Notice to US Food and Drug Administration of the Conclusion that the Intended Use of *Chlamydomonas reinhardtii* (THN 6) Dried Biomass Powder is Generally Recognized as Safe

Submitted by the Notifier:

Triton Algae Innovations
11558 Sorrento Valley Road, Suite 3
San Diego, CA 92121

Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc
2800 E. Madison, Suite 202
Seattle WA 98112

March 21, 2018

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Susan Carlson, PhD
Division Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health and Human Services
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Carlson:

In accordance with proposed regulation 21 CFR Part 170 Subpart E (Generally Recognized as Safe (GRAS) Notice), on behalf of Triton Algae Innovations (the notifier), the undersigned, Timothy S. Murbach, submits, for FDA review, the enclosed notice that *Chlamydomonas reinhardtii* (wild type), strain THN 6 dried biomass powder is GRAS for use in food.

Should you have any questions or concerns regarding this notice, please contact me at 253-286-2888 or tim@aibmr.com.

Sincerely,

(b) (6)

Timothy S. Murbach, ND, DABT (agent of the notifier)
Senior Scientific & Regulatory Consultant
AIBMR Life Sciences, Inc. ("AIBMR")

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Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

Triton Algae Innovations (the notifier) is submitting a new GRAS notice in accordance with 21 CFR Part 170, Subpart E, regarding the conclusion that *Chlamydomonas reinhardtii* (THN 6) dried biomass powder is Generally Recognized as Safe (GRAS) for its intended use, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act.

1.2 Name and Address of the Notifier and Agent of the Notifier

Notifier

David J. Schroeder
Director, Corporate & Regulatory Affairs
Triton Algae Innovations
11558 Sorrento Valley Road, Suite 3
San Diego, CA 92121
Tel: (202) 607-3461
dave@tritonai.com

Agent of the Notifier

Timothy S. Murbach, ND, DABT
Senior Scientific & Regulatory Consultant
AIBMR Life Sciences, Inc.
2800 E. Madison Street, Suite 202
Seattle, WA 98112
Tel: (253) 286-2888
tim@aibmr.com

1.3 Name of the Substance

Chlamydomonas reinhardtii (wild type), strain THN 6 dried biomass powder

1.4 Intended Conditions of Use

C. reinhardtii (THN 6) dried biomass powder is intended to be used as a nutritive ingredient in food to replace other dietary proteins. *C. reinhardtii* (THN 6) dried biomass powder is not intended for use in foods where standards of identity would

preclude such use, infant formula, meat, poultry, egg products, catfish, or any products that would require additional regulatory review by USDA.

1.5 Statutory Basis for GRAS Conclusion

The conclusion of GRAS status of *C. reinhardtii* (THN 6) dried biomass powder for its intended conditions of use, stated in Part 1.4 of this notice, has been made based on scientific procedures.

1.6 Not Subject to Premarket approval

Triton has concluded that *C. reinhardtii* (THN 6) dried biomass powder is GRAS for its intended conditions of use, stated in Part 1.4 of this notice, and, therefore, such use is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data and Information Availability Statement

The data and the information that serve as the basis for this GRAS conclusion will be available for review and copying during customary business hours at the offices of Triton Algae Innovations, 11558 Sorrento Valley Road, Suite 3, San Diego, CA 92121, Telephone: (202) 607-3461, email: dave@tritonai.com or will be sent to FDA upon request.

1.8 Exemption from Disclosure under the Freedom of Information Act

None of the information in Parts 2 through 7 of this GRAS notice are considered exempt from disclosure under the Freedom of Information Act (FOIA) as trade secret or commercial or financial information that is privileged or confidential.

1.9 Certification of Completion

We herby certify that, to the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of *C. reinhardtii* (THN 6) dried biomass powder.

(b) (6)

3/21/18

David J. Schroeder
Director, Corporate & Regulatory Affairs
Triton Algae Innovations
Notifier

Date

Part 2: Identity, Manufacture, Specifications, and Physical or Technical Effect

2.1 Identification

C. reinhardtii (wild type), strain THN 6 is a proprietary single-cell green algae of the Chlamydomonadaceae family. Taxonomically, the Chlamydomonadaceae belong to: order, Chlamydomonadales — class, Chlorophytina — phylum, Chlorophyta — kingdom, Plantae — domain, Eukaryota. In general, *C. reinhardtii* (wild type) are eukaryotic cells of approximately 10 microns in diameter with a seven layer, hydroxyproline-rich glycoprotein cell wall and two 10–12 micron anterior flagella.[1] Internally, the cells contain a single cup-shaped chloroplast, which comprises approximately two thirds of the cell volume, a nucleus, endoplasmic reticulum, one to four Golgi bodies, mitochondria, two basal bodies, and two contractile vacuoles. An eyespot that directs light to the photoreceptor gives the cell an asymmetric appearance due to its location at the distal end of one of the flagellar roots. *C. reinhardtii* (wild type) is capable of growing in dark conditions due to its ability to use acetate as a carbon source. Micrographs of healthy *C. reinhardtii* cells at different magnifications are shown in Figure 1.

C. reinhardtii was the first green algae species to have been subject to a genome project, and is the most studied of any algae, having served as a model organism for many years.[2] The genome consists of 121-megabase pairs with a GC content of 64%.[3] Genes for 259 tRNAs were identified, and 15,143 protein-coding genes were predicted, of which 7,476 could be placed into 6,968 families. Sixty-one classes of simple repeats, approximately 100 families of transposable elements, and 64 tRNA-related short interspersed elements were also identified. BLASTP (Basic Local Alignment Search Tool for searching protein collections) scores were used to plot best matches between the proteome of *C. reinhardtii* and those of humans and *Arabidopsis* (chosen to be representative of animals and angiosperms, respectively) for the purpose of phylogenomic exploration of the *C. reinhardtii* genome. Of the 6,968 protein families contained in the genome of *C. reinhardtii*, 2,282 were homologous to both humans and *Arabidopsis* while 706 were homologous to humans only and 1,879 were homologous to *Arabidopsis* only. Genes encoding proteins related to general functions, such as of nutrient acquisition, growth, metabolism, transport, and mating processes, as well as more specific functions, such as photosynthesis and flagellar function were identified. Two genetically determined gamete mating types, + and -, occur, which can pair to form a mixed diploid zygospore that, at germination, by meiosis, can produce four haploid vegetative cells as an unordered tetrad of two + and two – mating types.

Accurate identification of *C. reinhardtii* strains is possible by analysis of the internal transcribed spacer (ITS) subregion of the nuclear rDNA cistrons.[2] After sequencing the ITS1 and ITS2 regions of *C. reinhardtii* (THN 6), the sequences

were compared to a standard reference strain using Genebank® in order to confirm the identity of *C. reinhardtii* (THN 6) as a strain allotted to the species *C. reinhardtii*. In addition, each lot of *C. reinhardtii* (THN 6) is analyzed by sequencing ITS1 and ITS2 in order to validate the production strain identity (see Subpart 2.2.1 below).

