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BSc in Biology

POLYHYDROXYALKANOATES PRODUCTION FROM CO₂ USING PHOTOTROPHIC MIXED CULTURES

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Polyhydroxyalkanoates production from CO₂ using phototrophic mixed cultures

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Para a minha família.

ABSTRACT

Two of the current major environmental problems include the rise of CO₂ in the atmosphere and plastic pollution, creating the need to implement bio-based and biodegradable plastics on a wider scale. The use of Polyhydroxyalkanoates (PHAs) has emerged as a solution, but despite its advantages, the production costs are still too high compared to conventional plastics. This study involved keeping a lab-scale sequencing batch reactor (SBR) with a strictly anaerobic photosynthetic mixed culture (PMC), in order to try to reduce some of these costs, by removing the need of sterilization and aeration. The SBR was maintained with a continuous supply of inorganic carbon and a cyclic supply of sulfide under a feast and famine regime, attaining a biomass concentration of 0.7 ± 0.2 g/L of VSS. A representative full-cycle showed specific consumption rates for sulfides and inorganic carbon of 0.3 mmol SS/Cmmol Xi·d and 1 mmol IC/ Cmmol Xi·d, respectively. The glycogen and PHA contents detected were minimal, with an average of $6.2 \pm 2.2\%$ GLY/VSS, and a maximum of 3% PHA/VSS.

Parallel batch studies evaluated the impact of light intensity on the culture performance. These did not show a linear variation in the rates of consumption of inorganic carbon or sulfides, nor in the production of glycogen or PHA contents. However, in a test performed without light, higher PHA values were detected ($10.9 \pm 2.2\%$ PHA/VSS) and this could result from the anaerobic conversion of glycogen into PHA. This suggests that a possible means of increasing PHA production could occur through the implementation of dark/light cycles.

Additionally, two samples were tested for a possible new inocula for the SBR. One of them, collected from a creek in Covilhã, which waters are microbially rich in purple bacteria, seemed to be a good candidate as it grew with inorganic carbon and sulfides, and demonstrated visible PHA granules.

Finally, due to the limited availability of the gas chromatography (GC) equipment needed for PHA analysis, alternative protocols for detecting PHA were tested. Furthermore, to prevent equipment damage, a series of tests were performed to eliminate any possible sulfur residues

from the biomass samples and tried to identify any compounds that could damage the equipment. No compound capable of that was found.

Keywords: Bioreactor; Mixed microbial cultures; Phototrophic system; Biodegradable polymers; Polyhydroxyalkanoates; CO₂ capture; Purple bacteria.

RESUMO

Dois dos maiores problemas ambientais atuais incluem o aumento da concentração do CO₂ na atmosfera, assim como a grande acumulação de plásticos convencionais, havendo a necessidade de implementação de plásticos bio produzidos e biodegradáveis em larga escala. É neste contexto que a produção de particularmente, de polihidroxialcanoatos (PHAs) surge como possível solução. Apesar das suas vantagens os seus custos de produção ainda não conseguem competir com os dos plásticos convencionais.

Neste trabalho foi mantido um biorreator SBR em escala de laboratório, com a cultura mista fotossintética estritamente anaeróbia, de modo a reduzir os custos de operação, removendo a necessidade de esterilização e arejamento. O SBR foi mantido com presença constante de carbono, e num sistema cíclico de feast-famine de sulfuretos, com uma concentração de biomassa de 0.7 ± 0.2 g/L de VSS. Um full-cycle de referência apresentou velocidades específicas de consumo de sulfuretos e de carbono de 0.3 mmol SS/Cmmol Xi-d e de 1 mmol IC/ Cmmol Xi-d, respetivamente, e conteúdos de glicogénio e PHA mínimos, com uma média de $6.2 \pm 2.2\%$ de GLY/VSS, e um máximo de 3% de PHA/VSS.

Em paralelo, um estudo em testes batch, com um aumento gradual da intensidade de luz, demonstrou que esta não afeta linearmente as taxas de consumo de carbono inorgânico nem de sulfuretos, nem a produção de conteúdo de glicogénio nem de PHA. Num teste sem luz detetaram-se valores de PHA mais elevados ($10.9 \pm 2.2\%$ de PHA/VSS;), podendo resultar da conversão do glicogénio em PHA.

Foram ainda testadas duas amostras para um possível novo inóculo para o SBR. Uma coletada numa ribeira na Covilhã, cujas águas são ricas em bactérias púrpura, mostrou ser um possível candidato pois cresceu com carbono inorgânico e sulfuretos, e apresentou grânulos visíveis de PHA no final do estudo.

Por fim, devido a uma disponibilidade limitada com o equipamento de cromatografia gasosa (GC), necessário para a realização do protocolo de deteção de PHA, foram testados protocolos alternativos. Ademais, para prevenir a danificação do equipamento uma série de testes para

remoção de possíveis resíduos de enxofre na biomassa foram realizados, assim como tentativas de identificação de compostos, que pudesse danificar o equipamento. Tais compostos não foram encontrados.

Palavras chave: Birreator; cultura mista microbiana; sistema fototrófico, polímeros biodegradáveis, polihidroxicanoatos; captura de CO₂; bactérias purpura.

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Tubes 5 and 6: 1 wash with ethanol 50% and 1 wash with ethanol 98%. **Tubes 7 and 8:** 1 wash with ethanol 50% and 2 washes with ethanol 98%. All the washes were made for the attempt with no time of incubation, and for the attempt with 15 minutes of incubation. For the last attempt, with incubation overnight, the last tubes (7 and 8) were not prepared. 80

ACRONYMS

ATP	Adenosine triphosphate
APB	Anoxygenic photosynthetic bacteria
Bchl	Bacteriochlorophyll
CDW	Cell dry weight
Chl	Chlorophyll
EDTA	Ethylenediaminetetraacetic acid solution
ETC	Electron transport chain
F/F	Feast to Famine ratio
FF	Feast and Famine
FISH	Fluorescence <i>in situ</i> Hybridisation
GC	Gas chromatography
GLY	Glycogen
HAs	Hydroxyalkanoates
HD	Heptadecane
HD@HCl ₃	Chloroform with heptadecane as internal standard
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IC	Inorganic carbon
Lcl-PHAs	Long chain length polyhydroxyalkanoates
LHC	Light-harvesting complexes
Mcl-PHAs	Medium chain length polyhydroxyalkanoates
MMCs	Mixed microbial cultures
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NH ₄	Amonium
OD	Optical density
ORP	Oxidation-reduction potential

P(3HB-co-3HV)	Poly(3 -hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB)	Poly(3-hydroxybutyrate)
P(3HHx)	Poly (3-hydroxyhexanoate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(3HV)	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
PBS	Phosphate Buffer Saline
PCL	Polycaprolactone
PE	Polyethylene
PET	Polyethylene terephthalate
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PLA	Poly(lactic acid)-based
PMCs	Phototrophic mixed cultures
PNSB	Purple Non-Sulfur Bacteria
PO ₄	Phosphate
PP	Polypropylene
PPB	Phototrophic purple bacteria culture
PS	Photosystem
PSB	Purple Sulfur Bacteria
RC	Reaction center
SBR	Sequencing batch reactor
Scl-PHAs	Short chain length polyhydroxyalkanoates
SRT	Sludge retention time
SS	Soluble Sulfides
ST	Total Sulfides
TC	Total Carbon
TCA	Tricarboxylic Acid Cycle
TOC	Total Organic Carbon
TPS	Thermoplastics starch polymers
TSS	Total Suspended Solids
WWTP	Wastewater treatment plants
VFAs	Volatile fatty acids

VSS	Volatile Suspended Solids
X_i	Active Biomass
$-q_{SS}$	Specific substrate uptake rate for soluble sulfides
$-q_{IC}$	Specific substrate uptake rate for inorganic carbon

INTRODUCTION

1.1 CO₂ pollution

As we all know, the world population growth is increasing the demand for raw materials and leading to the overexploitation of Earth's natural resources. Furthermore, human activities like the burning of fossil fuels lead to emissions of vast amounts of CO₂ into the atmosphere, having a devastating impact on the ecosystems with severe consequences. Data shows that over the last 800,000 years there have always been fluctuations in CO₂ levels (Figure 1-1), yet, never exceeding 300 ppm [1]. However, this changed in the last century, and the latest reports show that a value of 419.25 ppm was achieved in July 2023 [8].

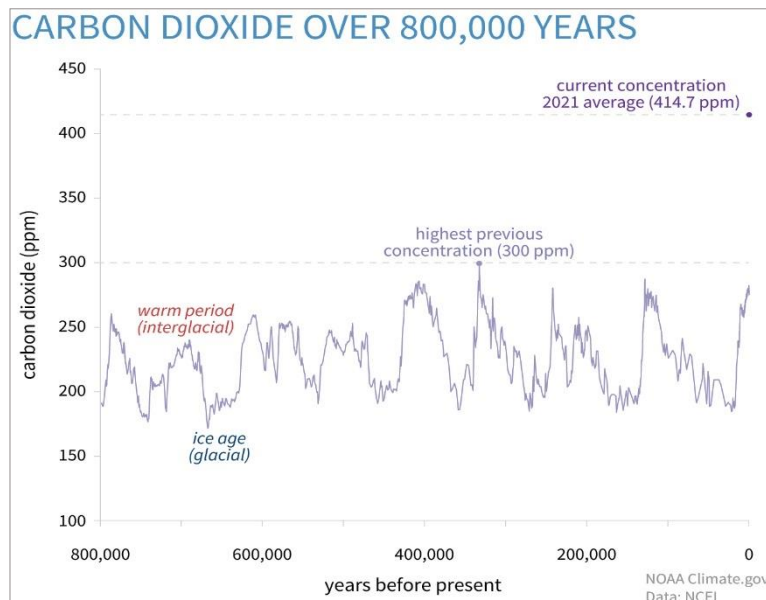


Figure 1-1: Global atmospheric carbon dioxide concentrations (CO₂) in parts per million (ppm) for the past 800,000 years [1].

CO₂ is one of the Earth's long-lived greenhouse gases of most concern since it absorbs and radiates heat gradually over time, and even though it absorbs less than other greenhouse

gases, like methane or nitrous oxide, it is much more abundant (80% of greenhouse gases in the air) and remains in the atmosphere for much longer [9]. For millions of years plants have captured carbon from the atmosphere, and yet we are releasing it in just a few hundred years by burning fossil fuels, such as coal and oil, for energy. So, each year we release more CO₂ than natural processes can remove, resulting in rising global amounts [1]. According to observations by the NOAA Global Monitoring Lab, in 2021 two-thirds of the total heating caused by human-produced greenhouse gases, can be attributed to CO₂ alone [1].

The amount of CO₂ in the atmosphere has reached a point where it's becoming more concerning with each passing day, and more important than ever, it is vital to create a more sustainable society, and to develop technologies that can tackle CO₂ emissions.

1.2 Plastic Pollution

Another huge concern in today's society is the accumulation of plastics. In the last decades, the growth of the plastic production industries increased exponentially, due to the attractive properties of plastics which can be easily molded into a variety of desired products [2]. Indeed, plastics present favorable properties like longevity, lightweight, water resistance, high elasticity, strength, durability, resistance to corrosion, ease of transport, and low cost of production [2].

According to Plastic Europe, 367 million tons of plastic were produced worldwide in 2020. In Europe alone, 55 million tons of plastic were produced to meet market demand of 49.1 million tons. The European plastics value chain, which consists of plastics producers, converters, recyclers, and machinery manufacturers, has experienced a decline both in demand and production over the past years as a result of the COVID crisis. Nonetheless, manufacturing plastic industries continued to grow, with the major end-use markets being packaging, and building and construction (Figure 1-2) [10].

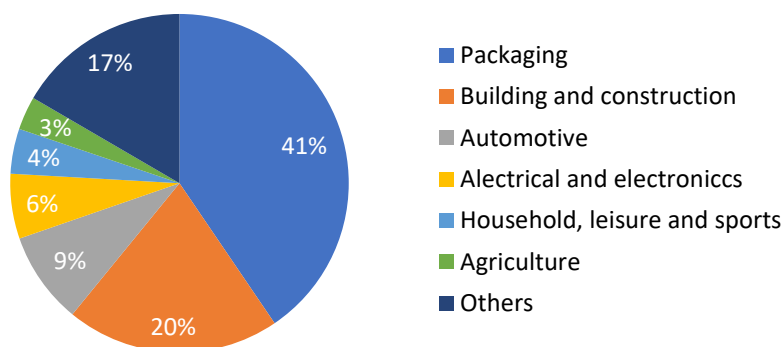


Figure 1-2: Distribution of European plastics demand by segment in 2020. (Others: plastic for furniture, medical applications, machinery, mechanical engineering, technical parts, etc.) [10].

However, coupled with the large production of plastics also comes a large accumulation of waste. It's needed to consider that petroleum products obtained through refining processes are frequently the most used raw materials in the production of conventional plastics, and these are non-biodegradable causing vast complications of environmental pollution [11]. The absence of proper disposal methods for this sort of material is critical as plastic waste is endangering human and wildlife health, and the environment by overflowing landfills, leaching into the soil, transferring through the air, and clogging up waterways [2], with an estimated 10 million tons of litter leaking into the marine environment every year [12].

Therefore, while durability is one of this material's most prized qualities, it also presents one of its biggest detrimental factors. Petroleum-derived plastics, in fact, never degrade completely, as they disintegrate into smaller pieces through hundreds of years [2], and when disposed of in landfills the degradation rates are tremendously slow [13]. Other methods like incineration generate toxic by-products and are expensive, and recycling can be done, but it is a very time-consuming process that requires sifting through a large range of discarded plastics and is constrained by the presence of additive substances (e.g. coverings, fillers and coloring materials) [13]. According to a report by the United Nations Environment Program, from the 300 million tons of plastic waste generated globally per year, only 9% of plastic waste is recycled (<https://www.unep.org/news-and-stories/story/plastic-recycling-underperforming-sector-ripe-remake>. Accessed 10 June 2023)

One way to overcome the lack of good disposal techniques is to use renewable materials, such as biodegradable polymers, with equivalent physicochemical properties, to replace traditional petroleum-based plastics [2].

1.3 Circularity of Resource Flow

The primary cause of plastic pollution is its linear life cycle, in which plastic is produced, utilized, and then discarded. Preventing these single-use plastics from entering the ecosystem in the first place would be a preferable strategy to counteract the environmental problems. Reusing and potentially recovering the resources that plastics are made of are some strategies that can be implemented to reverse this linearity, in other words, introducing a circularity of resource flow [14]. This can be accomplished by sustainably producing bio-sourced plastics from

renewable bio-based feedstocks, which reduces reliance on non-renewable resources (like fossil fuels). Or to use biodegradable plastics, that can be biodegraded under certain conditions by microorganisms without releasing harmful products into the environment [14].

1.4 Biodegradable plastics

The term "biodegradability" refers to the capacity of microorganisms to biodegrade organic matter, throughout their food chain, in a quick, safe, and efficient manner [3]. It consists of two stages, as shown in Figure 1-3: fragmentation of long polymer chains (under the effect of heat, humidity, sunlight, and/or enzymes) and mineralization (complete assimilation of the fragments by microbial populations in a particular environment) [3]. A plastic product must fully complete both phases to be considered biodegradable, and if not entirely assimilated by the microbial population in a short amount of time, it can present a potential risk to the environment [3].

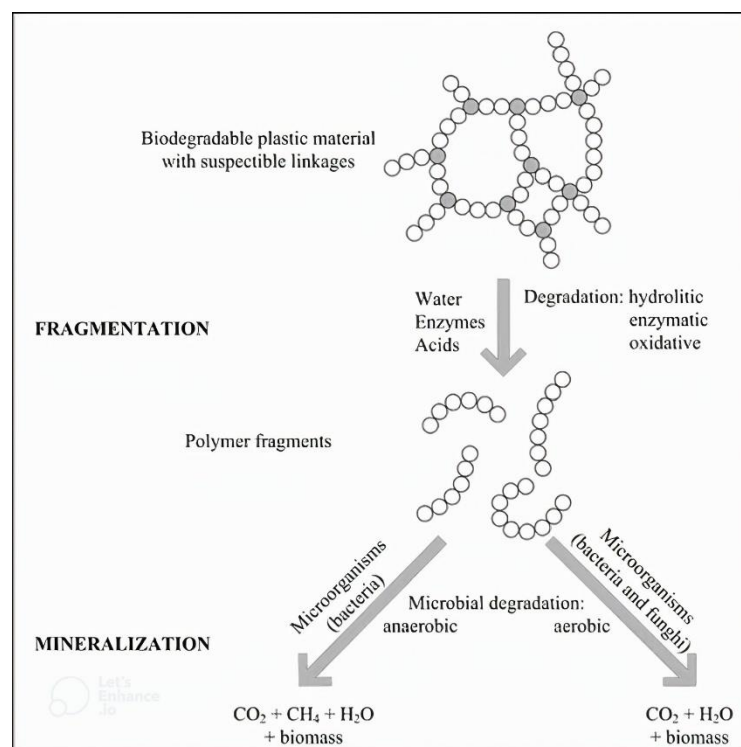


Figure 1-3: Biodegradation stages of biodegradable plastics [3].

There are various types of biodegradable plastics, and it should be noted that not all biobased polymers, such as biobased polyethylene [PE], are biodegradable, and there are biodegradable plastics that are not biobased (e.g., fossil-based polycaprolactone [PCL]) [3]. Some of the aspects that might impact the biodegradability of plastic are the raw materials utilized, the chem-

ical composition, and the structure of the final product. And to biodegrade, they require specific conditions, particularly the location and temperature, which are often industrial composting facilities that can achieve temperatures exceeding 50°C for extended periods of time [3].

1.4.1 Biobased biodegradable plastics

Within the biobased and biodegradable plastics there are some with higher importance, like starch-based (thermoplastics starch polymers [TPS]), polylactic acid-based (PLA), and polyhydroxyalkanoates (PHA)-based plastics [3].

Starch is a carbohydrate produced by most higher plants, that's highly available, has low costs of production, and has total biodegradability. However, natural starch has limited applications due to some of its mechanical properties (brittleness and hydrophilic character). To make TPS, the natural starch needs a pre-treatment, involving complex chemical, mechanical and thermal processing [3]. Starch can also be used as a filler and cross-linking agent to create a starch-plastic composite (e.g., starch-polyethylene [PE]), although it would only be partially biodegradable since only the starch degrades, leaving the remaining fragments in the environment [15].

PLA is the most used biodegradable aliphatic polyester, having tensile properties that vary depending on whether it is annealed or oriented, as well as the degree of crystallinity [16]. Lactic acid can be obtained through the fermentation of glucose, which is acquired from a variety of sugar sources (e.g., sugar cane, corn, potatoes, or tapioca). Although it has certain disadvantages such as brittleness and stiffness, it also has mechanical qualities similar to other conventional plastics such as polyethylene terephthalate (PET) and polypropylene (PP) [3].

PHAs are natural aliphatic polyesters of various hydroxyalkanoates (HAs), synthesized by a wide variety of microorganisms and stored intracellularly as carbon and energy reserve when growing in stressful conditions [3]. They have similar mechanical properties to thermoplastics such as PP and PE and can be used in their stead [15].

POLYHYDROXYALKANOATES (PHAs)

Thus far, more than 150 different types of PHAs have been identified, in more than 300 different microorganisms that can accumulate it both aerobically and/or anaerobically [17]. PHAs are structurally varied polymers with molecular weights ranging from 5×10^4 to 2×10^7 Da, depending upon the microbial species, and the growth conditions used (e.g., carbon source and its concentration, temperature, pH, and fermentation mode [batch, fed-batch, or continuous]) [13][4]. Figure 2-1 depicts its molecule configuration, which is typically composed of 600 to 35000 (*R*)-hydroxy fatty acid monomer units, connected by ester bonds, where each monomer unit has a side chain *R* group (e.g., an alkyl group or unsaturated alkyl group, a branched alkyl group, or substituted alkyl) [18]. PHAs are classified into three groups based on the structure and number of carbon atoms in the chain, as well as its branching:

- **Short chain length PHAs (Scl-PHA)**, contain 3-5 carbon atoms and include poly(3-hydroxybutyrate) P(3HB), poly(4-hydroxybutyrate) P(4HB), poly(3-hydroxyvalerate) P(3HV), and the heteropolymer including poly (3-hydroxybutyrate-copolymer-3-hydroxyvalerate) P(3HB-co-3HV) [19].
- **Medium chain length PHAs (Mcl-PHA)**, consists of 6-14 carbon atoms and comprise both homopolymers such as poly (3-hydroxyhexanoate) P(3HHx) and poly(3-hydroxyoctanoate) P(3HO), and heteropolymers such poly (3-hydroxyhexanoate-copolymer-3-hydroxyoctanoate) P(3HHx-co-3HO) [19].
- **Long chain length PHAs (Lcl-PHA)**, have more than 14 carbon atoms in the polymeric chain [19].

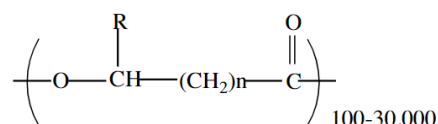


Figure 2-1: General formula of PHA [13].

The length of the side chain and its functional group influence the physical properties of the polymers [20]. So, by changing certain parameters (e.g., feedstock, bacterial source, fermentation conditions) the PHA structure can then be altered physically, chemically, or biologically in a way to fit specific applications [17][20]. Table 2.1 shows the main distinctions between Mcl-PHAs, Scl-PHAs, and PP (a conventional petrochemical-based plastic). In comparison to Scl-PHAs and PP, Mcl-PHAs exhibit a lower crystallinity, tensile strength, melting point, and glass transition temperatures, but higher elongation break. Scl-PHAs, however, have more tensile strength than PP [20].

Table 2.1: Comparison between physical properties of Mcl-PHAs, Scl-PHAs, and polypropylene [20].

Properties	Mcl-PHAs	Scl-PHAs	Polypropylene
Crystallinity (%)	20 - 40	40 - 80	70
Melting point (°C)	30 - 80	53 - 80	176
Density (g-cm ⁻³)	1.05	1.25	0.91
Tensile strength (MPa)	20	43 - 04	34
Glass transition temperature (°C)	- 40 - 150	-148 - 4	-10
Extension to break (%)	300 - 450	6 - 1000	400
UV light resistant	Good	Good	Poor
Solvent resistant	Poor	Poor	Good
Biodegradability	Good	Good	None

This ability to alter the polymer properties (e.g., mechanical characteristics, amphiphilic nature, surface structure, and rate of degradation) and make it suitable for particular applications, or useful for a wide range of them, is one of the main benefits of PHAs [17]. Above all, blending PHA with other polymers, altering the surface, or combining it with other inorganic materials is a way to further improve its mechanical properties and biocompatibility [20].

2.1 PHAs biosynthesis

PHAs are produced and accumulated intracellularly when there is an excess of carbon source, and an imbalance of essential growth nutrients. This confers an advantage for the microorganisms able to store them, as they can use them as carbon and energy sources during starvation conditions [19].

The catalytic mechanism for PHA production involves different pathways linked to the bacterium's central metabolic pathways, such as glycolysis, β -oxidation, tricarboxylic acid cycle (TCA), *de novo* fatty acids synthesis, amino acid catabolism, Calvin Cycle, and serine pathway [18]. Figure 2-2 shows the three naturally occurring PHA biosynthetic pathways.

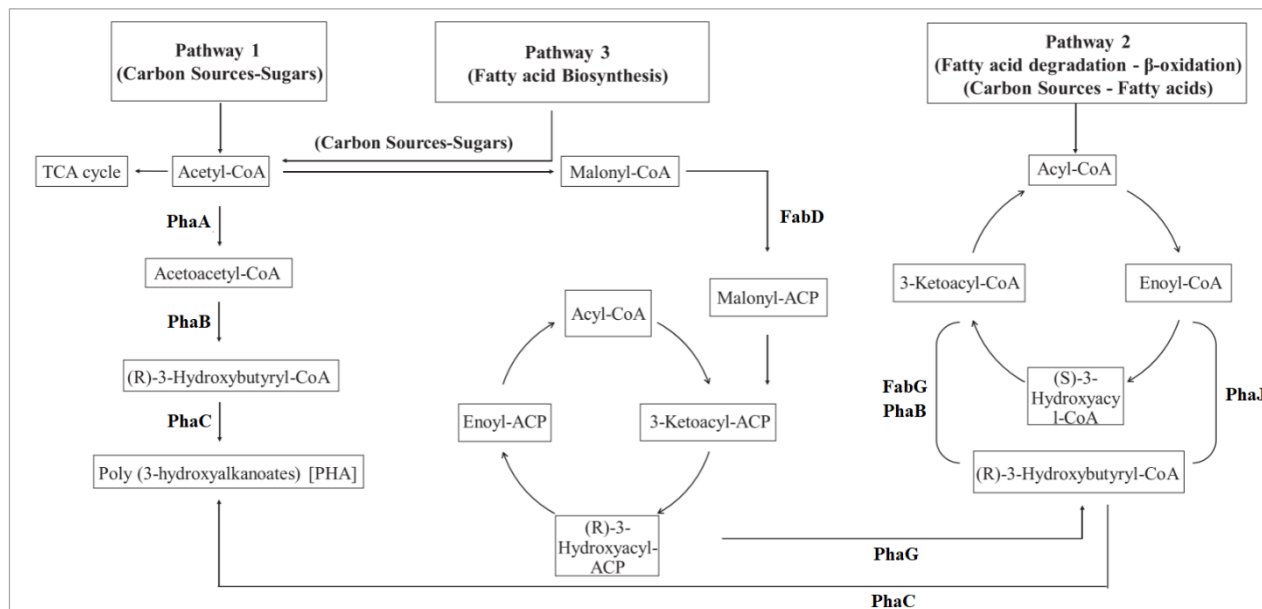


Figure 2-2: Representation of microbial biosynthesis pathways of PHAs (**PhaA** = 3-Ketothiolase, **PhaB** = Acetoacetyl-CoA reductase, **PhaC** = PHA synthase, **FabG** = 3-oxoacyl-[acyl-carrier-protein] reductase, **PhaJ** = (R)-specific enoyl-CoA hydratase, **PhaG** = (R)-3-hydroxydecanoyl-ACP:CoA transacylase, **FabD** = malonyl-CoA-ACP transacylase) [19].

• Pathway 1

This is the best-known pathway, represented by the model organism *Cupriavidus necator*, and is usually used to produce Scl-PHAs. Firstly, the carbohydrates are converted to acetyl-CoA molecules, in the TCA cycle. Two molecules of acetyl-CoA are then condensed into a molecule of acetoacetyl-CoA with the help of 3-ketothiolase enzyme (*PhaA*). Acetoacetyl-CoA is then converted into (R)-3-hydroxybutyryl-CoA by an NADPH dependent enzyme acetoacetyl-CoA reductase (*PhaB*); followed by the action of PHA synthase (*PhaC*) which finally catalyzes the ester bond formation in (R)-3-hydroxybutyryl-CoA to generate P(3HB). While carbon and nutrients are available in the media, bacteria grow normally and, there is an inhibition of the 3-ketothiolase (*PhaA*) due to the availability of free coenzyme-A from the TCA cycle. However, in the absence of essential nutrients, the access of acetyl-CoA into the TCA cycle is controlled, and the excess acetyl-CoA is directed toward the synthesis of PHB molecules. The PHA-accumulating microorganisms can then maximize nutrient resources in their adaptability to environmental conditions thanks to this metabolic regulation strategy [17][19].

- **Pathway 2**

In this pathway, the substrates originate from the β -oxidation pathway of fatty acids since these are a suitable carbon source. The first step is the oxidation of fatty acids that are subsequently converted to (R)-3-Hydroxyacyl-CoA by the action of (R)-enoyl-CoA hydratase (*PhaI*). The enzyme PHA synthase (*PhaC*) catalyzes then the polymerization of hydroxyalkanoate monomers [17].

- **Pathway 3**

This pathway is of significant interest because generates monomers for PHA synthesis from structurally simple and inexpensive carbon sources such as glucose, sucrose, and fructose. Microorganisms can obtain this carbon from environmental sources (wastewater, activated sludge, animal fats, hydrocarbons). The (R)-3-hydroxyacyl intermediates from the fatty acid *de novo synthesis* are converted from their acyl carrier protein (ACP) form to the CoA form by the enzyme acyl-ACP-CoA transacylase (*PhaG*). This enzyme is the key link between fatty acid synthesis and PHA [17][21].

2.2 PHAs biodegradability

One of the most crucial capabilities of PHAs is their ability to be biodegraded, intra or extracellularly, by PHA-producing bacteria, via PHA hydrolases or PHA depolymerases [22]. Their biodegradability is influenced by the polymer properties such as chemical composition, polymeric chain length, crystallinity, and complexity, as well as environmental conditions (e.g., temperature, pH, moisture, and oxygen content) [22]. Many microorganisms excrete PHA depolymerases that hydrolase PHA into water-soluble oligomers and monomers, which can be used as a carbon source for biomass growth [15]. And many can degrade them intracellularly, by using the same enzyme to break down P(3HB) to produce 3-hydroxybutyric acid, which is then oxidized to acetyl-acetate by a dehydrogenase and finally broken down to acetyl-CoA by a β -ketothiolase [21]. The biodegradation of PHAs can occur both in aerobic (forming water and CO₂) or anaerobic conditions (forming methane), without forming toxic products [22].

2.3 PHAs main properties and drawbacks

Due to their beneficial features (e.g., hydrophobicity, nontoxicity, biocompatibility, biodegradability, etc.), PHAs have been widely studied for decades, being established as a suitable substitute for traditional plastics [4]. They have excellent properties that make them ideal for

the packaging sector, including good tensile strength, printability, flavor and odor barriers, heat sealability, grease and oil resistance, temperature stability, and ease of dyeing [11]. Furthermore, life cycle analysis of their production has revealed that, in general, the manufacture and application of PHAs are more sustainable than synthetic polymers in terms of energy consumption and greenhouse gas emissions [19]. But, despite the success of industrial-scale production of PHAs, traditional methods involve pure aerobic cultures, associated with expensive carbon substrates, high-cost procedures such as fermentation, extraction, and purification processes, a large amount of freshwater consumption, and energy-intensive aeration [4][5]. As a result, the commercialization of PHAs continues to lag behind that of synthetic plastics, as PHAs produced by industrial biotechnology procedures cost 2.4-5.5 US\$/kg, which is several times higher than petroleum-based plastics (1.2 US\$/kg) [5]. To make bacterial PHAs manufacturing more cost-effective, there has been considerable interest, in recent years, in developing potential alternative procedures aimed at lowering polymer production costs. These include screening and selection of potential bacterial strains, synthesis pathways, carbon and nitrogen source, and cost-efficient downstream processes [17][19].

2.4 PHA Production: State of The Art

2.4.1 Single strain systems

To date, industrial production of PHA is traditionally based on the use of pure cultures of microorganisms in their wild form, or genetically engineered ones, that can accumulate PHA up to 90% of the cell dry weight [23][24]. The bacteria that are used for the production of PHAs can be divided into two groups: a group of bacteria that requires the limitation of an essential nutrient such as nitrogen, phosphorous, magnesium, or sulfur for the synthesis of PHA from an excess carbon source, and a group of bacteria that do not need this limitation and can accumulate polymer during growth. *A. eutrophus*, *Protomonas extorquens*, *Protomonas oleovorans*, and *Cupriavidus necator*, are examples of bacteria that belong to the first group, and *Alcaligenes latus*, *Paracoccus denitrificans*, a mutant strain of *Azotobacter vinelandii*, and recombinant *E. coli*, are examples of the second group of bacteria [15][25].

Single strain systems are currently used to produce PHAs at large-scale, with desired properties, to fabricate and commercialize biobased plastics, such as Nodax®, produced by Procter & Gamble, Biopol® by Metabolix in the US, and Biomer® by Biomer Co. in Germany [21]. As PHA can achieve the desired properties depending on the copolymer composition, companies

use that in their favor. Nodax® is a copolymer of P(3HB) and small amounts of Mcl units like 3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydecanoate. The incorporation of these Mcl monomers is reflected by lower crystallinity and melting temperature which in turn provide toughness and ductility, useful for commercial applications. This product is available as foams, fibers or nonwovens, films, and latex among others [21]. In turn, Biopol® is a copolymer of P(3HB-co-3HV), that can decrease the crystallinity and turn the polymer more elastic, by increasing the amount of 3HV. It can be used to coat paper and paperboards, is suitable for injection, blow molding, and film production, in addition, it has anti-static properties that can be exploited for electrical and electronical packaging [21]. Lastly, Biomer, a German company, owns the technology to produce P(3HB) on a large scale from *Alcaligenes latus*, which can accumulate up to 90% P(3HB) in dry cell weight, when growing the cells in a mineral medium with sucrose as carbon source. This polymer is exploited to make articles such as combs, pens, and bullets, and when commercialized as a melted polymer, with low viscosity, allows injection molding of objects with thin walls [21].

