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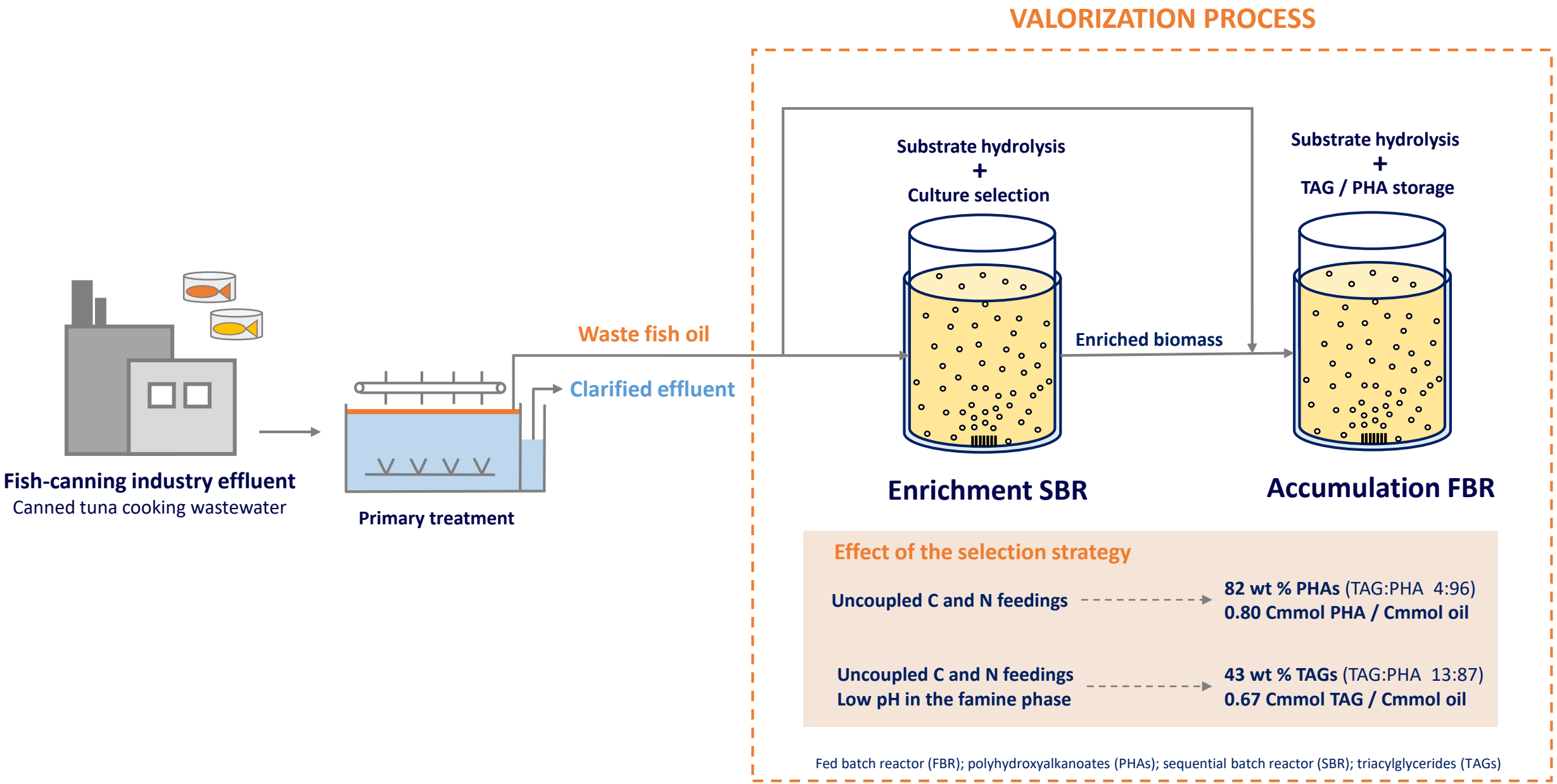
Highlights

- Preferent triacylglycerides or polyhydroxyalkanoates storage from waste lipids
- No waste oil pretreatment was needed before the enrichment and accumulation stages.
- Dominant metabolic pathways depended on the imposed selective pressures.
- Carbon and nitrogen (limited) uncoupling promoted polyhydroxyalkanoates storage.
- Low pH in the famine, an additional advantage for triacylglyceride-accumulators.



Fed batch reactor (FBR); polyhydroxyalkanoates (PHAs); sequential batch reactor (SBR); triacylglycerides (TAGs)

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ABSTRACT

Lipids are one of the main components of the organic matter present in the effluents of the food-processing industry. These waste streams can be biotransformed into valuable triacylglycerides (TAGs) and polyhydroxyalkanoates (PHAs), precursors of biofuels and biomaterials alternative to petroleum-based products. These compounds are yielded by mixed microbial cultures, and considering that both TAG and PHA accumulators may coexist within the community, it seems crucial to define those operational strategies that might control the selection of the dominant metabolic pathways (TAG or PHA accumulation). In this work, residual fish-canning oil wasused as a carbon source in a two-stage process (culture selection and intracellular compounds accumulation) in which the substrate was simultaneously hydrolyzed in these two stages without the need for a previous fermentation unit. It was pretended to maximise preferential TAG or PHA storage in the accumulation reactor by the imposition of certain selective pressures in the enrichment one. Uncoupling C and N feedings and limiting nitrogen availability in the medium, allowed to maximise PHA production (82.3 wt % of PHAs, 0.80 $Cmmol_{PHA}/Cmmol_{S}$). Besides, when low pH in the famine phase was considered as additional selective pressure, it was possible to shift the ratio TAG:PHA from 4:96 obtaining 43.0 wt % of TAGs (0.67 $Cmmol_{TAG}/Cmmol_{S}$). Therefore, this novel and simplified process demonstrated versatility and efficiency in the storage of TAGs and PHAs from a unique residual feedstock and using an open culture proving that product selection can be harnessed if choosing the right operational conditions in the enrichment stage.

Keywords: biotransformation; lipidic waste; mixed microbial culture; pH; uncoupled feeding.

ADF: aerobic dynamic feeding

C: carbon

COD: chemical oxygen demand

DO: dissolved oxygen (mg/L)

FBR: fed-batch reactor

F/F: feast/famine

FFA: free fatty acids

MMC: mixed microbial culture

N: nitrogen

PHA: polyhydroxyalkanoate

q_N: maximum specific nitrogen consumption rate ($Cmmol_N/Cmmol_X \cdot h$)

q_{PHA}: maximum specific polyhydroxyalkanoates production rate ($Cmmol_{PHA}/Cmmol_X \cdot h$)

q_S: maximum specific carbon consumption rate ($Cmmol_S/Cmmol_X \cdot h$)

q_{TAG}: maximum specific triacylglycerides production rate ($Cmmol_{TAG}/Cmmol_X \cdot h$)

q_X: maximum specific active biomass production rate ($Cmmol_X/Cmmol_S \cdot h$)

S: substrate

SBR: sequencing batch reactor

TAG: triacylglyceride

TN: total nitrogen (mg/L)

TS: total solids (g/g)

TSS: total suspended solids (g/L)

VS: volatile solids (g/g)

VSS: volatile suspended solids (mg/L)

WWTP: wastewater treatment plant

X: active biomass (Cmmol/L)

Y_{PHA}: maximum polyhydroxyalkanoates production yield ($Cmmol_{PHA}/Cmmol_S$)

Y_{TAG}: maximum triacylglycerides production yield ($Cmmol_{TAG}/Cmmol_S$)

Y_X: maximum active biomass production yield($Cmmol_X/Cmmol_S$)

1. INTRODUCTION

The food industry generates large volumes of solid and liquid waste types that suppose a significant environmental and economic concern. Among the industries causing a global major impact are seafood and fish-processing activities (Kosseva, 2013). Specifically, the fish-canning industry is characterized by the generation of large amounts of high strength liquid effluents (Cristóvaõ et al., 2015), which are rich in lipids, one of the main components of natural fish (Chipasa and Mędrzycka, 2006). These effluents are usually pre-treated before biological treatment, to separate solids and lipids using different physicochemical processes (Ahmad et al., 2020). Then, lipids are commonly composted or anaerobically digested (Cristóvaõ et al., 2015; Tamis et al., 2015). However, with the advancement of science and technology, wastewater treatment methods are constantly improving (Wu et al., 2020). In the last years, industrial waste fish oils have been identified as potential feedstocks for the obtention of value-added compounds such as renewable 2^{nd} generation biofuels (Sawangkeaw and Ngamprasertsith, 2013) or bioplastics (Surendran et al., 2020), which would contribute to cope with fossil fuel depletion and environmental problems (Leng et al., 2020).

Using single-cell microorganisms, the valorisation of waste oils is feasible since they are capable of storing important percentages of high-quality compounds that serve as raw materials or intermediates for a variety of applications (Garay et al., 2014; Gujjala et al., 2019). Special emphasis is given to triacylglycerides (TAGs) and polyhydroxyalkanoates (PHAs), excellent reserve materials that constitute the main compounds accumulated in eukaryotes and prokaryotes, respectively (Revellame et al., 2012). Their storage fulfils several roles in microorganisms such as cell growth and division (Garay et al., 2014), stress response against the pressure exerted by certain environments (Kumar et al., 2018), and energy reserve (Kumar et al., 2020).

Fungi and yeast are oleaginous microbes in their majority as they can accumulate over 20 wt % of intracellular TAGs (Alvarez and Steinbüchel, 2003). However, TAGs are not a common storage compound in bacteria and their accumulation was only described in a few prokaryotic species belonging to the actinobacterial group (Gujjala et al., 2019). Bacteria typically biotransform lipids into PHAs (Garay et al., 2014) although certain species are able to synthesize PHA and TAG together, as it is the case of *Rhodococcus Ruber* (Alvarez and Steinbüchel, 2003; Garay et al., 2014).

Although research on TAG and PHA production from lipidic feedstocks was traditionally focused on the use of edible plant oils as substrates and pure cultures with very high accumulation capacity (Basnett et al., 2018; Donot et al., 2014; Pérez-Arauz et al., 2019), the high production costs, mainly associated with substrate acquisition and culture conditions pushed for the development of cost-effective alternatives. These involved the use of waste feedstocks and mixed microbial cultures (MMCs) (Sabapathy et al., 2020) and not only reduced the costs of the process, but also increased resource efficiency and improved waste management performance contributing to the establishment of a circular economic model (Surendran et al., 2020; Yadav et al., 2020). Recently, it was developed studies regarding the use of animal fats for TAG (Lopes et al., 2018) and PHA (Sangkharak et al., 2020; Van Thuoc et al., 2019) production using pure cultures. Regarding MMCs, to the best of the author's knowledge, only the use of vegetable oils was explored so far. TAG accumulation has been demonstrated feasible through a two-stage process: (1) selection and enrichment of the MMC; and (2) maximization of the TAG storage before its extraction and purification (Tamis et al., 2015). In the case of PHA production, it has been proposed an analogous configuration that included a previous fermentation of the lipids contained in the residual streams

used as a substrate to obtain soluble organic acids (Campanari et al., 2014; Waller et al., 2012).

