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# Polyhydroxyalkanoates Production by Mixed Microbial Culture under High Salinity

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**Abstract:** The fishing industry produces vast amounts of saline organic side streams that require adequate treatment and disposal. The bioconversion of saline resources into value-added products, such as biodegradable polyhydroxyalkanoates (PHAs), has not yet been fully explored. This study investigated PHA production by mixed microbial cultures under 30 $g_{NaCl}$/L, the highest NaCl concentration reported for the acclimatization of a PHA-accumulating mixed microbial culture (MMC). The operational conditions used during the culture-selection stage resulted in an enriched PHA-accumulating culture dominated by the Rhodobacteraceae family (95.2%) and capable of storing PHAs up to 84.1% wt. (volatile suspended solids (VSS) basis) for the highest organic loading rate (OLR) applied (120 Cmmol/(L.d)). This culture presented a higher preference for the consumption of valeric acid (0.23 ± 0.03 $Cmol_{HVal}$/($Cmol_X$.h)), and the 3HV monomer polymerization (0.33 ± 0.04 $Cmmol_{HV}$/($Cmmol_X$.h) was higher as well. As result, a P(3HB-co-3HV)) with high HV content (63% wt.) was produced in the accumulation tests conducted at higher OLRs and with 30 $g_{NaCl}$/L. A global volumetric PHA productivity of 0.77 gPHA/(L.h) and a specific PHA productivity of 0.21 gPHA/(gX.h) were achieved. These results suggested the significant potential of the bioconversion of saline resources into value-added products, such as PHAs.

**Keywords:** saline resources; halotolerant; PHA-accumulating MMC; PHAs accumulation; biopolymer; P(3HB-co-3HV)



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## 1. Introduction

The fish and seafood segment from the agro-food industry is growing globally with a compound annual growth rate (CAGR) of 4.8% (2021–2026) towards a market value of EUR 518 billion by 2021 [1]. Although the fishery industry has been associated with high nutritional and commercial value, it has also generated a large amount of organic side streams that require adequate treatment and disposal [2,3]. Worldwide, 96.4 million tonnes of fish were generated in 2021, 90% of which came from marine environments, generating saline organic side streams that contained NaCl in the range of 3.5–46 $g_{NaCl}$/L, depending on the final product [4]. Companies have therefore been required to conduct expensive treatments of those saline streams before they can be safely disposed, as the conventional biological treatments for organic compound removal are less efficient due to their strong salt inhibition [5,6]. The ability to use these saline organic side streams as feedstock for biologic processes would be economically favourable for these industries by reducing treatment costs while also reducing the environmental burden caused by the commonly used chemicals during treatment [7]. Moreover, the conversion of these resources into value-added products, such as polyhydroxyalkanoates (PHAs), would also decrease the costs of treatment and contribute towards a circular economy.

PHAs are biodegradable polymers produced by certain microorganisms as intracellular carbon and energy reserves in nutrient-limited environments, and the production of this polymer is of growing interest in today's society as they are feasible substitutes of conventional plastics, due to similar physicochemical properties and a wide range of potential applications [8]. The uses of PHAs are therefore increasing, with a CAGR of 14.2%, and it is estimated that this sector will reach a global market value of EUR 107 million by 2025 [9]. Nonetheless, the production costs of PHAs are still limiting wider commercialization and industrialization [8] of this bioplastic as the production costs are estimated to be four times higher (EUR 4–5/kg) than conventional plastics.

The use of mixed microbial cultures (MMCs) is a strategy to reduce costs of production as: (i) aseptic conditions are not required, (ii) it allows the use of inexpensive/cheap raw materials such as saline waste streams [10], and (iii) the MMCs are more amenable to deal with complex matrices due to the diversity of microorganisms and pathways.

A typical PHA production process by MMCs comprises three stages [8]: (i) acidogenic fermentation of the organic resources for the production of volatile fatty acids (VFAs), which are precursors for PHA biosynthesis; (ii) culture selection, where the inoculum is enriched in PHA-accumulating MMC by applying selective pressure (e.g., feast and famine (F/f) regime); and (iii) PHA production, where the selected PHA-accumulating MMC from the second stage are fed with VFA-rich streams produced in the first stage to promote PHA accumulation of up to the culture's maximum capacity.

The selection stage is considered the core of the process [11], as the obtained PHA-accumulating MMC will determine the efficiency of the production process, namely in terms of PHA storage capacity and PHA productivity [8]. This stage focuses not only in obtaining an MMC enriched with PHA-accumulating organisms, capable of producing high amounts of PHAs, but also in the ability to sustain a high growth of the enriched MMC for the PHA production stage, in order to increase the volumetric productivity of the global PHA production process [8]. However, a low volumetric productivity is normally obtained due to low biomass concentrations reached in the selection reactor [10]. This is related to the fundamentals of the generally adopted F/f selection strategy, where the culture is subjected to periods of carbon substrate excess (feast phase) followed by periods of carbon starvation (famine phase), so that the organisms that do not store PHAs are not able to grow during the famine period and are eliminated from the reactor [10]. Nutrient supplementation is an important factor for the success of the selection reactor, as some feedstocks, although rich in carbon compounds, are frequently poor in nutrients or have reduced nutrient availability, namely in nitrogen that is required to support culture growth. The uncoupled feedings of carbon and nitrogen, where the latter is added after the exogenous carbon is exhausted, have shown to lead to a better selection performance with lower F/f ratios, higher PHA concentrations, and reduced culture instability [10,12].

The ability to use saline resources for PHA production could also promote the economic viability of PHA production. Moreover, the use of saline organic streams could open the possibility of using seawater (≈3.5% NaCl w/v) as a washing and diluting agent in the PHA production process. Previous works, using cheese whey as feedstock for the production of biodegradable food packaging with PHAs, have shown that the production of PHAs has been associated with excess freshwater usage [13]. This represents not only a significant consumption of freshwater, as the rapid decrease in freshwater availability is a major concern worldwide, but also brings surplus operation costs to the process. It has also been suggested that PHA production at high salinity concentrations can facilitate the recovery of produced PHAs from halotolerant microorganisms, as the cells can be lysed in distilled water, reducing purification costs by avoiding the utilization of commonly used organic solvents for PHA extraction [14]. Therefore, the proposed measures, besides being eco-friendly, can be economically advantageous for industries located at costal sites.

Overall, the use of MMCs for the production of PHAs at high saline concentrations has potential benefits; however, PHA production using MMCs in the presence of saline conditions has not yet been fully established.

Xiao et al. provided a comprehensive review of different strategies for the anaerobic treatment of saline wastewaters [15]. Among these studies, the bacterial consortia collected from saline environments (e.g., marine sediments) have been highlighted as promising in anaerobic processes for the treatment of saline wastewaters. Moreover, the successful conversion of saline substrates into VFAs at NaCl concentrations up to 70 g/L was recently indicated. He et al. tested the acidogenic fermentation of a mixture of food waste that was artificially salted in a NaCl concentration up to 70 g/L. The highest VFA production was obtained at 10 $g_{NaCl}$/L (0.54 $g_{VFA}/g_{Feedstock}$) but was still high at the highest salt concentration applied (0.44 $g_{VFA}/g_{Feedstock}$) [16]. In another study, a mixture of food waste, brine, and wastewater derived from a biodiesel production facility was used to produce VFAs. The acidogenic fermentation of this salted feedstock (12–18 $g_{NaCl}$/L) was feasible with an acidification degree up to 46% [17]. Fra-Vasquez et al. evaluated the conversion of wastewater from a cooked mussel processing factory (22 $g_{NaCl}$/L and pH 4.6) into VFAs. In this study, an extensive carbohydrate degradation of 96% and a maximum acidification degree of 43% were obtained [18].

