Juliana Roda Almeida

Licenciada em Bioquímica

Phototrophic bioplastic production from domestic and agro-industrial wastewaters

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Dra. Joana Costa Fradinho,
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Co-orientador: Prof. Maria da Ascensão C. F. Miranda Reis,
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Para os meus Pais…

“Ever tried. Ever failed. No matter. Try Again. Fail again. Fail better.”

Samuel Beckett

Expresso aqui os meus agradecimentos sinceros às muitas pessoas que direta e indiretamente me ajudaram a cumprir os meus objetivos e a realizar mais esta etapa da minha formação académica.

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ABSTRACT

Polyhydroxyalkanoates (PHA), are biodegradable polymers, naturally synthesized by several bacteria and with characteristics similar to conventional plastics. The phototrophic PHA production explores the capability of photosynthetic bacteria to accumulate PHA under open, non-aerated and illuminated conditions. These systems, surged as a means to lowering the operational costs commonly associated to the traditional PHA production via pure and aerobic cultures.

The principal objective of this thesis was to define operational conditions that can allow the retrofitting of High Rate Algae Ponds (HRAP), currently used in wastewater treatment into bacterial photosynthetic systems that can produce PHA while treating wastewater. In this specific work two different strategies to select bacterial Photosynthetic Mixed Cultures (PMCs) with the capacity to accumulate PHA were developed, with the cultures being fed with a fermented mixture of domestic wastewater and sugar molasses (WWM).

The first PMC was selected through a permanent feast regime, and operated for 224 days, under a 24 hours cycle with alternated light and dark periods, without aeration. Operation under low concentration of phosphorous and organic acids (OA) seems to be the best strategies to achieve PMCs with higher Bacteriochlorophylls/Chlorophylls ratio and higher PHA content, achieving a maximum PHA content of 19% PHA/VSS (20 Cmmol PHA/L) and 18,5% PHA/VSS (10 Cmmol PHA/L), for each strategy, respectively. It was also found that the presence of sugars in the fermented WWM solution during this permanent feast regime leads to negative effects on PMC's PHA accumulation capacity.

The second PMC operated in this work, was selected under a feast and famine regime under a 24 hours cycle with alternated light and dark periods. It was discovered that the high Feast/Famine ratios can lead to PMCs with higher PHA accumulation capacity, and that periodical feeding pulses during the initial light phase was the best strategy to achieve longer feast phases, attaining maximum PHA content of 29% PHA/VSS (25 Cmmol PHA/L).

PHA accumulation in both PMCs could be improved in separate accumulator reactors upon illuminations at higher light intensities, being registered a maximum value of 30% of PHA/VSS (17 Cmmol PHA/L) with the PMC selected under the feast and famine regime.

The obtained results show that both approaches appear to be promising strategies for selecting photosynthetic PHA accumulating bacteria and that are worthy of further development for application in wastewater treatment plants as means of valorising wastewater as carbon source for production of added-value bioproducts, like PHA, thus contributing to a circular economy.

Keywords: *Polyhydroxyalkanoates (PHA); Photosynthetic mixed cultures (PMC); Feast and Famine regime; Permanent Feast regime; Wastewater treatment; Added-value bioproduct*

Polihidroxialcanoatos (PHA) são polímeros biodegradáveis, sintetizados naturalmente por várias bactérias e com características semelhantes aos plásticos convencionais. A produção fototrófica de PHA explora a capacidade das bactérias fotossintéticas para acumular PHA em sistemas abertos, não arejados e que utilizam a luz solar como fonte de energia. Esses sistemas surgiram como um meio para diminuir os custos operacionais comumente associados à produção tradicional de PHA através de culturas puras e aeróbias.

O objetivo principal desta tese foi definir condições operacionais que permitam a adaptação das Lagoas de Algas de Alta Taxa (LAAT) que atualmente são utilizadas no tratamento de águas residuais, em sistemas fotossintéticos para produzir PHA a partir de bactérias enquanto se procede ao tratamento de águas residuais. Em especifico neste trabalho, foram desenvolvidas duas estratégias diferentes para selecionar Culturas Mistas Fotossintéticas (CMFs) de bactérias com a capacidade de acumular PHA, através da alimentação de culturas com uma solução fermentada de águas residuais e melaços.

A primeira CMF foi selecionada através de um regime de fartura permanente, ao longo de 224 dias, sob um ciclo de 24 horas com períodos alternados de luz e escuro, sem arejamento. A operação do reator sob baixa concentração de fósforo e ácidos orgânicos (OA) mostraram ser as melhores estratégias de seleção de CMFs com maior rácio de Bacterioclorofilas/Clorofilas e maior índice de PHA, atingindo um teor máximo de PHA de 19% de PHA/VSS (20 Cmmol PHA/L) e 18,5% de PHA/VSS (10 Cmmol PHA/L), para cada estratégia, respetivamente. Verificou-se também que a presença de açúcares na solução de alimentação durante este regime, conduz a efeitos negativos na capacidade de acumulação de PHA pela CMF.

A segunda CMF foi selecionada sob um regime de fartura e fome, num ciclo de 24 horas com períodos alternados de luz e escuro. Descobriu-se que os elevados rácios de Fartura/Fome levam a CMFs com maior capacidade de acumulação de PHA e que os pulsos periódicos de carbono, durante a fase inicial de luz foram a melhor maneira de alcançar fases de fartura mais longas, atingindo um teor máximo de PHA de 29% de PHA/VSS (25 Cmmol PHA/L). A acumulação de PHA em ambas as CMFs poderia ser melhorada, em reatores de acumulação à parte, após iluminação com intensidades de luz mais altas, sendo registado um valor máximo de 30% de PHA/VSS (17 Cmmol PHA/L) na CMF selecionada sob o regime de fome e fome.

Os resultados obtidos mostram que ambas as abordagens parecem ser estratégias promissoras para a seleção de bactérias fotossintéticas acumuladoras de PHA e que poderiam ser boas estratégias a ser aplicadas em estações de tratamento de águas residuais como meio de valorização de águas residuais como fonte de carbono para produção de bioprodutos de valor acrescentado, como PHA, contribuindo assim para uma economia circular.

Palavras-chave: *Polihidroxialcanoatos (PHA); Culturas mistas fotossintéticas (CMFs); Regime de fartura e fome; Regime de fartura permanente; Tratamento de água residuais; Bioproduto de valor acrescentado.*

3H2MV	3-hydroxy-2-methylvalerate
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
AcT	Accumulation Test
ADF	Aerobic Dynamic Feeding
ADP	Adenosine Diphosphate
AN/AE	Anaerobic feast / Aerobic famine
ATP	Adenosine Triphosphate
Bact/Chl	Bacteriochlorophyll to Chlorophyll ratio
Chiclana WWTP	Chiclana Wastewater Treatment Plant
CT	Control test
Cyt b6f	Cytochrome b6f
EBPR	Enhanced Biological Phosphorus Removal
EDTA	Ethylenediaminetetraacetic acid solution
F/F	Feast to Famine ratio
FF	Feast and Famine
FISH	Fluorescence in situ Hybridisation
GAO	Glycogen Accumulating Organisms
GC	Gas Chromatography
Gly	Glycogen
HPLC	High-Performance Liquid Chromatography
HRAP	High Rate Algae Pond
HRT	Hydraulic Retention Time
IC	Inorganic Carbon
k_La	Volumetric oxygen transfer coefficient
LH	Light Harvesting complex

mcl-PHAs	Medium chain length polyhydroxyalkanoates
MMC	Mixed Microbial Cultures
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NLR	Nitrogen Loading Rate
OA	Organic Acids
OLR	Organic Loading Rate
ORP	Oxidation Reduction Potential
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P3HB	Poly(3-hydroxybutyrate)
P3HV	Poly(3-hydroxyvalerate)
PAO	Polyphosphate Accumulating Organisms
PBS	Phosphate Buffered Solution
PHA	Polyhydroxyalkanoate
PLR	Phosphorous Loading Rate
polyP	Polyphosphate
PMC	Photosynthetic Mixed Culture
PNS	Purple Non-Sulphur
PSI	Photosystem I
PSII	Photosystem II
$\mathbf{q_{Carb}}$	Specific carbohydrate production rate
$\mathbf{q_{PHA}}$	Specific PHA production rate
$\mathbf{-q_{PO4}}$	Specific phosphate consumption rate
$\mathbf{-q_{S}}$	Specific substrate uptake rate
RC	Reaction Center
SBR	Sequencing Batch Reactor
SDS	Sodium Dodecyl Sulphate
scl-PHAs	Short chain length polyhydroxyalkanoates
SRT	Sludge Retention Time
TC	Total Carbon

TOC	Total Organic Carbon
TSS	Total Suspended Solids
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solids
WWM	Wastewater with molasses
X	Active biomass
$\mathbf{Y_{Carbs/PHA}}$	Yield of carbohydrates per PHA consumed
$\mathbf{Y_{Carbs/S}}$	Yield of carbohydrates per substrate consumed
$\mathbf{Y_{PHA/S}}$	Yield of PHA per substrate consumed
$\mathbf{Y_{X/S}}$	Yield of biomass per substrate consumed

CHAPTER 1.

MOTIVATION AND THESIS OUTLINE

1.1. THESIS MOTIVATION

In the present days, plastics have become an essential material for our society creating a wide variety of markets (agriculture, packaging, automotive, building and construction, etc.). However, the vast majority of plastics is produced from petroleum compounds, that are an exhausting source with increasing prices. In addition, the petroleum based plastics have also some negative characteristics such as non-degradability and its persistence in the environment, becoming a problem once it starts to accumulate as waste. To overcome this situation the development of plastics has been evolving from the use of synthetic molecules to new natural forms to produce biodegradable plastics. Polyhydroxyalkanoates, better known as PHAs, are biodegradable polymers, naturally synthesized by several bacteria that accumulate this polymer in the form of granules. In addition, PHAs also have similar characteristics to conventional plastics, which makes this material a potential substitute for traditional plastics.

Recently, phototrophic PHA production has also been achieved in photosynthetic mixed cultures (PMCs) under an open, non-aerated and using illuminated systems, as means of lowering the operational costs commonly associated to the PHA production by pure and aerated mixed cultures. The photo-bioreactors (PBR) are also commonly used as wastewater (WW) treatment systems with consortia of microalgae and bacteria communities.

This thesis is part of a collaborative project funded by the European Commission under the Horizon 2020 Research and Innovation programme, INCOVER, with to the goal of retrofitting High Rate Algae Ponds (HRAP) from the *Chiclana Wastewater Treatment Plant (WWTP)* used in WW treatment, to produce PHA using PMCs.

The main goals of this thesis were precisely to select a PHA accumulating PMC, under the same operational conditions of HRAPs from Chiclana WWTP, using artificial illumination and fed with real wastewaters, establishing the effect of using wastewater on the culture growth and PHA production stability, and furthermore, evaluating the impact of operational conditions (OLR, pH and light intensity) on the PHA production performance of the system. This is the first time that the PHA production using PMC is tested simulating demonstration scale systems and using real wastewater.

1.2. THESIS OUTLINE

The thesis includes six chapters, describing the work developed during this master thesis. In the current chapter - Chapter 1.- the motivation and objectives of this thesis is presented. Chapter 2. presents an introduction to the theme where is included some concepts about PHA production and photosynthetic systems. Chapter 3. describes the laboratorial methods utilized during this work, while Chapters 4. and 5. present the results and discussion of the research work performed during this project along with a general conclusion.

Chapter 4. presents the study regarding the effect of a permanent feast regime in the enrichment process of a PMC (henceforth named reactor SBR1), while Chapter 5. presents the study regarding the effect of a feast and famine regime in the enrichment process of a PMC (reactor SBR2). In these chapters, the effect of different nutrients and organic compounds concentrations are tested along with different light intensities to better understand the PHA accumulation capacity of the resulting PMC.

In chapter 6 the main conclusions of this Master thesis are presented, as well as some suggestions for future work.

CHAPTER 2.

INTRODUCTION

2.1. PLASTICS

Plastics, a polymeric material, have become a crucial part of our lifestyle, and the global plastic production has increased immensely since the 20th century. Their favourable properties like low density, high strength-to-weight ratio, high durability, ease of design and manufacture, and low cost, were the main reasons for the fast growth of this industry, as well as their facility to be moulded into a variety of products that find use in a wide range of applications (Gu and Ozbakkaloglu, 2016).

In 2016, according to *Plastic Europe*, the global plastics production was estimated at 322 million tons, which has been increasing every year. At the same time, in Europe with a market evaluated in 340 billion euros, 59 million tons of plastic were produced reaching a market demand of 47.8 million tons. Industries are getting more interested in the field of plastic manufacturing, creating a wide variety of markets, as we can see in Figure 2.1, where packaging reaches the highest demand percentage.

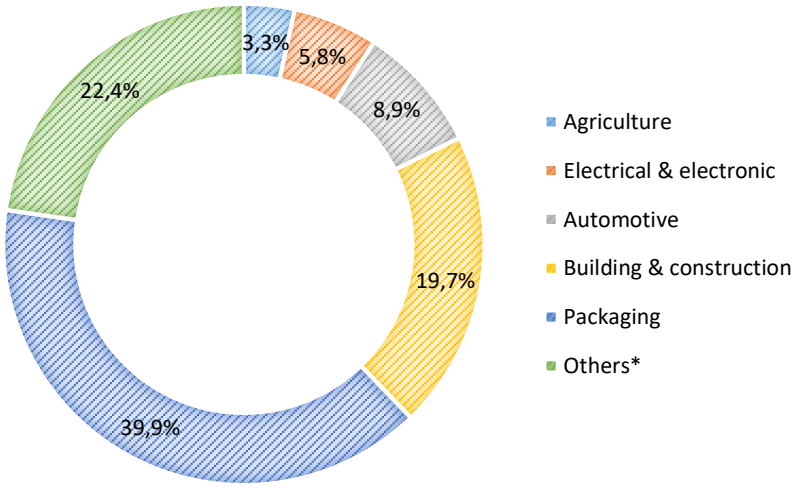

Figure 2. 1: Distribution of European plastics demand by segment in 2016 (Plastic Europe, 2016).

There are 25.8 million tons of post-consumer plastics waste that ended up in the waste stream. The traditional plastics are very resistant and not readily degraded in the ambient surroundings, for that reason the waste undergoes treatment process of recycling and energy recovery (29.7 % and 39.5% respectively), but still 30.8% of the wasted plastics went to landfills (Plastic Europe, 2016).

It is a fact that, most types of plastics will never degrade and will remain on landfills for several years. The synthetic polymers, need hundreds of years to degrade in normal environmental conditions, and if they have influence of the weathering, they give rise to smaller pieces of plastic debris without degrading the polymer itself. The end-of-life plastics accumulation in landfills and in natural habitats worldwide, has become a bigger problem in both waste-management issues and environmental damage (Hopewell et al., 2009). Nowadays, the basic materials used for making plastics are almost completely derived from petrochemicals produced from fossil oil and gas, that contribute to pollution and depletion of non-renewable natural resources (Álvarez-Chávez et al., 2012). Around 4% of annual petroleum production is directly used as a feedstock

to plastic production, and almost the same quantity is spent to providing the energy necessary to the manufacturing (Hopewell et al., 2009).

It leads us to a critical point, about the preservation of natural resources and recycling. There is a need to create renewed interest concerning biomaterials with the focus on renewable materials, like biodegradable polymers from which scientists can offer a possible solution to waste-disposal problems associated to traditional petroleum-derived plastics.

2.2. BIODEGRADABLE PLASTICS

Plastics such as polyethylene, polypropylene, polystyrene, are non-biodegradable, representing a huge concern for the environment due to their resistance to chemical, physical and biological degradation, and thus accumulating in the environment. To overcome this problem, it is necessary to develop biodegradable plastics (Luckachan and Pillai, 2011; Tokiwa et al., 2009). Research in this field has been growing, leading to the exploration of plastics susceptible to microbial attack which makes them degradable in a microbial active environment (Augusta et al.,1992; Witt et al., 1997).

There are two types of bioplastics, the biodegradable plastics that are produced from fossil materials but have the capability to be degraded by microorganisms and the bio-based plastics which consists in plastics synthesized from biomass or renewable resources (Tokiwa et al., 2009). The polyhydroxyalkanoates (PHA) are an example of a natural polymer produce by bacteria as an intracellular carbon and energy source. PHA has a large versatility that makes it a good candidate for applications as, a fully biodegradable polymer while presenting similar properties to the synthetic polymers (Khanna and Srivastava, 2005).

2.2.1. POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are polyesters composed of several units of hydroxyalkanoates, synthesized by many gram-positive and gram-negative bacteria from at least 75 different genera. These polyesters accumulate intracellularly as insoluble cytoplasmic inclusions, acting as a carbon/energy store under growth limiting conditions (Laycock et al., 2013; Panchal et al., 2013; Madison and Huisman, 1999).

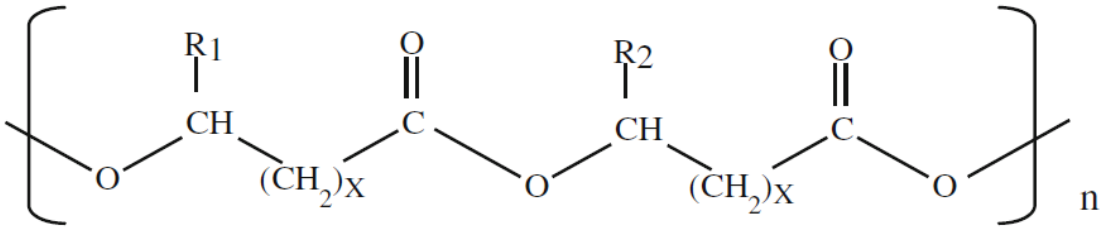

Figure 2. 2: General formula for PHAs. R_1/R_2: Alkyl groups C_1-C_{13}, x:1-4, n:100-30,000 (Panchal et al., 2013).

Figure 2.2 represents PHA's general formula where 'n' is the number of monomer units in each polymer chain, which varies between 100 and 30,000, 'R1' and 'R2' the side chain that includes alkyl groups with 1–13 carbons and 'x' in the main chain which ranges from 1 to 4 (Panchal et al., 2013).

PHAs can be classified into two main groups based on the number of carbon atoms in the monomers. There are the group of short chain length polyhydroxyalkanoates (scl-PHAs), which consist of monomers units with 3-5 carbon atoms (C_3-C_5), and the class of medium chain length polyhydroxyalkanoates (mcl-PHAs) that contain monomers units with 6-14 carbon atoms (C_6–C_{14}) (Singh et al., 2015; Panchal et al., 2013).

When a comparison of properties is made between PHAs and the traditional petrochemical based plastics such as polypropylene, it is seen that some *mcl*-PHAs have low crystallinity and tensile strength but high elongation to break, as also lower melting points and glass transition temperatures when compared with scl-PHAs and polypropylene. *scl*-PHAs on the other hand have higher tensile strength than polypropylene as we can see in Table 2.1 (Akaraonye et al., 2010).

Table 2. 1: Comparison of the physical properties of scl-PHAs and mcl-PHAs with polypropylene.

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

The most common PHAs are short chain length type such as poly(3-hydroxybutyrate) (P3HB) and the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)), having mechanical properties that are comparable to those of polypropylene (Laycock et al., 2013; Akaraonye et al., 2010). Therefore, polyhydroxyalkanoates represent a significant interest due to the 150 different monomers that have been reported until now, that creates a possibility to produce different types of biodegradable polymers with an extensive range of physical properties. The rich properties of PHAs depend on the bacterial species used and on the growth conditions that affect their synthesis and lead to various PHAs structures (Reddy et al.,2003; Chen, 2010).

2.2.2. POLYHYDROXYALKANOATES BIOSYNTHESIS

The synthesis of PHAs occurs under specific growth conditions, like excess of carbon along with deprivation of essential nutrients such as nitrogen (Gumel et al., 2012).

They can be synthesised by a wide variety of bacteria using different substrates such as renewable resources (sucrose, starch, cellulose, triacylglycerols), fossil resources (methane,

mineral oil, lignite, hard coal), agro-industrial by-products (molasses, whey, glycerol), chemicals (propionic acid, 4-hydroxybutyric acid) and carbon dioxide (Lee, 1996; Reddy et al.,2003). The produced microbial PHA depends on the carbon source used, giving rise to monomers that can or cannot resemble the structure of a particular carbon source. The cause for these differences can be explained by the metabolic pathways operating in the microorganisms. So far, biosynthesis of PHA can be describe trough eight pathways (Laycock et al., 2013; Philip et al., 2007).

Figure 2.3 shows two possible pathways. The best well studied pathway is Pathway I, that consists basically on the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, through the action of β-ketothiolase enzyme. Then the acetoacetyl-CoA is reduced by the acetoacetyl-CoA reductase to form 3-hydroxybutyryl-CoA, the building block of PHAs. At last, the PHA synthase enzyme polymerizes the monomers 3-hydroxybutyryl-CoA into poly(3-hydroxybutyrate) (P(3HB)) (Gumel et al., 2012; Philip et al., 2007*).*

There is also the fatty acid β-oxidation pathway, commonly known as Pathway II, that is associated with fatty acids production by microorganisms. The first step is the oxidation of fatty acids that give rise to acyl-CoA, subsequently the precursor is converted to 3-hydroxyacyl-CoA which can then form PHA under synthase catalysis (Philip et al., 2007).

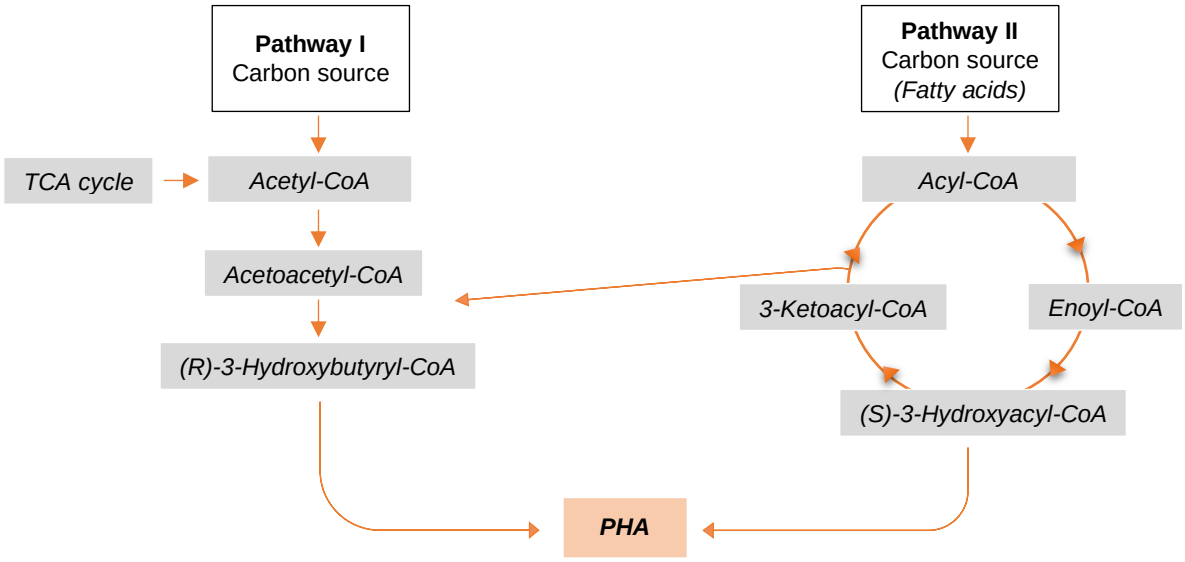

Figure 2. 3: Polyhydroxyalkanoates biosynthetic pathways. Adapted from Philip et al., 2007.