One of the most common enrichment strategies for PHA production in MMCs is the aerobic dynamic feeding (ADF), which was also demonstrated to provide a competitive advantage for TAG accumulators (Tamis et al., 2015). This strategy consists in the application of subsequent aerobic feast/famine (F/F) cycles in which the culture is initially subjected to an excess of carbon source and then submitted to carbon deficiency. Several variations of this enrichment strategy have been tested to optimize PHA production (Kourmentza et al., 2017). Uncoupling carbon (C) and nitrogen (N) availability in addition to the F/F regime was demonstrated to cause a positive impact in PHA accumulation (Lorini et al., 2020; Oliveira et al., 2017; Silva et al., 2017). These strategies favour its storage in the feast phase (exogenous C availability and N absence limiting, in turn, the development of non-accumulating microorganisms) and enhance the growth of populations with high PHA-storage ability in the famine (intracellular C and external N are available) (Oliveira et al., 2017; Silva et al., 2017). Variations in the ADF strategy to maximize TAG storage have not been reported yet. However, Santamauro et al. (2014) considered the possibility of using low pH combined with low temperature or high salinity to enrich an open culture in extremophile oleaginous microorganisms. But although it is known that oleaginous microorganisms can thrive relatively well in low pH environments (Donot et al., 2014), other species might be also selected under these conditions.

In the present research, it was pretended to valorise a residual fish-canning oily stream for the production of TAGs or PHAs using the same configuration, a two-stage process consisting of culture selection plus intracellular compounds accumulation in the enriched culture. Firstly, it was evaluated the feasibility of using the considered waste

stream as a substrate. Then, the versatility and robustness of the process were assessed by providing the most suitable operational conditions (N feeding strategy and pH) that promoted the follow-up of those metabolic routes concerning the accumulation of TAGs or PHAs, and hence the preferent development of TAG or PHA producers. Finally, it was determined the effect of the enrichment strategy over the process productivity and type of intracellular compounds accumulated.

2. MATERIALS AND METHODS

2.1. Substrate characteristics and metabolism

The substrate used in this study was residual oil from the cooking water of the industrial production of canned tuna (Table 1) removed in the primary treatment of the factory wastewater treatment plant (WWTP). It was characterized by high hydrophobicity identifying oleic and linoleic as the main fatty acids in its composition with a lower concentration of palmitic and stearic acids. These, have been previously found as the main fatty acids of several commercially available tuna products, especially the ones that are oil-packaged (Maqbool et al., 2011).

The residual oil used as a substrate was fed to a MMC in which different routes could be possible depending on the medium conditions due to the diversity of microorganisms potentially present in open cultures. The microbial conversion of non-soluble fats and oils is complex and starts with initial extracellular hydrolysis catalysed by lipases that yield free fatty acids (FFA) and glycerol. Soluble FFA are then available to be transported into the cell and once the substrate is taken up, a wide diversity of metabolic pathways are feasible (Becker, 2010; Garay et al., 2014; Jimenez-diaz et al., 2019; Tamis et al., 2015). FFA can directly support cell growth but under excess of carbon

and especially if a key nutrient is limited (typically nitrogen), certain microorganisms stop replication processes and utilize the available carbon to synthesize and store intracellular value-added compounds (Garay et al., 2014). On the one hand, certain oleaginous microorganisms can accumulate oils in the form of lipid droplets via the *ex novo* pathway when using a hydrophobic substrate (Patel and Matsakas, 2019) such as the waste fish oil considered in this research work. This metabolic route differs from the *de novo* pathway, which involves the consumption of hydrophilic substrates, in that it is entirely independent of nitrogen exhaustion in the medium since lipid accumulation and cell growth occur simultaneously (Huang et al., 2017). On the other hand, PHA producers can employ FFA as a substrate for the production of intracellular PHAs via β-Oxidation pathway (Jimenez-diaz et al., 2019) (Figure 1).

2.2 Experimental set-up

For the valorisation of the residual fish-canning oily stream, a two-unit process was considered: (1) a sequencing batch reactor (SBR) for the enrichment of the MMC and (2) a fed-batch reactor (FBR) to maximize the production of TAGs and PHAs. In both units, the substrate was simultaneously hydrolyzed not requiring a prior fermentation unit.

2.2.1 Selection of microorganisms with high accumulation capacity

The SBR for the enrichment of the MMC was inoculated with activated sludge from an urban WWTP. This unit consisted of a 4-L jacketed glass reactor aerobically operated as SBR under the F/F regime. Continuous aeration, provided by a couple of diffusers located at the bottom of the reactor, guaranteed the complete mixture of the system. The temperature was maintained at 30 ± 3 ºC through a thermostatic bath (Techne Inc, USA). The pH was only controlled off-line by $NaHCO_3$ buffer addition.

187 The SBR was operated in cycles of 12 h. After finishing each cycle, half of its total
188 volume (2 L) was exchanged resulting in hydraulic and solid retention times of 24 h. In
189 each cycle, 2 mL (equivalent to 114.5 Cmmol) of carbon source were added at the
190 beginning. The 2 L of dilution water were added at different times depending on the
191 feeding strategy (Figure S1). This stream contained: 0.60 g/L NH_4Cl, 0.70 g/L KH_2PO_4,
192 0.14 g/L $MgSO_4$, 0.10 g/L KCl, 0.65 – 1.00 g/L KH_2PO_4, 2 mL/L of a trace element
193 solution (Vishniac and Santer, 1957), and 0.0025 g/L allylthiourea to prevent
194 nitrification. In the uncoupled configurations, nitrogen was added separately as 20 mL
195 of a concentrated NH_4Cl solution, reducing the concentration of KH_2PO_4 in the dilution
196 water in the same proportion as the NH_4Cl added.

197 **2.2.2 Accumulation of storage compounds**

198 The storage capacity of the enriched culture was assessed by the performance of several
199 accumulation assays in a 4-L FBR inoculated with the effluent of the SBR. This
200 bioreactor was operated under the same conditions as those of the SBR except for the
201 feeding regime. To maximize the accumulation and prevent bacterial growth, no
202 nitrogen source was fed. The residual oil used as a substrate was added in pulses of 0.80
203 mL (45.8 Cmmol) following the dissolved oxygen (DO) concentration profile. The
204 maximum accumulation capacity was assumed when DO concentration remained at
205 saturation values after adding a substrate pulse (end of the experiment). The pH was
206 periodically measured and adjusted, if necessary, by adding different volumes of an 18
207 g/L $NaHCO_3$ solution.

208 **2.2.3 Operational strategy**

209 The SBR for the enrichment of the MMC was operated for 331 days with its operation
210 subdivided into five periods (I – V). During the whole operation six accumulation
211 assays in the FBR were performed (days 120, 176, 218, 245, 287, and 322) (Figure S2).

In the SBR, C and N were fed together at the beginning of the operation (period I, start-up – day 203). Afterward, two uncoupled C and N feeding strategies were tested: type A and B, in periods II (days 204 – 224) and III (days 225 – 251) respectively. In period IV (days 252 – 307), the pH was considered as an additional selective pressure in the SBR and finally, in period V (days 308 – end of the operation) the capacity of the system to recover the conditions of period III was explored. The coupled feeding configuration comprised the following stages: 1) feeding 2 mL of oil and 2 L of dilution water containing N (5 min); 2) aerobic reaction (708 min), and 3) 2 L of effluent withdrawal (7 min). The first uncoupling strategy (type A) consisted of 1) feeding 2 mL of oil (5 min); 2) aerobic reaction (180 min); 3) feeding 2 L of dilution water containing N (5 min); 4) aerobic reaction (523); and 5) effluent withdrawal (7 min). The other uncoupled configuration tested (type B) involved: 1) feeding 2 mL of oil and 2 L of dilution water without N (5 min); 2) aerobic reaction (120 – 180 min); 3) feeding 20 mL of a concentrated solution of N (5 min); 4) aerobic reaction (463 – 583 min); and 5) effluent withdrawal (7 min) (Figure S1). The length of the aerobic reaction before N addition was initially established according to the feast length previous to the system uncoupling and modified when necessary.

2.3. Sampling and analysis

For monitoring the SBR operation, the effluent discharged at the end of each cycle and the dilution water were analysed. Samples for solid-phase analysis were also taken at the end of the feast phase to determine the evolution of the TAGs and PHAs accumulated. The operation cycles were periodically characterized, and samples were taken in the 12 h of cycle operation. The accumulation assays performed in the FBR were also monitored and samples were taken before the addition of each pulse of substrate.

Temperature and DO concentration were measured on-line using a portable multimeter (HQ40d, Hach-Lange, USA). The raw samples of the SBR and FBR bioreactors were characterised on pH (pH and Ion-Meter GLP 22, Crison, Spain), total and volatile suspended solids (TSS and VSS, respectively) and also in the total chemical oxygen demand (COD) (APHA/AWWA/WEF, 2017). The COD of the supernatant obtained after single centrifugation of the samples was also determined (Centrifuge 5430 Eppendorf, USA). In the soluble fraction, after both centrifugation and filtration (0.45 μm pore size, cellulose-ester membrane, Advantec, Japan), soluble chemical oxygen demand (APHA/AWWA/WEF, 2017), lactic acid (measured by high-performance liquid chromatography, HP 1100 Agilent Technologies with RI detector 107 7A, USA), and total nitrogen (TN) were measured (TOC-L analyzer with the TNM- module, TOC-5000 Shimadzu, Japan). The dilution water was characterised on pH, TN, total organic carbon ,and inorganic carbon (TOC-L analyzer with the TNM- module, TOC-5000 Shimadzu, Japan). For the substrate characterization, the conductivity was determined with a portable conductivity meter (probe Sension + EC5 HACH, Spain), lipids were measured by the Soxhlet extraction method (Soxhlet extractor Jp Selecta 8001800, Spain) (APHA/AWWA/WEF, 2017) and the elemental composition was determined in an elemental analyser (FlashEA 1112 Thermo Scientific, USA). Total and volatile solids (TS and VS, respectively) were measured according to APHA/AWWA/WEF (2017).

Identification and quantification of storage compounds were carried according to the method described by Fra-Vázquez et al. (2018). For that purpose, fresh biomass samples were taken, centrifuged (Centrifuge 5430 Eppendorf, USA), frozen, and lyophilized to obtain a solid phase. Commercial calibration standards of TAGs and PHAs were used: palmitic-, stearic-, oleic- and linoleic acids, and a copolymer containing 88 %

hydroxybutyrate and 12 % hydroxyvalerate (Sigma Aldrich, USA). The monomer propyl esters were analysed by gas chromatography (HP innovax column equipped with a FID, Agilent, USA).

2.4 Calculations and statistical analysis

The percentage of intracellular compounds accumulated (TAGs or PHAs) expressed in dry weight (wt %), was calculated by dividing the measured mass of TAGs or PHAs by the mass of VSS present in the medium (in grams). Active biomass (X) was estimated as the difference between the measurements of VSS and storage compounds accumulated (TAG + PHA). The elemental composition of the biomass was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$.

Maximum specific conversion rates of carbon (– q_S, $Cmmol_S/(Cmmol_X \cdot h)$) and nitrogen (– q_N, $Cmmol_N/(Cmmol_X \cdot h)$) consumption, and TAG ($q_{TAG}$, $Cmmol_{TAG}/(Cmmol_X \cdot h)$) and PHA production ($q_{PHA}$, $Cmmol_{PHA}/(Cmmol_X \cdot h)$), were determined using the maximum slopes of the experimental data divided by the active biomass. Similarly, the specific biomass production rate was determined from the maximum slope of the produced biomass divided by the amount of substrate fed (q_X, $Cmmol_X/(Cmmol_S \cdot h)$). The production yield of the storage compounds (Y_{TAG} $Cmmol_{TAG}/Cmmol_S$; and Y_{PHA}, $Cmmol_{PHA}/Cmmol_S$) and the biomass (Y_X, $Cmmol_X/Cmmol_S$) were determined by dividing the related production rate ($Cmmol_{TAG}/h$, $Cmmol_{PHA}/h$, and $Cmmol_X/h$) by the carbon source consumption rate ($Cmmol_S/h$).

