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Dried aerobic heterotrophic bacteria from treatment of food and beverage effluents: Screening of correlations between operation parameters and microbial protein quality

Maarten Muys[1], Gustavo Papini[1], Marc Spiller[1], Myrsini Sakarika[1], Barbara Schwaiger[2], Céline Lesueur[2], Pieter Vermeir[3], Siegfried E. Vlaeminck[1,]*

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

[2] Lebensmittel Vertrauen Analysen LVA GmbH, Magdeburggasse 10, 3400 Klosterneuburg 236286 f, HG Wien, Oostenrijk

[3] Laboratory for Chemical Analysis, Department of green chemistry and technology, Ghent University, Valentin Vaerwyckweg 1, 9000 Gent, België

* Corresponding author: Siegfried.Vlaeminck@UAntwerpen.be

Abstract

Consortia of aerobic heterotrophic bacteria (AHB) have potential as sustainable microbial protein (MP) source in animal feed. A systematic screening of the nutritional value and safety of AHB biomass from full-scale activated sludge plants from 25 companies in the food sector was performed. The variable protein content (21-49%) was positively correlated with biomass-specific nitrogen loading rate and negatively with sludge retention time (SRT). Compared to the essential amino acid profile of soybean meal protein, AHB displayed an overall surplus of threonine and valine, and deficits in cysteine, histidine, lysine and phenylalanine. Histidine was positively correlated with $COD_{removed}$:$PO_4^{3-}{}_{influent}$ and valine, isoleucine and threonine with SRT. Most AHB samples were safe apropos heavy metals, polycyclic aromatic hydrocarbons and antibiotics. Some pesticides exceeded regulatory limits, necessitating mitigation. This work highlighted that the food sector can provide high-quality MP, while retrofitting existing activated sludge plants towards high-rate processes can increase AHB quality and productivity.

Keywords

High-rate activated sludge; resource recovery; nutrient recovery; industrial wastewater treatment

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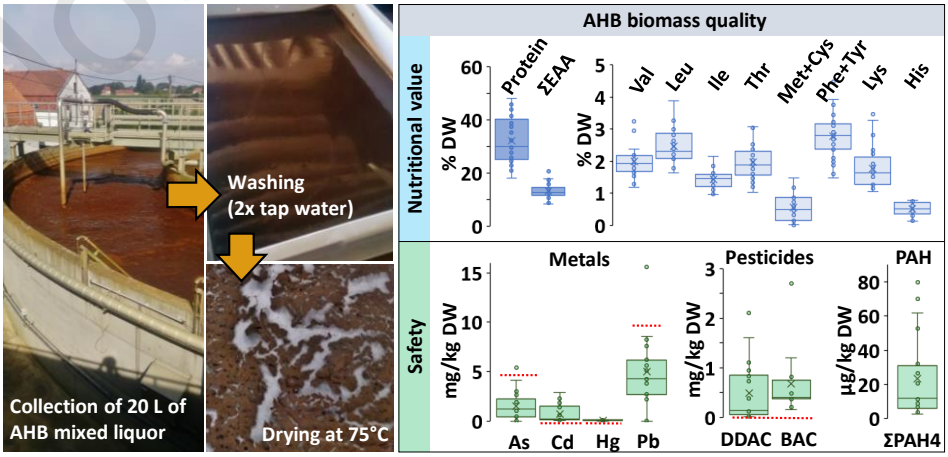

1. Introduction

The production of animal-derived products such as meat, dairy and eggs has a strong environmental impact. Conventional agriculture already consumes 30% of all ice-free land, 70% of all available freshwater and produces up to one-third of global greenhouse gas emissions (Vermeulen et al., 2012). Furthermore, resource depletion and environmental impacts rise rapidly due to population expansion and improving standards of living, which are predicted to result in a 50% higher protein demand by 2050 (FAO, 2018). To satisfy this demand, dairy and meat products are expected to increase by 82 and 102%, respectively (Boland et al., 2013). Besides livestock production, also aquaculture presents an increasing environmental impact. With an average annual growth rate of 8% since 1970 (Merino et al., 2012) and mainly depending on fishmeal as protein source, the pressure of aquaculture on fast declining ocean fisheries is alarming. With regard to Europe, currently 25 million ton soy is required to maintain livestock production of which 70% is imported from non-EU countries (Schreuder & De Visser, 2014). Because of this, soybean price fluctuations have a large impact on the European market, while Europe's environmental footprint increases in many developing countries where large-scale soy production results in deforestation and environmental degradation (Bosch, 2015).

To improve sustainability and guarantee self-sufficiency for protein-rich animal feed ingredients, the Europe stimulates the replacement of imported protein-rich animal feed with alternative local sources (European Parliament, 2018). A possible strategy is the production of "novel" protein sources (Boland et al., 2013) such as microbial protein (MP), synonym to single-cell protein (SCP). This is the biomass of microorganisms, typically dried, presenting a high protein content (40-80%, on dry weight), while

featuring functional components, e.g. vitamins, pigments and anti-oxidants (Anupama & Ravindra, 2000). A near-perfect nutrient conversion efficiency and high volumetric biomass productivity (Verstraete et al., 2016) enable the production of MP with a lower environmental footprint compared to conventional protein crops including soybean meal (Pikaar et al., 2017; Spiller et al., 2020). In addition, MP are capable to use low-value effluents as growth substrate, facilitating the recovery of the nutrients (e.g. N and P) and the carbon contained in it (Matassa et al., 2016).

The heterotrophic metabolism, that is the utilization of organic carbon as a C-source/ electron donor and the used of oxygen as an electron acceptor, is the metabolism with the highest biomass yield within the microbial community making up activated sludge (AS) in biological wastewater treatment plants (WWTP). In Europe, a large part of municipal AS is currently reused as organic fertilizer (37%), however, landfilling (40%) and incineration (12%) are still applied (Fytili & Zabaniotou, 2008; Mateo-Sagasta et al., 2015). In contrast, the end-use of industrial AS is variable and depends on potential contamination. Upcycling of low cost dried aerobic heterotrophic bacteria (AHB) biomass as MP source in animal feed manufacturing could increase resource efficiency, and enable higher value resource recovery and reuse, generating revenue, provided reasonable dewatering and drying costs. In the ‘60s AHB biomass was suggested as a potential protein source in animal feed (Grau, 1980). However, feeding trials with sewage AHB biomass often resulted in reduced weight gain. This appeared to be a consequence of the presence of several contaminants, such as metals, pesticides and pathogens, rather than a function of the deficiencies in protein quality (Clevenger, 1990; Shier & Purwono, 1994; Tacon & Ferns, 1979). Indeed, feeding trials with an inclusion rate of 5 to 60% AHB biomass from brewery effluent treatment on Nile tilapia

(Chuapoehuk et al., 1998) and cattle (Kienholz & Moreng, 1981), from dairy effluent treatment on lamb (Caton et al., 1989) and from cannery effluent treatment on cattle (Esvelt et al., 1976), all showed positive responses. Additionally, AHB biomass from selected companies in the food and beverage sector was observed to contain none or only low amounts of heavy metals and pesticides (Clevenger, 1990). Furthermore, the risk on fecal contamination is low as process effluents are often separated from sewage (Vriens et al., 1989). Additionally, the typically year-round supply of carbon-rich effluents makes continuous MP production on food and beverage industry effluents possible (Huige, 2006). Finally, the analysis of a limited number of AHB biomass samples originating from a limited amount of sectors, suggests that AHB biomass is rich in lysine and methionine, two essential amino acids (EAA) that are present in low quantities in many plant sources (Tacon et al., 2009; Vriens et al., 1989). Consequently, AHB biomass appears to be a promising high-quality protein source. However, comprehensive data on nutritional value and safety of AHB originating from the food and beverage sector is lacking.

Furthermore, there are a number of technological challenges to render dried AHB biomass a desirable MP product. Several contributors to variation exist: (1) the composition of water that serves as a growth substrate dependents on the type of industrial (sub)sector, (2) the composition of the water is function of the specific production processes (and companies) within a subsector, (3) other AHB treatment technologies and operational conditions, (4) temporal changes in water quality and quantity, for instance linked to batch production processes, cleaning cycles, changes in food product type and seasonalities, as well as (5) the dynamics of open microbial communities. These dynamics partly depend on process parameters with for instance

higher stability in high-rate systems (Meerburg et al., 2016), but even under constant process parameters open communities are intrinsically dynamic (Fernandez et al., 1999). To enable tailoring the nutritional value and maintain a consistent high-quality product over time, it is essential to understand the influences of wastewater characteristics and process parameters on the biochemical AHB composition, referring to challenge 2, 3 and 4. The influence of some parameters on AHB protein content has been described at lab-scale (Vriens et al., 1989). However, research on full-scale installations is missing, while the influence on protein quality was never assessed. Furthermore, available data on the effect of sludge retention time (SRT; dilution rate) are contradicting (Grau, 1980; Surucu et al., 1975) and other parameters such as food-to-microorganism rates (C, N and P loading rates), nutrient ratios, pH, temperature, conductivity and hydraulic retention time (HRT) were not considered before. Finally, although contaminant levels are expected to be low in food and beverage effluents, biomass safety has only been assessed for a few contaminants (heavy metals and selected organic pollutants), in biomass from few subsectors (dairy, potato, soybean and meat processing) (Clevenger, 1990).

