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Fine-tuning the flavor of *Tetraselmis chuii* by modifying nitrogen supply

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Abstract

Dried *Tetraselmis chuii* biomass has potential as flavoring agent for the development of plant-based seafood alternatives because of its seafood-like aroma and strong umami taste. Depending on the cultivation conditions, microalgae can adapt their metabolism, resulting in a change in biochemical composition. The aim of this study was to assess if the flavor of *T. chuii* could be modified by changing the nitrogen (N) supply in the cultivation medium in order to maximize the potential of *T. chuii* as flavoring agent. The sensory evaluation by a trained panel showed that the *T. chuii* biomass obtained from N starved cultivation conditions (N-deplete) is characterized by a significantly stronger odor intensity and earthy-like off-odor compared to *T. chuii* biomass obtained from N sufficient cultivation conditions (N-replete). The analysis of volatile organic compounds (VOCs) using SPME-GC-MS showed that these odor features of N-deplete biomass are attributed to an increased formation of odor-active VOCs including 2,3-butanedione, 3-methylbutanal, 3-methylbutanol and sulfur-containing dimethyl sulfide and dimethyl disulfide. In contrast, the *T. chuii* N-replete biomass possessed a significantly stronger taste intensity, umami and salty taste compared to the *T. chuii* N-deplete biomass. The higher umami is attributed to the significantly higher free glutamic acid (Glu) and adenosine monophosphate (AMP) concentrations in N-replete biomass compared to N-deplete biomass. This study illustrates that flavor and palatability of microalgae biomass is strongly affected by cultivating conditions and modifying these conditions can be an important tool in the development of plant-based seafood alternatives.

Keywords: Microalgae, *Tetraselmis chuii*, Nitrogen starvation, Sensory evaluation, Volatile Organic Compounds, Umami

1. Introduction

In recent years, plant-based food alternatives for meat, dairy and egg products have gained enormous attention among food producers because of the increasing demand for sustainable and healthy foods. While numerous plant-based alternatives have entered the food market, the development of plant-based alternatives for shellfish and fish is largely untapped [1]. Consumers can turn to plant-based seafood alternatives for animal welfare concerns, sustainability and ecological reasons (e.g. overfishing, spreading of anthropogenic residues from aquaculture to surroundings waters) as well as health concerns (e.g. bio-accumulation of marine pollutants in seafood, seafood allergies) [2]. To optimize consumer acceptance, the food industry is in need of natural flavorings that can mimic the seafood flavor. Flavor is defined as the overall sensation provided by the combination of odor (aroma), taste and textural sensations when consuming a particular food product [3]. The aroma of seafood is determined by a mixture of essential volatile organic compounds (VOCs) which include fatty-acid derived unsaturated aldehydes, ketones and alcohols, sulfur-containing volatiles (e.g. dimethyl sulfide (DMS)) and nitrogen-containing volatiles (e.g. trimethylamine (TMA)) [4]. An important feature of the taste of seafood is the umami aspect, which is determined by the free amino acids (FAAs) glutamic acid (Glu) and aspartic acid (Asp) and free 5'-nucleotides 5′-adenosine monophosphate (AMP), 5′-guanosine monophosphate (GMP) and 5′-inosine monophosphate acid (IMP) [5, 6].

Microalgae are a promising innovative food ingredient because of their rich nutritional characteristics, health benefits and sustainable cultivation methods [7]. Recently, Coleman et al. (2022) [8] reported that several marine microalgae including *Tetraselmis chuii* and *Rhodomonas salina* possess seafood-like flavors which could be valuable in the development of plant-based seafood alternatives. These marine microalgae share important odor-active volatiles with fish (coalfish and codfish) and shellfish (shrimp, lobster, crab and mussel) including VOCs originated from fatty acid oxidation (e.g. 2,6-nonadienal (E,E), 1-octen-3-ol and 3,5-octadien-2-one (E,E)), sulfur-containing VOCs (DMS, dimethyl disulfide (DMDS) and methanethiol (MeSH)) and nitrogen-containing TMA. Furthermore, *T. chuii* was also characterized by a strong umami taste because of the presence free Glu and 5'-nucleotide AMP. However, microalgae can also produce

certain odor-active compounds which do not contribute to the seafood flavor but generate off-flavors. These include buttery/cheesy smelling 2,3-butanedione, fruity smelling esters, floral smelling VOCs such as α-ionone and β-ionone that are derived from carotenoids and fatty/stale/grassy smelling VOCs such as 2,4-decadienal (E,E) which are derived from extended lipid oxidation [8]. These VOCs should be limited to reduce off-flavors when microalgae are used as flavoring agent in plant-based seafoods.

Several attempts have been made to incorporate *Tetraselmis* into food to increase its nutritional value (e.g. protein content). However, a substantial impact on the nutritional value is only obtained when *Tetraselmis* is incorporated in sufficient quantities (generally higher than 4% (w/w)), which has important implications for the sensory properties of the food. An intense fishy flavor was reported following the addition of 2% (w/w) *T. suecica* in cookies and crackers [9, 10]. The fishy flavors were also observed in crackers and breads containing 2.5% (w/w) *Tetraselmis* from an unspecified strain [11]. Lower appreciation scores were obtained in gluten-free bread containing 4% bead milled and freeze-dried *T. chuii* due to its strong fishy flavor [12]. However, whereas fishy flavors may be unwanted in many food products, we believe they may be valuable for improving the sensory properties of plant-based seafood alternatives.

Cultivating *T. chuii* under different growth conditions (e.g. nutrient availability, light intensity, cultivation temperature and pH) can highly impact the biochemical biomass composition [13]. By changing the cultivation conditions, it might also be possible to fine-tune the flavor properties of *T. chuii* which might increase the potential for *T. chuii* as a flavoring agent in food. So far, no studies have investigated the flavor properties of *T. chuii* biomass cultivated under different conditions. Nitrogen (N) stress is a commonly applied cultivation strategy that has a major impact on biomass composition [14]. This study evaluates whether these changes in biomass composition also impact the flavor profile of *T. chuii* biomass. We hypothesize that under N stress the umami intensity of the *T. chuii* biomass could reduce as N is the main element required for the synthesis of proteins and nucleic acids [15], which are the precursors for FAAs and 5'-nucleotides that contribute to the umami taste. Furthermore, under N stress, the carbon allocation in *T. chuii* is redirected from protein and pigment production towards starch accumulation, reaching up to 59% dry weight (DM) [16]. Pure starch is neither water-soluble nor volatile at ambient temperatures, which might result in modified flavor properties of the *T. chuii* biomass [17]. It is know that various stress conditions including nutrient stress induce the formation of VOCs that may serve as grazer deterrent or signal molecules

to other microalgae cells [18]. These VOCs include degradation products of carotenoids such as β-cyclocitral [19], degradation products of dimethylsulfoniopropionate (DMSP) such as DMS [20] and degradation products of polyunsaturated fatty acids (PUFAs) such as 2,4-decadienal [21]. As these VOCs have low odor threshold values (OTV), we hypothesize that N stress might also influence the aroma of the *T. chuii* biomass.

The aim of this study was to compare the flavor properties of *T. chuii* cultivated under nitrogen limited concentration (N-deplete) and nitrogen sufficient concentration (N-replete). For this purpose, both sensory evaluation and chemical profiling were carried out. The chemical profiling included an analysis of the odor-active VOCs and taste-active chemicals that contribute to the umami taste which include FAAs and 5'-nucleotides. *T. chuii* biomass could play an interesting role as flavoring agent in the development of plant-based seafood alternatives because of its seafood flavor properties. Fine-tuning of the taste and aroma of *T. chuii* by controlling the cultivation conditions could be the way forward to improve the consumer acceptance regarding this microalgae.