With respect to the selection of PHA-accumulating mixed cultures in saline conditions, only a few studies can be found with salinities up to 20 $g_{NaCl}$/L. Palmeiro-Sánchez et al. tested a PHA-accumulating MMC enriched using fermented tuna-processing wastewater in accumulation assays in the absence of NaCl and in the presence of 21.6 $g_{NaCl}$/L. Not only the PHA content decreased abruptly from 51.3% in the non-saline environment to 8.4% in the presence of NaCl, but also the HB–HV ratio changed, from 62:38 to 72:28 (% wt.) [19]. Conversely, in the work by Wen et al., the positive influence of NaCl on PHA storage in a culture-enriched reactor was reported [20]. Wen et al. studied the effects of NaCl at 0, 5.0, 10.0, and 15.0 $g_{NaCl}$/L on the enrichment of PHA-accumulating MMC using food waste. A maximum PHA content of 14.3% wt. was obtained under the condition at 15 $g_{NaCl}$/L, indicating the positive influence of NaCl; however, the maximum specific PHA production, biomass growth, and substrate consumption rates were found at 5 $g_{NaCl}$/L. In the PHA accumulation assays at the different NaCl concentrations with respective enriched cultures, the maximum PHA content of 50.5% wt. was also obtained at 5 $g_{NaCl}$/L and accumulations with higher NaCl led to lower PHA contents, as a PHA content of 42.6% wt. was obtained at 15.0 $g_{NaCl}$/L. Overall, Wen Q. et al. suggested that osmotic stress may trigger the synthesis of PHA; however, at higher NaCl contents, bacteria consume PHAs in response to high osmotic stress, leading to a lower maximum PHA content [20]. Alba Pedrouso A. et al. [21] were also able to obtain an MMC enriched in PHA-accumulating microorganisms at 5 $g_{NaCl}$/L using real feedstock obtained from the acidogenic fermentation of cooked mussel processing wastewater. This culture was able to reach a PHA content of 25% wt. using the same feedstock and with uncontrolled pH; however, when mimicking the feedstock using a synthetic mixture, the culture was able to reach 40% wt. Using the same feedstock, Argiz et al. [22] were able to select a more efficient PHA-accumulating MMC by removing the undesired substances (proteins and carbohydrates) with the addition of a settling stage and subsequent supernatant discharge after the end of the feast period of the cycle of an SBR. This culture was able to reach a higher PHA content of 60% wt. using a synthetic mixture as feedstock. In another work with the same feedstock but with high salinity levels, Roibás-Rozas et al. [23] were able to slowly adjust (throughout 100 days) the NaCl concentration in the selection reactor up to 20 $g_{NaCl}$/L, starting from a previously PHA-accumulating MMC selected at 5 $g_{NaCl}$/L. This culture was then also used for accumulation assays where the NaCl concentration was gradually increased throughout the assays until it reached 28 $g_{NaCl}$/L, reaching a PHA content of 41% wt.

The main aim of the present study was to assess the feasibility of selecting an efficient PHA-accumulating MMC under conditions of high salinity (30 $g_{NaCl}$/L). To date, this was the highest salinity value reported in the literature for the acclimatization of a PHA-accumulating MMC. This work was conducted at lab-scale, in a three-stage process for PHA production by MMC, where two reactors were operated for culture selection and PHA accumulation.

## 2. Materials and Methods

### *2.1. Culture Selection*

A sequence batch reactor (SBR) with a working volume of 2 L was used for culture selection. The SBR was inoculated with sediments collected from a saline area of Rio Tejo (Samouco, Portugal) that were passed through a filtration sieve (350 μm).

The SBR was operated under aerobic conditions with a hydraulic retention time (HRT) of 16 h, a solids retention time (SRT) of 3 days and 8 h cycles. Each cycle period consisted of 5 steps: (i) influent feeding/filling (5 min); (ii) aerated phase (434 min); (iii) purge (5 min); (iv) settling (30 min); and (v) supernatant withdrawal (6 min).

The reactor was operated in a temperature-controlled room (19–21 °C). Mixing was kept at 100 rpm using a one-blade impeller with a six-blade Rushton turbine, and pH was controlled at pH 8.4 ± 0.5 through automatic dosing of 0.5 M HCl. Air was supplied through fine bubble diffusers and the aeration rates (L/L) were adjusted throughout the operation so that the dissolved oxygen (DO) was not limited in the reactor. DO concentration and pH were monitored online.

A salted (30 $g_{NaCL}$/L) synthetic VFA mixture composed of acetic (HAc), propionic (HPro), butyric (HBut), and valeric (HVal) acids (25%Cmol each) was used as carbon source. Together with the synthetic mixture, a salted (30 $g_{NaCl}$/L) mineral solution was fed to the SBR in order to have the following concentrations of the following components (mg/L): ATU (10); EDTA-2Na (50); $MgSO_4$ (50); $CaCl_2$ (50); $FeCl_3 \cdot 6H_2O$ (1.5); $H_3BO_4$ (0.15); $CuSO_4 \cdot 5H_2O$ (0.03); KI (0.03); $MnCl_2 \cdot 4H_2O$ (0.12); $Na_2MoO_4 \cdot 2H_2O$ (0.06); $ZnSO_4 \cdot 7H_2O$ (0.12); and $CoCl_2 \cdot 6H_2O$ (0.15). The feeding was composed of 100 mL carbon solution plus 900 mL dilution mineral solution.

The SBR followed an aerobic dynamic feeding, namely F/f regime with uncoupling of the carbon and nitrogen availabilities in order to obtain selection pressure for PHA-accumulating organisms. A C/N/P (Cmol basis) ratio of 100:7.5:1 was initially applied; later, this ratio was changed to 100:10:1 and then to an optimal ratio value of 100:5:1. An ammonia solution was fed to the reactor 1 h after carbon feeding. Phosphorous was given through addition to the mineral solution. The OLR was initially 60 Cmmol/(L.d) and was increased to 120 Cmmol/(L.d) as a stable culture enriched in PHA-accumulating organisms was obtained. The study had a total duration of 145 days.

### *2.2. PHAs Accumulation*

MMC PHA-accumulation performance of the selected culture at two different OLRs (ACC-60 and ACC-120 for 60 and 120 Cmmol/(L.d), respectively), was assessed in a fed-batch reactor. A total of 1 L of biomass was collected from the corresponding SBR at the end of famine phase and used as inoculum. The accumulation reactor had a working volume of 1 L, and the accumulation procedure consisted in a pulse-wise feeding strategy of salted synthetic mixture (30 $g_{NaCl}$/L) under nitrogen-limiting conditions and using a food to microorganism (F/M) ratio of 1.5 times the one found in the SBR. Whenever the maximum PHA capacity of the selected culture was attained, biological activity was stopped by quenching to pH 2–3 using sulfuric acid. Assays were carried out with the same controlled conditions of pH, temperature, aeration, and stirring that were used for culture selection.

### *2.3. Analytical Procedures*

Total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to standard methods [24].

VFAs were quantified in filtered samples (0.20 μm) using a high-performance liquid chromatography (HPLC) in a VWR Hitachi Chromaster chromatographer equipped with a Pump 5160, an auto sampler 5260, a Column Oven 5310, a Diode Array Detector 5430, a RI Detector 5450, a Biorad 125-0129 30 × 4.6 mm pre-column, and an Aminex HPX-87H 300 × 7.8 mm column. The following conditions were used: column temperature 60 °C, 0.01 M $H_2SO_4$ eluent, flow rate 0.6 mL/min, and injection volume 99 μL. The

VFA concentrations were calculated from standard calibration curves (4–1000 mg/L of each compound).

Ammonia concentrations were determined in filtered samples (0.20 μm) using a segmented continuous flow analyser (Skalar SNA++). PHAs were extracted and hydrolyzed. The monomers of PHAs were esterified into 3-hydroxyacyl methyl esters to be quantified by gas chromatography (GC-FID, Brucker) using a method described in Oliveira et al. [11].