2.4.2 Mixed Microbial Cultures (MMCs)

One of the major causes of the elevated production costs of PHAs with pure cultures is the use of high-purity carbon substrates needed (e.g., glucose, sucrose, fructose), which can account for up to 45% of total production costs [24][25]. Therefore, there has been a recent increased interest in creating novel, less expensive ways for PHA production, with usage of low-value substrates (such as waste feedstocks) and microbial mixed cultures (MMC). These two new approaches offer a less expensive procedure due to unnecessary sterilization (energy savings), less expensive reactor construction materials, and reduced necessity of control (minimizes the need for control equipment). The fact these cultures can use a wide range of intricate and nutrient-rich substrates and continuously adapt to substrate changes is one of the main benefits of MMC processes. This is particularly advantageous when using feedstocks with undefined compositions, such as industrial waste [24][26].

MMCs are generally microbial populations of unknown composition capable of producing specific intra and extracellular reactions that can be selected by subjecting the biological systems to specific operational conditions [23]. It is known they are capable of accumulating large amounts of PHA under certain conditions, although still in lower amounts when compared to pure cultures, so the biomass must be enriched with PHA-producing microorganisms, in terms of enhancing the PHA production rate [26][27].

In MMC, PHA accumulation is triggered by internal metabolic limitations to growth. Indeed, MMC are often kept at low growth rates and therefore their enzymatic apparatus is rather limited (internal limitation). When exposed to an excessive amount of external substrate, MMC cannot grow at their maximum rate. However, the substrate can be actively consumed and stored intracellularly (e.g. as PHA) to be used as a substrate for growth in the future [27]. When producing PHA with MMC from industrial waste three stages are typically included (Figure 2-3). The first involves an acidogenic fermentation in which the organic carbon from the feedstock is converted to volatile fatty acids (VFAs), which are the precursors for PHA synthesis in MMC [28]. The VFAs are then fed to the MMC in the second stage using an SBR reactor (sequential batch reactor) which also applies a selective pressure, often a Feast and Famine (FF) regime, consisting of alternating excess and deprivation of the external substrate [27]. Under these conditions, microorganisms that can store substrates in the feast phase have a competitive advantage over others, since they can use the stored polymer as an internal carbon source during the famine phase [27]. During the SBR cycles the microorganisms that are not able to store PHA and are affected by the famine periods are eliminated from the reactor [28], and as result, employing this regime results in MMC enriched with PHA-producing microorganisms [27]. In the final stage, the culture selected from the SBR is fed with the VFA-rich stream produced in the acidogenic fermentation stage, thereby maximizing PHA accumulation [28]. Once the culture reaches maximum PHA content, it is harvested, and the PHA can be extracted and purified [25].

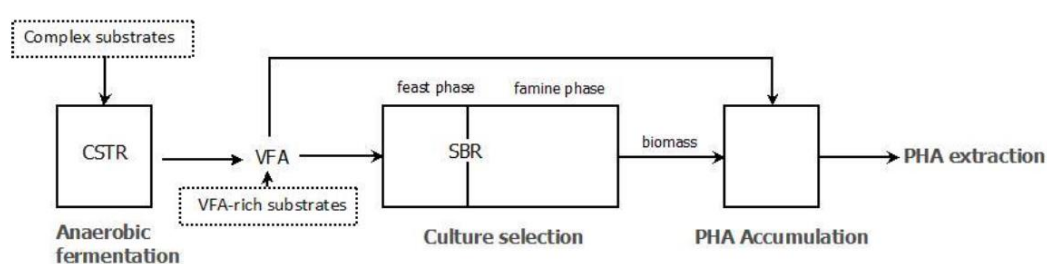


Figure 2-3: PHA production process by mixed microbial cultures. CSTR: continuous stirred tank reactor, SBR: Sequential Batch Reactor [25].

In MMC systems, VFAs like acetate, propionate, butyrate, and valerate are used as the precursors for PHA production since carbohydrates, and other substrates such as glycerol, tend to form glycogen besides PHA [25]. Butyric acid, which has been reported in multiple studies as the preferred VFA by MMCs due to its rapid uptake, has also been identified as the VFA with the greatest VFA to PHA conversion yields (up to 0.94 Cmol PHA/Cmol Butyrate) [25].

Nonetheless, the great majority of MMC studies to date involve aerobic cultures, and it is widely known that aeration is the most energy-intensive requirement, which requires high costs [6].

2.5 Phototrophic Mixed Cultures (PMC)

Recent studies proposed the use of Phototrophic Mixed Cultures (PMC), which can draw energy through the well-known process of phototrophy, and do not need oxygen to produce ATP, as a way to reduce production costs, as aeration is no longer essential [29]. PMCs can absorb light energy by trapping photons in membrane-bound oligomeric complexes and converting it into usable chemical energy, by transferring electrons to an acceptor [30]. Depending on the electron donor source, the photosynthetic pigments, and the number and locations of photosystems, there are two types of phototrophy: oxygenic and anoxygenic [31]. We are more interested in the anoxygenic process in this thesis since it does not require aeration and so reinforces the less expensive process.

2.5.1 Oxygenic Phototrophy

This process is primarily carried out by plants, algae, and cyanobacteria, to produce energy (in the form of ATP) and fixate CO_2 into carbohydrates while producing oxygen via water splitting [32]. Consists of light-harvesting complexes (LHC) grouped around a reaction center (RC), forming a photosystem (PS), capable of absorbing light and utilizing the energy to break the O-H bond in water molecules, releasing O_2 , protons (H^+), and electrons (e^-). The electrons subsequently enter an electron transport chain (ETC), as illustrated in Figure 2-4, and are ultimately stored in chemical bonds in energy carriers such as NADPH. At the same time, an electrochemical gradient is created across the membrane, by the protons, necessary for ATP production [32][33].

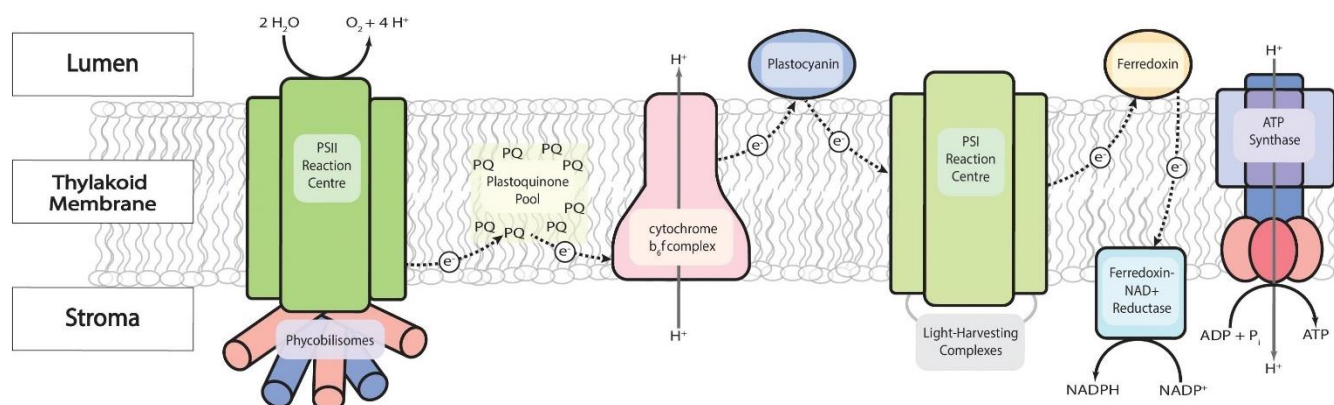


Figure 2-4: Scheme of light reactions in oxygenic photosynthesis [32].

LHC of different organisms contain different combinations of pigments, and therefore can absorb different ranges of light wavelengths [32]. Chlorophylls (Chl) are the pigments used by oxygenic phototrophic organisms; five photosynthetic chlorophylls have been characterized to date: Chl *a*, Chl *b*, Chl *c*, Chl *d*, Chl *e*, and Chl *f* [34]. Chl *a* absorption is restricted to two narrow bands in the Visible range, at $\approx 430\text{nm}$ and $\approx 680\text{nm}$, and Chl *b* absorption bands are at $\approx 460\text{nm}$ and $\approx 650\text{nm}$ [35].

2.5.2 Anoxygenic phototrophy

Anoxygenic phototrophy is a much simpler process, that uses only one PS and because it does not use water as an electron donor, it does not produce oxygen, a required aspect for this process, as oxygen represses the synthesis of the photosynthetic pigments needed [36]. Anoxygenic phototrophic bacteria mostly use sulfide, reduce sulfur compounds, hydrogen, and several small organic molecules as electron donors [36].

In the same way as oxygenic phototrophy, light energy is absorbed by the LHC and transferred to the RC to excite an electron. The electron is then transferred in a cyclical way to generate the proton motive force required for ATP production. The cyclic flow, as illustrated in Figure 2-5, involves the transference of an electron from the RC to a quinone pool, from which they are transferred, in pairs, to the cytochrome bc_1 complex to pump protons across the membrane, then to cytochrome c_2 , and finally back to the RC [32]. Another difference between the two phototrophic processes is that in the anoxygenic there can be a reverse electron flow when there is sufficient build-up in the quinone pool: electrons can travel against the thermodynamic gradient, driven by the energy of the proton motive force toward NADH dehydrogenase to generate NADH from NAD^+ . [32]

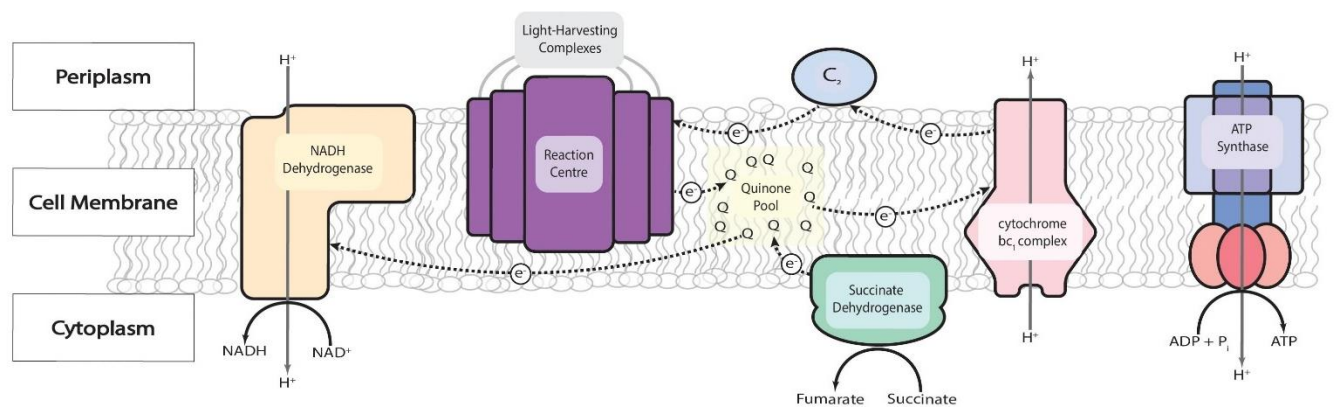


Figure 2-5: Scheme of light reactions in anoxygenic photosynthesis [32].

Anoxygenic phototrophic bacteria use bacteriochlorophylls (Bchl) and a variety of carotenoids as pigments, to capture and convert the light energy [36]. So far, a great variety of Bchl has been identified, and they all have absorption maximums in the IR region [37], giving them an advantage when competing with cyanobacteria or algae for the available light. However, their absorption spectrum is affected by a variety of factors, such as their location in membranes, meaning the same Bchl can absorb different wavelengths of light in different organisms [37].

Purple sulfur bacteria of the Gammaproteobacteria class, and purple non-sulfur bacteria of the Alpha and Betaproteobacteria classes, have Bchl *a* with an absorption wavelength range of 800/815-960nm. Some species of purple bacteria have Bchl *b* with absorption ranges of 835-850nm and 1010-1040nm. Green sulfur bacteria (Chlorobiaceae family) have mainly Bchl *c* (745-755nm), Bchl *d* (715-745nm), and Bchl *e* (710-725nm), and filamentous anoxygenic phototrophs, from the phylum Chloroflexi have Bchl *a* or *b* and *c/d*, absorbing at 720-878nm. [38] An overview of these various microorganisms is provided forthwith.

2.5.3 Green Bacteria

A shared feature of the two families of green bacteria, Chlorobiaceae (green sulfur bacteria) and Chloroflexaceae (filamentous anoxygenic phototrophs), is their type of light-harvesting bacteriochlorophyll and the organization of these pigments into chlorosomes. They are quite diverse and far apart in most other aspects, such as metabolism, photosynthetic apparatus, and phylogeny [39]. The filamentous anoxygenic phototrophs, formerly known as green gliding bacteria or green non-sulfur bacteria, were renamed since the term could be deceptive, as some microorganisms of this group are not green, and some can use sulfur [40].

Green sulfur bacteria (Chlorobiaceae) represented by *Chlorobium*, and filamentous anoxygenic bacteria (Chloroflexaceae) represented by *Chloroflexus*, can both grow photoautotrophically using CO₂ as the sole carbon source, and can both utilize organic compounds for growth [39]. All species from green sulfur bacteria are strictly anaerobic and obligatory phototrophic microorganisms, which only in the presence of CO₂ and sulfide as an inorganic electron donor, can assimilate organic compounds (such as acetate and pyruvate) to a limited extent. As a result, sulfide is employed as an electron donor and sulfur source, being oxidized to sulfate with the intermediate accumulation of elemental sulfur globules extracellularly [36]. These organisms are only able to store glycogen as a carbon reserve material [39], and PHA production has not been reported thus far for this phylum [41]. *Chloroflexus*, on the other hand, is a filamentous thermophilic anoxygenic phototrophic organism commonly found in neutral and alkaline hot springs. They can grow with various carbon sources (e.g. acetate, pyruvate, lactate, butyrate in addition to C₄ dicarboxylic acids, some alcohols, sugars, and amino acids) both anaerobically in the light or in the dark. Both sulfide and hydrogen can be used as electron donors for CO₂ fixation [39]. Some studies indicate that *Chloroflexus* can produce PHB as a reserve material during photoheterotrophic growth, in the presence of an inorganic hydrogen donor [39], [42], and during photoautotrophic growth, can produce, mainly, PHV [43].

2.5.4 Purple Bacteria

Purple bacteria are a major group of phototrophic gram-negative prokaryotes that inhabit aquatic and terrestrial environments. Their competitive success requires both light and anoxic conditions to perform anoxygenic photosynthesis [44]. This combination is most commonly found in lakes, ponds, estuaries, and other aquatic environments where H₂S is present. Physiochemical properties of these habitats, like sulfide concentration, pH, light quality and intensity, and temperature determine the abundance and diversity of bacteria [44].

These bacteria participate in the anoxic cycling of carbon both as primary producers (CO₂ fixation, photoautotrophy) and as light-stimulated consumers of reduced organic compounds (photoheterotrophy). In most illuminated sulfidic habitats, the role of purple bacteria as H₂S consumers is very important as H₂S is highly poisonous, and its oxidation yields nontoxic forms of sulfur, such as elemental sulfur (S₀) and sulfate (SO₄²⁻), allowing the upper waters to remain oxic and suitable for plants, animals, and other anaerobic bacteria [44].

Purple bacteria are divided into purple sulfur bacteria and purple non-sulfur bacteria, and their original difference was their tolerance and utilization of sulfide. Purple sulfur bacteria

were known as species that tolerated millimolar levels of sulfide, while purple non-sulfur bacteria could not. However, studies showed that at low levels of sulfide, typically less than 0.5 mM, most species of purple non-sulfur bacteria could in fact grow and in doing so, oxidize sulfide to S_0 , $S_4O_6^{2-}$, or SO_4^{2-} [44].

2.5.4.1 Purple Sulfur Bacteria (PSB)

This type of microorganism belongs to the *Gammaproteobacteria* class and falls into two families, the Chromatiaceae and the Ectothiorhodospiraceae. The formation of sulfur globules is the characteristic that creates the greatest distinction between them, and thus one of the most easily recognized. In the case of Chromatiaceae, they are formed within the cell wall when growing in the presence of sulfide, polysulfide, thiosulfate, or elemental sulfur, while in Ectothiorhodospiraceae these accumulate extracellularly (Figure 2-6) [45].

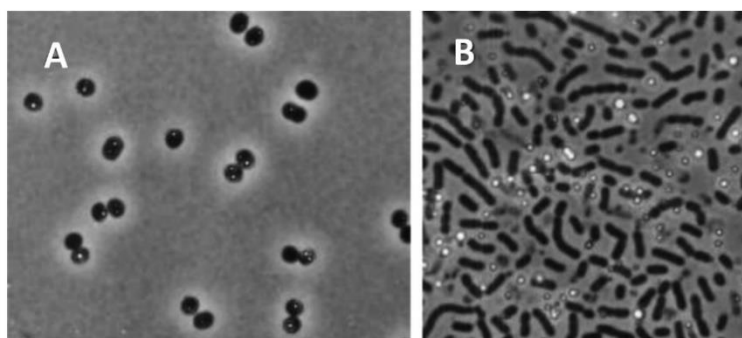


Figure 2-6: Phototrophic sulfur bacteria with intracellular sulfur globules (A), and extracellular sulfur globules (B). Adapted from [45].

Chromatiaceae species can be divided into two major physiological groups, metabolically specialized and versatile ones. The metabolic specialized are restricted anaerobes and obligatory phototrophic, that only photoassimilate acetate and pyruvate, in the presence of CO_2 and sulfide, and cannot use thiosulfate or hydrogen as electron donors. They don't grow with organic electron donors and cannot grow chemotrophically [36]. The versatile species, photoassimilate a greater number of organic substrates, grow in the absence of reduced sulfur sources with organic substrates as electron donors, and can grow under chemolithotrophic conditions [36]. During dark metabolism, this versatile species can use elemental sulfur as acceptors of electrons derived from oxidation of stored compounds [45]. Ectothiorhodospiraceae also differ from Chromatiaceae by lamellar intracytoplasmic membrane structures, significant differences in polar lipid composition, and by dependence on saline and alkaline growth conditions [36]. Under these alkaline conditions, polysulfides are stable intermediates during oxidation of sulfide to sulfur [45].

PSB are a type of microorganisms that can accumulate surplus substrates depending on the conditions they are growing [41]. Besides accumulating sulfur globules, they can also accumulate PHA, carbohydrates (glycogen), and polyphosphate. The accumulation of glycogen occurs with the production of sulfur in phototrophic growth on sulfide, although this storage rarely exceeds 10% of cell dry weight. PHA accumulation occurs intracellularly when growing in the light in the presence of organic acids, or in the dark, as a byproduct of the degradation of intracellularly stored glycogen with the most common polymer found being P(3HB) [41]. Most purple sulfur bacteria seem to only be able to synthesize this polymer regardless of the organic acid supplied, but there are some PSB that can synthesize a copolymer of β -hydroxybutyrate and β -hydroxyvalerate when incubated with organic acids with an odd number of carbons [41].

2.5.4.2 Purple Non-Sulfur Bacteria (PNSB)

Purple non-sulfur bacteria are the most varied group of phototrophic purple bacteria, belonging to the Proteobacteria phylum [46], containing as representatives the classes *Alpha* and *Betaproteobacteria*, whose major differences are represented by their diverse cell morphology, intracytoplasmic membrane structures, carotenoid content, carbon sources, and by the kind of electron donors used [45]. The *Alpha* subgroup contains species of the genera *Rhodospirillum*, *Rhodopila*, *Rhodopseudomonas*, *Rhodomicrobium*, and *Rhodobacter*, and the *Beta* subgroup includes species that have been rearranged into the genus *Rhodocyclus* [36]. PNSBs are a physiologically versatile group that can grow in light as well as in darkness. Under phototrophic (anaerobic/light) conditions they can grow photoautotrophically, with hydrogen or low levels of sulfide as electron donors (many of them do not oxidize sulfide completely to sulfate but instead form sulfur globules as the terminal sulfur product [45]), and CO₂ as the sole carbon source [36]. They do, however, grow preferentially photoheterotrophically in media containing easily accessible organic compounds, such as malate or pyruvate, as well as ammonia as nitrogen source [44]. They are then known for being able to employ all the known modes of metabolism: photolithoautotrophically, photoheterotrophically, chemolithoautotrophically, and chemoheterotrophically [47], and by being able to switch between them in a way to adapt to different environments [46]. Due to this ability, they are able to produce, and accumulate, various high-value by-products such as biohydrogen, polyphosphate, pigments (e.g., carotenoids and bacteriochlorophylls), single-cell proteins, coenzyme Q10, 5-aminolevulinic acid (5-ALA), and

PHA [48]. Various factors influence the production and storage of PHA by PNSB, so it is necessary to employ the right operation parameters that lead to their production. Such parameters include the carbon substrate, and the C/N ratio, where there should be an excess of C to trigger PHA accumulation; the limitation or absence of nutrients such as N, P, S, and Mg; the pH must be controlled between 6.5 and 7.0; the temperature maintained between 24 and 30°C, and since they are phototrophs, light availability also plays an important role [46][49].

2.6 PHA production from wastewaters

As already mentioned in section 2.4.2, the application of MMC is beneficial as they allow the use of low-value substrates. Several studies, [50]–[53], demonstrate that these cultures can be used in wastewater treatment plants (WWTP), which has a double benefit as it helps in the treatment of the wastewater at the same time it produces high-value products, as PHAs, with reduced production costs [49]. Besides, feeding MMC with 2nd generation feedstocks, as wastewaters promote waste valorization, therefore not competing with crops [54]. In line with cost reduction, there have been recent studies that evaluated the capacity of PHA production with Phototrophic Mixed Cultures (PMC) using real fermented domestic wastewater as feedstock [54], where under a permanent carbon feast regime they were able to obtain 20% PHA/VSS in the selector reactor, and under a feast and famine regime they obtained 26% PHA/VSS in the selector reactor and 30.8% PHA/VSS in the accumulation reactor under higher light intensity. Wastewaters contain, typically, high amounts of several nutrients, such as C, N, and P, allowing the growth of photosynthetic bacteria, and several wastewaters such as white spirit, poultry, food processing, oil-containing, brewery, dyeing, municipal and others, have been showed to be tolerated by PMC, while leading to the production of value-added products [55]. PMC can then be used to obtain a higher carbon conversion efficiency to biomass and PHA, since under anaerobic conditions they can obtain energy from light without losing CO₂, minimizing losses, and in the same way as aerobic systems of MMC, heterotrophically remove the organic carbon from the waste streams, with reduced costs, as they don't require aeration [56]. Therefore, integrating waste streams from WWTPs can help reduce the operational costs for PHA production, coupled with the use of PMC. Recent literature also shows that PMC enriched in purple bacteria can be used in the treatment of industrial and domestic wastewater, as well as for recovering bioresources, by selectively inhibiting some parts of the purple bacteria metabolism [57], [58].

2.7 PHA production by PMC

The various types of microorganisms that can compose the PMC have several metabolic pathways for producing cellular material and bioactive products [59]. External factors can regulate these pathways, making it necessary to choose the ideal conditions for culture growth, namely, to cause disruption (e.g., discontinuous feeding or variations on the electron donor/acceptor) in order to favor the desired pathway, leading to PHA production [26].

In purple bacteria, when there are sufficient amounts of nutrients the main mechanisms for electron dissipation, besides growth, is CO₂ fixation via Calvin cycle. However, when in nutrient-limiting conditions, the Calvin Cycle is not efficient, and other mechanisms need to be activated to maintain redox homeostasis. In this situation, phototrophic purple bacteria can dissipate the excess in the form of hydrogen or PHA, where both of which can serve as electron sinks [60]. Melnicki et al. proposed that regulating availability of S-nutrients could be a strategy for switching between pathways and deciding when to produce H₂ or PHA [61], which is of major importance since they are competitive pathways [62]. PNSB specifically, when growing photoheterotrophically with a carbon source more reduced than the biomass (e.g., propionate, butyrate, or longer-chain VFAs), suffer a redox imbalance which forces the cells to adopt strategies to dissipate electrons, through the central metabolic pathways as glycolysis and TCA cycle, fixing CO₂ or N₂, and producing H₂ and/or PHA, and by doing so returning to internal redox homeostasis [63]. Regarding green bacteria, namely the genera *Chloroflexus* it has been characterized the 3-hydroxypropionate cycle for autotrophic fixation of CO₂ [39], [64]. Nevertheless, CG Klatt et al. suggested that in natural environments these filamentous anoxygenic phototrophs must use the 3-hydroxypropionate, and the TCA cycle, as mixotrophic pathways, resulting in the simultaneous utilization of both organic and inorganic carbon sources, and both this pathways produce intermediates that can originate PHAs [65].

All phototrophic bacteria use light as an energy source for autotrophic, mixotrophic, or heterotrophic growth, and their carbon metabolism is essential for biomass growth. In photoheterotrophic growth their carbon metabolism is also essential to provide reducing equivalents for the photosynthetic electron transport. Due to the large amount of carbon metabolic pathways these microorganisms have and their versatility and/or uniqueness, the interest in exploring them has increased in recent years, [59] with new studies coming forth every year.

TECHNIQUES FOR DETECTION, QUANTIFICATION, AND CHARACTERIZATION OF PHA

There are several methods for detecting and analyzing intracellular microbial PHA, which can be used to identify novel PHA-producing microorganisms or for routine monitoring of PHA-producing bioprocesses. These methods include colony/cell staining (e.g., Nile Blue staining), polymerase chain reaction (PCR) gene detection, transmission electron microscopy (TEM), crotonic acid assay, fourier transform infrared spectroscopy (FTIR), liquid chromatography (LG), and gas chromatography (GC). The main detection methods (cell staining, PCR, and TEM) allow for quick screening of PHA-producing microorganisms, but they are also prone to detection errors, such as false positives in staining methods (staining other non-PHA lipid compounds) or non-specific PCR amplification. As a result, these two methods could only be used as a presumptive test of PHA production potential. In turn, TEM can provide direct visualization of PHA granules, confirming accumulation, but sample preparation is time-consuming and involves the use of radioactive chemicals, making it unsuitable for screening. Therefore, despite their ability to detect PHA, neither of these methods can quantify nor provide qualitative information about the PHA monomeric composition [18]. The crotonic acid assay is a viable method for quantifying and characterizing PHB in biomass, where P3HB is dissolved in concentrated sulphuric acid and converted into crotonic acid, which can be measured using a UV spectrophotometer due to its strong UV absorption at 235. While this method is simple and quick, it tends to overestimate polymer content and is limited to P3HB determination. Nonetheless, due to automated sample analysis and the ability to provide accurate PHA quantification and qualitative information about PHA monomeric composition, LC and GC-based methods are the most commonly used. At present, GC remains the preferred

method for qualitative and quantitative analysis of PHA monomers owing to its high separation power and detection sensitivity [18].

RESEARCH OBJECTIVES

4.1 Motivation and Thesis outline

With the emerging problems caused by plastic and CO₂ pollution and with new technologies needed, this master's thesis looked into the emerging field of CO₂-based plastics, where the use of microorganisms with CO₂ fixation biochemical pathways and technologies can enable CO₂ fixation into biodegradable plastics. The main objective of this research was to establish a phototrophic process that could be able to valorize CO₂ and convert it into PHA using mixed cultures enriched in anoxygenic photosynthetic bacteria (APB), while also optimizing PHA production, as a way of investigating ways to decrease the high costs of PHA-producing processes.

This study focused on maintaining a bench-scale photobioreactor with a PMC enriched in APB, operated in non-sterile conditions, while several operational parameters were controlled, including the rate of CO₂ consumption, the availability of nutrients, pH, temperature, and light. In order to optimize the PHA accumulation process, selection strategies based on different light intensities were tested in batch experiments to see how the culture would respond in terms of consumption and/or production rates.

In terms of the PHA detection methodology, there was a setback at the beginning of the internship as the GC detector had been damaged, by an accumulation of debris/particles from the injected samples, which left it inoperable for several months. It was hypothesized if the samples from our photobioreactor could be a source of this deterioration since sulfur is a metabolic intermediate of the culture, and is soluble in chloroform, a reagent employed in the group's protocol for PHA analysis. We made several attempts to wash and/or remove any

sulfur precipitates from our biomass, as well as testing different PHA quantification techniques.

In the following Chapter 5 the laboratorial methods utilized during this work will be described, including the operational methods of the photobioreactor and the batch tests, as well as all the analytical methods. Further, the methodology used to test a new inoculum for the purpose of creating a new photobioreactor is described. And lastly, the attempts performed to overcome the sulfur accumulation on our biomass, and the different PHA analysis methodologies experimented are presented.

In Chapter 6, all the results obtained are shown and discussed, in the same order as they are presented in Chapter 5.

EXPERIMENTAL METHODS

5.1 Selector Reactor (SBR)

The selection of the phototrophic mixed culture (PMC) was conducted in a sequencing batch reactor (SBR), inoculated with sludge from a phototrophic mixed culture enriched in purple bacteria operated under heterotrophic conditions (mixtures of VFAs from C2 to C5: acetate, propionate, butyrate, and valerate) from a previous work of Almeida et al. [54].

The selector reactor used was an acrylic reactor with a 2.7 L working volume, artificially illuminated by 2 halogen lamps (37W), one on each side of the reactor, at a light intensity of 180.6 W/m², corresponding to a volumetric light intensity of 2.09 W/L. The reactor was covered with a UV-Vis filter from LEE Filters Worldwide, USA - model 299 1.2ND, in order to illuminate the reactor only with infra-red light, preventing the growth of phototrophic oxygen producers, like microalgae and cyanobacteria (that require visible light to grow). The reactor lid had entrances that allowed the positioning of a sensor (to measure temperature, pH, and ORP), feeding/purging tubes, and argon inlet. In order to maintain the reactor in anaerobic conditions at all times, the system was closed through an O-ring between the lid and the reactor, and the reactor and all the flasks were connected to gas bags filled with argon to compensate liquid addition or removal from the reactor. The reactor was brushed every 2 weeks, when possible, to release the biomass attached to the reactor walls, and then immediately flushed with argon until it reached an ORP between -300 mV and -200 mV indicating that despite the opening of the reactor it returned to anaerobic conditions.

The reactor had two sampling ports located at middle height and at the bottom of the reactor, sealed with butyl rubber stoppers. Sampling was performed from the port at middle height. The reactor was continuously stirred with a magnetic stirrer and maintained at a temperature

of 28 - 32°C by recirculating water from a cooling bath through the reactor jacket. The pH was controlled at 6.5, at all times, by automated pumps that added an acid solution (HCl 2M) or a basic solution (NaOH 3M). A scheme of the reactor can be seen in Figure 5-1.

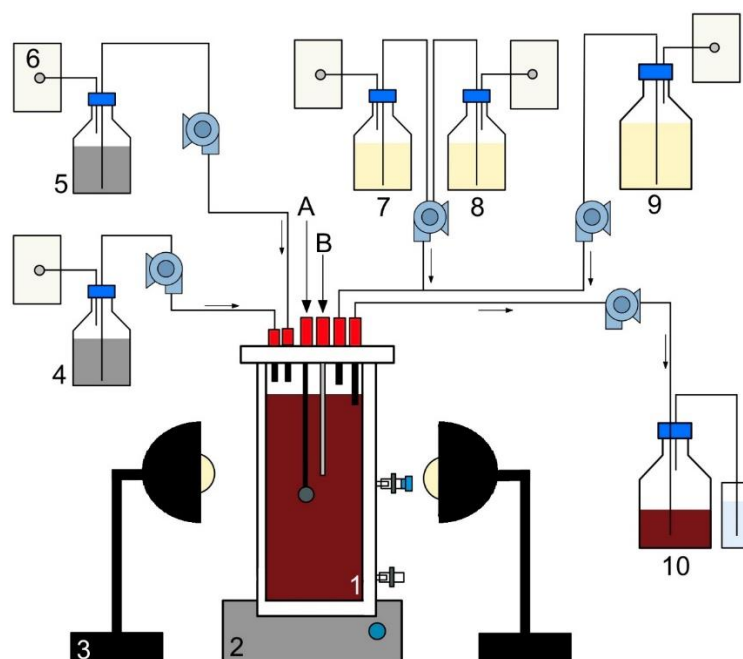


Figure 5-1: Scheme of the experimental setup of PMC selection SBR. 1 - Acrylic jacketed SBR covered with UV-VIS blocking filter; 2 - Magnetic stirrer; 3 - Halogen lamps; 4 and 5 - Acidic and basic solutions for pH control; 6 - Bags with argon to fill the headspace; 7 and 8 - Inorganic carbon and sulfide solutions; 9 - Synthetic mineral medium; 10 - Withdrawal; A - Argon inlet; B - Sensor to measure temperature, pH and ORP.

