Spherix Consulting Group

December 17, 2020

Rachel Morissette, Ph.D.
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
CPK-2 Building, Room 2092
5001 Campus Drive, HFS-225
College Park, MD 20740

Dear Dr. Morissette:

Enclosed please find a CD containing "GRAS Notification for Chlorella Powder in Selected Conventional Foods", Form 3667, and all corresponding references. The data and information that serve as the basis for this GRAS notification is available for review and copying at reasonable times at the office of Claire Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., 11821 Parklawn Drive, Suite 310, Rockville, MD 20852, Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or will be sent to FDA upon request.

We thank you for taking the time to review this GRAS notification. Should you have additional questions, please let us know.

Sincerely,

Claire L. Kruger, PhD, DABT, CFS
Managing Partner

Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310, Rockville, MD 20852
301-557-0375; info@spherixgroup.com

GENERALLY RECOGNIZED AS SAFE DETERMINATION FOR CHLORELLA POWDER IN SELECTED CONVENTIONAL FOODS

Prepared for:

Chlorella Industries Co., Ltd.
2-4-6, Shiba-daimon, Minato-ku
Tokyo 105-0012
Japan

Prepared by:

Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852

December 17, 2020

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2-AA: 2-aminoanthracene

A/G: Albumin/Globulin

ADI: Acceptable Daily Intake

AF-2: Furylfuramide

ALB: Albumin

ALP: Alkaline Phosphatase

ALT: Alanine Aminotransferase

AMY: Amylase

ANOVA: Analysis Of Variance

AOAC: Association of Official Agricultural Chemists

AST: Aspartate Aminotransferase

BW: Body Weight

CDC: Centers for Disease Control

CFR: United States Code of Federal Regulations

CK-22: Chlorella Powder

Cl: Chloride

CRE: Creatinine

DMSO: Dimethyl Sulfoxide

EDI: Estimated Daily Intake

FAAS: Flame Atomic Absorption Spectroscopy

FCC: Food Chemicals Codex

FFDCA: Federal Food, Drug and Cosmetic Act

FNDDS: Food and Nutrition Database for Dietary Studies

FOIA: Freedom of Information Act

GC-MS/MS: Gas Chromatograph-Mass Spectrometry

GFAAS: Graphite Furnace Atomic Absorption Spectroscopy

GLU: Glucose

GMP: Good Manufacturing Practice

GOT: Aspartate Aminotransferase

GPT: Alanine Aminotransferase

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

Hb: Hemoglobin

HPLC-MS/MS: High-Performance Liquid Chromatography-Mass Spectrometry

Ht: Hematocrit

IARC: International Agency for Research on Cancer

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy

IP: Inorganic Phosphorus

JHNFA: Japan Health and Nutrition Food Association

K: Potassium

LDH: Lactate Dehydrogenase

LOD: Limit Of Detection

LOQ: Limit of Quantitation

MCH: Mean Corpuscular Hemoglobin

MCHC: Mean Corpuscular Hemoglobin Concentration

MCV: Mean Corpuscular Volume

MEC: Mobile Examination Center

Na: Sodium

NCHS: National Center for Health Statistics

ND: Not Detected

NEFA: Non-Esterified Fatty Acid

Neg: Negative

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NITE: National Institute of Technology and Evaluation

NMRI: Naval Medical Research Institute

NOAEL: No-Observed-Adverse-Effect Level

OECD: Organization for Economic Cooperation and Development

PAHs: Polyaromatic Hydrocarbons

PCBs: Polychlorinated Biphenyls

PLT: Platelets

PSUs: Primary Sampling Units

RBC: Red Blood Cell Count

RNA: Ribonucleic Acid

RPM: Rotations Per Minute

rRNA: Ribosomal RNA

SD: Standard Deviation

SG: Specific Gravity

T-BIL: Total Bilirubin

TBILI: Total Bilirubin

TCH: Total Cholesterol

TCHOL: Total Cholesterol

TEF: Toxic Equivalency Factor

TEQ: Toxic Equivalent Quantity

TG: Triglyceride

TLC: Thin Layer Chromatography

TP: Total Protein

UA: Uric Acid

UPRO: Urinary Protein

USDA: United States Department of Agriculture

USEPA: United States Environmental Protection Agency

USFDA: United States Food and Drug Administration

USP: United States Pharmacopeia

VOL: Volume

WAF: Whole Algalin Flour

WBC: White Blood Cell Count

I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260

A. SUBMISSION OF GRAS NOTICE

Chlorella Industries Co., Ltd. is hereby submitting a GRAS notice in accordance with subpart E of part 170.

B. NAME AND ADDRESS OF THE SPONSOR

Chlorella Industries Co., Ltd.
2-4-6, Shiba-daimon, Minato-ku
Tokyo 105-0012
Japan

C. COMMON OR USUAL NAME

Chlorella Powder

Also known as Chlorella micro-powder, CK-22 powder, CK-22 micro-powder, *Chlorella* CK-22 powder, *Chlorella* CK-22 micro-powder, *Chlorella sorokiniana* CK-22 powder, *Chlorella sorokiniana* CK-22 micro-powder, *C. sorokiniana* CK-22 powder, *C. sorokiniana* CK-22 micro-powder

Previously known as *C. vulgaris* CK-22

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

This notification does not contain any trade secret or confidential information.

E. INTENDED USE

Chlorella Powder will be added to food as a source of macronutrients.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of Chlorella Powder for the intended use specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug and Cosmetic Act (FFDCA) as described under 21 CFR §170.30(b). The safety of the intake of Chlorella Powder has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of Chlorella Powder as an ingredient for the intended uses in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. Chlorella Powder is the spray-dried whole-cell biomass of *Chlorella sorokiniana* CK-22. Chlorella Micro Powder is jet pulverized Chlorella Powder. The only difference between Chlorella Powder and Chlorella Micro Powder is particle size. The particle size distribution of Chlorella Powder is 19 μm (10 percentile), 60 μm (50 percentile), and 134 μm (90 percentile). The particle distribution of the Chlorella Micro Powder is 4 μm (10 percentile), 12 μm (50 percentile), 22 μm (90 percentile). The percentiles reflect the amount of powder that can pass through increasing pan sieve sizes.

2. All manufacturing complies with current Good Manufacturing Practice.

3. Compliance with appropriate specifications and quality control parameters assures the production of a food-grade product.

4. All culture medium ingredients and food contact materials are food grade and comply with the conditions of use and specifications of the United States Code of Federal Regulations, Title 21 and/or Food Chemicals Codex.

5. The major constituents of Chlorella Powder are normal components of the human diet and are anticipated to be digested and metabolized in pathways similar to those occurring with the ingestion of other edible plants and microalgae.

6. Pivotal toxicology studies demonstrate the safety of *C. sorokiniana* CK-22. These studies were performed on the spray-dried biomass, Chlorella Powder.

    a. Ethanol and hot water extracts of Chlorella Powder did not induce DNA damage as assessed by an Umu genotoxicity assay (Himuro et al., 2014).

    b. An acute oral toxicity study was performed in male and female Wistar rats fed Chlorella Powder at 0, 1000, 2000, and 5000 mg/kg. No deaths and no differences in body weight were observed. No observable differences were noted between the treatment groups and controls (Himuro et al., 2014).

    c. A 28-day repeated oral dose study was performed in male and female Wistar rats fed Chlorella Powder in the diet at 0, 2.5, 5, and 10%. No significant treatment-related adverse effects were observed in the parameters evaluated (Himuro et al., 2014).

d. The safety of Chlorella Powder was evaluated in a published 90-day toxicology study performed with 0, 2.5, 5, and 10% Chlorella Powder mixed into the standardized commercial rodent feed for male and female Wistar rats. During the experimental period, no Chlorella Powder treatment-induced differences in general condition, body weight gain, feed and water consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, histopathology, or animal death were observed. The no observed adverse effect (NOAEL) was calculated to be 5.94 and 6.41 g/kg body weight/day for male and female rats, respectively (Himuro et al., 2017).

e. Chlorella Powder and Micro Powder differ only in particle size, and not other parameters that define composition; therefore, the safety will be the same between the two forms.

7. Additional studies in *Chlorella spp*. corroborate the pivotal safety studies conducted with the Chlorella Powder.

   a. A safety assessment of high lipid whole algalin flour from *C. protothecoides* was published in 2012 (Szabo et al., 2012). Whole algalin flour was not mutagenic in a bacterial reverse mutation assay (up to 5000 µg/plate) or clastogenic by in vivo chromosome aberration assay (2000 mg/kg in mice). The NOAEL was calculated to be 100000 ppm, the highest dose tested, corresponding to 4807 mg/kg body weight/day in male rats and 5366 mg/kg body weight/day in female rats.

   b. A second safety evaluation on whole algalin protein from *C. protothecoides* was published in 2013 (Szabo et al., 2013). Whole algalin protein was not mutagenic in a bacterial reverse mutation assay (up to 5000 µg/plate) or clastogenic by in vivo chromosome aberration assay (2000 mg/kg in mice). The NOAEL was calculated to be 100000 ppm, the highest dose tested, corresponding to 4805 mg/kg body weight/day in male rats and 5518 mg/kg body weight/day in female rats.

8. Application of a 100-fold safety factor to the NOAEL, determined in the pivotal 90-day toxicology study, results in an acceptable daily intake (ADI) for Chlorella Powder of 59.4 mg/kg/day or 3.56 g/day for a 60 kg human.

9. Clinical studies have reported that other products derived from *Chlorella* spp. are well-tolerated up to 6 g/day for up to 6 months.

10. The addition of Chlorella Powder or Chlorella Micro Powder to the intended foods will result in a mean estimated daily intake (EDI) of 522 mg/day (7.8 mg/kg/day) and a heavy consumer (90th percentile) intake of 870 mg/day (13.0 mg/kg/day).

11. The safety of Chlorella Powder and Chlorella Micro Powder is supported by appropriate documentation of the safety of the source organism, appropriate food grade specifications, a well-controlled production process, and demonstrated safety in pivotal genotoxicity and 90-day rodent bioassays. Ingestion of Chlorella Powder and Chlorella Micro Powder at the proposed EDI is determined to be safe and GRAS.

G. PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of intended use.

H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., at 11821 Parklawn Drive, Suite 310, Rockville, MD 20852. Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT (FOIA)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA.

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Chlorella Industries Co., Ltd and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Signature of Authorized Representative of
Chlorella Industries Co., Ltd.

October 22, 2020
Date

II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

A. COMMON OR USUAL NAME

Chlorella Powder

B. TRADE NAME

Chlorella Powder, Chlorella Micro Powder

C. DESCRIPTION OF CHLORELLA POWDER

Chlorella Powder is the spray-dried whole-cell biomass of *Chlorella sorokiniana* CK-22 (referred to as CK-22 in this notice). *Chlorella* spp. provide a dietary source of protein, dietary fiber, minerals (e.g., iron, magnesium, zinc) , and vitamins (e.g., vitamin B_2 (riboflavin), and chlorophylls (Kay, 1991). The spray-dried powder may then be further milled to form Chlorella Micro Powder.

Chlorella is a genus of eukaryotic, single-celled green algae found in freshwater, marine, and edaphic habitats (Bock, Krienitz, and Pröschold, 2011). Identification of species within this genus had historically relied on morphology and nutritional requirements, but more accurate identification using sequencing techniques is preferred (Huss et al., 1999). Although CK-22 was originally identified as *C. vulgaris* CK-22 by morphology, CK-22 has been redesignated as *C. sorokiniana* following *18S* rRNA sequencing. The CK-22 strain of *C. sorokiniana* was isolated from a pond in Saga-ken, Japan in 1973. Chlorella Industry Co., Ltd. maintains subcultures of CK-22 and frozen samples of CK-22 have been deposited in the National Institute of Technology and Evaluation, Japan (NITE, NITE SD 00247). CK-22 has not been subjected to any genetic manipulations. For taxonomy, see Table 1.

Table 1. Taxonomy of *Chlorella sorokiniana* CK-22

Domain	Eukaryota
(unranked)	Diaphoretickes
(unranked)	Archaeplastida
(unranked)	Viridiplantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	*Chlorella*
Species	*C. sorokiniana*
Ref.: (Bock, Krienitz, and Pröschold 2011; Rosenberg et al. 2014)	

1. Phenotypic Description of CK-22

Live CK-22 cells are spherical to slightly ovoid, measure 3-6 µm in diameter, and have a thin cell wall (Figure 1). CK-22 has cup-shaped chloroplasts with a starch grain-covered pyrenoid. These morphological characteristics also correspond with those of *C. vulgaris*. Although *C. vulgaris* and *C. sorokiniana* are morphologically identical, *C. sorokiniana* can be separated from *C. vulgaris* by hydrogenase activity, preference for warmer growth temperature, and the sequence of rRNA genes (Huss et al., 1999).

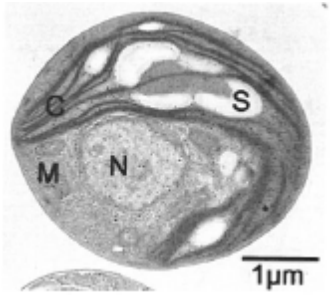

Figure 1. Electron Micrograph of *C. sorokiniana* CK-22
C: chloroplast, N: nucleus, M mitochondrion, S: Starch. Scale bar is 1 µm.

CK-22 is capable of growth in both the presence and absence of light. In the presence of light, CK-22 is photoautotrophic, producing its own organic compounds by photosynthesis. In the absence of light, CK-22 is heterotrophic, consuming glucose as a carbon source to produce organic compounds. The culture's light conditions influence color (chlorophyll production), lipid content, and growth rate; however, when there is a sufficient source of carbon, light conditions do not affect the rate of growth of CK-22. Although the light conditions do not affect the growth rate, there are some changes in morphology visible under a light microscope. During autotrophy, the chloroplasts are darker in color and more prominent (Figure 2).

