Boosting aerobic microbial protein productivity and quality on brewery wastewater: Impact of anaerobic acidification, high-rate process and biomass age

Gustavo Papini[a], Maarten Muys[a], Tim Van Winckel[a], Francis A. Meerburg[b], Wannes Van Beeck[a], Pieter Vermeir[c] and Siegfried E. Vlaeminck[a*]

[a] Research Group of Sustainable Energy, Air and Water Technology, Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium

[b] Independent scholar, Dijkstraat 8, 2630 Aartselaar, Belgium

[c] Laboratory of Chemical Analysis, Department of Green Chemistry and Technology, Gent University, Valentin Vaerwyckweg 1, 9000 Gent, Belgium

*Corresponding author e-mail: Siegfried.Vlaeminck@UAntwerpen.be

Keywords: resource recovery; single-cell protein; fermentation; contact stabilization; aquafeed

Abstract

Consortia of aerobic heterotrophic bacteria (AHB) are appealing as sustainable alternative protein ingredient for aquaculture given their high quality, and their production potential on feed-grade industrial wastewater. Today, the impact between pre-treatment, bioprocess choice and key parameter setting on AHB productivity and nutritional properties is unknown. This study investigated for the first time known the effects on AHB microbial protein production from (i) raw vs. anaerobically fermented brewery wastewater, (ii) high-rate activated sludge (HRAS) without vs. with feast-famine conditions, and (iii) three short solids retention times (SRT): 0.25, 0.50 and 1.00 d. High biomass (4.4-8.0 g TSS/L/d) and protein productivities (1.9-3.2 g protein/L/d) were obtained while achieving COD removal efficiencies up to 98% at SRT 0.50 d. The AHB essential amino acid (EAA) profiles were above rainbow trout requirements, excluding the S-containing EAA, highlighting the AHB biomass replacement potential for unsustainable fishmeal in salmonid diets.

1. Introduction

The World's population will reach 9.7 billion by 2050 (United Nations, 2019). At the same time, the predicted wealth increase will boost further animal-based protein demand in human diets (Godfray et al., 2010). In this period, fish production is projected to raise 21%, compared to 2018 (FAO, 2020). As virtually all fish stocks are overexploited (Godfray et al., 2010), this demand is expected to be covered by aquaculture (Rana et al., 2009). Nonetheless, aquaculture depends for one third on the limited provision of the increasingly expensive and unsustainable fish meal (FM) (Rana et al., 2009). Thus, alternative protein sources are needed and microbial protein (MP) is considered a promising feed ingredient for aquaculture.

MP are microorganisms, such as microalgae, bacteria, yeast or fungi, that have high nutritional quality and can be use as food or feed. They have several environmental benefits compared with traditional protein sources such as: high protein content (up to 83%), faster growth, no necessity of arable land, high nutrients assimilation efficiency and virtually nil water usage, as some MP can grow in liquid sidestreams (Verstraete et al., 2016). Most MP applications and developments as feed ingredients are based on dried microbial biomass of non-engineered bacteria, i.e. single-cell protein, which facilitates regulatory and end-user acceptance. Currently, microalgae and methane oxidizing bacteria (MOB) are the main relevant MP being supplied to the feed market (Ritala et al., 2017) but they rely on primary products while MPs production based on secondary resources are still missing. In this context, the most promising microbes are purple non-sulphur bacteria (PNSB), microalgae bacterial floc (MaB) and aerobic heterotrophic bacteria (AHB) (Verstraete et al., 2016). PNSB is a phototrophic anoxygenic microbe that, for wastewater treatment application, grows together with

other bacteria reaching growth rates up to 3.7 d^{-1} and yields close to 1 g of chemical oxygen demand (COD) as biomass/g $COD_{removed}$ (Alloul et al., 2019). MaB is a synergetic consortia between microalgae (phototrophic) and bacteria (heterotrophic) that have been tested at growth rates below 0.17 d^{-1} (Van Den Hende, 2014). Both MP have similar challenges such as tackling growth restraint due to light scattering and the poor settling properties (Van Den Hende, 2014). AHB are composed of a mixed microbial community that grows easily as flocs (settleable) in the aerobic reactor of the conventional activated sludge (CAS) processes for domestic and industrial wastewater treatment, and can achieve high growth rates and yields, up to 6 d^{-1} and 1 g $COD_{biomass}$/g $COD_{removed}$ respectively (Meerburg et al., 2016). Currently, this biomass is considered a waste stream although studies showed positive feeding trial results and that AHB quality is comparable to fish and soybean meal (Vriens et al., 1989). Still, the presence of heavy metals and faecal contamination in some wastewaters raises concerns about its safety. To preserve AHB quality and obtain public acceptability, the use of uncontaminated streams such as wastewater from food and beverage industries is recommended (Verstraete et al., 2016) and the development of a process able to maximize AHB production and quality while treating these streams is required.

In order to boost AHB productivities high-rate conventional activated sludge (HiCAS) and the high-rate contact stabilization (HiCS), are more appropriate. Under short SRT (< 2 day) and high specific loading rate (>2 g COD/g VSS/d), they maximize carbon redirection into biomass (Meerburg et al., 2015). The difference between both is that HiCAS only contains one phase, where COD is oxidized under aerobic conditions. In HiCS, a contact phase is included where the incoming substrate meets starved sludge coming from the stabilization step, under absence or low levels of oxygen (<0.5 mg

O_2/L). After settling, the return sludge is aerated in the stabilizer phase in absence of feed to starve the biomass till returning to the contactor, forming a feast-famine regime (Meerburg et al., 2015). There are several full-scale HiCAS in operation for domestic wastewater treatment (Meerburg et al., 2016), where the biomass is used for energy recovery instead, while HiCS remains a novel approach (Rahman et al., 2019). Here, HiCAS and HiCS were studied as potential AHB production processes.

Operational conditions can affect the AHB community composition (Gonzalez-Martinez et al., 2016; Valentín-Vargas et al., 2012), productivity and quality. SRT is one of the main parameters to design AS and shorter SRTs leads to higher observed cell yields (Vriens et al., 1989). Since maximal yield is desired, SRTs below 1 d were chosen to select for fast growing bacteria. Concomitantly, yield, rate of utilization, productivity of MP and microbial community are strongly dependent on the substrate (Fatemeh et al., 2019) which might affect biomass quality. Thus, simpler substrate could favour the growth of specific bacteria and, consequently, impact both productivity and MP quality. There is also a great knowledge gap on understanding the link between process parameters and the composition of the HRAS microbiome. Only two studies using HRAS in sewage treatment provided information about the microbial community (Gonzalez-Martinez et al., 2016; Meerburg et al., 2016). For industrial wastewater treatment and MP production this information is currently inexistent.

Hence, this study aims to determine the impact of different substrates, reactor operation and SRTs, to the COD fate in the process, COD removal efficiency, MP productivity, biomass quality, and AHB community composition. Therefore, 12 scenarios were tested using all combinations of substrate (raw and fermented synthetic brewery wastewater), reactor operation (HiCAS and HiCS) and short SRTs (0.25, 0.50 and 1.00 d).