The reactor was operated in 12 hours cycles (from 10 am to 10 pm), with a hydraulic retention time (HRT) and a sludge retention time (SRT) of 20 days. At the beginning of each cycle 67.5 mL were withdrawn, for 5 min, through the top of the reactor, and the SBR was then fed with 57.5 mL of synthetic medium (with pH adjusted at 6.5, whose composition is presented in Table 5.1), 5 mL of inorganic carbon source (1080mM of Na_2CO_3 solution for a 2mM carbon increment in the reactor), and 5 mL of reduced sulfur species (270mM of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ solution for a 0.5mM carbon increment in the reactor) as electron donor. All three solutions of the feed were added at the same time, for 1 minute.

The reactor was maintained in a permanent feast of inorganic carbon that due to its pH value of 6.5, kept a balance between bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3), as well as phosphorus, and nitrogen. The sulfide was fed in a feast/famine regime in each cycle.

Table 5.1: Synthetic inorganic medium composition

Compound	Concentration
KH ₂ PO ₄	0.3169 g/L
NH ₄ Cl	0.2504 g/L
MgCl ₂ ·6H ₂ O	0.2021 g/L
CaCl ₂ ·2H ₂ O	0.0261 g/L
NaCl	0.2488 g/L
Trace Elements	1 ml/L
EDTA (Ethylenediaminetetraacetic acid)	1 ml/L
Vit B12 (20 mg/mL)	1 ml/L

The trace elements solution was composed by 36.7 mg of Na₂MoO₄·2H₂O, 24.1 mg of NiCl₂·6H₂O, 17.4 mg of CuCl₂·2H₂O, 191.9 mg of CoCl₂·6H₂O, 74 mg of ZnCl₂, 110.8 mg of MnCl₂·4H₂O, and 1629.8 mg of FeCl₂·4H₂O in 1 L of distilled water. The EDTA was prepared by adding 5.2 g of ethylenediaminetetraacetic acid in 1 L of distilled water.

The withdrawal was maintained in anaerobic conditions, in a flask previously flushed with argon, and the collected biomass was stored at 4°C. Every solution, for pH control and feed, was flushed with argon during its preparation, to minimize oxygen input into the system.

5.2 Accumulation Batch tests

To study the light impact on the PMC behavior, and how different light intensities could alter the rates of consumption (of nutrients, inorganic carbon, and sulfides) and/or production (of VFAs, glycogen, PHAs, and bacteriochlorophylls), batch tests were performed in a separate batch flask. The flask contained 2 openings, one at the top and one at the bottom, as shown in Figure 5-2. Both openings were sealed with butyl rubber stoppers. Before the beginning of the SBR cycle, and therefore, before the feeding of the inorganic carbon and sulfide solutions, the synthetic media feed pump was turned on, and after this feed and stabilization of the pH at 6.5, 500 mL of biomass from the SBR were transferred to the sealed batch flask, previously de-aerated with argon. The biomass transfer was made using the withdrawal automated pump in order to maintain the most anaerobic conditions possible. The 500 mL of biomass in the batch flask were then flushed with argon for 10 minutes, to remove any oxygen that could have entered the system, and a bag with argon was added, to refill the headspace during the removal of the samples. In order to add the same concentrations of sulfide (0.5mM)

and inorganic carbon (2mM) solutions as in the SBR, 0.5 mL of each were added to the batch flask using a gas-tight syringe (1 mL, Supelco), by the top rubber stopper, and the pH was adjusted until it reached 6.5, with the addition of HCl 2M. The batch flask was then surrounded by a UV-VIS filter, in the same way as the SBR reactor, and illuminated by halogen lamps (70W). The batch flask was maintained in a room with controlled temperature of 30°C, agitated with a magnetic stirrer, and the pH was followed with pH strips and controlled at 6.5 with addition of HCl 2M when necessary. From here different conditions were tested.

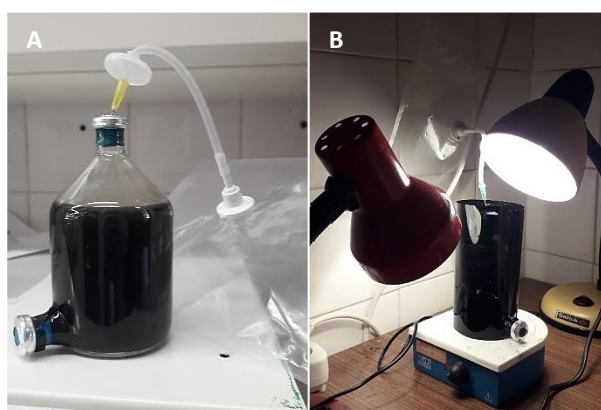


Figure 5-2: Batch flask used to perform the PHA accumulation tests. **A** - 500 mL of biomass in the batch flask, with the butyl rubber stoppers and metal caps, maintaining anaerobic conditions, and the argon bag used to refill the headspace. **B** - Example of a batch test, with the batch flask with the UV-VIS filter, the magnetic stirrer and two lamps to attain the required light intensity.

Five light intensities were tested 1.1 W/L (1.8 W/gVSS), 2.4 W/L (3.7 W/gVSS), 4.6 W/L (12.6 W/gVSS), 8.2 W/L (14.9 W/gVSS) and 16.4 W/L (24.3 W/gVSS). The sampling was proceeded in the same way as to the SBR, and samples were collected along 6 hours, from the bottom butyl rubber stopper. All the analytical methods were performed for each test. The tests for 1W/L and 16W/L were only made once, and the remaining ones in duplicates.

As a control an abiotic test was made, to evaluate sulfide and inorganic carbon losses through the system. 500 mL of the synthetic medium used in the SBR reactor were transferred to the batch flask, which was sealed with butyl rubber stoppers, flushed with argon for 10 minutes, and then 2mM of Na_2CO_3 and 0.5mM of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ were added, in the same way as for the batch tests. Samples were taken every hour, for 7 hours, in order to quantify the inorganic carbon, nutrients, and sulfides. This control was made in duplicate.

An additional test was made, in a condition with no light available (dark test). In duplicate, 50 mL of biomass from the SBR, right after feeding the rector, were transferred to sealed serum flasks, previously de-aerated with argon. The flasks were covered with aluminum foil and left, with stirring, in a room with controlled temperature at 30°C for 5.5 hours, in dark conditions.

A sample was taken initially on the SBR reactor, right after de feed, and a sample was taken from each serum flask, after de 5.5h. The concentration of sulfide, total organic and inorganic carbon (TOC and IC), nutrients (NH_4 and PO_4^{3-}), VFAs, and glycogen, the total suspended solids, and volatile suspended solids, were analyzed. The PHA determination was only performed in the end of the 5.5h of each replica, as well as a microbial characterization, using the Nile blue staining method.

5.3 Selection of new inocula for the SBR

Since the production of PHA via mixed cultures of anoxygenic phototrophic bacteria is still a novel approach, the search for new inocula capable of fixing CO_2 and performing this conversion is constantly necessary. Since these microorganisms grow in extreme habitats, and there can be more efficient species capable of performing this process than the ones we used, we explored two new possible inocula. One of them was a sample collected from a creek in Unhais da Serra, Covilhã, a region with various hot springs. The characteristics of these waters indicated this could be a place where microorganisms known for producing PHAs could grow. According to the "Direção Geral de Energia e Geologia", (<https://hidrogeologia.dgeg.gov.pt/agua-mineral-natural/unhais-da-serra>. Accessed 19 September 2022), these waters are sulfurous waters, with a primary component of sodium bicarbonate, a secondary composition of carbonate, fluoride, and sulfide, and are weakly mineralized.

The sample was collected in sterilized glass flasks and stored at 4°C . In the lab, the sample was centrifuged at 6000 rpm for 5 minutes, the pellet resuspended in a 100 mL sealed flask, containing 100 mL of an enriching growth medium (medium M1), prepared as described in [66], (composition in A.1). A sulfide solution was added in order to obtain a final sulfide concentration of 0.3%. The flask was left incubating, at 50°C , and covered with a UV-VIS filter to inhibit the growth of algae and microalgae.

The same procedure was applied to a sample collected from contaminants found in flasks growing a *Chloroflexus aurantiacus* strain (DSM-637). As these are photosynthetic cultures, resistant to sulfides, that can produce PHA from CO_2 , it was of interest to test if the contaminants found growing in the same medium were able to perform the same kind of process.

For 15 days the growth of the biomass in both flasks was followed by monitoring the macroscopic aspect of the culture. After this time, growth had been observed and the same inoculation procedure was repeated for both flasks, but this time to 250 mL flasks. The samples were left growing in the same conditions.

After another 15 days, the procedure was repeated, but this time for flasks containing 250 mL of the synthetic medium used in the SBR reactor (Table 5.1), pre-de-aerated with N₂. 20mM of Na₂CO₃ and 5mM Na₂S·9H₂O were added, with the intend to apply a selective pressure, and the pH settled at 6.5. The flasks were added, with the intention to apply a selective pressure, and the pH settled at 6.5. The flasks were left incubating for 7 weeks, with the UV-VIS filter, in a 30°C room, with agitation, with no control of pH, and with 24h light with a volumetric intensity around 2W/L.

During this time, the cultures didn't have organic carbon in the medium, and their growth depended on the consumption of bicarbonate. A sample from both samples was preserved using the cryopreservation protocol as described in [66].

5.3.1 Follow-up of the new inocula

2 flask containing 250 mL of the synthetic medium used in the SBR reactor (Table 5.1) pre-de-aerated with N₂, with 20mM of Na₂CO₃ and 5mM Na₂S·9H₂O with pH at 6.5, were prepared. An initial sample was collected to measure the initial concentrations of sulfide, total organic and inorganic carbon (TOC and IC), and nutrients (NH₄ and PO₄³⁻).

The flasks with the cultures that were growing for 7 weeks were then centrifuged in the same conditions as previously (6000 rpm for 5 minutes), and the procedure of inoculation was repeated to the new 250 mL flasks. The 2 flasks were left incubating with the UV-VIS filter, in the 30°C room, with agitation, and pH controlled at 6.5 with pH strips and addition of HCl 2M when necessary. The flask containing the sample derived from the creek had a volumetric light intensity of 1.71 W/L and the one derived from the *Chloroflexus* contaminants had a volumetric light intensity of 0.46 W/L.

For 7 days, samples were taken each day to analyze the optical density (OD) at 620 nm, and to control the pH, maintaining it at 6.5. Samples to analyze the concentration of sulfide, total organic and inorganic carbon (TOC and IC), nutrients (NH₄ and PO₄³⁻), VFAs, and glycogen, the TSS and VSS, and pigments (chlorophylls and bacteriochlorophylls), were taken at the day of incubation (day 0), and at days 2, 4 and 7 of incubation.

The microbial characterization was made using the Nile blue staining protocol, on the first (day 0) and last day (day 7) of incubation for both samples.

5.4 Analytical Methods

5.4.1 Sampling procedure

When taking samples from the SBR reactor, an average of 12 samples were collected during the 12 hours of the cycle (as far as possible), with the first one being collected before the feed. Analysis for determination of substrate and nutrient consumption, and polymer production profiles were carried out approximately once a month. For each sample a total of 23 mL of reactor mixed liquor were collected and distributed for the various analysis. 10.5 mL were distributed in 7 eppendorfs, one immediately frozen, and the other 6 were centrifuged at 10 000 g for 3 minutes. The supernatant was collected for VFAs (section 5.4.2), and ammonium and phosphate analysis (section 5.4.3), being filtered and stored at - 4°C, or in the freezer, respectively. The pellets were frozen for posterior lyophilization to perform glycogen and PHA analysis (sections 5.4.6, and 5.4.7). 3 mL were distributed between two eppendorfs containing a drop of NaOH 6M, centrifuged in the same conditions and the supernatant collected for total organic carbon (TOC) analysis (section 5.4.4), 3 mL were used to determine the sulfides concentration, in a protocol explained in section 5.4.5. 4.5mL were distributed for 3 eppendorfs to analyze the pigments (section 5.4.9). The remaining 2 mL were for losses during filtration steps needed for some protocols. The total suspended solids (TSS) and volatile suspended solids (VSS) analysis (section 5.4.8) were performed in the first and the last sample of each cycle and required an extra 20 mL of mixed liquor per sample.

5.4.2 Volatile Fatty Acids Analysis

Volatile fatty acids (VFAs), namely acetic acid (HAc), propionic acid (HPr), isobutyric acid, butyric acid (HBu), isovaleric acid, and valeric acid (HVa) were determined by high-performance liquid chromatography (HPLC) described by Wang et al. [67]. A VWR Hitachi Chromaster equipment with an RI detector, a Biorad pre-column (125- 0129 30 x 4.6 mm), and Biorad Aminex HPX-87H column (300x7.8 mm) was used. Sulphuric acid (H₂SO₄) 0,01 N was used as the eluent at a flow rate of 0.5 mL/min and 30°C operating temperature. The organic acid concentrations were calculated through a calibration curve using 4-1000 mg/L standards.

1mL sample was filtered with a 0.2 μm syringe filter into an HPLC vial. No dilutions were applied for this analysis.

5.4.3 Nutrients Quantification

The nutrients (NH_4^+ and PO_4^{3-}) concentrations were analyzed by a colorimetric method implemented in a continuous flow analyzer (Skalar San++, Skalar Analytical, The Netherlands). The continuous flow analysis allows to perform two analytical measurements on a single sample simultaneously. 1.5mL of sample was filtered with a 0.2 μm syringe filter and a dilution of 1:3 was applied with Milli-Q water.

5.4.4 Total Organic Carbon

The Total Organic Carbon (TOC) was analyzed in a TOC-VCSH Analyzer (Shimadzu) by Total Carbon (TC) analysis with a combustion catalytic oxidation at a temperature of 680°C and high purity air as a carrier gas at a flow rate of 150mL/min, and by Inorganic Carbon (IC) analysis that consists in a pre-acidification of the solution with hydrochloric acid (2M). The TOC values are obtained by subtracting the IC from TC ($\text{TOC} = \text{TC} - \text{IC}$). Samples for this method were basified with a drop of NaOH (6M), during the sampling procedure, maintaining the pH above 9, to shift dissolved CO_2 equilibrium in water towards carbonate, thus preventing CO_2 losses during analysis and enabling a representative analysis of sample IC. 3 mL of sample were filtered with a 0.2 μm syringe filter and a dilution of 2.5:11.5 was applied with Milli-Q water.

5.4.5 Sulfide analysis

The sulfide analysis was based on Sugahara et al. protocol [68], consisting of a colorimetric determination of the sulfide in solution. The principle is based on the reaction between N,N-diethyl-p-phenylenediamine plus ferric chloride (the coloring reagent) and sulfide to give methylene blue, a colored compound. A calibration curve was built with sulfide standards. To do this a known sulfide concentrated solution was prepared, adding 13g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ into 200 mL distilled water, previously degassed from O_2 with N_2 flow, in a Schott bottle. Using a gas-tight syringe (1 mL, Supelco), 1 mL from the concentrated sulfide solution was transferred into a 100 mL serum flask, filled with 100 mL of distilled water, previously flushed with N_2 to remove O_2 in the headspace. From this flask, another dilution was applied, transferring 1mL into another 100 mL serum flask with 100 mL MilliQ water flushed previously with N_2 , in the same way. The exact concentration of dissolved sulfide in the second 100 mL serum flask was

measured with a sulfide LCK 653 cuvette test kit, with a measuring range of 0.1 - 2 mg/L of S^{2-} .

For the standard sulfide solutions preparation, successive dilutions were applied in sealed glass serum flasks containing MilliQ water, all previously degassed with N_2 flow. The first standard was prepared by adding 0.2 mL of the previously prepared concentrated sulfide solution using a gas-tight syringe (1 mL, Supelco), and transferring it into a 50 mL serum flask, with 50 mL MilliQ water. The successive standards contain only 2 mL of MilliQ water, and they were prepared by transferring 2 mL from the previous standard, using a gas-tight syringe (5 mL, Supelco), until standard 7. Once the standards were ready, 0.2 mL from each standard were transferred, using a gas-tight syringe, to glass tubes, containing 0.2 mL of the coloring agent, and kept closed for 15 minutes. Then, 5 mL of HCl (0.1M) were added and the absorbance was measured at 666 nm. The coloring solution was prepared by adding 0.4g of N, N-diethyl-p-phenylenediamine and 0.6g of iron (III) chloride to 100 mL of HCl 6M. The blank solution was prepared by adding to the glass tube only 0.2 mL of iron (III) chloride reagent without N, N-diethyl-p-phenylenediamine, 0.2 mL of a sample, and 5 mL of HCl (0.1M).

For the preparation of the samples, the same protocol was adopted, whereas, at the time of sampling, a disposable 5 mL syringe containing 1 mL of HCl (6M) was used to take 3 mL of sample, to analyze the Total Sulfides (ST). With the decrease in pH caused by the HCl, all the components with sulfur present were solubilized and could be detected. After mixing, the mixture was filtered with a 0.45 μm syringe filter into a 1.5 mL eppendorf containing 1 drop of zinc acetate. Exactly 1 mL was collected with a micropipette and added to another eppendorf containing 0.1 mL of zinc acetate solution and 0.4 mL of NaOH (6 M). To analyze the Soluble Sulfides (SS), only the sulfides that were soluble at pH 6.5 (pH of the SBR), 1.5 mL of sample was filtered with a 0.45 μm syringe filter into a 1.5 mL eppendorf, and 1 mL of this was transferred to another eppendorf containing, in the same way, 0.1 mL of zinc acetate solution and 0.4 mL of NaOH (6M). This procedure allowed the sample to be stored in the freezer preventing loss of sulfide until the analysis. The analysis were performed using the same protocol as the standards.

5.4.6 Carbohydrates analysis

Carbohydrates hydrolysable to glucose were determined using the method described by Latham et al. [69], with some modifications, where the carbohydrates quantification can be performed by acidic hydrolysis of the biomass with diluted hydrochloric acid, which allows the

simultaneous extraction and hydrolysis to glucose which can be further determined by chromatography. For this procedure, biomass pellets were lyophilized in a CoolSafe freeze drier (ScanVac) overnight. After weighing the pellets, 2 - 4 mg of biomass were mixed with 2 mL of 0.6 M HCl and digested for 2h at 100°C in a dry-heat thermo-block. The samples were then cooled in an ice bath, filtered (0.45 µm syringe filter) to an HPLC vial, and glucose was analyzed by HPLC in a VWR Hitachi Chromaster equipment with an RI detector, a Biorad pre-column (125- 0129 30 x 4.6 mm), and Biorad Aminex HPX-87H column (300x7.8 mm) was used. Sulphuric acid (H₂SO₄) 0.01 N was used as the eluent at a flow rate of 0.5 mL/min and 30°C operating temperature.

The standard used for the quantification of glucose was D-glucose anhydrous (Scharlau), dissolved in HCl 0.6 M. A calibration curve of six points was used to find the concentration of glucose in samples. The six standards were prepared through sequential dilution from a mother solution of 1000 mg/L. By dividing the concentration of glucose in each sample by the weighted amount of lyophilized biomass, it was possible to obtain the percentage of glucose in the total solids. This percentage, divided by the ratio VSS/TSS, allowed the determination of the percentage of carbohydrates on VSS.

5.4.7 Polyhydroxyalkanoates determination

The protocol adopted in this thesis refers to Lanham et al. [70] with minor modifications. 2 mg of lyophilized pellets were digested for 2h at 100°C (dry-heat thermo-block) with 1 mL of acidic methanol (5% sulfuric acid) and 1 mL of chloroform containing 0.5 g/L heptadecane as internal standard. The tubes were then cooled in ice until room temperature. 1 mL of water was added, and the tube was mixed using the vortex for 1 min and left to rest for 1 hour to complete the phase separation. Glass Pasteur pipettes were used to remove and discard the upper water phase. The process of water addition, mixing and water phase removal was repeated one more time (these water additions promote the removal of methanol, sulfuric acid and other compounds from the chloroform). The chloroform phase was then extracted into a GC vial containing molecular sieves to remove traces of water and agitated. Immediately after (to prevent chloroform evaporation) the sample was filtered with a PTFE 0,22 µm filter to a new GC vial and sealed with aluminum capped PTFE/rubber septum. At last, 1,5 µL of sample were injected in a gas chromatographer (from Perkin Elmer) equipped with an FID detector and a Restek, crossbond Stabilwax column (60 m, 0,53 mm internal diameter, 1 µm film thickness) coupled with a guard-column (0,32 mm internal diameter). Samples were analyzed using

helium as a carrier gas, at constant pressure (14,5 psi). The temperature of injection was 280°C, the temperature of the detector is 230°C and the temperature ramp started at 40°C, then increased at a rate of 20°C/min until 100°C, then increased at a rate of 3°C/min until 175°C, then increased again at 20°C/min until 220°C for ensuring cleaning of the column after each injection.

Samples were calibrated using a commercial co-polymer of PHB-PHV (88:12% molar / 86:14 % w/w) for calibrating PHB, and PHV.

5.4.8 Total Suspended Solids and Volatile Suspended Solids

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined according to standard methods described by APHA et al. [71]. The first step consisted in removing volatile compounds from the glass-fiber filter disks (VWR, glass fiber 1.2 µm, 47 mm) by placing them for a minimum of 30 minutes at 500 °C muffle furnace. Sets of 2 filters were then weighed ($W1$, g), and stored. Per analysis 0.01 L (V) of sample were filtered with the set of 2 filters on a filtration vacuum apparatus. The filter is then placed in a 100°C oven for at least 24 hours. After drying, the filter is weighed ($W2$, g). In this step, we obtained the TSS, which is determined by taking the initial weight ($W1$, g) of the filter to the second weight ($W2$, g) of the filter and dividing by the total volume (V , L) of the sample:

$$TSS (g/L) = \frac{W2 - W1}{V}$$

At last, to obtain the VSS, the filter is placed into a 500°C muffle furnace for a minimum of 2 hours. After cooling in a desiccator, the filter is weighed ($W3$, mg). By subtracting the third weight ($W3$, mg) from the second weight ($W2$, mg) and dividing by the total volume (V , L) of the sample we get the Volatile Suspended Solids:

$$VSS (g/L) = \frac{W2 - W3}{V}$$

5.4.9 Chlorophylls and Bacteriochlorophylls

To evaluate the total pigment content (Bchl $a + b$, Bchl c , and carotenoids), samples of 1.5 mL were collected and centrifuged at 10 000 g for 3 minutes. After the centrifugation, the supernatant was discarded and the pellet resuspended in 1.5 mL of ethanol 98%, which was then left at room temperature overnight, in the dark, to solubilize the pigments. The day after, the sample was centrifuged, the supernatant transferred to a quartz cuvette (Hellma Analytics,

QS-10mm), and the absorbance spectrum measured in a single beam scanning UV-Vis spectrophotometer M509T. The baseline was performed with ethanol 98% and the wavelength window was set in an interval of 190 - 900 nm. Bchl *a* presence was determined by a peak at 365 and 775nm, Bchl *a + b* at 610 and 750 nm, Bchl *c* at 670 nm, and the presence of carotenoids with a peak between 400 and 500 nm.

5.4.10 Microbial Characterization of the PMC

Biomass samples were collected for microscopic morphological observation and Nile blue staining examination, and for bacterial population analysis by fluorescence *in situ* hybridization (FISH).

5.4.10.1 Nile blue Staining Method

Nile blue staining was conducted according to the method of Bengtsson et al. [72]. To begin, 1 mL of sample was taken directly from the reactor, a drop of Nile blue solution was added, and incubated in a thermostatic water bath at 50°C for 15 minutes. The sample was then washed with Phosphate Buffer Saline (PBS) 1x until the supernatant presented no color. Thereafter, slides were prepared with about 20 µL of the stained sample, covered with a glass cover slip, and observed at the microscope using fluorescent filters in bright and phase contrast field. An Olympus U-RFL-T epifluorescence microscope was used, with a 100x objective for qualitative observation of the internal PHA granules.

5.4.10.2 Fluorescence *in situ* Hybridization Method

In this work, it was performed sample fixation with paraformaldehyde (PFA) that consists in adding 3 volumes of PFA to 1 volume of biomass sample. Then, the sample was incubated for 2 - 3 hours at 4°C. The sample fixation was also performed by adding 1 volume of ethanol 98% to 1 volume of sample, with a period of incubation of 4 - 16 hours at 4°C. After the period of incubation, both samples fixated with PFA and ethanol were centrifuged (10 000 g, 3 min) and the pellet was washed twice with 1mL of PBS 1x. Finally, the cells were resuspended in 0.5 mL of PBS and 0.5 mL of ethanol absolute, and the samples were stored at -20°C, until further analysis.

The first step in Fluorescence *in situ* hybridization was the sample application and dehydration, which consisted in applying the sample (3 to 30 µL) in specific glass microscope slides for FISH.

After the sample dried naturally, or in the oven at 46°C, a serial dehydration in ethanol (50%, 80%, and 98%) was performed where the slide stayed 3 minutes in each ethanol solution. The probe hybridization step starts with the preparation of the hybridization buffer (0.9 M NaCl, 0.01% Sodium Dodecyl Sulphate (SDS), 20 mM Tris/HCl, pH 7.2, and with a formamide percentage specific for the probe used), that would be applied in each well (8 µL). After that, 1 µL of the specific probe was added and it was necessary to gently mix the probe in the buffer solution. The remaining buffer was applied to the moisturizing tissue in the hybridization tube to keep the chamber with the same atmosphere, and the slide was placed inside the tube, which would be in the oven at 46°C for 1,5 - 3 hours.

At the end of the incubation time, it was necessary to prepare the 50 mL of washing buffer with NaCl 5M, 1mL of Tris-HCL 1M, EDTA 0.5M, 50 µL SDS 10%, and fill till 50mL with MilliQ water, and to place it in a bath to reach 48°C. The slides were then gently washed with the buffer and placed inside the washing buffer tube into the bath at 48°C for 10 - 15 minutes. Afterward, the slide was washed with cold MilliQ water and dried. To finish the procedure, a few drops of Vectashield were added to the dried slides, and a cover slip was placed forcing Vectashield to cover all wells. Nail polish was applied to the board of the cover slip to prevent its movement, and the slides were stored at -20°C protected from light. A *Zeiss Imager D2 epifluorescence* microscope was used for the microscopic observations of biomass samples. The following specific oligonucleotide probes were employed:

Table 5.2: Oligonucleotide probes utilized in FISH analysis.

Probe Name	Fluorochrome	Formamide	Specificity
EUBmix (Bacteria)	FitC	35%	Domain
ARC915 (Archaea)	Cy3	35%	Domain
ALF969 (Alphaproteobacteria)	Cy3	35%	Class
GAM42a (Gammaproteobacteria)	Cy3	35%	Class
Rhodopseud (<i>Rhodopseudomonas</i>)	Cy3	30%	Genus

5.4.11 Light intensity measurements

Light intensity of the lamps, for both the SBR and the batch tests, was measured using a Li-COR light meter LI-250A equipped with a pyranometer sensor LI-200SA. The sensor measures the average light intensity in W/m^2 over a span of time of 15 seconds.

The weighted average of light intensity per area, obtained from the light meter was then multiplied by the illuminated area and divided by the volume of the reactor/batch flask, to obtain the volumetric light intensity (W/L). For the batch tests, the lamps were placed at a distance that allowed us to obtain the desired light intensity for each test. The measurements were made with the sensor behind the UV-VIS filter to acquire the accurate intensity the PMC would be receiving, for both the SBR and the batch tests.

5.4.12 Calculations of kinetic and stoichiometric parameters

The biomass PHA content was determined in terms of percentage of VSS on mass basis [$\text{PHA} (\%) = 100 \times (\text{PHA}/\text{VSS}), \text{gPHA/gVSS}$]. The VSS used for this calculations were the obtained values from the total suspended solids (TSS) and volatile suspended solids (VSS) analysis.

The specific substrate uptake rates for soluble sulfides and inorganic carbon [$-q_{\text{S(Soluble)}}$ in $\text{mmolS}/(\text{Cmmol Xi} \cdot \text{d})$, and $-q_{\text{IC}}$ in $\text{mmolC}/(\text{Cmmol Xi} \cdot \text{d})$], were determined by dividing the volumetric substrate uptakes [in $(\text{mmolS/L} \cdot \text{d})$ and $(\text{mmolC/L} \cdot \text{d})$] for the initial concentration of active biomass (Cmmol Xi/L) at the beginning of each cycle, where Xi was calculated through a general biomass chemical formula of $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}\text{P}_{0.02}$, according to Almeida et. al [54].

5.5 Analytical development to overcome the PHA analysis interferences

Since this work involved the utilization of sulfide as an electron donor, it was hypothesized that some sulfur could be stored intracellularly or attached extracellularly to the biomass, and partially become solubilized in the chloroform used for PHA analysis. Since the injection of non-volatile compounds in the GC can affect its performance (or even lead to deterioration), several tests were conducted to determine if S was present in the lyophilized biomass and if it could be removed by washing, or if alternative PHA analytical procedures could be applied to this specific biomass.

5.5.1 Crotonic Acid Method

In order to try an alternative approach protocol to quantify the PHAs, besides the GC method, we tested the protocol described by Law et. al [73], with minor modifications. This method allows the quantitative conversion of poly- β -hydroxybutyric acid to crotonic acid by heating in concentrated sulfuric acid and the acid determination by UV absorption at 235nm (as the absorption maximum of crotonic acid is shifted to this wavelength when in concentrated sulfuric acid). Lyophilized samples from the SBR reactor were compared with lyophilized samples from other mixed cultures operated in the lab that are not fed with sulfide, namely an aerobic mixed microbial culture. The protocol was: 10 mL of sulfuric acid were added to each lyophilized sample (>2mg per sample) and incubated for 10 min at 100°C in a dry-heat thermoblock. After cooling to room temperature, a sample was transferred to a quartz cuvette and the absorbance at 235nm was measured against a sulfuric acid blank.

5.5.2 Washing the samples with NaCl solutions

With this approach, we had the goal to remove S precipitates adsorbed to the cell walls, by successively washing the biomass with NaCl solutions. Through the results of the TSS/VSS protocol, we attempted to detect any notable change in the TSS, possibly confirming the S removal.

36 mL of mixed liquor were collected from the SBR reactor and distributed in 6 tubes with 6 mL each.

- **Tube 1 and 2 (control):** the samples were centrifuged, and the pellets washed with 6 mL NaCl 0.9%.
- **Tube 3 and 4:** the samples were centrifuged, the pellets washed with 6 mL NaCl 0.9%, and then with 6 mL NaCl 2.5%.
- **Tube 5 and 6:** the samples were centrifuged, the pellets washed with 6 mL NaCl 0.9%, then with 6 mL NaCl 2.5%, and finally with 6 mL NaCl 4%.

All the centrifugations were made at 10 000 g for 3 minutes. For all the washes the samples were agitated by hand and left for 15 minutes with the respective solutions. After the last wash, 1 mL of each supernatant was collected to measure the absorbance. The absorbances were determined using a quartz cuvette (Hellma Analytics, QS-10mm) and the absorbance spectrum was measured in a single beam scanning UV-Vis spectrophotometer M509T, with a wavelength window set in an interval of 190 - 600 nm. The baseline was performed using the

adequate solution. The rest of the supernatants were discarded, and all the final pellets were resuspended in 6 mL NaCl 0.9%. 5 mL of each final sample were used to perform the Total Suspended and Total Volatile Solids protocol. All the results presented were calculated using the averages of the two replicates.

5.5.3 Washing the samples with ethanol

For this attempt, we tried in the same way, to remove S precipitates by solubilizing them in ethanol, and with consecutive washes trying to remove the higher amount of S possible. Since, in ethanol, sulfur has its maximum and minimum absorbances at 264.0nm and 249.3nm [74], we were expecting to see a variation in the absorbance spectrums obtained, and possible changes in the TSS values obtained.