Statistical analysis was performed by SPSS software, IBM Corp Released 2017 (IBM SPSS Statistics for Windows, Version 25.0 Armonk, NY). One-way variance analysis (ANOVA) was used to test the existence of statistically significant differences between the composition of the intracellular compounds accumulated in the SBR during the different operational periods (five independent groups). To determine which of these

287 groups differed from each other, the Turkey post hoc test was applied. On the other
288 hand, to compare the type of intracellular compounds accumulated in SBR and FBR
289 reactors during the same operational periods (comparison of two unrelated groups on
290 the same dependent variable), the independent-samples t-test was considered. A 5 %
291 significance level was assumed in both cases.

292

293 **3. RESULTS AND DISCUSSION**

294 **3.1 Feasibility of using fish-canning oil to enrich a MMC in TAG and PHA**
295 **producers**

296 During the first 203 days (Period I), the SBR was operated under a coupled feeding
297 strategy (simultaneous C and N addition) to enrich conventional activated sludge in
298 microorganisms with high storage ability. It is known that lipids, especially long-chain
299 fatty acids with saturated carbon chains, are less responsive to biodegradation than other
300 organic substrates (Chipasa and Mędrzycka, 2006). However, the typical F/F profile
301 was observed after 3 enrichment cycles (Figure S3.a), which indicated that the culture
302 was able to consume the substrate despite its high hydrophobicity and organic matter
303 composition (Table 1).

304 The operation of the reactor became stable after 50 days. During this period, the feast
305 phase length presented wide variability (from 1.3 to 5.5 hours), and the VSS
306 concentration sharply decreased due to culture selection (from 2.13 ± 0.10 g/L to 0.32 ±
307 0.04 g/L between days 1 and 44). From day 45 onwards, the length of the feast phase
308 followed a downward trend and reduced its variability. Nevertheless, even after more
309 than 100 days it still ranged between 2 to 4 hours (feast/cycle ratio of 0.21 ± 0.07).
310 According to Dionisi et al. (2005), ratios lower than approximately 0.25 allow for the
311 selection of microorganisms with a good storage ability. These variations in the feast

312 phase length could be due to the low solubility of the substrate and grease accumulation
313 in the walls of the reactor, which difficulted mass transfer and limited the microbial
314 uptake.
315 The pH was maintained close to neutrality (Figure S3.b) by $NaHCO_3$ addition in the
316 dilution water. With the progress of the operation, $NaHCO_3$ addition was reduced (from
317 1 to 0.64 g/L) until reaching stationary state. Occasionally, excess of $NaHCO_3$ increased
318 the pH in the liquid bulk above neutral (e.g. on day 50 of operation, 0.7 g/L $NaHCO_3$
319 were added in the feeding, and a pH of 7.9 was observed at the end of the cycle) (Figure
320 S3.c). In these conditions, the FFA produced in a first hydrolysation of the substrate by
321 lipase-catalysed reactions, saponified and became unavailable for microbial
322 metabolization. Once the pH was optimised, the possible saponification did not occur
323 anymore. Although after feeding, the pH slightly increased during the feast (e.g. from
324 7.0 to 7.4 on day 72 and from 7.0 to 7.3 on day 171), it decreased to the initial values
325 remaining almost constant during the whole cycle in period I (Figure 2.a, 2.b and 2.c).
326 In period I, the ratio C/N was maintained at 16.7 ± 2.4 g COD/g TN, and about 274 ± 28
327 g of TN were fed in each cycle. Neither the COD nor the TN were fully consumed
328 during the SBR cycle and average concentrations of 162 ± 40 mg COD/L (measured in
329 the centrifuged and non-filtered sample) and 81 ± 17 mg TN/L were detected in the
330 effluent (end of the cycle). All the consumed nitrogen was assumed to be used for
331 growth since nitrification was inhibited by allylthiourea addition. The presence of
332 remnant COD at the end of each cycle is explained by the nature of the substrate and the
333 presence of slowly biodegradable or recalcitrant compounds. The composition of the
334 substrate and in special its hydrophobicity (slow diffusion) could have limited microbial
335 growth (as no N limiting conditions were imposed) and explain the low VSS
336 concentrations obtained at steady-state, 0.58 ± 0.06 g/L (Figure S3.d).

During the feast phase of period I, microbial growth due to extracellular substrate consumption, along with intracellular accumulation was observed (Figure 2.a, 2.b and 2.c), but the progressive enrichment of the culture enhanced the use of intracellular substrate for growth during the famine phase. For example, on day 50, microorganisms grew during the feast while 13.7 wt % TAGs and 0.9 wt % PHAs was accumulated and no significant growth was observed during the famine phase (Table 2). However, once these percentages increased on day 78 up to 20.6 wt % and 1.6 wt %, respectively, growth took place during both phases (Table 2).

The percentage of intracellular compounds accumulated (as a sum of PHAs and TAGs) did not significantly vary after day 78 onwards (period I), although changes in the composition were observed, which suggested alterations in the cellular metabolism due to a slight enrichment of the culture in PHA-accumulators (Silva et al., 2017). For example, on day 171 the highest PHA production (5.3 wt %) was reached in period I but concomitantly, TAGs accumulated decreased (10.8 wt %) in comparison with days 78, 111, and 118 (Figure 3.a).

Results obtained in period I showed the feasibility of the culture to metabolize the fish-canning waste oil. However, the carbon source was mainly used for growth as a consequence of the high N availability present in the medium. Once the substrate was hydrolyzed, FFA weretransported into the cell and mainly degraded for biomass production (respiration via the Krebs Cycle) (Figure 1). At the same time, part of the FFA were directly accumulated as lipid reserve (TAGs with a composition proportional to that of the substrate (Table 1, Table 2)) via the *ex novo* fatty acids pathway (Figure 1), a primary anabolic process occurring simultaneously with the production of lipid-free biomass regardless of the C/N ratio in the medium (Carsanba et al., 2018; Lopes et al., 2018; Pérez-Arauz et al., 2019;). Besides, results obtained in period I (Table 2)

362 evidenced that the presence of too high N concentrations blocked PHA biosynthesis, 3-
363 Hydroxyacyl-CoA instead of being polymerized, was further transformed into acetyl-
364 CoA and channelled into the Krebs cycle (Figure 1) (Tan et al., 2014).
365 Consequently, the enrichment stage of the process needed to be optimized to improve
366 culture selection and maximize TAG or PHA storage.

367 **3.2 Optimization of the SBR enrichment strategy**

368 In this section, shifts in the SBR enrichment strategy were explored to improve
369 selection and establish the operational conditions for the preferential development of
370 TAG or PHA-storing populations. Thus, although both storage compounds are
371 considered valuable potential sources (Garay et al., 2014), their mixture seems not and
372 might hinder downstream extraction and purification processes.

373 **3.2.1 Channelling lipids metabolism towards PHA production**

374 **3.2.1.1 Nitrogen availability limitation**

375 In periods II and III, N availability was limited during the feast phase (unbalanced
376 nutrient conditions) to favour the PHA accumulation pathway (Figure 1) and maximise
377 PHA storage (Tan et al., 2014). For that purpose, two different strategies were tested in
378 the SBR and its cycle configuration was changed on day 204 to set uncoupling type A,
379 (period II) and on day 225 to set uncoupling type B (period III). In both cases, C and N
380 feedings were uncoupled to supply the N source at the end of the feast ensuring
381 unbalanced nutrient conditions (C excess in the presence of N limitation). The amount
382 of N added was limited to the minimum needed for microbial growth. Therefore, N was
383 controlled maintaining a minimum concentration in the effluent of the SBR. The
384 quantity of TN added was reduced from 274 ± 28 to 141 ± 10 g of TN per cycle, and
385 consequently, about 12 ± 4 mg TN/L were measured in the effluent, which supposed a
386 reduction of more than 85 % respect to the values observed in the coupled

configuration. After modifying the N feeding strategy, specific nitrogen consumption and biomass production rates were substantially lower during the feast phase when compared with period I, especially in the case of the type B configuration (period III) (Table 2). In periods II and III, the specific N consumption rate significantly decreased during the feast phase and biomass growth mainly occurred in the famine phase, favouring culture selection (Table 2). Consequently, a positive effect over PHA accumulation was observed (Figure 3.a), which confirmed that N limitation and C and N uncoupling allowed for lipids biotransformation into PHAs and promoted the enrichment of the culture in PHA producers.

After C and N uncoupling, the ratio between the feast phase length and the length of the whole cycle decreased (Figure S3.a). This result evidenced the enrichment of the microbial culture in species with high storage ability (Dionisi et al., 2005). The ratio was reduced from 0.21 ± 0.07 in period I (coupled system, accumulation + extracellular growth), to 0.10 ± 0.05 and 0.11 ± 0.04 in periods II and III (single accumulation).

The F/F profile also changed when C and N were uncoupled. Initially, microorganisms used organic matter as an electron donor and oxygen as an acceptor (DO concentration decreased). But after this, a lag phase was observed where DO concentration progressively increased. Then, the addition of nutrients reduced the DO again because of respiration for microbial growth. Therefore, the DO profiles presented two slopes, although when the feast length was too short they overlapped (similar DO profiles were observed by Lorini et al. (2020)). The sum of both slopes showed that accumulation plus intracellular growth (0.35 ± 0.07 and 0.35 ± 0.06 in periods II and III, respectively) took longer than simultaneous accumulation and extracellular growth (0.21 ± 0.07, period I) (which is in agreement with the observed by Silva et al. (2017) and Oliveira et al. (2017)) (Figure S4).

412 **3.2.1.2 Initial carbon availability**

413 The higher C availability at the beginning of the cycle in the case of the uncoupling type
414 A cycle (equal amount of COD added in half of the volume) lead to differences in
415 composition and amount of storage compounds accumulated between the two
416 uncoupling strategies tested (Figure 3.a; Table 2).

417 During the feast phase, uncoupling type B cycle caused the enhancement of PHA
418 accumulation at the expense of TAGs (32.1 wt % PHAs and 5.8 wt % TAGs on day
419 238) in comparison to the uncoupling type A cycle (18.4 wt % PHAs and 25.8 wt %
420 TAGs on day 218). The total quantity of storage compounds accumulated at the end of
421 the feast was lower in type B for similar food to microorganism ratios (values of 4.4 and
422 4.6 g COD/g VSS for A and B, respectively). In the uncoupling strategy A, 22.6 wt %
423 TAGs and 2.12 wt % PHAs remained at the end of the cycle whereas in type B only 8.0
424 wt % TAGs and 0.48 wt % PHAs were measured. The higher carbon availability might
425 be selecting for microorganisms that consume it faster, using shorter pathways, which
426 could explain the higher TAG than PHA accumulation at the end of the feast phase in
427 period II. Moreover, due to the high initial concentration in the feast phase, extracellular
428 carbon was not fully consumed and therefore, it was also available in the famine phase.
429 This explains the high percentage of TAGs accumulated measured at the end of the
430 cycle (Vasiliadou et al., 2018).

431 The strategy of uncoupling type B seems to be the most efficient to maximize PHA
432 production. It favours a higher PHA storage during the feast and avoids the presence of
433 extracellular substrate during the famine limiting the growth of non-storing populations.

