Title:

High variability in nutritional value and safety of commercially available Chlorella and Spirulina biomass indicates the need for smart production strategies

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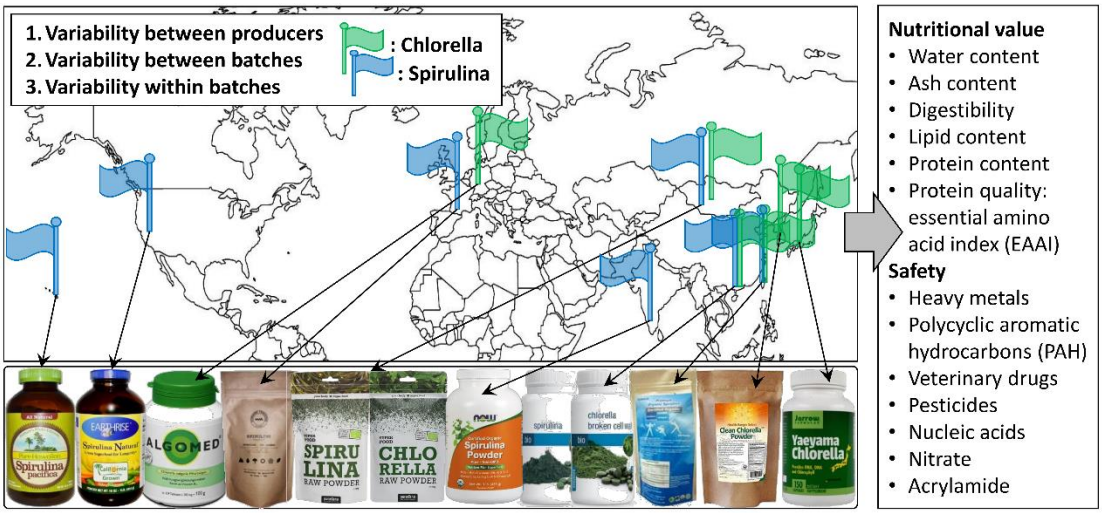
Abstract

Microalgal biomass production is a resource-efficient answer to the exponentially increasing demand for protein, yet variability in biomass quality is largely unexplored. Nutritional value and safety were determined for Chlorella and Spirulina biomass from different producers, production batches and the same production batch. Chlorella presented a similar protein content (47±8%) compared to Spirulina (48±4%). However, protein quality, expressed as essential amino acid index, and digestibility were lower for Chlorella (1.1±0.1 and 51±9%, respectively) compared to Spirulina (1.3±0.1 and 61±4%, respectively). Generally, variability was lower between batches and within a batch. Heavy metals, pesticides, mycotoxins, antibiotics and nitrate did not violate regulatory limits, while polycyclic aromatic hydrocarbon levels exceeded the norm for some samples, indicating the need for continuous monitoring. This first systematic screening of commercial microalgal biomass revealed a high nutritional variability, necessitating further optimization of cultivation and post-processing conditions. Based on price and quality, Spirulina was preferred above Chlorella.

Keywords

Arthrospira platensis, *Chlorella vulgaris*, nucleic acids, food supplement, cyanobacteria

Graphical abstract



Highlights

- Variability in Chlorella and Spirulina products demands process optimization
- A high protein or lipid content does not necessarily imply a high nutritional value
- Safe consumption doses indicate capacity as protein source rather than as supplement
- Potential PAH contamination requires systematic control to guarantee product safety
- Based on price and nutritional quality, Spirulina was preferred above Chlorella

1. Introduction

Microalgal biomass is an emerging source of sustainable protein that could meet predicted global protein requirements. However, microalgae have not gained significant importance as food protein source (Draaisma et al., 2013; OECD, 2013). Major obstacles are the rather high production costs as well as technical difficulties to incorporate dried algal powder into generally accepted conventional food (Becker, 2007). Interestingly, recent technical improvements in reactor design, production and post-processing techniques and successful research towards high-value compounds resulted in a more efficient microalgae production at lower cost (Enzing et al., 2014). In addition, increasing awareness of environmental problems related to the demographic explosion, as well as the high ecological footprint of conventional agriculture, resuscitated the interest in microalgae as a sustainable protein source with additional functional quality, in food and feed applications (Verstraete et al., 2016; Vigani et al., 2015). This translates in a considerable growth expectation of the global microalgae market in the years to come (Pulz & Gross, 2004).

Biomass of the cyanobacterium *Arthrospira* spp., known as "Spirulina", and the green microalga *Chlorella* spp. has been commercially produced at large scale for food and feed applications since the early 1960s. *A. platensis, A. maxima, C. vulgaris* and *C. pyrenoidosa* are the most commonly utilized species at a commercial level. Currently, the estimated global production volumes of Chlorella and Spirulina are 6600 and 12000 tons of dry matter per year, respectively (Frost & Sullivan, 2015; Garcia et al., 2017). The global Chlorella market price was estimated to be 28.7 €/kg in 2014 with a 28.4% compound annual growth rate (CAGR) (Frost & Sullivan, 2015), while the market price of Spirulina was 24€/kg in 2014, growing at a CAGR of 10% (Garcia et al., 2017).

Furthermore, Chlorella and Spirulina gain increasing attention as a protein source in regenerative life support systems (RLSS). Examples are the MELiSSA concept of the European Space Agency (ESA) in which Spirulina plays a vital role to upgrade nutrients to a high-value dietary protein source while providing the crew of oxygen (Clauwaert et al., 2017), and the PBR@LSR concept of the German Aerospace Center (DLR) applying Chlorella for similar purposes (Keppler et al., 2018).

Variability of nutritional value exists not only among species and strains but also within the same strain (Chacon-Lee & Gonzalez-Marino, 2010; Hu, 2004). Depending on cultivation parameters such as temperature, pH, nutrient concentrations, light quality, light intensity and photoperiod, protein values are recorded between 7 and 70% dry weight (DW) for *C. vulgaris* and between 17 and 73% DW for *A. platensis* (Figure 1). Protein data should, however, always be interpreted carefully as many researchers overestimate protein content based on a total nitrogen (N) or Kjeldahl-N measurement, also including non-protein nitrogen (Maehre et al., 2018). In literature, species-specific nitrogen-to-protein conversion factors are suggested, even though it was shown that these factors cannot be considered constant (Safi et al., 2013). Besides protein, also lipid content depends on cultivation conditions with observed values between 12 and 53% DW for *C. vulgaris* and between 9 and 17% DW for *S. platensis* (Piorreck et al., 1984). Finally, biomass post-processing can have adverse effects on nutritional quality. An example is freeze-drying which can result in a 5% protein loss, and convective drying with a potential 27% protein loss (Desmorieux & Decaen, 2005). Most commercial production systems for microalgae are open ponds, harder in control compared to closed photobioreactors. Only when the exact effects of production parameters and process

conditions on nutritional quality are known, fine-tuning is possible to alter the microalgal metabolism in favor of the particular compound of interest.