2.3. BACTERIAL CULTURES USED IN PHA PRODUCTION

In the past few years, PHA has been industrially produced by pure cultures such as *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans*, recombinant *Alcaligenes eutrophus* and recombinant *Escherichia coli* (Salehizadeh and Loosdrecht, 2004).

PHA production is normally associated to high costs which are related to the substrates costs and extraction of the polymer from the cells, which implies higher costs of manufacture and consequently raise the polymer price (Reis et al., 2003). There are several industries, since 1980s, that produce PHAs under the trade names of Biopol™ that is a co-polymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), Nodax™ is PHA copolymer that consist on 3-hydroxybutyrate and a comparatively small quantity of medium chain length monomers such as

3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydecanoate, Biocycle™, Biomer™, and BioGreen™ (Anjum et al., 2016; Możejko-Ciesielska et al., 2016). However, the polyhydroxyalkanoates co-polymers are sold at a higher price – \$9 Kg^{-1} P(3HB) - than the conventional plastics - < \$1 Kg^{-1} – (Reis et al., 2003).

In the past decades, the interest on potential alternative for PHA production has been rising, and efforts have been made towards decreasing the polymer production costs, such as improvements on the process technology, specifically the downstream processing, and by increasing the volumetric production capacity of reactor systems. Despite the improvements made by the implementation of new process, the utilization of different substrates and a variety of microbial cultures, the main cost factor is still the quality of the substrate and the sterile processes associated to the pure cultures PHA production. The possibility of utilization open mixed microbial cultures (MMCs) facilitates the use of substrates of lower quality, without needing sterilization methods (Reis et al., 2003; Kleerebezem and Loosdrecht, 2007).

A new process for the PHA production based on waste organic carbon show that the production cost of PHA could decrease for less than €4/kg (cost reduction of 85% lower, due to the substrate used) when compared with €8/kg cost of the a traditional PHA production in pure culture process (Reis et al., 2003).

The typical PHA production system by MMCs can be divided into three-stages, starting with the substrate and finishing with the biopolymer. The process includes (1) acidogenic fermentation of the substrate, (2) selection of PHA-accumulating cultures, (3) PHA batch accumulation using the enriched sludge and fermented substrates, as can be seen in Figure 2.4 (Duque et al., 2013).

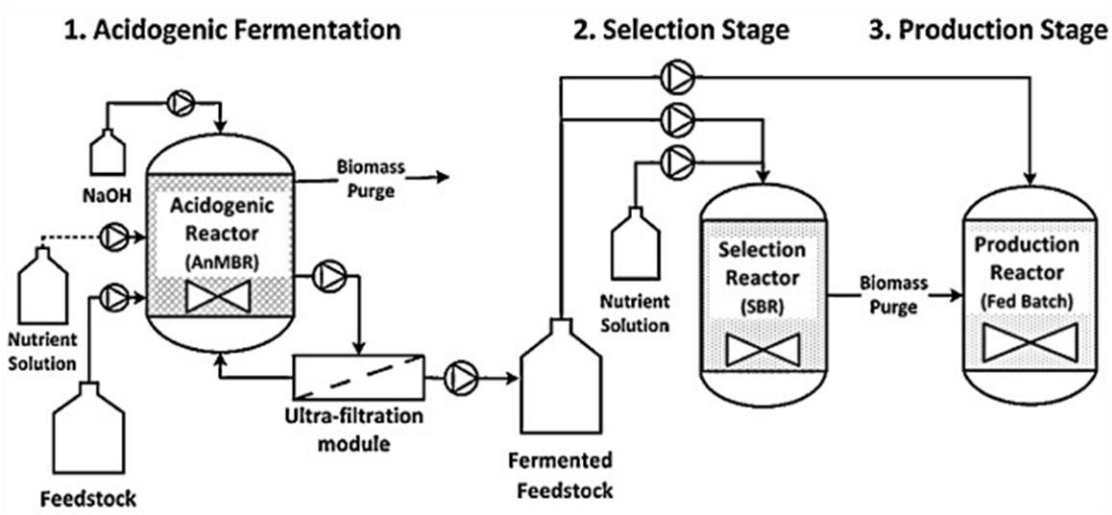

Figure 2. 4: Experimental setup of the 3-stage PHA production process. (Duque et al., 2013)

The utilization of open mixed microbial cultures enables the use of complex agro-industrial wastes and other substrates of low quality as feedstock. After fermentation step these substrates give rise to volatile fatty acids (VFAs) (e.g., acetate, propionate, butyrate, and valerate), that are the best precursors for PHA production in MMCs (Albuquerque et al., 2011).

A disadvantage of the MMCs is the fact that microbial populations are of unknown composition, for this reason it is important to implement some methods to select the desired population with

the required characteristics. The second step is the main challenge for the production system, since it is responsible for selecting a stable culture with a high PHA storage capacity under operational conditions imposed on the biological system (Albuquerque et al., 2007). Processes that exposed the MMCs to a transient carbon supply, are commonly known for selecting microbial populations with enhanced capacity to store PHA. The Feast and Famine (FF) process is often called as unbalanced growth and is characterized by successive periods of excess of carbon (feast), where storage of polymer occurs, alternating with periods of substrate limitation (famine), where the stored polymer can be used as carbon and energy source for growth and maintenance. The ideal Feast/Famine ratio should be comprised between 0.21 – 1.1 as tested by Albuquerque et al. (2010a).

The famine phase occurs under carbon limitation. It is thought that the lack of an external substrate during a considerable period of time lead to a reduction of cells activities to a minimum of cellular maintenance processes, that consequentially diminish intracellular components responsible for cell growth, such as enzymes synthesis and RNA transcription (Serafim et al., 2008; Albuquerque et al., 2010a).

The feast phase requires a physiological adaptation of the microorganisms due to the previous lack of carbon source. After the starvation period, the microbial culture growth is delayed, due to the low amount of enzymes available in the cells, entering in an adaptation period where the substrate uptake is mainly driven toward polymer storage. It is important to say that, usually in this type of situations, the storage phenomena dominate over cell growth, giving the ability to store internal reserves and a competitive advantage to those without this skill, but when the cells are under conditions where the substrate is continuously present, for a long period of time, the growth became more visible (Salehizadeh and Loosdrecht, 2004). The FF strategy is usually carried out by two different approaches, that can select distinct PHA accumulating organisms, the Aerobic dynamic feeding (ADF) and the Anaerobic feast / Aerobic famine (AN/AE).

The Anaerobic feast / Aerobic famine strategy has been used in diverse systems. In the anaerobic phase (AN), there is an external growth limitation due to the absence of an electron acceptor, such as oxygen or nitrate. When in the presence of carbon, this restriction allows the storage of the carbon by PHA accumulating bacteria, instead of biomass growth. Still, in the aerobic phase (AE), the electron acceptor is present and there is no carbon available, which means that only PHA accumulating bacteria have the ability to survive. The repeated cycles of AN feast/AE famine can lead to a selection of polyphosphate and glycogen accumulating organisms (PAOs and GAOs) (Serafim et al., 2008; Pereira et al., 1996).

Polyphosphate-accumulating organisms have the capability to take up carbon sources, primarily volatile fatty acids (VFAs), during the anaerobic phase, and convert them in intracellular poly-hydroxyalkanoates (PHAs). The energy necessary to do the VFAs uptake came from the degradation of intercellular storage polymers such as polyphosphate (polyP), that release inorganic phosphate, and glycogen, that provide reducing power required for the production of PHAs. In the aerobic phase, PHA is oxidised, gaining energy for growth and providing energy for the inorganic phosphate uptake and glucose production, thus regenerating the internal polymers

- polyphosphate and glycogen -. Glycogen-accumulating organisms (GAOs), are another group of microorganisms capable of storing carbon sources in the anaerobic phase, through the hydrolysis of glycogen via glycolysis, without the capacity to release phosphorous (Pereira et al., 1996; Oehmen, et al., 2006; Saunders, et al., 2003; Zhou et al., 2008).

The Aerobic dynamic feeding (ADF) is the most well-studied strategy of transient feeding in MMCs, consisting in long periods without substrate supply (famine period) alternated with short periods of substrate excess (feast period), in a fully aerobic reactor (Wang et al., 2017; Serafim et al., 2008). ADF is a widely-used strategy to enrich aerobic mixed cultures in PHA producing organisms, and in Jonhson et al. (2009) accumulation levels of 89% with an acetate-fed culture was achieved, which is the highest value reported in mixed cultures, and the closest to the best accumulation values of the pure cultures (80-90%) (Dias et al., 2006). There are also interesting values on cultures using low cost agro-industrial surplus feedstocks for example, the utilization of fermented sugar molasses led to a PHA accumulation values of approximately 75% (Albuquerque et al., 2010a) and 61% (Albuquerque et al., 2010b). In the past, many other fermented agro-industrial wastes were tested as possible substrates for PHA production, such as fermented paper mill effluents (Bengtsson et al., 2008), industrial wastewaters (Dionisi et al., 2006), fermented olive oil mill effluents (Dionisi et al., 2005; Beccari et al., 2009), fermented tomato cannery wastewater (Lui et al., 2008) and palm oil mill effluents (Mumtaz et al., 2010).

There are some studies which shows that the accumulated polymer contained at least, five types of monomers, with different ratios depending on the VFA composition of the substrate (Bengtsson et al., 2010a,b). It is known that n-alkanoates of even carbon number produce the homopolymer of poly(3-hydroxybutyrate) [P(3HB)], while the n-alkanoates of odd carbon numbers are responsible for the production of the poly(3-hydroxyvalerate). This way, the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate units [P(3HB-co- 3HV)] was produced only in the presence of both even and odd alkanoates (Akiyama et al., 1992). This is one of the biggest advantages of the MMCs that are fed with fermented feedstocks containing mixtures of organic acids (acetate, propionate, butyrate and valerate), that had the possibility to produce PHA polymers with a big diversity of hydroxyalkanoates monomers – 3-hydroxybutirate (3HB), 3-hydroxyhexanoate (3HHx), 3-hydroxyvalerate (3HV), 3-hydroxy-2-methyl-valerate (3H2MV) – (Takabatake et al., 2000; Lemos et al., 2006; Bengtsson et al., 2010b).

However, the vast majority of studies conducted to improve the production of PHAs always involves aerobic cultures, which require high costs with aeration. As an alternative to the high cost of aeration, there are other cultures, such as Photosynthetic Mixed Cultures (PMCs) that obtain energy from light, not requiring aeration.

2.4. PHOTOSYNTHETIC MIXED CULTURE PHA PRODUCTION

A large number of microorganisms are not restricted to obtaining energy from the oxidation of organic and inorganic compounds to grow, there are some that have the ability to capture light energy in order to synthesize ATP and NADH or NADPH, thus being usually called photosynthetic microorganisms.

There are two large groups of photosynthetic microorganisms: those that produce energy by oxygenic photosynthesis, which include microorganisms such as eukaryotes (e.g: algae) and cyanobacteria; and the anoxygenic microorganisms group, of which the green and purple photosynthetic bacteria are part (Prescott, 1999).

Photosynthesis, is the process by which photosynthetic organisms covert light energy from the sun into a chemically useful form, and is performed by pigment-protein complexes - reaction centres (RC) and light-harvesting complexes (LH) - that are localized in the photosynthetic membrane. This process is yet divided into two phases, the ***light phase*** and the ***dark phase***. In the light phase the energy is collected by the light-harvesting complex and converted in chemical energy, whereas in the dark phase the energy produced in the presence of light is used to reduce or fix CO_2 and to synthesize new cellular components (Kühlbrandt, 1995; Prescott, 1999).

2.4.1. OXYGENIC PHOTOSYNTHESIS PROCESS

The oxygenic photosynthesis is one of the most important process on earth, not only because it can convert light energy into chemical energy but also because it is responsible for producing molecular oxygen.

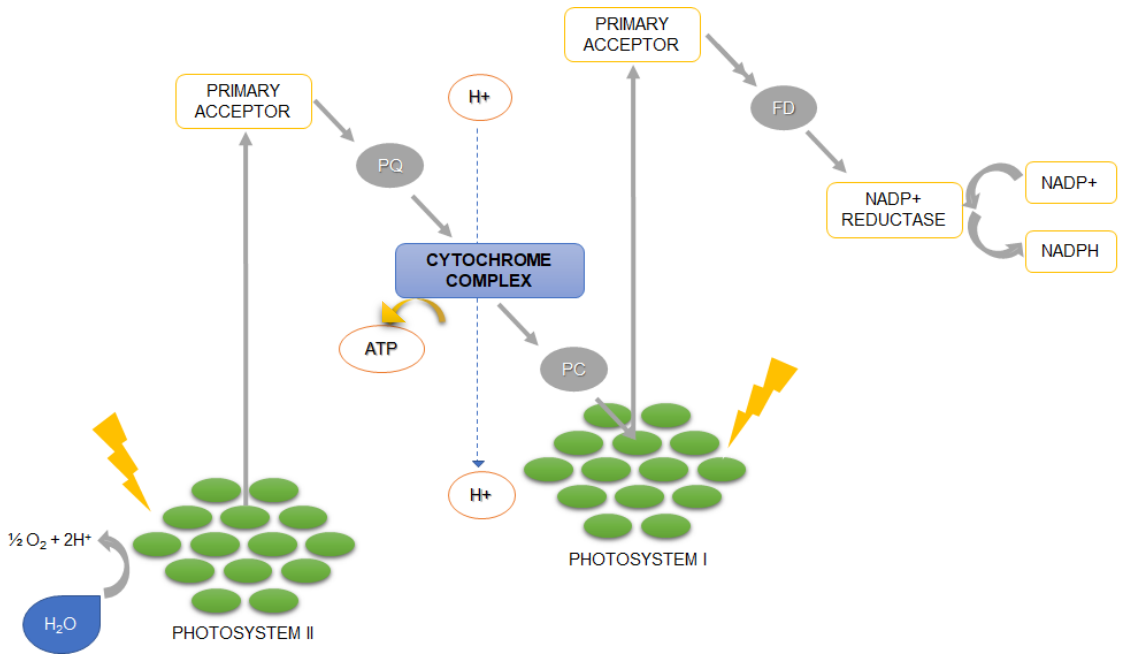

Figure 2. 5: The Z scheme. A model to illustrate the flow of electrons through the two photosystems (PSI and PSII) of the light reactions, in a noncyclic photophosphorylation. Adapted from Govindjee & Govindjee, 1974; Prescott et al. 1999.

It is conducted by two different photosystems – Photosystem I (PSI) and Photosystem II (PSII) -, that are both constituted by a light-harvesting complex, usually called by antenna, that consist in an assembling of chlorophylls and accessory pigments that are responsible for collecting photons, and a reaction-center responsible for the electrons transport. Both photosystems are electronically connected by an intermediate membrane called Cytochrome *b*$_6$*f* (Cyt *b*$_6$*f*). There are, at least, two types of chlorophylls involved in this process, the *chlorophyll a* that has a light

absorption peak at 680nm and at 430 nm, and the *chlorophyll b* that has a light absorption peak at 645nm (Prescott et al. 1999).

When the PSII antenna absorbs light, electrons in the reaction-center chlorophyll (P680) are excited to a higher energy level and travel through the cytochrome b6f to PSI via an electron transport chain where ATP can be formed. To replenish the deficit of electrons in the reaction center, electrons are extracted from oxidation of water to O_2. After reaching the PSI, the photoelectrons fill the electron deficit of the reaction-center chlorophyll of photosystem I (P700). Due to photo-excitation, electrons are again trapped in an electron acceptor molecule that reduces $NADP^+$ to NADPH. This is called a non-cyclic photophosphorylation.

2.4.2. ANOXYGENIC PHOTOSYNTHESIS PROCESS

The anoxygenic photosynthesis is performed by a cyclic photophosphorylation process. It is conducted in only one photosystem – Photosystem I (PSI) -, that has a similar constitution to the PSI from the oxygenic photosynthesis. Similarly, to the oxygenic photosynthesis process, there are also at least two types of chlorophylls involved in this process, the *bacteriochlorophyll a* that has a light absorption peak at 850-910 nm, and the *bacteriochlorophyll b* that has a light absorption peak at 1020-1035 nm. When the photon reaches the reaction center (P870) the electrons are excited to a higher energy level and transferred in an electron transport chain, ATP being formed near the region of the Cytochrome b6f (Cyt b6f). In the end of the cycle the electron returns to the oxidized reaction-center (P870) thus being described as a cyclic process (Prescott et al. 1999).

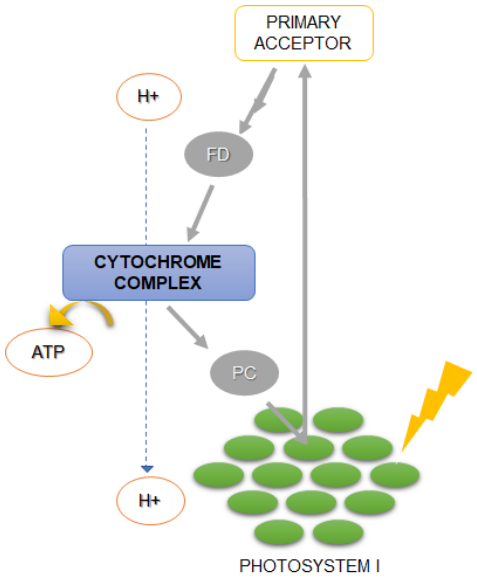

Figure 2. 6: The Z scheme. A model to illustrate the flow of electrons through the photosystem one (PSI) of the light reactions, in a cyclic photophosphorylation. Adapted from Prescott et al. 1999.

2.4.3. STUDIES PERFORMED IN PHOTOSYNTHETIC MICROORGANISMS

Despite the several studies performed in heterotrophic bacteria for wastewater treatment and PHA production, the photosynthetic organisms are being explored by various research groups for the same applications.

Recently, microalgae have gained considerable attention as a sustainable means to produce biofuels and bioproducts. Because they play a vital role in recycling carbon by converting CO_2 into organic compound through photosynthesis, they can be used to produced biofuel and biodiesel, while producing oxygen (Chia et al., 2017; Chisti, 2007). The capability of microalgae to do the uptake of nitrogen and phosphorus allows their growth and at the same time to remove these inorganic nutrients from the wastewater. There are also some microalgae that shown tolerance and bioremediation capabilities to certain heavy metals being very useful in bioremediation applications (Rahman et al., 2014; Pinto et al., 2003).

Other photosynthetic microorganism with great interest to study is the cyanobacteria. They are known to be capable of synthesizing poly(3-hydroxybutyrate) (PHB) under photoautotrophic (exploit CO_2 as a raw material for cellular growth and light for energy) and chemoheterotrophic (use organic carbon substrates) conditions. Several studies of PHA accumulation with chemoheterotrophic cyanobacterium have been made such as Samantaray and Mallick (2011), which have recently reported an 85% PHB content obtained by *Aulosira fertilissima* under 0.26% citrate, 0.28% acetate, and 5.58mg/L K_2HPO_4 supplementation. Also Sharma and Mallick (2005) obtained 35% PHB accumulation using acetate supplementation and Bhati and Mallick (2015) attained 71% polymer content, in a N_2-fixing cyanobacterium. On the other hand, the cyanobacteria *Synechococcus sp. MA19*, achieved a maximum of 55% PHB content under photoautotrophy and phosphate limited condition at 50°C, in a process that might contribute to the reduction of both organic carbon source and O_2 supply which are typically high demanding in PHA production processes (Nishioka et al., 2001).

The green bacteria are photoautotrophic microorganisms that use CO_2 as the principal carbon source, but in addition they also utilize organic compounds for growth, being able to form two types of carbon reserves – glycogen and poly(3-hydroxybutyrate) – (Blankenship et al., 1995). In 1974, Pierson and Castenholz, reported that *Chloroflexus aurantiacus*, a green non-sulfur bacteria, had the capability to store poly(3-hydroxybutyrate) during photoheterotrophic growth. Some years later, Sirevåg and Castenholz (1979), proved that the same bacteria not only accumulate significant amounts of PHB when in presence of acetate and an inorganic hydrogen donor (H_2), but also has the capability to form polyglucose in the absence of the hydrogen donor.

Various purple bacteria have shown great variation in their capability to photoassimilate organic carbon compounds and to grow photoheterotrophically.

Purple sulphur bacteria are photoautotrophs where some species have a limited capability of photoheterotrophy, and are poorly equipped for metabolism and growth in the dark (Hunter et al., 2009). As reviewed by Liebergesell et al.; 1991, studies have shown that some purple sulphur bacteria such as *Chromatium, Lamprocystis roseopersicina, Amoebobacter, Thioeystis, Ectothiorhodospira,* when in presence of acetate and under illumination conditions, are capable of accumulating PHB. The *Chromatium vinosum, Thioeystis violacea* and the *Ectothiorhodospira mobilis* were the bacteria that reached the best PHB accumulation values, of 58%, 83% and 57,5% respectively, under a concentration of 20mM of acetate.

Purple Non-Sulphur (PNS) bacteria are versatile microorganisms that can grow as photoheterotrophs, photoautotrophs or chemoheterotrophs, depending on available conditions, and at the same time possess diverse capacities for dark metabolism and growth (Hunter et al., 2008).

The *Rhodobacter* and the *Rhodopseudomonas* are the two most well studied species of PNS bacteria, being able to synthesize and accumulate PHA, in the presence of light and organic carbon. Khatipov et al. (1988) studied poly-3-hydroxybutyrate accumulation by *Rhodobacter sphaeroides*, achieving 40% PHB using ammonium medium supplemented with acetate.

Recently a new process consisting in a mixture of photosynthetic microorganisms, commonly called PMC, was explored concerning its capability to accumulate PHA. A consortium of microalgae and bacteria that was selected under illuminated conditions in a FF regime, reached PHA accumulation values of 20% under continuous illumination (Fradinho et al. 2013a). In alternating light/dark conditions, the selected PMC achieved a PHA content of 30% PHA/VSS (Fradinho et al., 2013b). Fradinho et al. (2014), also show that the PMC selected under acetate feeding could also use butyrate and propionate to produce PHA, with the propionate enabling the production of HV monomers.

In these studies, the culture selection occurred using the FF strategy, but Fradinho et al., (2016) proposed a new selection strategy for photosynthetic mixed cultures, consisting in keeping the microbial culture under the continuous presence of a carbon source, called Permanent Feast regime**.** In this regime, organisms are selected for their capability of internally regulating the cell's reducing power through PHA formation.