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3.2.2 Channelling lipids metabolism towards TAG production

The pH was maintained stable during the SBR cycles at almost neutral values during period I (Figure 2.a, 2.b and 2.c) showing an average of 7.1 ± 0.3 at the end of the cycle. However, after uncoupling, this parameter became unstable. In period II (uncoupling A) the pH decreased but it was recovered by $NaHCO_3$ addition (Figure 2.d) reaching values of of 7.5 ± 0.2 at the end of the cycle. In period III (uncoupling B) the pH increased at the beginning of the cycle due to $NaHCO_3$ supply, but it decreased during the famine phase (Figure 2.e) (average of 6.4 ± 0.5 at the end of the cycle). This reduction was a consequence of the microbial growth sustained by the intracellular carbon stored. In the respiration of the intracellular carbon, CO_2 is released (Figure 1) acidifying the medium. In the coupled system, it was not observed a decrease of the pH since $NaHCO_3$ was added immediately before the organic matter oxidation, which was mainly extracellular and took place during the feast phase.

Considering that respiration acidifies the medium and low pH environments constitute a competitive advantage for oleaginous microorganisms, it was explored the feasibility of selecting a culture with high TAG-storage capacity by the imposition of a double selective pressure in the SBR (period IV, days 254 – 307): N limitation during the feast phase and low pH during the famine. For nitrogen limitation, the same feeding strategy of period III was imposed (uncoupling type B). The pH was reduced by lowering the IC in the dilution water (Figure S3.b) to reduce the buffering capacity of the system and allow for greater acidification in the famine phase. The pH at the beginning of the cycle increased over 7.0 as a consequence of dilution water addition. However, during the feast phase, this parameter sharply decreased since the system buffering capacity was very low. For example, on day 287 of operation (Figure 2.f), the pH at the beginning of the cycle increased up to 7.6 but then decreased reaching a value of 3.8 at the end of the

462 cycle. On average, the pH at the end of the cycle decreased from 6.5 ± 0.4 to 4.0 ± 0.3
463 between periods III and IV.

464 In period IV, the highest percentages of TAGs and the lowest of PHAs were observed at
465 the end of the feast phase (Figure 3.a). For example, on day 287 of the enrichment
466 cycle, a maximum of 30.6 wt % TAGs and 0.7 wt % PHAs were achieved, whereas at
467 day 238 of the enrichment cycle (period III) only a 5.8 wt % TAG was accumulated.

468 These results showed that nitrogen limitation during the feast phase favoured the
469 accumulation of storage compounds (advantage for accumulators), but low pH
470 conditions during the famine only allowed for the survival of those species able to grow
471 on the intracellular carbon source at low pH environments and resist these extreme
472 conditions for a long time (TAG accumulators). The ratio between the length of the
473 feast and the length of the cycle (feast/cycle ratio) was in period IV (0.12 ± 0.03) similar
474 to the one measured in periods II and III (0.11 ± 0.04 and 0.12 ± 0.03, respectively) and
475 also indicated an enrichment of the culture in microorganisms with high storage ability
476 (Dionisi et al., 2005). Accumulation together with intracellular growth (0.24 ± 0.06),
477 took longer than simultaneous accumulation and extracellular growth (0.21 ± 0.07,
478 period I). The sum of accumulation and intracellular growth in periods II and III (0.35 ±
479 0.07 and 0.35 ± 0.06, respectively) was higher than in period IV. This result suggested
480 that the use of internal TAGs was easier than the use of internal PHAs for growth. In
481 fact, VSS significantly increased, from 0.50 ± 0.16 g/L and 0.48 ± 0.08 g/L in periods II
482 and III to 0.94 ± 0.13 g/L in period IV.

483 **3.2.3 Evaluation of the system recovery capacity**

484 During period V (days 308 – 331) the selective pressure imposed by low pH during the
485 famine phase was removed to test if it was possible to return to similar conditions to
486 those of period III and recover the PHA-accumulation capacity of the system. For that

purpose, the IC of the dilution water was increased obtaining a pH of 6.2 ± 0.4 at the end of the feast phase (similar to that of period III, 6.5 ± 0.4). With it, the VSS concentration decreased, the feast/cycle ratio increased and the evolution of the different parameters monitored during the whole cycle (Figure 2.g) presented a similar trend to the observed in period III. From day 317 onwards an increasing PHA proportion in the storage compounds was observed (Figure 3.a), reaching a 21.6 wt % at the end of the feast phase on day 322 (Figure 2.g).

3.2.4 Statistic comparison of the SBR enrichment strategy

The effect of imposing certain selective pressures in the enrichment SBR was statistically analysed to determine their impact over the preferent development of TAG or PHA producers, which correlated with the type of intracellular compounds accumulated. For that purpose, it was considered the composition of the intracellular compounds stored at the end of the feast phase in the SBR (operating at stationary state) during periods I – V.

Applying one-way ANOVA and Turkey post hoc tests (Table S1), it was observed statistically significant differences in the type of intracellular compounds accumulated when C and N feedings were uncoupled and N availability was limited to promote preferential PHA biosynthesis (same amount of C per unit of time) (periods I vs III). Besides, low pH imposition (period IV) maintaining the same conditions of those of period III, was also demonstrated to have a statistically significant impact over the preferent storage of TAGs.

3.3 TAG and PHA accumulation

3.3.1 Effect of the strategy of enrichment over the maximum storage capacity of the enriched culture and the storage compounds composition

In period I, the SBR was operated under excess of nitrogen and consequently, the effluent of the enrichment reactor used as inoculum in the FBR contained high nitrogen concentrations (up to 100 mg TN/L in the worst-case situations). In the accumulation assays performed on days 120 and 176 of operation (period I), the initial TN concentration was consumed during 9 – 10 hours due to biomass growth (Figure 4.a, 4.b). Therefore, in period I, lipid accumulation was not the dominant process since the rate of accumulation was lower than the one of respiration (Papanikolaou and Aggelis, 2010). The maximum accumulations of storage compounds measured were 21.7 wt % (19.7 wt % TAG, 2.0 wt % PHA) and 22.1 wt % (17.1 wt % TAG and 5 wt % PHA) reached on days 120 and 176, respectively. The maximum percentages of intracellular storage and production yields obtained in the FBR were not significantly different from those reached at the end of the feast phase in the SBR during period I (Figure 3.a). This was mainly related to the high N availability in the medium and the subsequent pathways used by the microorganisms to metabolize the substrate.

The maximum accumulation capacity of the MMC greatly increased after uncoupling C and N supply (periods II and III). Accumulations of 84.2 wt % (23 wt % TAGs and 61.2 wt % PHAs) and 85.6 wt % (3.3 wt % TAGs and 82.3 wt % PHAs, respectively) were measured respectively on days 218 (type A) and 245 (type B) (Figure 3.b). The TAG:PHA ratio varied from 27:73 (day 218, type A) to 4:96 (day 245 type B), (Figure 3.b; Table 3) showing that although intracellular storage was similar, type B configuration enhanced the enrichment of the culture in PHA accumulators. Lower biomass production yields, and higher Y_{PHA} and Y_{TAG} in comparison to those reached in

537 period I were obtained in the FBR assays performed during periods II and III (Table 3).
538 Production yields were much lower in type A than in type B feeding strategy, which
539 might be explained due to higher TAG and PHA accumulations measured at the
540 beginning of the experiment.

541 An accumulation assay using the TAG-accumulating culture selected in the SBR during
542 period IV (day 287) was carried. To possess comparable results and only evaluate the
543 effect of low pH in the SBR over the maximum accumulation capacity, the pH in the
544 FBR was increased by the addition of $NaHCO_3$ avoiding possible effects of low pH
545 over accumulation (Figure 4.e). The highest percentage of intracellular TAG and TAG
546 production yield (Figure 3.b; Table 3) were obtained in this assay. On day 287, it was
547 accumulated 43.0 wt % TAGs and only 6.3 wt % PHAs implying a TAG:PHA ratio of
548 87:13. Concomitant lactic acid production occurred with TAG accumulation at the end
549 of the assay, 330 mg/L (equivalent to 11 Cmmol/L) of lactic acid were present in the
550 reaction medium. This proved that the excess of carbon under oxygen and N limiting
551 conditions pushed the microbial community to ferment the intracellularly accumulated
552 TAGs to lactic acid.

553 Contrary, in the last accumulation assay performed on day 322 in period V (Figure 4.f),
554 the capacity of PHA accumulation was recovered (Figure 3.b). The PHAs accumulated
555 increased from 6.3 wt % (day 287, period IV) up to 34.8 wt %, and the ratio TAG:PHA
556 varied from 87:13 to 11:88. Potentially, more operation cycles would increase the
557 enrichment capacity until the levels achieved in period III (Table 3).

558 Therefore, the implementation of different selection strategies in the SBR allowed for
559 the enrichment of the culture in microorganisms with a high TAG or PHA storage
560 ability and promoted the subsequent preferential accumulation of one or another storage
561 compound. In fact, the composition of the compounds stored at the end of the

562 accumulation assays was proportional to that of the enrichment reactor at the end of the
563 feast phase when comparing the same operational periods (Figure 3, Table 2, Table 3).
564 No statistically significant differences were observed between both pair of data (t-test
565 for independent samples, t(5) = 0.331, p = 0.749 (see details in Table S1)). Operating
566 the SBR with type B uncoupling strategy, N limitation during the feast phase and pH
567 6.4 ± 0.4 at the end of the cycle was proven the best strategy to obtain intracellular
568 compounds rich on PHAs (85.6 wt % PHAs, Y_{PHA} = 0.80, and TAG:PHA = 4:96). The
569 same operational conditions were proven optimal to maximise TAG accumulation but
570 maintaining the pH low during the famine phase (4.0 ± 0.3) to select for TAG
571 accumulators (43.0 wt % TAGs, Y_{TAG} = 0.67, and TAG:PHA = 87:13) (Figure S5)

572 **3.3.2 Comparison with literature**

573 After reviewing available literature concerning the microbial valorization of lipidic
574 feedstocks for TAG or PHAsproduction (information summarised in Table 4), it was
575 observed that most of the research works performed to date were focused on the use of
576 pure cultures and vegetable oils as a substrate. Very few studies considered the use of
577 animal fats (Herrero et al., 2018; Lopes et al., 2018; Riedel et al., 2015; Sangkharak et
578 al., 2020; Van Thuoc et al., 2019), specifically, only Van Thuoc et al. (2019) and
579 Sangkharak et al. (2020) explored the production of PHAs from feedstocks rich in fish
580 lipids. Overall, these works showed that the use of animal fats, which are complex
581 lipidic substrates, compromised the productivity of the system.

582 The maximum quantity of TAGs accumulated in this study (period IV), was comparable
583 or even higher than the one obtained in previous experiments using both pure (Chan et
584 al., 2018; Lopes et al., 2018) and mixed cultures (Tamis et al., 2015) (Table 4).
585 Regarding PHA production, the results reached during period III (82.3 %wt PHAs,
586 $Y_{PHA/S}$ = 0.80 Cmmol/Cmmol) were very similar to those obtained when using soybean

587 oil for PHA biosynthesis by a pure culture (*Cupriavidus necator* 81 %wt of PHA, Y_{PHA}
588 0.85 g/g (da Cruz Pradella et al., 2012)) (Table 4).

589 None of the experiments presented in Table 4 showed the possibility of storing TAGs or
590 PHAs in the same system. However, their simultaneous accumulation has been proven
591 with other non-lipidic substrates: Hori et al. (2009) investigated the simultaneous
592 production of TAGs and PHAs by *Rhodococcus aetherivorans* (Hori et al., 2009)*;*
593 Castro et al. (2018) obtained 33 wt % of neutral lipids containing 77 % TAG and 4 wt
594 % of PHA in a previous enriched culture using lubricant-based wastewater (Castro et
595 al., 2018); and Fra-Vázquez et al. (2018) obtained 46 wt % TAG and 7.4 wt % PHA in a
596 MMC when using crude glycerol as a substrate (Fra-Vázquez et al., 2018).

