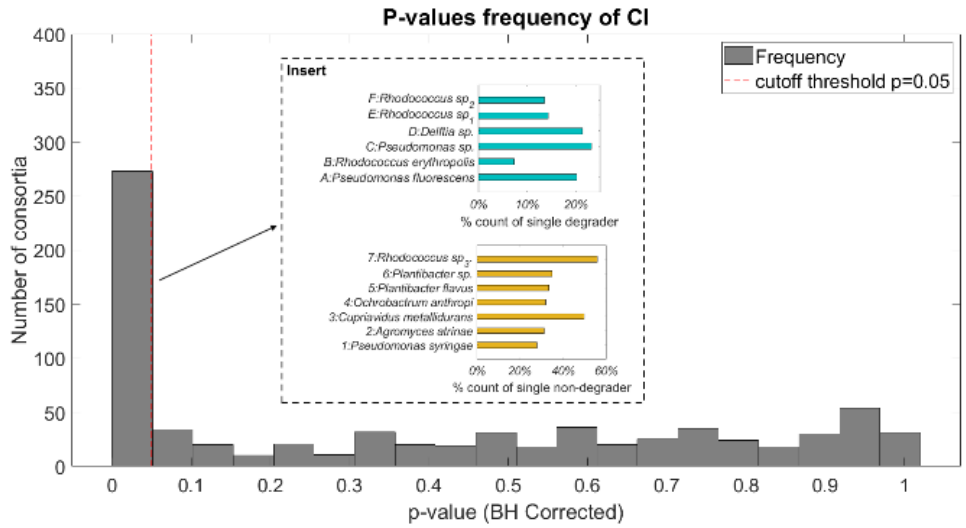


Fig.3.11(Continued) Significance test on all possible combinations for ***GS*** (a), ***CI*** (b), and ***FS*** (c), where histograms show the number of consortia hitting the p-values<0.05 threshold (HB-corrected).

b



c

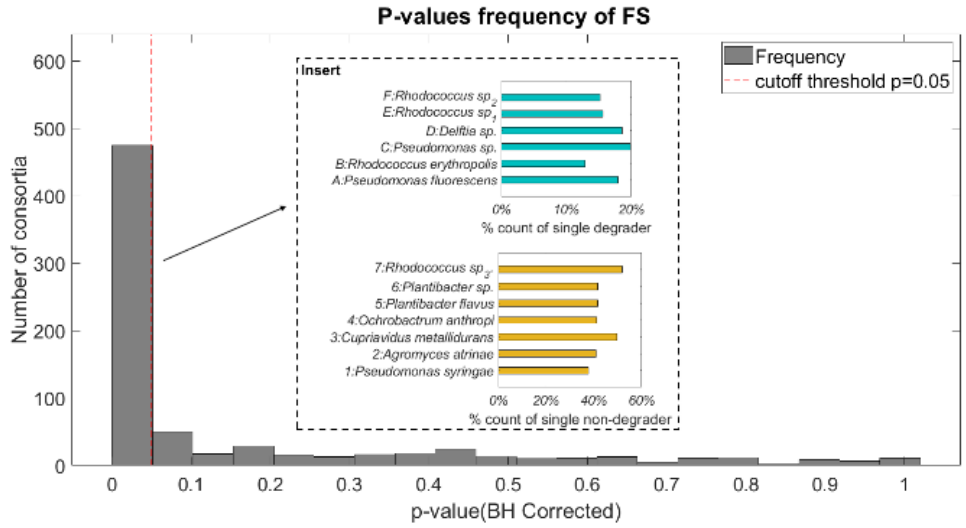
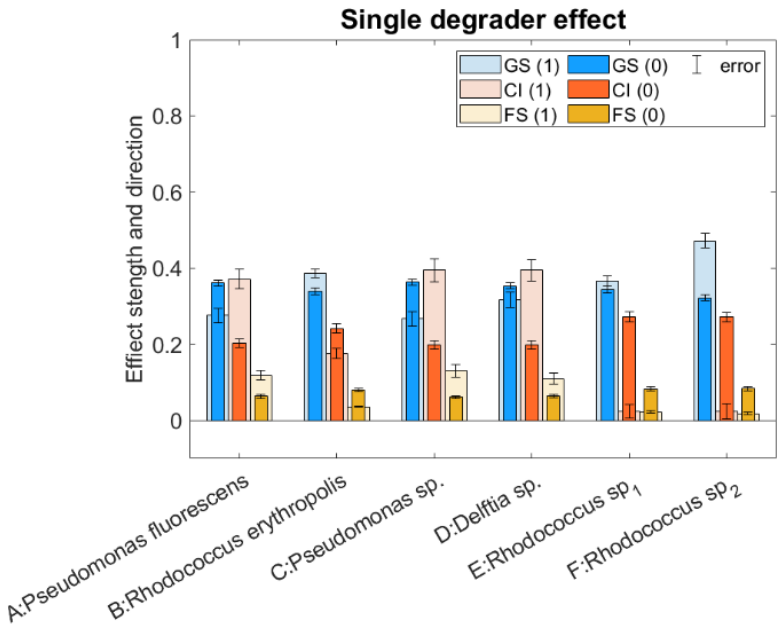


Fig.3.11 Significance test on all possible combinations for ***GS*** (a), ***CI*** (b), and ***FS*** (c), where histograms show the number of consortia hitting the p-values<0.05 threshold (HB-corrected).

The effect of individual microbes (Fig.3.12) indicates that two *Pseudomonas* (degrader *A, C*) had a very similar effect on all three assessment indexes. Specifically**,** consortia involving *A* and *C* tend to have lower values ***GS*** but higher values for both ***CI*** and ***FS*** compared to consortia that do not contain the two bacterial members. This trend opposes what was observed in consortia containing *Rhodococcus* degrader (degrader *B*, *E* and *F*) which are featured by higher ***GS*** and significantly lower ***CI*** and ***FS***. *Delftia* (degrader *D*) had a similar effect on ***CI*** and ***FS*** but higher ***GS*** compared to the two *Pseudomonas* degraders. Among non-degraders, *C. metallidurans* had the strongest effects on the consortia, evidenced by significantly higher ***GS*** and ***CI*** and reduced ***FS*** values associated with its presence. Also, non-

degrader *7* was found to result in higher ***CI*** and ***FS*** values. The rest of the non-degrader had a weak influence on ***GS*** and ***CI,*** albeit with no obvious effect on the ***FS.***

a



c

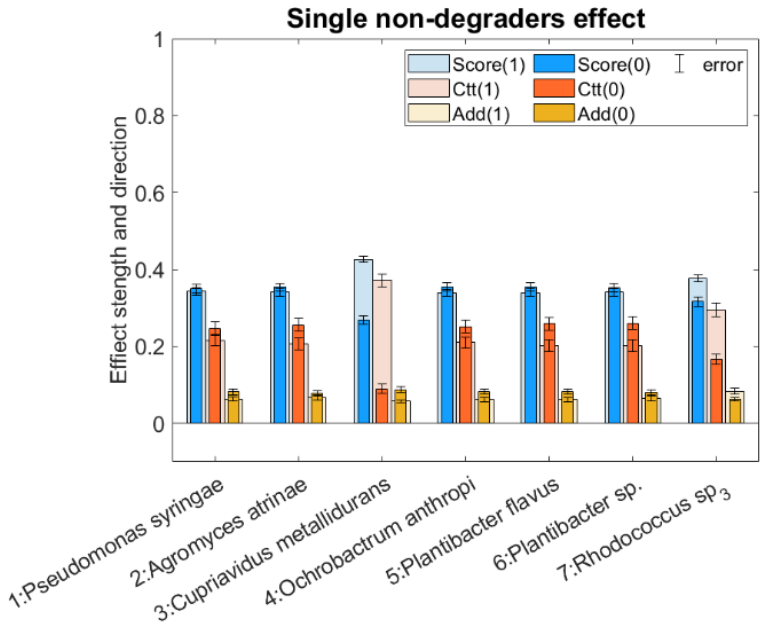


Fig.3.12 Combination significance test result. a, b: Effects on the three parameters. The bar charts presented the normalised value of the three parameters with (light colour, marked as logic 1) and without (dark colour, marked as logic 0) specific bacterial members.

3.4 Discussion

3.4.1 Data validity

By dividing characterised bacteria into VOC degraders and non-degraders, the screening magnitude was significantly reduced while balancing high throughput and analysis rate. The data gathered from these combinations exhibit a strong correlation between the two biological replicate screens. The RMSE value was 5.6153, with an RMSE percentage of 13.06 %, and this is considered relatively low under the complex biological factors and potential experimental errors, such as inaccuracies of manual pipetting and optical reflection caused by the sealing materials of 96-well plates. Furthermore, the strong correlation between fluorescent data and actual cell density underscores very high accuracy in the quantification of algae biomass by directly measuring chlorophyll fluorescence. Given this evidence, it is reasonable to believe that the fluorescence data is consistent and valid to quantify algae growth, ensuring reliability and reproducibility of the subsequent consortia screening and results interpretation.

3.4.2 Monitoring algae growth as a proxy for consortia performance in the screen

In terms of energy and mass contribution, the VOC mixture provides approximately 27.16 mmol of carbon source and a theoretical maximum of only 16.12 kJ of chemical energy per litre of culture media to an entire consortium under aerobic conditions, estimated using combustion enthalpy data of each VOC compound (NIST chemistry Webbook, 1998). This carbon is equivalent to about 0.61 L of gaseous CO_2 at standard temperature and pressure (STP), the fixation of which by algal photosynthesis requires at least 127.11 kJ of solar energy under the maximum theoretical photosynthetic efficiency (PE), i.e. 10%, that microalgae can achieve (Roux, 2016; Masojídek et al., 2021).

Since the screening experiment was performed within a closed environment (microplate wells) for the individual consortium, the VOCs in the media are seen as the main carbon sources of the entire consortium which have a high likelihood of being transferred into algal biomass or secondary bacteria metabolite derived from the photosynthetic product if mutualist interaction exists within the consortia. However, as a continuous illumination

scheme served as infinite energy input, the cycle of VOCs- derived carbons within such consortia is more likely to stay in the form of algae biomass, i.e. photosynthetic units according to the maximum power principle (MPP) that shapes the ecosystem (Odum, 1983; DeLong, 2008; Dillon et al., 2020). For this reason, actual algae growth is not only anticipated to be a strong and straightforward reflection of VOCs degradation but also robust features such as energy-capturing ability, primary production, and suitability for scale-up.

3.4.3 Interpretation of screening parameters with bacteria properties

Since the overall ***GS*** value was based on a direct comparison of chlorophyll accumulation in tested consortia versus axenic algae control, its distribution showed that most consortia had more algae growth than the control (Fig.3.8 d). Consortia with some of the highest algae growths were found to be associated with two *Rhodococcus* (Degraders *B* and *F*) and contrastingly, two *Pseudomonas* (Degraders *A* and *C*), were found in some of the consortia with the lowest growth. The combination significance test (Fig.3.11 a) against ***GS*** revealed that the selection and combination of bacteria had no obvious effect on algae growth, except for a few combinations involving *C. metallidurans* (non-degrader *3*). In addition, single microbe effect analysis (Fig.3.12 a) also showed that the three *Rhodococcus* degraders (*B*, *E* and *F*) and two *Pseudomonas* degraders (*A* and *C*) had different biological behaviours and influence on the consortia which suggested their species-level difference.

The distribution of ***CI*** (Fig.3.9 d) spanned both negative and positive values, with a slight trend of being centred around zero. This suggests the existence of both algae growth promotion and inhibition across the screened consortia, a greater number of which, had neutral effects on algae growth. Unlike ***GS***, the comparative nature of ***CI*** allows it to uncover the behaviour of a consortium against the potential influence of different co-cultured bacteria while also improving the measurement accuracy due to an increased number of observations, which led to very distinct patterns between ***GS*** and ***CI*** of the same consortia. Specifically, the average ***CI*** values of consortia containing degraders *E* and *F* were significantly lower than the rest of the degraders, despite having the highest average ***GS***. This could be explained by their high algae growth promotion effects being dominant and leaving little room for further effects beyond their original contribution. Interestingly, consortia based on another *Rhodococcus* (degrader *B*) were found to have much higher ***CI***

values despite already having high ***GS*** values. This suggests the potential existence of special positive interactions associated with this degrader being synergistically added up. Apart from the great difference caused by the degraders, the combination significance test (Fig.3.11 b) revealed that ***CI*** values of consortia were also affected by the selection and combination of non-degraders among which *C. metallidurans* and another *Rhodococcus* (non-degrader *7*) were found to be most influential.

FS provides extra information about the stability of a consortium by assessing the difference between its ***CI*** and ***GS.*** Theoretically, stable consortia are relatively independent systems with unchanged behaviour when co-cultured with different bacteria and their ***GS*** and ***CI*** values tend to be close to each other, thus having ***FS*** values close to 1. In contrast, ***FS*** values deviating from 1 indicate consortia of weaker resilience which are more subject to the influence of additional bacteria and end up in either algae growth promotion (***FS***>1) or growth inhibition (***Fs***<1) effects. Most of the screened consortia had low stability, which was shown as antagonist results on algae growth as the combinations of non-degraders varied, evidenced by that ***FS*** peaked around 0 and 0.6 (Fig.3.10 d).

The trailing pattern of ***FS*** (Fig.3.10 d) towards higher values greater than 1 indicates that fewer consortia had synergistic algae growth promotion effects. Notably, extreme outliers (far from 1) in ***FS*** values suggest the significant modification of behaviour or emergence of extra functions. However, these were only found in consortia based on degraders *A*, *C* and *D* (Fig.3.10 a, b). ***FS*** value in consortia based on the three *Rhodococcus* degraders (*B*, *E* and *F*) were homogeneously below 1, indicating that they were not significantly affected by any synergistic effects caused by non-degraders. Stable consortia were found to be strongly related to degrader *B*, which was found to have an average ***FS*** close to 1. Additionally, some two-bacteria and five-bacteria consortia containing *C. metallidurans* also exhibited high stability.

Based on the observation of the three parameters, degraders appear to be the fundamental functional unit in the consortia although their effects vary with the specific microbes. *Rhodococcus* has stronger growth-promoting effects on *C. terrestris* evidenced by their association with high ***GS*** values, which could be a result of the efficient single-ring aromatics metabolic systems in this genus (McLeod et al., 2006; Patrauchan et al., 2008; Táncsics et al., 2008). However, due to the same reason, *Rhodococcus* could easily dominate the VOC

degrading processes thus outcompeting and shedding the effect of other non-degraders and thus leading to enhanced functional stability (Coyte et al., 2015).

Contrary to this, two *Pseudomonas* (degrader *A, C*) and *Delftia* (degrader *D*) were considered to have lower performance in supporting algae photosynthesis, evidenced by their frequent presence in low-***GS*** consortia despite many species in these genera having been recognised as strong aromatic hydrocarbon degraders (Kauppi et al., 1998; Shigematsu et al., 2003). Conversely, while these bacteria may be less efficient in VOC degrading, their reduced competitiveness left niches for the activities of non-degraders which in turn, may have improved the overall consortia performance, as reflected by high ***FS***.

Among the 7 non-degraders, *C. metallidurans* and *Rhodococcus Sp3* seem to be the only two bacteria affecting the consortia behaviour and thus were very likely to directly participate in the VOCs degradation process due to their frequent presence in consortia of high ***GS*** and ***CI*** value and potential VOCs metabolic capability reported in other studies (Cébron et al., 2008; Alviz-Gazitua et al., 2019, 2022a). Other non-degraders including *Pseudomonas syringae*, *Agromyces atrinae*, *Ochrobactrum anthropic* and two *Plantibacter* were seen as 'weak' bacteria members in the consortia with little effect on any of the parameters regardless of their presence or absence, nor the variation of their combinations.

3.5 Conclusion

Shortlisted environmental isolates were combined to form algae-bacteria consortia and were screened for their potential VOCs degradation ability via a high-throughput method based on 96-well plate formats. Instead of investigating the actual VOCs degrading process or any complex microbial interactions, algae-bacteria consortia were determined as the singular analytical units. Based on this, a hypothesis was proposed and discussed, wherein algae biomass accumulation was determined to be indicative of VOCs degradation. Algal chlorophyll fluorescence data showed a strong correlation with algae cell density, which serves as a proxy for the VOCs degrading ability of the consortia. This provides a reliable foundation for further analysis.

The screening method, by taking advantage of the hierarchical structure of bottom-up combinations, gave rise to three consortia assessment parameters, growth scores (***GS***) algae growth contribution index (***CI***) and functional stability (***FS***), in achieving rapid and effective consortia according to their VOCs degrading potential. The interpretation of these parameters, together with their significance test was also able to reveal the role of certain of the key bacteria such as *Rhodococcus erythropolis* and *Cupriavidus metallidurans*, that are potentially involved in VOCs catabolism within consortia with high algae growth. Overall, this study served as an efficient filtering and predictive tool for identifying VOCs degrading consortia. However, the significance of these findings requires further validation through other research methods which will be discussed in the subsequent chapter.

Chapter 4: VOCs Degradation in Algae-bacteria Consortia

Aims and objectives

- *Present a case study on selected consortia with distinctive performance, as identified in Chapter 3, serving as a validation of the screening method.*
- *Develop experimental methodologies for VOC biodegradation quantification using GC-FID.*
- *Compare the results with screening outcomes to assess the validity and predictive accuracy of the screening method.*

4.1 Introduction

Chapter 1 presented a comprehensive review of VOCs existing in combustion processes and their impact on ecosystems and human health, giving a foundational understanding of the objective of this project. The preceding chapters (Chapters 2 and 3) explored the novelty of combining environmentally isolated microalgae and bacteria for VOCs bioremediation, targeting benzene, phenol, toluene, and tetrahydrofuran as representative compounds. While the bottom-up consortia design and screening method exhibited rapidity and high throughput in assessing and discovering consortia with bioremediation potential using algae growth as a proxy, the actual occurrence of VOCs degradation remains to be confirmed by analytical approach.

Chromatography is a method for the separation of individual components from a mixture according to the difference in speeds of analytes migrating on the mobile phase. Gas chromatography (GC), and High-performance liquid chromatography (HPLC) are two of the most sophisticated chromatography technology used in bioremediation studies. The development of materials for the mobile phase (columns) extended versatility in quantifying a diverse range of compounds (McNair et al., 2019). When coupled with different detecting technologies such as mass spectrometry (MS), chromatography has even wider applications such as identifying and quantifying the various chemical components which is extremely useful in mapping degradation pathways. These facilitate studies dealing with complex samples containing multiple mixed analytes (Ogata and Taguchi, 1987; Trigueros et al., 2010; Mukherjee and Bordoloi, 2012; Jahanshahi et al., 2022).

Due to the volatile nature of the representative VOCs used in this project, GC was determined to be the best option for their analysis to ensure resolution and sensitivity (Guidetti Vendruscolo et al., 2019). Also, numerous studies exist regarding the biodegradation of these compounds and their analytical protocol using GC, including column and solvent selections which are well established (Reardon et al., 2000b; Yao et al., 2009; Chen et al., 2013b; Jiang et al., 2015).

This chapter presented the case study on exemplar consortia, selected according to their distinctive performance identified by the screening method in Chapter 3. To verify their

VOCs biodegradation ability, Gas Chromatography with Flame ionization detector (GC-FID) was adopted to quantify the residual VOCs concentration in the culture media where the exemplar consortia were cultured in 100 mL bottles. Also, algae biomass in these consortia was measured and examined for any correlation with VOCs degradation, which serves as a validation test on the preceding hypotheses. Furthermore, the performance of exemplar consortia in VOCs degradation as well as algae biomass production, were compared with the screening results as a final verification to assess the validity and predictive accuracy of the screening method.

4.2 Methodology

4.2.1 Exemplar consortia selection

Primarily using growth score (***GS***) and contribution index (***CI***) as selection criteria, consortia identified as having 'high', 'moderate' and 'low' VOC degradation performance, were selected as exemplar consortia. Their categorization schemes are outlined in Table 4.1 below. Given the absence of analytical evidence to validate the efficacy of ***GS*** and contribution index ***CI***) in anticipating the actual performance of consortia, this preliminary scheme was utilised to select representative test subjects which subsequently served to validate the predictive ability of the screening method.

Table 4.1 Exemplar consortia and their categorising scheme

Consortia	*GS*		*CI*		Category		Reason*
B14	7.19	↑	2.72	↑	H	↑↑	high ***GS***, high ***CI***
E14	9.86	↑	-0.45	↓	M	↑↓	high ***GS***, low ***CI***
B23	6.86	↑	3.25	↑	H	↑↑	high ***GS***, high ***CI***
E23	12.80	↑	-1.42	↓	M	↑↓	High ***GS***, low ***CI***
B27	3.03	↓	1.25	↓	L	↓↓	Low ***GS***, low ***CI***
E27	10.97	↑	-2.16	↓	M	↑↓	high ***GS***, low ***CI***
B123	9.19	↑	6.30	↑	H	↑↑	high ***GS***, high ***CI***
E123	8.49	↑	1.85	↓	M	↑↓	high ***GS***, low ***CI***
B125	10.15	↑	4.00	↑	H	↑↑	high ***GS***, high ***CI***
E125	10.59	↑	-0.13	↓	M	↑↓	high ***GS***, low ***CI***
B123456	5.23	↓	4.35	↑	M	↓↑	low ***GS***, high ***CI***
E123456	5.13	↓	4.07	↑	M	↓↑	low ***GS***, high ***CI***
B124567	5.41	↓	1.02	↓	L	↓↓	low ***GS***, low ***CI***
E124567	6.31	↑	0.27	↓	M	↑↓	high ***GS***, low ***CI***

Note: Consortia names correspond to bacteria names listed in Table 3.1; Median ***GS*** (5.60) and ***CI*** (1.97) were used as thresholds for preliminary categorization. Consortia were ranked based on ***GS*** and ***CI*** values: high in both (↑↑) as high performance (H), mixed (↑↓ or ↓↑) as moderate performance (M), and low in both (↓↓) as low performance (L)

4.2.2 Consortia cultivation and sample preparation

Selected exemplar consortia were incubated in 100 mL gas-tight glass bottles assembled with PTFE septum aluminium crimp seals (Fig.4.1). A 1 mL volume of inoculant algae *C. terrestris*, obtained from a 7-day culture in VOCs free medium, were inoculated into the incubating bottle. Each bottle contained approximately 3×10^5 *C. terrestris* cells. The bacteria inoculant was obtained from overnight cultures pre-grown in LB broth. To avoid the contamination of non-VOC carbon source, the bacteria culture was subjected to centrifugation and pellet washing twice using PBS buffer before a final volume of 20 µL bacteria suspensions was pipetted separately into the incubating bottle, following the combination scenario of the corresponding exemplar consortium.

Each incubating bottle was filled with 60 mL of 3N-BBM+V medium containing the same synthetic VOCs mix as described in the preceding chapter, i.e. 100 mg/L of benzene, toluene, phenol and THF (each compound at 100 mg/L). To ensure aerobic conditions, each bottle had approximately 40 mL of headspace volume. Inoculated bottles were incubated at 25 °C

in continuous illumination and orbital shaking conditions at a speed of 120 rpm/min. All consortia were inoculated as duplicates.

Fig.4.1 Exemplar consortia in up-scaled bottles

Daily sampling was conducted by taking 3 mL of liquid culture samples from each flask daily using sterile syringes and needles after vortexing. To separate residual VOCs from the aqueous phase, 1 mL aliquots were passed through 0.22 um glass fibre syringe filters and transferred into glass vials, followed by liquid-liquid extraction using 0.5 mL of HPLC grade dichloromethane (ThermoFisher) through adequate mixing on a tube rotator for 12 h. The organic phase containing analytes VOCs was transferred into 2 mL autosampler vials before further processing. Apart from this, algae growth in the consortia was monitored as OD at 685nm absorbance using a spectrophotometer (Spectronic 200E, ThermoScientific™).

4.2.3 Gas Chromatography

Samples were processed by Gas Chromatography and Flame ionization detector (GC-FID) platform equipped with HP-5 column (Inner Diameter:0.32 mm, Film thickness:0.25 µm, length 30 m, Agilent). The chromatography programme was set up as follows: initial oven temperature 35 °C for 15 seconds, then increased to 185 °C at a ramp of 80 °C per minute. The injector and detector temperature was set as 250 °C and 300 °C respectively. Samples were injected as 1 µL aliquots in split mode with a slit ratio of 8.0:1. To enhance separation

resolution, high-purity helium was used as carrier gas with a flow rate of 1.0 mL/min in the first 45 seconds after sample injection, then increased to 2 mL/min with a ramp of 5 mL/min.

A series of VOCs standards were established for analysing the GC data. The standards were prepared by dissolving known amounts of benzene, toluene, phenol and THF in dichloromethane (DCM) to achieve 100 mg/L, 66.7 mg/L 33.3 mg/L of VOCs-solvent mixture. The actual VOC concentrations in the samples were calculated using their GC data measured against the standard curves.

4.2.4 VOCs concentration determination

Notably, VOC concentration data obtained from GC analysis may not accurately reflect the true VOC quantities in liquid samples, due to the variation of the extraction efficiencies of different VOC compounds transmitting from the medium into the organic solvent. However, the extraction efficiency, also known as the Partition Coefficient (*P*), is inherently dependent on the properties of the solvent system and the analyte to be extracted (Moldoveanu and David, 2015a). While data of Partitioning Coefficient in water/DCM systems remain limited, Octanol-Water is a well-established for solvent systems wherein the Octanol-Water Partition Coefficients (P_{ow}) of different analytes are available. Also, linear dependence exists between the logarithm of P_{ow} for a solute and the logarithm of Partition Coefficient for the same solute in an similar solvent systems (Moldoveanu and David, 2015b; Amézqueta et al., 2020). It is therefore reasonable to hypotheses that for a specific analyte in this study, the extraction efficiency is a constant and is equal to the Partitioning Coefficient in water/DCM systems which can be obtained by:

$$P_{H_2O/DCM} = \frac{C'_{aq_0}}{C_{aq_0}} \qquad \text{Eq.4.1}$$

Where C'_{aq_0} and C_{aq_0} are concentrations of a specific VOC detected by GC analysis and its initial concentration in the medium (i.e. 100 mg/L), respectively.

Assuming that all samples taken in this experiment were at partitioning equilibrium, for day i the true quantity of this specific VOC can be estimated via $P_{H_2O/DCM}$ using the following equation:

$$C_{aq_i} = 2 \times C'_{aq_i} \times P_{H_2O/DCM} \qquad \text{Eq.4.2}$$

In addition, due to the volatile nature of the VOCs involved in this study, their distribution in aqueous medium and headspace air was considered. Since the volume ratio of DCM to medium in the extraction is 0.5:1 mL, C_{aq_i} is supposed to time 2.

Using C_{aq_i} data, the quantity of analyte in the medium can be calculated via Eq.4.3

$$N_{aq_i} = \frac{C_{aq_i} \times V_{aq_i} \times 6.022 \times 10^{23}\ mol^{-1}}{M} \qquad \text{Eq.4.3}$$

Where N_{aq_i} is the quantity of analyte in the aqueous phase, M is the molecular weight of the analyte VOC compound ($g \cdot mol^{-1}$). V_{aq_i} Is the volumes of the aqueous medium on day i,

The distribution of the volatile analyte in water and headspace air obeys Henry's Law (Schwardt et al., 2021) using the following equation:

$$P_i = \frac{C_{aq_i}}{k_H} \qquad \text{Eq.4.4}$$

where P_i is the partial pressure of the analyte in the gas phase on day i ; k_H is the Henry's Law constant ($mol \cdot m^{-3} \cdot Pa^{-1}$) of the analyte (when water is used as solvent) under certain atmospheric pressure and temperature.

The amount of analyte in the headspace air can be determined using Ideal Gas Law via the following equation

$$N_{gs_i} = \frac{P_i \times V_{gs_i}}{k_B \times T} \qquad \text{Eq.4.5}$$

Where the $N_{gs_i}$and V_{gs_i} are the amount of analyte in the gas phase and the volume of headspace air. k_B is the Boltzmann constant ($k_B = 1.380649 \times 10^{-23}\ J/K$). T demotes the kelvin temperature (K).

Given that the incubation bottles were gas-tight and were seen as closed systems, the mass balance principle can be applied using:

$$N_i = N_{aq_i} + N_{gs_i} \qquad \text{Eq.4.6}$$

In addition, the changes in liquid-headspace volume ratio as a result of daily sampling are expected to alter the VOCs distributions between the aqueous and gas phases. Considering this factor, the total VOCs quantities within the incubator bottle were subject to correction using the following equation:

$$\begin{cases} Dg_i = N_i - N_{i+1} - N_{loss_i} \\ N_{loss_i} = V_{sample} \times C_{aq_i} \\ V_{aq_{i+1}} = V_{aq_i} - V_{sample} \end{cases} \qquad \text{Eq.4.7}$$

where Dg_i is the amount (mole) of a specific VOC analyte being degraded biologically on day i , N_i and $N_{i+1}$are its amount in the entire incubation bottle on day i and $i+1$. C_{aq_i} is the concentration of analyte in the aqueous medium determined by GC analyses. $V_{aq_{i+1}}$is the volumes of the aqueous medium on day i and $i+1$. V_{sample} refers to the volume of liquid samples taken daily. This correction was also validated using a computational simulation. The Henry's Law constants for the four VOCs adopted in this study are listed in Table 4.2.