In addition to nutritional characteristics, biomass quality is based on the level of potentially hazardous components such as heavy metals, polycyclic aromatic hydrocarbons (PAH), toxins, pathogens and pesticides. European legislation sets maximum residue levels for contaminants in food supplements for heavy metals (cadmium, mercury and lead) and PAH (PAH4: benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene). Allergens, toxins, pathogens and pesticides were also detected in microalgal biomass (van der Spiegel et al., 2013), however, no maximum levels are set. Multiple sources of these hazardous components exist. Since microalgae production often takes place using surface or groundwater and nutrients are supplied from commercial fertilizers, microalgae can accumulate toxic compounds present in these resources (Al-Dhabi, 2013). Additionally, open pond cultivation allows pathogens to occur (van der Spiegel et al., 2013). Further, microalgae contain nucleic acids (DNA and RNA), of which human overconsumption causes increased levels of uric acid in the blood, leading to gout (Edozien et al., 1970). Lastly, improper post-processing (e.g. thermal treatment, drying) can be a potential source of PAH contamination (Zelinkova & Wenzl, 2015).

Current research that determines nutritional value or safety of full-scale produced Spirulina and Chlorella (Al-Dhabi, 2013; Campanella et al., 1999; Kent et al., 2015; Ortega-Calvo et al., 1993) investigated only a limited amount of products. In addition, a systematic approach to determine the exact magnitude of nutritional variability in industrial quality microalgae is lacking. Furthermore, some biomass characteristics are rarely determined such as protein quality (i.e. essential amino acid profile), digestibility

and the content of heavy metals, PAH, nucleic acids and nitrate. Some contaminants such as pesticides, mycotoxins and antibiotics were even never determined before in commercial microalgal biomass. Finally, the variation between production batches and within the same batch produced at one company was never researched.

In view of this knowledge gap, this study aims at defining the variability in nutritional quality and safety of microalgae originating from different companies situated worldwide. Doing so, the viability of process optimization was assessed to increase product quality (i.e. nutritional value and safety), while also the nutritional parameters with a large potential improvement were determined. Furthermore, nutritional variability was defined between production batches and within a production batch from one company. The analyzed parameters were also used to evaluate package information and to make a price-quality comparison between Chlorella and Spirulina. Finally, safe consumption doses were determined based on measured contaminants and their legal limits in food.

2. Material and Methods

2.1. Sample collection

In total 11 Chlorella and 11 Spirulina samples in the form of powder were obtained from shops in Belgium, retailers in the Benelux or directly from the producing companies (Table 1). Within each group of 11 samples, 5 samples originated from the same company having a different expiration date (different production batch) or the same expiration date (same production batch).

2.2. Nutritional parameters

Biomass dry weight (Total Solids, TS), water content, organic (Volatile Solids, VS) and inorganic (ash fraction) contents were determined gravimetrically in triplicate on 300 mg sample by drying at 105 °C until constant weight and incineration at 550 °C for 2 hours, respectively.

Human digestibility was determined in-vitro following the harmonized protocol of Minekus et al. (2014). A triplicate aliquot of 0.05 g was mixed with simulated gastric fluid (SGF), containing pepsin (2000 U/mL), and incubated for 2 hours at 37 °C at 1200 rpm (Grant-Bio PHMT PSC24). Subsequently, simulated intestinal fluid (SIF) containing pancreatin (100 U trypsin activity/mL) and bile salt (10 mM) was added before the sample was incubated for 2 hours as described earlier. After centrifugation, the pellet was analyzed for Kjeldahl nitrogen (KjN) (AOAC International., 1995). Digestibility was determined by subtracting KjN in the pellet after digestion (undigested fraction) from the KjN content of the sample before digestion.

Total lipid content of all samples was measured according to Bligh and Dyer (1959). A triplicate aliquot of 0.05 g sample was mixed with 0.2 mL demineralized water and 0.75 mL mixed solvent containing 2:1 chloroform:methanol. The mixture was homogenized using a thermoshaker for 10 min (Grant-Bio PHMT PSC24). After centrifugation at 5000 g for 5 min, the supernatant was carefully transferred and mixed with a 50% chloroform solution. After centrifuging at 5000 g for 5 min, the bottom chloroform phase was evaporated at 40 °C for at least 20 hours, after which the remaining lipids were determined gravimetrically. In parallel, a control sample with sunflower oil and a blank sample were included.

Protein content was determined in two ways, based on a Kjeldahl nitrogen measurement on 0.025 g biomass with a conversion factor of 6.25 as described above and based on Markwell et al. (1978), an adaptation of Lowry et al. (1951). Subsequent to protein extraction on 5 mg biomass with trichloric acid following Slocombe et al. (2013), part of the extract was used to determine biomass protein and part was used for essential amino acid (EAA) analysis.

Prior to EAA analysis, protein extracts were hydrolyzed with 6M HCl for 24 hours at 110 °C in vacuum-sealed hydrolysis tubes (Wilmad Labglas). To avoid amino acid oxidation, hydrolysis and subsequent acid evaporation were performed under a vacuum atmosphere, alternating with nitrogen gas flushing. After evaporation and dissolution in 0.75 mM HCl, samples were stored at -20 °C. EAA were derivatized with propyl chloroformate following the Phenomenex EZ:faast amino acid analysis procedure (solid phase extraction, derivatization and liquid/liquid extraction), after which separation was performed with gas chromatography (Agilent HP6890 Series GC system Plus) and detection with mass spectrometry (HP 5973 Mass selective detector). Bovine Serum Albumin (BSA) was used as a control to determine amino acid recovery after hydrolysis. Norvaline was used as an internal standard during EZ:faast sample preparation.

EAA data were normalized based on the WHO/FAO/UNU (2007) established human reference pattern, with a value of 100 representing the best match between the sample EAA content and the consumer's needs. The essential amino acid index (EAAI) was calculated according to the following equation (Oser, 1959):

186 $$\mathrm{EAAI} = \sqrt[n]{\frac{\mathrm{aa1}}{\mathrm{AA1}} \times \frac{\mathrm{aa2}}{\mathrm{AA2}} \times \ldots \times \frac{\mathrm{aa}n}{\mathrm{AA}n}}$$

187 Here, aa*n* represents the percentage of the EAA content in the sample and AA*n*
188 represents the FAO/WHO established human reference content (WHO/FAO/UNU,
189 2007). Finally, the digestible essential amino acid index (DEAAI) was calculated by
190 multiplying EAAI with the analyzed in-vitro digestibility.

191 **2.3. Safety parameters**

192 For heavy metal analysis (Pb, Hg, Cd, As, Zn, Cu, Ni, and Cr) an aliquot of 0.5-1 g was
193 weighted in digestion tanks (CEM Mars Express). Around 0.6g internal standard
194 solution, 10 mL of 65% nitric acid and 1.5 mL 30% HCl was added. After digestion,
195 each container was filled with Millipore water to approximately 60 g. Around 3 g of the
196 digested solution was mixed with 3.25% nitric acid to around 9 g, after which the
197 sample was analyzed with ICP-MS (Agilent ICP-MS 7500cx Series).

198 Samples for polycyclic aromatic hydrocarbons (PAH) analysis were homogenized
199 (Robot Coupe Retsch GRINDOMIX) after which 5 g was supplemented with internal
200 standard and extracted using acetonitrile. Further, Bekolut citrate kit 01 was added and
201 the homogenate was centrifuged for 5 min at 6000 rpm. The upper phase was removed,
202 followed by a dispersive solid phase cleanup (d-SPE) (Bekolut PSA-Kit-04). After
203 mixing and centrifugation, the supernatant was evaporated with nitrogen gas.
204 Acetonitrile was used to reconstitute the sample, after which analysis took place using
205 GC-MS/MS (Agilent Technologies GC 7890A and 7000 Triple Quad MS/MS; Agilent
206 Technologies Select PAH). Measured PAH included benzo[a]anthracene,
207 benzo[a]pyrene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene,
208 chrysene, cyclopenta[cd]pyrene and triphenylene. Both, benzo[a]pyrene and the sum of

four PAH's (ΣPAH4: benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene) were used as an indicator for contamination (see supplementary material).

