Alma Mater Studiorum – University of Bologna

SCHOOL OF SCIENCE
Department of Industrial Chemistry "Toso Montanari"

Second cycle degree in

Low Carbon Technologies and Sustainable Chemistry

Classe LM-71 - Scienze e Tecnologie della Chimica Industriale

Process optimization for polyhydroxyalkanoate production by mixed microbial cultures within the B-PLAS project

Experimental degree thesis

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Academic Year 2020-2021

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Abstract

Polyhydroxyalkanoates (PHA) are biodegradable polyesters with comparable properties to some petroleum-based polyolefins. Within the context of the B-PLAS process, the PHA production is integrated with a wastewater treatment process aimed at recovering energy and C through platform molecules such as volatile fatty acids (VFAs). These compounds are eventually provided as a fermentation broth to selected mixed microbial cultures (MMCs) under a dynamic feeding regime, producing PHA-enriched biomass. In this work, an anoxic/aerobic selection and enrichment process has been explored and compared on a bench scale with the most studied, completely aerobic alternative. A final setup for both the bench-scale experiments and the B-PLAS pilot plant has also been proposed, allowing to achieve, on-demand, a higher dried biomass PHA content (>50%) compared to the analogous aerobic setup. In addition, the selected population has been proven to respond positively to reduced oxygenation conditions, opening new possibilities to maximise the production while reducing the oxygenation-related costs and streamlining the process scheme.

1 Introduction

1.1 The problem of single-use packaging

In 2019, it was reported that plastics and plastic derived material production annual figure exceeded 360 million tons, equivalent to trillions of dollars in global economic value. Packaging is the largest sector of the plastics industry, representing more than 40 % of total plastic consumption. Plastic waste generation is strongly influenced by primary use, but also the product lifetime. Therefore, the packaging sector is the dominant generator of plastic waste due to the reduced lifetime of the related items, which are responsible for almost half of the global total. However, none of the commonly used plastics is biodegradable. As a result, they accumulate in landfills or natural environments rather than decompose. The only way to permanently eliminate plastic waste is by destructive thermal treatments, such as combustion or pyrolysis. Thus, near-permanent contamination of the natural environment with plastic waste is a growing concern. Based on the alarming increase in thermoplastic commodities production and demand, it has been projected that about 12 billion metric tons of plastic waste will be in landfills by 2050. In addition, plastic waste enters the ocean at a rate of 11 million metric tons per year, harming marine life and damaging habitats [1] [2]. Thermoplastic commodities occupy a high-volume fraction in municipal landfills because of their relatively low density (0,9-0,95 g/cm^3). Some estimations forecast that the substitution of actual thermoplastic-based packaging by biodegradable plastics reduces almost 20% of the total sectorial waste by volume and 12% by weight [3].

Currently, thermoplastics such as polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP), and polystyrene (PS) make up a total of 60% of the overall plastic demand in the world. While these plastics are traditionally petrochemically derived, there is a growing demand to produce plastics using renewable resources (so-called “bioplastics”) as alternatives to their petrochemically derived counterparts (Figure 1). All bioplastics are produced starting from natural resources. However, not all are biodegradable. Bioplastics are a family of plastics that can be divided into two categories, biodegradable and non-biodegradable (Table 1). The diversity of biodegradable bioplastics is found in the variation in biodegradation rates and routes. Such plastics include polylactic acid (PLA), PHAs, cellulose, and starch.

Similar to oil-based plastics, biodegradable biobased plastics can be recycled or incinerated, but they are not widely recycled, as they are seen as contaminants in the current recycling

system. They may also be microbially degraded, allowing for alternative end-of-life management, such as industrial and home composting, anaerobic digestion, depending on the plastics' type, facilitating the development of a circular economy. Non-biodegradable bioplastics include bio-polyethene terephthalate (bio-PET), polyol-polyurethane, and bio-polyethene (Bio-PE) [4]. These polymers have the same chemical structure as their non-biobased counterpart, thus possessing the same properties.

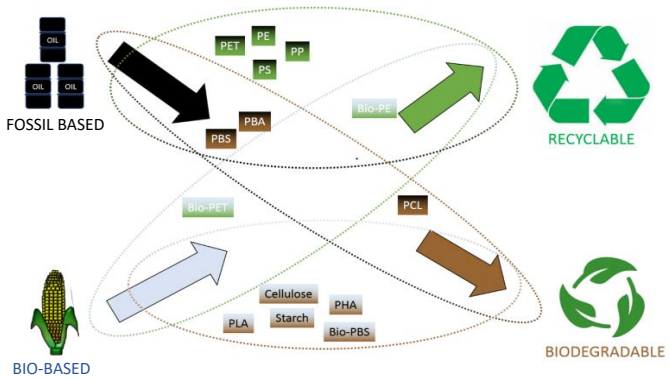

Figure 1. Four-way diagram of origin vs end-of-life management for different polymers

PHAs, together with PLA and polybutylene succinate (PBS), are considered the green polymers of the future since they are expected to gradually substitute conventional plastics with similar physicochemical, thermal, and mechanical properties such as PP and low-density polyethylene (LDPE). While PLA and PBS are produced upon polymerisation of lactic and succinic acid, respectively, PHA polymerisation is performed naturally by bacteria. At present, PHAs cover a niche in the field of the bioplastic market (around 1.4% of the biopolymers globally produced in 2018), mainly because of the non-competitive process costs if compared with cheaper biobased and biodegradable plastics such as PLA (2,0-2,5 €/kg) [5] [6].

Life cycle analyses (LCA) have shown that PHAs, depending on how they are produced, can outperform polyethylene or polypropylene in impact categories that include CO2 production, ecological footprint, and environmental toxicity [7], as well as global warming potential and non-renewable energy consumption [8]. However, several reports have indicated that the

production of PHA requires more fossil fuels than petrochemical plastics [9]. The impact appears to be largely dependent on the available carbon feedstock as well as the nature of the electricity source that is used in the production process. Despite mixed reports on life cycle analysis, there is still much optimism around PHAs, and it is anticipated that as production technology evolves, LCA will increasingly favour PHAs [10].

The current PHA price also depends on monomer composition, and it is generally higher for copolymers; overall, it ranges from 2.5 to 6 €/kg, which is less than the typical range of 10–12 € /kg reported at the beginning of the past decade. Despite the burden of costs and the environmental impacts of plastic trash, the current PHA prices are not commercially competitive compared to conventional petroleum-based polymers, which typically cost around 1.0 € /kg. Although the price of PHAs is high, several companies are producing PHA products worldwide, trying to meet the market's demand, including in the UK, Japan, US, Germany, Brazil, Italy, and China [11].

1.2 The problem of sewage sludges (SS) from wastewater treatment

In the last two decades, the European Commission adopted the Urban WasteWater Treatment (UWWT) Directive 91/271/EC (CEC, 1991) to guarantee an advanced wastewater collecting and treatment system. Wastewater treatment is unequivocally associated with the production of a large amount of sewage sludge, which requires suitable and environmentally accepted management before final disposal. The amount of sewage sludge produced in 2005 by the 27 EU Member States was about 11 Mton of dry matter. A great variety of sludge treatment technologies is available in the EU, among which anaerobic and aerobic digestions are the most widespread stabilisation methods, whereas thermal drying is the preferred dewatering approach. Sludge reuse (including direct agricultural application and composting) is the most popular final disposal strategy (53% of produced sludge), followed by incineration (21%) and landfilling (15%). However, the most common disposal method in the new Member States that joined the EU after 2004 is still landfilling. More stringent legislations than the Sewage Sludge Directive 86/278/EC (that encouraged the use of treated sewage sludge, or biosolids, in agriculture) have been adopted by several European countries for managing sludge disposal in soil, setting lower limit values for heavy metals, pathogens, and organic and inorganic toxic micropollutants. However, most of the current final disposal strategies neglect the potential use of sludge as a

feedstock for producing value-added chemicals or materials, losing C atoms and chemical energy stored inside this waste [5].

SS are an important source of biological matter commonly produced in the primary and secondary processes of WWT plants (WWTPs) (11 Mt/y in EU-27). SS handling is one of the most challenging and expensive operations for a WWTP since it accounts for 30–40% of capital costs and 50% of operating costs of the plant. Anaerobic digestion (AD), with its four steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis, is one of the most common and widely adopted methods for SS treatment and stabilisation for large-scale operation plants. In fact, in addition to reducing the overall amount of biosolids to be disposed of by about 40% and stabilising organic substances, AD produces biogas, the two main components of which are methane and carbon dioxide, which can be exploited for energy recovery. Other beneficial features of the AD process include inactivation and reduction of pathogens and improvement of sludge dewaterability [12].

1.3 PHA structure and properties

PHAs are biologically synthesised polyesters, which are fully biobased and biodegradable, presenting thermoplastic properties similar to some commodity polyolefins. Over 150 different types of hydroxyalkanoate (HA) monomers have been identified, synthesised by over 300 bacterial species as a carbon and energy storage reserve. PHA molecular weights range from $1x10^4$ to $3x10^6$. The properties of PHA depend mainly on monomer composition, microstructure (randomly distributed monomers or organised as block copolymers), and molecular weight distribution [13]. PHAs have a semicrystalline structure (degree of crystallinity between 40% and 80%). Varying the chain length of PHA monomers affects the polymers' thermal properties, such as melting and glass transition temperatures and degree of crystallinity. Many different monomer units can be incorporated into PHA polymers, and an example of the structures of these monomers is shown in Figure 2. In general, PHA monomers may be divided up into three main classes: (i) short-chain-length (SCL)PHA, which consist of monomers with chain lengths of 3–5 carbon units; (ii) medium-chain-length (MCL) PHA, which consist of monomers with chain lengths between 6 and 14 carbon units; and (iii) long-chain-length (LCL) PHA, which are composed of monomers with carbon chain lengths greater than 14 units. These monomers can be incorporated to form homopolymers or copolymers with various physical properties. Polymers composed solely of SCL monomer units generally have

thermoplastic properties, while polymers composed of MCL subunits generally have elastomeric properties. PHA copolymers with a relatively high mol% of SCL monomers and low mol% of MCL monomers have properties similar to the bulk commodity plastic polypropylene [14].

The poly-3-hydroxybutyrate (P(3HB)) or PHB is by far the most observed PHA. It exhibits functional thermoplastic properties; its melting temperature is close to 180 °C, and the glass transition temperature is around 4 °C. However, due to its high crystallinity (55–80% crystalline), P(3HB) is fairly stiff and brittle, limiting its applications, especially as a sole packaging material. The elongation to break (flexibility) is about 2–10% for P(3HB), compared to 400% for polypropylene. Copolymers of PHAs vary in the type and proportion of monomers. Incorporating SCL units other than 3-hydroxybutyrate (3HB), such as 3-hydroxyvalerate (3HV), reduces polymer crystallinity by disturbing the crystal lattice. These copolymers exhibit lower crystallinity and superior mechanical properties over P(3HB). They also generally present lower melting and glass transition temperatures compared to P(3HB). Because the temperature at which degradation of PHA occurs is rather insensitive to composition, the lower melting temperatures of these copolymers enable them to be processed at lower temperatures than P(3HB), reducing molecular weight losses. These copolymers also present a higher melt viscosity, which is a desirable property for extrusion blow moulding. PHA copolymers that include medium-chain monomers, such as 3-hydroxyhexanoate (3HHx), for example, P(3HB-co-3HHx), are elastomers and have a much lower melting point and glass transition temperature than the P(3HB) homopolymer. Their molecular structure is analogous to soft polypropylene. This is due to chain defects that cause crystal disruption and enhanced molecular entanglement, resulting in a highly amorphous material [15]. Examples of other PHA polymers made with fluorinated side chains derived from synthesised nonanoic acid and fluorinated acid co-substrates are also available. Monomers with conjugated sidechains, once incorporated into a PHA polymer chain, can also be chemically modified to increase the polymer's functionality and the number of potential applications [16] [17].

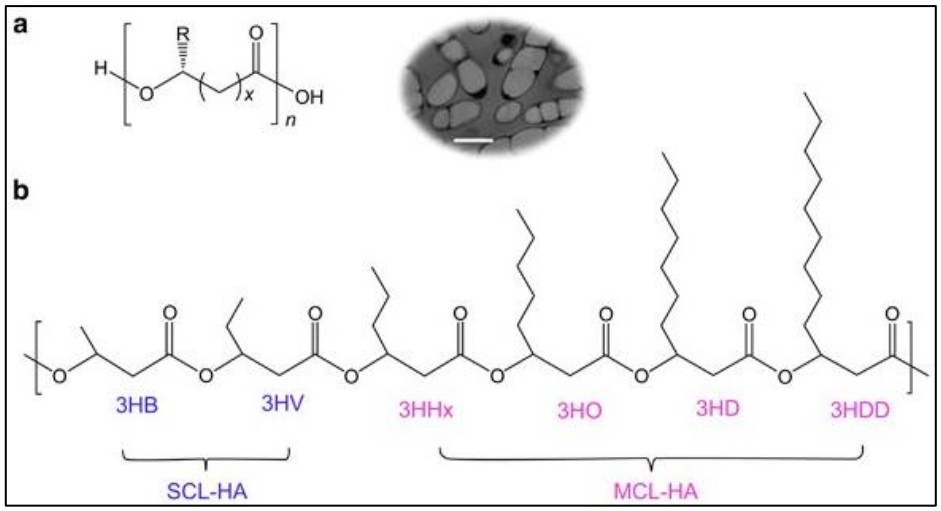

*Figure 2. (**a**) General molecular formula of PHAs. Typically, x = 1–8, and n ranges from 100 to 1000 s. (**b**) Some commonly synthesised short-chain-length PHA monomers (SCL-HA) and middle-chain-length PHA monomers (MCL-HA)* [18].

1.4 Key factors in the production of PHAs

Typically the industrial-scale production of PHA has been carried out via microbiological pathways [19]. When considering a sustainable large-scale production system of microbial polymers, the following criteria must be considered: economic, ethical, environmental, and engineering. Considering these constraints, the production of biopolymers should be based on (i) the use of co-products and wastes as a substrate; (ii) cheap or inexpensive microbial cultivations, for example, the use of open mixed microbial cultures (MMC) instead of sterile, pure cultures; (iii) finely tuned microorganism cultivation strategies and cultivation variables, (iv) extraction of biopolymers by using low cost and environmentally friendly processes [20]. Each of these considerations has been make clear in this section.

- The choice of feedstock for the production of biopolymers consists of several fundamental principles: (i) suitability for storage and transport; (ii) constant availability in the vicinity of the production plant and no competition with other applications; (iii) high content of organic matter with low toxicity to microorganisms and, if possible, a high specific ratio between chemical oxygen demand (COD) and nitrogen (N) or phosphorus (P) in order to match with metabolic requirements for producing biopolymers (i.e. nutrient limitation triggers biopolymer production). Moreover, the price of substrates strongly impacts the selling price of biopolymers, accounting for almost 30% of the production cost [21]. The main substrates used for the selective growth of PHA-storing organisms and PHA production have been volatile fatty acids

(VFAs) since VFAs are efficiently converted into PHAs [22]. In contrast, carbohydrates are preferably stored as polysaccharides [23]. It is worth mentioning that the distribution of VFAs is known to affect the PHA monomer composition. VFAs with an even number of carbon atoms tend to produce PHB, while VFAs with odd carbon atoms tend to produce PHBV copolymers with different % HV molar fractions. Based on this fact, many studies have suggested the possibility of regulating the PHA composition by manipulating the fermentation conditions in the preceding acidogenesis step [24] [25] [26]. At present, commercial production of VFA is mainly accomplished by chemical routes. However, the use of non-renewable petrochemicals as raw materials and the increasing price of oil have renewed the interest in biological routes of VFA production. In biological VFA production, pure sugars such as glucose and sucrose have been commonly employed as the main carbon source, raising the ethical concern on using food to produce chemicals. This issue can be resolved by utilising organic-rich wastes such as sludges generated from wastewater treatment plants, food waste, organic fraction of municipal solid waste and industrial wastewater for VFA production via anaerobic digestion. Such transformation of waste into VFA also provides an alternative route to reduce the ever-increasing amount of waste generated. A complete review of the production of VFA from waste biomasses and a general overview of waste feedstocks for PHA production can be found in the bibliography [11] [27] [28].

- Concerning the bacterial cultivation type, the main distinction has to be made between pure and mixed microbial cultures. Currently, industrial PHAs production is conducted using natural isolates or engineered strains and pure substrates. An alternative scenario that would contribute to the reduction of the PHA production cost is to employ mixed culture biotechnology [29]. This approach uses open (under non-sterile conditions) MMC and ecological selection principles, where microorganisms able to accumulate PHA are selected by the operational conditions imposed on the biological system. Thus, the principle is to engineer the ecosystem, rather than the strains, combining the methodology of environmental biotechnology with the goals of industrial biotechnology [30]. MMC have shown several advantages over pure cultures, such as (i) the possibility of using a wide range of substrates (included low or negative valued feedstocks) due to the broadening of metabolic potentials; (ii) the degradation of certain recalcitrant compounds due to synergies between microbial species; (iii) the tolerance to non-axenic conditions; (iv) the ability to better resist to potential inhibitors, present in the feedstock or produced during the culture; (v) an increase in growth yield; (vi) better protection against contaminants, since all ecological niches are already occupied; (vii) increased resistance to

fluctuating environmental conditions; (viii) the overall process simplicity and cost reduction. In certain circumstances, MMC can also represent an alternative to genetic manipulation (for example, the construction of long and complex metabolic pathways, which can be time-consuming and costly). Besides these advantages, the use of MMC also presents major challenges which have to be addressed. Indeed, the most often highlighted difficulties in scientifically investigating such complex mixed cultures and controlling the optimum balance between microorganisms involved in the community. In terms of biopolymer production, one of the biggest challenges is to identify the specific conditions that must be applied to the culture to promote only the microbial community able to produce targeted biopolymers while achieving community stability over time [20]. For a complete study on the possibilities of producing PHA from pure cultures, the reader can look at his work [19].