The principle of this enrichment process resides in the anoxygenic photosynthetic bacteria capabilities. Previously, it was mentioned that under illuminated environments, these bacteria do not release oxygen during the photosynthesis process, but they have the capability to produce ATP, necessary to a normal cell metabolism (e.g. substrate uptake, growth). In order to maintain cell homeostasis, it is necessary to oxidize reduced molecules (e.g. NADH, NADPH) produced during the cell metabolism, and in the absence of an electron acceptor (e.g. O_2), cells have to activate internal mechanisms that enable that oxidation. The PHA accumulation process, is a mechanism that requires reduced precursors for polymer formation. Under anaerobic conditions, it is expected that only the bacteria that can dissipate reducing power through PHA formation will have the capability to grow.

The major advantage of the feast regime in relation to the FF regime would be the possibility of simultaneous growth and PHA accumulation continuously throughout the cycle. Fradinho et al., (2016) used this enrichment process for PHA accumulating bacteria, achieving a 60% PHA content, the maximum value ever reported for a Photosynthetic Mixed Culture (PMC).

In this work, both FF regime and the permanent feast regime will be approached, in order to study the effects of both enrichment processes in the selection of a PHA accumulating PMC. Adding to the possibility of using direct sunlight these systems is the use of organic waste as the substrate source, two important factors that enables the decrease of PHA production costs.

CHAPTER 3.

EXPERIMENTAL METHODS

3.1. ASSEMBLY OF REACTORS

Initially, in order to operate representative systems of the HRAP – *Jet Mixed* and *Paddle Wheel* - it was necessary to build systems with specific characteristics. The process started with the replication of the operating conditions of two HRAP from the *Chiclana Wastewater Treatment Plant*. In summer, Chiclana WWTP has on average an illumination of 285 W/m^2 in 24h. In this work, it was assumed that the illumination would simulate the day light and occur for 12h implying an average illumination of 570 W/m^2 during these 12h. Table 3.1 summarizes the operational conditions of the Jet Mixed and Paddle Wheel Ponds that were simulated in this work at laboratory scale.

Table 3. 1: Operational working conditions of the Jet Mixed and Paddle Wheel Ponds of Chiclana WWTP.

	Jet Mixed Pond	Paddle Wheel Pond
AREA	32 m^2	32 m^2
WORKING HEIGHT	50 cm	30 cm
LIGHT INTENSITY	1,14 W/L	1,9 W/L
AGITATION	Jet pump	Paddle wheel
k_La	0,0032 min^{-1}	0,0068min^{-1}

Two reactors (SBR1 that simulate the Jet Mixed Pond and SBR2 that simulate the Paddle Wheel Pond) with the same dimensions were built, with an internal diameter of 20cm and with a total height of 26cm (Figure 3.1). The reactors maximum working liquid height was 20cm which corresponded to a total volume of 5,8L. To set the light intensity in the reactors, several measurements of light were made at a height of 17cm in order to obtain the light intensity most similar to the HRAP that each reactor simulates. The lamp of the *SBR1* was set at 8,5cm of the top of the reactor and the *SBR2* was set at 1cm of the top of the reactor. At last, oxygen-transfer tests were conducted in order to obtain k_La values in the reactors similar to the Ponds ones. These tests followed Ahmad and Boyd (1988) protocol. It consists in filling the reactor with water and by the addition of sodium sulphite (10 mg liter^{-1} for 1mg liter^{-1} of dissolved oxygen) and cobalt chloride (0,0075mg Co liter^{-1}) the water became deoxygenated. Stirring was set at different velocities until the water mixing provided the desired k_La in the SBR1. For the SBR2, it was used the same mixing/stirring set in the Jet Mixed reactor but higher k_La was obtained by adding an air disperser at half height of the reactor with an air flow of 7,3mL/min.

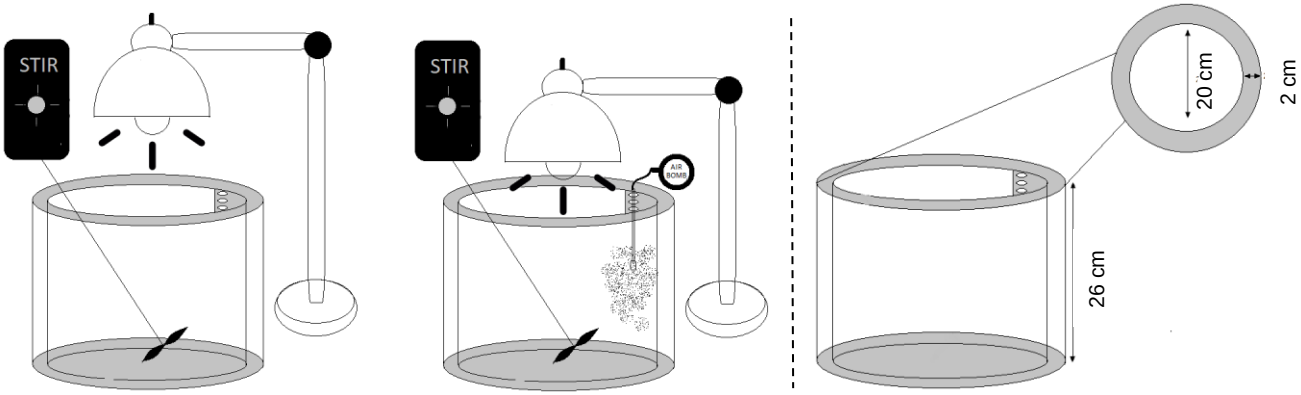

Figure 3. 1: Scheme of the reactors. Left – Reactor SBR1 simulating Jet Mixed HRAP; Right – Reactor SBR2 simulating Paddle Wheel HRAP.

3.2. PHOTOSYNTHETIC MIXED CULTURE ENRICHMENT

3.2.1. REACTOR SIMULATING JET MIXED HRAP

A sequencing batch reactor (SBR1) was inoculated with algae sludge from a HRAP from Chiclana Wastewater Treatment Plant at Andalucia, Spain. The reactor's working volume was 5,8L and was operated for 8 months in 24 hours cycles, with alternating periods of light (12h) and dark (12h) with external illumination with a halogen lamp (60 W) at a light intensity of 193 W/m^2, which corresponds to a volumetric light intensity of 1,14 W/L. SBR1 was operated with a hydraulic retention time (HRT) and sludge retention time (STR) of 6 days and the temperature was controlled at 25°C. During each cycle, SBR1 was continually fed during the 12 hours light (permanent feast regime) with real fermented wastewater that contained, amongst other compounds, organic acids (OAs). This fermented wastewater was diluted with varying proportions of mineral medium (containing per liter 0,68 g NH_4Cl and 0,78 g KH_2PO_4) during the operation period to adjust the OLR of the reactor, since the organic compounds concentration of the fermented feed also varied.

3.2.2. REACTOR SIMULATING PADDLE WHEEL POND

A second sequencing batch reactor (SBR2) was operated in conditions similar to the ones described above with the exception that it was illuminated with a light intensity of 315,6 W/m^2, which corresponds to a volumetric light intensity of 1,90 W/L, and it was aerated with the dispersion set at half height of the reactor (12cm) with an air flow rate of 7,3 mL/min. The SBR2 was operated under a feast and famine regime and was fed under two different methods: (1) semi continuous feed along 4hours and (2) pulse feed every hour during 4 – 5 hours.

3.2.3. FERMENTED SOLUTION

SBR1 and SBR2 were both fed with a fermented mixture of wastewater supplemented with 1% v/v of molasses obtained from laboratory-scale UASB reactors that were being operated by a project partner to produce organic acids (OAs). Since raw wastewater in Chiclana WWTP has low carbon oxygen demand (COD), it was necessary to add an additional carbon rich source to obtain enough OAs during the anaerobic step to enable PHA production in this work.

3.3. ANALYTICAL METHODS

3.3.1. ORGANIC ACIDS

The concentrations of the organic acids (e.g. acetate, lactate, propionate, butyrate, valerate…) were determined by high performance liquid chromatography (HPLC) using a IR detector, a Biorad 125-0129 30x4.6mm pre-column and a Aminex HPX-87H 300x7.8MM (Biorad) column. Sulphuric acid 0.01 N was used as the eluent at a flow rate of 0.5 mL/min and 30°C operating temperature. The organic acids concentrations were calculated through a calibration curve using 31,25 – 1000 mg/L standards.

3.3.2. TOTAL CARBOHYDRATES

The total carbohydrates were determine using the method described by Lanham et al. (2012). The biomass used in this procedure, was previously centrifuged at 10 000×g during 3 minutes, washed with 0,9% NaCl and lyophilized in a *CoolSafe* freeze-drier (ScanVac) overnight. After weighing the pellets, the biomass was mixed with 2mL of 0,6 M HCl and digested for 2h at 100°C. The samples were then cooled in an ice bath, filtered (0.45 µm membrane) and glucose was analysed by HPLC using a IR detector, a Biorad Aminex HPX-42A pre-column and a VARIAN Metacarb 87H column, with 0,01N H_2SO_4 as eluent with a flow rate of 0,5 mL/min and 30°C operating temperature.

3.3.3. POLYHYDROXYALKANOATES DETERMINATION

Polyhydroxyalkanoates determination was performed according to Lanham et al. (2013) with minor modifications. The culture samples were centrifuged at 10 000×g, the supernatant was discarded and the pellet was lyophilized in a *CoolSafe* freeze-drier (ScanVac) overnight. The lyophilized pellet (2 - 4 mg) was digested for 2h at 100°C (dry-heat thermo-block) with 1 mL of acidic methanol (5% sulfuric acid) and 1 mL of chloroform that contained heptadecane as internal standard. To separate the organic from inorganic phase 0,5 mL of water was added and then vortexed for 30 seconds. The lower phase that contains the chloroform and the PHA's monomer, was extracted to a GC vial and dried using molecular sieves to remove traces of water. At last, 2 µL of the sample were injected into a gas chromatograph (Gas Chromatograph 430-GC, Bruker) equipped with a Restek column (60 m, 0.53 mm internal diameter, 1 µm film thickness, Crossbond, Stabilwax).

Helium was the carrier gas at an elution rate of 1 mL/min and heptadecane was used as the internal standard during gas chromatography. The PHV and PHB polymers concentrations were calculated through a calibration curve using an initial standard of poly(3-hydroxybutyric-co-3-hydroxyvaleric) copolymer (Sigma-Aldrich*),* that has a 12 mol% HV, which corresponds to approximately 14 wt% HV.

3.3.4. NUTRIENTS QUANTIFICATION

Ammonia and phosphate were analysed by a colorimetric method implemented in a flow Segmented Flow Analyser (Skalar 5100, Skalar Analytical, The Netherlands).

3.3.5. TOTAL ORGANIC CARBON

The Total Organic Carbon (TOC) was analysed in a TOC-V_{CSH} Analyser (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 (TOC = TC – IC). Samples for this method were basified with NaOH (5M), during the sampling of the reactor 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.

3.3.6. TOTAL SUSPEND SOLIDS AND VOLATILE SUSPEND SOLIDS

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined according to standard methods (APHA et al., 1995). The first step is removing volatile compounds from the glass-fiber filters disk (VWR, glass fiber 1,2µm, 47mm) by placing them for a minimum of 30 minutes at 500 °C muffle furnace. The clean filters were then weighed (w_1, *mg*) and inserted on the filtration vacuum apparatus and usually 5 – 10 L of sample (V_T, *L*) was filtered. Next, the filter is place into a 103-105ºC oven to dry overnight. After drying, the filter is remove from the oven to cool in a desiccator, and when a constant temperature was reached the filter is weighed (w_2, *mg*). In this step, we obtained the TSS, that is determined by taking the initial weight (w_1, *mg*) of the filter to the second weight (w_2, *mg*) of the filter and divide by the total volume (V_T, *L*) of the sample ($\frac{w_1 - w_2}{V_T} = TSS$). At last, to obtain the VSS, the filter is placed into a 500ºC muffle furnace for a minimum of 2 hours. After cooling in a desiccator the filter is weighed (w_3, *mg*). By subtracting the third weight (w_3, *mg*) to the second one (w_2, *mg*) and dividing by the total volume (V_T, *L*) of the sample ($\frac{w_2 - w_3}{V_T} = VSS$) we get the Volatile Suspended Solids.

3.3.7. CHLOROPHYLLS AND BACTERIOCHLOROPHYLLS QUANTIFICATION

The biomass used in this method was taken directly from the reactors (8mL) and centrifuged at 8000_xg during 5 minutes. Then de supernatant was discarded and the biomass was dissolved in 8 mL of ethanol (99,9%), and incubated at room temperature, in dark conditions, overnight. After this, the biomass was centrifuged again at 8000_xg during 5 minutes, and the supernatant was used to measure the absorbance spectre of the sample in a GE Healthcare Ultrospec™ 2100 pro UV/Visible Spectrophotometer, with a cuvette quartz (Hellma Analytics, QS-10mm). The base line was made with ethanol 99,9% and the wavelength window was set in an interval of 190-900 nm.

3.3.8. SUGAR AND SULPHATES QUANTIFICATION

The quantification of sugars and sulphates was performed at *Requimte* - Laboratory of Analysis. The concentrations of sugars (Glucose and Frutose) was determined by high performance liquid chromatography (HPLC - Dionex ICS-3000) using an amperometric detector (PAD – Pulse amperometric detection), a CarboPAC-PA10 250x4mm column and an Amino-trap pre-column. Sodium hydroxide 0,018 M was used as the eluent at a flow rate of 1mL/min and 30°C operating temperature. The quantification of sulphates was also performed by HPLC (Dionex ICS-3000) using a conductivity detector, an ionPAC AS9-HC 250x4mm column and a AG9-HC pre-column. A solution with sodium carbonate 0,008 M and sodium hydroxide 0,0015 M was used as eluent at a flow rate of 1 mL/min and 25°C operating temperature.

3.3.9. LIGHT INTENSITY MEASUREMENTS

The light intensity was measured using a Li-COR light meter LI-250 A equipped with a pyranometer sensor LI-200 SA.

3.4. PHA ACCUMULATION - BATCH TESTS UNDER DIFFERENT ILLUMINATION

To evaluate de PHA accumulation capacity of the PMCs enriched in SBR1 and SBR2, several experimental trials were carried out to evaluate the best accumulation conditions for each reactor (Table 3.2).

Table 3. 2: Operating conditions of batch accumulation tests performed with PMCs enriched in SBR1 and SBR2.

	TESTS	CONDITIONS			
		TYPE OF TEST	LIGHT INTENSITY	AMMONIA	PHOSPHOROUS
JET MIXED (PMC FROM SBR1)	A	Control test (CT) in the main reactor	1,14 W/L	Present	Present
	B	CT in accumulation reactors	1,14 W/L	Present	Present
	C	Accumulation test (AcT)	1,14 W/L	Limited	Limited
	D	AcT	1,90 W/L	Limited	Limited
	E	CT under winter conditions	0,494 W/L	Present	Present
	F	AcT under winter conditions	0,494 W/L	Limited	Limited
	G	AcT under maximum light	3,47 W/L	Limited	Limited
PADDLE WHEEL (PMC FROM SBR2)	A	CT in the main reactor	1,90 W/L	Present	Present
	B	CT in accumulation reactors	1,90 W/L	Present	Present
	C	AcT	1,90 W/L	Limited	Limited
	D	AcT (at minimum operating height of the *Paddle Wheel HRAP, 15cm)*	3,8 W/L	Limited	Limited
	E	CT under winter conditions	0,82 W/L	Present	Present
	F	AcT under winter conditions	0,82 W/L	Limited	Limited
	G	AcT under maximum light	6,17 W/L	Limited	Limited

For these tests, smaller replicas of the original reactors were used, with a total height of 22 centimeters, and with an internal diameter of 8 centimeters (Figure 3.2). The tests were performed

at a liquid height of 20 centimeters, corresponding to a total volume of 1 liter, and operated during 8 hours and externally illuminated with a halogen lamp (30 W).

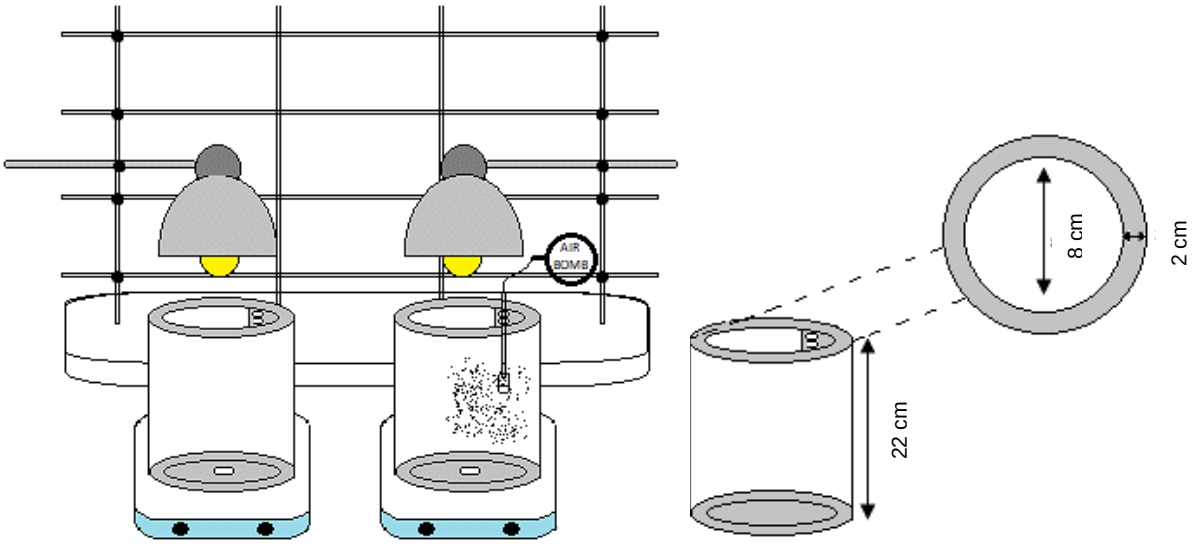

Figure 3. 2: Scheme of the accumulation reactors. Left – Reactor simulating SBR1 Jet Mixed HRAP; Right – Reactor simulating SBR2 Paddle Wheel HRAP.

3.5. MICROBIAL CHARACTERIZATION OF THE PMC

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

3.5.1. NILE BLUE STAINING METHOD

Nile Blue staining was conducted according to the method of Bengtsson et al. (2008). To begin, 1 mL of wet sample was taken directly from the reactor and 50µL of Nile Blue solution was added and incubated at 55°C for 10 minutes. After that, 30µL of the sample was transferred to a glass slide, covered with coverslip and examined under the microscope (*Olympus BX51* epifluorescence). This method allows the visualization of intracellular PHA granules that can be observed by fluorescence microscopy.

3.5.2. 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, sample is incubated for 2 – 3 hours at 4°C. The sample fixation was also performed adding 1 volume of ethanol 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 (10,000xg, 3min) and the pellet was washed twice with 1mL of phosphor buffer solution (PBS) 1x. Finally the cells were resuspended in 0,5mL of PBS and 0,5mL of ethanol and the samples were stored at -20°C, until further analysis.

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

After that the sample is dried naturally or in the oven at 46°C. Once the sample is dry, a serial dehydration in ethanol (50%, 80% and 98%) is performed where the slide stays 3 minutes in each ethanol solution. The probe hybridisation step starts with the preparation of the hybridisation 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 will be applied in each well (8 µL). After that, 1 µL of the specific probe is added and it is necessary to gently mix the probe in the buffer solution. The remaining buffer is applied to the moisturising tissue in the hybridisation tube to keep the chamber with the same atmosphere, and the slide is placed inside the tube, that will be in the oven at 46°C for 1,5 – 3 hours.

At the end of the incubation time it is 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 H_2O, 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. Afterwards, the slide is 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 is placed forcing Vectashield to cover all wells. Nail polish is applied to the board of the cover slip to prevent its movement, and the slide is 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 3. 3: Oligonucleotide probes utilized in FISH analysis.

Probe Name	Fluorochrome	Formamide	Specificity
Eubmix (EUB338 and EUB338-II and III) (Bacteria)	FitC	35%	Domain
Arc915 (Archea)	Cy3	20%	Domain
Alf969 (Alphaproteobacteria)	Cy3	35%	Class
Bet42a (Betaproteobacteria)	Cy3	35%	Class
Gam42a (Gammaproteobacteria)	Cy3	35%	Class
Delta495 (Delta-Proteobacteria)	Cy3	35%	Class
LGC354 (Firminutes)	Cy3	35%	Phylum
Grb *(Rhodobacter* and *Roseobacter)*	Cy3	35%	Genus
Rhodo-2 (Rhodoespirilaceae)	Cy3	20%	Family
RHC_439 (Rhodocyclaceae)	Cy3	35%	Family
DSBAC357 (Desulfobacteriaceae)	Cy3	35%	Family
DSB706 (Desulfobulbaceae)	Cy3	35%	Family
DSV687 *(*Desulfovibrionales*)*	Cy3	20%	*Order*

3.6. CALCULATION OF KINETIC AND STOICHIOMETRIC PARAMETERS

The PHA content corresponds to the addition of PHB and PHV in Cmmol/L. The biomass PHA content is determined in terms of percentage of VSS on mass basis [PHA (%) = 100 × (PHA/VSS), gPHA/gVSS], considering that VSS is constituted by active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS and to determine X in Cmol a general biomass chemical formula of $CH_{1.8}O_{0.5}N_{0.2}$ was considered.

The specific substrate uptake rate ($-q_S$ in Cmol OA/Cmol·d), specific PHA storage rate (q_{PHA} in Cmol PHA/Cmol X·d) and specific carbohydrate production rate (q_{Carbs} in Cmol Carbs/Cmol X·d) were determined from the linear regression of the experimental data over time, and dividing the slope for the initial concentration of active biomass. The total organic acids (OA) concentration corresponds to the sum of lactic acid, acetic acid, butyric acids, isovaleric acid, propionic acid and valeric acid concentrations (OA, in terms of Cmol/L)

The yields of PHA and total carbohydrates per substrate consumed ($Y_{PHA/S}$ in Cmol PHA/Cmol OA; $Y_{Carbs/S}$ in Cmol Carbs/Cmol OA) was calculated by dividing the q_{PHA} and the q_{Carb} for q_S.

CHAPTER 4.

SBR1_JET MIXED HRAP RESULTS AND DISCUSSION

4.1. MIXED PHOTOSYNTHETIC CULTURE ENRICHMENT AT LAB SCALE REACTOR SIMULATING THE JET MIXED HRAP (SBR1)

The enrichment of the PMC used in this study was carried out under a permanent feast regime, with alternated periods of 12 hours of light and 12 hours of dark. The reactor was fed with a fermented mixture of wastewater with 1%(v/v) sugar molasses containing a mixture of different organic acids – lactate, acetic, propionic, butyric and valeric -. Since the fermentation process was being optimized in parallel with this work the percentage of each acid fluctuated along the time, and sometimes the feeding also contained considerable amounts of sugars, namely – glucose and fructose (monomers of sucrose)-. The different compositions of the feed solutions can be seen in the Appendix AP1. Figure 4.1. shows the behaviour of the reactor in a period of 8 months subjected to fluctuating concentrations of OAs, NH_4 and PO_4, and different sugar concentrations in the feed solution.