Nucleic acid (DNA and RNA) content was determined in triplicate by absorbance at 260 nm of phenol/chloroform extracts. A volume of 500 mL lysis buffer (10 mM Tris, 10 mM EDTA, 0.1 M NaCl, 2% SDS, pH 8.0) was added to 5-20 mg mg sample and vortexed for at least 10 minutes (Vortex Genie). Next, 500 ml of a mixture of 2.3:1 phenol:chloroform (pH 7) was added. The sample was vortexed as before, incubated for 30 minutes at -80 °C and centrifuged for 30 minutes at 15000g at 4 °C. The watery layer on top was transferred and 0.6 times the volume of ice cold isopropanol was added. After incubation at -80 °C for 30 minutes and centrifugation for 30 sec at 15000g at 4 °C, the supernatant was discarded. The nucleic acid pellet was then washed with 500 mL of ice cold EtOH (70%). The samples were incubated for 30 minutes at -20 °C and centrifuged for 20 minutes at 15000g at 4 °C. The supernatant was discarded, and the pellet was air-dried. Once all ethanol was evaporates, the pellet was suspended in 100 µL H_2O and stored at -20 °C upon analysis with a HTX Synergy, using a Take3 plate (Biotek). For every sample, the DNA concentration and quality of the samples was determined based on the absorbance at 260 nm, 280 nm, and 320 nm. All samples showed adequate quality.

Nitrate was extracted from 5-6 g with 50-70 mL water for 15 min in a water bath at 80 °C. After cooling to 20 °C, water was added up to 100 mL, shaken and filtered through a fluted filter. Part of the solution was filtered (0.45 µm) and measured with ion chromatography (Dionex ICS 3000; Ion Pac AS 17-C) with UV-detection (VWD 5000).

231 For antibiotics analysis (full list in supplementary material), a sample of 2 g ± 0.1 g was
232 homogenized with 100 µL of antibiotics internal standard solution and 2 mL of
233 Na_2EDTA-McIlvaine buffer. For protein precipitation, 8 mL of acetonitrile was added.
234 After centrifugation, the resulting supernatant was purified by means of mixing with
235 around 500 mg C18EC bulk sorbent. After the bulk sorbent settled using centrifugation,
236 5 mL of supernatant was evaporated with nitrogen gas at 45 °C, reducing the residual
237 volume to less than 0.5 mL. The residue was reconstituted with 2 mL HPLC mobile
238 phase (initial conditions), vortexed and centrifuged for 5 min at 6000 rpm. Finally, the
239 supernatant was filtered (PTFE, 0.2 µm) and analyzed using HPLC-MS/MS (Agilent
240 Technologies HPLC 1290; RRHD Eclipse Plus C18 column; Agilent 6490 Triple Quad
241 LC/MS).

242 Pesticides (chlorpyrifos, ametryn, benzalkonium chloride (BAC) C12, C14 and C16,
243 didecyldimethylammonium chloride (DDAC) C10, tebuconazole) and mycotoxins
244 (aflatoxin B1, B2, G1 and G2, deoxynivalenol, fumonisin B1 and B2, HT-2 toxin,
245 ochratoxin-A, T-2 toxin, zearalenone) were measured by subjecting the homogenized
246 sample to an acetonitrile liquid-solid partition extraction in the frozen state.
247 Triphenylphosphate was added as internal standard together with acetonitrile.
248 Subsequently, a citrate salt kit (Bekolut Citrate-Kit-01) was added, whereby excess
249 water was separated and the acetonitrile phase stabilizes at pH 5-5.5. After shaking and
250 centrifugation (5 min at 6000 rpm), an aliquot of the acetonitrile phase was filtered and
251 pesticides were measured by GC-MS/MS (Agilent Technologies GC-QQQ-MS 7890A;
252 G7000B Triple Quadrupole), mycotoxins by HPLC-MS/MS (Agilent Technologies
253 HPLC 1290; RRHD Eclipse Plus C18 column; Agilent 6490 Triple Quad LC/MS).

For acrylamide analysis, a sample of 2.0 g ± 0.1 g was homogenized and mixed with 50 µl of C13-acrylamide working solution, 5 mL of n-hexane, 5 mL of water and 10 mL of acetonitrile. Thereafter, a citrate salt kit (Bekolut Citrate-Kit-01) was added, mixed well and centrifuged. A 2 mL aliquot of the acetonitrile extract was filtered (0.45 µm) and measured using HPLC-MS/MS (Agilent Technologies 1200 QQQ-HPLC; 6460 Triple Quadrupole).

3. Results and discussion

3.1. Nutritional value

3.1.1. Water and organic matter

To understand the content of water, organic matter and minerals in the microalgal biomass, figure 2 presents the variability in VS/TS ratio and water content between different producers (Figure 2A), production batches (Figure 2B) and within the same production batch (Figure 2C). Biomass water content was below 10% for all Chlorella and Spirulina samples, which enables safe storage (Hosseinizand et al., 2017). Chlorella biomass originating from different producers presented on average a 36% lower water content (3.7%) compared to Spirulina (5.0%), which could be due to producer dependent drying methods and drying times (Show et al., 2013). As expected, the variability between producers was higher for both species compared to the variability between different production batches and within a batch (Figure 2B, C).

Concerning the average VS/TS ratio, slightly higher values were observed for Chlorella (0.94) compared to Spirulina (0.92), indicating a higher ash content in Spirulina biomass. Elevated ash fractions can be positive since it typically includes essential minerals (e.g. Ca^{2+} and K^{+}), however, careful monitoring is advised since the ash fraction also contains toxic heavy metals (e.g. Hg^{2+}) (Campanella et al., 1999). As

discussed further, the total heavy metal content represents only 0.04–0.13% of the ash fraction, which indicates the predominance of non-risky minerals. The higher Spirulina ash fraction could be due to the higher salt content of the cultivation medium. Depending on the washing method applied, the biomass can contain residual salts (Zhu & Lee, 1997). Tokusoglu and Unal (2003) also measured a higher total ash content for the washed biomass of three freshwater Spirulina of 7.4, 7.5 and 10.4%, compared to freshwater Chlorella with a 6.3% ash content. Similar to the variability in water content, biomass VS/TS ratio variability (comparing minimum to maximum) for Chlorella (60%) and Spirulina (55%) was higher between producers compared to the variability between different production batches and within a batch (Figure 2B, C). Except for the variability between Chlorella production batches a similar variability in VS/TS ratio of 60% was observed. This indicates the possible influence of cultivation conditions, providing that post-processing conditions are not subjected to changes. Costard et al. (2012) also observed an ash content variability of 66% in one species of *Chlorella* sp. with an increase from exponential to stationary growth phase.