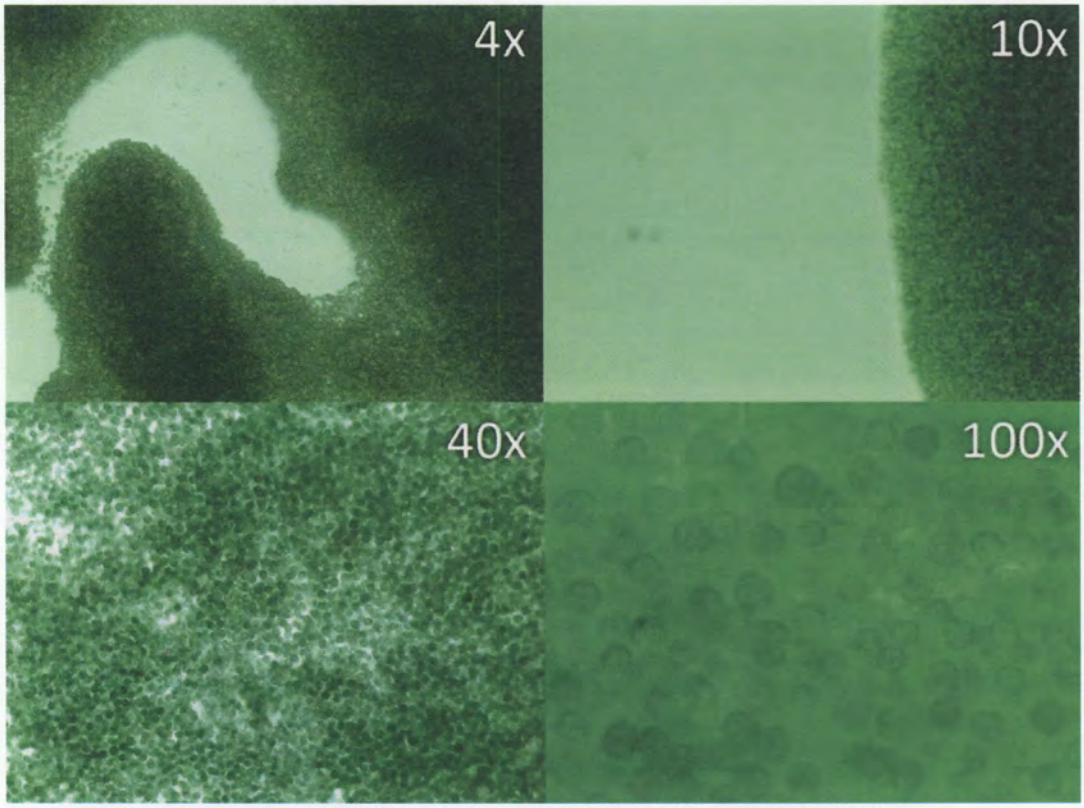

Figure 1. Microscope Photographs of Healthy *C. reinhardtii* Cells

The nutritional profile of *C. reinhardtii* (THN 6) biomass powder has been evaluated, and in addition to ≥30% complete protein content, the biomass contains complex carbohydrates and fiber, omega 3, 6, and 9 fatty acids, vitamins, minerals, and chlorophyll (see also Table 2—Specifications below).[4]

2.2 Manufacturing

C. reinhardtii (THN 6) dried biomass powder is produced using a closed aerobic heterotrophic fermentation process as described in the flowchart (Figure 2) and process description below.

2.2.1 Manufacturing Overview

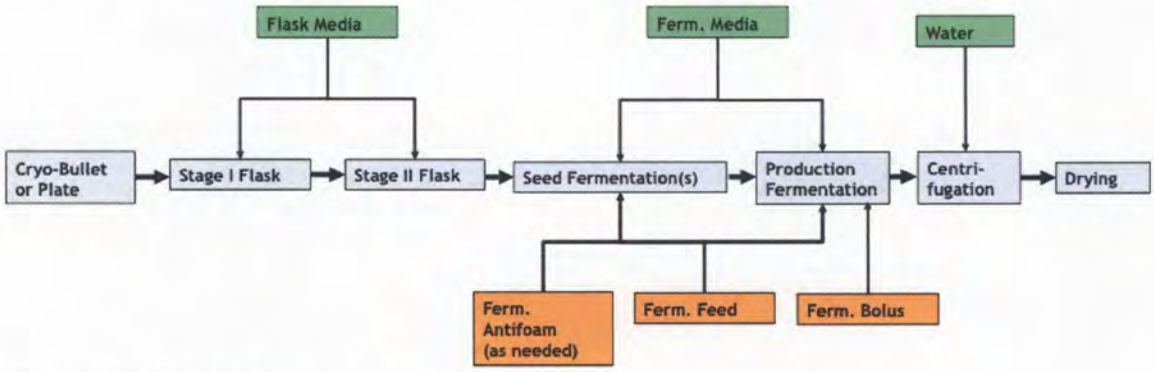

Figure 2. Manufacturing Flowchart

Preparation of Media and Feed: Flask and fermentation media are simple salt formulations with an added trace elements solution that are pH adjusted using 98% glacial acetic acid and sterilized by autoclaving. Feed for fermentation is a concentrated fermentation medium with acetic acid added as the primary carbon source; the feed is sterilized by filtration.

Strain Selection: In a biological safety cabinet, a colony is taken from a single agar plate streaked with *C. reinhardtii* (THN 6) using a sterile loop and used to inoculate the prepared Stage 1 flask. Alternatively, the inoculum may be sourced from a cryobullet. In the alternative case, the cryobullet is removed from storage and transferred on dry ice to a water bath for thawing. Cells are quickly thawed by gently swirling the vial in the water bath, and the thawed cells are used to inoculate the prepared Stage 1 flask.

Flask Propagation: Flask propagation of the cell mass employs a two-stage process, with all transfers taking place in a biological safety cabinet. The inoculum is added to 150 mL of sterile flask medium in the Stage I flask (baffled 500 mL shake flask with vented closure), and the flask is placed on an orbital shaker under controlled RPM, temperature, lighting, and time. At set times, samples are aseptically removed and tested for contamination (check-time 1) by microscopy and photography (see Figure 1) and quantification (check-time 2) of optical density (OD) and dry cell weight concentration (DCW). Finally, during the Stage I

propagation, the strain is validated by sequencing of ITS regions 1 and 2. Upon the three criteria of a negative contamination test, a DCW in the range of acceptance, and the validation of the strain identity, the Stage II inoculation may proceed. The Stage II flask (baffled 3000 mL shake flask with vented closure) is prepared by adding 1050 mL of sterile flask medium, and the Stage I broth is added to inoculate the prepared Stage II flask; the flask is placed on an orbital shaker under controlled RPM, temperature, lighting, and time. Checks for contamination and quantification, as described for Stage I are also performed during Stage II as acceptance criteria for inoculation of the seed fermentation.

Seed Fermentation(s): The fermentation medium is prepared in the fermentation vessel and steam sterilized. Seed inoculum is aseptically transferred in a biological safety cabinet from the Stage II flask(s) to a sterile seed inoculum vessel and then to the seed fermentor, and the pH is manually adjusted to the appropriate set point, after which changes in pH automatically control feed delivery. Seed fermentation is run aerobically and in the absence of light with control of temperature, pH, airflow, air pressure, feed rate, agitation, and total fermentation time. In the event of significant foam formation, a sterile antifoam bolus is added. OD and DCW are measured at regular set intervals throughout the fermentation process and a contamination check is conducted once at a set check-point. The seed fermentation in harvested when DCW is within the acceptable range.

Production Fermentation: The production fermentation is also run aerobically and in the absence of light, and the process, including control points, is similar to that described in the seed fermentation step except for the greater initial concentration of cell mass and the transfer of inoculum from the seed fermentor instead of the shake flask with process controls remaining enabled in the seed fermentor during inoculum transfer. There is no "hard target" for harvesting of the production cell mass as the overall goal is to produce as much as possible within specification.

Downstream Processing: Upon completion of production fermentation, according to the Standard Operating Procedures (SOP) of the contract manufacturing operation, algal cell mass is separated from liquid via centrifugation, and the resulting cell mass cream is transferred to the spray-drying infrastructure.

Cell Mass Quality Control: Spray-dried cell mass is evaluated against the specifications and if acceptable, transferred to plastic-lined drums, together with the Certificate of Analysis (CoA), for shipping and storage. In the event of an out of specification (OOS) event, including the potential for an OOS production lot, steps will be taken to identify the issue(s) and take appropriate corrective action(s), including steps to bring the lot within specification where possible. In the event that a specific lot cannot be corrected to meet the specification, the lot will be destroyed in accordance with SOP and cGMP.

2.2.2 Good Manufacturing Practice

C. reinhardtii (THN 6) dried biomass powder from Triton is produced and stored in an FDA registered facility under strict adherence to current GMP standards set to comply with the U.S. Code of Federal Regulations, 21 CFR Part 110.