SBR1 operation started by being inoculated with algae sludge from a HRAP from Chiclana WWTP, and was fed with fermented waste water with molasses (WWM) (Feed A.1_Appendix AP1). The initial adaptation period between 0 and 11th day started with a feeding pulse that set the initial organic acids concentration in 15 Cmmol OA/L, and as the organic acids were consumed it is visible the continually growing of the biomass – Figure 4.1_(B) – reaching a biomass concentration of 0,8 g VSS/L.

After this period, the reactor started be continuously fed, during the 12 hours' light period, with a corresponding organic loading rate (OLR) of 12 – 14,5 mmol C/L·d. The SBR1 worked at a pH between 7 and 6,5 and at an ORP between -400mV and -300mV which indicates that despite the open operating conditions and the stirring, the reactor was under anaerobic conditions. The imposed operating conditions led to the enrichment of a bacterial culture, namely of purple bacteria, as it can be seen by the ratio of Bacteriochlorophylls/Chlorophylls (Bact/Chl) that reached a value of 0,88, four times higher than the initial ratio obtained with the HRAP inoculum – Figure 4.1_(C) -. However, until the end of December, the PHA content only reach residual values of 1-3%PHA/VSS (0,06 Cmmol PHA/L).

In January, the same continuous alimentation method was maintained and the reactor reached a stable phase with a biomass concentration of 1,8 $\pm$ 0,2 g/L of VSS, a ratio of Bact/Chl of 1 and PHA content near the 19% of PHA/VSS (20 Cmmol PHA/L; 81%HB and 19%HV). However, the organic acids began to accumulate at the end of the month, reaching values near 35 Cmmol OA/L that could be inhibitory for the well function of the microbial culture.

In the period correspondent to February, day 75 to 87, Figure 4.1. shows the response of the selected PMC in the presence of a considerable amount of sugar - 53 Cmmol sugars/L, which correspond to a 20% of the total organic carbon of the Feed B.1 (Appendix AP1) -. The high concentrations of sugar in the feed solution along with the anaerobic conditions in the reactor led to a fermentation process that consequently led to a rise of the organic acids concentrations.

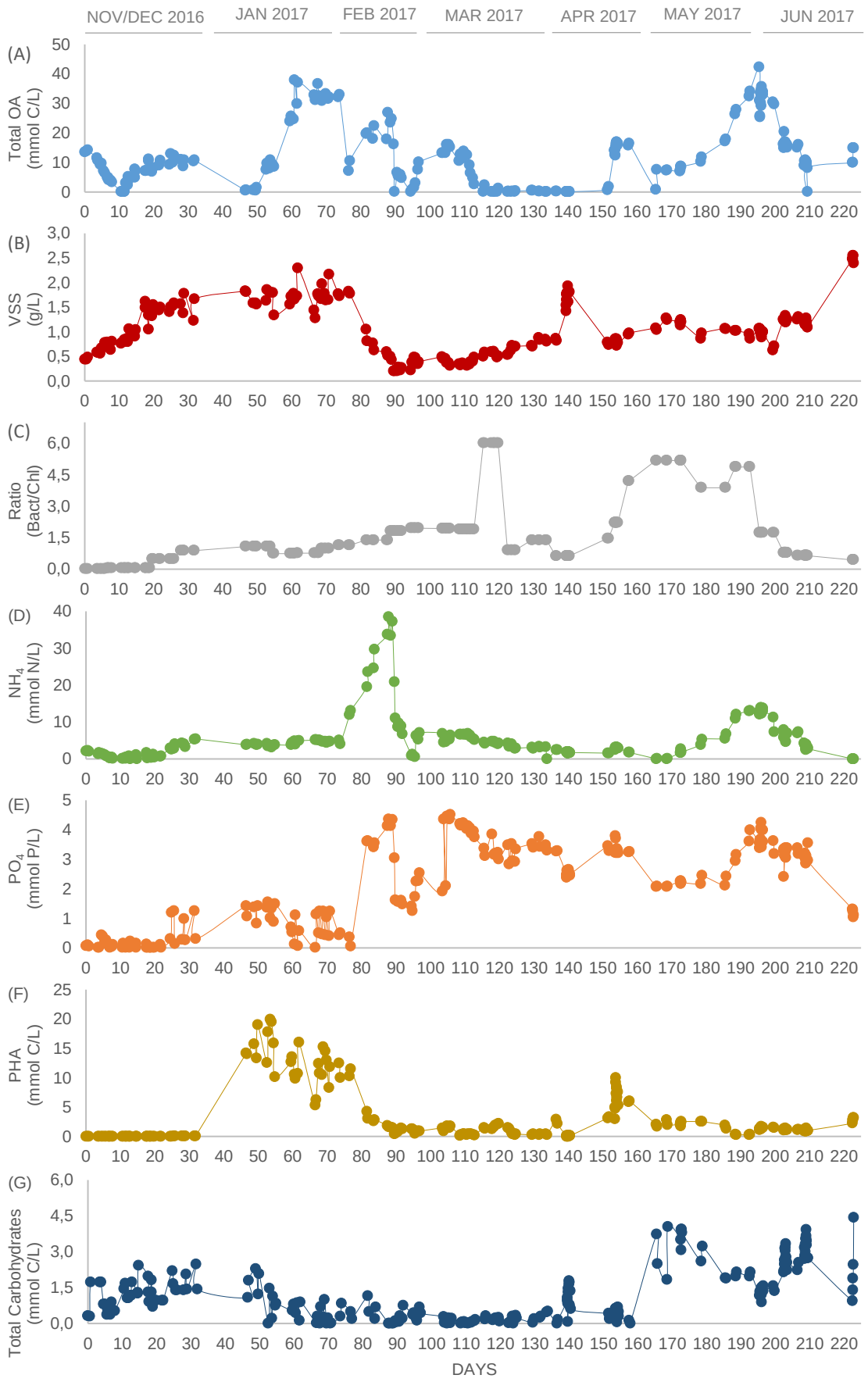

Figure 4. 1: Profile of SBR1 over 224 days of operation. **(A)** Total Organic Acids; **(B)** Total Volatile Suspended Solids; **(C)** Ratio Bacteriochlorophylls / Chlorophylls. **(D)** Ammonium (NH_4) **(E)** Phosphate (PO_4); **(F)** Polyhydroxyalkanoates (PHA) **(G)** Total Carbohydrates.

The presence of sugars along with the anaerobic conditions may favour the appearance of fermentative bacteria, which may explain why the microbial culture lost its capability to accumulate PHA (residual values). Moreover, the excess of organic acids led to a diminishment of the pH in the medium, reaching pH 4, that also led to a large decline in the biomass concentration (0,3 $\pm$ 0,2 g/L of VSS) (Figure 4.1_(B); day 75 to 87).

The reactor entered in a phase of decay, and by the analysis of the ratio of Bact/Chl, that reached a 1,9 value, the decay was more accentuated in the algae/cyanobacteria. This might be due to the fact that pH is a very important factor in algae and cyanobacteria growth, which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth (Li et al. 2014; Nayaka and Prasanna 2007), while the purple bacteria can be found in extreme environments, such as acidic conditions (Hunter et al. 2008).

Since the biomass concentration decreased dramatically, there was a need to change the feed solution for another one with lower concentrations of sugar (Feed C.1 and C.2_Appendix AP1) and to lower the OLR to approximately 4 mmol C/L·d (3,5 times lower). As the biomass concentration increased with the culture recovery, the OLR was increased along the time, avoiding an excessive accumulation of organic acids. At the beginning of April, the reactor reestablished the previous biomass concentration reaching a 1,9 g VSS/L concentration, and the Bact/Chl ratio was 0,63, indicating a growing content of microalgae. Later in this month, the Bact/Chl reached a ratio of 5,2 while the PHA content reached the 18,5% of PHA/VSS (10 Cmmol PHA/L; 65% HB and 35% HV).

At the very ending of April and beginning of May, the feed solution available contained sugars again, but with a lower sugar concentration (11 $\pm$ 3 mmol C/L which correspond to an 8% of the total organic carbon of the Feed D.1 (Appendix AP1)). This time the reactor did not suffer a biomass loss, but similarly to what happened before, the microbial culture also lost is capability to accumulate PHA, which is in line with the previous findings: that the presence of sugar under anaerobic conditions leads to fermentative metabolism and to the loss of PHA accumulation capability by the PMC. This time the pH medium ranged 5 – 5,5, but since the purple bacteria were the dominant microorganisms in the reactor, the biomass did not suffer a decrease in the concentration as before.

Relatively to the total carbohydrates, it is possible to see that since beginning of May (165th day) the carbohydrates suffer an increasing in their concentration from 0,2 Cmmol Carbs/L to 3,5 Cmmol Carbs/L. At the 175th day, the ratio of Bact/Chl started to decrease until the last day of operation and the PMC lost the capability to accumulate PHA, favouring the carbohydrates accumulation. A plausible explanation for this behaviour alteration of the PMC could be the increasing of algae content, which are known for accumulate carbohydrates.

Moreover, there is a recurring behaviour detected regarding the carbohydrates: they were mostly produced during the light phase and consumed during the dark phase. This carbohydrate profile was observed in the initial and final months of operation that coincides with the lower ratios of Bact/Chl meaning a bigger incidence of the microalgae in the reactor. This is a characteristic behaviour of the microalgae that was studied by Ernst et al. (1984). The method utilized to quantify

carbohydrates not only quantifies the glucose derived from bacterial glycogen but the total glucose present in the sample, such as starch from the microalgae. For that reason, it should be noted that the changes in carbohydrate concentrations could be due to the activity of both bacteria and algae (Lanham et al. 2012; Fradinho et al. 2013a).

Regarding to the TOC results (data not shown), the organic acid concentration detected by the HPLC in the fed solution corresponds to 70-80% of the total organic carbon. However, in the SBR1 the organic acids detected by HPLC only corresponded to 20% - during the initial phase - and 50% - at the end of the 8months - of the total organic carbon detected. The discrepancy observed between the two methods might be due to the fact that HPLC results presented in this work only correspond to the sum of the concentrations of lactic acids, acetic acid, propionic acid, valeric acid, isovaleric acid and butyric acid. While the TOC have a nonspecific nature that allows the detection of contamination from any source, regardless of its nature. In a general view, the TOC is a less specific technic when compared with the HPLC analysis.

4.1.1. OPERATING CONDITIONS THAT FAVOR PHA ACCUMULATION

There were two periods – January and April – when the SBR1 presented considerable amount of PHA, which suggests that two distinct selection processes were occurring in complement with the permanent carbon feast selection strategy.

The first selection process observed focused on the effect of ammonium and phosphate in the selection of a PMC with the capacity to accumulate PHA. During the period between 0 and 19th day, N and P concentrations registered in the reactor were very low, leading to limitation of N and P. At day 19, the reactor began to be fed with a mineral solution (NLR 1,4 mmol N/L·d / PLR 0,6 mmol P/L·d) that led to higher concentrations of ammonium and phosphate in the medium. From this point on, it was observed that the culture began to perform uptake of phosphorous during the light phase and release of phosphorus during the dark phase. The observed P cycling may be an indication of the presence of polyphosphate-accumulating organisms (PAOs). Also, these organisms have the capability to accumulate PHA (Oehmen, et al., 2006) and indeed, during this period the PHA content reached values near 19% of PHA/VSS (20 mmol C/L), the highest value registered during the 8 months' analysis. These results indicated that the operating conditions were likely leading to the selection of PHA accumulating bacteria. After the previously mentioned decay phase in February, the microbial culture lost this ability, and the N and P concentrations registered were maintained at constant levels.

To better understand the uptake and release of phosphorous mechanism and its influence in PHA production some studies that followed the behaviour of the microbial culture along the 24hours cycle were performed. Each reactor cycles were performed under constant presence of ammonia and growth of biomass (Cycle A – NLR 2,9 mmol N/L·d; Cycle B – NLR 2,9 mmol N/L·d; Cycle C – NLR 1,6 mmol N/L·d). In Figure 4.2 the behaviour of the reactor during different days and in different operating conditions is presented. The Figure 4.2_(A), shows the cycle representative of the period of November and December, when the culture had a balanced concentration of organic acids and phosphate. On the other hand, in Figure 4.2_(B) and (C)

(January) it is possible to follow the behaviour of the culture when it had lower values of OAs and phosphate, respectively.

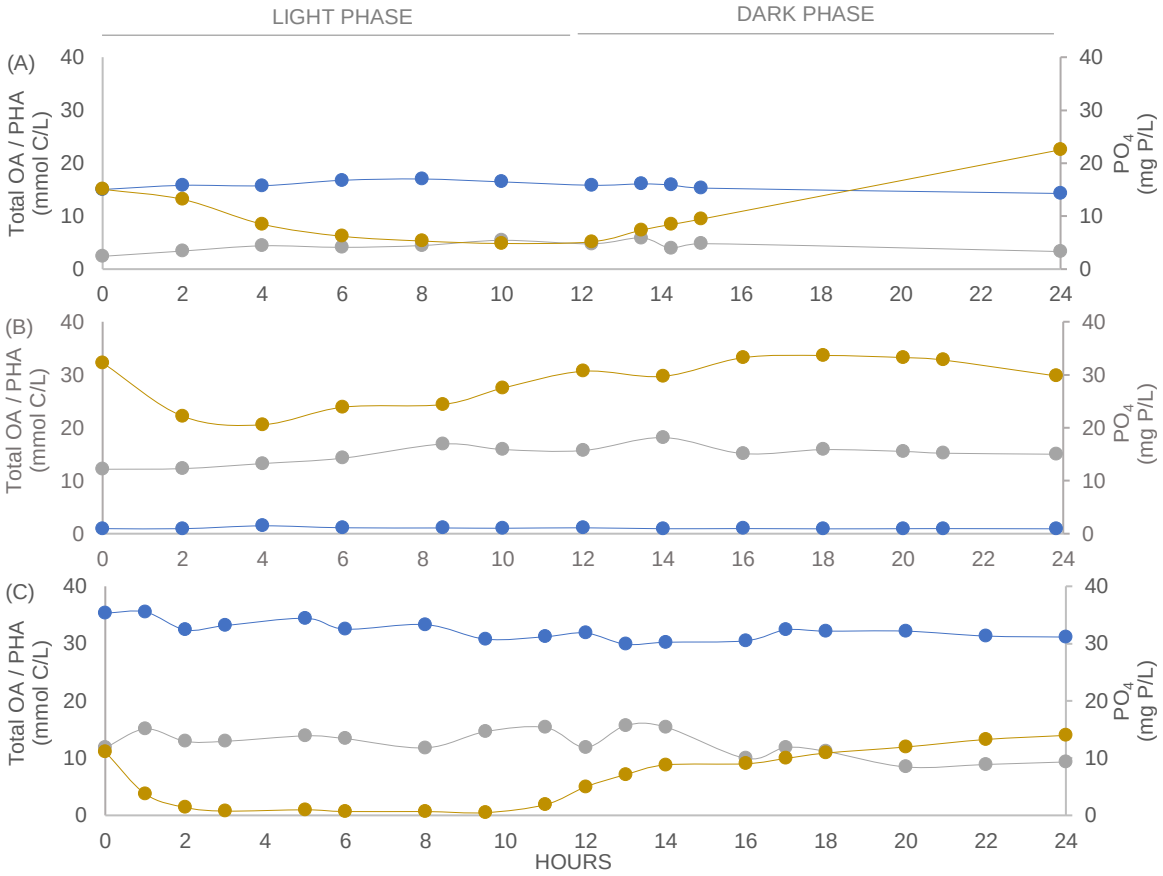

Figure 4. 2: Evolution of the culture behaviour in the periods of November/December and January under 12hours continuous feeding during the light phase operational conditions. (A) Reactor cycle on 26th day with an OLR of 12,52 mmol C/L·d and a PLR of 1,3 mmol P/L·d; (B) Reactor cycle on 47th day with an OLR of 7,84 mmol C/L·d and a PLR of 1,3 mmol P/L·d; (C) Reactor cycle on 74th day with an OLR of 12,6 mmol C/L·d and a PLR of 1,3 mmol P/L·d. (●) PO_4 (●) PHA (●) Total Organic Acids.

It can be observed that the phosphate uptake in the first hours of the cycle suffered some alterations along the days. In day 26 (Figure 4.2_(A)) the phosphate uptake rate was 13,2 mg P/ g X·d and phosphate consumption became stable at the end of 6 hours (notice that the reactor was continuously fed during the 12h of light phase). In day 47, the phosphate uptake rate was 16,7 mg P/g X·d (Figure 4.2_(B)) and 16,3 mg P/g X·d (Figure 4.2_(C)) on the 74th day, becoming stable after 2 hours. The slight rate decrease observed between the 47th and 74th days, might be due to the lack of phosphorous present in the medium, which prevented the continuation of the phosphorous uptake by the culture. The PHA production efficiency of the microbial culture along the cycles can also be inferred from the values of PHA content. The initial 6% of PHA/VSS (5 ± 0,5 mmol C/L) increased to a maximum value of 15% of PHA/VSS (15 ± 0,5 mmol C/L) (Figure 4.2_(B)), and then decreased to 12% PHA/VSS (13 ± 0,5 mmol C/L).

The phosphorous uptake rates obtain in this work, are similar to the ones obtain in Fradinho et al. (2016) – 17,4 mg P/g X·d – in a PMC under continuous light that present phosphorus uptake at the beginning of the cycle. As previously mentioned, this mechanism can be associated to PAOs, which are known as microorganisms that have the capability to store inorganic phosphate (poly-P) and PHA as internal polymers (Oehmen, et al., 2006). In the reactor cycles presented in

Figure 4.2, there was release of P but there seemed to be so few that would not allow uptake of acids and accumulation of PHA. In the reactor cycles presented in the Figure 4.2, it is not possible to conclude for sure that the release of phosphorous during the dark phase allows the organic acids uptake to produce PHA. However, it is possible to say that any microorganisms that could accumulate poly-P as a reserve of energy, during the light phase, can take advantage of it during the dark phase for cell maintenance, even if it only serves for maintenance it allows these organisms to be more active and competitive over each cycle.

Another important factor that seemed to play an important role in the enrichment of the culture in PHA accumulating organisms, was the continuous presence of carbon in the reactor, but at very low concentrations – 0,02 – 2 Cmmol OA/L -, under the continuous presence of N and P. This occurrence is well observed in the period between the months of March and April, where the reactor came from an initial phase where the organic acids were scarce, and after an increase of the organic acids concentration, the PHA content achieved 18,5% of PHA/VSS (10 Cmmol PHA/L). The low availability of organic acids seemed to favour the growth of bacteria that had the capability to accumulate PHA as an internal carbon reserve. This lower PHA accumulation value, when compared with the previously selection method, might be due to the lower concentrations of organic acids presents in the medium or just because it might be a less efficient method. In any case, the reactor could be operated solely as a selector reactor and a further increase of culture PHA content could be achieved in a separate accumulator reactor. In practice, the wastewater treatment plant could operate two ponds: one for culture selection and other for PHA accumulation with higher OLR.

In Figure 4.2_(B), it is also possible to observed the behaviour of the SBR1 culture under the two previously selection method mentioned in simultaneous. In the presence of low organic acids concentration (≈ 1 Cmmol OA/L) and of P cycling, the PMC achieved a PHA accumulation of – 15,5% of PHA/VSS; 15 $\pm$ 0,5 mmol C/L, the higher value registered in these three reactor cycles - which might indicate that the combining of the two methods could be beneficial for the selection of PHA accumulating bacteria.

4.2. PMC CARBOHYDRATES METABOLISM

It is also known that intracellular carbohydrates can have an important role in the PHA production. For this reason, it was also important to study the evolution of the carbohydrates content throughout the reactor selection process.

Figure 4.3 shows the behaviour of the total carbohydrates mechanism along the 8 months. It is possible to observe a typical photosynthetic behaviour of the carbohydrates metabolism, where during the light phase the carbohydrates are synthesized, and in the dark phase the carbohydrates are degraded (Ernst et al.,1984; Díaz-Troya et al. 2014). This behaviour could be associated with PHA production in cyanobacteria. Under dark anoxic conditions, the cyanobacterial are capable of fermenting glycogen, and one possible fermentation product could be PHA (Stal et al. 1992; Wu et al., 2001). However, this is not the case observed in the reactor. In the light phase carbohydrates are synthesised, but once in the dark phase, while carbohydrates

are consumed, the PHA content remains constant, suggesting that the carbohydrates are not involved in the PHA production.

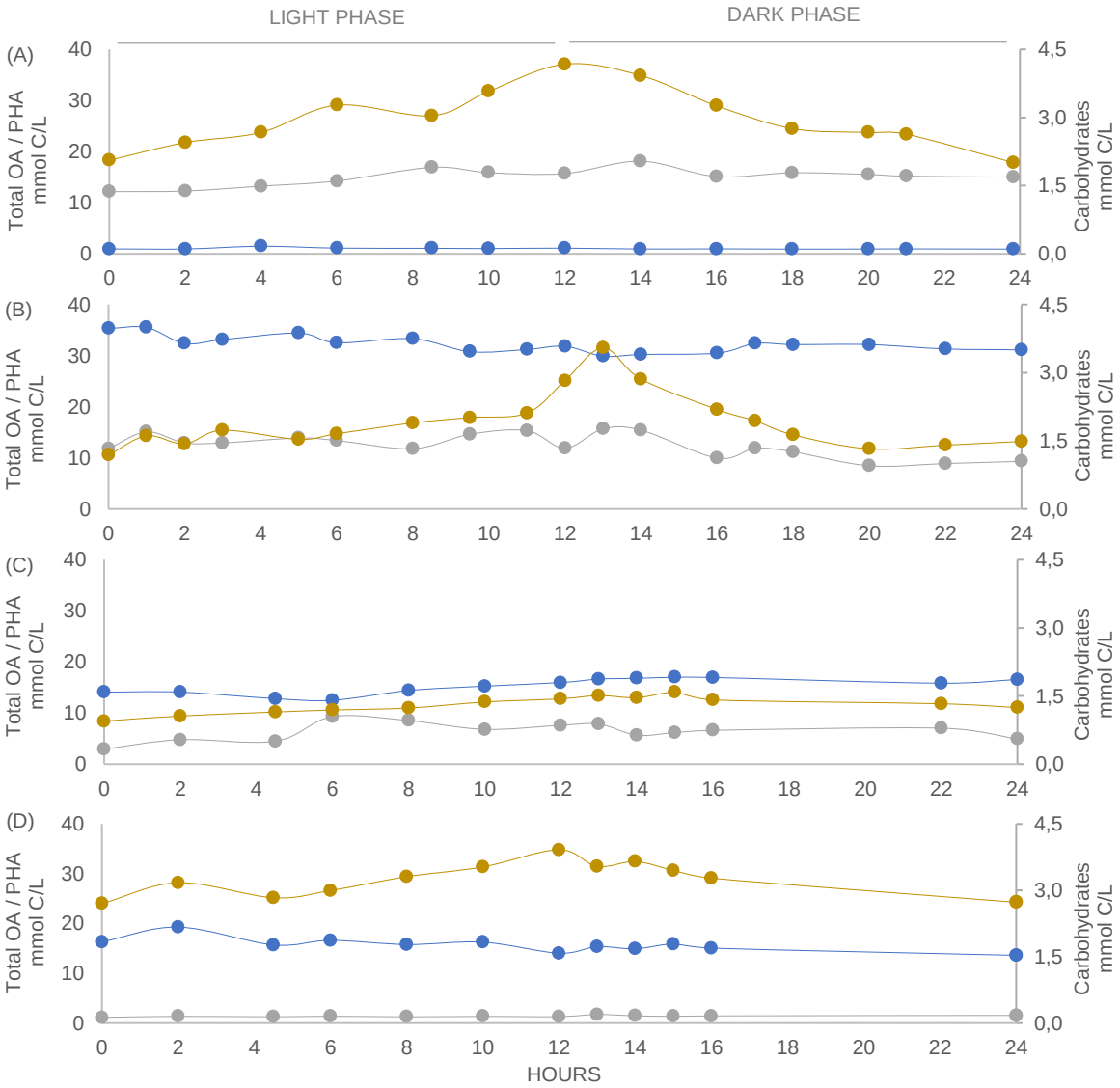

Figure 4. 3: Evolution of the culture behaviour in the periods of January, April and June under 12hours continuous feeding during the light phase operational conditions. (A) Reactor cycle on 47th day with an OLR of 7,84 mmol C/L·d; (B) Reactor cycle on 74th day with an OLR of 7,84 mmol C/L·d; (C) Reactor cycle on 153rd day with an OLR of 6,3 mmol C/L·d; (D) Reactor cycle on 208th day with an OLR of 9,3 mmol C/L·d. (●) Total Carbohydrates (●) PHA (●) Total Organic acids.