Lyophilized biomass was weighted and incubated with 1 mL chloroform and 1 mL acidic methanol (20% $H_2SO_4$) (for methanolysis) through digestion at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (2 μL) into a gas chromatograph equipped with a flame ionization detector (Bruker 430-GC) and a BR-SWax column (60 m, 0.53 mm internal diameter, 1 mm film thickness, Bruker, USA), using helium as carrier gas at 1.0 mL/min. The temperature regime started at 40 °C and increased stepwise to 100 °C at a rate of 20 °C/min, to 175 °C at a rate of 3 °C/min, and to 220 °C at a rate of 20 °C/min (cleaning step of the column after each injection). Injector and detector temperatures were 280 °C and 230 °C, respectively. Determination of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) concentrations was made through the use of two calibration curves, one for 3HB and other for 3HV, using standards (0.1–10 g/L) of a commercial P(3HB-co-3HV) (88%: 12%, Sigma) and corrected using heptadecane as internal standard (concentration of ≈1 g/L). Intracellular PHA granules were identified using Nile blue staining as described in Oliveira et al. [11] and observed with epifluorescence microscope Olympus BX51 equipped with an Olympus XM10 camera (Cell-F software). Microbial community assessment samples were collected for microbial community analysis at the beginning of the study (inoculum), from cycles throughout operation of the reactors and from the accumulation assays.

Biomass samples from sediments collected from a saline area of Rio Tejo (Samouco, Portugal) (inoculum) and from the culture selected at OLR of 60 and 120 Cmmol/(L.d) were collected and were phylogenetically characterized on high-throughput sequencing of the 16S V1-3 rRNA gene. DNA extraction, gene sequencing and bioinformatics processing was carried out by DNASense (Aalborg, Denmark) as described in [25].

### *2.4. Calculations*

The F/f ratio was calculated as the ratio between the period lengths of feast and famine phases of the SBR cycle. The PHA content in VS ($g_{PHA}/g_{VS}$) was calculated by multiplying the PHA content in TS ($g_{PHA}/g_{TS}$, given by GC analysis) by the VS/TS ratio obtained for the lyophilized pellets used for GC analysis. The PHA content in VSS ($g_{PHA}/g_{VSS}$) was calculated by multiplying the PHA content in VS by the VSS/VS ratio obtained in the reactor (VSS/VS ≈ 1). The PHA content in the biomass was determined in terms of percentage of VSS on mass basis (% wt., $g_{PHA}/g_{VSS}$). VSS were considered to be constituted of active biomass (X) and PHA. For determining cell growth, the generic chemical formula for MMC ($CH_{1.8}O_{0.5}N_{0.2}S_{0.02}P_{0.02}$) [26], with a molecular weight (MW) of 25.30 g/Cmol, was used. The ΔPHAs was determined as the maximum PHA content ($PHA_{max}$,% wt.) minus the PHA content at the beginning ($PHA_0$,% wt.) of the experiment. Stoichiometric and kinetic performance parameters were determined for the SBR and PHA accumulation assays in pseudo-steady-state conditions with the reactors operated at both OLRs.

The specific substrate consumption rates (-qVFA, $Cmol_{VFA}/(Cmol_X.h)$); specific PHA storage (qPHA, $Cmol_{PHA}/(Cmol_X.h)$) and consumption (-qPHA, $Cmol_{PHA}/(Cmol_X.h)$) rates; and specific growth rates (qX, $Cmol_X/(Cmol_X.h)$) were determined from the linear regression of the experimental data of the substrates, PHA, and X-specific concentrations (i.e., instant concentration divided by the biomass concentration), respectively, and plotted over time. Specific storage yield ($Y_{PHA/S}$, $Cmol_{PHA}/Cmol_{VFA}$) was calculated as the ratio between qPHAs and the -qVFAs.

Growth yields on stored PHAs ($Y_{X/PHA}$, $Cmol_x/Cmol_{PHA}$) were calculated as the ratios between the specific growth rate during the famine phases ($qX_{famine}$) and -qPHA.

In the accumulation assays, the specific rates and yields were calculated, as described before, for each pulse. In the accumulation tests, the first three pulses' average values of each parameter were considered. Volumetric PHA productivity ($g_{PHA}/(L.h)$) was calculated as the ratio of cumulative produced PHAs (ΔPHA) in 1 L of working volume per unit of time (hour). Specific PHA productivity ($g_{PHA}/(g_X.h)$) was calculated as the ratio of cumulative produced PHAs per X at the beginning of the assay ($X_0$) per unit of time (hour).

Standard errors associated with the determined parameters were estimated using standard errors propagation formulae.

## 3. Results and Discussion

### *3.1. Culture Selection: PHA-Accumulating MMC*

The culture selection stage for a PHA-accumulating mixed culture under conditions of high salinity (30 $g_{NaCl}/L$) could indicate the potential value of saline resources as well as the benefit of using seawater as a washing and diluting agent in the PHA production process.

In order to select a halotolerant PHA-accumulating MMC, sediments collected from a saline area (Samouco, Portugal) were used to inoculate the SBR with halotolerant organisms and then submitted to an SRT for three days and to an aerobic dynamic feeding, namely the conventional F/f regime and the decoupled carbon and nitrogen feeding, throughout the operation. An artificially salted (30 $g_{NacL}/L$) equimolar (Cmol basis) synthetic mixture was used as saline-fermented feedstock. The F/f ratio was continuously monitored (Figure 1) since it is a useful performance indicator of the culture selection process as an F/f below 0.2 h/h has been thought to boost the selection of an efficient PHA-accumulating culture [8].

The culture selection was started with an OLR of 60 Cmmol/(L.d) and a C/N/P ratio of 100 Cmol:7.5 Nmol:1 Pmol. An F/f ratio below 0.2 h/h was obtained after 14 days of operation, suggesting that the selective pressure was correctly being applied. However, the selected culture was unable of complete nitrogen consumption at the initial (100 Cmol: 7.5 Nmol) or higher (100 Cmol: 10 Nmol) C/N applied. The C/N ratio was then optimized to 100 Cmol: 5 Nmol (Figure 1), and a stable PHA-accumulating MMC was attained (F/f < 0.2 h/h). With regards to suspended solids (VSS and TSS), a strong increase in the TSS over VSS was observed for the first period of acclimatization (Figure 1), resulting in a VSS/TSS ratio of 29 ± 5% wt. for the culture selected at an OLR of 60 Cmmol/(L.d), which suggested the intracellular accumulation of inorganic compounds. Several halophile microorganisms have been reported to accumulate a variety of small molecules, both inorganic (e.g., $Na^+$, $K^+$, $Cl^-$) and organic (e.g., ectoine, glycine betaine, 3-hydroxybutyric acid), in the cytoplasm to counteract the external osmotic pressure [27].

The selected culture enriched at OLR 60 Cmmol/(L.d) showed a cycle with a typical profile for the aerobic dynamic feeding strategy (F/f regime and uncoupled carbon and nitrogen availabilities) (Figure 2A). As carbon, in the form of VFAs, was added to the reactor, cells began consuming the VFAs and storing carbon in the form of PHAs. As the VFAs were completely consumed (end of the feast), PHA production halted, and a small decrease in PHA concentration in the reactor occurred due to cell metabolic activity. After 1 h of feeding, nitrogen, in the form of ammonia, was added to the reactor, allowing the cell growth of PHAs. This caused a decrease in PHA content as it was consumed for growth and other metabolic activity. As nitrogen in the media was depleted, the PHA consumption rates decreased throughout the remaining cycle. The VSS concentration increased with the increase in PHA content during the feast period, but as the VFAs were depleted and the PHAs were consumed for cell maintenance and growth, the VSS concentration decreased.

In order to further increase volumetric PHA productivity, the OLR was doubled to 120 Cmmol/(L.d) whilst maintaining selective pressure for PHA-accumulating organisms (Figure 1). Subsequently, the concentration of active biomass (X) increased ≈1.7 times from 1.92 ± 0.04 to 3.26 ± 0.34 g/L (Figure 1) while also increasing the PHA storage capacity from 35 ± 2% wt. to 49 ± 3% wt., indicating a good culture adaptation to higher organic load and even leading to better culture performance. The obtained PHA contents at the end of the feast period were already quite high and comparable with other reported

accumulation assays [23], which suggested the possibility of skipping the third stage of the traditional MMC PHA-accumulating process in order to lower operating costs. This culture showed a cycle profile very similar to that obtained in an OLR of 60 Cmmol/(L.d) (Figure 2B), whereas the VSS/TSS ratio shifted to 52 ± 0% wt.