2. Materials and methods

2.1. Synthetic brewery wastewater

Synthetic raw and fermented wastewater recipes were designed to obtain similar characteristics of the real brewery wastewater before and after fermentation. Synthetic wastewater was used to guarantee reproducibility and prevent the occurrence of confounding factors due to the quality oscillation of the real brewery wastewater. Samples from four Flemish breweries were analysed and data from the literature were gathered in order to define the typical COD speciation, pH and temperature of brewery wastewater, that were then used as target values for the raw synthetic brewery wastewater recipe (Driessen and Vereijken, 2003; Okoli and Okonkwo, 2016). Easily biodegradable organic components such as sugars, soluble starch, volatile fatty acids (VFA), and ethanol are typically present in brewery wastewater (Driessen and Vereijken, 2003). To simulate these compounds, yeast, malt, peptone, beer, acetic acid, propionic acid and butyric acid were included in the recipe. Most of the ingredients are fully soluble, except yeast and malt extract. From the total COD content per gram of product, 70% of yeast's COD content is particulate and for malt extract, only 3%. So in both, raw and fermented medium, around 90% of the particulate COD was provided by the yeast. Regarding VFA concentration, reports from Palm Brewery ('personal communication') showed that VFA corresponded to around 14% of the soluble COD in the raw brewery wastewater. The COD from the VFA was divided between acetic acid (40%), propionic acid (50%) and butyric acid (10%) (Ahn et al., 2001; Scampini, 2010). To avoid any potential limitation of the AHB growth, nitrogen (N) and phosphorus (P) both were dosed in excess. N and P target values were set by calculating maximum biomass production assuming 100% COD removal efficiency, maximum observed yield

(Y_{obs}) of 1 g $COD_{biomass}$/g $COD_{removed}$ (Meerburg et al., 2016) and a typical activated sludge biomass composition $C_{50}H_{87}O_{23}N_{12}P$. A safety factor of +50% was included and the final COD:N:P ratio was defined, 42.0:5.4:1.0. For other macronutrients, such as Ca, Cl, Fe, Mg, Na, K and S, literature was used to define the recommended levels (Grady Jr. et al., 2011). Micronutrients were not dosed assuming that tap water used in the preparation of the media would contain the amount necessary for the microbial growth. The fermented brewery wastewater was calculated based on the COD concentration of the raw influent and literature data about the amount of COD lost and converted into VFA during the fermentation process. Based on real wastewater fermentation experiments performed by Ahn et al. (2001) and Alexiou (1998), it was assumed a conversion ratio of 0.42 g VFA as COD/g total $COD_{influent}$ and that 15% of the total incoming COD would be lost because microbial growth was assumed. Thus, the VFA concentration would correspond to 49% of the total COD in the fermented wastewater and the same VFA speciation, as in raw wastewater, was used. Table 1 resumes all the main target values used to formulate raw and fermented synthetic brewery wastewater. It was decided to work with a 20 times concentrated medium. In this way, small volumes could be kept in a fridge (4°C) during the tests, safeguarding media quality. The concentrated medium was prepared every week. To prepare it, 2 L of demi water was added to a 5 L recipient. Under thorough mixing by a magnetic stirrer, each inorganic compounds was added per time till complete dissolution. Next, the organic products were added one by one. After that pH was corrected to 7 using 2 M NaOH and finally 1 mL of antifoam (SE-15, Sigma Aldrich) was added per liter of concentrated medium. Finally, the demi water was added to the medium reaching a final volume of 5 L and then the effluent was stored at 4°C for usage. A 150L vessel containing tap water

153 and a 0.03M phosphate buffer solution was used to dilute the medium to the final
154 concentration and to keep the pH stable in the experiments. The buffer was the main
155 source of P for the bacteria. Information on brewery wastewater characteristics,
156 ingredients' composition and media recipe is provided (see Supplementary Material).

157 **2.2. Reactor setup and operation**

158 The main characteristics of the scenarios are shown in Table 2. Two 2 L acrylic
159 sequencing batch reactors (SBR) were operated simultaneously. One reactor was always
160 fed with synthetic raw wastewater while the other with synthetic fermented substrate.
161 Meanwhile, both reactors were kept at the same SRT (0.25, 0.50 or 1.00 d) and
162 operation mode (HiCAS or HiCS). Biomass was collected from a CAS plant treating
163 brewery wastewater from AB InBev (Leuven, Belgium) and stored at 4°C for
164 (re)inoculation whenever it was necessary to reach 1.5 g VSS/L. Concentrated medium
165 and tap water with buffer solution was added to reach 2 L volume and then the reactor
166 was started in batch mode for at least 1-2 d, then continuous operation was started. An
167 acclimation period of 3-5 days was respected, to reach steady-state, prior to resuming
168 sampling. Samples were taken daily.
169 The reactors were placed in a temperature-controlled room (20°C), and pH was kept
170 automatically at 7.00 ± 0.40 by a controller (Consort, Belgium). Overhead stirrer was
171 used for mixing, air pumps for aeration and peristaltic pumps controlled were used to
172 add influent (concentrated medium plus water), harvest biomass and remove the treated
173 effluent. Timers were used to set the exact time of equipment operation according to the
174 reactor phases. Total cycle time in both reactors was 120 min. For HiCAS the phases
175 were: feed and react (100 min aerated), settle (15 min), withdraw (4 min) and idle (1
176 min). In the HiCS system the cycle was divided as follows: feed and contact (28 min,

unaerated), stabilization (72 min, aerated), settle (15 min), withdraw (4 min) and idle (1 min). The contact to stabilization time ratio used was 0.38, the optimal found by Meerburg et al. (2016). The harvesting of the biomass was performed during the react phase in HiCAS and during the contact phase in HiCS.

2.3. Analytical procedures

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (Greenberg et al., 1992) and used to quantify biomass in the system. Photometric test kits (Merck, Germany) were used to measure COD fractions, total (CODt) and soluble (CODs). Prior to CODs determination, the sample was filtered using a 0.2 μm syringe filter (Chromafil PTFE, Macherey-Nagel) to remove particles. COD particulate (CODp) was obtained by the difference between CODt and CODs. Colloidal COD, represented by the solids with size between 0.20-25 μm, were therefore deliberately accounted together with the CODp and the fate of these particles were not investigated although recommended for future studies.

Protein content was determined according to a modified Lowry method as described in Markwell et al. (1978). The protein values were expressed as a fraction of total suspended solids (TSS). Amino acid (AA) residuals measurements started with a hydrolysis step, which was performed with 6M HCl for 24 hours at 110°C, in vacuum-sealed hydrolysis tubes (Wilmad Labglas). To avoid AA oxidation, hydrolysis and subsequent acid evaporation were performed under vacuum atmosphere, alternating with nitrogen gas flushing. After evaporation and dissolution in 0.75 mM HCl, samples were stored at -20°C. AA were derivatised with propyl chloroformate following the EZ:faast kit amino acid analysis procedure (Phenomenex, 2003). Injection volume was 2 μL with a split ratio of 1:25 and the injection temperature of 250°C. Helium at a

constant flow of 1.1 mL/min was used as a carrier gas. The oven temperature was set to increase 30 °C/min from 110 °C to 320 °C. The separation of the sample was done using a using a Zebron TM EZ-AAA Amino Acid GC 10 m x 0.25 mm column (Phenomenex) and the analyses were performed with gas chromatography mass spectrometry (Agilent HP6890 Series GC system Plus; HP 5973 Mass selective detector). The MSD Chemstation E.02.01.117 (Agilent Technologies. Inc., Santa Clara, CA) software was used for the data analysis. Parallel analysis of Bovine Serum Albumin (BSA) at a known concentration was performed to determine AA recovery. Arginine was not measured, and tryptophan was destroyed during acid hydrolysis. Essential amino acids (EAA) data were normalized by the rainbow trout's EAA requirements in order to compare the scenarios.

2.4. Calculations

SRT was calculated as total biomass in the reactor divided by the sum of the daily biomass harvested and the biomass present in the effluent, while Y_{obs} was calculated as in Meerburg et al. (2015). COD mass balance was calculated for each scenario, using the mean daily values obtained during steady state, in order to determine the fate of COD during the process. The mass balance takes in consideration the influent COD, as soluble ($COD_{s,inf}$) and particulate ($COD_{p,inf}$), and the outgoing COD fractions of the particulate biomass harvested ($COD_{p,harv}$), and particulate and soluble effluent COD ($COD_{p,eff}$ and $COD_{s,eff}$). The unaccounted COD, the portion that enters the process and does not leave in the effluent, was assumed to be oxidized to CO_2 through microbial respiration, here represented as $COD_{Oxidized}$. The sum of the outgoing particulate COD, harvested and in the effluent, corresponds to the COD redirected, assuming that all solids after the process are potential MP. COD removal efficiency was calculated as the

difference between the incoming total COD and soluble COD divided by the total COD influent while COD removal rate was calculated using the formula:

$$COD_{removal\ rate} = Flow\ x\ (total\ COD_{influent} - soluble\ COD_{effluent})/(0.83\ x\ Reactor\ volume)$$