597 **3.3.3 Opportunities of the process: TAG vs PHA production**

598 The present research demonstrated that it was possible to drive microbial metabolism
599 towards the preferential production of two different storage materials in the same
600 system using a unique feedstock: TAGs mainly formed by oleic and linoleic acids, and
601 PHAs composed of hydroxybutyrate units.

602 TAG accumulation via *ex novo* pathway from waste fish oil did not lead to the
603 transformation of the storage lipids into upgraded fatty acid profiles (Carsanba et al.,
604 2018). Intracellular TAGs presented a composition proportional to that of the substrate
605 (Table 1) and the lack of specificity is expected to hinder their use in industries such as
606 pharmaceutical, cosmetic, or food-processing. However, the mixture of FFA obtained
607 appeared suitable for biodiesel production (Bušić et al., 2018; Sawangkeaw and
608 Ngamprasertsith, 2013). Therefore, it should be evaluated if the recovery of microbial
609 TAGs would be more sustainable and economically favourable than the waste fish oil
610 pretreatment needed before transesterification (Sawangkeaw and Ngamprasertsith,
611 2013).

On the other hand, polyhydroxybutyrate (PHB) biopolymers are a valuable feedstock for bioplastics production due to their material properties, similar to those of polypropylene (Sudesh et al., 2000), and appear as a higher way of valorization than energy production from TAGs (Wallace et al., 2017). PHB obtention using MMCs, saving an operation unit, and considering a waste stream as a substrate, would strongly reduce production costs, the major restriction for wide PHA commercialization and industrialization (Surendran et al., 2020).

4. CONCLUSIONS

This process demonstrated versatility and efficiency in non-pre-fermented residual fish-canning oil valorization into TAGs and PHAs using a MMC. The implementation of different selection strategies for the enrichment of the culture in microbial species with high TAG or PHA storage ability had a significant impact on the selection of the dominant metabolic pathways in the system, which allowed to increase and control the quantity and type of compounds accumulated. When carbon and nitrogen were fed separately and the amount of nitrogen added was limited, PHA accumulation and productivity substantially increased compared to that obtained in the coupled system (from 5.0 wt % to 82.3 wt % and from 0.03 $Cmmol_{PHA}/Cmmol_{S}$ to 0.80 $Cmmol_{PHA}/Cmmol_{S}$) whereas TAG production was insignificant (TAG:PHA ratio of 4:96). Furthermore, when low pH conditions in the famine were considered as an additional selective pressure in the uncoupled system, TAG accumulation dominated and it was possible to reach a maximum 43.0 wt % TAG in the FBR (TAG:PHA ratio of 87:13) obtaining a production yield of 0.67 $Cmmol_{TAG}/Cmmol_{S}$.

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844

Figure 1. Simplified metabolic pathways describing TAG and PHA production from lipids in microbial cultures. Free fatty acids (FFA) yielded after the substrate hydrolysis can be catabolized (direct respiration) or accumulated as reserve materials (TAGs or PHAs produced by the ex *novo* or PHA biosynthesis pathways, respectively).

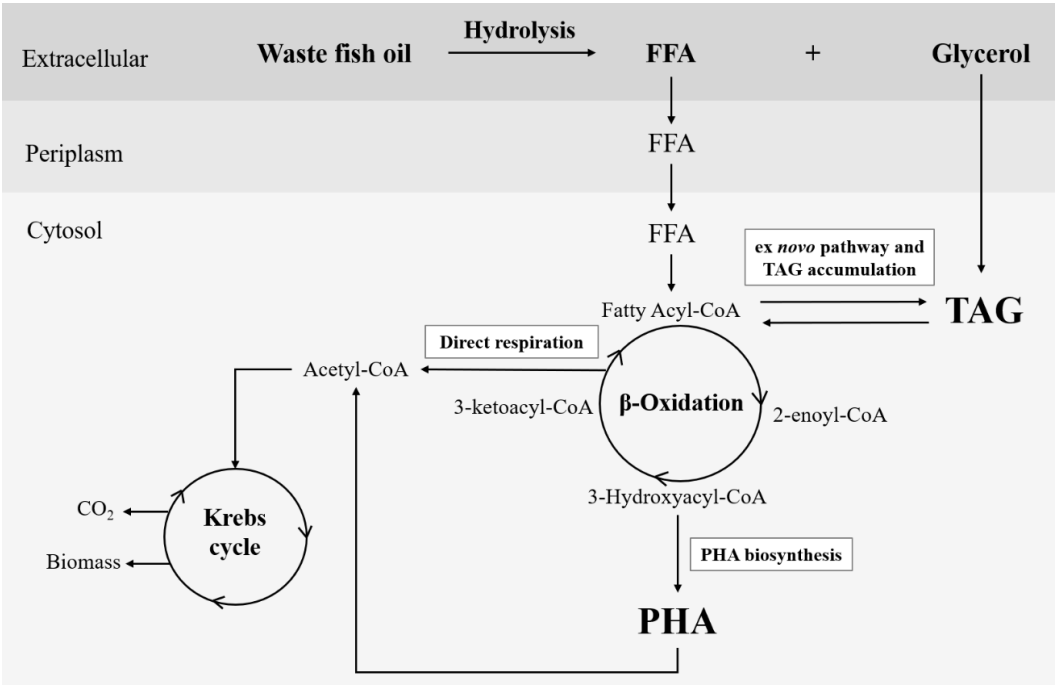

Figure 2. Characterization of the SBR enrichment cycles. Concentrations of DO (—), X (---), pH (●), TN (♦), TAG + PHA (○).

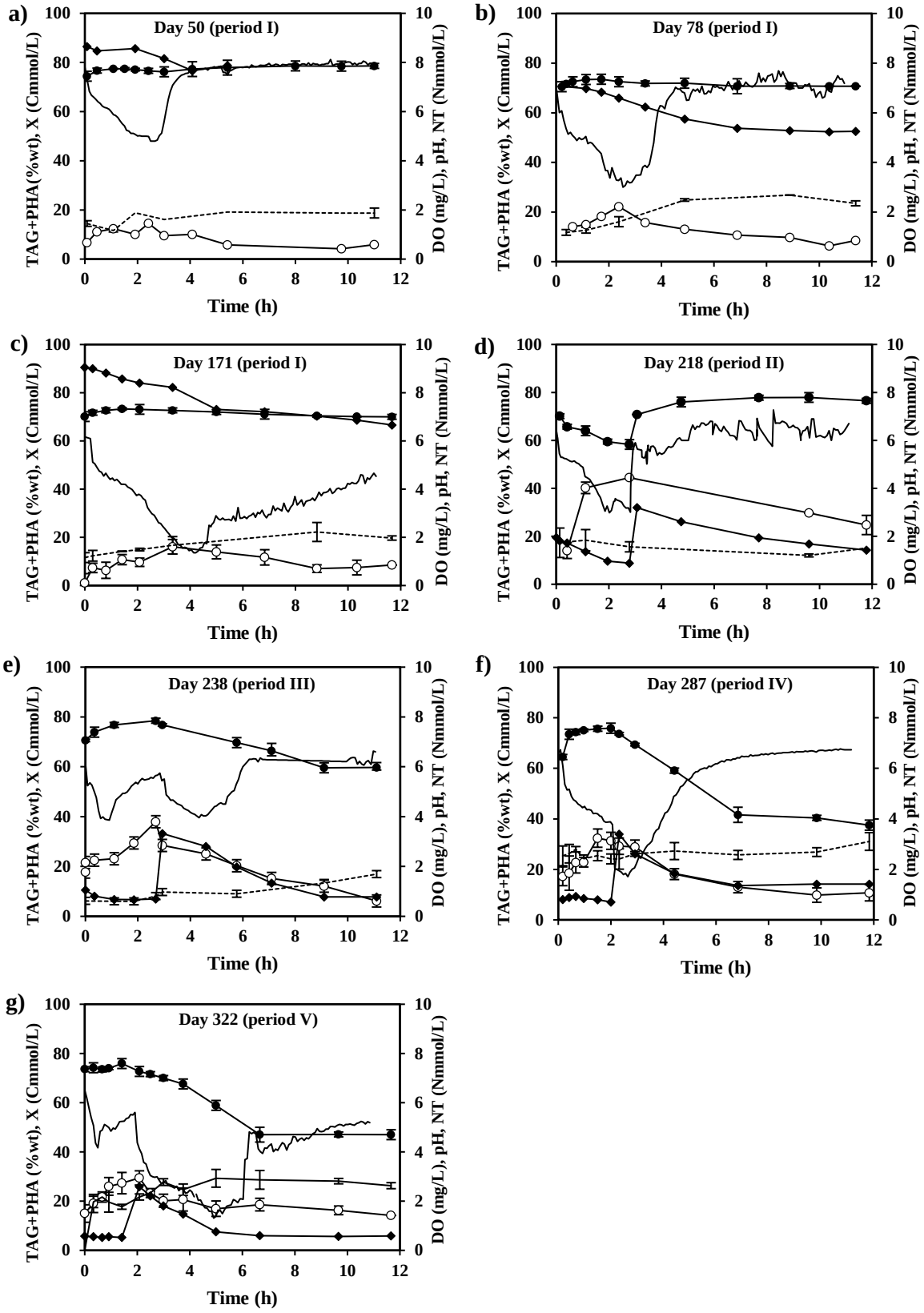

Figure3. Maximum intracellular storage and composition: a) at the end of the feast phase in the SBR; b) at the end of the accumulation assays in the FBR. Percentages of intracellular TAGs (Δ), and PHAs (×) in the biomass. Composition expressed in percentages of palmitic (□), stearic (■), oleic (▩), linoleic (■) and PHB (⚃).