Table 4.2 Henry's Law Constants of VOCs

VOC Name	k_H $(\frac{mol}{m^3 Pa})$	reference	k_H in this study
Benzene	1.7×10^{-3}	Kim and Kim, (2014)	1.75×10^{-3}
	1.8×10^{-3}	Hiatt, (2013)	
	1.7×10^{-3}	Sieg et al., (2009)	
	1.8×10^{-3}	Jian, (2008)	
Toluene	1.5×10^{-3}	Kutsuna and Kaneyasu, (2021)	1.55×10^{-3}
	1.5×10^{-3}	Kim and Kim, (2014)	
	1.7×10^{-3}	Lee et al., (2013)	
	1.5×10^{-3}	Kish et al., (2013)	
Phenol	3.0	Harrison et al., (2002)	3.2
	4.2	Duchowicz et al., (2020)	
	3.6	Wang et al., (2017)	
	2.0	Mackay et al., (2006)	
THF	2.2×10^{-1}	Signer et al., (1969)	1.45×10^{-1}
	1.4×10^{-1}	Cabani et al., (1971)	
	1.1×10^{-1}	Hilal et al., (2008)	
	1.1×10^{-1}	Abraham et al., (1990)	

Note: Henry's Law constants listed above are under 298.15 K (25°C). Constants adopted in this study derive from average values of the most recent studies with experimental evidence whenever available.

4.2.5 Data analysis and statics

In this study, Pearson correlation coefficients (PCC) were used to check any potential linear correlation between the results. Unless specified otherwise, p=0.05 was set as the significant threshold. All data processing, screening parameters calculations and statistical calculations for the above-mentioned tests and equations were performed using MATLAB 2023 (MathWorks Inc., United States).

4.3 Result

4.3.1 VOCs quantification

GC results of VOCs standards showed that the four representative VOCs can be adequately separated by the current choice of solvent and oven program, characterised by four distinct peaks with fair resolution (Fig.4.2 a). Also, the established standard curves exhibited high alignment, with R^2 values >0.99, attesting to the accuracy of quantification and reliability of the subsequent results (Fig.4.2 b-e).

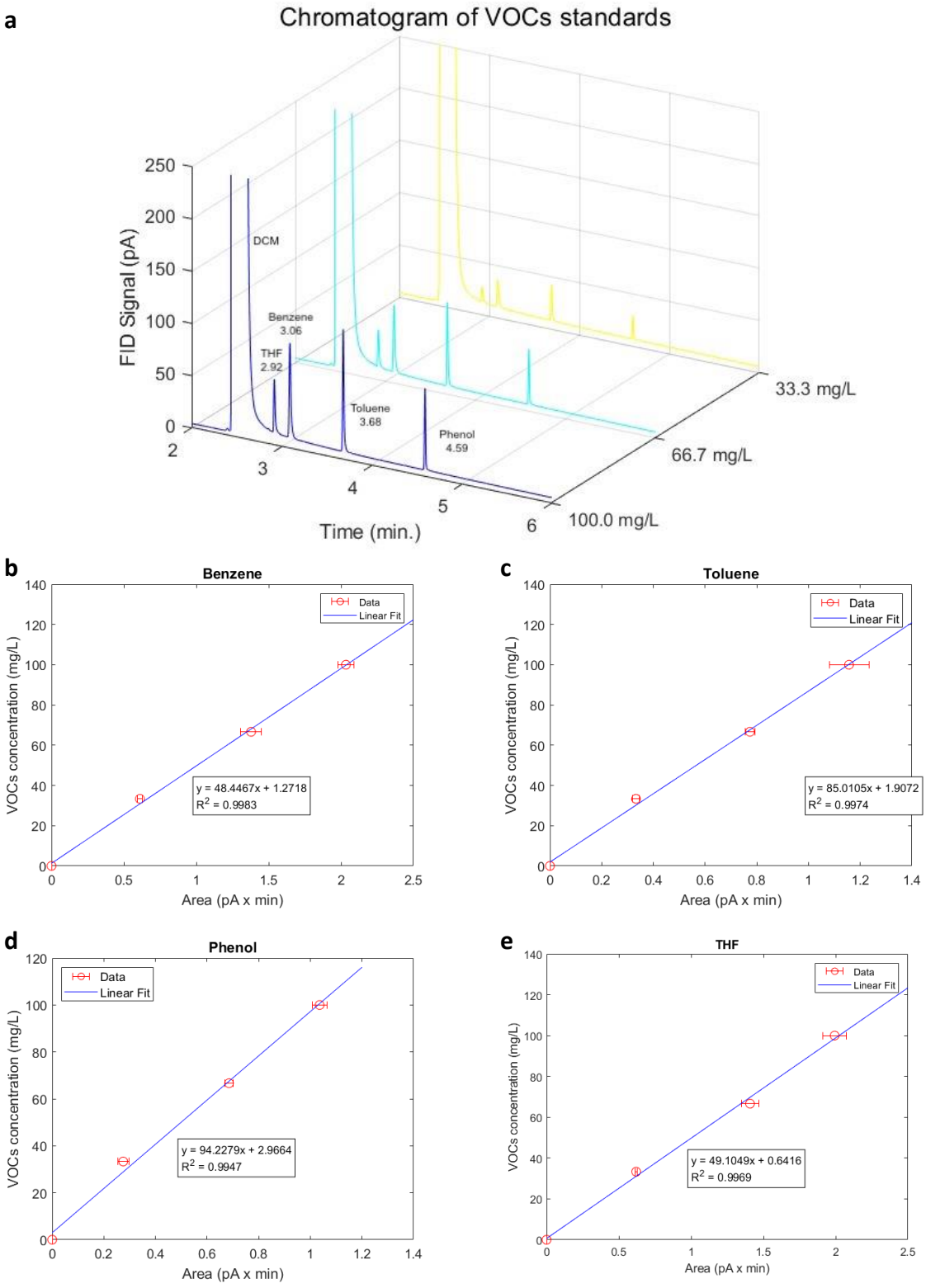


Fig.4.2 GC-FID chromatograph (a) of VOCs and standard curves of VOCs standard; b: Benzene, c: Toluene, d: Phenol, e: THF.

4.3.2 VOCs degradation in exemplar consortia

The residual VOCs concentration in culture media containing different exemplar consortia were presented in Fig.4.3. It is clear that the incubation bottles deployed in this study were able to prevent VOCs escaping and the medium-headspace volume ratio change caused by the daily sampling scheme did not have significant impact on the concentration of any VOCs compounds. Also, the VOCs degradation observed in any consortium was completely a result of the biological process since no natural degradation was observed in the blank groups despite their high volatility and even light-sensitivities (phenol). This was evidenced by the relatively consistent VOCs concentrations in the blank samples (Fig.4.3 (1)). For axenic *C. terrestris* culture, i.e. the control (Fig.4.3 (2))., a minimal decrease of concentration in benzene (16.5 mg/L) and toluene (26.1 m/L) was observed on day 7, while the other two compound concentrations remained relatively unchanged. Given the slightly larger variations in data of benzene and toluene on day 7, it is not clear whether *C. terrestris* can cause the change of these two compounds.

Compared to other VOCs, phenol seems to be the most bioavailable VOC, exhibiting noticeable degradation in all samples and its concentration in the medium rapidly approached zero in most consortia within 4 days. Benzene and toluene shared a very similar pattern of concentration changes across the 14 consortia, characterised by the rapid decrease in B23, E23, B123, E123, B123456 and E123456 (Fig.4.3 (5) (6) (9) (10) (13) (14)). Remarkably, THF showed no sign of declining in any of the consortia as suggested by its steady concentration.

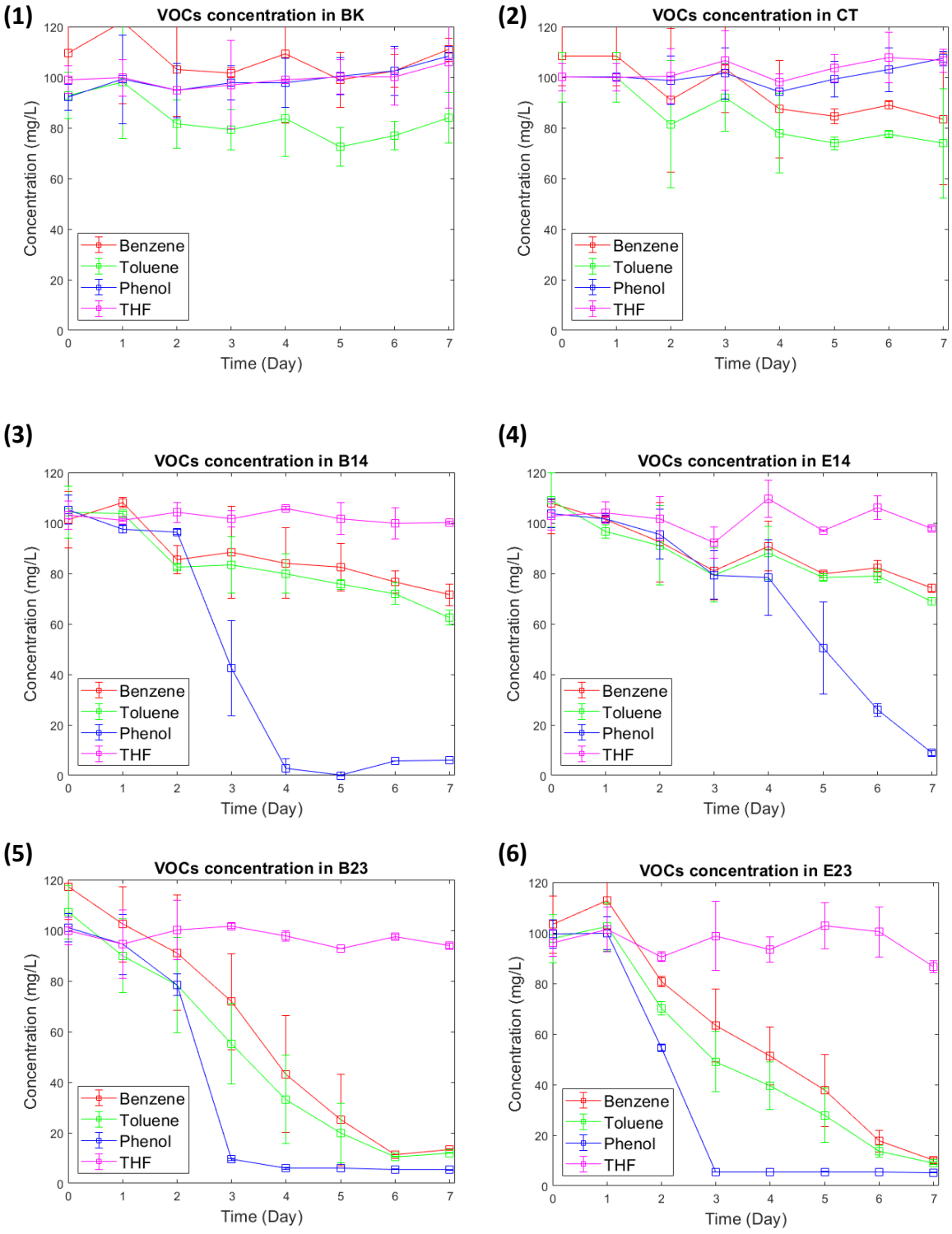


Fig.4.3 (Continued) Residual concentrations of VOCs in the culture media from (1): Blank (BK), (2): Control (CT), (3): consortium B14; (4): consortium E14, (5) consortium B23, (6): consortium E23, (7): consortium B27, (8): consortium E27, (9): consortium B123, (10): consortium E123; (11): consortium B125, (12): consortium E125; (13): consortium B123456; (14): consortium E123456; (15): consortium B124567, (16): consortium E124567.

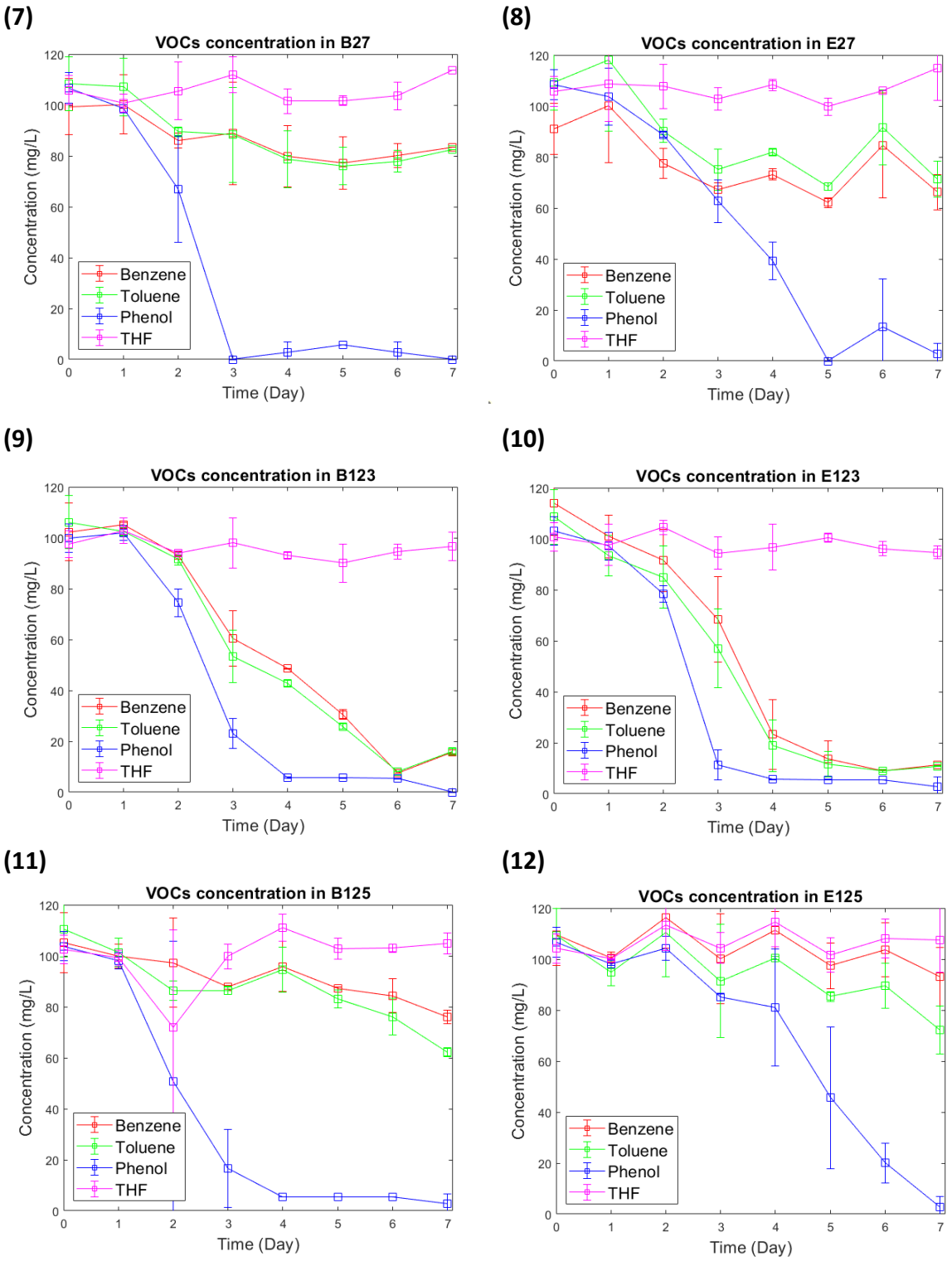


Fig.4.3 (continued) Residual concentrations of VOCs in the culture media from (1): Blank (BK), (2): Control (CT), (3): consortium B14; (4): consortium E14, (5) consortium B23, (6): consortium E23, (7): consortium B27, (8): consortium E27, (9): consortium B123, (10): consortium E123; (11): consortium B125, (12): consortium E125; (13): consortium B123456; (14): consortium E123456; (15): consortium B124567, (16): consortium E124567.

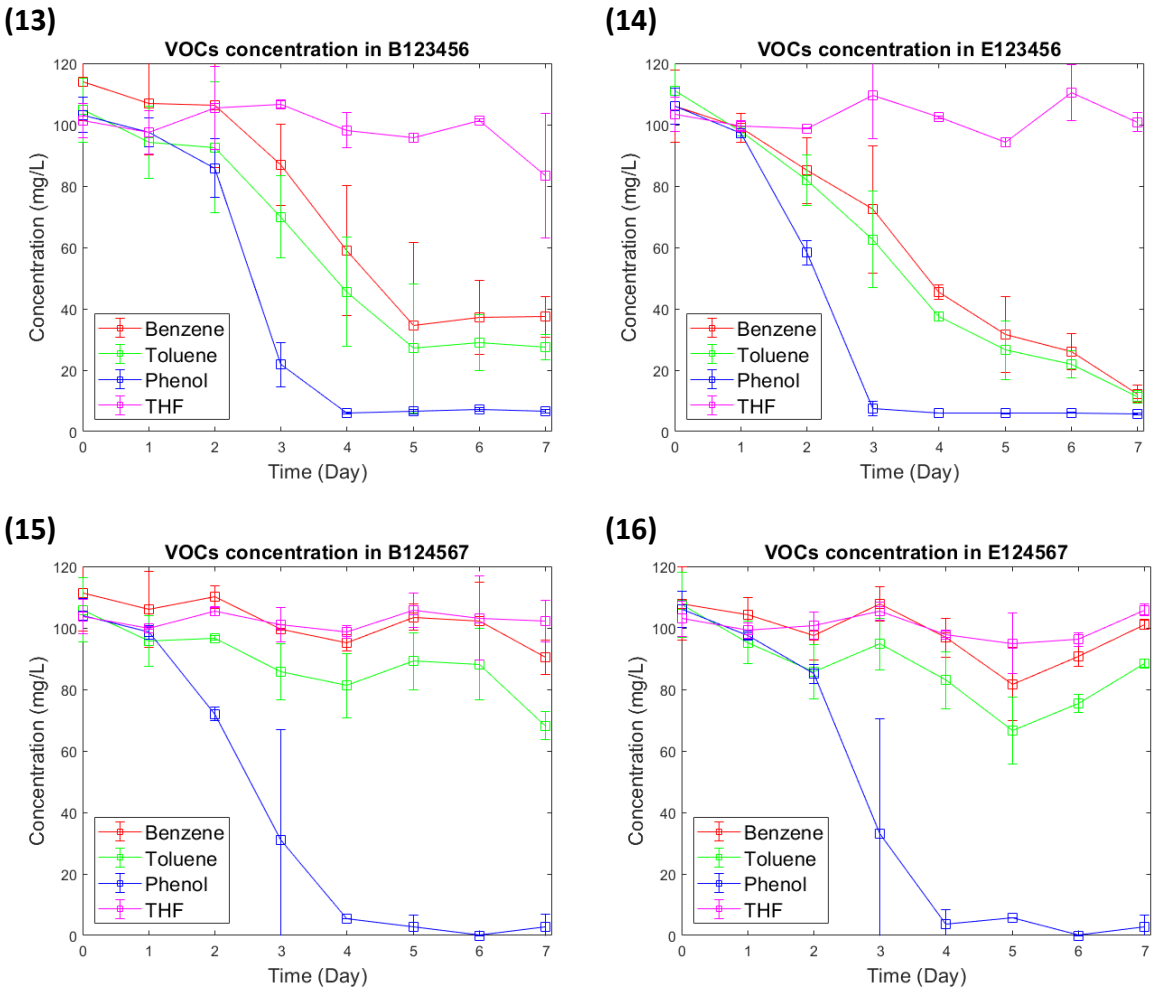


Fig.4.3 Residual concentrations of VOCs in the culture media from (1): Blank (BK), (2): Control (CT), (3): consortium B14; (4): consortium E14, (5) consortium B23, (6): consortium E23, (7): consortium B27, (8): consortium E27, (9): consortium B123, (10): consortium E123; (11): consortium B125, (12): consortium E125; (13): consortium B123456; (14): consortium E123456; (15): consortium B124567, (16): consortium E124567.Note: All samples began with an initial VOCs concentration of 100 mg/L per compound; any variations, such as concentrations >100 mg/L, are due to the intricacies in the liquid-liquid extraction process

The loss of VOCs loss due to sampling is presented in Fig.4.4. Despite being able to dynamically re-distribute between the aqueous media and headspace air, all four VOCs exhibited a continuous sampling loss, especially for the blank (BK) and control (CT) bottles which exhibit a near-linear loss. Phenol (Fig.4.4 c) and THF (Fig.4.4 d), with higher affinity to water, underwent more pronounced sampling loss. However, consortia with major aqueous VOCs concentration reductions observed in the GC-FID analysis demonstrated a decelerated VOCs sampling loss ranging from 2-6% depending on the types of VOCs.

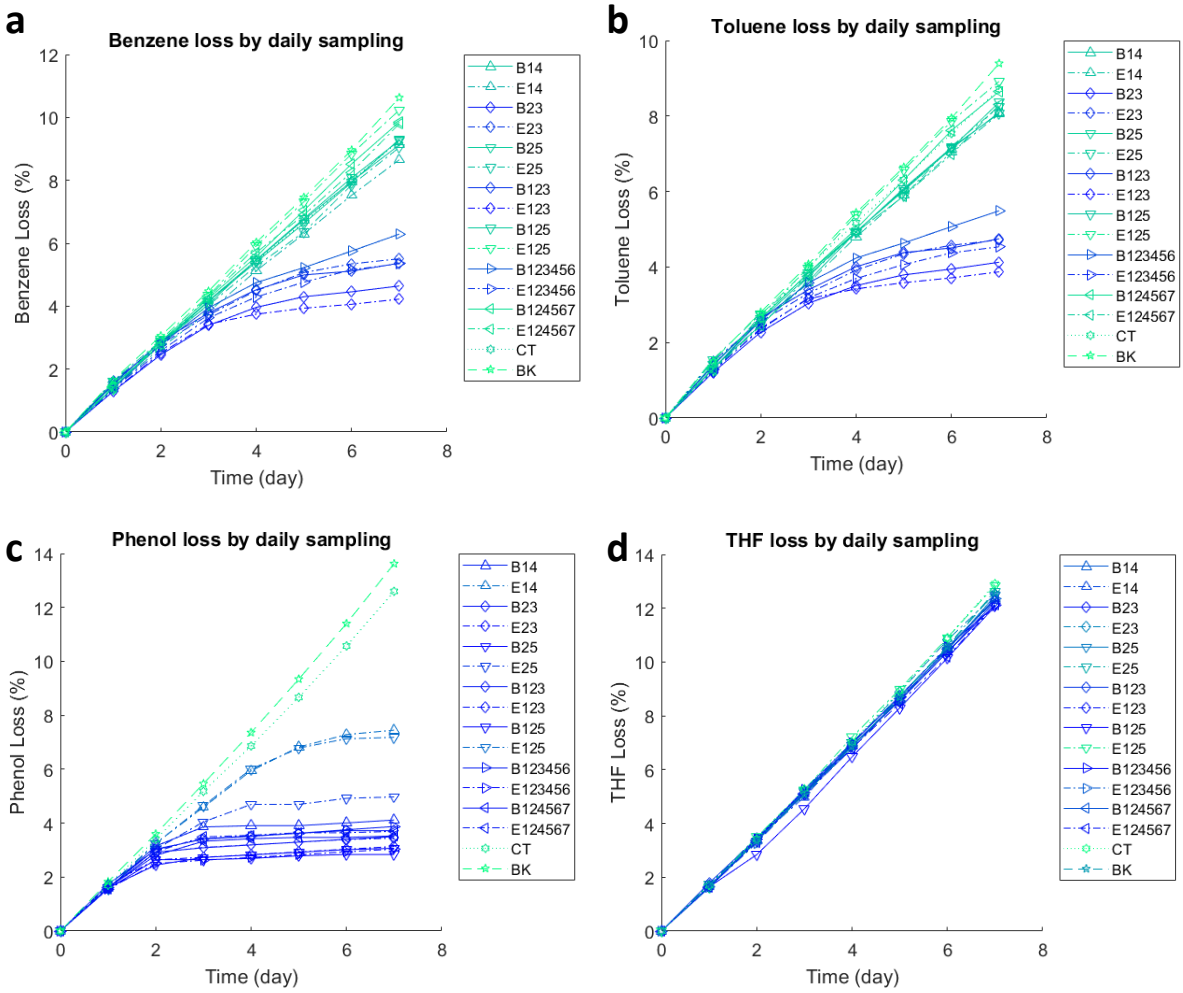


Fig.4.4 VOCs sampling loss in each consortium (calculated via Henry's Law); a: Benzene, b: Toluene, c: Phenol and d: THF.

After accounting for sampling loss and aqueous-gas distributions, the total amount of degraded VOCs within the incubation bottles was visualised and aligned with the algae growth images for each exemplar consortium (Fig.4.5 a). Rapid degradations of benzene and toluene mainly occurred between day 2 and day 4, with benzene having the daily degradation of 20.61 mg·L-1·D-1 to 39.86 mg·L-1·D-1 and 18.58 mg·L^{-1}·D-1 to 35.45 mg·L^{-1}·D^{-1} respectively. Consortia B23, E23, B123 E123, B123456 and E123456 had some of the highest degradations of benzene with 63.41% to 95.71% removal over the 7 days. A similar pattern was also observed for toluene which had 65.62 to 92.70% removal in the same consortia. In contrast, no more than 34.48% of benzene and 40.43% of toluene were

degraded in the remaining consortia. The degradations of phenol were more rapid in most consortia with > 90% removals observed as early as day 4. Despite having a milder rate, the degradation of phenol in consortia E14, E27, and E125 was thorough, achieving 84% to nearly 100%. Particularly, for THF, while up to 33.38% removal was obtained, actual degradation remains unlikely and the removal rate could be a result of measurement error or pipetting error introduced during GC analysis being added up and reflected in the data processing, which is suggested by the large error bar in some of the GC result (Fig.4.3). This assumption is also applied to benzene and toluene in CT and BK. However, despite this data variation, the true VOCs degradations remain distinctly and evidently distinguishable due to their magnitude in data compared to noise.

Consortia with high VOCs removal ability, including B23, E23, B123, E123, B123456 and E123456, also had some of the highest algae biomass content as reflected by their OD_{685}. Furthermore, a strong correlation ($R= 0.82$, $P<0.001$) was found between total VOCs degradation and OD_{685} data. Notably, the performance of consortia, in terms of VOCs degradation and OD_{685} were affected by the combination of non-degraders especially the presence of *C. metallidurans*, while no significant difference was found in terms of VOCs degradation between consortia containing the two different degraders ($p=0.71$).

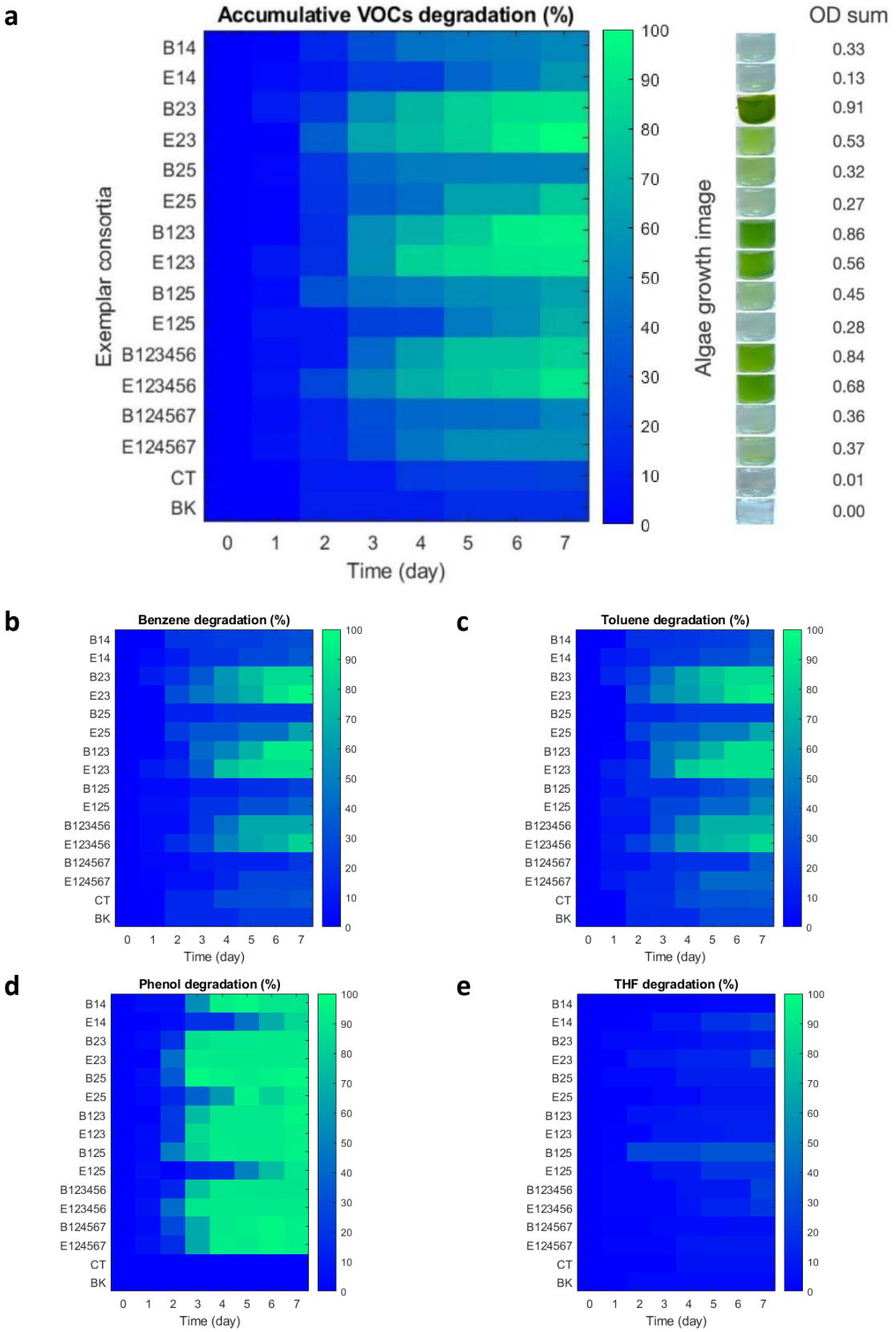


Fig.4.5 Visualisation of total VOCs degradation in exemplar consortia and alignment with algae growth (a), and the degradation of individual VOC across time (b: Benzene, c: Toluene, d: Phenol, e: THF).