The factor 0.83 used in the COD removal rate is added to discount the settling time, withdraw and idle, from the total reaction phase (i.e., contact, stabilization). The final AHB product was considered as the sum of biomass harvested and present in the effluent, expressed as TSS. The daily AHB biomass and protein volumetric productivities were calculated as:

$$AHB\ productivity = Flow\ x\ (TSS_{harvested} + TSS_{effluent})/\ Reactor\ volume$$

$$Protein\ productivity = AHB\ productivity\ x\ AHB\ protein\ content\ \%$$

2.5. Microbial community analysis

One sample per each different scenario was taken and stored at -20°C in order to preserve the genetic material until further processing. Total DNA content extraction was done using the Powerfecal kit (Qiagen, Germany) and following the manufacturers protocol. The DNA extracts were sent to Novogene Biotech Co., Ltd (United Kingdom) for amplicon sequencing analysis. Amplification of the V3-V4 hypervariable region of the 16S rRNA was performed using the primers forward 341f (CCTAYGGGRBGCASCAG) and reverse 806r (GGACTACNNGGGTATCTAAT) and carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The amplicon sequencing libraries were pooled and sequenced in an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed, and the amplicon sequence variants (ASVs) were obtained using DADA2 (Callahan et al., 2016) and ASVs were classified using the EzTaxon database (Chun et al., 2007). Downstream microbial community analysis was performed using

249 tidyamplicons (https://github.com/SWittouck/tidyamplicons) in the R (v.4.0.2) for
250 windows (R Core Team, 2020). Alpha and beta diversity were calculated using Inverse
251 Simpson and Bray-Curtis dissimilarity indices respectively. Principal Coordinate
252 Analysis (PCoA) was performed to visualize the dissimilarities between microbial
253 communities in the tested scenarios. Bray-Curtis distance between samples followed by
254 hierarchical average linkage clustering of samples was done. Then, Permutational
255 Multivariate Analysis of Variance (PERMANOVA), with number of permutations set to
256 999, was made to identify significant effect of the independent variables on the
257 microbial structure. The top 11 most common ASV, at phylum and genus level, were
258 plotted in a stacked bar graph. The raw data generated from 16S rRNA gene
259 sequencing is available at the European nucleotide archive (https://www.ebi.ac.uk/) via
260 the accession number: PRJEB55306.

261 **2.6. Statistical analyses**

262 Normality of data residuals was tested using the Shapiro–Wilk test and homogeneity of
263 variances using Levene's test. In case the null hypothesis of normality was accepted,
264 multiple comparisons were performed using analysis of variance (ANOVA) test and
265 post-hoc pairwise comparison Student's *t*-test. In case of unequal variances, Welch's
266 correction for ANOVA was applied followed by a Games-Howell pairwise comparison.
267 If normality was rejected, the non-parametric Kruskal–Wallis rank sum test and a post-
268 hoc pairwise Wilcoxon Rank Sum test was performed. Pairwise comparisons were just
269 used in case the null hypothesis of equal means was rejected and *p*-values were
270 corrected using the Bonferroni method. Significant differences were considered for *p*-
271 values below 0.05. Multiple linear regression (MLR) was done using a stepwise
272 backwards modelling strategy starting with the full model containing all three

independent variables (substrate, reactor and SRT), all pairwise interactions and the triple interaction term. The main dependent variables of interest were included in the model. This model was simplified by removing the least significant term, based upon the *p*-value, starting with the highest order terms. Cut-off for significance was 0.01 to account for multiple hypothesis testing and to reduce the chance of type I error (see Supplementary Material). All statistical tests were performed with the software R (v.4.0.2) for Windows (R Core Team, 2020).

3. Results and discussion

3.1. COD redirection and observed yield

The process applied needs to work concomitantly as a AHB production system and wastewater treatment. Redirecting COD from the liquid stream to the MP final product is the preferred COD removal pathway, thus minimizing COD oxidation to CO_2 is desired. The remaining suspended and soluble COD in the treated effluent should be reduced if possible as they represent biomass and substrate losses respectively. To reveal the COD fate for each scenario, a COD mass balance was calculated using the mean daily values during steady state conditions (Fig.1) and values are presented as a percentage of the total COD influent.

Type of reactor had a more evident impact on $COD_{p,harv}$ and COD redirection (the sum of $COD_{p,harv}$ and $COD_{p,eff}$) than type of substrate and SRT (Fig.1). MLR showed that the type of reactor alone ($p = 1.08E-12$) and the interaction of medium with SRT ($p = 8.64E-4$) had a significant effect in the COD redirection. HiCAS' values for redirection ranged between 46-72% while HiCS' remained between 40-47%, with overall means of 60% and 45% respectively. The highest COD redirection was obtained in HiCAS reactor treating raw substrate under SRT of 0.50 d (72%) and 1.00 d (70%). These COD

297 redirection values are comparable to the results in other HRAS studies on domestic
298 wastewater (Jimenez et al., 2015; Meerburg et al., 2015; Rahman et al., 2016), equaling
299 the highest redirection value (72%) obtained by Rahman et al. (2019). Per SRT, the
300 ranges for COD redirection means for 0.25 d, 0.50 d and 1.00 d were respectively 40-
301 59%, 47-72%, 44-70%. Within the same type of substrate and reactor, processes at SRT
302 0.50 d had always higher COD redirection than SRT 1.00 d while both had superior
303 values than SRT 0.25d. The same trend was found for COD_{harv}. Thus, in this study,
304 among the SRTs tested, scenarios using SRT 0.50d can be considered the best for COD
305 redirection and harvesting. The highlight was the HiCAS reactor at raw substrate and
306 SRT 0.50 d that reached 71% of COD_{harv} with an extremely low COD particulate
307 fraction ending in the effluent (1%). Considering that 10% of the total influent COD is
308 particulate, and that 90% of the CODp comes from the yeast added to the media, it is
309 possible that this product would be completely or partially hydrolysed, or end up in the
310 final AHB product. Assuming that all CODp would form part of the final biomass, this
311 would represent 12-26% of the total COD harvested at maximum. As both ingredients
312 are feed grade, the potential presence of these compounds in the final product is not a
313 problem, in fact, it might contribute to its quality as spent yeast is typically used as feed
314 for animals. Understanding the impact of yeast on the final MP product is out of the
315 scope in this research, thus, further investigation would be necessary.
316 Regarding the particulate COD in the effluent, most of the reactors obtained below 7%
317 with an average value of 5%. The only exception to this range was found in the
318 condition raw, HiCAS, SRT 1.00 d, where the mean particulate COD losses was 17%.
319 Thus, in almost all the tested cases, most of the redirected COD is harvested indicating a
320 satisfactory bioflocculation process favouring settling. Interestingly, good settleability is

often a problem in HRAS systems treating low-strength wastewater (Rahman et al., 2016) where CODp losses to the effluent can go up to 54% (Meerburg et al., 2015). Nevertheless, care should be taken when comparing these results as several factor can affect the bioflocculation, and therefore settleability, such as the type of reactor operation (i.e. SBR, plug-flow, HiCS, HiCAS), specific-loading rate, shear force, etc. (Meerburg et al., 2015; Rahman et al., 2019). MLR pointed to a triple interaction between substrate, reactor and SRT causing a significant impact on $COD_{p,eff}$ ($p = 7.2E-7$, $R^2 = 62\%$) showing the complexity of this process. The most noticeable differences between the literature and this study, are the strength and composition of the substrate, and the substrate fill time. It is known that the influent composition affect microbial growth (Fatemeh et al., 2019) thus it can have an impact on the microbial community, floc structure and settleability. Hence it can be expected that studies using different substrates could lead to a distinct biomass settling behavior. Moreover, the quick and intermittent feeding regime in the SBR, lasting only 5 min per cycle, favours the formation of a large COD gradient between the beginning (high COD level) and end of the cycle (low COD level). The presence of a pronounced substrate gradient, due to the short fill time, can have a strong positive effect on biomass settleability (Martins et al., 2003). Although these two factors may have contributed to the low biomass presence in the effluent in this study, the precise mechanisms are unknown.