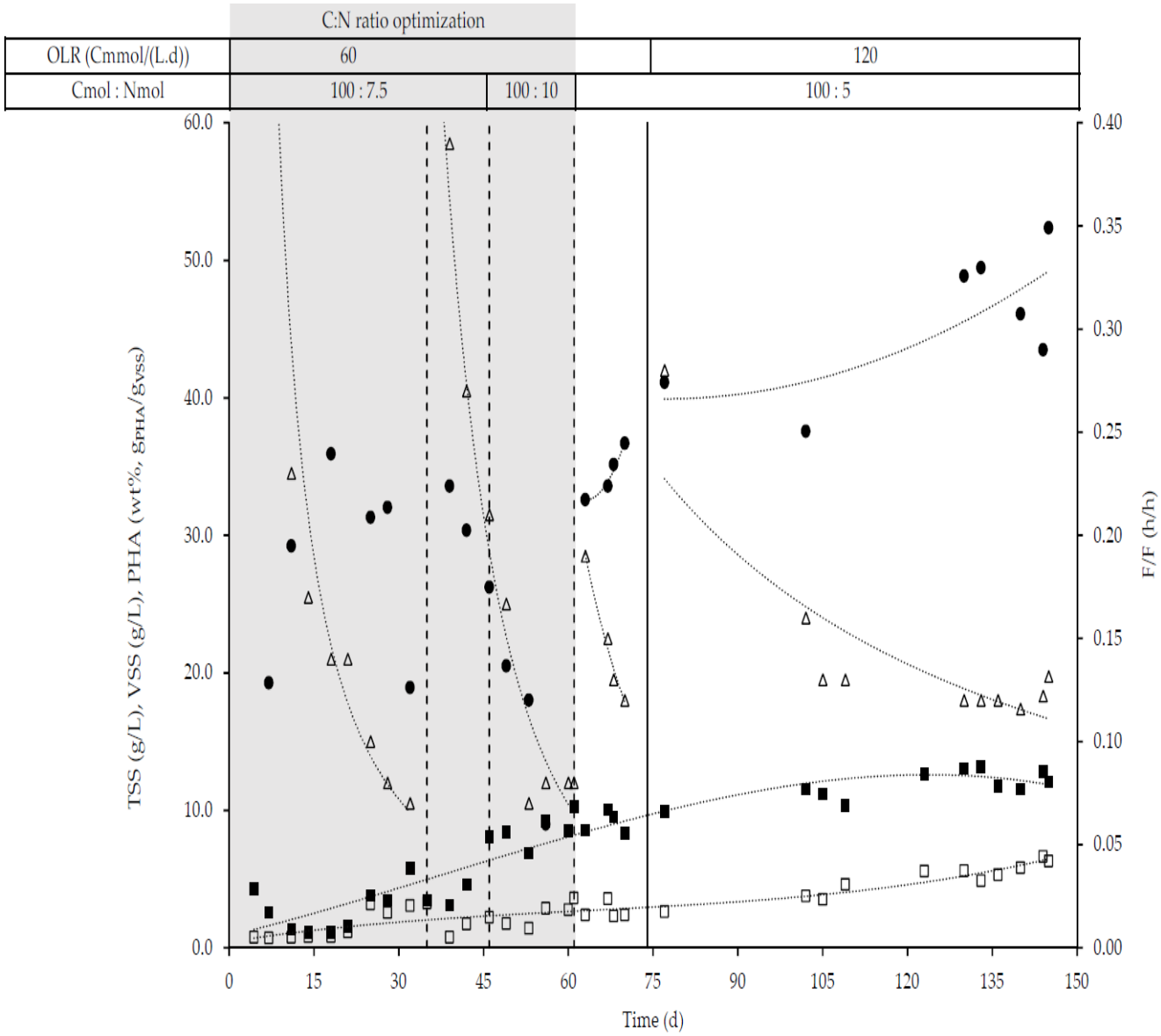


**Figure 1.** Profile of the mixed culture adaptation throughout the operation. A feast and famine (F/f) below 0.2 h/h is considered to boost the selection of an efficient PHA-accumulating culture. Gray-filled areas represent different C/N ratios used and the white area is reactor operation at optimal C/N. The vertical solid line represents the organic loading rate increase. TSS (■); VSS (□); F/f(Δ); and PHAmax (●).

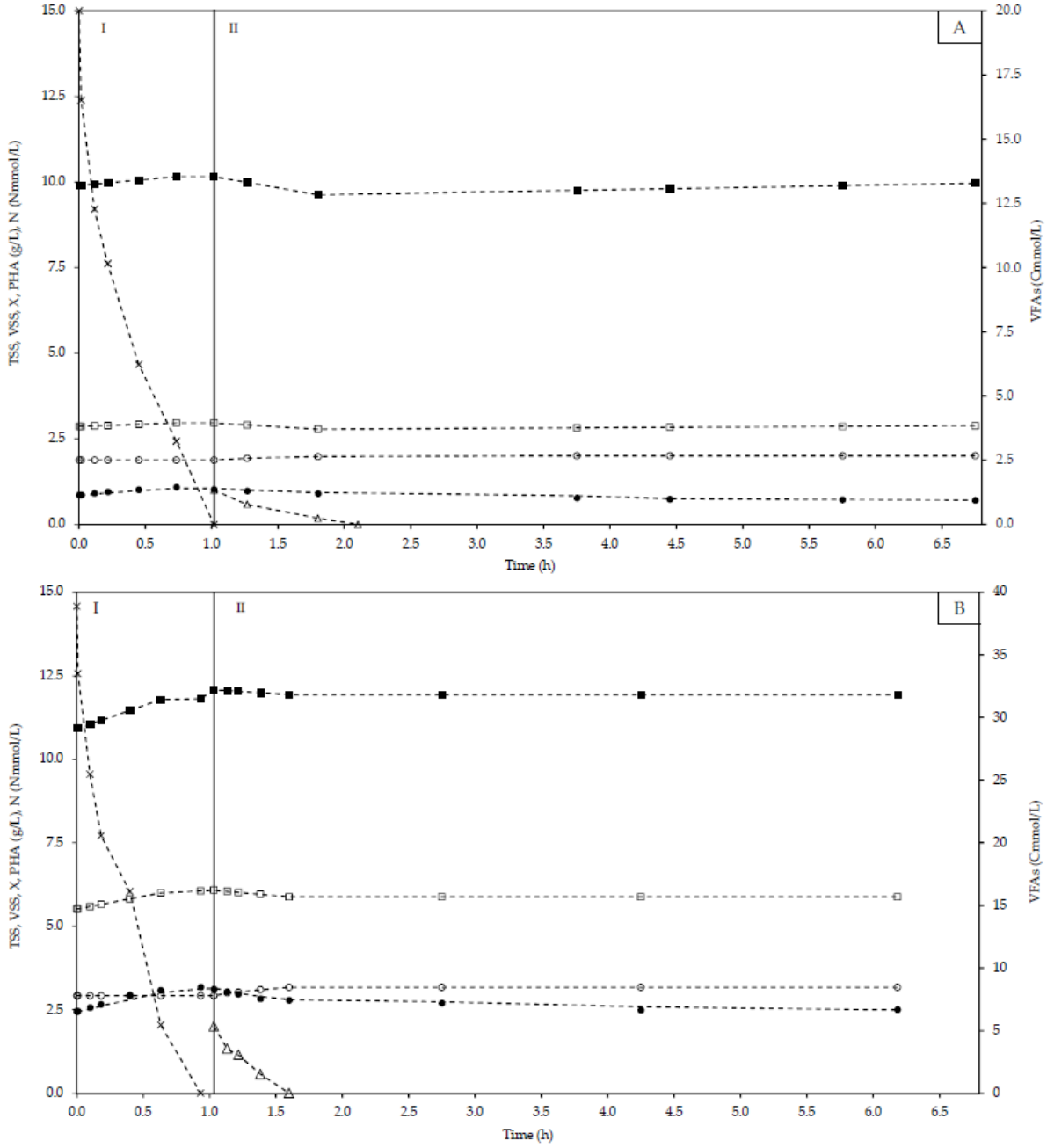


**Figure 2.** Representative SBR cycle conducted at organic loading rate (OLR) 60 Cmmol/(L.d) (**A**) and 120 Cmmol/(L.d) (**B**). The vertical solid line limit feast (I) and famine (II) periods. TSS (■); VSS (□); $NH_4^+$(Δ); PHAs (●); X (o); and VFAs (X).