A comparison between screening results (***GS***, ***CI***, ***FS***) of the exemplar consortia versus their VOCs degradation rates and OD_{685} results were presented in Table 4.2. Surprisingly, no significant correlation was found between total algae biomass and ***GS*** (R=-0.18, P=0.54) of these bottle-grown exemplar consortia. One possible cause of this deviation is the different sensing mechanisms from which data were generated since the fluorescence signal is measured more sensitively with less interference on the optical pathway caused by suspended bacteria cells (Lakowicz, 2006). Also, larger flask-scale culture volume may influence the growth of algae due to differences in factors such as growth space, nutrient availability and luminance patterns.

Conversely, ***CI*** demonstrated a higher level of efficacy when predicting the algae growth in flask scale exemplar consortia, evidenced by its strong positive correlation with OD_{685} (R=0.73, P<0.001), which could be attributed to its comparative nature, as well as its extended number of observations. A moderate positive correlation lies between OD_{685} and ***FS*** (R=0.58, P=0.003) which, reflects the stability of a consortium against the potential influence of additional bacteria, which could serve as an important reference for the consortia robustness.

Table 4.2: Comparison of screening results, VOCs degradation rate and algae growth of exemplar consortia

Consortia	Screening Parameters			VOCs degradation rate (mg/L)					OD_{685}
	GS	***CI***	***FS***	**B**	**T**	**P**	**THF**	**Total**	
B14	7.19	2.72	0.62	30.85	32.79	96.54	*3.20	163.39	0.33
E14	9.86	-0.45	0.19	34.49	37.76	84.28	*25.09	181.61	0.13
B23	6.86	3.25	0.72	85.49	85.81	91.38	*10.95	273.62	0.91
E23	12.80	-1.42	0.07	95.72	92.70	93.76	*26.74	308.91	0.53
B27	3.03	1.25	0.92	20.98	23.02	100	*11.36	157.99	0.32
E27	10.97	-2.16	-0.03	63.41	65.62	100	*9.17	243.14	0.27
B123	9.19	6.30	0.84	92.14	87.48	100	*11.97	291.77	0.86
E123	8.49	1.85	0.45	87.89	87.80	93.92	*12.11	281.71	0.56
B125	10.15	4.00	0.56	25.03	43.86	94.56	*33.38	196.83	0.45
E125	10.59	-0.13	0.20	38.46	54.83	97.68	*19.81	210.78	0.28
B123456	5.23	4.35	0.69	65.26	69.67	91.22	*25.55	251.71	0.84
E123456	5.13	4.07	0.67	82.52	84.86	91.86	*21.09	280.34	0.68
B124567	5.41	1.02	0.30	21.69	37.06	96.53	*5.48	160.76	0.36
E124567	6.31	0.27	0.19	26.90	40.43	98.45	*9.06	174.84	0.37
CT	0	N/A	N/A	*32.35	*34.09	*5.60	*6.87	*78.92	0.01
BK	N/A	N/A	N/A	*23.13	*27.23	*3.06	*3.28	*56.70	0.00

Note: VOCs abbreviation B: benzene, T: toluene, P: phenol, THF: tetrahydrofuran; VOCs degradation rate was calculated as 7-day mean values using endpoint data. Results marked with * are estimated results based on experimental fact, for example, a 'negative' VOC concentration indicates zero degradation.

4.4 Discussion

The high alignment of the standard curves of GC data established on VOCs standards and the steady concentrations of residual VOCs in the blank confirms the dependability of incubating bottles in preserving the analytes. Computational analysis based on Henry's law suggested that the VOCs loss due to sampling only accounts for a small proportion (<14%) of the total VOCs in the entire incubation bottles. It is evident that consortia with a faster reduction of VOCs retain fewer VOCs in the bottles, consequently resulting in even lower VOCs loss through sampling (2%-6%). Therefore, any major VOCs concentration reduction observed in GC-FID analysis is expected to be the result of biological degradation. Also given that only a minor decrease of benzene and toluene was observed in the axenic algae control which is a sign of very limited VOCs utilisation in the axenic algae control. It is a plausible hypothesis that the degradation of all four VOCs observed in all 14 exemplar consortia was a result of bacteria activity. The strong correlation between total VOCs degradation rate and algae growth in exemplar consortia suggested that VOCs degradation acted as the primary carbon source for algae growth, which was attributed to bacteria metabolism. This provided direct evidence supporting the hypothesis in Chapter 3, further validating the screening method.

In terms of the degradation efficiency of individual VOCs compounds, phenol appears to be the most readily degradable VOC for all 14 exemplar consortia due to its relatively central position on aromatic compounds catabolic pathways of associated bacteria (Alviz-Gazitua et al., 2022b; Jiang et al., 2015; Selvakumaran et al., 2011) and thus served as the basic carbon sources for algae growth in all 14 exemplar consortia. However, further algae growth largely depends on the degradation of benzene and toluene, which affects the availability of carbon sources (CO_2) for photosynthesis.

Surprisingly, bottle-grown exemplar consortia had very different patterns between actual algae growth (OD_{685}) and their ***GS*** from screening. One possible cause of this deviation is the different sensing mechanisms from which data were generated since the fluorescence signal is measured more sensitively without the interfering beams that may introduce error, especially with the presence of suspending bacteria cells on the optical pathway (Lakowicz, 2006). Also, larger flask-scale culture volume may influence the growth of algae due to changes such as space, nutrient availability and luminance patterns. Conversely, ***CI***

demonstrated a higher level of efficacy when predicting the algae growth in flask scale exemplar consortia, which could be attributed to its comparative nature, as well as its extended number of observations. Furthermore, ***FS*** reflect the stability of a consortium against the potential influence of additional bacteria, which is an additional but important reference for the evaluation and prediction of the consortia performance.

Although there is no evidence showing that the two degraders affect the total VOCs degradation in its containing consortia, the presence of degrader *B* seems to result in higher algae growth suggesting that when a similar amount VOCs is degraded, either degrader *E* yields less carbon which is biologically available for algae *C. terrestris* or degrader *B* has more pronounced growth-promoting effect on the algae due to unknown mechanism. While all consortia metabolize benzene and toluene more slowly, the presence of the bacteria non-degrader *3*, *C. metallidurans* made the degradation of the two VOCS faster and more thorough, suggesting that the aromatic compounds catabolic traits associated with *C. metallidurans* reported in other studies were highly likely to have taken effect in the consortia although it was not identified as a degrader in the pre-screening experiment (Chapter 2). Finally, these consortia were found to have relatively high ***CI*** values and ***FS*** values closer to 1, which further evidences the predictive potential of the two parameters for algae growth and stability.

4.5 Conclusion

The core hypothesis behind the screening method was validated with GC analyses on the degradation of VOCs. Certain robust consortia achieved 90.7%, 92.15 % and nearly 100% removal (7-day) for benzene, toluene and phenol respectively, starting each with initial concentrations of 100 mg/L. VOCs degradation by consortia was predominantly attributed to certain bacteria such as degrader *B* (*Rhodococcus erythropolis*) and non-degrader *3* (*Cupriavidus metallidurans*) This degradation was believed to have contributed to the growth of microalgae *Coelastrella terrestris*, as suggested by the strong correlation between the two results ($R=0.82$, $P<0.001$).

The efficiency of VOCs degradation could be predicted by the two screening parameters, i.e. algae growth contribution index (***CI***) and functional stability (***FS***) as defined in Chapter 3. Consortia containing the two *Rhodococcus* degraders (*B* and *E*) showed no significant difference in the VOCs degradation, while degrader *B* results in higher algae growth. Specifically, non-degrader *3*, *C. metallidurans* was identified as a key bacteria involved in VOCs catabolism within high-performance consortia. This potential role triggers interest for further investigation.

Chapter 5: Exemplar Algae-bacteria Consortia Community Structure Analysis

Aims and objectives

- *Determine the end point microbial community structure of the exemplar algae-bacteria consortia examined in Chapter 4.*
- *Detail the design of primers and assays to validate the primer specificity.*
- *Interpretation of consortia communities structure results under the context of VOCs degradation capability.*

5.1 Introduction

Insight into the structure of microbial community is essential for the study of microbial systems, especially in the context of synthetic microbial systems which are usually created to achieve certain biological tasks. Understanding the structure of these communities is instrumental for evaluating their stability, monitoring functionality succession, as well as assessing the robustness of their efficacy for biological tasks.

Culture-based methods are often used for the convenient identification of specific microorganisms. However, these methods lack speciation. For example, when profiling the microbial communities with conventional agar plate cultivation and colony forming unit (CFU) counting, while it allows easy estimation and quantification of the bacterial population, this method is usually subject to a long turnaround time and may introduce cultivation bias due to the variation of bacteria growth speed. Also, suitable selective media is often required to distinguish bacteria with similar colony morphologies (Peyroux et al., 2023).

Microscopy is another method that allows for immediate imaging of microbial cell morphology. However, it is typically constrained by limited resolution and lack of the ability to provide direct insights into microbial activities. While advanced microscopic techniques such as fluorescence microscopy and confocal laser scanning microscopy (CLSM) enable direct observation of microbial cells and differentiation of microbial species based on their fluorescence patterns and even their spatial arrangement within microbial systems (Lutz et al., 2019; Schlundt et al., 2020), these technologies often require the selection of suitable fluorescent stains or fluorophores, making prior knowledge of the microbe essential.

To gain deeper insight into interactions and even functional roles, a combination of more resource-intensive molecular technologies such as Fluorescence in situ Hybridization (FISH) (Lukumbuzya et al., 2019) is sometimes needed. These methods may not be ideal for studying the population structure of some simple microbial communities with unknown members of species.

Sequencing-based methods have been a pivotal tool in the studies of microbiomes and offer insights into the microbe diversity, functional capabilities, interactions, and ecology of microbial communities. Common techniques include ribosomal RNA (rRNA) gene

sequencing, shotgun metagenomics and microbial whole-genome sequencing (WGS). By balancing high resolution and coverage, the sequencing-based method can be used to detect pathogenic microorganisms (Yu et al., 2019), and survey microbial phylogeny and taxonomy in complex naturally occurring microbiomes in soil (Naylor et al., 2022), water (Naylor et al., 2022) or human body (Bikel et al., 2015). However, one major feature of the sequencing-based method is the complexity of the data generated and the substantial computational requirements for analysis and interpretation purposes. The cost and accessibility of sequencing facilities also remain substantial barriers.

For the methodological framework of this research, it is essential to employ a method that is not only cost-effective and time-efficient but also one which offers good specificity and feasible integration with the high-throughput screening methods defined in Chapter 3. Given these criteria, Quantitative Polymerase Chain Reaction (qPCR) presents an option suiting this purpose. qPCR is a well-established technique for the detection and quantification of target DNA. It is known for its rapidity, sensitivity and high-throughput features (Kralik and Ricchi, 2017). Depending on the choice of fluorescent methods, i.e. dye-based or probe-based, qPCR can achieve high specificity on the amplification of target DNA template even in the presence of background non-target DNA, making it suitable for multiplexed assays (Kubista et al., 2006). Given the known 16S rRNA gene sequences of bacteria as described in the previous chapter, it is possible to design specific primers tailored to these bacteria to estimate their populations within the consortia.

This chapter presents a subsequent analysis of the microbial community structure of the same exemplar algae-bacteria consortia examined in Chapter 4 using dye-based qPCR. By designing primers primarily targeting 16s rRNA genes with a resolution up to genus level, this study achieved the quantification of bacteria population in the consortia. The results profiled the bacterial community structure in these consortia, particularly showing their persistence before and after a 7-day VOCs degradation experiment. These findings also revealed some of the core bacteria within the consortia and provided a more defined study objective for more in-depth future work, such as proteomics studies focusing on their interactions or functional roles.

5.2 Methodology

5.2.1 Consortia sample processing

Algae populations were quantified via optical density set as absorbance at 685 nm (OD_{685}) using a spectrophotometer. To enhance comparability with screening results, daily OD_{685} data of flask-grown exemplar consortia were normalised using the control data and then summed to reflect the accumulative feature of the total algae biomass in 7 days.

The population of individual bacteria members in each exemplar consortium were quantified via qPCR using the remaining 2 mL sample aliquots. Consortia biomass pellets were obtained by high-speed centrifugation (10000 g, 1 min) and were washed using nuclease-free water (QIAGEN) to remove salts and residual phenolic compounds. Full consortia DNA extraction was performed using PCRBIO Rapid Extract Lysis Kit (PCR Biosystems) following the kit instructions. To ensure thorough DNA extraction, an excessive amount of reagents was used, combined with extended incubation time (30 min) for both the cell lysis (75 °C) and proteinase deactivation (95 °C) with additional vortex steps in between. Obtained consortia genomic DNA extracts were adjusted to equal volumes of 100 µl before qPCR analysis.

5.2.2 qPCR standards

This experiment was designed to analyse bacteria composition in the consortia using target genes copy number as a proxy for bacteria cell number through absolute quantification approach, rather than relative quantification which typically requires internal control. This involves series of standard samples containing known quantities of the target genes being included in each qPCR experiment which serves as reference for the abundances of target genes.

To establish the quantification standards, bacteria adopted in the screening and VOCs degradation experiment (Table 3.1) were cultured in 100 mL of LB media at room temperature overnight. For slower-growing bacteria such as *Rhodococcus* (non-degrader *7*, and degrader *B*, *E*, *F*) the culturing time was extended to 48 hours to ensure sufficient biomass yield. For algae, 200 mL axenic culture was cultured for 7 days. Algae and bacteria

biomasses were harvested through centrifugation (4800 g, 10 min) and washed using type1 water (processed by Milli-Q® Type 1 Ultrapure Water System) to remove salts and residual medium. Bacteria pellets obtained were weighed and underwent DNA extraction using the same extraction kit.

Crude bacterial genomics DNA yielded from this step was subjected to further cleaning and purification using the ethanol acetate precipitation method described by Sambrook et al., (1989). 300 µl aliquots of each DNA sample were transferred into 2 mL Eppendorf™ tubes to which 30 µl of 3M Sodium acetate (pH 5.2, Merck) was then added followed by the addition of 1200 µl of 99.8% ethanol (absolute, Fisher Scientific). The tubes were gently inverted to ensure mixing while avoiding shearing damage to DNA molecules. To facilitate the DNA precipitation, all the samples were incubated at - 80℃ for 1 hour followed by high-speed centrifugation (15000 g, - 10 ℃) for 15 min. After removing the supernatant, the DNA pellet obtained in each sample was washed using 1 mL 70% ethanol and re-centrifuged (15000 g, - 10 ℃) for 15 min to remove residual salts. The re-pelleted DNA sample was then placed in the laminar flow biosafety cabinet to completely air dry and remove residual ethanol before then storing at - 20℃ for further use.

Each of the purified, dried DNA pellets was re-suspended as 100 µl aliquots in nuclease-free water followed by an undisturbed 1-hour settling time. After completely dissolving, the DNA concentration in each sample was measured in ng/µL using a microvolume spectrophotometer (NanoDrop™, Thermo Scientific) and their DNA purity was assessed by the ratio of absorbance at 260 and 280 nm. Subsequently, DNA concentration results were converted into molecular concentration, i.e. number of DNA molecules per µL of the sample, using Eq.5.1 which was described by Karunakaran et al., (2016).

$$N = \frac{6.022 \times 10^{23} \times C}{660 \times L} \qquad \text{Eq.5.1}$$

N : Number of DNA molecules per µL of sample; C: DNA concentration (g/µL); L : Genome length of the known bacterium (bp).

Using nuclease-free water, the DNA aliquots were adjusted to equal molecule concentration of an initial 10^7 molecules/µL. These samples were then subjected to serial dilution (10^{-1} to

10^{-5}) to produce a concentration gradient, which served as the qPCR standards for quantifying the bacterial population. Instead of water, multiplex DNA matrices were used as the dilution buffer for this step. These matrices were prepared by mixing equal volumes of DNA from non-target bacteria and were adjusted to a final DNA concentration of 50 ng/µL. The purpose of this was to generate serially diluted standards containing 10^6 to 10^2 target DNA molecules, which are always in the presence of 50 ng/µL of background non-target DNA to simulate the presence of multiple genomic DNA from different bacteria in real consortia samples. It is also important to note that the dilution matrices excluded DNA from bacteria of the same genus as the target bacteria, which ensures only one type of target DNA exists in each standard thus eliminating the potential interference from the DNA of closely related bacteria. In addition, the 10^{-2} diluted DNA samples were also used in subsequent primer specificity assays.

5.2.3 Primer design

Primers were designed using the NCBI Primer designing tool. To enhance the specificity of primers, 16s rDNA sequences of all bacteria in this study were uploaded into a custom database. Notably for the four *Rhodococcus* (non-degrader *7*, degrader *B*, *E* and *F*), additional primer was designed based on catechol 1,2-dioxygenase gene (CatA) gene due to the possible inefficiency of 16s rDNA for distinguishing bacteria in this genus (Zörn et al., 2022) and the prevalent application of assay based on catA gene in differentiating species within this genus (Táncsics et al., 2008; Iminova et al., 2022). The catA gene sequence of degrader *B*: *Rhodococcus erythropolis* (GenBank ID: AJ605581.1) as well as two other species *Rhodococcus rhodochrous* (GeneBank ID: AF043741.1) and *Rhodococcus opacus* (GenBank ID: X99622.2) (Strachan et al., 1998; Cai et al., 2020) were used for CatA primers designing.

For the primer parameters, the melting temperature (Tm) of primers was set between 55℃ and 65℃ with a maximum allowed Tm difference of 3℃. The PCR product size was within the 150 to 500 bp range. The primer specificity stringency setting was used as many total mismatches to unintended range as possible, including 1 mismatched within the last 1 bps at the 3’ end. Due to the phylogenetic closeness and high similarity of 16S rDNA sequences, it might be challenging to design primers specific to bacteria from the same genus. As a result,

these bacteria shared the same primer sets. The finalised primer sequences were ordered from Integrated DNA Technologies Inc. (Coralville, IA, USA.).

5.2.4 Primer validation and specificity test

The designed primers were first validated with single-template assay wherein these primers were used for basic PCR involving genomic DNA from target bacteria and non-target bacteria respectively. This assay was conducted using PCRBIO® Taq Mix Red kit in a thermocycler (Applied Biosystems™) following the protocol in the instruction manual and the annealing temperatures as determined from the T_m of the primers being used. These assays were performed in replicates and the PCR products were checked by gel electrophoresis on 1% ultrapure agarose gel (Invitrogen™) prepared using TAE buffer stained by ethidium bromide (1%). The presence of distinct bands with the expected size in the target sample and the absence of specific bands in the non-target sample was viewed as a sign of ideal primers.

Validated primer sets were tested for specificity via multiplexed PCR assays wherein mixtures of genomic DNA from different non-target bacteria were used as background DNA. The extension temperature of this PCR assay was taken as the average T_m of the forward and reverse primer set being tested. The PCR products were checked by gel electrophoresis and the specific primer sets were determined by the simultaneous satisfaction of the following criteria 1) yielding negative PCR results on the background DNA without the presence of target DNA; 2) successful amplification of the target DNA amidst the background DNA. The detailed configuration of the DNA template matrix for this test is listed in Table 5.1.

Table 5.1 Template DNA matrices for primer specificity test

Target bacteria	**Target-positive template DNA mix**							**Target-negative template DNA mix**						
(label and name)	**TP1^{+}**	**TP2^{+}**	**TP3^{+}**	**TP4^{+}**	**TP5^{+}**	**TP6^{+}**	**TP7^{+}**	**TP1^{-}**	**TP2^{-}**	**TP3^{-}**	**TP4^{-}**	**TP5^{-}**	**TP6^{-}**	**TP7^{-}**
A: *Pseudomonas fluorescens*	×	×	×	×	×	×	×	Null	×	×	×	×	×	×
B: *Rhodococcus erythropolis*	×	×	×	×	×	×	×	×	×	×	×	×	Null	×
C: *Pseudomonas sp.*	×	×	×	×	×	×	×	Null	×	×	×	×	×	×
D: *Delftia sp.*	×	×	×	×	×	×	×	×	×	×	×	×	×	Null
E: *Rhodococcus sp$_1$*	×	×	×	×	×	×	×	×	×	×	×	×	Null	×
F: *Rhodococcus sp$_2$*	×	×	×	×	×	×	×	×	×	×	×	×	Null	×
1: *Pseudomonas syringae*	×	×	×	×	×	×	×	Null	×	×	×	×	×	×
2: *Agromyces atrinae*	×	×	×	×	×	×	×	×	Null	×	×	×	×	×
3: *Cupriavidus metallidurans*	×	×	×	×	×	×	×	×	×	Null	×	×	×	×
4: *Ochrobactrum anthropi*	×	×	×	×	×	×	×	×	×	×	Null	×	×	×
5: *Plantibacter flavus*	×	×	×	×	×	×	×	×	×	×	×	Null	×	×
6: *Plantibacter sp.*	×	×	×	×	×	×	×	×	×	×	×	Null	×	×
7: *Rhodococcus sp$_3$.*	×	×	×	×	×	×	×	×	×	×	×	×	Null	×
Algae: *Coelastrella terrestris*	×	×	×	×	×	×	×	×	×	×	×	×	×	×

Note: The template DNA mix was prepared in target-positive (+) and target-negative (-) groups which are featured by the presence (×) and the absence (Null) of target DNA (marked in green background). The template DNA matrices were prepared by directly mixing 10 times diluted pre-extracted DNA samples. The DNA concentration in each matrix was kept at 10^6 molecules per µl.

5.2.5 Annealing temperature optimisation

After validation and specificity testing, the selected primers were further optimised for their annealing /extension temperatures to eliminate any potential non-specific amplification in the qPCR. This was achieved by interchangeably applying the following two steps 1): increasing the annealing temperature by 2℃ if non-specific DNA amplification occurred; and 2) decreasing the annealing temperature by 1 ℃ if the PCR product disintegrates. After each temperature adjustment, subsequent gel electrophoresis checks were conducted until the optimal annealing temperature was determined.

5.2.6 qPCR programs

All qPCR reactions were run in 96-well PCR plates (Sarstedt) using qPCRBIO SyGreen Mix Lo-ROX kit (PCR Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems®, Thermo Scientific) with optimal specificity primer pairs. To increase the limit of detection, the PCR program for all primer pairs was set to 80 cycles with an initial denaturing at 95°C for 30 seconds, 5 seconds of denaturing at 95°C followed by Primer annealing and extension at optimal annealing temperatures for 30 seconds per cycle, unless specified otherwise. The reaction volumes were all set as 10 µL.

5.2.7 Statistics and data processing

Based on the qPCR result, the population of each bacteria in the consortia was calculated using Eq.5.2

$$\boldsymbol{N = \frac{1}{GCN} \times 10^{\frac{C_t - b}{a}} \times \frac{V_{ext}}{V_{pcr} \times V_s}} \qquad \text{Eq.5.2}$$

Where $\boldsymbol{N}$ is the population size of target bacteria in the consortia per millilitre of culture. $\boldsymbol{a}$ and $\boldsymbol{b}$ are constants determined using the qPCR standard of each target bacteria. $\boldsymbol{V_{ext}}$, $\boldsymbol{V_{pcr}}$

and $\boldsymbol{V_s}$ are volumes of template DNA in the consortia extract, PCR reactions, and culture sample of consortia respectively. Given that the 16S rRNA gene copy number (16S GCN) in different bacteria species might vary largely which may introduce quantification bias (Louca et al., 2018; Gao and Wu, 2023), the qPCR standard was corrected for Ribosomal RNA operon copy numbers in each genus or species (Table 5.2).

Table 5.2 Bacteria GCN for qPCR standards correction

Bacteria label	**Identities**	**GCN**	**Gene**	**Data type**
A	*Pseudomonas fluorescens*	5.3	16s	Median
B	*Rhodococcus erythropolis*	1	CatA	Estimation
C	*Pseudomonas sp.*	4.8	16s	Mean
D	*Delftia sp.*	5	16s	Absolute
E	*Rhodococcus sp$_1$.*	1	CatA	Estimation
F	*Rhodococcus sp$_2$.*	1	CatA	Estimation
1	*Pseudomonas syringae*	5	16s	Absolute
2	*Agromyces atrinae*	2.3	16s	Mean
3	*Cupriavidus metallidurans*	4	16s	Absolute
4	*Ochrobactrum anthropi*	4.8	16s	Mean
5	*Plantibacter flavus*	2	16s	Absolute
6	*Plantibacter sp.*	2	16s	Absolute
7	*Rhodococcus sp$_3$.*	1	CatA	Estimation
Algae	*Coelastrella terrestris*	N/A	N/A	N/A

Note: 16s GCN data were obtained from the *rrnDB* database developed by Stoddard et al., (2015). 'Absolute' refers to GCN data that are directly available in the database. 'Mean' denotes bacteria without GCN data at the species level and average GCN values from the same-genus bacteria were used as substitutes. For all the *Rhodococcus*, the GCNs for the CatA gene were estimated to be 1 according to Táncsics et al., (2008).

5.3 Results

5.3.1 Primer validation and specificity

A total of 46 primers were obtained, and 39 combinations of forward-reverse primer sets were tested, with some forward primers sharing the same reverse primers (primer sequences detailed in Table S4, Supplementary Material 1). The validation and test filtered out 17 primer sets, identifying 7 optimal primer sets which exhibited the ability to successfully amplify target DNA (image result not shown). The details of these primers are listed in Table 5.3.

Table 5.3 Primer selected for qPCR

Name	Sequence(5'-3')	Target gene	T_m	Amp. length
1F	CTTCGGGCCTTGCGCTATCA	16s	60.2°C	449 bp
1R	CTCTAGCTTGCCAGTTTTGG	16s	53.5°C	
2F	TGAAGGAGAGCTTGCTCTTT	16s	53.9°C	415 bp
2R	AGCCGGTGCTTTTTCTGCAA	16s	58.3°C	
3F	AGTAGCTGGTCTGAGAGGAC	16s	55.4°C	193 bp
3R	CCACGCCAGGTATTAACCAG	16s	55.6°C	
4F	CGGGGGAAAGATTTATCGGC	16s	56°C	364 bp
4R	AAATCCGAACAACGCTAGCC	16s	55.7°C	
5F	GAGCTTCCACCGCATGGTGA	16s	59.9°C	480 bp
5R	CCGCTACACCAGGAATTCCAA	16s	57.3°C	
BF	GTACGGCACCCACCCGGTAA	CatA	62°C	400 bp
BR	ACTTGGCAGGCAACGTCTTG	CatA	58.5°C	
DR	GGCCTTCGGGTTGTAAACTG	16s	56°C	338 bp
DF	GTCAGTACAGGTCCAGGGGA	16s	58.2°C	

The multiplexed assay on these primers showed that all 7 primer sets were able to successfully amplify target DNA with high specificity using the average T_m of forward and reverse primers as the extension temperature, to yield a distinct single DNA band on the gel

(Fig.5.1 a). However, at this temperature setting, primer sets 1F-1R, 4F-4R, BF-BR and DF-DR exhibited non-specific amplification of non-target background DNA (Fig.5.1 b).

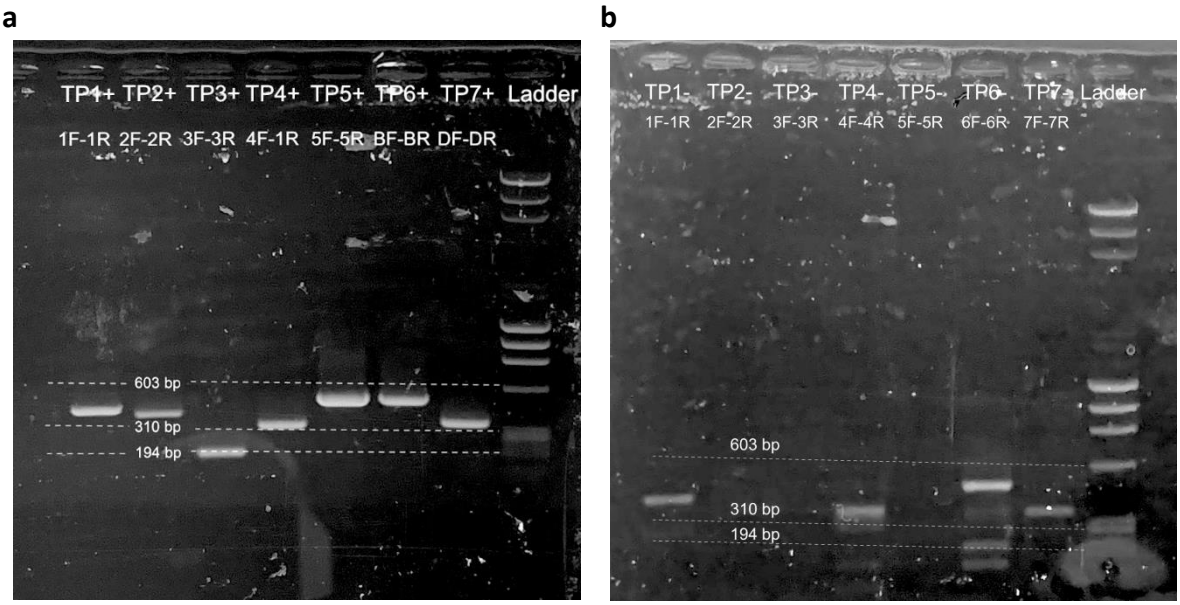
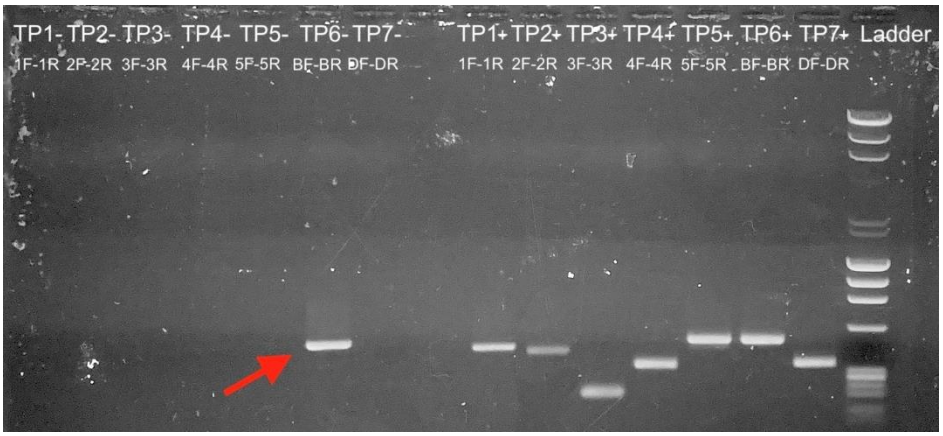


Fig.5.1 Gel electrophoresis results of primer specificity tests. a) Specific amplification of test primer sets when target-positive template matrixes were used. b) Non-specific amplification in target-negative template matrixes, lanes 1, 4, 6 and 7. Ladder used: Quick-Load DNA Marker, Broad Range (New England Biolabs®).

