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A novel approach for the protein determination in food-relevant microalgae

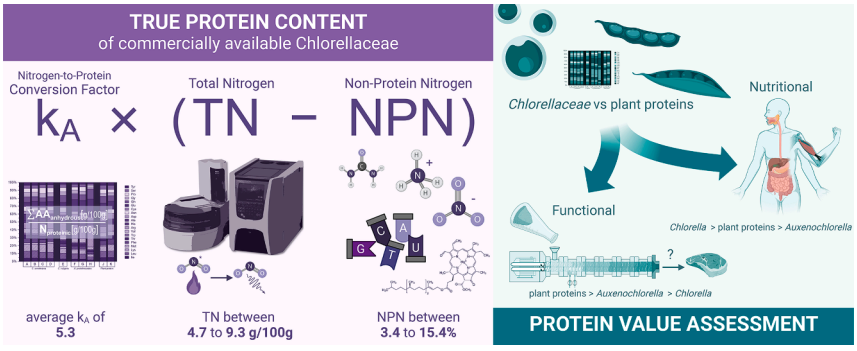
Corina Sägesser [a], Johanna M. Kallfelz [a], Samy Boulos [b,*], Laila Hammer [c,d,e], Lukas Böcker [a], Reto Portmann [c], Laura Nyström [b], Alexander Mathys [a,*]

[a] Sustainable Food Processing Laboratory, Department of Health Sciences and Technology, ETH Zurich, Schmelzbergstrasse 9, 8092 Zürich, Switzerland
[b] Laboratory of Food Biochemistry, Department of Health Sciences and Technology, ETH Zurich, Schmelzbergstrasse 9, 8092 Zürich, Switzerland
[c] Development and Analytics Research Division, Agroscope, Schwarzenburgstrasse 161, 3003 Bern, Switzerland
[d] Department of Health, Swiss Distance University of Health (FFSH), Zollstrasse 11, 8005 Zürich, Switzerland
[e] Division of Human Nutrition and Health, Wageningen University and Research, Stippeneng 4, 6708 Wageningen, Netherlands

HIGHLIGHTS

- Neither crude protein ($k_p \times$ TN) nor sum of amino acids accurate for protein content.
- Average k_A of eight commercially available Chlorellacea biomasses was 5.3.
- Confirmation of k_p of 4.78 for crude protein of microalgae.
- Considerable variation of non-protein nitrogen & amino acid profiles between species.
- *C. sorokiniana* with comparable indispensable-amino-acid-in-protein levels to egg.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae are gaining interest as food ingredient. Assessments of functional and nutritional properties are necessary to forward their implementation. In this study, protein content and composition of eight commercially available microalgae biomasses were determined and compared to conventional food proteins. A novel procedure for the determination of the true protein content was proposed: Multiplication of proteinic nitrogen with a sample-specific nitrogen-to-protein conversion factor k_A. The proteinic nitrogen was derived from the difference of total nitrogen minus non-protein nitrogen. The average k_A for microalgae was 5.3 and considerable variation between different microalgae biomasses were detected. In addition, the content of non-protein nitrogen varied between 3.4% and 15.4%. The amino acid profiles of *Chlorella* samples were nutritionally superior to the tested plant proteins but indicated lower protein interaction tendency, potentially limiting their structuring functionality. In contrast, *Auxenochlorella* contained lower amounts of indispensable amino acids while showing comparable interaction potential to plant proteins.

* Corresponding authors at: Department of Health Sciences and Technology, ETH Zurich, Switzerland.
E-mail addresses: samy.boulos@hest.ethz.ch (S. Boulos), alexander.mathys@hest.ethz.ch (A. Mathys).

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1. Introduction

Due to population growth and socio-economical effect, the demand for proteins is expected to keep rising (FAO, 2018). While animal sources are a major driver of climate change, a trend towards non-animal protein is emerging (Zhao et al., 2022). In this context, microalgae stand out with protein contents of up to 70 %, balanced amino acid profiles and ecological benefits such as their ability of being cultivated on non-arable land without freshwater and their higher productivity compared to terrestrial crops (Caporgno & Mathys, 2018; Wang et al., 2021). Especially in urban areas where arable land is scarce, such as Singapore, highly productive novel protein sources are sought-after (Mok et al., 2020). Because of population growth and soil depletion, such protein sources will gain importance in the coming years. Beyond that, microalgae are rich in vitamins, minerals and essential fatty acids and can consequently be utilized as functional food ingredients (Nazih & Bard, 2018).

Animal-free protein sources seem to gain highest consumer acceptance if processed into novel products that mimic animal products (Kyriakopoulou et al., 2018). Meat-like textures can be formed via extrusion of plant protein extracts. This research aims to assess the potential of microalgal biomass as extrusion ingredient. However, the determination of protein quantity and quality is not straightforward. The protein content of food is commonly quantified indirectly based on the total nitrogen (TN), which is determined via either elemental analysis or Kjeldahl. The nitrogen content is then multiplied with a nitrogen-to-protein conversion factor (k_p). 6.25 is generally used, even though, it had been known for long that this leads to systematic overestimations for biomaterials since it is based on an average nitrogen content of pure protein and therefore does not take account of non-protein nitrogen (NPN) which is present in food materials (Jones, 1941). Microalgae have been reported to contain up to 54 % NPN (Templeton & Laurens, 2015). Consequently, systematic overestimation of protein content is a known issue in the sector. Apart from overestimation, discussions about the validity of using one factor for all foodstuffs are on-going since early last century. Jones (1941) propounded that the nitrogen content of food proteins varied between 13 and 19 % due to different amino acid compositions and suggested to use specific factors for certain common foodstuff. According to FAO (2003), specific factors are to be used for grains, nuts, soybean products, milk and cheese. However, the soy industry and many analytical labs refuse to adapt their practice (Krul, 2019). Scientists propose to finally standardize the procedure to define and implement specific k_p for all relevant protein sources or to develop new direct methods to determine the protein content in food via amino acid analysis (Krul, 2019).