3.1.2. Digestibility

Although a higher biomass digestibility is not adding nutritional value in a direct manner, it determines the availability of nutritional compounds for further uptake by the body. Because Chlorella features a rigid cellulosic cell wall, which is lacking in cyanobacteria like Spirulina, a lower in-vitro digestibility of Chlorella can be expected (Becker, 2004). Indeed, compared to the average digestibility of Chlorella samples from different producers (51%), the average digestibility of Spirulina (61%) was 19% higher (Figure 2A). Literature data for Chlorella and Spirulina in-vitro digestibility presents a wide range, but most researchers use different in-vitro protocols which makes

302 comparison difficult (Tibbetts et al., 2015). Reported in-vitro protein digestibility ranges
303 between 27 and 70% for Chlorella (Hedenskog et al., 1969; Morris et al., 2008) and
304 between 70 and 85% for Spirulina (Devi et al., 1981). The variability in biomass
305 digestibility between producers was 74% for Chlorella and 23% for Spirulina
306 (comparing minimum to maximum). To increase digestibility, many Chlorella
307 producing companies apply physical or chemical cell wall disruption techniques, which
308 can be the reason for the larger observed variability within Chlorella samples. Cell wall
309 disruption methods found for the samples in this study are the patented low-pressure
310 flash expansion (sample C1) and high-impact, jet-spray drying (sample C3).
311 Additionally, processing can alter digestibility as was observed by Becker (2007) who
312 reported digestibility coefficients of 59 and 89 for air and drum dried Chlorella and
313 values of 84 and 76 for drum and sun-dried Spirulina, respectively. Finally, lower
314 variabilities in digestibility were observed between production batches of Chlorella
315 (19%) and Spirulina (12%) and within a production batch of Chlorella (10%) and
316 Spirulina (13%) (Figure 2B, C). Hence, a similar trend in decreasing variability between
317 producers, between batches and within a batch was observed, similar to the trend for
318 water content and ash fraction.

319 **3.1.3. Lipids**

320 Chlorella samples present an average lipid content of 7.4% while the average lipid
321 content of Spirulina is slightly higher with 10% (Figure 2A). Chlorella lipid content
322 presents the largest variability between producers with the highest lipid content (12%)
323 more than double the value of the lowest (3.6%), while the variability in Spirulina lipid
324 content is lower with 43% (between minimum and maximum). Due to the importance of
325 microalgae in biofuel production, the influence of cultivation conditions on the lipid

content has been researched extensively. It was found that nitrogen limitation is an effective method to increase lipid content, mostly at the expense of protein (Piorreck et al., 1984). However, cyanobacteria do not show significant changes in their lipid content and fatty acid composition in response to nitrogen supply (Becker, 2004). This was also reflected in the larger variability in lipid content between different production batches and within a production batch of Chlorella (19% and 9%, respectively) compared to that of Spirulina (6% and 1%, respectively). Finally, Chlorella lipid content is rather underestimated on the package, while Spirulina lipid content is overestimated.

Although not measured in this study, abundant data on lipid quality (fatty acid composition) is available in literature. Two essential fatty acids (EFA), α-linolenic acid (18:3n-3; ALA) and linoleic acid (18:2n-6; LA), determine lipid quality. Furthermore, the conversion products of ALA, eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA), are also considered important. Otles and Pire (2001) observed that commercial *C. pyrenoidosa* (n=3) lipids exist out of 14-16% ALA, 11-22% LA and 0-0.53% DHA+EPA, while *S. platensis* (n=3) lipids contain no ALA or DHA, 16-17% LA and 0-0.19% EPA. This species dependent variability in EFA composition indicates the potential for lipid quality improvement.

3.1.4. Protein and essential amino acids

Despite the general assumption that Spirulina contains a higher protein content compared to Chlorella, both species contain a similar average amount of protein of 48%. However, the average digestible protein content is lower for Chlorella (24%) compared to Spirulina (29%), due to the lower digestibility of Chlorella biomass. Comparing minimum to maximum protein content, Chlorella presents 55% variability between producers, which is higher compared to the variability in Spirulina biomass of

23% (Figure 2A). The cultivation parameter dependent variability in protein content reported by different authors (as presented in Figure 1) is reflected in the variability in this study for both Chlorella and Spirulina. Figure 1 shows an even larger variability in literature compared to the measured variability in this study. This can be explained by the inclusion of experiments under unfavorable conditions (e.g. nitrogen limitation) and by the use of different analytical methods based on total nitrogen (Maehre et al., 2018). In contrast, microalgae producing companies strive for the highest possible biomass productivity and quality, avoiding nutrient limitations or other harmful cultivation conditions. Furthermore, since not all intracellular nitrogen is present in protein but also in other nitrogenous constituents like nucleic acids, amines, glucosamides and cell wall material, a total nitrogen measurement overestimates the real protein content. This is also observed in this study, where a higher average protein content based on KjN was obtained (60% for Chlorella and 67% for Spirulina), compared to the protein measured based on the Markwell essay. Additionally, the ratio Markwell-protein over KjN-protein is larger for Chlorella compared to Spirulina, indicating the higher Spirulina non-protein nitrogen content. Indeed non-protein nitrogen amounts to 11.5% in Spirulina (Becker, 2004) and 10.3% in Chlorella (Fowden, 1952). Values for package match of KjN-protein verify that KjN measurements are standard practice for protein determination in the food industry. Although this easy KjN is used as standard protein measurement, still up to 37% difference in package match between producers can be observed. This might suggest that protein content is not measured for every batch but an average value is displayed on the package. Finally, the variability comparing minimum and maximum protein content between production batches (6% for Chlorella and 22% for Spirulina; Figure 2B) and within a batch (8% for Chlorella and 3% for Spirulina; Figure 2C) is

smaller compared to the variability between producers, but still indicates the room for nutritional optimization within one company.

In addition to bulk protein content, its quality in terms of EAA is a core marker for nutritional value (Figure 3). Humans are limited to the biosynthesis of certain amino acids only (non-essential amino acids) while the remaining (essential) amino acids have to be provided through food. Despite the similar average protein content in Chlorella and Spirulina samples originating from different producers, Spirulina contains a more favorable EAA composition according to human requirements. This is reflected in a higher EAAI for Spirulina (1.25), compared to Chlorella (1.05) (Figure 3G). Spirulina originating from different producers presents the largest variability in EAA with EAAI values between 1.01 and 1.45. Considering the separate amino acids, Chlorella biomass was mainly short in the sulfur containing amino acids (methionine and cysteine) with an average value of 14±3 mg (met+cys)/g protein compared to the required 22 mg (met+cys)/g protein (Figure 3A; supplementary material). Furthermore, also histidine content (11±2 mg his/g protein) was limiting compared to the required 15 mg his/g protein. Lysine was only short in some samples (C2, 3, 4 and 7), with a minimum of 33 mg lys/g protein. Spirulina also contained deficiencies in the sulfur containing amino acids (18±5 mg (met+cys)/g protein) and histidine (10±3 mg his/g protein) (Figure 3D; supplementary material). In contrast with Chlorella, Spirulina did not present a deficiency in lysine (53±7 mg lys/g protein). Taking into account digestibility, the DEAAI dropped below the optimal score of 1 for most samples, indicating an EAA shortage compared to the required reference intake (Figure 3G). In general, EAA profiles found in literature of most studied microalgae are favorably compared to the reference EAA profile, with minor deficiencies among the sulfur-containing amino