A broad and systematic quality comparison was performed of dried AHB biomass of large food and beverage industries (potato and starch processing, dairy, beer brewing and other sectors). Dried AHB biomass from the activated sludge process of 25 food-processing companies was analyzed for nutritional value and safety, and the variability of these features was also monitored over time for 9 companies. Comparisons were made with soybean meal, as most generally applied protein source in animal feeds, and feed ingredient regulations. Furthermore, the influence of process parameters and water characteristics on the nutritional biomass value was statistically investigated, to distill

generic insights for high-quality AHB biomass production. Sampled plants focused only on rendering water dischargeable and not on the production of MP. This enabled to identify the baseline potential of MP production in current wastewater treatment plants, along with room for improvement for retrofitted or greenfield installations.

2. Materials and Methods

2.1. Biomass collection and operational parameters at the treatment plants

Variability in biomass quality was assessed in AHB biomass originating from the AS process, treating the effluent from 25 different companies in the food and beverage sector (Table 1). Target companies belonged to the potato and starch processing subsector, the dairy subsector and the brewery subsector, all presenting global abundance and high effluent volumes, linking up to large quantities of AHB biomass produced. The fourth category includes subsectors in which maximum 2 companies were sampled, including production of yeast (O1), enzymes (O2), plant-based dairy (O3), plant-based oils and fats (O4, O5), cookies and cake (O6) and soft drinks (O7). Additionally, the nutritional value was determined over time for 9 of the 25 companies (selected within a radius of 50 km from the laboratory), at 4 additional time points over a period of 323 days. Samples of 10 L were collected from the aerobic reactor during aeration to ensure sample homogeneity. Supernatant was discarded, after which the thickened biomass was washed two times with tap water with intermittent centrifugation at 4000 g. Subsequently, the AHB biomass was dried during 14 – 20 hours in layers of c.a. 0.5 cm thickness at 75°C, with air ventilation to ensure fast drying. Dried samples were stored in closed containers in the dark at 20°C.

Process parameters and water characteristics were acquired up to one month before the time of sampling, including flow rate, chemical oxygen demand (COD), biodegradable

COD (bCOD), total N, total P, PO_4^{3-}, electrical conductivity (EC), pH and temperature (Table 1). Additional samples were collected from the AHB reactor influent, treated effluent and return activated sludge (RAS) to validate companies' information and to determine process parameters. Samples were collected and stored according to the APHA (2012) guidelines. Influent and effluent samples of the AHB reactor were analyzed for COD (Spectroquant; NOVA 60), ammonium and ortho-phosphate (San++ Automated Continuous Flow Analyzer) and Kjeldahl nitrogen (AOAC International., 1995). Sample pH and electrical conductivity (EC) were measured immediately after sampling (Hanna instruments HI2020; HI230).

2.2. Biomass nutritional value

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined on a 5-15 mL suspended sludge sample according the standard methods (APHA, 2012). Protein content was determined based on Markwell et al. (1978). Amino acid residue analysis was initiated by protein hydrolysis, with 6M HCl for 24 hours at 110°C, in vacuum-sealed hydrolysis tubes (Wilmad Labglass). To avoid amino acid oxidation, hydrolysis and subsequent acid evaporation were performed under vacuum atmosphere, alternating with nitrogen gas flushing. Amino acid residues were derivatized with propyl chloroformate following the EZ:faast amino acid analysis procedure (Phenomenex, 2003), after which separation and analysis was performed with GC-MS (Agilent HP6890 Series GC system Plus; HP 5973 Mass selective detector). Bovine Serum Albumin (BSA) was analyzed in parallel to determine amino acid recovery. Arginine was not measured, while tryptophan was destroyed during acid hydrolysis.

2.3. Comparison of protein quality with soybean meal

AHB biomass EAA composition was compared with that the conventional high-protein feed ingredient, soybean meal. Specifically, the EAA deficit or surplus was calculated as the difference between soybean meal and AHB biomass. Soybean meal protein content was recalculated from reported crude protein levels of 44 – 49% (on dry weight) (Tacon et al., 2009) and the N-to-protein conversion factor of 5.5 found by Mariotti et al. (2008), instead of the general factor of 6.25, which was observed to lack accuracy and overestimate protein content. Applying this factor to the reported values of Tacon et al. (2009), a true protein content is found of 39 – 43%, or 41% (on dry weight) on average. The monetary AHB biomass surplus value compared to soybean meal, was calculated by multiplying deficits and surpluses with the average market price of commercial feed-grade EAA used in animal feed formulation (lysine, methionine, threonine and valine) (supplementary material).

2.4. Biomass safety parameters

Heavy metals (Cr, Ni, Cu, Zn, As, Cd, Hg, Pb) were analyzed in samples of 0.5-1.0 g (European standard method EN 15763). Each sample was weighted in digestion tanks (CEM Mars Express). Around 0.6 g internal standard solution and 10 ml of 65% nitric acid and 1.5 ml of 30% HCl was added. After digestion, each container was filled with Millipore water to approximately 60 g. Around 3 g of the digested solution was mixed with 3.25% nitric acid to around 9 g, after which the sample was analyzed for heavy metals with ICP-MS (Agilent ICP-MS 7500cx Series).

Samples for polyaromatic hydrocarbons (PAH) analysis (benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene) were homogenized (Robot Coupe Blixer or Retsch GRINDOMIX) after which 5 g was supplemented with internal

standard and extracted using acetonitrile (European standard method EN 16619:2015). Bekolut citrate kit 01 was added and the homogenate was centrifuged for 5 min at 6000 rpm. The upper phase was removed, followed by a dispersive solid phase cleanup (d-SPE) (Bekolut PSA-Kit-04). After mixing and centrifugation, the supernatant was evaporated using nitrogen gas. Acetonitrile was used to reconstitute the sample, after which analysis took place using GC-MS/MS (Agilent Technologies GC 7890A; Agilent Technologies 7000 Triple Quad MS/MS; Agilent Technologies Select PAH; pre-column Agilent Technologies Select PAH).

For pesticide and mycotoxin analysis (see supplementary material for a full list), the homogenized sample was subjected to an acetonitrile liquid-solid partition extraction in the frozen state (European standard method EN 15662). Triphenylphosphate was added as internal standard together with acetonitrile. Subsequently, a citrate salt kit (Bekolut Citrat-Kit-01) was added, whereby excess water separates out and the pH of the acetonitrile phase stabilizes at 5-5.5. After shaking and centrifugation (5 min at 6000 rpm), an aliquot of the acetonitrile phase was filtered and pesticides were measured by GC-MS/MS (Agilent Technologies GC-QQQ-MS 7890A; G7000B Triple Quadrupole), mycotoxins by HPLC-MS/MS (Agilent Technologies HPLC 1290; RRHD Eclipse Plus C18 column; Agilent 6490 Triple Quad LC/MS).

For antibiotics analysis (see supplementary material for a full list) according Schwaiger et al. (2018), a sample of 2.0 ± 0.1 g was homogenized with 100 µl of antibiotics internal standard solution and 2 ml of Na_2EDTA-McIlvaine buffer. For protein precipitation, 8 ml of acetonitrile was added. After centrifugation, the resulting supernatant was purified by means of mixing with around 500 mg C18EC bulk sorbent. After the bulk sorbent settled using centrifugation, 5 ml of supernatant was evaporated

with nitrogen gas at 45°C, reducing the residual volume to less than 0.5 ml. The residue was reconstituted with 2 ml HPLC mobile phase (initial conditions), vortexed and centrifuged for 5 min at 6000 rpm. Finally, the supernatant was filtered (PTFE, 0.2 μm) and analyzed using HPLC-MS/MS (Agilent Technologies HPLC 1290; RRHD Eclipse Plus C18 column; Agilent 6490 Triple Quad LC/MS).

2.5. Statistical analysis

Data comparison was performed using an independent sample t-test assuming normality, at a significance level of $p < 0.05$ (IBM SPSS statistics 24). Furthermore, the main process parameters and water characteristics influencing biomass protein content were identified applying multi-linear regression analysis (IBM SPSS statistics 24). The model was built based on protein content expressed as %VSS, to avoid the influence of non-organic material present in the AHB biomass (e.g. sand particles from potato washing). The assumptions of linearity and homogeneity of variance were assessed with scatter plots of observed versus predicted values and standardized residuals versus predicted values, respectively. The assumption of normality was confirmed with a QQ-plot of the residuals. To present the outcome of the multiple linear regression in a comprehensive manner, the variables in the final model were z-transformed, after which the regression model was build and the parameters estimated. Finally, Pearson correlation analysis was performed (IBM SPSS statistics 24) between EAA contents and all independent variables, after which principle component analysis (PCA) was applied in JMP pro 14 to study the relation between EAA composition and selected process parameters and water characteristics presenting a significant Pearson correlation ($p < 0.01$).