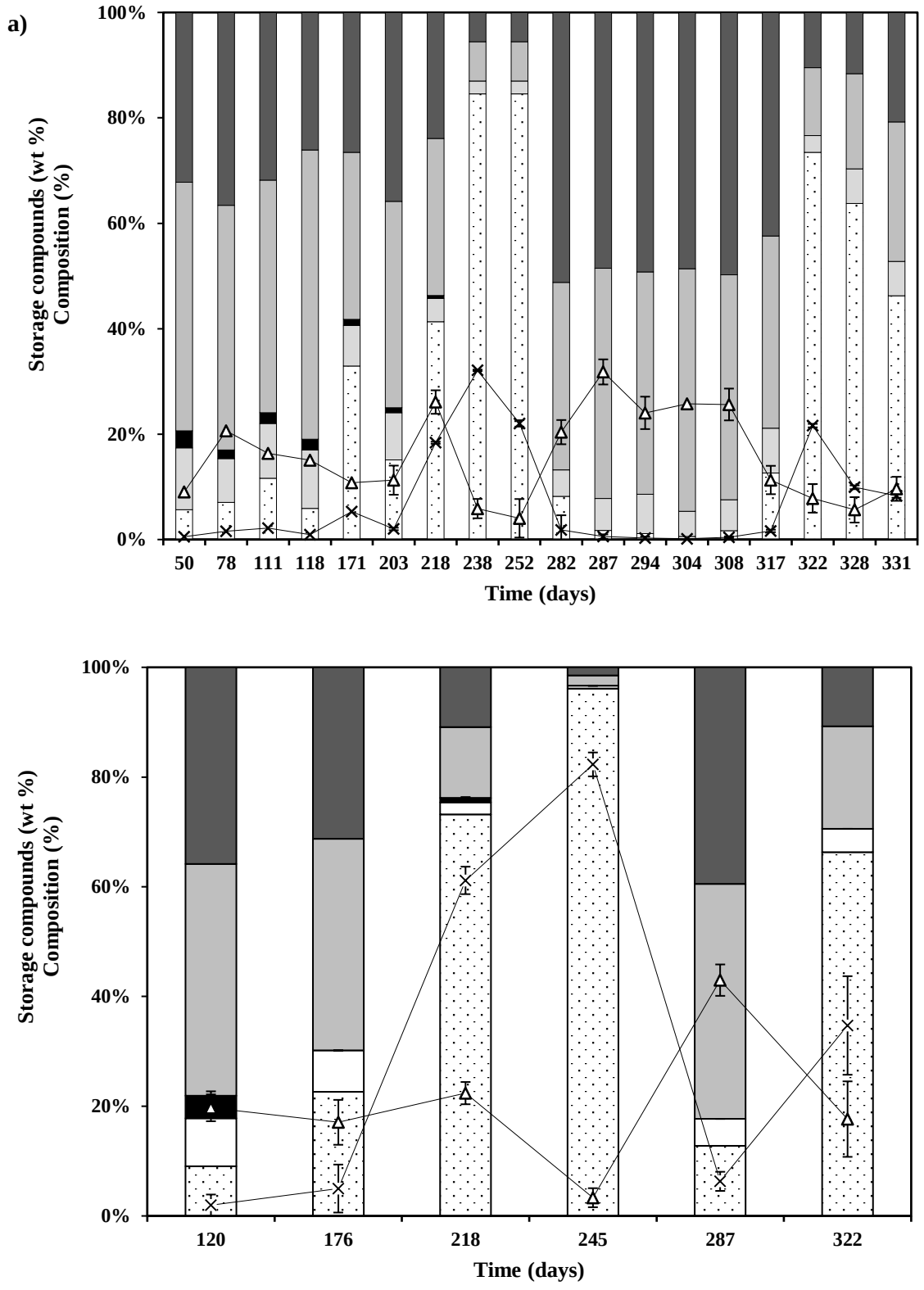

Figure 4. Complete characterization of the accumulation assays performed in the FBR. X (---), pH (●), TN (♦), TAGs (Δ), PHAs (×), oil (■).

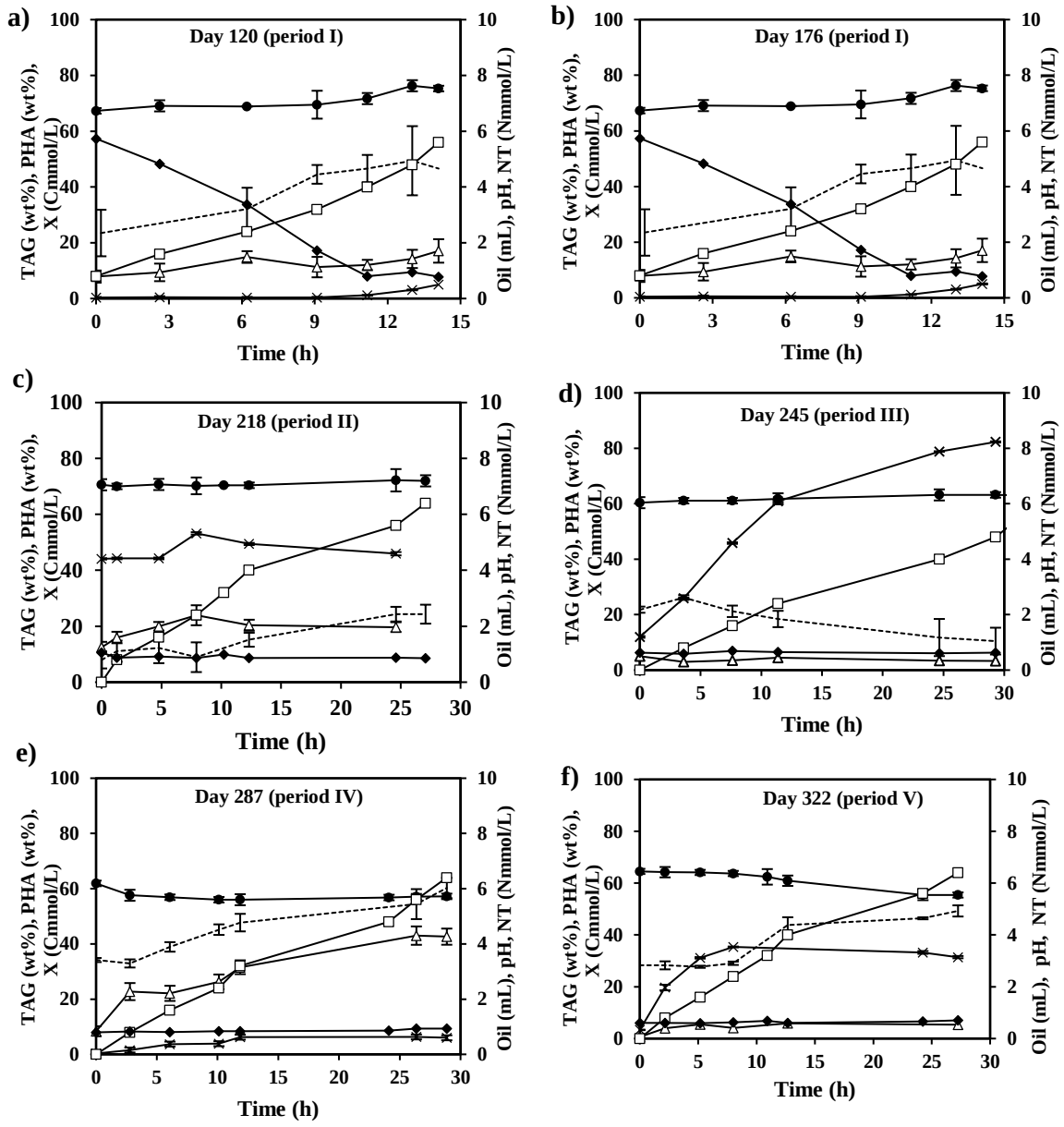

Table 1. Characteristics of the fish-canning industry oil used as a substrate.

Parameter	Value
pH	3.75 ± 0.20
Conductivity (µS/cm)	0.60 ± 0.10
Density (g/L)	900 ± 10
TS (g/g)	0.853 ± 0.018
VS (g/g)	0.852 ± 0.019
VS/TS (%)	99.89
COD (g/g)	2.50 ± 0.11
Lipids (g/g)	0.823 ± 0.089
Composition (wt%)	Palmitic acid: 10.3 ± 0.4 Stearic acid: 2.5 ± 1.0 Oleic acid: 38.3 ± 1.6 Linoleic acid: 31.2 ± 1.5 Others: 17.7 ± 4.2

Table 2. Intracellular storage, kinetic parameters and yields determined in different cycles monitored in the enrichment SBR.

Operational period	**I**	**I**	**I**	**II**	**III**	**IV**	**V**
Day of operation	Day 50	Day 78	Day 171	Day 218	Day 238	Day 287	Day 322
% wt max.							
TAGs	13.7	20.6	10.8	25.8	5.8	30.6	7.8
PHAs	0.9	1.6	5.3	18.4	32.1	0.7	21.6
Composition							
TAG:PHA	94:6	93:7	67:33	42:58	15:85	98:2	27:73
Palmitic:Stearic:Oleic:Linoleic	14:4:44:33	9:2:50:39	11:2:47:40	8:0:51:41	16:0:48:36	6:0:47:47:	12:0:49:40
PHB:PHV	100:0	100:0	100:0	100:0	100:0	100:0	100:0
q (Cmmol/Cmmol·h)							
$q_{TAG/X \cdot h}$	0.018	0.089	0.034	0.030	0.000	0.131	0.000
$q_{PHA/X \cdot h}$	0.001	0.005	0.024	0.065	0.069	0.001	0.048
- $q_{N/X \cdot h}$ [(1)]	0.053 – 0.022	0.196 – 0.029	0.079 – 0.031	0.002 – 0.000	0.005 – 0.003	0.000 – 0.002	0.000 – 0.005
$q_{X/S \cdot h}$ [(1)]	0.002 – 0.000	0.002 – 0.012	0.005– 0.015	0.019 – 0.017	0.000 – 0.027	0.000 – 0.017	0.000 – 0.017
Y (Cmmol/Cmmol)							
$Y_{TAG/S}$	0.053	0.098	0.127	0.168	0.000	0.467	0.000
$Y_{PHA/S}$	0.002	0.006	0.041	0.078	0.115	0.001	0.147
$Y_{X/S}$ [(1)]	0.035 – 0.000	0.035 – 0.310	0.075 – 0.112	0.052 – 0.224	0.000 – 0.289	0.000 – 0.171	0.000 – 0.449

[(1)] Data regarding feast (left) and famine (right) phases: feast – famine.

Table 3. Intracellular storage, kinetic parameters and yields determined in the batch assays performed in the accumulation FBR.

Operational period	**I**	**I**	**II**	**III**	**IV**	**V**
Day of operation	120	176	218	245	287	322
% wt max.						
TAGs	19.7	17.1	23.0	3.3	43.0	17.6
PHAs	2.0	5.0	61.2	82.3	6.3	34.8
Composition						
TAG:PHA	91:9	77:23	27:73	4:96	87:13	34:66
Palmitic:Stearic:Oleic:Linoleic	10:5:46:39	10:0:50:40	8:3:46:43	12:0:48:39	6:0:49:45	13:0:56:32
PHB:PHV	100:0	100:0	100:0	100:0	100:0	100:0
q (Cmmol/Cmmol·h)						
$q_{TAG/X \cdot h}$	0.003	0.002	0.002	0.000	0.002	0.000
$q_{PHA/X \cdot h}$	0.000	0.000	0.003	0.004	0.000	0.001
$- q_{N/X \cdot h}$	0.001	0.001	0.000	0.000	0.000	0.000
$q_{X/S \cdot h}$	0.056	0.022	0.000	0.000	0.009	0.001
Y (Cmmol/Cmmol)						
$Y_{TAG/S}$	0.234	0.129	0.158	0.022	0.667	0.163
$Y_{PHA/S}$	0.014	0.027	0.240	0.803	0.080	0.244
$Y_{X/S}$	0.679	0.317	0.000	0.000	0.253	0.038

Table 4. TAGs and PHAs production from oily fractions using both pure culture and MMCs: a comparison with literature.