2.2.3 Raw Materials

Raw materials used in the production of Triton's *C. reinhardtii* (THN 6) dried biomass powder are food grade and/or suitable for use in the production of food grade products for human consumption. Triton carefully reviews and verifies the CoA provided by the suppliers of all food-grade compounds utilized in the production process and also confirms the food-grade qualify of all packaging materials used for storage and shipping of the finished product. No material of human or animal origin is used. *C. reinhardtii* (THN 6) dried biomass powder is non-GMO and not irradiated.

2.3 Specifications

The specifications for the food-grade product *C. reinhardtii* (THN 6) dried biomass powder, along with the specification methods, are listed in Table 1 below.

Table 1. *C. reinhardtii* (THN 6) Dried Biomass Powder Specifications

Test Items	Specification	Method
Physical Characteristics		
Appearance	Green powder	Visual inspection
Moisture	NMT 10%	AOAC Variable
Composition		
Protein (crude)	30–70%	AOAC 990.03
Fat (crude)	NMT 10%	AOAC 945.16
Fiber (acid detergent)	1–25%	AOAC 991.43
Ash	NMT 5%	AOAC 942.05
Chlorophyll	NMT 25%	Knap 1996[5]
Heavy Metals		
Arsenic	NMT 0.2 ppm	USP<233>
Cadmium	NMT 0.2 ppm	USP<233>
Lead	NMT 0.2 ppm	USP<233>
Mercury	NMT 0.2 ppm	EPA 7471
Microbiological Tests		
Total Aerobic Microbial	NMT 1000 CFU/g	AOAC 990.12
Total Yeast & Mold	NMT 1000 CFU/g	BAM Ch. 18
Total Coliforms	NMT 100 CFU/g	AOAC 991.14
E. coli	Negative (absent/1 g)	AOAC 991.14
Salmonella	Negative (absent/25 g)	AOAC 030301

Staphylococcus aureus	Negative (absent/1 g)	AOAC 2003.7

Abbreviations: BAM, US FDA Bacteriological Analytical Manual; CFU, colony forming units; EPA, US Environmental Protection Agency; NMT, not more than; ppm, parts per million; USP, United States Pharmacopeia

2.3.1 Batch Analysis

As Triton's *C. reinhardtii* (THN 6) dried biomass powder is a novel product that has not yet been placed in the food supply, a history of batch analyses for commercial production lots has not yet been generated. However, production conformity and consistency will be tested per the product specification for each production lot. The specifications have been developed and finalized as reported above, and results of a single batch analysis (lot # TAI-1215-01, manufactured December 15, 2016) are shown in the table below and meet the product specifications for identification, physical characteristics, composition, heavy metals, and microbial analyses.

Table 2. *C. reinhardtii* (THN 6) Dried Biomass Powder Batch Analyses

Test Items	Specification	TAI-1215-01
Physical Characteristics		
Appearance	Green powder	Green powder
Moisture	NMT 10%	5.4%
Composition		
Protein (crude)	30–70%	36.0%
Fat (crude)	NMT 10%	2.0%
Fiber (acid detergent)	1–25%	7.3%
Ash	NMT 5%	4.8%
Chlorophyll	NMT 25%	0.49%
Heavy Metals		
Arsenic	NMT 0.2 ppm	ND[a]
Cadmium	NMT 0.2 ppm	0.1 ppm[b]
Lead	NMT 0.2 ppm	ND[a]
Mercury	NMT 0.2 ppm	ND[a]
Microbiological Tests		
Total Aerobic Microbial	NMT 1000 CFU/g	ND
Total Yeast & Mold	NMT 1000 CFU/g	190 CFU/g
Total Coliforms	NMT 100 CFU/g	ND
E. coli	Negative (absent/g)	Negative
Salmonella	Negative (absent/25 g)	Negative
Staphylococcus aureus	Negative (absent/g)	Negative

Abbreviations: CFU, colony forming units; ND, not detected; NMT, not more than; ppm, parts per million.

[a], Limit of Detection (LoD) = 0.10 ppm; [b], LoD = 0.020 ppm

2.4 Physical or Technical Effect

C. reinhardtii (THN 6) dried biomass powder is not intended to produce any physical or other technical effects that are relevant to safety of the ingredient.

Part 3: Dietary Exposure

3.1 Intended Use

C. reinhardtii (THN 6) dried biomass powder is intended to be used as a nutritive ingredient in food to replace other dietary proteins. *C. reinhardtii* (THN 6) dried biomass powder is not intended for use in foods where standards of identity would preclude such use, infant formula, meat, poultry, egg products, catfish, or any products that would require additional regulatory review by USDA.

3.2 Exposure Estimates

Exposure to *C. reinhardtii* (THN 6) biomass powder from the intended use was estimated based on complete dietary protein replacement for the U.S population (ages 2+) using food consumption data from the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES). Protein concentrations were assigned to all relevant NHANES (2013–2014) food codes using composition data from the United States Department of Agriculture (USDA)'s Food and Nutrient Database for Dietary Studies (FNDDS). The FNDDS database provides information on the concentration of approximately 60 food constituents (including protein) for each NHANES food code and accounts for both naturally occurring and added protein levels in food. The protein exposure data was then derived using analysis by Creme Food Safety software 3.6 (www.cremeglobal.com).

The WWEIA/NHANES survey contains data from two non-consecutive 24-hour dietary recall interviews of 7,574 individuals. Creme software is a probabilistic modeling tool that uses high-performance computing to predict intake (including total aggregate exposure) of food codes or groups and/or individual food ingredients. Creme Food Safety performs calculations using large-scale food consumption data sets. It bases the calculated estimates on each individual's body weight from the survey, as opposed to averaged body weights. Calculations also incorporated the NHANES assigned sample weights for each individual in the survey, which measure the number of people in the population represented by that specific subject and help to ensure that the results statistically represent the entire U.S. population. Sample weights for NHANES participants incorporate adjustments for unequal selection probabilities and certain types of non-response, as well as an adjustment to independent estimates of population sizes for specific age, sex, and race/ethnicity categories. The data is shown for the total population background protein exposure. Results are given as both absolute exposure (g/day), as well as exposure relative to body weight (g/kg bw/day).

Data estimated directly from the NHANES short 2-day survey do not necessarily adequately represent individual usual long-term intake due to the large amount of

random error. This is because it may not capture infrequent consumers. It assumes that subjects who consumed a product on a survey day consume it every day of the year, and it does not adjust for potential day-to-day variation in intake (i.e., intra-individual variation over time is not accounted for). Thus estimation of "usual" or "lifetime" exposure was also added to the model based on methodologies developed by Nusser et al., 1996, at Iowa State University.[6] These lifetime data are considered the most relevant data, as GRAS exposure estimates should be based on expected regular exposure over the lifespan. The technique of estimating usual/lifetime intakes relies on the ability to transform the input daily average data (from food consumers) into normality, which is tested using the Anderson-Darling test statistic within the Creme Global software.

Estimated total protein exposure results are shown in Tables 1 and 2 below for the total population (ages 2 years and older).

Table 1. Estimated Absolute Exposure to Protein (g/day) by the U.S. Population (ages 2+)

Population Group	Age in yrs	N (% of total)	Absolute Protein Consumption Daily Average (g/day)				Lifetime 90th% Exposure Estimates (g/day)
			Mean	Mean SE	90th%	90th% SE	
Total Population	2+	7067 (100)	79.92	0.6867	122.6	2.073	114.8

SE = standard error
Creme #255

Table 2. Estimated Exposure to Protein Relative to Body Weight (g/kg bw/day) by the U.S. Population (ages 2+)

Population Group	Age in yrs	N (% of total)	Protein Consumption Relative to Body Weight Daily Average (g/kg bw/day)				Lifetime 90th% Exposure Estimates (g/kg bw/day)
			Mean	Mean SE	90th%	90th% SE	
Total Population	2+	7067 (100)	1.259	0.01276	2.220	0.04370	2.092

SE = standard error
Creme #255

Given the *C. reinhardtii* (THN 6) biomass powder specification range of 30–70% (w/w) protein, total 90th percentile lifetime absolute and relative to bodyweight exposure to *C. reinhardtii* (THN 6) biomass powder based on total replacement of background protein exposure are calculated to be 164.0 to 382.6 g/day (114.8/0.7 to 114.8/0.3) and 2.989 to 6.974 g/kg bw/day (2.092/0.7 to 2.092/0.3), respectively. This assessment is extremely conservative as it includes the assumption that 100% of all protein consumed is from *C. reinhardtii* THN 6 biomass powder.