3. Results and discussion

3.1. Variability in AHB biomass nutritional value

The AHB biomass protein content of the 25 sampled companies was situated between 21 and 49% on dry weight (25-64% VSS) (Figure 1). These values are comparable to AHB protein data in literature (30-50% on dry weight), however, the majority of research focusses on sewage AHB biomass (Smith & Rothman, 1981; Vriens et al., 1989). Few studies mention the protein content in AHB biomass in the brewery subsector, reporting values of 38-50% (on dry weight) (Vriens et al., 1989). Further, although no significant difference between sectors was observed ($p < 0.05$) (Figure 1 A2), the variability in protein content within each sector, comparing minimum to maximum, was substantial with a value of 131% for the potato and starch subsector, 34% for the dairy subsector, 78% for the brewery subsector and 106% within the other subsectors. In contrast, the temporal variability within a company was slightly lower with an average of 26%, between minimum and maximum, and values ranging between 6% (company O1.1) and 58% (company B2.2) (Figure 1 B1). Literature data on temporal variability in the food and beverage sector is scarce, however, one study reported dried dairy AHB protein contents with a variability of 42% (29-41% crude protein) over an unspecified period (Clevenger, 1990). Another study monitored sewage AHB biomass over a period of 1 year reporting a variability in protein content of 65% (17-28% protein) (Tacon & Ferns, 1979). Similar to AHB biomass protein content, no significant influence of subsector on total EAA content was noticed ($p < 0.05$), with values of 11 ± 2.6% (on dry weight) for the potato and starch, 11 ± 2.4% for the dairy, 11 ± 2.7% for the brewery and 12 ± 2.6% for the other subsectors (Figure 1 A2). Further, the temporal variability between the minimum and maximum biomass EAA

content was company dependent with values between 3% (company D1) and 163% (company B1). This observation indicates that EAA content depends on company specific water characteristics and process parameters.

To get a better view on AHB protein quality and variability of individual EAA, Figure 2 presents the EAA residue profiles of all analyzed AHB (in mg EAA/ g protein). In agreement with the variable EAA content, individual EAA also present a large variability, with the largest value observed for the sulfur containing amino acids (methionine and cysteine) with values between 0.3 and 28 mg (met+cys)/g protein. Similarly, lysine and histidine content varied considerably, between 7 and 86 mg Lys/g protein and between 4 and 27 mg His/g protein, respectively (Figure 2). As expected, the temporal variability within the individual EAA per company was lower compared to the variability between different companies. Overall, the largest temporal change was observed for methionine and cysteine, with minimum values as low as 0.4 mg (met+cys)/g protein and maximum values as high as 21 mg (met+cys)/g protein (Figure 2). Furthermore, phenylalanine and tyrosine, as well as lysine and histidine presented a large temporal variability up to 492% (sample B2.2: between 15 and 86 mg lys/g protein), comparing minimum to maximum. The magnitude of the observed variability was highly dependent on the individual EAA and on company. AHB biomass with a relatively stable EAA composition over time were PS1, D3, B3 and O1.2 presenting a median EAA variability of 27%, 61%, 49% and 22%, respectively.

AHB biomass samples in this study originated from plants not optimized for MP production, focusing on rendering water dischargeable. Hence, the considerable variability in both protein content and quality at subsector level as well as at company level, is a logical consequence of the large range of influent characteristics (e.g. due to

specific production processes and temporal changes in water quality and quantity), resulting in different operational parameters. These changing parameters are known to influence microbial community dynamics (Vriens et al., 1989), and in extension, biochemical biomass composition. The observed variability in nutritional value identifies the baseline potential of current installations along with the potential to develop new systems aiming at a stable production of AHB biomass with a high nutritional value.

3.2. Influence of process parameters on nutritional value

To better understand the exact contribution of all influencing parameters on AHB biomass protein content (%VSS), a multiple linear regression analysis was performed (Figure 3; supplementary material) including relevant AHB reactor parameters (pH, temperature, salinity, VS/TS), influent characteristics (total COD, bCOD/N, N/P) and process characteristics (N loading rate, bCOD loading rate, HRT and SRT). The three variables that contributed significantly to the final model ($p < 0.05$, $R^2 = 0.572$), were pH, biomass specific N loading rate and SRT. It can be observed from Figure 3 that SRT has the largest contribution to the model, while the confidence intervals, indicating the precision, are similar for all parameters. Furthermore, the negative regression coefficient for SRT and positive regression coefficients for biomass specific N loading rate and pH, indicate that a higher SRT results in a lower protein content, while the opposite is true for the other two parameters.

To verify the influence of SRT (i.e. bacteria age) on protein content, a batch growth experiment (materials and methods in supplementary material) was performed using AHB biomass originating from a pilot-scale MP reactor (Avecom, Belgium). The AHB biomass protein content presented values between 31 and 51% (on dry weight) during

only 12 hours of cultivation (supplementary material). In agreement with the multi-linear model, highest protein content was measured for younger cells in exponential growth phase. Similarly, Grau (1980) reports the lab-scale observation of a negative relation between SRT and protein content. AHB biomass cultivated in semi-continuous mode presented an average protein content of 51% at an SRT of 2 days and 35% at an SRT of 20 days. Furthermore, Vriens et al. (1983) observed at lab-scale an increase from 42% to 62% protein and a four-times higher daily protein production at SRT of 4-6 days compared to an SRT of 20 days. In contrast, Surucu et al. (1975) observed a linear decrease in biomass protein content with decreasing SRT, with a value of 45% at 10 days SRT and a value of 36% at an SRT of 0.75 days. However, these experiments were performed at 58°C, which might be an indication of the inverse effect in thermophilic conditions, compared to the mesophilic conditions in this study (maximum 36°C).

The positive correlation between N loading rate and protein content could not be verified in literature. Most studies focus on the influence of COD loading rate. In current study, COD loading rate was not found to significantly influence protein content, however, Heddle (1979) reported a decrease in biomass crude protein content with decreasing COD loading rate. Closely related to loading rates are influent nutrient ratios, which were not found to significantly influence protein content in this study. In contrast, generally a higher C/N ratio typically results in a lower protein and higher lipid content (Goldberg, 1985). Also, if nitrogen supply is too low to maintain the required COD/N ratio of 20/1 in wastewater treatment, N addition in the aerobic reactor is general practice. For a decrease in COD/N ratio from 100/2.19 to 100/5.31, Vriens et al. (1983) observed at lab-scale an increase from 43% to 62% protein in AHB biomass

protein content at an SRT of 4 days. In terms of COD/P ratio, Bates and Torabian (1981) reported at lab-scale a minimum AHB protein content of 42% at a COD/P ratio of 100/0.1, with an increase up to 63.8% at a COD/P ratio of 100/2.

Other potentially influencing process parameters are pH, oxygen concentration, and temperature. In current study, only pH contributed to the linear model, positively correlating to protein content, however no literature was found to compare, leaving potential for further investigation. The effect of O_2 concentration in the aerobic reactor was hard to determine since this control parameter fluctuates between certain pre-set values, resulting in complex O_2 concentration dynamics in large-scale aerated reactors. However, in terms of the more general aerobic-to-anoxic time ratio, no correlation with AHB protein content was observed. Also at lab-scale, Gaudy and Turner (1964) did not observe a change in sewage AHB protein content for a range of dissolved oxygen (DO) concentrations from 1.4 to 7.1 mg/L. Concerning AHB reactor temperature, no considerable influence on AHB protein content was noticed, while literature to verify this observation is lacking, leaving room for further study.

Finally, principle component analysis was performed between EAA contents and the variables that presented a significant ($p < 0.05$) Pearson correlation with one or more individual EAA (supplementary material). The first two principal components, however, explained only 36.6 and 16.3% of the total variance, which was found too low to draw conclusions (data not shown). This was in line with the correlation analysis between process parameters and biomass EAA composition, presenting relatively low Pearson correlation coefficients between 0.28 and 0.43 ($p < 0.05$) (supplementary material). The trends with a significant correlation at the 0.01 level, are the positive correlations between histidine and CODrem/PO_4inf ($r = 0.369$) and between valine,

isoleucine and threonine with SRT (r = 0.391, 0.392 and 0.428, respectively). No literature could be found to explain these relations for AHB biomass, however, Sueoka (1961) did not observe a significant influence of medium composition, temperature or growth phase on *E. coli* EAA composition. The specific influence of operational parameters on EAA content presents potential for further research.

3.3. Safety of AHB biomass as feed ingredient

Similar to protein content, AHB mineral content varied depending on the company, with values of 6-111 mg/kg for chromium, 6-108 mg/kg for nickel, 7-166 mg/kg for copper and 137-548 mg/kg for zinc (figure 5A). The brewery subsector presented the highest average values for all minerals. The minerals copper, zinc and chromium have an important function within the metabolism of animals (Moral et al., 2008), however, limits within feed are set. The maximum tolerance limit for copper in animal feed is 40 mg/kg as recommended by NRC (2005). For zinc, the maximum allowable content in pig diets in Europe is 250 mg/kg, based on environmental concerns. For soluble chromium Cr(III) the NRC maximum tolerable level is 100 mg/kg. Although nickel is not recognized as an essential mineral, deprivation has been shown to have adverse effects, while higher levels of nickel are toxic (EFSA-CONTAM, 2015), resulting in the maximum tolerable level for cattle of 50 mg Ni/kg diet (NRC, 2005). In case AHB biomass is blended at the recommended 10% level (Clevenger, 1990), none of the analyzed minerals exceeded the limits.