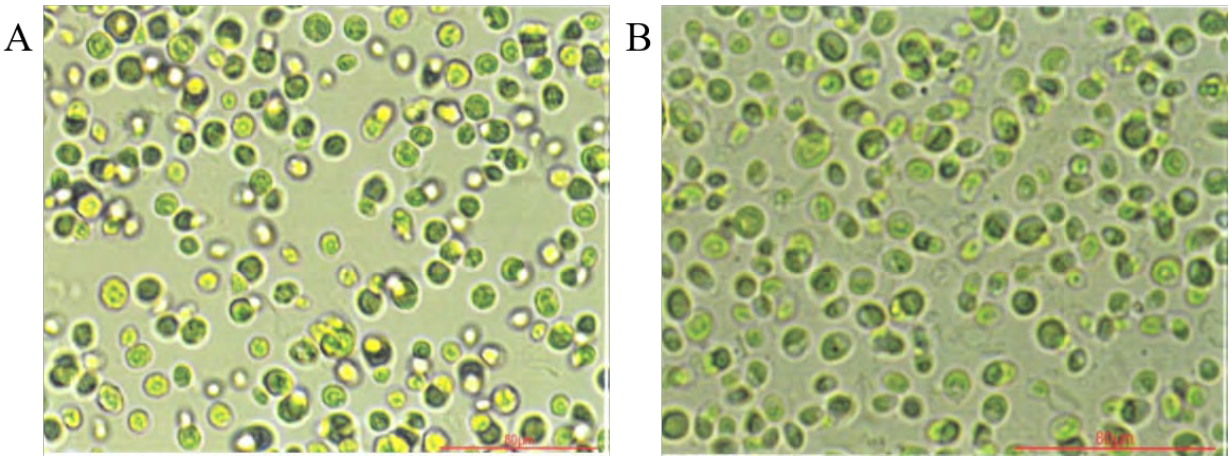

Figure 2. Heterotrophic and Autotrophic Culture Morphology for CK-22

The scale bar represents 80 µm. A.) Heterotrophic morphology, typified by changes in cell shape. B.) Autotrophic morphology, chloroplasts are more prominent as darker green spots inside the cells.

2. Genotypic Description of CK-22

Although morphological characteristics have been historically used to differentiate between species, ribosomal RNA (rRNA) sequencing now provides a more accurate means for identifying members of the genus *Chlorella* (Huss et al., 1999). Comparison of the *18S* rRNA sequences of CK-22 with *C. sorokiniana* SAG 211-8k (accession number X62441, Culture Collection of Algae at the University of Göttingen, Germany) and *C. vulgaris* SAG 211-11k (accession number X13688) shows that the CK-22 *18S* rRNA sequence is 99.8% similar to the *C. sorokiniana*, SAG 211-8k sequence and 99.5% similar to *C. vulgaris* SAG 211-11b (Table 2).

Table 2. Summary of *18S* rRNA Sequence Alignment Results Comparing *C. vulgaris* and *C. sorokiniana* to CK-22

Comparison	Bases conserved	%Sequence Similarity
CK-22 vs. *C. vulgaris*[a]	1714/1723	99.5%
CK-22 vs. *C. sorokiniana*[b]	1720/1723	99.8%

[a] *C. vulgaris* SAG 211-11b (accession number X13688)
[b] *C. sorokiniana* SAG 211-8k (accession number X62441)

D. PRODUCTION PROCESS

CK-22 is cultured in a series of indoor cultures and then expanded to outdoor pools at Chlorella Industry Co., Ltd. Kyushu production plant in Chikugo, Japan. Chlorella Industry Co. Ltd. is registered for In-House Safety Assessment of Health Foods by Japan Health and Nutrition Food Association (JHNFA) and the production plant complies with JHNFA's Good Manufacturing Practice (GMP) program for dietary supplements. All fermentation vessels, food contact materials, raw materials, and processing aids are U.S. food grade. Additionally, both Chlorella Powder and Micro Powder are Halal-certified foods in Japan.

One batch of CK-22 seed culture corresponds to one lot of intermediate product, yielding 600 kg. Many lots of intermediate product are mixed together to form one lot of finished Chlorella Powder. Chlorella Powder may be further processed to produce Chlorella Micro Powder. One lot of finished Chlorella Powder is between 1000-1500 kg and approximately 100 finished lots are produced a year. Each lot is further packaged into 10 kg packs. Both the intermediate product and finished product are stored in a warehouse located at the Kyushu Chikugo Factory, with the temperature ranging from 1.8-38^{o}C and humidity between 20-90%.

1. CK-22 Culture

CK-22 is cultured and sequentially expanded in the following steps: slant culture, flask culture, jar culture, seed culture, tank culture, and finally, outdoor pool culture. The quality of each culture step is assessed by the morphology of the CK-22 cells and the green color of the

culture. The growth of CK-22 is dependent on the availability of glucose and oxygen in the medium and exposure to light. Chlorophyll content, glucose content, and dissolved O_2 levels are monitored daily to determine when the culture is ready to proceed to the next step in production (Figure 3). All culture media, culture vessels, and transport lines are sterilized before use. If contamination is observed at any step, the culture is discarded and all culture vessels are washed and sterilized.

Slant culture: A stock of CK-22 is maintained in slant culture. The slant culture is initiated from the original frozen stock strain once a month. An agar slant culture is inoculated using a sterile loop. This slant culture is managed by successive subculture at Chlorella Industry Co., Ltd. The slant culture is considered successful if the CK-22 culture is dark green in color and proliferating within 1-2 weeks under constant fluorescent light. The slant culture is then used to inoculate the flask culture.

Flask, jar, and seed culture: All culture vessels and culture medium for the indoor culture steps are sterilized prior to inoculation at each step. The flask culture is inoculated under sterile conditions and cultured under constant mechanical agitation for 1-2 weeks in the presence of glucose with constant exposure to a light source. A portion of the flask culture is then expanded to the jar culture. The remaining culture is discarded. The jar culture is grown with agitation and ambient light. A transport line for seed culture inoculation is connected to the jar culture and the entire jar culture biomass is transferred when cell growth plateaus. After inoculation from the jar culture, the CK-22 culture is grown with constant agitation, and under controlled temperature and pH. The seed culture is grown in the presence of glucose and the absence of light. A portion of the seed culture is collected and used for the next step, tank culture. The biomass is replaced with fresh culture medium that is added to the remaining biomass in the seed culture. The process is repeated for approximately 5-6 months, on the condition that the culture meets quality control parameters. If these parameters decrease over time, the culture is discarded and a new culture is immediately resumed by inoculation from the jar culture.

Quality of the flask, jar, and seed cultures is assessed daily by chlorophyll content, and microscopic inspection for appearance, color, and absence of microbial contamination. The pH of the indoor culture steps is controlled to prevent contamination.

Tank culture: A portion of seed culture is transferred to a tank culture vessel containing sterile medium with constant agitation, in the presence of glucose, and the absence of light. Temperature and pH are controlled during tank culture. Once the biomass reaches the appropriate chlorophyll content, the entire tank culture is moved to the outdoor pool culture via a sterile, food-grade pipeline.

The quality of the culture at this step is assessed daily by chlorophyll content and microscopic examination, assessing the morphology of the CK-22 cells (Figure 2). Microscopic examination is also performed to screen for any microbial contamination. If contamination is detected, the culture is discarded.

Outdoor pool culture: The tank culture is then expanded to a shallow, outdoor pool by pipeline and cultured in the presence of ambient light and temperature with agitation. The nitrogen concentration and pH of the culture medium are monitored during the outdoor pool culture as a functional readout of the health of the culture. Accordingly, agitation speed may be increased to maintain the health of the culture. The culture is also monitored through microscopic examination of cell shape, lack of aggregation, and for potential microbial contamination. In the event of microbial contamination, the culture is discarded. After the appropriate chlorophyll content is reached, the cultured is transferred via a pipeline to be harvested for processing.

2. Processing and Production of Chlorella Powder

Processing and production of Chlorella Powder from the outdoor culture include washing, filtering, and sterilizing the algae (Figure 3). Due to the nature of the outdoor culture step, it is possible that foreign bodies, such as sand or dirt, may be present. Multiple filtration steps are included in the production process to remove these potential contaminants and prevent damage to the production machinery.

After the culture process is complete, the CK-22 biomass is collected by filtering the culture through 800 µm and 550 µm filters. The culture is then washed and concentrated by three separate centrifugation steps to remove the culture medium. Each wash is done with sterile water, and the culture is passed through a final 350 µm filter. The entire washing and concentration steps last approximately 2 hours. The final slurry is passed through a magnetic strainer, cooled to 2-5^{o}C, and stored for up to 24 hours before undergoing heat inactivation for 3 minutes at 100^{o}C.

The heat sterilized CK-22 is then spray-dried. The spray-dried intermediate product is sieved through a magnetic strainer, passed through a 20-mesh strainer, and then packaged into 100 kg bags. One batch of seed culture corresponds to one lot of intermediate product. The intermediate product is then inspected for quality and, upon passing inspection, is stored in a warehouse. The quality parameters assessed in the intermediate product are the same as the product specifications (see Table 3): appearance, foreign substance, water, ash content, chlorophyll, iron, total plate count, and coliforms. Once a month, the intermediate product is assessed for chlorophyll b, protein, and vitamin B_2 content. The intermediate spray-dried CK-22 may be stored in a warehouse (between 1.8-30^{o}C and 20-90% humidity) up to two years prior to being incorporated into the final product.

The final product consists of multiple lots of intermediate product that are mixed to yield one 1000-1500 kg finished lot. After mixing, the product goes through a magnetic strainer. The Chlorella Powder is then inspected for quality before being packaged into 10 kg packs in food-grade aluminum laminate bags. The particle distribution of the Chlorella Powder is: 19 μm (10 percentile), 60 μm (50 percentile), 134 μm (90 percentile).

Spray-dried Chlorella Powder consists of aggregated particles of CK-22 cells, due to aggregates in water droplets. To produce the micro powder, the Chlorella Powder is jet-pulverized and then packaged into food-grade aluminum bags, which are heat-sealed. The finished Chlorella Micro Powder particles pass through a 30 μm filter as a quality control step. The particle distribution of the Chlorella Micro Powder is 4 μm (10 percentile), 12 μm (50 percentile), 22 μm (90 percentile).

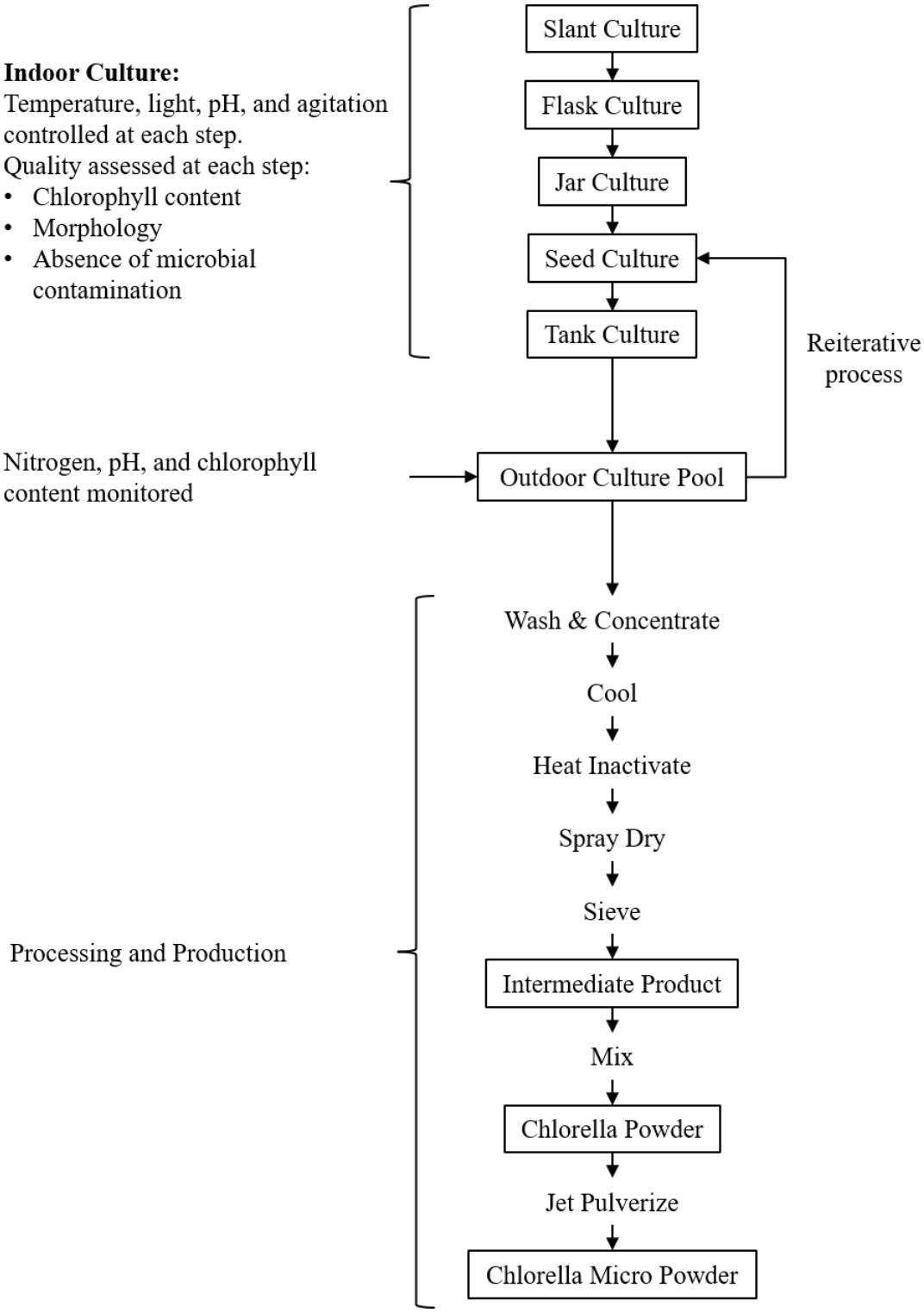

Figure 3. Flow Diagram of Production Process for Chlorella Powder

CK-22 is cultured and expanded through multiple indoor culturing steps. The last three culture steps (seed, tank, and outdoor culture), are repeated for ~5-6 months. The culture is then washed and concentrated before being cooled and heat inactivated. The biomass is then spray-dried, mixed, and/or pulverized before packaging.

3. Processing Aids

There are no excipients in either Chlorella Powder or Chlorella Micro Powder. All medium components are compliant with 21 CFR and/or FCC. The final product is stored in olefin lined aluminum laminate bags that are compliant with Japanese Food Hygiene Law Notification No. 370 or No. 201, announced by the Health and Welfare Ministry 1959 and the US Code of Federal Regulations 21 §177.1520, Olefin polymers. The water used in the production of Chlorella Powder is well and tap water, both of which adhere to the municipal drinking water standards. Water quality, based on these standards, is assessed twice a year.

E. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. Product Specifications

Chlorella Industry Co. evaluates each lot of Chlorella Powder and Chlorella Micro Powder against a set of product specifications that establish the physical characteristics, nutrient content, potential metal and microbial contaminants, and the pheophorbide content of the finished product. Pheophorbide is included as it is a natural degradation product of chlorophyll that can cause photosensitive dermatitis among sensitive individuals (Jitsukawa et al., 1984). The omega 3 fatty acid content product specification is the sum of all omega 3 fatty acids as measured by the compendial method Association of Official Agricultural Chemists (AOAC) 966.23.

a. Chlorella Powder

Data from three representative lots of Chlorella Powder demonstrate that the production process produces a product that reproducibly meets the product specifications (Table 3).

Table 3. Product Specifications and Lot Data for Chlorella Powder

Parameter	Method	LOD	Specification	Chlorella Powder Lot No.		
				170725	180926	190805
Physical Characteristics						
Appearance	Visual Inspection	-	Green Powder	Green Powder	Green Powder	Green Powder
Foreign body	Visual Inspection	-	Not observed	Not observed	Not observed	Not observed
Moisture (%)	AOAC 930.15	-	<7.0	5.1	5.0	5.2
Ash (%)	AOAC 942.05	-	<11.0	7.0	7.2	7.1
Nutrition						
Chlorophyll (%)	Alkaline-pyridine method[a]	-	≥1.5	3.0	2.5	2.8
Chlorophyll b	TLC[b]	0.08 g/100 g	Detectable	Detectable	Detectable	Detectable

Table 3. Product Specifications and Lot Data for Chlorella Powder

Parameter	Method	LOD	Specification	Chlorella Powder Lot No.		
				170725	180926	190805
Vitamin B_2 (mg/100 g)	AOAC 970.65	-	≥4.0	5.9	5.7	5.9
Protein (%)	AOAC 984.13	-	≥55.0	64.4	63.1	65.0
Iron (mg/100 g)	AOAC 999.10	-	40-100	57	60	63
Fat (%)	AOAC 954.02	-	≥8.8	13.1	11.4	11.5
Carbohydrate (%)	Calculated	-	≥7.0	10.4	13.5	11.2
Omega 3 Fatty Acids (%)	AOAC 991.39	-	≥1.00	1.64	1.39	1.67
Metals						
Arsenic (ppm)	AOAC 999.10	0.5 ppm	<1.0	N.D.	N.D.	N.D.
Lead (ppm)	AOAC 999.10	0.2 ppm	<1.0	N.D.	N.D.	N.D.
Cadmium (ppm)	AOAC 999.10	0.02 ppm	<0.20	N.D.	N.D.	N.D.
Mercury (ppm)	AOAC 971.21	0.01 ppm	<0.10	N.D.	N.D.	N.D.
Chromium (ppm)	USP<730>	0.5 ppm	<2.0	N.D.	N.D.	N.D.
Microbial Characteristics						
Aerobic plate count (cfu/g)	AOAC 966.23	-	<1000	100	100	100
Coliforms	Deoxycholate Agar Method[c]	-	Negative/1 g	Negative	Negative	Negative
E. coli	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Staphylococcus	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Salmonella	USP<62>	-	Negative/10 g	Negative	Negative	Negative
Pseudomonas aeruginosa	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Mold (cfu/g)	USP<62>	-	<100	<100	<100	<100
Yeast (cfu/g)	USP<62>	-	<100	<100	<100	<100
Other						
Total Pheophorbide (mg/100 g)	Kanshoku No.99b[d]	-	<50	10	15	25

Abbreviations: AOAC - Association of Official Agricultural Chemists; LOD - limit of detection; TLC - thin layer chromatography; FAAS - flame atomic absorption spectroscopy; GFAAS - Graphite furnace atomic absorption spectroscopy; N.D - not detected; ICP-AES - Inductively coupled plasma atomic emission spectroscopy; USP - United States Pharmacopeia; ppm - parts per million; cfu - colony forming units.
[a]Analytical Biochemistry 57,255-267(1974)
[b]J. Chromatogr.1977 Apr 11;134(2):359-64.
[c]14th ed., APHA Inc., New York, pp.58-59, Standard Methods for the Examination of Water and Wastewater, 1976
[d]Res. Bd Canada 25 (3) 523-540, 1968

b. Chlorella Micro Powder

Data from three representative lots of Chlorella Micro Powder demonstrate that the production process produces a product that reproducibly meets the product specifications (Table 4).

Table 4. Product Specifications and Lot Data for Chlorella Micro Powder

Parameter	Method	LOD	Specification	Chlorella Micro Powder Lot No.		
				170728	180914	190805
Physical Characteristics						
Appearance	Visual Inspection	-	Light green Powder	Light green Powder	Light green powder	Light green powder
Particle size (% through 200 mesh)	Jet Sieve Method	-	≥95	≥ 99	≥ 99	≥ 99
Foreign body	Visual Inspection	-	Not observed	Not observed	Not observed	Not observed
Moisture (%)	AOAC 930.15	-	<7.0	4.7	4.7	5.0
Ash (%)	AOAC 942.05	-	<11.0	6.9	7.0	7.0
Nutrition						
Chlorophyll (%)	Alkaline-pyridine method[a]	-	≥1.5	2.9	2.8	2.9
Chlorophyll b (g/100 g)	TLC[b]	0.08 g/100 g	Detectable	Detectable	Detectable	Detectable
Vitamin B_2 (mg/100 g)	AOAC 970.65	-	≥4.0	6.0	5.6	6.0
Protein (%)	AOAC 984.13	-	≥55.0	64.4	63.8	65.0
Iron (mg/100 g)	AOAC 999.10	-	40-100	58	58	61
Fat (%)	AOAC 954.02	-	≥8.8	12.7	12.1	12.0
Carbohydrate (%)	Calculated	-	≥7.0	11.3	12.4	11.0
Omega-3 Fatty Acids (%)	AOAC 991.39	-	≥1.00	1.84	1.69	1.73
Metals						
Arsenic (ppm)	AOAC 999.10	0.5 ppm	<1.0	N.D.	N.D.	N.D.
Lead (ppm)	AOAC 999.10	0.2 ppm	<1.0	N.D.	N.D.	N.D.
Cadmium (ppm)	AOAC 999.10	0.01 ppm	<0.20	N.D.	N.D.	N.D.
Mercury (ppm)	AOAC 971.21	0.01 ppm	<0.10	N.D.	N.D.	N.D.
Chromium (ppm)	(USP<730>)	0.5 ppm	<2.0	N.D.	N.D.	N.D.
Microbial Characteristics						
Aerobic plate count (cfu/g)	AOAC 966.23	-	<1000	100	100	100
Coliforms	Desoxycholate Agar Method[c]	-	Negative/1 g	Negative	Negative	Negative
Eschericia coli	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Staphylococcus	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Salmonella	USP<62>	-	Negative/10 g	Negative	Negative	Negative
Pseudomonas aeruginosa	USP<62>	-	Negative/1 g	Negative	Negative	Negative
Mold (cfu/g)	USP<62>	-	<100	<100	<100	<100
Yeast (cfu/g)	USP<62>	-	<100	<100	<100	<100
Other						
Total Pheophorbide (mg/100 g)	Kanshoku No.99b[d]	-	<50	17	21	23

Abbreviations: AOAC - Association of Official Agricultural Chemists; LOD - limit of detection; TLC - thin layer chromatography; N.D. - not detected; USP - United States Pharmacopeia; ppm - parts per million; cfu - colony forming units.

[a]Analytical Biochemistry 57,255-267(1974)

[b]J. Chromatogr.1977 Apr 11;134(2):359-64.

[c]14th ed., APHA Inc., New York, pp.58-59

[d]Res. Bd Canada 25 (3) 523-540, 1968

2. Additional Quality Parameters

Additional parameters, such as pesticides, aflatoxins, microcystins, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and radioactive isotopes, further demonstrate the quality of Chlorella Powder and Chlorella Micro Powder.

a. Chlorella Powder

Residual pesticides. Four lots of Chlorella Powder were subjected to a pesticide screen with a limit of quantitation of 0.01 ppm (Table 5). None of the pesticides were detected above the limit of quantitation and were therefore deemed not detected. Chlorella Industry Co., Ltd. performs the residual pesticide screen on Chlorella Powder annually.

Table 5. Pesticides Screened in Four Lots of Chlorella Powder

Not Detected Pesticides, as Screened by GC-MS/MS		
1,1-Dichloro-2,2-bis(4-ethylphenyl) ethane	2,4-DB	2-Phenylphenol
Acetochlor	Acrinathrin	Alachlor
Aldrin/dieldrin	Ametryn	Atrazine
Azaconazole	Azinphos methyl	Benalaxyl
Bendiocarb	Benfluralin	Benoxacor
BHC	Bifenox	Bioresmethrin
Bitertanol	Bromacil	Bromobutide
Bromophos ethyl	Bromopropylate	Bupirimate
Butafenacil	Butamifos	Cadusafos
Cafenstrole	Captan	Carfentrazone ethyl
Chinomethionat	Chlorbufam	Chlordane
Chlorfenson	Chlorfenvinphos	Chlorobenzilate
Chloroneb	Chlorpropham	Chlorpyrifos
Chlorpyrifos methyl	Chlorthal dimethyl	Chlozolinate
Cinidon ethyl	Clodinafop propargyl	Clomazone
Clomeprop	Cloquintocet mexyl	Cyanazine
Cyanophos	Cyclosulfamuron	Cyfluthrin
Cyhalofop butyl	Cyhalothrin	Cypermethrin
Cyproconazole	DCIP	Deltamethrin/tralomethrin
Demeton-S-methyl	Desmedipham	Diazinon
Dichlobenil	Dichlofenthion	Dichlofluanid
Diclocymet	Diclofop methyl	Diclomezine
Dicloran	Dicofol	Dicrotophos
Diethofencarb	Difenoconazole	Diflufenican
Dimethametryn	Dimethenamid	Dimethipin
Dimethoate	Diniconazole	Dioxathion
Disulfoton	Endosulfan	Endrin
EPN	EPTC	Esprocarb
Ethalfluralin	Ethiofencarb	Ethion
Ethofumesate	Etobenzanid	Etofenprox
Etoxazole	Etridiazole	Etrimfos
Fenamidone	Fenamiphos	Fenarimol
Fenbuconazole	Fenchlorphos	Fenitrothion
Fenoxycarb	Fenpropathrin	Fenpropimorph

Table 5. Pesticides Screened in Four Lots of Chlorella Powder		
Fensulfothion	Fenvalerate	Flamprop methyl
Fluacrypyrim	Flucythrinate	Fludioxonil
Flufenpyr ethyl	Flumioxazin	Fluquinconazole
Fluridone	Flusilazole	Flutolanil
Fluvalinate	Folpet	Formothion
Fthalide	Halfenprox	Heptachlor
Hexaconazole	Hexavhlorobenzene	Hexazinone
Imazamethabenz methyl ester	Imazaquin	Isazophos
Isocarbophos	Isofenphos	Isoprothiolane
Isoxathion	Lactofen	Lenacil
Malathion	Mecarbam	Mefenpyr diethyl
Mepronil	Metalaxyl/mefenoxam	Metconazole
Methabenzthiazuron	Methacrifos	Methidathion
Methoprene	Methoxychlor	Metolachlor
Metominostrobin	Metribuzin	Mevinphos
Molinate	Monocrotophos	Monolinuron
Myclobutanil	Napropamide	Nicotine
Norflurazon	Oxadixyl	Oxpoconazole fumurate
Oxyfluorfen	Paclobutrazol	Parathion
Parathion methyl	Penconazole	Pendimethalin
Pentoxazone	Permetrin	Phenothrin
Phenthoate	Phorate	Phosalone
Phosphamidon	Picolinafen	Piperonyl butoxide
Pirimiphos methyl	Pretilachlor	Prochloraz
Procymidone	Profenofos	Prohydrojasmon
Prometryn	Propachlor	Propanil
Propaphos	Propargite	Propazine
Propiconazole	Propoxur	Propyzamide
Prothiofos	Pyraclofos	Pyraflufen ethyl
Pyrazophos	Pyrethrins	Pyridaben
Pyridafenthion	Pyrifenox	Pyrimethanil
Pyrimidifen	Pyriproxyfen	Quinalphos
Quinoclamine	Quinoxyfen	Quintozene
Resmethrin	Simazine	Simetryn
Spirodiclofen	Tebuconazole	Tebufenpyrad
Tecnazene	Tefluthrin	Tepraloxydim
Terbacil	Terbufos	Terbutryn
Tetrachlorvinphos	Tetraconazole	Tetradifon
Thifluzamide	Thiobencarb	Thiometon
Tiadinil	Tolclofos methyl	Tolfenpyrad
Triadimefon	Triadimenol	Triallate
Triazophos	Trichlamide	Trifluralin
Uniconazole P	Vamidothion	Vinclozolin
XMC	Zoxamide	
Not Detected Pesticides, as Screened by HPLC-MS/MS		
1-Naphthalene acetic acid	Acetamiprid	Alanycarb
Anilazine	Anilofos	Aramite
Azafenidin	Azimsulfuron	Azoxystrobin
Bensulfuron methyl	Bensulide	Benthiavalicarb isopropyl
Bispyribac sodium	Boscalid	Buprofezin
Carbaryl	Carpropamid	Chlorantraniliprole
Chloridazon	Chlorimuron ethyl	Chlorsulfuron

Table 5. Pesticides Screened in Four Lots of Chlorella Powder		
Chromafenozide	Cinosulfuron	Clofencet
Clofentezine	Cloransulam methyl	Clothianidin
Cumyluron	Cyazofamid	Cycloate
Cycloprothrin	Cyenopyrafen	Cyflufenamid
Cyprodinil	Diallate	Diflubenzuron
Dimethirimol	Dimethomorph	Diuron
Epoxiconazole	Ethametsulfuron-methyl	Ethoxysulfuron
Ethychlozate	Famoxadone	Fenhexamid
Fenobucarb	Fenpyroximate	Flazasulfuron
Florasulam	Flufenoxuron	Flumetsulam
Fluometuron	Fluopicolide	Flutriafol
Fosthiazate	Halosulfuron methyl	Hexythiazox
Imazosulfuron	Imicyafos	Imidacloprid
Indoxacarb	Iodosulfuron methyl	Iprovalicarb
Isoprocarb	Isouron	Linuron
Mandipropamid	Mesosulfuron methyl	Metamitron
Methoxyfenozide	Metosulam	Metsulfuron methyl
Naptalam	Nicosulfuron	Oxamyl
Oxaziclomefone	Oxycarboxin	Pencycuron
Penoxsulam	Phenmedipham	Phosmet
Phoxim	Pirimicarb	Propaquizafop
Pyraclonil	Pyraclostrobin	Pyrazolynate
Pyrazosulfuron ethyl	Pyrazoxyfen	Pyriftalid
Rimsulfuron	Silafluofen	Simeconazole
Sulfosulfuron	Sulfotep	Sulprofos
Tebufenozide	Tebupirimfos	Tebuthiuron
Thiacloprid	Thiamethoxam	Thifensulfuron methyl
Tolyfloxysulfuron	Tralkoxydim	Tribenuron methyl
Tricyclazole	Triflumuron	
Lots screened: 160805, 170725, 180704, 23160 Quantitation Limit: 0.01 ppm. A not detected result is defined as below the limit of quantitation HPLC-MS/MS: High-performance liquid chromatography-mass spectrometry GC-MS/MS: Gas chromatography-mass spectrometry		

Aflatoxins. Three lots of Chlorella Powder were screened for Aflatoxins B_1, B_2, G_1, and G_2 with a limit of quantitation of 1 µg/kg (1 ppb). No aflatoxins were found above the limit of quantitation in any of the screened lots and were therefore deemed not detected, Table 6. Chlorella Industry Co., Ltd. performs the aflatoxin screen on Chlorella Powder twice a year.