- One of the critical steps in PHA production processes with open mixed cultures is the selection/enrichment of superior PHAs accumulating bacteria in a mixed culture. Activated sludge with significant PHA storage capacity was first observed in aerobic WWTPs where selectors for bulking control were introduced. This process configuration creates periods of excess and lack of external carbon substrate. Conditions of carbon excess and limitation, named feast and famine (FF) or aerobic dynamic feeding (ADF), were simulated in lab-scale reactors, and the selected microbial population showed an enhanced capacity to store PHA (PHA accumulation potential, PAP). It has been proven that the amount of intracellular components required for cell growth (RNA and enzymes) tend to decrease after a certain period in the absence of an external carbon substrate. Thus, when carbon substrate becomes available again, these components may not be enough to ensure a maximum growth rate [31]. Oppositely, fewer enzymes are required for PHA storage, which can occur faster than cell growth, providing the cells with a means of rapidly consuming the available external substrate. The need for physiological adaptation (replenishment of intracellular compounds required for growth) following each famine period is considered the primary mechanism inducing PHA storage in microorganisms subjected to FF conditions [32]. Following long periods of starvation, fully aerobic activated sludge cultures were shown to convey much more carbon toward storage than toward cell growth and maintenance [33]. After the depletion of the external carbon substrate, the stored PHA is the only carbon and energy source available for these processes. Consequently, microorganisms that are quicker to store and reuse the substrate for growth possess a competitive advantage in such ecosystems [34]. ADF allows for selecting cultures

with a high and stable capacity of PAP during the feast and good capacity to grow on PHA during the famine phase.

PHA storage is also observed in Enhanced Biological Phosphorus Removal (EBPR) systems which are run under alternating anaerobic/aerobic cycles. The main groups of bacteria selected are polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs). In anoxic conditions, PAOs and GAOs take up the carbon substrate and store it as PHA (they can produce copolymers of HB, HV and 3-hydroxy-2-methyl valerate even using acetate as a sole C source [35]). At the same time, glycogen or polyphosphates, second storage polymers, are consumed. PHA is used in the subsequent aerobic phase for cell growth, maintenance, and glycogen/polyphosphate pool replenishment [36]. Under anoxic conditions, GAOs derive their ATP requirements and reducing equivalents from glycogen catabolism through glycolysis [15]. Such cultivation conditions allowed to achieve a PHA content of up to 30% of total suspended solids (TSS) [37]. Although culture enrichment under dynamic conditions of oxygen availability (Figure 3a) differs from fully ADF processes (Figure 3b) in terms of availability of the final electron acceptor, it still falls under the definition of an FF regime. Indeed, the external carbon substrate is provided only in the absence of the final electron acceptor, thus limiting growth during this period. Only in the subsequent aerobic stage, the cells can grow using the then available oxygen for oxidative phosphorylation. However, at this stage, the external substrate has already been depleted. Just as for fully aerobic FF, the transient availability of carbon substrate, and consequent internal growth limitation, create strong pressure for PHA storage. The sole imposition of anoxic/aerobic phases (in the presence of external carbon substrate throughout the full cycle) would not select PHA-storing cultures. The two main differences between anoxic/aerobic FF and fully aerobic FF relate to (i) cell growth rate and (ii) polymer composition. In anoxic/aerobic FF, cell growth occurs only from the intracellular polymer, and so the observed growth rate is typically much lower than in ADF [38], in which cell growth can occur from the external substrate together with PHA storage. Regarding polymer composition, the presence of a second storage compound such as glycogen (whose degradation produce different precursors for PHA synthesis) usually allows for a copolymer production starting from a single C source.

Generally, anoxic/aerobic FF systems select cultures with lower productivities than those reported for ADF systems but with a broader range of polymer compositions [39] [40]. A complete summary regarding the main characteristics of selection and enrichment techniques applied for MMCs is available in the bibliography [41].

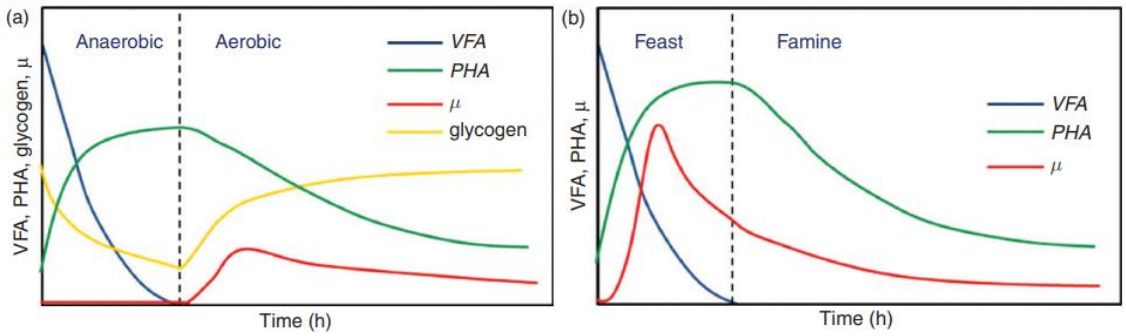

Figure 3. Mechanisms for PHA storage by mixed microbial cultures under (a, left) anoxic/aerobic feast and famine and (b, right) fully aerobic feast and famine. μ represent the cell growth rate in h^{-1} [15].

PHA production processes from MMC is heavily affected by the operating parameter imposed on the selection bioreactor. In principle, the selection process could be operated at so high organic load rate (OLR) that the PHA content in the biomass at the end of the feast phase is high enough to be already harvest for PHA extraction. Regardless, the need to impose FF conditions limits the organic load. Thus, most MMC PHA production studies are usually operated in two separate stages: culture selection and PHA accumulation. Physical separation of the culture selection and PHA production stages allows for their individual optimisation, as different conditions were shown to be required in each stage (shown in Table 1).

Table 1. Common values of operating parameters used for the selection of PHA accumulating culture [11].

Operating parameter	*Range*
Related to reactor operating parameters	
Sludge retention time	1–20 day
Hydraulic retention time	1–3 day
Length of SBR cycle	2–12 h
pH	7–9.5
T	20–30 °C
Operation mode	SBR or two continuous reactors sequentially disposed
Related to feedstock	
Organic loading rate	1.8–31.25 g-COD l^{-1} day^{-1}
Substrate concentration	0.9–31.25 g-COD l^{-1}
C/N ratio	9–120 g-C g N^{-1}
Resultant from both feeding and reactor parameters	
F/F ratio	0.1–1.15

The culture selection stage aims to develop reactor operating strategies that can maintain the selective pressure given by FF conditions toward cultures with high PAP while operating at an OLR and short retention time (i.e., high growth capacity). The selection of microbial cultures

that could exhibit a high growth rate without compromising a high storage capacity would allow the reactor to be operated at higher cell concentration, thus allowing the subsequent accumulation stage to be carried out with a more concentrated biomass inoculum. Despite the knowledge gathered on culture selection, operating the reactor to select for a stable culture over time with both a high growth rate and a high PAP is still a major challenge [42]. In addition, most of the available agro-industrial waste/surplus biomass feedstocks cannot be directly converted to PHA because MMCs do not store carbohydrates as PHA but rather as glycogen. Thus, PHA production by mixed cultures from waste and surplus-based feedstocks requires a previous anaerobic fermentation stage to convert their organic content into VFAs. The resulting process usually comprises three stages that need to be separately perfectionated (Figure 4): (1) the acidogenic fermentation stage, (2) the culture selection stage, and (3) the PHA production stage [43].

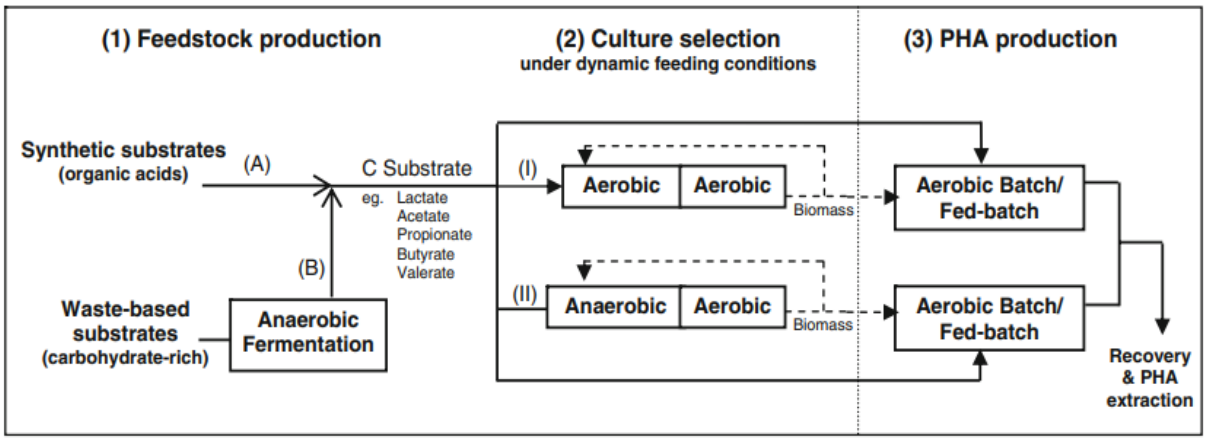

Figure 4. Three-steps PHA production process by mixed microbial cultures using either synthetic (A) or waste-based substrates (B) and conducting PHA-accumulating culture selection using Aerobic/Aerobic (I) or Anaerobic/Aerobic (II) dynamic feeding strategies. PHA production (step 3) is carried out in batch/fed-batch mode using the cultures enriched in step 2 and the feedstock produced in step 1 [39].

As in the enrichment step, also in the PHA-productive step several operational parameters impact the PHA accumulation. However, reactor configuration and culture mode are the most pivotal. High substrate concentrations supplied under batch mode should be avoided since they can cause substrate inhibition and thus limit PHA productivity.

In order to circumvent that, several sequencing batch reactors (SBR) and fed-batch strategies have been suggested, especially in bench-scale studies. Pulsed fed-batch cultivation modes are adopted when synthetic VFA mixtures are used [44]. However, due to an increase occurring in the working volume after substrate addition, the feed should be very concentrated. Nevertheless, this is rarely the case with fermentation effluents since they hardly exceed 20 gCOD/L. Discharge of the exhaust supernatant has been suggested as an alternative yet; this approach requires a settling step between batches, which severely limits the productivity [45]. Continuous feeding processes have shown the best performances until now, given that they can attain sustained productivity; still, they are not the most studied systems at bench scale due to their relative difficult process control, while they possibly be the best option for industrial scale production [41] [46]. Pulsed fed-batch production may result in high PHA productivity, but as the substrate is being consumed, PHA productivity eventually decreases. This phenomenon might be avoided by continuously supplying substrate. Continuous substrate addition has been successfully performed using the pH as an indicator, given that the pH increases with VFA consumption [47] [48]. On the other hand, less successful results were obtained when the substrate was supplied taking into account previously observed substrate uptake rates, resulting in either accumulation or limitation of the substrate in the reactor [44]. Alternatively, an on-demand continuous addition of substrate, based on change of dissolved oxygen (DO), has been proven efficient in maintaining optimal amounts of carbon substrate in the reactor [49].

Overall, the economic viability of the PHA production process by mixed cultures is highly dependent on the ability to select a microbial population with both a high cell growth rate (to achieve high cell concentration) and a high PHA storage rate. Only then volumetric productivities become sufficiently competitive for large-scale production. Up to the last studies, the ideal configuration would be a small SBR to enrich the culture broth in organisms with a high cell growth rate and high PHA storage capacity. The biomass produced per cycle should match the biomass required to inoculate a larger bioreactor for production purposes. The latter reactor should be operated in fed-batch mode, similarly to pure cultures, for example, with a cell growth phase followed by a PHA production phase with cell growth limitation. The operational strategy is certainly different in these two reactors. The best strategy for sludge enrichment seems to be adopting a feast and famine strategy with ammonia in excess. The excess of ammonia increases the selective pressure of organisms with a high cell growth rate, and, when combined with the feast and famine regimen, it also favours the organisms with a high PHA storage capacity [43]. Recent summaries showing the state-of-art operating

conditions and single-unit performances of two and three-stage processes are available in the bibliography [41] [46].

- So far, significant research efforts in the field of MMCs have been focused on: (i) the use of cheap wastes and feedstock as carbon sources (e.g., poultry litter, glycerol, molasses, grass), (ii) the improvement of PHA storage capacity of the bacteria (by adopting multi-steps cultivation strategies), (iii) the development of continuous process at lab or pilot-scale. However, little information is available on the characterisation of PHA extracted from MMCs and, above all, on the efficiency of the polymer recovery in the extraction step [26]. As well described in the literature, obtaining PHA from bacteria through a series of downstream steps (e.g. microbial biomass pre-treatment, polymer extraction and post-treatment purification) could be challenging and expensive [50]. Specifically, the downstream cost can cover up to 50% of the total production costs, involving extensive use of non-recyclable (and sometimes highly toxic) chemicals/materials and high energy consumption [51]. Moreover, it is worth mentioning that operating with MMCs introduces a further issue. MMCs are claimed to be more resistant to cell hydrolysis than pure cultures in which genetic manipulation and/or cell constraints, due to high polymer amount, increase cellular fragility. Thus, the effort required for PHA extraction/purification can become much more relevant than in the case of single strains. Researchers have hypothesised the existence of a PHA fraction difficult to be extracteddue to a and complex Non-Polymer Cellular Matrix/Mass (NPCM) that surrounds the polymer, preventing the access of the solvent to polymer granules [26]. Comprehensive reviews about PHA downstream/purification processes and extraction economics can be found in the bibliography [52] [53] [54].

1.5 Chemical Oxygen Demand (COD) as a unit of measure to monitor biomass and organic concentration

Chemistry and biology are relatively distant research areas. To help in the comprehension of this work, it is helpful to use a standard unit of measure of chemical energy that should be readily applicable in both aqueous biological systems and thermochemical processes.

COD measure of the oxidizability of a substance, expressed as the equivalent amount in oxygen of an oxidising reagent consumed by the substance under fixed laboratory conditions. COD is commonly used to measure the concentration of all the organics in wastewater treatment. However, it is also a direct and valuable measurement of chemical energy stored in biomass or

solutions (as COD concentration). 1 kg of COD corresponds to a certain amount of organic matter that needs 1 kg of oxygen to be completely oxidised to CO_2. From the theory, COD is proportional to the number of electrons transferred from water to organic molecules during biomass production. It is commonly expressed as the mass of oxygen consumed over the volume of solution in grams per litre (g/L).

An approach for a precise but time-consuming estimation of the biomass in wastewater is calculating it in terms of Volatile Suspended Solid (VSS). To overcome the problems related to this kind of measurement, several studies [55] [56] [57] suggested measuring the biomass concentration in terms of COD. In addition, COD can be used for the mass balance of three-stage processes, evaluating overall performances from the fermentation of waste streams to the production of PHAs [58].

1.6 B-PLAS project and process: Linking the need for PHA bioplastics and the treatment of ADSS

B-PLAS aims to realise 1 to 1 scale plants to convert agro-industrial waste, food waste and other exhausted feedstocks from anaerobic digestion into PHA biopolymers, developing a robust and reliable industrial process. The process is made to produce PHAs bioplastics at competitive market costs using a sequence of consolidated and innovative technologies that aim to valorise the residual carbon fraction stored in negative value feedstocks (disposal cost: 5-120 €/wet ton) after biomethane production. Multiple goals are achievable within the context of this project, including the volumetric reduction of the organic waste to dispose of, the production of platform molecules like VFAs and the obtainment of PHA from MMC. In addition, the different process units can be individually optimised depending on different physical-chemical properties of the waste feedstocks to treat, possibly meeting the requirements of several industrial sectors and companies [59].

The process is based on different technologies (Figure 5): (i) a hydrothermal carbonisation unit (HTC) to thermally increase the C fraction of the process inlet available for VFA production, (ii) an anaerobic acidogenic digestor converting the soluble COD into VFAs, (iii) a solid-liquid separation unit (filter press) separating the fermentation broth enriched in VFAs, (iv) bioreactors for PHA production from MMCs and (v) the PHA extraction unit. At the moment, the inlet stream of the process is represented by the semi-solid, viscous fraction resulting from

anaerobic digestion and centrifugation of agro-industrial wastes at Caviro Extra's facilities (Faenza, RA), the same location of the described pilot plant.