It is possible that the carbohydrates may be starch from algae (non-PHA producers), thus not leading to PHA production during the dark phase. However, if the carbohydrates are glycogen, bacteria may not be converting it to PHA because they are not in complete anaerobic conditions. Sulphates resent in the feed may be used as electron acceptors during the dark, thus preventing PHA production. Indeed, the negative oxidation reduction potential (ORP) present in the SBR1, between -300 and -400 mV could facilitate the reduction of the sulphates, since redox potentials below -200/-217 mV enable the reduction reaction of the SO_4^{2-} to S^{2-} (Barton and Hamilton, 2007).

The highest carbohydrate contents achieved were at 47th and 208th day, 7.0% and 9.5% of Carbs/VSS, respectively. While at 74th and 153rd day, were registered the lowest carbohydrate

contents, 5.6% and 5.3% of Carbs/VSS, respectively. Comparing these results with the profile of the Bact/Chl ratio, it is possible to conclude that the highest carbohydrates contents corresponded to the lower Bact/Chl ratio, and contrariwise. This was expected once the quantification of the total carbohydrates not only quantifies the glucose derived from bacterial glycogen but the total glucose present in the sample, such as starch from the microalgae. The carbohydrate content obtained by the PMC are slightly higher than the carbohydrate content obtained in Fradinho et al. (2016) (3-5% of Carbs/VSS), by a photosynthetic mixed culture under a permanent feast regime and continuous illumination. This discrepancy could also be related to different algae contents present in the PMCs of this work and in Fradinho et al. (2016).

4.3. PMC PHA ACCUMULATION CAPACITY

To evaluate the PHA accumulation capacity of the PMC a series of batch tests were carried out under different light intensities and nutrients limitations. These tests were performed in June, at a low Bact/Chl ratio and during a stable phase of the reactor and continuous presence of organic acids, N and P, and with a constant biomass concentration of 1,02 $\pm$ 0,1 g X/L.

Figure 4.4_(A) represent the control test made in the main reactor, commonly known as the selection reactor, serving as a reference point to evaluate the PMC behaviour in the accumulation reactors, where the batch tests were performed.

In the control tests – Test B and E -, the OLR was maintained according to the main reactor as well as the NLR and PLR, and in the accumulation tests – Test C, D and F -, the NLR and PLR were zero, in order to create growth limitation and promote the polymer accumulation. Also, in these same accumulation tests, the higher concentration of organic acids was sustained by multiple pulses of feed solution in order to maintain the presence of organic acids that are easily converted into PHA (like acetate) (See Methods section, Table 3.2).

In Figure 4.4_(A) and (B), and in the Table 4.1 that presents the kinetic and stoichiometric parameters of the PMC in each trial, it is possible to see the differences between the two control tests (A) and (B). The control test (B) that was made in the accumulation reactor has a higher consumption rate of organic acids 0,45 Cmmol OA/Cmmol X.d, as well as a higher PHA production rate of 0,08 Cmmol PHA/Cmmol X.d, when compared with the test (A) performed in the selection reactor that has a consumption rate of organic acids of 0,18 Cmmol OA/Cmmol X.d, and a PHA production rate of 0,02 Cmmol PHA/Cmmol X.d. These results suggest that the batch tests, performed in the accumulation reactor, were subjected to operating conditions different than the ones in the selection reactor, despite our efforts to simulate the selection reactor operation at a lower scale. Agitation might be one of the factors affecting the downsized operation. Because agitation is connected to oxygen dispersion, the higher oxygenation of the system could affect the organic acid consumption and consequently the PHA accumulation. Although, the tests performed in the accumulator reactors have an error associated with the change of the operating system, it is possible to compare the batch tests in between them and in relation to the batch control test – Test B.

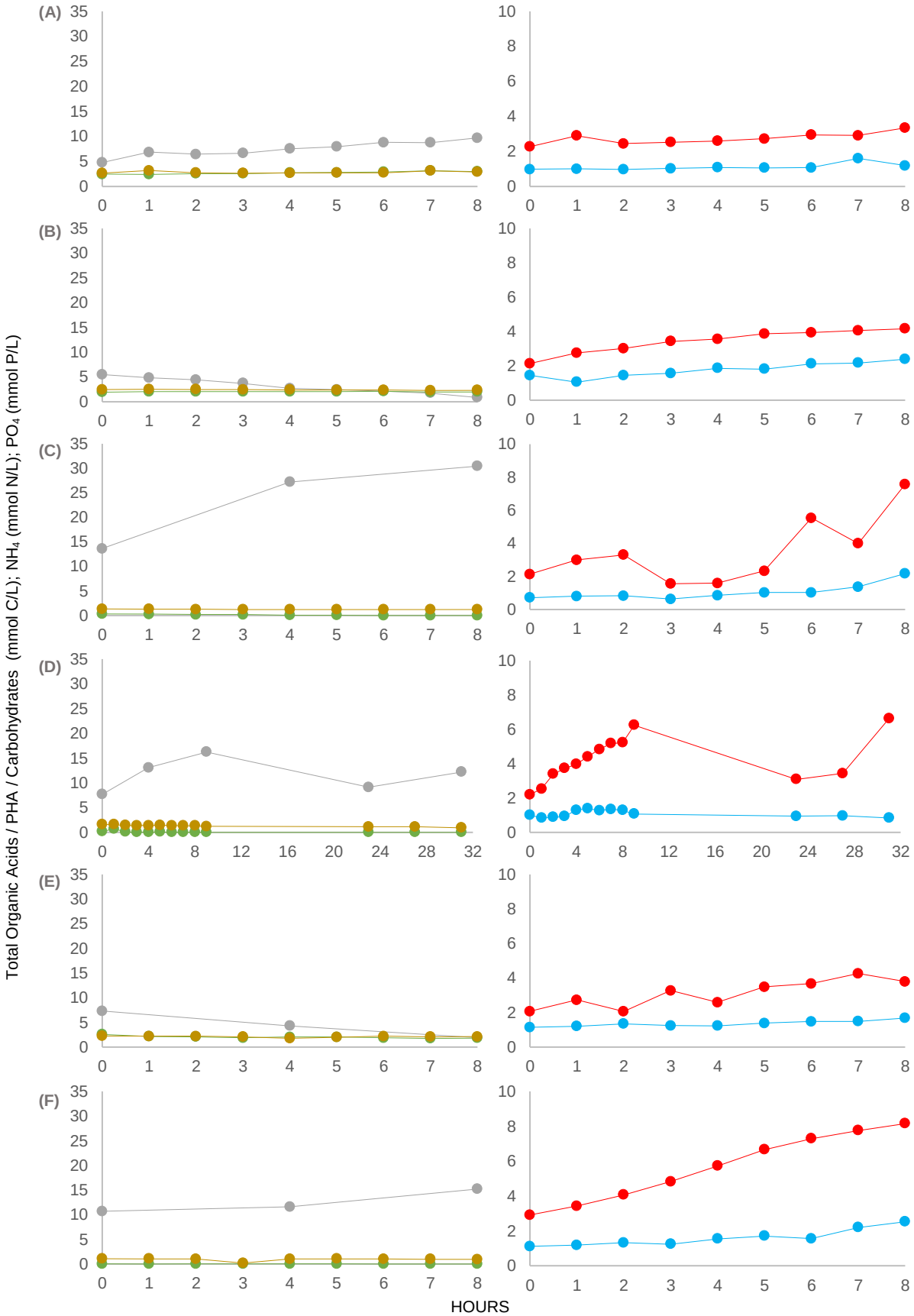

Figure 4. 4: Batch tests performed to evaluate the SBR1 PMC capability to accumulate PHA under different conditions. **(A)** CT of SBR1; **(B)** CT performed in the accumulation reactor under the same conditions of SBR1; **(C)** AcT performed at a light intensity of 1,14W/L, under N and P limitation; **(D)** AcT performed at a light intensity of 1,9W/L, under N and P limitation; **(E)** CT performed at a light intensity of 0,5W/L (winter illuminations); **(F)** AcT performed at a light intensity of 0,5W/L, under N and P limitation (winter illumination). (●) Total Organic Acids (●) PO_4 (●) NH_4 (●) Total Carbohydrates (●) PHA.

An interesting detail was observed, in tests carried out under the presence of ammonia and phosphate, the control tests (B) and (E), the biomass concentration was maintained practically constant, presenting the lowest production rates of PHA and Carbohydrates (Table 4.1). However, in the accumulation tests - (C), (D) and (F) - despite de continuous presence of phosphorous, it was possible to observe the impact of the absence of ammonia on the behaviour of the PMC. In the Test C, the ammonia concentrations start at 0,32 mmol N/L, and after the total consumption (4th hour, Figure 4.4_(C)), the internal polymers production starts to increase. The same phenomenon could be observed in Test D and F, that were permanently performed under ammonia limitation, and that also presented a constant production of the internal polymers. It is then possible to conclude that there was no cell growth registered and that the privation of the external nutrients lead to higher production of the internal polymers as it could be confirmed in the Table 4.1.

Table 4. 1: Kinetic and stoichiometric parameters of batch tests performed with the PMC in different experimental conditions. **Test (A)** CT of SBR1; **Test (B)** CT performed in the accumulation reactor under the same conditions of SBR1; **Test (C)** AcT performed at a light intensity of 1,14W/L, under N and P limitation; **Test (D)** AcT performed at a light intensity of 1,9W/L, under N and P limitation; **Test (E)** CT performed at a light intensity of 0,5W/L (winter illuminations); **Test (F)** AcT performed at a light intensity of 0,5W/L, under N and P limitation (winter illumination).

	q_{PHA}	q_{Carb}	$-q_S$	$Y_{PHA,S}$	$Y_{Carb,S}$
Test (A)	0,02	0,04	0,18	0,12	0,22
Test (B)	0,08	0,13	0,45	0,18	0,29
Test (C)	0,13	0,24	0,45	0,30	0,53
Test (D)	0,10	0,21	0,65	0,15	0,32
Test (E)	0,00	0,07	0,68	0,00	0,10
Test (F)	0,07	0,30	0,49	0,13	0,61

q_{PHA} Cmmol PHA/Cmmol X·d, **q_{Carb}** Cmmol Carb/Cmmol X·d, **$-q_S$** Cmmol OA/Cmmol X·d,**$Y_{PHA,S}$** Cmmol PHA/Cmmol OA, **$Y_{Carb,S}$** Cmmol Carb/Cmmol OA.

Concerning the control test (E) which represents the PMC behaviour under winter light intensities, results indicate a lower capacity of the culture to produce internal polymers, when compared to test (B) (summer illumination). Indeed, practically no PHA was produced under low light availability and only 10% of the consumed substrate was used for carbohydrates production ($Y_{Carbs/S}$ of 0,10 Cmmol Carb/Cmmol OA). However, contrary to what was expected, under lower energy availability, the substrate consumption rate is higher in the test (E) than the test (B), and there is no logical explanation for this behaviour. This result can be associated with analysis errors, but since replicated tests have not been performed, it can not be corrected.

In the accumulation test some values registered appear to be contradictory, since it was expected that under higher light intensities that provide higher energy availability, the yield of the intern polymers per substrate would register higher values.

In Table 4.1, it can be seen that the PHA rate production in the test (C) is higher than the q_{PHA} in the test (D). Still, during the test (C) higher yields of PHA and carbohydrates per substrate were obtained, 0,30 Cmmol PHA/Cmmol OA and 0,53 Cmmol Carb/Cmmol OA, when compared with test (D), that was performed under higher light intensities. A possible explanation could be the

different OLR use in each trial, 2,24 Cmmol/L·h in the test (C) and 1,99 Cmmol/L·h in test (D), where the higher OLR lead to increased availability of specific acids that play an important role in the production of the internal polymers. Fradinho et al. (2014), showed that the acetic acid, the butyric acid and the propionic acid enable the PHA production, and that in the presence of acetic acid the consumption rate of the butyric and propionic acids was increased. In the feed solution used in the present tests, there were six organic acids, in different proportions, however only the acetic, propionic, butyric and valeric acids had significant percentages that could affect the production of the PHA. During the tests (C) and (D), the acetic acid and the propionic acid were totality consumed in-between pulses (data not show), with the butyric and valeric acids accumulating in the reactor and being responsible for OA accumulation observer in the Figure 4.4_(C) and (D). These results indicate that the higher OLR, that leads to higher content of acetic and propionic acids, present in test (C), likely favoured the higher PHA production observed.

The test (D) also evaluate the capacity of the PMC to accumulate PHA over two days under accumulation conditions. However, at the end of the second day (31th hour) the PHA content did not increase in relation to the PHA observer at the end of the first day (10th hour), indicating that the PMC reached the maximum PHA content after one day of accumulation, and that a second day of accumulation did not enabled the achievement of higher PHA content.

During the batch test, the valeric and butyric acid were the organic acids less consumed by the culture (data not show), being the acetic and propionic acids the preferred carbon source and likely responsible for the PHA formation. The even carbon number lead to P3HB formation, while odd numbered OA allow the production of 3HV monomers, and because of that the PHA polymer produced in the batch tests by the PMC is the co-polymer P(3HB-co-3HV) maintaining a constant ratio of nearly 60% of the HB monomer and 40% of HV monomer.

Despite all of these conclusions, the accumulation tests were performed after a decreased in the ratio of Bact/Chl, in June, indicating that the PMC was not well enriched in PHA accumulating bacteria. Indeed, the maximum PHA content obtained was near 3% of PHA/VSS, which indicates that the culture was not accumulating PHA. In the presence of a non-PHA accumulating PMC, despite the optimization/conditions tested, the PHA production did not suffered an increase. Comparing the yields from the PHA with Carbohydrates, it is notable that the carbohydrate had always a higher yield when compared with the PHA, and that organic acids uptake is mostly used for carbohydrate storage.

In order to better understand the behaviour of the PMC in the presence of sugars in the medium, some accumulation tests were performed in May, at 160th and 166th day, to see the PHA accumulation capacity of the PMC enriched and fed with sugar. These tests were performed under a higher Bact/Chl (5,18) ratio when compared with the Bact/Chl ratio during the tests performed in June (0,64).

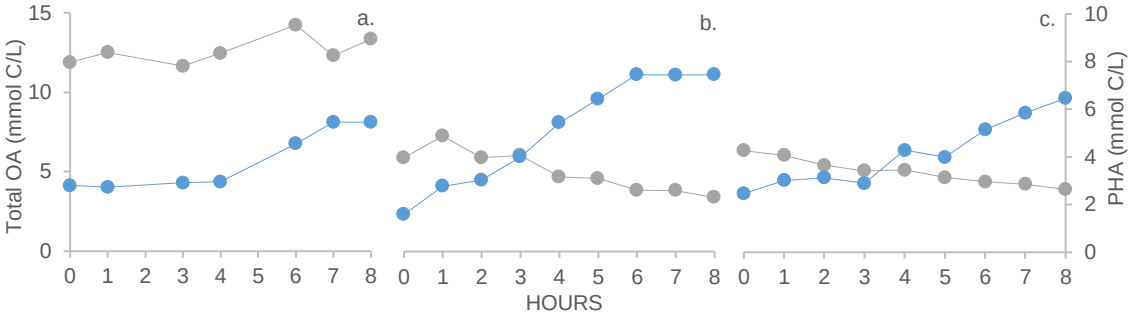

Figure 4. 5 : Batch test performed in a PMC enriched and fed with sugars (Feed D.1) operated under higher light. **a.** Control test in SBR1; **b.** Test performed under 6,17 W/L light intensity and ammonium limitation; **c.** Test performed under 3,47 W/L light intensity and ammonium limitation. (●) Total Organic Acids (●) PHA/VSS

In the Figure 4.5 it is possible to observe the PHA accumulation efficiency of the PMC selected and fed in the presence of sugars (Appendix AP1_Feed D.1_14,6 Cmmol sugar/L), which as it was already mentioned in this chapter in topic 4.1_Mixed photosynthetic culture enrichment at lab scale reactor simulating the jet mixed HRAP (SBR1), leads to a decrease in the PHA accumulation. The tests presented in Figure 4.5_a. was performed under the same light intensity of SBR1 and under no limitation of N and P. The specific PHA accumulation rate in the SBR1 is 0,22 Cmmol PHA/Cmmol X·d, and the microbial culture can only achieve a PHA content of 5% PHA/VSS (5,5 Cmmol PHA/L). However, to study the polymer accumulation efficiency of the PMC selected in the presence of sugars, two batch tests were performed under higher light intensity in order to determine the maximum PHA production capacity. The test performed at a light intensity of 6,17 W/L (Figure 4.5_b.) achieved higher specific substrate consumption rate (3,52 Cmmol OA/Cmmol X·d) and specific PHA accumulation rate (0,54 Cmmol PHA/Cmmol X·d) obtaining a content of 9,5% PHA/VSS (7,6 Cmmol PHA/L). While the test performed at 3,47 W/L (Figure 4.5_c.) obtain a PHA content of 8,5% PHA/VSS (6,5 Cmmol PHA/L) with a specific substrate consumption rate and PHA accumulation rate of, 2,69 Cmmol OA/Cmmol X·d and 0,26 Cmmol PHA/Cmmol X·d, respectively. It was concluded that the higher light intensities increased not only the PHA accumulation, but also the specific rates.

The highest PHA content registered along the 8 month in the main reactor was 19% PHA/VSS, that was obtained during the period under phosphorous cycling enrichment method. The fact that the batch tests did not achieved such high values is likely due to the operative enrichment process at the time which led to a PMC with less PHA production efficiency.

The discrepancy between the 19% of PHA/VSS registered and the maximum value ever reported for PMC operated in a feast regime, 60% of PHA/VSS, obtained by Fradinho et al. (2016), is due to the completely different operational conditions utilized. Fradinho et al. (2016) operated a PMC with 24h illumination (1,8W/L) using synthetic medium with acetate as the sole carbon source. In the present work, the PMC was selected under light/dark cycles (only 12h illumination at 1,14W/L) using real fermented wastewater/molasses composed of a very complex mixture of compounds, where some could even be inhibitory for the PHA production (e.g. sugars, sulphates). Also, the permanent change of the feed composition due to upstream optimizations, affected the culture stability and the possibility of achieving higher PHA content. Still, the changing

of ratio of each organic acid in the feed solution could lead to the consumption of the preferential acids and to the accumulation of some non-preferential organic acids that could reach inhibitory values. Eventually, higher PHA content could be obtain with the applications/optimization of the best strategies for PHA accumulation as long with a stable feed solution that depends on the optimization of upstream processes by our partners.

4.4. MICROBIAL CHARACTERIZATION

The initial sludge that was used in this work was originally composed almost by microalgae, in the Figure 4.6_A1, it is possible to observer some microorganisms (signed with orange arrows) that had autofluorescence which are a characteristic of the algae. In the adaptation period, day 0 to 11, some bacteria started to appear, as it could be seen in the Figure 4.6_B1, by the lack of the autofluorescence in some microorganisms. It was possible to notice the presence of different algae including *Chlorellas* which had a round shape and green colour, and some *Diatom*, in specific it was observed a *Phaeodactylum tricornutum* diatom, with a fusiform shape and brown colour. In the bacterial population, it was possible to observe the presence of some granules, that correspond to the PHA granules (Figure 4.6 – B3).

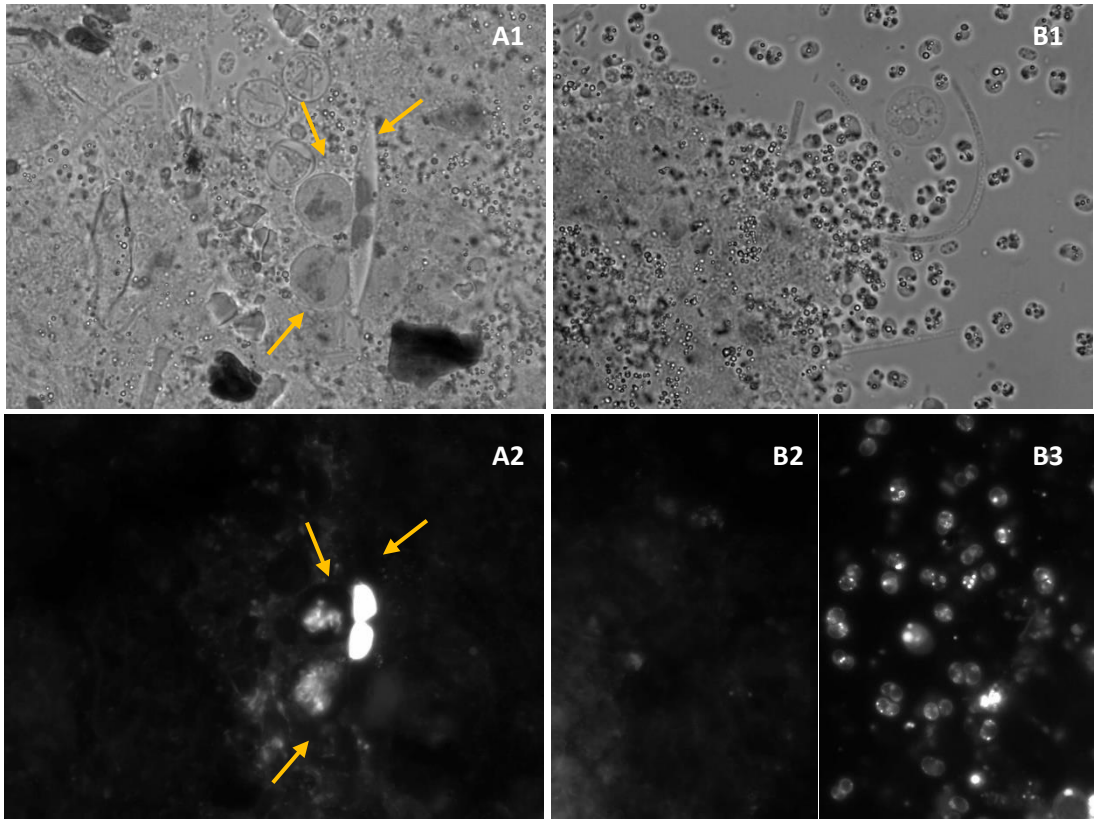

Figure 4. 6: Microscopic images of the PMC at 4th day. **A1** and **B1** – Bright field; **A2** and **B2** – Fluorescence images; **B3** – Fluorescence images of Nile blue staining indicating PHA granules; → Algae.