Table 6. Aflatoxins Screened in Chlorella Powder			
Aflatoxin	**Chlorella Powder Lot No.**		
	160426	**170417**	**180219**
Aflatoxin B_1	ND	ND	ND
Aflatoxin B_2	ND	ND	ND
Aflatoxin G_1	ND	ND	ND
Aflatoxin G_2	ND	ND	ND
Method: High-performance liquid chromatography. Quantitation limit: 1.0 µg/kg (1 ppb). ND: not detected. A not detected result is defined as below the limit of quantitation			

Microcystin residues. Three lots of Chlorella Powder were screened for Microcystin LR, RR, and YR with a limit of quantitation of 0.1 ppm. No microcystins were found above the limit of quantitation in any of the screened lots and were therefore deemed not detectable, Table 7. Chlorella Industry Co., Ltd. performs the microcystin screen on Chlorella Powder yearly.

Table 7. Microcystin screening in Chlorella Powder

Microcystin	Chlorella Powder Lot No.		
	160426	170417	180219
Microcystin LR	ND	ND	ND
Microcystin RR	ND	ND	ND
Microcystin YR	ND	ND	ND

Method: Liquid chromatography-mass spectrometry. Quantitation limit is 0.1 ppm.
ND: not detected. A not detected result is defined as below the limit of quantitation

Polycyclic aromatic hydrocarbon residues. Polycyclic aromatic hydrocarbons (PAHs) are monitored yearly. No PAH residues have been detected above the limit of quantitation in Chlorella Powder and were therefore deemed not detected, Table 8.

Table 8. Polycyclic Aromatic Hydrocarbons in Chlorella Powder

Residue	Toxic Equivalency Factor (TEF)	Chlorella Powder Lot No.					
		160426		170417		180219	
		Conc.	TEQ	Conc.	TEQ	Conc.	TEQ
Benzo[a]pyrene	1	ND	0	ND	0	ND	0
Benzo[b]fluoranthene	0.1	ND	0	ND	0	ND	0
Benz[a]anthracene	0.1	ND	0	ND	0	ND	0
Chrysene	0.01	ND	0	ND	0	ND	0

Method: Gas chromatography-mass spectrometry
Quantitation Limit: 1 ppb
TEF: toxic equivalency factor, Nisbet et al., 1992. TEQ: toxic equivalent quantity. TEQ = TEF x Concentration
ND: not detected. A not detected result is defined as below the limit of quantitation
Conc.:concentration

Dioxins and polychlorinated biphenyl residues. Polychlorinated biphenyls (PCBs) are monitored yearly. No PCBs have been detected above the limit of quantitation (0.01 ng/g) in three non-consecutive lots of Chlorella Powder and were therefore deemed not detected (Table 9).

Table 9. Polychlorinated biphenyl (PCB) in Chlorella Powder

PCB	LOQ	Chlorella Powder Lot No.		
		180903	181030	190124
2,4,4'-Trichlorobiphenyl (2,4,4'-TrCB #28)	0.01 ng/g	ND	ND	ND
2,2',5,5'-Tetrachlorobiphenyl (2,2',5,5'-TeCB #52)	0.01 ng/g	ND	ND	ND
2,2',4,5,5'-Pentachlorobiphenyl (2,2',4,5,5'-PeCB #101)	0.01 ng/g	ND	ND	ND
2,2'4,4',5,5'-Hexachlorobiphenyl (2,2',4,4',5,5'-HxCB #153)	0.01 ng/g	ND	ND	ND
2,2',3,4,4'5'-hexachlorobiphenyl (2,2',3,4,4',5'-HxCB #138)	0.01 ng/g	ND	ND	ND
2,2',3,4,4',5,5'-Heptachlorobiphenyl (2,2',3,4,4',5,5'-HpCB #180)	0.01 ng/g	ND	ND	ND

Method: Gas chromatograph-high resolution mass spectrometer, performed by JFRL (Japan Food Research Laboratories)
LOQ: limit of quantitation
ND: not detected. A not detected result is defined as below the limit of quantitation

Radioactivity monitoring. Radioactive contaminants are monitored every six months. No radioactive isotopes were detected in three lots of Chlorella Powder (Table 10).

Table 10. Radioactive Isotopes Screened in Chlorella Powder

Isotope	Chlorella Powder Lot No.		
	170112	170725	180129
Iodine-131 (LOD Bq/kg)	ND (0.70)	ND (0.64)	ND (0.62)
Cesium-134 (LOD Bq/kg)	ND (0.82)	ND (0.99)	ND (0.88)
Cesium-137 (LOD Bq/kg)	ND (0.86)	ND (0.96)	ND (0.90)
LOD: limit of detection ND: not detected. Method: Germanium semiconductor detector			

b. Chlorella Micro Powder

Residual pesticides. Three lots of Chlorella Micro Powder were subjected to a pesticide screen (Table 11). No pesticides were found above the limit of quantitation in any of the screened lots and were therefore deemed not detected, Table 11. Chlorella Industry Co., Ltd. performs the residual pesticide screen on Chlorella Micro Powder annually.

Table 11. Pesticides Screened in Three Lots of Chlorella Micro Powder

Not Detected Pesticides, as screened by GC-MS/MS		
1,1-Dichloro-2,2-Bis(4-ethylphenyl) ethane	2-(1-Naphthyl) acetamide	2,4-DB
2-Phenylphenol	Acetochlor	Acrinathrin
Alachlor	Aldrin/dieldrin	Ametryn
Atrazine	Azaconazole	Benalaxyl
Bendiocarb	Benfluralin	Benoxacor
BHC	Bifenox	Bifenthrin
Bioresmethrin	Bitertanol	Bromacil
Bromobutide	Bromophos ethyl	Bromopropylate
Bupirimate	Butafenacil	Butamifos
Cadusafos	Cafenstrole	Captan
Carfentrazone ethyl	Chinomethionat	Chlorbenside
Chlorbufam	Chlordane	Chlorethoxyphos
Chlorfenapyr	Chlorfenson	Chlorfenvinphos
Chlorobenzilate	Chloroneb	Chlorpropham
Chlorpyrifos	Chlorpyrifos methyl	Chlorthal dimethyl
Chlozolinate	Cinidon ethyl	Cinmethylin
Clodinafop propargyl	Clomazone	Clomeprop
Cloquintocet mexyl	Cyanazine	Cyanophos
Cyclosulfamuron	Cycloxydim	Cyfluthrin
Cyhalofop butyl	Cyhalothrin	Cypermethrin
Cyproconazole	DCIP	Deltamethrin/tralomethrin
Demeton-S-methyl	Desmedipham	Diazinon
Dichlobenil	Dichlofenthion	Dichlofluanid
Diclocymet	Diclofop methyl	Diclomezine
Dicloran	Dicofol	Dicrotophos
Diethofencarb	Difenoconazole	Difenzoquat
Diflufenican	Dimepiperate	Dimethametryn

Table 11. Pesticides Screened in Three Lots of Chlorella Micro Powder		
Dimethenamid	Dimethipin	Dimethoate
Diniconazole	Dioxathion	Disulfoton
Endosulfan	Endrin	EPN
EPTC	Esprocarb	Ethalfluralin
Ethiofencarb	Ethion	Ethofumesate
Ethoprophos	Etobenzanid	Etofenprox
Etoxazole	Etridiazole	Etrimfos
Fenamidone	Fenamiphos	Fenarimol
Fenbuconazole	Fenchlorphos	Fenitrothion
Fenoxycarb	Fenpropathrin	Fenpropimorph
Fensulfothion	Fenthion	Fenvalerate
Flamprop methyl	Fluacrypyrim	Flucythrinate
Fludioxonil	Flufenpyr ethyl	Flumioxazin
Fluquinconazole	Fluridone	Flusilazole
Flutolanil	Fluvalinate	Folpet
Formothion	Fthalide	Halfenprox
Heptachlor	Hexachlorobenzene	Hexaconazole
Hexazinone	Imazamethaben methyl ester	Imazaquin
Imibenconazole	Isazophos	Isocarbophos
Isofenphos	Isoprothiolane	Isoxathion
Kresoxim methyl	Lactofen	Lenacil
Malathion	Mecarbam	Mefenpyr diethyl
Mepronil	Metalaxyl mefenoxam	Metconazole
Methabenzthiazuron	Methacrifos	Methidathion
Methoprene	Methoxychlor	Metolachlor
Metominostrobin	Metribuzin	Mevinphos
Molinate	Monocrotophos	Monolinuron
Myclobutanil	Napropamide	Nicotine
Norflurazon	Oxadixyl	Oxpoconazole fumarate
Oxyfluorfen	Paclobutrazol	Parathion
Parathion methyl	Penconazole	Pendimethalin
Pentoxazone	Permethrin	Phenothrin
Phenthoate	Phorate	Phosalone
Phosphamidon	Picolinafen	Piperonyl butoxide
Pirimiphos methyl	Pretilachlor	Prochloraz
Procymidone	Profenofos	Prohydrojasmon
Prometryn	Propachlor	Propanil
Propaphos	Propargite	Propazine
Propiconazole	Propoxur	Propyzamide
Prothiofos	Pyraclofos	Pyraflufen ethyl
Pyrazophos	Pyrethrins	Pyridaben
Pyridafenthion	Pyrifenox	Pyrimethanil
Pyrimidifen	Pyriproxyfen	Quinalphos
Quinoclamine	Quinoxyfen	Quintozene
Resmethrin	Simazine	Simetryn
Spirodiclofen	Tebuconazole	Tebufenpyrad
Tecnazene	Tefluthrin	Tepraloxydim
Terbacil	Terbufos	Terbutryn
Tetrachlorvinphos	Tetraconazole	Tetradifon
Thifluzamide	Thiobencarb	Thiometon
Tiadinil	Tolclofos methyl	Tolfenpyrad
Tolylfluanid	Triadimefon	Triadimenol

Table 11. Pesticides Screened in Three Lots of Chlorella Micro Powder		
Triallate	Triazophos	Tribuphos
Trichlamide	Trifluralin	Uniconazole p
Vamidothion	Vinclozolin	XMC
Zoxamide	-	-
Not Detected Pesticides, as screened by HPLC-MS/MS		
Acetamiprid	Alanycarb	Anilazine
Anilofos	Aramite	Azafenidin
Azimsulfuron	Azoxystrobin	Bensulfuron methyl
Bensulide	Benthiavalicarb isopropyl	Bispyribac sodium
Boscalid	Buprofezin	Carbaryl
Carpropamid	Chlorantraniliprole	Chloridazon
Chlorimuron ethyl	Chlorsulfuron	Chromafenozide
Clofencet	Clofentezine	Cloransulam methyl
Clothianidin	Cumyluron	Cyazofamid
Cycloate	Cycloprothrin	Cyenopyrafen
Cyflufenamid	Cyprodinil	Diallate
Diflubenzuron	Dimethirimol	Dimethomorph
Diuron	Epoxiconazole	Ethametsulfuron methyl
Ethoxysulfuron	Ethychlozate	Famoxadone
Fenhexamid	Fenobucarb	Fenpyroximate
Flazasulfuron	Florasulam	Flufenoxuron
Flumetsulam	Fluometuron	Fluopicolide
Flutriafol	Fosthiazate	Halosulfuron methyl
Hexythiazox	Imazosulfuron	Imicyafos
Imidacloprid	Indoxacarb	Iodosulfuron methyl
Iprovalicarb	Isoprocarb	Isouron
Linuron	Mandipropamid	Mesosulfuron methyl
Metamitron	Methoxyfenozide	Metosulam
Metsufuron methyl	Naptalam	Nicosulfuron
Oxamyl	Oxaziclomefone	Oxycarboxin
Oxydemeton methyl	Pencycuron	Penoxsulam
Phenmedipham	Phosmet	Phoxim
Pirimicarb	Propaquizafop	Pyraclonil
Pyraclostrobin	Pyrazolynate	Pyrazosulfuron ethyl
Pyrazoxyfen	Pyriftalid	Rimsulfuron
Silafluofen	Simeconazole	Sulfosulfuron
Sulfotep	Sulprofos	Tebufenozide
Tebupirimfos	Tebuthiuron	Thiacloprid
Thiamethoxam	Thifensulfuron methyl	Tolyfloxysulfuron
Tralkoxydim	Tribenuron methyl	Tricyclazole
Triflumuron	Triflusulfuron methyl	

Lots analyzed: 160708, 170728, 180705
GC-MS/MS: gas chromatograph-mass spectrometry
HPLC-MS/MS: High-performance liquid chromatography-mass spectrometry
ND: not detected. A not detected result is defined as below the limit of quantitation

Aflatoxins. Three lots of Chlorella Micro Powder were screened for Aflatoxins B_1, B_2, G_1, and G_2 with a limit of quantitation of 1.0 µg/kg (1 ppb). No aflatoxins were found above the limit of quantitation in any of the screened lots and were therefore deemed not detected (Table 12). Chlorella Industry Co., Ltd. performs the aflatoxin screen on Chlorella Micro powder every six months.