The culture was then subjected to another OLR increase to 180 Cmmol/(L.d); however, the system became unstable as the biomass settling characteristics suffered a change, increasing the sludge volume index up to a point that the used reactor could not support, and the operation was stopped.

With regards to PHA polymer, a composition of an average HB/HV ratio of 48%:52% wt. (46%:54%Cmol) was obtained for an OLR of 60 Cmmol/(L.d), whereas a polymer with higher 3HV content, 35%:65% wt. (33%:67%Cmol), was produced at a higher OLR. Considering that HV monomers can be produced by a single molecule of valeric acid or by a combination of acetic and propionic acids in a ratio of 1:1 molecules [28], approximately 46% ± 2% (≈6.1 $Cmol_{HV}$/13.3 $Cmol_{HVprecursors}$) and 82% ± 2% (≈21.9 $Cmol_{HV}$/26.7 $Cmol_{HVprecursors}$) of the HV precursors were used towards HV synthesis for OLR 60 and 120 Cmmol/(L.d),

respectively. The recruitment of 3HB as a compatible solute to counteract the high external osmotic pressure and to be a potent compatible solute with chaperoning activity [27,29] could be the explanation/promoter for the higher HV polymerization and the obtained HB:HV ratio.

Overall, the general behaviour of the different OLRs was similar; however, the specific consumption and production rates found for the tested conditions had noticeable differences (Table 1). The consumption rates of acetic and propionic acids were similar, 0.08 ± 0.00 vs. 0.10 ± 0.01 $Cmol_{HAc/}(Cmol_X.h)$ and 0.10 ± 0.02 vs. 0.11 ± 0.03 $Cmol_{HPro/}(Cmol_X.h)$, whereas consumption rates of butyric and valeric acids were higher under conditions with higher OLR, increasing to 78% from 0.09 ± 0.01 to 0.16 ± 0.03 $Cmol_{HBut/}(Cmol_X.h)$ and to 77% from 0.13 ± 0.02 to 0.23 ± 0.03 $Cmol_{HVal}/(Cmol_X.h)$ (OLR 60 vs. OLR 120). The higher consumption-specific rates of these VFAs led to a slight difference in the overall VFA consumption rates, which increased from 0.40 ± 0.03 $Cmol_{VFA}/(Cmol_X.h)$ at an OLR of 60 Cmmol/(L.d) to 0.60 ± 0.04 $Cmol_{VFA}/Cmol_X$ at an OLR of 120 Cmmol/(L.d). With regards to PHA production, there was a considerable difference in the specific production rate of the 3HV monomer, being three times higher (0.11 ± 0.01 and 0.33 ± 0.04 $Cmol_{HV}/(Cmol_X.h)$), whereas the 3HB production was slightly higher, 0.09 ± 0.00 and 0.15 ± 0.02 $Cmol_{HB}/(Cmol_X.h)$, which could explain the sizeable differences in the composition of the copolymer produced by the culture. The determined PHA production yield was also considerably higher for the higher OLR (0.60 ± 0.01 vs. 0.75 ± 0.04 $Cmol_{PHA}/Cmol_{VFA}$).

**Table 1.** Operating conditions applied and performance parameters determined for the culture selection stage considering the average of two monitored batches carried out at organic loading rate (OLR) 60 and 120 Cmmol/(L.d).

| **Parameter (Unit)** | **Average ± Standard Deviation** | |
|---|---|---|
| OLR ($Cmmol_{VFA}/(L.d)$) | 60 | 120 |
| FP profile (HAc/HPro/HBut/HVal,% Cmol basis) | 25:25:25:25 | 25:25:25:25 |
| Feast/famine (h/h) | 14.0 ± 0.00 | 0.13 ± 0.01 |
| X @cycle start ($g_X/L/Cmol_X/L$) | 1.92 ± 0.04/75.8 ± 1.70 | 3.26 ± 0.34/129 ± 13.6 |
| $PHA_{max}$ (% wt., VSS basis) | 35.1 ± 1.56 | 49.2 ± 3.13 |
| ΔPHAs ((% wt., VSS basis) | 5.70 ± 0.93 | 9.6 ± 1.76 |
| HB/HV ratio (% wt. basis/Cmol basis) | 48:52/46:54 | 35:65/33:67 |
| -qVFA ($Cmmol_{VFA}/(C\text{-}mmol_X.h)$) | 0.40 ± 0.03 | 0.60 ± 0.04 |
| -qHAc ($Cmmol_{HAc}/(Cmmol_X.h)$) | 0.08 ± 0.00 | 0.10 ± 0.01 |
| -qHPro ($Cmmol_{HPro}/(Cmmol_X.h)$) | 0.10 ± 0.02 | 0.11 ± 0.03 |
| -qHBut ($Cmmol_{HBut}/(Cmmol_X.h)$) | 0.09 ± 0.01 | 0.16 ± 0.03 |
| -qHVal ($Cmmol_{HVal}/(Cmmol_X.h)$) | 0.13 ± 0.02 | 0.23 ± 0.03 |
| qPHAs ($Cmmol_{PHA}/(Cmmol_X.h)$) | 0.20 ± 0.01 | 0.46 ± 0.01 |
| qHB ($Cmmol_{HB}/(Cmmol_X.h)$) | 0.09 ± 0.00 | 0.12 ± 0.01 |
| qHV ($Cmmol_{HV}/(Cmmol_X.h)$) | 0.11 ± 0.01 | 0.33 ± 0.04 |
| $qX_{famine}$ ($Cmmol_X/(Cmmol_X.h)$) | 0.06 ± 0.00 | 0.15 ± 0.02 |
| -qPHAs ($Cmmol_{PHA}/(Cmmol_X.h)$) | 0.10 ± 0.03 | 0.36 ± 0.01 |
| -qHB ($Cmmol_{HB}/(CmmolX.h)$) | 0.08 ± 0.02 | 0.13 ± 0.02 |
| -qHV ($Cmmol_{HV}/(Cmmol_X.h)$) | 0.09 ± 0.03 | 0.23 ± 0.03 |
| $Y_{PHA/VFA}$ ($Cmmol_{PHA}/Cmmol_{VFA}$) | 0.60 ± 0.01 | 0.75 ± 0.04 |
| $Y_{X/PHAs}$ ($Cmmol_X/Cmmol_{PHA}$) | 0.70 ± 0.18 | 0.44 ± 0.03 |

During the famine phase, the specific consumption of PHAs (-qPHA) and the biomass growth ($q_{Famine}$) were higher in the higher OLR, being 0.36 ± 0.01 $Cmmol_{PHA}/(Cmmol_X.h)$ and 0.15 ± 0.02 $Cmmol_X/(Cmmol_X.h)$ vs. 0.10 ± 0.03 $Cmmo_{PHA}/(Cmmol_X.h)$ and 0.06 ± 0.00 $Cmmol_X/(Cmmol_X.h)$, respectively, for the culture with an OLR of 60 Cmmol/(L.d). However, the obtained yields for the biomass growth were 0.70 ± 0.18 $Cmol_X/Cmol_{PHAs}$ and 0.44 ± 0.03 $Cmol_X/Cmol_{PHAs}$ for the higher OLR condition. In this respect, the recruitment of 3HB, as a player in the osmotic balance, could also explain the differences found in growth yields.