Because of the large number and variety of protein containing foods, it is nearly impossible that an individual will randomly or intentionally consume a product containing *C. reinhardtii* (THN 6) biomass powder every single time that he/she consumes a protein-containing food daily over a lifetime. While food labels will list the dried algae as an ingredient and may even highlight the ingredient occasionally in marketing, it is assumed that many consumers will not always realize that the ingredient is present in the food. In other words, it will be an "invisible" ingredient to many consumers, which decreases the chance that only food products that contain the ingredient will be chosen by those consumers. Additionally, there will be cost and market share limitations of adding this specialty ingredient to foods in general, making it even less likely that an individual would consume it in all foods consumed daily. Furthermore, most foods contain protein as an endogenous constituent, and in many foods, protein is a major component (e.g., meat, fish, poultry, dairy, eggs). Such foods are excluded from substitution by *C. reinhardtii* (THN 6) biomass powder for the protein they inherently contain; in reality only foods containing added protein have the potential to contain this ingredient. Additionally, even if it were possible to replace endogenous proteins in such foods, many of them are excluded from the intended use of *C. reinhardtii* (THN 6) biomass powder by virtue of standards of identity and/or other categorical exclusions listed in Subparts 1.4 and 3.1. Thus, considering *C. reinhardtii* (THN 6) biomass powder as total dietary protein replacement in our estimates is enormously conservative.

More realistic estimates of protein replacement can be calculated based on the assumption that *C. reinhardtii* (THN 6) biomass powder will replace only 10% of dietary protein consumed. In light of the limitation to add *C. reinhardtii* (THN 6) biomass powder only to foods that contain added protein and market share limitations due to the cost of the ingredient, market competition, and natural variation in food choices from day to day, this assumption is still considered highly conservative. This calculation results in lifetime 90th percentile *C. reinhardtii* (THN 6) biomass powder consumption estimates of 16.4–38.3 g/day or 0.299–0.697 g/kg bw/day, respectively (equivalent to 11.5 g protein/day or 0.209 g protein/kg bw/day, respectively).

Part 4: Self-limiting Levels of Use

There are no known inherent self-limiting levels of use.

Part 5: Experience Based on Common Use in Food Prior to 1958

The GRAS conclusion for *C. reinhardtii* (THN 6) dried biomass powder is based on scientific procedures, and thus, experience based on common use in food prior to 1958 is not considered pivotal information. To the best of our knowledge, *C. reinhardtii* (THN 6) dried biomass powder was not commonly used in foods prior to 1958.

Part 6: Narrative

6.1 Absorption, Distribution, Metabolism, and Excretion (ADME)

No studies investigating pharmacokinetic parameters of *C. reinhardtii* (THN 6) dried biomass powder were located. However, as the ingredient is considered primarily as a nutritive macro-ingredient in foods and is comprised of at least 30% protein, up to 25% fiber, and up to 10% fat as well as up to 5% vitamins and minerals and small amounts of chlorophyll and without appreciable amounts of other bioactive compounds or known toxic compounds,[4] the body is expected to act upon it through similar physiological processes of digestion and ADME common to other edible plant-derived foodstuffs commonly consumed in the human diet.

6.2 Toxicology Studies

Triton investigated the potential mutagenic activity and 28-day subchronic repeated-dose oral toxicity of its *C. reinhardtii* (THN 6) dried biomass powder.[4] These studies were conducted in Good Laboratory Practice (GLP) certified facilities (Toxi-Coop Zrt., Hungary) and in compliance with GLP according to Hungarian GLP regulations, Joint Decree No 9/2001 (III. 30) and are described in the summaries below. No additional published toxicological studies were identified in searches of the scientific literature.

6.2.1 Bacterial Reverse Mutation Assay

Purpose: To evaluate the mutagenic potential of *C. reinhardtii* (THN 6) dried biomass powder, a bacterial reverse mutation test was conducted in compliance with OECD Guidelines for the Testing of Chemicals, No. 471 (adopted 21 July 1997).

Methods: Four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2 *uvrA*) were used in the presence and absence of metabolic activation using rat liver microsome preparations (S9-mix) with appropriate positive and negative controls. The study included a preliminary solubility test, a preliminary range finding test, an initial mutation test (IMT) utilizing a standard plate incorporation procedure, and a pre-incubation procedure performed as a confirmatory mutation test (CMT). Concentrations of *C. reinhardtii* (THN 6) dried biomass powder used for the IMT and CMT were: 5000, 1600, 500, 160, 50, 16, and 5 µg /plate. Three replicates were investigated for each concentration level and the controls (untreated, vehicle (DMSO), and positive reference) in the IMT and CMT.

Results: The positive controls induced the expected responses. Both the increases in revertant colony numbers induced by the positive controls and the spontaneous

revertant colony numbers of the vehicle control agreed with historical control data. No concentration-related or biologically relevant increases were seen in revertant colony numbers of any of the five bacterial strains upon treatment with the test item at any of the concentration levels either in the presence or absence of an S9 activation system. No growth inhibition was observed at any test concentration and revertant colony numbers remained within the range of biological variability of the test system; background lawn development was unaffected. All results were unequivocally negative according to the study criteria for both positive and biologically relevant responses.

Conclusions: Under the experimental conditions applied, *C. reinhardtii* (THN 6) dried biomass powder failed to induce gene mutations by base pair changes or frameshifts in the genomes of the strains used at concentrations up to the maximum recommended test concentration of 5000 µg/plate.

6.2.2 In vitro Mammalian Chromosomal Aberration Study

Purpose: To evaluate the clastogenic potential of *C. reinhardtii* (THN 6) dried biomass powder, an in vitro mammalian chromosomal aberration test was conducted in compliance with OECD Guidelines for the Testing of Chemicals, No. 473 (adopted 26 September 2014).

Methods: *C. reinhardtii* (THN 6) dried biomass powder was suspended in Dulbecco's modified Eagle's medium, and three concentrations were chosen for use with or without metabolic activation (see below) on the basis of preliminary cytotoxic investigations. The chromosomal aberration assays were conducted in two independent experiments (each in duplicate) using V79 male Chinese hamster lung cells. The cells were exposed to the negative control or each test item concentration with and without metabolic activation with S9-mix. Groups of cells were also exposed to the respective positive controls for use with or without S9-mix. Exposure and sampling times were as follows:

- Experiment A: 3h treatment/20h sampling time.
    - Without S9-mix: 100, 200, and 400 µg/mL
    - With S9-mix: 150, 300, and 600 µg/mL
- Experiment B: 20h treatment without S9-mix/20 and 28h sampling times.
    - Without S9-mix: 50, 100, 200, and 400 µg/mL
- Experiment B: 3h treatment with S9-mix/28h sampling time.
    - With S9-mix: 150, 300, and 600 µg/mL

Following treatment (exposure) and sampling (expression) time, cells were exposed to a selection agent, colchicine (0.2 µg/mL), 2.5 hours prior to harvesting

and fixing for slide preparation. Chromosomal aberration frequencies were then scored blind for 300 well-spread metaphase cells.

Results: In both experiments, A and B, the concurrent negative and positive controls were compatible with the respective historical controls. Structural aberrations without gaps were less than 5% in the concurrent negative controls and the concurrent positive controls induced biologically and statistically significant increases in cells with aberrations compared to the concurrent negative controls. No statistically significant or concentration-related increases compared to the concurrent or historical negative controls in numbers of cells with chromatid or chromosome aberrations or in the rate of polyploidy and endoreduplicated metaphases were observed after treatment with the different concentrations of *C. reinhardtii* (THN 6) dried biomass powder with or without metabolic activation in either experiment. Inclusion or exclusion of gap-type aberrations did not alter the results.