	Inoculum	Feedstock	Production process	wt % max.	$Y_{TAG/S}$ - $Y_{PHA/S}$	Reference
TAGs pure culture						
	Mucor circinelloides	Cheese whey	Erlenmeyer flasks	34	0.04 g / g	Chan et al. (2018)
	Rhodococcus opacus *Rhodococcus Jostii* *Rhodococcus wratislaviensis*	Olive oil mill waste	Rotary shaker Petri plates	77 – 83	-	Herrero et al. (2018)
	Yarrowia lipolytica	Pork lard	Batch bioreactor	27 – 58	-	Lopes et al. (2018)
PHAs pure culture						
	Pseudomonas mendocina	Coconut oil	Fermentation	58	-	Basnett et al. (2018)
	Cupriavidus necator	Soybean oil	Batch bioreactor	80	0.85 g /g	da Cruz Pradella et al. (2012)
	Cupriavidus necator	Peanut oil	Fermentation	51	0.56 g / g	Pérez-Arauz et al. (2019)
	Ralstonia eutropha	Waste frying oil Tallow	Batch fermentation	70 63	-	Riedel et al. (2015)
	Cupriavidus necator	Tuna condensate from the fish-canning industry	Batch fermentation	42	0.42 g / g	Sangkharak et al. (2020)
	Pseudomonas sp.	Corn oil Waste vegetable oil	Cultivation in a shaker	37 24	-	Song et al. (2008)
	Salinivibrio sp.	Waste fish oil and glycerol	Batch bioreactor	42	0.32 g / g	Van Thuoc et al. (2019)

Table 4 (Continuation)

	Inoculum	Feedstock	Production process	wt % max.	$Y_{TAG/S}$ - $Y_{PHA/S}$	Reference
TAGs MMC						
	Activated sludge	Soybean oil	SBR + FBR	54	-	Tamis et al. (2015)
PHAs MMC						
	Activated sludge	Olive oil pomace	Pretreatment + SBR	39	0.36 g / L	Waller et al. (2012)
	Activated sludge	Olive oil mill wastewater	Pretreatment + Acidification + SBR + FBR	30	0.56 g COD / g COD	Campanari et al. (2014)
	Activated sludge (10 %) and palm oil mill effluent (90 %)	Palm oil mill wastewater	Single SBR for growth and accumulation	45	-	Md Din et al. (2006)
	Activated sludge for aerobic granules formation	Palm oil mill wastewater	Pretreatment + SBR	68	0.66 g /g COD	Gobi and Vadivelu (2014)
PHAs + TAGs MMC						
	Activated sludge	The oily fraction of a fish-canning industry effluent	SBR + FBR	(1) TAG: 43 (2) PHA: 82	(1) 0.67 $Cmmol_{TAG}$ / $Cmmol_S$ (2) 0.80 $Cmmol_{PHA}$ / $Cmmol_S$	This research work

(1) Data concerning Period IV, highest TAGs accumulation and production yield. (2) Data regarding Period III, highest PHAs accumulation and production yield.

A novel strategy for triacylglycerides and polyhydroxyalkanoates production using waste lipids

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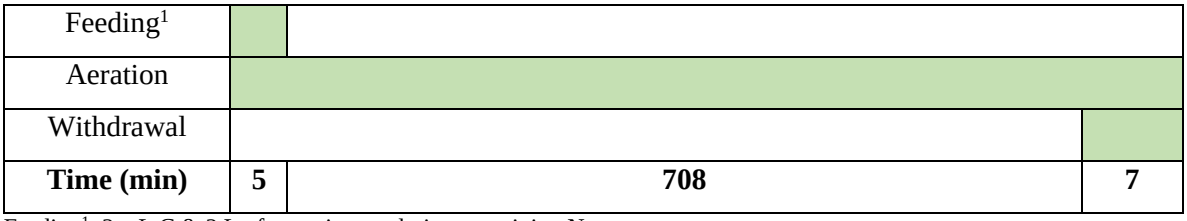
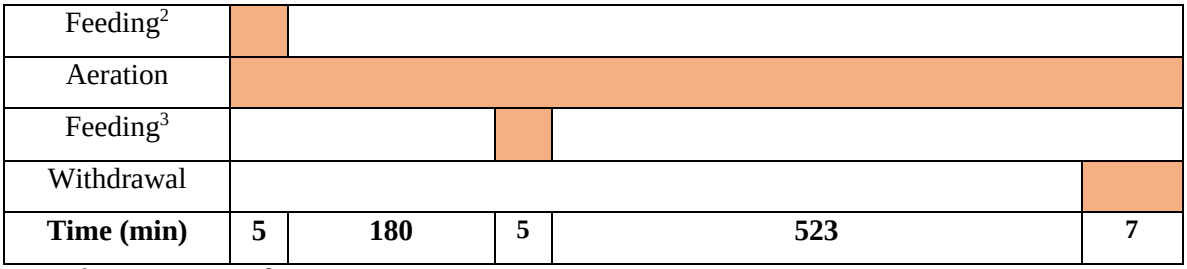
Figure S1. Configuration of the SBR cycles during the different operational periods (I – V).

Coupled system configuration (period I)



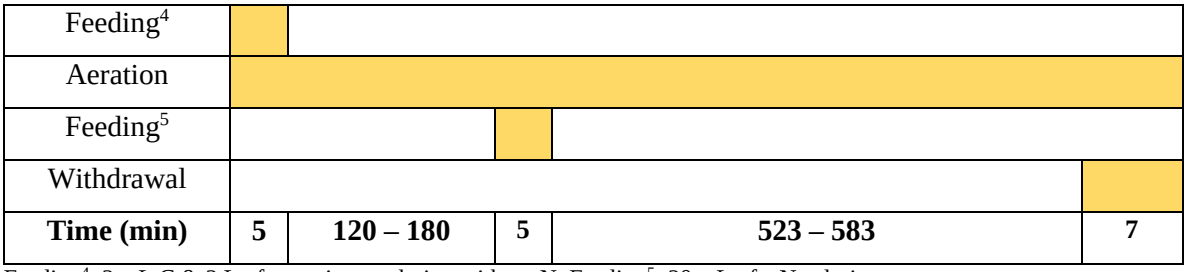
Feeding[1]: 2 mL C & 2 L of a nutrients solution containing N.

Uncoupled system configuration type A (period II)



Feeding[2]: 2 mL C; Feeding[3]: 2 L of a nutrients solution containing N.

Uncoupled system configuration type B (periods III, IV and V)



Feeding[4]: 2 mL C & 2 L of a nutrients solution without N; Feeding[5]: 20 mL of a N solution.

Figure S2. Distribution of the different operational periods in the SBR (I – V) and accumulation assays performed in the FBR.

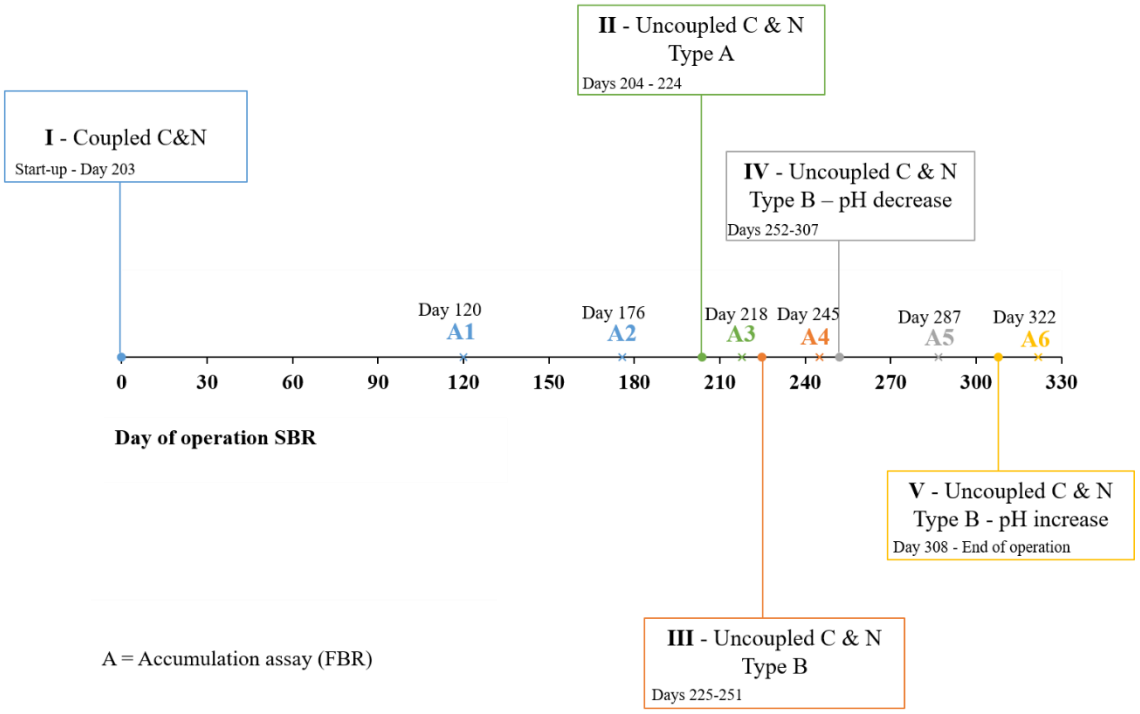


Figure S3. Evolution of different parameters in the SBR during periods I – V. a) Feast phase length considering accumulation + growth (○), single accumulation (●); b) Inorganic carbon (×) and pH (●) of the nutrients solution, pH at the end of the cycle (●); c) TN of the nutrients solution (●), TN at the end of the cycle (●); d) VSS at the end of the cycle (●).

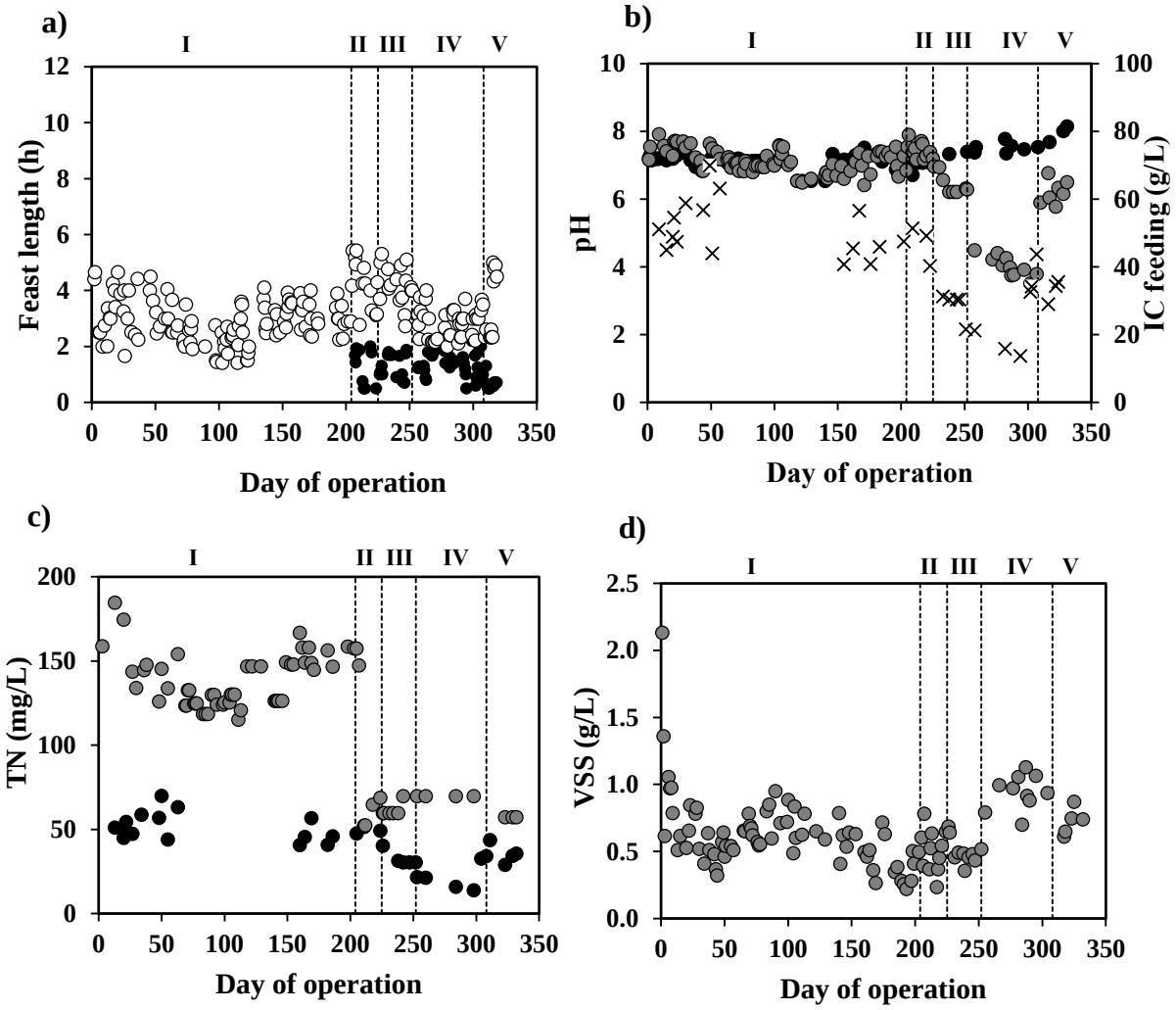


Figure S4. Comparison of the evolution of the dissolved oxygen (DO) profiles in the coupled and uncoupled systems and feast phase length quantification.