Various authors have studied the true protein content of microalgae via amino acid quantification and identified significant variations in k_p. According to a recent review, k_p for microalgae ranges between 3.00 and 6.35 and even within the genus of *Chlorella*, variations of 3.66 to 6.35 were reported (Acquah et al., 2020). The main reason for these variations lies in inconsistent amount of NPN. Lourenço et al. (2004) cultivated *Chlorella minutissima* under different conditions and analysed the NPN contents at different growth stages. They could demonstrate that NPN varied between 8.6 % and 35.3 % and it was generally highest in the exponential growth and under aeration. The major fraction of NPN was inorganic nitrogen which is known to act as cell-internal nitrogen storage. The second important fraction was nucleic acids. Chlorophyll was only of minor importance. On the other hand, the amino acid profile is reported to be relatively constant for different microalgae species and in different growth phases under nutrient replete conditions (Templeton & Laurens, 2015). However, metabolic stress has the potential to affect the profile. Despite all mentioned variations, scientist generally agree to use $k_p = 4.78$ to determine "crude protein" for microalgae as it results in much more realistic estimates than the general default factor of 6.25, which is still the industry standard (Acquah et al., 2020; Laurens et al., 2020; Lourenço et al., 2004; Tibbetts et al., 2015). Nevertheless, the importance of analysing the true protein content in scientific context is stressed and it is generally agreed that amino acid quantification is the most accurate method (Wang et al., 2021).

Amino acids are detected after hydrolysis via HPLC. Hydrolysis conditions are always a compromise between incomplete hydrolyses and amino acid degradation. It is recommended to run six different hydrolyses to optimize the amino acid recovery (Laurens et al., 2020; Templeton & Laurens, 2015). However, most commercial labs only execute two to three different hydrolysis. Internal standards can be included to estimate and correct losses (Deutsches Institut für Normung, 2005). In the end, the protein content is calculated as the sum of the detected amino acids in anhydrous form. Even though, in microalgae up to 40 % of the amino acids can be present as free monomers (Dortch et al., 1984). All in all, inaccuracies may be expected.

Besides k_p, the protein-internal nitrogen-to-protein conversion factor (k_A) can be determined in dividing total protein by overall nitrogen within protein (PN). Mossé (1990) suggested accounting for the losses during amino acid quantification by building the factor k as average of k_p and k_A. This elegant approach may be feasible for plant proteins but is not applicable for microalgae proteins due to the high and inconstant amounts of NPN. In contrast to k_p, k_A of microalgae protein generated under nutrient replete conditions is comparable to k_A of other food proteins and more consistent over various samples (Templeton & Laurens, 2015).

Hereinafter, true protein is estimated by multiplying k_A with the proteinic nitrogen derived from the difference of TN minus NPN. With this approach, losses of hydrolysis do not falsify results and NPN is accounted for. The true protein contents and amino acid compositions of microalgae biomasses are then compared to those of conventional extrusion raw materials to evaluate their suitability as future food ingredient. Protein content and composition influence not only the nutritional value of the final product but also the raw material's potential to form intermolecular bonds and thereby generate networks during processing. Different interactions between amino acids define the protein's interaction potential. The strongest protein–protein interaction is the covalent disulfide bridge between two cysteines (Mojab & Marcey, 2021). At neutral pH and low ionic strength, the salt bridge interaction between acidic and alkaline amino acids is the second strongest interaction, followed by the cation-interaction between protonated and aromatic amino acids as well as the amide bridge interaction in-between amidic amino acids (Xie et al., 2015). Besides, conventional interactions such as hydrogen bonds, Van der Waals forces and hydrophobic interactions play a role (Mojab & Marcey, 2021).

In the present work, commercially available Chlorellacea powders were screened for high protein content and low coloration. Many suppliers only focus on one of the mentioned criteria. Samples that only matched one criterion were therefore also included. It can be assumed that high protein products were grown under mild C-starvation (Molazadeh et al., 2019). For low coloration, chlorophyll-deficient strains can be cultivated heterotrophically (Schüler et al., 2020). Protein content, amino acid profile and NPN components of the selected samples were compared and their suitability for inclusion into food structures was judged from a functional and a nutritional perspective.

2. Materials & methods

2.1. Raw materials

All screened raw materials were received in form of powders. Photoautotrophic *C. sorokiniana* biomass was sourced from Roquette Klötze GmbH (Klötze, Germany; lot code: 2C26.09.24, received in March 2022) hereinafter referred to as powder A. Heterotrophically cultivated *C. sorokiniana* with different pigmentations were obtained from Langyatai Group Co. Ltd (Qindao, China; lot code: HKQ421075, received in April 2022), Duplaco B.V. (Oldenzaal, Netherslands; lot code: PL21E31-01Y, received in December 2021) and Allmicroalgae-Natural Products

SA (Pataias, Portugal; lot code: L2021WC007, received in February 2022) hereinafter referred to as powder B, C and D, respectively. Heterotrophically cultivated *C. vulgaris* biomass was received from Algenuity Limited (Bedfordshire, UK; lot code: BF100-01–2020, received in May 2021 hereinafter referred to as powder E. Yellow biomass of *A. protothecoides* was delivered from Daesang Corporation (Seoul, South-Korea; lot code: SCA24001, received in May 2022), from Sophie's Bionutrients (Wageningen, Netherlands, lot code: SBN202101, received in December 2021) and from Alver AG (Chardonne, Switzerland; lot code: C2B07-C318234, received in December 2018) hereinafter referred to as powder F, G and H, respectively. The soy protein concentrate Alpha 8 (Solae LLC, Missoursi, USA; lot code: R880002119) and pea protein isolate Nutralys F85M (Roquette, Lestem, France; lot code: W007T) were used as references due to their well-known structuring-ability during extrusion, hereinafter referred to as powder J and K, respectively. The powders' colours varied from whitish over yellow to green (see supplementary material). Species were determined via strain identification executed by GreenCoLab (Faro, Portugal). To this end, GreenCoLab developed specific primer pairs (see supplementary material) to amplify the rcbL and ITS gene sequences of the *Chlorella* genus. After DNA extraction, rbcL and ITS genes were amplified and Sanger-sequenced. For both sequences, phylogenetic trees using Maximum Likelihood were constructed based on literature research and bioinformatic analysis (see supplementary material).