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2. Materials and methods

2.1. Strain and pre-cultivation

The marine chlorophyte *Tetraselmis chuii* RCC128 was acquired from Roscoff Culture Collection (Roscoff, France). This microalgae culture was gradually scaled up from 50mL to 10L in autoclaved (20min at 121°C) glass bottles which contain pre-filtered (1µm pore size, Golantech) and autoclaved natural seawater from the Eastern Scheldt (The Netherlands). Food grade nutrients ($NaNO_3$ and $NaH_2PO_4*2H_2O$) were autoclaved, whereas trace elements and vitamins were filter sterilized (0.2µm pore size, Sartorius stedim Sartoclean CA 0.2µm 5625307A9--00). The cultivation medium was based on F/2 [22], containing 75mg/L $NaNO_3$ (13444-1kg ≥99.5%), 5.65mg/L $NaH_2PO_4*2H_2O$ (04269-1kg ≥99.0%), 3.15mg/L $FeCl_3*6H_2O$ (31232-250g ≥99%), 0.01mg/L $CuSO_4*5H_2O$ (12849-100g 99-101%), 0.01mg/L $CoCl_2*6H_2O$ (255599-100g 98%), 0.006mg/L $Na_2MoO_4*2H_2O$ (A7302-100g 81-83%) which were purchased from Honeywell Fluka. 4.16mg/L NaH_2EDTA (E1001-125g 99-101 %), 0.18mg/L $MnCl_2*4H_2O$ (M1106-125g 101%) and 0.5µg/L biotin (BI115 100,5%) were purchased from Spectrum chemical. 0.022mg/L $ZnSO_4*7H_2O$ (438401-500g 99-108,7%) and 0.1mg/L thiamine HCl (411005-100g 98-102%) were acquired from J.T.Baker. 0.5µg/L cyanocobalamin (V2876-100mg ≥98.0%) was obtained from Sigma-Aldrich. Cultures were continuously illuminated at a photon flux density of 150 µmol photons/m^2 s provided by cool white fluorescent tubes (white F3 S7, Sanlight). The temperature in the incubator (Polar Refrigerator Model CW193) was maintained at 20 ± 0.5°C and the medium was aerated with 5L/min filtered air (Durr Technik GBWA062ABKM13013 Air Pump) and buffered with HEPES buffer (CL0008540500-500g 99.5+%, Chem-Lab NV) to maintain a pH 8.

2.2. Cultivation setup

Four sterilized glass bubble columns of 40L with internal diameter of 20cm (Phyco-Conical PBR Varicon Aqua solutions, United Kingdom) were placed in a temperature controlled room at 20 ± 0.5°C for the batch cultivation of *T. chuii* (Figure 1). The photobioreactors (PBRs) were filled with 36L of seawater which was pre-filtered (1µm pore size), UV treated and filter sterilized (0.2µm pore size). Afterwards, each PBR was inoculated with 2L of pre-cultured *T. chuii* in the exponential phase. The nitrogen (N) concentration in the

culture medium was adjusted to 100mg NO_3-N/L in two PBRs (labelled as "N-replete") and 30mg NO_3-N/L in the other two PBRs (labelled as "N-deplete"). All PBRs were provided with $NaH_2PO_4*2H_2O$ to a concentration of 20mg PO_4-P/L to avoid phosphorus (P) limitation. The PBRs were continuously illuminated from three sides using the same light settings as the pre-cultivation. The cultures were aerated (5L/min) and the pH was maintained at 8.0 ± 0.5 by a pH controller using food-grade CO_2 (CO_2 Premier 26263, Air products). This cultivation setup was repeated after 3 months, resulting in four biological replicates of *T. chuii* cultivated in both N-replete and N-deplete conditions. *T. chuii* grown in N-deplete PBRs were N starved for 7 days, while N was not limited during the cultivation of the *T. chuii* in the N-replete PBRs. Due to different adaption time after inoculating in the PBRs and biological variations, the *T. chuii* cultures were harvested after 12 days in the first run and after 15 days in the second run (repetition) to achieve 7 days of N starvation. All PBRs were harvested in the declining phase (late-exponential phase) using a lamella centrifuge (4000g, R. Van Houte). Recently, we showed that storage and processing have major effect on the flavor profile of microalgae [23]. In order to study solely the effect of cultivation, freshly harvested *T. chuii* pastes were immediately frozen at -20°C, freeze-dried within 3 days (Labconco, cat no. 7934031) and subsequently stored in airtight glass jars at -80°C until analysis. The microalgae growth was monitored by cell count which was measured using a Bürker chamber under the microscope. To ensure N limitation for 7 days, the NO_3-N concentration was followed with a Skalar Flow Injection Analysis (FIA). To ensure no phosphorus limitation occurred during the growth, the P concentration was followed with an Inductive Coupled Plasma-Optical Emission Spectrometry (ICP-OES) analysis.

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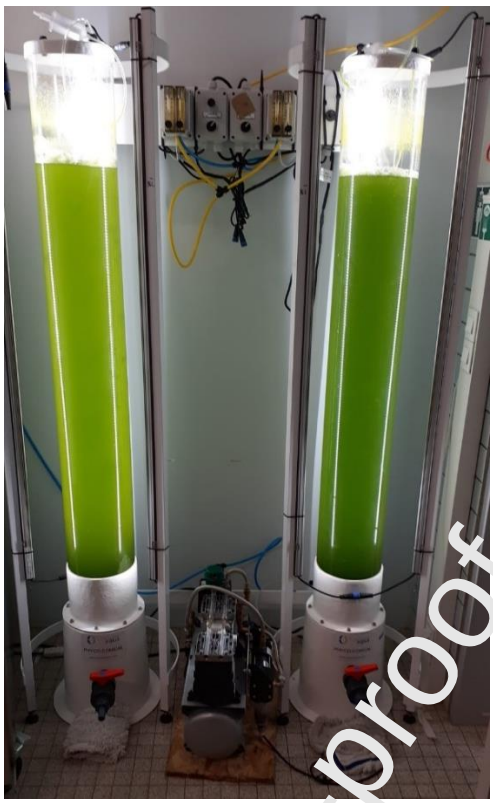

Figure 1: Experimental setup of T. chuii cultivation. The PBR on the left was cultivated in N-deplete conditions (30mg NO3-N/L) and the PBR on the right was cultivated in N-replete conditions (100mg NO3-N/L).

2.3. Biochemical composition

Protein content was determined by the Kjeldahl's method [24]. A conversion factor of 4.78 was used to convert total nitrogen to the protein percentage in the microalgae [25]. Carbohydrate content was determined by the Dubois method [26]. Fatty acids (FAs) were extracted and quantified based on the AOAC official method 996.6. Shortly, 1g of freeze-dried material was hydrolyzed by 10mL HCl (25% v/v) and 2mL ethanol (CL00.0507.2500, Chem-lab NV) in a hot water bad for 40min at 80°C. To limit oxidative degradation of the fatty acids, 100mg pyrogallol (P0381 ≥98%, Sigma-Aldrich) was added. Subsequently, 25mL of diethyl ether (CL00.0405.2500, Chem-lab NV) and 25mL of petroleum ether (CL00.1608.2500, Chem-lab NV) were added for the extraction and the organic ether layer was separated from the alcoholic water layer after centrifugation (600g for 5min). Next, the ether was evaporated and lipid residue was redissolved in 3mL of chloroform (102445, Sigma-Aldrich). The fatty acids were methylated with 2mL of boron trifluoride (7% v:v in methanol) (B1252, Sigma-Aldrich) and 1mL of toluene (CL00.2028.1000, Chem-lab NV) and subsequently incubated at 100°C for 45min. The fatty acid methyl esters (FAME) were analyzed and quantified by means of gas chromatography (GC) mass spectrometry using a Gerstel MPS sampler coupled

to an Agilent 8890 GC and a 5977B MSD mass spectrometer with a Agilent Select FAME column (50 m x 0.25 mm x 0.25µm). Injector temperature was 250°C and a split ratio of 180:1 was used. The oven temperature program was set as follows: start at 50°C, hold for 2min, then raised to 180°C at a rate of 20°C/min. Next, the temperature was raised to 240°C at a rate of 14°C/min. FAME were identified and quantified by comparing the retention times and relative response (using triglyceride triundecanoin (C11:0) (T5534, Sigma-Aldrich) as internal standard) with those of authentic standards (18919-1AMP, Supelco).The fatty acids (total FA) were divided in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Details on the complete fatty acid profile can be found in Supplementary Information. Chlorophyll and carotenoid content were determined according to Kulkarni & Nikolov (2018) [27]. In short, 0.02g freeze-dried biomass was extracted with 10mL ethanol (CL00.0507.2500, Chem-lab NV) in a ultra-sonication bath, followed by incubation in a warm water bath at 45°C for 30min. Next, the supernatant was collected after centrifuging (10min at 3000g). This extraction procedure was repeated a second time. Absorbance (A) at 664nm, 649nm and 470nm was measured using UV–vis spectroscopy (Shimadzu UV 1700) and following equations were used for the calculation of chlorophyll a, chlorophyll b and carotenoids:

(1) Chlorophyll a (µg/mL)=(13.36$*A$664)−(5.19$*A$649)

(2) Chlorophyll b (µg/mL)=(27.43$*A$649)−(8.12$*A$664)

(3) Carotenoids (µg/mL)=((1000$*A$470)−(2.13$*$ Chlorophyll a)−(97.64$*$ Chlorophyll b))/209

The color of the *T. chuii* biomass was determined by measuring the reflectance using a CM-5 reflectance spectrophotometer (Konica Minolta Sensing Americas Inc). *T. chuii* samples were diluted to a concentration of 40 mg DM/mL and 2mL of the sample was placed in a small petri dish. Every sample was measured nine times and the average color values were calculated for the N-deplete and N-replete samples. The results were expressed in terms of L*, lightness (from 0 to 100%); a*, redness to greenness (60 to −60); b*, yellowness to blueness (60 to −60), following CIELAB system.