Three approaches were tested: washing the samples with no incubation time; washing with 15 minutes of incubation per wash; and leaving the samples incubating overnight with ethanol. For the first two approaches, 48 mL of mixed liquor were collected from the SBR reactor and distributed for 8 tubes, with 6 mL each, and for the last approach, 36 mL were collected and distributed for 6 tubes, with 6 mL each.

The procedure for the washes was the same for all the approaches, and can be seen in Figure 5-3, where:

- **Tubes 1 and 2 (control):** 6 mL were centrifuged, the supernatant discarded, the pellet resuspended in 6 mL NaCl 0.9%, and the VSS/TSS protocol performed.
- **Tubes 3 and 4:** 6 mL were centrifuged, the supernatant discarded, the pellets washed with 6 mL ethanol 50%, centrifuged, 1 mL of the supernatant was collected to measure the absorbance and the rest discarded. The pellets were resuspended in 6 mL NaCl 0.9%, and the VSS/TSS protocol performed.
- **Tubes 5 and 6:** 6 mL were centrifuged, the supernatant discarded, the pellets washed 1 time with 6 mL ethanol 50% and 1 time with 6 mL ethanol 98%, and then centrifuged. 1 mL of the final supernatant was collected to measure the absorbance and the rest discarded. The pellets were resuspended in 6 mL NaCl 0.9%, to perform the VSS/TSS protocol.
- **Tubes 7 and 8 (only performed for the first two approaches):** 6 mL were centrifuged, the supernatant discarded, and the pellets washed 1 time with 6 mL ethanol 50% and 2 times with 6 mL ethanol 98%, and then centrifuged. 1 mL of the final supernatant

was collected to measure the absorbance and the rest discarded. The pellets were re-suspended in 6 mL NaCl 0.9%, to perform the VSS/TSS protocol.

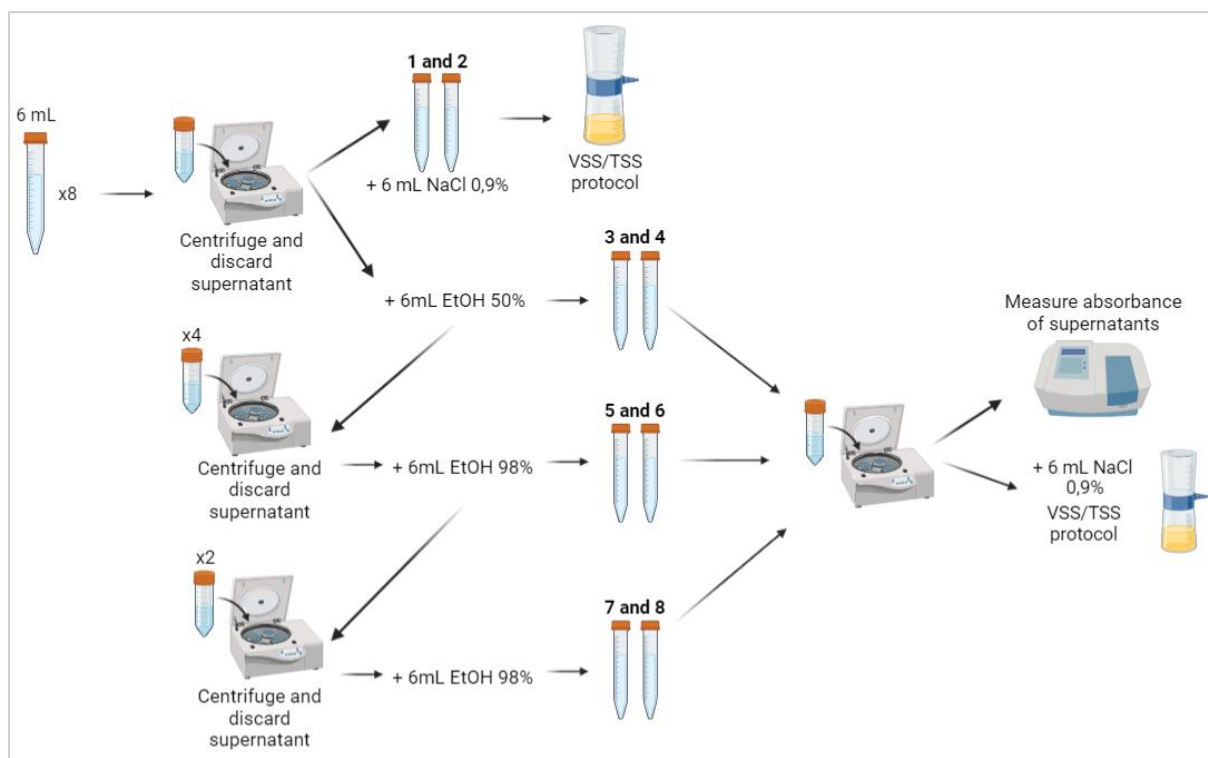


Figure 5-3: Scheme of the protocol followed to wash the samples by solubilizing the S in ethanol. 3 approaches were tested, all with the same protocol, differing in the incubation time for washing the samples (first approach: no incubation time, second approach: 15 minutes, and last approach: incubating overnight). Tubes 7 and 8 were prepared only for the first two approaches. For the last approach, the initial number of tubes was 6.

All the centrifugations were made at 10 000 g for 3 minutes. Every time ethanol or NaCl 0.9% was added, the sample was mixed using the vortex for a minimum of 30 seconds.

Regarding the incubation times: for the first approach, as there was no incubating time, after adding the ethanol and vortexing, the next step of the protocol was done right away; for the second approach after adding the ethanol and vortexing, the samples were left incubating for 15 minutes (in every wash) before continuing the protocol. The last approach was slightly different: as for tubes 3 and 4, after adding the ethanol 50% and vortexing the samples were left incubating overnight before continuing the protocol, and for tubes 5 and 6 after adding the ethanol 50% (first wash) and vortexing, the samples were left incubating for 1 hour, and for the second wash (with ethanol 98%), after adding the ethanol and vortexing the samples were left incubating overnight before continuing with the protocol.

To perform the Total Suspended Solids and Volatile Suspended Solids protocol, 5 mL of each final sample were used. The absorbances were determined using a quartz cuvette (Hellma Analytics, QS-10mm) and the absorbance spectrum was measured in a single beam scanning

UV-Vis spectrophotometer M509T, with a wavelength window set in an interval of 190 - 600 nm. The baseline was performed using the adequate solution.

All the results presented were calculated using the averages of the two replicates.

5.5.4 PHA extraction with chloroform at 10°C

The previous attempts had the goal of washing S precipitates which could be interfering with the analysis. The next attempt had the goal to solubilize PHA instead, as we tried a different way to analyze PHA.

A protocol described by Filippi et al. [75] with minor modifications was performed, where a minimum of 2 mg of lyophilized samples of biomass from the SBR reactor, and biomass from a PHA producing aerobic mixed microbial culture (not fed with sulfide), were suspended in 1mL of chloroform, in duplicate. The mixtures were left with gentle stirring for 24h at 10°C. According to the author, this treatment can dissolve PHA and the cell membrane is not solubilized. (It is expected that PHA will solubilize into de chloroform but not any eventual S present inside the cells, while the low temperature minimizes S solubility in the chloroform.) After the 24h, the suspensions were filtered using a 0.2 µm syringe filter (previously filled with chloroform), to GC vials, and the absorbances of all samples measured using a quartz cuvette (Hellma Analytics, QS-10mm) in a single beam scanning UV-Vis spectrophotometer M509T, with a wavelength window set in an interval of 190 - 600 nm.

For further comparison, after the treatment, the usual protocol of PHA digestion, described in 5.4.7, was performed. A scheme of this attempt can be seen in Figure 5-4.

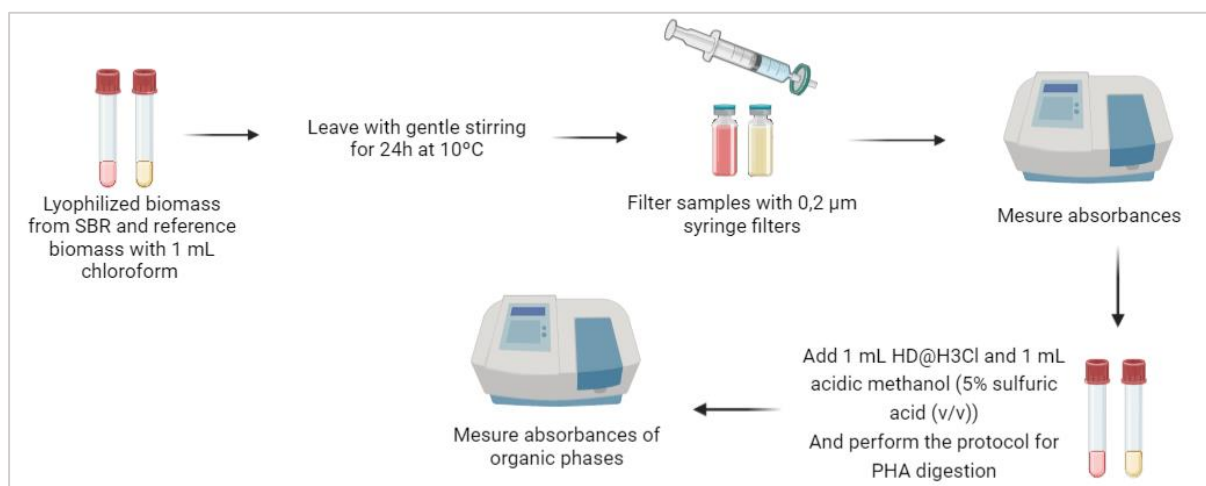


Figure 5-4: Scheme of the protocol followed to solubilize PHA in chloroform at 10°C. All the samples were prepared in duplicate (not shown in the scheme).

The controls were made by using the same amounts of lyophilized samples both from the SBR reactor and from the reference biomass. A normal digestion protocol with lyophilized biomass, from the SBR, and from the reference biomass, was performed, on duplicate, without any kind of pre-treatment and the absorbance of the organic phases measured.

To further compare the spectrums, experiments with MilliQ water, ethanol 98%, and chloroform were made, adding 1 mL of each to lyophilized biomass, from the SBR, and from the reference biomass, in duplicate, and left with gentle stirring for 24h at room temperature. After the 24h, all samples were filtered using 0.2 µm syringe filters, previously filled with the respective solutions, and the absorbances measured in the same conditions, with the respective blanks.



Figure 5-5: Photo of the apparatus for the lyophilized samples, from the SBR and the reference biomass, with 1mL of each solvent (MilliQ water, ethanol 98%, or chloroform) stirring at room temperature for 24h.

5.5.4.1 Pre-wash the samples with NaCl 0.9% or with ethanol 98%

Due to some interferences in the absorbance spectrums of the experiments with MilliQ water, ethanol 98%, and chloroform, new ones were made adding a step of a pre-treatment.

To remove interferences caused by organic acids, samples from the SBR reactor and the reference biomass were centrifuged, and the absorbance of the supernatant measured against a blank of distilled water. The pellets were washed 2 times with NaCl 0.9%, and the absorbance of both supernatants measured against a blank of NaCl 0.9%. The final pellets were lyophilized. The experiments were prepared in the same way: adding 1 mL of MilliQ water, ethanol 98%, or chloroform to the lyophilized biomass, in duplicate, and left with gentle stirring for 24h at room temperature. After the 24h all samples were filtered using 0.2 μm syringe filters, previously filled with the respective solutions, and the absorbances were measured in the same conditions, with the respective blanks. A scheme of this protocol can be seen in Figure 5-6.

All the centrifugations were made at 10 000 g for 3 minutes.

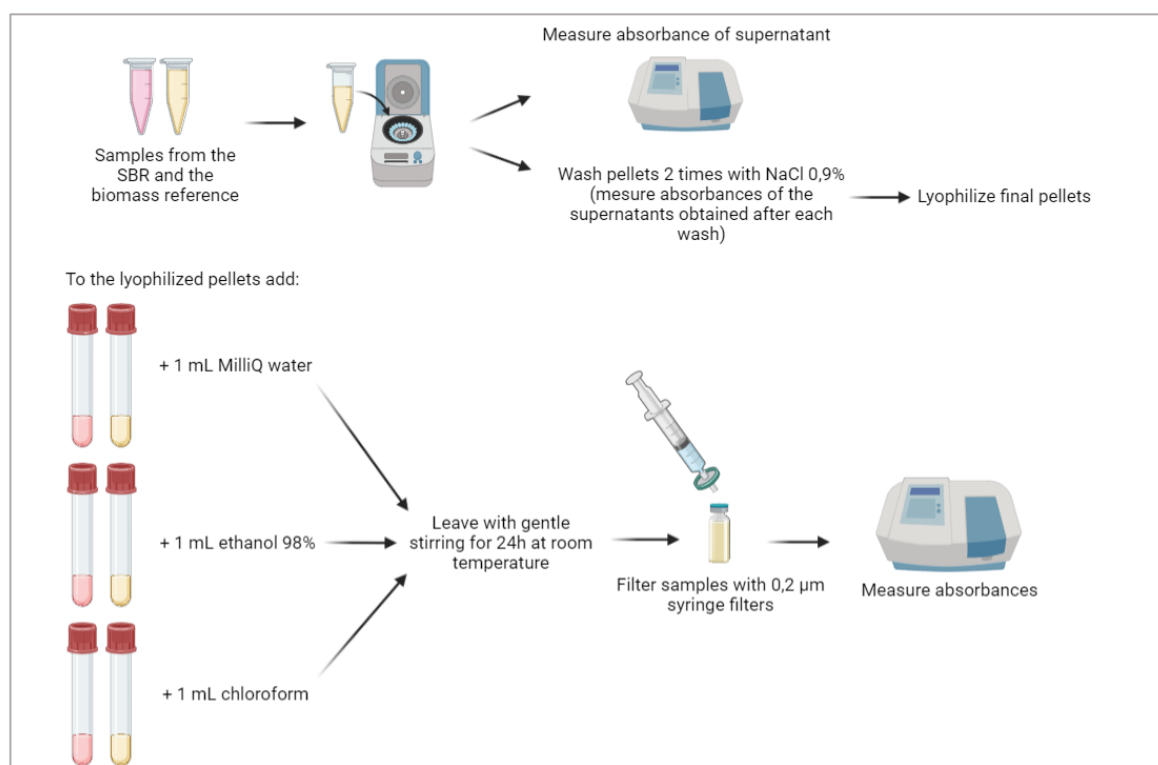


Figure 5-6: Scheme of the protocol followed, with a pre-wash with NaCl 0.9% to remove organic acids interferences. All the samples were prepared in duplicate before leaving for 24h with gentle stirring (not shown in the scheme).

To remove interferences caused by pigments on the experiment with chloroform, samples from the SBR reactor and from the reference biomass were centrifuged, the supernatants discarded, and the pellets left overnight with ethanol 98%. The day after the samples were centrifuged in the same conditions, washed with MilliQ water, and the pellets lyophilized. At this moment, the pigments were removed, and a new experiment with chloroform was performed:

it was added 1 mL chloroform to the lyophilized pellets, in duplicate, and left with gentle stirring for 24h at room temperature. After the 24h, the samples were filtered using 0.2 μ m syringe filters, previously filled with chloroform, and the absorbances measured under the same conditions. All the centrifugations were made at 10 000 g for 3 minutes. A scheme of this protocol can be seen in Figure 5-7.

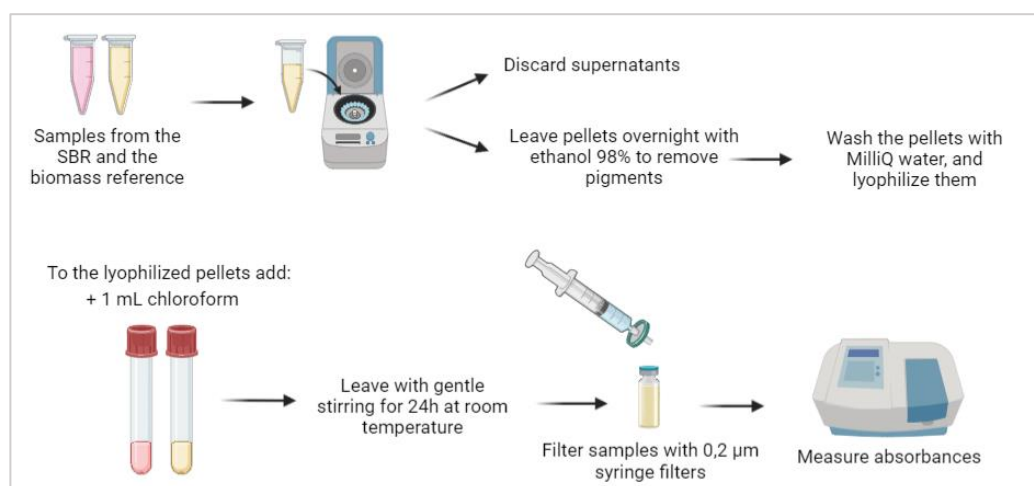


Figure 5-7: Scheme of the protocol followed for the chloroform experiment, with a pre-wash with ethanol 98% to remove pigment interferences. The samples were prepared in duplicate before leaving for 24h with gentle stirring (not shown in the scheme).

Lastly, since the experiments were made at room temperature, and the protocol for PHA digestion has a step of incubating the samples at 100°C for 2 hours, we wanted to confirm this difference in temperatures was not affecting the compounds being extracted to chloroform. For this purpose, the PHA digestion protocol was performed with lyophilized biomass from the SBR, previously left with ethanol 98% overnight to remove pigment interferences in the same way. The organic phase, obtained after phase separation, was stored in a GC vial, and the absorbance measured in the same way as in the previous protocols. The absorbance spectrums obtained from both protocols, (stirring 24h at room temperature with chloroform, and from the PHA digestion [2h at 100°], both performed with lyophilized biomass, from the SBR, previously washed with ethanol 98%) were compared.

RESULTS AND DISCUSSION

6.1 SBR

6.1.1 Full-cycle characterization

During the period from September 2021 to July 2022, the SBR was maintained in the same conditions, with a biomass concentration of 0.7 ± 0.2 g/L of VSS (value obtained by calculating the average of the initial biomass concentrations measured in 11 full-cycles monitored between October and June). Despite attempts to keep all parameters stable, the system proved to be highly sensitive and with long recovery times between instabilities (presented in the following section 0), emphasizing the need for extreme caution when operating it. For this reason, only a few full-cycles had identical and comparable profiles (5 full-cycles), while the remaining 7 showed non-viable results, mostly for sulfide analysis. Nonetheless, the profiles between them remained as identical as possible over the months, for all the analyzed parameters. A general graph showing the initial values for the various parameters analyzed in the full-cycles is shown in Figure 6-1. As can be noted, even when trying to keep the feeding solutions identical, and despite having the feeding pumps calibrated, there was a constant variation in the initial concentrations of the feeding solutions, mainly for the sulfides. This could be due to losses of sulfides in the preparation of the feeding solution, during the sampling procedure, or even during the preparation of the samples for analysis. This can also be the case for some of the inorganic carbon values detected. Furthermore, it is noteworthy that the maximum glycogen values observed were below 30% GLY/VSS, with the exception of 3 instances where they exceeded 70% (Figure 6-1, D). These values were unexpected and could be attributed to a potential high production by the culture or analysis errors.

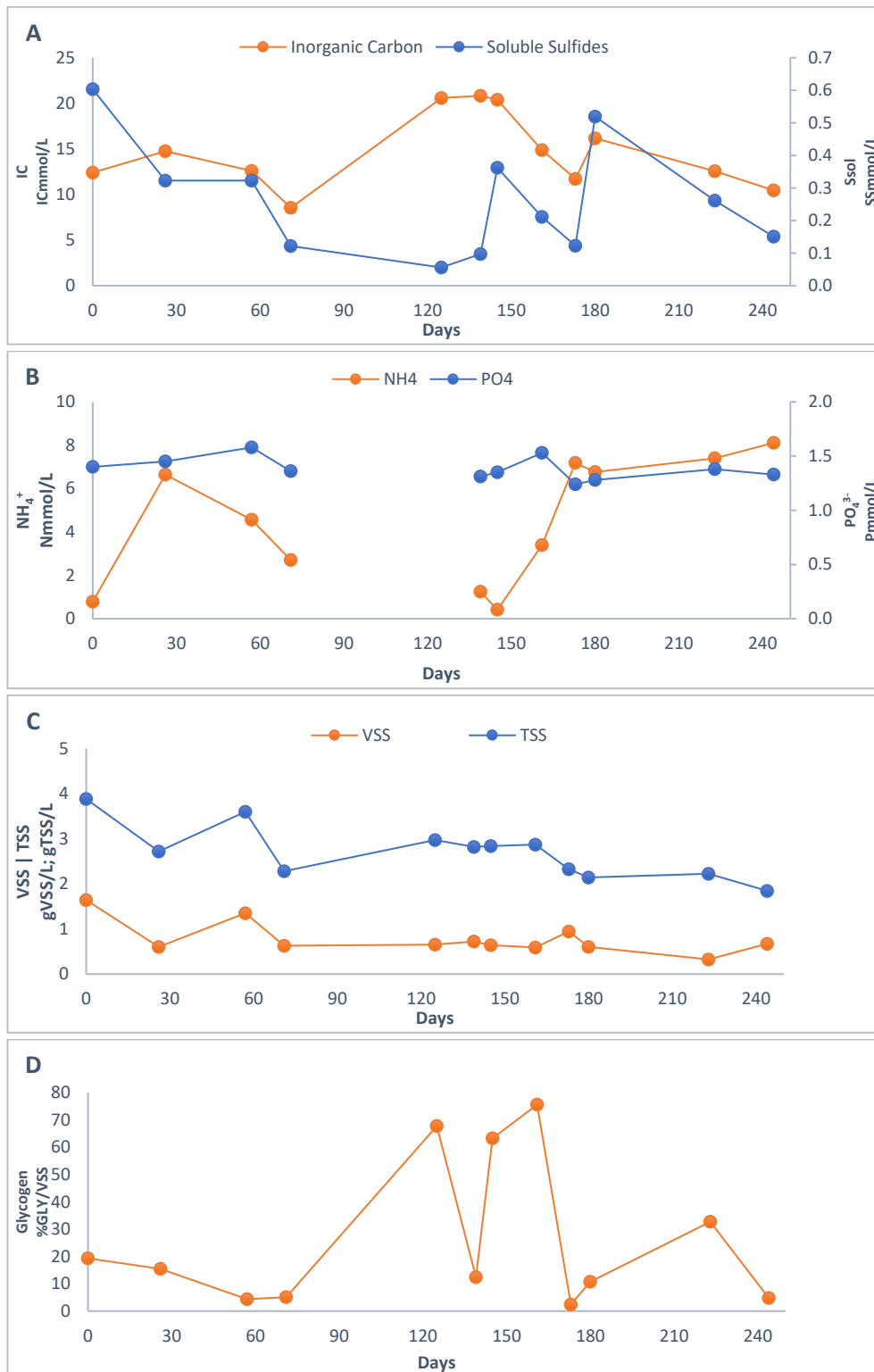


Figure 6-1: Values obtained from the 12 full-cycles analyzed between September 2021 to July 2022. **A:** first value obtained post-feeding for Inorganic Carbon (IC in ICmmol/L) and Soluble Sulfides (SS in SSmmol/L); **B:** first value obtained post-feeding for nutrients NH₄⁺ (Nmmol/L) and PO₄³⁻ (Pmmol/L). There is a gap due to the loss of data from 1 full cycle; **C:** biomass concentration at the beginning of each full-cycle (gVSS/L) and the correspondent total suspended solids (gTSS/L); **D:** maximum value of glycogen (%GLY/VSS) obtained.

6.1.1.1 Reference Full-cycle

Due to the pre-existing problem with the GC equipment, it was only possible to analyze a restricted number of samples for the detection and quantification of PHA. Therefore, we chose the full-cycle that showed the best results for all parameters analyzed (performed in 15 march, 2022) and 4 PHA samples were analyze in this full-cycle. This full-cycle is referred throughout this thesis as the "reference full-cycle" and the values obtained in the calculations of its results are the ones used as a method of comparison from now on.

The following graphs refer to this reference full-cycle and demonstrate the usual behavior of the SBR. With regard to sulfide consumption, it could be monitored through the measured ORP values (Figure 6-2). When sulfide is added to the reactor, the ORP drops to -300mV due to the highly reductive nature of sulfide. During the first 2h of the cycle, sulfide is oxidized by the culture into S^0 , and when sulfide becomes depleted, S^0 is oxidized to sulfate and we observe an ORP increase until reaching a plateau, which likely marks the depletion of S^0 . It is possible to observe this variation in the Figure 6-2, with a noticeable rise in the ORP values after 1.8h (when the 0.5mM of added sulfides were depleted). This variation allowed us to confirm daily if the SBR remained in a stable condition, as the ORP value started to change to higher values after an average of 2 ± 0.4 hours (average of 5 full-cycles).

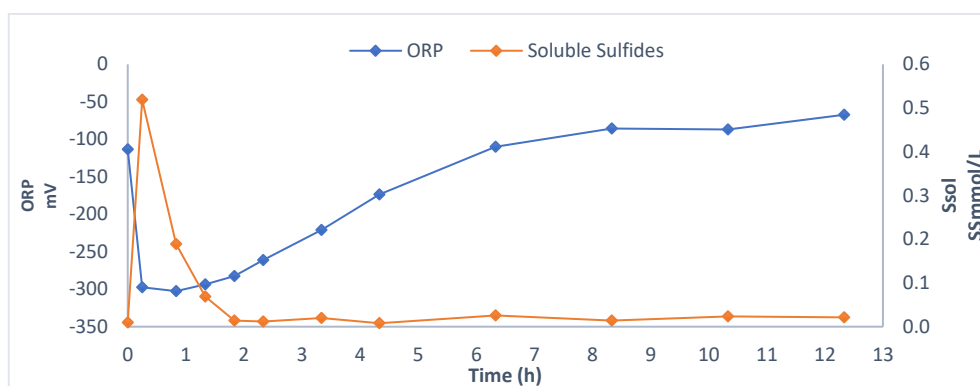


Figure 6-2: Relation between soluble sulfide (SS) consumption (mM) and ORP values (mV), from the SBR reactor, in a reference SBR full-cycle analysis.

The reference full-cycle presented a specific sulfide uptake rate of 0.3 mmol SS/Cmmol X_{i-d} . Regarding the inorganic carbon (Figure 6-3), the 2mM IC added were consumed, although never bringing the system to a state of total depletion, with IC always being in excess. It's possible to notice, in Figure 6-3, that during the first 1.8 hours, corresponding to the period when SS were being consumed, there was a more rapid consumption of IC reaching a maximum specific IC uptake rate of 2.5 mmol IC/Cmmol X_{i-d} . Nevertheless, the consumption of

IC extends until the 4 hours of the cycle, with a rate of 1 mmol IC/ Cmmol Xi-d. This consumption was expected since this is a mixed culture with multiple CO₂ fixation processes present.

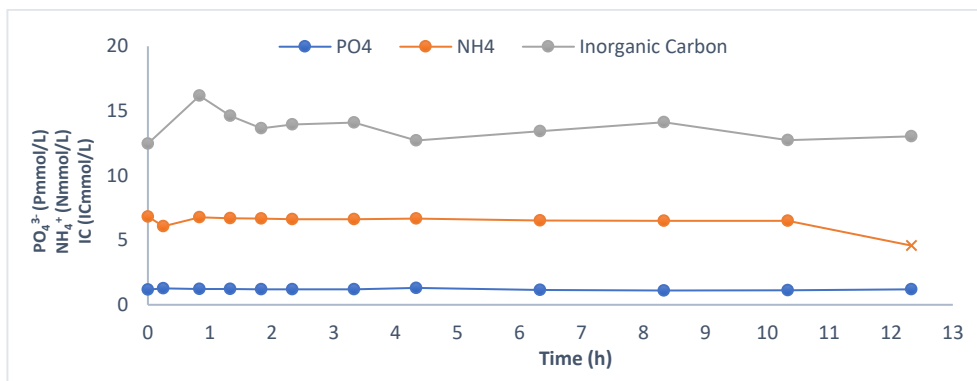


Figure 6-3: Concentration, in mM, of inorganic carbon (IC), and nutrients (PO₄³⁻ and NH₄⁺), in the reference SBR full-cycle analysis.

In regard to nutrient consumption, it is nearly non-existent as the variation were minimal due to the very slow biomass growth. With an SRT of 20 days and 12-hour cycles, biomass productivity is only 0.03 gVSS/L·d, so there is no noticeable consumption of ammonia in particular. The concentration of the nutrients (NH₄⁺ and PO₄³⁻) remained with an average of 6.6 ± 0.2 Nmmol/L, and 1.2 ± 0.1 Pmmol/L, during the whole cycle. The last point in the NH₄⁺ analysis was not included in the calculations as it was assumed to be an error in the analysis.

In terms of glycogen content, there were no significant variations as well, as the values were constant during the cycle with an average value of $6.2 \pm 2.2\%$ of GLY/VSS (1.5 ± 0.5 Cmmol/L), reaching a maximum value of 10.7% of GLY/VSS (2.7 Cmmol/L).

As for PHA content, only 4 samples were analyzed: right after the feed (0.5 h), at the moment of sulfur depletion (1.8 h), at 4.3 h, and at 8.3 h. As it is possible to observe in Figure 6-4, there is a slight increase in the production of PHA in the first 2 hours (from 2.7% of PHA/VSS; 0.7 Cmmol/L to 3% of PHA/VSS; 0.9 Cmmol/L), corresponding to the moment of sulfide and inorganic carbon consumption. However, since the values are too similar, and no more samples could be analyzed, it is impossible to determine if the variation was due to any production during the feast phase, or analysis errors. No conclusions can be drawn from this analysis, only that PHA levels detected were very low, indicating that the culture was not accumulating significant levels.

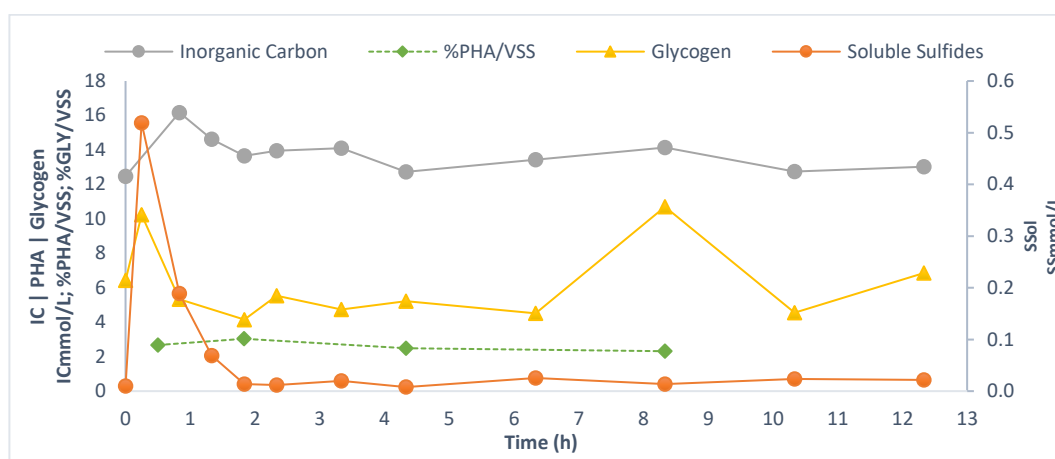


Figure 6-4: Concentrations, in mM, of inorganic carbon (IC) and soluble sulfides (SS), values of glycogen (in %GLY/VSS), and the four samples of PHA (in %PHA/VSS) analyzed at: 0.5; 1.8; 4.3 and 8.3 hours, in the reference SBR full-cycle. The value for the point at 1.3h from glycogen is missing due to an outlier value that was identified as an error.

Results obtained through HPLC analysis using a Refractive Index detector indicated the absence of organic acids, alcohols, or other short-chain organic compounds (aldehydes, ketones). This suggests that either no compound excretion was occurring within the reactor or that any produced compounds were being consumed by the bacterial consortia. Since we are dealing with a mixed culture, it is reasonable to expect phototrophic organisms in the consortium to fix CO₂ and excrete compounds that non-phototrophic organisms may immediately consume.

6.1.2 Pigments characterization

As for the typical pigment profile in ethanol solvent, the absorption spectra obtained for the reference full-cycle can be seen in Figure 6-5. It is objectively observed that there are peaks at 365 and 775 nm, indicating the presence of Bchl *a*, and a peak between 400 and 500 nm indicating the presence of carotenoids. These pigments are indicative of the presence of purple bacteria in the bacterial consortia. On the other hand, green bacteria containing Bchl *c* (visible at a wavelength of 670 nm) are less abundant since a peak at this wavelength is hardly observed.