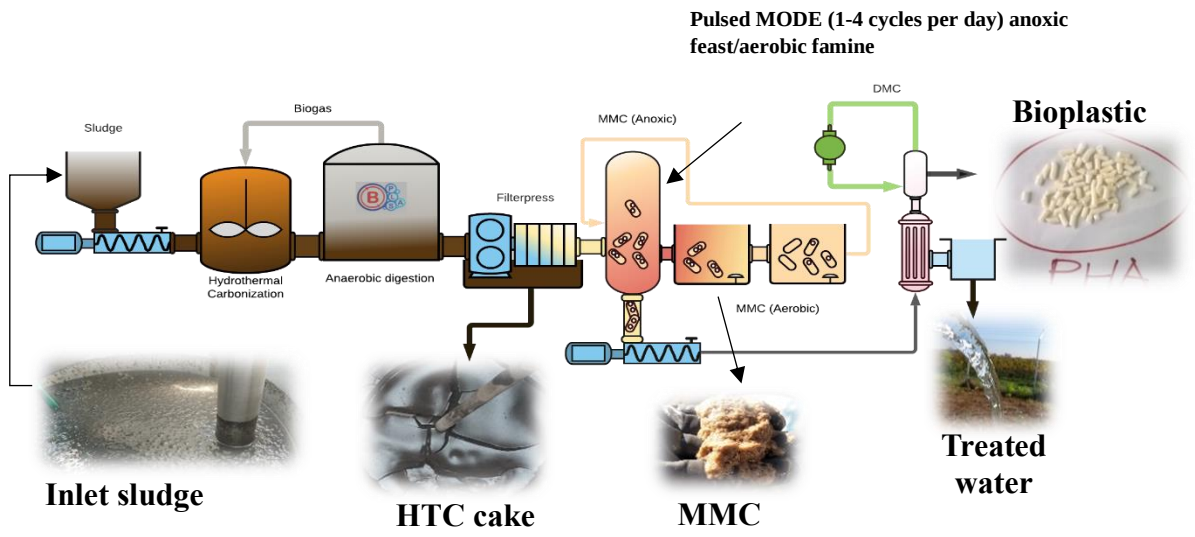

Figure 5. B-PLAS graphical process scheme.

- *HTC unit*

This thermochemical unit aims at increasing the bioavailability of the residual organic fraction in the inlet sludge thanks to high pressures and temperatures (up to 18 bar and 200 °C) under agitation (to avoid the precipitation of the solid component present in the inlet). By doing so, almost half of the total COD can be solubilised in the liquid fraction, increasing the readily fermentable substrate otherwise unretrievable. The sensible heat of the output liquid is then recovered with a counterflow heat exchanger. This unit allows for an increased Yield of VFAs COD/inlet total COD in the following fermentation step.

- *Anaerobic acidogenic digestor*

This bioreactor allows for selective conversion of soluble COD in the effluent coming from HTC into VFAs. Thanks to low residence times and thermophilic conditions created by two electrical resistors, it is possible to minimise biogas production. For the initialising phase, the inoculum was taken from the inlet stream of the B-PLAS process since the anaerobically digested sewage sludge already contains useful bacteria for acidogenic digestion (Figure 6).

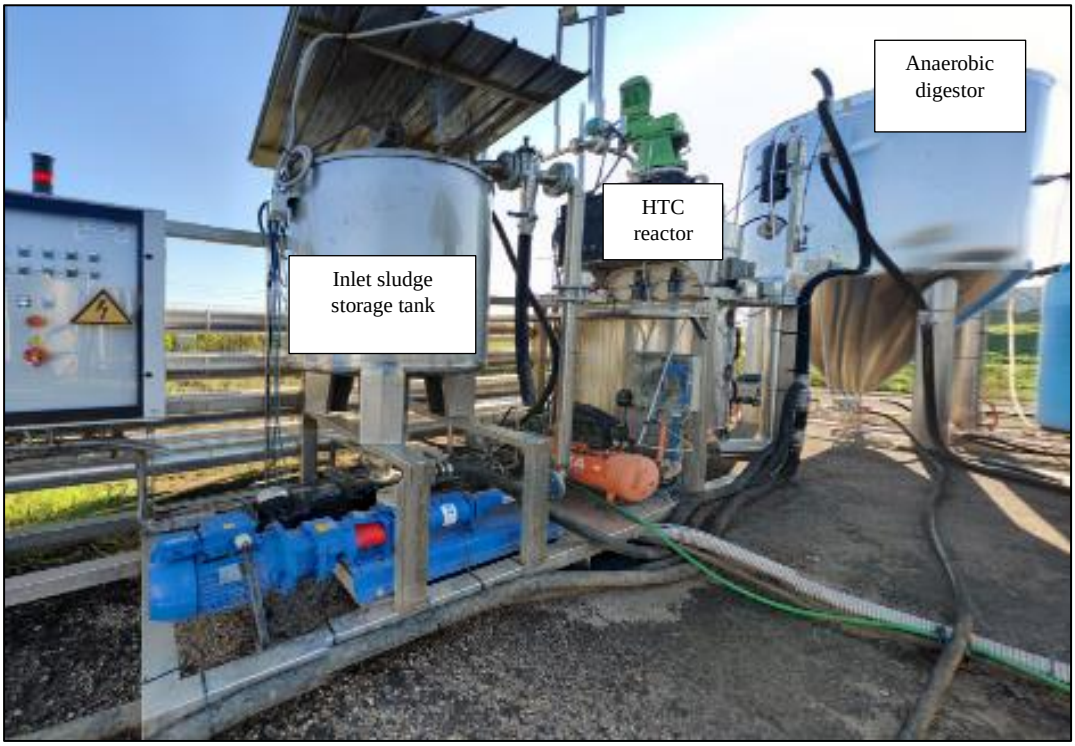

Figure 6: HTC unit and anaerobic digestor.

- *S-L separation unit*

The filter press can separate in situ the effluent stream of anaerobic acidogenic digestion. The VFA-rich fermentation broth is going to be fed into the aerobic bioreactors, while the solid cake has been proven to be suitable for agricultural spreading or composting.

- *Aerobic bioreactors for MMC cultivation (Figure 7a)*

The first 5m^3 metallic vessel (R1) has been used for the selection and the acclimatisation of a culture broth with high PAP. Spargers and stirrers guarantee aeration. The feeding is composed of the stream containing the VFAs collected in the previous step, a synthetical solution of macro and micronutrients and slightly alkaline water. The FF feeding strategies and the operational parameters determine the characteristics of the selected PHA-storing biomass. It is worth mentioning that the first inoculation was done with a lab-selected activated sludge, which was dominated by *Azoarcus sp., Amaricoccus sp., Tahuera sp., Rhodobacter sp.* (unpublished data).

Acclimatised MMCs have eventually shown comparable characteristics to activated sludges used in WWT.

Concomitantly to the feed pulses in the first reactor, the selected suspended biomass overflows in the following 1 m^3 polyethylene tank (PHA enrichment bioreactor, R2). Here, the MMC is fed again with the same VFA-enriched solution, but it is subjected to Phosphorous limitation. The batch-like configuration allows for a PHA-enrichment up to 50% w/ dry w of the cell dried matter. Surprisingly, the highest contents in PHA were obtained during summer, when the dissolved oxygen concentration (DO) in the accumulation reactor decreased due to environmental conditions and process issues.

The active biomass in the fermentation broth is then concentrated via S-L separation (first by sedimentation that a tangential filtration). The resulting viscous slurry is mixed with Dimethyl carbonate (DMC) and conveyed under pressure to the extraction unit.

- *PHA extraction unit*

This system includes several sub-units (Figure 7b):

(i) a reactor for the pressurised solvent extraction of the slurry biomass with DMC (110 °C, 5-10 minutes) that selectively dissolves the microbial PHA
(ii) a Solid-Liquid separation system of the aqueous (centrate) and organic (filtrate) phases via filtration with sintered metal filters (40 °C)
(iii) an air ejector (Venturi centrifugal pump) that creates the vacuum necessary for the following sub-units and collects the vapour streams resulting from them
(iv) two batch vacuum dryers in which the filtrate organic fraction is dried, the DMC/residual moisture mix is recovered, and the PHA residues are manually collected
(v) a 5L vacuumed stripping chamber where the biomass in the centrate is separated from residual DMC
(vi) an 8L phase-separation tank which collects supersaturated vapour from the ejector and allows for gravitational separation of water and DMC

The products composing the output of the B-PLAS process are here listed in order of mass relevance: (i) a dried sludge resulting from the filter press, (ii) an aqueous effluent from the

MMC bioreactor, (iii) dried PHA pellets or chips, (iv) biogas stream from the anaerobic acidogenic digestor (up to 98% of CO_2).

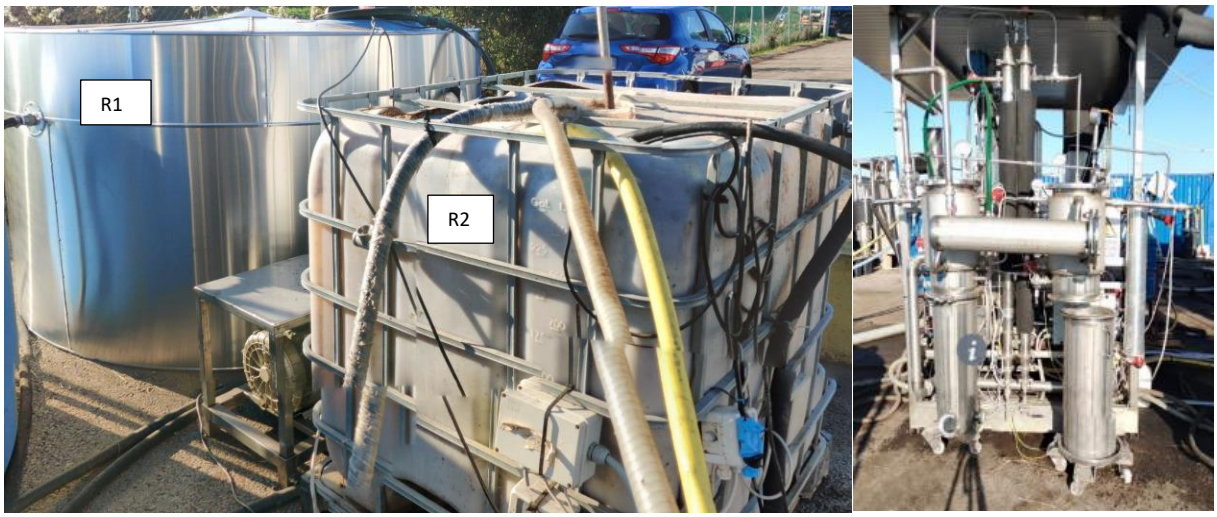

Figure 7. Aerobic bioreactors for the selection and production of PHA from MMC (a, left) and polymer extraction unit (b, right).

1.6.1 Unexpected results during summer 2020

Promising results were recorded during August 2020 when a lower DO concentration due to spargers' fouling and high temperatures led to interesting performances in R1 SBR. The concentration of %PHA in the selection reactor R1 reached 30-50% PHA content w/dry w (with 0,18-0,32% yield of gCOD consumed VFA /gCOD produced PHA). R2 has worse performances of 20-30% PHA content with a maximum yield of 0,2 PHA/VFA. The polymer's composition also shows the presence of HV monomers (a typical monomer that GAO/PAO can synthesise). In the created anoxic or microaerophilic (MAA) conditions, a selective pressure favouring a particular species of bacteria in the selection reactor could have occurred, thus leading to a substantial change in the composition of the MMC (>85% *Azospirillum sp*.). In any case, this behaviour has not been studied over a prolonged time span.

The fact that such uncontrolled conditions have led R1 (SBR) to accumulate more PHA than R2 (batch-like reactor) triggered this research indeed.

1.7 Aim of the thesis

Starting from the evidence that emerged from the pilot plant, two goals were set for this work.

1) Anoxic/aerobic selection reactor.

Test if a lab-scale, single-stage selection SBR can be implemented under anoxic/aerobic FF, to consistently achieve some 25-35% w PHA/dry w content at the end of the feast phase, minimum values for the economics of the extraction process. The anoxic zone is devoted to substrate storage as PHA, while the aerobic zone is required for cell maintenance and growth. This goal had to be proven by providing a simulated fermentation broth made of a synthetic solution of Acetic acid in tap water (C substrate) and real wastewater (the centrate of an agro-industrial AD) as a macronutrients source. Other process parameters that will be tuned are the aeration condition, OLR and P availability. Finally, the performances of this setup will be compared with an identical SBR subjected to aerobic FF cycles, which is known to allow for a max of 20% w PHA/dry w at the end of the feast phase [60].

2) Process optimization of the B-PLAS pilot-scale plant.

On the result of the lab-scale study, develop an on-demand PHA accumulation process to enable up to 45-50% w PHA /dry w content using an extended anoxic feast in the absence of nutrients. This process optimization will be tested on both bench-scale (in the single-stage reactor) and possibly on pilot-scale at the. In this latter test, a real fermentation broth (both VFA and nutrients from real waste streams) will be used to perform the MMC enrichment in PHA-accumulating microorganisms and the production of PHA-enriched biomass (>45%) after a prolonged period of anoxic feast. This approach may extend the application and process reliability compared with the conventional three-step process. In addition, it could simplify the process while reducing requirements such as balancing the influent nutrients and the aeration-related expenses.

2 Materials and methods

2.1 Solvents and reactants

All the following chemicals have been used without further purification.

- 2-Ethylbutyric acid ($C_6H_{12}O_2$) >99%, Sigma Aldrich;
- 3-Methylbutanoic acid ($(CH_3)_2CHCH_2COOH$) >99%, Sigma Aldrich;
- Acetic acid (CH_3CO_2H), ≥99.5%, Sigma Aldrich;
- Acetone (CH_3COCH_3) ≥99.5%, Sigma Aldrich;
- Acetonitrile (CH_3CN) 99,8%, Sigma Aldrich;
- Butyric acid ($CH_3CH_2CH_2COOH$) >99%, Sigma Aldrich;
- *trans*-2-butenoic acid (Crotonic acid), ($CH_3CH=CHCOOH$), 98%, Sigma Aldrich
- Dimethyl carbonate ($C_3H_6O_3$) >99%, Sigma Aldrich;
- Dipotassium hydrogen phosphate (K_2HPO_4) ≥ 99%, Sigma Aldrich;
- Hexanoic acid ($CH_3(CH_2)_4COOH$) >99%, Sigma Aldrich;
- Isobutyric acid ($(CH_3)_2CHCO_2H$) >99%, Sigma Aldrich;
- Methanol (CH_3OH), 99.8% Sigma Aldrich;
- Poly-3-hydroxybutyrate, MW: 0,8 MDa, PDI: 5,9 , Biomer;
- Potassium hydrogen sulfate ($KHSO_4$) reagent grade, Sigma Aldrich;
- Propionic acid (CH_3CH_2COOH) ≥99.5%, Sigma Aldrich;
- Sodium bicarbonate ($NaHCO_3$) ≥ 99.5%, Sigma Aldrich;
- Sodium Chloride (NaCl) 99,8 %, Sigma Aldrich;
- Sodium hydroxide (NaOH) > 97%, Sigma Aldrich;

2.2 Reactor set-up and operations

Laboratory operations started on January 25, 2021, and were stopped on July 8, 2021 (164 days). The whole set-up has been iteratively developed trying to replicate the B-PLAS process and with the intention to be eventually applied back to the same pilot plant.

2.2.1 RANA and RAE

Two identical lab-scale plastic bioreactors with a working volume of 5L (Ø: 28 cm) were studied in parallel. They were individually inoculated with 2 g of freeze-dried PHA-producing aerobic sludge collected from the B-PLAS pilot plant. The first bioreactor was operated under fully aerobic conditions (for the purpose of this work, it will be called RAE, light blue colour in the figures). It was used as a control reactor to test out the performances of the second anoxic-aerobic reactor (here called RANA, green colour in the figures). RANA has been run under the same conditions as RAE, except for the aeration cycles it received and for a plastic cap that limited the air exchange with the surroundings.

Both RANA and RAE reactor (Figure 8) were equipped with (i) a piston compressor (2000 L air/h, Hailea ACO 208) controlled by an analogic timer (15-minute intervals mechanical timer, Goodbay) which provided aeration in excess through a circular, porous stone sparger (Ø: 11 cm, ATPWONZ), (ii) a small submersible water pump used for culture broth recirculation and stirring purposes (200 L/h) and (iii) a pH and a T probe. In addition, a bellow metering pump regulated by an analogic timer was used to supply both the C substrate and the nutrient solution to the reactors. The stirring capacity was due both to the sparger and the liquid recirculation pumps; the bioreactors were assumed perfectly mixed; thus, the outlet concentration was assumed equal to the concentration inside the reactors. To simulate the outdoor B-PLAS bioreactors, the temperature ranged from 18 °C to 33 °C throughout the operation months. The pH was not regulated too, so the only processes able to vary the pH of the reactors were the addition of VFA stock, the addition of nutrient solution and the consumption of C source by the MMC.