Comparing the obtained results with the ones obtained by Oliveira et al., using real non-saline feedstock and an uncoupled feast and famine strategy, the culture in the present study had higher qPHAs ($0.33 \pm 0.04$ vs. $0.24 \pm 0.0$ $Cmol_X/Cmol_{PHA}$) even though it was in presence of 30 $g_{NaCl}/L$ [10]. However, the real feedstock used also had in the composition a small amount of ammonia and proteins that may have contributed to the slightly lower qPHAs values. The obtained values in the present study were also higher, as compared to a study by Alba Pedrouso et al., where the culture that had acclimatized at 5 $g_{NaCl}/L$ reached a qPHA of 0.24 $Cmmol_{PHA}/(Cmmol_X.h)$ [21]. It was also considerably higher, as compared to the slowly adapted culture to 20 $g_{NaCl}/L$ in the work of Roibás-Rozas et al., where the qPHA was only 0.05 $Cmmol_{PHA}/(Cmmol_X.h)$ [23]. The use of an inoculum obtained from a saline environment, a strategy that other studies have not followed, may have played a major role in the swiftness and favourable outcome of the enrichment reactor as the microorganisms were already adapted to their natural saline environments and were, therefore, able to outperform cultures that had not been naturally adapted.

The differences found between the PHA storage capacity, the specific rates of the two applied OLRs, as well as the shift of the HB/HV ratio of the produced polymer suggested a slight culture change in the OLR of 120 Cmmol/(L.d). In this respect, culture samples were taken for the identification of the selected microorganisms just before the change of the OLR and at the end of the operation. The 16S rRNA gene sequencing revealed the presence of a diverse, mixed microbial community in the sediments collected from the saline area of Rio Tejo and that the applied selective pressure for PHA-accumulating organisms had led to a shift in the mixed microbial profile (Supplementary Material, Figure S1). The culture selected at an OLR of 60 Cmmol/(L.d) was enriched on a microorganism from the Alphaproteobacteria phylum, dominated by a species from Rhodobacteraceae (74.5%) as well as a few species from the Phyllobacteriaceae (13.3%) family, whereas at the highest OLR operation (120 Cmmol/(L.d)), the Rhodobacteraceae family dominated even more, representing 95.2% of the selected culture. Rhodobacteraceae are aquatic bacteria that have a vast global distribution [30] and frequently thrive in marine environments, which was in accordance with the origin of the used inoculum and the saline applied conditions. Rhodobacteraceae family members are known to have PhaC class I PHAs synthase, producing short-chain length PHAs based on VFA precursors availability [31,32]. It has also been reported that the synthesis of both P3HB and P(3HB-co-3HV) polymers were from unrelated carbon sources [33].

Overall, an efficient and stable halotolerant PHA-accumulating culture was successfully selected under high salinity conditions (30 $g_{NaCl}/L$), and to the best of our knowledge, this may be the first report of culture selection for PHA-accumulating MMCs at such a high salt concentration.

### *3.2. PHAs Accumulation Assays*

After the cycles were characterized, half of the biomass of the selection reactor at the end of the famine phase was harvested for an accumulation assay in a fed-batch reactor using a pulse-wise feeding strategy (Figure 3). Pulses of the VFA mixture without ammonia and with a food to microorganism ratio (Cmol/gVSS) of 1.5 times the one found in the selection reactor were given to the culture as the DO concentration started to increase, so that the culture was consistently consuming VFAs and accumulating PHAs. For both accumulation assays, the biomass concentrations were considered constant as no nitrogen was fed to the system, and the specific rates of the three initial pulses were aligned with the presented averages. The obtained results are presented in Table 2.

The accumulation reactor that operated at an OLR of 60 Cmmol/(L.d) (ACC-60, Figure 3A) was fed with 4 pulses, reaching a maximum PHA content of 55.3% wt. with a polymer composition of 49%: 51% (% wt./wt., HB/HV) was similar to the one found in the preceding monitored cycles.

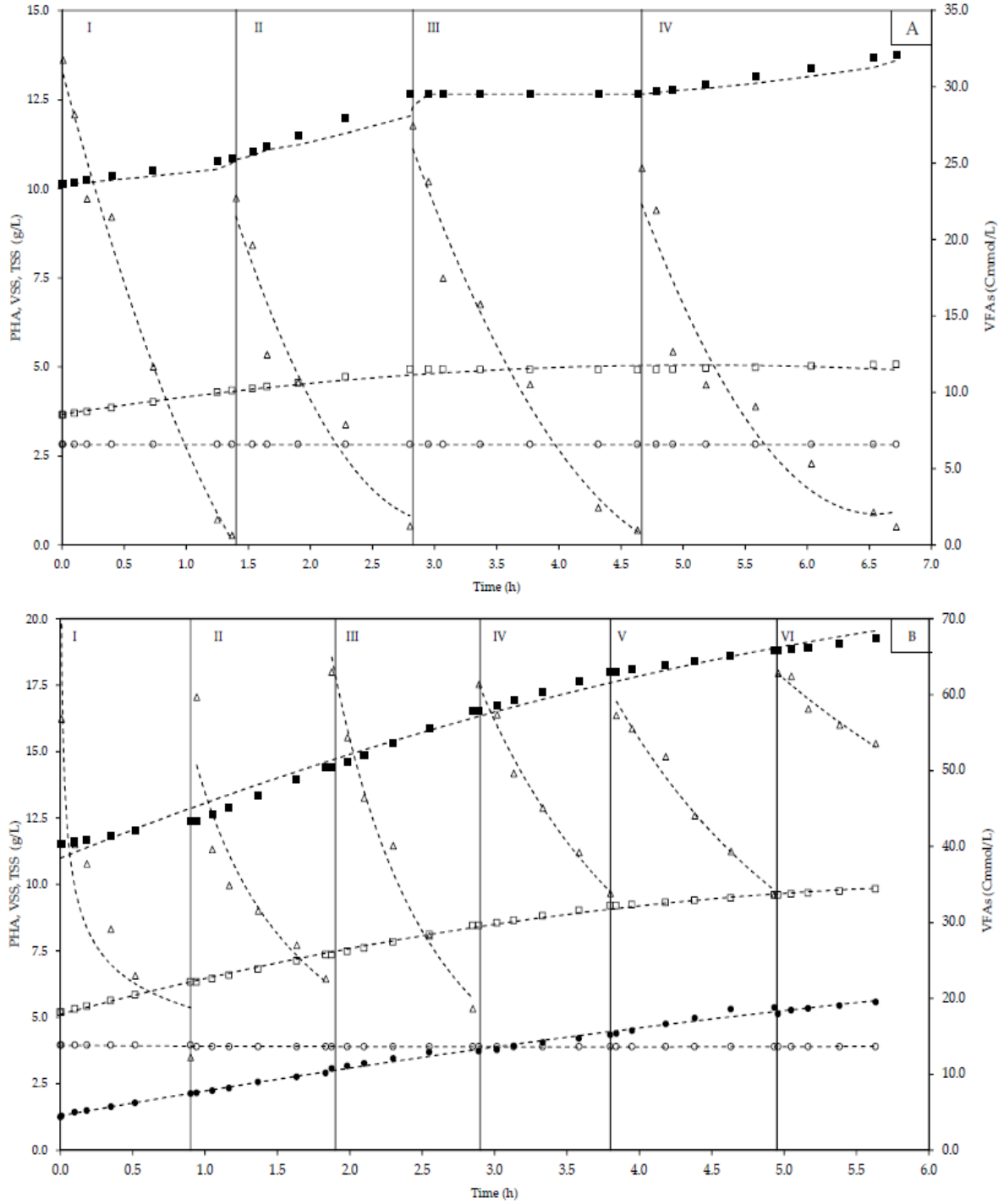


**Figure 3.** Profile of fed-batch accumulations conducted with the cultures selected at OLRs of 60 Cmmol/(L.d) (**A**) and 120 Cmmol/(L.d) (**B**). Pulses limited by the vertical solid lines and numbered between I and IV for ACC-60 and I and VI for ACC-120. TSS (■); VSS (□); PHAs (●); X (o); and VFAs (Δ).