2.4. Volatile organic compounds analysis

Volatile organic compounds (VOCs) were determined using an automated headspace solid-phase microextraction (HS-SPME) – gas chromatography-mass spectrometry (GC-MS) method according to Coleman et al. (2022) [8]. For extraction, 1.2g DM of *T. chuii* was dissolved in 12mL mineral water (Cristaline) and was hermetically sealed in a 20mL amber colored SPME vial to avoid photodegradation. The samples were kept refrigerated until 10min before the start of the incubation to limit oxidation or degradation reactions prior to the extraction. The vials were incubated for 30min at 40°C, followed by 30min extraction at 40°C using a Gerstel MPS sampler coupled to an Agilent 7890A GC. The loaded Supelco 50/30µm DVB/CAR/PDMS fiber was desorbed in splitless mode (250°C, 3min) and compounds were separated on a DB-5MS column (30m x 250µm x 1µm; Agilent) using a helium flow rate of 1mL/min. The oven temperature program was set as follows: start at 35°C, hold for 3min, then raised to 220°C at a rate of 3.5°C/min. The mass spectra in the electron impact ionization (EI) mode were generated at 70eV and recorded in full scan mode (35 – 250 m/z) utilizing a 5975C Inert XL mass spectrometer (Agilent).

The identification of aroma compounds via Unknown analysis of Masshunter (Agilent) was performed based on (1) spectral match, compared to the NIST (v2, 2011) and an in-house spectral database, and (2) retention index (RI), compared to the Aroma office 2D (v3.00.00, 2017, Gerstel) and an in-house RI database. RI calibration of the chromatogram was accomplished using an C8-C20 alkane standard solution (04070-1ML, Sigma-Aldrich). Aroma compounds were identified if following criteria were met: (1) peak height is more than 10^3 (counts), (2) signal-to-noise ratio is higher than 3, (3) spectrum match probability is higher than 75% , and (4) calculated experimental RI differs less than 15 from library RIs. Experimental Kovats RI, library RI and match factor information used for identification can be found in Supplementary Information.

Semi-quantitative determination of the volatiles was performed by spiking 10µL of the internal standard mix to each sample prior to the SPME-GC-MS analysis. A internal standard stock solution was prepared at 8.16ng/µL 2-methyl-3-heptanone (103128-5G, Sigma-Aldrich) and 87.5ng/µL methyl nonanoate (76368-1ML, Sigma-Aldrich) in methanol (CL00.1377.2500, Chem-Lab Analytical). Similar to Isleten Hosoglu et al. (2020) [28], the area of the chromatographic peak of each identified volatile was divided by the area corresponding to the internal standard 2-methyl-3-heptanone. Areas corresponding to identified esters were divided by the area of internal standard methyl nonanoate. To calculate semi-quantitative concentrations of each volatile, the obtained responses were multiplied with the concentration of internal standard in the

samples (6.8µg/L for 2-methyl-3-heptanone and 72.9µg/L for methyl nonanoate), assuming that all of the response factors were equal to one. Subsequently, these concentrations were divided by their individual OTV determined in water to calculate the odor activity values (OAVs) of each volatile. Since both concentration in the sample and OTV are taken into account, OAVs provide more insight on which volatile compounds contribute to the flavor. Because of the high OTVs of alkanes and alkenes [29], we assumed their contribution to the aroma was negligible and were not further considered [30]. This approach for calculating semi-quantitative results is comparable to the approach described by Giri et al. (2010) [31] and Van Durme et al. (2013) [32]. The four biological replicates of N-replete and N-deplete were measured in triplicate.

2.5. Free Amino acid analysis

Free amino acids (FAAs) were determined according to Coleman et al. (2022) [8]. Dried algae sample (0.2g) is extracted with 8.5mL UHPLC-MS water using a vortex for 15s. Prior the extraction, 0.5mL of the internal standard mixture was added containing 134.9µg/mL methionine-3,3,4,4-d_4; 34.1µg/mL N-methyl-L-valine; 146.3µg/mL histidine-$^{13}C6^{15}N3$; 282.7µg/mL L-glutamine $^{13}C5$; 1259.2µg/mL L-glutamic acid $^{13}C5$; 294.4µg/mL DL-Lysine-$^{13}C1,2$; 435.7µg/mL L-aspartic acid-^{15}N-2,3,3-d_3; 634.1µg/mL L-aspargine-$^{15}N2$; 316.8µg/mL L-alanine-2,3,3,3-d_4 and 430.2µg/mL homoarginine in UHPLC-MS water. FAAs were separated from proteins and peptides by precipitation using 1mL 35% sulfosalicylic acid (S2130-100G, Sigma-Aldrich). After 15min of extraction at room temperature, samples were centrifuged for 10min at 4000g. Aliquots of 500µL of the supernatants were diluted with 100µL acidified (1%v/v formic acid) ammonium formate buffer (4M) and 400µL acetonitrile (ACN). Amino acids were separated according to van ’t Land (2019) [33] by hydrophilic interaction chromatography (HILIC) using a LC-MS system. 3µL of aqueous extract was injected onto an Intrada HILIC column (100mm x 3mm x 3µm) maintained at 37°C. A binary gradient at a flow rate of 0.6mL/min was used, consisting of acidified (0.3% v/v formic acid) ACN and 20/80 (ACN (≥ 99.95%) /ammonium formate (≥ 99%)). Amino acids were detected using a Shimadzu triple quadrupole mass spectrometer (LCMS-8040) equipped with an electrospray ionization source (ESI). Nitrogen was used as nebulizer and argon as collision gas. Most amino acids were ionized in positive ESI and measured in multiple reaction monitoring (MRM), except for glycine in selected-ion monitoring (SIM). Aspartic acid was detected after negative ESI and SIM. Details on binary gradient, MRM and SIM can be

found in SI. The quantification was performed using calibration curves of 20 amino acids at 6-point external calibration curve close to their taste threshold.

2.6. Free 5'-nucleotides analysis

Free 5'-nucleotides were extracted and analyzed according to Moerdijk-Poortvliet et al. (2022) [34]. In brief, 50mg dried sample and 5mL Milli-Q were homogenized and extracted for 15min at 35˚C followed by centrifugation (3700g, 20min). The supernatant of the samples was supplied with 125µL concentrated H_2SO_4 for acid precipitation followed by centrifugation (3700g, 20min). The supernatant was analyzed by means of High Performance Liquid Chromatography (HPLC) using a DIONEX Ultimate 3000 HPLC system equipped with a SIELC PrimeSep D mixed-mode column (150mm x 4.6mm x 5µm) with a corresponding guard column (10mm x 4.6mm x 5µm) and detected by Ultraviolet (UV) (DAD 3000). The injection volume was 10µL and elution was performed isocratically at 0.8mL/min, with 10mM H_2SO_4 (pH 1.95) as the mobile phase; the detection wavelength was 260nm. Quantification of nucleotides was achieved using a 7-point external calibration curve (5 to 1000µM).

2.7. Equivalent umami concentration

The intensity of the umami taste of microalgae can be estimated by measuring the equivalent umami concentration (EUC) expressed as monosodium glutamate (g MSG/100g). The synergy effect between the umami amino acids and 5'-nucleotides is represented by the following equation [35]:

$$Y = \sum a_i b_i + 1218 \left(\sum a_i b_i\right) \left(\sum a_j b_j\right)$$

where Y is the EUC of the mixture expressed in g MSG/100g; 1218 is a synergistic constant; a_i is the concentration (g/100g) of each umami amino acid, aspartic acid (Asp) or glutamic acid (Glu); a_j is the concentration (g/100g) of each umami 5′-nucleotide, inosine monophosphate (IMP), guanosine monophosphate (GMP) or adenosine monophosphate (AMP); b_i is the relative umami concentration (RUC) for each umami amino acid compared to MSG (1 for Glu and 0.077 for Asp) and b_j is the RUC for each umami 5′-nucleotide compared to IMP (1 for IMP; 2.3 for GMP and 0.18 for AMP).