5.3.2 Primer annealing temperature optimisation.

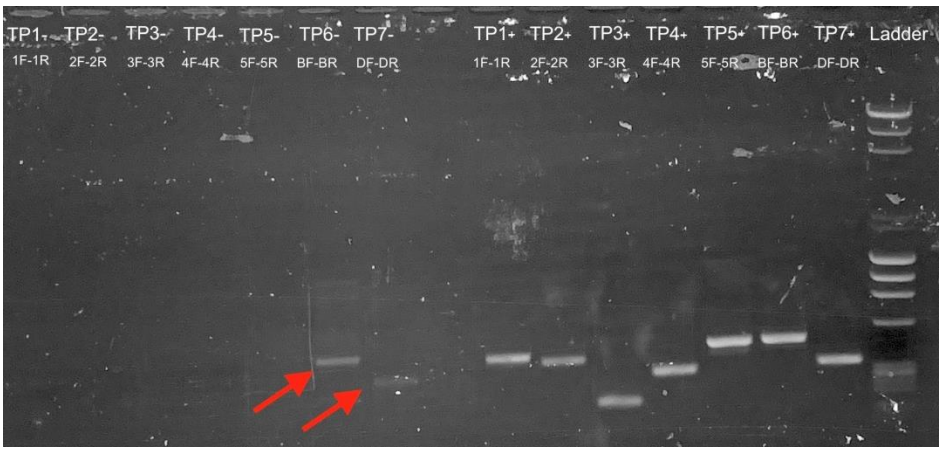
Upon increasing the annealing temperature of all primer sets to 63°C, most of the previously observed non-specific bands were effectively eliminated (Fig.5.2). Notably, two persistent non-specific bands were still observed in primer sets BF-BR and DF-DR (Fig.5.2 a,b). However, as the temperature increased, these bands progressively reduced in intensity till they were finally hardly visible as the temperature was set to 72°C (Fig.5.2 d). When the annealing temperature was set to 75°C, all DNA bands in both target-positive and target-negative samples disappeared, suggesting PCR termination at this temperature. While changes of temperature between 63°C to 72°C showed no significant effect on the PCR efficacy of tested primers as a whole (since single DNA bands remained distinctly visible across the temperature changes), inhibited amplification was observed for primer set 2F-2R at temperatures of 69°C and above. Therefore, the optimal annealing temperatures were determined as 68°C for 2F-2R and 72°C for remaining primers sets.

a



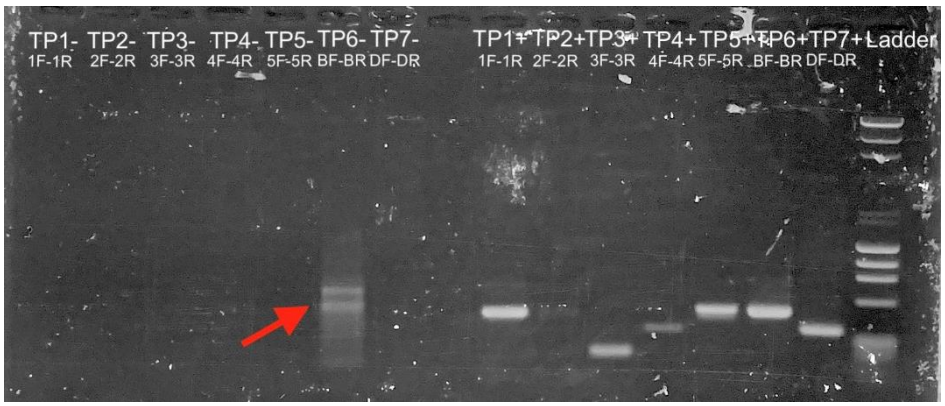
63°C

b



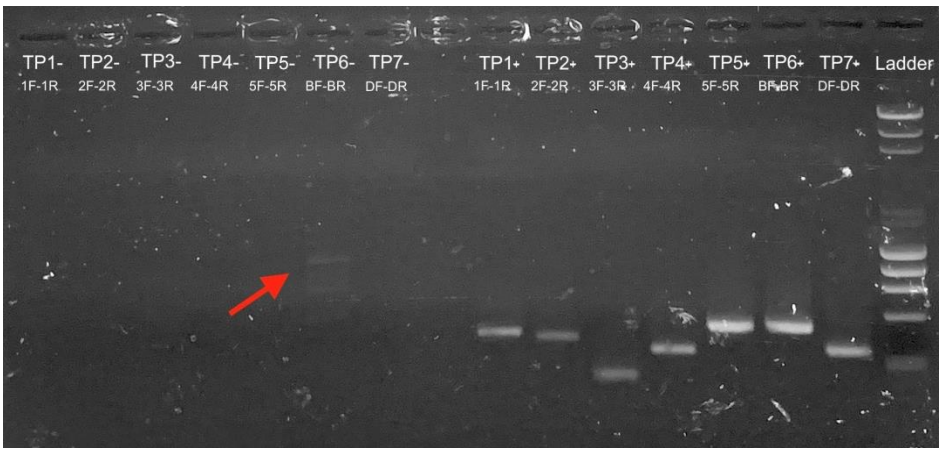
66°C

c



69°C

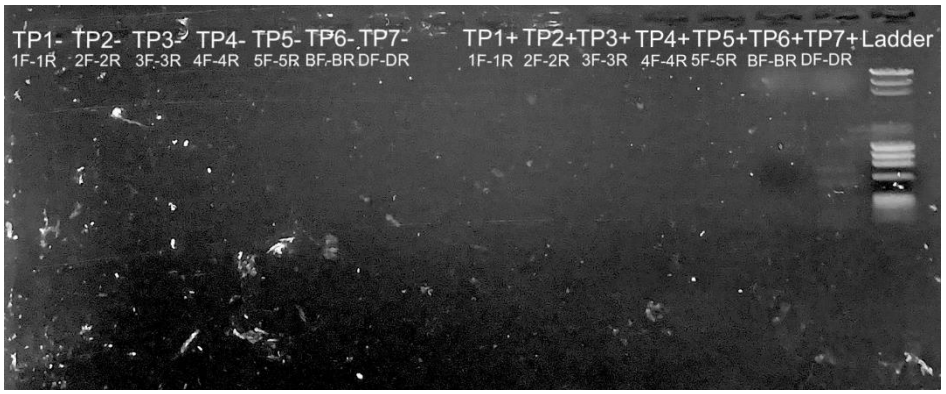
d



72°C

Fig.5.2 (continued) Gel electrophoresis results after each temperature adjustment.

e



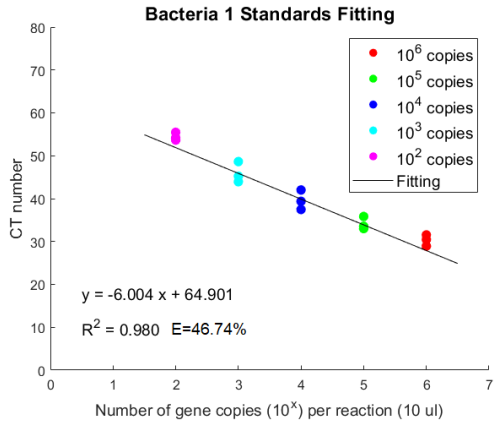
75°C

Fig.5.2 Gel electrophoresis results after each temperature adjustment.

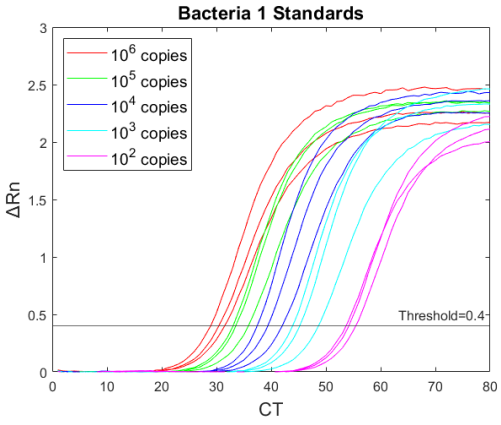
5.3.3 qPCR analysis

Using the established standards, the result of qPCR analysis on bacteria DNA quantities in the consortia are presented in Fig.5.3-Fig.5.9. Based on the standard curves, all 7 designed primer sets showed successful amplification of the target DNA despite the presence of non-target DNA. The melting curves indicated that these amplifications were specific, with the exception of non-degrader *4* in consortia B14, B123456, E123456, B124567, and E124567, where the population of bacteria *4* appeared to be extremely low (data not shown). However, large variations of the PCR efficiency were observed in different targets, ranging from as low as 46.75 % for bacteria 1 (Fig.5.3) to as high as 91.18% for bacteria B (Fig.5.8). Variation was also found in amplification curves for the PCR replicates in standards especially those which are highly diluted (less than 10^3 DNA molecules per PCR reaction). Notably, different PCR efficiencies were observed in target DNA from bacteria B (91.18%) and bacteria E (62.19%) even when the same primer set BF-BR was used. Overall, the established standards showed robust fitting with R^2 values close to 1. Amplification results for specific consortia revealed variations both among the three qPCR replicates and the two biological replicates. These variations were not consistently associated with any particular sample as some samples displayed similar amplification curves for one bacterium but showed marked differences for another, especially for those with large CT values (≥40).

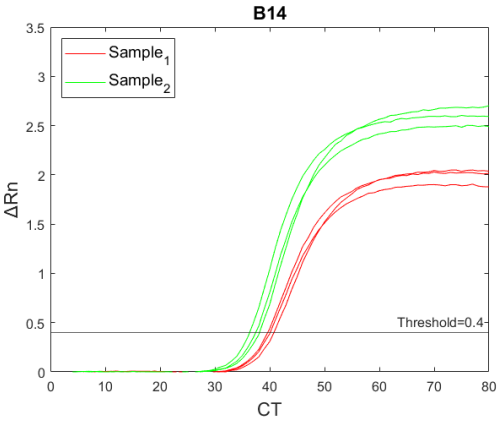
1)



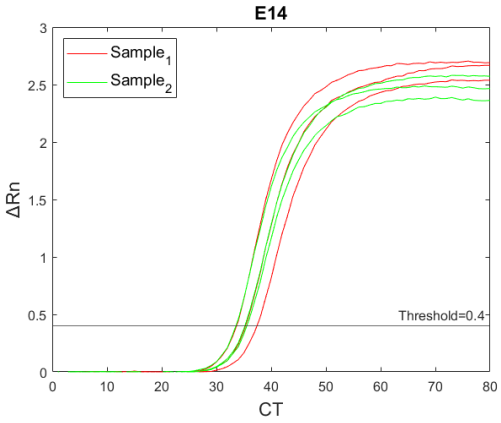
2)



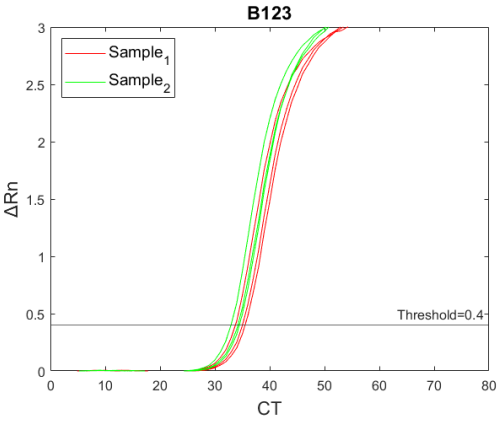
3)



4)



5)



6)

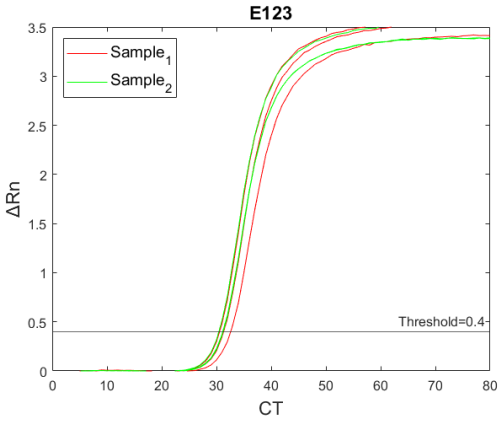
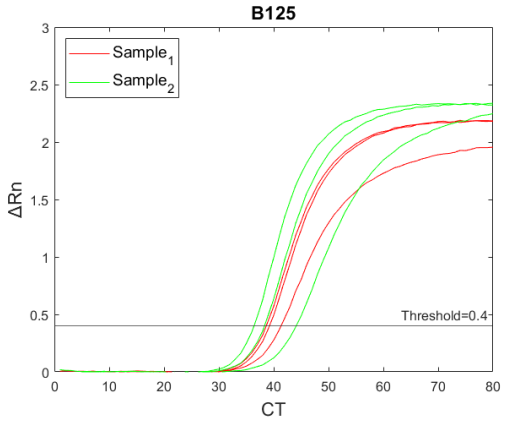
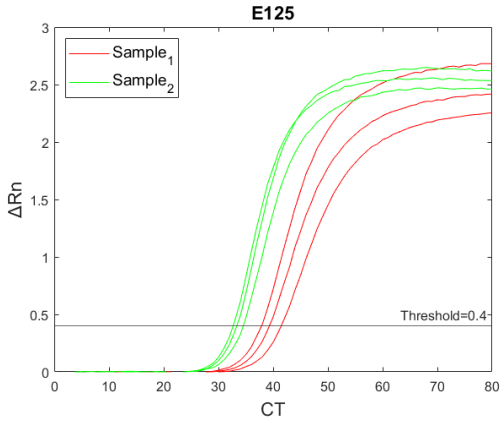


Fig.5.3 (Continued) qPCR results of bacteria 1 (*Pseudomonas syringae*) in exemplar consortia

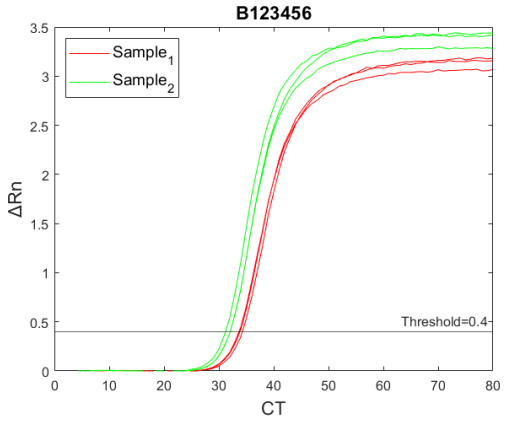
7)



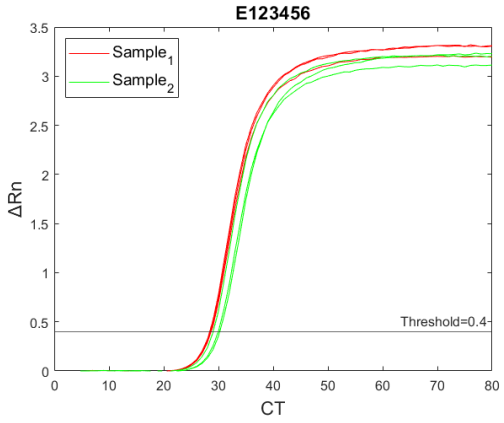
8)



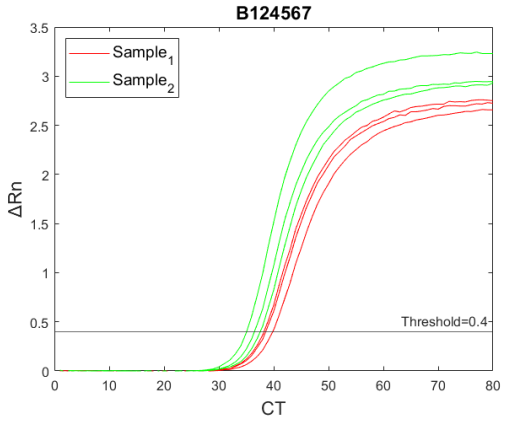
9)



10)



11)



12)

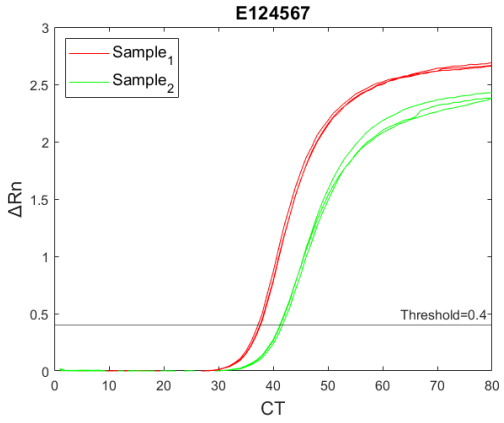
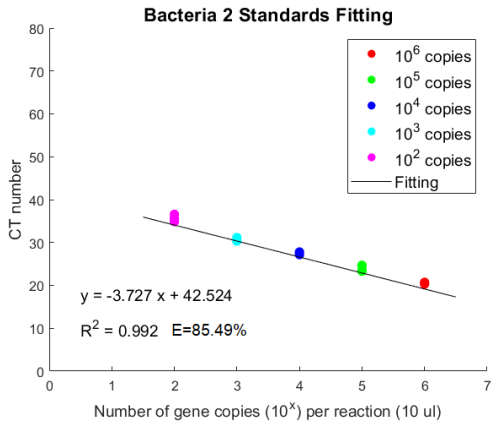
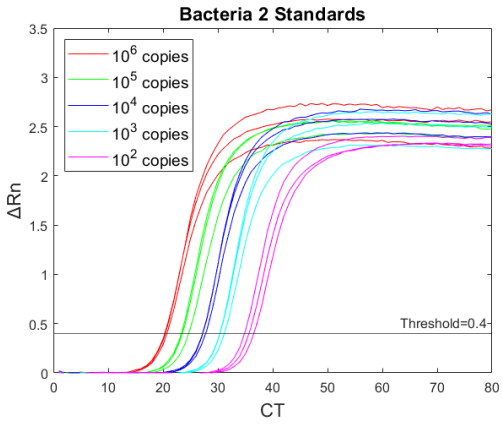


Fig.5.3 qPCR results of bacteria 1 (*Pseudomonas syringae*) in exemplar consortia

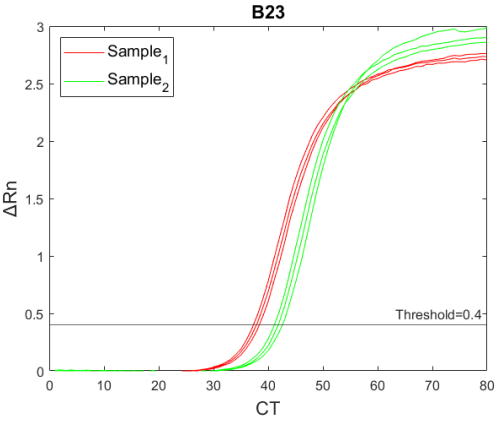
1)



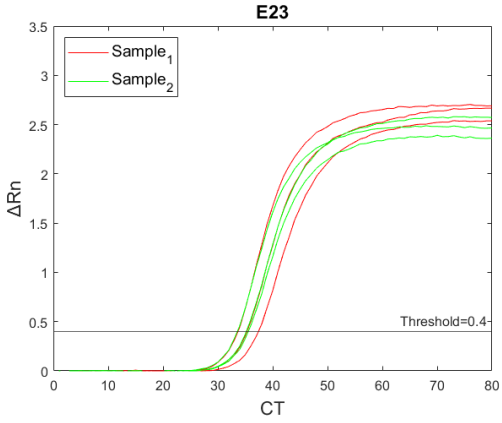
2)



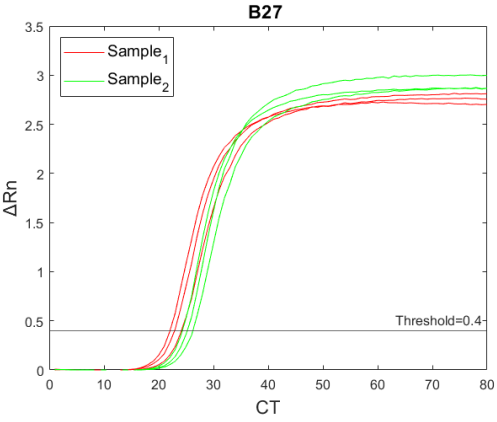
3)



4)



5)



6)

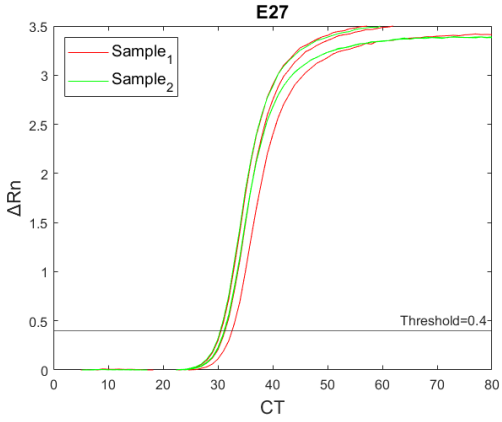
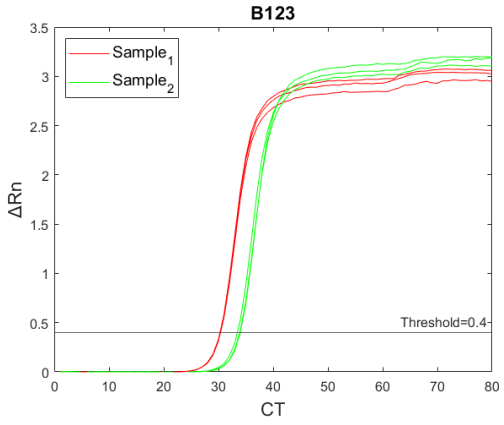
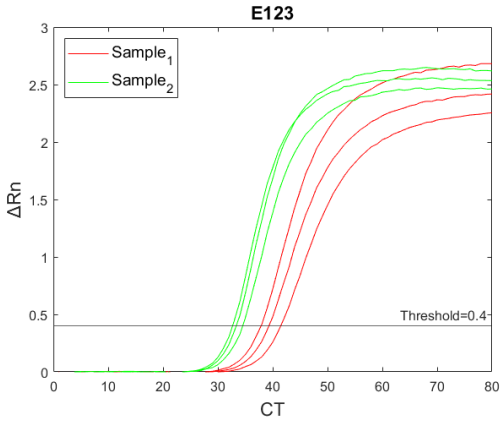


Fig.5.4 (Continued) qPCR results of bacteria 2 (*Agromyces atrinae*) in exemplar consortia

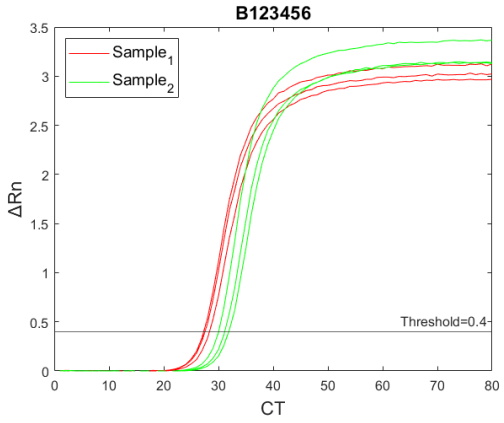
7)



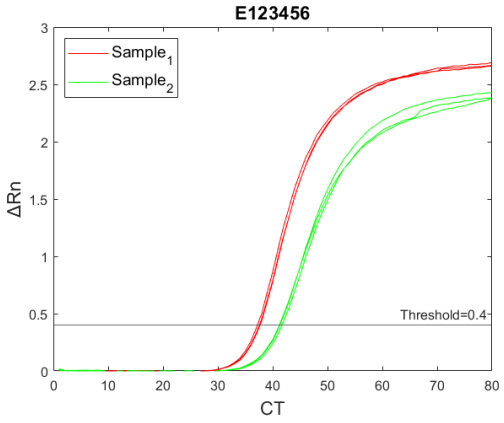
8)



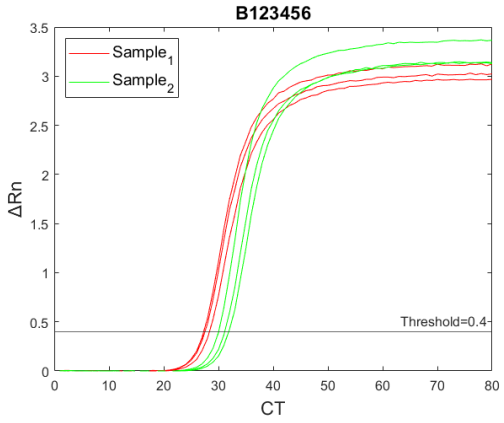
9)



10)



11)



12)

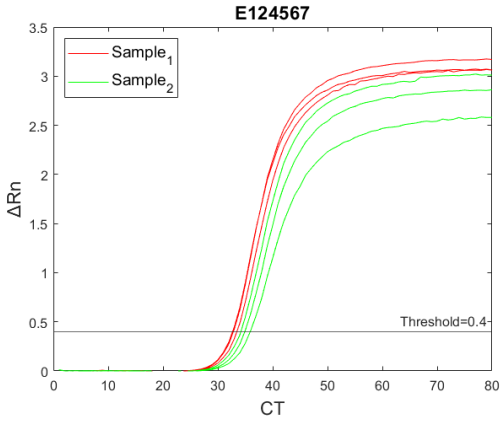


Fig.5.4 qPCR results of bacteria 2 (*Agromyces atrinae*) in exemplar consortia

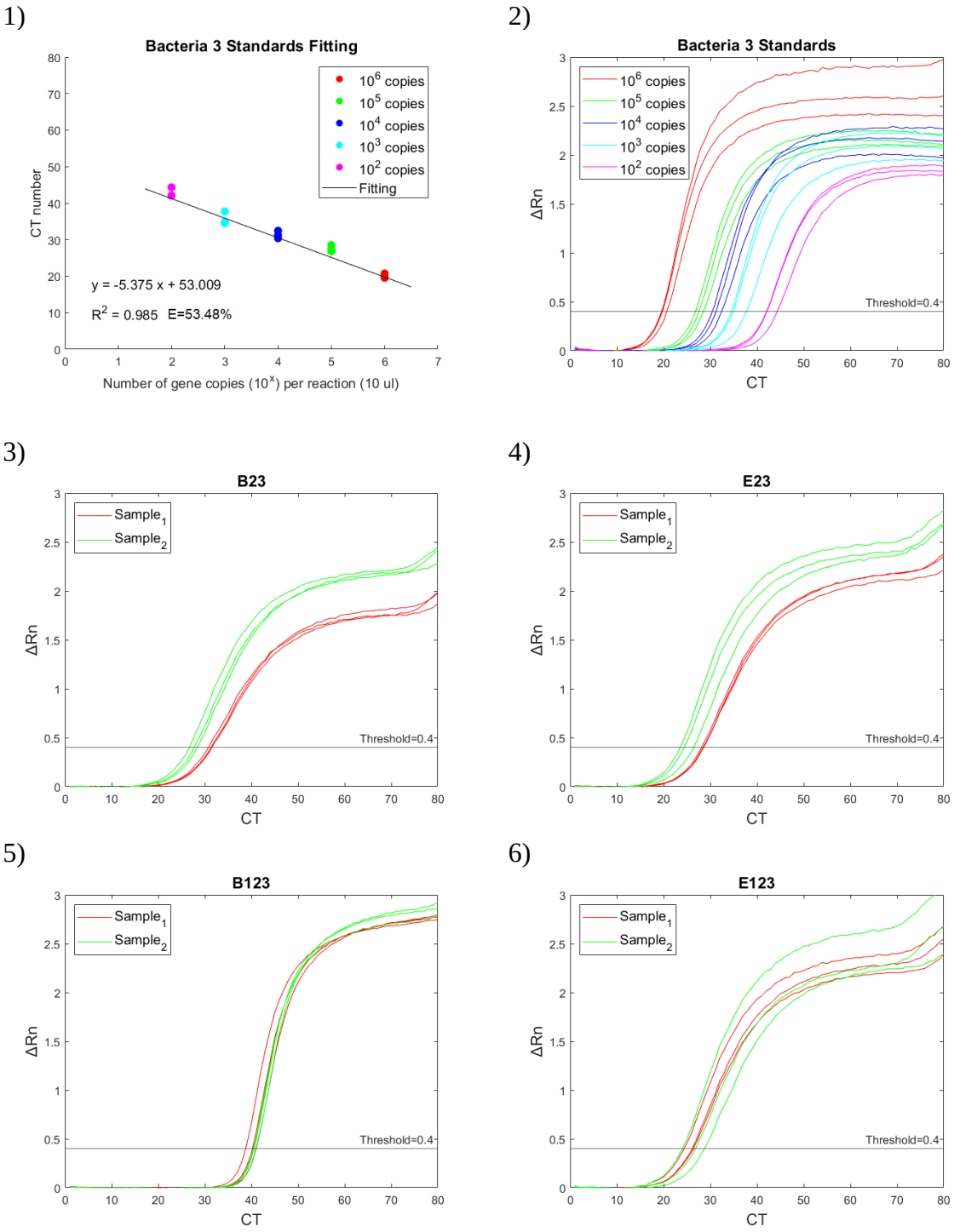
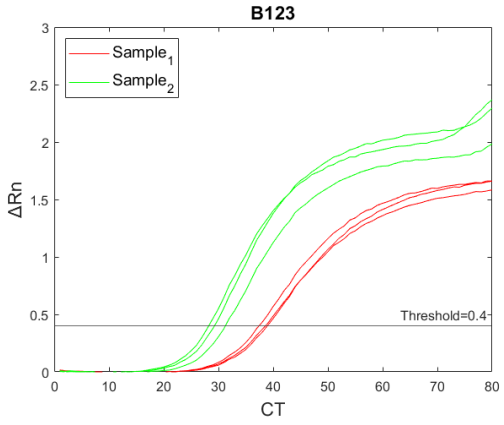