The accumulation with the culture selected at an OLR 120 Cmmol/(L.d) (ACC-120) had a slightly different behavior and provided carbon pulses closer to the main PHA precursors depletion (valeric and butyric acids) and, therefore, completed a 6-pulse accumulation assay in less time than the 4 pulses of ACC-60. The higher consumption rates of valeric and butyric acids obtained for the culture selected at an OLR 120 Cmmol/(L.d) (Table 1) allowed us to identify their real-time depletion based on the DO profile (Supplementary Material,

Figure S2). However, due to the feeding strategy applied, acetic and propionic acids were accumulated throughout the assay at approximately 95% of the total remnant VFA concentration (53 Cmmol/L) in the effluent when the assay was stopped. The accumulation at an OLR of 120 Cmmol/(L.d) (ACC-60, Figure 3B) was able to reach a PHA content of 84.1% wt.

**Table 2.** Operating conditions applied and performance parameters determined for the PHA-accumulation stage considering one monitored batch carried out with the culture selected at OLRs of 60 and 120 Cmmol/(L.d). Only the first four pulses were considered for the average values, as during the fifth pulse, the exogenous carbon was mainly channelled for growth.

| Parameter (Unit) | Average ± Standard Deviation | |
|---|---|---|
| OLR ($Cmmol_{VFA}$/(L.d)) | 60 | 120 |
| FP profile (HAc/HPro/HBut/HVal,% Cmol basis) | 25:25:25:25 | 25:25:25:25 |
| X @inoculum ($g_X$/L/$Cmol_X$/L) | 2.73/108 | 3.72/147 |
| PHAs @inoculum (% wt., VSS basis) | 22.3 | 35.4 |
| $PHA_{max}$ (% wt., VSS basis) | 55.3 | 84.1 |
| HB/HV ratio (% wt. basis/Cmol basis) | 49:51/47:53 | 37:63/35:65 |
| -qVFA [b] ($Cmmol_{VFA}$/(C-$mmol_X$.h)) | 0.41 ± 0.02 | 0.45 ± 0.04 |
| qPHA [b] ($Cmmol_{PHA}$/($Cmmol_X$.h)) | 0.24 ± 0.01 | 0.30 ± 0.01 |
| $Y_{PHA/VFA}$ ($Cmmol_{PHA}$/$Cmmol_{VFA}$) | 0.60 ± 0.05 | 0.67 ± 0.05 |
| Volumetric PHA productivity ($g_{PHA}$/(L.h)) | 0.30 [a] (0.32 [b]) | 0.77 [a] (0.84 [b]) |
| Specific PHA productivity($g_{PHA}$/($g_X$.h)) | 0.11 [a] (0.12 [b]) | 0.21 [a] (0.28 [b]) |

[a] considering 4 pulses for ACC-60 and 6 pulses for ACC-B. [b] considering the first 3 pulses of the accumulation test.

The HB/HV polymer composition of ACC-120 was also similar to the one found in the cycle monitorizations that preceded the accumulation at 37%:63% (% wt./wt., HB/HV) and 35%:65% (Cmmol basis; HB/HV). As seen in the comparison of the monitorizations at the different OLRs, the qPHA was higher in the ACC-120 with a value of 0.22 ± 0.03 $Cmol_{HV}$/($Cmol_X$.h) than the 0.16 ± 0.01 $Cmol_{HV}$/($Cmol_X$.h) obtained for ACC-60. The PHAs storage yield was also higher in ACC-120 with a value of 0.67 ± 0.05 $Cmol_{PHA}$/$Cmol_{VFA}$ while 0.60 ± 0.05 $Cmol_{PHA}$/$Cmol_{VFA}$ was the yield found for ACC-60. Overall, a higher volumetric (0.77 $g_{PHA}$/(L.h)) and specific productivity (0.21 $g_{PHA}$/($g_X$·h)) were obtained in ACC-120, as compared to ACC-60 (0.3 $g_{PHA}$/(L.h)) and 0.11 $mg_{PHA}$/($g_X$.h), respectively). The obtained values, namely in ACC-120, were considerably higher than the presented results of Roibas-Rozas et al., where an adapted MMC to a lower NaCl concentration (close to 20 $g_{NaCl}$/L) was able to reach a PHA concentration of 41.5% wt. with a specific productivity of 68.1 $mg_{PHA}$/($g_X$.h) [23]. These results were, however, obtained by using a real feed, rich in nitrogen, and the salt concentration was increased throughout the assay, starting from 5 $g_{NaCl}$/L, and the VFA mixture used was 43:7:42:8 (Cmol basis) acetic/propionic/butyric/valeric acids [23]. The obtained values in the present study were also significantly higher, as compared to those obtained by Oliveira et al. of 33% wt. using the acclimatized culture with an uncoupled F/f strategy and real feedstock (as previously described). Nevertheless the qPHAs and specific productivity were slightly higher than those obtained in the present study with 0.41 $Cmol_{PHA}$/($Cmol_X$.h) and 0.25 $g_{PHA}$/($g_X$.h), respectively [10].

The high HV content in the copolymer (Table 2) obtained at the highest OLR was unusually high and surpassed the lowest melting temperature range obtained for P(3HB-co-3HV) accordingly to the work by Chan et al. [34]. In that study the melting temperature decreased to 60–70 °C for copolymers with an HV content up to 35–44% Cmol and increased to a range of 80–100 °C for higher HV contents. The morphological and physical properties of the obtained polymer enriched in HV will be determined in future studies, which will give insights about potential applications of the polymer.

## 4. Conclusions

The bioconversion of VFAs into PHAs by MMC under high salinity was shown to be feasible in our study. An efficient halotolerant PHA-accumulating MMC dominated by Rhodobacteraceae (93%) was selected at a salinity of 30 $g_{NaCl}/L$ by submitting the culture to an aerobic dynamic feeding, namely an F/f regime with uncoupled carbon and nitrogen availabilities throughout the operation. This culture showed good adaptation to high salinity, likely due to the activation of the metabolic pathways to counteract the osmotic pressure, namely through the assimilation of inorganic compounds and the recruitment of 3HB as osmolytes.

The culture demonstrated a high PHA storage capacity, reaching 84.1% wt. of P(3HB-co-3HV) with an HV content of 63% wt. The produced sort-chain-length copolymer was unusual due to its high HV content. This efficient culture also presented a notable volumetric productivity of 0.77 $g_{PHA}/(L.h)$ and specific productivity of 0.21 $g_{PHA}/(gX.h)$.

To best of our knowledge, this is the first study of a successful acclimatization of an efficient PHA-accumulating MMC with near-sea-water salinity. It suggested the potential for using saline side streams as feedstock for PHA production as well as for using sea water in the process, which could lower production costs and the environmental burden as well as promote the integration of this biopolymer in the market.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14031346/s1, Figure S1: Heatmap of the most abundant bacterial microbial communities present in the inoculum and the selected cultures for OLR of 60 and 120 Cmmol/(L.d).; FigureS2: Representative oxygen profile consumption for a cycle at OLR of 60 Cmmol/(L.d) (dash line) and at OLR of 120 Cmmol/(L.d) (full line).