2.8. Salt analysis

Analysis of Na, K, Mg and Ca was performed by ICP-OES (Varian 720, Varian, Mulgrave, Australia) after acid mineralization with HNO_3 in a microwave oven (CEM MARS Xpress, Matthews, USA). Aliquots of 0.250g dry sample were weighed in triplicate in polytetrafluoroethene (PTFE) microwave vessels. After the addition of 4mL nitric acid and 4mL bidistilled water, the vessels were closed and placed into a microwave system. The samples were heated to 180°C in 15min and maintained at that temperature for 30min. After cooling, samples were diluted on a weight basis (2g sample solution + 8g bidistilled water) in analytical 15mL tubes. Quantification of the elements in the digests was performed using an 8-point external calibration curve (calibration range: 0.01ppm - 100ppm).

2.9. Sensory Evaluation

All sensory research performed in this study was in accordance with Ethical Standards of the Commission Flavor and Odor of ILVO (ECSG-ILVO). Prior to the sensory sessions, the *T. chuii* biomasses were tested on microbial quality and safety (coagulase positive *S. aureus* enumerations, *E. coli* enumerations, *Salmonella* detection, *Listeria* spp. and *Listeria monocytogenes* detection) and trace element analysis (Cd, Pb, Hg, As and I). Afterwards, a risk evaluation was made and evaluated by the ECSG-ILVO and can be found in Supplementary Information. Informed consent was obtained from all participants of the sensory evaluation. The flavor of the *T. chuii* samples were evaluated by a sensory panel which was trained according to the ISO8586:2012 standard. All panelists had experience in evaluating algae (> 2 years). The taste lab is equipped according to the ISO standard 8589:2007 using the Fizz (Fizz Biosystèmes) software. A free choice profiling (FCP) session was organized in which the sensory attributes of the N-replete and N-deplete *T. chuii* biomass were identified. Based on a panel discussion, the sensory attributes and reference products were selected. Afterwards, two sensory tests were organized in which the intensity of the sensory attributes (Table 1) were scored using a scale ranging from 0 (absent) to 10 (very strong). In the first sensory test, two N-replete and two N-deplete samples were evaluated by a panel group consisting of 11 judges. In the second sensory test, one N-replete and one N-deplete sample were assessed by a different panel group consisting of

8 judges. All *T. chuii* samples were dissolved in mineral water (Cristaline) to a concentration of 40g/L. Small amounts (20mL) were presented in randomly coded and closed 30mL screw-capped amber colored glass vials.

Table 1: Sensory attributes associated to the T. chuii biomass, their descriptions and used reference products.

Sensory attributes	Description	Reference product
Odor intensity	/	/
Grassy odor	The odor associated with sweet, freshly cutted grass	0.2g/L 1-hexen-3-ol solution
Earthy odor	The odor associated with beetroot odor	Pure beet root
Shellfish odor	The odor of mussel, crab, seafood	Mix 5g/L mussel and 5g/L crab powder[a]
Taste intensity	/	/
Umami	The taste associated with monosodium glutamate (MSG)	0.595g/L MSG
Salt	The taste on the tongue associated with salt	1.19g/L NaCl
Sweet	The taste on the tongue associated with sucrose	0.195g/L sucrose
Bitter	The taste associated with caffeine	0.195g/L caffeine
Sour	The taste associated with citric acid	0.43g/L citric acid
Shellfish taste	The taste of mussel, crab, seafood	Mix 5g/L mussel and 5g/L crab powder[a]

[a]crab powder (*Cancer pagurus*) and mussel powder (*Mytilus edulis*) were obtained from Flandor Flavors International (Zulte, Belgium)

2.10. Statistical Analysis

Using R (version 4.0.5), sensory scores proved to fit a normal distribution, based on the evaluation of the Quantile Quantile plot and a histogram of the residuals. A linear mixed-effect model was conducted for each sensory attribute (lmer function, “lsmeans” package). The treatment (N-replete and N-deplete) was considered as fixed factor and the effect of the assessors were considered random. To determine significant differences between the sensory attributes of the treatments, ANOVA (Type III) was used at an α risk of 5%. This method is commonly applied to data from descriptive analysis [36]. Wilcoxon rank sum tests were used to determine significant differences within all chemical data of all N-replete and N-deplete biological replicates (n = 4), applying an α risk of 5%. Principal component analysis (PCA) was performed using MetaboAnalyst (version 5.0) to identify the similarities and differences in the volatile data (mean-centered and log transformed) and sensory data (mean-centered) of the different *T. chuii* biomasses. The correlations

between odor sensory scores and the VOCs detected by SPME-GC-MS were studied using partial least-squares regression (PLSR) (plsr function, “pls” package). This technique is robust and can deal with small sample size, missing values and multicollinearity [37]. For the PLSR, the mean odor sensory scores (averaged over the panelists) and the mean chemical OAVs (average of 3 chemical replicates) were used for each biological replicate of N-replete and N-deplete. Chemical OAVs were mean-centered and log transformed. To determine the number of components in the PLSR analysis, the leave-one-out cross-validation was used. The regression coefficients obtained from the PLSR-model were visualized in a heat plot.

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3. Results and discussion

3.1. Effect of nitrogen supply on the biochemical composition of *T. chuii* biomass

Major differences in biochemical composition are observed between the *T. chuii* biomass obtained from N-replete and N-deplete conditions (Table 2). The protein content of N-replete biomass (35.0% DM) is twice as high compared to the N-deplete biomass (16.4% DM). On the contrary, the carbohydrate content is drastically reduced in the N-replete biomass (10.3% DM) compared to the N-deplete biomass (36.3% DM). These findings are coherent with the study of Bondioli et al. (2012) [38] in which *T. suecica* (genetically close to *T. chuii*) was grown in 16L outdoor PBRs under N starved and N sufficient conditions for 14 days. N starvation of *T. suecica* caused a strong decrease in protein content compared to the *T. suecica* grown in N sufficient cultivation medium (from 51% to less than 10% DM) which was compensated by an increased carbohydrate content (from 15% to more than 50% DM). Similar changes in biochemical composition were observed for *T. suecica* grown in 20L PBR under N limited conditions for 10 days [39] and *T. subcordiformis* grown in 0.5L PBR under N starvation conditions for 7 days [40].

A shift from polyunsaturated fatty acids (PUFAs) to mono-unsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) is observed in the N-deplete *T. chuii* biomass. This shift is mainly attributed to the significant reduction of linoleic acid (C18:2, n-6c) and alpha-linolenic acid (C18:3, n-3c) and the increase of palmitic acid (C16:0) and elaidic acid (C18:1, n-9t) in the N-deplete biomass (Supplementary Table A.4). Souza & Kelly (2000) [39] also found higher amounts of SFAs and lower amounts of PUFAs in *T. suecica* grown under N limited conditions compared to *T. suecica* cultures grown under N sufficient conditions.

Finally, a strong increase of the chlorophyll and carotenoid content in the N-replete *T. chuii* biomass is observed. These findings are in line with the study of Dammak et al. (2017) [41] in which the highest chlorophyll and carotenoid content in *Tetraselmis* sp. biomass corresponded with the highest N concentration in the cultivation medium. In our study, the difference in chlorophyll content between N-replete and N-deplete is also reflected in the color analysis. Higher L* (lightness) and b* (yellow axis) values are obtained in the N-deplete biomass, explaining its yellowish green color. The N-replete biomass, on the contrary, possesses a deep dark green color which is associated with the higher chlorophyll content.

Table 2: Biochemical composition and colorimetric values of T. chuii grown in N-replete and N-deplete conditions. Data are expressed as the average and standard deviation of the four biological replicates. Significant differences between the N-replete and N-deplete biomass are indicated by p < 0.05.