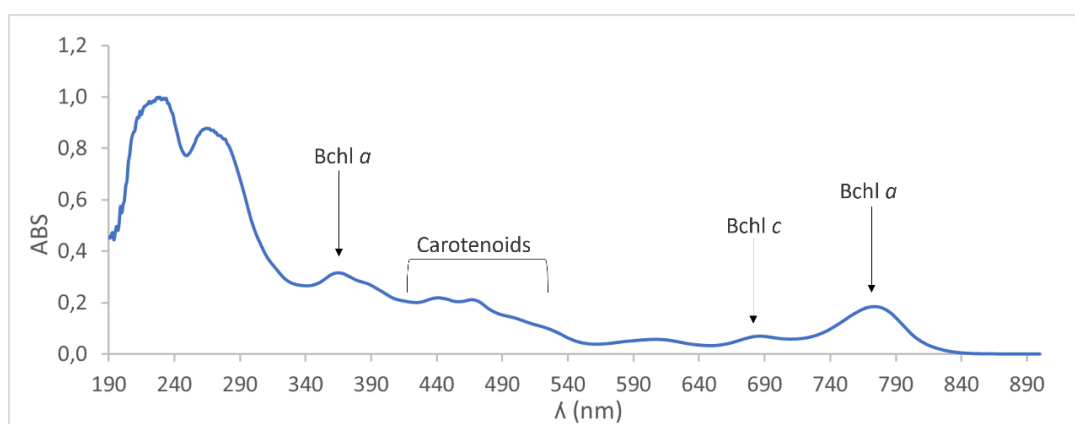


Figure 6-5: Pigment UV-Vis absorption spectra, after extraction with ethanol 98%, from the reference full-cycle. It is also possible to compare the profiles of the UV-Vis absorption spectra of 7 full-cycles performed. This comparison is shown in Figure 6-6, where we can see that over time the ABS of the entire spectra has decreased. Given that the mixed culture's gVSS/L remained constant over time and considering the general decrease in absorbance values, it can be inferred that the culture experienced a reduction in microorganisms with photosynthetic capabilities over time.

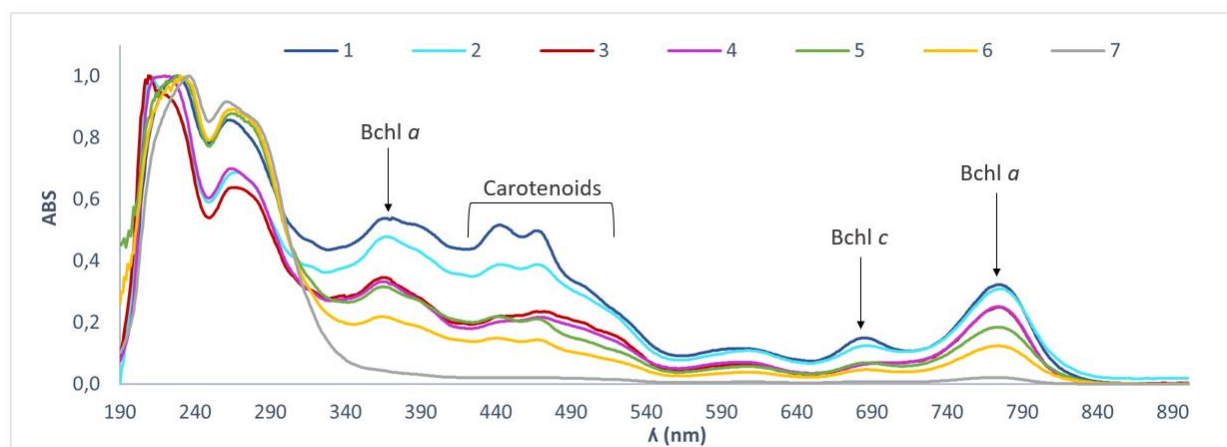


Figure 6-6: Comparison of the pigment UV-Vis absorption spectra, after extraction with ethanol 98%, of 7 full-cycles. The full-cycles are indicated in the figure with the numbers 1 to 7, in order of performance. The line represented by the number 6 corresponds to the reference full-cycle.

6.1.3 Microbial characterization

From microscope observation (Figure 6-7, A1-A3) of the SBR biomass it was possible to observe that the majority of the PMC was constituted by rod-shaped bacteria. Some of these bacteria are in a "star shape" (signed with orange arrows) and can possibly be identified as *Rhodospseudomonas*, a PNSB with PHA production capacity. The biomass typically presented a dispersed appearance, with some large clumps, with a visible example in Figure 6-7-A3. When

observed with Nile blue staining, it was possible to confirm that several bacteria have fluorescent granules in their interior, possibly PHA granules. The "star-shaped" bacteria, however, did not usually show fluorescence, indicating that these bacteria were possibly not contributing to the production of the polymer.

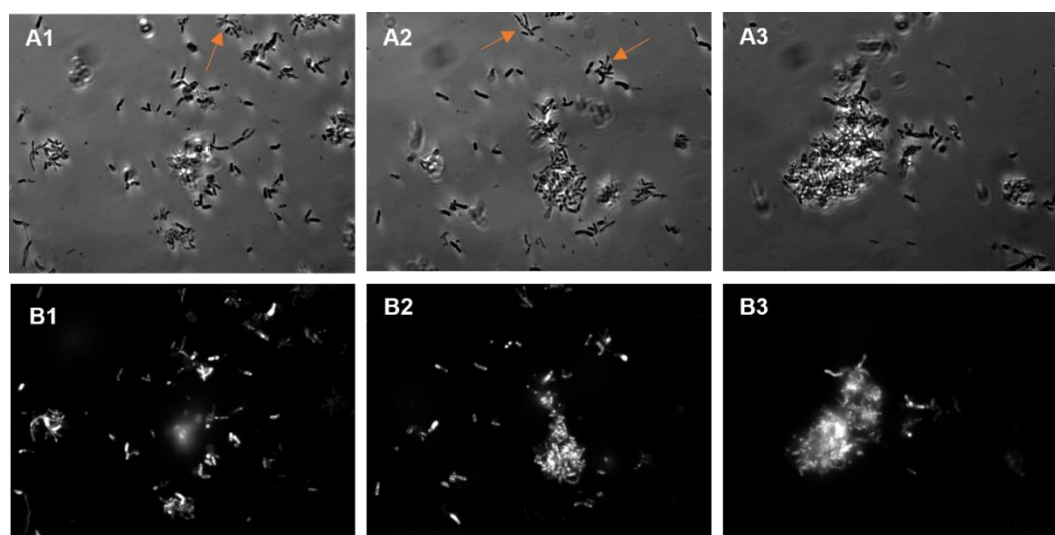


Figure 6-7: **A1-A3** - Microscopic images of the PMC. **B1-B3** - Fluorescence images of Nile blue staining, possibly indicating PHA granules, corresponding to the microscopic images (A1-A3).

With regard to FISH analysis, the samples were from the same day as the Nile blue results presented, and the results obtained are shown in Table 6.1. In a general way the Alphaproteobacteria class was the most dominant, with almost no presence of the Gammaproteobacteria class. Archaea were not found. *Rhodopseudomonas* were only detected when the sample fixation was performed with PFA (to fixate Gram -), the lack of detection in the Gram + samples (using ethanol to perform the sample fixation) could potentially stem from errors in the sample preparation.

Table 6.1: Qualitative results from FISH analysis from the PMC of the SBR. **EUBmix** - Bacteria; **ARC915** - Archaea; **ALF969** - Alphaproteobacteria; **GAM42a** - Gammaproteobacteria; **Rhodopseud** - *Rhodopseudomonas*.

<i>Samples</i>	EUBmix	ARC915	ALF969	GAM42a	Rhodopseud
Gram -	(+++)	(-)	(++)	(●)	(++)
Gram +	(+++)	(-)	(+)	(●)	(-)

(-) Non present; (●) Almost non-existent; (+) Present; (++) Abundant; (+++) Extremely abundant.

FISH images revealed an elevated presence particularly of rod-shaped and coccus, using the universal bacterial probe EUBmix (Figure 6-8, A). From the ALF969 probe (Figure 6-8, B) the majority of the cells were rod-shaped, with some "star-shaped" bacteria present. This "star-shaped" bacteria can also be detected in the images obtained with Rhodopseud probe (Figure

6-8, D1-D3). Since *Rhodopseudomonas* belong to the Alphaproteobacteria subgroup, it seems to reason that a substantial proportion of the rod-shaped cells detected with the ALF969 probe could be *Rhodopseudomonas*. Nonetheless, it is important to note that while the ALF969 probe detected a significant proportion of bacteria, many of them did not hybridize with the Rhodopseud probe. This finding suggests that a vast number of this remaining bacteria belongs to a different genera. Regarding the GAM42a probe, it is worth noting that the majority of hybridizing cells were cocci-shaped (Figure 6-8, C). Moreover, these bacteria do not typically exhibit any fluorescence when observed through Nile blue staining, indicating that they were not producing any PHA.

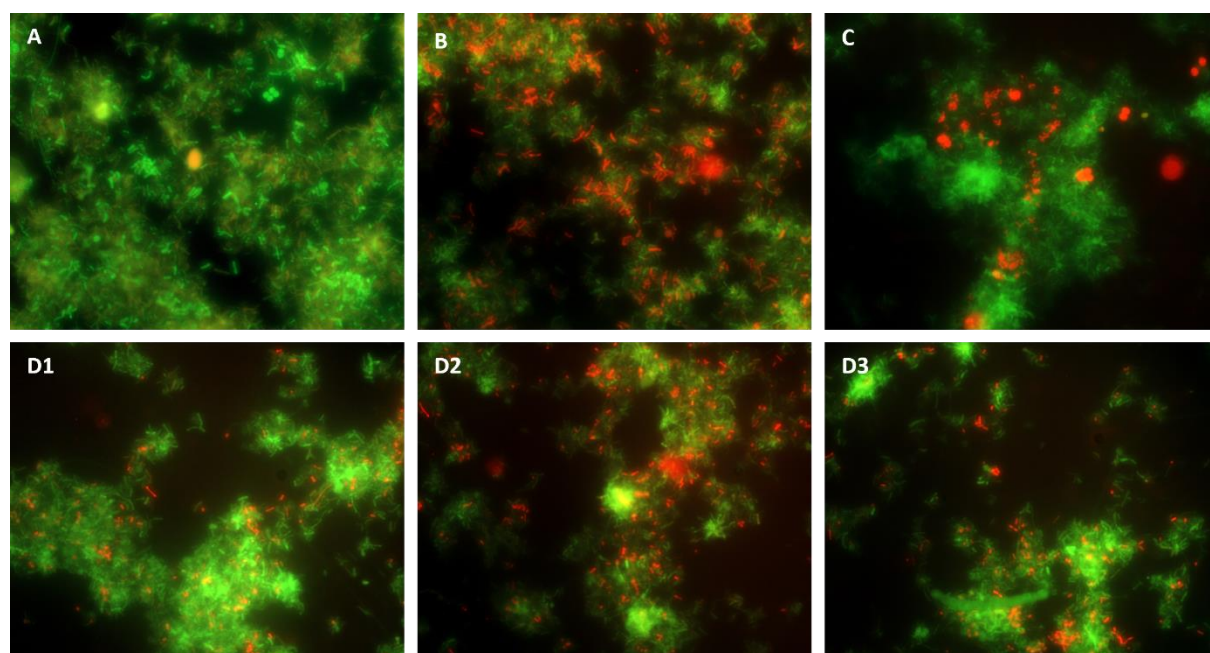


Figure 6-8: Pictures obtained from FISH analysis from the PMC of the SBR. **A** - EUBmix (bacteria); **B** - ALF969 (Alphaproteobacteria); **C** - GAM42a (Gammaproteobacteria); **D1-D3** - Rhodopseud (*Rhodopseudomonas*).

A remarkable aspect of the bacterial characterization performed is that the *Rhodopseudomonas* (star-shaped cells), which seem to constitute a large part of the Alphaproteobacteria present, show no fluorescence in the Nile blue analysis, and so, do not seem to be contributing to the accumulation of PHA in the SBR. It can be hypothesized that PHA production within the SBR was not due to Gammaproteobacteria or *Rhodopseudomonas*. Instead, it could possibly be associated with another alphaproteobacterial genus.

6.1.4 Instabilities

During the whole time the SBR was operated, we encountered numerous reasons that caused instabilities in the PMC behavior, which most times lead to a loss of activity from the culture,

reflected by static ORP values (between -350mV and -300mV). These values indicated a non-consumption of sulfides, and therefore no consumption of inorganic carbon. Until the SBR returned to a stable operation we could not perform the batch tests, which preceded a delay in the planning of the experimental design.

By the time this thesis started, the system had already been operated for 253 days under the same conditions. Therefore, in this sub-section and consequently in Figure 6-9, the day 253 corresponds to this thesis first day of operation with this system.

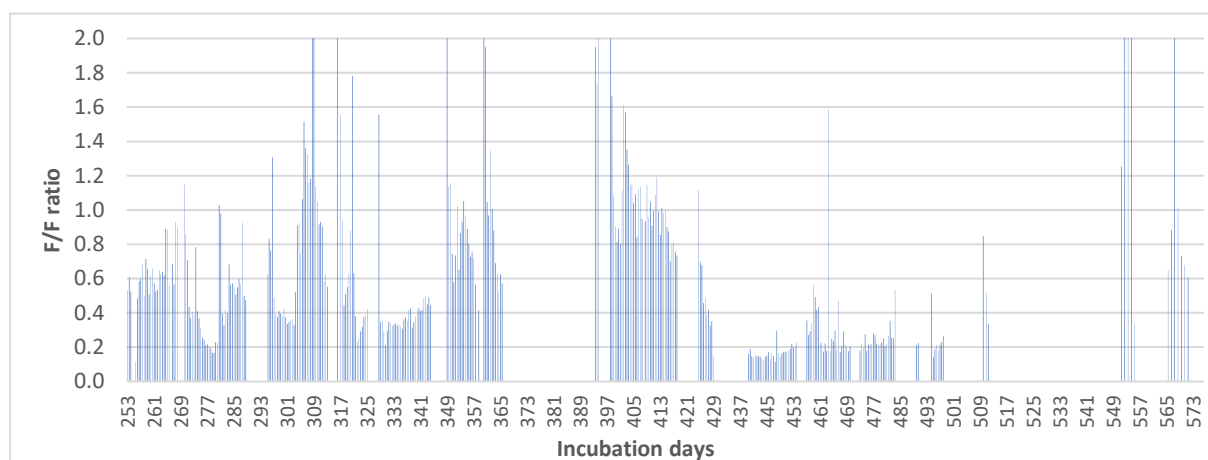


Figure 6-9: Feast to Famine (F/F) ratio of sulfides of the SBR, for 323 days, from 1 September 2021 to 21 July 2022.

As it is possible to observe in **Erro! A origem da referência não foi encontrada.**, the Feast to Famine (F/F) ratio of sulfides was not always constant, despite all the attempts to maintain the system in the same conditions for the whole period. On September 1, 2021, the SBR was already at 253 days of incubation, and the system remained stable for 61 days. During this period 2 full-cycles and 2 batch tests were performed. The gap in data from day 289 to day 295 was due to a lack of signal from the data collector device, but the system was maintained stable. At day 304 the reactor was brushed, and the system was slightly affected, resulting in a slowly consumption of sulfides, proven by longer feast periods, thereby resulting in higher F/F ratio values. When the culture was starting to stabilize, a problem with the HCl pump resulted in pH instabilities, at day 314, and in a stagnant culture behavior that lasted 4 days. The SBR returned then to the stable state, a batch test was performed, and at day 326 the HCl pump malfunctioned again, leaving the culture unstable. At day 329 the culture was stable and remained in that state for 19 days, during which 2 full-cycles were analyzed. At day 345 biomass was removed for a batch test, and the SBR was brushed, resulting in system instability, where the feast period was more than double the normal value, reaching an average of $5.6 \pm 1.2h$,

until day 366. From this point until day 397, a gap in data can be observed, due to the loss of data from the collection device, that could not be recovered. Regarding the data loss it is possible to observe that the system was becoming progressively slower at sulfide consumption, shown by higher F/F ratio values, and at day 397, 500 mL of biomass were removed from the SBR and 500mL of recent purge, which was stored at 4°C, were added. Until day 419 the system was as stable as possible, and a full-cycle analysis and a batch test were performed. At day 419 the SBR was brushed, but the culture did not react well, making the system unstable, for 5 days. After stabilizing, a full-cycle and a batch test were performed on day 429, and the following day the NaOH pump malfunctioned. Probably due to the high value of removed volume and the pH instability, the SBR became unstable and remained with no activity until day 440. From here, until day 499 the system was stable, with a slight decrease in the F/F ratio value, and 2 full-cycle analyses and 6 batch tests were performed. During this period some instabilities happened to the system, which can be seen by the gaps in the graph, caused by different motives, but the culture has returned to a stable F/F ratio after 2 to 3 days each time. However, on day 499, after the removal of biomass for a batch test, and brushing the SBR, the culture became unstable, and after 4 days, there was a problem with the stirrer that reflected in a longer period of stabilization. At day 512 the culture had returned to a stable state, but the data from this day to day 538 were lost, due to the same reason as previously. However, in these 26 days, 4 batch tests were performed. On day 539 a power failure in the lab shut down the pumps and the collector data device, leaving the culture with no activity, that could not be restored until the end of the internship. Attempts to return to an active culture were made, such as adding purge and reducing the sulfide feeding, but all failed, as, during these 37 days, several other occurrences, such as pH pump malfunctions have contributed to a constant state of non-activity in the culture.

The various instabilities that occurred throughout the period are explained in further detail in the following.

6.1.4.1 Controllable instabilities

Certain instabilities were caused by our decisions towards the SBR reactor, such as the moment of the brushing, as the opening of the reactor resulted in an oxygen entrance into the system. Even with the argon flushing afterwards, to remove any existing oxygen presence, sometimes the PMC was already overly affected, needing a few days to recover and get back to the normal feast and famine profile. Another factor that led to instabilities was the removal

of additional biomass from the SBR to perform the batch tests. At a certain point, around 490 days of incubation, it was observed a washout of the purple bacterial culture, resulting in white-colored mass (without pigments) possibly resulting from performing several consecutive batch assays, not allowing the SBR culture to recover.

Some possible suggestions to avoid the mentioned problems could be the brushing of the reactor with constant presence of bubbling argon, to reduce oxygen entrance, and wider spacing in the conduct of batch tests. Even using the 67.5 mL of withdrawal collected prior to the beginning of the cycle of the SBR, an extra 432.5 mL were required to reach the 500 mL needed for the batch tests. As biomass growth was already slow due to the 20-day SRT, it was further affected by the large amounts of volume harvested, each time, for the performance of the batch tests. To overcome this problem the volume used for the batch tests could be adjusted to a smaller one, or the tests could be spaced further apart, to allow the SBR to recover.

6.1.4.2 Incontrollable instabilities

In addition to the factors already mentioned, there were several times when the SBR became unstable due to reasons beyond our control. Within these are mainly failures in the pH adjustment and failures in the light intensity received by the reactor. The reasons that caused the pH irregularities were several, such as malfunctions of the pumps, caused by power failures in the laboratory or reasons beyond our knowledge in which the pumps did not turn on at the set time, or uncontrolled addition of acid and base solution, due to electrode deregulation. Faults in stirring also led to inaccurate data detection by the electrode, resulting in the uncontrolled addition of pH solutions. As for the failures in light intensity supplied to the reactor, the main reasons were power failures in the laboratory and the burning of lamps, leaving the reactors without light, or with the wrong intensity, mainly during the weekends, when the SBR was unsupervised.

These causes showed us the importance of paying close attention to certain parameters, such as pH, agitation, and light intensity, to try to avoid instabilities in the SBR profile, and consequent delays in test planning. It also led us to the conclusion that it is, in fact, an extremely sensitive system with highly demanding operating conditions. A much stricter control of these parameters is essential to prevent these difficulties.

6.2 Batch tests

An overview of each batch test performed will be presented, followed by a comparison between them, with the respective specific uptake rates of soluble sulfides and inorganic carbon as well as the production rates of glycogen and PHAs. Furthermore, the results obtained from the batch tests performed without light availability will be presented and discussed.

6.2.1 Abiotic controls

Firstly, it is important to note that during the abiotic tests, a decline in sulfide levels was observed within the initial 2 hours, ultimately depleting entirely at the 7-hour mark (Figure 6-10, left). The decrease in sulphides was attributed to losses that occurred during sampling, which are unavoidable, even with the best efforts to minimize oxygen input into the system. However, as the majority of sulfide consumption occurred within the first 2-hour interval in all the biotic batch tests conducted, and assuming consumption outweighed sampling losses, it was decided to conduct the batch tests even in the presence of these possible losses. Nevertheless, the concentrations of the other components (IC, N, and P) remained constant (Figure 6-10, right), indicating an absence of contamination in the medium.

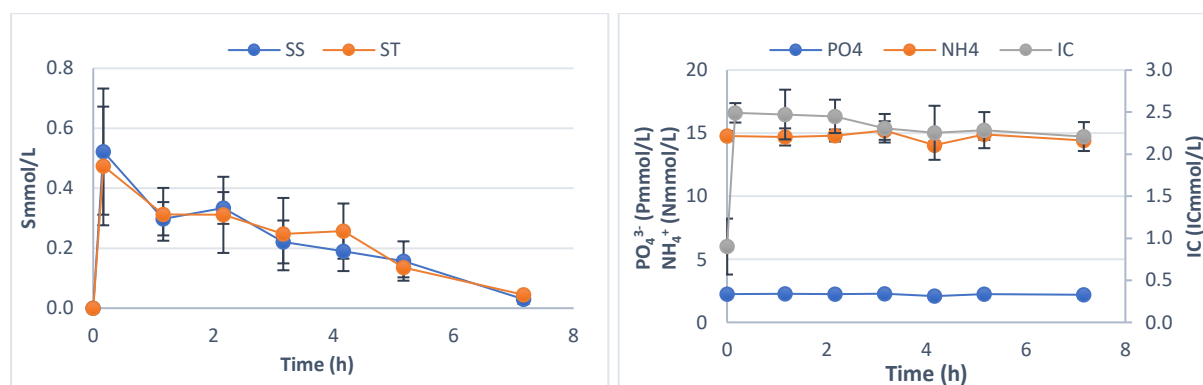


Figure 6-10: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM (left), and concentrations of nutrients (PO₄³⁻ and NH₄⁺) in mMP and mMN, and Inorganic Carbon (IC), in mMC (right), in the abiotic control performed. Error bars calculated from duplicate tests.

6.2.2 1.8 W/gVSS

The first batch test was performed with the lowest light intensity (1.8 W/gVSS) corresponding to a light intensity of 26.5 W/m², and a volumetric light intensity of 1.1 W/L (half of the intensity supplied to the SBR). During the 8 hours in which the test was performed no total depletion of SS was observed (Figure 6-11, left). The point corresponding to 8h corresponds to a loss

of data, but we can assume there was no consumption of SS since no alteration in ST is observed. This was the only batch test where total depletion of soluble sulfides was not observed. It was anticipated that the culture would have slower activity due to the lower amount of light supplied compared to the SBR.

Regarding the uptake of inorganic carbon, consumption can also be observed, particularly during the first 6 hours (see Figure 6-11, right). This consumption is significantly greater during the first two hours when a higher amount of SS is also being consumed.

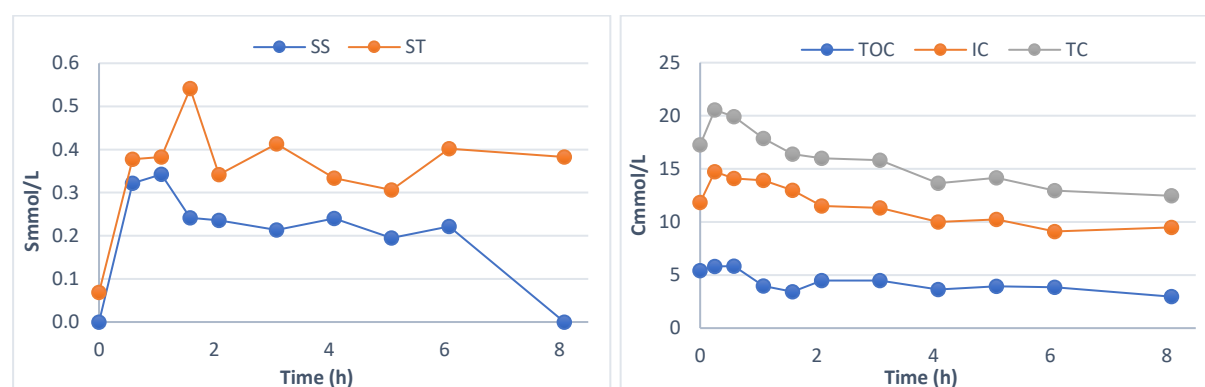


Figure 6-11: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM (left), and concentrations of Total Organic Carbon (TOC), Inorganic carbon (IC), and Total Carbon (TC), in mM (right), during the batch test with 1.8 W/gVSS.

As for nutrient concentrations (N and P), there were no significant variations during the 8-hour period. This lack of variation raises questions regarding what is happening in the culture, as the concentration of soluble sulfides and inorganic carbon decreases. It remains to be determined whether the decrease in SS concentration could be attributed to sampling losses, as is known to occur from the abiotic controls, or whether it is actually being consumed by the culture. As there is no decrease in IC concentration in the abiotic controls, we can infer that its decrease is due to the culture's consumption.

Regarding glycogen content, the culture had an average $54.2 \pm 16.4\%$ of GLY/VSS (11.2 ± 3.4 Cmmol/L), reaching a maximum of 61.1% of GLY/VSS (12.9 Cmmol/L), at the end of the first hour, two times the initial value, detected before feeding, of 31.9% of GLY/VSS (6.6 Cmmol/L). This high value could be an error in the analysis, but since there is no replicate for this condition it cannot be confirmed. A possible alternative explanation for the high percentage is that the culture could be in fact accumulating higher percentages of GLY/VSS by the time this batch test was performed, and as the following batch tests were conducted, the biomass capable of accumulating high concentrations of glycogen was gradually removed, and the SBR was not given time to recover. This hypothesis is supported by the fact that a full-

cycle realized at the same day as this batch test showed an average %GLY/VSS content of $41.3 \pm 14.8\%$ of GLY/VSS (9.4 ± 3.4 Cmmol/L). In the same way, the following full-cycles did not detect such high amounts of glycogen, even though we maintained the same conditions throughout the whole study.

As for PHA, at the end of the test (8h), it reached 5.3% of PHA/VSS (1.4 Cmmol/L). Since it was only feasible to analyze 1 sample for PHA content per batch test, it is not possible to determine whether the concentration increased or decreased throughout the test. The only feasible comparison is with the PHA value at the beginning of the reference full-cycle since this is the only full-cycle analysis we were able to conduct. Comparing these values, the percentage of PHA/VSS obtained in this batch test (5.3%) exceeded that in the reference full-cycle (2.7%). However, since the culture was achieving high percentages in glycogen production, it can be assumed that it could also be producing higher amounts of PHA.

6.2.3 3.7 W/gVSS

The second condition tested was at a light intensity of 3.7 W/gVSS, similar to the one provided to the SBR (3.5 W/gVSS). The light intensity provided for this batch test corresponded to 2.2 W/L, and a volumetric light intensity of 54.8 W/m². For this condition we verified total sulfide depletion at 4.6 hours (Figure 6-12, left). During this period a consumption of inorganic carbon is also observable (Figure 6-12, right), particularly during the first 3 hours, which links with the time period of sulfide consumption. The nutrients concentration remained constant during the whole test.

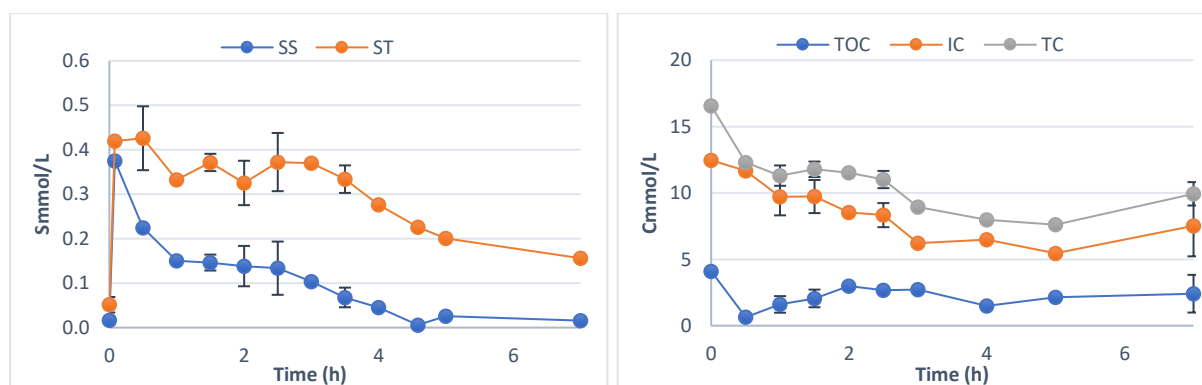


Figure 6-12: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM (left), and concentrations of Total Organic Carbon (TOC), Inorganic carbon (IC), and Total Carbon (TC), in mM (right), during the batch test with 3.7 W/gVSS. Error bars calculated from duplicate tests.

Regarding the content of glycogen, it maintained an average of $8.98 \pm 0.26\%$ of GLY/gVSS (2.74 ± 0.22 Cmmol/L), with the highest value obtained in the initial sample, corresponding to 9.56

$\pm 3.13\%$ of GLY/gVSS (3.10 ± 1.72 Cmmol/L). It is possible that this decrease could be an indication of glycogen consumption by the culture. Nevertheless, the data is inconclusive as the values are very low, and do not exhibit significant variation. As for PHA, at the end of the 7 hours, it presented $2.37 \pm 0.05\%$ of PHA/VSS (0.75 ± 0.08 Cmmol/L), a value identical to that of the reference full-cycle (2.7%).

6.2.4 12.6 W/gVSS

The third condition tested (12.6 W/gVSS), corresponded to a light intensity of 4.6 W/L, doubling the exposure provided to SBR (2.09 W/L), and a volumetric light intensity of 113.8 W/m². Two replicates were evaluated, but one was excluded due to a lower concentration of sulfides added than planned (possibly due to the loss of sulfides in the feeding solution), and the TOC analysis containing too many errors. Hence, the presented results are based on a single batch test. Once again, the culture took near 5 hours to be depleted of sulfides, and a consumption of inorganic carbon can be observed during the whole period of testing, as seen in Figure 6-13. The TOC results presented errors in the analysis of the 3 first samples, so the consumption rates were calculated using the point at 1h as the initial value (12.6 mM IC). It is important to note that this result will have a high degree of error due the use of the result obtained at the firsts hour as the initial value since the highest consumption occurs precisely in the first hours of the test. The concentration of nutrients remained constant during the whole experiment.

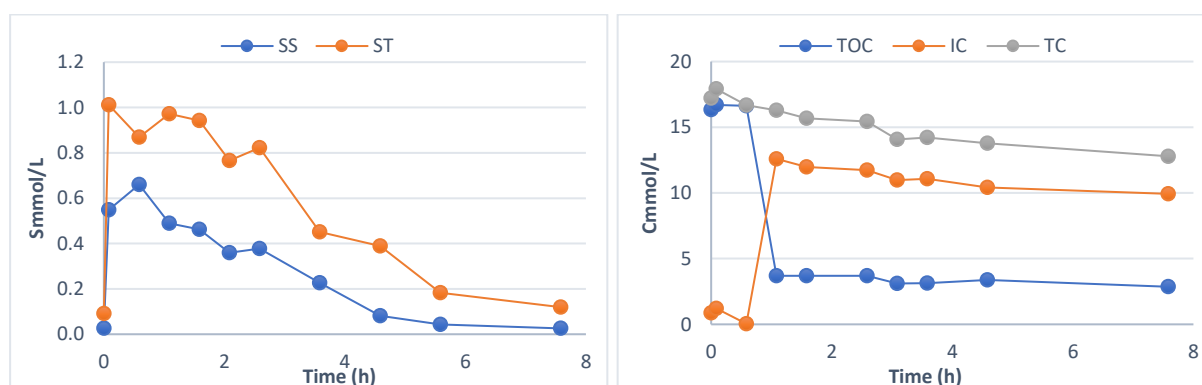


Figure 6-13: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM/L (left), and concentrations of Total Organic Carbon (TOC), Inorganic carbon (IC), and Total Carbon (TC), in mM/L (right), during the batch test with 12.6 W/gVSS.