Table 4.2 show the FISH analysis results. In a general view the Alphaproteobacteria and the Gammaproteobacteria are the dominant classes along the enrichment process, despite the significant appearance of Deltaproteobacteria after the first sugar feeding period.

Table 4. 2: Qualitative results from FISH analysis from PMC at 47th day, 74th day, 83rd day, 97th day, 139th day, 186th day and 208th day. **ALF969** - Alphaproteobacteria; **BET42a** - Betaproteobacteria; **GAM42a** – Gammaproteobacteria; **Delta42a** – Deltaproteobacteria; **LGC0354** – Firmicutes; **Grb** - *Rhodobacter* and *Roseobacter*; **RHC439** – Rhodocyclus; **DSBAC357** – Desulfobacteraceae; **DSB706** – Desulfobulbaceae; **DSV687** - Desulfovibrionales; **Rhodo-2** - Rhodospirillaceae; **ARC915** - Archaea.

Samples	ALF 969	BET 42a	GAM 42a	Delta 42a	LGC 0354	GRb	RHC 439	DSBAC 357	DSB 706	DSV 687	Rhodo 2	ARC 915
47th day FEED A1	inc.	(●)	(++)	(●)	(●)	(+)	(-)	(-)	(●)	(-)	(-)	(●)
74th day FEED A2	(++)	(●)	(+++)	(●)	(●)	(++)	(-)	(-)	(●)	(-)	(-)	(●)
83rd day FEED B1	inc.	(-)	(++)	(+)	(●)	inc.	(-)	(●)	(●)	(-)	(-)	(●)
97th day FEED C1	inc.	(++)	(+++)	(+)	(●)	(●)	(●)	(●)	(-)	(-)	(●)	(+)
139th day FEED C2	(+++)	(++)	(+)	(++)	(●)	(+++)	(●)	(●)	(+)	(-)	(-)	(●)
186th day FEED D1	(++)	(+)	(++)	(+)	(●)	(++)	(-)	(●)	(●)	(●)	(-)	(+)
208th day FEED E1	(+++)	(●)	(++)	(++)	(●)	(+++)	(-)	(-)	(+)	(-)	(●)	(+)

(-) Non-present; **(●)** Almost non-existent; **(+)** Present; **(++)** Abundant; **(+++)** Extremely Abundant; inc. – inconclusive.

During the FISH analysis, it was possible to observer a change in the shape of which class group, for instances the Alphaproteobacteria appear mostly as rod shaped bacteria. The two genera of Alphaproteobacteria tested – Rhodobacter and Roseobacter – correspond to the purple bacteria, and seemed to be the majority of the Alphaproteobacteria present in the reactor. Regarding the Gammaproteobacteria, this class suffers some changes along the enrichment process. The samples from the 47th and 74th day present Gammaproteobacteria as cocci bacteria, in the sample from the 83rd day this class was constituted by rod shaped and cocci bacteria, and finally at the remaining days (97th, 139th, 186th and 208th) the Gammaproteobacteria appear only as rod shaped bacteria.

The appearance of the Deltaproteobacteria could be related to the increase of the sulphates concentration on the fed solution (Appendix AP1_Since Feed B.1). This class comprise a branch of strictly anaerobic genera, which contains most of the known sulphate-reducing bacteria (SBR). The SRB are known to be capable to obtain energy by oxidizing organic compounds while reducing sulphate (SO_2^{-4}) to hydrogen sulphide (H_2S) (Pereira et al. 2011). Despite the negative results regularly obtain for the three SBR families of the Deltaproteobacteria tested - Desulfobacteraceae, Desulfobulbaceae and Desulfovibrionales -, the presence of considerable amounts of sulphate in the medium could promote the growth of Deltaproteobacteria.

At last, regarding the Archaebacteria results, it was possible to see a population increasing after the decay phase, which indicates greater resistance of these microorganisms under unfavourable condition.

Crossing the results obtain in the FISH analysis with the Nile Blue observations it was possible to conclude that the PHA accumulating bacteria follows the evolution of the Gammaproteobacteria, being this the main bacterial group responsible for PHA accumulation.

4.5. CONCLUSIONS

This chapter evaluated the possibility of selecting a PHA accumulating photosynthetic mixed culture under a feast regime. Results show that there are two strategies to select a PMC under a mixed organic acids feeding solution, under low concentrations of phosphorus and of organic acids in the medium. The low phosphorus concentration proved to be the best enrichment method utilized along the 8 months, with a PHA content of almost 19% of PHA/VSS (20mmol C/L) (the highest value registered), when compared with the 18,5% of PHA/VSS (10mmol C/L) obtain during the low OA concentrations. The PHA formed is the copolymer P(3HB-co-3HV), the HV percentage (20-40%) presented along the 8month allows the extracted polymer to becomes more ductile and easier to process and thus possesses a wider range of industrial applications when compare with a PHB polymer. Also, a considerable negative impact was noticed every time that the reactor was fed with a solution with high concentration of sugar. It is thought that the presence of sugar in the medium favoured a fermentative metabolism, leading to less effective PHA producing PMCs. In this way, the constant upstream control of the fermentation process of WWM is imperative, to guarantee a stable and appropriated composition of the feed solution. Furthermore, microbial analysis indicates a constant predominance of the Alphaproteobacteria and Gammaproteobacteria species in the reactor, and with the last ones as the principal PHA accumulating bacteria.

CHAPTER 5.

SBR2_PADDLE WHEEL HRAP RESULTS AND DISCUSSION

5.1. MIXED PHOTOSYNTHETIC CULTURE ENRICHMENT AT LAB SCALE REACTOR SIMULATING THE PADDLE WHEEL HRAP (SBR2)

The enrichment of the PMC used in this study was carried out under a feast and famine regime, with alternated periods of 12 hours of light and 12 hours of dark. The SBR2 was fed with same fermented mixture of wastewater with 1% (v/v) sugar molasses that fed the SBR1 (Appendix AP1).

During the 8 months, efforts were being made to achieve the optimal ratio between the Feast and Famine periods to potentiate the PHA accumulating bacteria's growth. The main obstacle found during the selection process was to find the proper feeding methodology to create a greater balance (of hours) between the feast and famine phases. Despite the fact that the main objective of this work is to achieve PMC with higher PHA accumulating bacteria content, the microalgae present in the SBR2 are very important during a feast and famine enrichment process, due to their competence to produce oxygen that will be necessary to promote the oxidation of reduced molecules from the PHA consumption during the famine phase allowing the cell maintenance.

The oxidative-reduction potential (ORP) is a standard measure of the activity of electrons involved in oxidation reactions in aqueous environments and can be used to monitor biological processes. According to Ndegwa et al. (2007), the ORP and the dissolved oxygen (DO) have strong linear relations, and in the presence of easily degradable organic compounds, both ORP and DO decrease, and increase rapidly thereafter before the total depletion of the organic compounds. For instances, the ORP profile of SBR 2 (Appendix AP2), had a similar behaviour to what Ndegwa et al. (2007) reported. During the feast phase the ORP decreased and after the depletion of the organic substrate the ORP started to increase up to positive values, which indicates the presence of oxygen during the famine phase (aerobic phase). On the other hand, in the permanent feast regime, the ORP maintained a constant profile in the negative values (Appendix AP2), without the existence of oxygen.

Figure 5.1 shows the evolution of the culture's performance during the enrichment period of 224 days. Similarly to the SBR1, the SBR2 also had an adaptation period between the day 0 and 11, achieving a biomass concentration of 0,89 $\pm$ 0,2 g/L of VSS, after this adaptation period, two fed methods were tested with an OLR of 3,4 mmol C/L·d.

The first method tested consisted of feeding the reactor with a single pulse of fermented WWM, at the beginning of the light phase. However, since the microbial population was still in adaptation to the feast and famine regime the ratio between the two phases Feast/Famine (F/F) started to decrease every day, which indicates a better performance of the microbial culture. Therefore, in order to extend the feast phase, a second method was tested where the reactor was continuously fed during 4 hours. As it could be seen in the Figure 5.1_(A), the lower concentration of the organic acids observed between the period of November/December to January, caused by the feeding methods mentioned led to low organic acids availability. The lack of organic acids present in the reactor and the higher light operating intensity in the reactor favoured the microalgae growth, which was responsible for the low Bact/Chl ratio obtain (< 0,2), and the absence of PHA.

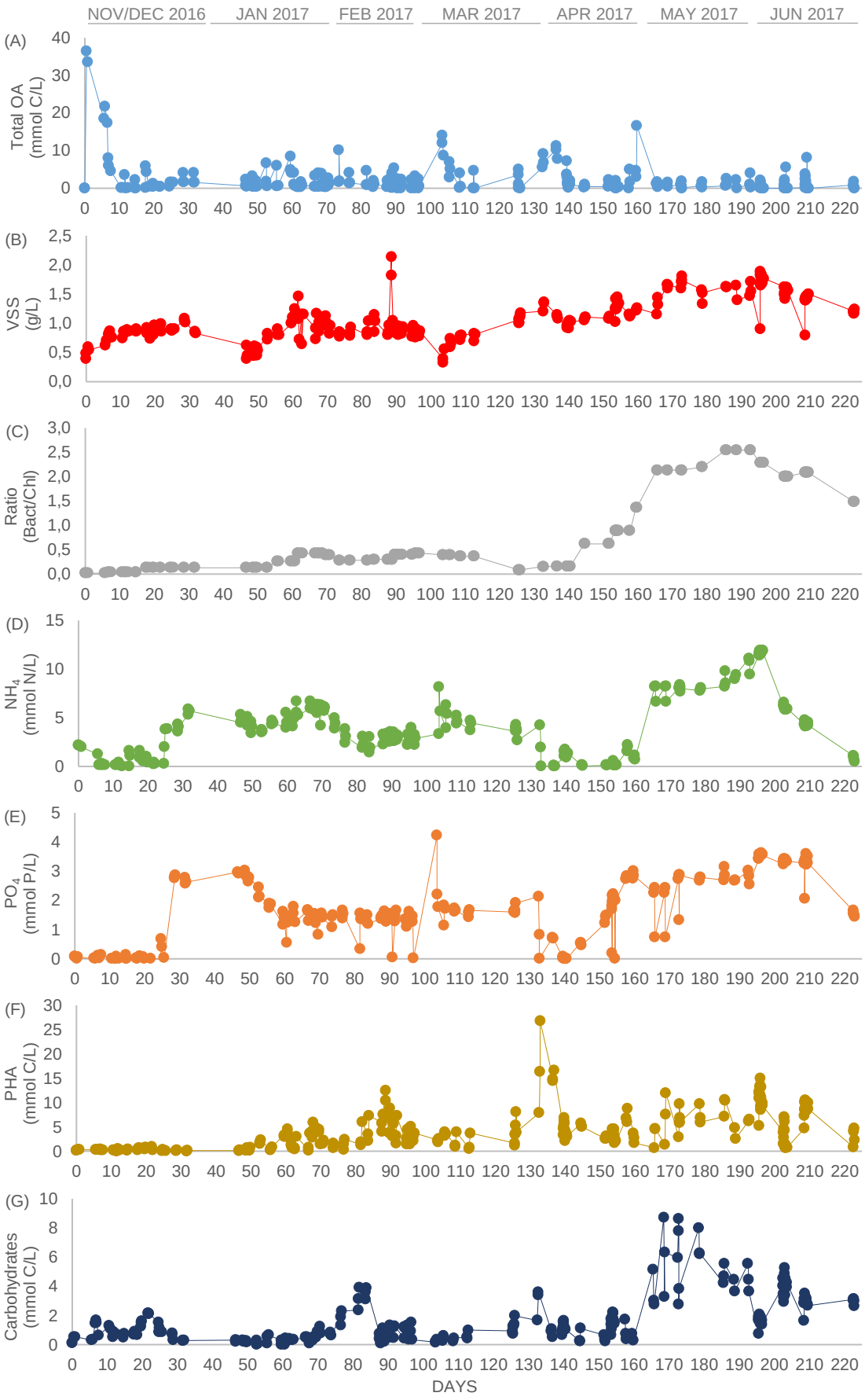

Figure 5. 1: Profile of SBR2 over 224 days of operation. **(A)** Total Organic Acids; **(B)** Total Volatile Suspended Solids; **(C)** Ratio Bacteriochlorophylls / Chlorophylls. **(D)** Ammonium (NH_4) **(E)** Phosphate (PO_4); **(F)** Polyhydroxyalkanoates (PHA) **(G)** Total Carbohydrates.

During the period between January and March, new feeding processes were tested to ensure the presence of a feast phase. It became clear that in order to extend the feast phase the solution was not to increase the organic load in the pulse given at the beginning of the light period. Since sometimes in pulses with an OLR $\geq$ 9,5 mmol C/L·d the pH dropped to inhibitory values, the consumption rate of the microbial culture decreased, leading to a permanent feast regime. Periodical pulses with an approximately OLR of 2 mmol C/L·d each was the solution found to increase the feast period without the pH limitation. This feeding method was implemented at the end of March, where it started being noticed the growth of the purple bacteria (increase of Bact/Chl ratio), an increase in the PHA content, and a constant growth of the biomass, achieving a concentration of 1,8 g/L of VSS.

However, the highest PHA content obtained was 29% of PHA/VSS (25 mmol C/L) at the end of March. The lower values registered after this period was due to the fact that between the feeding pulses the reactor entered in small starvation phases where the PHA started being consumed and for this reason it was continuously necessary to control and adjust the pulses accordingly to the end of each feast phase.

Unlike what happened in SBR1, the presence of high concentrations of sugar in the feed solution (beginning of February and end of April), did not affected significantly the reactor performance. During this period both biomass and the PHA content maintained their behaviour.

Similar to what happened in the SBR1, the N and P concentrations registered in the reactor, until the 19th day, were very low, leading to limitation of N and P. At day 19, the reactor began to be fed with a mineral solution (NLR 1,4 mmol N/L·d / PLR 0,6 mmol P/L·d) that leads to higher concentrations of ammonium and phosphate in the medium. However, this time the culture maintained the phosphorous concentrations constants. In fact, it was in the ammonium concentrations that was observed an interesting behaviour. It was observed that during the feast phase the ammonium concentrations was maintained constant, and once in the famine phase the N content starts to decrease, suggesting that the microbial culture underwent for a physiological adaptation after a starvation period, having only the capability to grow during the famine period, as it was observed by Albuquerque et al. (2010a). This subject will be further discussed in the next topic.

During the enrichment process, besides the PHA accumulation the selected PMC was also capable to accumulate carbohydrates as an internal polymer. It was observed two different accumulations processes. The first one was detected in the low Bact/Chl ratio period where the carbohydrates were produced during the light phase and consumed during the dark phase, a behaviour previously observed in the SBR1 and it was due to the presence of algae. However, at 202nd day a different behaviour was observed, where the consumption of the carbohydrates occurred during the feast phase, and once in the famine phase the carbohydrates synthesis occurred. This suggests that the carbohydrates were used as an additional energy source that could lead to PHA accumulation.

Regarding to the TOC results (data not shown), the organic acid concentration detected by the HPLC in the fed solution and in the SBR2 bulk were similar to the SBR1.

5.2. AMMONIUM METABOLISM

As mentioned before, there were some particularities observed regarding the ammonium metabolism along the enrichment process of the PCM. Figure 5.2 shows the behaviour of the reactor in three reactor cycles made at different times and for each one, different operating condition were present. The Figure 5.2_(A), shows a cycle performed in the beginning of January, with a Feast/Famine ratio of 0,5 (considering only the light hours). In the other hand, Figure 5.2_(B) represents the reactor behaviour at the end of February, with a F/F ratio of 7, which indicates that the reactor only entered in the famine phase 1,5 hours before the beginning of the dark phase. At last, in Figure 5.2_(C) it is possible to follow the behaviour of the reactor in March, under a F/F ratio of 0.7.

In the Figure 5.2_(A), it is possible to see that, in January, during the single carbon pulse method, the ammonia consumption only starts in the famine phase (4th hour). After the OA depletion, the ammonium starts being consumed with PHAs enabling the cell growth.

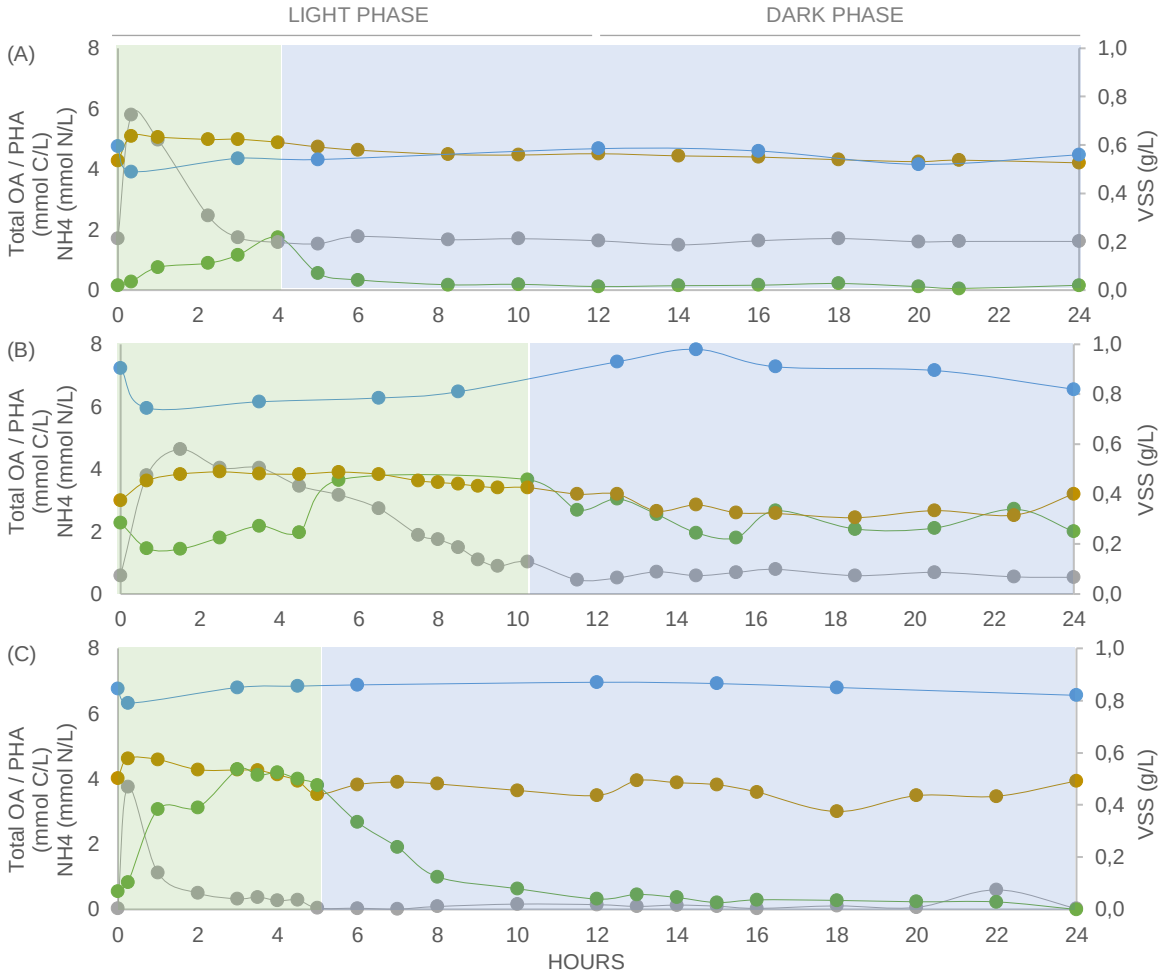

Figure 5. 2: Evolution of the ammonium metabolism in the periods of January, February and March (A) Reactor cycle on 47th day with an OLR of 4,4 mmol C/L·d and a NLR of 1,2 mmol N/L·d; (B) Reactor cycle on 97th day with an OLR of 3 mmol C/L·d and a NLR of 1,5 mmol N/L·d; (C) Reactor cycle on 116th day with an OLR of 3,7 mmol C/L·d and a NLR of 0,8 mmol N/L·d. Feast phase; Famine phase; (●) NH_4 (●) Total Organic Acids (●) VSS (●) PHA.

In February, a new behaviour was observed. Figure 5.2_(B), shows that the ammonium consumption begins in the feast phase as well as the biomass growth, after the PHA achieved a stable point. After this point, the organic acids and ammonia consumption were responsible for the biomass growth until the end of the feast phase. After that it could be possible to observe a decrease in the PHA content that also led to cell growth. This behaviour suggests that at 6th hour, the available carbon is used for cell growth and the PHA accumulation process stops, reaching a stable point. This performance could also have been observed during the cycle performed on 116th day (Figure 5.2_(C)), however the internal polymer does not reach a stable phase. The ammonia's lag phase, observed in the Figure 5.2_(A) and (B), potentiate the PHA's accumulation process since it works as an internal limitation that prevents the OAs uptake for cell growth.

In general, the ammonia uptake seemed to be coupled to the substrate consumption, however the initial stationary phase observed in the ammonium metabolism, here called by lag phase, could be due to the necessity of the microorganisms of the PMC to physiologically adapt to growth conditions following a long starvation period. The constant presence of ammonia trough the feast and famine cycles allows the degradation of the internal polymer (PHA) for cell growth, as it has also observed by Albuquerque et al. (2010a).

These results allow to conclude that under absence of ammonia consumption the PMC choose to accumulate PHA in order to create energy reserves for posterior cell growth. In a feast and famine strategy, it will be advantageous to create limitation of ammonia during the feast phase in order to increase PHA accumulation. However, in the famine phase, the ammonia presence would be necessary to allows the degradation of the internal polymer (PHA) for cell growth, this way only the bacteria that accumulate the PHA could proliferate.