398 acids methionine and cysteine. In contrast to the EAA variability of 4-56% and EAA
399 differences between Spirulina and Chlorella observed in this study, Brown (1991)
400 observed a rather similar AA composition in 12 genera (16 different species), however,
401 excluding Spirulina and Chlorella. In terms of growth conditions, James et al. (1989)
402 observed the temperature dependency of *Chlorella* sp. AA composition. Most of the
403 EAA such as threonine, valine, methionine, isoleucine, leucine and lysine were present
404 more at 30 and 35 °C compared to 15 °C and cystine and methionine showed an
405 increasing trend with increasing temperature up to 30 °C. Compared to this study, the
406 sufficient cysteine and methionine content in sample S1 could indicate that cultivation
407 temperature was optimal. Furthermore, Ogbonda et al. (2007) also observed an
408 influence of temperature and pH on the AA composition of *Spirulina* sp. with the
409 highest EAA content at pH 9 and 30 °C. At 25 °C, the EAAI was only 0.4 while at 30
410 °C a value of 1.0 was obtained, while the presented amino acids show a relative
411 standard deviation between 24 and 75%, indicating the significant room for EAA profile
412 altering. Choi et al. (2003) determined the amino acid composition of *S. platensis*
413 cultivated with ammonium, nitrate, nitrite and urea as nitrogen source. After 30 days,
414 urea resulted in the highest amino acid content (174 mg/g dry weight), while the amino
415 acid profile was similar for all N sources. Further, within the ammonium treatment, the
416 highest amino acid content (127 mg/g dry weight) was reached after 16 days, compared
417 to only 73 mg/g dry weight after 30 days. Since it is not known which nitrogen source
418 or harvesting time was applied to cultivate the biomass in this study, the exact
419 magnitude of EAA variation due to these parameters cannot be determined.

3.2. Contamination and safe consumption

3.2.1. Heavy metals

Heavy metals end up in microalga biomass due to their presence as trace contaminants in fertilizers (Al-Dhabi, 2013) and because microalgae are known to bioaccumulate metals (Arunakumara & Xuecheng, 2008). While some metals are toxic (i.e. As, Cd, Hg, Pb, Ni), others are considered essential in human nutrition (Cu, Zn, Cr) but become hazardous when a certain intake value is exceeded. With the advice of the European Food Safety Authority (EFSA), the European Union (EU) dictates maximum residue levels for toxic trace elements in food and recommends daily intake levels for essential trace elements (see supplementary material).

No violations of the EU regulation for food supplements were observed for cadmium, mercury and lead (Figure 4A, B). The measured mercury, cadmium and arsenic content in Chlorella ranged between 0.02 and 0.10 mg/kg, 0.01 and 0.10 mg/kg and 0.59 and 1.1 mg/kg, respectively, while no lead was detected (Figure 4A). In the Spirulina samples, mercury and cadmium levels were similar, ranging between 0.02 and 0.11 mg/kg and between 0.01 and 0.17 mg/kg, respectively, while no arsenic or lead was detected (Figure 4B). Nickel was mainly found in the Spirulina samples in concentrations between 1.1 and 3.4 mg/kg. These (heavy) metal contents are in the same range as those reported in other studies except for lead, which is often observed in a concentration between 0.1 and 15 mg/kg (Al-Dhabi, 2013; Al-Homaidan, 2006; Campanella et al., 1999; Ortega-Calvo et al., 1993). For inorganic mercury, EFSA's Scientific Panel on Contaminants in the Food Chain (CONTAM) determined a tolerable weekly intake (TWI) level of 4 µg/kg body weight, corresponding with a daily safe consumption quantity of 444-2000 g Chlorella and 364-2000 g Spirulina (see

supplementary material). For cadmium, a TWI level of 2.5 µg/kg body weight indicates a safe daily consumption quantity of 313-2500 g Chlorella and 313-2083 g Spirulina. For arsenic, no maximum levels are established for food, however, based on the benchmark dose lower confidence limit ($BMDL_{01}$) of 0.3-8 µg/kg body weight/day a daily consumption of 20-36 g Chlorella (only detected in C7a, C7b and C7c) can be considered safe (see supplementary material). For nickel the TDI is set at 2.8 µg Ni/kg body weight, permitting a consumption of 163 g per day for Chlorella (detected only in C7c1) and between 58 and 178 g/d Spirulina (detected in S1, S3, S4, S5, S7c2 and S7c3).

Copper and zinc were present in both types of microalgae as they are common fertilizers in microalgae cultivation, while chromium was only detected in Spirulina between 2.1 and 22.3 mg/kg. Copper content ranged between 1.2 and 22.3 mg/kg in the Chlorella samples while a content between 0.94 and 6.4 was measured in Spirulina. Zinc was present in larger concentrations between 14 and 69 mg/kg in Chlorella and between 17 and 50 mg/kg in Spirulina. Because copper and zinc are essential to sustain the health and function of the human body, an adequate daily intake (ADI) is advised of 1.6 mg copper/day, while the average requirement (AR) of zinc is set at 7.3 and 5.5 mg zinc/day for males and females, respectively. Finally, no average requirements are set for chromium, however, the TDI of 300 µg/day should not be exceeded, indicating a daily safe consumption dose of at least 940 g Spirulina (S4, S7c2,3; see supplementary material).

3.2.2. Polycyclic aromatic hydrocarbons

PAH can originate from natural and anthropogenic processes, mainly by incomplete combustion of organic matter. Because microalgae undergo a drying process PAH

contamination is possible. Benzo(a)pyrene and ΣPAH4 levels exceeded the EU norm of 10 µg/kg and 50 µg/kg, respectively (Figure 4C, D; supplementary material). The samples of Chlorella that were highly contaminated with values between 538 and 873 µg/kg benzo(a)pyrene and between 2323 and 3423 µg/kg PAH4, were originating from the same batch (C7c1, C7c2 and C7c3). Different production batches of Chlorella from the same company (C7a and C7b) did not violate the limits. Within the Spirulina samples originating from the same company, a violation of the ΣPAH4 norm was observed for samples S7a, S7b, S7c2 and S7c3, with values between 56 and 84 µg/kg. Benzo(a)pyrene concentrations were safe with values between 3 and 4 µg/kg. Considering the average exposure of the European population to benzo(a)pyrene (0.24 µg/d) and PAH4 (1.17 µg/d), a safe daily consumption dose of 39-1700 g Chlorella and 25-1900 g Spirulina can be determined (see supplementary material). Zelinkova and Wenzl (2015) analyzed several food supplements, including 1 Chlorella and 9 Spirulina samples, for the occurrence of ΣPAH4 and detected levels between 17 and 68 µg/kg benzo(a)pyrene and between 97 and 275 µg/kg PAH4 in 3 Spirulina samples.

The potential violation of PAH limits and the observation that violations are not constant over time but batch specific, makes periodic monitoring essential. Special attention should be given towards downstream processing (e.g. drying), a known source of PAH. Sources of pollutants should be identified for highly contaminated products and remediating measures taken.

3.2.3. Other potentially hazardous components

Nucleic acids (DNA and RNA) are sources of purines that may cause an elevated uric acid level in the blood and increased urinary excretion of uric acid (Edozien et al., 1970). The measured nucleic acid content for Chlorella (1.4±0.8 %DW) and Spirulina

(1.6±0.4 %DW) was lower compared to the reported values by Ortega-Calvo et al. (1993) of 5.4%DW for one commercial Chlorella sample and between 4.8 and 5.7%DW for three commercial Spirulina samples. Considering the tolerable daily intake of nucleic acids from unconventional sources of 2 g, the measured nucleic acid contents permit a safe consumption dose between 73 and 425 g/d Chlorella and between 106 and 265 g/d Spirulina (Figure 4E, F; supplementary material).