Conclusions: *C. reinhardtii* (THN 6) dried biomass powder was not clastogenic in this test system.

6.2.3 In vivo Mammalian Micronucleus Study

Purpose: To evaluate the genotoxic potential of *C. reinhardtii* (THN 6) dried biomass powder, an in vivo mammalian erythrocyte micronucleus test was conducted in mice in compliance with OECD Guidelines for the Testing of Chemicals, No. 474 (adopted 26 September 2014). The Institutional Animal Care and Use Committee (IACUC) of Toxi-Coop Zrt. permitted the conduct of the study according to Standard Operating Procedures (SOP) for animal protection. Additionally, care and use of study animals was in accordance with the National Research Council Guide for Care and Use of Laboratory Animals 8th Edition (published 2011) and in compliance with the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

Methods: A single dose of *C. reinhardtii* (THN 6) dried biomass powder was administered by gavage to male Crl:NMRI BR mice at test concentrations of 0 (vehicle-control), 500, 1000, and 2000 mg/kg bw. The high-dose is the limit dose for mammalian erythrocyte micronucleus tests. The negative control/vehicle was 1% aqueous methylcellulose solution. The positive control, cyclophosphamide 60 mg/kg bw, was administered by intraperitoneal injection. All treatments were administered at a uniform volume of 10 mL/kg bw. The negative control and high-dose groups consisted of 10 analyzable animals each, and all other groups consisted of five animals each. The main micronucleus test was conducted at the doses described above in males only based on the results of a preliminary toxicity test that was conducted using a single dose of *C. reinhardtii* (THN 6) dried

biomass powder, by gavage, at a concentration of 2000 mg/kg bw in two animals/sex/group in order to determine the high-dose and assess gender differences. No mortality, signs of toxicity, or gender specific effects were observed in the preliminary test.

All animals were observed immediately following dosing and at regular intervals until sacrifice for mortality, signs of toxicity, or adverse reactions to treatment. Bone marrow smears were prepared in duplicate on standard microscope slides, which were coded for blind evaluation, from samples obtained from the femurs of five animals from each dose group immediately following sacrifice according to the following schedule: half the mice from the negative control and high-dose groups and all mice from the low-dose, mid-dose, and positive control groups were sacrificed 24 hours post treatment; the remaining negative control and high-dose animals were sacrificed at 48 hours. The proportion of polychromatic erythrocytes (PCE) to total erythrocytes per animal was determined by counting at least 500 erythrocytes per animal. Four thousand PCE per animal were scored for frequency of micronuclei.

Results: No mortality, clinical signs of toxicity, or adverse reactions to treatment were observed in any animals during the study. In the concurrent negative and positive controls, observed frequencies of micronucleated PCE (MPCE) were compatible with the respective historical control data; the increase observed for the concurrent positive control was statistically significant compared to the concurrent negative control. No statistically significant differences were observed in proportion of PCE to total erythrocytes in the three dose groups compared to the concurrent and historical negative controls, but a slight non-significant decrease in the ratio observed in the high-dose group at both sampling times was considered an indication of bone marrow exposure to the test item. No statistically significant dose-related increases were observed in frequency of MPCE in the treated groups compared to the concurrent and historical negative controls. Although a statistically significant decrease in MPCE frequency compared to the concurrent negative control was observed in the mid-dose group, MPCE frequency remained within the distribution of historical negative control data at all sampling times in all dose-groups.

Conclusions: *C. reinhardtii* (THN 6) dried biomass powder, at concentrations up to the limit dose of 2000 mg/kg bw, was unequivocally negative for producing micronuclei under the conditions of this in vivo mouse micronucleus test.

6.2.4 Twenty Eight-Day Repeated-Dose Oral Toxicity Study

Purpose: To evaluate the potential health hazards, including identification of toxic effects and target organs, of repeated oral exposure to *C. reinhardtii* (THN 6) dried biomass powder in male and female rats, and to determine a NOAEL, a 28-day repeated-dose oral toxicity study in rats was performed in compliance with the test procedure recommendations of OECD Guidelines for the Testing of Chemicals, No. 407 (adopted 3 October 2008). The IACUC of Toxi-Coop Zrt. permitted the conduct of the animal studies according to SOP for animal protection. Additionally, care and use of study animals was in accordance with the National Research Council Guide for Care and Use of Laboratory Animals 8th Edition (published 2011) and in compliance with the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection.

Methods: Ten SPF Hsd.Han Wistar rats/sex/group were administered *C. reinhardtii* (THN 6) dried biomass powder dissolved in distilled water (vehicle) at concentrations to provide for uniform administration by gavage of a dose volume of 10 mL/kg bw. Four groups were administered doses of 0 (vehicle-control), 1000, 2000, or 4000 mg/kg bw/day for 28 consecutive days. The high-does was chosen as the highest feasible dose due to solubility limits of the ingredient.

Table 3. Group designation

Dose (mg/kg bw/day)		Males/Females
Control	0	10/10
Low–dose	1000	10/10
Mid–dose	2000	10/10
High–dose	4000	10/10

All tests and examinations were conducted according to study protocols and in compliance with above stated guidelines:

- Observations of mortality and clinical signs and measurements of food intake and body weight were conducted.
- A functional observation battery (FOB) was conducted during the final exposure week.
- Ophthalmological examinations were conducted prior to beginning dosing and again during the final exposure week.
- Evaluation of hematology and clinical chemistry parameters were conducted on blood samples drawn on the day of sacrifice.
- Measurement of organ weights (absolute and relative) and gross pathological examinations were conducted on all animals at necropsy.

- Full histopathological examinations were conducted on the preserved organs and tissues of all control and high-dose animals.
- Histopathological examinations of organs in which gross lesions or other abnormalities were observed in animals of the lower dose groups were also conducted.
- All quantitative data was subjected to statistical analysis.

Results: No mortality; clinical signs or abnormal behavior; alterations in grip strength, motor activity, and reactions to stimuli; or ophthalmologic alterations were observed during the study. No statistically significant differences in mean body weight, mean body weight gain, mean daily food consumption, or mean feed efficiency were observed in the male groups compared to controls. In the females, slight, transient, statistically significant decreases in mean body weight gain (all dose groups, Days 0–4) and feed efficiency (mid-dose group, Days 0–7) and a slight, transient, statistically significant increase in feed efficiency (high-dose group, Days 21–27) were observed compared to controls, but no significant differences were observed in mean body weights or food consumption of females and cumulative body weight gain and feed efficiency were not affected.

Several statistically significant alterations in clinical pathology parameters were observed among the sexes and dose groups. These changes were low in magnitude (within the historical control ranges of the laboratory), were largely without clear dose relationships (exceptions in females only were a dose-wise increase in bilirubin becoming statistically significant compared to controls at the high-dose and a dose-wise increase in glucose becoming statistically significant compared to controls at the mid- and high-doses), and lacked any correlating histopathology; therefore, they were considered normal biological variations and not test item-related.

Sporadic, statistically significant differences in absolute and relative organ weights observed among the sexes and groups compared to relevant controls were: decreased mean heart weights (absolute and relative to brain weight; low-dose group males), decreased mean weights of the prostate with seminal vesicles and coagulating glands attached (absolute, relative to body, and relative to brain; high-dose group males), and decreased mean absolute brain weight in the high-dose group females. These changes were within historical control ranges and lacked correlating histopathology. A statistically significant difference compared to controls was observed in liver weight relative to body weight in the high-dose male group, and while this change had the appearance of a dose relationship, it remained well within the historical control range and lacked correlating clinical chemistry or histopathology and, therefore, was not considered toxicologically relevant.