Fig.5.5 (Continued) qPCR results of bacteria 3 (*Cupriavidus metallidurans*) in exemplar consortia

7)



8)

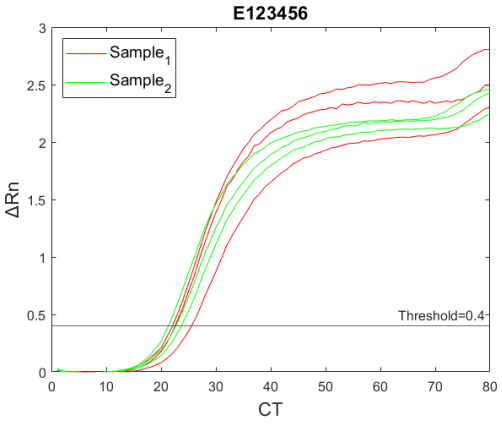
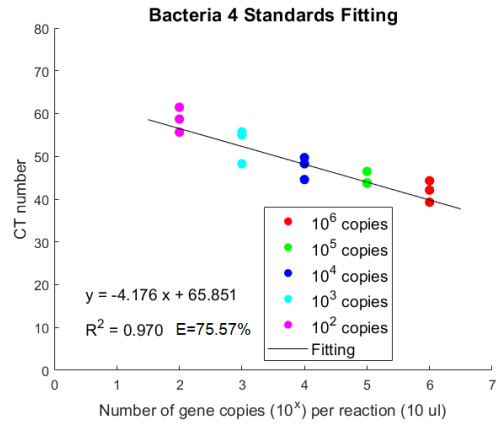
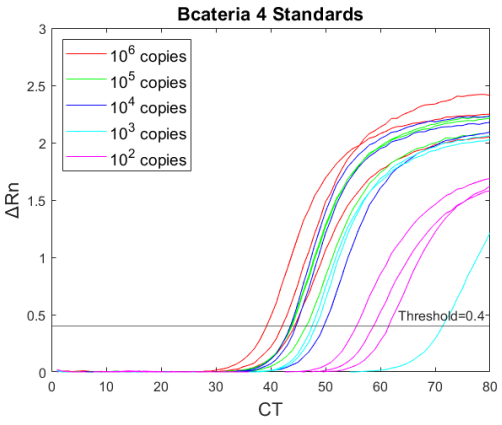


Fig.5.5 qPCR results of bacteria 3 (*Cupriavidus metallidurans*) in exemplar consortia

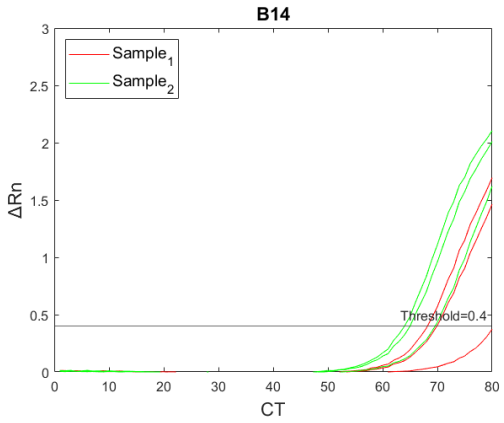
1)



2)



3)



4)

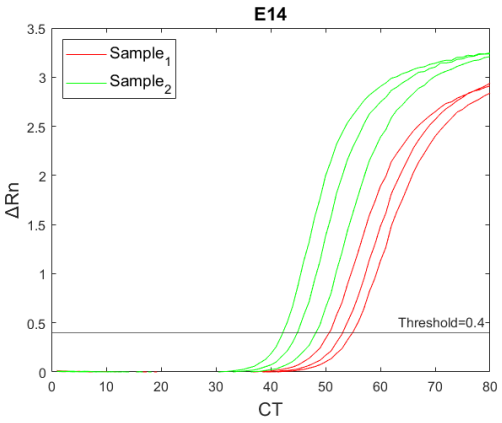


Fig.5.6 (Continued) qPCR results of bacteria 4 (*Ochrobactrum anthropi*) in exemplar consortia

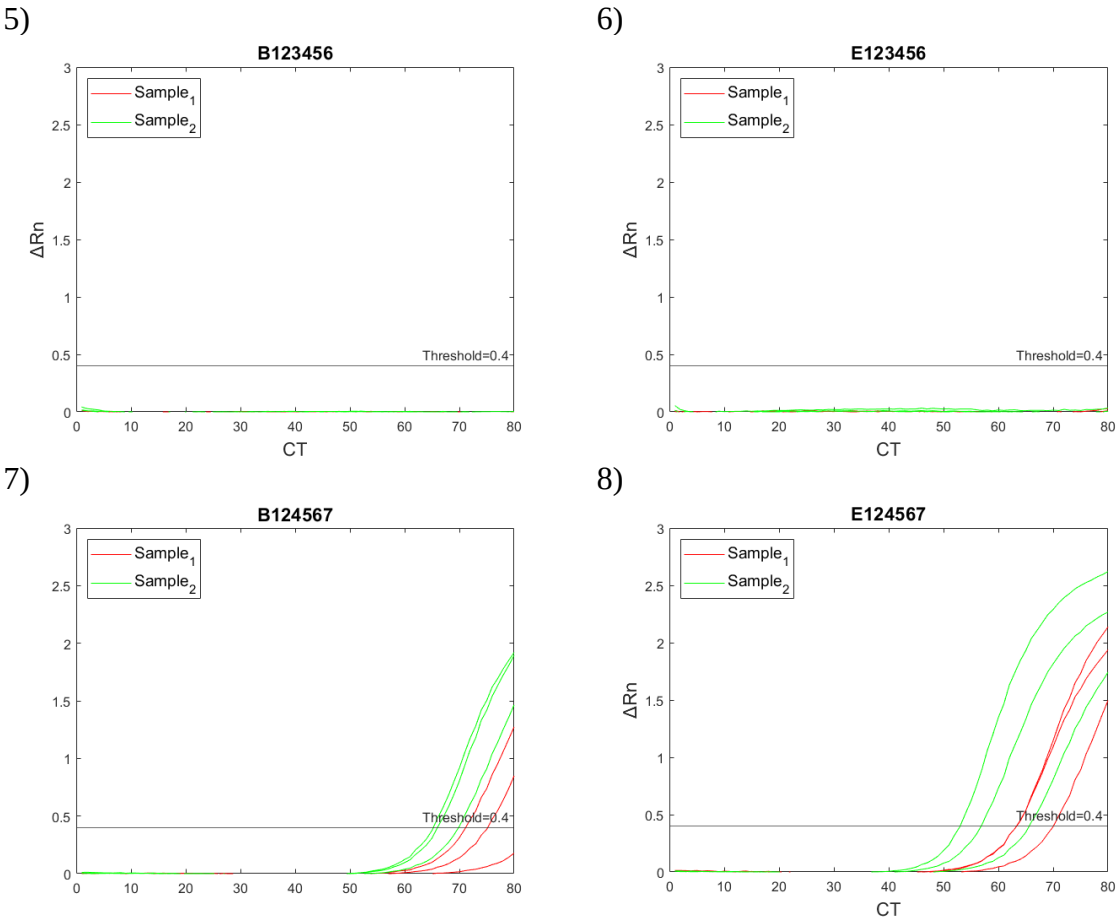


Fig.5.6 qPCR results of bacteria 4 (*Ochrobactrum anthropi*) in exemplar consortia

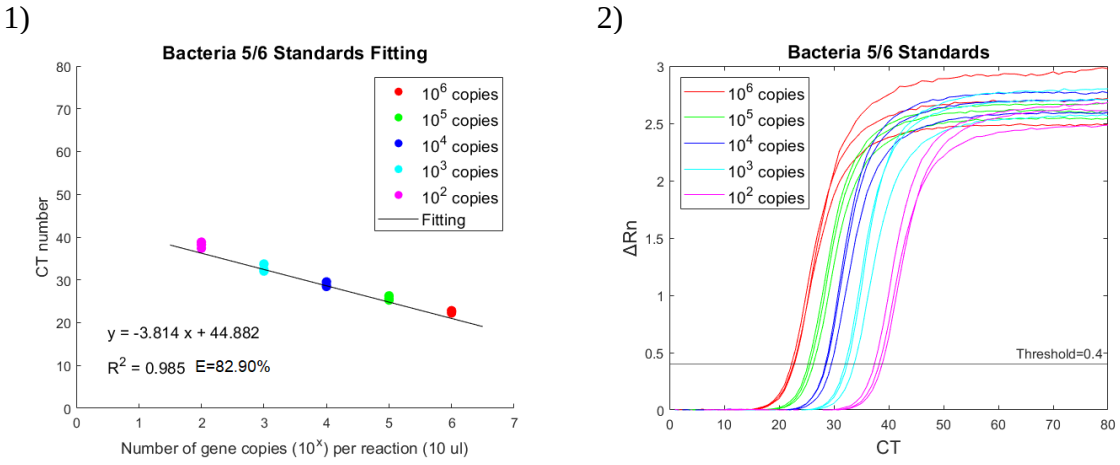


Fig.5.7 (Continued) qPCR results of bacteria 5/6 (*Plantibacter flavus /Plantibacter sp.*) in exemplar consortia

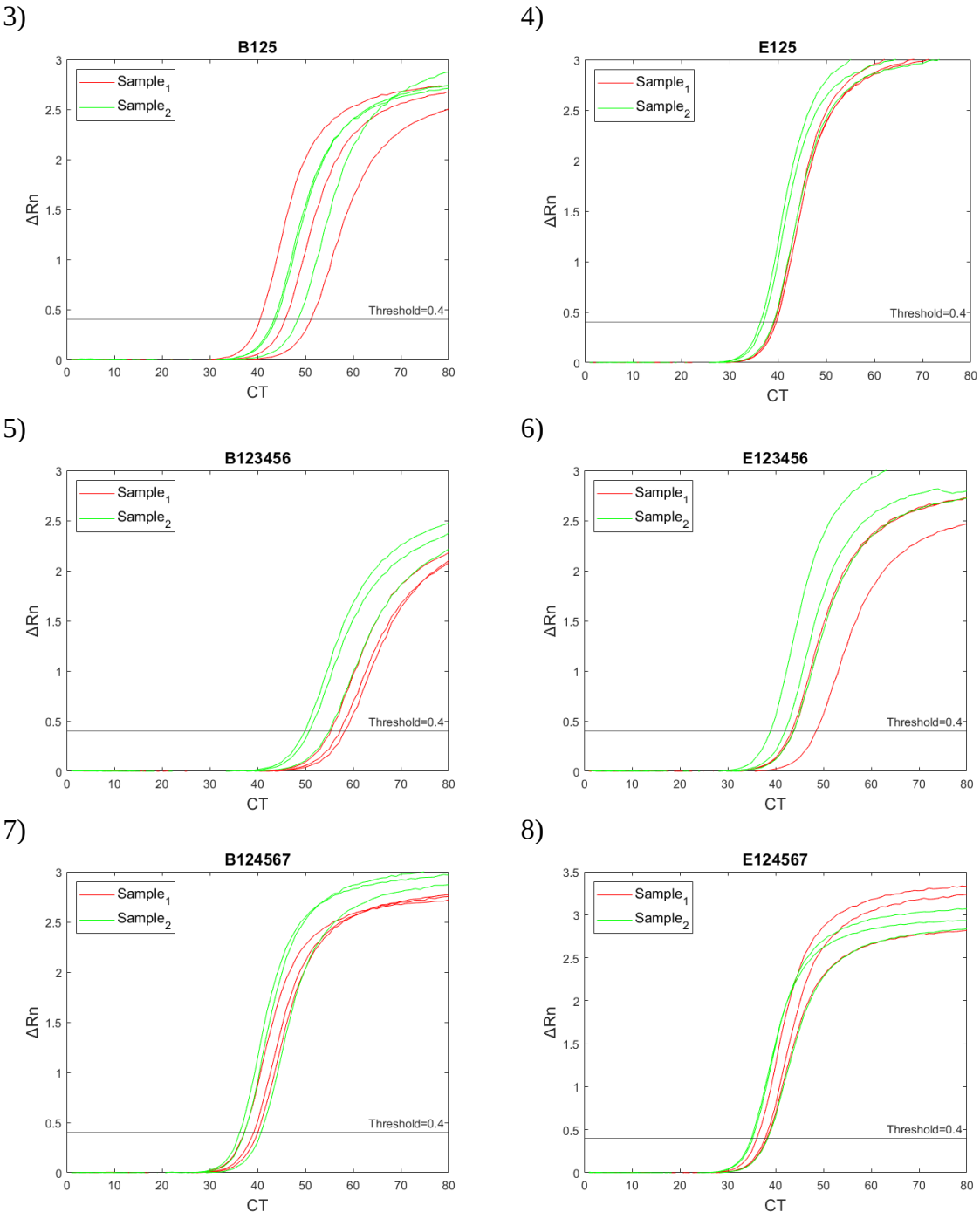
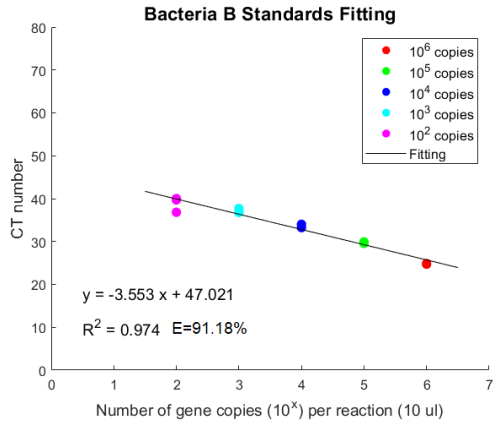
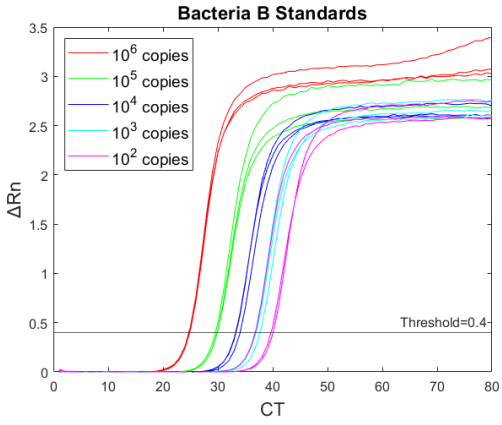


Fig.5.7 qPCR results of bacteria 5/6 (*Plantibacter flavus /Plantibacter sp.*) in exemplar consortia

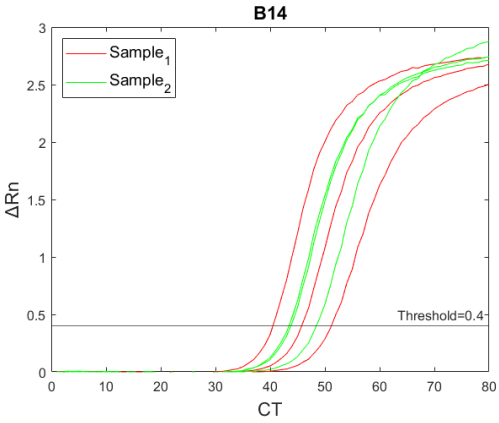
1)



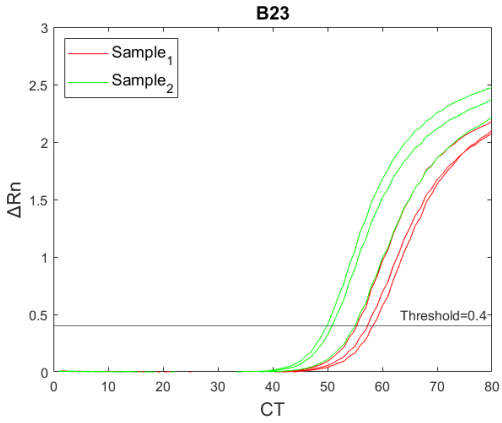
2)



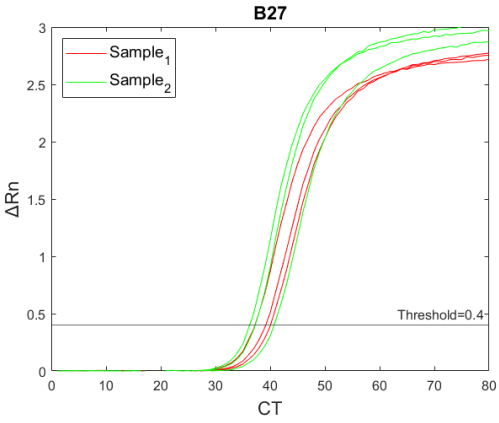
3)



4)



5)



6)

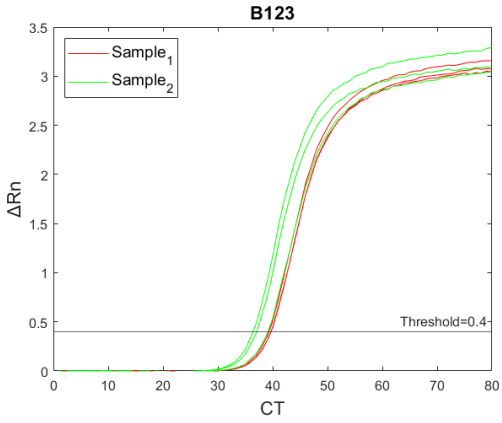
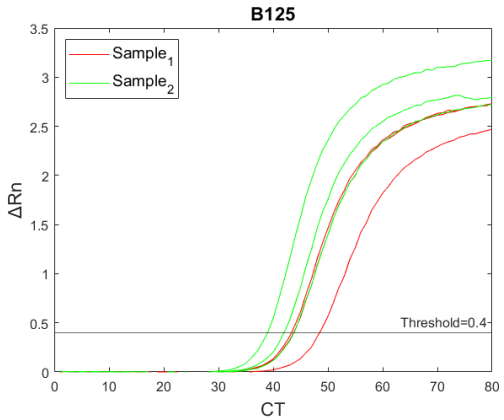
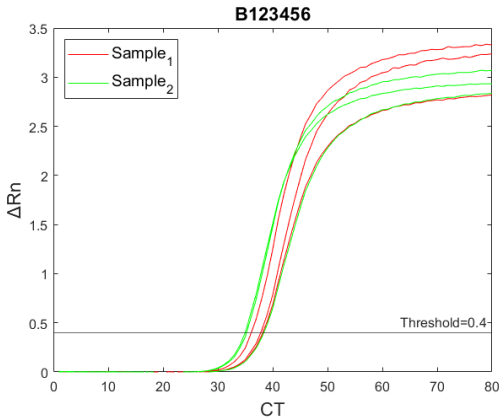


Fig.5.8 (Continued) qPCR results of bacteria B/7 (*Rhodococcus erythropolis/ Rhodococcus sp3*) in exemplar consortia

7)



8)



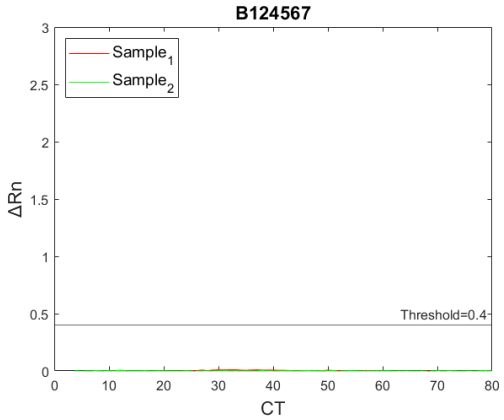


Fig.5.8 qPCR results of bacteria B/7 (*Rhodococcus erythropolis/ Rhodococcus sp$_3$*) in exemplar consortia.

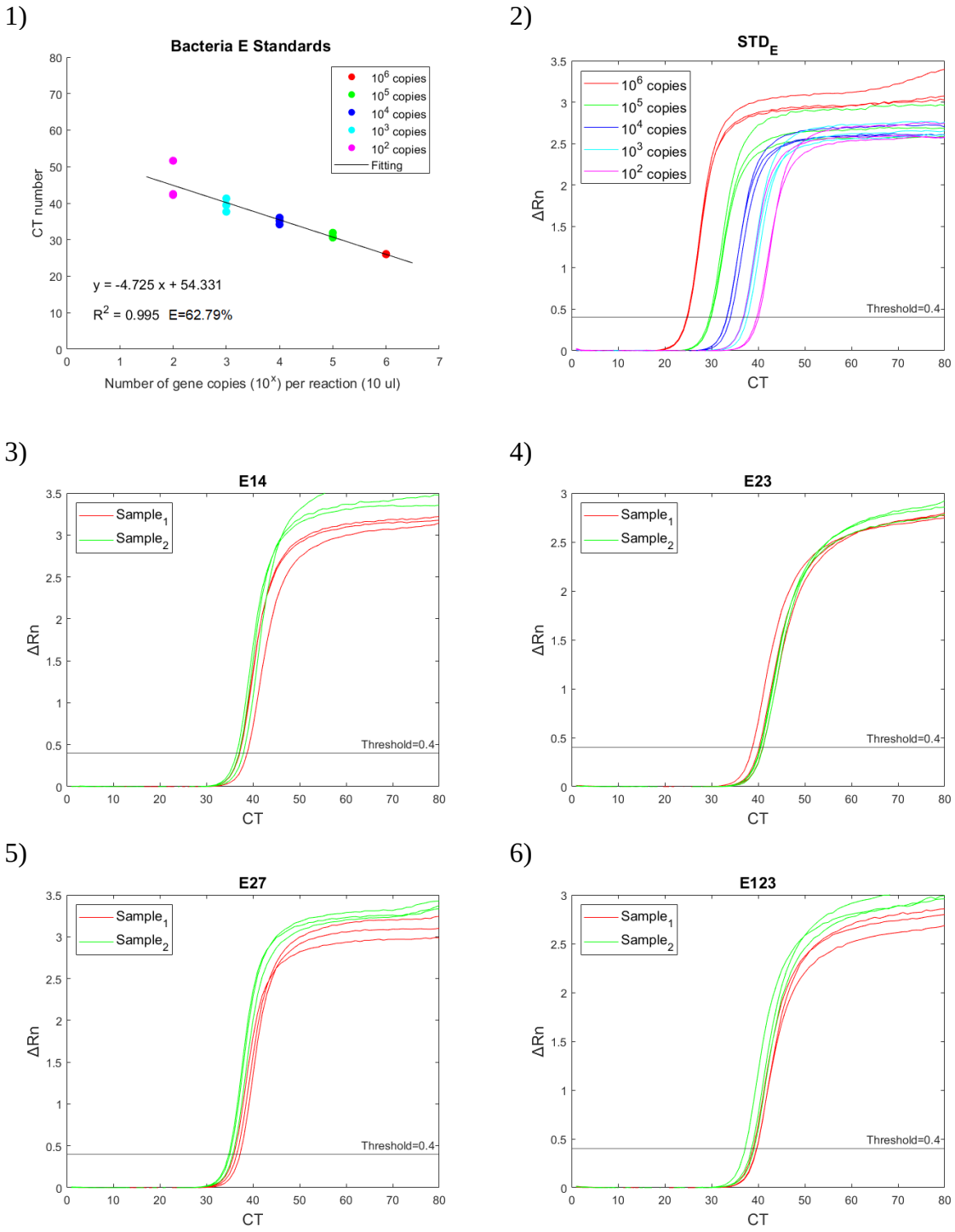
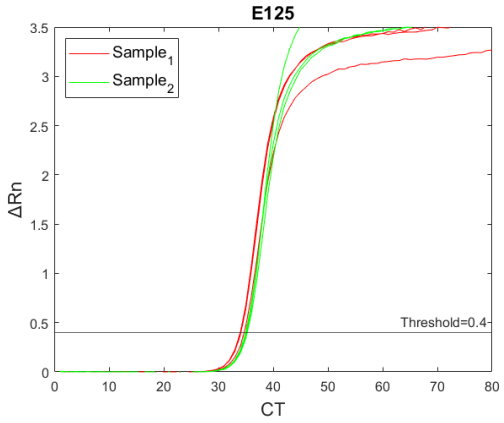
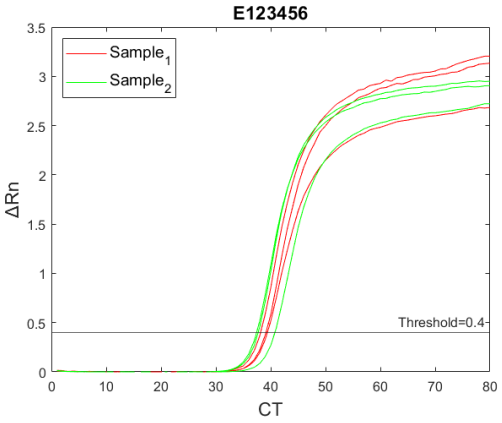


Fig.5.9 (Continued) qPCR results of bacteria E/7 (*Rhodococcus sp₁/ Rhodococcus sp₃*) in exemplar consortia

7)



8)



9)

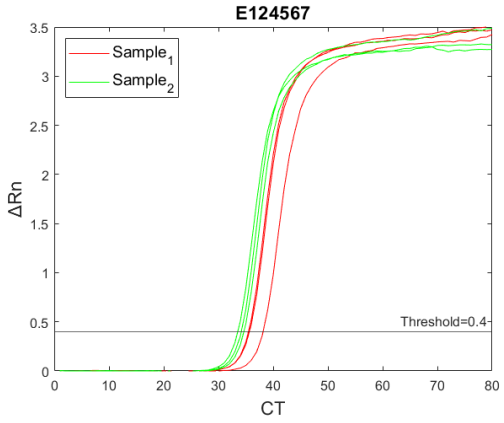


Fig.5.9 qPCR results of bacteria E/7 (*Rhodococcus sp$_1$/ Rhodococcus sp$_3$*) in exemplar consortia

5.3.4 Consortia structure analysis

Based on the qPCR results, the GCN-corrected estimation of the bacterial population in each consortium is presented in Fig.5.10. The bacterial population size in different consortia on day 7 exhibited great variety, ranging from as low as 208 cells per mL in B14, to approximately 7090 cells per mL in B124567, regardless of the number of bacteria members initially inoculated into the consortia. Despite having positive amplification of their DNA in qPCR, certain bacteria members were almost not detected by qPCR such as the two *Plantibacter* (non-degrader *5* and *6*) with population size < 10 cells /mL. Compared to degrader *B*, the presence of degrader *E* generally resulted in a larger overall bacterial population in the consortia with the exception of B124567.

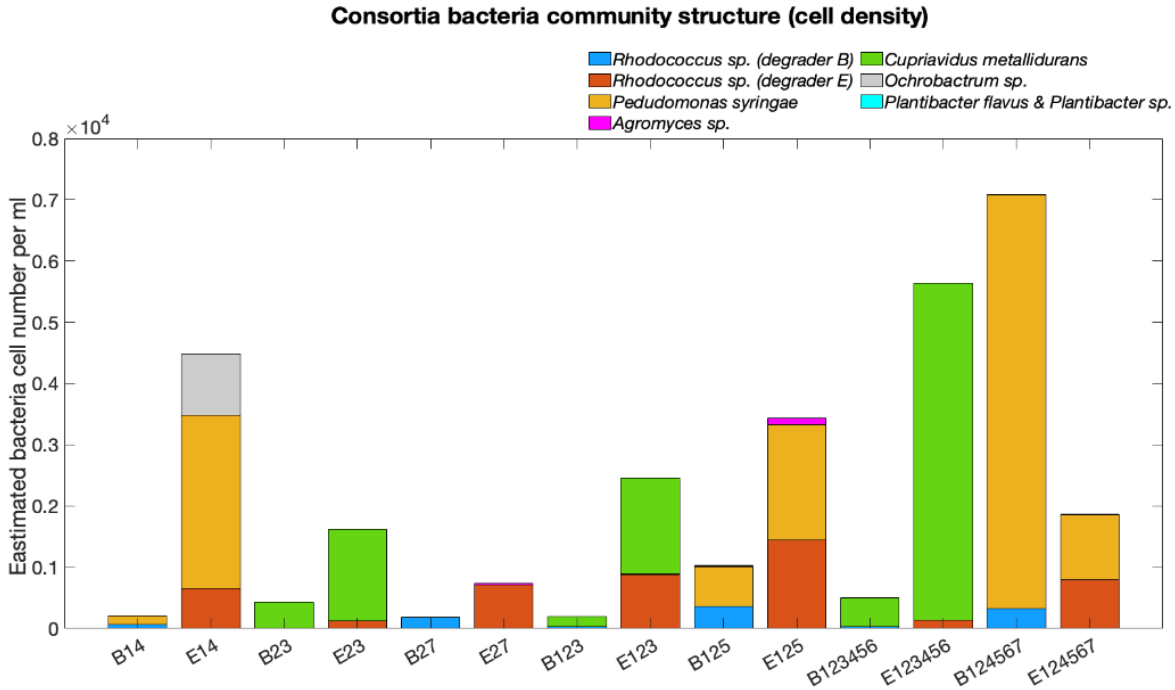


Fig.5.10 Bacteria community structure of exemplar consortia on day 7.