When considering AHB biomass as protein ingredient in feed, animal and consumer safety are crucial. For this reason, European legislation sets maximum limits for ‘undesirable substances’ in animal feed for heavy metals, dioxins, aflatoxins, pesticides, botanicals and mycotoxins (in particular ochratoxin A, deoxynivalenol, zearalenone,

fumonisins, T-2 and HT-2 toxin). The analyzed AHB biomass contained heavy metals with values of 1.8 ± 1.4 mg arsenic/kg, 0.7 ± 0.9 mg cadmium/kg, 0.07 ± 0.03 mg mercury/kg and 5.6 ± 3.2 mg lead/kg (figure 5B). Compared to the EU limits for heavy metals in feed of 2 mg arsenic/kg, 10 mg lead/kg, 0.1 mg mercury/kg and 1 mg cadmium/kg, most heavy metal violations were observed in AHB biomass from the potato and starch subsector, with arsenic (2.6 ± 2.0 mg/kg), cadmium (1.7 ± 0.9 mg/kg) and lead (7.9 ± 4.7 mg/kg) as main contaminants (Figure 5B). Mercury content (0.06 ± 0.04 mg/kg) did not violate the limits. Although, most of the soil resulting from potato washing is returned to the field, it was tested if agricultural fertilizers could be the source of heavy metals in AHB biomass. AHB biomass was analyzed that was cultivated on only the potato cutting water from company PS6 (Avecom). Indeed, resulting AHB biomass contained a lower arsenic level of 0.5 mg As/kg compared to 4.1 mg As/kg for cultivation on the complete processing effluent. However, cadmium, mercury and lead were present in higher concentrations (4.3, 12.2 and 5 mg/kg, respectively), compared to the AHB grown on the complete processing effluent. Some heavy metals are hypothesized to originate from metal salts (e.g. $FeCl_3$), dosed to remove residual phosphorus, that are potentially contaminated with heavy metals such as cobalt (~20 mg/L) (personal communication). To avoid these contaminants, residual phosphorus can be precipitated after AHB production, during a polishing step.

Remarkable was that in all samples, traces of pesticides were observed (Figure 5C) and although most were present in low or non-detectable concentrations, in all analyzed samples the EU pesticide maximum residue levels (MRL) for food and feed (supplementary material) were exceeded for one or more pesticides. The highest concentrations were measured for didecyldimethylammonium chloride (DDAC-C10)

(0.5±0.6 mg/kg) and the group of alkylbenzyl dimethylammonium chlorides ('Sum BAC') (0.7 ± 0.7 mg/kg). Both are quaternary ammonium compounds, used in the dairy industry for disinfection. Flutolanil was detected only in the AHB biomass originating from potato processing, logically considering its function as disinfectant of potato seedlings against black scurf. Chlorpropham, known as potato sprout suppressant, was mainly found in AHB biomass originating from potato processing, while metalaxyl, known as fungicide for crop diseases control was mainly found in AHB biomass from breweries. Although the MRL exceedance was low for most samples, pesticide levels should be closely monitored when MP production is intended.

Sources of PAH in AHB biomass in the food and beverage sector are diffuse, e.g. air depositions on the production site ending up in run-off rainwater. Currently, no regulations exist for PAH, mainly because transfer rate data from feed to animal products are limited. Average values for benzo(a)anthracene (4.6 ± 5.1 µg/kg), chrysene (11.5 ± 13.5 µg/kg), benzo(b)fluoranthene (4.0 ± 4.8 µg/kg) and benzo(a)pyrene (3.1 ± 3.6 µg/kg) (Figure 5D), were similar to median levels in feed ingredients (0.61-8.2 µg BaA/kg; 0.25-9.15 µg Chr/kg; 0.36-5.2 µg BbF/kg and 0.29-7.9 µg BaP/kg) (Bulder et al., 2006). The companies presenting the highest PAH content were PS2, PS3, B1 and B2.2 with values between 53 and 80 mg PAH4/kg. No literature was found presenting PAH data in AHB biomass from food and beverage processing. For sewage AHB biomass values of 2-29 µg BaA /kg, 3-94 µg CHR/kg, 3-48 µg BbF/kg and 2-26 µg BaP/kg are reported, which are higher compared to the values in this study (Liu et al., 2017; Stevens et al., 2003), therefore suggesting the food and beverage sector is no specific source of PAH.

Finally, no veterinary drugs were found in the AHB biomass, while in one sample (D1) the mycotoxins alternariol and beauvericin were detected in trace amounts of 82.36 µg/kg and 10.34 µg/kg, respectively.

In most research considering MP, nucleic acids are also mentioned as a safety risk (Anupama & Ravindra, 2000), because after breakdown by intestinal enzymes, guanine and adenine are transformed to uric acid of which high blood levels lead to gout. However, because most mammals can oxidize uric acid to the more soluble allantoin, eliminating the safety risk, nucleic acid content was not measured in this study. Finally, pathogens are a risk when sanitary waste is co-treated with processing effluents, however, because AHB biomass application in animal feed requires decoupling between these streams, pathogens were not considered here.

3.4. AHB biomass quality and valorization potential

Due to its potentially high protein content (up to 49%; on dry weight), AHB biomass is mainly regarded as (partial) substitute for high-protein ingredients such as soybean meal. AHB biomass quality was observed to be suboptimal compared to soybean meal in terms of EAA content (in mg AA/g dry weight), with only threonine presenting a surplus (Figure 4A). This is mainly a consequence of the lower average protein content of 34 ± 7.3% (on dry weight), compared to the average soybean meal protein content of 39–43% (see materials and methods). Based on protein quality (in mg AA/g protein) (Figure 4B), most AHB biomass can compete with soybean meal as protein source. However, depending on the individual EAA, deficits or surpluses occur. AHB biomass presents overall a surplus for threonine and valine, while for cysteine, histidine, lysine and phenylalanine an overall deficit was observed (Figure 4B). For tyrosine, methionine, isoleucine and leucine, a deficit or surplus was dependent on subsector.

Furthermore, AHB biomass originating from the dairy subsector was the least complete EAA source and only presented a surplus for threonine. Considering the potential market biomass surplus value of AHB produced in current plants, based on the applied EAA in animal feed formulation, it can be observed that within each subsector some companies contain a higher value and some a lower value compared to soybean meal (Figure 4C). The overall average surplus value for AHB biomass was slightly positive (0.03 €/kg protein). Taking into account the potential protein content (up to 50% on dry weight) under optimal SRT and N loading rate, AHB biomass has the potential to compete with soybean meal. Production costs should be evaluated to assess equivalence to the market price of soybean meal.

Looking at the different subsectors, dairy industry presented an inferior protein quality compared to the others. A possible explanation is the lower average N loading rate (4.1 ± 2.8 g N/kgVSS/d) within the dairy subsector, however, the difference was not significant ($p < 0.05$). AHB biomass EAA profile from the potato and starch subsector was mainly rich in tyrosine, threonine and valine, while brewery biomass was richer in methionine, threonine and valine. In general, literature observation that AHB protein quality compares well with soybean meal was not confirmed in the present study (Vriens et al., 1989). Depending on company and timing, AHB biomass was observed to contain higher or lower EAA levels compared to soybean meal. This variability in protein quality indicates the suitability to blend AHB biomass from different origin to answer the EAA demand of the target animal. However, current variability for the limiting EAA makes this a difficult task. In retrofitting existing treatment plants or in greenfield installations aiming at MP production, a stable high-quality product should be the target.

Finally, AHB biomass presents additional advantages over conventional protein sources. A recent study provided evidence for a lower environmental impact of AHB protein production than soybean meal production in the LCA endpoint categories human health and ecosystems (Spiller et al., 2020). Another study observed more orange egg yolks from hens fed diets containing dried AHB from citrus processing plants (Angalet et al., 1976). Furthermore, compared to soy protein, AHB biomass does not contain endogenous anti-nutrients such as protease trypsin inhibitor (El sayed et al 1999; Tacon et al. 1993). Finally, AHB biomass was observed to contain high vitamin B12 levels (Hoover et al., 1951), essential in several basic metabolic enzyme systems.

3.5. Process optimization in function of MP production

Current treatment plant operation in the food and beverage sector is established based on economic and environmental constraints. Firstly, plant operation needs to assure that the discharge limits are reached. Secondly, sludge production and aeration are minimized to limit treatment costs. Because low AHB biomass production demands a high SRT, while high SRTs have high aeration needs, aerobic reactors are generally designed with an intermediate SRT between 15 and 25 days. In contrast, MP production aims at maximizing biomass yield and optimal protein productivity and quality. As was observed in this study, shorter SRTs and higher N loading rates align with these goals. Shorter SRTs result in higher cell yields due to the lower maintenance energy necessary and the lower cell death. Additionally, auto-oxidation of the biomass decreases, leading to a decrease in oxygen consumption per quantity of protein produced (Surucu et al., 1975). An adverse effect of short SRTs is, however, that in contrast to current intermediate SRTs, they potentially result in insufficient COD removal (Vriens et al., 1989). To avoid this potentially compromising tradeoff between high-quality biomass

and sufficient treatment, a polishing step can be implemented after the MP production stage to reach discharge limits (Lee et al., 2015; Verstraete et al., 2016).

Based on the aforementioned information, high-rate activated sludge (HRAS) appears to be a promising process to produce AHB biomass at a low SRT (< 2 days) and at high biomass specific loading rate (> 2 kg COD/kgVSS/d). Different types of HRAS have been applied for domestic wastewater treatment. Meerburg et al. (2015) introduced two types of reactors that obtained biomass yields, up to 1 g $COD_{biomass}$/g $COD_{removed}$, a characteristic that could be valorized in MP production by increased AHB productivities. The use of HRAS for protein production was not described and studied yet, offering potential for further research. Specially, it should be validated by full-scale measurements if SRT values lower than 2 days will indeed result in increased AHB protein content.

Finally, AHB biomass drying should guarantee an acceptable shelf life. Drying methods that are commonly used include spray drying, drum drying and freeze-drying (Goldberg, 1985). Further assessment of dewatering and drying methods should indicate which strategy consumes the least energy.

4. Conclusions

The food and beverage sector is in pole position to provide sustainable and high-quality MP for the animal feed industry. The broad range in nutritional quality of currently produced AHB biomass determines the baseline potential along with room for improvement for retrofitted or greenfield installations. Residual risk management is essential by careful monitoring and eliminating of heavy metals, PAH and pesticides. Optimization of treatment plants towards protein production can result in a feed value

that is on par with soybean meal, while designing for increased AHB biomass quality should aim at higher rate processes, thereby also increasing AHB productivity.