2.2. Nitrogen

The total nitrogen content was determined via chemiluminescence of combusted suspensions by a total organic carbon analyser coupled with a total nitrogen module (TNM-L, Shimadzu, Japan) and via Kjeldahl. Measurements were executed in at least triplicates for TNM-L and in duplicates for Kjeldahl. Moreover, nitrogen solubility was assessed by TNM-L-analysis of nitrogen in the supernatant of centrifuged powder suspensions. For that, powders were suspended at 1 % (w/w) and hydrated for 90 min at room temperature. 45 mL of these suspensions were then centrifuged in 50 mL tubes at 10′000 rcf for 15 min. The nitrogen solubility index (NSI) was calculated as the quotient of the nitrogen in the supernatant to the (total) nitrogen in the uncentrifuged suspension. Hence, soluble nitrogen components that were trapped in intact cells or attached to insoluble cell debris are not accounted for in this index.

2.3. Amino acid analysis

All powders were sent to Eurofins Scientific AG (Schönenwerd, Switzerland) to get their amino acid contents analysed. Eurofins executed an alkaline hydrolysis for tryptophan based on the ISO method 13904:2016 and an oxidative hydrolysis for cysteine and methionine as well as an acidic hydrolysis for all remaining amino acids based on the ISO method 13903:2005. The hydrolysed amino acids were quantified via ion exchange chromatography (IC-UV) resp. liquid chromatography (LC-FLD) for tryptophan. Eurofins did single determinations without use of internal standards. Three samples were chosen to be analysed in duplicates to get an idea of the variability.

The Eurofins analysis does not differentiate between amidic and acidic amino acids but provides the sum of both (Asx = Asn + Asp and Glx = Gln + Glu). The degree of amidation was later analysed in our labs. Amide nitrogen (N_{amide}) from the side chains of Gln and Asn was quantified according to Mossé et al. (1985). In short, 1 g of powder and 20 mL of 2 M HCl were incubated under stirring in a sealed Pyrex tube at 115 °C for 3 h to liberate the amide ammonia. The whole suspension was transferred into the steam distillation unit B-324 (Büchi Labortechnik AG, Flawil, Switzerland) with the help of 30 mL H_2O and neutralized with 10 mL of 4 M NaOH. 10 mL of 1 M carbonate buffer (pH 10.5) were added, followed by a steam distillation of 5.5 min whereby approximately 100 mL of distillate were collected in 30 mL of 40 g/L boric acid solution. 15 drops of Tashiro indicator were added. 0.025 M HCl was then titrated into the mixture until the colour changed from green to pink. Based on the amount of HCl required for the colour change, the total ammonia after hydrolysis could be calculated, which is the sum of ammonia released from amidic sidechains and free ammonia. Free ammonia was determined by means of an adapted procedure: 1 g of powder was suspended in 60 mL of water and 10 mL of 1 M carbonate buffer. The pH of the suspension was adjusted to 10 by addition of NaOH. The suspension was distilled again into 30 mL boric acid without prior incubation and the amount of free ammonia determined according to the method described above. N_{amide} was calculated as difference of both procedures described. The degree of amidation was determined by the quotient of molar amount of N_{amide} to the sum of the molar amounts of Glx and Asx. All N_{amide} quantifications were executed in triplicates. In addition, control experiments confirmed high recovery of Asn and Gln amide nitrogen and no ammonia release of other amino acids.

Due to discrepancies between total nitrogen detected and the sum of nitrogen from all amino acids, it was decided to repeat amino acid analysis internally including internal standards to control for the losses during hydrolysis. The tryptophan analysis was performed as described in Walther et al. (2022), which involved alkaline hydrolysis at 110 °C for 20 h and subsequent detection by UHPLC-UV. All other amino acids were measured according to ISO 4214 | IDF 254:2022 (Jaudzems & Fuerer, 2022), with modifications described elsewhere (Sousa et al., 2023). Briefly, after acidic hydrolysis at 110 °C for 24 h, hydrolysates were neutralized, derivatized with AccQTag Ultra reagent (Waters, Switzerland), and amino acids measured by UHPLC. The internal standards methyl-tryptophan (for tryptophan) and L-Norvaline (for all other amino acids) were used to correct for losses. Since Asn and Gln are converted to Asp and Glu, only their sums Asx and Glx were determined. Four samples were analysed in triplicates. The others were analysed in duplicate.

2.4. Non-protein nitrogen

Apart from inorganic nitrogen, urea can be used as nitrogen source for *Chlorella* cultivation (Ribeiro et al., 2020). There was only little information available on cultivation and harvesting of the commercial biomasses. Thus, media residues cannot be excluded. It was therefore decided to not only determine the content of ammonia and nitrate but also urea in the commercial biomasses. 10 % (w/v) suspensions of biomass in 1 M carbonate buffer were prepared (pH 10) and heated at 100 °C for 10 min under stirring for cell disruption. The suspension was then centrifuged at 10′000 rcf for 15 min to remove cell debris. Urea was determined spectrophotometrically in the supernatant according to Zawada et al. (2009) after neutralization with 1 M HCl. In short, a reagent mix consisting of 100 mg/L phthaldialdehyde, 513 mg/L primachin-diphosphate, 2.5 g/L boric acid, 245.2 g/L sulfuric acid and 300 mg/L Brij-35 as well as urea standard solutions with concentrations of 10, 30, and 50 mg/L were prepared. 50 μL of neutralized biomass suspension supernatant were mixed with 200 μL of reagent mix in clear flat-bottom 96-well-plate, incubated in the dark for 3 h at room temperature and its optical density (OD) was determined at 430 nm with a plate reader (Tecan Group AG, Männedorf, Switzerland). Blank measurements were executed correspondingly but without phthaldialdehyde and primachin-diphosphate in the solvent mix and their OD was subtracted from OD of the reagent mix. Concentrations were calculated based on the standard curve. Spiking with urea indicated high linearity of results.

Ammonia and nitrate content were estimated via test stripes MQuant from Merck Supelco (Darmstadt, Germany). Supernatants of suspensions of untreated as well as disintegrated biomass (alkaline heat treatment mentioned above) were evaluated. Treated suspensions were coloured and no conclusive estimations for ammonia test stripes were possible. Nitrate test stripes also worked for treated suspensions if they did not contain substantial amounts of chlorophyll (powder D, E, F, G, H). For these samples, the results for untreated and treated suspensions were

equal. Besides, nitrate is presumed to passively cross biological membranes in form of nitrous acid (Samouilov et al., 2007). Thus, equilibrated concentration inside and outside cells is expected. It was therefore decided to use the nitrate test stripes in unheated suspensions. In contrast, ammonia was measured, as mentioned above, as part of the degree of amidation determination via titration.