Thymic hemorrhages were observed in two low-dose males and dilatation of the uterine lumen was observed without a dose relationship in one to three females of each group (controls and treated animals). No other gross lesions were observed in any group. On microscopic examination of the two low-dose males, acute thymic hemorrhages were observed. Acute thymic hemorrhages were also observed in control females, and pulmonary hemorrhages and acute alveolar emphysema were observed with similar frequencies among the sexes in the control and high-dose groups. These are findings observed commonly in untreated laboratory animals and were considered due to hypoxia associated with the exsanguination procedure. Because the uterine dilatation occurred with similar frequency in all groups and without a dose relationship and was also observed microscopically with similar frequency in control and high-dose females, it was considered a normal estrogenic effect of the estrous cycle, and microscopic examinations were not extended to the low- and mid-dose females. Hyperplasia of the bronchus associated lymphoid tissue is an antigenic response that also occurs in untreated animals and was not associated with inflammatory lesions in either of the single control and high-dose group males in which it was observed in this study. No microscopic changes considered test item-related were observed; all microscopic changes are summarized in Table 8.

Table 4. Summary of Histopathology Findings (reproduced with permission from Murbach et al., 2017)

Organs	Dose group (mg/kg bw/day) Observations	Control n=10	1000 N/A	2000 N/A	4000 n=10
Male					
	Animals with no microscopic findings	7/10	N/A	N/A	8/10
Lungs:	Alveolar emphysema, minimal	1/10	—	—	1/10[a]
	Acute pulmonary hemorrhage, minimal	1/10	—	—	1/10[a]
	Hyperplasia of BALT, mild	1/10	—	—	1/10
Thymus:	Acute hemorrhage, mild to moderate	0/10	2/2	—	0/10
Female					
	Animals with no microscopic findings	6/10	N/A	N/A	7/10
Lungs:	Alveolar emphysema, minimal	0/10	—	—	1/10
	Acute pulmonary hemorrhage, minimal	2/10	—	—	0/10
Thymus:	Acute hemorrhage, minimal	1/10	—	—	0/10
Uterus:	Dilatation	1/10	—	—	2/10

Abbreviations: —, not examined; BALT, bronchus associated lymphoid tissue; N/A, not applicable (only animals with gross lesions were examined).
Data represent incidence of the observation (number of animals with observation per number of animals observed).
Organs without lesions in 10/10 control or high-dose animals not shown unless low- or mid-dose animals were also examined.
a = minimal alveolar emphysema and minimal acute pulmonary hemorrhage observed in same animal.

Conclusions: Repeated administration by gavage of 0, 1000, 2000, and 4000 mg/kg bw/day of *C. reinhardtii* (THN 6) dried biomass powder for 28 consecutive days did not cause adverse effects or signs of toxicity in male or female Hsd.Han Wistar rats; a NOAEL was determined as 4000 mg/kg bw/day; the highest dose tested.

6.3 Unpublished Growth Studies in Piglets

Triton has conducted unpublished studies on the effects of *C. reinhardtii* (THN 6) dried biomass powder consumption in piglets. In a growth study in groups (n=3/group) of 14-day-old sow-reared piglets, a nutritionally complete cow milk protein-based sow milk replacer formula (LiquiWean, MSC, Dundee, IL) was provided with or without *C. reinhardtii* (THN 6) dried biomass powder (300 mg/L). Mean body weight gain and mean intestinal weights were comparable and slightly higher over eight-days in the *C. reinhardtii* fed animals than in the formula alone fed animals. Statistical significance was not reported. Results were similar in another piglet study conducted in groups of six 14-day-old piglets over 14-days. Body and intestinal weights were statistically significantly increased on postpartum Day 28 (Day 14 of treatment) compared to baseline (postpartum Day 14) in both the control and *C. reinhardtii* groups. Between the groups, body weight gains and intestinal weights were unaffected in the *C. reinhardtii* fed animals compared to conventional sow milk replacer formula. In both groups, immune system development was comparable and considered normal based on assessment of parameters such as peripheral blood mononuclear cell populations and serum cytokines. In a third study, groups of eight piglets weaned after 21 days were fed a control diet (standard nursery diet except that it did not contain spray-dried plasma, antibiotics, or zinc oxide for reasons related to efficacy outcomes of the study) or a diet containing 0.33% *C. reinhardtii* (THN 6) dried biomass powder (approximately 6 g algal dried biomass daily). Compared to the control diet, the algae supplemented diet had no statistically significant effect on body weight gain over a 6-day treatment period. In these studies, *C. reinhardtii* (THN 6) dried biomass powder was well tolerated and did not have adverse effects on growth or immune development in young pigs.

While the above piglet studies are not toxicological studies, they do corroborate, in an additional species, the lack of adverse effects on body weight and body weight gain and immune cells and organs observed in the 28-day repeated-dose oral toxicity study in rats summarized in Subpart 6.2.4.

6.4 Non-pathogenicity and Non-toxicogenicity

Our searches of the scientific literature and public health-related databases did not find any evidence of pathogenicity or toxicogenicity of any members of the class Chlorophyceae. Several toxin producing alga are known to occur within the subphylum Chlorophytina as follows[7]: *Caulerpa* spp. are marine benthic green macroalga (i.e., a seaweed) consumed as food in the Philippines and known to produce the toxins caulerpicin (a long-chain saturated hydroxy amide) and caulerpin (a pyrazine derivative) during the rainy season. An organic lipid extract from *Chaetomorpha minima*, another marine benthic green macroalgae, is a known ichthyotoxin and hemolysin. *Ulva* spp. (sea lettuces), a group of edible green macroalgae, are also known to product hemolysins. For example, two water soluble (thought to be a galactolipid and a sulfolipid) and one lipid soluble (palmitic acid) hemolysins have been isolated from *Ulva australis* Areschoug (synonym *Ulva* pertusa). In addition, the author gave a secondhand report alleging toxin-producing members of Chlorophytina genera *Chlorella* and *Scenedesmus*.

Some members of more distantly related algal species, such as seaweeds of the phylum, Rhodophyta (red algae) and class, Chrysophyceae (golden algae); microalgae of the classes, Coccolithophyceae and Bacillariophyceae (diatoms); and especially, members of the superclass, Dinoflagellata are also known to produce toxins.[7-9] Nonetheless, these organisms are only very distantly taxonomically related to *C. reinhardtii* at the levels of kingdom or domain. Additionally some members of the phylum Cyanobacteria are well known toxin producers that are sometimes confused with algae due to widespread use of the common name 'blue-green algae'; however, these organisms are not taxonomically related to *C. reinhardtii*, except at the level of all life, as they belong to domain Bacteria and are prokaryotic microorganisms rather than eukaryotes.

Finally, if *C. reinhardtii* (THN 6) dried biomass powder possessed pathogenic or toxicogenic potential, some indication of this would have been expected in the toxicological investigations summarized in Subpart 6.2, yet these investigations were unequivocally negative for toxic effects. Thus, based on our searches of the public domain and the extensive body of scientific literature on *C. reinhardtii* as a model laboratory organism and potential recombinant protein production platform, the absence of toxic effects in formal toxicological investigations, and the fact that most closely related toxicogenic organisms diverge at the taxonomic level of subphylum, there is no reason to suspect a pathogenic or toxicogenic potential of *C. reinhardtii* (THN 6).

6.5 Allergenicity

C. reinhardtii (THN 6) dried biomass powder does not contain or have added, and is manufactured in a facility free of, all eight major allergens (milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, and soybeans) identified, and required to be disclosed in labeling, in the Food Allergen Labeling and Consumer Protection Act (FALCPA).