E-supplementary material of this work can be found in the online version of the paper.

5. Acknowledgement

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6. References

1. Angalet, S.A., Fry, J.L., Damron, B.L., Harms, R.H. 1976. Evaluation of Waste Activated-Sludge (Citrus) as a Poultry Feed Ingredient .2. Quality and Flavor of Broilers, Egg-Yolk Color and Egg Flavor. *Poultry Science*. **55**(4). 1219-1225.
2. Anupama, Ravindra, P. 2000. Value-added food: Single cell protein. *Biotechnology Advances*. **18**(6). 459-479.
3. AOAC International. 1995. Official methods of analysis of AOAC International, AOAC International. Arlington, USA.
4. APHA. 2012. Standard Methods for the Examination of Water and Wastewater. American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF).
5. Bates, M.H., Torabian, A. 1981. The Effects of the Cod - P-Ratio on Laboratory Activated-Sludge Systems. *Water Research*. **15**(8). 999-1004.
6. Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P.M., Fischer, A.R.H., van Boekel, M.A.J.S., Rutherfurd, S.M., Gruppen, H., Moughan, P.J., Hendriks, W.H. 2013. The future supply of animal-derived protein for human consumption. *Trends in Food Science & Technology*. **29**(1). 62-73.
7. Bosch, G. 2015. Novel protein sources for pig feed. in: *VIII Jornadas de Suinicultura da IAAS-UTADAt.* Vila Real, Portugal.
8. Bulder, A.S., Hoogenboom, L.A.P., Kan, C.A., van Raamsdonk, L.W.D., Traag, W.A., Bouwmeester, H. 2006. Initial risk assessment of polycyclic aromatic hydrocarbons (PAHs) in feed (materials). Animal Sciences Group Wageningen UR; RIKILT - Institute of Food Safety. Report 2006.001.
9. Caton, J.S., Williams, J.E., Beaver, E.E., May, T., Belyea, R.L. 1989. Effects of Dairy Biomass Protein on Ruminal Fermentation and Site and Extent of Nutrient Digestion by Lambs. *Journal of Animal Science*. **67**(10). 2762-2771.
10. Chuapoehuk, W., Piadang, S., Tinnungwattana, W. 1998. Recycling of sewage sludge: Feeding Nile tilapia, Oreochromis niloticus (Linn), with irradiated and dried sludge from beer industry. International Atomic Energy Agency (IAEA). 1011-4289. IAEA-TECDOC--1023.
11. Clevenger, T.E. 1990. Safety and Efficacy of Food-Processing Sludges as Animal Feed - Chemical Characterization. *Research Journal of the Water Pollution Control Federation*. **62**(6). 820-827.
12. EFSA-CONTAM. 2015. Scientific Opinion on the risks to animal and public health and the environment related to the presence of nickel in feed. *EFSA Journal*. **13**(4). 4074.
13. Esvelt, L.A., Hart, H.H., Heinemann, W.W. 1976. Cannery waste biological sludge disposal as cattle feed. *J Water Pollut Control Fed*. **48**(12). 2778-90.
14. European Parliament. 2018. Report on a European strategy for the promotion of protein crops – encouraging the production of protein and leguminous plants in the European agriculture sector (2017/2116(INI)). European Parliament.
15. FAO. 2018. FAOSTAT Database, Vol. 2018, Food and Agriculture Organization of the United Nations. Rome, Italy, http://faostat3.fao.org/home/E.
16. Fernandez, A., Huang, S.Y., Seston, S., Xing, J., Hickey, R., Criddle, C., Tiedje, J. 1999. How stable is stable? Function versus community composition. *Applied and Environmental Microbiology*. **65**(8). 3697-3704.

17. Fytili, D., Zabaniotou, A. 2008. Utilization of sewage sludge in EU application of old and new methods - A review. *Renewable & Sustainable Energy Reviews*. **12**(1). 116-140.
18. Gaudy, A.F., Turner, B.G. 1964. Effect of Air-Flow Rate on Response of Activated Sludge to Quantitative Shock Loading. *Journal Water Pollution Control Federation*. **36**(6). 767-781.
19. Goldberg, I. 1985. Single cell protein. Springer-Verlag, Berlin ; New York.
20. Grau, P. 1980. Utilization of Activated-Sludge as Fodder Supplement. *Environmental Technology Letters*. **1**(12). 557-570.
21. Heddle, J.F. 1979. Activated-Sludge Treatment of Slaughterhouse Wastes with Protein Recovery. *Water Research*. **13**(7). 581-584.
22. Hoover, S.R., Jasewicz, L.B., Porges, N. 1951. Vitamin B12 in activated sewage sludge. *Science*. **114**(2956). 213.
23. Huige, N. 2006. Brewery by-products and effluents. in: *Handbook of Brewing*, (Eds.) F.G. Priest, G.G. Stewart, Taylor & Francis Group. Boca Raton, FL, USA, pp. 656-707.
24. Kienholz, E.W., Moreng, R.E. 1981. A New Brewery by-Product for Turkey Diets. *Poultry Science*. **60**(8). 1879-1883.
25. Lee, J.Z., Logan, A., Terry, S., Spear, J.R. 2015. Microbial response to single-cell protein production and brewery wastewater treatment. *Microbial Biotechnology*. **8**(1). 65-76.
26. Liu, Z., Li, Q., Wu, Q., Kuo, D.T.F., Chen, S., Hu, X., Deng, M., Zhang, H., Luo, M. 2017. Removal Efficiency and Risk Assessment of Polycyclic Aromatic Hydrocarbons in a Typical Municipal Wastewater Treatment Facility in Guangzhou, China. *Int J Environ Res Public Health*. **14**(8).
27. Mariotti, F., Tome, D., Mirand, P.P. 2008. Converting nitrogen into Protein - Beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition*. **48**(2). 177-184.
28. Markwell, M.A.K., Haas, S.M., Bieber, L.L., Tolbert, N.E. 1978. Modification of Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Analytical Biochemistry*. **87**(1). 206-210.
29. Matassa, S., Boon, N., Pikaar, I., Verstraete, W. 2016. Microbial protein: future sustainable food supply route with low environmental footprint. *Microbial Biotechnology*. **9**(5). 568-575.
30. Mateo-Sagasta, J., Raschid-Sally, L., Thebo, A. 2015. Global Wastewater and Sludge Production, Treatment and Use. in: *Wastewater Economic Asset in an Urbanizing World*, (Eds.) P. Drechsel, M. Qadir, D. Wichelns, Springer.
31. Meerburg, F.A., Boon, N., Van Winckel, T., Vercamer, J.A.R., Nopens, I., Vlaeminck, S.E. 2015. Toward energy-neutral wastewater treatment: A high-rate contact stabilization process to maximally recover sewage organics. *Bioresource Technology*. **179**. 373-381.
32. Meerburg, F.A., Vlaeminck, S.E., Roume, H., Seuntjens, D., Pieper, D.H., Jauregui, R., Vilchez-Vargas, R., Boon, N. 2016. High-rate activated sludge communities have a distinctly different structure compared to low-rate sludge communities, and are less sensitive towards environmental and operational variables. *Water Research*. **100**. 137-145.
33. Merino, G., Barange, M., Blanchard, J.L., Harle, J., Holmes, R., Allen, I., Allison, E.H., Badjeck, M.C., Dulvy, N.K., Holt, J., Jennings, S., Mullon, C., Rodwell, L.D.

2012. Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? *Global Environmental Change-Human and Policy Dimensions*. **22**(4). 795-806.
34. Moral, R., Perez-Murcia, M.D., Perez-Espinosa, A., Moreno-Caselles, J., Paredes, C., Rufete, B. 2008. Salinity, organic content, micronutrients and heavy metals in pig slurries from South-eastern Spain. *Waste Management*. **28**(2). 367-371.
35. NRC. 2005. Mineral tolerance of animals. *2nd rev. ed*. National Academies Press, Washington, D.C.
36. Pikaar, I., Matassa, S., Rabaey, K., Bodirsky, B.L., Popp, A., Herrero, M., Verstraete, W. 2017. Microbes and the Next Nitrogen Revolution. *Environmental Science & Technology*. **51**(13). 7297-7303.
37. Schreuder, R., De Visser, C. 2014. EIP-AGRI Focus Group Protein Crops: final report.
38. Schwaiger, B., König, J., Lesueur, C. 2018. Development and Validation of a Multi-class UHPLC-MS/MS Method for Determination of Antibiotic Residues in Dairy Products. *Food Analytical Methods*. **11**(5). 1417-1434.
39. Shier, W.T., Purwono, S.K. 1994. Extraction of Single-Cell Protein from Activated Sewage-Sludge - Thermal Solubilization of Protein. *Bioresource Technology*. **49**(2). 157-162.
40. Smith, S.H., Rothman, H. 1981. Recycling Sewage-Sludge as a Food for Farm-Animals - Some Ecological and Strategic Implications for Great-Britain. *Agricultural Wastes*. **3**(2). 87-108.
41. Spiller, M., Muys, M., Papini, G., Sakarika, M., Buyle, M., Vlaeminck, S.E. 2020. Environmental impact of microbial protein from potato wastewater as feed ingredient: Comparative consequential life cycle assessment of three production systems and soybean meal. *Water Research*. **171**. 115406.
42. Stevens, J.L., Northcott, G.L., Stern, G.A., Tomy, G.T., Jones, K.C. 2003. PAHs, PCBs, PCNs, organochlorine pesticides, synthetic musks, and polychlorinated n-alkanes in U.K. sewage sludge: survey results and implications. *Environ Sci Technol*. **37**(3). 462-7.
43. Sueoka, N. 1961. Correlation between Base Composition of Deoxyribonucleic Acid and Amino Acid Composition of Protein. *Proceedings of the National Academy of Sciences of the United States of America*. **47**(7). 1141-&.
44. Surucu, G.A., Engelbrecht, R.S., Chian, E.S.K. 1975. Thermophilic microbiological treatment of high strength wastewaters with simultaneous recovery of single cell protein. *Biotechnology and Bioengineering*. **17**(11). 1639-1662.
45. Tacon, A.G.J., Ferns, P.N. 1979. Activated Sewage Sludge, a Potential Animal Foodstuff .1. Proximate and Mineral-Content - Seasonal-Variation. *Agriculture and Environment*. **4**(4). 257-269.
46. Tacon, A.G.J., Metian, M., Hasan, M.R. 2009. Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. Food and Agriculture Organization of the United Nations, Rome.
47. Vermeulen, S.J., Campbell, B.M., Ingram, J.S.I. 2012. Climate Change and Food Systems. *Annual Review of Environment and Resources, Vol 37*. **37**. 195-+.
48. Verstraete, W., Clauwaert, P., Vlaeminck, S.E. 2016. Used water and nutrients: Recovery perspectives in a 'panta rhei' context. *Bioresource Technology*. **215**. 199-208.
49. Vriens, L., Nihoul, R., Verachtert, H. 1989. Activated Sludges as Animal Feed - a Review. *Biological Wastes*. **27**(3). 161-207.