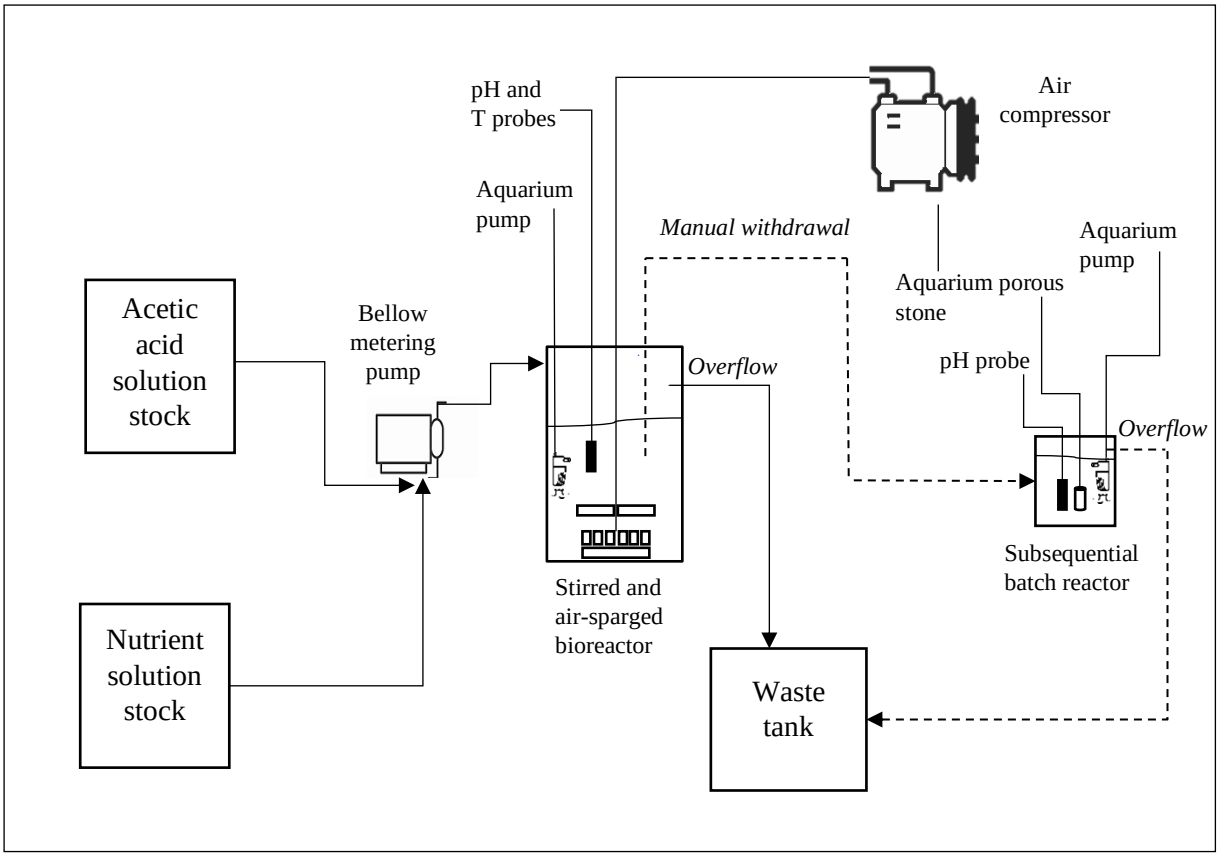

Figure 8. Schematic process flow diagram of the bioreactors studied in this work. Discontinuous arrows indicate batch processes.

Selection of a PHA-accumulating culture was carried out in such SBR-like reactors subjected to ADF without intermediate settling or idle phase (continuous reaction period) [41]. Consequently, the solid retention time (SRT) was equal to the hydraulic retention time (HRT); these parameters varied depending on the number of ADF cycles per day. Each SBR cycle consisted of 15 min influents. The C substrate and the nutrient solution were provided in couple to the systems, a variable feast and famine period length (depending on the number of cycles per day) and a withdrawal phase (1 min). The end of the feast phase was evaluated as explained in section 2.3.3. The end of the famine period (so the end of the cycle) was assumed to be the last moment before the following influent addition (also called 'Prefeed'). During period 1 (53 days), RAE and RANA were subjected to 2 feed cycles per day with 1L of total influent per reactor per cycle (HRT = 60 h). RAE was fully aerated during the 12 h of FF cycle, RANA was not aerated for the first 4 h (simulated anoxia), then aeration started for the final 8 h. During period 2 (107 days), the length of the SBR cycles was set at 8h accounting for 3 feed cycles per day (HRT = 40 h), while the influent volume for each feeding cycle was kept the same (1L).

Here RANA was subjected to 2 h 30 min of anoxia right after the influent addition, followed by 5 h 30 min of full aeration. Following the outcomes of some interesting papers [61] [62], during period 2 RANA was also subjected to MAA-like oxygen limitation, since this condition was proven to allow for a PHA content up to 60% of TSS [37]. Specifically, during a week of test, the aeration was set at 1 h anoxia followed by 2 intermittent sparging of air (15 min compressor on - 45 min compressor off) and then 5 h of aerobiosis. In this condition of oxygenation, the accumulation potential of an MMC enriched in GAO (selected by period 1 and period 2 conditions) should increase at the expense of the process of biomass growth.

2.2.2 Sampling and maintenance

Every working day, mixed liquor samples of approximately 30 mL were collected from RANA and RAE at the end of the famine phase and the end of the following feast phase. Total and soluble COD, Acetic acid residual concentration, and PHA content were extensively monitored throughout Period 1 and Period 2. In addition, on workdays, the reactor was cleaned daily during the famine phase by manually passing a spatula along the walls and all the instruments inside the culture broth to prevent biofilm formation and obtain homogeneous conditions inside the reactor. Reactor cleaning was not performed during the weekend; this involved some biomass growth on the SBR walls. At the end of the experimental period, the content of the reactors was individually centrifugated, and the biomass was freeze-dried to be conserved for some following studies.

2.2.3 Batch experiments

During period 2, final batch experiments (both aerobic and microaerophilic) were carried out to investigate the PAP of the cultivation broth (Figure 9). For every test, a sample of 1000 mL of mixed liquor was directly withdrawn from the parent reactor (either RAE or RANA) at the end of the respective feast phase so that most of the initial C substrate provided was depleted. As the parent reactor, the batch reactor (Ø: 1L of working volume) was equipped with T and pH probes, submersible water recirculation pump (200 L/h) and air compressor (1500 L/h) connected with two sintered stone spargers in parallel (Ø: 2 cm). Different feeding and aeration conditions were used depending on the experiment type, but the C source was always in excess (1,5-2 gCOD/L) compared to the same influent in the parent reactors (0,5gCOD/L). In addition, 20 mL of batch culture broth were usually sampled at the beginning of the experiment (after

the feeding), at intermediate times and at the end. For the RAE aerobic accumulation tests, the same amount of C source that the parent reactor received was provided to the batch reactor (250 mL), but the nutrient influent was not provided; aeration was kept always switched on for the whole test. Microaerophilic tests on RAE and RANA were characterised by an aeration cycle of 1h anoxia followed by rounds of 15 min of aeration - 45 min of anoxia. According to the parent reactors, the total influent was 1L of Acetic acid and nutrient solutions combined.

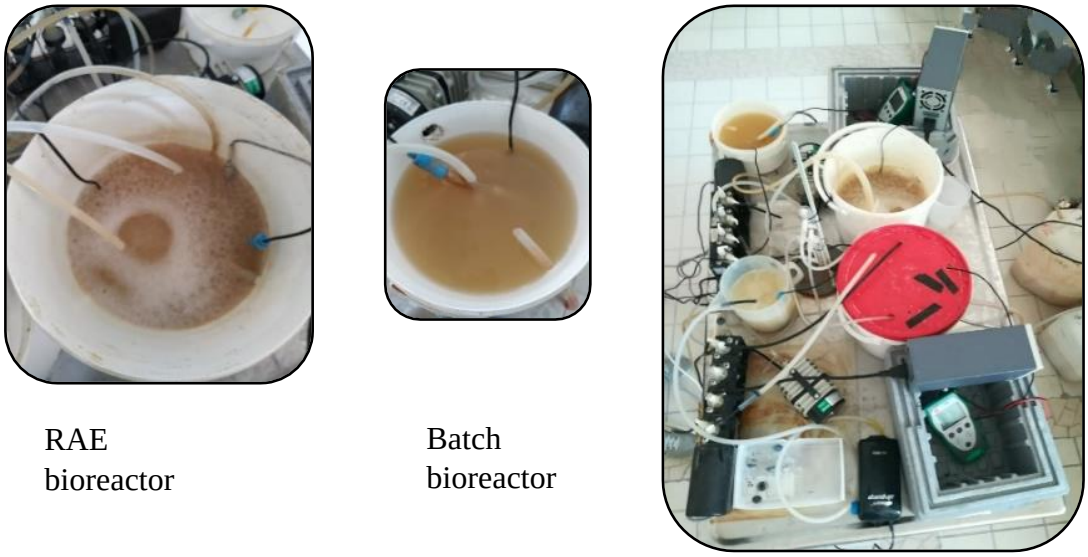

Figure 9. Configuration of RANA, RAE, batch bioreactors and final experimental setup.

2.2.4 Cultivation medium and influent solutions

For each feeding cycle, RANA and RAE reactors received 1L of total influent, comprising 250 mL of a synthetic aqueous solution of Acetic acid (no buffer pH) and a real wastewater solution resulting from the centrifugation of ADSS agro-industrial wastes as a source of macro nutrients. This wastewater was collected from the B-PLAS site and was analysed by an external laboratory. The analysis is available from the 8th of February ongoing. However, since results were received after some weeks from the wastewater collection, it was impossible to control the composition of this influent solution in real-time. In any case, only COD and inorganic molecules were measured; no further analysis on the chemical composition has been carried out.

The wastewater was vacuum filtrated on a Gooch filter to block solid residues and used at it is, except for a short period in which synthetic K_2HPO_4 was added. The pH of the filtered solution was 9 ± 0,3 for all the weekly measurements. Since the wastewater composition changed over time, the quantification of C, N and P entering the two bioreactors is reported in Table 2. Remarkably, the used wastewater has always shown a high ratio C to P ratio and a low C to N ratio (low P and high N concentration compared to the optimum values from the last studies) [45] [63]. However, depending on the P concentration of the wastewater, the concentration of Acetic acid solution has to be kept indicatively above certain levels ([Acetic acid] higher than 50 C/P approx.) to allow for both effective biomass growth and PHA storing but cannot be increased at will, risking losing the biomass physiological adaptation due to lack of a macronutrient ([Acetic acid] lower than 200 C/P approx.). In that case, the composition of the population is assumed to change drastically, and a sharp drop in biomass concentration and accumulation potential can occur. In such a situation, a single addition of freeze-dried inoculum (0,5 g) and a prolonged K_2HPO_4 addition has occurred. In Period 2, the influent Acetic acid solution concentration was kept around 10 g/L, a similar value of the effluent from the real acidogenic fermented stream in the pilot plant.

To understand to which extend ammonia contributes to the soluble COD of the culture broth, two stripping tests on the real fermented wastewater were also conducted (addition of NaOH to pH 11, heating at 60 °C under vigorous stirring at 500 rpm for 1h to eliminate NH_4^+ as NH_3) [64]. COD was measured before and after the stripping test; the ammonia-related COD was then calculated as the difference between the two values.

Table 2. Nominal concentrations of target substances in the nutrient solution stock. The COD here measured was probably primarily due to ammonia and other organic macromolecules. INLET = in the 1L feed; dividing these values by 5 returns the influent concentration inside the reactors.

Date	C.O.D/ ppm O_2	Ammonia/ ppm N-NH4+	Chlorides/ ppm Cl^-	Nitrates/ ppm N-$NO3^-$	Sulphates/ ppm $SO4^{2-}$	Phosphates/ ppm P-$PO4^{3-}$	mMol P/ INLET	gCOD/ INLET	mMol C/ INLET	OLR gCOD L^{-1} day^{-1}	mMol N/ INLET	mol COD/ mol P inlet	mol COD/ mol N inlet	comments
9.02								5,00	156	2,00				
12.02	919	670	1104	3,46	69	16,4	0,40	6,15	192	2,30	36	483	5	**2 feed/day**
16.02	522	570	1023	0,01	55	12,5	0,30	4,25	132	1,70	31	438	4	
22.02	959	775	1120	2,86	38	16,3	1,69	4,25	132	1,70	42	79	3	**0,3 g/L K_2HPO_4**
01.03	955	785	1149	2,00	29	16,4	1,69	4,25	132	1,70	42	78	3	**0,3 g/L K_2HPO_4**
08.03	1055	860	1128	0,44	38	17,1	1,71	4,25	132	1,70	46	78	3	**0,3 g/L K_2HPO_4**
15.03	1079	780	1165	0,45	35	16,0	1,03	4,25	132	1,70	42	128	3	**0,15 g/L K_2HPO_4**
22.03	995	715	1189	0,06	50	15,1	0,37	2,50	78	1,50	38	213	2	**3 feed/day**
06.04	1448	1150	1219	0,26	12	21,6	0,52	2,50	78	1,50	62	149	1	
12.04	1002	835	1159	0,11	40	16,4	0,40	2,50	78	1,50	45	196	2	
19.04	1604	1380	1166	0,00	61	21,5	0,52	2,50	78	1,50	74	150	1	
26.04	1576	1090	1097	1,79	188	21,5	0,52	2,50	78	1,50	58	150	1	
17.05	1132	990	964	2,35	316	21,4	0,52	2,50	78	1,50	53	150	1	
24.05	1094	1090	950	0,72	203	19,6	0,47	2,50	78	1,50	58	164	1	
31.05	1240	940	961	0,45	174	21,3	0,52	2,50	78	1,50	50	151	2	
07.06	993	900	1015	2,44	82	22,0	0,53	2,50	78	1,50	48	146	2	
14.06	1052	960	1041	1,04	22	21,7	0,53	2,50	78	1,50	51	148	2	
21.06	873	680	1080	18,43	60	15,4	0,37	2,50	78	1,50	36	209	2	
28.06	870	605	1037	0,03	29	16,1	0,39	2,50	78	1,50	32	200	2	
average	1076	876	1087	2,05	83	18								
sd	254	204	80	4	80	3								
rsd	24%	23%	7%	201%	95%	16%								

2.3 Analytical methods and calculations

In this chapter, all the methods used for this thesis are presented. The names of each analysis here reported are thereafter used as a reference to indicate the method further described

2.3.1 pH and temperature

Online measurements of the bioreactors' pH were obtained with a pH 70+ DHS meter (XS instruments), equipped with a consumer-grade electrode (Atlas scientific) and a temperature probe. Data were downloaded and logged using the elaboration software Datalink v2.0 (Giorgio Bormac).

2.3.2 COD analysis

COD was measured by thermal oxidation at 1200 °C with detection of the oxygen consumption using a COD analyser QuickCODLab (LAR Process Analyzer AG, Figure 10) following the ASTM D6238-98 method. This is a chemicals-free technique that tolerates high concentrations

of dissolved salts (up to 10 g/L) that usually pose problems to COD measurements. After proper dilution, the sample was injected with a precision micro syringe directly into the injection port of the instrument. Then, a 2-zone oven allows for thermal oxidation of all organics at 1200 °C under air/nitrogen flow in only 3 minutes. The outflow is analysed with an O_2 detector, and the COD was calculated as mgO_2/L, comparing the signal areas (O_2 consumption) with the proper calibration curve. Standard glucose solutions were used to build two calibration curves between 250-2500 mgCOD/L and 1000-10000 mgCOD/L knowing that 1 g of glucose is converted to CO_2 and H_2O, demanding 1,067 gO2. All analyses were performed in duplicate or triplicate; results are shown as the average of measurements with an RSD < 5 %. Total COD analyses were performed by direct injection of 100 µL of well-mixed culture broth freshly sampled. For soluble COD determination, 10 mL of mixed sample were centrifuged at 5000 RPM for 8 minutes in a plastic tube; then the supernatant was filtered on glass microfibre filters GF/C grade (pore size 0.45 µm, Whatman) and 100 µL of the filtrate were analysed with the method mentioned above. This measurement can be an indication of the C source available in the solution at the moment of analysis. It was possible to evaluate the ΔCOD (also called by difference (Equation 1) with these two values. This value estimates the total biomass concentration in the reactor, which is composed of active catalytic biomass (Xc) and PHA combined.

$$\mathbf{\Delta COD = total\ COD - soluble\ COD}$$

Equation 1. Evaluation of the total biomass concentration in terms of COD.