Lower SRT typically results in a higher soluble COD fraction in the effluent and lower fraction of COD oxidized (Meerburg et al., 2015). HiCAS clearly obtained lower CODs results, between 2-41% than HiCS, 24-44%. Both trends can be seen in most of the cases (Fig.1) within the same reactor and substrate. MLR confirms that CODs results were significantly affected by the interaction between reactor and SRT ($p = 4.3E-5$, $R^2 =$

345 74%) while $COD_{Oxidized}$ was influenced by SRT (p = 2.6E-6) and by the interaction
346 between substrate and reactor (p = 7.2E-3). When comparing the $COD_{Oxidized}$ at the
347 same reactor and SRT, raw wastewater had always lower values than fermented. This
348 indicates that different types of substrates could lead to different COD oxidation levels
349 and recovery. HiCS had always higher CODs and lower $COD_{Oxidized}$ meaning that an
350 important fraction of the substrate was not oxidized to CO_2 due to the absence of oxygen
351 in the contact phase which prevented the growth of the aerobic bacteria. Keeping the
352 contact phase unaerated was a strategy selected to promote the biosorption (Meerburg et
353 al., 2015), which is an important route for organic matter capture and to increase the
354 observed yield of the biomass. Rahman et al. (2019) managed to obtain microbial
355 growth and biosorption by maintaining a low concentration of oxygen (around 0.5 mg
356 O_2/L) in the contact phase and this approach is suggested for future investigations.
357 Statistical analysis did not find any difference between the Y_{obs}, probably due to the
358 high variation of the data. The values remained between 0.56-0.81 g $COD_{biomass}$/g
359 $COD_{removed}$ for HiCAS and 0.64-0.78 g $COD_{biomass}$/g $COD_{removed}$ for HiCS. The obtained
360 results indicate that a timid biosorption may have occurred in the scenarios where Y_{obs}
361 were above the maximum theoretical biological yield of 0.4-0.7 g COD/g $COD_{removed}$ for
362 heterotrophic growth (Metcalf & Eddy, 1991), knowing that HRAS systems can reach
363 up to 1 g COD/g $COD_{removed}$ (Meerburg et al., 2016). Still, the results are considered
364 satisfactory for carbon recovery, specially under raw substrate processed by HiCAS in
365 SRT between 0.5-1.0 d, where high yields (0.76-0.77 g COD/g $COD_{removed}$) and COD
366 redirection (70-72 %) were obtained. Therefore, based on all the results above, it is
367 possible to affirm that HiCAS had a better performance than HiCS, due to its higher
368 COD redirection and harvesting and lower CODs in the effluent.

3.2. COD removal rate and efficiency

The COD removal rate and efficiency determines the performance of the tested conditions in the wastewater treatment perspective (Fig. 2). The reactors at SRT of 0.50 d had the highest COD removal rate, up to 16 g COD/$L_{reactor}$/d in HiCAS and HiCS reactors at raw substrate. COD removal rates were always lower at SRT 0.25 d. At such a low SRT, high specific loading rate are applied causing deterioration of the COD removal but typically can bring the advantage of obtaining higher observed yield (Vriens et al., 1989), thus increasing the efficiency of carbon capture. As mentioned above, Y_{obs} did not differ significantly between SRTs, thus the 0.25 d SRT did not pose an advantage to the process. Next, at the same substrate and reactor type, the mean COD removal rate at SRT 0.50 was 13-19% higher than at SRT 1.00. This difference was not statistically significant in almost all the cases, except at raw and HiCAS, but this was caused by the lower COD loading rate applied in these reactors in order to keep the biomass in levels in which the sludge blanket could settle and compress thus avoiding significant losses as CODp in the effluent.

HiCAS reactors obtained high removal efficiencies, both with raw substrate (98% for HiCAS SRT 0.50 d and 94% for HiCAS SRT 1.00 d), as well as fermented (97% for HiCAS SRT 0.5 and 96% for HiCAS SRT 1.00 d). In fact, it is known that low SRT (below 2 d) is sufficient to allow a substantial removal of soluble COD (Grady Jr. et al., 2011). Brewery wastewater typically contains high fractions of readily degradable organics, thus, high removal efficiencies can be expected even at a low SRT. The lower COD removal efficiency in HiCS reactors, between 56-75%, and the reasons were discussed in the previous section. Thus, results show that HiCAS reactors operating at 0.50 d was the optimal strategy to reach high COD removal rates and efficiencies.

3.3. AHB protein content and productivities

From the MP production perspective, reaching high protein content is a desirable feature. In Fig.3A, it is possible to verify that AHB's mean protein content among all the tested scenarios remained between 37.0-47.5% on dry weight, which is within the interval found in literature. Vriens et al. (1989) found protein levels ranging between 30-50% (dry weight basis). Muys et al. (2020) sampled AHB biomass from 25 companies from the food and beverage industry obtaining a protein levels between 21-49 % on dry weight. Protein levels within the same type of substrate were statistically similar, independent of the type of reactor and SRT. MLR analysis showed that only the substrate had a significant effect on protein levels ($p = 5.1E-5$). The mean protein content obtained in the reactors fed with raw and fermented wastewater were 39% and 45% on dry weight, respectively. Thus, there is an absolute mean difference of 6% that is incremented to the protein level of AHB biomass produced on fermented wastewater. In fact, Dohanyos et al. (1978) showed that the type of influent composition had an impact on AHB's protein level but there is still a lack of information about the effect of complex substrate matrix to the MP biomass quality and it poses an opportunity to increase protein productivity requiring further investigation. Other factors were suggested to influence AHB protein levels such as SRT, COD/N ratio, COD/P ratio, N-specific loading rate and pH (Muys et al., 2020; Vriens et al., 1989). From these, only SRT could had caused an effect in protein levels as pH was kept stable at 7.00 using a controller, nutrients (N and P) were dosed in excess and COD:N:P ratios were the same in both substrates. COD:N:P was kept the same in both substrates to avoid confounding factors that could hamper the results. Thus if substrate would cause a significant effect to the dependent variables (i.e., biomass productivity, protein level), this can be only

417 explained by the difference in COD level and fractionation (i.e. presence of VFA). The
418 effect of SRT on the protein level was not detected likely due to low and narrow range
419 (0.25-1.00 d) applied compared with other studies, 2-66 d (Muys et al., 2020; Tucek et
420 al., 1977), where a negative relation between SRT and protein content was found.
421 MLR showed that AHB and protein productivity were affected significantly by type of
422 substrate in combination with SRT levels. Biomass productivity (Fig.3B) was higher in
423 reactors operated under raw wastewater (6.14-7.97 g TSS/L/d) compared with the ones
424 under fermented substrate (4.37-6.92 g TSS/L/d). When comparing the same type of
425 reactor and SRT between raw and fermented scenarios, the mean biomass productivity
426 was higher in reactors processing raw wastewater in almost all the cases, except for
427 HiCAS at SRT 0.25 d. However, the higher protein level for scenarios using fermented
428 substrate raised protein productivities to levels equivalent to those obtained under raw
429 wastewater. The maximum biomass and protein productivity was attained in HiCAS
430 reactor fed with raw wastewater at SRT of 0.50 d, respectively 7.97 g TSS/L/d and 2.95
431 g protein/L/d, followed by HiCAS under fermented wastewater at SRT of 0.25 d
432 reaching biomass and protein production of 6.92 g TSS/L/d and 3.21 g protein/L/d,
433 respectively. Results show that protein productivities were typically lower at SRT 1.00
434 d, compared to 0.25 and 0.50 d. The best productivity was achieved at SRT 0.50 d,
435 making this the preferred operational setpoint.
436 Fermented substrate had a positive impact on protein levels and productivity.
437 Nevertheless, the inclusion of an anaerobic fermentation step would increase the
438 complexity of the production process as this extra biological step would require
439 additional operational care to guarantee stable fermentation conditions and substrate
440 quality for the aerobic process. This would, consequently, reduce variabilities to the

AHB biomass production. Although it would result in an additional operational burden, similar protein productivities can be achieved with 13% less biomass, difference found when comparing the highest AHB productivity using raw substrate versus the highest productivity using fermented (Fig. 3B). This means a lower demand for the downstream processing which represents by far the highest operation costs in MP production (Spiller et al., 2020) thus the economics remain to be evaluated. Compared to other MP, AHB outperformed PNSB and MaB. Alloul et al. (2019) obtained a PNSB productivity of 1.7 g DW/L/d (46% protein content), the maximum reported for this bacteria. The maximum MaB productivity reached by Van Den Hende (2019) was 0.26 g DW/L/d (28% protein level).