The total amount of nucleic acids (NA) was also determined spectrophotometrically by measuring the absorption at 260 nm with a NanoDrop 8000 (Thermo Fisher Scientific Inc., Waltham, USA). Hence, NA needed to be isolated from the biomass. First, 5 to 20 mg of biomass and 750 µL of DNA/RNA Shield (1X from Zymo Research Europe GmbH, Freiburg, Germany) were weighed into a ZR BeadBashing Lysis Tube with 0.1–0.5 mm beads (Zymo Research Europe GmbH, Freiburg, Germany), cooled in an ice bath and 4x bead-bashed at 8 m/s for 2 cycles of 20 s with 20 s pause, always cooled in ice in between. The subsequent isolation was executed with and according to the Quick DNA/RNA Viral Kit from Zymo Research Europe GmbH (Freiburg, Germany). Briefly, 300 µL of supernatant of bead-bashed, centrifuged biomass suspension was mixed with 600 µL of Viral DNA/RNA buffer of which 800 µL were transferred into Zymo-Spin IIC-XLR Column and centrifuge. Column was twice washed with 500 µL of Viral Wash Buffer plus 500 µL of ethanol. NA were collected within two washes of 100 µL nuclease-free water. Control measurements of a second passage of the biomass-supernatant-buffer-mix through a fresh column and a third rinse of the column with nuclease-free water indicates that the procedure could isolate a major fraction of all NA. The nitrogen content of NA equates to 16.84 % for DNA resp. 16.12 % for RNA under the assumption of equal fractions of all for nucleotides (Dortch et al., 1984). The average of 16.48 % was used for the calculation of NA-N.

Chlorophyll (Chl) was not analytically determined since it is reported to only make up for a small fraction within the NPN. Lourenço et al. (2004) reported 0.2–1.8 % Chl-N for *C. minutissima* under mixotrophic growth condition. This is negligible compared to cumulative uncertainty of the sum of all NPN determined in this work. Nevertheless, rough assumptions based on the green cast were executed. Dark green samples were assumed to contain 1 %, bright green 0.4 % while yellow as well as white samples lack chlorophyll-N.

All analytical NPN determination were executed in triplicates except for nitrate content quantification which was run in duplicates. The whole analytical process was illustrated in Fig. 1.

2.5. Protein content

k_p and k_A were both determined based on the sum of anhydrous amino acids (AAA), divided by TN (from elemental analysis) and PN (from amino acids), respectively as indicated in the following equations:

$$k_p = \sum AAA/TN \quad (1)$$

$$k_A = \sum AAA/PN \quad (2)$$

The true protein content was calculated according to the following equations:

$$Protein\,content = k_A{}^*(TN - NPN) \quad (3)$$

Whereby the nitrogen-to-protein conversion factor k_A was determined individually for every powder. Thus, true protein is not determined as sum of AAA but taking more factors into account. It was reasoned that this is the more accurate way due to discrepancies in total AAA content.

The nutritional quality of the protein was evaluated by assessing the amino acid composition. Digestibility was not included since it has not been properly examined for most of the analysed raw materials, yet. The indispensable amino acid score (IAAS) was calculated after identifying the limiting amino acid via equation (4) based on the WHO requirement pattern (WHO, 2007).

$$IAAS = \frac{share\ of\ limiting\ AA\ test\ protein}{share\ of\ limiting\ AA\ in\ requirement\ pattern} \quad (4)$$

2.6. Data analysis

Due to small sample size of $n \leq 3$ no statistical analyses were executed in this study. The results were compared using mean and standard deviation.

3. Results & discussion

The nitrogen solubility index (NSI) serves as estimation of protein solubility which is dependent on protein composition and conformation.

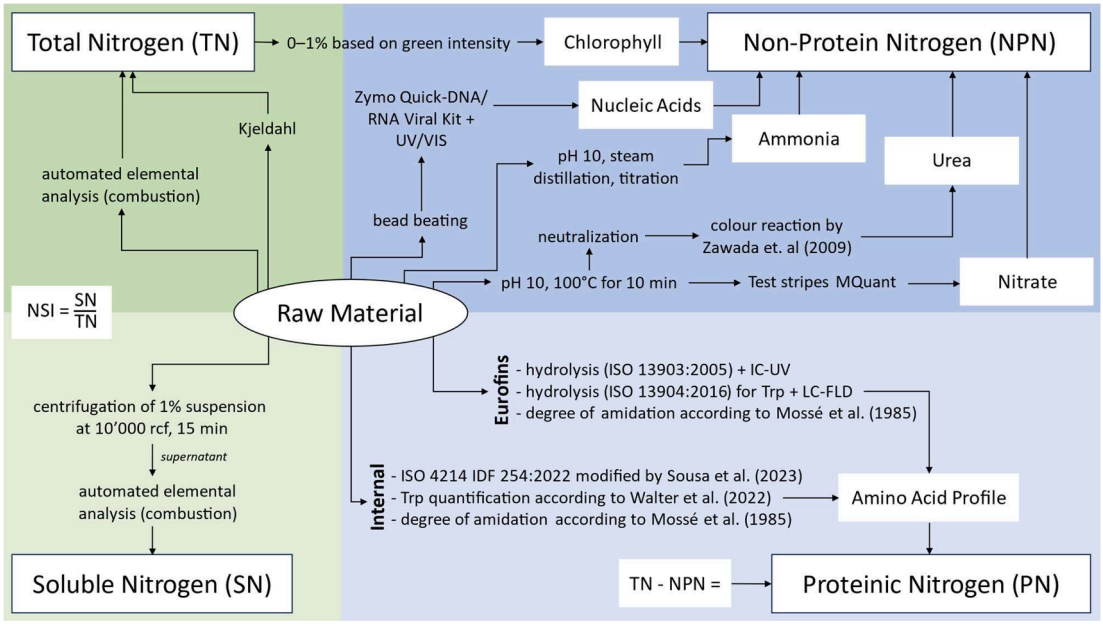

Fig. 1. Schematic illustration of analytical steps for the determination of total nitrogen (TN), soluble nitrogen (SN), non-protein nitrogen (NPN), proteinic nitrogen (PN) and nitrogen solubility index (NSI).