Finally, low concentrations of some pesticides were measured in one Chlorella sample (C1: 0.017 mg/kg chlorpyrifos) and in three Spirulina samples (S1: 0.014 mg/kg chlorpyrifos, 0.014 mg/kg ametryn; S4: 0.13 mg/kg BAC-C12, 0.13 mg/kg BAC-C14, 0.01 mg/kg BAC-C16, 0.11 mg/kg DDAC-C10; S7a: 0.007 mg/kg tebuconazole). Only Chlorella sample C4, originating from India, contained traces of the antibiotic sulfadoxine, present in antimalarial medication, with a concentration of 135 µg/kg. No mycotoxins or acrylamide was detected. Finally, nitrate content varied between 9 and 188 mg/kg DW for Chlorella and between 8 and 368 mg/kg DW for Spirulina (data in supplementary material). Although these values hardly contribute to the total amount of N in the biomass, the highest values could indicate that nitrate was used as nitrogen source during cultivation. Considering the ADI for nitrate of 3.7 mg/kg body weight, the highest nitrate content (368 mg/kg DW in sample S4) accord to the consumption of 700 mg biomass.

3.3. Overall appreciation based on nutritional quality and price

The systematic nutritional and safety analysis, including protein content, EAA composition (protein quality), lipid content and in-vitro digestibility, on a significant amount of industrial Chlorella and Spirulina samples, indicate the superiority of Spirulina compared to Chlorella. With respect to potential hazardous contamination

516 mainly heavy metal, PAH and nucleic acid contents were determining. Based on these
517 contaminants, Spirulina would be the overall safer choice. Figure 5 presents the price
518 for the purchased microalga biomass, bought in typical food supplement volumes
519 between 100 and 350 g for Chlorella and between 100 and 453 g for Spirulina. A large
520 difference in price for the same product can be observed between 50 and 267 €/kg for
521 Chlorella and between 48 and 191 €/kg Spirulina. Based on total biomass, Chlorella is
522 on average 18% more expensive compared to Spirulina and based on protein content,
523 Chlorella is 15% more expensive. Given the fact that the average Spirulina biomass
524 digestibility and EAAI are both on average 19% higher compared to Chlorella, it is the
525 preferred purchase. Additionally, Spirulina would be the most interesting species to
526 research for RLSS applications. Currently, microalgae are produced as a food
527 supplement and manufacturers report a recommended dose on the package between 2
528 and 9 gram per day (table 1). However, if microalgae are consumed as full or partial
529 protein source the consumed doses increase, as well as the risk on contaminant
530 exposure. Aside from the samples containing an exceptionally high PAH content,
531 calculated safe consumption doses indicate the capacity as protein source rather than as
532 food supplement (see supplementary manterial).

533 **4. Conclusion**

534 The revealed variability in nutritional quality within one microalgal type originating
535 from different producing companies, and from different batches within a company,
536 indicates the importance of growth parameter optimization. Furthermore, a high total
537 protein or lipid content does not imply a high overall nutritional quality, since the EAA
538 profile could still be unfavorable or a low digestibility could result in a lower nutrient
539 availability. Furthermore, current package information lacks often accuracy and product

safety is not always guaranteed. This necessitates careful and continuous monitoring of nutritional quality and safety. Finally, based on price and nutritional quality, Spirulina was preferred above Chlorella.

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

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7. Figure captions

Table 1. Overview of examined Chlorella and Spirulina samples (all in powder form, except for S6, which were fine rods). The expiration date was used as a proxy for production batch. The reported recommended dose was given on the package. Empty cells for the samples C7 and S7 indicate that the same info is applicable as for C7a and S7a.

Figure 1. Variability in protein content of *Chlorella* spp. and *Spirulina* spp. based on literature research on the influence of cultivation parameters (autotrophic cultivation; lab scale) (see supplementary material). Dotted line: average; full line: median.

Figure 2. Overview of the variability in several markers for nutrition quality for Chlorella (green) and Spirulina (blue). 1A. Variability between producers; 1B. Variability between different batches; 1C. Variability within the same batch. Digestibility is measured in-vitro. Package match is expressed as 'measured content/package content'. VS: volatile solids; TS: total solids; KjN: Kjeldahl nitrogen. Dotted line: average; full line: median.

Figure 3. Essential amino acid (EAA) profiles for Chlorella (green; A, B and C) and Spirulina (blue; D, E and F) samples normalized for human essential amino acid requirements (circle indicates a value of 100 which is a perfect match with human requirements according FAO/WHO). Essential amino acid index (EAAI) and digestible essential amino acid index (DEAAI) variability for Chlorella and Spirulina (G, H and I) representing protein quality (a value of 1 represents a perfect match with human requirements). Used digestibility values are presented in figure 2. Data expressed as mg AA/g protein are presented in supplementary materials.

Figure 4. Safety parameters: heavy metal (4A. Chlorella; 4B. Spirulina), polycyclic aromatic hydrocarbon (PAH) content (4C. Chlorella; 4D. Spirulina) and nucleic acid content (4E. Chlorella; 4F. Spirulina). Dotted lines represent the limits in food supplements according to the European regulation for food supplements (see supplementary material). If no dotted line is displayed, no European limits are established for food supplements.

Figure 5. Variability in price per kg biomass and per kg protein for Chlorella (green; left) and Spirulina (blue; right). Dotted line: average; full line: median.

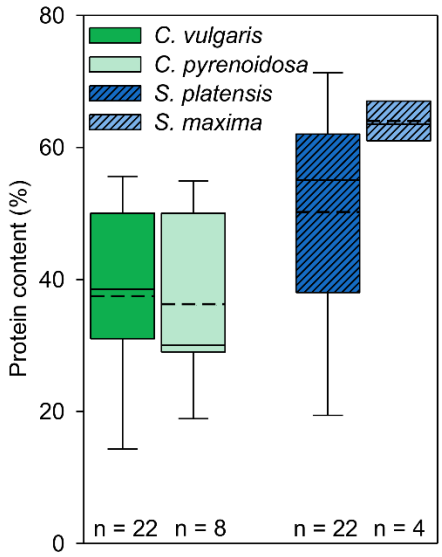
8. Tables

Table 1. Overview of examined Chlorella and Spirulina samples (all in powder form, except for S6, which were fine rods). The expiration date was used as a proxy for production batch. The reported recommended dose was given on the package. Empty cells for the samples C7 and S7 indicate that the same info is applicable as for C7a and S7a.