Table 12. Aflatoxins Screened in Chlorella Micro Powder

Aflatoxin	Chlorella Micro Powder Lot No.		
	150725	160708	170728
Aflatoxin B_1	ND	ND	ND
Aflatoxin B_2	ND	ND	ND
Aflatoxin G_1	ND	ND	ND
Aflatoxin G_2	ND	ND	ND
Method: High performance liquid chromatography. Quantitation limit is 1.0 µg/kg (1 ppb). ND: not detected. A not detected result is defined as below the limit of quantitation			

Microcystin residues. Three lots of Chlorella Micro Powder were screened for Microcystin LR, RR, and YR with a limit of quantitation of 0.1 ppm. No microcystins were found above the limit of quantitation in any of the screened lots and were therefore deemed not detected (Table 13). Chlorella Industry Co., Ltd. performs the microcystin screen on Chlorella Micro Powder annually.

Table 13. Microcystin Screening in Chlorella Micro Powder

Microcystin	Chlorella Micro Powder Lot No.		
	150725	160708	170728
Microcystin LR	ND	ND	ND
Microcystin RR	ND	ND	ND
Microcystin YR	ND	ND	ND
Method: Liquid chromatography-mass spectrometry. The quantitation limit is 0.1 ppm. ND: not detected. A not detected result is defined as below the limit of quantitation			

Polycyclic aromatic hydrocarbon residues. Three lots of Chlorella Micro Powder were analyzed for PAH content by gas chromatography coupled with mass spectrometry, with a limit of quantitation (LOQ) of 1 part per billion (ppb). Benzo[b]fluoranthene and chrysene were detected in one lot at the LOQ (Table 14). The no significant risk levels (NSRLs) established by the California Environmental Protection Agency for benzo[b]fluoranthene and chrysene are 0.096 µg/day and 0.35 µg/day, respectively (California Environmental Protection Agency 2004). Assuming the 90th percentile user would consume 870 mg/day of Chlorella Powder and benzo[b]fluoranthene and chrysene are present at 1 ppb, the 90th percentile user would be exposed to 0.00087 µg/day; therefore, the presence of these PAHs is not at a level that approaches the NSRLs for these PAHs. The presence of PAHs is monitored annually.

Table 14. Polycyclic Aromatic Hydrocarbons in Chlorella Micro Powder

Residue	Toxic Equivalency Factor (TEF)	Chlorella Micro Powder Lot No.					
		150725		160708		170728	
		Conc.	TEQ	Conc.	TEQ	Conc.	TEQ
Benzo[a]pyrene	1	ND	0	ND	0	ND	0
Benzo[b]fluoranthene	0.1	ND	0	ND	0	1 ppb	0.1 ppb
Benz[a]anthracene	0.1	ND	0	ND	0	ND	0
Chrysene	0.01	ND	0	ND	0	1 ppb	0.01 ppb

Method: Gas chromatography-mass spectrometry
Quantitation Limit: 1 ppb
TEF: toxic equivalency factor, Nisbet et al., 1992
TEQ: toxic equivalent quantity. TEQ = TEF x Concentration
ND: not detected. A not detected result is defined as below the limit of quantitation

Dioxins and polychlorinated biphenyl residues. Polychlorinated biphenyls (PCBs) are monitored annually. To document that the PCBs are not present in the Chlorella Micro Powder, Chlorella Industries analyzed three lots by gas chromatograph-high resolution mass spectrometry with a limit of quantitation of 0.01 ng/g (ppb). Although 2,4,4'-Trichlorobiphenyl was detected in one lot of Chlorella Micro Powder at the limit of quantitation, no other PCBs were detected above the limit of quantitation in the same lot and were therefore deemed not detected (Table 15). Additionally, no PCBs were detected in the other two lots that were tested.

Table 15. Polychlorinated Biphenyl in Chlorella Micro Powder

Polychlorinated Biphenyl	Chlorella Micro Powder Lot No.		
	180914	181119	190129
2,4,4'-Trichlorobiphenyl (2,4,4'-TrCB #28)	0.01	ND	ND
2,2',5,5'-Tetrachlorobiphenyl (2,2',5,5'-TeCB #52)	ND	ND	ND
2,2',4,5,5'-Pentachlorobiphenyl (2,2',4,5,5'-PeCB #101)	ND	ND	ND
2,2'4,4',5,5'-Hexachlorobiphenyl (2,2',4,4',5,5'-HxCB #158)	ND	ND	ND
2,2',3,4,4'5'-hexachlorobiphenyl (2,2',3,4,4',5'-HxCB #138)	ND	ND	ND
2,2',3,4,4',5,5'-Heptachlorobiphenyl (2,2',3,4,4',5,5'-HpCB #180)	ND	ND	ND

Method: Gas chromatograph-high resolution mass spectrometer, testing performed at JFRL (Japan Food Research Laboratories)
Limit of quantitation = 0.01 ng/g.
ND: not detected. A not detected result is defined as below the limit of quantitation

Radioactivity monitoring. Radioactive contaminants are monitored every six months. To demonstrate that the Chlorella Micro Powder does not contain radioactivity, the amount of radioactivity in three lots of Chlorella Micro Powder was determined using Germanium semiconductor detector. No radioactive isotopes were detected in Chlorella Micro Powder (Table 16).

Table 16. Radioactive Isotopes Screened in Chlorella Micro Powder			
Isotope	**Chlorella Micro Powder Lot No.**		
	170210	**170728**	**180221**
Iodine-131 (LOD Bq/kg)	ND (0.82)	ND (0.90)	ND (0.75)
Cesium-134 (LOD Bq/kg)	ND (0.99)	ND (0.92)	ND (0.90)
Cesium-137 (LOD Bq/kg)	ND (0.92)	ND (0.98)	ND (0.86)
LOD: limit of detection ND: not detected. Method: Germanium semiconductor detector			

F. STABILITY OF CHLORELLA POWDER

1. Genotypic Stability

The *18S* rRNA sequence obtained from spray-dried CK-22, the intermediate product is compared to the *18S* rRNA sequence of the original stock and seed stocks on an annual basis. Sequencing results from three individual lots indicate that there is 100% sequence similarity between the *18S* rRNA sequence obtained from three lots of Chlorella Powder and the original/seed stock and *C. sorokiniana*.

2. Stability of Chlorella Powder and Chlorella Micro Powder

To determine the shelf life of the Chlorella Powder and Chlorella Micro Powder products, Chlorella Industries monitored the appearance, moisture, chlorophyll, vitamin B_2, protein, iron, omega-3 fatty acid, aerobic plate count, and coliform content of Chlorella Powder (Table 17) and Chlorella Micro Powder (Table 18) under warehouse storage conditions (between 1.8^{o}C and 30^{o}C, 20-90% humidity) up to 50 months. The methods used for these parameters are the same as those described in the product specification tables (Table 3 and Table 4). Both Chlorella Powder and Chlorella Micro Powder complied with product specifications for at least 4 years, supporting the current shelf life of 3 years.

Table 17. Chlorella Powder Stability Study Results							
Parameter	**Specification**	**Lot No. 140616**		**Lot No. 140626**		**Lot No. 140717**	
		Months		**Months**		**Months**	
		0	**50**	**0**	**49**	**0**	**48**
Appearance	Green powder	normal	normal	normal	normal	normal	normal
Moisture (%)	<7.0	4.9	4.7	4.6	4.6	5.9	5.4
Chlorophyll (%)	≥2.0	2.9	2.6	2.8	2.6	2.9	2.6
Vitamin B_2 (mg/ 100 g)	≥4.0	5.9	5.8	5.7	5.9	5.9	5.8
Protein (%)	≥55.0	62.4	62.1	62.7	62.4	63.5	64.0
Iron (mg/100 g)	≥40-100	52	54	55	54	61	61
Omega 3 Fatty Acids (%)	≥1.00	1.65	1.53	1.82	1.75	1.66	1.63
Aerobic Plate Count (cfu/g)	<3000	100	100	100	100	100	100
Coliforms (cfu/g)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Neg.: negative							

Table 18. Chlorella Micro Powder Stability Study Results

Parameter	Specification	Lot No. 140510 Months		Lot No. 140529 Months		Lot No. 140703 Months	
		0	49	0	48	0	48
Appearance	Light green powder	normal	normal	normal	normal	normal	normal
Moisture (%)	<7.0	4.5	4.1	4.8	4.2	4.5	4.3
Chlorophyll (%)	≥2.0	3.0	2.7	3.1	2.7	3.0	2.6
Vitamin B_2 (mg/ 100 g)	≥4.0	5.8	5.7	5.7	5.7	5.8	5.7
Protein (%)	≥55.0	62.1	62.9	62.7	63.3	62.4	62.7
Iron (mg/100 g)	≥40-100	52	51	55	54	56	54
Omega 3 Fatty Acids (%)	≥1.00	1.69	1.69	1.65	1.51	1.56	1.47
Aerobic Plate Count (cfu/g)	<3000	100	100	200	100	100	100
Coliforms (cfu/g)	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Neg.: negative

III. DIETARY EXPOSURE

A. INTENDED EFFECT

Chlorella Powder and Chlorella Micro Powder will be added to food as a source of macronutrients.

B. HISTORY OF USE

Commercial production of *Chlorella* spp.-based products occurs in Japan, Taiwan, Korea, and Indonesia. Chlorella products (powders and oils) are widely consumed throughout the world, particularly in Asian counties as supplements, food additives, and pharmaceutical additives. In the United States, a flour derived from *C. protothecoides* strain S106 containing 40-75% protein (based on Kjeldahl method with a conversion factor of 6.25) is GRAS for use as a source of protein in baked goods and mixes, breakfast cereals, meal replacements, cheeses, milk products, dairy and nondairy products, egg products, fish products, plant protein products, grain products and pastas, gravies and sauces, salad dressings, margarines, processed vegetables and vegetable juices, fresh and processed fruit juices, nonalcoholic beverages, gelatins and puddings, frozen dairy, soups, nut products, snack foods, and soft candy at a level intended to provide up to 5.5g/day (GRN 519). Additionally, a flour derived from *C. protothecoides* strain S106 containing 40-70% lipid is GRAS for use as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening (GRN 496). Algal flour is intended for use in baked goods, beverages and beverage bases, breakfast cereals, cheese, non-dairy, egg products, fats and oils (including salad dressing and mayonnaise), frozen dairy products, puddings and custards, meal replacements, milk and milk products, snack foods, vegetable and seafood soups, and sweet sauces at levels ranging from 5 to 120 g/kg food, and an oil derived from *C. protothecoides* strain S106 is GRAS as a substitute for vegetable oil for cooking at levels ranging from 1.8-26% in specified foods (GRN 469 and GRN 384, respectively). Chlorella products manufactured by Chlorella Industry Co., Ltd. are currently sold in Taiwan, South Africa, the Czech Republic, Malaysia, Singapore, and Israel.

C. INTENDED USE

Chlorella intends to add Chlorella Powder or Chlorella Micro Powder to selected foods in the U.S. food supply. The individual proposed food uses and use levels for Chlorella Powder or Chlorella Micro Powder employed in the current intake analysis are summarized in Table 19.

D. ESTIMATED DAILY INTAKE

1. Introduction

Spherix Consulting has completed an assessment of the consumption of both the Chlorella Powder and Chlorella Micro Powder, referred to collectively as Chlorella Powders, by the U.S. population resulting from the proposed uses of Chlorella Powders, Table 19. Estimates for the intake of Chlorella Powders were based on the proposed food uses and maximum use level in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2015-2016 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2018; USDA, 2018; U.S. Department of Agriculture, Agricultural Research Service, 2016.). Calculations for the mean and 90th percentile intakes were performed for all proposed food uses of Chlorella Powders combined. A fixed concentration of Chlorella Powders was used for each food, rather than a fixed mass to accommodate variations in serving size for the intended food products. In order to estimate intake of Chlorella Powders from the proposed uses in food, food codes representative of each approved use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS) for the corresponding biennial NHANES survey. The total expected daily intake was calculated using the amounts of Chlorella Powders listed in Table 19 for each food category. The intakes were reported for the following population groups:

- Newborns, ages 0-1 year
- Infants, ages 1-2 years
- children, ages 2 to 5 years
- children, ages 6 to 12 years
- teenagers, ages 13 to 19 years
- adults, ages 20 years and up
- total population (all age groups combined, excluding infants of 0-2 years)

Table 19. Summary of Proposed Food Use and Estimated Intake for Chlorella Powders

Food Category	Proposed Food Use	Use Level (mg/g)	Serving Size (g/serving)	Estimated Intake (mg/serving)
Baked Goods	Yeast breads	10	50	500
	Doughnuts	5	30	150
Snack Food	Nutrition bars	5	100	500
Drinks	Protein and nutritional powders	10	50	500
Grains	Pasta	10	50	500
	Noodles	10	65	650

2. Food Consumption Survey Data

a. Survey Description

The most recent National Health and Nutrition Examination Surveys (NHANES) for the years 2015-2016 are available for public use. NHANES are conducted as a continuous, annual survey, and are released in 2-year cycles. In each cycle, approximately 10,000 people across the U.S. completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Hayes et al., 2014). Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2015-2016 survey, these data were used to generate estimates for the current intake analysis.

The NHANES provide the most appropriate data for evaluating food use and food consumption patterns in the United States, containing 2 years of data on individuals selected via stratified multistage probability sample of civilian non-institutionalized population of the U.S. NHANES survey data were collected from individuals and households via 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person in the Mobile Examination Center (MEC), and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For example, in the 2009-2010 NHANES, there were 13,272 persons selected; of these 10,253 were considered respondents to the MEC examination and data collection. 9754 of the MEC respondents provided complete dietary intakes for Day 1 and of those providing the Day 1 data, 8,405 provided complete dietary intakes for Day 2. The release data do not necessarily include all the questions asked in a section. Data items may have been removed due to confidentiality, quality, or other considerations. For this reason, it is possible that a dataset does not completely match all the questions asked in a questionnaire section. Each data file has been edited to include only those sample persons eligible for that particular section or component, so the numbers vary.

In addition to collecting information on the types and quantities of foods being consumed, the NHANES surveys collect socioeconomic, physiological, and demographic

information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population.

Sample weights are incorporated with NHANES surveys to compensate for the potential under representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2016; USDA, 2012).

b. Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer in Octave and used to generate estimates for the intake of Chlorella Powders by the U.S. population. Estimates for the daily intake of Chlorella Powders represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights in order to provide representative intakes for the entire U.S. population. "All-user" intake refers to the estimated intake of Chlorella Powders by those individuals consuming food products containing Chlorella Powders. Individuals were considered users if they consumed 1 or more food products containing Chlorella Powders on either Day 1 or Day 2 of the survey.

3. Food Survey Results

The estimated "all-user" total intakes of Chlorella Powders from all proposed food uses of Chlorella Powders in the U.S. by population group is summarized in Table 20. Table 20 describes the "all-user" expected daily intake by age group in servings of 287 selected foods supplemented with the described levels of Chlorella Powders in the United States.