For this condition a maximum of 11.3% of GLY/VSS (1.3 Cmmol/L) was detected, presenting a slight increase compared to the initial value (7.47% of GLY/VSS (0.87 Cmmol/L)). The PHA analysis was performed on the last sample collected (7.6h), detecting 3.7% of PHA/VSS (0.6

Cmmol/L), also being identical to the one obtained in the reference full-cycle analysis. It is unclear whether this value represents the maximum PHA produced by the culture or if some of the PHA produced was already consumed, since the analysis was conducted with the last sample. This would be normal behavior since the culture was depleted of SS around the fifth hour of the test.

6.2.5 14.9 W/gVSS

This batch test (14.9 W/gVSS) corresponded to a light intensity of 8.2 W/L (four times the intensity supplied to the reactor), and a volumetric light intensity of 199.6 W/m².

In this test the SS were consumed during the 8-hour period (Figure 6-14, left). The point around 5 hours was deemed to be an analysis error as the IC continued to be consumed during the whole test period. The nutrient concentration remained, in the same way as previous, constant the whole test.

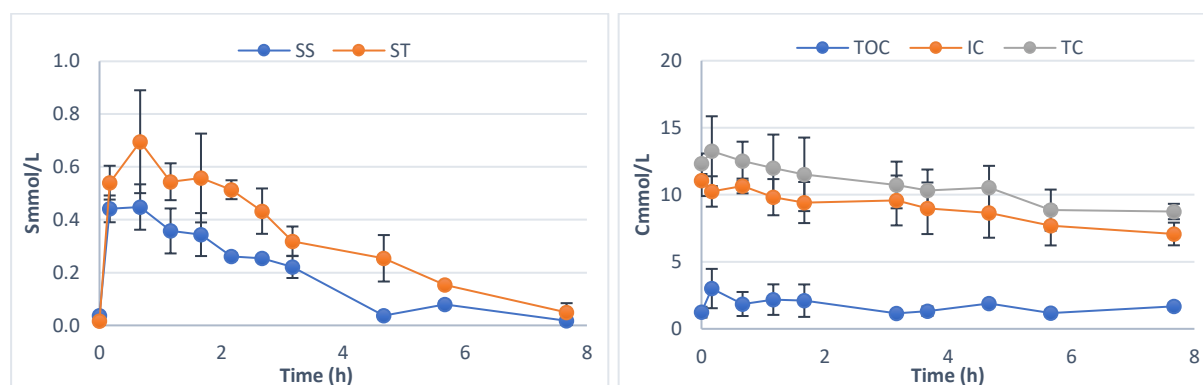


Figure 6-14: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM/L (left), and concentrations of Total Organic Carbon (TOC), Inorganic carbon (IC), and Total Carbon (TC), in mM/L (right), during the batch test with 14.9 W/gVSS. Error bars calculated from duplicate tests.

As for glycogen we obtained fairly low values, reaching a maximum of only $2.48 \pm 0.67\%$ of GLY/VSS (0.53 ± 0.33 Cmmol/L), being the lowest values obtained so far in a batch test. Compared to the initial sample ($2.34 \pm 0.73\%$ GLY/VSS; 0.52 ± 0.35 Cmmol/L), there were no variations in the maximum value obtained. It can be inferred that no glycogen production occurred during this test.

As for PHA, at the moment of soluble sulfide depletion (4.7 hours) it was detected $2.9 \pm 0.6\%$ of PHA/VSS (0.5 ± 0.1 Cmmol/L), and at the end of the batch test (7.7 hours) $2.8 \pm 0.5\%$ of PHA/VSS (0.5 ± 0.1 Cmmol/L). During the 3 hours where the culture didn't have soluble sulfides available, no PHA was consumed, but it could be explained by almost residual values.

6.2.6 24.3 W/gVSS

The last condition tested, was made without a replicate (as the first one [1.8 W/gVSS]), and corresponded to a light intensity of, 16.4 W/L (eight times the intensity provided to the SBR), and a volumetric light intensity of 401.8 W/m². In this test the soluble sulfides were depleted in only 2.2 hours (Figure 6-15), the shortest time obtained from all the batch tests.

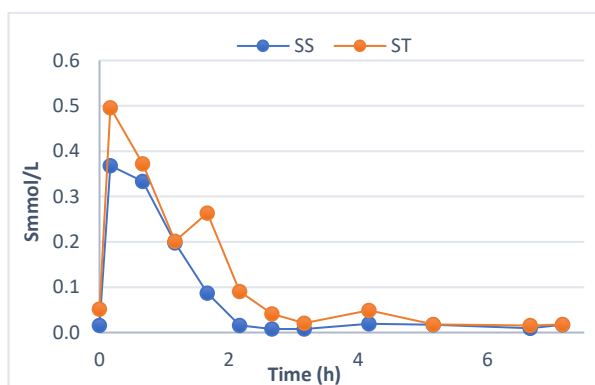


Figure 6-15: Concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM/L, during the batch test with 24.3 W/gVSS.

Regarding the inorganic carbon, there was an error during the TOC analysis, as the TOC values obtained were below zero, and therefore not viable. Since there were no more samples available to repeat the analysis, and there was no replicate, we have no information regarding the inorganic carbon consumption, and can only analyze the sulfide consumption. As for the nutrients, the concentrations remained constant during the 7 hours of experiment, as in previous tests. Since we don't have the data for IC consumption, we can't determine whether the pronounced decline in SS concentration was due to consumption by the culture or whether it was due to losses during sampling. There is also the question of whether the high light intensity could have affected the SS concentration inside the flask. An abiotic test using this light intensity could answer this question.

Concerning the content of glycogen and PHA, this batch test obtained very low, almost residual results for both. For glycogen the maximum value obtained was 0.78% of GLY/VSS (0.18 Cmmol/L), and for PHA was of 0.57% of PHA/VSS (0.13 Cmmol/L). The value of the PHA obtained corresponded to the last point of the test (7.2 hours). So, although this was the batch test with more light intensity provided, and with the fastest sulfide consumption, it was also the batch test with the lowest concentrations of glycogen and PHA produced.

Nile blue staining was only performed on this last batch test. The results show some fluorescing granules visible in Figure 6-16 (B1, B2), even though the results of the glycogen, and PHA, accumulation analysis showed the lowest results among all batch tests. Since there are

no other results from the batch tests, there is no way to compare the fluorescence. The only comparison possible is with the reference full-cycle, where the percentages of glycogen and PHA produced were higher, but the Nile blue staining results showed a lower amount of fluorescence granules.

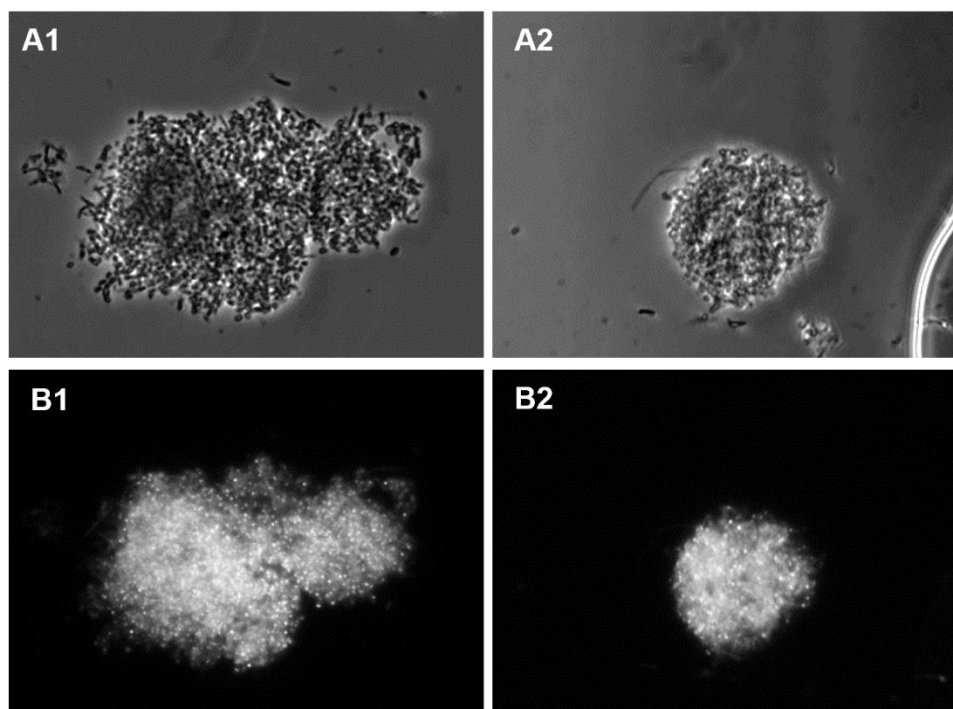


Figure 6-16: **A1-A2** - Microscopic images of biomass from the batch test with 24.3 W/gVSS of light available. **B1-B2** - Fluorescence images of Nile blue staining indicating PHA granules, corresponding to the microscopic images (A1-A2).

6.2.7 Comparing all the batch tests

When comparing all the batch tests, we can see in Figure 6-17 that the specific uptake rates of Soluble Sulfides (qSS) were lower in the first two batch tests, most likely due to an insufficient light intensity. The rates, however, increased from 12.6W/gVSS onward, having stagnated around the same values. We can assume that increasing light intensity only seems to have an effect up to 12.6W/gVSS, with regard to sulfide consumption. There was a significant difference between the batch tests and the reference full-cycle when comparing specific uptake rates, where the batch tests had visibly lower results.

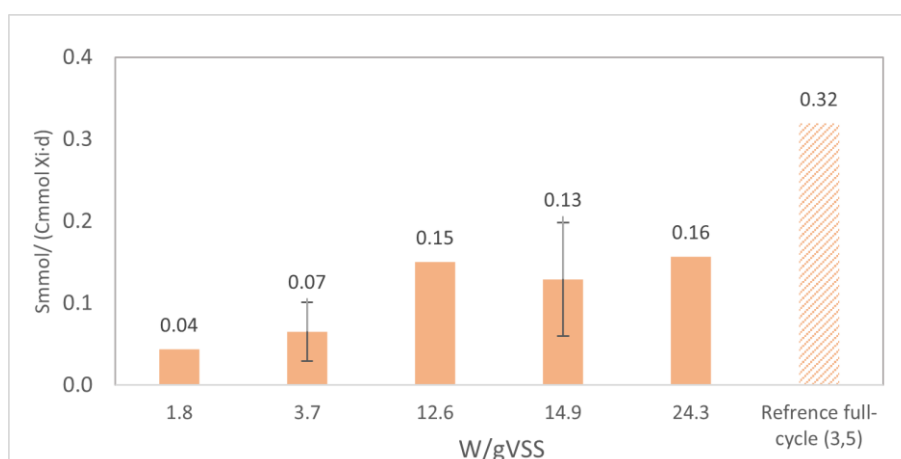


Figure 6-17: Specific uptake rates of Soluble Sulfides (SS), in Smmol/ (Cmmol Xi · d), for the batch tests with different light intensities, and the reference full-cycle.

As for the specific uptake rate of Inorganic Carbon (qIC), presented in Figure 6-18, there was no significant changes with the increase in light intensity, and the values obtained were similar to the reference full-cycle result. So, despite the increase in sulfide uptake rate, the rate of inorganic carbon consumption remained constant.

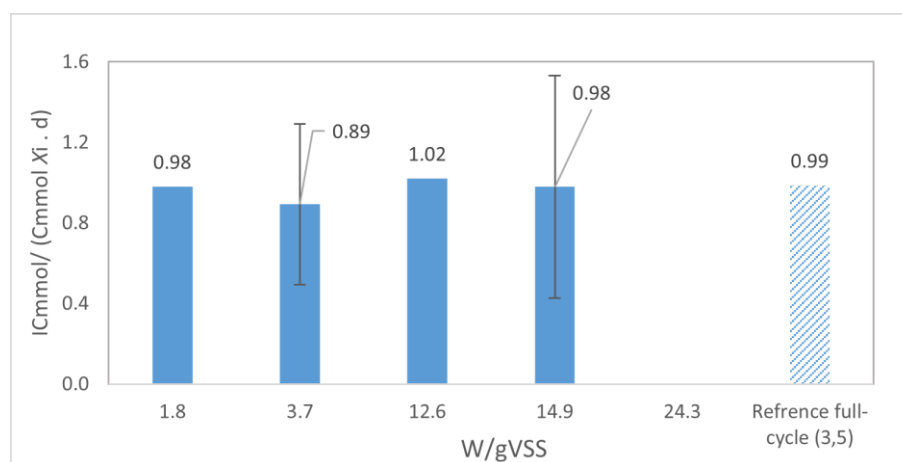


Figure 6-18: Specific uptake rates of Inorganic Carbon (IC), in ICmmol/ (Cmmol Xi · d), for the batch tests with different light intensities, and the reference full-cycle. The batch test performed with 24.3W/gVSS presented errors in the TOC analysis that made the results invalid.

As for nutrients consumption, the concentrations of N and P were always constant during the duration of all the batch tests, showing nearly no intake. An average of the concentrations of nutrients in all batch tests can be seen in Figure 6-19. Comparing all the tests, the concentration of P (mM) was around the same for all (around 1.2 mM), but the concentration of N slightly changed, especially in the first batch tests (1.8 W/gVSS) most probably caused by an error in the feeding inorganic medium. Nonetheless, there was never any shortage of nutrients during the tests.

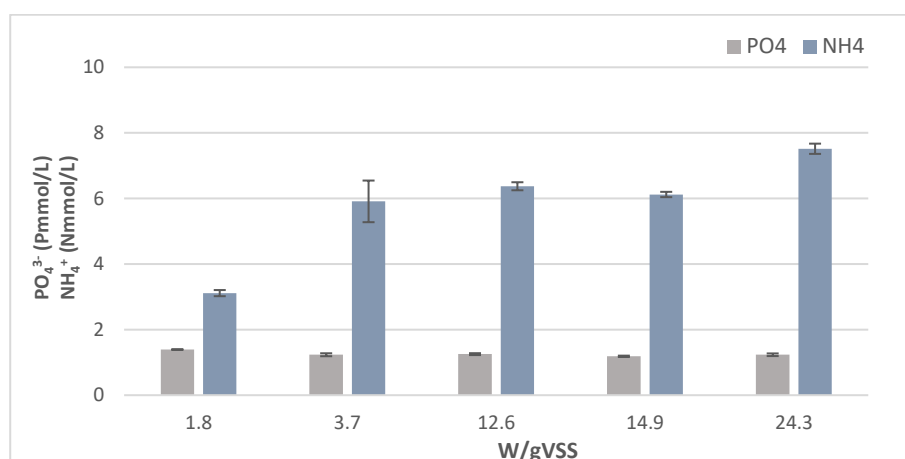


Figure 6-19: Average of the concentrations, in mM, of the nutrients present during the whole duration of the batch tests, for each condition, in W/gVSS.

As for the productions, it is possible to compare in Table 6.2, and remark that there is no linear correlation between the increase of light intensity and glycogen or PHA production. The test conducted at the lowest light intensity (1.8W/gVSS) resulted in the highest levels of glycogen and PHA produced. Objectively speaking, based solely on the results reported in Cmmol/L, it can be inferred that the production of both products decreased consistently with an increase in light intensity.

Table 6.2: Maximum values obtained, for each batch test performed, in percentage of glycogen/VSS, and the respective value in Cmmol/L. Percentage of PHA/VSS and the respective Cmmol/L of PHA, analysed on the last point for each batch test.

Batch test conditions	Glycogen		PHA	
	% GLY/VSS	Cmmol/L	% PHA/VSS	Cmmol/L
1.8 W/gVSS	61.1	12.9	5.30	1.36
3.7 W/gVSS	9.56 ± 3.13	3.10 ± 1.72	2.37 ± 0.05	0.75 ± 0.08
12.6 W/gVSS	11.26	1.32	3.75	0.58
14.9 W/gVSS	2.48 ± 0.67	0.53 ± 0.33	2.80 ± 0.46	0.49 ± 0.13
24.3 W/gVSS	0.78	0.18	0.57	0.13

This decline may have resulted from a potential decrease in the culture's response to the elevated light intensity, diverting the consumed carbon for cell preservation. However, it is more plausible that the concentration of biomass capable of producing high percentages of glycogen and PHA in the SBR declined due to constant collection for batch tests, leading to a culture with a decreasing ability to accumulate. It was shown in a previous work of Fradinho et al. an increase in consumption rates and linearly higher values of carbohydrate and PHB content, in purple bacteria, with increasing light intensity [29]. These results seem to indicate that the

decreased polymer production with increasing light intensity, may in fact be due to a loss of photosynthetic polymers producers within the mixed culture.

We analyzed all samples using HPLC, but no VFAs were detected, and as previously mentioned for the reactor, it may indicate that no compounds excretion was occurring, or any that could have been produced were also consumed.

As for the pigments, we only have the analysis for 4 of the conditions, as represented in Figure 6-20. The presence of a peak at around 775 nm in all tests indicates the presence of Bchl *a* + *b*, confirming the presence of anoxygenic bacteria, as expected, as well as the presence of carotenoids (peak between 400-500 nm). The graph shows that the tests conducted under lower light intensity had a more distinct peak at 775 nm, suggesting greater pigment production. Nevertheless, as mentioned in section 6.1.2, the culture of the SBR experienced a decline in microorganisms with photosynthetic capabilities over time. Since the tests were conducted in chronological order, the last tests were performed when the SBR culture had the lowest amount of photosynthetic organisms. Therefore, it is unclear whether the reduction in pigment presence resulted from the effect of light intensity in the batch tests or a consequence of the state of the SBR culture at the time.

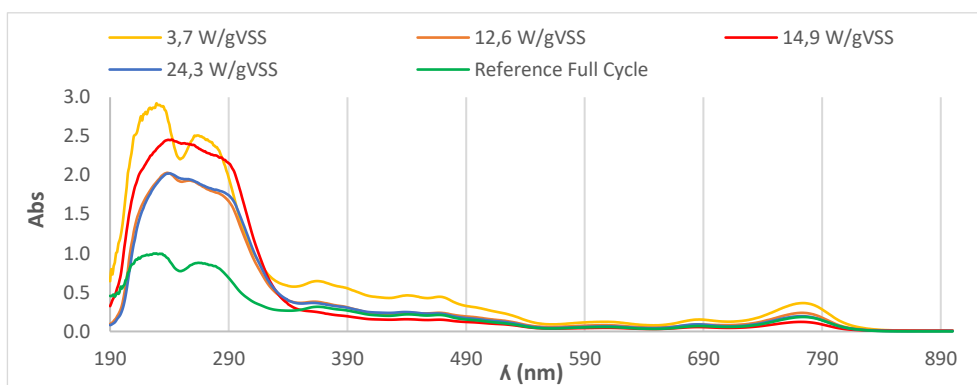


Figure 6-20: Absorbance spectra obtained from pigment extraction with ethanol 98%, for 4 of the batch tests, and for the reference full-cycle.

Looking at all the results, we can see that for this mixed culture, increasing the light intensity doesn't seem to be the best parameter to change in order to obtain higher contents of PHAs. The specific uptake rates of inorganic carbon didn't increase with increasing light intensity, even though the specific uptake rate of soluble sulfides did. Additionally, the production of glycogen and PHA did not show any positive effect induced by the increase in light intensity. The results of the maximum values detected for glycogen and PHA also showed very low values, which posed difficulties in their analysis, as their small variations could have been caused by culture changes or analysis errors.

6.2.8 No light available

When testing the culture under non-light conditions, interesting results were obtained. It is worth noting that the soluble sulfides were depleted by the end of the test, as depicted on the left in Figure 6-21, for both replicates. However, it is probable that an error occurred while transferring the biomass from the SBR to the serum flasks, despite efforts to maintain necessary anaerobic conditions. The inorganic carbon values remained constant without any consumption, as shown in Figure 6-21 (right), where the concentration values decrease from 12,56 mM to 11,62 mM. This outcome was expected since the operation was performed in the dark.

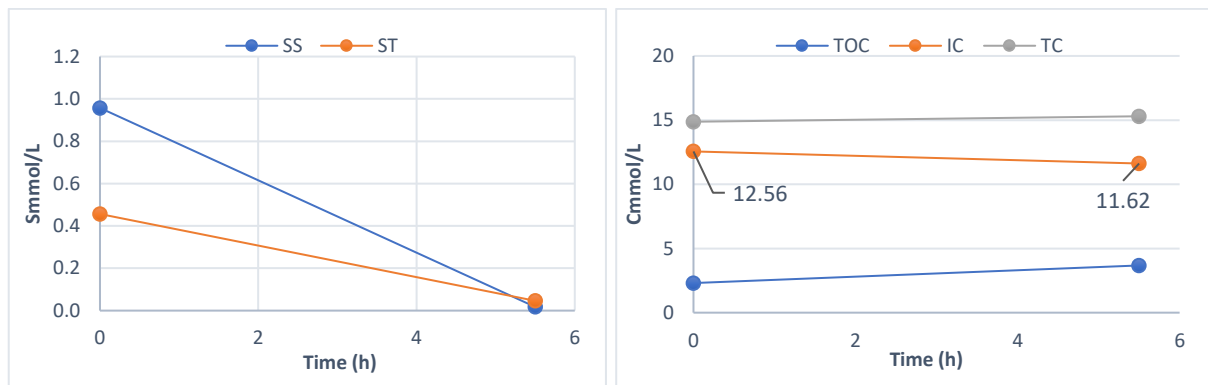


Figure 6-21: **Left:** initial and final concentrations of Soluble Sulfides (SS) and Total Sulfides (ST), in mM. **Right:** initial and final concentrations of Total Organic Carbon (TOC), Inorganic carbon (IC), and Total Carbon (TC), in mM, for the batch test in dark conditions.

Nutrient intake was also not observed, as it is possible to confirm with the results shown in Table 6.3.

Table 6.3: Initial and final concentrations of nutrients: PO_4^{3-} and NH_4^+ , in mM of P and N, respectively, for the batch test in dark conditions.

Nutrients concentration		
Time (h)	mM P	mM N
0.0	1.21	5.84
5.5	1.19 ± 0.01	5.98 ± 0.25

Regarding the production and accumulation of glycogen, the values detected were merely residual. On the other hand, PHA was detected (Table 6.4) with values of percentage of PHA/VSS, three times higher than those detected in the reference full-cycle (3% of PHA/VSS; 0.9 Cmmol/L). As demonstrated in previous studies on PHA-producing PMCs, these bacteria can convert glycogen reserves into PHB under non-light conditions [6]. In our study, there was no intake of inorganic carbon. Therefore, we can assume that the PHA content could be resulting from the activation of some internal bacterial mechanisms, and the consequent conversion of reserves. However, it is important to point out that the glycogen levels at the beginning of

the study were residual (0.91% of GLY/VSS). It was not possible to confirm whether there was an increase in glycogen levels early in the study and its subsequent conversion to PHB, due to the lack of samples throughout the study. Additionally, it was also not possible to verify whether the culture from the SBR collected for this test had higher levels of PHA content, as the starting point (at 0h) could not be analyzed due to the limited number of samples we were able to analyze on the GC equipment. It is unclear whether the results were due to an activation of some internal mechanism caused by the dark condition, or a pre-existing condition from the SBR biomass. To confirm these results, further studies involving constant sampling, confirmation of no sulfide loss during sampling, and verification of the initial and final glycogen and PHA contents would be necessary.

Table 6.4: Initial and final concentrations of glycogen % of GLY/VSS, and in Cmmol/L, and final concentration of PHA in % of PHA/VSS, and in Cmmol/L, for the batch test in dark conditions.

Time (h)	Glycogen		PHA	
	% GLY/VSS	Cmmol/L	% PHA/VSS	Cmmol/L
0.0	0.91	0.15	-	-
5.5	0.59	0.06	10.9 ± 2.2	1.5 ± 0.3

By observing the images obtained through Nile blue staining, it is possible to observe fluorescence, very visible in the clumps of biomass, confirming the possible accumulation of granules of PHA (Figure 6-22). However, the appearance of fluorescent granules in the star-shaped cells (*Rhodopseudomonas*) is also notable (Figure 6-23), unlike what was observable in the full-cycle observations.

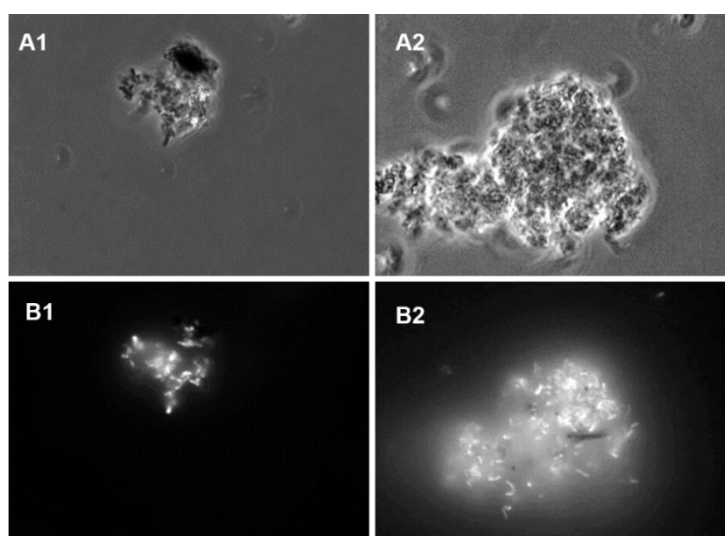


Figure 6-22: **A1, A2** - Microscopic images of clumps of biomass on the batch test with no light available. **B1, B2** - Fluorescence images of Nile blue staining indicating PHA granules, corresponding to the microscopic images (A1-A2).

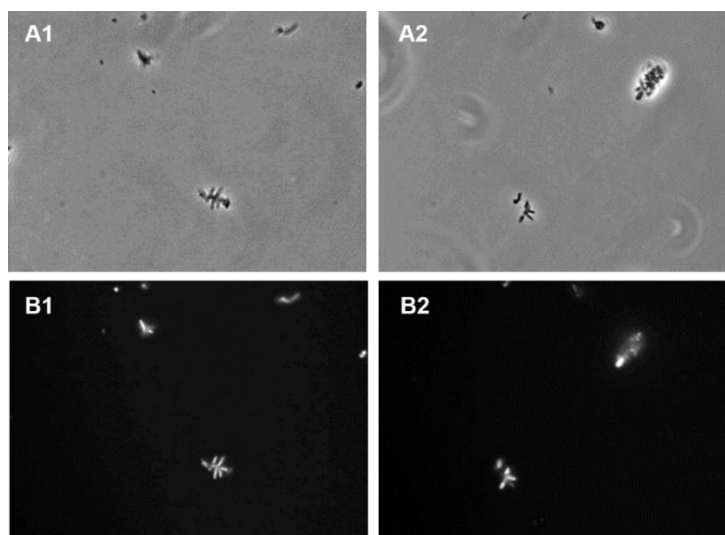


Figure 6-23: **A1, A2** - Microscopic images of star-shaped cells from the batch test with no light available. **B1, B2** - Fluorescence images of Nile blue staining indicating PHA granules, corresponding to the microscopic images (A1-A2).

6.3 New possible inocula

With the aim of testing different microbial mixed cultures for possible new inoculum for an SBR reactor, two samples were tested. The sample collected from the creek will be referred to in this work as the "creek sample", and the sample derived from a contamination found in *Chloroflexus* growth flasks will be referred to as the "sample from contamination".

6.3.1 Creek Sample

During the 7 days of growth (in 250mL of the same mineral medium as the SBR, with 20mM of Na_2CO_3 , and 5mM $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$), during which the samples were analyzed, the optical density (OD) at 620nm was measured. These results are displayed in Table 6.5, where it can be seen that the OD remained constant from beginning to end, with slightly increase on days 3 and 4. This could mean the culture had reached a stationary phase, where no growth was observed. During this period the biomass concentration obtained values of 0.29 ± 0.04 g/L of VSS, and 0.40 ± 0.05 g/L of TSS.

Table 6.5: Optical Density (OD) at 620 nm, from samples collected during the 7 days of growth of an inoculum from a creek.

Day	OD (620 nm)	Day	OD (620 nm)
0	0.597	3	0.641
1	0.541	4	0.607
2	0.574	7	0.597

Regarding the consumption of sulfides and inorganic carbon, the initial concentrations detected were lower than expected, as 5 mM $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and 20 mM of Na_2CO_3 were intended to be added. It is assumed it was an error in the preparation or on the addition of the solutions into the flask. Nonetheless, the following table indicates that there was a consumption of both compounds during the 7-day period. Although consumption was low, this demonstrates that the inoculum can grow in the same medium as the SBR culture and can also fixate CO_2 .

Table 6.6: Soluble Sulfides (SS) and Inorganic Carbon (IC) concentrations (mM) detected on the inoculum from a creek.

Day	SS (mM)	Day	IC (mM)
0	1.97	0	18.3
2	1.45	2	16.2
4	1.45	4	15.7
7	1.35	7	14.1

As for nutrients, there was no uptake, as the concentrations of ammonium and phosphate remained constant during the 7 days, with $14.3 \pm 0.1\text{mM}$ of N, and $2.4 \pm 0.1\text{mM}$ of P, respectively. Regarding the products, no glycogen was detected in the samples, and the PHA detection methodology wasn't followed due to time constraints and the limited number of samples that could be analysed. Nevertheless, according to the observations performed to characterize the culture using the Nile blue staining protocol, at day 0 the biomass appeared dispersed but had clearly identifiable granules with fluorescence. At the end of the experiment, on day 7, there was an identical amount of cells with prominently displayed fluorescing granules. These fluorescing granules were visible in dispersed individual cells as in large biomass clusters. Due to a problem between the microscope and the computer during the analysis, it was regrettably not possible to preserve the images acquired during the study. There is a possibility that these granules could be PHA, but without the determination analysis it is impossible to confirm. Lastly, as for pigment detection, it is possible to see, in Figure 6-24, that the amount of pigments decreased during the experiment, as the absorbance values were lower every day. Since the OD values were constant, meaning no variation of number of cells, the decreasing could be explained or by the loss of pigments, or by the loss of phototrophic organisms, and the

growth of other non-phototrophic ones. This could be possible through anaerobic hydrolysis and fermentation of these dying phototrophic organisms, which would serve as nutrient source for the non-phototrophic organisms.

By analysing the profile of pigments, extracted with ethanol, shown in Figure 6-24, it is possible to determine the existence of Bchl *a* (with visible peaks at 365), Bchl *a* + *b* (at 610 nm and 750) and the presence of carotenoids (400-500 nm). The existence of these pigments confirms the presence of purple bacteria in the consortium.

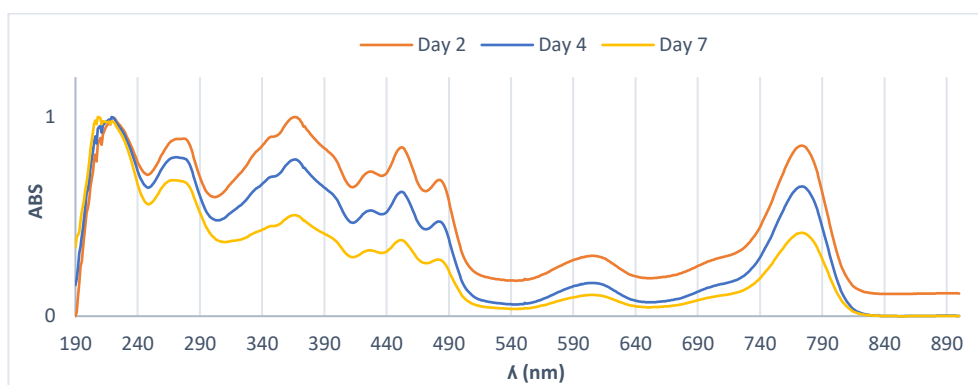


Figure 6-24: Absorbance spectra of pigments extracted with ethanol 98%, derived from a sample of a creek, at day 2, 4 and 7 of incubation.

We can predict that some of these purple bacteria could belong to the *Gammaproteobacteria* class, based on a study of the natural microbiome in the waters where the sample was collected. The study, which was conducted from 2017 to 2018, determined that *Gammaproteobacteria* was the predominant class, comprising 70.76% of the microbiome (source: <https://hidrogenoma.dgeg.gov.pt/agua-mineral-natural/unhais-da-serra>, Accessed 19 September 2022). It could be possible that these bacteria are responsible for producing some PHA (visible granules in the Nile blue analysis). However, it would require additional testing to determine whether this sample would make a viable new inoculum for an SBR for PHA production, as a longer period of growth and sampling, and the realization of the PHA determination protocol. Nonetheless, due to its ability to consume the supplied sulfides and inorganic carbon, it could be a possible candidate for acclimatizing a new SBR, with the aim of producing PHAs.