50. Vriens, L., Vandeneynde, E., Verachtert, H. 1983. Parameters Affecting Protein-Production from Brewery Wastewater in a Multi-Channel Laboratory-Scale Activated-Sludge System. *European Journal of Applied Microbiology and Biotechnology*. **18**(1). 52-59.

7. Table and figure captions

Table 1. AHB biomass samples from 25 companies in potato and starch industry, dairy industry, brewery industry and from other industries. All companies were located in Flanders (Belgium). A complete data overview as well as company classification based on reactor configuration is presented in supplementary material.

Figure 1. Protein, total amino acid (AA) and essential amino acid (EAA) residue content (% on dry weight) and protein content (%VSS) in AHB biomass from 25 companies (A1) and the average per subsector (A2). The variability over time at 5 moments, within a selection of 9 companies (B1 and B2) ($\Delta t_{(sample\ 1–2)}$ = 30 weeks and $\Delta t_{(sample\ 2–3)}$ = 2 weeks).

Figure 2. Essential amino acid (EAA) composition (mg EAA/g protein) in AHB biomass from 25 companies distributed over potato and starch, brewery, dairy and other industries and the temporal variability within 9 companies, presented as 3 different samples ($\Delta t_{(sample\ 1–2)}$ = 30 weeks; $\Delta t_{(sample\ 2–3)}$ = 2 weeks).

Figure 3. Standardized regression coefficients with their confidence interval for the three variables that contributed significantly to protein content variability in the multi-linear regression model ($p < 0.05$). The distance of the standardized coefficients from zero represents their relative importance and their confidence interval indicates the precision.

Figure 4. Surplus in AHB biomass EAA composition, compared with that of soybean meal (41% true protein (on dry weight); see materials and method section for assumptions) per kg dry weight of biomass (A) and per kg protein (B). Panel (C) presents the AHB biomass surplus value, which is the monetized EAA surplus for the 5 commercially blended amino acids in animal feed (lysine, methionine, cysteine, threonine and valine).

Figure 5. Mineral (A), heavy metal (B), pesticide (C) and polycyclic aromatic hydrocarbon (PAH) (D) content in AHB biomass from 19 companies. Horizontal lines represent the limits for heavy metals in feed according to the EU regulation; numbers in (B) present the exceedance of flutolanil (1), chlorpropham (2), DDAC-C10 (3), sum BAC (4), propham (5) and metalaxyl (6) of EU limits (see supplementary information). (Sample PS1 was not analyzed for pesticides and PAH).

8. Tables

Table 1. AHB biomass samples from 25 companies in potato and starch industry, dairy industry, brewery industry and from other industries. All companies were located in Flanders (Belgium). A complete data overview as well as company classification based on reactor configuration is presented in supplementary material.

			AHB reactor				*Influent characteristics*			*Process characteristics*			
Subsector	**Company code**	**Pre-treatment**	**Aerobic/ Anoxic ratio**	**TSS (g/L)**	**VS/ TS**	**pH**	**Total COD (g/L)**	**bCOD/N**	**N/P**	**HRT (d)**	**SRT (d)**	**N loading rate (g/kgVSS/d)**	**bCOD loading rate (g/kgVSS/d)**
Potato & Starch	PS1*	Anaerobic	2.76	7.9	0.66	6.7	2.31	6.6	6.1	5.6	21.5 [c]	10.7	69
	PS2*	Anaerobic	1	6.3	0.84	7.2	1.38	26.7	1.1	1.1	8.3 [c]	8.6	233
	PS3	Anaerobic	1.22	9.7	0.57	8.2	2.81	11.6	10.7	4.7	30.0 [d]	5.8	83
	PS4	Anaerobic	3.17	3.2	0.80	7.7	2.93	11.3	6.8	3.3	14.0 [b]	28.7	332
	PS5	Anaerobic	-	12	0.52	7.5	3.50	17.3	-	6.3	27.0 [d]	4.9	93
	PS6	Anaerobic	1.25	11	0.48	7.8	4.06	10.2	13.2	11.0	28.1 [a]	6.1	68
	PS7	-	-	3.5	0.73	7.6	3.27	50.0	3.5	4.6	13.6 [b]	5.1	262
Dairy	D1*	Fat removal	10.8	5.8	0.77	8.2	0.69	14.2	4.5	1.3	13.3 [a]	5.9	102
	D2*	-	1.86	5.5	0.81	8.7	2.32	77.8	4.6	4.0	26.8 [b]	0.3	116
	D3*	Fat removal	5.25	7.3	0.89	7.9	1.97	46.3	5.1	4.9	42.6 [c]	1.5	62
	D4	Fat removal	-	7.6	0.59	8.5	0.72	7.5	-	1.0	13.9 [a]	10.6	140
	D5	Fat removal	2.69	9.8	0.98	6.7	4.00	15.7	8.3	4.0	39.2 [a]	6.1	102
Brewery	B1*	Anaerobic	1	4.8	0.63	8.2	0.82	15.4	7.8	1.8	17.0 [a]	10.0	147
	B2.1*	-	2	3.0	0.75	7.9	2.35	30.2	5.5	2.3	10.0 [c]	6.3	415
	B2.2*	-	1.27	5.2	0.70	8.1	0.76	27.7	6.6	1.7	16.2 [c]	5.0	119
	B3*	Anaerobic	2	6.9	0.74	7.8	1.89	37.6	6.5	2.0	17.1 [c]	4.7	188
	B4	-	3	11	0.70	7.8	6.01	120.4	3.8	3.4	48.0 [a]	3.1	215
	B5	Anaerobic	-	4.4	0.61	8.0	1.33	29.2	3.1	1.5	5.1 [b]	10.3	321
	B6	-	-	15	0.81	8.0	3.50	30.2	8.4	2.0	14.3 [a]	4.4	127
Other	O1.1*	-	1.22	4.6	0.95	8.2	1.93	18.1	11.2	1.6	9.8 [c]	12.2	260
	O2.2*	-	1.22	4.2	0.93	8.1	0.49	10.4	7.9	1.2	28.3 [c]	6.4	95
	O2	-	-	8.6	0.66	7.4	2.65	11.2	-	3.3	28.1 [a]	11.3	133
	O3	Anaerobic	-	6.0	0.72	8.3	0.70	6.3	5.8	1.2	31.3 [c]	17.9	129
	O4.1	Fat removal	2.00	4.1	0.91	7.1	2.70	140.9	2.8	1.7	32.7 [a]	2.0	345
	O4.2	Fat removal	1.65	3.6	0.90	7.2	0.86	167.6	2.8	1.4	58.5 [a]	0.9	177
	O5	Fat removal	3.17	2.5	0.86	6.7	0.44	23.1	2.1	0.9	17.0 [a]	5.9	187
	O6	-	4.11	2.8	0.96	7.1	6.97	96.6	10.8	12.9	66.4 [a]	1.2	174
	O7.1	-	2.00	3.2	0.67	7.4	1.82	48.0	5.3	6.6	20.6 [d]	1.9	122
	O7.2	-	1.34	2.8	0.65	7.4	0.56	28.4	6.8	4.4	42.2 [d]	1.0	59

* Companies that were sampled 5 times to assess variations in time: data are the average of 5 different samples in time (a complete overview of the data is presented in supplementary material)