In this study, the best overall scenario was operated with raw brewery wastewater, HiCAS reactor and SRT 0.50 d. This scenario obtained the highest mean COD redirection and harvesting with the lowest effluent soluble and particulate COD, while the protein levels and observed yield did not differ substantially among the 12 tested treatments. Raw substrate has higher potential based on its COD redirection with lower COD oxidation, however, it did lead to poorer soluble and particulate COD effluent. Further research into solids separation is therefore needed. Fermented effluent had a statistically higher protein level content, thus a trade-off between redirection and specific protein production is apparent. Further investigation is consequently necessary.

3.4. AHB essential amino acids profile

The EAAs cannot be synthetized by the target organism and therefore need to be obtained through the diet. Here, rainbow trout was chosen as the target organism as salmonids are the largest consumers of FM , 15% of the total FM used in aquaculture in 2019 (EUMOFA, 2021), and have the biggest share in value of the global fish market

since 2013 (FAO, 2020). For salmonids there are ten EAA and eight of them were measured: histidine, isoleucine, leucine, lysine, methionine + cysteine (met+cys), phenylalanine + tyrosine, threonine, and valine. In Fig.4, the EAA composition of the AHB, from the 12 scenarios tested, and FM (Heuzé et al., 2015) were normalized to the EAA requirements of rainbow trout (Ogino, 1980). All scenarios yielded an AHB biomass with an outstanding EAA profile, comparable to FM, and matching rainbow trout requirements. Only the levels of the sulphur-containing EAA (met+cys) were deficient in all scenarios reaching a mean value of 25±22% of rainbow trout's requirement although a broad range was found, from 1.7 to 22.5 mg (met+cys)/g of protein. The coefficient of variance of the others EAA amongst all the scenarios remained between 12-26%, way lower compared to the sulphur-containing EAA (86%). Muys et al. (2020) investigated the AHB biomass quality of conventional activated sludge from 25 different food industries and also found that methionine + cysteine were the EAA with highest variability with values ranging between 0.3-28 mg (met+cys)/g of protein which are quite similar to the ones obtained in this study. These EAAs are known to be limiting in AHB biomass and other microorganisms (Vriens et al., 1989). Currently, one way of dealing with this problem is to blend AHB biomass to methionine-rich products or to add synthetic methionine. In both cases, economic and sustainable issues need to be taken in consideration. Overall, reactors treating raw wastewater had a total EAA value between 361-534 mg EAA/g of protein while fermented had 300-434 mg EAA/g of protein, thus raw reactors were on average 18% higher than fermented. This fact, together with the high variability of individual EAA, indicate a potential to steer amino acid profile by changing operational conditions and should be further investigated.

3.5. Microbial community

Revealing the predominant composition of the microbial community and understanding how the different operational conditions affect it is essential for the process development. As most of the operational parameters were controlled just substrate, type of HRAS and SRT could influence the AHB community. PCoA analysis on the Bray-Curtis distance matrix (Fig. 5) showed the similarities between the bacterial community composition at genus level for each of the tested scenarios. Samples ordinated closer to one another are more similar regarding their bacterial community structure than those that are more distant. Three clusters could be observed: i) cluster 1 was formed by all the four scenarios testing SRT 0.25 d plus the fermented HiCS 1.00 d sample; ii) cluster 2 was formed by all the four samples operated at SRT 0.50 d plus the raw HiCS 1.00 d sample; iii) cluster 3 was represented by the two HiCAS reactor at SRT 1.00 d samples, under raw and fermented substrate. PERMANOVA analysis confirmed that only SRT had a significant influence ($p = 2.2E-2$) over the AHB bacterial community in our set-up. Strong correlations between SRT and microbial community changes were previously reported in both high and low-rate AS (Gonzalez-Martinez et al., 2016; Valentín-Vargas et al., 2012). HiCS at SRT 1.00 d under fermented (cluster 1) and raw substrate (cluster 2) did not group with their correspondent SRT (cluster 3) as it would be expected. These two strayed scenarios were operated in the same period, in parallel, and had the same inoculum. Nevertheless, laboratory-scale reactors operated under similar conditions had no reproducibility in the microbial community and shifts occurred over time in Boon et al. (2000). Chance-driven processes could force the bacterial community to evolve differently to similar perturbations (Kaewpipat and

512 Grady, 2002). This effect is to be more pronounced in small-scale reactors (Curtis et al.,
513 2003), and therefore could had happened in this study.

514 Alpha-diversity and relative abundances were also obtained (see Supplementary
515 material). Studies pointed that lower SRT leads to lower diversity (Gonzalez-Martinez
516 et al., 2016; Meerburg et al., 2016). Here, alpha-diversity did not follow a clear trend
517 based on any parameter although scenarios using raw substrate had typically a higher
518 Inverse Simpson index. The Inverse Simpson (richness and evenness) and observed
519 species (richness) for all the scenarios remained between 3-33 (mean = 11) and 404-957
520 (mean = 571) respectively. The limited number of samples and the short range of SRT
521 (0.25-1.0 d) are a potential bias to these results. Taxonomic assignment of ASV
522 identified 47 phyla among all the scenarios while just 10 were present in all the
523 samples. The relative abundance of the top three dominant phyla were varying between
524 20-81% for Proteobacteria (mean = 60%), 5-73% for Bacteroidetes (mean = 25%) and
525 1-44% for Firmicutes (mean = 8%). Together these phyla represented between 78-98%
526 (mean = 93%) of the total reads. Proteobacteria and Bacteroidetes are by far the most
527 common phylum in high and low rate AS (Gonzalez-Martinez et al., 2016; Meerburg et
528 al., 2016). In total, 923 genera were found only 23 were present in all the tested
529 scenarios. The relative abundance of the top 11 genera remained between 48 and 82%
530 (mean = 66%) of the total counts comprising strictly and facultative aerobic organisms.
531 Some genera such as *Acinetobacter*, *Arcobacter* and *Leadbetterela*, obtained a relative
532 abundance close or higher than 50% in at least one scenario.

533 ## 4. Conclusions

534 • HiCAS and the novel HiCS can achieve high AHB and protein productivities, along
535 with great EAA profiles.

- High observed yields and outstanding COD removal were obtained in HiCAS.
- The best performance was obtained using raw substrate, HiCAS and SRT 0.50 d.
- More studies in AHB community and biomass quality steerability are essential.
- Necessity to evaluate process techno-economic feasibility.
- HRAS can be the key process to turn AHB MP product into reality.

Declaration of competing interest

None.

Acknowledgements

The authors kindly acknowledge the Research Foundation Flanders (FWO-Vlaanderen) for supporting G.P. with a doctoral fellowship (strategic basic research: 1S38917N). Special thanks to AB InBEV NV, Duvel Moortgat NV and Palm Breweries NV for providing samples to this work. Graphical abstract used images from Freepik.com.

References

1. Ahn, Y.H., Min, K.S., Speece, R.E., 2001. Pre-acidification in anaerobic sludge bed process treating brewery wastewater. Water Res. 35, 4267–4276.
2. Alexiou, I.E., 1998. A study of Pre-Acidification Reactor Design for Anaerobic Treatment of High Strength Industrial Wastewater. Newcastle University.
3. Alloul, A., Wuyts, S., Lebeer, S., Vlaeminck, S.E., 2019. Volatile fatty acids impacting phototrophic growth kinetics of purple bacteria: Paving the way for protein production on fermented wastewater. Water Res. 152, 138–147.
4. Boon, N., Goris, J., De Vos, P., Verstraete, W., Top, E.M., 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline- degrading Comamonas testosteroni strain, I2gfp. Appl. Environ. Microbiol. 66, 2906–2913.