5.3. PMC CARBOHYDRATES METABOLISM

In Chapter 4., it was mentioned that the carbohydrates metabolism could have an important role on the polyhydroxyalkanoates production, however the PMC selected under a permanent feast regime did not show that capability. Figure 5.3 reveals the evolution of the carbohydrates metabolism along the enrichment process of the PMC of SBR2, and as it can be seen, in this case the PHA production could be affected by the presence of the carbohydrates.

Similarly, to what happened in SBR1, in the Figure 5.3_(A)**,** is possible to observe the carbohydrates synthesis during the light phase and their consumption during the dark phase, a characteristic behaviour of the microalgae and confirmed by the low ratio of Bact/Chl of 0,13. It was also suggested by Fradinho et al. (2013a), that once the PMC photosystem became active under illumination, ATP became available and the microbial culture no longer required an extra source for energy generation, and for that same reason the carbohydrates consumption was not observed during the light phase. Since the reactor cycle represented in the Figure 5.3_(A) was performed after the 47th day under a FF regime during light and dark cycles, the PMC photosystem was already selected, allowing to concluded that during the light phase the selected photosynthetic mixed culture use the light to obtain energy, and in the absence of light, the consumption of carbohydrates provide the necessary energy for cell maintenance. In addition, the Figure 5.3_(B) shows that despite the carbohydrates were present they did not vary in

abundance, which refutes the idea that in any of the previous situation the carbohydrates were playing a role in PHA production.

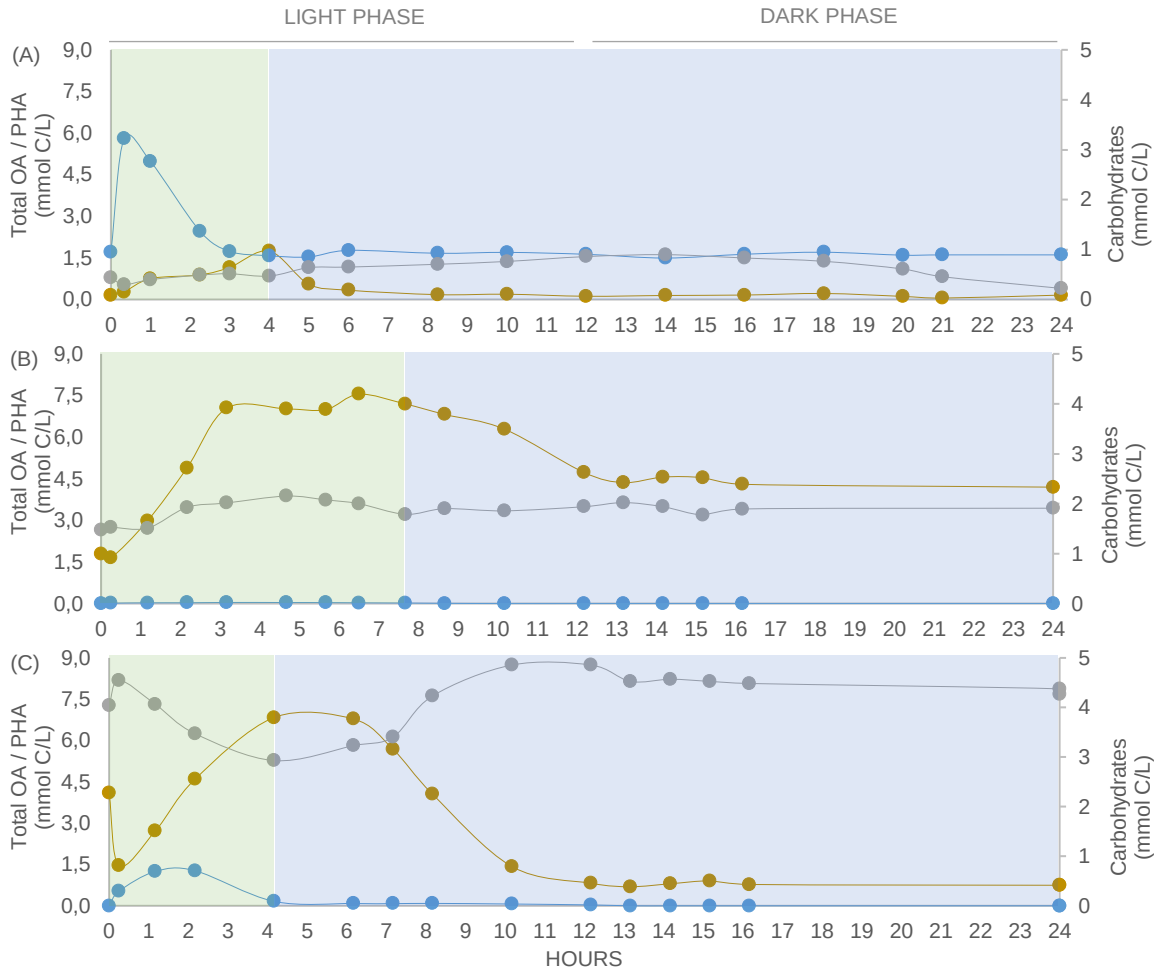

Figure 5. 3: Evolution of the PMC carbohydrates metabolism in the periods of January, May and June. (A) Reactor cycle on 47th day with an OLR of 4,4 mmol C/L·d; (B) Reactor cycle on 179th day with an OLR of 6,8 mmol C/L·d; (C) Reactor cycle on 202nd day with an OLR of 6 mmol C/L·d. Feast phase; Famine phase; (●) PHA (●) Total Carbohydrates (●) Total Organic Acids.

On the other hand, in Figure 5.3_(C) a new carbohydrates profile was observed after an increase of the Bact/Chl ratio along with an increase in the active biomass content to 1,39 ± 0,07 g X/L, for instances the carbohydrates consumption was performed during the feast phase, while their synthesis occurred during the famine phase. Once in the aerobic phase, PHA is oxidised, and the PMC gain energy for growth and for the glucose uptake regenerating the internal polymer - glycogen – as it was observed by Fradinho et al. (2013a).

The microbial culture's carbohydrate consumption rate was 0,15 Cmol Carbs/Cmol X·d, and the $Y_{Carb,S}$ calculated in period between the 0 and 2 hours was 0,18 Cmol Carb/Cmol OA, which are a similar value when compared to the value of acetate-fed PMC cultures operated under light feast and dark famine obtain by Fradinho et al. (2013a) (0,17 ± 0,09 Cmol Carb/Cmol Acet). During the light phase, the PMC use energy light to performed OA uptake. However, the microorganisms that also use the glycogen hydrolyses to get energy, will have a competitive advantage, once they can take energy not only from the light but also from the consumption of carbohydrates.

5.4. PMC PHA ACCUMULATION CAPACITY

To evaluate the PHA accumulation capacity of the PMC a series of batch tests were carried out under different light intensities and nutrient limitations. However, the tests were performed during an unstable phase of the reactor, which means that besides the changed behaviours observed due to the use of different reactors, mentioned in the last chapter, the tests were performed under different conditions in the main reactor.

The three control tests – Figure 5.4_(A), (B) and (E) – were conducted between days 214 and 216 when the culture presented a F/F ratio of 0,8, i.e. long feast and very short famine. As it can be seen in Figure 5.4, the initial PHA content of these trials starts at higher values which indicates that the culture in the main reactor did not had the capability to consume all the internal polymer until the beginning of the new light cycle, leading to lower PHA accumulation rates.

By looking into the organic acids profile – Figure 5.4 and Table 5.1 - of each control tests, it is possible to conclude that the tests performed in the accumulation reactors, test (B) and (C), had a lower organic acids consumption rate when compared with the control test performed in the main reactor (test A). This might be due to the air flux installed in the accumulation reactors. Probably it was lower than the air flux in the main reactor, despite the efforts made to obtain the same conditions in the batch tests. However, and as expected the control test with the same light intensity (test (B)) had a higher substrate consumption rate when compared with the control test carried out under the winter light intensity (test (E)).

The accumulation tests – Figure 5.4_(C), (D) and (F) – were performed a few days later (between 220th day and 223rd day) when the culture presented a F/F ratio of 0,42. This time, the main reactor recovered the capability to consume the PHA, starting the new light cycle with residual PHA values, becoming possible to correctly evaluate the capacity of the PMC to store the internal polymer. These changes made it impossible to compare the control assays with the accumulation assays to see if the nitrogen and phosphorus limitation could favour PHA accumulation, however it still possible to study the influence of the light intensity.

Table 5.1, shows that the test (D), which represents the trial performed under the maximum light intensity that the Paddle Wheel Pond could be operated, achieved the highest substrate consumption rate as well as PHA production rate in the accumulation tests, reaching a PHA content of 14,5% PHA/VSS (8 Cmmol PHA/L). This time, it was also possible to confer that with winter illumination – test (F) – the reactor did not suffer negative consequences, in fact it was capable to achieve the same PHA content obtain under the normal operating conditions (Test (F) – 9 Cmmol PHA/L (9% PHA/VSS); Teste (C) – 8 Cmmol PHA/L (10% PHA/VSS)).

During the control test (B) and (E), and like discussed in the previous topic, after the PMC entered in the famine phase (3rd hour) the consumption of PHA led to the carbohydrates synthesis. Another interesting fact, about the carbohydrates metabolism, was observed during the batch tests with limited nutrient conditions. In Figure 5.4_(C) and (D), after a depletion of ammonia, it is possible to see an increase on the carbohydrates uptake rate, suggesting that the organic acids that were being used for the cell growth while ammonia was available (data not show) were now being stored as glycogen.

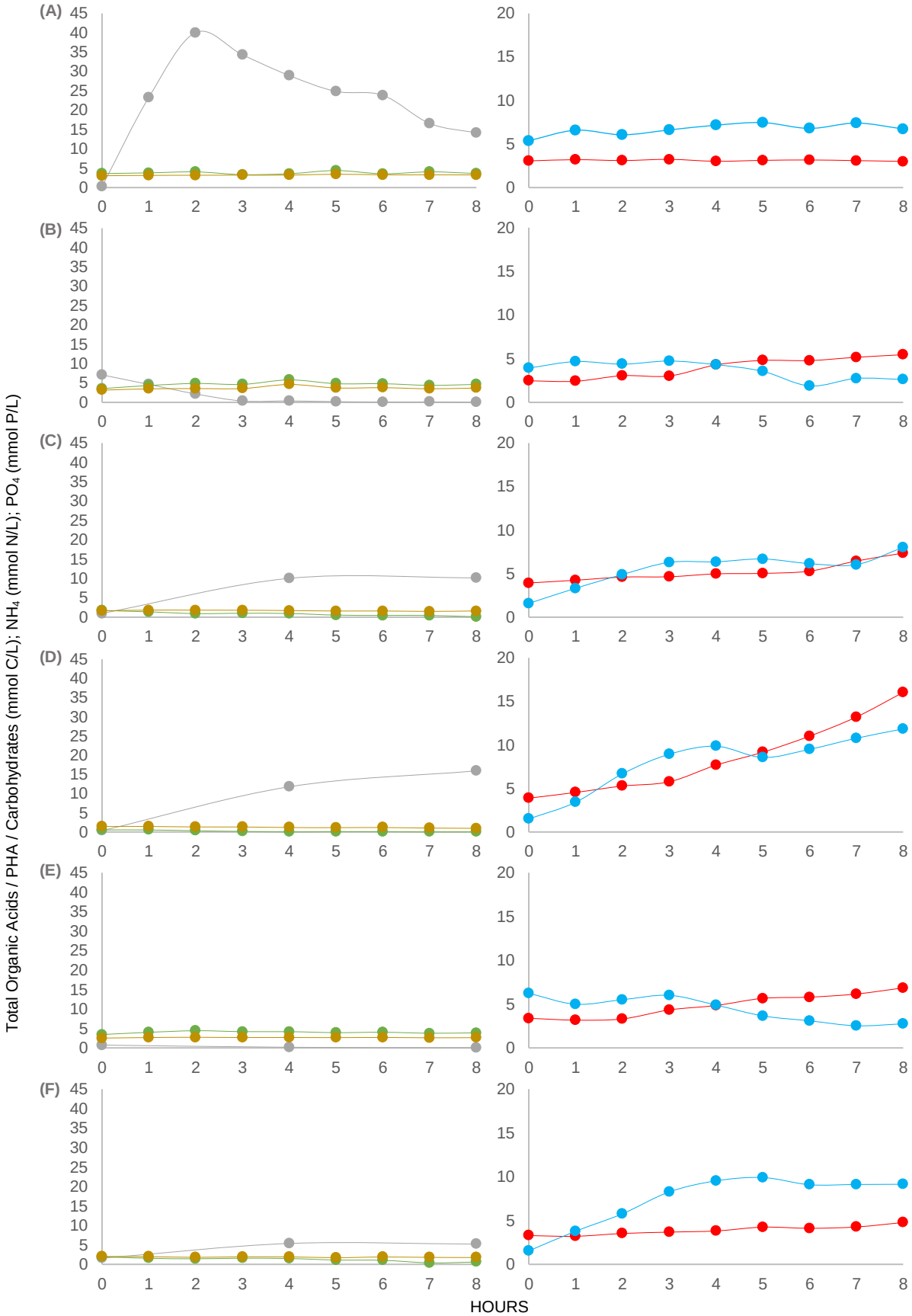

Figure 5. 4: Batch tests performed to evaluate the SBR2 PMC capability to accumulate PHA under different conditions. **(A)** CT of SBR2; **(B)** CT performed in the accumulation reactor under the same conditions of SBR2; **(C)** AcT performed at a light intensity of 1,90W/L, under N and P limitation; **(D)** AcT performed at a light intensity of 3,8W/L, under N and P limitation; **(E)** CT performed at a light intensity of 0,8 W/L (winter illumination) **(F)** AcT performed at a light intensity of 0,8W/L (winter illuminations), under N and P limitation. (●) Total Organic Acids (●) PO_4 (●) NH_4 (●) Carbohydrates (●) PHA.

Table 5. 1: Kinetic and stoichiometric parameters of batch tests performed with the PMC in different experimental conditions. **Test (A)** CT of SBR2; **Test (B)** CT performed in the accumulation reactor under the same conditions of SBR2; **Test (C)** AcT performed at a light intensity of 1,90W/L, under N and P limitation; **Test (D)** AcT performed at a light intensity of 3,8W/L, under N and P limitation; **Test (E)** CT performed at a light intensity of 0,8 W/L (winter illumination) **Test (F)** AcT performed at a light intensity of 0,8W/L (winter illuminations), under N and P limitation.

	q_{PHA}	q_{Carb}	$-q_S$	$Y_{PHA,S}$	$Y_{Carb,S}$
Test (A)	0,2374	0,0273	2,6772	0,0887	0,01
Test (B)	0,2774	0,2679	1,8719	0,1482	0,14
Test (C)	0,2234	0,1746	0,8562	0,2609	0,20
Test (D)	0,2920	0,5129	1,6481	0,1772	0,31
Test (E)	0,0575	0,3190	0,6704	0,0858	0,48
Test (F)	0,2887	0,0664	0,6699	0,4311	0,10

q_{PHA} Cmmol PHA/Cmmol X·d, **q_{Carb}** Cmmol Carb/Cmmol X·d, **q_S** Cmmol OA/Cmmol X·d, **$Y_{PHA,S}$** Cmmol PHA/Cmmol OA, **$Y_{Carb,S}$** Cmmol Carb/Cmmol OA.

The accumulation tests were performed after an increase in the ratio of Bact/Chl, indicating that PMC was enriched in purple bacteria. Also, the maximum PHA content obtained was near the 14,5% of PHA/VSS (9,3 Cmmol PHA/L) – test (D) -, which indicates that when a selected microbial culture is submitted to higher light intensities, the PHA accumulating bacteria became more efficient, in this particular case the PMC achieved a PHA content twice as much as the main reactor during the same period (Main reactor – 7,5% PHA/VSS (5 Cmmol PHA/L); Test D – 14,5 % PHA/VSS (9,3 Cmmol PHA/L)). However, in none of the batch tests was observed a stable period of PHA content, which indicates that the culture never reached the maximum accumulation capacity.

In order to better understand the behaviour of the PMC when selected and fed with medium containing sugars accumulation tests were performed, at 181th and 193rd day. In Figure 5.5 it is possible to observe two batch tests performed with a) the PMC was selected under significant sugar concentrations (Feed D.1_14,6 Cmmol sugar/L), presenting a F/F ratio of 0,5 (Figure 5.5_a.), and b) the PMC was selected without sugar, and with a F/F ratio of 0,63 (Figure 5.5_b.). The tests indicated in Figure 5.5_a. and b. were performed under high light intensity in order to determine the maximum PHA accumulating capacity of each PMC, and to compare the effects of the presence of sugar in the enrichment process. Similar to what happen in Chapter 4., the higher light intensities lead to higher PHA accumulation with an outcome of 30% PHA/VSS (16 Cmmol PHA/L), for both essays, the maximum value registered along the 8 months of the enrichment process, and comparable to the PHA content obtain by Fradinho et al. (2013b) (30% PHB/VSS) in a PMC selected under a FF regime and alternated light/dark cycles. Contrary to what happens in the permanent feast regime, the presence of a considerable amount of sugar in a feast and famine enrichment process seems not affect the PHA accumulation capacity of the photosynthetic mixed culture. In Chapter 4., it was mentioned that the presence of significant amounts of sugar in the feed solution favoured the appearance of fermentative bacteria that are also favoured by anaerobic environments, conditions on which the SBR1 was being operated. However the SBR2 seems to be operating under an anaerobic feast/aerobic famine regime, which could be the

principal cause for the fact that the presence of sugars in a FF regime does not affect the PMC behaviour, since the presence of oxygen in the medium does not favour the growth of fermentative bacteria.

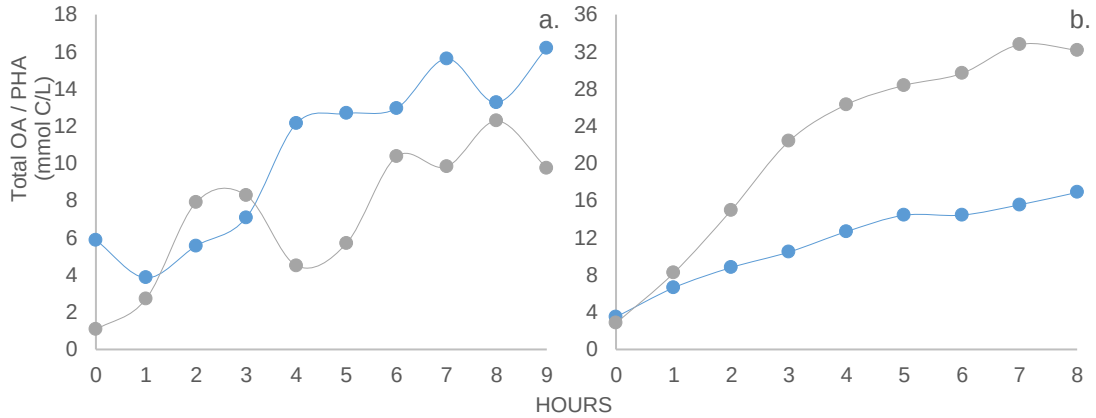

Figure 5. 5: Batch accumulation test operated under higher light availability. **a.** Test performed under 6,17W/L light intensity and fed solution with sugar; **b.** Test performed under 6,17 W/L light intensity and fed solution without sugar. (●) Total Organic Acids (●) PHA

Figure 5.6 represents three reactor cycles performed in SBR 2, during a low Bact/Chl ratio (Figure 5.6_a) and after a rise in the Bact/Chl ratio (Figure 5.6_b. and c.), meaning a decrease in the algae content. Comparing the Figure 5.6_a. and b. that were operating under the same F/F ratio, but with different Bact/Chl ratios, it is possible to see that under a lower algae content – Figure 5.6_b. – the photosynthetic culture achieved higher PHA contents. However, the PHA consumption rate during the famine phase decreased (Figure 5.6_a. – 1,13 Cmmol PHA/Cmmol X·d and b. – 0,13 Cmmol PHA/Cmmol X·d). In Figure 5.6_b. a 6,5 Cmmol PHA/L content took 8 hours to reach residual percentages, so it was to expect that in Figure 5.6_c. with a feast phase of 10hours, the PMC would not be able to consume the 11 Cmmol PHA/L content until the end of the light.

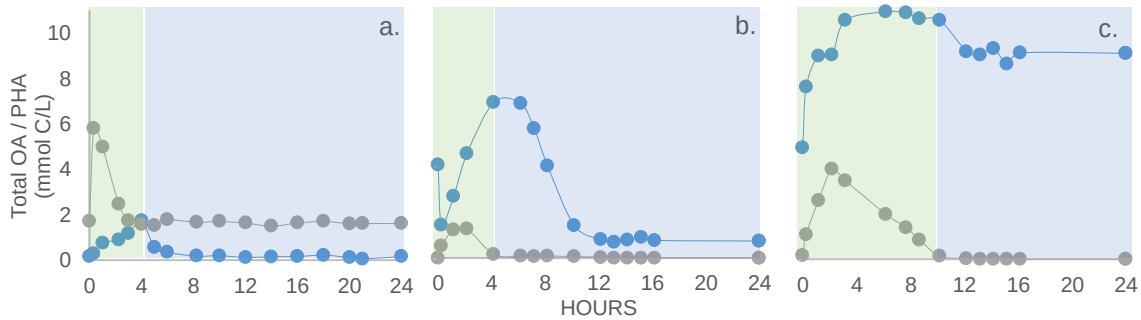

Figure 5. 6: Evolution of the PMC behaviour under different F/F and Bact/Chl ratios. a. Reactor cycle performed on 47th day, under a 0,33 F/F and 0,13 Bact/Chl ratios; **b.** Reactor cycle performed on 202nd day, under a 0,33 F/F and 2,01 Bact/Chl ratios; **c.** Reactor cycle performed on 208th day, under a 0,83 F/F and 2,08 Bact/Chl ratios. Feast phase; Famine phase; (●)Total Organic Acids (●)PHA

Without the decrease of the PHA content the reactor starts behaving as permanent feast regime. It was possible to conclude that, although a high Bact/Chl ratio in the PMC, promoted by higher F/F ratios, was likely responsible for higher PHA content, the diminishment of the algae content leads to oxygen limitation. Since this is necessary to consume the internal polymer, without it the culture does not fully consumes the PHA and starts losing its capability to accumulate the polymer. A possible and viable solution could be the aeration of the system after

the feast phase that will increase the oxygen availability in the medium and promote the PHA consumption.

At last, during the six batch tests performed – Figure 5.4 – the PHA polymer was composed by 70% of PHB and 30% of PHV. However, this composition varied with the composition of the feed solution. For instances in the batch tests performed under high light intensity, the feed solution with sugar originated a polymer with 60% of PHB and 40% of PHV, and the feed solution without sugar led to a polymer equivalent to the other batch tests, 70% of PHB and 30% of PHV. It was noticed that the PHB and PHV content altered in consonance with the percentages of acid acetic and butyric, and acid propionic and valeric, respectively.