6.3.2 Sample from a contamination

As for the sample derived from contamination found in *Chloroflexus* growth flasks, the OD values are represented in Table 6.7. Contrary to the previous samples, these values had a slightly increase, representing an increase of density of cells in the liquid culture. The biomass

concentration values obtained were, likewise, higher in relation to the previous ones reaching 0.36 ± 0.08 g/L of VSS, and 0.57 ± 0.06 g/L of TSS.

Table 6.7: Optical Density (OD) at 620 nm, from samples collected during the 7 days of growth of an inoculum from a contaminant found in flasks growing *Chloroflexus* strains.

Day	OD (620 nm)	Day	OD (620 nm)
0	0.884	3	0.879
1	0.891	4	0.912
2	0.913	7	0.917

In the same way as before the initial concentrations were lower than planned. Although there was an increase in the OD, and the VSS values, no consumption of inorganic carbon was observed, as shown in Table 6.8, and the minimal decrease in soluble sulfides concentration (of 0.66 mM), were probably due to losses during the sampling procedure.

Table 6.8: Soluble Sulfides (SS) and Inorganic Carbon (IC) concentrations (mM) detected on the inoculum from a contaminant found in flasks growing *Chloroflexus* strains.

Day	SS (mM)	Day	IC (mM)
0	1.97	0	18.3
2	2.17	2	17.6
4	1.52	4	18.2
7	1.32	7	19.5

As for nutrient consumption, in the same way as the other sample, the concentrations remained constant during the 7 days, with average values of 13.3 ± 0.3 mM of N, and 2.5 ± 0.2 mM of P. PHA determination protocol was not performed in these samples as well, however glycogen was detected. On days 2 and 4, respectively, residual values of 0.52% of GLY/VSS (0.08 mmolC/L) and 0.36% GLY/VSS (0.04 mmolC/L) were detected.

Regarding the pigments detection, contrary to the creek samples, the absorbance values remained constant, possibly indicating no loss of pigments during the 7 days, as for this sample there was an increase in cell density throughout the experiment. It was possible to detect the same pigments as in the other sample: Bchl *a* (at 365 and 375 nm) Bchl *b* (358 nm), Bchl *a + b* (610 nm) and carotenoids (400-500 nm). It is noteworthy the higher production of carotenoids, comparing to the Bchl *a* production, which differs from the creek sample where it is nearly the same. From this we can hypothesize that the creek culture could be richer in purple bacteria than the one from the contamination sample.

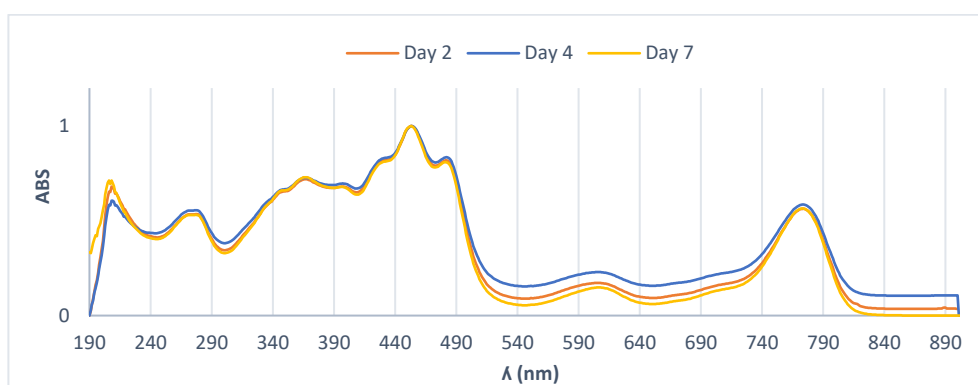


Figure 6-25: Absorbance spectra of pigments extracted with ethanol 98%, derived from the growth of a contamination found in flasks growing *Chloroflexus*, at day 2, 4 and 7 of incubation.

When the samples were examined under a microscope using the Nile blue staining characterization methodology, it was feasible to see that on day 0, the biomass had a dispersed look with small clusters (similar to the creek samples), but with few fluorescent granules that were poorly defined. When the biomass was re-observed after 7 days, it was less dispersed, but this time the granules were essentially non-existent, and it was very challenging to detect fluorescence. For the same motive as before, images of the observation could not be saved. As a result, we could assume that this culture wasn't producing PHA granules and therefore it was no longer a viable option for a new SBR inoculum. More specific tests could confirm the PHA production capacity of this culture, as it appears to be composed of purple bacteria. Suggested tests include batch tests using inorganic (containing CO_2 and H_2S) and organic (like acetate) mediums.

6.4 Analytical development to overcome the PHA analysis interferences

As indicated in the sub-section 5.5, the potential sulfur buildup from the culture could be dissolving into the chloroform (matrix employed in the PHA detection protocol), possibly being the cause of GC deterioration. For this reason, we conducted different alternative protocols to detect PHA, as described below, and subsequently various tests to try to verify the presence and rinse any potential sulfur from our samples.

6.4.1 Crotonic Acid Method

As a first attempt we tried a different approach to quantify poly- β -hydroxybutyric by converting it into crotonic acid. However, after heating the samples in concentrated sulfuric acid for

10 minutes at 100°C, they all exhibited a brown color (Figure 6-26). When measuring the absorbances at 235 nm, all the values obtained were too high and could not be read by the equipment or interpreted. This color change could be explained by a brown-colored compound known to appear in this reaction, possibly from the degradation of PHB or crotonic acid, due to the concentrated sulfuric acid [76].

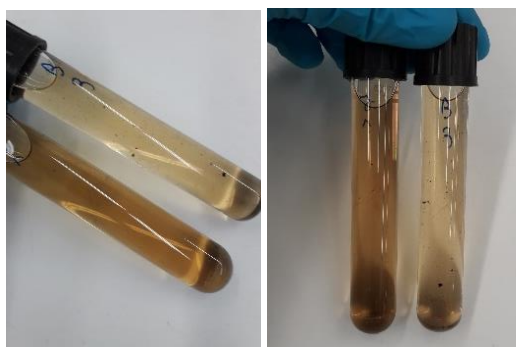


Figure 6-26: Photos of the lyophilized samples from the SBR and from other mixed culture not fed with sulfide, presenting a brown coloration after incubation at 100°C in a dry-heat thermo-block with 10 mL of sulfuric acid.

As we were unable to analyze the results, we discarded this protocol and proceeded to the next experiments. However, upon further investigation, it was found in another paper, that the starting solution with concentrated sulfuric acid cannot be read by a UV spectrophotometer, requiring a series of dilutions with sterile distilled water, prior to PHB measurement [77]. They also mention the color change of the solution to brown caused by polymer conversion, and it could also be avoided by performing the dilutions. Repeating this protocol with the additional steps could be a viable alternative for the PHB determination. However, this information came too late to confirm its feasibility.

6.4.2 Washing the samples with NaCl solutions

With this attempt we tried to wash any remains of sulfur from the biomass with successive washes with NaCl. After the 3 different washes we obtained the results from the Total Suspended and Total Volatile Solids protocol, presented in Table 6.9. As it is possible to observe, the successive washes did not have the expected impact, since the results obtained were identical for all washes. That is, even when increasing the NaCl solution concentration, we were not able to remove any significant amounts of sulfur residues, as the TSS values remained constant, when we expected a decrease in this value with its possible removal. When comparing the % of VSS/TSS with the full-cycles, the results were also identical (e.g.: in the reference full-cycle 28% VSS/TSS were detected at the beginning of the cycle).

Table 6.9: Results obtained from the Total Suspended and Total Volatile Solids analysis, after performing successive washes of the biomass from the SBR with NaCl. **Tubes 1 and 2:** 1 wash with NaCl 0.9%. **Tubes 3 and 4:** 1 wash with NaCl 0.9%, and 1 wash with NaCl 2.5%. **Tubes 5 and 6:** 1 wash with NaCl 0.9%, 1 wash with NaCl 2.5%, and 1 wash with NaCl 4%.

	VSS (g/L)	TSS (g/L)	VSS/TSS (%)
Tubes 1 and 2	0.63 ± 0.07	2.35 ± 0.21	27
Tubes 3 and 4	0.60 ± 0.00	2.11 ± 0.07	28
Tubes 5 and 6	0.68 ± 0.11	2.38 ± 0.34	29

When observing the absorbance readings of the different supernatants presented in Figure 6-27, and considering the detection of sulfur with a visible peak near 260 nm [74], it can be seen that none of the three spectra show such peak. Therefore, it can be assumed that sulfur was not being extracted into the supernatant after the NaCl washes, resulting in the determination of non-functionality of this protocol.

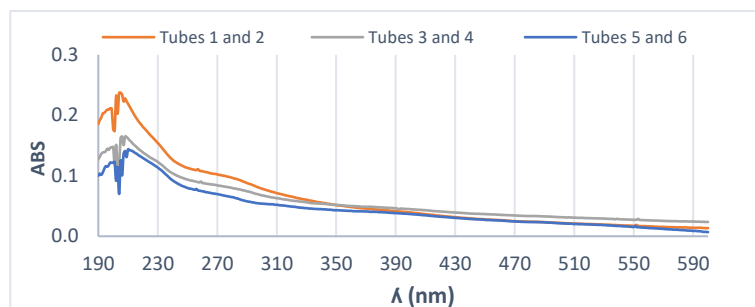


Figure 6-27: Absorbance of the supernatant's spectrums obtained, after performing successive washes of the biomass from the SBR with NaCl: **Tubes 1 and 2:** 1 wash with NaCl 0.9%. **Tubes 3 and 4:** 1 wash with NaCl 0.9%, and 1 wash with NaCl 2.5%. **Tubes 5 and 6:** 1 wash with NaCl 0.9%, 1 wash with NaCl 2.5%, and 1 wash with NaCl 4%.

6.4.3 Washing the samples with ethanol

Since the attempt with NaCl did not show an increase in the VSS/TSS ratio, nor did it exhibit a representative sulfur peak in the absorbance spectra of the supernatants, we attempted a different approach. We attempted to solubilize sulfur residues in ethanol, and then removing them through washes of the biomass. We thus expected to see a reduction in the TSS values, and an increase in the percentage of VSS/TSS. In addition, we expected to see a decrease in the absorbances obtained between 249 and 264 nm, with the consecutive washes, as these are the minimum and maximum wavelengths of ultraviolet absorption for aqueous sulfur solutions, when extracted with ethanol [74].

When analyzing the absorbances obtained, we can see that for the approach with no incubation time (Figure 6-28-A), with only 1 wash with ethanol 50% (tubes 3 and 4) the amount of sulfur extracted to the solvent and detected was non-significant. With 1 wash with ethanol

50% followed by 1 wash with ethanol 98% (tubes 5 and 6) we obtained higher values of abs, possibly indicating that the second wash was able to remove some sulfur to the supernatant, however, this increase was most likely due to the extraction of pigments into the ethanol. In the tubes 7 and 8, with 1 wash with ethanol 50% followed by 2 washes with ethanol 98%, the absorbances were lower than before, suggesting that a higher amount of sulfur and, mostly pigments, could be extracted in the first wash with ethanol 98% and the second wash was not necessary.

The absorbances of the supernatants for the approach with 15 minutes of incubation (Figure 6-28-B) were very similar to the ones from the approach with no incubation time, which could mean that the 15 minutes incubation were not sufficient to have a bigger impact on the extraction of the sulfur to the solvent.

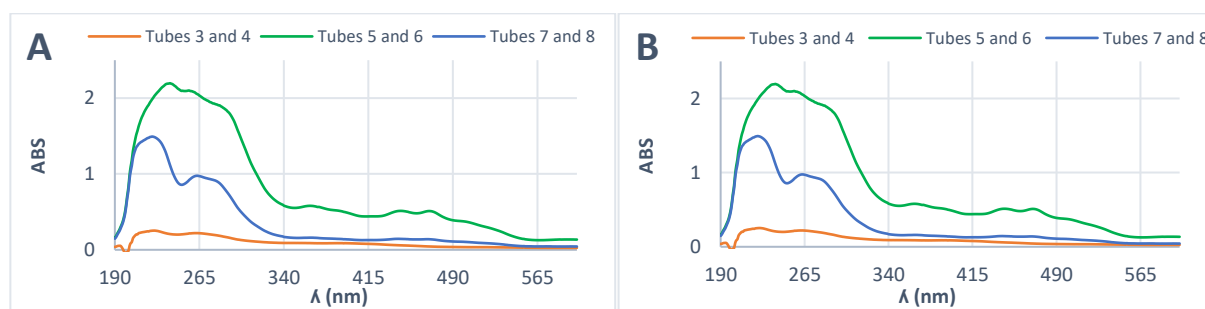


Figure 6-28: Absorbance of the supernatant's spectra obtained after ethanol washes with no incubation time (A), and with 15 minutes incubation time (B) to remove aqueous sulfur from SBR biomass. **Tubes 3 and 4:** 1 wash with ethanol 50%. **Tubes 5 and 6:** 1 wash with ethanol 50% and 1 wash with ethanol 98%. **Tubes 7 and 8:** 1 wash with ethanol 50% and 2 washes with ethanol 98%.

When leaving the samples incubating overnight (Figure 6-29) it was observed that with 1 wash with ethanol 50% (tubes 3 and 4), a very light peak appears around 260 nm, and for the samples with 1 wash with ethanol 50% and 1 wash ethanol 98% (tubes 5 and 6) there was a more perceptible peak at that wavelength.

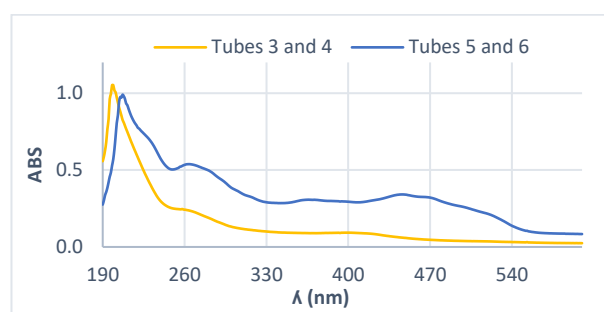


Figure 6-29: Absorbance of the supernatant spectrums obtained after ethanol washes with incubation overnight to remove aqueous sulfur from SBR biomass. **Tubes 3 and 4:** 1 wash with ethanol 50%. **Tubes 5 and 6:** 1 wash with ethanol 50% (1 hour incubation) and 1 wash with ethanol 98% (incubation overnight).

Although the absorbances differ between different tubes and different attempts, when looking at the results from the Total Suspended and Total Volatile Solids analysis, the percentages of VSS/TSS didn't have significant variations between samples, and the discrepancy could not be proved to be caused by the washes (Table 6.10). In fact, we were expecting that with the sequential washes with a more concentrated ethanol a higher amount of sulfur was being extracted to the solvent, and we would see a higher percentage of VSS/TSS in the tubes 7 and 8, which was not the case. Even when comparing the washed samples with the control (tubes 1 and 2) no significant differences were observed, leaving the question of whether in fact any amount of sulfur was being removed with the washes. From here we concluded that the washes with ethanol were not the best approach since no significant variations were observed. The differences observed in the obtained spectrums could potentially be attributed to pigment interference, as they are also extracted into the ethanol solution.

Table 6.10: Results of VSS/TSS (%), obtained from the Total Suspended and Total Volatile Solids analysis, after performing successive washes of the biomass from the SBR with ethanol. **Tubes 1 and 2:** control, no wash was performed. **Tubes 3 and 4:** 1 wash with ethanol 50%. **Tubes 5 and 6:** 1 wash with ethanol 50% and 1 wash with ethanol 98%. **Tubes 7 and 8:** 1 wash with ethanol 50% and 2 washes with ethanol 98%. All the washes were made for the attempt with no time of incubation, and for the attempt with 15 minutes of incubation. For the last attempt, with incubation overnight, the last tubes (7 and 8) were not prepared.

	No incubation	15 min. incubation	Overnight incubation
1 and 2	38%	33%	30%
3 and 4	40%	37%	45%
5 and 6	27%	31%	34%
7 and 8	33%	34%	-

6.4.4 PHA extraction with chloroform at 10°C

For this attempt, we utilized an alternative strategy by trying to dissolve the PHA in chloroform, while avoiding any solubilization of the cell matrix and sulfur residues. The author suggested that decreasing the temperature of the chloroform would hinder this solubilization [75].

So, after leaving the samples with sulfur (from our SBR) and samples without sulfide, in chloroform, with gentle stirring, at 10°C for 24 hours, they were filtered to retain any insoluble components. The absorbance of the solutions was then measured. Since we are trying to confirm the removal of sulfur residues in the solution, we are specifically analyzing any peak that comes out between 250 and 265 nm (wavelengths at which aqueous sulfur is absorbed when dissolved in chloroform) [74]. When analyzing the spectrums obtained, represented in Figure 6-30, there is a visible peak at 265 nm in both samples. However, we know that the sample represented by the orange line on the graph does not contain any sulfides. Therefore, the peak in this sample could not be sulfur, but some other compound that was extracted into the chloroform. This raises the question of whether the peak of the SBR sample (blue line), also visible at 265 nm, is an interference as well, or if it is actually some sulfur that may have dissolved in the chloroform.

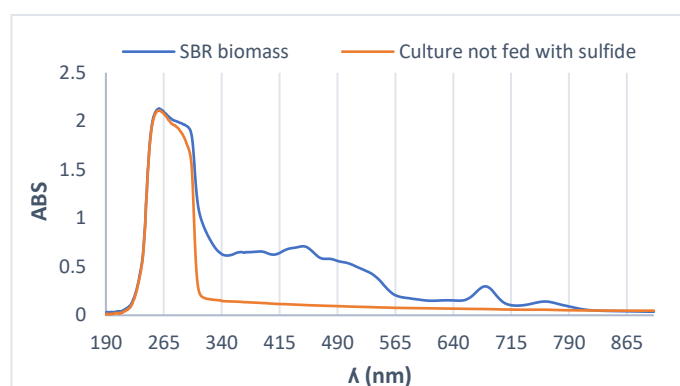


Figure 6-30: Absorbance spectrums obtained from the filtrated samples from the SBR - blue line, and from a PHA producing aerobic mixed microbial culture (not fed with sulfide) - orange line, after leaving the lyophilized biomass gentle stirring with 1mL of chloroform for 24 hours at 10°C.

To confirm that treating the samples with chloroform at 10°C had been successful in removing sulfur residues and that our SBR samples could then pass in the GC equipment, the absorbances of the organic phases of both samples (with and without sulfides) were measured after going through the PHA digestion protocol. It is evident from the results depicted in the figure that the samples possess nearly identical spectrums. This suggests that the application of chloroform treatment at a 10°C may have successfully eliminated any sulfur residues that could have been present in our SBR sample.

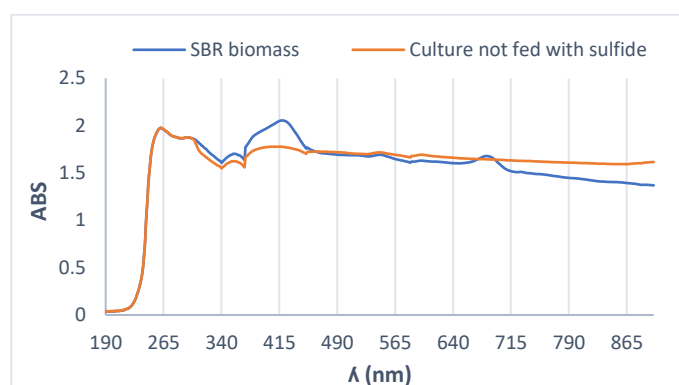


Figure 6-31: Absorbance spectrums obtained from the organic phases from both samples, after the PHA digestion protocol, afterward the pre-treatment with chloroform at 10°C.

However, to confirm the initial differences between the samples (one containing sulfides and the other not), we performed a control by reading the absorbances of the organic phases of both samples after the PHA digestion protocol without any pre-treatment. A significant dilemma emerged since, as can be seen in Figure 6-32, the spectra obtained were again nearly identical.

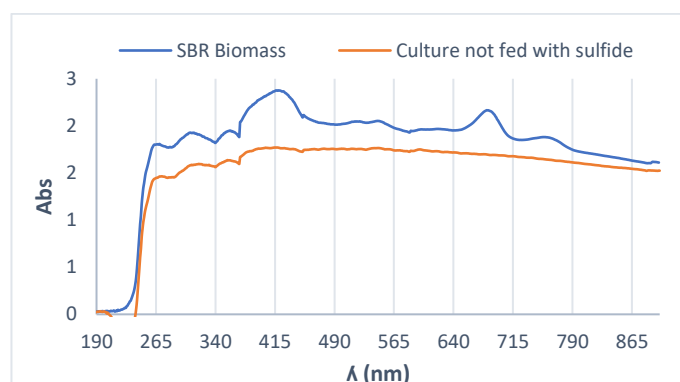


Figure 6-32: Control - absorbance spectrums obtained from the organic phases from both samples, after the PHA digestion protocol.

We began to question whether the peak repeatedly detected at 265nm in our SBR samples was actually sulfur, and what the peak at that same wavelength in the samples without sulfur represented.

To clarify these questions, the procedure was repeated, but this time at room temperature, and both samples (with and without sulfides) were left for 24 hours with gentle stirring in 1 mL of chloroform, 1 mL of ethanol 98%, and 1 mL of MilliQ water. The results for the experiment performed with chloroform showed, in both samples, a peak at 260 nm (Figure 6-33 - A). When in ethanol 98%, the SBR samples showed two distinct peaks, one at 230nm, and other at 260 nm (Figure 6-33 - B). Since the maximum absorption wavelengths for aqueous sulfur in ethanol is 264nm [74], the peak at 260nm could indicate the presence of sulfur in the samples. This

peak was not detected in the samples from the culture not fed with sulfides, as expected. Thus, we continued to wonder what the compound present in both samples was, that absorbed at 260 nm in chloroform. The experiment with MilliQ water showed only one peak at 200 nm on the culture not fed with sulfides (Figure 6-33 - C), which could come from organic acids.

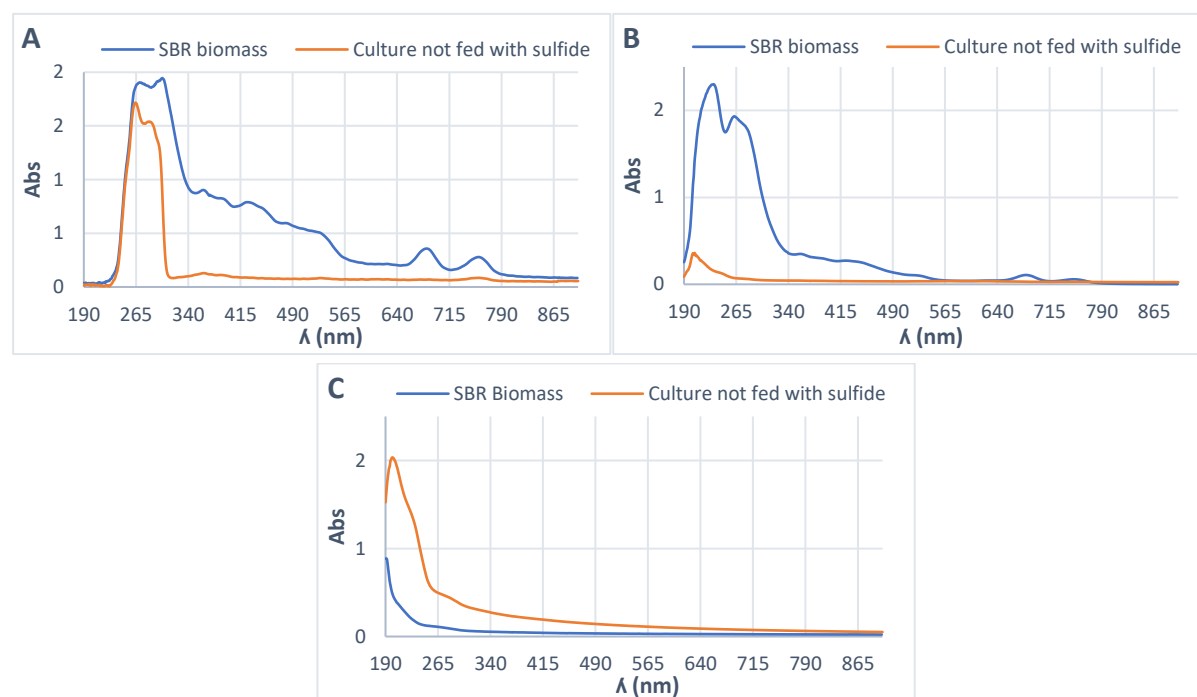


Figure 6-33: Absorbance spectrums obtained from the filtrated samples from the SBR biomass, and from a PHA producing aerobic mixed microbial culture (not fed with sulfide), after leaving the lyophilized biomass gentle stirring for 24 hours at room temperature with 1 mL of chloroform (A), 1 mL of ethanol 98% (B), and 1 mL of Milli Q water (C).

6.4.4.1 Removal of interferences

To eliminate the interferences that could be occurring, these experiments were repeated with two prewashes with 0.9% NaCl. During the washes, absorbance readings were taken (Figure 6-34). For both samples it can be seen that the peaks present at 200 nm disappeared, indicating the washing away of the organic acids.

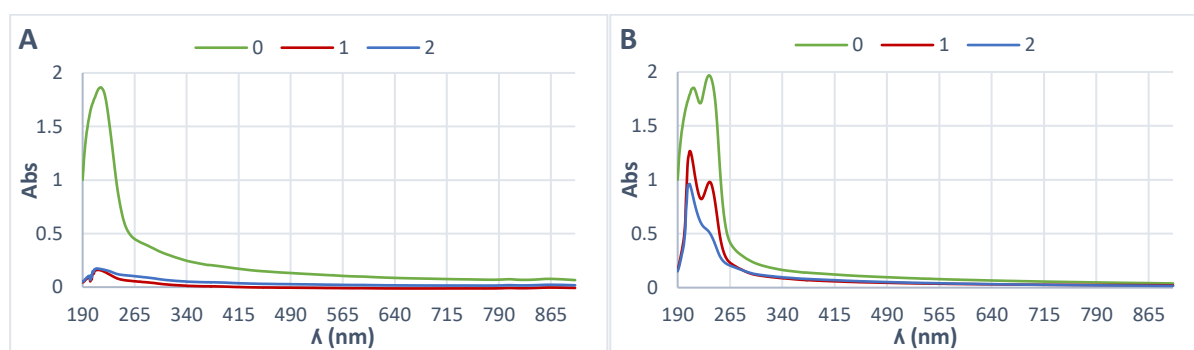


Figure 6-34: Absorbance spectrums obtained from SBR biomass (A), and from a PHA producing aerobic mixed microbial culture (not fed with sulfide) (B). 0: supernatant absorbance before any wash. 1: supernatant absorbance after washing the biomass with NaCl 0.9%. 2: supernatant absorbance of the biomass after a second wash with NaCl 0.9%

After freeze-drying the samples and repeating the experiments, it was possible to observe that all the results were remarkably similar. In the experiment with Milli Q water, a peak appears at 190 nm for the SBR samples and a peak at 220 nm for the samples with no sulfides (Figure 6-35 - C). In the one with chloroform the peak at 260 nm still appears (Figure 6-35 - A), although the interferences were eliminated. The samples in ethanol 98%, (Figure 6-35 - B), still present a peak that could be sulfur, at 260 nm. Even when removing some of the interferences, the major peaks that made us question if sulfur was or was not present remained, so no direct conclusions could be made.

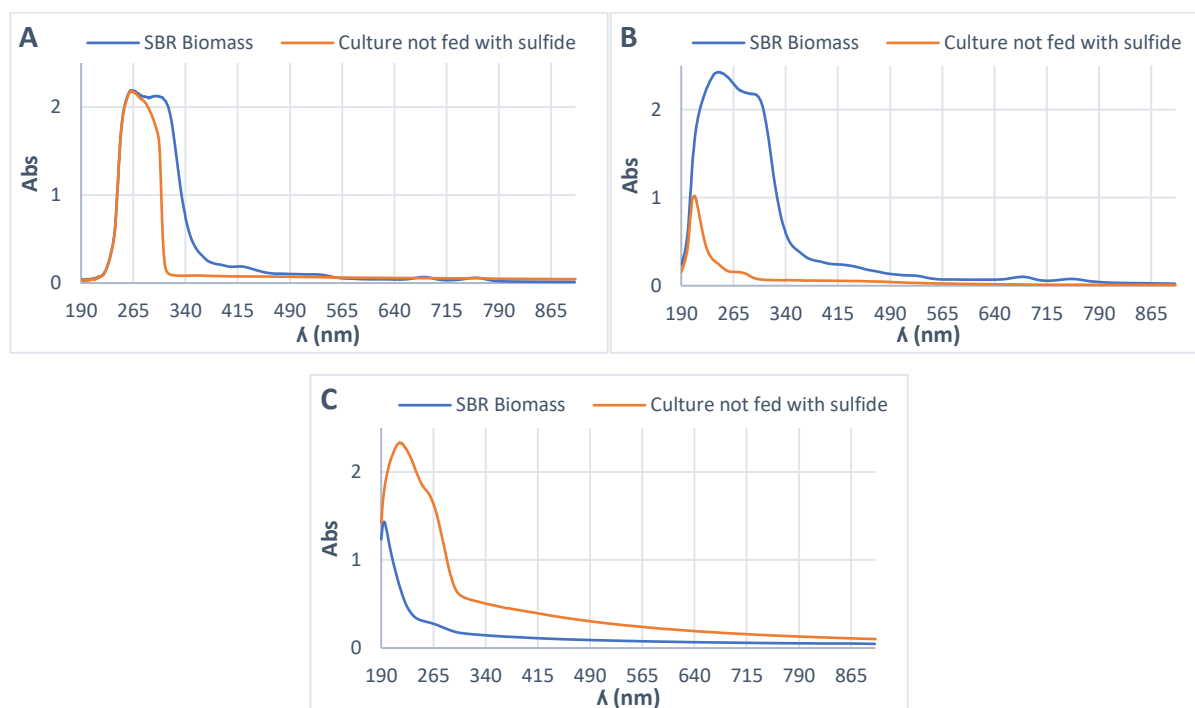


Figure 6-35: Absorbance spectrums obtained from the filtrated samples from the SBR biomass, and from a PHA producing aerobic mixed microbial culture (not fed with sulfide), after leaving the lyophilized biomass gentle

stirring for 24 hours at room temperature with 1 mL of chloroform (A), 1 mL of ethanol 98% (B), and 1 mL of MilliQ water (C). All samples were subjected to two prewashes with NaCl 0.9%.

To make sure no interferences, in the chloroform experiment, were being caused by pigments, a new attempt was made. Before leaving the biomass from both, the SBR and the culture with no sulfides for 24 hours with chloroform with gentle stirring, all the samples were washed with ethanol 98%. After the 24 hours, the absorbance spectrums presented in Figure 6-36 were obtained. From here we can see that even after removing the pigments, both samples continue to present a peak at 260 nm.

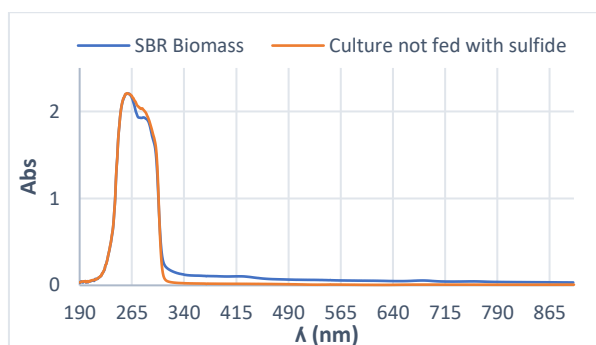


Figure 6-36: Absorbance spectra of samples from the SBR biomass and the biomass from a PHA producing aerobic mixed microbial culture (not fed with sulfides), after being washed with ethanol 98%, lyophilized and left with gentle stirring for 24 hours at room temperature, with 1mL of chloroform.