For the observed samples, it varied between 12 % and 58 %. *Chlorella sorokiniana* samples had the lowest NSI with 12 % to 19 %, while *Auxenochlorella protothecoides* had the highest NSI with 40 % to 58 %. The *Chlorella vulgaris* sample had an NSI of 34 % which was similar to the NSI of plant proteins, that ranged from 22 % to 34 %. For plant proteins, the NSI is dependent on the native protein composition as well as the extraction process (Ebert et al., 2020). During extraction, protein denaturation and aggregation decrease solubility (Grossmann & McClements, 2023). For NSI of microalgae, another contributing factor is the cell integrity. Intact cells enclose soluble proteins (Machado et al., 2022). Therefore, not only NSI was analysed but also microscopic images were studied (see supplementary material). For powders A, B and C cell debris is visible in representative pictures while NSI was below 20 %. This indicates that proteins were either denatured or are still entangled to cells and debris. Most damaged cells are visible for powder E. Only few broken cells are visible for powder F, G and H whereas their NSI was above 40 %. The presence of potential exoproteins or cell wall poration may explain the higher NSI. The NSI seems to correlate more with the species than with the cultivation and harvesting. For all microalgae samples, intact cells seem to be present which emphasized the importance of cell disintegration for compositional analytics. On the other hand, no more intact cells are visible for the plant proteins and especially powder K was very homogeneous. However, the NSI of plant proteins was not higher but comparable. Thus, protein solubility does not seem to bear a functional advantage for structuring.

3.1. Nitrogen content

Elemental analysis and Kjeldahl lead to comparable results for total nitrogen (TN). However, the proteinic nitrogen (PN) from the amino acids detected by Eurofins was notably higher than TN for two of the samples (powder K and J). It was therefore decided to repeat the amino acid analysis internally. The PN of the internal amino acid analysis was similar or lower than TN, as illustrated in Fig. 2. Lower PN can be explained by presence of non-protein nitrogen (NPN). PN of Eurofins was in average 12 % higher than PN determined internally. The discrepancy between PN of the two analyses may arise from different hydrolysis efficiency, as hydrolysis conditions are a compromise between incomplete hydrolysis and degradation (Boulos et al., 2020).

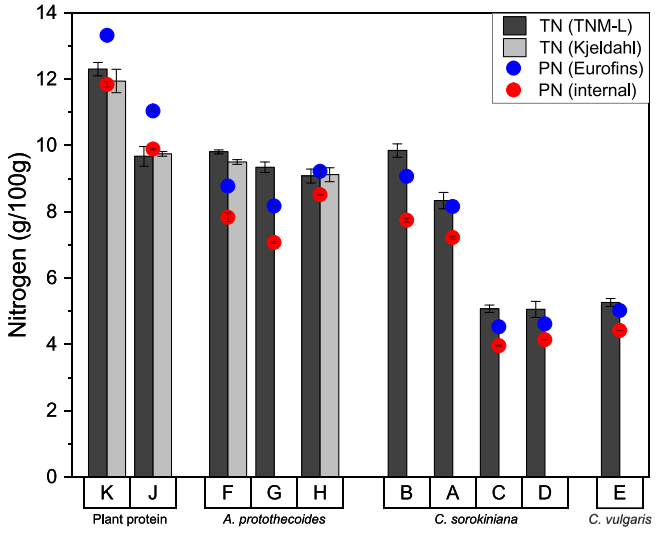

Fig. 2. Total nitrogen (TN) of sample weight analysed via elemental analysis (TNM-L) and Kjeldahl compared to nitrogen from protein (PN) analysed by Eurofins Scientific AG and internal analysis. Error bars represent deviation between duplicate measurement respectively standard deviations of triplicate measurements (n = 3 for TN_{TNM-L} and $PN_{internal}$ of F and G; n = 2 for $TN_{Kjeldahl}$, $PN_{Eurofins}$ and all other $PN_{internal}$ determinations).

Nevertheless, the relative amino acid compositions of both analyses were very similar (see supplementary material). Thus, k_A from both determinations were comparable as well.

3.2. Nitrogen-to-protein conversion factors

The protein conversion factors k_p and k_A are shown in Fig. 3. There was less variation of k_A than of k_p. This finding is consistent with the review of Templeton et al. (2015). With an average of 5.3, microalgal k_A were lower than reported in previous studies, while k_p was generally higher (Templeton & Laurens, 2015). This implies a lower NPN content than previously observed. Powder H had a considerably lower k_A than the other *A. protothecoides* samples, which indicates major differences in amino acid profile. It is not clear if this difference was strain-specific or induced by external stress.

3.3. Non-protein nitrogen (NPN)

Detected NPN ranged from 3.4 % to 15.4 % for microalgal biomass and was below 2 % for plant proteins, as illustrated in Fig. 4a. The results are in line with the rule of thumb from Becker (2013), stating that the NPN content is around 10 % for microalgae. However, Templeton et. al (2015) reported NPN values above 36 % for *C. vulgaris* at various growth stages. In contrast, Lourenço et al. (2004) detected variation of NPN between 8.6 % and 35.3 % for *C. minutissima* under different cultivation conditions. They reported that NPN was generally lower under carbon limitation as well as in the stationary growth phase. Carbon starvation results in increased C/N assimilation ratio while C/N ratio of the biomass decreases simultaneously (Myers & Cramer, 1948). Combining these findings, carbon starvation leads to biomass with high protein and low inorganic nitrogen contents. Since for this study, biomass with high protein content were screened, it is well possible that the NPN contents are at the lower edge of the range. Besides, the content of organic compounds such as nucleic acids and chlorophyll is most probably correlated to total biomass and thereby decreases relatively if the protein content rises.