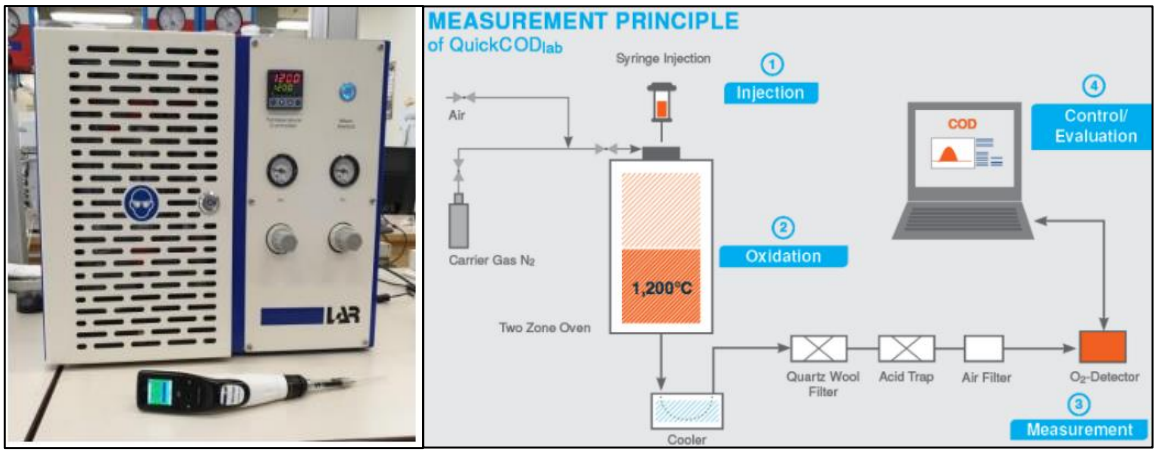

Figure 10. COD analyser QuickCODLab (left), block scheme of the machine (right).

2.3.3 Feast and Famine period length

For most of the time, the length of the feast and famine periods were indirectly estimated by the consumption of soluble COD to pre-feed levels and the visual change in the dynamic pattern of pH measurement (Figure 11) [65]. Only during the last three weeks, an arbitrary method was developed; the pH data were tabulated retrospectively to monitor how the FF length varied during the experimental months. An Excel spreadsheet has been used to calculate the end of the feast phase as the time at which the total negative pH pulse was dissipated to an arbitrary 50% extent. However, in most other studies, the length of feast and famine periods is measured via DO concentration changes. In particular, the famine phase usually starts with the VFA influent addition. It terminates with a net rising in the DO profile, associated with a reduced bacterial metabolic activity after the depletion of the C source.

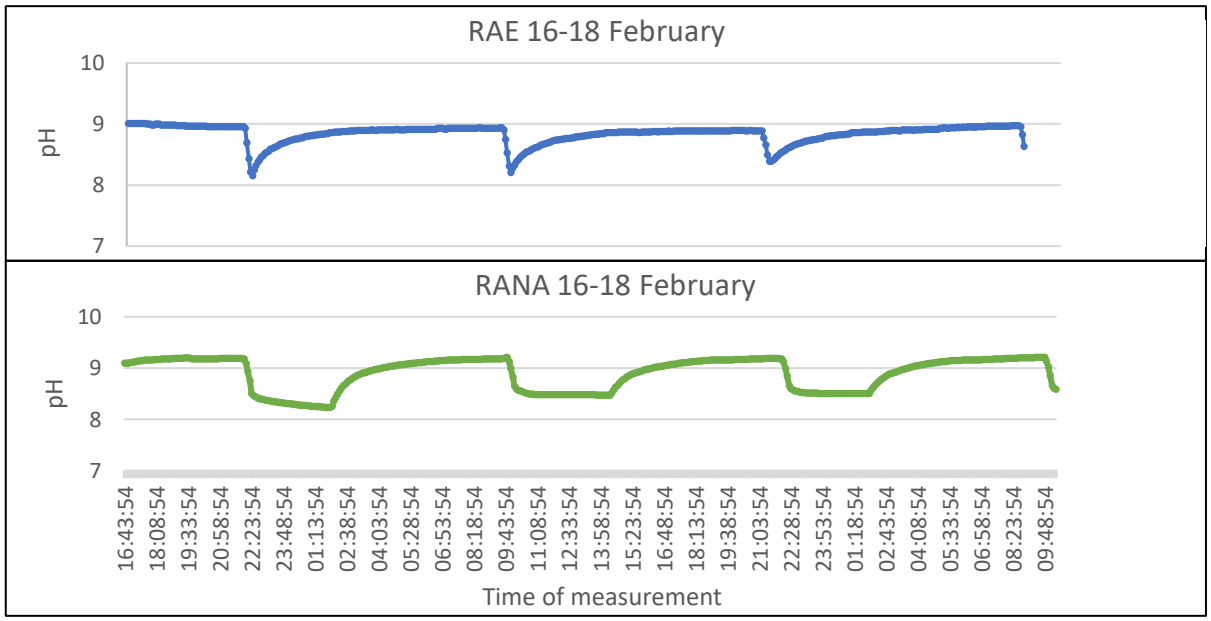

Figure 11. Typical pH patterns recorded in RAE and RANA bioreactor. Each negative spike is due to substrate addition.

2.3.4 VFA analysis

Volatile fatty acids (VFA) analysis was performed from the 12th of February using the method suggested in this paper with slight modifications [66]. In a 2 mL volume GC vial (Thermo Fischer Scientific) were added: 100 µL of the centrifugated and filtered sample (as for soluble COD), 100 µL saturated solution of $KHSO_4$ 100 µL saturated solution of NaCl, 100 µL of 2-

Ethylbutyric acid at 1 mg/mL in deionised water as Internal Standard (IS), 1 mL of Dimethyl carbonate (DMC). The solution was hand-shaken, and 100 µL of the supernatant was injected at 250 °C in splitless mode in a GC-6850A coupled with MSD 5975 mass quadrupole spectrometer (Agilent Technologies). Column type: Nitroterephthalic-acid-modified Polyethylene glycol DB-FFAP 30 m, 0.25 mm, 0.25 µm (Agilent Technologies). The method starts from 50 °C for 10 min then 10 °C/min up to 250 °C without holding time, with Helium as carrier gas (at constant pressure, 33 cm/s linear velocity at 200 °C). The injection port temperature was set at 280 °C. Detection was performed by MSD 5977E detector under electron ionisation at 70 eV with full scan mode acquisition at 1 scan/s in the 29-450m/z range. Acetic acid's response factor (RF) was extrapolated as the linear regression slope between 250 and 2000 mg/L (Equation 2), resulting from the analysis of standard solutions prepared from the pure substance. Three technical replicates have been analysed for each standard solution (RSD < 5%). The concentrations of Acetic acid (x) in gCOD/L were obtained with Equation 3.

$$\mathbf{RF_x} = \frac{\mathbf{A_x} * \mathbf{m_{IS}}}{\mathbf{A_{IS}} * \mathbf{m_x}}$$

Equation 2. Calculation of the response factors. A indicates the peak area; m indicates the mass of a specific compound in solution.

$$[\boldsymbol{Acetic\ acid}] = \frac{\mathbf{A_x} * \mathbf{m_{IS}} * \mathbf{COD_x}}{\mathbf{A_{IS}} * \mathbf{RF_x} * \mathbf{V_{sample}}}$$

Equation 3. Calculation of the Acetic acid concentration in solution in terms of gCOD/L. COD_x indicates the grams of COD per gram of compound.

2.3.5 Indirect PHA quantification

The resulting pellet obtained via centrifugation of 10 mL of broth at 5000 RPM for 8 minutes was separated from the supernatant and frozen for at least 90 min. Samples were then freeze-dried (at -55 °C, 1 mbar) with an Alpha 1-2 LD plus lyophiliser (Martin Christ) to remove residual moisture.

Following the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) simplification philosophy, a fast and simple In-Vial Thermolysis (IVT) method was used for the quantification

of PHA in the solid pellet [67]. With this treatment, PHAs are thermochemically degraded in the absence of oxygen into their corresponding marker 2-alkenoic acid as Crotonic acid (CA) from PHB with high yield (Y_{AC} > 40% w_{AC}/w_{PHB}). A proposed mechanism for thermal degradation has been reported as in Figure 12. The results from the IVT procedure were already correlated to those obtained with the traditional methanolysis approach for PHA analysis in a previous work [67]. Every batch of IVT included a positive control sample of pure PHB (1-1,5 mg) to keep track of the average Y_{AC} over time, thus detecting possible deviation to the average percentage value (45 ± 5) % due to experimental errors or instrumental issues.

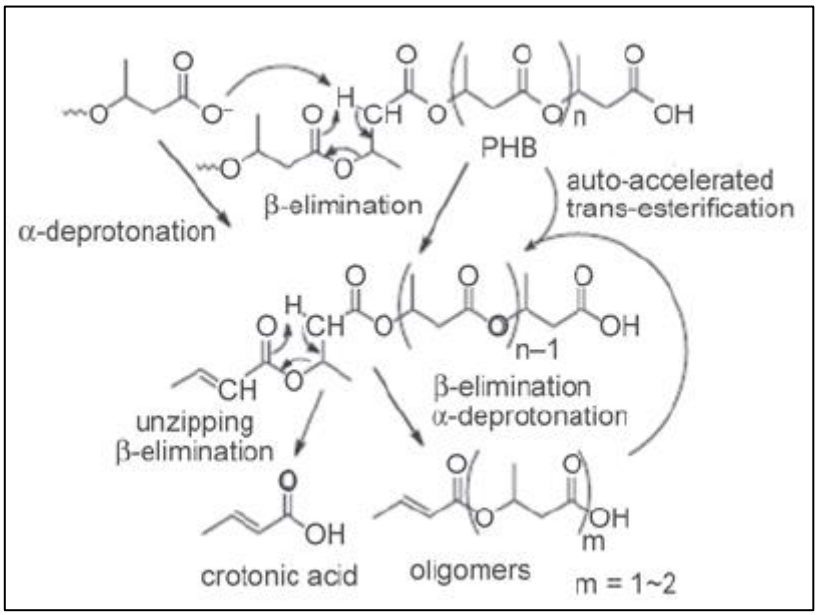

Figure 12. Mechanism of thermal degradation for PHB [68].

Operatively, freeze-dried pellets were weighted and powdered inside a screw cap glass vial (4mL, 50 mm high) and heated on a hot plate for an analytical meta-pyrolysis (IVT, Figure 13) at 350 °C for 20 min. The temperature was monitored with an infrared thermometer. The vial plastic cap was wrapped in wet paper to avoid its melting. The resulting products (biochar, bio-oil, and a gaseous fraction) were cooled down to room temperature. A spike of internal standard solution (100 µL of a solution 5 mg/mL of 2-Ethylbutyric acid in Acetonitrile) was added to the pyrolysis vial and eluted with Acetonitrile (2 mL), which can solubilise the CA and other 2-alkenoic acids. After that, 1 mL of clear solution was moved into a GC-MS vial and analysed with the same GC-MS method and instrumentation (Figure 13) abovementioned. The %CA in the dry weight of the biomass subjected to IVT was calculated on a mass basis (Equation 4).

Then, the %PHA (w polymer /dry w biomass) in the sample was assessed indirectly via Y_{AC} (Equation 5). The calibration has been performed with standard solutions of CA in Acetonitrile prepared in laboratory, within a linear interval between 500 and 10000 mg/L. Three technical replicates have been analysed for each standard solution (RSD ≤ 5%). RF for CA has been extrapolated as explained in the previous section (the only difference is the IS solution). The quantification of PHA content started on the 12th of February.

$$\%\mathbf{AC} = \frac{\mathbf{A_x} * \mathbf{m_{IS}}}{\mathbf{A_{IS}} * \mathbf{RF}} * \frac{\mathbf{1}}{\mathbf{m_{IVT\ sample}}}$$

Equation 4. Quantification of CA in the IVT sample.

$$\%\mathbf{PHA} = \frac{\%\mathbf{AC}}{\mathbf{Y_{AC}}}$$

Equation 5. Indirect quantification of PHB content in the biomass dry weight.

As a comparison with GC-MS analysis, a colourimetric titration method for quantifying PHB was sometimes used, allowing to overcome some intrinsic bottlenecks of the IVT/GC-MS (e.g., instrument faults, higher time demand, cost-intensity). For this purpose, 2-alkenoic acids produced by IVT are the primary source of weak acidity in the sample subjected to this procedure. Thus, the amount of PHAs can be quantified by simple methanolysis and titration of CA equivalent with an alkali solution [67]. In the meta-pyrolysis vial, 2 mL of a solution methanol-water (1:1 v/v) and 50 μL of a solution containing 1 mg/ml of bromothymol blue were added, giving a light-yellow solution. Then 1mL of this solution was transferred to a closed vial and spiked with aliquots of 10 μL of titrant ($NaHCO_3$, 5 mg/mL) under vigorous stirring until the solution became light green and then light blue at rest. This method allowed for a reliable PHB quantification in the range of 10–50% PHA with 5-15 mg microbial samples. Despite the loss of information for PHA monomer composition, IVT-titration method can be directly applied in PHA producing plants without the need for special equipment. Furthermore, it can also be used when specific analytical instrumentation is not available in close proximity to bacteria production plants.

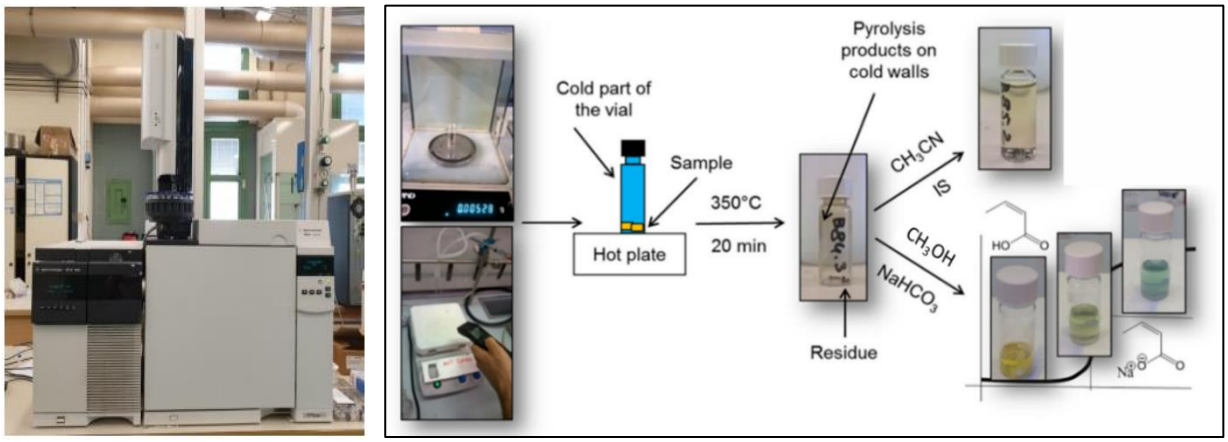

Figure 13. GC-MS system utilised for the VFA and PHB analysis (left) and the sequence of the IVT steps and following analysis, visual representation [67] *(right).*

2.3.6 Calculation of concentrations and yields

Knowing the total biomass concentration (Equation 1) and the %PHA in the dried biomass (Equation 5), PHA and active biomass concentration were calculated in gCOD/L as in Equations 6 and 7. The conversion factors resulting from oxidation stoichiometry of PHB and active catalytic biomass were respectively 1,67 gCOD/gPHB and 1,42 gCOD/gXc [41] [69]. The ΔCOD is assumed to only be composed of PHA inclusions and active biomass (excluding PHA); in this way is possible to calculate the concentration of the two components (on a COD basis) knowing their weight in the dried biomass sample and their conversion factors.

$$[\mathbf{PHA}] = \mathbf{\Delta COD} * \%\mathbf{PHB} * \frac{\mathbf{COD_{PHB}}}{\mathbf{COD_{Xc}}}$$

Equation 6. Calculation of the concentration of PHB in gCOD/L.

$$[\mathbf{Xc}] = \mathbf{\Delta COD} - [\mathbf{PHB}]$$

Equation 7. Calculation of the concentration of Xc gCOD/L.

To convert these concentrations on a mass basis in g/L it is necessary to divide each value for the corresponding conversion factor. The [PHA]/[Xc] has been evaluated to understand the

portion of influent COD dedicated to biomass maintenance and growth and the amount stored as a biopolymer.

The storage yields ($Y_{\Delta PHA/\Delta VFA}$) and growth yields ($Y_{\Delta Xc/\Delta VFA}$) per unit of consumed COD (gCOD/gCOD) were measured at the end of the feast phase. When measured for following days, an inter-day average has been considered. Biopolymer storage yield is a crucial parameter for commercial PHA production viability (g PHA/ gVFA consumed). A high biopolymer storage yield indicates that a large portion of valuable feedstock (e.g., organic acids) is consumed for polymer production rather than being lost as oxidation products or used to form new cells.

Specific PHA storage rate (q_p) and substrate removal rate ($-q_s$) have been measured as gCOD/(gCOD*h) in reference to the initial [Xc].

3 Discussion of results and system improvements

3.1 pH control and feast/famine length determination

For the duration of the study, the constant pH monitoring allows for a continuous evaluation of some processes happening in the reactor, especially the consumption of soluble COD due to microbial uptake. In some situations, changes in the usual pH trend allowed for detecting fails in the inlet solution pumping process primarily due to clogging of the nutrient wastewater pipes or power blackout, as in Figure 14. It was possible to discriminate between tube clogging and power blackout because the C source was still provided in the first case. At the same time, the nutrients were less than the theoretical value, simulating a classical accumulation test with reduced nutrient availability. In the second case, the culture did not receive any influent, the negative pH-spike is missing, and the following biomass drop could be expected.