Table 20. Estimated "All-user" Daily Intake (EDI) of Chlorella Powders in Targeted Foods by Population Group (2015-2016 NHANES Data)

Population Group	N users	N population	% Users	Mean mass (kg)	Mean EDI (g)	90th % EDI (g)	Mean EDI (g/kg)	90th % EDI (g/kg)
ages 2-5	277	915	30.3	16.9	0.37	0.61	0.022	0.036
ages 6-12	493	1505	32.8	36.6	0.49	0.82	0.013	0.022
ages 13-19	405	1143	35.4	67.4	0.54	0.96	0.0080	0.014
ages 20 and up	2372	5748	41.3	80.0	0.53	0.88	0.0067	0.011
ages 2 and up	3547	9311	38.1	67.0	0.52	0.87	0.0078	0.013

4. Conclusions

In summary, the mean intakes of Chlorella Powders by the all Chlorella Powders consumers ages 2 and up ("all-user") from all proposed food uses were estimated to be 522 mg/person/day or 7.8 mg/kg body weight/day from the added Chlorella Powders of the proposed servings/day. The heavy consumer (90^{th} percentile all-user) intakes of Chlorella Powders from all proposed food uses were estimated to be 870 mg/person/day or 13.0 mg/kg body weight/day from the added Chlorella Powders of the proposed servings/day.

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The safety of Chlorella Powder (and Chlorella Micro Powder, as it is generated from the Chlorella Powder product) is supported by a pivotal published 90-day toxicology study in rats, which resulted in a No Observed Adverse Effect Level (NOAEL) of at least 5.94 g/kg body weight/day, the highest dose tested (Himuro et al., 2014). An Acceptable Daily Intake (ADI) of 59.4 mg/kg/day (3.6 g/day for a 60 kg person) is calculated utilizing a 100-fold safety factor. Additional published pivotal safety information is provided in an Umu assay, an acute oral toxicity study, and a 28-day repeated oral toxicity study (Himuro et al., 2014). The results of these studies found that hot water and ethanol extracts of Chlorella Powder are not genotoxic. Chlorella Powder has an LD_{50} of greater than 5000 mg/kg body weight and does not result in mortality or treatment-related adverse effects in rats fed up to 10% Chlorella Powder in the diet, the highest dose used. The safety of intake is also supported by corroborating published clinical studies of *Chlorella* spp. ingestion with no adverse events reported. *Chlorella* spp. have not been reported to produce marine toxins and finished lots of Chlorella Powder and Chlorella Micro Powder do not contain these toxins. Furthermore, algal flours and oils from other *Chlorella* spp. have been notified as GRAS (GRN 384, 569, and 519).

Based on these data, Chlorella Industries concludes that there is reasonable certainty of no harm from the ingestion of Chlorella Powder/Chlorella Micro Powder in accordance with the intended uses and use levels defined in Section III and is therefore GRAS.

A. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

The major constituents of Chlorella Powder, the spray-dried biomass of CK-22, are normal components of the diet and are anticipated to be digested and metabolized in pathways similar to those occurring with the ingestion of other plants and microalgae.

B. GENOTOXICITY STUDIES

1. Summary

As assessed by Umu assay, hot water and ethanol extracts of Chlorella Powder were found to not be genotoxic. Two corroborative studies using the Ames assay and a chromosomal aberration test on the dried biomass of *Chlorella protothecoides* are described in published reports (Szabo et al., 2012 and Szabo et al., 2013) as well as in two GRAS notifications (GRN 469 and GRN 519). These studies reported that the dried biomass of *C. protothecoides* is not mutagenic or genotoxic, supporting the absence of genotoxicity of Chlorella Powder.

2. Umu Assay for Chlorella Powder

Chlorella Industries Co., Ltd conducted a genotoxicity test on ethanol and hot water Chlorella Powder extracts using the Umu assay (Himuro et al., 2014). The *Salmonella Typhimurium* TA 1535 gene *umuC* is induced following the SOS response, a global response to DNA damage originally described in bacteria (Oda et al., 1985). The Umu assay utilizes the *umuC* gene fused to the lacZ operon to quantify DNA damage through the expression of β-galactosidase. Importantly, studies have shown that the results from the Umu assay are statistically equivalent to the Ames test (McDaniels et al., 1990) and similar conclusions regarding carcinogens in animals may be reached (Reifferscheid et al., 1996). The Umu test can detect genotoxicity of substances regardless of the type of DNA damage but is not ideal for detecting genotoxicity of anthracyclines (Yasunaga et al., 2006). A result is considered positive if it is 2x or greater than the β-galactosidase activity of the negative control.

Compared to the negative control (10% dimethyl sulfoxide, DMSO), ethanol extracts of Chlorella Powder incubated both with and without S9 did not increase the activity of β-galactosidase (Table 21). In contrast, hot water extracts of Chlorella Powder increased the activity of β-galactosidase 1.5-fold, although this increased activity was still less than the positive controls, 2-aminoanthracene (2-AA) and furylfuramide (AF-2). In this test, values that are more than twice the negative control are considered to indicate genotoxicity. Therefore, both the hot water and ethanol extracts of Chlorella Powder were not genotoxic as assessed by Umu assay. Although these extracts do not exactly represent the powder, the results confirm that ethanol and hot water extractable substances from CK-22 are not genotoxic under the conditions of the assay.

a. Methods

Samples of Chlorella Powder extracted in either ethanol or hot water were used for the Umu Assay. To generate the ethanol extract, 500 mg of Chlorella Powder was suspended in 10 mL 80% ethanol, and then homogenized for one minute at 10,000 rotations per minute (RPM). It was then centrifuged for 20 minutes at 3000 RPM and the supernatant fraction was diluted to 50 mL with 10% DMSO (approximately 5-fold dilution). To generate the hot water extract, 500 mg of Chlorella Powder was suspended in 25 mL of distilled water and heated to 100ºC for 10 minutes. After cooling to room temperature, it was then centrifuged for 20 minutes at 3000 RPM and the supernatant fraction was diluted to 50 mL with distilled water (approximately 2-fold dilution). A comparison between samples treated with or without S9 from rat liver was included. All samples were diluted in 10% DMSO for absorbance measurements.

An Umulac AT Umu test kit (Protein Purify) was used to determine genotoxicity. The test was carried out in accordance with kit instructions, and β-galactosidase activity was measured using a plate reader at 630 nm. The positive controls for this assay were 2-AA and AF-2. AF-2 was used to produce a positive result in the presence and absence of S9. 2-AA was used because it becomes mutagenic only when metabolically activated. A positive result is defined as a sample with more than twice the absorbance of the control solvent (10% DMSO).

b. Results

The positive control 2-AA produced 3.5 times more β-galactosidase activity than the negative control at 0.3 µg/mL in the presence of S9 (Table 21). The positive control AF2 produced at least 2x more β-galactosidase activity than the negative control at 0.1 µg/mL and 0.3 µg/mL in the presence and absence of S9. None of the Chlorella Powder samples demonstrated more than 2x the β-galactosidase activity of the negative control in the presence or absence of S9. Mutagenicity was not observed in ethanol or hot water extracts of Chlorella Powder.

Table 21. Results of Umu Assay with Chlorella Powder

		S9	β-galactosidase Activity (A_{630})	Sample/ Control †	Mutagenicity Result
10% DMSO (Negative Control)		-	0.346	1.0	Negative
		+	0.442	1.0	Negative
2-AA (Positive Control)	0.033 µg/mL	-	0.412	1.2	Negative
		+	0.534	1.2	Negative
	0.1 µg/mL	-	0.408	1.2	Negative
		+	0.760	1.7	Negative
	0.3 µg/mL	-	0.424	1.2	Negative
		+	1.535	3.5	Positive
AF2 (Positive Control)	0.033 µg/mL	-	0.579	1.7	Negative
		+	0.679	1.5	Negative
	0.1 µg/mL	-	0.873	2.5	Positive
		+	0.950	2.1	Positive
	0.3 µg/mL	-	1.207	3.5	Positive
		+	1.376	3.1	Positive
Chlorella Powder ethanol extract		-	0.301	0.9	Negative
		+	0.389	0.9	Negative
Chlorella Powder hot water extract		-	0.521	1.5	Negative
		+	0.564	1.3	Negative

† All samples are normalized to the negative control. A 2-fold increase over the negative control was deemed positive.
DMSO: Dimethyl sulfoxide
2-AA: 2-aminoanthracene
AF2: furylfuramide

3. Corroborative Genotoxicity Studies in *Chlorella protothecoides*

The corroborative genotoxicity studies that also support the safe use of CK-22 as a food ingredient include Organisation for Economic Co-operation and Development (OECD)-compliant Ames and chromosome aberration assays conducted on a high lipid whole algalin flour derived from *C. protothecoides,* and OECD-compliant Ames and chromosome aberration assays conducted on a whole algalin protein derived from *C. protothecoides* (Szabo et al., 2012; Szabo et al., 2013). Although the test articles used in these studies are not identical to Chlorella Powder and/or Chlorella Micro Powder, the results suggest that *Chlorella* as a genus does not appear to produce genotoxic metabolites.

In an OECD-compliant Ames assay, Szabo et al. (2012) evaluated the genotoxicity of a high lipid whole algalin flour derived from *C. protothecoides* using *S. typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* WP2uvrA cultured with and without S9 metabolic activation at doses up to 5000 µg/plate using the standard plate incorporation and pre-incubation methods. Cytotoxic effects of the test substance were observed in test strain TA1537 at 316 µg/plate without S9 metabolic activation and at 2500 µg/plate with S9 metabolic activation in the experiment using the plate incorporation method. In the experiment using the pre-incubation method, strains TA98 and TA100 also demonstrated cytotoxic effects at 316 µg/plate and higher without S9 metabolic activation. In the same experiment, strain TA1537 had an increase in revertant colonies at 10.0 µg/plate and higher without S9 metabolic activation and at 316 µg/plate and higher with S9 metabolic activation. Since these increases were not greater than twice the number of revertant colonies as the negative control and did not demonstrate a dose-response relationship, the authors concluded that the *C. protothecoides* high lipid algalin flour was not mutagenic.

In an OECD-compliant in vivo chromosome aberration assay, Szabo et al. (2012) administered the high lipid whole algalin flour derived from *C. protothecoides* blended with cottonseed oil via oral gavage at 2000 mg/kg body weight to 10 male and 10 female Naval Medical Research Institute (NMRI) mice. The vehicle control was administered to an equal number of mice of both sexes. The positive control was 40 mg/kg body weight cyclophosphamide in saline, administered by intraperitoneal (i.p.) injection to 5 male and 5 female mice. The mice were given 40 µg Colcemid® to arrest metaphase 24- and 48-hours post-treatment. Bone marrow cells were harvested and analyzed for cytogenic damage (breaks, fragments, deletion exchanges, chromosomal disintegrations, and gaps). Treatment with high lipid whole algalin flour from *C. protothecoides* did not significantly enhance the number of aberrant cells 24- or 48-hours post-dose, and therefore, did not induce cytotoxicity.

In an OECD-compliant Ames assay, Szabo et al. (2013) evaluated the genotoxicity of a whole algalin protein from *C. protothecoides* using *S. typhimurium* strains TA98, TA100,

TA1535, and TA1537, and *E. coli* WP2uvrA cultured with and without S9 metabolic activation at doses up to 5000 µg/plate using the standard plate incorporation and pre-incubation methods. Whole algalin protein-related cytotoxic effects appeared in test strain TA1537 at 2500 and 5000 µg/plate with S9 metabolic activation using the plate incorporation method, and at 2500 µg/plate without S9 metabolic activation in the experiment performed with the pre-incubation method. Although a reduction in the number of revertants in test strain TA1537 at the 316 µg/plate concentration in the preincubation method without S9 metabolic activation met the criteria for cytotoxicity, no dose-response relationship was evident, so the authors deemed the finding not biologically relevant. Since whole algalin protein did not induce a dose-dependent relationship in this assay and because no treatment yielded twice the number of colonies observed in the negative control, the authors concluded that whole algalin protein from *C. protothecoides* was not mutagenic.

In an OECD-compliant in vivo chromosome aberration assay, Szabo et al. (2013) administered whole algalin protein from *C. protothecoides* via oral gavage at 2000 mg/kg body weight to 10 male and 10 female NMRI mice. The vehicle control was administered to an equal number of mice of both sexes. The positive control was 40 mg/kg body weight cyclophosphamide in saline, administered by intraperitoneal injection to 5 male and 5 female mice. The mice were given 40 µg Colcemid® to arrest metaphase 24- and 48-hours post-treatment. Bone marrow cells were harvested and analyzed for cytogenic damage (breaks, fragments, deletion exchanges, chromosomal disintegrations, and gaps). Treatment with whole algalin protein did not significantly enhance the number of aberrant cells 24- or 48-hours post-dose. The mean mitotic index value for the 48-hour male test group was significantly lower than the negative control; however, the effect was determined to be due to biologically variability within the animals and it was not considered relevant by the authors. Whole algal protein from *C. protothecoides* was not clastogenic in this assay.

C. TOXICOLOGY STUDIES

1. Summary

The safety of Chlorella Powder has been determined in OECD-compliant rat acute toxicity, 28-day dietary toxicity, and 90-day dietary toxicity studies (Himuro et al., 2014; Himuro et al., 2017). Additional corroborative studies that support the safe use of Chlorella Powder and Micro Powder include 90-day toxicity studies performed with other *Chlorella* spp. products.

2. Pivotal Toxicology Studies

a. Acute Toxicity (Himuro et al., 2014)

i. Methods

Wistar rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The acute toxicity test was carried out according to OECD guideline 420 (OECD, 2001). Before the tests, all animals were acclimated for 7 days and had free access to water and pelleted rodent diet (CE-2 rodent feed from CLEA Japan, Inc., Tokyo, Japan). Animals were housed in individual cages (one rat/cage) with a 12-hour light cycle (6:00-18:00) at 23 ± 0.5C in 55 ± 5% relative humidity. The animals were cared for according to the NIH published guideline. The vehicle group was administered 2 mL saline by gavage, while the exposed groups were administered a single dose of 1000 mg/kg (low-dose), 2000 mg/kg (middle-dose), and 5000 mg/kg (high-dose) body weight of Chlorella Powder in 2 mL distilled water by gavage. All animals had free access to water and feed for 14 days.