The composition of NPN varied upon all samples and does not seem to be species-specific. It more probably depends on cultivation and harvesting conditions. Major amounts of ammonia were determined for powder G and F, indicating the presence of intra-cellular storage pools. In contrast, the third *A. protothecoides* biomass, powder H, contained nearly no inorganic nitrogen. Nitrate was only detected in powder C and K. As discussed previously, the chosen nitrate quantification method may only detect free nitrate and it was assumed that the concentration inside and outside intact cells equilibrates due to passive transport. Assuming that no equilibration took place, but free nitrate ratio corresponds to NSI, based on the detection limit of the chosen method, the maximal nitrate level the other analysed microalgae samples could contain is 0.4 % NO_3^--N in total N, which is still negligible. Under these assumptions, powder C could contain up to 6 % NO_3^--N in total N. Thus, it would make sense to reassess the total nitrate and nitrite content of powder C with a more suitable method. The detected levels of urea were low in all samples. The detected nitrogen from nucleic acids (NA-N) in total N ranged from 1 % to 5.8 % for microalgae and were generally lower for plants. This difference is expected as single cell organism especially in the exponential phase are reported to contain high levels of NA (Lourenço et al., 1998; Nasseri et al., 2011). The presence of NA does not only reduce the real k_p, but may also impose adverse health effects as risk factor for kidney stones and gout (Gantar & Svirčev, 2008). A healthy adult should not consume more than 2 g per day and Nasseri et al. (2011) argues that food ingredient shall not contain more than 2 % (w/w). All samples of this study except powder A with 2.5 % and powder G with 2.1 % were below this threshold.

Summing up, it seems crucial to test for NPN in microalgal biomasses and to develop more streamlined NPN determination protocols as biological variability does not allow to perform accurate estimations.

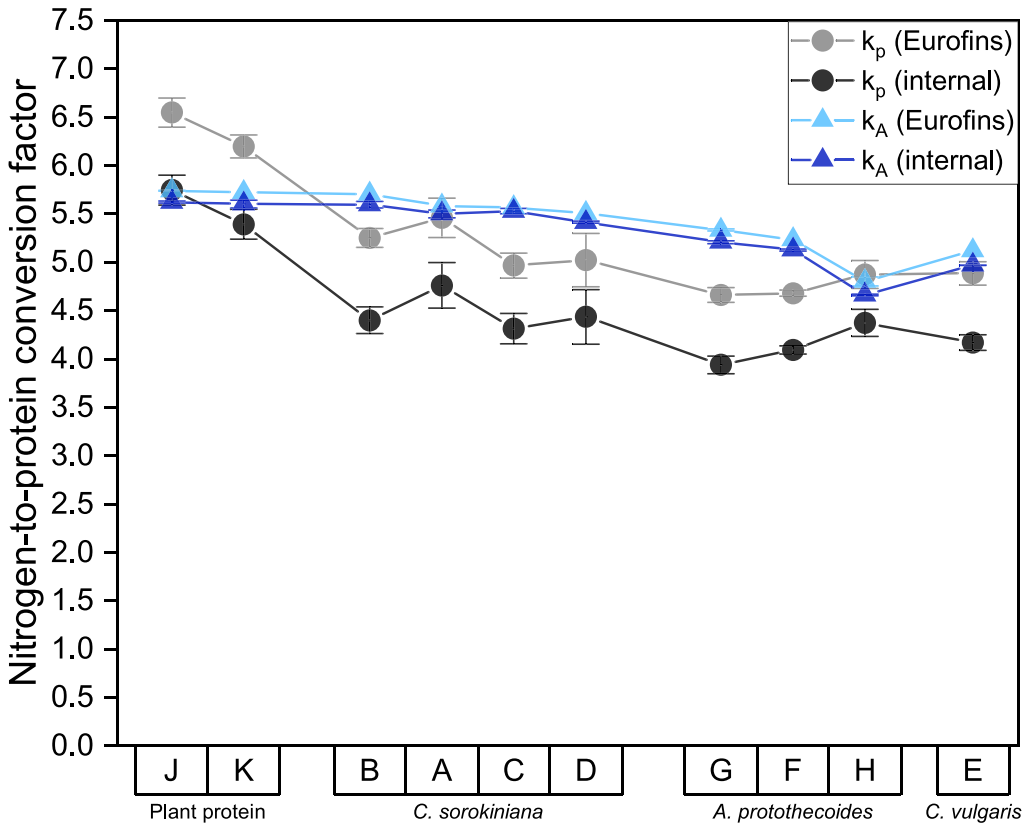

Fig. 3. Nitrogen-to-protein conversion factors k_p and k_A from two independent amino acid quantifications (Eurofins vs. internal analysis) of the same samples. Error bars represent deviation between duplicate measurement respectively standard deviations of triplicate measurements (n = 3 for internal k_A and k_p determination of G and F, n = 2 for the rest).

In Fig. 4b, protein nitrogen (PN) was determined as the difference between TN (from elemental analysis) and NPN. The derived values were on average 4 % lower and 7 % higher than the PN based on Eurofins' amino acid profile and our internal amino acid quantification, respectively (displayed in Fig. 2). The lower values of our internal amino acid quantification may be explained by incomplete hydrolysis. This underlines that true protein cannot be accurately determined by just summing up all detected amino acids.

3.4. Protein content

The final protein content, henceforth referred to as true protein, was calculated based on Equation (3), whereby the sample-specific k_A was the average of the k_A(Eurofins) and k_A(internal). It was then compared to the crude protein estimation based on TN (from elemental analysis), using the generic k_p of 4.78 suggested for microalgae by Lourenço et al. (2004), illustrated in Table 1.

In this study, the variation of crude protein to true protein was in the range of ± 10 %. On average, crude protein matched true protein very well (36.9 vs 37.7 g/100 g, respectively). Thus, our data reinforces that a k_p of 4.78 should be used for microalgae in absence of other more accurate quantification methods. However, we advise to execute more detailed analysis such as the determination of NPN for reliable protein estimations.