The relative abundance of these bacteria (Fig.5.11) showed that the two degraders (*B* and *E*) remained consistently detected across all samples at day 7. However, their relative abundance varied based on the non-degraders they were co-cultured with. Among non-degraders, *C. metallidurans* exhibited the strongest dominance, evidenced by its high relative abundance and the absence of other co-cultured degraders. Non-degraders like *Agromyces sp*. and *Ochrobactrum sp*. only contributed small proportions of the overall

community populations in consortia where *P. syringes* and *C. metallidurans* were absent (except in consortium E14). In consortia containing *C. metallidurans*, *B* or *E* accounted for less than 10% of the bacterial population. Bacteria 1, a *Pseudomonas*, was the second dominant non-degrader, appearing in B14, E14, B125, E125, B14567, and E14567 but was absent when bacteria *3* (*C. metallidurans*) was present.

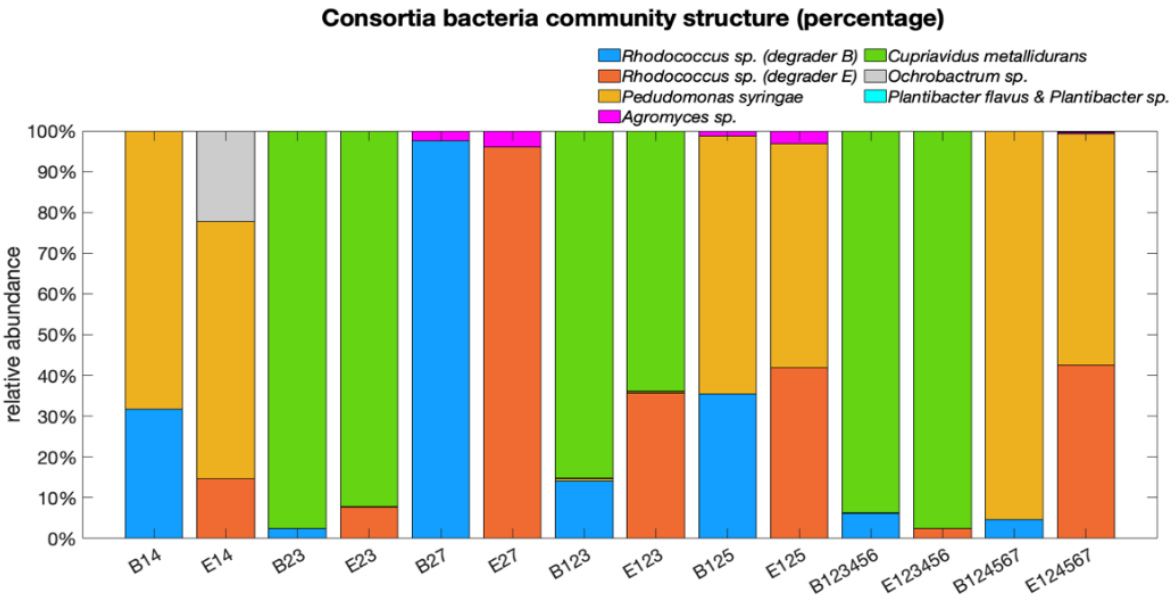


Fig.5.11 Bacteria relative abundance in exemplar consortia.

The algae growth (OD_{685}) data shows no clear correlation between bacterial load and algae growth. However, in consortia with greater algae growth, the total bacterial population tends to be smaller (< 0.25×10^3 cells per mL). This trend is more distinct in consortia containing degrader *B* and non-degrader *3*.

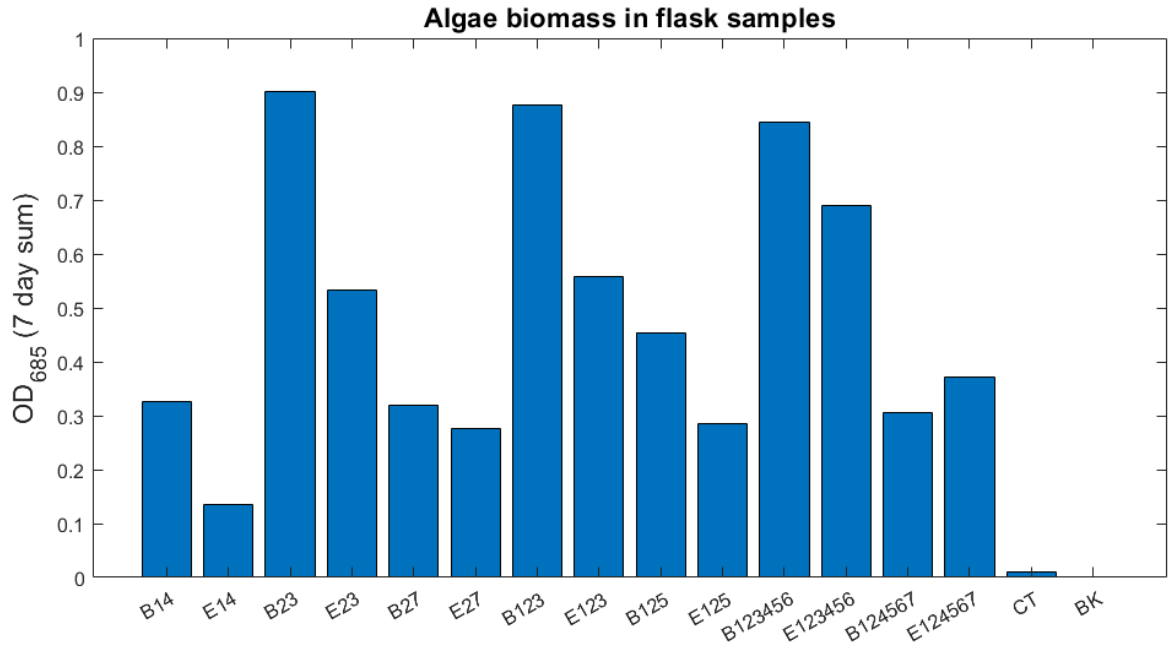


Fig.5.12 Algae growth in the consortia. Calculated as the sum of daily OD_{685} values across 7 days.

5.4 Discussion

5.4.1 Primers validation

From the initial design of 39 primer sets targeting the 16s DNA and CatA gene of bacterial members in the consortia, only 7 sets were shortlisted according to the follow-up validation assays. Although most of the 7 primer sets exhibited relatively high specificity, sets 1F-1R, 4F-4R, BF-BR, and DF-DR showed non-specific amplification which could be explained by the existence of alternative non-target binding sites since bacteria genomics were used. However, this issue was addressed by the annealing temperature optimisation experiment where higher temperatures restrict non-specific binding (Kralik and Ricchi, 2017). Additionally, the incorporation of multiplex background DNA in the tests minimised the possibility of false positives in subsequent analyses and further reduced non-specific amplification.

5.4.2 PCR efficiency:

The observed PCR efficiency in this study ranged from 46.74% to 91.18%. While some reactions fell within the acceptable range, others were below the ideal efficiency. This suggests that amplification under certain primer-template combinations might be in suboptimal conditions. Typically, efficiencies between 80% and 110% are recognized as optimal for qPCR assays (Rocha et al., 2016) and deviations outside this range can indicate potential issues with the reaction. These issues might include primer-dimer formation, suboptimal reaction conditions, or problems with the template DNA. However, it's essential to note that PCR efficiency can be instrument-dependent. While it may vary across different platforms, it remains reproducibly stable on a single platform (Svec et al., 2015). By employing standards with a known amount of analyte DNA established on a DNA matrix in the presence of background non-target DNA, a relatively valid comparison and quantification can be achieved.

5.4.3 Consortia structure interpretation

In the 14 exemplar consortia, the population size of different bacteria varied significantly and surprisingly not all bacteria persisted throughout the study, indicating a dynamic succession across the 7-day cultivation.

Based on the findings in the VOCs degradation studies (Chapter 4), the VOC removal performance between consortia with degrader *B* and degrader *E* shared high similarities. Consortia with degrader *B* had an overall smaller bacteria population (except for B124567) and this aligned with the higher algae biomass in the same consortia. A possible explanation for this is that more VOC-originated carbon was made available for algae photosynthesis when the VOCs degradation rates were similar, according to the ecosystem model introduced in Chapter 3. Also, as observed in Chapter 4, the presence of non-degrader *C. metallidurans* accelerated benzene and toluene degradation, implying its aromatic compounds catabolic traits influenced the consortia, even if it was not identified as a degrader initially. While other non-degraders did not directly affect the VOCs degradation, their persistence in the consortia remains enigmatic which suggests adverse interactions exist.

Degraders *B* and *E*, although both belonging to the *Rhodococcus* genus, led to different community structures. Consortia with degrader *B* tend to have smaller bacterial populations than those with degrader *E*, indicating a more competitive nature of degrader *B* which could inhibit the growth of co-cultured bacteria while promoting algae growth. This competitive trait of degrader *B*, which was identified as *R. erythropolis*, may be attributed to its ability to produce a wide range of antibiotic compounds effective against both gram-positive and negative bacteria, even including other *Rhodococcus* species (Kitagawa and Tamura, 2008). Also, several other *Rhodococcus* species are known for synthesizing antibiotic compounds in co-cultures (Kurosawa et al., 2008; Ward et al., 2018). This competitive nature suggests the potential existence of more complex interactions beyond competition for VOCs as bacteria growth carbon source, which influenced the bacterial population structure observed. However, the limited resolution of 16s rDNA sequencing makes it challenging to identify the exact species of the two *Rhodococcus* (degrader *E* and non-degrader *7*) and the qPCR analysis made it difficult to distinguish between bacteria *7*, *B*, and *E* in consortia B27, E27,

B14567, and E14567. Therefore, a metabolic profile analysis is recommended for deeper insights.

Consortia with high VOC degradation tend to have smaller bacterial populations and reduced species diversity, regardless of the initial bacteria members that were introduced into them, which might be due to the result of competitive interaction such as competition for VOCs as carbon sources or the secreting of antibiotic compounds. However, algae as the 'flora' of the consortia, seemed to benefit from these interactions because a smaller bacterial population is expected to lead to more VOC-originated carbons being fixed by algae, which reinforces the 'ecosystem' model defined in Chapter 3.

Also, the screening experiment, although unable to provide direct insights into the consortia population structure, was able to demonstrate its predictive ability as evidenced by the observation in this study. Consortia with high VOC degradation and smaller bacterial populations, particularly those with both degrader *B* and non-degrader *3*, had a higher ***CI*** index. Additionally, their ***FS*** index was closer to 1, indicating greater functional stability. This also matches the modelling of Coyte et al., (2015) where a stable microbial system was dominated by competitive interactions. These findings merit further investigation, especially through targeted proteomic studies focused on interactions. The screening method employed could prove valuable in narrowing down the targets for such studies.

5.5 Conclusion

This study achieved the profiling of bacterial population structure in the exemplar consortia using qPCR by designing primers targeting each genus of bacteria. From an initial 39 primer sets, 7 sets were shortlisted and further optimised for annealing temperature to reduce non-specific amplification. The specificity of these primers was also validated by multiplexed assays using matrices of background DNA. While the observed PCR efficiency varied and some reactions deviated from the optimal range, the establishment of qPCR standard curves was designed to reduce the likelihood of false positives and enhance the accuracy of the results.

The population size of bacteria showed significant variation, with only certain bacteria persisting throughout the 7-day culturing. Among the two degraders introduced into the consortia, degrader *B* exhibited a greater competitive nature which could be a result of antibiotic-producing properties of bacteria in this genus. The study also revealed that the size of the bacteria population affects algae growth in closed-system scenarios. These findings not only reinforced the 'ecosystem' model defined in Chapter 3 but also exhibited the potential of the qPCR method being high-throughput and cost-effective for providing additional population information supplementary to the screening method, which can help narrow down the targets for further metabolic or proteomic studies to generate deeper insights.

Chapter 6: Conclusion and Future Work

Aims and objectives

- *Summarise the experimental findings and conclusions throughout the entire project.*
- *Address the encountered limitations in this project and define the objectives for future work.*

6.1 Conclusion

The integration of microbial communities, specifically algae and bacteria, has been recognized for its potential in various fields related to biotechnology. While previous efforts have attempted to harness the synergy between algae and bacteria to enhance functionality and promote the production of bioproducts, persistent challenges such as specificity due to the overwhelming possibility of interactions, and predictability issues arising from the complexity of multi-species systems, have necessitated further exploration and innovation in this area. The study sought to address these challenges through a bottom-up approach, engineering artificial consortia for the biodegradation of selected VOCs.

The experiment demonstrated the construction of algae-bacteria consortia using environmentally isolated microalgae and bacteria that are either able to degrade or were resistant to VOCs. The high-throughput screening method developed in this work facilitated the identification of effective consortia combinations without the need for intricate knowledge about individual bacteria species, strains or complex microbial interactions. Exemplar consortia showcased high degradation performance, i.e., >90% removal of benzene, toluene, and phenol in 7 days. The degradation was primarily attributed to the bacteria *Rhodococcus erythropolis* and *Cupriavidus metallidurans*, which concurrently contributed to the growth of the microalgae *Coelastrella terrestris*. This research has underscored the potential of converting VOC waste into valuable algal biomass through carefully constructed algae-bacteria consortia. The findings highlight an efficient screening tool for engineering more stable and efficient microbial systems for bioremediation.

Expanding these findings beyond the context of the current project, the screening method shows the potential for broader applications. Firstly, apart from the VOCs involved in this project, the same methodology can be directly adopted for the bioremediation of a variety of pollutants by simply isolating microbes that are tolerant and able to degrade the pollutant compounds of interest. This should include choosing specific polluted sites exposed to compounds of interest or introducing selective stress by directly adding the compound to enrich functional strains. Secondly, apart from bioremediation, the screening method exhibits high efficiency in identifying bacteria with algae growth-promoting effects. When the microbial candidates, i.e. algae and bacteria of interest, are identified, this method can

be applied to quickly evaluate synthetic consortia that are tailored for specific biological tasks, such as producing desired bioproducts. Finally, the method exhibited high throughput and great potential to handle and assess a vast number of combinations of microbes in polymicrobial systems, among which novel interactions may occur and thus be identified. These interactions could serve as the starting point for metabolic engineering or other omics studies that aim to further enhance the microbial system functionality, or reveal unknown interactions.

6.2 Limitations and future work

Despite the experimental findings presented in this thesis, the study had certain limitations. Firstly, the identification of environmentally isolated microbes using 16S rRNA gene sequencing had low resolution. Enhancements to distinguish microbes at species or strain levels could reduce the chance of repeated strain collection. Also, the study was constrained to a select group of pollutants and bacterial isolates. Future work could expand the scope by exploring a wider array of pollutants and microbial isolates capable of degrading them. Furthermore, the study focused on 14 exemplar consortia with two different *Rhodococcus* as degraders. A more exhaustive exploration of exemplar consortia could provide a comprehensive understanding of the degradation capabilities and interactions within the consortia.

Secondly, the role of non-degraders and their population changes over time were not fully captured. This limitation can be addressed by more frequent sampling for qPCR analysis, or by using advanced visualization techniques such as Fluorescence in situ Hybridization (FISH) to directly visualise the real-time structure of communities. Also, the current work could benefit from adopting alternative techniques such as flow cytometers to reveal exemplar consortia community structure which work may work as cross-verification to the qPCR results. Also, quantitative omics studies such as proteomics and metabolomics, could provide valuable insights into the interactions within these consortia.

Since only chlorophyll content served as the proxy for monitoring VOCs degradation in the consortia, the screening method may benefit from adopting alternative parameters that target specific microbial interactions beyond VOC degradation. Identifying these interactions could involve employing omics studies to identify molecular barcodes or other 'proxy' molecules associated with specific consortia behaviour. Also, while growth data were collected over 7-day periods in the consortia screening experiment, the screening method processed these data into single data points. More frequent data collection could provide a more solid time series data set and incorporating time series data processing may lead to deeper insights into the time-dependent dynamics of microbial consortia during VOC removal. Furthermore, the screening method took advantage of the hierarchical structure consortia combinations. By integrating sophisticated biological models and more robust

algorithms, coupled with appropriate computational power, this method could be further refined to improve predictive capabilities and scalability, which allows broader applications.

Additionally, by performing the screening, distinct characteristics observed between algae *C. terrestris* and bacteria *Rhodococcus* and *C. metallidurans*, highlight the potential to capture key microbial interactions. Although this study was not aimed to uncover all possible interactions, developing a microbial interactions database would be a valuable resource for documenting these behaviours, providing a reference point for future studies involving microbial co-culturing.

Finally, the project only utilised synthetic VOC feeds and did not explore the real fume gas derived from the industrial site, nor did it consider other factors such as CO_2 concentration or executive heat and operation time. This left a gap in understanding the long-term performance of the consortia in an industrial setting. Future research could also focus on investigating the scalability of the process using real industrial VOC waste streams. Moreover, while this project was primarily applied to VOCs bioremediation, the screening process may include a pool of candidate strains known to produce high-value bioproducts, thus enhancing the commercial prospects of the design of future projects.

In summary, this study was a pioneering effort in algae-bacteria consortia engineering, established on a bottom-up approach and there remain opportunities for future research to build upon this foundation.

Reference

Abbasian, F., Lockington, R., Mallavarapu, M., et al. (2015) A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria. *Applied Biochemistry and Biotechnology*, 176 (3): 670–699. doi:10.1007/s12010-015-1603-5.

Abe, K., Hattori, H. and Hirano, M. (2007) Accumulation and antioxidant activity of secondary carotenoids in the aerial microalga Coelastrella striolata var. multistriata. *Food Chemistry*, 100 (2): 656–661. doi:10.1016/j.foodchem.2005.10.026.

Abinandan, S., Subashchandrabose, S.R., Venkateswarlu, K., et al. (2019) Soil microalgae and cyanobacteria: the biotechnological potential in the maintenance of soil fertility and health. *Critical Reviews in Biotechnology*, 39 (8): 981–998. doi:10.1080/07388551.2019.1654972.

Abraham, M.H., Whiting, G.S., Fuchs, R., et al. (1990) Thermodynamics of solute transfer from water to hexadecane. *Journal of the Chemical Society, Perkin Transactions 2*, 2 (2): 291–300. doi:10.1039/P29900000291.

Abuhamed, T., Bayraktar, E., Mehmetoğlu, T., et al. (2004) Kinetics model for growth of Pseudomonas putida F1 during benzene, toluene and phenol biodegradation. *Process Biochemistry*, 39 (8): 983–988. doi:10.1016/S0032-9592(03)00210-3.

Aburai, N., Ohkubo, S., Miyashita, H., et al. (2013) Composition of carotenoids and identification of aerial microalgae isolated from the surface of rocks in mountainous districts of Japan. *Algal Research*, 2 (3): 237–243. doi:10.1016/j.algal.2013.03.001.

Adroer, N., Casas, C., de Mas, C., et al. (1990) Mechanism of formaldehyde biodegradation by Pseudomonas putida. *Applied Microbiology and Biotechnology*, 33 (2): 217–220. doi:10.1007/BF00176528.

Ahmad, A.L., Yasin, N.H.M., Derek, C.J.C., et al. (2011) Microalgae as a sustainable energy source for biodiesel production: A review. *Renewable and Sustainable Energy Reviews*. 15 (1) pp. 584–593. doi:10.1016/j.rser.2010.09.018.

Aiyar, P., Schaeme, D., García-Altares, M., et al. (2017) Antagonistic bacteria disrupt calcium homeostasis and immobilize algal cells. *Nature Communications*, 8 (1): 1–13. doi:10.1038/s41467-017-01547-8.

Alemzadeh, E., Haddad, R., Ahmadi, A.R., et al. (2014) Identification of Chlorophyceae based on 18S rDNA sequences from Persian Gulf. *Iranian Journal of Microbiology*, 6 (6): 437–442. Available at: http://ijm.tums.ac.ir (Accessed: 8 October 2020).

Alviz-Gazitua, P., Durán, R.E., Millacura, F.A., et al. (2022a) Cupriavidus metallidurans CH34 Possesses Aromatic Catabolic Versatility and Degrades Benzene in the Presence of Mercury and Cadmium. *Microorganisms*, 10 (2). doi:10.3390/MICROORGANISMS10020484/S1.

Alviz-Gazitua, P., Durán, R.E., Millacura, F.A., et al. (2022b) Cupriavidus metallidurans CH34 Possesses Aromatic Catabolic Versatility and Degrades Benzene in the Presence of Mercury and Cadmium. *Microorganisms*, 10 (2). doi:10.3390/MICROORGANISMS10020484/S1.

Alviz-Gazitua, P., Fuentes-Alburquenque, S., Rojas, L.A., et al. (2019) The response of

cupriavidus metallidurans CH34 to cadmium involves inhibition of the initiation of biofilm formation, decrease in intracellular c-di-GMP levels, and a novel metal regulated phosphodiesterase. *Frontiers in Microbiology*, 10 (JULY): 1499. doi:10.3389/FMICB.2019.01499/BIBTEX.

Amézqueta, S., Subirats, X., Fuguet, E., et al. (2020) Octanol-Water Partition Constant. *Liquid-Phase Extraction*, pp. 183–208. doi:10.1016/B978-0-12-816911-7.00006-2.

Amin, S.A., Green, D.H., Hart, M.C., et al. (2009) Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (40): 17071–17076. doi:10.1073/pnas.0905512106.

Amin, S.A., Hmelo, L.R., Van Tol, H.M., et al. (2015) Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature*, 522 (7554): 98–101. doi:10.1038/nature14488.

Ammar, E.E., Aioub, A.A.A., Elesawy, A.E., et al. (2022) Algae as Bio-fertilizers: Between current situation and future prospective. *Saudi Journal of Biological Sciences*, 29 (5): 3083–3096. doi:10.1016/J.SJBS.2022.03.020.

Antizar-Ladislao, B. (2010) Bioremediation: Working with bacteria. *Elements*, 6 (6): 389–394. doi:10.2113/gselements.6.6.389.

Arora, N. and Philippidis, G.P. (2023) The Prospects of Algae-Derived Vitamins and Their Precursors for Sustainable Cosmeceuticals. *Processes 2023, Vol. 11, Page 587*, 11 (2): 587. doi:10.3390/PR11020587.

Arzul, G., Seguel, M., Guzman, L., et al. (1999) Comparison of allelopathic properties in three toxic alexandrium species. *Journal of Experimental Marine Biology and Ecology*, 232 (2): 285–295. doi:10.1016/S0022-0981(98)00120-8.

Ask, J., Karlsson, J., Persson, L., et al. (2009) Whole-lake estimates of carbon flux through algae and bacteria in benthic and pelagic habitats of clear-water lakes. *Ecology*, 90 (7): 1923–1932. doi:10.1890/07-1855.1.

Azarian, G.H., Mesdaghinia, A.R., Vaezi, F., et al. (2007) *Algae Removal by Electro-coagulation Process, Application for Treatment of the Effluent from an Industrial Wastewater Treatment Plant*. Available at: https://ijph.tums.ac.ir/index.php/ijph/article/view/2091 (Accessed: 9 April 2021).

Becker, E.W. (2007) Micro-algae as a source of protein. *Biotechnology Advances*, 25 (2): 207–210. doi:10.1016/J.BIOTECHADV.2006.11.002.

Becker, E.W. (2013) Microalgae for Human and Animal Nutrition. *Handbook of Microalgal Culture: Applied Phycology and Biotechnology: Second Edition*, pp. 461–503. doi:10.1002/9781118567166.CH25.

Bedia, C., Cardoso, P., Dalmau, N., et al. (2018) "Applications of Metabolomics Analysis in Environmental Research." In *Comprehensive Analytical Chemistry*. Elsevier B.V. pp. 533–582. doi:10.1016/bs.coac.2018.07.006.

Bell, W. and Mitchell, R. (1972) *the Biological Bulletin Chemotactic and Growth Responses of Marine*., 143 (2).

Benjamini, Y. and Hochberg, Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, 57 (1): 289–300. doi:10.1111/J.2517-6161.1995.TB02031.X.

Bérard, A., Dorigo, U., Humbert, J.F., et al. (2005) Microalgae community structure analysis based on 18S rDNA amplification from DNA extracted directly from soil as a potential soil bioindicator. *Agronomie*, 25 (2): 285–291. doi:10.1051/agro:2005004.

Bhatia, S.K., Bhatia, R.K., Choi, Y.-K., et al. (2018) Biotechnological potential of microbial consortia and future perspectives. *Critical Reviews in Biotechnology*, 38 (8): 1209–1229. doi:10.1080/07388551.2018.1471445.

Bikel, S., Valdez-Lara, A., Cornejo-Granados, F., et al. (2015) Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. *Computational and Structural Biotechnology Journal*, 13: 390–401. doi:10.1016/J.CSBJ.2015.06.001.

Brennan, L. and Owende, P. (2010) Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, 14 (2): 557–577. doi:10.1016/J.RSER.2009.10.009.

Cabani, S., Conti, G. and Lepori, L. (1971) Thermodynamic study on aqueous dilute solutions of organic compounds. Part 2.—Cyclic ethers. *Transactions of the Faraday Society*, 67 (0): 1943–1950. doi:10.1039/TF9716701943.

Cai, C., Xu, Z., Xu, M., et al. (2020) Development of a Rhodococcus opacus Cell Factory for Valorizing Lignin to Muconate. *ACS Sustainable Chemistry and Engineering*, 8 (4): 2016–2031. doi:10.1021/ACSSUSCHEMENG.9B06571/ASSET/IMAGES/LARGE/SC9B06571_0008.JPEG.

Cai, S., Hu, C. and Du, S. (2007) Comparisons of Growth and Biochemical Composition between Mixed Culture of Alga and Yeast and Monocultures. *Journal of Bioscience and Bioengineering*, 104 (5): 391–397. doi:10.1263/jbb.104.391.

Cape, J.N. (2003) Effects of airborne volatile organic compounds on plants. *Environmental Pollution*, 122 (1): 145–157. doi:10.1016/S0269-7491(02)00273-7.

Cébron, A., Norini, M.P., Beguiristain, T., et al. (2008) Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHDα) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *Journal of Microbiological Methods*, 73 (2): 148–159. doi:10.1016/j.mimet.2008.01.009.

Cembella, A.D. (2003) Chemical ecology of eukaryotic microalgae in marine ecosystems. *Phycologia*, 42 (4): 420–447. doi:10.2216/I0031-8884-42-4-420.1.

Chakraborty, U., Chakraborty, B.N., Basnet, M., et al. (2009) Evaluation of Ochrobactrum anthropi TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. *Journal of Applied Microbiology*, 107 (2): 625–634. doi:10.1111/j.1365-2672.2009.04242.x.

Chandrasekhar, T., Varaprasad, D., Gnaneswari, P., et al. (2023) Algae: The Reservoir of Bioethanol. *Fermentation 2023, Vol. 9, Page 712*, 9 (8): 712. doi:10.3390/FERMENTATION9080712.

Chao, C.Y.H., Kwong, P.C.W., Wang, J.H., et al. (2008) Co-firing coal with rice husk and bamboo and the impact on particulate matters and associated polycyclic aromatic hydrocarbon emissions. *Bioresource Technology*, 99 (1): 83–93. doi:10.1016/j.biortech.2006.11.051.

Chen, C.Y., Yeh, K.L., Aisyah, R., et al. (2011) Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*, 102 (1): 71–81. doi:10.1016/J.BIORTECH.2010.06.159.

Chen, C.Y., Zhao, X.Q., Yen, H.W., et al. (2013a) Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal*, 78: 1–10. doi:10.1016/J.BEJ.2013.03.006.

Chen, D.Z., Fang, J.Y., Shao, Q., et al. (2013b) Biodegradation of tetrahydrofuran by Pseudomonas oleovorans DT4 immobilized in calcium alginate beads impregnated with activated carbon fiber: Mass transfer effect and continuous treatment. *Bioresource Technology*, 139: 87–93. doi:10.1016/J.BIORTECH.2013.04.037.

Cheng, D., Li, D., Yuan, Y., et al. (2017) Improving carbohydrate and starch accumulation in Chlorella sp. AE10 by a novel two-stage process with cell dilution. *Biotechnology for Biofuels*, 10 (1): 1–14. doi:10.1186/S13068-017-0753-9/TABLES/2.

Le Chevanton, M., Garnier, M., Bougaran, G., et al. (2013) Screening and selection of growth-promoting bacteria for Dunaliella cultures. *Algal Research*, 2 (3): 212–222. doi:10.1016/j.algal.2013.05.003.

Cho, D.H., Ramanan, R., Heo, J., et al. (2015) Organic carbon, influent microbial diversity and temperature strongly influence algal diversity and biomass in raceway ponds treating raw municipal wastewater. *Bioresource Technology*, 191: 481–487. doi:10.1016/J.BIORTECH.2015.02.013.

Chronakis, I.S. and Madsen, M. (2011) Algal proteins. *Handbook of Food Proteins*, pp. 353–394. doi:10.1533/9780857093639.353.

Cirri, E. and Pohnert, G. (2019) Algae−bacteria interactions that balance the planktonic microbiome. *New Phytologist*, 223 (1): 100–106. doi:10.1111/nph.15765.

Collins, J.D. (2001) “Tuberculosis in cattle: New perspectives.” In *Tuberculosis*. 1 February 2001. Churchill Livingstone. pp. 17–21. doi:10.1054/tube.2000.0262.

Comolli, L.R. (2014) Intra- and inter-species interactions in microbial communities. *Frontiers in Microbiology*, 5 (NOV): 1–3. doi:10.3389/fmicb.2014.00629.

Contreras-Angulo, J.R., Mata, T.M., Cuellar-Bermudez, S.P., et al. (2019) Symbiotic Co-Culture of Scenedesmus sp. and Azospirillum brasilense on N-Deficient Media with Biomass Production for Biofuels. *Sustainability 2019, Vol. 11, Page 707*, 11 (3): 707. doi:10.3390/SU11030707.

Coyte, K.Z., Schluter, J. and Foster, K.R. (2015) The ecology of the microbiome: Networks, competition, and stability. *Science*, 350 (6261): 663–666. doi:10.1126/SCIENCE.AAD2602/SUPPL_FILE/COYTE.SM.PDF.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., et al. (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature 2005 438:7064*, 438 (7064): 90–93.

doi:10.1038/nature04056.