559 5. Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes,
560 S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data.
561 Nat. Methods 2016 137 13, 581–583.
562 6. Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K., Lim, Y.W., 2007.
563 EzTaxon: a web-based tool for the identification of prokaryotes based on 16S
564 ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57, 2259–2261.
565 7. Curtis, T.P., Head, I.M., Graham, D.W., 2003. Theoretical ecology for engineering
566 biology. Environ. Sci. Technol. 37, 64A-70A.
567 8. Dohanyos, M., Chudoba, J., Tucek, F., Grau, P., 1978. The influence of substrate and
568 micronutrients on formation of protein in activated sludge. Tech. Res. Rep.
569 9. Driessen, W., Vereijken, T., 2003. Recent developments in biological treatment of
570 brewery effluent. Inst. Guild Brew. Conv. Livingstone, Zambia, March 2-7 10.
571 10. Eddy, M.&, 1991. Wastewater engineering: treatment, disposal and reuse, 3rd ed.
572 McGraw-Hill, New York.
573 11. EUMOFA, 2021. Fishmeal and fish oil: production and trade flows in the EU.
574 Publications Office of the European Union, Luxembourg.
575 12. FAO, 2020. The State of World Fisheries and Aquaculture - Sustainability in Action.
576 Food and Agriculture Organization of the United Nations.
577 13. Fatemeh, S., Reihani, S., Khosravi-Darani, K., 2019. Influencing factors on single-cell
578 protein production by submerged fermentation: A review. Electron. J. Biotechnol. 37,
579 34–40.
580 14. Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F.,
581 Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C., 2010. Food security: The
582 challenge of feeding 9 billion people. Science (80-.). 327, 812–818.

583 15. Gonzalez-Martinez, A., Rodriguez-Sanchez, A., Lotti, T., Garcia-Ruiz, M.J., Osorio,
584 F., Gonzalez-Lopez, J., Van Loosdrecht, M.C.M., 2016. Comparison of bacterial
585 communities of conventional and A-stage activated sludge systems. Sci. Reports 2016
586 61 6, 1–11.
587 16. Grady Jr., C.P.L., Daigger, G.T., Love, N.G., Filipe, C.D.M., 2011. Biological
588 Wastewater Treatment, 3rd ed. CRC Press; IWA Publishing.
589 17. Heuzé, V., Tran, G., Kaushik, S., 2015. Fish meal. Feedipedia, a programme by INRA,
590 CIRAD, AFZ and FAO [WWW Document].
591 18. Kaewpipat, K., Grady, C.P.L., 2002. Microbial population dynamics in laboratory-
592 scale activated sludge reactors. Water Sci. Technol. 46, 19–27.
593 19. Markwell, M.A.K., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of
594 the Lowry procedure to simplify protein determination in membrane and lipoprotein
595 samples. Anal. Biochem. 87, 206–210.
596 20. Martins, A.M.P., Heijnen, J.J., Van Loosdrecht, M.C.M., 2003. Effect of feeding
597 pattern and storage on the sludge settleability under aerobic conditions. Water Res. 37,
598 2555–2570.
599 21. Meerburg, F., Boon, N., Van Winckel, T., Vercamer, J., Nopens, I., Vlaeminck, S.,
600 2015. Toward energy-neutral wastewater treatment: A high-rate contact stabilization
601 process to maximally recover sewage organics. Bioresour. Technol. 179, 373–381.
602 22. Meerburg, F., Vlaeminck, S.E., Roume, H., Seuntjens, D., Pieper, D.H., Jauregui, R.,
603 Vilchez-Vargas, R., Boon, N., 2016. High-rate activated sludge communities have a
604 distinctly different structure compared to low-rate sludge communities, and are less
605 sensitive towards environmental and operational variables. Water Res. 100, 137–145.
606 23. Meerburg, F.A., Boon, N., Van Winckel, T., Pauwels, K.T.G., Vlaeminck, S.E., 2016.

607 Live fast, die young: Optimizing retention times in high-rate contact stabilization for
608 maximal recovery of organics from wastewater. Environ. Sci. Technol. 50, 9781–
609 9790.
610 24. Muys, M., Papini, G., Spiller, M., Sakarika, M., Schwaiger, B., Lesueur, C., Vermeir,
611 P., Vlaeminck, S.E., 2020. Dried aerobic heterotrophic bacteria from treatment of food
612 and beverage effluents: Screening of correlations between operation parameters and
613 microbial protein quality. Bioresour. Technol. 307, 123242.
614 25. Ogino, C., 1980. Requirements of Carp and Rainbow Trout for Essential Amino
615 Acids. Nippon SUISAN GAKKAISHI 46, 171–174.
616 26. Okoli, C.S., Okonkwo, P.C., 2016. Fluidized- Fluidized - Bed Reactor for Treatment
617 of Brewery Wastewater 35, 91–96.
618 27. R Core Team, 2020. A language and environment for statistical computing.
619 28. Rahman, A., De Clippeleir, H., Thomas, W., Jimenez, J.A., Wett, B., Al-Omari, A.,
620 Murthy, S., Riffat, R., Bott, C., 2019. A-stage and high-rate contact-stabilization
621 performance comparison for carbon and nutrient redirection from high-strength
622 municipal wastewater. Chem. Eng. J. 357, 737–749.
623 29. Rahman, A., Meerburg, F.A., Ravadagundhi, S., Wett, B., Jimenez, J., Bott, C., Al-
624 Omari, A., Riffat, R., Murthy, S., De Clippeleir, H., 2016. Bioflocculation
625 management through high-rate contact-stabilization: A promising technology to
626 recover organic carbon from low-strength wastewater. Water Res. 104, 485–496.
627 30. Rana, K.J., Siriwardena, S., Hasan, M.R., 2009. Impact of rising feed ingredient prices
628 on aquafeeds and aquaculture production, FAO Fisheries and Aquaculture Technical
629 Paper.
630 31. Ritala, A., Häkkinen, S.T., Toivari, M., Wiebe, M.G., 2017. Single cell protein-state-

631 of-the-art, industrial landscape and patents 2001-2016. Front. Microbiol. 8, 2009.
632 32. Scampini, A.C., 2010. Upflow anaerobic sludge blanket reactors for treatment of
633 wastewater from the brewery industry. Massachussetts Institute of Technology.
634 33. Spiller, M., Muys, M., Papini, G., Sakarika, M., Buyle, M., Vlaeminck, S.E., 2020.
635 Environmental impact of microbial protein from potato wastewater as feed ingredient:
636 Comparative consequential life cycle assessment of three production systems and
637 soybean meal. Water Res. 171, 115406.
638 34. Tucek, F., Chuboda, J., Dohanyos, M., Grau, P., 1977. The influence of sludge age on
639 formation of protein in activated sludge. Prague Inst. Chem. Tech. Res. Rep.
640 35. United Nations, Department of Economic and Social Affairs, P.D., 2019. World
641 Population Prospects 2019: Press Release.
642 36. Valentín-Vargas, A., Toro-Labrador, G., Massol-Deyá, A.A., 2012. Bacterial
643 community dynamics in full-scale activated sludge bioreactors: Operational and
644 ecological factors driving community assembly and performance. PLoS One 7.
645 37. Van Den Hende, S., 2014. Microalgal bacterial flocs for wastewater treatment: from
646 concept to pilot scale.
647 38. Van Winckel, T., Liu, X., Vlaeminck, S.E., Takács, I., Al-Omari, A., Sturm, B.,
648 Kjellerup, B. V., Murthy, S.N., De Clippeleir, H., 2019. Overcoming floc formation
649 limitations in high-rate activated sludge systems. Chemosphere 215, 342–352.
650 39. Verstraete, W., Clauwaert, P., Vlaeminck, S.E., 2016. Used water and nutrients:
651 Recovery perspectives in a “panta rhei” context. Bioresour. Technol. 215, 199–208.
652 40. Vriens, L., Nihoul, R., Verachtert, H., 1989. Activated Sludges as Animal Feed : A
653 Review 27, 161–207.
654

655 Table 1. Characteristics of the synthetic raw and fermented brewery wastewater

Parameter	Raw (g/L)	Fermented (g/L)
CODtotal	3.700	3.145
CODparticulate	0.369	0.350
CODsoluble	3.331	2.795
CODsoluble as VFA	0.473	1.554
CODsoluble as non-VFA	2.858	1.241
BOD_5	2.442	2.076
Total nitrogen, as N	0.478	0.406
Total phosphorus, as P	0.088	0.075