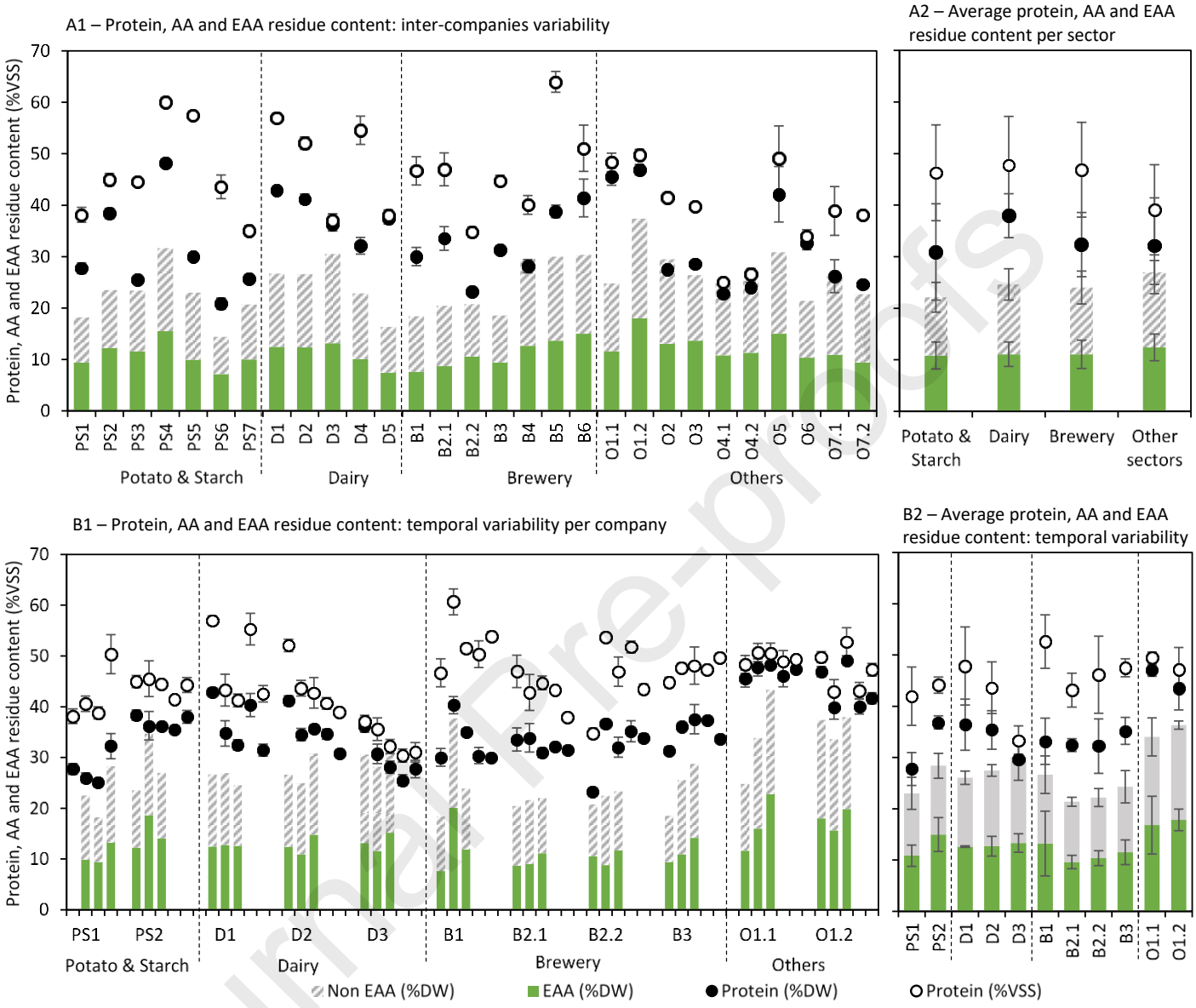
a: SRT calculation based on company data for the amount of sludge wasted

b: SRT calculation based on company data of Qwas (waste activated sludge flow rate) and Xwas (sludge density of WAS)

c: average of SRT calculation based on company data for the amount of sludge wasted and the SRT calculation based on company data of Qwas (waste activated sludge flow rate) and Xwas (sludge density of WAS)

d: SRT calculation as theoretical biomass yield, based on influent COD concentrations and aerobic/anoxic ratio (see supplementary material)

9. Figures



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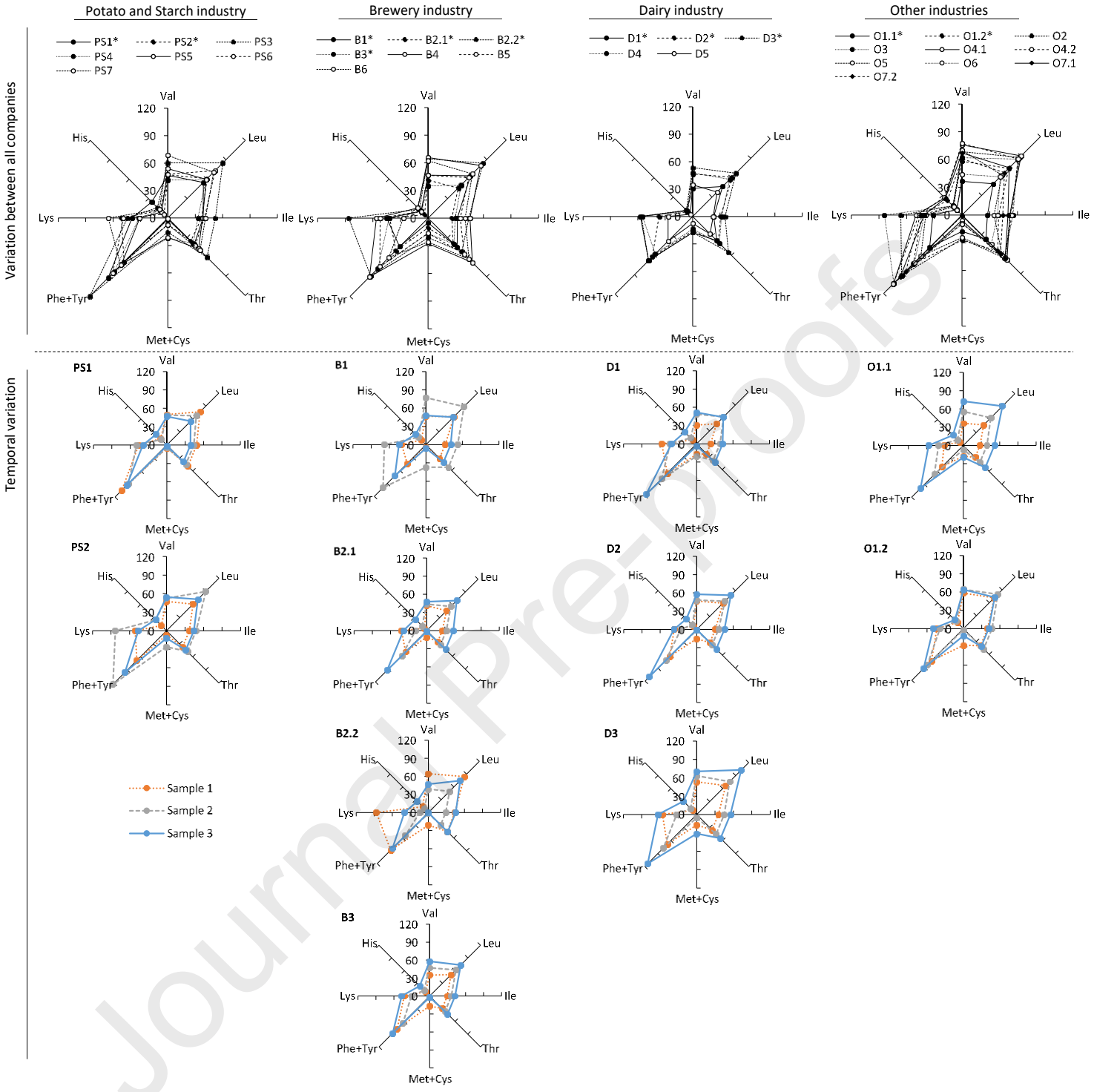

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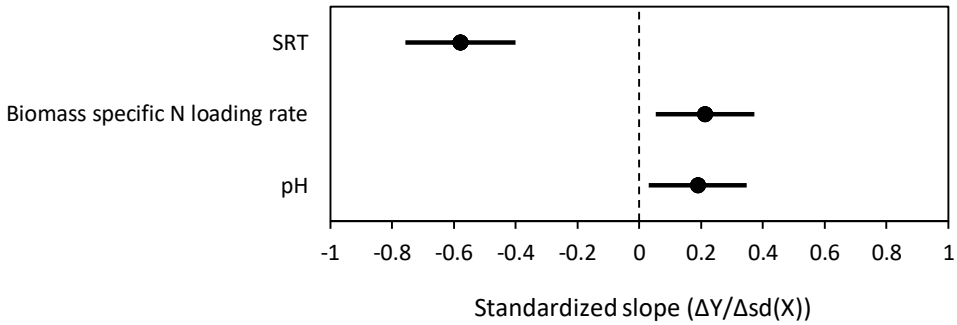