To the best of our knowledge, *C. reinhardtii* proteins will be a novel introduction to the human diet. In addition proteins, toxins produced by microorganisms have occasionally been implicated in allergic reactions; however, as discussed in Subpart 6.4, there is no reason to suspect *C. reinhardtii* produces any toxins. Species belonging to several genera of subphylum Chlorophytina have been demonstrated to be a cause of inhalant allergic sensitivity.[10] It is difficult to determine how closely related some of the microalgal species studied by McElhenney et al., in 1962 are to *C. reinhardtii* because the authors only list genera and there has been subsequent reclassification of microalga since 1962. Nonetheless, the potential that some of the studied organisms could be related to *C. reinhardtii* at the order level cannot be entirely ruled out based on our review of the scientific literature and AlgaeBase (a database of information on algae; www.algaebase.org). For example, some species of genus *Neochloris* (studied by McElhenney at al.) have been reclassified to genus *Ettlia*, which belong to order, Chlamydomonadales, although we were unable to confirm that any of the species reclassified to *Ettlia* are found in Hawaii, which was the reported source of McElhenney's *Neochloris* species. *Chlorosarcinopsis* spp. (also studied by McElhenney) are currently classified as belonging to the Chlamydomonadales order; however, some *Chlorosarcinopsis* spp. have been reclassified to other genera that are more distantly related. As was the case with *Neochloris* spp., we were not able to confirm that any currently and formerly classified *Chlorosarcinopsis* spp. are found in South Dakota, the reported source of McElhenney's *Chlorosarcinopsis* species.

A single report of an allergic reaction to *Chlorella*, related to *C. reinhardtii* at the level of phylum, has also been reported.[11] There have been several reports of allergic reactions to Cyanobacteria[12-15]; however, Cyanobacteria belong to domain Bacteria and are not taxonomically related to *C. reinhardtii*.

Finally, our searches of the scientific literature and public health-related databases did not find any indication of potential allergenicity of any member of the family, Chlamydomonadaceae, to which *C. reinhardtii* belongs, and specifically, as expected because the ingredient has not yet been marketed for use in food, no reports of allergic reactions to *C. reinhardtii* (THN 6) dried biomass powder were found in our investigations. Thus, while allergic potential of several microorganisms of subphylum Chlorophytina have been documented (and

potentially of order Chlamydomonadales), there is no reason to suspect an allergenic concern related to members of the Chlamydomonadaceae family.

Notwithstanding the above, in order to further investigate the allergenic potential of *C. reinhardtii* (THN 6) dried biomass powder, Triton has conducted an in vitro simulated gastric fluid digestibility study using a standardized assay. The test system used porcine pepsin in simulated gastric fluid (comprised of sodium chloride, water, and hydrogen chloride) at pH 2.0. A standardized flour of the known allergen, raw soybean, was used as a positive control. The reaction mixture was preheated to body temperature (37 °C) and the test substance or positive control were added based on fixed amounts of test protein. Fixed volumes of the digesting protein reaction mixture were withdrawn at predetermined times (between zero and 60 minutes), digestion was halted using a buffer mixture, and the mixtures were analyzed via SDS-PAGE (i.e, gel electrophoresis) using appropriate negative controls and standards to assure the reliability of the assay. Destained gels were visualized in a UVP image station under white light transillumination. The experiments were run in duplicate.

Under the simulated conditions, *C. reinhardtii* (THN 6) proteins exhibited partial degradation to random sized proteins of less than 15 kDa after 30 seconds. Degradation continued with the low molecular weight smears getting lighter and smaller over time and by 20 minutes, only a faint smear smaller than 5 kDa was observable. In contrast, a very distinct band of approximately 47 kDa as well as two bands at approximately 17 kDa and a band at 7 kDa were stable throughout the experiment (up to 60 minutes) in the soybean positive control experiments.

Based on these results it was concluded that *C. reinhardtii* (THN 6) proteins were rapidly digested (i.e., < 10% stainable bands by five minutes) under the experimental conditions, suggesting a low likelihood of allergenic potential, while three major proteins from soy, a known modest allergenic source, were resistant to digestion.

6.6 History of Consumption

There is no known history of use of *C. reinhardtii* as a food or food ingredient.

6.7 Past Sales and Reported Adverse Events

To our knowledge, no products containing *C. reinhardtii*-derived ingredients have yet been introduced into the food supply. Accordingly, no FDA letters regarding concern for safety of *C. reinhardtii*-derived ingredients were located, and a search of MedWatch, FDA's adverse event reporting program, FDA's Recalls, Market Withdrawals, & Safety Alerts search engine, and FDA's Center for Food Safety

and Applied Nutrition Adverse Event Reporting System did not uncover any mention of *C. reinhardtii* products. All databases were accessed on March 2, 2018.

6.8 Basis for the GRAS Conclusion

The scientific procedures establishing the safety of *C. reinhardtii* (THN 6) dried biomass powder comprise the technical element of the GRAS standard. The common knowledge element is comprised of the general availability and general acceptance, throughout the scientific community of qualified experts, of the technical element. Together, the technical element and the common knowledge element form the basis for Triton's conclusion of GRAS status of *C. reinhardtii* (THN 6) dried biomass powder for its intended use.

6.8.1 Technical Element

C. reinhardtii (THN 6) dried biomass powder has been the subject of a thorough safety assessment as described above. The totality of evidence supporting the safety of *C. reinhardtii* (THN 6) dried biomass powder is comprised of data and information that establish the safety of *C. reinhardtii* (THN 6) dried biomass powder under the conditions of its intended use (the technical element) and data and information that are corroborative of safety. The scientific data, information, and methods forming the technical element of this conclusion are:

- The establishment of identity, demonstrating that the organism is a pure strain of *C. reinhardtii*, which was among the first green algae subjected to a genome project, as well as the taxonomic information and absence of any reports in the literature that would suggest that *C. reinhardtii* is pathogenic, toxicogenic or allergenic;
- The method of manufacture and specifications, demonstrating the safe production and the high quality control standards of *C. reinhardtii* (THN 6) dried biomass powder;
- The results of the bacterial reverse mutation test, in vitro mammalian chromosomal aberration test, and in vivo mammalian micronucleus test, establishing the lack of genotoxic potential of *C. reinhardtii* (THN 6) dried biomass powder;
- The 28-day repeated-dose oral toxicity study in rats, establishing the lack of adverse health effects and/or target organs of repeated exposure to *C. reinhardtii* (THN 6) dried biomass powder in rats; and
- The composition of *C. reinhardtii* (THN 6) dried biomass powder (which is without appreciable amounts of non-nutritive bioactive compounds or known toxic compounds) consisting of macro- and micronutrients common

to edible plant-derived foodstuffs upon which the body is expected to act through similar physiological processes of digestion and ADME to such foodstuffs that are commonly consumed in the human diet.

Because the ingredient is intended as a nutritive replacement for conventional macronutrients, its use in foods will necessarily be at relatively high levels. It is not possible to test such uses in laboratory animals at doses many fold greater than the level of exposure in humans; nonetheless, we sought to test as high a level of exposure in rats as possible based on the maximum feasible dose due to solubility of the test item. As such the high dose group of the 28-day study was selected as 4000 mg/kg bw/day in male and female Wistar rats, and this level was concluded to be the NOAEL, indicating the safety of the ingredient at the highest feasible dose in rats.

Because of the food-like nature of the ingredient, being essentially composed of conventional macro (protein, fat, and carbohydrate) and micro (vitamins and minerals) nutrients as well as chlorophyll, all of which are typical components of plant foods commonly found in the human diet, *C. reinhardtii* (THN 6) dried biomass powder is expected to be acted upon by the body through similar physiological processes and, as such, presents no cause for concern when consumed as a replacement for added dietary protein. The totality of evidence supporting the safety of the ingredient as described in this Subpart supports a conclusion that the intended use of *C. reinhardtii* (THN 6) dried biomass powder is reasonably certain to be safe.

The safety of *C. reinhardtii* (THN 6) dried biomass powder is corroborated by a series of unpublished growth studies in piglets demonstrating no adverse effects of *C. reinhardtii* (THN 6) dried biomass powder on lean body weight gain and/or mean intestinal weights and/or immune system development and an in vitro simulated gastric fluid digestibility study indicating the low allergenic potential of *C. reinhardtii* (THN 6) dried biomass powder.