Immediately after dosing, the animals were observed for toxicity signs, mortality, and morbidity at hours 1, 2, 3, and 4. They were then kept under observation for toxicity signs throughout the test period. Individual body weights were recorded on days 1, 4, 7, and 14, and water intake was measured by weighing the drinking bottles on days 1, 7, and 14. At the end of the administration, all rats were fasted for 16 hours, after which blood was collected from the abdominal aorta. Animals were terminated under pentobarbital anesthesia. Following termination, a thorough necropsy was performed on animals, and the following organs were weighed after dissection: heart, lungs, brain, liver, spleen, kidneys, adrenals, thymus, thyroid, testes, epididymides, seminal vesicle, ovary, and uterus.

Statistical analysis was carried out using Ekuseru-Toukei 2012 software (version 1.00, Social Survey Research Information Co., Ltd., Tokyo Japan). Variance in data for body weight, feed intake, water intake, hematology, serum biochemistry, and organ weight was checked for homogeneity by Bartlett’s procedure. When the data were homogeneous, a one-way analysis of variance (ANOVA) was applied. In the heterogeneous cases, the Kruskal-Wallis test was used. When statistically significant differences were found, Dunnett’s multiple comparison test was employed for comparison between control and Chlorella-administered groups.

ii. Results

No deaths were observed in any group. No mortality or adverse clinical signs were observed in any animal. Chlorella Powder-administered groups showed no significant differences in body weight compared to the control group (Figure 4). The absolute and relative (organ to body weight ratio) weight of all organs showed no statistically significant differences between control and treated groups (Table 22). In necropsy, no abnormalities were found in the Chlorella Powder treated groups.

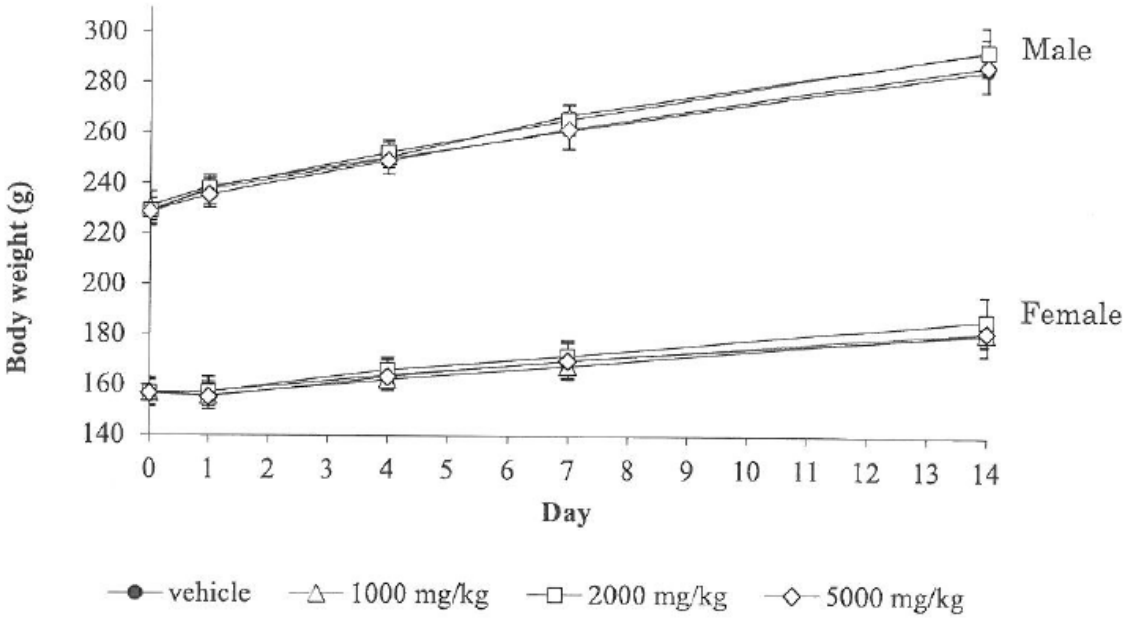

Figure 4. Body Weight of Wistar Rats Administered Chlorella Powder
Each data point represents the mean ± standard deviation, n = 5/sex/group. No significant differences in body weights were observed in either sex 14 days following Chlorella Powder administration (Himuro et al., 2014).

Table 22. Organ Weights in Rat Acute Toxicity Study (Himuro et al., 2014)								
Chlorella Powder (g/kg)	**Males (n = 5/group)**				**Females (n = 5/group)**			
	0	**1**	**2**	**5**	**0**	**1**	**2**	**5**
Brain (g)	2.0 ± 0.09	2.0 ± 0.1	2.0 ± 0.09	2.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.06	1.8 ± 0.04	1.8 ± 0.04
Thymus (g)	0.4 ± 0.07	0.4 ± 0.03	0.4 ± 0.02	0.5 ± 0.08	0.3 ± 0.05	0.3 ± 0.3	0.3 ± 0.02	0.4 ± 0.08
Heart (g)	0.8 ± 0.09	0.9 ± 0.05	09 ± 0.04	0.8 ± 0.04	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.01	0.5 ± 0.03
Lung (g)	1.1 ± 0.09	1.0 ± 0.03	3.6 ± 0.1	0.7 ± 0.07	0.7 ± 0.09	0.7 ± 0.08	0.7 ± 0.07	0.8 ± 0.06
Liver (g)	12.5 ± 0.4	12.0 ± 0.6	12.5 ± 0.7	12.0 ± 0.3	6.6 ± 0.6	6.7 ± 1.1	6.6 ± 0.5	6.5 ± 0.7
Spleen (g)	0.6 ± 0.04	0.6 ± 0.07	0.6 ± 0.04	0.6 ± 0.04	0.4 ± 0.05	0.4 ± 0.08	0.4 ± 0.01	0.4 ± 0.02
Kidney (g)	2.2 ± 0.09	2.3 ± 0.2	2.1 ± 0.2	2.2 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.04	1.4 ± 0.1
Adrenal gland (g)	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00
Testes and epididymis (g)	3.5 ± 0.1	3.6 ± 0.1	3.7 ± 0.08	3.7 ± 0.2	-	-	-	-
Seminal vesicle (g)	0.6 ± 0.02	0.7 ± 0.07	0.7 ± 0.03	0.6 ± 0.02	-	-	-	-
Prostate gland (g)	0.4 ± 0.08	0.5 ± 0.06	0.5 ± 0.08	0.5 ± 0.07	-	-	-	-
Ovaries, ovarian duct and uterus (g)	-	-	-	-	0.4 ± 0.07	0.4 ± 0.05	0.4 ± 0.07	0.4 ± 0.05
Mean ± Standard Deviation b.w.: body weight								

iii. Discussion

The toxicity of Chlorella Powder was assessed in an OECD-compliant acute oral toxicity study in 8-week old male and female Wistar rats. Twenty male and twenty female rats were randomly divided into four groups, each with 5 males and 5 females. Each group was administered one dose of 0, 1, 2, or 5 grams of Chlorella Powder in phosphate-buffered saline by gavage. Body weight and water consumption were monitored up to termination, 14 days post-treatment. No animals died as a result of Chlorella Powder administration and following the observation period, the animals were terminated and gross pathology was conducted. No observable differences or symptoms were noted and organ weights were not significantly different in the Chlorella Powder treated groups compared to the control group. There were also no differences in body weights among groups over the course of the study.

b. Repeated Oral Toxicity Studies

i. 28-day Dietary Toxicity Study in Rats (Himuro et al., 2014)

Methods. The 28-day dietary toxicity test was carried out according to OECD guideline 407 (OECD, 2008). Eighty Wistar rats (aged 6 weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were randomly divided (10 animals/sex/group). All rats were acclimated for 7 days prior to treatment. The rats had free access to water and pelleted rodent feed (CE-2, CLEA Japan, Inc., Tokyo, Japan) during the acclimation and test periods. The control group was fed rodent feed and separate treatment groups were fed the same rodent feed containing 2.5% (low-dose), 5% (middle-dose), and 10% (high-dose) Chlorella Powder for 28 days.

The animals were observed for toxicity signs, mortality, and morbidity twice a day during the test period. Individual body weights, feed and water intakes were measured on days 7, 14, 21, and 28. After observation of external appearance on the day following the last dose, blood was collected from the abdominal aorta under pentobarbital anesthesia after 16-hour fasting. Hematological parameters measured at Kurume Clinical Laboratories (Fukuoka, Japan) included white blood cell count (WBC), WBC differential counts (neutrophils, lympohycytes, monocytes, eosinophils, and basophils), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT). Serum biochemical parameters were also measured at Kurume Clinical Laboratories and included total protein (TP), albumin (ALB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMY), total bilirubin (T-BIL), creatinine (CRE), uric acid (UA), glucose (GLU), total cholesterol (TCH), non-esterified fatty acid (NEFA), triglyceride (TG), sodium (Na), potassium (K), chloride (Cl), and inorganic phosphorus (IP).

Following termination, a thorough necropsy was performed, and heart, lungs, brain, liver, spleen, kidneys, adrenals, thymus, thyroid, testes, epididymides, seminal vesicles, ovaries, and uterus were weighed after dissection. After macroscopic examination, a histopathological examination was performed on the collected organs from the control and high-dose groups of both sexes. All samples were fixed in 10% neutral buffer formalin and stained with hematoxylin and eosin.

Statistical analysis was carried out using Ekuseru-Toukei 2012 software (version 1.00, Social Survey Research Information Co., Ltd., Tokyo Japan). Variance for body weight, feed intake, water intake, hematology, serum biochemistry, and organ weight was assessed for homogeneity by Bartlett's method. When the data were homogeneous, a one-way analysis of variance (ANOVA) was applied. In the heterogeneous cases, the Kruskal-Wallis test was used. When statistically significant differences were found, Dunnett's multiple comparison test was employed for comparison between control and the Chlorella Powder administered groups.

Results. No mortality or adverse clinical signs were observed. The average daily Chlorella Powder intake in the 2.5, 5, and 10% groups were 2.12, 4.06, and 8.57 g/kg/day for males and 2.12, 4.21, and 8.62 g/kg/day for females, respectively. Body weight in the 10% (high-dose) female group showed a significant increase compared to the controls at the end of the test (Figure 5). Compared to the controls, daily feed intake was significantly increased in the 5 (middle-dose) and 10% (high-dose) females (Table 23). Daily water intake significantly increased in 10% (high-dose) males compared to the controls. The increases in body weight, water, and feed intake were within the normal range for Wistar rats throughout the test period. These findings were considered test-article related, but not adverse.

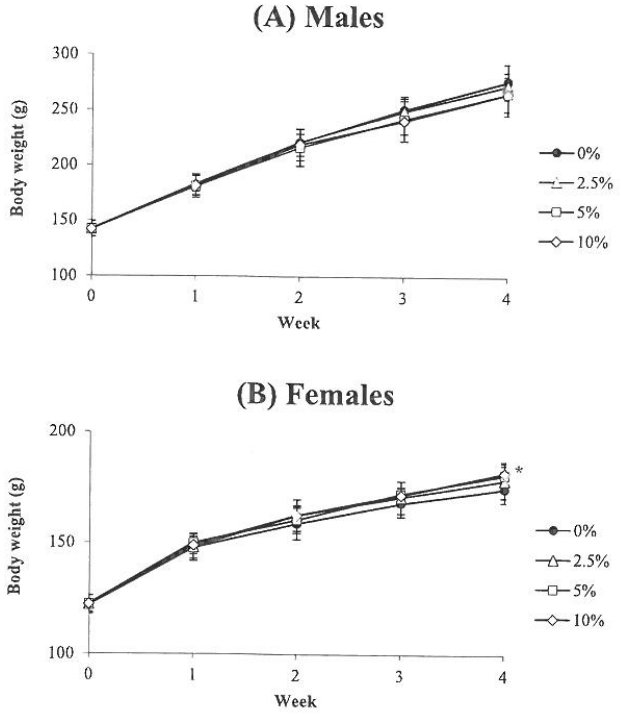

Figure 5. Body Weight Changes of Rats Given Chlorella Powder in the Feed for 28 Days
Male (A) and female (B) rats were administered 0, 2.5, 5, and 10% Chlorella Powder. *Significantly different from the control group at $p < 0.05$. Each point represents the mean ± standard deviation, n=10/group. (Himuro et al., 2014).

Table 23. Body Weight, Feed and Water Intake In 28 Day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2014)

	Males			
	% Chlorella Powder in Feed			
	0% (n = 10)	**2.5% (n = 10)**	**5% (n = 9)**	**10% (n = 10)**
Final body weight (g)	290.3 ± 22.9	288.1 ± 13.1	284.6 ± 28.7	287.5 ± 22.1
Feed intake (g/day)	18.3 ± 1.2	19.2 ± 1.1	17.7 ± 1.3	18.8 ± 1.0
Water intake (g/day)	28.4 ± 1.9	30.1 ± 2.1	29.2 ± 2.2	31.8 ± 1.9*
	Females			
	% Chlorella Powder in Feed			
	0% (n = 10)	**2.5% (n = 10)**	**5% (n = 10)**	**10% (n = 10)**
Final body weight (g)	180.6 ± 7.8	181.7 ± 8.0	186.4 ± 4.2	192.5 ± 6.0*
Feed intake (g/day)	13.2 ± 0.07	13.8 ± 0.5	13.9 ± 0.3*	14.2 ± 0.3**
Water intake (g/day)	22.7 ± 1.5	24.2 ± 0.6	24.2 ± 1.1	24.1 ± 1.0

Values are mean ±standard deviation.
* Significantly different from the control group at $p < 0.05$.
** Significantly different from the control group at $p < 0.01$.

One male rat in the 5% (low-dose) group died after receiving anesthesia. The fatality was connected to human error in anesthesia administration and was therefore not considered to be test article related. In females, there were no significant changes in hematological and serum biochemical parameters among all groups (Table 24 and 25). In males, significant increases of neutrophils and RBCs were observed in the 10% (high-dose) and 5% (middle-dose) groups, respectively. A significant decrease of serum ALP and a significant increase of serum K were observed in all male Chlorella Powder-administered groups. A significant increase of serum TCH and a significant decrease of serum Cl were observed in the 2.5% (low-dose) group. Importantly, none of the changes of neutrophils, RBC, ALP, K, TCH, and Cl levels in males were dose-dependent. Because these differences were not observed in females and lacked significant correlative changes in other parameters, they were not considered test-article related.

A significant decrease in serum ALP was observed in all Chlorella Powder-administered males, but the values remained within the range observed in normal controls (Roy et al., 2010). The significant decrease of serum Cl in 2.5% males was within the normal range in rats (Delaney et al., 2003).