	N-replete	**N-deplete**	**p-value**
Biochemical composition			
Carbohydrates	10.3 ± 0.9% DM	36.3 ± 2.6% DM	<0.05
Proteins	35.0 ± 4.7% DM	16.4 ± 0.9% DM	<0.05
Total Fatty acids	7.1 ± 0.4% DM	6.5 ± 0.3% DM	0.057
Saturated fatty acids (SFA)	2.0 ± 0.1% DM	2.2 ± 0.1% DM	<0.05
Mono-unsaturated fatty acids (MUFA)	0.9 ± 0.2% DM	1.2 ± 0.2% DM	0.100
Polyunsaturated fatty acids (PUFA)	4.2 ± 0.0% DM	3.1 ± 0.2% DM	<0.05
Carotenoids	4.0 ± 0.5mg/g DM	2.6 ± 0.6mg/g DM	<0.05
Chlorophyll a	12.3 ± 1.8mg/g DM	4.9 ± 1.3mg/g DM	<0.05
Chlorophyll b	8.3 ± 1.2mg/g DM	3.2 ± 0.9mg/g DM	<0.05
Colorimetric values			
L*: Lightness (0 - 100 %)	10.0 ± 2.3	20.4 ± 2.3	<0.05
a*: Greenness (-60) to Redness (+60)	-6.4 ± 1.1	-9.1 ± 0.7	<0.05
b*: Blueness (-60) to Yellowness (+60)	9.4 ± 2.9	20.2 ± 3.1	<0.05

3.2. Effect of nitrogen supply on the odor and volatile profile of *T. chuii* biomass

Both the N-replete and N-deplete *T. chuii* biomass are characterized by a dominant shellfish-like aroma (Figure 2) which is an interesting flavoring feature for the development of plant-based seafood alternatives. However, the intensity of the odor features of *T. chuii* is strongly influenced by the N supply in the cultivation medium. The N-deplete *T. chuii* biomass possesses an overall stronger odor intensity ($p < 0.0001$) due to the more pronounced earthy-like odor ($p < 0.05$) and shellfish-like odor ($p = 0.0509$). The stronger earthy-like odor of the N-deplete *T. chuii* is undesirable and might reduce its applicability in food.

From the 27 identified VOCs, only few exceeded their OTV and therefore contribute to the flavor properties of *T. chuii*. These VOCs include DMS, DMDS, MeSH, TMA, 2,3-butanedione, 3-methylbutanol, 3-methylbutanal, isophorone and benzaldehyde (Table 3). Based on the OAVs, the odor of the N-deplete and N-replete *T. chuii* is strongly determined by the sulfur-containing compound DMS. Sulfur-containing volatiles are essential contributors to the aroma of several shellfish products such as mussel, oyster and crab [42, 43], which could explain the strong shellfish-like odor in both N-replete and N-deplete *T. chuii* biomass (Figure 2). The stronger odor intensity of the N-deplete biomass is explained by the significantly higher sum of the OAVs of all VOCs found in the N-deplete biomass (OAV 4288 ± 1039) compared to the N-replete

biomass (OAV 1714 ± 569). Similar to our study, the VOC emission of cyanobacteria *Microcystis flos-aquae* increased when the N was depleted in the cultivation medium [44].

Partial least-squares regression (PLSR) analysis was performed to determine the relationship between the OAVs and the sensory scores of the odor attributes. The first three components of the PLSR model explain 97.6% of the variance in the volatile profile and explain 99.3%, 97.7% and 97.0% of the variance of the shellfish, grassy and earthy odor, respectively. The correlation (regression) coefficients obtained from the PLSR model are presented in a heat plot (Figure 3).

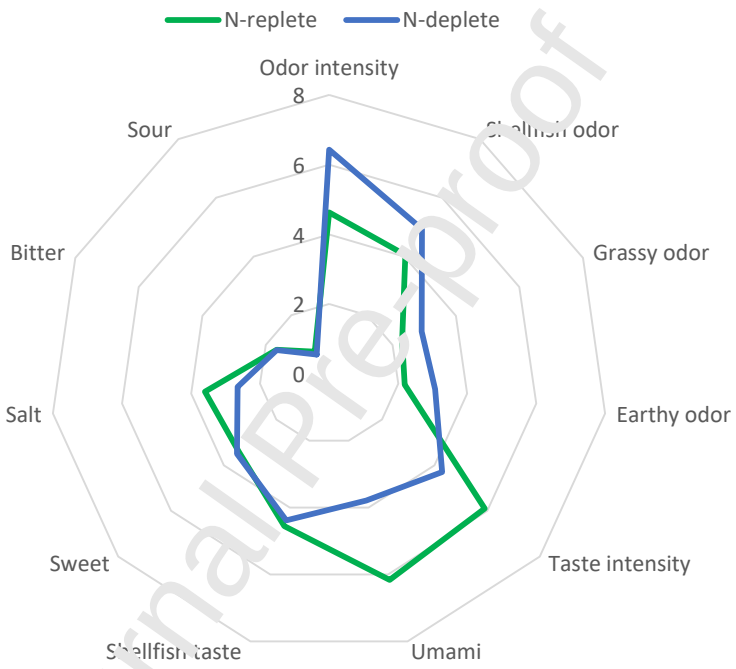

Figure 2: The sensory evaluation of the N-replete T. chuii biomass (green) and the N-deplete T. chuii biomass (blue) visualized in a radar chart. Average scores over the biological replicates (n = 3) were used.

A strong correlation is found between the shellfish-like odor, the sulfur-containing compounds DMS, DMDS and MeSH, and the nitrogen-containing compound TMA (Figure 3). Similarly, Van Durme et al. (2013) [32] linked the cooked shrimp and marine-like odor in *Tetraselmis* sp. paste to the presence of sulfur-containing compounds. Furthermore, Coleman et al. (2022) [8] showed that DMS, MeSH as well as TMA are important for the seafood-like flavors of microalgae. TMA originates from trimethylamine N-oxide degradation and is generally present in marine seafood contributing to their flavor. However, TMA is an early indicator of seafood spoilage as the accumulation of this VOC could result in an unwanted putrid odor [45]. The concentration of TMA in N-deplete and N-replete *T. chuii* biomass did not significantly differ. In contrast,

the sulfur-containing volatiles DMS, DMDS and MeSH increased when *T. chuii* was N starved which could explain the higher observed odor intensity and shellfish-like odor in the N-deplete biomass.

Marine microalgae such as *Tetraselmis* can accumulate high intracellular concentrations of DMSP, depending on the growth phase and cultivation conditions such as light intensity and salt concentrations [46]. In a stressful environment such as in N limitation conditions, proteins are broken down for the regeneration of intracellular nitrogen. Amino acid methionine is transformed into DMSP which can be further degraded by DMSP lyase to DMS and acrylate [47]. Sunda et al. (2007) [48] found a 70% increase of the DMPS/C ratio under N limitation of the diatom *Skeletonema costatum* compared to N sufficient conditions. Furthermore, the authors also observed an increased activity of the DMSP cleavage enzyme, resulting in a 20-fold increase of DMS in the culture per unit of cell volume of *Emiliania huxleyi*. Sunda & Huntsman (2002) [46] highlighted the importance of the antioxidant function of DMSP and its breakdown products DMS and acrylate. Possibly, the lower photosynthetic efficiency (lower chlorophyll content) and reduced antioxidant capacity (lower carotenoid content) in the N deplete *T. chuii* might have resulted in increased oxidative stress due to the generation of reactive oxygen species (ROS). In response, the N-deplete *T. chuii* culture might have up-regulated its DMSP/DMS-system as a protect (antioxidant) mechanism to scavenge the harmful ROS, resulting in higher DMS formation. In addition, both DMDS and MeSH can be generated from DMS or by the (microbial) catabolism of sulfur-containing compounds such as methionine, cysteine and derivatives [49].

Based on the PLSR results, the earthy odor is strongly correlated with 3-methylbutanal, 3-methylbutanol and benzaldehyde (Figure 3). Higher OAVs were calculated for VOCs 3-methylbutanal and 3-methylbutanol in the N-deplete *T. chuii* biomass. The odor feature of 3-methylbutanal is described as musty, cocoa, coffee and nutty-like and the odor of 3-methylbutanol is described as fermented, cognac and whiskey-like [50]. Alkyl aldehyde 3-methylbutanal can be generated from 2-keto acid α-ketoisocaproate, an intermediate in the branched chain amino acid pathway of leucine, via keto-acid decarboxylases. Subsequently, 3-methylbutanal can be converted into alkyl alcohol 3-methylbutanol by alcohol dehydrogenases [51]. In the study of Hasegawa et al. (2012) [52], the alkyl alcohols (e.g. 2-methylbutanol, 3-methylbutanol and 2-phenylethanol) emitted by the freshwater cyanobacteria *Microcystis aeruginosa* raised when the N was depleted in the cultivation medium.