Daly, G., Ghini, V., Adessi, A., et al. (2022) Towards a mechanistic understanding of microalgae–bacteria interactions: integration of metabolomic analysis and computational models. *FEMS Microbiology Reviews*, 46 (5): 1–19. doi:10.1093/FEMSRE/FUAC020.

Dash, H.R., Mangwani, N., Chakraborty, J., et al. (2013) Marine bacteria: Potential candidates for enhanced bioremediation. *Applied Microbiology and Biotechnology*, 97 (2): 561–571. doi:10.1007/S00253-012-4584-0/TABLES/2.

Datt, B. (1998) Remote sensing of chlorophyll a, chlorophyll b, chlorophyll a+b, and total carotenoid content in eucalyptus leaves. *Remote Sensing of Environment*, 66 (2): 111–121. doi:10.1016/S0034-4257(98)00046-7.

DeLong, J.P. (2008) The maximum power principle predicts the outcomes of two-species competition experiments. *Oikos*, 117 (9): 1329–1336. doi:10.1111/J.0030-1299.2008.16832.X.

Deshmukh, S., Kumar, R. and Bala, K. (2019) Microalgae biodiesel: A review on oil extraction, fatty acid composition, properties and effect on engine performance and emissions. *Fuel Processing Technology*, 191: 232–247. doi:10.1016/J.FUPROC.2019.03.013.

Dillon, M.L., Hawes, I., Jungblut, A.D., et al. (2020) Energetic and Environmental Constraints on the Community Structure of Benthic Microbial Mats in Lake Fryxell, Antarctica. *FEMS Microbiology Ecology*, 96 (2): 207. doi:10.1093/FEMSEC/FIZ207.

Dion, L.M., Lefsrud, M. and Orsat, V. (2011) Review of CO2 recovery methods from the exhaust gas of biomass heating systems for safe enrichment in greenhouses. *Biomass and Bioenergy*, 35 (8): 3422–3432. doi:10.1016/j.biombioe.2011.06.013.

Dittami, S.M., Eveillard, D. and Tonon, T. (2014) A metabolic approach to study algal-bacterial interactions in changing environments. *Molecular Ecology*, 23 (7): 1656–1660. doi:10.1111/mec.12670.

Dong, Q.L. and Zhao, X.M. (2004) “In situ carbon dioxide fixation in the process of natural astaxanthin production by a mixed culture of Haematococcus pluvialis and Phaffia rhodozyma.” In *Catalysis Today*. 14 December 2004. Elsevier. pp. 537–544. doi:10.1016/j.cattod.2004.09.052.

Dragone, G., Fernandes, B.D., Abreu, A.P., et al. (2011) Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Applied Energy*, 88 (10): 3331–3335. doi:10.1016/J.APENERGY.2011.03.012.

Duchowicz, P.R., Aranda, J.F., Bacelo, D.E., et al. (2020) QSPR study of the Henry’s law constant for heterogeneous compounds. *Chemical Engineering Research and Design*, 154: 115–121. doi:10.1016/J.CHERD.2019.12.009.

Duong, V.T., Li, Y., Nowak, E., et al. (2012) Microalgae isolation and selection for prospective biodiesel production. *Energies*, 5 (6): 1835–1849. doi:10.3390/en5061835.

Ecol Prog Ser, M., Ada, M. and an no, T. (n.d.) *h4ARINE ECOLOGY PROGRESS SERIES Promotion of cyst formation in the toxic dinoflagellate Alexandrium (Dinophyceae) by natural bacterial assemblages from Hiroshima Bay, Japan*.

El-Saied, H., Basta, A.H. and Gobran, R.H. (2007) Research Progress in Friendly Environmental Technology for the Production of Cellulose Products (Bacterial Cellulose and Its Application). *https://doi.org/10.1081/PPT-120038065*, 43 (3): 797–820. doi:10.1081/PPT-120038065.

Elazzazy, A.M., Abdelmoneim, T.S. and Almaghrabi, O.A. (2015) Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi Journal of Biological Sciences*, 22 (4): 466–475. doi:10.1016/J.SJBS.2014.11.018.

Englen, M.D. and Kelley, L.C. (2000) A rapid DNA isolation procedure for the identification of Campylobacter jejuni by the polymerase chain reaction. *Letters in Applied Microbiology*, 31 (6): 421–426. doi:10.1046/j.1365-2672.2000.00841.x.

Environmental Protection (2010) *The Air Quality Standards Regulations 2010*., 3 (1001): 1–49. Available at: http://www.legislation.gov.uk/uksi/2010/1001/made/data.pdf.

Estrada-de los Santos, P., Vacaseydel-Aceves, N.B., Martínez-Aguilar, L., et al. (2011) Cupriavidus and Burkholderia species associated with agricultural plants that grow in alkaline soils. *Journal of Microbiology*, 49 (6): 867–876. doi:10.1007/s12275-011-1127-9.

Evtyugina, M., Alves, C., Calvo, A., et al. (2014) VOC emissions from residential combustion of Southern and mid-European woods. *Atmospheric Environment*, 83: 90–98. doi:10.1016/J.ATMOSENV.2013.10.050.

Fabris, M., Abbriano, R.M., Pernice, M., et al. (2020) Emerging Technologies in Algal Biotechnology: Toward the Establishment of a Sustainable, Algae-Based Bioeconomy. *Frontiers in Plant Science*, 11: 515802. doi:10.3389/FPLS.2020.00279/BIBTEX.

Fernandez, L., Mercader, J.M., Planas-Fèlix, M., et al. (2014) Adaptation to environmental factors shapes the organization of regulatory regions in microbial communities. *BMC Genomics*, 15 (1): 1–12. doi:10.1186/1471-2164-15-877.

Ferreira, G.F., Ríos Pinto, L.F., Maciel Filho, R., et al. (2019) A review on lipid production from microalgae: Association between cultivation using waste streams and fatty acid profiles. *Renewable and Sustainable Energy Reviews*, 109: 448–466. doi:10.1016/J.RSER.2019.04.052.

Fierer, N., Nemergut, D., Knight, R., et al. (2010) Changes through time: integrating microorganisms into the study of succession. *Research in Microbiology*, 161 (8): 635–642. doi:10.1016/J.RESMIC.2010.06.002.

Gao, Y. and Wu, M. (2023) Accounting for 16S rRNA copy number prediction uncertainty and its implications in bacterial diversity analyses. *ISME Communications 2023 3:1*, 3 (1): 1–9. doi:10.1038/s43705-023-00266-0.

Gilbert, J.A. and Henry, C. (2015) Predicting ecosystem emergent properties at multiple scales. *Environmental Microbiology Reports*, 7 (1): 20–22. doi:10.1111/1758-2229.12258.

Gralka, M., Szabo, R., Stocker, R., et al. (2020) Trophic Interactions and the Drivers of Microbial Community Assembly. *Current Biology*, 30 (19): R1176–R1188. doi:10.1016/J.CUB.2020.08.007.

Griffin, A.S., West, S.A. and Buckling, A. (2004) Cooperation and competition in pathogenic bacteria. *Nature*, 430 (7003): 1024–1027. doi:10.1038/nature02744.

Guidetti Vendruscolo, R., Bittencourt Fagundes, M., Jacob-Lopes, E., et al. (2019) Analytical strategies for using gas chromatography to control and optimize microalgae bioprocessing. *Current Opinion in Food Science*, 25: 73–81. doi:10.1016/J.COFS.2019.02.008.

Guieysse, B., Hort, C., Platel, V., et al. (2008) Biological treatment of indoor air for VOC removal: Potential and challenges. *Biotechnology Advances*. 26 (5) pp. 398–410. doi:10.1016/j.biotechadv.2008.03.005.

Guiry, M.D. (2012) HOW MANY SPECIES OF ALGAE ARE THERE? *Journal of Phycology*, 48 (5): 1057–1063. doi:10.1111/J.1529-8817.2012.01222.X.

Guo, H., Yang, Y., Liu, K., et al. (2016) Comparative Genomic Analysis of Delftia tsuruhatensis MTQ3 and the Identification of Functional NRPS Genes for Siderophore Production. *BioMed Research International*, 2016. doi:10.1155/2016/3687619.

Hallenbeck, P.C., Grogger, M., Mraz, M., et al. (2016) Solar biofuels production with microalgae. *Applied Energy*, 179: 136–145. doi:10.1016/J.APENERGY.2016.06.024.

Hamed, T.A., Bayraktar, E., Mehmetoğlu, T., et al. (2003) Substrate interactions during the biodegradation of benzene, toluene and phenol mixtures. *Process Biochemistry*, 39 (1): 27–35. doi:10.1016/S0032-9592(02)00293-5.

Harrison, F., Paul, J., Massey, R.C., et al. (2008) Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. *ISME Journal*, 2 (1): 49–55. doi:10.1038/ismej.2007.96.

Harrison, M.A.J., Cape, J.N. and Heal, M.R. (2002) Experimentally determined Henry's Law coefficients of phenol, 2-methylphenol and 2-nitrophenol in the temperature range 281–302 K. *Atmospheric Environment*, 36 (11): 1843–1851. doi:10.1016/S1352-2310(02)00137-1.

He, S. and Xue, G. (2010) Algal-based immobilization process to treat the effluent from a secondary wastewater treatment plant (WWTP). *Journal of Hazardous Materials*, 178 (1–3): 895–899. doi:10.1016/j.jhazmat.2010.02.022.

He, Z., Kan, J., Mansfeld, F., et al. (2009) Self-Sustained Phototrophic Microbial Fuel Cells Based on the Synergistic Cooperation between Photosynthetic Microorganisms and Heterotrophic Bacteria. *Environmental Science & Technology*, 43 (5): 1648–1654. doi:10.1021/es803084a.

Health and Safety Executive (2020) List of Workplace Exposure Limits (WELS) (Fourth Edition 2020). *Hse*, 2002: 1–61. Available at: https://www.hse.gov.uk/pubns/books/eh40.htm.

Hiatt, M.H. (2013) Determination of Henry's law constants using internal standards with benchmark values. *Journal of Chemical and Engineering Data*, 58 (4): 902–908. doi:10.1021/JE3010535/SUPPL_FILE/JE3010535_SI_002.PDF.

Hibbing, M.E., Fuqua, C., Parsek, M.R., et al. (2009) Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology 2009 8:1*, 8 (1): 15–25. doi:10.1038/nrmicro2259.

Higuchi, Y., Kamimura, N., Takenami, H., et al. (2022) The Catabolic System of Acetovanillone and Acetosyringone in Sphingobium sp. Strain SYK-6 Useful for Upgrading Aromatic Compounds Obtained through Chemical Lignin Depolymerization. *Applied and Environmental Microbiology*, 88 (16). doi:10.1128/AEM.00724-22/SUPPL_FILE/AEM.00724-22-S0001.PDF.

Hilal, S.H., Ayyampalayam, S.N. and Carreira, L.A. (2008) Air - liquid partition coefficient for a diverse set of organic compounds: Henry's law constant in water and hexadecane. *Environmental Science and Technology*, 42 (24): 9231–9236. doi:10.1021/ES8005783/SUPPL_FILE/ES8005783_SI_001.PDF.

Hirano, A., Ueda, R., Hirayama, S., et al. (1997) CO2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. *Energy*, 22 (2–3): 137–142. doi:10.1016/S0360-5442(96)00123-5.

Hodač, L., Hallmann, C., Spitzer, K., et al. (2016) Widespread green algae Chlorella and Stichococcus exhibit polar-temperate and tropical-temperate biogeography. *FEMS Microbiology Ecology*, 92 (8). doi:10.1093/femsec/fiw122.

Hu, C.W., Chuang, L. Te, Yu, P.C., et al. (2013) Pigment production by a new thermotolerant microalga Coelastrella sp. F50. *Food Chemistry*, 138 (4): 2071–2078. doi:10.1016/j.foodchem.2012.11.133.

Humenik, F.J. and Hanna, G.P. (1971) Algal-Bacterial Symbiosis for Removal and Conservation of Wastewater Nutrients. *Journal (Water Pollution Control Federation)*, 43 (4): 580–594. Available at: http://www.jstor.org/stable/25037357.

Iglina, T., Iglin, P. and Pashchenko, D. (2022) Industrial CO2 Capture by Algae: A Review and Recent Advances. *Sustainability 2022, Vol. 14, Page 3801*, 14 (7): 3801. doi:10.3390/SU14073801.

Illman, A.M., Scragg, A.H. and Shales, S.W. (2000) Increase in Chlorella strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*, 27 (8): 631–635. doi:10.1016/S0141-0229(00)00266-0.

Iminova, L., Delegan, Y., Frantsuzova, E., et al. (2022) Physiological and biochemical characterization and genome analysis of Rhodococcus qingshengii strain 7B capable of crude oil degradation and plant stimulation. *Biotechnology Reports*, 35: e00741. doi:10.1016/J.BTRE.2022.E00741.

Inoue, K. and Uchida, T. (2013) Microalgae cultured by sewage and organic constituents. *Chemosphere*, 93 (7): 1442–1445. doi:10.1016/j.chemosphere.2013.07.088.

IPCC (2023) Summary for Policymakers: Synthesis Report. *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*, pp. 1–34.

Jacob-Lopes, E., Maroneze, M.M., Deprá, M.C., et al. (2019) Bioactive food compounds from microalgae: an innovative framework on industrial biorefineries. *Current Opinion in Food Science*, 25: 1–7. doi:10.1016/J.COFS.2018.12.003.

Jahanshahi, S., Badiefar, L., Khodabandeh, M., et al. (2022) Bioremediation of a salty petrochemical wastewater containing bisphenol A by a novel indigenous Pseudomonas

pseudoalcaligenes. *RSC Advances*, 13 (1): 388–398. doi:10.1039/d2ra06206b.

Jeong, S.W., Nam, S.W., Hwangbo, K., et al. (2017) Transcriptional Regulation of Cellulose Biosynthesis during the Early Phase of Nitrogen Deprivation in Nannochloropsis salina. *Scientific Reports 2017 7:1*, 7 (1): 1–11. doi:10.1038/s41598-017-05684-4.

Jian, L. (2008) Dynamic Measurements of Henry's Law Constant of Aromatic Compounds Using Proton Transfer Reaction Mass Spectrometry. *Acta Physico-chimica Sinica*.

Jiang, B., Zhou, Z., Dong, Y., et al. (2015) Biodegradation of Benzene, Toluene, Ethylbenzene, and o-, m-, and p-Xylenes by the Newly Isolated Bacterium Comamonas sp. JB. *Applied Biochemistry and Biotechnology*, 176 (6): 1700–1708. doi:10.1007/S12010-015-1671-6/FIGURES/4.

Jiang, L., Li, Y. and Pei, H. (2021) Algal–bacterial consortia for bioproduct generation and wastewater treatment. *Renewable and Sustainable Energy Reviews*, 149: 111395. doi:10.1016/J.RSER.2021.111395.

Jiang, L.L., Zhou, J.J., Quan, C.S., et al. (2017) Advances in industrial microbiome based on microbial consortium for biorefinery. *Bioresources and Bioprocessing*. 4 (1). doi:10.1186/s40643-017-0141-0.

Jiménez-Vázquez, K.R., García-Cárdenas, E., Barrera-Ortiz, S., et al. (2020) The plant beneficial rhizobacterium Achromobacter sp. 5B1 influences root development through auxin signaling and redistribution. *Plant Journal*, 103 (5): 1639–1654. doi:10.1111/tpj.14853.

Jiménez, D.J., Dini-Andreote, F., DeAngelis, K.M., et al. (2017) Ecological Insights into the Dynamics of Plant Biomass-Degrading Microbial Consortia. *Trends in Microbiology*, 25 (10): 788–796. doi:10.1016/J.TIM.2017.05.012.

Jiménez, J.I., Miñambres, B., García, J.L., et al. (2002) Genomic analysis of the aromatic catabolic pathways from Pseudomonas putida KT2440. *Environmental microbiology*, 4 (12): 824–841. doi:10.1046/J.1462-2920.2002.00370.X.

Jin, P., Wang, H., Liu, W., et al. (2017) Bactericidal metabolites from Phellinus noxius HN-1 against Microcystis aeruginosa. *Scientific Reports*, 7 (1): 1–10. doi:10.1038/s41598-017-03440-2.

Joh, G., Choi, Y.S., Shin, J.K., et al. (2011) Problematic algae in the sedimentation and filtration process of water treatment plants. *Journal of Water Supply: Research and Technology - AQUA*, 60 (4): 219–230. doi:10.2166/aqua.2011.035.

Johnstone, T.C. and Nolan, E.M. (2015) Beyond iron: Non-classical biological functions of bacterial siderophores. *Dalton Transactions*. 44 (14) pp. 6320–6339. doi:10.1039/c4dt03559c.

Jouanneau, Y., Martin, F., Krivobok, S., et al. (2011) Ring-hydroxylating dioxygenases involved in PAH biodegradation : structure, function, biodiversity. *undefined*.

Juárez-Jiménez, B., Manzanera, M., Rodelas, B., et al. (2010) Metabolic characterization of a strain (BM90) of Delftia tsuruhatensis showing highly diversified capacity to degrade low molecular weight phenols. *Biodegradation*, 21 (3): 475–489. doi:10.1007/s10532-009-9317-4.

Karunakaran, E., Vernon, D., Biggs, C.A., et al. (2016) Enumeration of sulphate-reducing bacteria for assessing potential for hydrogen sulphide production in urban drainage systems. *Water Science and Technology*, 73 (12): 3087–3094. doi:10.2166/WST.2016.026.

Kauppi, B., Lee, K., Carredano, E., et al. (1998) Structure of an aromatic-ring-hydroxylating dioxygenasenaphthalene 1,2-dioxygenase. *Structure*, 6 (5): 571–586. doi:10.1016/S0969-2126(98)00059-8.

Kazamia, E., Czesnick, H., Nguyen, T.T. Van, et al. (2012) Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environmental Microbiology*, 14 (6): 1466–1476. doi:10.1111/J.1462-2920.2012.02733.X.

Kesaano, M. and Sims, R.C. (2014) Algal biofilm based technology for wastewater treatment. *Algal Research*. 5 (1) pp. 231–240. doi:10.1016/j.algal.2014.02.003.

Khaw, Y.S., Khong, N.M.H., Shaharuddin, N.A., et al. (2020) A simple 18S rDNA approach for the identification of cultured eukaryotic microalgae with an emphasis on primers. *Journal of Microbiological Methods*, 172: 105890. doi:10.1016/j.mimet.2020.105890.

Kim, B.H., Ramanan, R., Cho, D.H., et al. (2014) Role of Rhizobium, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction. *Biomass and Bioenergy*, 69: 95–105. doi:10.1016/J.BIOMBIOE.2014.07.015.

Kim, J., Lyu, X.M., Lee, J.J.L., et al. (2018) Metabolomics analysis of Pseudomonas chlororaphis JK12 algicidal activity under aerobic and micro-aerobic culture condition. *AMB Express*, 8 (1): 131. doi:10.1186/s13568-018-0660-x.

Kim, J.M., Le, N.T., Chung, B.S., et al. (2008) Influence of soil components on the biodegradation of benzene, toluene, ethylbenzene, and o-, m-, and p-xylenes by the newly isolated bacterium Pseudoxanthomonas spadix BD-a59. *Applied and Environmental Microbiology*, 74 (23): 7313–7320. doi:10.1128/AEM.01695-08.

Kim, S.C. and Shim, W.G. (2010) Catalytic combustion of VOCs over a series of manganese oxide catalysts. *Applied Catalysis B: Environmental*, 98 (3–4): 180–185. doi:10.1016/j.apcatb.2010.05.027.

Kim, Y.H. and Kim, K.H. (2014) Recent advances in thermal desorption-gas chromatography-mass spectrometery method to eliminate the matrix effect between air and water samples: Application to the accurate determination of Henry's law constant. *Journal of Chromatography A*, 1342: 78–85. doi:10.1016/J.CHROMA.2014.03.040.

Kim, Y.S., Son, H.J. and Jeong, S.Y. (2015) Isolation of an algicide from a marine bacterium and its effects against the toxic dinoflagellate Alexandrium catenella and other harmful algal bloom species. *Journal of Microbiology*, 53 (8): 511–517. doi:10.1007/s12275-015-5303-1.

Kimura, N. and Kamagata, Y. (2009) Impact of dibenzofuran/dibenzo-p-dioxin amendment on bacterial community from forest soil and ring-hydroxylating dioxygenase gene populations. *Applied Microbiology and Biotechnology*, 84 (2): 365–373. doi:10.1007/s00253-009-2046-0.

Kish, J.D., Leng, C., Kelley, J., et al. (2013) An improved approach for measuring Henry ' s law coef fi cients of atmospheric organics. *Atmospheric Environment*, 79: 561–565. doi:10.1016/j.atmosenv.2013.07.023.

Kitagawa, W. and Tamura, T. (2008) Three Types of Antibiotics Produced from Rhodococcus erythropolis Strains. *Microbes and Environments*, 23 (2): 167–171. doi:10.1264/JSME2.23.167.

Kitcha, S. and Cheirsilp, B. (2014) Enhanced lipid production by co-cultivation and co-encapsulation of oleaginous yeast Trichosporonoides spathulata with microalgae in alginate gel beads. *Applied Biochemistry and Biotechnology*, 173 (2): 522–534. doi:10.1007/s12010-014-0859-5.

Kralik, P. and Ricchi, M. (2017) A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. *Frontiers in Microbiology*, 8 (FEB): 239909. doi:10.3389/FMICB.2017.00108/BIBTEX.

Kubista, M., Andrade, J.M., Bengtsson, M., et al. (2006) The real-time polymerase chain reaction. *Molecular Aspects of Medicine*, 27 (2–3): 95–125. doi:10.1016/J.MAM.2005.12.007.

Kurosawa, K., Ghiviriga, I., Sambandan, T.G., et al. (2008) Rhodostreptomycins, antibiotics biosynthesized following horizontal gene transfer from Streptomyces padanus to Rhodococcus fascians. *Journal of the American Chemical Society*, 130 (4): 1126–1127. doi:10.1021/JA077821P/SUPPL_FILE/JA077821P-FILE001.PDF.

Kutsuna, S. and Kaneyasu, N. (2021) Henry ' s law constants and hydration equilibrium constants of n -hexanal and their temperature dependence as determined by the rectangular pulse method. *Chemical Engineering Science*, 239: 116639. doi:10.1016/j.ces.2021.116639.

Lakowicz, J.R. (2006) *Principles of fluorescence spectroscopy*. Springer. doi:10.1007/978-0-387-46312-4/COVER.

Lam, M.K. and Lee, K.T. (2012) Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnology Advances*. 30 (3) pp. 673–690. doi:10.1016/j.biotechadv.2011.11.008.

Lamont, I.L., Martin, L.W., Sims, T., et al. (2006) Characterization of a gene encoding an acetylase required for pyoverdine synthesis in Pseudomonas aeruginosa. *Journal of Bacteriology*, 188 (8): 3149–3152. doi:10.1128/JB.188.8.3149-3152.2006.

Larkin, M.J., Kulakov, L.A. and Allen, C.C.R. (2005) Biodegradation and Rhodococcus - Masters of catabolic versatility. *Current Opinion in Biotechnology*, 16 (3 SPEC. ISS.): 282–290. doi:10.1016/j.copbio.2005.04.007.

Le, T.T., Eymann, C., Albrecht, D., et al. (2006) Differential gene expression in response to phenol and catechol reveals different metabolic activities for the degradation of aromatic compounds in Bacillus subtilis. *Environmental Microbiology*, 8 (8): 1408–1427. doi:10.1111/J.1462-2920.2006.01034.X.

Lee, S., Mukherjee, S., Brewer, B., et al. (2013) *A Laboratory Experiment To Measure Henry ' s Law Constants of Volatile Organic Compounds with a Bubble Column and a Gas Chromatography Flame Ionization Detector (GC-FID)*.

Lee, Y.K. (2001) "Microalgal mass culture systems and methods: Their limitation and potential." In *Journal of Applied Phycology*. 2001. Springer. pp. 307–315. doi:10.1023/A:1017560006941.

Liu, H., Jeong, J., Gray, H., et al. (2012) Algal uptake of hydrophobic and hydrophilic dissolved organic nitrogen in effluent from biological nutrient removal municipal wastewater treatment systems. *Environmental Science and Technology*, 46 (2): 713–721. doi:10.1021/es203085y.

Louca, S., Doebeli, M. and Parfrey, L.W. (2018) Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome*, 6 (1): 1–12. doi:10.1186/S40168-018-0420-9/FIGURES/4.

Lovejoy, C., Bowman, J.P. and Hallegraeff, G.M. (1998) Algicidal effects of a novel marine Pseudoalteromonas isolate (class Proteobacteria, gamma subdivision) on harmful algal bloom species of the genera Chattonella, Gymnodinium, and Heterosigma. *Applied and Environmental Microbiology*, 64 (8): 2806–2813. doi:10.1128/aem.64.8.2806-2813.1998.

Lu, N., Pei, J., Zhao, Y., et al. (2012) Performance of a biological degradation method for indoor formaldehyde removal. *Building and Environment*, 57: 253–258. doi:10.1016/j.buildenv.2012.05.007.

Lukumbuzya, M., Schmid, M., Pjevac, P., et al. (2019) A Multicolor Fluorescence in situ Hybridization Approach Using an Extended Set of Fluorophores to Visualize Microorganisms. *Frontiers in Microbiology*, 0 (JUN): 1383. doi:10.3389/FMICB.2019.01383.

Lutz, H.L., Ramírez-Puebla, S.T., Abbo, L., et al. (2019) A Simple Microbiome in the European Common Cuttlefish, Sepia officinalis . *mSystems*, 4 (4). doi:10.1128/MSYSTEMS.00177-19/SUPPL_FILE/MSYSTEMS.00177-19-ST002.XLSX.

Mackay, D., Shiu, W.-Y., Shiu, W.-Y., et al. (2006) Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals. *Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals*. doi:10.1201/9781420044393.

Maeda, A.H., Nishi, S., Hatada, Y., et al. (2020) Chemical and genomic analyses of polycyclic aromatic hydrocarbon biodegradation in Sphingobium barthaii KK22 reveals divergent pathways in soil sphingomonads. *International Biodeterioration & Biodegradation*, 151: 104993. doi:10.1016/J.IBIOD.2020.104993.

Mahapatra, D.M., Chanakya, H.N., Joshi, N. V., et al. (2018) *Algae-Based Biofertilizers: A Biorefinery Approach*., pp. 177–196. doi:10.1007/978-981-10-7146-1_10.

Maoka, T. (2020) Carotenoids as natural functional pigments. *Journal of Natural Medicines*, 74 (1): 1. doi:10.1007/S11418-019-01364-X.

Marchi, B., Zanoni, S. and Pasetti, M. (2018) Industrial Symbiosis for Greener Horticulture Practices: The CO2 Enrichment from Energy Intensive Industrial Processes. *Procedia CIRP*, 69: 562–567. doi:10.1016/J.PROCIR.2017.11.117.

Marsland, R., Cui, W., Goldford, J., et al. (2019) Available energy fluxes drive a transition in the diversity, stability, and functional structure of microbial communities. *PLoS Computational Biology*, 15 (2): e1006793. doi:10.1371/journal.pcbi.1006793.

Martínková, L., Uhnáková, B., Pátek, M., et al. (2009) Biodegradation potential of the genus Rhodococcus. *Environment International*, 35 (1): 162–177. doi:10.1016/j.envint.2008.07.018.

Masojídek, J., Ranglová, K., Lakatos, G.E., et al. (2021) Variables Governing Photosynthesis

and Growth in Microalgae Mass Cultures. *Processes 2021, Vol. 9, Page 820*, 9 (5): 820. doi:10.3390/PR9050820.

Matsumoto, M., Sugiyama, H., Maeda, Y., et al. (2010) Marine diatom, Navicula sp. strain JPCC DA0580 and marine green alga, Chlorella sp. Strain NKG400014 as potential sources for biodiesel production. *Applied Biochemistry and Biotechnology*, 161 (1–8): 483–490. doi:10.1007/s12010-009-8766-x.

Mayer, E., de Quadros, P.D. and Fulthorpe, R. (2019) Plantibacter flavus, Curtobacterium herbarum, Paenibacillus taichungensis, and Rhizobium selenitireducens endophytes provide host-specific growth promotion of Arabidopsis thaliana, basil, lettuce, and bok choy plants. *Applied and Environmental Microbiology*, 85 (19). doi:10.1128/AEM.00383-19.

McLeod, M.P., Warren, R.L., Hsiao, W.W.L., et al. (2006) The complete genome of Rhodococcus sp. RHA1 provides insights into a catabolic powerhouse. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (42): 15582–15587. doi:10.1073/pnas.0607048103.

McNair, H.M., Miller, J.M. and Snow, N.H. (2019) Front Matter. *Basic Gas Chromatography*, pp. i–xvii. doi:10.1002/9781119450795.FMATTER.

Meng, X., Yan, D., Long, X., et al. (2014) Colonization by endophytic Ochrobactrum anthropiMn1 promotes growth of jerusalem artichoke. *Microbial Biotechnology*, 7 (6): 601–610. doi:10.1111/1751-7915.12145.

Metting, F.B. (1996) Biodiversity and application of microalgae. *Journal of Industrial Microbiology and Biotechnology*, 17 (5–6): 477–489. doi:10.1007/BF01574779/METRICS.

Min, M., Wang, L., Li, Y., et al. (2011) Cultivating chlorella sp. in a pilot-scale photobioreactor using centrate wastewater for microalgae biomass production and wastewater nutrient removal. *Applied Biochemistry and Biotechnology*, 165 (1): 123–137. doi:10.1007/s12010-011-9238-7.