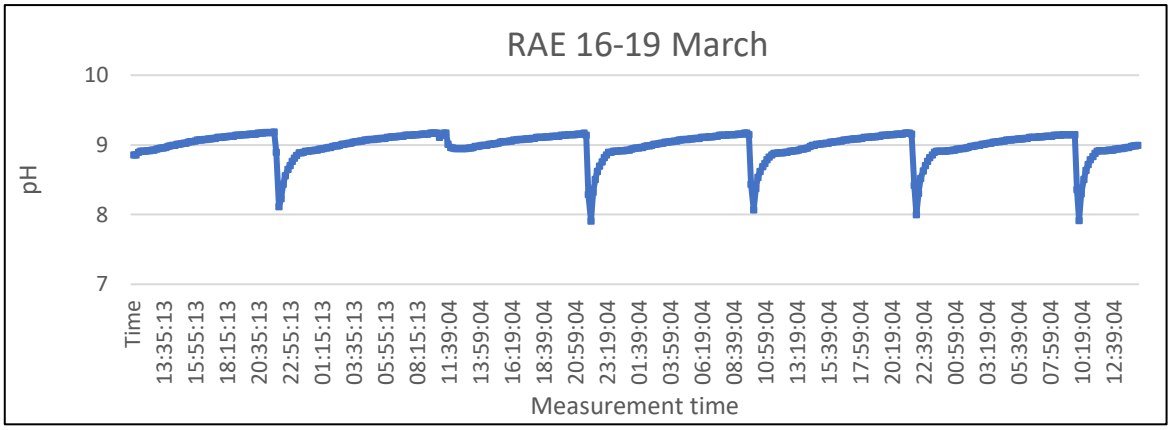

Figure 14: Detection of pump failures via pH measurements.

Another indication that can be derived from the measured pH at the end of the famine phase is the working pH of the culture. During the study, both bioreactors under scrutiny returned to a pH between 8,6 and 9.2 (depending on the influent composition) before a new substrate addition. In case the nutrient influent was not reaching the reactors for more than one day, the minimum pH measured before the new substrate addition was 8,2. After the set point conditions were established again, the nominal pH returned around 9 before the feast phase, showing a general resilience. Studies on the effect of pH on PHA production by activated sludge obtained higher sludge PHB content when the pH was controlled at 8.0 or 9.0, which is in line with the working conditions of the present work. So, It can be affirmed that PHB production is improved if the reactor pH is not controlled or controlled at high pH values [70]. From the process point of view, reactor operation without pH control reduces both process control complexity and operating costs (fewer chemicals used). Overall, the pH stability may prove some culture resistance to variations of external conditions.

Concerning the determination of feast and famine duration, an alternative approach to the classical DO concentration was developed based on the culture's pH variation. At this scope, the point at which the negative pH pulse was halved has been calculated. Experimentally this point corresponded to a 60-70% reduction (depending on the working pH) in the concentration of acidity in the culture broth. Arbitrarily this point was assumed as the end of the feast phase.

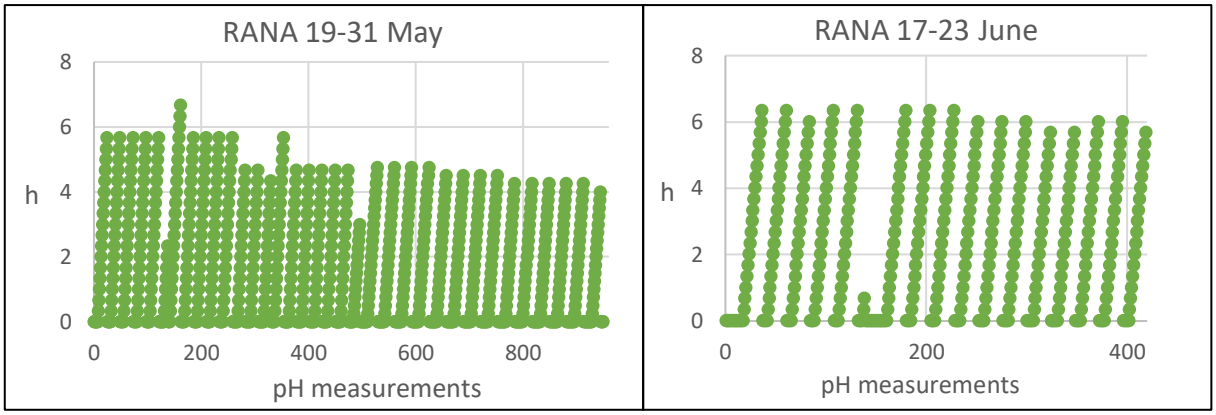

Figure 15. Measurement of the famine phase comparing two different moments.

Figure 15 shows the famine phase's length in 2 different moments during Period 2. The feast phase is calculated by subtracting the famine length from the whole cycle length (8h). Each column represents one cycle (8h); visibly lower columns are due to data downloading and logging and can be discarded.

On the left, data from the MAA test are shown. The famine phase started to increase during the days, even if the imposed reactor conditions were the same. The F/F ratio (length of the feast/length of the famine), which is one of the factors driving the selection in the culture, increased from 0,33 (2 feast – 6h famine) to almost 1. The graph on the right is relative to a moment of relative cycle stability, and the F/F ratio is stable around 0,33.

Comparing these results with the aeration cycles (3h MAA – 5h aerobiosis), it turned out that a substantial part of the C substrate was not consumed during the MAA test. Suppose that amount of Acetic acid becomes available in the aerobic phase. In this case, non-storing microbial species may have endured and survived these shorter famine phases, generally competing for the substrate with bacteria selected in MAA conditions. In addition to that, bacteria selected under aerobic conditions can generally uptake acetate faster than the MAA-selected ones [65]. A decreased duration of the famine phase was then occurring (reduced to no lack of substrate); thus, the selective pressure was strongly reduced, and both PAP and biomass concentration decreased. Such behaviour of increased famine length also occurred on other occasions (RANA accumulation in Periods 1 and 2). It again resulted in a shift in the culture composition with sudden changes in the culture performances [32].

Overall, a similar pH-based method for determining the FF lengths can be used when DO probes are not available. In addition, the construction of trends over time may result helpful in case of long-term identification of the system status.

3.2 Evaluation of the soluble COD baseline

Three ammonia NH_3 stripping tests were conducted, as explained in section 2.2.4. Each analysis returned similar results (Table 3); interestingly, the COD attributed to the ammonia is half of the total wastewater's COD.

Table 3. Resulting COD measurements from NH_3 stripping tests. Data in gCOD/L.

	tCOD	**sCOD**	**deltaCOD**
16.04	1,58	0,79	0,79
19.05	1,35	0,65	0,7
30.06	1,12	0,54	0,58

The measurements of soluble COD at the beginning of the feast (Prefeed) always returned values between 0,5 and 1 gCOD/L. Indeed, if such a high COD could be attributed to residual C substrate, the famine phase did not occur, highlighting a problem in the system. Thus, this test allowed an approximation of what prefeed soluble COD values can be expected, establishing a baseline for the soluble COD due to the nutrient solution.

3.3 Timeline of monitored events

Throughout this study, as it becomes clear from Table 2, because of the fluctuation in the nutrient solution's P content, the C/P ratio of the feed was not always as planned. This deviation and some operative issues led to variable cultivation conditions, especially during period 1. In period 2, the only variable to this parameter was the nutrient solution's composition since it originated from a real digested waste. Due to the described inconsistency of cultivation

conditions during the experimental weeks, selecting a stable and productive RANA culture was hardly possible.

Since this work also aims to assess the effect of some experimental conditions in fostering PAP in mixed cultures, only the most relevant results are presented here. This section will discuss the following events, which are here listed for the sake of clarity.

- Unexpected RANA accumulation (February and April)

- Microaerophilic test (19 - 31 May)

- Final batch tests (1-8 July)

3.3.1 RANA accumulation in February and April

In Period 1, an unexpected increase in stored PHA occurred. Due to a pump failure, the nutrient solution was provided at around 1/4 of the nominal value for three days (13.02-16.02). After that failure, the nutrient inlet was restored to the nominal value as in Table 2.

From the 12.02 to the 16.02, the C input per cycle was 6,15 gCOD, with an OLR of 2,30 gCOD/(L*day). As shown in Figure 16, the residual VFA concentration on the prefeed of 12.02 was 3 gCOD/L, when an expected value should be lower than 1 gCOD/L approx. (soluble COD baseline).

What was happening simulates a batch PHA accumulation test in terms of inlet composition (high C substrate, absence of a nutrient. On 15.02, the PHA content reached 35%. Then the following day, the %PHA and the [PHA] reached respectively 53% and 2,69 gCOD/L, the highest values ever recorded for these reactors. Such a high polymer concentration is the result of combined high biomass concentration and high PHA content. At that moment also the ratio [PHA]/[Xc] was the highest recorded (1,67), meaning that most of the C substrate was stored as biopolymer instead of being used for biomass growth. According to the literature, such behaviour is expected due to the lack of nutrients that prevent biomass maintenance, which led to a sharp drop in both PAP and active biomass in the last days presented [71].

The $Y_{\Delta PHA/\Delta VFA}$ and $Y_{\Delta Xc/\Delta VFA}$ were not evaluated during this week because the precise amount of C substrate consumed during each cycle was hardly quantifiable.

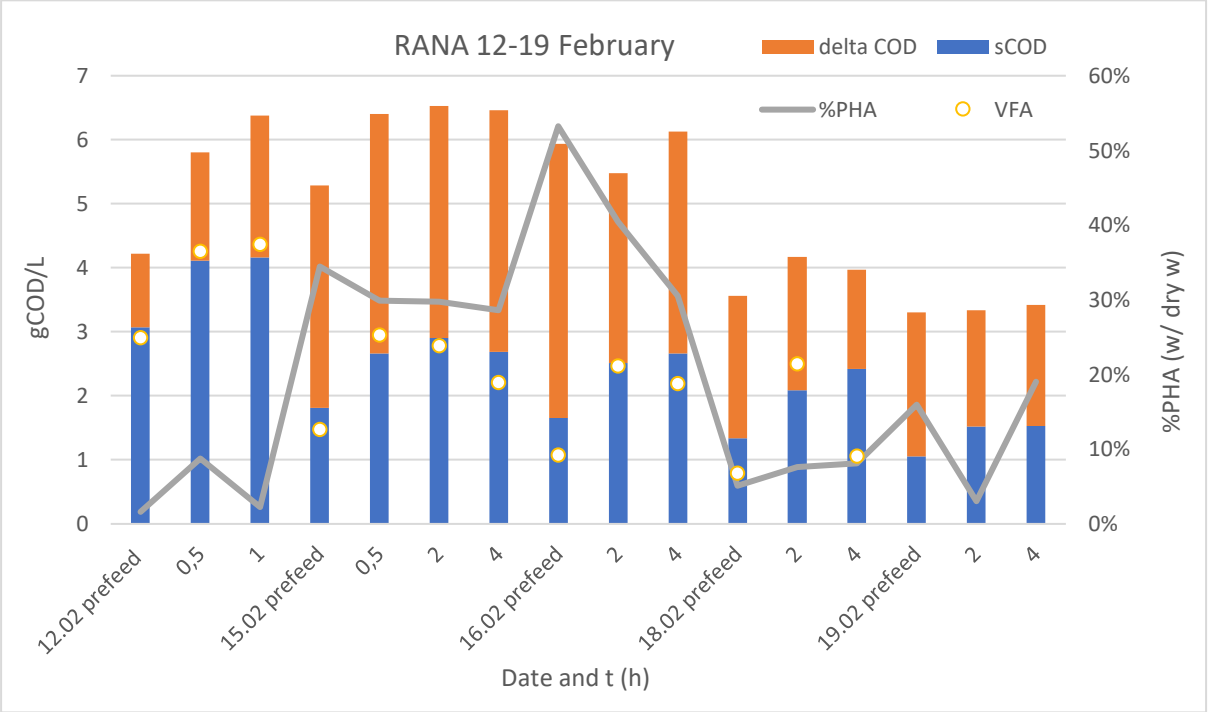

Figure 16. Performances of RANA, February. Standard deviations for the day 15.02 and 16.02 were measured on the mean value of two technical replicates. Max SD: 14%.

Only during the two days of highest PHA accumulation (15.02-16.02), the quantifications via IVT/GC-MS also revealed the Pentenoic acid peaks, and the measured HV accounted for up to 2% of the overall PHA amount. As suggested in section 1.4, this could be evidence of GAO presence in the MMC; indeed, GAO can be selected with anoxic/aerobic FF cycles, and they are able to produce HB-HV copolymers with acetate as the sole C source. Being aware they were present in the RANA reactor could have helped to stabilise the MMC selection process.

Unfortunately, the high OLR combined with an extremely low P influent for some days led to a prolonged system instability and performances drop.

During the same time span, RAE was not subjected to nutrient limitation. Anyhow it showed a similar trend to RANA. It is hard to define the behaviour of the aerobic reactor since it was expected a much higher polymer content due to a concentrated C substrate influent and scarcity

of nutrients. It is possible that both the following instability and performances drop of RAE were due to the same problem of P deficiency since the reported optimal growth C/P ratio for aerobic culture is around 100 [46].

In any case, these storage performances of RAE (Figure 17) reflected what was going to consistently happen in the next months; in fact, the %PHA fluctuated from 5-10% (end of the famine) to around 20% (end of the feast). The maximum [PHA] and [PHA]/[Xc] were respectively 0,44 gCOD/L and 0,32. Even if these performances cannot be compared with RANA, they are similar to values found in the literature for selection reactors subjected to aerobic FF conditions [60] [70].

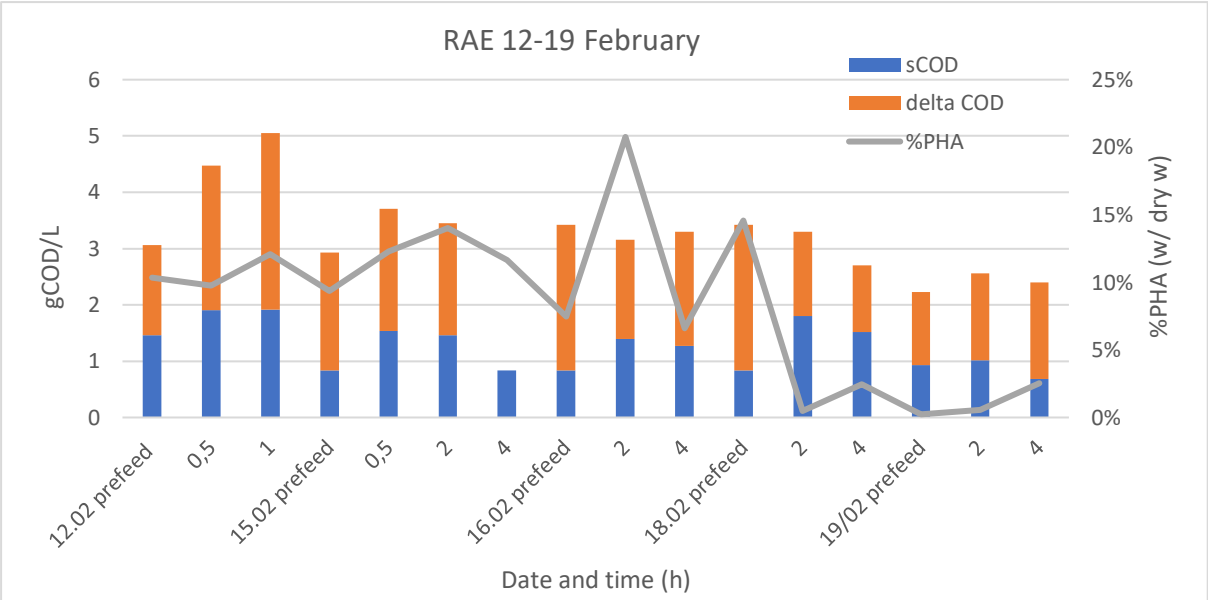

Figure 17. Performances of RAE, February.

The following event of interest happened in Period 2 and was similar to the first one listed above. From 24.04 to 26.04 a nutrient deficit (pump-related) was responsible for a transient PHA storage peak (29%) followed by a severe drop in performances which perdures for some days (Figure 18). Before this date, the typical RANA performances were lower than expected, with a PHA content that hardly exceeds 20% at the end of the feast.

Due to the lower C substrate per cycle (2,5 gCOD) and different microbial compositions, the accumulation %PHA and the biomass increase were limited compared to what happened in

February. The best performances in terms of [PHA] and [PHA]/[Xc] were 0,70 gCOD/L and 0,53.

Even in the April event, after a prolonged deficit of P, the [Xc] and the PAP strongly decreased. The population behaviour and the PHA content achieved during this second accumulation test were highly similar to what happened in a previous similar experiment [72].