Finally, 2,3-butanedione (diacetyl) is also an important contributor of the odor of N-deplete *T. chuii* biomass, whereas this VOC is completely absent in the N-replete *T. chuii* biomass. Its strong buttery aroma contributes to the stronger odor intensity observed in N-deplete *T. chuii* biomass. 2,3-butanedione can be produced from α-acetolactate, an intermediate in branched chain amino acid pathway of valine and leucine, via oxidative decarboxylation [31]. Most likely, due to the protein degradation in the N-deplete *T. chuii* biomass, amino acids such as leucine and valine are broken down, resulting in higher amounts of 2,3-butanedione, 3-methylbutanal and 3-methylbutanol. These VOCs were also generally found in several microalgae and seaweeds species [8, 32, 36]. 2,3-butanedione is found in alcoholic beverages and dairy products as a by-product during fermentation by yeast and lactic acid bacteria (LAB) [53]. It is unlikely that the 2,3-butanedione in the N-deplete PBR is formed by fermentation reactions by yeast or LAB as the PBR were continuously aerated and no elevated numbers of yeast and LAB were observed in the N-deplete biomass (data not shown). Jaffrès et al. (2011) [54] indicated that the formation of 3-methylbutanal and 2,3-butanedione is associated with microbial activity during seafood spoilage. Although no contamination was observed in the *T. chuii* cultures, bacterial activity in the N-deplete PBR cannot be ruled out as microalgae in a symbiotic relationship with bacteria [55]. The N starved conditions in the *T. chuii* culture could have caused more cell death which might have increased the microbial activity in the N-deplete PBRs, explaining the higher amount of VOCs compared to the N-replete *T. chuii* biomass. To conclude, this study shows that N stress during the cultivation of *T. chuii* generates higher amounts of odor-active VOCs, affecting the odor properties of the microalgae biomass. The earthy-like odor of the N-deplete *T. chuii* biomass can be perceived as an off-flavor when using *T. chuii* as flavoring agents in plant-based seafood.

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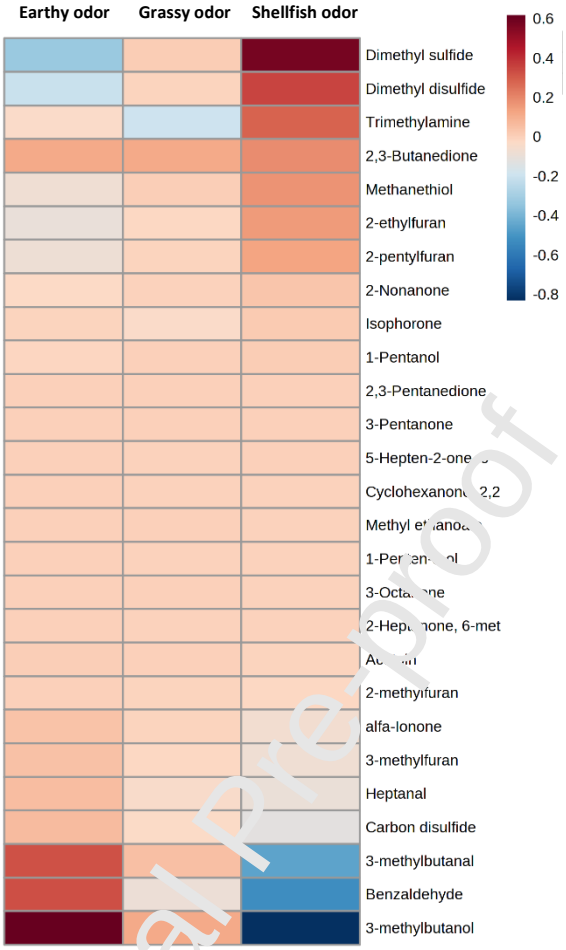

Figure 3: Heat plot of the correlation coefficient between each volatile and the odor attributes obtained by PLSR analysis. High correlations are indicated in red, low correlations are indicated in blue

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Table 3: Odor activity values (OAV) of all individual volatiles and odor threshold value (OTV) determined in water (in ppb). OAVs are expressed as the average and standard deviation of the four biological replicates of the N-replete and the N-deplete T. chuii biomass. Library retention index, experimental Kovats retention index and spectral match factor information used for compound identification can be found in Supplementary Information. Significant differences between the N-replete and N-deplete biomass are indicated by $p < 0.05$.

Volatile Organic Compound	OTV	N-replete	N-deplete	p-value
Sum		1714 ± 569	4288 ± 1039	<0.05
Sulfur-containing compounds				
Methanethiol (MeSH)	0.02 [c]	21.7 ± 6.8	37.5 ± 9.9	0.057
Dimethyl sulfide (DMS)	0.12 [a]	1662 ± 563	4172 ± 1067	<0.05
Dimethyl disulfide (DMDS)	0.29 [a]	0.44 ± 0.16	1.11 ± 0.53	<0.05
Carbon disulfide	16 [a]	1.21 ± 0.62	0.45 ± 0.22	0.057
Nitrogen-containing compounds				
Trimethylamine (TMA)	0.37 [b]	12.8 ± 16.0	11.8 ± 9.6	0.442
Aldehydes				
3-methylbutanal	1.1 [d]	3.90 ± 1.35	7.28 ± 3.75	0.057
Heptanal	0.8 [b]	0.62 ± 0.74	0.11 ± 0.22	0.204
Benzaldehyde	1.5 [a]	2.64 ± 5.07	0.96 ± 0.70	0.243
Alcohols				
3-methylbutanol	1.69 [a]	0.45 ± 0.38	4.92 ± 6.03	<0.05
1-Pentanol	5.5 [a]	0.03 ± 0.04	0.00 ± 0.00	0.155
1-Penten-3-ol	358.1 [d]	0.003 ± 0.004	0.001 ± 0.001	0.322
Alcohol / ketone				
Acetoin	800 [b]	0.003 ± 0.002	0.074 ± 0.020	<0.05
Ketones				
2,3-Butanedione	0.059 [d]	0.00 ± 0.00	48.2 ± 30.0	<0.05
2,3-Pentanedione	9.8 [a]	0.00 ± 0.00	0.01 ± 0.02	0.093
3-Pentanone	28 [a]	0.02 ± 0.00	0.04 ± 0.01	<0.05
3-Octanone	21.4 [d]	0.02 ± 0.01	0.04 ± 0.01	<0.05
2-Nonanone	5 [b]	0.01 ± 0.02	0.09 ± 0.12	0.204
Carotenoids-derived compounds				
5-Hepten-2-one, 6-methyl-	50 [b]	0.03 ± 0.02	0.10 ± 0.04	<0.05
2-Heptanone, 6-methyl-	50 [b]	0.05 ± 0.02	0.05 ± 0.01	0.557
Cyclohexanone, 2,2,6-trimethyl-	100 [b]	0.005 ± 0.002	0.005 ± 0.001	0.343
Isophorone	0.3 [b]	2.23 ± 0.89	2.00 ± 0.65	0.443
α-Ionone	3 [b]	0.09 ± 0.09	0.03 ± 0.01	0.100
Furans				
3-methylfuran	2.3 [d]	0.54 ± 0.15	0.31 ± 0.10	<0.05
2-methylfuran	2.3 [d]	0.65 ± 0.39	0.37 ± 0.13	0.171
2-ethylfuran	2.3 [d]	0.54 ± 0.14	0.53 ± 0.32	0.243
2-pentylfuran	5.8 [d]	0.12 ± 0.02	0.18 ± 0.14	0.343
Esters				
Methyl ethanoate	170 [a]	0.005 ± 0.002	0.002 ± 0.000	0.171

Odor detection threshold values: [a][56]; [b][57]; [c][50]; [d][31]; [e][58]

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A principal component analysis (PCA) was carried out to visualize the effect of N starvation on the sensory properties of *T. chuii* (Figure 4). The first two principal components (PC) are sufficient to explain 84.6% of the variance of the data, with component PC1 contributing 66.7% and component PC2 17.9%. From this PCA plot, it is clear that the N-deplete and N-replete *T. chuii* samples are separated by PC1. PC2 shows higher variation in the odor properties within the N-deplete samples, whereas the N-replete samples are more clustered together. In addition, the PCA of the volatile profile explains 90.6% of the variance of the data, with component PC1 contributing 63.3% and component PC2 27.3% (Figure 4). Similar to the PCA of the sensory properties, more consistency in the volatile profile was observed within the N-replete *T. chuii* samples than within the N-deplete *T. chuii* samples. Possibly, the severity and duration of the N stress was not identical in every N-deplete PBR due to biological variation in the *T. chuii* cultures. This might explain the more inconsistent VOCs formation, resulting in a more inconsistent odor profile of the N-deplete samples compared to the N-replete samples. However, the severity and duration of the N stress on the flavor properties of *T. chuii* should be further investigated.