	Code	Brand	Country of origin (city)	Retailer	Cultivation system	Reported species	Expiration date (DD/MM/YY)	Recommended dose (g/d)
Chlorella	C1	Febico	Taiwan (Ping-Tung)	Febico	Outdoor pond	*Chlorella pyrenoidosa*	04/03/19	3
	C2	Not specified	China (Hainan)	pit-pit	Outdoor pond	Not specified	16/09/18	Not specified
	C3	Jarrow formulas	Japan (Ishigaki)	Jarrow	Outdoor pond	*Chlorella vulgaris*	01/07/17	2
	C4	Clean Chlorella	South Korea	Health Ranger Select	Not specified	Not specified	Not specified	5
	C5	Algomed	Germany (Klötze)	Algomed	Indoor tubular photobioreactor	*Chlorella vulgaris*	14/12/18	3
	C6	Iswari	China (Hainan)	Iswari	Not specified	Not specified	01/03/17	6
	C7a	Purasana	Mongolia	Bioplanet	Not specified	*Chlorella vulgaris* Beijerinck	30/03/19	9
	C7b						30/04/19	
	C7c1			Origin'O			31/05/18	
	C7c2							
	C7c3							
Spirulina	S1	Febico	Taiwan (Ping-Tung)	Febico	Outdoor pond	*Spirulina platensis*	14/01/19	3
	S2	Not specified	China (Hainan)	pit-pit	Outdoor pond	Not specified	11/09/18	Not specified
	S3	Parry Nutraceutical	India (Chennai)	Now foods	Not specified	Not specified	01/11/17	3.3
	S4	Nutrex Hawaii	USA (Hawaii)	Cyanotech Nutrex	Outdoor pond	*Spirulina platensis*	01/01/19	3
	S5	Earthrise	USA (Irvine)	Earthrise	Outdoor pond	*Spirulina platensis*	01/01/19	3
	S6	Domaine traverse*	France (Toulon)	NA	Greenhouse pond	Not specified	Not specified	3 – 5
	S7a	Purasana	Mongolia	Origin'O	Not specified	*Spirulina platensis*	31/05/18	9
	S7b						30/03/19	
	S7c1			Bioplanet			30/04/19	
	S7c2							
	S7c3			Origin'O				

* Fine rods (extrusion process); NA: Not applicable

9. Figures



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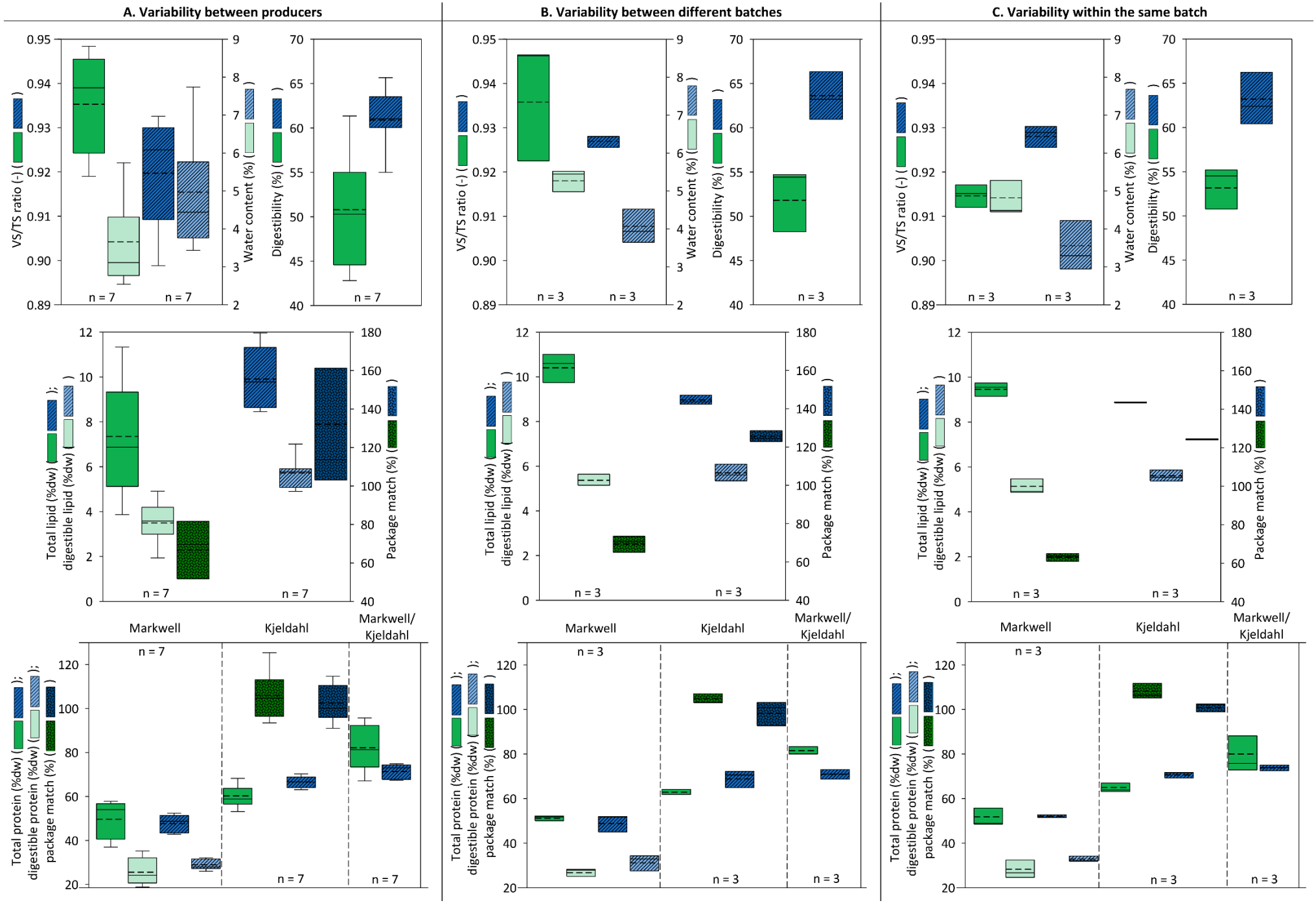

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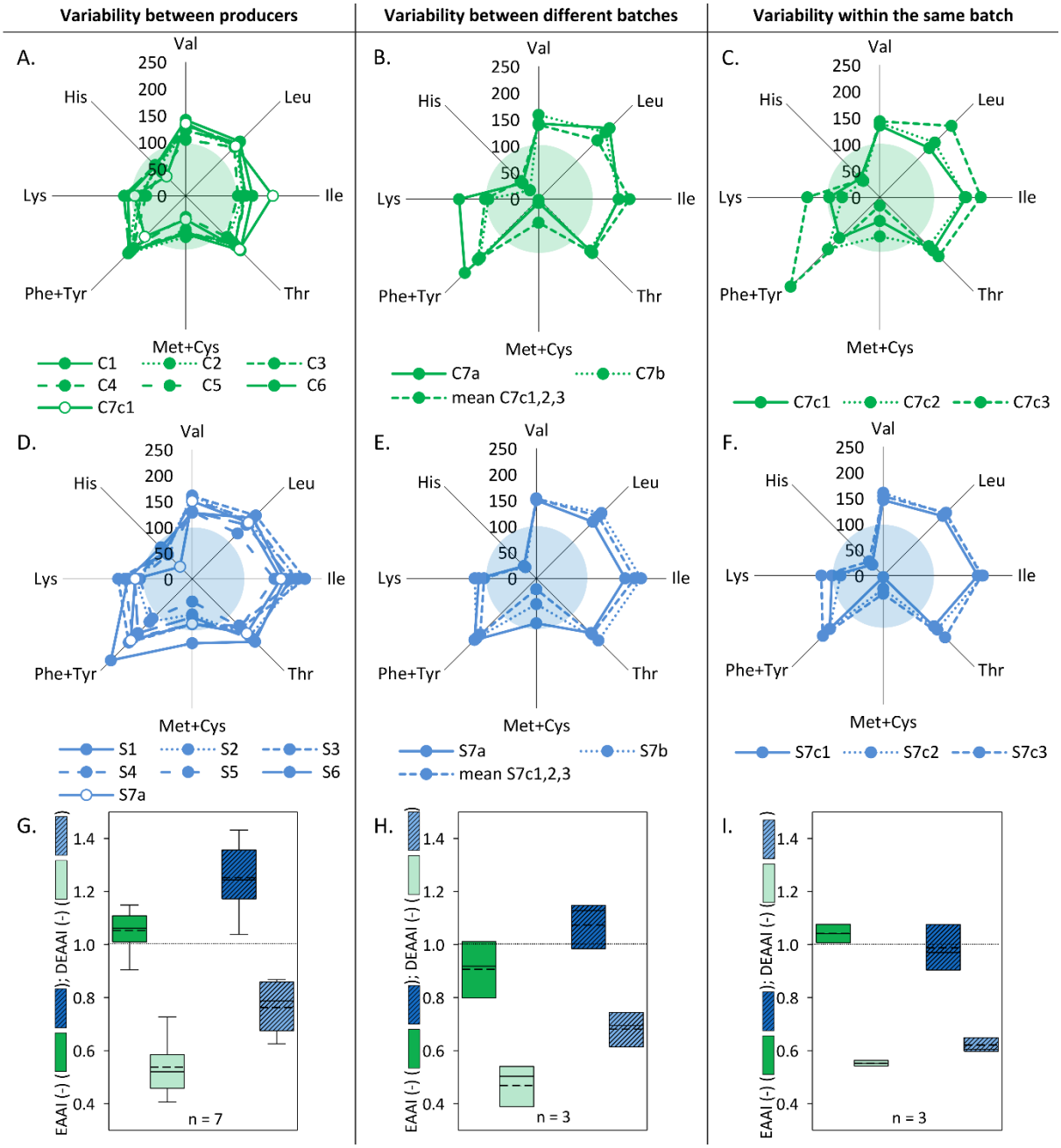