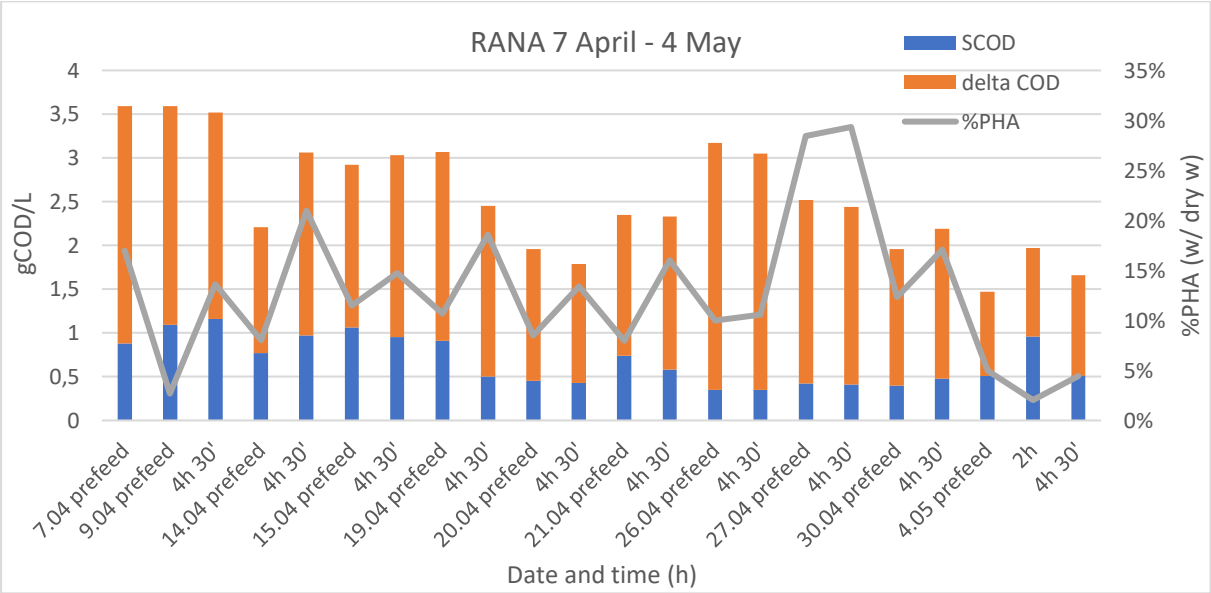

Figure 18. Performances of RANA, Period 2.

In summary, the potential PAP has been evaluated in two different moments. It can be said that higher polymer concentrations were achieved with young biomass (few days from inoculation) at high OLR coupled with an absence of P. The max PHA content recorded (53%) and PHA concentration (2,69 gCOD/L) were comparable to the ones obtained in another batch study with acclimatised biomass under similar conditions [62] [71]. The major unsolved problem is still maintaining such performances over a more extended time and reproducing this phenomenon on command. For sure, measuring the F/F ratio and the concentration of C source before each new feeding cycle is essential to detect if the famine phase occurred. In addition, a finer control on the P limitation in an anoxic/aerobic process can lead to highly different results [71]. Nonetheless, obtaining a 25-35% w PHA/ dry w consistently during the weeks was not possible, and further trials are necessary to achieve this goal.

An advancement to the current reactor setup may include implementing a liquid recirculation system after a biomass settling phase. Changing the liquid medium composition with fresh nutrient solution, water, or a mix of the two can eliminate residual acetate in the starvation period, allowing the process to work at high OLR without the risk to prolong the feast phase. In turn, the selective pressure during starvation phases should favour PHA-storing organisms, which can uptake the C substrate faster.

Another effort to stabilise the system and obtain prolonged high performances can be made in terms of feed regimen. A fed-on-demand approach controlled by the oscillation of DO has been reported to eliminate the initial substrate inhibition and return higher PHB contents without an initial lag phase [44]. This improvement would also eliminate the problem of prolonged feast duration because a new substrate spike is provided only when the previous one has been consumed. This approach could help to increase the OLR that can be provided to the reactors, possibly increasing the biomass concentration and thus the overall productivity [42].

The poor reproducibility of RANA results reflects the several systems adjustments and changes that were made trying to optimise the selection process. Nevertheless, some trends occurring in the system have been clarified, and some comparisons with other studies can be made. Specifically, it has been assessed how and how fast the RANA populations reacted to a sudden drop of C/P ratios, simulating the PHA-productive reactor of a three-stage process. The effects of the C/P parameter on the accumulation performances of anoxic/aerobic FF cultures has already been studied [72]; the outcomes of these two works are similar indeed.

It is almost certain that the RANA populations in the two events shown above were different, being selected over different conditions of OLR and influent composition, number of feeding cycles, FF duration and others. Being open systems, the composition of MMC is likely to vary in response to transient conditions. The compositional variation can result in a variable performance, which is undesirable for industrial applications. Therefore, understanding the microbial community structure and its dynamics under different conditions can facilitate building an adequate environment for optimal microbial selection [73].

In addition to the one-time accumulation events, both single-stage reactors were able to select MMC with average PAP, obtaining performances comparable to similar setups [62] [74]. Furthermore, the fast response (around one day) to transient conditions (such as the absence of one nutrient) is a valuable feature to trigger a PHA accumulation steadily.

3.3.2 MAA test

During Period 2, the effects of a simulated MAA/aerobic selection process were tested on RANA bioreactor starting from the first feed cycle on 19.05. On the 27.05 after a week of stability and a spike in PHA content, a performance drop occurred, similarly to what is presented in the previous section. Figures 19 and 20 are shown the pH trends and the performances of PHA accumulation.

The imposed aeration conditions stimulate a significant substrate consumption (rising segments) in the 15' of active air sparging in the reactor. However, when the aeration was turned off, the uptake of Acetic acid seemed slowed or stopped (flat segments). This behaviour was not in line with the assumptions of a slow but visible pH increase during the MAA phase. Indeed, it was assumed that if GAO were present in the MMC (in whatever fraction of the total), they would be able to uptake (slowly) the substrate even when the air was not sparged in the reactor, exploiting glycogen as an energy source and PHA as an electron sink.

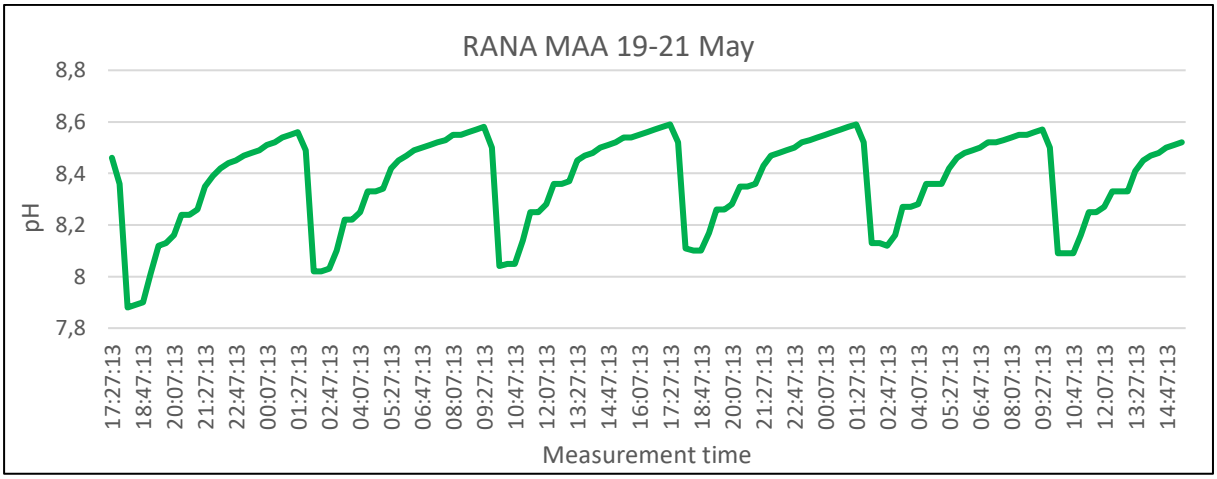

Figure 19. Typical pH trend during the MAA test.

Looking at Figure 20, a pattern in the content of PHA can be recognised. On the 20.5, the culture already responded with an increased PHA content at the end of the feast phase compared to the correspondent value in the standard anoxic/aerobic setup. Performance stability over the cycles was established in the following days (25-30% of polymer content four hours after the feed). On 27.05, the PHA content peaked at 47%, but unfortunately, no explanation has been provided justifying the recorded data. Again, the following days were marked by a drop in accumulation

potential. An almost equal situation (precisely 48% accumulation, then biomass and PAP drop) has been already experienced in the study here suggested [75].

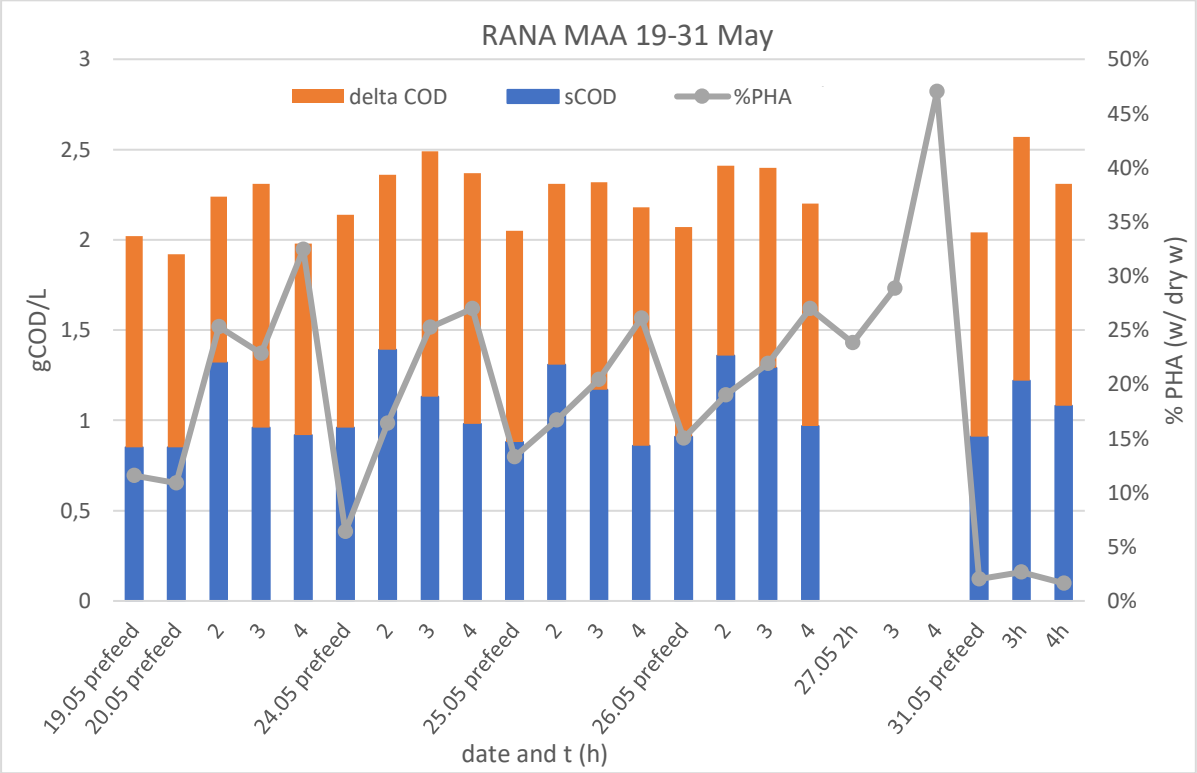

Figure 20. Performances of RANA under MAA/aerobic F, May. COD data on the 27th of May are not available.

The only correlation with this event was made afterwards. As shown in section 3.1, the famine length started to decrease from the second day of the test, going from 6h to 4h. Most likely, the variation in the F/F ratio had a negative effect on the cultivation, as already explained.

The decreased oxygen availability seemed to have affected only the PAP of the population, while the [Xc] remains almost stable throughout every 8h cycle. Until 27.05 average values of $Y_{\Delta PHA/\Delta VFA}$ were 0,48 ± 0,13 gCOD/gCOD, while q_p and $-q_s$ were 0,06 ± 0,01 and 0,14 ± 0,01 gCOD/gCOD/*h. These values are comparable or even superior to similar studies [62] [75] [76]. It can be derived that the biomass is able to store C substrate as PHA quickly. Oppositely the $Y_{\Delta Xc/\Delta VFA}$ had a negative value (-0,28 ± 0,17) gCOD/gCOD, meaning that active biomass decreased while C substrate was uptake.

In MAA/aerobic condition, some authors reported an increased PAP (up to 63% PHA in weight) compared to MMC acclimatised under anoxic/aerobic feast and famine cycles (max 35% in

weight) [37]. The explanation relies on the assumption that, for the anoxic/aerobic selected organism, the PHA-storage process is faster than the glycogen production process. Suppose a limited amount of oxygen is provided to the culture. At some low oxygen concentrations, assimilative activities from oxidative degradation of part of the substrate may be suppressed. At the same time, microorganisms may bring acetate inside the cell (less energy-requiring process), storing it as PHA. In the following aerobic phase, the microorganism may be able to grow, consuming the stored PHA. Therefore, that metabolic model will select PHA-accumulators without the ability to utilise energy reserve material like glycogen since there is no reason to have it. This feature may result in an advantage because glycogen production deviates a part of the C substrate that may be used for PHA storage. However, an extended metabolic explanation is available in the literature [37] [77].

Having a low (but uncontrolled) DO concentration in the culture broth seems to affect the MMC's PAP similarly to a sudden increase in OLR or a decrease in nutrient content. Interestingly, the PHA content increased (25-30%) compared with stable anoxic/aerobic selection cycling under consistent P limitation (15-20%), but the active biomass concentration stayed still or decreased. Using acetate as the substrate (as the model above), the aerobic acetate uptake and its conversion to PHB are strictly oxygen-dependent. In the total absence of oxygen, ATP cannot be synthesized, and the transport of acetate into the cell is not enabled, except in the presence of another ATP source in solution (e.g., glycogen).On the other hand, results have also shown that an oversupply of oxygen compromises PHB production (regular RAE).

Overall, this test wanted to go beyond the present knowledge on the B-PLAS unusual behaviour that has been addressed with this study. The low control over the oxygen concentration made these results just a starting point for further assessments. In any case, some system improvements can be easily drafted.

In the first place, better control of the DO can help define the most productive oxygen limitation condition compared to an entire aerobic cycle. For example, it has been shown that better results can be obtained at 70% saturation compared to 20% O_2 saturation [75].

Other tests may involve reaching the same OLR feeding more shots of diluted C substrate, possibly avoiding any substrate inhibition, and selecting more competitive microorganisms able to uptake acetate faster. Eventually, since glycogen is probably not involved in MAA enrichment, the air supply may be slightly changed, with shorter but more frequent periods of aeration during the MAA phase (e.g., repetition of shorter cycles 5' sparger on/15' sparger off).

3.3.3 Final batch accumulation tests

During the last week, some batch tests (section 2.2.3) were performed to define the final PAP of the two reactors. At the beginning of the trials, the C substrate concentration was between 1,5 and 2 gCOD/L (3-4 times higher than the parent reactors), probably reducing the overall performances of batches because of substrate inhibition.

All batch experiments were carried twice; both results are reported here, giving an idea of the two biological systems' inter-day variability. Only RANA under MAA conditions was tested once. To remove any doubt about the impossibility of accumulating PHA under complete anoxic conditions, the usual Period 2 feed was added to 1L of RANA and RAE. The two cultures were kept shaken inside a sealed flask for up to 24h. No PHA accumulation nor VFA consumption has been registered after 4 and 24 h (results are not presented).

Then, a MAA accumulation batch test was carried out on RANA, simulating what happened in a feast phase of the MAA test on the parent reactor, but with substrate much in excess. Only a fraction of the Acetic acid in excess was consumed, probably because of the lack of a full aeration period (only the feast was simulated). A modest PHA content and concentration were achieved (16% and 0,21 gCOD/L) while the [Xc] stayed stable around 0,9 gCOD/L. Even if this test was carried out for a shorter time than what happened in May in the parent reactor, the performances were much poorer (Figure 21).

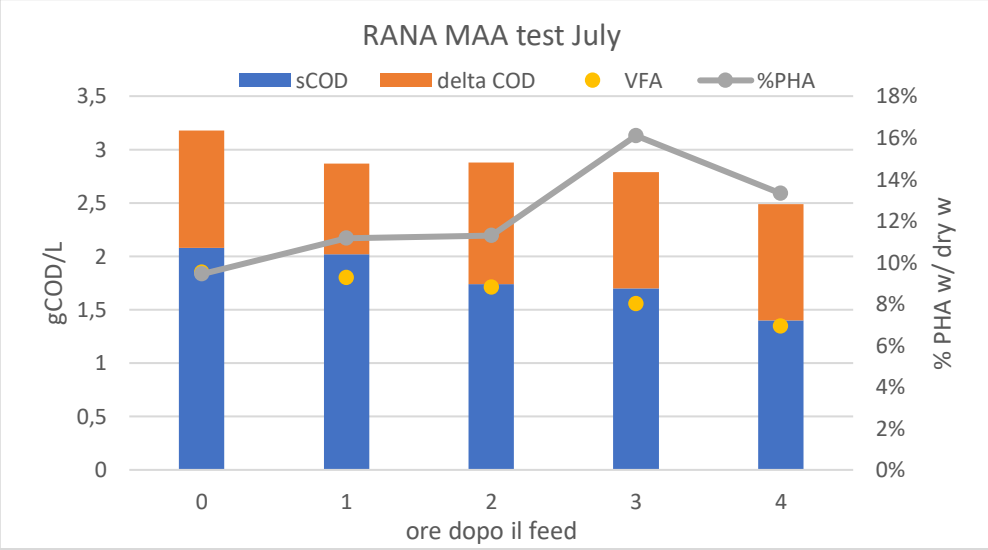

Figure 21. Performances of RANA in the last MAA batch test, July.