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## References

1. Fish & Seafood—Worldwide | Statista Market Forecast. Available online: https://www.statista.com/outlook/cmo/food/fish-seafood/worldwide (accessed on 29 November 2021).
2. Ching, Y.C.; Redzwan, G. Biological treatment of fish processing saline wastewater for reuse as liquid fertilizer. *Sustainability* **2017**, *9*, 1062. [CrossRef]
3. Cleaner Production Assessment in Fish Processing. Available online: https://digitallibrary.un.org/record/441680 (accessed on 21 May 2021).
4. Anh, H.T.H.; Shahsavari, E.; Bott, N.J.; Ball, A.S. Options for Improved Treatment of Saline Wastewater from Fish and Shellfish Processing. *Front. Environ. Sci.* **2021**, *9*, 236. [CrossRef]
5. Lefebvre, O.; Moletta, R. Treatment of organic pollution in industrial saline wastewater: A literature review. *Water Res.* **2006**, *40*, 3671–3682. [CrossRef] [PubMed]
6. Dan, N.P.; Visvanathan, C.; Basu, B. Comparative evaluation of yeast and bacterial treatment of high salinity wastewater based on biokinetic coefficients. *Bioresour. Technol.* **2003**, *87*, 51–56. [CrossRef]

7. Srivastava, A.; Parida, V.K.; Majumder, A.; Gupta, B.; Gupta, A.K. Treatment of saline wastewater using physicochemical, biological, and hybrid processes: Insights into inhibition mechanisms, treatment efficiencies and performance enhancement. *J. Environ. Chem. Eng.* **2021**, *9*, 105775. [CrossRef]
8. Kourmentza, C.; Plácido, J.; Venetsaneas, N.; Burniol-Figols, A.; Varrone, C.; Gavala, H.N.; Reis, M.A.M. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering* **2017**, *4*, 55. [CrossRef]
9. Polyhydroxyalkanoate (PHA) Market Global Forecast to 2025 | MarketsandMarkets. Available online: https://www.marketsandmarkets.com/Market-Reports/pha-market-395.html?gclid=CjwKCAjw9MuCBhBUEiwAbDZ-7tH0vuR1VsKfgUvzT6jPuDry18A_H8vWVihQJFN_iN7OotkL2PVH7xoCDykQAvD_BwE (accessed on 29 November 2021).
10. Oliveira, C.S.S.; Silva, C.E.; Carvalho, G.; Reis, M.A. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities. *New Biotechnol.* **2017**, *37*, 69–79. [CrossRef]
11. Oliveira, C.S.S.; Silva, M.O.D.; Silva, C.E.; Carvalho, G.; Reis, M.A.M. Assessment of protein-rich cheese whey waste stream as a nutrients source for low-cost mixed microbial PHA production. *Appl. Sci.* **2018**, *8*, 1817. [CrossRef]
12. Silva, F.; Campanari, S.; Matteo, S.; Valentino, F.; Majone, M.; Villano, M. Impact of nitrogen feeding regulation on polyhydroxyalkanoates production by mixed microbial cultures. *New Biotechnol.* **2017**, *37*, 90–98. [CrossRef]
13. Ecoefficient Biodegradable Composite Advanced Packaging | ECOBIOCAP Project | FP7 | CORDIS | European Commission. Available online: https://cordis.europa.eu/project/id/265669 (accessed on 20 November 2020).
14. Koller, M.; Niebelschütz, H.; Braunegg, G. Strategies for recovery and purification of poly[(R)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. *Eng. Life Sci.* **2013**, *13*, 549–562. [CrossRef]
15. Xiao, Y.; Roberts, D.J. A review of anaerobic treatment of saline wastewater. *Environ. Technol.* **2010**, *31*, 1025–1043. [CrossRef] [PubMed]
16. He, X.; Yin, J.; Liu, J.; Chen, T.; Shen, D. Characteristics of acidogenic fermentation for volatile fatty acid production from food waste at high concentrations of NaCl. *Bioresour. Technol.* **2019**, *271*, 244–250. [CrossRef] [PubMed]
17. Duarte, M.S.; Oliveira, J.V.; Pereira, C.; Carvalho, M.; Mesquita, D.P.; Alves, M.M. Volatile Fatty Acids (VFA) Production from Wastewaters with High Salinity—Influence of pH, Salinity and Reactor Configuration. *Fermentation* **2021**, *7*, 303. [CrossRef]
18. Fra-Vázquez, A.; Pedrouso, A.; Val del Rio, A.; Mosquera-Corral, A. Volatile fatty acid production from saline cooked mussel processing wastewater at low pH. *Sci. Total Environ.* **2020**, *732*, 139337. [CrossRef]
19. Palmeiro-Sánchez, T.; Oliveira, C.S.S.; Gouveia, A.R.; Noronha, J.P.; Ramos, A.M.; Mosquera-Corral, A.; Reis, M.A.M. NaCl presence and purification affect the properties of mixed culture PHAs. *Eur. Polym. J.* **2016**, *85*, 256–265. [CrossRef]
20. Wen, Q.; Ji, Y.; Hao, Y.; Huang, L.; Chen, Z.; Sposob, M. Effect of sodium chloride on polyhydroxyalkanoate production from food waste fermentation leachate under different organic loading rate. *Bioresour. Technol.* **2018**, *267*, 133–140. [CrossRef]
21. Pedrouso, A.; Fra-Vazquez, A.; Del Rio, A.V.; Mosquera-Corral, A. Recovery of Polyhydroxyalkanoates from Cooked Mussel Processing Wastewater at High Salinity and Acidic Conditions. *Sustainability* **2020**, *12*, 10386. [CrossRef]
22. Argiz, L.; Fra-Vázquez, A.; del Río, Á.V.; Mosquera-Corral, A. Optimization of an enriched mixed culture to increase PHA accumulation using industrial saline complex wastewater as a substrate. *Chemosphere* **2020**, *247*, 125873. [CrossRef]
23. Roibás-Rozas, A.; Val del Rio, A.; Hospido, A.; Mosquera-Corral, A. Strategies for the valorisation of a protein-rich saline waste stream into polyhydroxyalkanoates (PHA). *Bioresour. Technol.* **2021**, *334*, 124964. [CrossRef]
24. APHA/AWWA. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; APHA American Public Health Association: Washington, DC, USA, 1998.
25. Wang, X.; Oehmen, A.; Freitas, E.B.; Carvalho, G.; Reis, M.A.M. The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production. *Water Res.* **2017**, *112*, 269–278. [CrossRef]
26. Heinzle, E.; Biwer, A.P.; Cooney, C.L. *Development of Sustainable Bioprocesses*; John Wiley & Sons, Ltd: Chichester, UK, 2006.
27. Obruca, S.; Sedlacek, P.; Koller, M.; Kucera, D.; Pernicova, I. Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: Biotechnological consequences and applications. *Biotechnol. Adv.* **2018**, *36*, 856–870. [CrossRef] [PubMed]
28. Prados, E.; Maicas, S. Bacterial Production of Hydroxyalkanoates (PHA). *Univers. J. Microbiol. Res.* **2016**, *4*, 23–30. [CrossRef]
29. Soto, G.; Setten, L.; Lisi, C.; Maurelis, C.; Mozzicafreddo, M.; Cuccioloni, M.; Angeletti, M.; Ayub, N.D. Hydroxybutyrate prevents protein aggregation in the halotolerant bacterium Pseudomonas sp. CT13 under abiotic stress. *Extremophiles* **2012**, *16*, 455–462. [CrossRef] [PubMed]
30. Pujalte, M.J.; Lucena, T.; Ruvira, M.A.; Arahal, D.R.; Macián, M.C. The family *Rhodobacteraceae*. In *The Prokaryotes*; Springer: Berlin/Heidelberg, Germany, 2014; pp. 439–512. [CrossRef]
31. Mezzolla, V.; D'Urso, O.F.; Poltronieri, P. Role of PhaC type I and type II enzymes during PHA biosynthesis. *Polymers* **2018**, *10*, 910. [CrossRef]
32. Perez-Zabaleta, M.; Atasoy, M.; Khatami, K.; Eriksson, E.; Cetecioglu, Z. Bio-based conversion of volatile fatty acids from waste streams to polyhydroxyalkanoates using mixed microbial cultures. *Bioresour. Technol.* **2021**, *323*, 124604. [CrossRef]

33. Van-Thuoc, D.; Huu-Phong, T.; Minh-Khuong, D.; Hatti-Kaul, R. Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) Production by a Moderate Halophile *Yangia* sp. ND199 Using Glycerol as a Carbon Source. *Appl. Biochem. Biotechnol.* **2015**, *175*, 3120–3132. [CrossRef]
34. Chan, C.M.; Vandi, L.J.; Pratt, S.; Halley, P.; Ma, Y.; Chen, G.Q.; Richardson, D.; Werker, A.; Laycock, B. Understanding the effect of copolymer content on the processability and mechanical properties of polyhydroxyalkanoate (PHA)/wood composites. *Compos. Part A Appl. Sci. Manuf.* **2019**, *124*, 105437. [CrossRef]