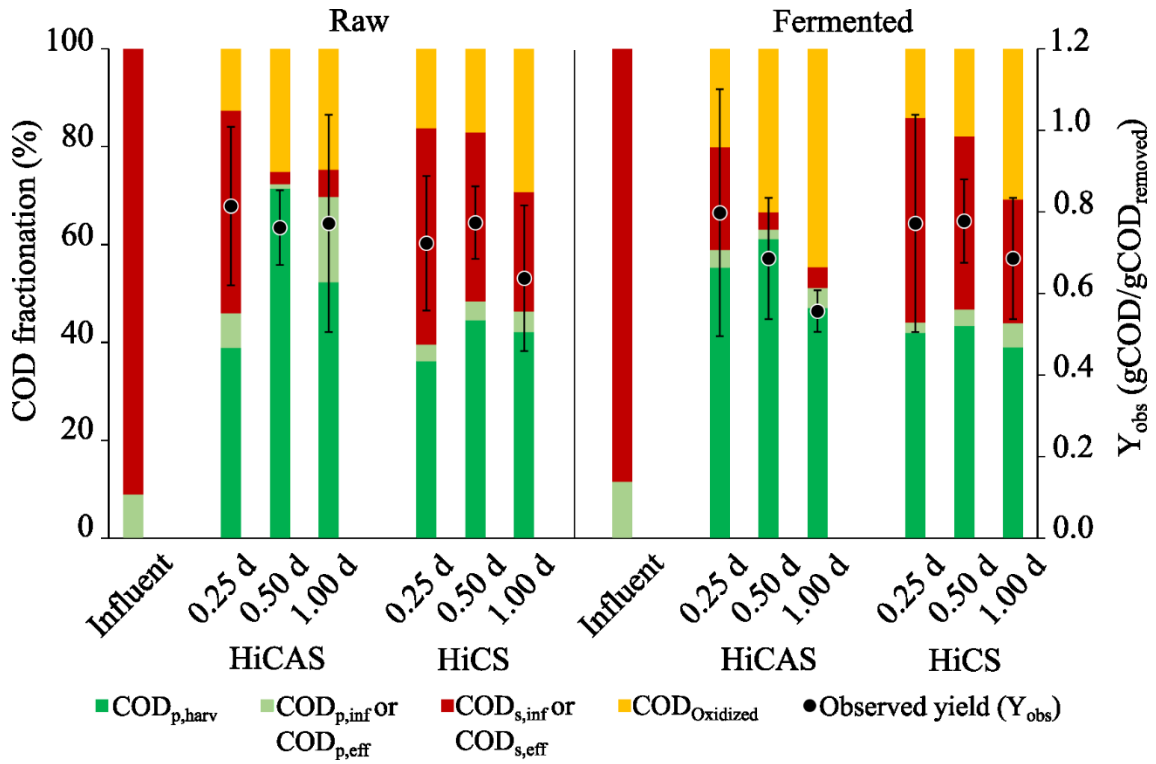
656

657 Table 2. Overview of reactor operation characteristics, biomass concentration and COD

658 influent. SLR: specific loading rate; VER: volume exchange ratio

Substrate	Reactor	SRT d	SLR g COD_T/g VSS/d	$COD_{influent}$ g COD_T/L	VER %	VSS g/L	VSS/TSS %
Raw	HiCAS	0.22 ± 0.01	13.1 ± 1.2	2.76 ± 0.16	44 ± 0	1.39 ± 0.18	87 ± 3
		0.50 ± 0.02	4.5 ± 0.4	3.67 ± 0.38	30 ± 1	3.48 ± 0.35	90 ± 2
		0.87 ± 0.12	2.7 ± 0.4	3.52 ± 0.24	27 ± 1	5.30 ± 0.60	86 ± 3
	HiCS	0.23 ± 0.01	14.4 ± 1.8	3.13 ± 0.36	46 ± 2	1.54 ± 0.36	91 ± 3
		0.49 ± 0.03	7.4 ± 1.1	3.55 ± 0.28	48 ± 2	3.32 ± 0.44	87 ± 5
		1.12 ± 0.07	2.9 ± 1.0	3.67 ± 0.41	36 ± 6	6.09 ± 0.67	89 ± 3
Fermented	HiCAS	0.24 ± 0.01	11.2 ± 2.3	2.59 ± 0.10	42 ± 0	1.35 ± 0.38	87 ± 5
		0.50 ± 0.02	5.3 ± 0.8	3.16 ± 0.30	31 ± 1	2.67 ± 0.24	88 ± 3
		1.17 ± 0.1	2.7 ± 0.3	3.17 ± 0.13	27 ± 1	4.68 ± 0.33	85 ± 3
	HiCS	0.24 ± 0.01	14.1 ± 3.4	2.67 ± 0.39	44 ± 1	1.33 ± 0.27	87 ± 4
		0.48 ± 0.03	7.5 ± 1.8	3.04 ± 0.33	46 ± 2	2.78 ± 0.61	88 ± 4
		1.09 ± 0.13	3.6 ± 0.9	2.75 ± 0.94	35 ± 5	4.21 ± 0.55	88 ± 5

659



660

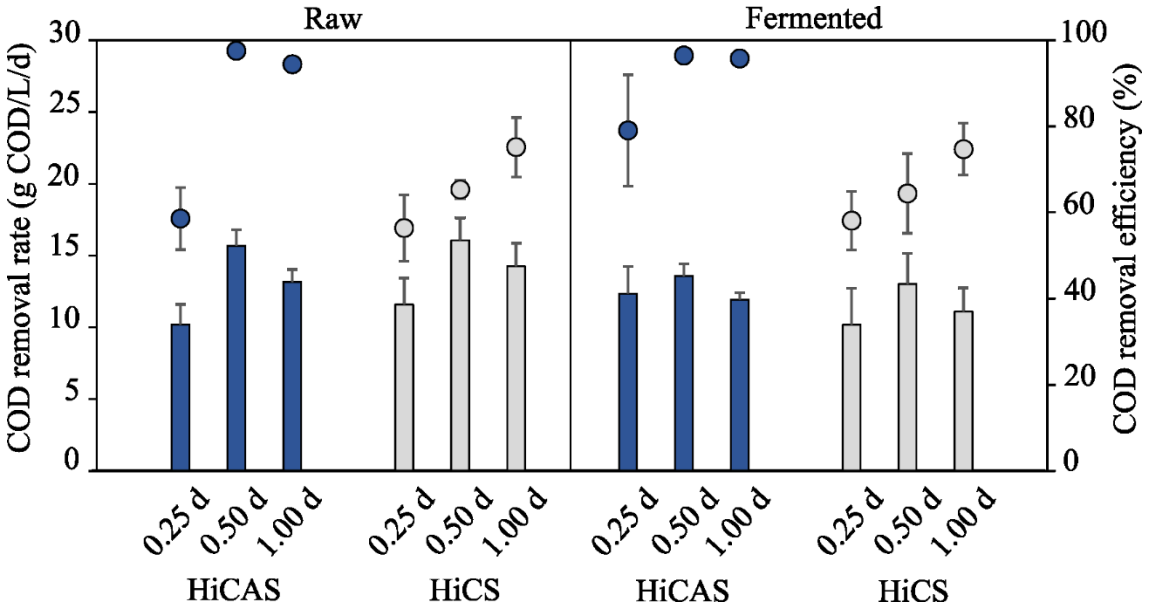
661 Fig.1. Fate of COD and observed yield (Y_{obs}) in the different scenarios based in the

662 mean values obtained in a daily mass balance. Subscript "p" and "s" stands to

663 particulate and soluble respectively, while "harv" represents the harvested fraction of

664 the biomass, "inf" COD influent, and "eff" COD effluent of the process. Error bars

665 correspond to the 95% confidence interval.



666

667 Fig.2. COD removal rate (bars) and efficiency (dots) in the 12 tested scenarios. HiCAS
668 and HiCS are represented in blue and grey color respectively. Error bars correspond to
669 the 95% confidence interval.