In conclusion, it can be affirmed that the final composition of RANA did not display any interesting features for the aim of the study.

During the same week, RAE was tested under both MAA conditions (Figure 22) and full aerobic conditions (Figure 23) to study the influence of oxygen concentration on the aerobic culture. It is worthwhile mentioning that polymer content at the beginning of each experiment was around 20%, the threshold value for completely aerobic selection reactors at the end of the feast phase. Although the initial culture broths have three days of difference, the recorded performances are almost equal.

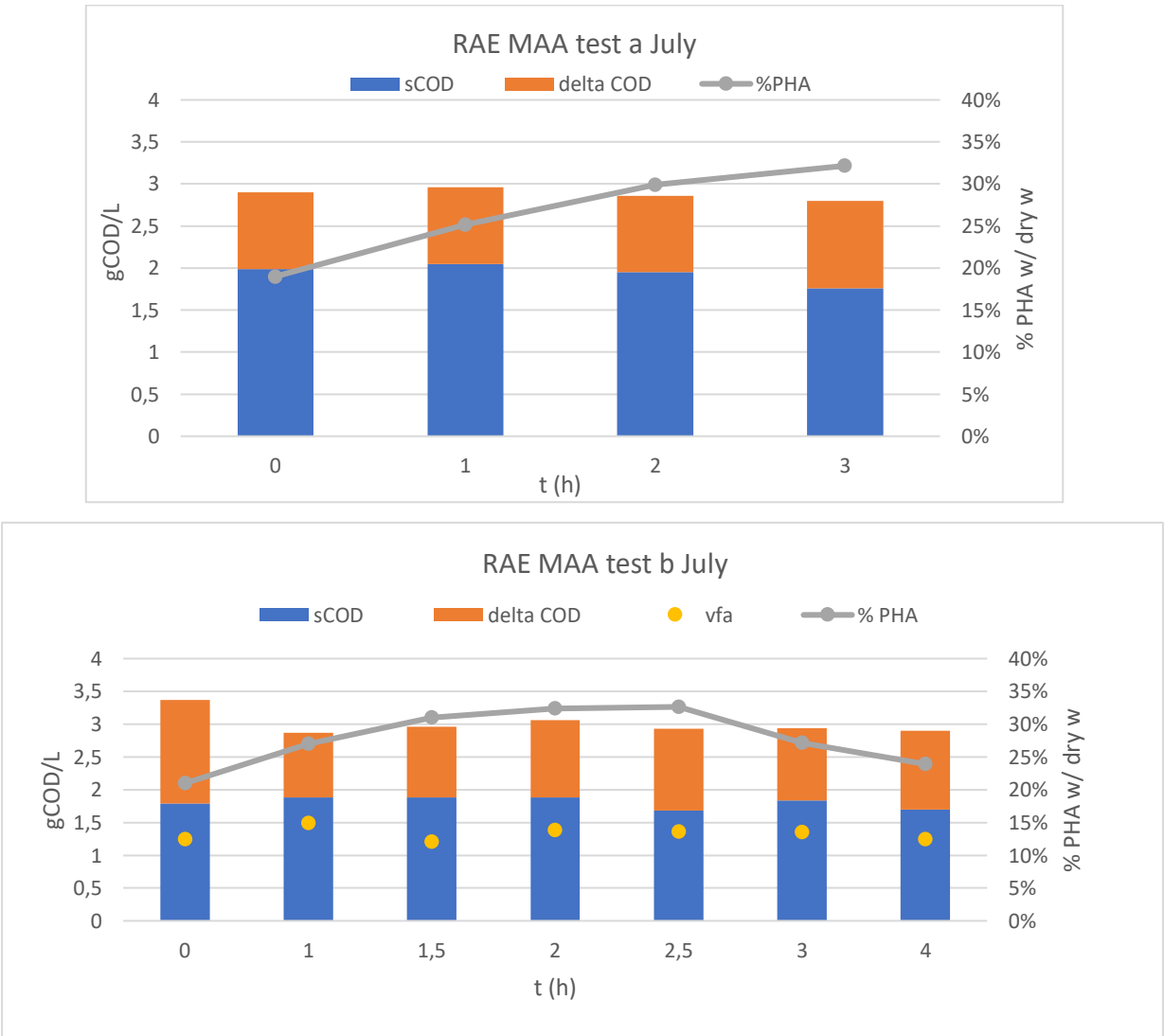

Figure 22. Performances of two RAE accumulation batch tests obtained under MAA conditions, July.

Both trials return a max of 33% PHA content after 3h and 2,5h, but no biomass increase has been registered. Maximum [PHB] and [Xc] were 0,48 gCOD/L and 0,86 gCOD/L. The irregular and non-consistent consumption of Acetic acid prevents the evaluation of storage yields and growth yield. However, even if the C substrate concentration could be measured with higher accuracy, the storage yield could be expected at positive values. In contrast, the growth yield could be next to zero or even negative, as shown with previous MAA tests. These batch tests have shown once more that MAA conditions can allow for a substantial increase in the PHA content of the biomass (a 50% relative increase from 21% to 33%) while reducing the biomass concentration. Even if MAA-related literature is not abundant, very similar values for PHA content under P limitation (max 35-37%) are reported in this study [62]. Under 20% of DO saturation, the same values have been achieved [75].

With this kind of assumption, if a culture broth with a high biomass concentration undergoes MAA conditions, the overall PHA content has the potential to increase a lot, providing that a sufficient oxygen concentration allows for substrate uptake. Thus, the storage yield and storage rate could be increased substantially compared to a regular aerobic/aerobic process, decreasing the need for highly concentrated C substrate influent. If needed, at a specific time (that need to be carefully evaluated for each culture), the residual, non-consumed substrate may be recirculated in other systems or for another accumulation batch. If proven on lab-scale, this could be a promising feature to be scaled up. Before that, a quantitative study on the DO concentration must be carried out to return an optimal value allowing for a constant [Xc] but substantial PHA enrichment in terms of increasing [PHA]

At the end of the study, two completely aerobic accumulation batch tests have been carried out to evaluate RAE's potential when accumulating Acetic acid in the absence of P.

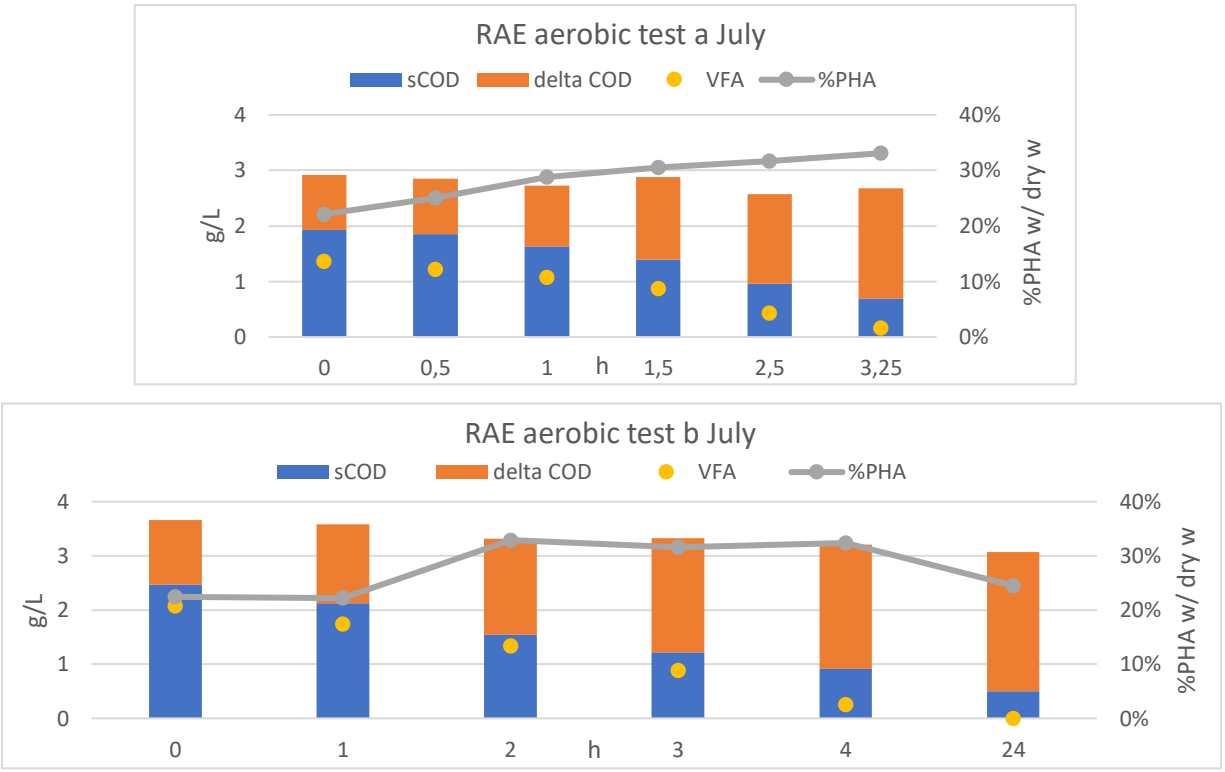

Figure 23. Performances of two RAE accumulation batch tests obtained under aerobic conditions, July.

As for MAA accumulation tests, the peak in polymer content was 33% after 2-3 hours. This measure was established as the last accumulation performance of RAE. When this value has been reached, the C substrate concentration is still half or 1/3 of the initial one, suggesting that further COD consumption has been devoted only to biomass growth (without PHA drop). If the goal is to maximise the [PHA] in the reactor, this could be an interesting feature. However, if the ultimate goal is to efficiently use C substrate (see the storage yield), anoxic/aerobic or MAA/aerobic conditions can be a better solution.

During the first test, RAE a shown a maximum [PHB] of 0,77 at 3h 25min, an $Y_{\Delta PHA/\Delta VFA}$ of 0,43 gCOD/gCOD and an $Y_{\Delta Xc/\Delta VFA}$ of 0,4 gCOD/gCOD; -qs were 0,22 gCOD/(gCOD*h) and 0,5 gCOD/(gCOD*h) respectively.

After 4h RAE b displayed a maximum [PHB] of 0,88, an $Y_{\Delta PHA/\Delta VFA}$ of 0,31 gCOD/gCOD and an $Y_{\Delta Xc/\Delta VFA}$ of 0,30 gCOD/gCOD; q_p and -qs were 0,16 gCOD/(gCOD*h) and 0,52 gCOD/(gCOD*h) respectively.

Comparing the obtained performances with similar aerobic batch experiments found in common reviews, it can be affirmed that the $Y_{\Delta PHA/\Delta VFA}$ are in the average (0,4-0,5

gCOD/gCOD). Values of q_p around 0,2 gCOD/(gCOD*h) are slightly lower than average values (0,15-0.6), even if the available results are highly scattered[42][46][70].Still, the PHA content in % w/ dry w reached lower values than classical accumulation batch tests (up tp 70%)

Overall, the batch tests commented in this section have shown that biomass growth and PHA storage can be influenced by manipulating oxygen concentration, although the final PHA content is independent of oxygen concentration. Therefore, decoupling these two phenomena by manipulating DO instead of limiting nitrogen or phosphate availability represents a significant opportunity for PHA production processes that utilise nutrient-rich feedstocks. Also, a major advantage of operating at low DO is a reduction in aeration requirements, which represents a reduction in operating costs.

4 Conclusions

This thesis shows that, by means of an anoxic feast, it is possible to obtain high PHA (>30%, higher than that obtained with an aerobic feast) content within a single reactor for a reduced time span. However, it was impossible to obtain a stable performance over time (more than two weeks). Achieving a long-term stabilization of some main parameters regulating the anoxic feast/aerobic famine cycle (mainly F/F ratio and C/P ratio) could lead to a reproducible MMC's behaviour and thus performances. Nonetheless, starting from the theory of PHA accumulation in MMC, it can be foreseen that the achievement of such a high peak in PHA content is not compatible with the required stress-based selection of MMC for PHA accumulation. Nonetheless, this work has proven that an anoxic environment acts similarly to nutrient depletion, which is the typical strategy for obtaining high polymer content in the PHA production reactor. According to these findings, the most straightforward way to utilise the acclimatised biomass is to withdraw a portion of the total culture broth (e.g., $1m^3$ out of the $5m^3$ available) and impose a second (or even a third) anoxic/aerobic feast using the same fermentation broth without decoupling nor modifying the nutrient stream as in conventional three-stage processes. A process scheme comparison is provided in Figure 24.

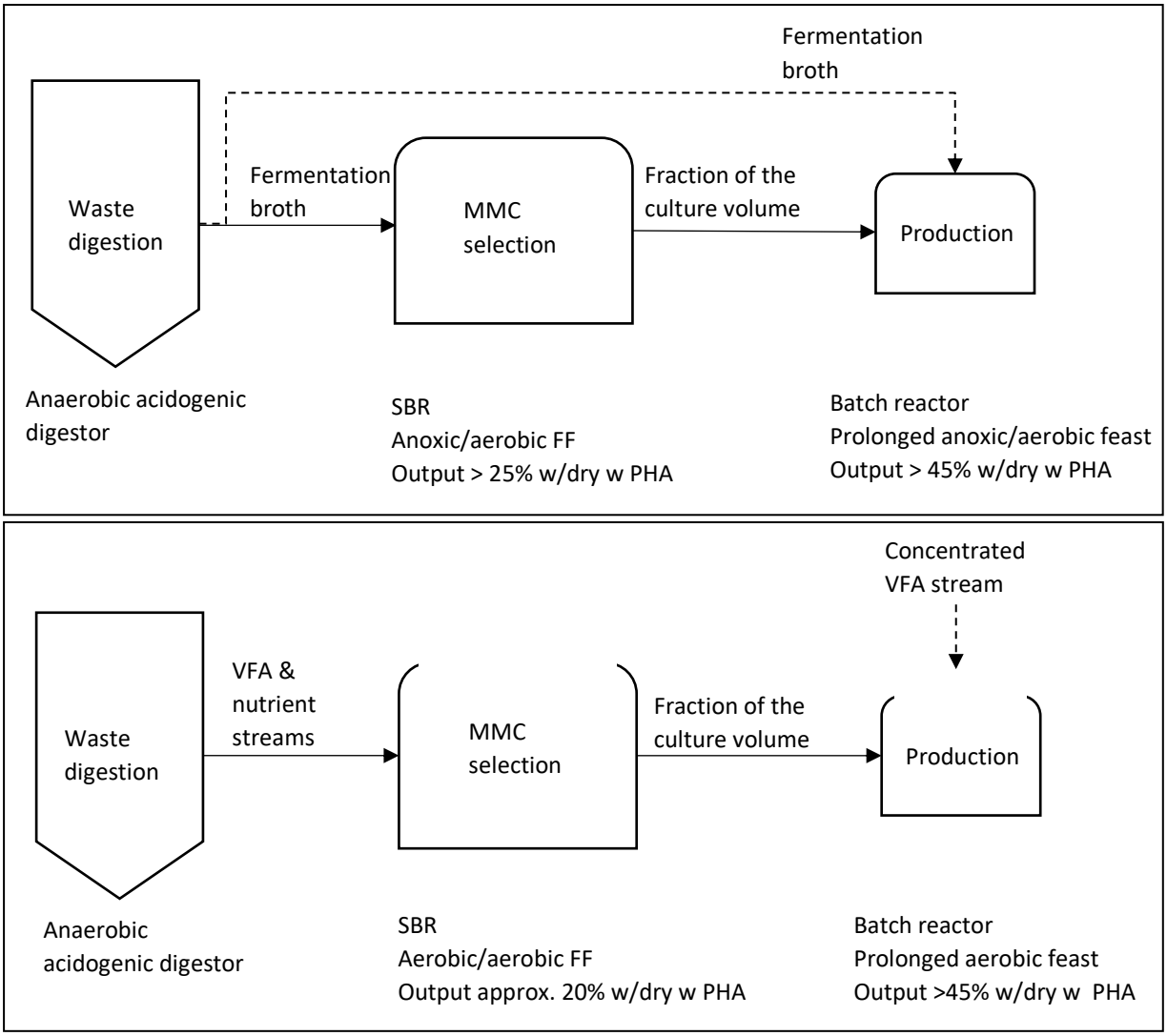

Figure 24. Comparison between the modified (above) and conventional (below) process scheme for on-demand PHA accumulation.

In this way, the parent tanks continue to produce adapted biomass through ADF without suffering significant destabilization. At the same time, in the following batch reactor, high PHA concentration may be achieved one-shot. This modified setup was proven to deliver final bacterial biomass with high PHA content (up to 53%); most important, this setup could be easily implemented in the full-scale plant, thus minimising the aeration expenses and streamlining the production process.

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