Mirizadeh, S., Nosrati, M. and Shojaosadati, S.A. (2020) Synergistic Effect of Nutrient and Salt Stress on Lipid Productivity of Chlorella vulgaris Through Two-Stage Cultivation. *Bioenergy Research*, 13 (2): 507–517. doi:10.1007/S12155-019-10077-8/TABLES/7.

Moldoveanu, S. and David, V. (2015a) *Chapter 6 Solvent Extraction*. Elsevier B.V. Available at: http://www.sciencedirect.com:5070/book/9780444543196/modern-sample-preparation-for-chromatography (Downloaded: 10 October 2023).

Moldoveanu, S. and David, V. (2015b) Phase Transfer in Sample Preparation. *Modern Sample Preparation for Chromatography*, pp. 105–130. doi:10.1016/B978-0-444-54319-6.00005-0.

Montagnolli, R.N., Lopes, P.R.M. and Bidoia, E.D. (2015) Screening the Toxicity and Biodegradability of Petroleum Hydrocarbons by a Rapid Colorimetric Method. *Archives of Environmental Contamination and Toxicology*, 68 (2): 342–353. doi:10.1007/S00244-014-0112-9/FIGURES/4.

Moody, J.D., Freeman, J.P., Doerge, D.R., et al. (2001) Degradation of Phenanthrene and Anthracene by Cell Suspensions of Mycobacterium sp. Strain PYR-1. *Applied and Environmental Microbiology*, 67 (4): 1476–1483. doi:10.1128/AEM.67.4.1476-

1483.2001/ASSET/FD2E13ED-7F37-4642-AC0B-72AD34D55221/ASSETS/GRAPHIC/AM041159403B.JPEG.

Moriarty, D.J.W., Pollard, P.C., Hunt, W.G., et al. (1985) Productivity of bacteria and microalgae and the effect of grazing by holothurians in sediments on a coral reef flat. *Marine Biology*, 85 (3): 293–300. doi:10.1007/BF00393250.

Mortensen, L.M. (1987) Review: CO2 enrichment in greenhouses. Crop responses. *Scientia Horticulturae*, 33 (1–2): 1–25. doi:10.1016/0304-4238(87)90028-8.

Mouget, J.L., Dakhama, A., Lavoie, M.C., et al. (1995) Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved? *FEMS Microbiology Ecology*, 18 (1): 35–43. doi:10.1016/0168-6496(95)00038-C.

Mujtaba, G., Rizwan, M. and Lee, K. (2017) Removal of nutrients and COD from wastewater using symbiotic co-culture of bacterium Pseudomonas putida and immobilized microalga Chlorella vulgaris. *Journal of Industrial and Engineering Chemistry*, 49: 145–151. doi:10.1016/J.JIEC.2017.01.021.

Mukherjee, A.K. and Bordoloi, N.K. (2012) Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by Bacillus subtilis and Pseudomonas aeruginosa strains and a formulated bacterial consortium. *Environmental Science and Pollution Research*, 19 (8): 3380–3388. doi:10.1007/s11356-012-0862-8.

Müller, C., Scapini, T., Rempel, A., et al. (2023) Challenges and opportunities for third-generation ethanol production: A critical review. *Engineering Microbiology*, 3 (1): 100056. doi:10.1016/J.ENGMIC.2022.100056.

Musialik-Piotrowska, A., Kordylewski, W., Ciolek, J., et al. (2010) Characteristics of air pollutants emitted from biomass combustion in small retort boiler. *Environment Protection Engineering*, 36 (2): 123–131.

Naik, S.N., Goud, V. V., Rout, P.K., et al. (2010) Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews*. 14 (2) pp. 578–597. doi:10.1016/j.rser.2009.10.003.

Naylor, D., McClure, R. and Jansson, J. (2022) Trends in Microbial Community Composition and Function by Soil Depth. *Microorganisms 2022, Vol. 10, Page 540*, 10 (3): 540. doi:10.3390/MICROORGANISMS10030540.

Nemali, K. (2022) History of Controlled Environment Horticulture: Greenhouses. *HortScience*, 57 (2): 239–246. doi:10.21273/HORTSCI16160-21.

Ni, Z., Wang, Y., Wang, Y., et al. (2020) Comparative Life-Cycle Assessment of Aquifer Thermal Energy Storage Integrated with in Situ Bioremediation of Chlorinated Volatile Organic Compounds. *Environmental Science and Technology*, 54 (5): 3039–3049. doi:10.1021/ACS.EST.9B07020/ASSET/IMAGES/LARGE/ES9B07020_0002.JPEG.

Niehus, R., Picot, A., Oliveira, N.M., et al. (2017) The evolution of siderophore production as a competitive trait. *Evolution*, 71 (6): 1443–1455. doi:10.1111/evo.13230.

NIST chemistry Webbook (1998). doi:10.5860/choice.35-2709.

Numberger, D., Ganzert, L., Zoccarato, L., et al. (2019) Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. *Scientific Reports*, 9 (1): 1–14. doi:10.1038/s41598-019-46015-z.

Obayori, O.S., Ilori, M.O., Adebusoye, S.A., et al. (2008) Pyrene-degradation potentials of Pseudomonas species isolated from polluted tropical soils. *World Journal of Microbiology and Biotechnology*, 24 (11): 2639–2646. doi:10.1007/s11274-008-9790-7.

Odum, H.T. (1983) Maximum power and efficiency: A rebutttal. *Ecological Modelling*, 20 (1): 71–82. doi:10.1016/0304-3800(83)90032-7.

Ogata, M. and Taguchi, T. (1987) Quantitation of urinary metabolites of toluene, xylene, styrene, ethylbenzene, benzene and phenol by automated high performance liquid chromatography. *International Archives of Occupational and Environmental Health 1987 59:3*, 59 (3): 263–272. doi:10.1007/BF00377738.

Okurowska, K., Karunakaran, E., Al-Farttoosy, A., et al. (2021) Adapting the algal microbiome for growth on domestic landfill leachate. *Bioresource Technology*, 319: 124246. doi:10.1016/J.BIORTECH.2020.124246.

Olivieri, G., Gargano, I., Andreozzi, R., et al. (2013) Effects of photobioreactors design and operating conditions on Stichococcus bacillaris biomass and biodiesel production. *Biochemical Engineering Journal*, 74: 8–14. doi:10.1016/j.bej.2013.02.006.

Olivieri, G., Marzocchella, A., Andreozzi, R., et al. (2011) Biodiesel production from Stichococcus strains at laboratory scale. *Journal of Chemical Technology and Biotechnology*, 86 (6): 776–783. doi:10.1002/jctb.2586.

Olson, D.G., McBride, J.E., Joe Shaw, A., et al. (2012) Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology*. 23 (3) pp. 396–405. doi:10.1016/j.copbio.2011.11.026.

Papone, T., Kookkhunthod, S. and Leesing, R. (2012) Microbial Oil Production by Monoculture and Mixed Cultures of Microalgae and Oleaginous Yeasts using Sugarcane Juice as Substrate. *undefined*.

Parker, M.S., Mock, T. and Armbrust, E.V. (2008) Genomic Insights into Marine Microalgae. *Annual Review of Genetics*, 42 (1): 619–645. doi:10.1146/annurev.genet.42.110807.091417.

Patrauchan, M.A., Florizone, C., Eapen, S., et al. (2008) Roles of ring-hydroxylating dioxygenases in styrene and benzene catabolism in Rhodococcus jostii RHA1. *Journal of Bacteriology*, 190 (1): 37–47. doi:10.1128/JB.01122-07.

Peyroux, J., Almahmoudh, I., Prebe-Coquerel, E., et al. (2023) Rapid and automated screening of carbapenemase- and ESBL-producing Gram-negative bacteria from rectal swabs using chromogenic agar media and the ScanStation device Thompson, C.M. (ed.). *Microbiology Spectrum*. doi:10.1128/SPECTRUM.02723-23.

Pinyakong, O., Habe, H. and Omori, T. (2003) The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). *The Journal of general and applied microbiology*, 49 (1): 1–19. doi:10.2323/JGAM.49.1.

Poddar, T.K., Majumdar, S. and Sirkar, K.K. (1996) Membrane-Based Absorption of VOCs

from a Gas Stream. *AIChE Journal*, 42 (11): 3267–3282. doi:10.1002/aic.690421127.

Pogorzelska, E., Godziszewska, J., Brodowska, M., et al. (2018) Antioxidant potential of Haematococcus pluvialis extract rich in astaxanthin on colour and oxidative stability of raw ground pork meat during refrigerated storage. *Meat Science*, 135: 54–61. doi:10.1016/J.MEATSCI.2017.09.002.

Pohnert, G. (2004) "Chemical Defense Strategies of Marine Organisms." In *Topics in current chemistry*. Top Curr Chem. pp. 179–219. doi:10.1007/b95453.

Pröschold, T. and Darienko, T. (2020) The green puzzle Stichococcus (Trebouxiophyceae, Chlorophyta): New generic and species concept among this widely distributed genus. *Phytotaxa*, 441 (2): 113–142. doi:10.11646/PHYTOTAXA.441.2.2.

Prosser, J.I., Bohannan, B.J.M., Curtis, T.P., et al. (2007) The role of ecological theory in microbial ecology. *Nature Reviews Microbiology*, 5 (5): 384–392. doi:10.1038/nrmicro1643.

Qu, W., Zhang, C., Chen, X., et al. (2021) New concept in swine wastewater treatment: development of a self-sustaining synergetic microalgae-bacteria symbiosis (ABS) system to achieve environmental sustainability. *Journal of Hazardous Materials*, 418: 126264. doi:10.1016/J.JHAZMAT.2021.126264.

Ramanan, R., Kim, B.H., Cho, D.H., et al. (2016) Algae-bacteria interactions: Evolution, ecology and emerging applications. *Biotechnology advances*, 34 (1): 14–29. doi:10.1016/J.BIOTECHADV.2015.12.003.

Rauytanapanit, M., Janchot, K., Kusolkumbot, P., et al. (2019) Nutrient deprivation-associated changes in green microalga coelastrum sp. TISTR 9501RE enhanced potent antioxidant carotenoids. *Marine Drugs*, 17 (6): 1–12. doi:10.3390/md17060328.

Reardon, K.F., Mosteller, D.C. and Bull Rogers, J.D. (2000a) Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for Pseudomonas putida F1. *Biotechnology and Bioengineering*, 69 (4): 385–400. doi:10.1002/1097-0290(20000820)69:4<385::AID-BIT5>3.0.CO;2-Q.

Reardon, K.F., Mosteller, D.C. and Bull Rogers, J.D. (2000b) *Biodegradation Kinetics of Benzene, Toluene, and Phenol as Single and Mixed Substrates for Pseudomonas putida F1*. doi:10.1002/1097-0290.

Del Rio-Chanona, E.A., Cong, X., Bradford, E., et al. (2019) Review of advanced physical and data-driven models for dynamic bioprocess simulation: Case study of algae–bacteria consortium wastewater treatment. *Biotechnology and Bioengineering*, 116 (2): 342–353. doi:10.1002/BIT.26881.

Rizwan, M., Lee, J.H. and Gani, R. (2015) Optimal design of microalgae-based biorefinery: Economics, opportunities and challenges. *Applied Energy*, 150: 69–79. doi:10.1016/j.apenergy.2015.04.018.

Roca, A., Rodríguez-Herva, J.J., Duque, E., et al. (2008) Physiological responses of Pseudomonas putida to formaldehyde during detoxification. *Microbial Biotechnology*, 1 (2): 158–169. doi:10.1111/j.1751-7915.2007.00014.x.

Rocha, A.J., Miranda, R. de S., Sousa, A.J.S., et al. (2016) Guidelines for Successful

Quantitative Gene Expression in Real- Time qPCR Assays. *Polymerase Chain Reaction for Biomedical Applications*. doi:10.5772/65850.

Roux, J. (2016) *Migration from fossils to CO2 lifecycle management industries : recommendations to policy makers based on analysis of profitability , ROI and plant economics*., (June).

Rudi, K., Skulberg, O.M., Larsen, F., et al. (1997) Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. *Applied and Environmental Microbiology*, 63 (7): 2593–2599. doi:10.1128/aem.63.7.2593-2599.1997.

Safafar, H., Uldall Nørregaard, P., Ljubic, A., et al. (2016) Enhancement of Protein and Pigment Content in Two Chlorella Species Cultivated on Industrial Process Water. *Journal of Marine Science and Engineering*, 4 (4): 84. doi:10.3390/jmse4040084.

Samarakoon, K. and Jeon, Y.J. (2012) Bio-functionalities of proteins derived from marine algae — A review. *Food Research International*, 48 (2): 948–960. doi:10.1016/J.FOODRES.2012.03.013.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Labortaroy Manual. *Society*, 68: 1232–9. doi:10.1128/AEM.68.3.1232.

Sánchez-Guerrero, M.C., Lorenzo, P., Medrano, E., et al. (2005) Effect of variable CO2 enrichment on greenhouse production in mild winter climates. *Agricultural and Forest Meteorology*, 132 (3–4): 244–252. doi:10.1016/J.AGRFORMET.2005.07.014.

Saxena, P., Sangela, V. and Harish (2020) Toxicity evaluation of iron oxide nanoparticles and accumulation by microalgae Coelastrella terrestris. *Environmental Science and Pollution Research*, 27 (16): 19650–19660. doi:10.1007/s11356-020-08441-9.

Schlundt, C., Mark Welch, J.L., Knochel, A.M., et al. (2020) Spatial structure in the “Plastisphere”: Molecular resources for imaging microscopic communities on plastic marine debris. *Molecular Ecology Resources*, 20 (3): 620–634. doi:10.1111/1755-0998.13119.

Schulze-Makuch, D., Haque, S., De Sousa Antonio, M.R., et al. (2011) Microbial life in a liquid asphalt desert. *Astrobiology*, 11 (3): 241–258. doi:10.1089/ast.2010.0488.

Schwardt, A., Dahmke, A. and Köber, R. (2021) Henry’s law constants of volatile organic compounds between 0 and 95 °C – Data compilation and complementation in context of urban temperature increases of the subsurface. *Chemosphere*, 272: 129858. doi:10.1016/J.CHEMOSPHERE.2021.129858.

Segev, E., Wyche, T.P., Kim, K.H., et al. (2016) Dynamic metabolic exchange governs a marine algal-bacterial interaction. *eLife*, 5 (NOVEMBER2016). doi:10.7554/eLife.17473.

Selvakumaran, S., Kapley, A., Kashyap, S.M., et al. (2011) Diversity of aromatic ring-hydroxylating dioxygenase gene in Citrobacter. *Bioresource Technology*, 102 (7): 4600–4609. doi:10.1016/j.biortech.2011.01.011.

Seyedsayamdost, M.R., Carr, G., Kolter, R., et al. (2011) Roseobacticides: Small molecule modulators of an algal-bacterial symbiosis. *Journal of the American Chemical Society*, 133 (45): 18343–18349. doi:10.1021/ja207172s.

Shade, A. and Handelsman, J. (2012) Beyond the Venn diagram: The hunt for a core microbiome. *Environmental Microbiology*, 14 (1): 4–12. doi:10.1111/J.1462-2920.2011.02585.X.

Sharma, M., Sharma, P. and Kim, J.N. (2013) Solvent extraction of aromatic components from petroleum derived fuels: A perspective review. *RSC Advances*. 3 (26) pp. 10103–10126. doi:10.1039/c3ra00145h.

Sheludchenko, M.S., Kolomytseva, M.P., Travkin, V.M., et al. (2005) Degradation of aniline by Delftia tsuruhatensis 14S in batch and continuous processes. *Applied Biochemistry and Microbiology*, 41 (5): 465–468. doi:10.1007/s10438-005-0083-8.

Shi, Y., Wang, J. and Zhou, R. (2021) Pt-support interaction and nanoparticle size effect in Pt/CeO2–TiO2 catalysts for low temperature VOCs removal. *Chemosphere*, 265: 129127. doi:10.1016/j.chemosphere.2020.129127.

Shigematsu, T., Yumihara, K., Ueda, Y., et al. (2003) Delftia tsuruhatensis sp. nov., a terephthalate-assimilating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology*, 53 (5): 1479–1483. doi:10.1099/ijs.0.02285-0.

Shou, W., Ram, S. and Vilar, J.M.G. (2007) Synthetic cooperation in engineered yeast populations. *Proceedings of the National Academy of Sciences of the United States of America*, 104 (6): 1877–1882. doi:10.1073/pnas.0610575104.

Si, G., Liu, B., Liu, Y., et al. (2022) Light-introduced partial nitrification in an algal-bacterial granular sludge bioreactor: Performance evolution and microbial community shift. *Bioresource Technology*, 354: 127226. doi:10.1016/J.BIORTECH.2022.127226.

Sieg, K., Starokozhev, E., Schmidt, M.U., et al. (2009) Inverse temperature dependence of Henry's law coefficients for volatile organic compounds in supercooled water. *Chemosphere*, 77 (1): 8–14. doi:10.1016/J.CHEMOSPHERE.2009.06.028.

Signer, R., Arm, H. and Daeniker, H. (1969) Untersuchungen über das Verhalten organischer Mischphasen 8. Mitteilung. Dampfdrücke, Dichten, thermodynamische Mischungsfunktionen und Brechungsindices der binären Systeme Wasser-Tetrahydrofuran und Wasser-Diäthyläther bei 25°. *Helvetica Chimica Acta*, 52 (8): 2347–2351. doi:10.1002/HLCA.19690520816.

da Silva Ferreira, V. and Sant'Anna, C. (2017) Impact of culture conditions on the chlorophyll content of microalgae for biotechnological applications. *World Journal of Microbiology and Biotechnology*, 33 (1): 1–8. doi:10.1007/S11274-016-2181-6/TABLES/2.

Singh, U.B. and Ahluwalia, A.S. (2012) Microalgae: a promising tool for carbon sequestration. *Mitigation and Adaptation Strategies for Global Change 2012 18:1*, 18 (1): 73–95. doi:10.1007/S11027-012-9393-3.

Sivakumar, G., Jeong, K. and Lay, J.O. (2014) Bioprocessing of Stichococcus bacillaris strain siva2011. *Biotechnology for Biofuels*, 7 (1). doi:10.1186/1754-6834-7-62.

Sluijs, I., Cadier, E., Beulens, J.W.J., et al. (2015) Dietary intake of carotenoids and risk of type 2 diabetes. *Nutrition, Metabolism and Cardiovascular Diseases*, 25 (4): 376–381. doi:10.1016/J.NUMECD.2014.12.008.

Solymosi, K. and Mysliwa-Kurdziel, B. (2016) Chlorophylls and their Derivatives Used in Food Industry and Medicine. *Mini-Reviews in Medicinal Chemistry*, 17 (13). doi:10.2174/1389557516666161004161411.

Spolaore, P., Joannis-Cassan, C., Duran, E., et al. (2006) Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, 101 (2): 87–96. doi:10.1263/jbb.101.87.

Stachowiak, B. and Szulc, P. (2021) Astaxanthin for the Food Industry. *Molecules 2021, Vol. 26, Page 2666*, 26 (9): 2666. doi:10.3390/MOLECULES26092666.

Stoddard, S.F., Smith, B.J., Hein, R., et al. (2015) rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic acids research*, 43 (Database issue): D593–D598. doi:10.1093/NAR/GKU1201.

Strachan, P.D., Freer, A.A. and Fewson, C.A. (1998) Purification and characterization of catechol 1,2-dioxygenase from Rhodococcus rhodochrous NCIMB 13259 and cloning and sequencing of its catA gene. *Biochemical Journal*, 333 (3): 741–747. doi:10.1042/BJ3330741.

Lo Surdo, J. and Bauer, S.R. (2012) Quantitative approaches to detect donor and passage differences in adipogenic potential and clonogenicity in human bone marrow-derived mesenchymal stem cells. *Tissue Engineering - Part C: Methods*, 18 (11): 877–889. doi:10.1089/ten.tec.2011.0736.

Svec, D., Tichopad, A., Novosadova, V., et al. (2015) How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification*, 3: 9. doi:10.1016/J.BDQ.2015.01.005.

Takemura, A.F., Chien, D.M. and Polz, M.F. (2014) Associations and dynamics of vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology*. 5 (FEB) p. 38. doi:10.3389/fmicb.2014.00038.

Táncsics, A., Szoboszlay, S., Kriszt, B., et al. (2008) Applicability of the functional gene catechol 1,2-dioxygenase as a biomarker in the detection of BTEX-degrading Rhodococcus species. *Journal of Applied Microbiology*, 105 (4): 1026–1033. doi:10.1111/J.1365-2672.2008.03832.X.

Taylor, C.R., Hardiman, E.M., Ahmad, M., et al. (2012) Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. *Journal of Applied Microbiology*, 113 (3): 521–530. doi:10.1111/J.1365-2672.2012.05352.X.

Therien, J.B., Zadvornyy, O.A., Posewitz, M.C., et al. (2014) Growth of Chlamydomonas reinhardtii in acetate-free medium when co-cultured with alginate-encapsulated, acetate-producing strains of Synechococcus sp. PCC 7002. *Biotechnology for Biofuels*, 7 (1): 1–8. doi:10.1186/s13068-014-0154-2.

Timmins, M., Thomas-Hall, S.R., Darling, A., et al. (2009) Phylogenetic and molecular analysis of hydrogen-producing green algae. *Journal of Experimental Botany*, 60 (6): 1691–1702. doi:10.1093/jxb/erp052.

Torsvik, V., Sørheim, R. and Goksøyr, J. (1996) Total bacterial diversity in soil and sediment communities—A review. *Journal of Industrial Microbiology and Biotechnology*, 17 (3–4): 170–178. doi:10.1007/BF01574690.

Trigueros, D.E.G., Módenes, A.N., Espinoza-Quiñones, F.R., et al. (2010) The evaluation of benzene and phenol biodegradation kinetics by applying non-structured models. *Water Science and Technology*, 61 (5): 1289–1298. doi:10.2166/wst.2010.034.

Tsukahara, K. and Sawayama, S. (2005) Liquid fuel production using microalgae. *Journal of the Japan Petroleum Institute*. 48 (5) pp. 251–259. doi:10.1627/jpi.48.251.

TURNER, S., PRYER, K.M., MIAO, V.P.W., et al. (1999) Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis. *The Journal of Eukaryotic Microbiology*, 46 (4): 327–338. doi:10.1111/j.1550-7408.1999.tb04612.x.

Udayan, A., Pandey, A.K., Sirohi, R., et al. (2023) Production of microalgae with high lipid content and their potential as sources of nutraceuticals. *Phytochemistry Reviews*, 22 (4): 1. doi:10.1007/S11101-021-09784-Y.

Ullah, K., Ahmad, M., Sofia, et al. (2014) Algal biomass as a global source of transport fuels: Overview and development perspectives. *Progress in Natural Science: Materials International*, 24 (4): 329–339. doi:10.1016/j.pnsc.2014.06.008.

Vadiee, A. and Martin, V. (2012) Energy management in horticultural applications through the closed greenhouse concept, state of the art. *Renewable and Sustainable Energy Reviews*, 16 (7): 5087–5100. doi:10.1016/J.RSER.2012.04.022.

Vaillancourt, F., Bolin, J. and Eltis, L. (2006) The ins and outs of ring-cleaving dioxygenases. *Critical Reviews in Biochemistry and Molecular Biology*. 41 (4) pp. 241–267. doi:10.1080/10409230600817422.

Velupillaimani, D. and Muthaiyan, A. (2019) Potential of Bacillus subtilis from marine environment to degrade aromatic hydrocarbons. *Environmental Sustainability 2019 2:4*, 2 (4): 381–389. doi:10.1007/S42398-019-00080-2.

Villacorte, L.O., Ekowati, Y., Neu, T.R., et al. (2015) Characterisation of algal organic matter produced by bloom-forming marine and freshwater algae. *Water Research*, 73: 216–230. doi:10.1016/j.watres.2015.01.028.

Vishnuvardhan Reddy, S., Thirumala, M., Kishore Reddy, T. V., et al. (2008) Isolation of bacteria producing polyhydroxyalkanoates (PHA) from municipal sewage sludge. *World Journal of Microbiology and Biotechnology*, 24 (12): 2949–2955. doi:10.1007/S11274-008-9839-7/FIGURES/1.

Wackett, L.P., Kwart, L.D. and Gibson, D.T. (1988) Benzylic monooxygenation catalyzed by toluene dioxygenase from Pseudomonas putida. *Biochemistry*, 27 (4): 1360–1367. doi:10.1021/BI00404A041.

Wang, A., Lv, J., Wang, J., et al. (2022) CO2 enrichment in greenhouse production: Towards a sustainable approach. *Frontiers in Plant Science*, 13: 1029901. doi:10.3389/FPLS.2022.1029901/BIBTEX.

Wang, C., Yuan, T., Wood, S., et al. (2017) Uncertain Henry's law constants compromise equilibrium partitioning calculations of atmospheric oxidation products. *Atmospheric Chemistry and Physics*, 17 (12): 7529–7540. doi:10.5194/ACP-17-7529-2017.

Wang, E.X., Ding, M.Z., Ma, Q., et al. (2016) Reorganization of a synthetic microbial consortium for one-step vitamin C fermentation. *Microbial Cell Factories*, 15 (1). doi:10.1186/s12934-016-0418-6.

Wang, Y., Tibbetts, S.M. and McGinn, P.J. (2021) Microalgae as Sources of High-Quality Protein for Human Food and Protein Supplements. *Foods*, 10 (12). doi:10.3390/FOODS10123002.

Ward, A.L., Reddyvari, P., Borisova, R., et al. (2018) An inhibitory compound produced by a soil isolate of Rhodococcus has strong activity against the veterinary pathogen R. equi. *PLoS ONE*, 13 (12). doi:10.1371/JOURNAL.PONE.0209275.

Weinberger, F., Friedlander, M. and Gunkel, W. (1994) A bacterial facultative parasite of Gracilaria conferta. *Diseases of Aquatic Organisms*, 18 (2): 135–141. doi:10.3354/dao018135.

Williams, P.J.L.B. and Laurens, L.M.L. (2010) Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. *Energy and Environmental Science*. 3 (5) pp. 554–590. doi:10.1039/b924978h.

Wirth, R., Lakatos, G., Maróti, G., et al. (2015) Exploitation of algal-bacterial associations in a two-stage biohydrogen and biogas generation process Philippe Soucaille. *Biotechnology for Biofuels*, 8 (1): 59. doi:10.1186/s13068-015-0243-x.

Xia, M., Liu, Y., Taylor, A.A., et al. (2017) Crude oil depletion by bacterial strains isolated from a petroleum hydrocarbon impacted solid waste management site in California. *International Biodeterioration and Biodegradation*, 123: 70–77. doi:10.1016/j.ibiod.2017.06.003.

Xie, B., Bishop, S., Stessman, D., et al. (2013) Chlamydomonas reinhardtii thermal tolerance enhancement mediated by a mutualistic interaction with vitamin B12-producing bacteria. *ISME Journal*, 7 (8): 1544–1555. doi:10.1038/ismej.2013.43.

Yao, Y., Lv, Z., Min, H., et al. (2009) Isolation, identification and characterization of a novel Rhodococcus sp. strain in biodegradation of tetrahydrofuran and its medium optimization using sequential statistics-based experimental designs. *Bioresource Technology*, 100 (11): 2762–2769. doi:10.1016/J.BIORTECH.2009.01.006.

Yoon, S.R., Kim, D.H. and Lee, O. (2020) *Deuterostichococcus epilithicus , and Pseudostichococcus monallantoides in Korea*., 9 (3): 181–190. doi:10.12651/JSR.2020.9.3.181.

Yu, X., Jiang, W., Shi, Y., et al. (2019) Applications of sequencing technology in clinical microbial infection. *Journal of Cellular and Molecular Medicine*, 23 (11): 7143. doi:10.1111/JCMM.14624.

Zanchetta, E., Damergi, E., Patel, B., et al. (2021) Algal cellulose, production and potential use in plastics: Challenges and opportunities. *Algal Research*, 56: 102288. doi:10.1016/J.ALGAL.2021.102288.

Zhang, S.J., Du, X.P., Zhu, J.M., et al. (2020) The complete genome sequence of the algicidal bacterium Bacillus subtilis strain JA and the use of quorum sensing to evaluate its antialgal ability. *Biotechnology Reports*, 25: e00421. doi:10.1016/j.btre.2020.e00421.

Zhang, Y., Su, H., Zhong, Y., et al. (2012) The effect of bacterial contamination on the heterotrophic cultivation of Chlorella pyrenoidosa in wastewater from the production of soybean products. *Water Research*, 46 (17): 5509–5516. doi:10.1016/j.watres.2012.07.025.

Zhu, S., Wang, Y., Huang, W., et al. (2014) Enhanced Accumulation of Carbohydrate and Starch in Chlorella zofingiensis Induced by Nitrogen Starvation. *Applied Biochemistry and Biotechnology*, 174 (7): 2435–2445. doi:10.1007/S12010-014-1183-9/FIGURES/4.

Zhu, X., Han, Y., Feng, Y., et al. (2022) Formation and emission characteristics of intermediate volatile organic compounds (IVOCs) from the combustion of biomass and their cellulose, hemicellulose, and lignin. *Atmospheric Environment*, 286: 119217. doi:10.1016/J.ATMOSENV.2022.119217.

Zhuang, L., Zhao, L. and Yin, P. (2018) Combined algicidal effect of urocanic acid,: N - acetylhistamine and l-histidine to harmful alga Phaeocystis globosa. *RSC Advances*, 8 (23): 12760–12766. doi:10.1039/c8ra00749g.

Zomorrodi, A.R. and Segrè, D. (2016) Synthetic Ecology of Microbes : Mathematical Models and Applications. *Journal of Molecular Biology*, 428 (5): 837–861. doi:10.1016/j.jmb.2015.10.019.

Zörn, G., Vázquez-Boland, J.A., Navas Néstor Ladrón, J., et al. (2022) Rapid identification of Rhodococcus equi by a PCR assay targeting the choE gene. *Journal of Clinical Microbiology*, 41 (7): 3241. doi:10.1128/JCM.41.7.3241-3245.2003.