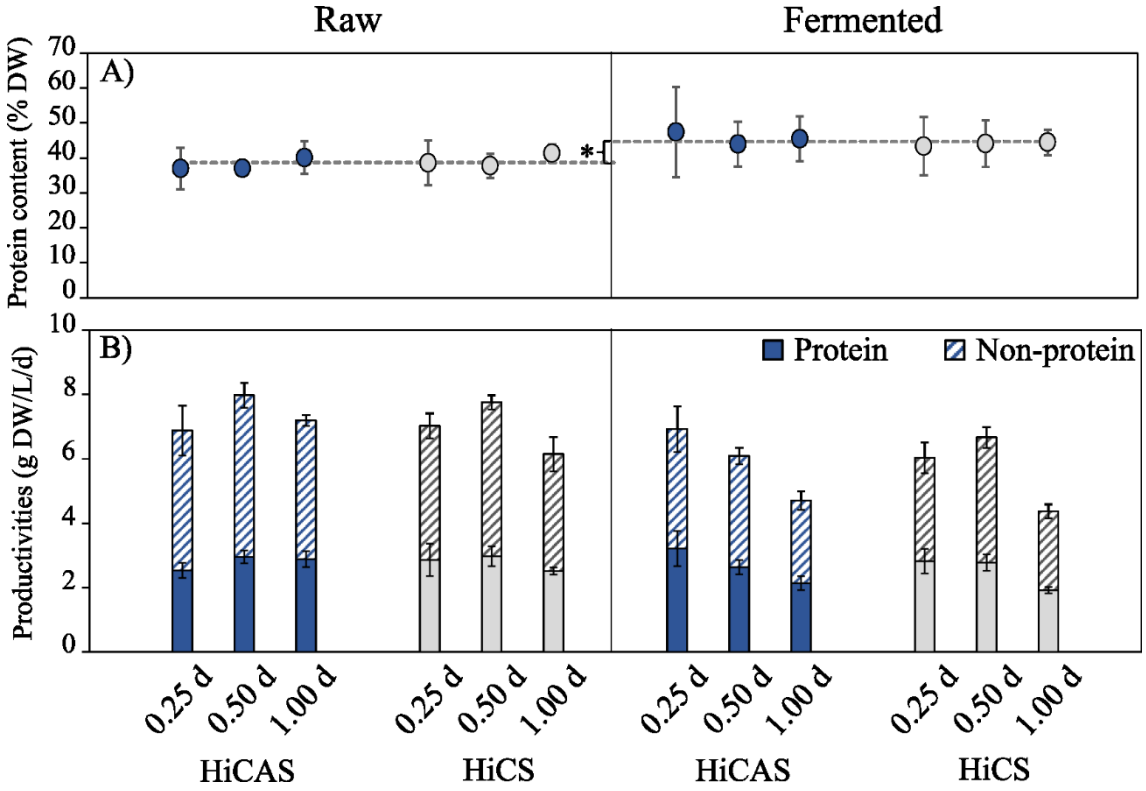
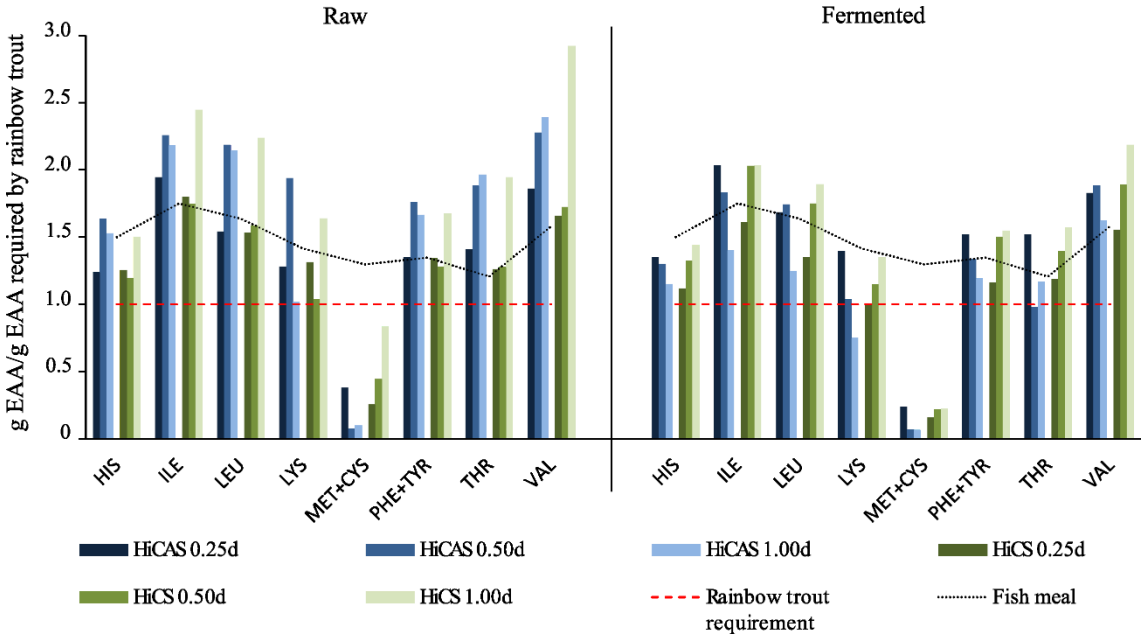
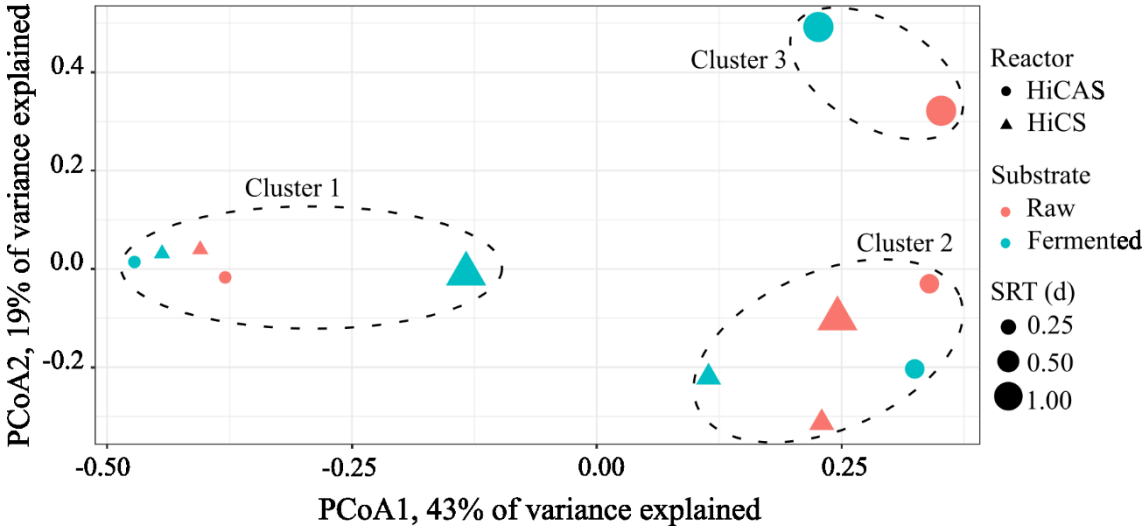


Fig. 3. A) Protein level as dry weight (DW), B) biomass productivities as g DW/L/d, separated into protein and non-protein fraction. Error bars correspond to the 95% confidence interval. Asterisk "*" denotes a significant difference between the means ($p<0.05$).



675

676 Fig. 4. Essential amino acids (EAA) residuals of the AHB biomass in the 12 scenarios
677 and fish meal normalized by the rainbow trout EAA requirement, represented by the red
678 dashed line. The values equal or above 1 would meet or exceed rainbow trout's
679 requirement. EAA abbreviations, HIS: histidine; ILE: isoleucine; LEU: leucine; LYS:
680 lysine; MET+CYS: methionine+cysteine; PHE+TYR: phenylalanine+tyrosine; THR:
681 threonine; VAL: valine.



682

683 Fig. 5. Principal Coordinate Analysis (PCoA) using Bray-Curtis distance for the
684 comparison of the bacterial community similarities between the 12 tested scenarios.
685 Each point represents one sample for a specific scenario. The closer the points are one
686 another, higher the similarities between the microbial communities. The percentage of
687 variation explained by each PCoA is indicated in axis.