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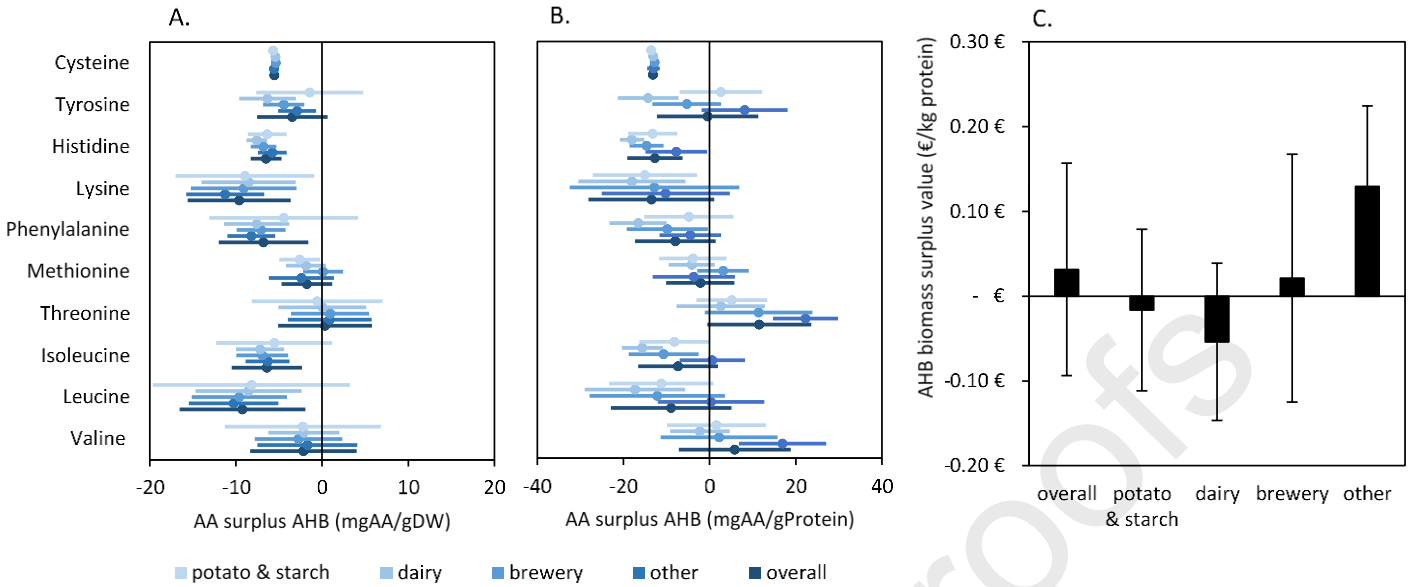


Figure 4. Surplus in AHB biomass EAA composition, compared with that of soybean meal (41% true protein (on dry weight); see materials and method section for assumptions) per kg dry weight of biomass (A) and per kg protein (B). Panel (C) presents the AHB biomass surplus value, which is the monetized EAA surplus for the 5 commercially blended amino acids in animal feed (lysine, methionine, cysteine, threonine and valine).

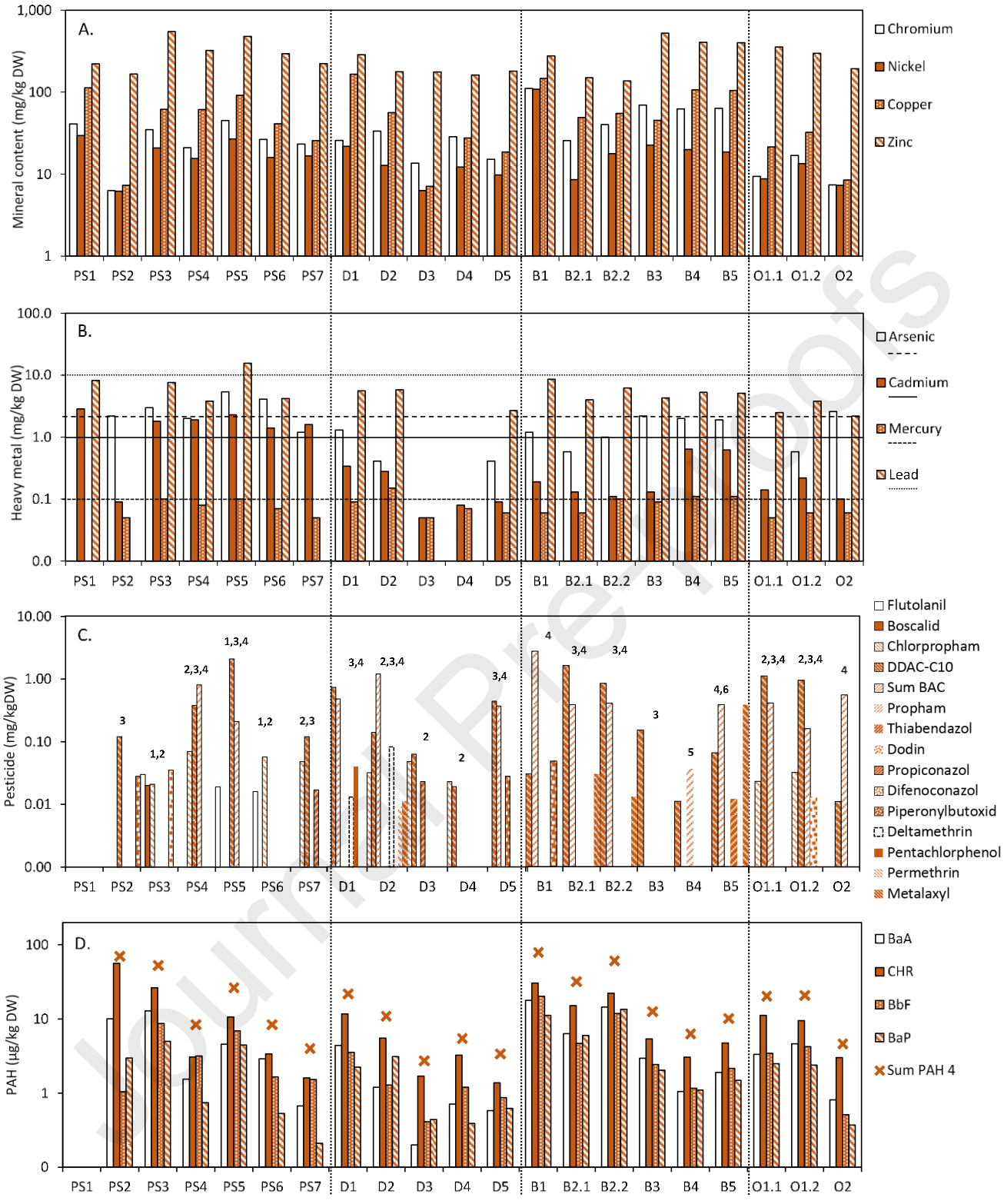
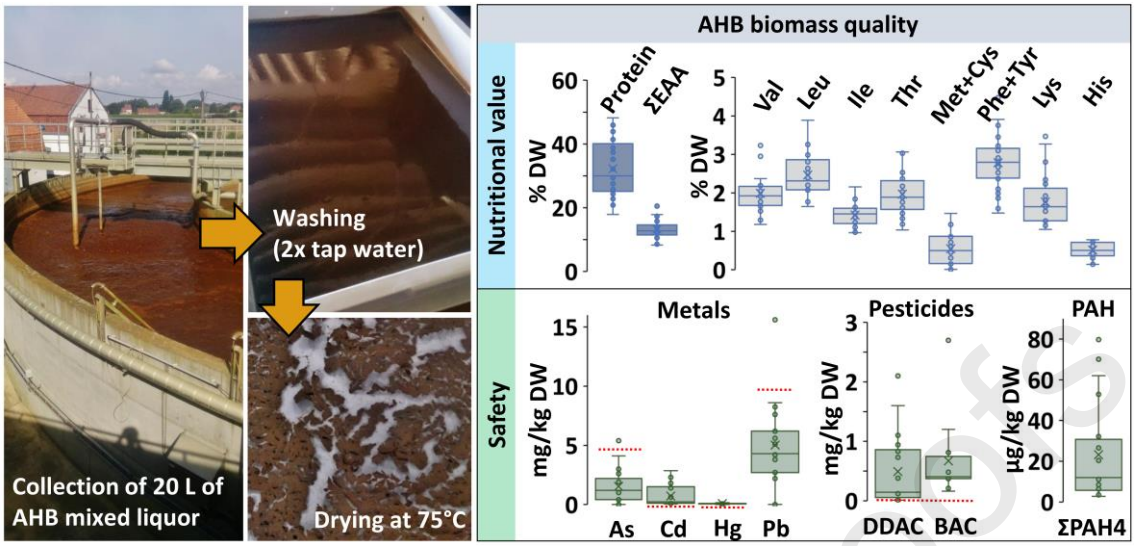


Figure 5. Mineral (A), heavy metal (B), pesticide (C) and polycyclic aromatic hydrocarbon (PAH) (D) content in AHB biomass from 19 companies. Horizontal lines represent the limits for heavy metals in feed according to the EU regulation; numbers in (B) present the exceedance of flutolanil (1), chlorpropham (2), DDAC-C10 (3), sum BAC (4), propham (5) and metalaxyl (6) of EU limits (see supplementary information). (Sample PS1 was not analyzed for pesticides and PAH).



Highlights

- Temporal and inter-company variability in AHB biomass quality was considerable
- The essential amino acid profiles show high quality protein for animal feed
- To guarantee full safety, some contaminants should be eliminated
- Protein levels increase with increasing N loading rate and decreasing sludge age
- While optimization potential exists, some plants already show high quality

CRediT author statement

Maarten Muys: Supervision, Conceptualization, Methodology, Investigation, Writing, Reviewing and Editing, Data Curation, Visualization

Gustavo Papini: Methodology, Writing, Investigation

Marc Spiller: Methodology, Reviewing and Editing, Writing, Investigation

Myrsini Sakarika: Methodology, Investigation

Barbara Schwaiger: Resources, Writing

Céline Lesueur: Resources, Writing

Pieter Vermeir: Conceptualization, Supervision, Resources, Funding acquisition

Siegfried E. Vlaeminck: Conceptualization, Supervision, Reviewing and Editing, Writing, Resources, Funding acquisition