5.5. MICROBIAL CHARACTERIZATION

The initial sludge that was used in this work was originally composed almost by microalgae. After the initial adaptation period (day 0 to 11), the continuously presence of organic substrates favoured the appearance of bacteria (Figure 5.7).

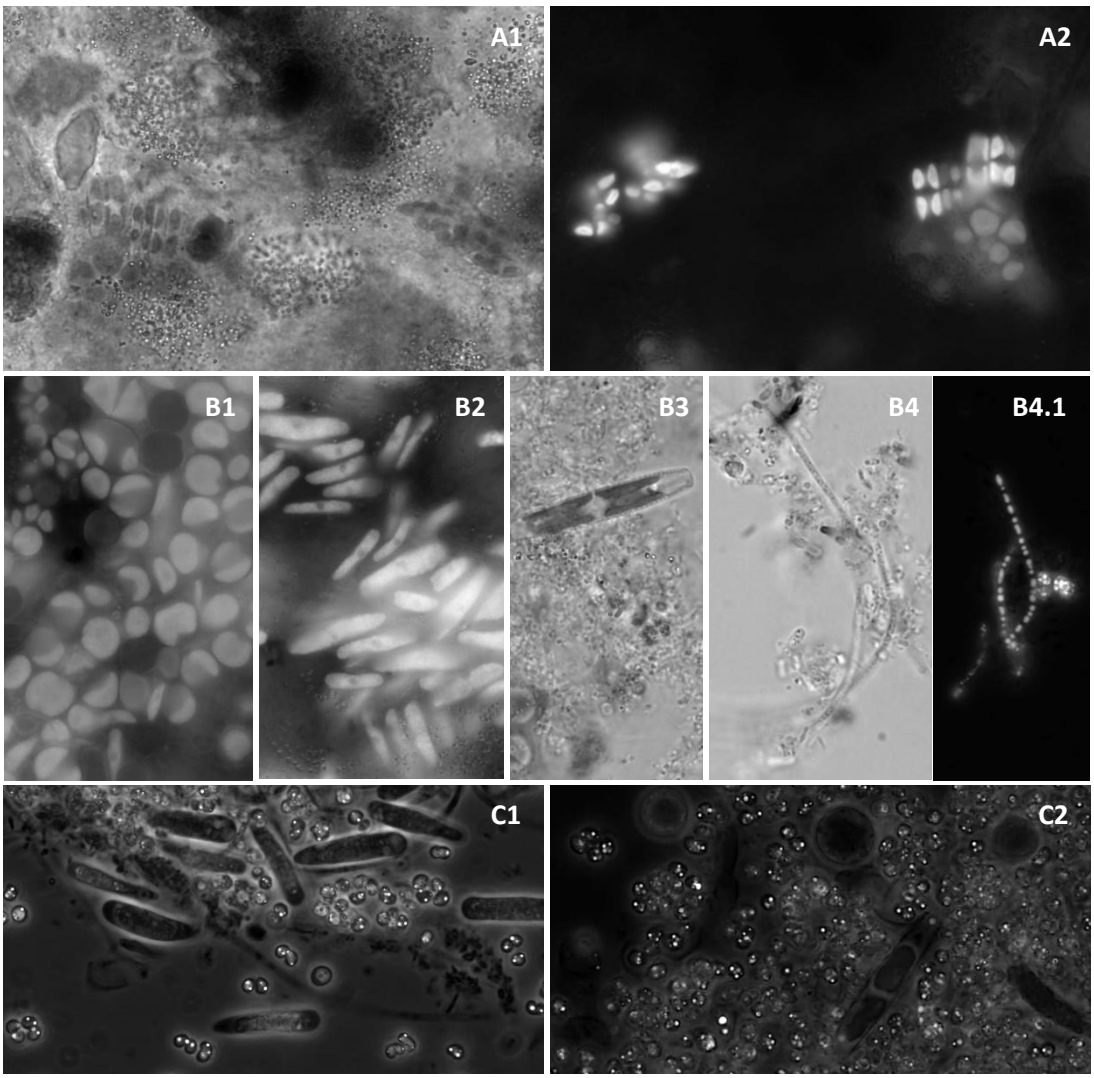

Figure 5. 7: Microscopic images of the PMC during at 4th operation day. **A1, B3** and **B4** – Bright field; **A2, B1, B2** and **B4.1** – Fluorescence images; **C1** and **C2** – Fluorescence images of Nile blue staining indicating PHA granules.

The presence of some bacteria that has the capability to accumulate PHA, was detected at 4th day, as it can be seen in the Figure 5.7, C1 and C2, by the fluorescence of the internal PHA granule caused by the Nile blue staining. There were also detected a majority group of microorganisms which possess autofluorescence when irradiated with UV light (A2, B1, B2 and B4.1), allowing to conclude that the algae are still the dominant specie after the adaptation period. The algae are presents in a variety of forms, including Chlorellas which had a round shape and green colour (B1), some Diatom with a fusiform shape and green colour (B2 and B3), and some filamentous green algae (B4 and B4.1).

It was noticed that, during the period between 0 and 11th day, SBR 1 and SBR 2 appeared to have similar culture developments. However, SBR 2 presented a higher algae content which might be due to the high operating light intensities, favouring the prevalence of the microalgae.

Table 5.2 shows the FISH analysis results. Similar to what happens in the SB1, in SBR2 the Alphaproteobacteria and the Gammaproteobacteria are also the dominant class, and the Deltaproteobacteria also appear after the first sugar feeding period.

During the FISH analysis, it was possible to observer the evolution of each class group. In the SBR2 the shape of each class change along the 8months. For instances the Alphaproteobacteria probe show that between 47th day and 97th day the Alphaproteobacteria appear as rod shaped bacteria, while between 139th day and 202nd day this class appear in both rod and cocci shaped bacteria, being the cocci shape the dominant one. The two genera tested for purple bacteria – *Rhodobacter* and *Roseobacter* – belongs to the Alphaproteobacteria class. The probe that test these two genera obtain a positive result and follow the same evolution as ALF969, corresponding to the majority of their population.

The Gammaproteobacteria also suffers some changes along the enrichment process, as it was possible to observer with the different shapes in the reactor samples (47th, 139th and 186th day: cocci bacteria; 74th and 83rd day: rod shaped bacteria; 97th and 202nd

day: cocci and rod shaped bacteria), while the Betaproteobacteria and the Deltaproteobacteria maintained their rod shape along the enrichment process. The Deltaproteobacteria is related to the increase of the sulphates concentration on the fed solution (Appendix AP1), once the presence of considerable amounts of sulphate in the medium could promote their growth, but at the same time the three genera tested - Desulfobacteraceae, Desulfobulbaceae and Desulfovibrionales – had a negative result.

The Betaproteobacteria are present along the eight months of the enrichment process, and are known to comprise aerobic microorganisms capable to derive energy from light, and playing a role in the nitrification process (Dang et al., 2010). These bacteria are also referred in literature as PHA accumulating bacteria in ADF systems (Lemos et al. 2008; Queirós et al. 2014)

The Archaebacteria results are also similar to the SBR1 indicating greater resistance of the microorganisms under unfavourable condition.

Table 5. 2: Qualitative results from FISH analysis of SBR2 during the 8months. **ALF969** - Alphaproteobacteria; **BET42a** -Betaproteobacteria; **GAM42a** – Gammaproteobacteria; **Delta42a** – Deltaproteobacteria; **LGC0354** – Firmicutes; **Grb** - *Rhodobacter* and *Roseobacter*; **RHC439** – Rhodocyclus; **DSBAC357** – Desulfobacteraceae; **DSB706** – Desulfobulbaceae; **DSV687** - Desulfovibrionales; **Rhodo-2** - Rhodospirillaceae; **ARC915** - Archaea.

Samples	ALF 969	BET 42a	GAM 42a	Delta 42a	LGC 0354	GRb	RHC 439	DSBAC 357	DSB 706	DSV 687	Rhodo 2	ARC 915
47th day FEED A1	(+++)	(++)	(++)	(++)	(●)	(+++)	(●)	(●)	(+)	(●)	(-)	(●)
74th day FEED A2	(++)	(++)	(++)	(++)	(●)	(++)	(●)	(-)	(+)	(●)	(-)	(+)
83rd day FEED B1	(++)	(+)	(+)	(+++)	(●)	(++)	(●)	(●)	(●)	(●)	(-)	(+)
97th day FEED C1	(+++)	(++)	(+)	(++)	(●)	(+++)	(●)	(●)	inc.	(+)	(●)	(++)
139th day FEED C2	(++)	(+)	(+++)	(+)	(●)	(++)	(-)	(●)	(●)	(●)	(-)	(●)
186th day FEED D1	(++)	(●)	(++)	(++)	(●)	(++)	(+)	(●)	(++)	(●)	(-)	(●)
208th day FEED E1	(++)	(+)	(+)	(++)	(-)	(++)	(●)	(-)	(+)	(●)	(-)	(+)

(-) Non-present; **(●)** Almost non-existent; **(+)** Present; **(++)** Abundant; **(+++)** Extremely Abundant; inc - inconclusive.

The differences observed in the microbial culture along a feast and famine enrichment process when comparing to the permanent regime, allows to conclude that the different operational conditions may lead to possible alterations in the culture's species compositions, both in algae and bacterial domain. Crossing the results obtain in the FISH analysis with the Nile Blue observations it was possible to conclude that the PHA accumulating bacteria follows the evolution of the Alphaproteobacteria, being this the main bacterial group responsible for PHA accumulation while in the SBR1 the Gammaproteobacteria are the class responsible for the PHA accumulation. Another difference observed is the higher algae and Betaproteobacteria content in the SBR2. The high algae content could be due to the higher light intensity operational conditions, while the considerable presence of the Betaproteobacteria could be to the fact that the reactor also operate under aerobic conditions favouring the growth of this class.

5.6. CONCLUSIONS

This chapter evaluated the possibility of selecting a PHA accumulating photosynthetic mixed culture under a feast and famine regime. Results show that low F/F ratios leads to low Bact/Chl ratios, and with that to low PHA accumulation efficiencies, and the best way to prolong the feast phase is with intermittent pulses (each hour) that allows to extend the presence of the organic matter in the reactor without leading to substrate inhibitions. The light intensity also proved to be an important factor to rise the PHA content in the microbial culture, leading to the maximum registered value of 30% of PH/VSS. The HV percentage (30-40%) of the copolymer P(3HB-co-3HV) produced along the 8month is responsible for the formation of a polymer more ductile and easier to process. It was also noticed that the PMC under a FF regime does not suffer a negative

impact every time that the reactor was fed with a solution with high concentration of sugar, it is believed that the presence of an aerobic phase after the feast phase disfavour the growth of fermentative bacteria, which do not have the capability to accumulate PHA. Furthermore, microbial analyses indicate an alteration in the PMC species composition along the operating reactor period, with a constant predominance of the Gammaproteobacteria and Alphaproteobacteria which was the principal PHA accumulating bacteria. The algae content is always superior in SBR2 than in SBR1 until the end of March, time where the successive feeding pulses method was applied promoting the growth of purple bacteria in the SBR2 and a decrease in the algae content.

CHAPTER 6.

CONCLUSIONS AND FUTURE WORK

Ponds represent one of the oldest forms of biological wastewater treatment, having been used as the only means of wastewater treatment prior to water discharge to surface waters and for pre-treatment or storage prior to wastewater treatment in conventional WWTP. A wide range of industrial and municipal wastewaters have been treated in pond systems (Mahaprata et al., 2013).

The set goals of this work were to transform WW from a waste stream into a source of new added-value bioproducts contributing to a circular flow economy. Both High Rate Algae Pond (HRAP) and photo-bioreactors (PBR) can be WW treatment systems which utilize microalgae and bacteria consortia that grow in a symbiotic relationship performing a series of reactions that eliminate pollutants from WW. As an alternative to the cost-intensive element of sanitation of the WW, the phototrophic PHA production surged exploring the capability of photosynthetic bacteria to accumulate PHA under open, non-aerated and using light, thereby lowering operational costs.

In Chapter 4. and 5. two PBR were tested under different light intensities and feeding regimes, replicating real operating conditions of HRAPs from Chiclana WWTP. Results show that under a permanent feast regime there are two possible ways to select a PMC with higher Bact/Chl ratios, such as under low phosphorous concentration and/or low concentrations of organic acids in the medium, obtaining a 19% PHA/VSS (20 Cmmol PHA/L) and 18,5% PHA/VSS (10 Cmmol PHA/L) respectively. Despite the phosphorus limitation seeming to lead to the best enrichment method, it will be interesting to study the effect of the combination of the two methods in the PMC behaviour. In the future, it will be necessary to subject the PMC to low phosphorus and OA concentration, to evaluate PMC's evolution and the respective PHA accumulation efficiency.

On the other hand, the microbial culture enriched under a feast and famine regime made it possible to conclude that low F/F ratios leads to low Bact/Chl ratios, which indicates low PHA accumulating efficacy. The best way to achieve higher F/F ratios without causing substrate limitations, and at the same time increase the Bact/Chl ratio was by giving several pulses along the initial hours of the light phase, with an interval between each pulse that was set by the presence of organic acids, obtaining a PHA content of 29% of PHA/VSS (25 Cmmol PHA/L). Although high F/F and Bact/Chl ratios lead to higher PHA content, it was observed that at the same time the microbial culture lost the capability to quickly degrade the PHA until de end of the light phase. This behaviour is correlated to the diminishment of the algae content that leads to an oxygen limitation, that is necessary to consume the internal polymer. Hereafter, it will be necessary to test the aeration of the system after the feast phase, in order to increase the oxygen availability in the medium and promote the PHA consumption until the end of the light phase.

In both reactors, the light intensity proved to be an important factor to rise the PHA content in the microbial culture, leading to the maximum registered value of 30% of PHA/VSS in the PMC under the feast and famine regime. However, the influence caused by the presence of sugar during the enrichment of both PMCs showed different impacts in each one. Under a permanent feast regime, the presence of sugar caused a negative impact, with the culture losing its capability to accumulate PHA. Under a FF regime the PMC did not suffer a negative impact when the reactor was fed with a solution with high concentration of sugar. This can be explained by the fact that the combination of an anaerobic environment (continuously present in the permanent feast

regime) with the presence of sugar in the medium led to the growth of fermentative bacteria, which do not have the capability to accumulate PHA, leading to less effective PMCs. In this way, the constant control of the fermentation process of molasses is imperative, to guarantee a better composition of the feed solution.

The PHA content observed in each reactor seemed to be accordingly with other studies performed under similar conditions, however there still the need to optimize the operation conditions of the reactors. The best strategies to selected PHA accumulating bacteria only operate in a short period of time, and not under the optimal conditions, surging the need to optimize the same strategies before forward to demo-scale.

Furthermore, microbial analysis indicates an alteration in the PMC species composition along the operating reactor period in the both regimes. The Alphaproteobacteria and Gammaproteobacteria were the main classes present in both reactors, despite the fact that in the SBR1 the Gammaproteobacteria was the principal PHA accumulating bacteria, and in the SBR 2 the PHA accumulating bacteria were the Alphaproteobacteria. This allows to conclude that the PMC enrichment under different regimes leads to significant differences in the microbial culture. Along the enrichment process different metabolisms were detected in both reactors that are usually associated to PAOs. It will be important to confirm their presence in future FISH analysis or to reveal the possibility of some photosynthetic organisms presenting a similar behaviour.

A direct comparison between the Feast and Famine regime with the Permanent feast regime is difficult to made, since the light intensities provided to each reactor were different, and as previously mentioned, the light availability is an important factor that can affect the PHA accumulation. During the batch accumulation tests, it was possible to compare the behaviour of the two PMCs under the same conditions, however the PMCs utilized in the accumulation tests for the SBR1 were not selected under the two best methods tested along the 8 months, leading to lower PHA productivities and lower contents when compared with SBR 2 accumulation tests. However, when comparing the reactors at their best enrichment period, despite de fact that the higher PHA content was obtained in the reactor operated in a feast and famine regime, 29% of PHA/VSS (25 Cmmol PHA/L), the highest PHA content obtained with the Permanent feast regime, 19% of PHA/VSS (20 Cmmol PHA/L) was not so distant from the first.

The permanent feast regime had the advantage to only need one reactor to performed the PMC enrichment and at the same time the PHA accumulation, while the feast and famine necessitates of one reactor to do the enrichment of the PMC and another one to accomplish the PHA accumulation. Considering the purpose of this work and its final application in HRAP from the Chiclana WWTP, the permanent feast regime became a valuable strategy since it requires less operation space and at the same time less control, while the feast and famine enrichment process requires a constant control of the feast phase in each pulse and requires distinct pond facilities to execute the selection and accumulation stages. However, the sensibility of a PMC selected under a permanent feast regime to the presence of a considerable amounts of sugar is a drawback if the feeding solution is inconstant.

Summing up all these considerations, both strategies appear to be promising strategies for selecting photosynthetic PHA accumulating bacteria while treat wastewater (allowing to remove of organic matter as well as nutrients presents in wastewater). Therefore, future work should focus on optimizing the enrichment process of both reactors under the same light intensities to better understand the best and rentable approach for the photosynthetic PHA production in Chiclana's Ponds.

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AP.1: *Characterization of each fed solution.*

PERIOD	DAY	FEED	TOTAL CARBON		PERCENTAGE FROM HPLC DATA							SUGARS	SULPHATES	MINERAL	
			TOC (mmol C/L)	HPLC (mmol C/L)	Lactate	Acetic	Propionic	Butiric	Isovaleric	Valeric	Ethanol	Glucose + Frutose (mmol C/L)	mg SO_4^{-2}/L	NH_4 $mg.L^{-1}$	PO_4 $mg.L^{-1}$
					% C										
From 0 to 47th day	0		22	172	2	35	17	22	1	11	12	0,4	27,5	20	6
	47	A.1	138	95	2	27	10	11	0	5	0	0,0	19,3	59	7
	55		166	117	12	30	11	12	0	6	0	0,0	23,4	69	6
From 48 to 74th day	60		266	257	1	33	43	14	1	7	0	0,0	34,8	101	11
	67	A.2	203	245	1	33	44	15	0	7	0	0,0	34,8	101	11
	74		226	124	5	43	28	24	0	23	0	0,0	34,8	65	6
From 75 to 87th day	87	B.1	204	221	6	33	28	15	0	19	0	53,7	76,2	2	0
From 87 to 111th day	87		239	241	3	36	29	13	0	18	0	2,9	74,2	10	0
	97	C.1	199	126	0	22	33	16	0	28	0	1,5	78,8	9	0
	111		231	223	3	20	25	12	1	19	20	2,5	80,3	12	2
From 112 to 150th day	112		213	189	5	19	27	15	0	23	12	3,4	68,0	1	1
	122	C.2	200	214	2	17	23	17	0	25	15	0,0	80,5	9	1
	144		201	35	35	2	1	8	21	3	31	1,9	67,2	0	0
From 151 to 177th day	151		259	97	48	1	1	1	6	0	43	14,6	152,5	2	0
	157	D.1	213	143	2	18	35	22	0	23	2	6,1	129,7	2	0
	172		215	156	12	26	19	20	0	23	0	10,5	125,6	1	1
From 178 to 184th day	178	D.2	218	140	8	31	18	16	0	27	0	11,5	101,6	1	0
From 185 to 224th day	185		212	154	6	34	18	17	0	25	0	13,8	94,0	2	0
	199	E.1	275	150	6	20	7	37	0	30	0	0,0	47,7	39	1
	223		249	227	3	18	13	24	0	23	18	0,0	73,4	49	2

AP2: *ORP behaviour of SBR1 and SBR2.*

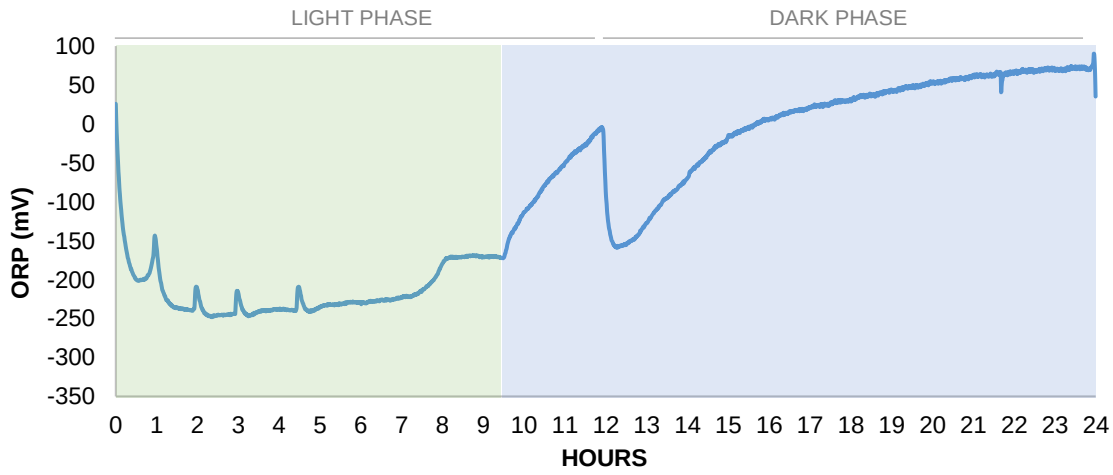

Figure AP. 1: *ORP profile of SBR2 along 24hours cycle with 12h light and 12h dark. Feast phase; Famine phase.*

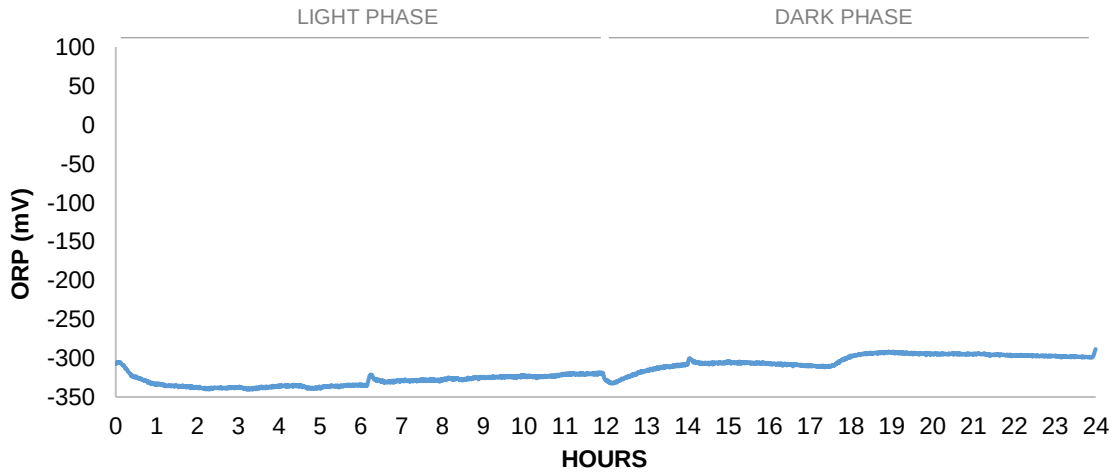

Figure AP. 2: *ORP profile of SBR1 along 24hours cycle with 12h light and 12h dark.*