3.5. Protein composition

The full amino acid (AA) profile was determined by averaging two independent amino acid analysis and comparing it to indispensable AA (IAA) requirement for healthy adults (see Fig. 5). Glx and Asx were divided into their respective amidic and acidic AA assuming equal amidation in both fractions. Contrary to the literature, AA profiles of different microalgae samples varied considerably, with less differences of AA composition within a species than between species. *C. sorokiniana* had similar profiles to those reported by Templeton et al. (2015). In that same study, N-starvation was reported to increase Ala by 30 %, decrease Glx by 20 % and slightly decrease Lys and Arg. In the present work, powder A contained lowest levels of Glx and Arg. Powder A was also the only biomass cultivated photoautotrophically. On the other hand, powder E to H contained highest amounts of Glx and Arg. This might be the result of C-starvation which is known to have opposed effects to N-starvation on biomass composition (Cai et al., 2022). However, powder H contained 3.8 and 3.2 times more Arg and Glx, respectively, than powder A. To our knowledge, such deviations have not been reported before in microalgal amino acid profiles. Even under copper stress, Arg is reported to only decrease by 10 %, while Gly increased by 20 % (Shakya et al., 2022). As noted earlier, powder H did not contain relevant amounts of inorganic nitrogen which could be an indication of severe C-starvation that might explain the abnormal amino acid profile (Cai et al., 2022). Further research is required to understand the role of genetics, cultivation conditions and harvesting procedure on the AA profile.

C sorokiniana samples contained with above 39.8 g / 100 g protein the highest levels of indispensable amino acids (IAA). These levels are comparable to the ones of egg protein of 40.5–42.7 g / 100 g (Attia et al., 2020). Egg is generally known to be an excellent source of IAA (Puglisi & Fernandez, 2022). *C. vulgaris* had comparable levels to the two plant proteins. *A. protothecoides* had lower amounts of IAA than all other analysed samples. Compared to IAA requirements defined by WHO (2007), all examined *Chlorella* samples had no limiting IAA. In contrast, the amino acid score of powder F to K are all limited by sulphur-containing AA. For powder F, G and J the score is around 80 % while

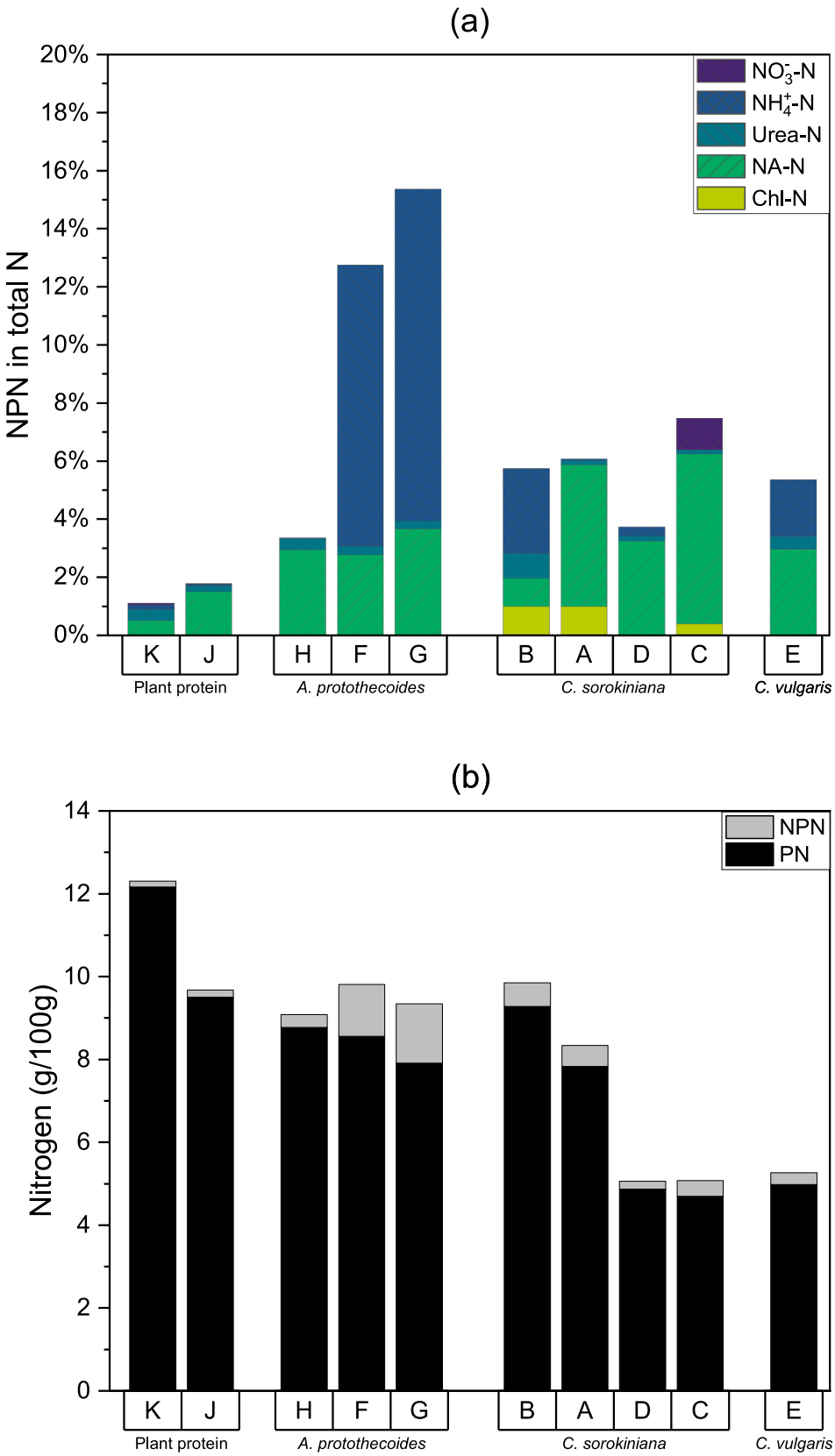

Fig. 4. Proportion of non-protein nitrogen (NPN) from NO_3^-, NH_4^+, urea, nucleic acids (NA) and chlorophyll (Chl) in subfigure a. Protein nitrogen (PN) content in sample as difference of total nitrogen (TN) based on elemental analysis minus determined non-protein nitrogen (NPN) in subfigure b. (n = 3).

Table 1
Protein content (g/100 g) based on protein-nitrogen (PN = TN – NPN) multiplied with sample-specific k_A, here called "true protein", vs. total nitrogen multiplied by generic k_p of 4.78 suggeted for microalgae by Lourenço et al. (Lourenço et al., 2004) here called "crude protein".