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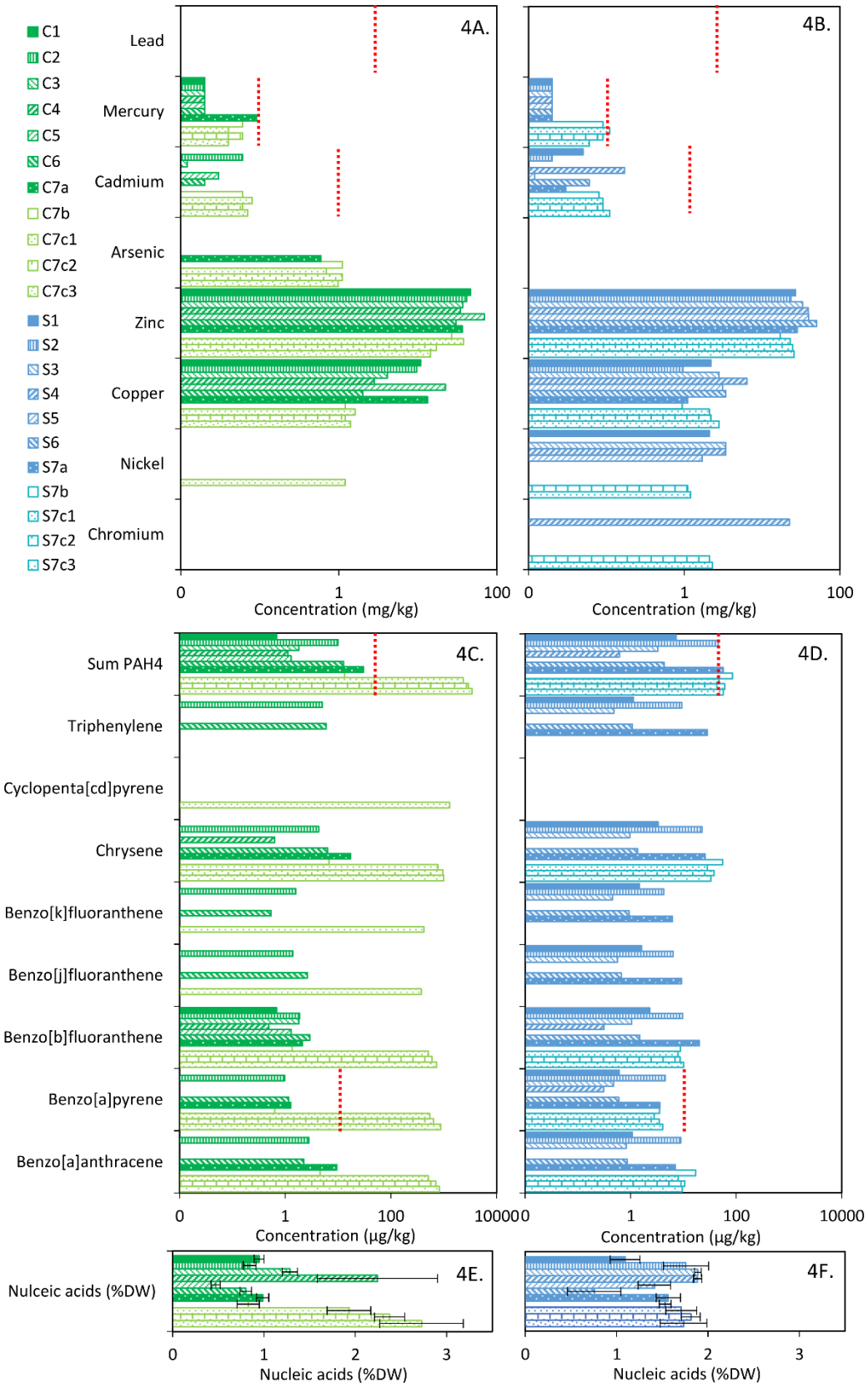
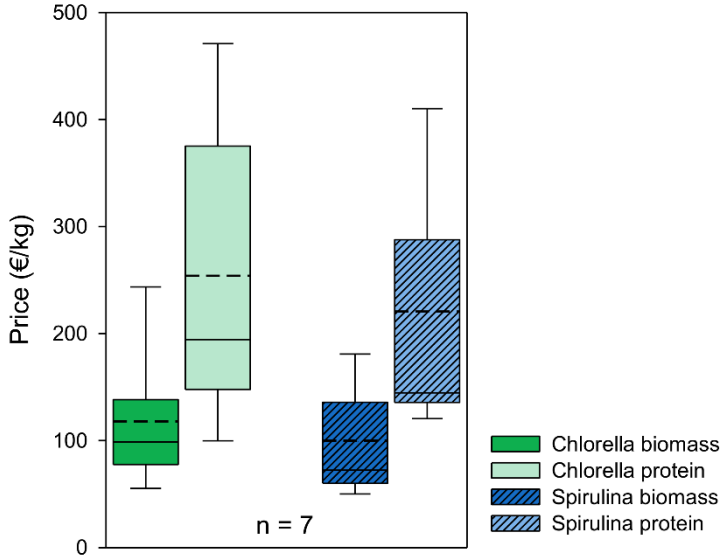


Figure 4. Safety parameters: heavy metal (4A. Chlorella; 4B. Spirulina), polycyclic aromatic hydrocarbon (PAH) content (4C. Chlorella; 4D. Spirulina) and nucleic acid content (4E. Chlorella; 4F. Spirulina). Dotted lines represent the limits in food supplements according to the European regulation for food supplements (see supplementary material). If no dotted line is displayed, no European limits are established for food supplements.



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754 Figure 5. Variability in price per kg biomass and per kg protein for Chlorella (green; left) and
755 Spirulina (blue; right). Dotted line: average; full line: median.

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