Finally, to exclude any other possibility of interference, we wanted to confirm that the high temperature that the samples are subjected to in the PHA digestion protocol didn't affect the extraction of compounds to chloroform. After washing the sample from the SBR with ethanol 98%, the lyophilized biomass was subjected to the PHA digestion protocol, and the absorbance of the organic phase was measured. This result is presented in Figure 6-37. When comparing to Figure 6-36 (extraction to chloroform at room temperature), we can confirm that no other compounds are being extracted into the chloroform when it reaches higher temperatures. However, both still show a peak at 260 nm, which requires further evaluation (literature and experimental) to determine to what it can be attributed.

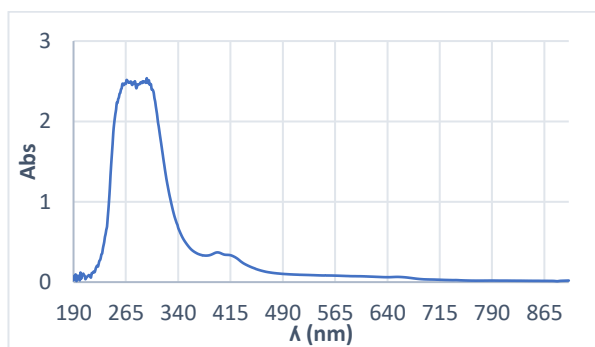


Figure 6-37: Absorbance spectra of samples of the organic phase, after the PHA digestion protocol, of the SBR Biomass (pre-washed with ethanol 98% to remove pigment inferences).

As all the SBR samples analyzed so far did not show any differences compared to the sulfide-free culture samples, the hypothesis that the SBR samples could have sulfur residues that could damage the GC was discarded. Further studies could be performed to try to identify the compound extracted in chloroform at 260 nm, but we did not continue due to time and resource management constraints. Regarding the PHA analyses, required both for characterization of the SBR and batch tests, they were conducted with limited samples due to time constraints, since no reason was found to prevent them from passing the GC equipment.

CONCLUSIONS AND FUTURE WORK

This thesis was separated into various projects, conducted simultaneously over the course of several months. Despite encountering numerous obstacles, we were able to draw conclusions from each of them.

One of our primary aims was to maintain a bench-scale photobioreactor enriched with a PMC containing APB in a stable state. While this was attainable to some extent, numerous instabilities resulted in decreased or cessation of activity on the part of the culture. Maintaining a mixed culture system under strict anaerobic conditions was found to be, indeed, challenging, as the constitution/activity of the culture varied over time in an uncontrollable manner. In particular, the initial concentration of sulfides, present in the feed solution, proved to be one of the parameters that undesirably varied over time, which may have contributed to changes in the behavior of the mixed culture. Greater rigor in the preparation of these solutions was proven crucial, as with any other solution containing volatile components, such as the bicarbonate solution. The preparation of samples containing these components, for the respective analyses, also proved challenging, as the possible loss of sulfides/bicarbonate through volatilization could be the justification for inconsistent results. Based on the results obtained from the referenced full cycle, it was confirmed that the culture was rich in purple bacteria, with the absence of oxygenic bacteria. Microbial characterization analyses indicated that a large part of this purple bacterial community consisted of organisms from the *Alphaproteobacteria* subgroup, especially *Rhodopseudomonas*. The culture exhibited consumption of sulfides and bicarbonate, as anticipated. However, PHA production was minimal, with a maximum detection of 3% of PHA/VSS (0.9 Cmmol/L). This percentage is significantly lower in comparison with other published studies, as is the case from a work from Fradinho et al. where 60% of PHA content were reached when growing a PMC in a SBR with continuous illumination and a feast regime, using acetate as carbon source [78]. A prolonged SRT time (20 days) appeared to be one of

contributors to the culture's inability to accumulate PHA, as it was not under sufficient selective pressure, and the ongoing collection of biomass for the batch tests and the instabilities that occurred throughout the course of the work period also seem to have had a strong impact.

At the same time, batch tests were conducted to confirm the culture's reaction to changes in light intensity. The tests revealed no significant relationship between the two variables. The culture demonstrated an increase in specific uptake rates of Soluble Sulfides (qSS) with rising light intensity. However, there were no significant variations in the specific uptake rate of Inorganic Carbon (qIC). Regarding the production of glycogen and PHA, both exhibited higher maximum values during the test conducted at lower light intensities. However, the results appear to have been influenced by external factors outside the scope of the batch test. All other trials showed low accumulation values, and there was no discernible correlation with the light intensities employed. It was not possible to draw definitive conclusions about this study except that light intensity has showed little effect on the culture's activity. During the batch test conducted without light, a higher detection of PHA values was observed (10.9 ± 2.2 of %PHA/VSS; 1.5 ± 0.3 Cmmol/L). This may have resulted from the conversion of glycogen to PHA due to the activation of mechanisms in the purple bacteria that allow this process to occur in a dark environment. Since glycogen values were not detected at the beginning of the test (0.59% of GLY/VSS; 0.15 Cmmol/L), it's unclear if there were any analysis errors in both the glycogen and/or PHA quantification protocols. To answer these questions, it would be crucial to conduct further tests in dark conditions, with samples taken over time, not just at a start and end point, and with analysis for PHA also at the start of the test.

A parallel study testing two potential new inocula for PHA production in the photobioreactor was also performed. The culture derived from *Chloroflexus* growth flasks contamination was discarded as a potential candidate, despite growing in a medium containing bicarbonate and sulfides. In spite of having a pigment profile typical of purple bacteria (rich in Bchl *a* and *b*), it did not show any fluorescence when observed using the Nile Blue protocol. Therefore, we concluded that this culture was not producing any PHA. Further testing utilizing PHA detection methods could corroborate this conclusion. On the other hand, the culture collected from a creek in Unhais da Serra, Covilhã, demonstrated stronger potential as a candidate. This culture was able to grow in a medium rich in bicarbonate and sulfides and showed some consumption of both compounds. Its pigment profile was typical of a culture rich in purple bacteria, but this culture, differently from the other one, exhibited the presence of fluorescent granules when visualized with Nile Blue after 7 days of growth. These findings suggest that

this culture may be converting bicarbonate into PHA reserves. Further studies with extended incubation durations and larger culture volumes, implementing all characterization protocols, would be necessary to confirm that this culture is indeed suitable for inoculation into a new SBR for PHA production.

Finally, another study was also carried out in parallel to determine whether or not biomass samples from the photobioreactor could contain sulfur and deteriorate the GC equipment. Attempts to cleanse our samples of any sulfur residue were unsuccessful, as the washing with NaCl and ethanol did not reduce the VSS/TSS percentages in any of the tests. Regarding alternative detection methods for PHA detection, the protocol with the aim to convert PHB into crotonic acid was found to introduce a substantial error and was ultimately discarded. More interesting results were obtained by extracting PHA to chloroform at 10°C, with the aim of not solubilizing the sulfur. After extracting the PHA into the chloroform matrix, the results were identical between our samples and those from a culture that was not fed with sulfides. This may suggest that the protocol was executed effectively and that the sulfur did not dissolve. However, results indicating no differences between samples, when the extraction was made at room temperature (and not at 10°C), raised questions about the existence of a significant difference between samples. After conducting multiple tests, extracting into various matrices (ethanol, chloroform, and MilliQ water) with or without pre-washing (ethanol for removing pigment interferences or NaCl for removal of organic acid interferences), we observed that there were no significant differences between our SBR samples and the sulfide-free culture samples. We concluded that our samples would not be a risk for the GC equipment. However, since we didn't have the time or resources to carry out more extensive and detailed tests to try to identify the compounds present in our samples, we were only able to run a limited number of samples through the equipment, which proved to be not ideal. Despite the challenges and obstacles faced throughout this study, we were able to derive some insights into each of the topics covered.

BIBLIOGRAPHY

- [1] Rebecca Lindsey, "Climate.gov," Climate Change: Atmospheric Carbon Dioxide. Accessed: Jul. 06, 2023. [Online]. Available: <https://www.climate.gov/news-features/understanding-climate/climate-change-atmospheric-carbon-dioxide>
- [2] P. Lamba, D. P. Kaur, S. Raj, and J. Sorout, "Recycling/reuse of plastic waste as construction material for sustainable development: a review," *Environmental Science and Pollution Research*, vol. 29, no. 57, pp. 86156–86179, 2022, doi: 10.1007/S11356-021-16980-Y.
- [3] M. R. Havstad, "Biodegradable plastics," in *Plastic Waste and Recycling*, Academic Press, 2020, pp. 97–129. doi: 10.1016/B978-0-12-817880-5.00005-0.
- [4] D. Tan, Y. Wang, Y. Tong, and G.-Q. Chen, "Grand Challenges for Industrializing Polyhydroxyalkanoates (PHAs)," *Trends Biotechnol*, vol. 39, no. 9, pp. 953–963, 2021, doi: 10.1016/j.tibtech.2020.11.010.
- [5] D. Crutchik, O. Franchi, L. Caminos, D. Jeison, M. Belmonte, A. Pedrouso, A. Val Del Rio, A. Mosquera-Corral, J. Campos, "Polyhydroxyalkanoates (PHAs) Production: A Feasible Economic Option for the Treatment of Sewage Sludge in Municipal Wastewater Treatment Plants?," *Water (Basel)*, vol. 12, no. 4, 2020, doi: 10.3390/w12041118.
- [6] J. C. Fradinho, J. M. B. Domingos, G. Carvalho, A. Oehmen, and M. A. M. Reis, "Polyhydroxyalkanoates production by a mixed photosynthetic consortium of bacteria and algae," *Bioresour Technol*, vol. 132, pp. 146–153, 2013, doi: 10.1016/j.biortech.2013.01.050.
- [7] A. Alexandra Barbosa Lanham, "Full-scale biological phosphorus removal: quantification of storage polymers, microbial performance and metabolic modelling," PhD Thesis, Universidade NOVA de Lisboa, 2012.
- [8] Global Monitoring Laboratory, "National Oceanic and Atmospheric Administration," Carbon Cycle Greenhouse Gases. Accessed: Jul. 06, 2023. [Online]. Available: <https://gml.noaa.gov/ccgg/>
- [9] "UCAR - Center for Science Education," Some Greenhouse Gases Are Stronger than Others. Accessed: Jul. 06, 2023. [Online]. Available: <https://scied.ucar.edu/learning-zone/how-climate-works/some-greenhouse-gases-are-stronger-others>

- [10] PlascticsEurope, "Plastics - the Facts 2021 - An analysis of European plastics production, demand and waste data," 2021. Accessed: Apr. 04, 2022. [Online]. Available: <https://plasticseurope.org/knowledge-hub/plastics-the-facts-2021/>
- [11] V. Katiyar, S. S. Gaur, A. K. Pal, and A. Kumar, "Properties of Plastics for Packaging Applications," in *Polymers for Packaging Applications*, 1st ed., S. Alavi, S. Thomas, K. P. Sandeep, N. Kalarikkal, J. Varghese, and S. Yaragalla, Eds., CRC Press, 2014, pp. 03–38.
- [12] J. Boucher and G. Billard, "The challenges of measuring plastic pollution," *Field Actions Sci Rep*, no. Special Issue 19, pp. 68–75, 2019, Accessed: Jun. 01, 2022. [Online]. Available: <http://journals.openedition.org/factsreports/5319>
- [13] A. Anjum, M. Zuber, K. M. Zia, A. Noreen, M. N. Anjum, and S. Tabasum, "Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements," *International Journal of Biological Macromolecules*, vol. 89. Elsevier B.V., pp. 161–174, Aug. 01, 2016. doi: 10.1016/j.ijbiomac.2016.04.069.
- [14] D. G. Bucknall, "Plastics as a materials system in a circular economy," *Philosophical Transactions of the Royal Society A*, vol. 378, no. 2176, Jan. 2020, doi: 10.1098/rsta.2019.0268.
- [15] S. Khanna and A. K. Srivastava, "Recent advances in microbial polyhydroxyalkanoates," *Process Biochemistry*, vol. 40, no. 2, pp. 607–619, 2005, doi: 10.1016/j.procbio.2004.01.053.
- [16] N. A. A. B. Taib, M. Rahman, D. Huda, K. Kuok, S. Hamdan, M. Bakri, M. Julaihi, A. Khan, "A review on poly lactic acid (PLA) as a biodegradable polymer," *Polymer Bulletin*, vol. 80, no. 2, pp. 1179–1213, Feb. 2023, doi: 10.1007/s00289-022-04160-y.
- [17] V. Sharma, R. Sehgal, and R. Gupta, "Polyhydroxyalkanoate (PHA): Properties and Modifications," *Polymer (Guildf)*, vol. 212, Jan. 2021, doi: 10.1016/j.polymer.2020.123161.
- [18] G. Y. A. Tan, C. Chen, L. Li, L. Wang, I. Razaad, Y. Li, L. Zhao, Y. Mo, J. Wang, "Start a research on biopolymer polyhydroxyalkanoate (PHA): A review," *Polymers*, vol. 6, no. 3. Molecular Diversity Preservation International, pp. 706–754, 2014. doi: 10.3390/polym6030706.
- [19] M. Kumar, R. Rathour, R. Singh, Y. Sun, A. Pandey, E. Gnansounou, K. Andrew Lin, D. Tsang, I. Thakur, "Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects," *J Clean Prod*, vol. 263, 2020, doi: 10.1016/j.jclepro.2020.121500.

- [20] E. Akaraonye, T. Keshavarz, and I. Roy, "Production of polyhydroxyalkanoates: The future green materials of choice," *Journal of Chemical Technology and Biotechnology*, vol. 85, no. 6, pp. 732–743, Jun. 2010. doi: 10.1002/jctb.2392.
- [21] S. Philip, T. Keshavarz, and I. Roy, "Polyhydroxyalkanoates: Biodegradable polymers with a range of applications," *Journal of Chemical Technology and Biotechnology*, vol. 82, no. 3, pp. 233–247, Mar. 2007. doi: 10.1002/jctb.1667.
- [22] R. A. J. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams, and I. Radecka, "Bacterial synthesis of biodegradable polyhydroxyalkanoates," *J Appl Microbiol*, vol. 102, no. 6, pp. 1437–1449, 2007, doi: 10.1111/j.1365-2672.2007.03335.x.
- [23] J. M. L. Dias, P. Lemos, L. Serafim, C. Oliveira, M. Eiroa, M. Albuquerque, A. Ramos, R. Oliveira, M. Reis, "Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product," *Macromol Biosci*, vol. 6, pp. 885–906, 2006, doi: 10.1002/mabi.200600112.
- [24] L. S. Serafim, P. C. Lemos, M. G. E. Albuquerque, and M. A. M. Reis, "Strategies for PHA production by mixed cultures and renewable waste materials," *Applied Microbiology and Biotechnology*, vol. 81, no. 4, pp. 615–628, Dec. 2008. doi: 10.1007/s00253-008-1757-y.
- [25] C. Kourmentza, J. Plácido, N. Vnetsaneas, A. Burniol-Figols, C. Varrone, H. Gavala, M. Reis, "Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production," *Bioengineering*, vol. 4, no. 2, 2017, doi: 10.3390/bioengineering4020055.
- [26] M. A. M. Reis, L. S. Serafim, P. C. Lemos, A. M. Ramos, F. R. Aguiar, and M. C. M. Van Loosdrecht, "Production of polyhydroxyalkanoates by mixed microbial cultures," *Bioprocess Biosyst Eng*, vol. 25, no. 6, pp. 377–385, 2003, doi: 10.1007/s00449-003-0322-4.
- [27] D. Dionisi, M. Beccari, S. D. Gregorio, M. Majone, M. P. Papini, and G. Vallini, "Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate," *Journal of Chemical Technology and Biotechnology*, vol. 80, no. 11, pp. 1306–1318, Nov. 2005, doi: 10.1002/jctb.1331.
- [28] C. S. S. Oliveira, C. E. Silva, G. Carvalho, and M. A. Reis, "Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities," *N Biotechnol*, vol. 37, pp. 69–79, Jul. 2017, doi: 10.1016/J.NBT.2016.10.008.

- [29] J. C. Fradinho, A. Oehmen, and M. A. M. Reis, "Improving polyhydroxyalkanoates production in phototrophic mixed cultures by optimizing accumulator reactor operating conditions," *Int J Biol Macromol*, vol. 126, pp. 1085–1092, 2019, doi: 10.1016/j.ijbiomac.2018.12.270.
- [30] P. C. Hallenbeck, C. Z. Lazaro, and E. Sagir, "Photosynthesis and Hydrogen from Photosynthetic Microorganisms," in *Microalgal Hydrogen Production: Achievements and Perspectives*, Royal Society of Chemistry, 2018, pp. 1–30. doi: 10.1039/9781849737128-00001.
- [31] D. Fleischman, "Photosynthesis," in *Cell Physiology Source Book*, Fourth Edition., Elsevier, 2012, pp. 909–924. doi: <https://doi.org/10.1016/B978-0-12-387738-3.00051-2>.
- [32] S. Stephens, R. Mahadevan, and D. G. Allen, "Engineering Photosynthetic Bioprocesses for Sustainable Chemical Production: A Review," *Frontiers in Bioengineering and Biotechnology*, vol. 8. p. 1469, 2021. doi: 10.3389/fbioe.2020.610723.
- [33] J. H. A. Nugent', "Oxygenic photosynthesis Electron transfer in photosystem I and photosystem II," *Eur. J. Biochem*, vol. 237, pp. 519–531, 1996, doi: <https://doi.org/10.1111/j.1432-1033.1996.00519.x>.
- [34] M. Chen, "Chlorophyll Modifications and Their Spectral Extension in Oxygenic Photosynthesis," *Annu Rev Biochem*, vol. 83, pp. 317–340, 2014, doi: 10.1146/annurev-biochem-072711-162943.
- [35] H. Scheer, "An Overview of Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications," in *Chlorophylls and Bacteriochlorophylls. Advances in Photosynthesis and Respiration*, vol. 25, B. Grimm, R. J. Porra, W. Rüdiger, and H. Scheer, Eds., Springer, Dordrecht, 2006, pp. 1–26. doi: 10.1007/1-4020-4516-6_1.
- [36] J. F. Imhoff, "Taxonomy and Physiology of Phototrophic Purple Bacteria and Green Sulfur Bacteria," in *Anoxygenic Photosynthetic Bacteria. Advances in Photosynthesis and Respiration*, vol. 2, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, Eds., Kluwer Academic Publishers, 1995, pp. 1–15.
- [37] A. K. Singh, H. K. Rana, and A. K. Pandey, "Analysis of chlorophylls," in *Recent Advances in Natural Products Analysis*, Elsevier, 2020, pp. 635–650. doi: 10.1016/B978-0-12-816455-6.00019-6.
- [38] D. M. George, A. S. Vincent, and H. R. Mackey, "An overview of anoxygenic phototrophic bacteria and their applications in environmental biotechnology for sustainable Resource recovery," *Biotechnology Reports*, vol. 28, Dec. 2020, doi: 10.1016/J.BTRE.2020.E00563.

- [39] R. Sirevåg, "Carbon Metabolism in Green Bacteria," in *Anoxygenic Photosynthetic Bacteria. Advances in Photosynthesis and Respiration*, vol. 2, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, Eds., Kluwer Academic Publishers, 1995, pp. 871–883.
- [40] B. K. Pierson and R. W. Castenholz, "Taxonomy and Physiology of Filamentous Anoxygenic Phototrophs," in *Anoxygenic Photosynthetic Bacteria. Advances in Photosynthesis and Respiration*, vol. 2, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, Eds., Kluwer Academic Publishers, 1995, pp. 31–47.
- [41] J. Mas and H. Van Gernerden, "Storage Products in Purple and Green Sulfur Bacteria," in *Anoxygenic Photosynthetic Bacteria. Advances in Photosynthesis and Respiration*, vol. 2, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, Eds., Kluwer Academic Publishers, 1995, pp. 973–990.
- [42] R. C. Fuller, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, "Polyesters and Photosynthetic Bacteria," in *Anoxygenic photosynthetic bacteria*, vol. 2, R. E. Blankenship, M. T. Madigan, and C. E. Bauer, Eds., Springer, Dordrecht, 1995, pp. 1245–1256.
- [43] M. T. J. Van Der Meer, S. Schouten, B. Van Dongen, W. Rijpstra, G. Fuchs, J. Sinnighe Damsté, J. De Leeuw, D. Ward, "Biosynthetic Controls on the ¹³C Contents of Organic Components in the Photoautotrophic Bacterium *Chloroflexus aurantiacus*," *Journal of Biological Chemistry*, vol. 276, no. 14, pp. 10971–10976, Apr. 2001, doi: 10.1074/jbc.M009701200.
- [44] M. T. Madigan and D. O. Jung, "An Overview of Purple Bacteria: Systematics, Physiology, and Habitats," in *The Purple Phototrophic Bacteria*, vol. 28, C. N. Hunter, F. Daldal, M. C. Thurnauer, and J. T. Beatty, Eds., Springer, Dordrecht, 2009, pp. 1–15.
- [45] N. U. Frigaard and C. Dahl, "Sulfur Metabolism in Phototrophic Sulfur Bacteria," in *Advances in Microbial Physiology*, vol. 54, Robert K. Poole, Ed., Academic Press, 2008, pp. 103–200. doi: 10.1016/S0065-2911(08)00002-7.
- [46] S. Sali and H. R. Mackey, "The application of purple non-sulfur bacteria for microbial mixed culture polyhydroxyalkanoates production," *Reviews in Environmental Science and Biotechnology*, vol. 20, pp. 959–983, 2021. doi: 10.1007/s11157-021-09597-7.
- [47] S. Romagnoli and F. Robert Tabita, "Carbon Dioxide Metabolism and its Regulation in Nonsulfur Purple Photosynthetic Bacteria," in *The Purple Phototrophic Bacteria. Advances in Photosynthesis and Respiration*, vol. 28, C. N. Hunter, F. Daldal, M. C. Thurnauer, and J. T. Beatty, Eds., Springer, Dordrecht, 2009, pp. 563–576.

- [48] V. Montiel-Corona and G. Buitrón, "Polyhydroxyalkanoates from organic waste streams using purple non-sulfur bacteria," *Bioresource Technology*, vol. 323. 2021. doi: 10.1016/j.biortech.2020.124610.
- [49] I. Monroy and G. Buitrón, "Production of polyhydroxybutyrate by pure and mixed cultures of purple non-sulfur bacteria: A review," *Journal of Biotechnology*, vol. 317. pp. 39–47, 2020. doi: 10.1016/j.jbiotec.2020.04.012.
- [50] S. Bengtsson, A. Karlsson, T. Alexandersson, L. Quadri, M. Hjort, P. Johansson, F. Morgan-Sagastume, S. Anterrieu, M. Acros-Hernandez, L. Karabegovic, P. Magnusson, A. Werker, "A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale," *N Biotechnol*, vol. 35, pp. 42–53, 2017, doi: 10.1016/j.nbt.2016.11.005.
- [51] F. Morgan-Sagastume, S. Heimersson, G. Laera, A. Werker, and M. Svanström, "Techno-environmental assessment of integrating polyhydroxyalkanoate (PHA) production with services of municipal wastewater treatment," *J Clean Prod*, vol. 137, pp. 1368–1381, 2016, doi: 10.1016/j.jclepro.2016.08.008.
- [52] F. Morgan-Sagastume, M. Hjort, F. Gérardin, S. Lacroix, G. Gaval, L. Karabegovic, T. Alexandersson, P. Johansson, A. Karlsson, S. Bengtsson, M. Arcos-Hernández, P. Magnusson, A. Werker, "Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale," *Bioresour Technol*, vol. 181, pp. 78–89, 2015, doi: 10.1016/j.biortech.2015.01.046.
- [53] M. Reis, M. Albuquerque, M. Villano, and M. Majone, "Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks," in *Comprehensive Biotechnology, Second Edition*, vol. 6, 2011, pp. 669–683. doi: 10.1016/B978-0-08-088504-9.00464-5.
- [54] J. R. Almeida, E. Serrano, M. Fernandez, J. C. Fradinho, A. Oehmen, and M. A. M. Reis, "Polyhydroxyalkanoates production from fermented domestic wastewater using phototrophic mixed cultures," *Water Res*, vol. 197, no. 117101, Jun. 2021, doi: 10.1016/j.watres.2021.117101.
- [55] H. Lu, G. Zhang, Z. Zheng, F. Meng, T. Du, and S. He, "Bio-conversion of photosynthetic bacteria from non-toxic wastewater to realize wastewater treatment and biore-source recovery: A review," *Bioresource Technology*, vol. 278. pp. 383–399, 2019. doi: 10.1016/j.biortech.2019.01.070.

- [56] J. Fradinho, L. D. Allegue, M. Ventura, J. A. Melero, M. A. M. Reis, and D. Puyol, "Up-scale challenges on biopolymer production from waste streams by Purple Phototrophic Bacteria mixed cultures: A critical review," *Bioresource Technology*, vol. 327. 2021. doi: 10.1016/j.biortech.2021.124820.
- [57] D. Puyol, V. Monsalvo, E. Marine, R. Rogalla, J. Melero, F. Martínez, T. Hülsen, D. Batstone, "Purple phototrophic bacteria as a platform to create the next generation of wastewater treatment plants: Energy and resource recovery," in *Wastewater Treatment Residues as Resources for Biorefinery Products and Biofuels*, 2019, pp. 255–280. doi: 10.1016/B978-0-12-816204-0.00012-6.
- [58] D. Puyol, T. Hülsen, B. Padrino, D. J. Batstone, F. Martinez, and J. A. Melero, "Exploring the inhibition boundaries of mixed cultures of purple phototrophic bacteria for wastewater treatment in anaerobic conditions," *Water Res*, vol. 183, Sep. 2020, doi: 10.1016/j.watres.2020.116057.
- [59] K.-H. Tang, Y. J. Tang, R. E. Blankenship, T. E. Hanson, U. Kappler, and O. Brookings, "Carbon metabolic pathways in phototrophic bacteria and their broader evolutionary implications," *Front Microbiol*, vol. 2, no. 165, 2011, doi: 10.3389/fmicb.2011.00165.
- [60] G. Capson-Tojo, D. Batstone, M. Grassino, S. Vlaeminck, D. Puyol, W. Verstraete, R. Kleerebezem, A. Oehmen, A. Ghimire, I. Pikaar, J. Lema, T. Hülsen, "Purple phototrophic bacteria for resource recovery: challenges and opportunities," *Biotechnol Adv*, vol. 43, no. 107567, 2020.
- [61] M. R. Melnicki, E. Eroglu, and A. Melis, "Changes in hydrogen production and polymer accumulation upon sulfur-deprivation in purple photosynthetic bacteria," *Int J Hydrogen Energy*, vol. 34, no. 15, pp. 6157–6170, Aug. 2009, doi: 10.1016/j.ijhydene.2009.05.115.
- [62] S. Ghosh, U. K. Dairkee, R. Chowdhury, and P. Bhattacharya, "Hydrogen from food processing wastes via photofermentation using Purple Non-sulfur Bacteria (PNSB) – A review," *Energy Conversion and Management*, vol. 141. pp. 299–314, 2017. doi: 10.1016/j.enconman.2016.09.001.
- [63] A. Alloul, N. Blansaer, P. Cabecas Segura, R. Wattiez, S. E. Vlaeminck, and B. Leroy, "Dehazing redox homeostasis to foster purple bacteria biotechnology," *Trends Biotechnol*, 2022, doi: 10.1016/j.tibtech.2022.06.010.
- [64] G. Strauss and G. Fuchs, "Enzymes of a novel autotrophic CO₂ fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle," *Eur J Biochem*, vol. 215, no. 3, pp. 633–643, 1993.

- [65] C. G. Klatt, Z. Liu, M. Ludwig, M. Kühn, J. Jensen, D. Bryant, D. Ward, "Temporal meta-transcriptomic patterning in phototrophic Chloroflexi inhabiting a microbial mat in a geothermal spring," *ISME Journal*, vol. 7, no. 9, pp. 1775–1789, Sep. 2013, doi: 10.1038/ismej.2013.52.
- [66] G. Farnocchia, "Evaluating the PHA storage capacity and the impacts of growth conditions on Chloroflexus aurantiacus, a green non-sulphur phototrophic bacterium," Master Thesis, Alma Mater Studiorum – University of Bologna.
- [67] X. Wang, A. Oehmen, E. B. Freitas, G. Carvalho, and M. A. M. Reis, "The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production," *Water Res*, vol. 112, pp. 269–278, Apr. 2017, doi: 10.1016/J.WATRES.2017.01.064.
- [68] S. Sugahara, M. Suzuki, H. Kamiya, M. Yamamuro, H. Semura, Y. Senga, M. Egawa, Y. Seike, "Colorimetric determination of sulfide in microsamples," *Analytical Sciences*, vol. 32, no. 10. Japan Society for Analytical Chemistry, pp. 1129–1131, 2016. doi: 10.2116/analsci.32.1129.
- [69] A. B. Lanham, A. Ricardo, M. Coma, J. Fradinho, M. Carvalheira, A. Oehmen, G. Carvalho, M. Reis, "Optimisation of glycogen quantification in mixed microbial cultures," *Bioresour Technol*, vol. 118, pp. 518–525, Aug. 2012, doi: 10.1016/j.biortech.2012.05.087.
- [70] A. B. Lanham, A. Ricardo, M. Albuquerque, F. Pardelha, M. Carvalheira, M. Coma, J. Fradinho, G. Carvalho, A. Oehmen, M. Reis, "Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems," *Process Biochemistry*, vol. 48, no. 11, pp. 1626–1634, Nov. 2013, doi: 10.1016/j.procbio.2013.07.023.
- [71] APHA, "Standard Methods for the Examination of Water and Wastewater," 1999.
- [72] S. Bengtsson, A. Werker, M. Christensson, and T. Welander, "Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater," *Bioresour Technol*, vol. 99, no. 3, pp. 509–516, Feb. 2008, doi: 10.1016/j.biortech.2007.01.020.
- [73] J. H. Law and R. A. Slepecky, "Assay of poly-3-hydroxybutyric acid," 1960.
- [74] H. L. Friedman and M. Kerker, "Ultraviolet Absorption of Aqueous Sulfur Solutions," 1952.
- [75] S. Filippi, P. Cinelli, A. Mezzetta, P. Carlozzi, and M. Seggiani, "Extraction of polyhydroxyalkanoates from purple non-sulfur bacteria by non-chlorinated solvents," *Polymers (Basel)*, vol. 13, no. 23, Dec. 2021, doi: 10.3390/polym13234163.

- [76] M. Darshan and D. Nishith, "Recovery and characterization of poly(3-Hydroxybutyric acid) synthesized in *Staphylococcus epidermidis*," *Afr J Environ Sci Tech*, vol. 8, no. 6, pp. 319–329, Jun. 2014, doi: 10.5897/ajest2014.1645.
- [77] F. Sabbagh Mojaveryazdi, I. I. Muhamad, S. Rezaia, and H. Benham, "Importance of Glucose and *Pseudomonas* in Producing Degradable Plastics," *J Teknol*, vol. 69, no. 5, 2014, doi: <https://doi.org/10.11113/jt.v69.3194>.
- [78] J. C. Fradinho, M. A. M. Reis, and A. Oehmen, "Beyond feast and famine: Selecting a PHA accumulating photosynthetic mixed culture in a permanent feast regime," *Water Res*, vol. 105, pp. 421–428, Nov. 2016, doi: 10.1016/j.watres.2016.09.022.

APPENDIX

A.1 Methods - Selection of new inocula for the SBR

MEDIUM 1			
yeast extract	1.00	g	
glycyl-glycine	1.00	g	
Na ₂ HPO ₄ ·7H ₂ O	0.10	g	
MgSO ₄ ·7H ₂ O	0.10	g	
KNO ₃	0.10	g	
NaNO ₃	0.50	g	
NaCl	0.10	g	
CaCl ₂ ·2H ₂ O	0.05	g	
Fe(III) citrate sol.	5.00	ml	
Trace element sol. SL-6	1.00	ml	
Distilled water	1050.00	ml	
Neutralized sulphide solution		Trace element solution SL-6	
Na ₂ S·9H ₂ O	3.00	g	
distilled water	100.00	ml	
		Fe(III) citrate solution	
		Fe (III) citrate	0.10 g
		distilled water	100.00 ml

Figure A.1-0-1: Composition of the enriching growth medium M1 from [66]. After preparing the 1050mL of medium with all the components, the Fe (III) citrate solution and the trace element solution, and setting the pH to 8.2, the medium was transferred to 100mL serum flasks, bubbled with N₂, and 1mL of neutralized sulfide solution was added, in order to obtain an sulfide end concentration of 0.03%.



(2023)

INÊS CARDOSO

POLYHYDROXYALKANOATES PRODUCTION FROM CO₂ USING PHOTOTROPHIC MIXED CULTURES