		True protein (PN × k_A)	Crude protein (TN × 4.78)	Crude/true protein
Plant proteins	K	**68.9** ± 2.6		
	J	**54.0** ± 2.3		
A. protothecoides	F	**44.3** ± 1.0	46.9 ± 0.3	106 %
	G	**41.7** ± 1.6	44.7 ± 0.6	107 %
	H	**41.7** ± 2.0	43.4 ± 0.7	104 %
C. sorokiniana	B	**52.5** ± 1.8	47.1 ± 0.8	90 %
	A	**43.4** ± 2.3	39.9 ± 1.5	92 %
	D	**26.6** ± 1.8	24.2 ± 1.2	91 %
	C	**26.0** ± 1.1	24.3 ± 0.6	93 %
C. vulgaris	E	**25.1** ± 1.4	25.2 ± 0.7	100 %
Microalgal average		**37.7**	36.9	98 %

it is between 60 and 65 % for powders H and K. Powder H does not meet the requirements for four other IAA besides sulphur-containing AA. Thus, while the *A. protothecoides* samples had highest protein content at relatively neutral colour, their protein is less nutritionally valuable regarding the amino acid profile. This rough analysis of the IAA balance provides a good initial indicator to quickly assess nutritional protein quality. However, digestibility must be considered for more adequate evaluation. This is especially relevant since several cell-wall containing microalgae are known to be hard to disintegrate (Acquah et al., 2020). Thus, further research is needed to assess bioaccessibility.

Last but not least, AA sidechains influence the interaction potential between proteins. In this study, only the general composition was studied, not the individual proteins. This can only provide indications of potential interactions. Cys is known to form the strongest intermolecular interactions (Mojab & Marcey, 2021). Apart from powder H, all samples contained comparable amounts of cysteine of approx. 1.2 %. Powder H contained 0.8 % and is therefore expected to have lower interaction potential than the other powders. The main difference between examined *C. sorokiniana* samples and conventional meat analogue raw materials was a higher Ala and lower Glx content. Ala is mainly involved in weak hydrophobic interaction. Glx may build amide bridges (Xie et al., 2015). Thus, low Glx level reduces the amide bridge building potential. The *C. vulgaris* sample had higher amounts of Ala and Arg than the plant proteins. Arg may interact via ionic bridges with acidic AA while Ala participates in weak hydrophobic interactions (Xie et al., 2015). *A. protothecoides* samples mainly contained more Arg and Glx compared to conventional raw materials, while all other AA were lower. Thus, more amide and ionic bridges are expected on the expense of all other interactions. In summary, *C. sorokiniana's* interaction potential seems to be generally lower compared to plant proteins, while *C. vulgaris* and *A. protothecoides* samples contained more ionic and amide bridge-building AA on the cost of other potentially interactive sidechains. However, the real effect of these compositional variation on structuring needs to be studied during food processing trials, such as extrusion.

4. Conclusion

The present work illustrated that neither crude protein nor the sum of anhydrous amino acids is a reliable protein quantification method for microalgal biomass. Instead, the multiplication of k_A with proteinic nitrogen was proposed. Since k_A of different samples ranged from 4.8 to 5.7, sample-specific k_A values had to be used. Future identification of

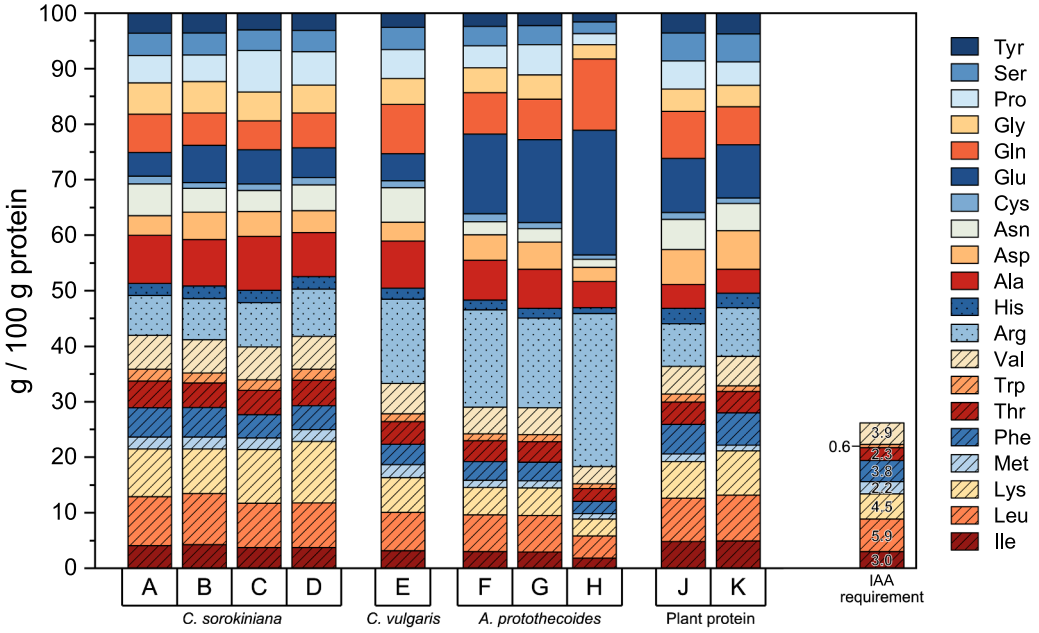

Fig. 5. Amino acid profiles of algal biomass and plant protein extracts, indispensable and semi-indispensable AA marked with dashes and dots, respectively (n = 3). On the right side, indispensable AA requirement for healthy adults defined by WHO (WHO, 2007) are illustrated for comparison.

generic k_A for specific species-cultivation combination may economise this quantification. Additionally, streamlined non-protein nitrogen quantification would accelerate the process. Accurate protein analysis is essential for evaluating functionality and nutritional value of food ingredients and will become increasingly relevant as microalgae gain prominence in the food industry.

CRediT authorship contribution statement

Corina Sägesser: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Johanna M. Kallfelz:** Investigation, Writing – review & editing. **Samy Boulos:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Laila Hammer:** Investigation, Validation, Writing – review & editing. **Lukas Böcker:** Conceptualization, Supervision, Writing – review & editing. **Reto Portmann:** Funding acquisition, Writing – review & editing. **Laura Nyström:** Funding acquisition, Writing – review & editing. **Alexander Mathys:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129849.

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