6.8.2 Common Knowledge Element

The scientific data, information, and methods herein reported, that provide the basis of this GRAS conclusion by scientific procedures are published and available in the public domain. Part 7 of this GRAS notice contains the citations for the published studies. This publicly available data and information fulfills the requirement for general availability of the scientific data, information, and methods relied on to establish the technical element of the GRAS standard. The peer-review of the published studies and lack of Letters to the Editor or other dissenting opinions provides ample evidence of consensus among qualified experts that there is reasonable certainty that consumption of *C. reinhardtii* (THN 6) dried biomass powder for its intended use is not harmful. The general

availability and acceptance of this scientific data, information, and methods satisfies the common knowledge element of this GRAS conclusion.

6.9 Data and Information that is Inconsistent with the GRAS Conclusion

We have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status.

6.10 Information that is Exempt from Disclosure under FOIA

There is no data or information in this GRAS notice that is considered exempt from disclosure under FOIA as trade secret or commercial or financial information that is privileged or confidential.

Part 7: Supporting Data and Information

Initial literature searches for the safety assessment described in Part 6 of this GRAS notice were conducted during May, 2016. Additional literature searches were conducted during the course of time spanning July 2017 through November 2017 and again on March 2, 2018.

7.1 Data and Information that are *not* Generally Available

The studies in piglets described in Subpart 6.3 and the in vitro digestibility study described in Subpart 6.5 of this GRAS notice are not generally available. They are included because the safety assessment of this GRAS conclusion should evaluate all relevant safety-related data pertaining to the intended use of *C. reinhardtii* (THN 6) dried biomass powder. However, this information is merely corroborative to the toxicological studies described in Subpart 6.2, the taxonomic classification of the species described in Subparts 6.4 and 6.5, and other information relating to allergenicity described in Subpart 6.5. We believe the safety conclusion can still be made even if qualified experts throughout the scientific community do not generally have access to this information.

7.2 References that *are* Generally Available

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From: Tim Murbach
To: Bewry, Nadine
Subject: Re: GRN 000773 ((dried C. reinhardtii): FDA"s comments - please respond by COB 06/21/2018
Date: Thursday, June 21, 2018 3:33:21 PM
Attachments: image010.png
image.png

Dear Nadine,

Please find our responses to your June 6, 2018 questions below in blue:

1. Please provide information on the source of the Chlamydomonas reinhardtii THN6 strain or strain lineage.

Response 1: The lineage of this strain is that it is a natural wild-type isolate - which is not genetically modified - acquired from the Chlamydomonas Resource Center at the University of Minnesota.

2. Please provide information on any genetic modifications made to produce strain THN6.

Response 2: There have been no genetic modifications to THN6; THN6 is Triton Algae Innovations' (the notifier) internal company name for the wild-type strain identified and described in Question 1.

3. (As revised by FDA on June 14, 2018) Please provide narrative to confirm that your strain (THN6) is similar to those that are the subjects of the referenced literature on the species and standard laboratory strains mentioned in the GRAS notice, and state if there are any differences that impact safety.

Response 3: THN6 is the wild-type strain described in the literature (as outlined in the response to Question #1) which was the subject of the safety studies supporting this GRAS conclusion. THN6 - which is Triton Algae Innovations' internal name for this wild-type strain - is the exact same genus and species as those referenced in the literature, and contains some minor genetic variation in the form of single nucleotide polymorphisms (SNP), as is the case with all organisms. The SNP variations in this strain do not impact the safety findings underpinning this conclusion of GRAS status, especially given, as noted above, that the toxicological investigations described and cited in Part 6 (as well as the unpublished digestibility study) were conducted using THN6 specifically. Thus, to our knowledge, Triton Algae Innovations' internal THN6 wild type strain is the only strain of *C. reinhardtii* that has been subjected to formal toxicological investigations.

Please let me know if clarification of further information is needed with respect to any of the above responses. Also, please let me know if you require the above responses in a separate document.

Kind Regards,

Tim Murbach, ND, DABT
Senior Scientific & Regulatory Consultant
AIBMR Life Sciences, Inc.
tim@aibmr.com
Ph. (253) 286-2888

www.aibmr.com
www.toxicoop.com

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On Thu, Jun 14, 2018 at 2:56 PM, Bewry, Nadine <Nadine.Bewry@fda.hhs.gov> wrote:

> Hi Tim,
>
> Thank you for your follow-up call earlier. I have been out of the office since Monday.
>
> Our revised comment is below:
>
> > Please provide narrative to confirm that your strain (THN6) is similar to those that are the subjects of the referenced literature on the species and standard laboratory strains mentioned in the GRAS notice, and state if there are any differences that impact safety.
>
> I hope that this statement clarifies our previous one (question #3). Please let me know.
>
> Best regards,
>
> Nadine
>
> ———————————————————
>
> **Nadine Bewry, Ph.D., M.P.H.**
> Consumer Safety Officer | Toxicology Reviewer
>
> **From:** Tim Murbach [mailto:tim@aibmr.com]

Sent: Tuesday, June 12, 2018 8:50 PM

To: Bewry, Nadine <Nadine.Bewry@fda.hhs.gov>
Subject: Re: GRN 000773 ((dried C. reinhardtii): FDA's comments - please respond by COB 06/21/2018

HI Nadine,

Just following up on the review team's response in case I may have missed an email from you.

Kind Regards,

Tim Murbach, ND, DABT
Senior Scientific & Regulatory Consultant

AIBMR Life Sciences, Inc.

tim@aibmr.com

Ph. (253) 286-2888

www.aibmr.com

www.toxicoop.com

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On Fri, Jun 8, 2018 at 6:46 AM, Bewry, Nadine <Nadine.Bewry@fda.hhs.gov> wrote:

> Hi Tim,
>
> As a follow-up, I shared your questions and comments with the review team. Our goal is to provide a response to you within the next two business days.
>
> Best regards,
>
> Nadine
>
> _____
>
> **Nadine Bewry, Ph.D., M.P.H.**
> Consumer Safety Officer | Toxicology Reviewer
>
> **From:** Tim Murbach [mailto:tim@aibmr.com]
> **Sent:** Thursday, June 7, 2018 11:00 AM
> **To:** Bewry, Nadine <Nadine.Bewry@fda.hhs.gov>
> **Subject:** Re: GRN 000773 ((dried C. reinhardtii): FDA's comments - please respond by COB 06/21/2018
>
> Hi Nadine,
>
> Can you please provide some additional clarification regarding question #3? By safety narrative, do you mean Part 6 of the notice? The only *Chlamydomonas reinhardtii* strain that was described in the literature cited in Part 6 was *Chlamydomonas reinhardtii* THN6 specifically. In other words, the Part 6 literature pertains strictly to the article of commerce. I did provide some general background discussion regarding characteristics of *Chlamydomonas reinhardtii* based on standard laboratory strains in Subpart 2.1 (Identification, citations 1–3), so I just want to verify that this is the literature to which you want a comparison and contrast of *Chlamydomonas reinhardtii* THN6.
>
> Kind Regards,
>
> Tim Murbach, ND, DABT
> Senior Scientific & Regulatory Consultant

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On Wed, Jun 6, 2018 at 2:46 PM, Bewry, Nadine <Nadine.Bewry@fda.hhs.gov> wrote:

> Dear Dr. Murbach,
>
> In reviewing GRN 000773, our review team would like Triton Algae Innovations (Triton) to address the following:
>
> 1. Please provide information on the source of the *Chlamydomonas reinhardtii* THN6 strain or strain lineage.
>
> 2. Please provide information on any genetic modifications made to produce strain THN6.
>
> 3. Please provide a narrative on how the THN6 strain is similar or different from the wild-type described in the literature that was used as part of your safety narrative.

To facilitate the timely review of the notice, please email Triton's response within 10 business days (by COB Thursday, June 21, 2018).

Please let me know if you have any questions.

Best regards,

Nadine Bewry, Ph.D., M.P.H.
Consumer Safety Officer | Toxicology Reviewer

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

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