Low amounts of fatty acid-derived VOCs are detected in both N-deplete and N-replete *T. chuii* biomass (Table 3). Similar to our study, Van Durme et al. (2013) [32] detected low amounts of fatty acid-derived VOCs in the *Tetraselmis* paste. In contrast to the present study and the study of Van Durme et al. (2013) [32], the studies of Moran et al. (2022) [30] and Coleman et al. (2022) [8] observed higher amount of fatty-acid derived VOCs in *Tetraselmis* biomass. Moran et al. (2022) [30] detected several fatty acids-derived VOCs such as hexanal, 1-octen-3-ol, 3,5-octadien-2-one (E,E) and 2-octenal (E) in the *Tetraselmis* biomass. Coleman et al. (2022) [8] detected fatty acids-derived VOCs such as 2,6-nonadienal (E,E), hexanal, heptanal, 4-heptenal (Z) and 2-nonenal (E) in *T. chuii* biomass obtained from an industrial producer. These VOCs originate from fatty acid oxidation, possessing fishy, fatty and grassy odor features [14]. Coleman et al. (2022) [8] described the odor of the freeze-dried *T. chuii* biomass as shellfish-like, however, in contrast to the present study and the study of Van Durme et al. (2013) [32], also pronounced grassy and fishy odor features were observed.

The difference in volatile profile and odor features of the *T. chuii* biomass between studies might be explained by a difference in processing and storage conditions of the microalgae biomass. In the present study, the harvested microalgae pastes were immediately frozen, freeze-dried and stored airtight at -80°C.

These post-harvesting processing steps may be delayed in the *T. chuii* production on an industrial scale. Recently, we showed that post-harvesting conditions such as wet storage of the concentrated microalgae paste has major effect on the aroma profile of the microalgae [23]. Possibly, lipid oxidation and carotenoid degradation occurred during post-harvesting conditions of the industrial produced *T. chuii* biomass, resulting in grassy and fishy-like odors. Whereas the lipid oxidation and carotenoid degradation in the *T. chuii* biomass produced in this study might be limited. However, the effect of processing and storage conditions on the flavor profile of *T. chuii* biomass should be further investigated. Beside the post-harvesting conditions, other cultivation conditions such as controlled indoor vs outdoor conditions, batch vs continuous cultivation and harvesting time might also influence the volatile profile and odor features of microalgae. As cultivation and post-harvesting conditions can strongly influence the volatile profile of microalgae, controlling these conditions may significantly improve the consistency of the microalgae flavor profile and enhance the palatability of the microalgae.

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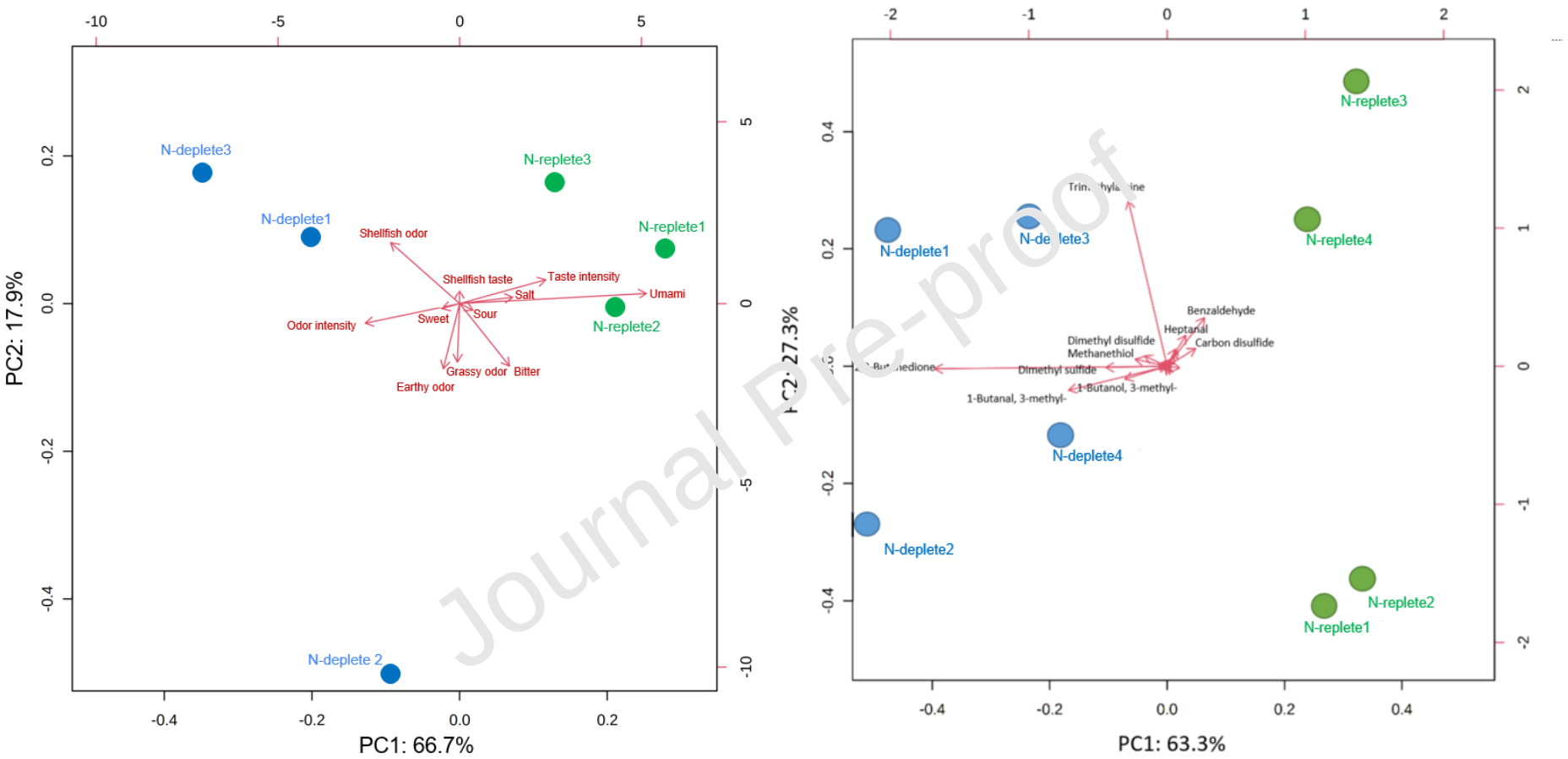

Figure 3: (Left) The PCA plot of the sensory evaluation of all individual N-replete T. chuii (green) and the N-deplete T. chuii (blue) samples, using the average scores of the assessors. (Right) The PCA plot of the volatile profile of all individual N-replete T. chuii (green) and the N-deplete T. chuii (blue) samples, using the average scores of the chemical measurements (n =3)

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3.3. Effect of nitrogen supply on the taste profile of *T. chuii* biomass

Both N-deplete and N-replete *T. chuii* biomass are characterized by a strong shellfish-like taste. The N supply in the cultivation medium did not influence this interesting flavor property of the *T. chuii* biomass. However, a stronger taste intensity ($p < 0.0001$), umami taste ($p < 0.0001$) and salty taste ($p < 0.05$) are observed in the N-replete *T. chuii* biomass (Figure 2). Generally, salt and umami taste improve the acceptability and palatability of food [59], which makes the N-replete *T. chuii* biomass more preferable as flavoring agent compared to N-deplete *T. chuii* biomass. Significantly higher amounts of FAAs and 5'-nucleotides are detected in the N-replete *T. chuii* biomass (Table 4). Similarly, Flynn (2007) [60] observed a decrease of 82% in total FAAs content after 72h of N limitation in *T. marina*. In N stressed conditions, microalgae can recycle N and ammonia via the deamination of nitrogen rich compounds such as proteins and nucleotides [61]. In contrast, if higher N supply is available, microalgae are capable of N uptake beyond the requirement for immediate growth. Khalid et al. (2019) [62] indicated that N can be stored as intracellular ions (nitrate, nitrite and ammonia) and FAAs, which explains the higher FAA concentrations in N-replete *T. chuii* biomass. This 'luxury' uptake and cellular storage of N can be considered as a survival strategy for possible upcoming nutrient stress [63]. The higher FAA and 5'-nucleotide concentrations could explain the higher observed taste intensity in the N-replete biomass compared to the N-deplete *T. chuii* biomass. In addition, if N is depleted from the cultivation medium, microalgae *T. chuii* can accumulate starch as energy reserve to survive in unfavorable conditions [16]. Although not measured directly in our study, one can expect that the majority of the measured carbohydrate content of N-deplete *T. chuii* consists of starch. In theory, starch is tasteless and odorless [17], which could be an additional reason for the lower taste intensity observed in the N-deplete biomass.