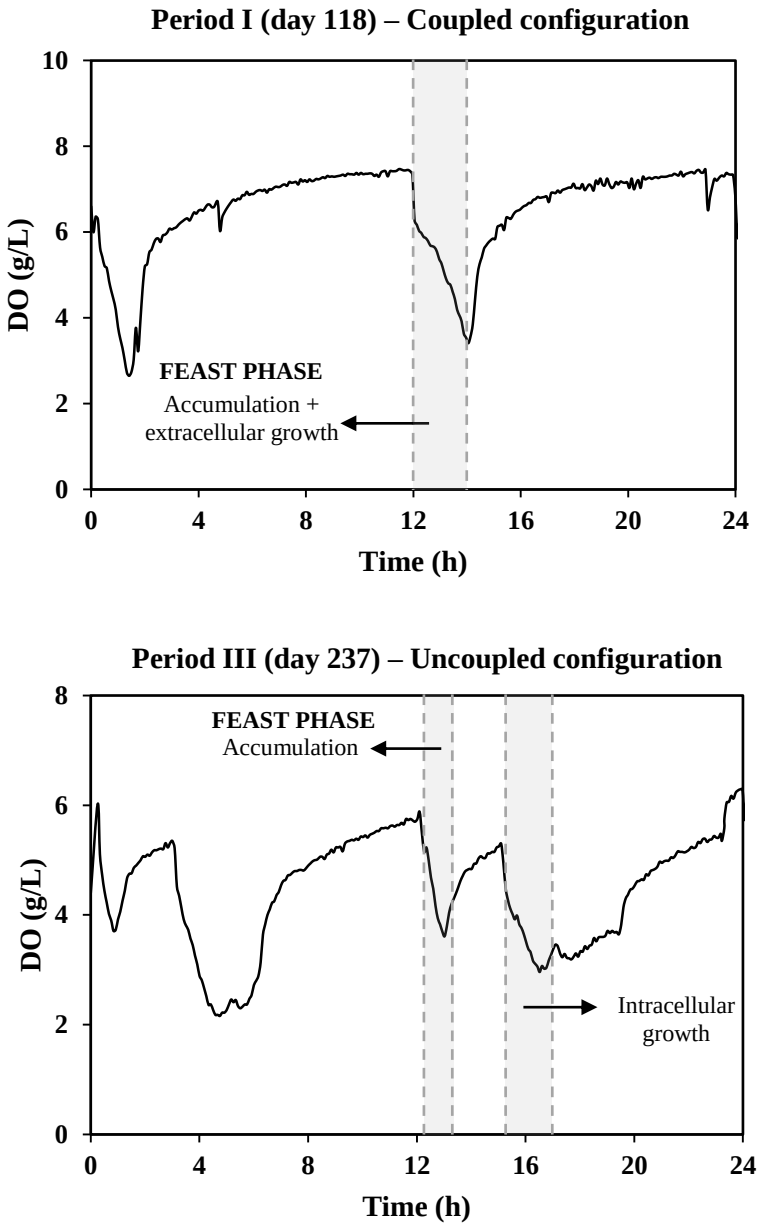


Figure S5. Simplified flowchart of the research methodology and operational periods.

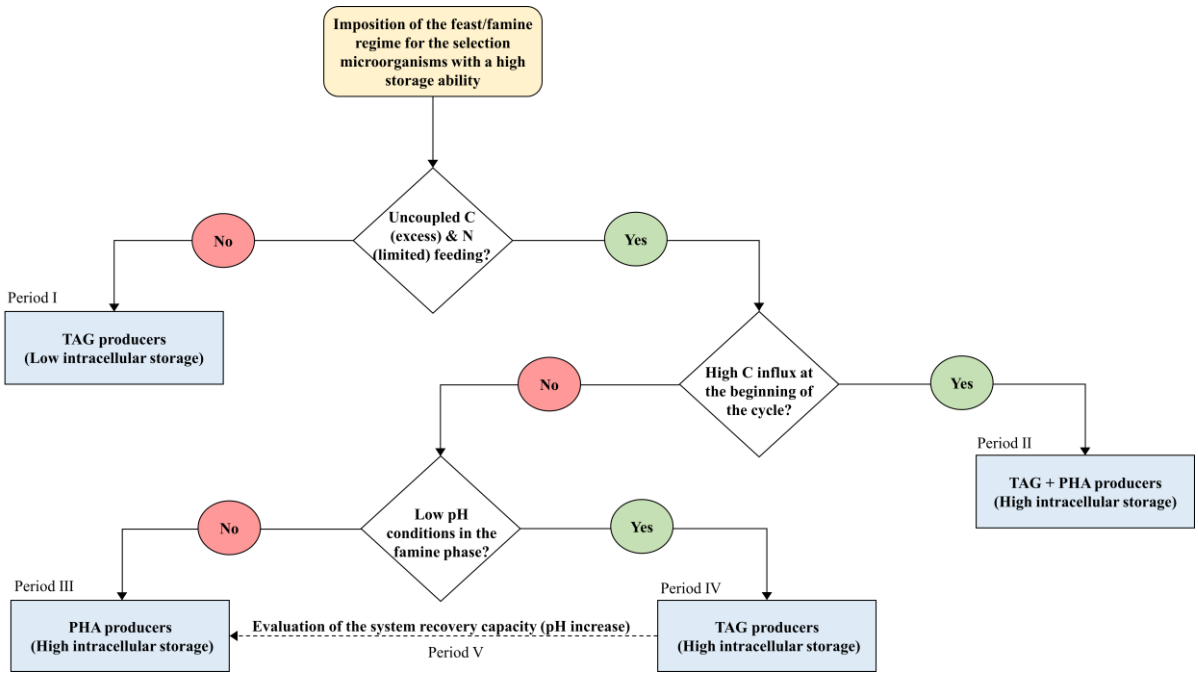


Table S1. Results of the statistical analysis. a) One-Way ANOVA, Turkey post hoc test considering TAG and PHA as independent variables: comparison of the storage compounds composition at the end of the feast phase in the enrichment SBR during different operational periods. b) t-test for independent samples: comparison of the composition of the intracellular compounds accumulated during the same operational periods in SBR and FBR reactors.

a)

One-Way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TAG	Between Groups	11273.204	4	2818.301	8.731	.001
	Within Groups	4519.156	14	322.797		
	Total	15792.360	18			
PHA	Between Groups	11273.204	4	2818.301	8.731	.001
	Within Groups	4519.156	14	322.797		
	Total	15792.360	18			

Turkey post hoc tests (TAG as an independent variable)

Period (i)	Period (j)	Mean Difference (i – j)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
I	II	28.29000	14.66963	.348	-17.4197	73.9997
	III	(1) 71.51000	14.66963	(2) .002	25.8003	117.2197
	IV	-10.11000	11.59736	.903	-46.2467	26.0267
	V	26.52000	10.87929	.162	-7.3792	60.4192
II	I	-28.29000	14.66963	.348	-73.9997	17.4197
	III	43.22000	17.96655	.171	-12.7628	99.2028
	IV	-38.40000	15.55949	.154	-86.8825	10.0825
	V	-1.77000	15.03189	1.000	-48.6085	45.0685
III	I	(1) -71.51000	14.66963	(2) .002	-117.2197	-25.8003
	II	-43.22000	17.96655	.171	-99.2028	12.7628
	IV	(1) -81.62000	15.55949	(2).001	-130.1025	-33.1375
	V	-44.99000	15.03189	.062	-91.8285	1.8485
IV	I	10.11000	11.59736	.903	-26.0267	46.2467
	II	38.40000	15.55949	.154	-10.0825	86.8825
	III	(1) 81.62000	15.55949	(2).001	33.1375	130.1025
	V	36.3000	12.05233	.057	-.9244	74.1844
V	I	-26.2000	10.87929	.162	-60.4192	7.3792
	II	1.7000	15.03189	1.000	-45.0685	48.6085
	III	44.99000	15.03189	.062	-1.8485	91.8285
	IV	-36.63000	12.05233	.057	-74.1844	.9244

Table S1 (continuation)

a)

Turkey post hoc tests (TAG as an independent variable)

Period (i)	Period (j)	Mean Difference (i – j)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
I	II	-28.29000	14.66963	.348	-73.9997	17.4197
	III	[1] -71.51000	14.66963	[2].002	-117.2197	-25.8003
	IV	10.11000	11.59736	.903	-26.0267	46.2467
	V	-26.52000	10.87929	.162	-60.4192	7.3792
II	I	28.29000	14.66963	.348	-17.4197	73.9997
	III	-43.2000	17.96655	.171	-99.2028	12.7628
	IV	38.40000	15.55949	.154	-10.0825	86.8825
	V	1.77000	15.03189	1.000	-45.0685	48.6085
III	I	[1] 71.51000	14.66963	[2] .002	25.8003	117.2197
	II	43.22000	17.96655	.171	-12.7628	99.2028
	IV	[1] 81.62000	15.55949	[2] .001	33.1375	130.1025
	V	44.99000	15.03189	.062	-1.8485	91.8285
IV	I	-10.11000	11.59736	.903	-46.2467	26.0267
	II	-38.40000	15.55949	.154	-86.8825	10.0825
	III	[1] -81.62000	15.55949	[2] .001	-130.1025	-33.1375
	V	-36.63000	12.05233	.057	-74.1844	.9244
V	I	26.52000	10.87929	.162	-7.3792	60.4192
	II	-1.77000	15.03189	1.000	-48.6085	45.0685
	III	-44.99000	15.03189	.062	-91.8285	1.8485
	IV	36.63000	12.05233	.057	-.9244	74.1844

[1] The mean difference is significant at the 0.05 level. [2] Significatively different (sig. < 0.05).

b)

Group statistics

Reactor	Nº of samples	Mean	Std. deviation	Std. error deviation
SBR	5	53.0204	32.98924	14.75324
FBR	5	45.8840	35.20856	15.74575

Levene's test for equality of variances

F	Sig.
0.134	0.724

Table S1 (continuation)

b)

t-test for Equality of Means

	t	Degrees of freedom	Sig (2-tailed)	Mean difference	Std. Error Difference	95 % Confidence Interval of the Difference	
						Lower	Upper
Equal variances	.331	8	.749	7.13640	21.57745	-42.62130	56.89410
Equal variances not assumed	.331	7.966	.749	7.13640	21.57745	-42.65793	56.93073