Importantly, the taste contribution of the FAAs in the N-deplete and N-replete *T. chuii* is mainly determined by the umami-tasting Glu. The Glu concentration detected in the N-replete *T. chuii* (7.85 ± 0.99mg Glu/g DM) is more than 5 times higher compared to the Glu concentration measured in the N-deplete *T. chuii* (1.48 ± 0.25mg Glu/g DM). In addition, the free 5'-nucleotide AMP concentration is significantly higher in the N-replete biomass (0.84 ± 0.17mg AMP/g DM) compared to the N-deplete biomass (0.30 ± 0.02mg AMP/g DM). As a result, significantly higher equivalent umami concentration (EUC) values are calculated in the N-replete samples (79.1 ± 73.4g MSG/100g DM) compared to the N-deplete samples (6.8 ± 6.5g MSG/100g

DM), which explains its higher observed umami taste. Coleman et al., (2022) [8] calculated an EUC value of 21g MSG/100g DM for industrial produced *T. chuii* which explained its high umami taste compared to several other microalgae species. Notably, the free 5'-nucleotide GMP has a strong impact on the outcome of the EUC values, despite its low concentration (N-replete: 0.30 ± 0.34mg GMP/g DM and N-deplete: 0.12 ± 0.13mg GMP/g DM). However, no differences in umami taste were observed within the N-replete and N-deplete *T. chuii* biomass. The high variation in the calculated EUCs values within the N-replete and N-deplete *T. chuii* biomass is attributed to the high variation in the GMP concentration. Yamaguchi et al. (1971) [35] mentioned that a small amount of 5'-nucleotides may greatly increase the umami taste of free Glu by reducing its taste threshold value. Latsos et al. (2022) [64] also showed that the pH of the cultivation medium had an effect on the taste of *Rhodomonas salina*. A higher EUC value was calculated in the biomass cultivated at pH 7 (13.5g MSG/100g DM) compared to the biomass cultivated at pH 8.5 (7.77g MSG/100g DM), explaining the difference in umami taste. Modifying the cultivation parameters has therefore important consequences for the umami features and taste intensity of microalgae.

Next to the stronger taste intensity and umami taste, the N-replete biomass is also characterized by a significantly stronger salty taste compared to the N-deplete biomass (Figure 2). Interestingly, no significant differences in the concentration of sodium (Na) are found between the N-replete and N-deplete biomass. Most likely, the increased salty taste of the N-replete *T. chuii* biomass is explained by its higher umami taste, as umami can enhance saltiness, palatability and taste intensity of the food products [65, 66]. Therefore, the strong umami taste of the N-replete *T. chuii* biomass might be useful as a healthy salt replacer to reduce the sodium intake. Notably, the concentration of magnesium (Mg) is significantly higher in the N-replete samples (8.10 ± 2.59mg Mg/ g DM) compared to the N-deplete samples (5.47 ± 0.42mg Mg/ g DM). This is linked to the higher chlorophyll content in the N-replete samples as chlorophyll contains Mg in its chemical structure. Although the presence of Mg is often linked to bitterness [67], the higher Mg concentration in N-replete *T. chuii* biomass did not cause any difference in bitter taste. Interestingly, umami compounds such as Glu and AMP in the presence of Na were reported to suppress bitterness [68]. Utilizing the taste-enhancing properties of umami in the N-replete *T. chuii* biomass could be useful to suppress bitterness in plant-based food.

Table 4: Free amino acids Glu and Asp and free 5-'nucleotide AMP, GMP and IMP in N-replete and N-deplete T. chuii biomass as well as the calculated equivalent umami concentration (EUC) values calculated according the equation by Yamaguchi et al. (1971) [35]. Full free amino acids and 5-'nucleotide profile can be found in Supplementary Information. Average and standard deviation of the four biological replicates for the N-replete and the N-deplete T. chuii biomass are given. Significant differences between the N-replete and N-deplete biomass are indicated by $p < 0.05$.

Taste Compounds		Unit	N-replete	N-deplete	p-value
Free amino acids					
Total sum	/		31.20 ± 3.80	5.27 ± 1.36	<0.05
Glutamic acid	Glu	mg/g DM	7.85 ± 0.99	1.48 ± 0.25	<0.05
Aspartic acid	Asp	mg/g DM	1.05 ± 0.32	0.08 ± 0.02	<0.05
Free 5'-nucleotides					
Total sum	/	mg/g DM	2.45 ± 0.77	1.18 ± 0.20	<0.05
Adenosine monophosphate	AMP	mg/g DM	0.84 ± 0.17	0.30 ± 0.02	<0.05
Guanosine monophosphate	GMP	mg/g DM	0.30 ± 0.34	0.12 ± 0.13	0.4429
Inosine monophosphate	IMP	mg/g DM	/	/	/
Equivalent umami concentration	EUC	g MSG/100g DM	79.1 ± 73.4	6.8 ± 6.5	<0.05
Salts					
Sodium	Na	mg/g DM	16.36 ± 0.55	15.15 ± 1.80	0.1714
Potassium	K	mg/g DM	21.03 ± 2.89	24.11 ± 2.80	0.3429
Magnesium	Mg	mg/g DM	8.10 ± 2.59	5.47 ± 0.42	<0.05
Calcium	Ca	mg/g DM	25.88 ± 7.42	28.04 ± 2.91	0.2326

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4. Conclusion

The nitrogen supply in the cultivation medium strongly impacts the flavor properties of the *Tetraselmis chuii* biomass which has important implications for its palatability and consumer acceptance.

Despite the dominant shellfish-like aroma of the *T. chuii* biomass obtained from nitrogen starved cultivation conditions (N-deplete), some of its flavor features may be undesirable when using this biomass as flavoring. The N-deplete biomass possesses a stronger odor intensity compared to the *T. chuii* biomass obtained from nitrogen sufficient cultivation conditions (N-replete) due to the increased formation of VOCs. The higher amount of odor-active VOCs 3-methylbutanal, 3-methylbutanol and 2,3-butanedione in the N-deplete biomass cause an earthy-like off-odor. Furthermore, due to the lower amount of FAAs and 5'-nucleotides, the taste intensity of the N-deplete biomass is lower compared to the N-replete biomass. These results indicate that the N-deplete biomass is less suitable as flavoring agents for plant-based seafood alternatives.

Nitrogen sufficient cultivation conditions (N-replete) positively influence the flavor properties of the *T. chuii* biomass. The N-replete *T. chuii* biomass is characterized by a stronger umami and salty taste compared to the N-deplete *T. chuii* biomass which is attributed to increased concentrations of free Glu and 5'-nucleotides AMP. These taste features in combination with its shellfish-like flavor provided by the presence of aroma-active sulfur-containing volatiles DMS and DMDS and nitrogen-containing volatile TMA gives this *T. chuii* biomass high potential as flavoring agent for plant-based seafood alternatives.

Our study illustrates the potential of modifying the growth conditions for optimizing the flavor properties of microalgae. Besides adjusting the nitrogen supply in the cultivation medium, further flavor optimization might be possible as growth phase and other cultivation parameters such as light intensity and salt concentration might also influence the flavor properties. Furthermore, to reduce possible off-flavors, food processors should be aware of the effect of post-harvesting conditions such as storage and drying on the flavor of microalgae biomass. Finally, the behavior and stability of the microalgae flavor during processing of the final food product should also be investigated. Optimizing and controlling the cultivation and post-harvesting conditions is needed to obtain microalgae with a desired flavor profile for certain food application. Our approach of modifying the cultivation conditions contributes to the improvement of the

consumer acceptance of microalgae biomass and might help in the development of plant-based seafood alternatives.

Declarations of 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.

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Statement of informed consent, human/animal rights

No conflicts or animal rights applicable. The sensory research procedures in this study followed the guidelines that constrain the use of human subjects from the Nuremberg code of ethics in medical research and the declaration of Helsinki (as revised in 2013).

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CRediT authorship contribution statement

Bert Coleman: Conceptualization, Formal analysis, Investigation, Software, Methodology, Visualization, Data curation, Writing - original draft. **Christof Van Poucke:** Conceptualization, Investigation, Methodology, Data curation, Validation, Visualization, Software, Supervision, Writing - review & editing. **Bavo De Witte:** Conceptualization, Investigation, Methodology, Data curation, Software, Supervision, Validation, Visualization, Writing - review & editing. **Valentina Casciaro:** Formal analysis, Writing - review & editing. **Tanja Moerdijk-Poortvliet:** Formal analysis, Writing - review & editing. **Koenraad Muylaert:** Conceptualization, Investigation, Methodology, Supervision, Visualization, Writing - review & editing. **Johan Robbens:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing - review & editing.

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Highlights:

- Nitrogen supply in growth medium impacts the sensory quality of *Tetraselmis chuii*
- N-deplete *T. chuii* biomass possesses a stronger odor intensity and earthy-like odor
- N-replete *T. chuii* biomass possesses a stronger umami and salty taste
- Nitrogen sufficient conditions positively influence the seafood flavor of *T. chuii*

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