Table 24. Hematology Results from 28 Day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2014)

Hematology Parameters	Males				Females			
	% Chlorella Powder in Feed				% Chlorella Powder in Feed			
	0% (n=10)	2.5% (n=10)	5% (n=9)	10% (n=10)	0% (n=10)	2.5% (n=10)	5% (n=10)	10% (n=10)
WBC ($x10^3/\mu L$)	3.3 ± 0.7	3.6 ± 0.8	3.4 ± 0.9	3.2 ± 0.7	3.0 ± 0.4	3.1 ± 0.6	2.7 ± 0.9	2.7 ± 0.8
Neutrophils (%)	20.4 ± 4.4	24.1 ± 5.4	26.4 ± 6.1	27.9 ± 6.7*	20.5 ± 3.5	20.4 ± 4.8	23.3 ± 3.9	23.2 ± 4.0
Lymphocytes (%)	77.0 ± 5.1	72.9 ± 5.7	70.6 ± 6.1	69.1 ± 6.7	76.8 ± 4.4	77.1 ± 5.0	73.4 ± 3.8	73.7 ± 4.6
Monocytes (%)	1.6 ± 0.9	1.9 ± 0.8	1.9 ± 0.8	1.9 ± 0.6	1.6 ± 0.9	1.3 ± 1.0	2.2 ± 0.8	1.8 ± 1.3
Eosinophils (%)	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.3	1.3 ± 0.4
Basophils (%)	0.03 ± 0.09	0.03 ± 0.09	0.08 ± 0.2	0.08 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
RBC ($x10^6/\mu L$)	9.2 ± 0.3	9.5 ± 0.3	9.8 ± 0.5*	9.3 ± 0.3	8.3 ± 0.3	8.4 ± 0.3	8.4 ± 0.3	8.6 ± 0.3
Hemoglobin (g/dL)	16.2 ± 0.5	16.6 ± 0.4	17.1 ± 1.1	16.5 ± 0.5	15.1 ± 0.8	15.4 ± 0.7	15.4 ± 0.6	15.7 ± 0.5
Hematocrit (%)	49.0 ± 1.8	50.3 ± 1.1	51.0 ± 1.7	49.0 ± 2.0	46.3 ± 1.7	47.4 ± 1.8	47.5 ± 1.7	48.4 ± 1.9
MCV (μm^3)	53.1 ± 1.2	53.2 ± 1.8	52.5 ± 2.3	52.5 ± 2.0	56.3 ± 0.5	56.3 ± 1.5	56.5 ± 1.0	56.3 ± 1.3
MCH (pg)	17.3 ± 0.5	17.7 ± 0.5	17.6 ± 0.5	17.6 ± 0.5	18.3 ± 0.5	18.3 ± 0.5	18.2 ± 0.4	18.0 ± 0.0
MCHC (%)	33.1 ± 0.9	33.0 ± 0.8	33.5 ± 1.9	33.5 ± 1.2	32.6 ± 0.9	32.7 ± 0.5	32.4 ± 0.7	32.5 ± 0.7
Platelets ($x10^5/mm^2$)	5.8 ± 0.7	5.9 ± 0.6	5.5 ± 0.7	5.8 ± 0.6	5.0 ± 0.6	5.0 ± 0.4	5.0 ± 0.4	4.9 ± 0.4

Values are mean ±standard deviation.
*Significantly different from the control group at $p < 0.05$.
Abbreviations: WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Table 25. Serum Biochemical Results from 28 Day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2014)

Serum Biochemistry Parameters	Males				Females			
	% Chlorella Powder in Feed				% Chlorella Powder in Feed			
	0% (n=10)	2.5% (n=10)	5% (n=9)	10% (n=10)	0% (n=10)	2.5% (n=10)	5% (n=10)	10% (n=10)
TP (g/dL)	6.2 ± 0.3	6.2 ± 0.2	6.0 ± 0.3	6.1 ± 0.3	5.5 ± 0.2	5.7 ± 0.4	5.5 ± 0.2	5.6 ± 0.5
ALB (g/dL)	4.7 ± 0.1	4.6 ± 0.2	4.4 ± 0.2	4.5 ± 0.2	4.2 ± 0.2	4.3 ± 0.2	4.2 ± 0.1	4.2 ± 0.3
LDH (IU/L)	3382.7 ± 820.0	3255.8 ± 407.1	3526.9 ± 449.7	3395.4 ± 696.0	3036.6 ± 478.6	3027.3 ± 313.9	2846.2 ± 361.8	2730.7 ± 459.8
AST (IU/L)	108.3 ± 31.8	1604.3 ± 14.1	171.0 ± 20.56	202.5 ± 90.4	160.3 ± 19.3	155.0 ± 14.5	157.0 ± 20.5	146.4 ± 19.4
ALT (IU/L)	48.1 ± 8.1	47.5 ± 5.8	42.7 ± 4.7	50.2 ± 10.9	41.1 ± 5.0	42.0 ± 6.7	40.4 ± 6.9	43.0 ± 10.1
ALP (IU/L)	933.7 ± 131.7	729.4 ± 61.4**	712.1 ± 74.0**	725.9 ± 75.2**	537.0 ± 71.7	547.0 ± 91.5	586.0 ± 94.0	535.5 ± 101.9
AMY (IU/L)	1472.5 ± 167.0	1704.0 ± 124.7	1671.6 ± 82.3	1736.1 ± 118.2	750.1 ± 139.5	794.2 ± 102.2	758.0 ± 114.8	797.1 ± 93.4
T-BIL (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ±0.0	0.1 ± 0.0	0.1 ± 0.0
CRE (mg/dL)	0.3 ± 0.03	0.3 ± 0.02	0.3 ± 0.1	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.06	0.3 ± 0.03	0.3 ± 0.02
UA (mg/dL)	2.7 ± 1.6	1.8 ± 0.8	1.8 ± 0.8	1.8 ± 0.8	1.9 ± 0.9	1.7 ± 0.6	1.5 ± 0.5	1.3 ± 0.6
GLU (mg/dL)	73.0 ± 22.8	91.5 ± 16.5	78.4 ± 10.0	78.4 ± 10.0	76.9 ± 25.3	85.9 ± 22.1	84.0 ± 6.5	74.2 ± 10.1
TCH (mg/dL)	50.8 ± 7.0	64.6 ± 12.8**	59.4 ± 8.8	59.4 ± 8.8	77.0 ± 9.3	77.9 ± 11.1	76.6 ± 9.5	80.7 ± 9.1
NEFA (mmol/L)	604.0 ± 112.8	711.8 ± 141.3	656.4 ± 94.8	656.4 ± 94.8	763.4 ± 198.3	678.2 ± 115.8	625.4 ± 48.6	628.6 ± 116.6
TG (mg/dL)	51.7 ± 27.7	91.7 ± 40.5	74.4 ± 23.1	74.4 ± 23.1	40.7 ± 14.7	33.0 ± 11.0	34.4 ± 8.0	42.2 ± 12.73
Na (mmol/L)	145.5 ± 1.3	144.9 ± 1.7	144.8 ± 2.0	144.8 ± 2.0	144.7 ± 0.9	144.3 ± 0.8	144.4 ± 0.7	145.3 ± 1.4
K (mmol/L)	4.6 ± 0.3	5.1 ± 0.3*	5.1 ± 0.4*	5.1 ± 0.4*	4.3 ± 0.2	4.3 ± 0.1	4.4 ± 0.3	4.3 ± 0.3
Cl (mmol/L)	100.6 ± 1.6	99.0 ± 1.3*	100.1 ± 1.5	100.1 ± 1.5	100.3 ± 1.3	100.4 ± 1.3	101.3 ± 1.6	100.3 ± 2.0
IP (mmol/L)	10.4 ± 1.2	10.2 ± 0.8	10.0 ± 1.4	10.0 ± 1.4	8.7 ± 1.3	8.3 ± 1.3	8.5 ± 1.0	9.7 ± 1.1

Values are mean ±standard deviation.
TP: total protein, ALB: albumin, LDH: lactate dehydrogenase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, AMY: amylase, T-BIL: total bilirubin, CRE: creatinine, UA: uric acid, GLU: glucose, TCH: total cholesterol, NEFA: non-esterified fatty acid, TG: triglyceride, Na: sodium, K: potassium, Cl: chloride, IP: inorganic phosphate.
*Significantly different from the control group at $p < 0.05$.
** Significantly different from the control group at $p < 0.01$.

One rat in the 5% (middle-dose) females showed hydrops in the ovary upon necropsy. Because it was incidental, it was not considered test article-related. There were no macroscopic abnormalities reported.

Absolute renal weights were significantly increased in 10% (high-dose) males and females (Table 26). Because renal weights of 10% (high-dose) males and females increased compared to the controls, histopathological examination of kidneys of the control and 10% (high-dose) groups in both sexes was performed. These histopathological examinations produced no remarkable findings. The absolute renal weights in 10% (high-dose) males and females were statistically significantly increased as compared to controls; however, since this finding was not dose dependent, it was not considered test article-related. Furthermore, CRE, UA, Na, K, Cl, and IP, as measures of renal function, were within the normal range, such that no abnormality of kidney functional parameters was found.

Table 26. Absolute Organ Weights from 28 Day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2014)

Organ	Males				Females			
	% Chlorella Powder in Feed				% Chlorella Powder in Feed			
	0% (n=10)	2.5% (n=10)	5% (n=9)	10% (n=10)	0% (n=10)	2.5% (n=10)	5% (n=10)	10% (n=10)
Body weight (g)	276.6 ± 15.9	272.8 ± 11.9	265.5 ± 19.4	265.6 ± 15.7	174.7 ± 6.0	178.5 ± 7.8	181.0 ± 4.4	181.9 ± 5.2
Heart (g)	0.8 ± 0.06	0.8 ± 0.05	0.8 ± 0.07	0.8 ± 0.05	0.6 ± 0.02	0.6 ± 0.03	0.6 ± 0.03	0.6 ± 0.03
Lung (g)	1.0 ± 0.2	1.0 ± 0.04	0.9 ± 0.08	1.0 ± 0.1	0.8 ± 0.05	0.8 ± 0.05	0.8 ± 0.07	0.8 ± 0.04
Brain (g)	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.07	2.0 ± 0.08	1.8 ± 0.08	1.8 ± 0.1	1.8 ± 0.08	1.9 ± 0.08
Liver (g)	10.4 ± 1.0	10.4 ± 0.8	10.0 ± 1.6	10.3 ± 0.9	5.8 ± 0.2	6.1 ± 0.3	6.1 ± 0.4	6.2 ± 0.3
Spleen (g)	0.7 ± 0.08	0.7 ± 0.05	0.7 ± 0.09	0.7 ± 0.07	0.5 ± 0.04	0.5 ± 0.03	0.5 ± 0.03	0.5 ± 0.04
Kidneys (g)	2.5 ± 0.2	2.6 ± 0.2	2.5 ± 0.2	2.7 ± 0.3*	1.5 ± 0.09	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1*
Adrenals (g)	53.6 ± 9.5	55.7 ± 13.6	45.9 ± 6.8	53.8 ± 10.2	69.1 ± 3.6	66.3 ± 6.2	67.4 ± 6.2	68.7 ± 7.4
Thymus (g)	0.4 ± 0.07	0.4 ± 0.07	0.3 ± 0.05	0.3 ± 0.09	0.3 ± 0.05	0.3 ± 0.06	0.3 ± 0.05	0.3 ± 0.04
Thyroid (mg)	14.9 ± 3.6	14.7 ± 2.4	13.9 ± 2.0	15.6 ± 3.6	11.9 ± 2.5	13.0 ± 1.6	10.6 ± 2.7	11.7 ± 1.7
Prostate (g)	0.4 ± 0.07	0.4 ± 0.1	0.3 ± 0.09	0.4 ± 0.09	-	-	-	-
Testes, epididymis (g)	3.8 ± 0.3	3.8 ± 0.2	3.8 ± 0.4	3.7 ± 0.2	-	-	-	-
Seminal vesicle (g)	0.9 ± 0.09	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	-	-	-	-
Ovaries and uterus (g)	-	-	-	-	0.5 ± 0.09	0.5 ± 0.06	0.5 ± 0.05	0.5 ± 0.09

Values are mean ± standard deviation.
*Significantly different from the control group at $p < 0.01$.

Discussion. No toxicological differences were observed in an OECD-compliant 28-day dietary toxicity test of 2.5, 5, and 10% of Chlorella Powder in the feed fed to in male and female rats. One fatality occurred in the males fed 5% Chlorella Powder. The fatality was connected to human error and was therefore not considered to be test article-related. Although some significant differences were observed in body weight, water, and feed intake, these differences were within the normal historical control range for Wistar rats at the testing facility and were not considered adverse. In females, there were no significant changes in hematological and serum biochemical parameters. There were some changes in hematology and serum biochemistry parameters in the males, but these changes were not dose dependent and were within historical control ranges at the testing facility. These differences were not considered test article-related. Absolute renal weights were significantly increased in 10% (high-dose) males and females, but no remarkable findings were observed upon histopathological examinations and serum biochemistry parameters indicative of renal injury were within the normal range. The repeated dose 28-day dietary toxicity study reported no test article-related toxicity.

ii. 90-day Dietary Toxicity in Rats (Himuro et al., 2017)

Methods. The Chlorella Powder was mixed into the basal diet at concentrations of 2.5, 5.0, and 10.0% to ensure comparable protein and dietary fiber content across dose groups. The basal diet was used as the control diet. The diets were prepared weekly, or more frequently as needed, and were refrigerated until use.

The 90-day toxicity test was based on OECD Guideline 408 (OECD, 1998), with the exception that histopathological examination was limited to liver and kidney from control and 10% Chlorella Powder fed rats. Six-week old male and female Wistar rats (Japan SLC Inc., Shizuoka, Japan) were fed a pelleted rodent diet and water ad libitum for a 1-week acclimation period.

Forty male and forty female rats were randomly divided into 4 groups (n=10/sex/groups) and were housed individually with a 12-hour light cycle (6:00-18:00) at 23 ± 0.5ºC in 55 ± 5% relative humidity. The animals were cared for according to the published NIH guideline.

Each group of animals was fed one of the Chlorella Powder-containing diets for 13 weeks. Mortality and clinical signs were examined twice a day, and body weight and feed/water intake were measured once a week during the experimental period.

An ophthalmoscopy examination was performed during the last week of the experimental period. Ophthalmological examinations of the fundus, refractive media, iris, and conjunctivae were performed on all animals using binocular indirect ophthalmoscopy (Welch Allyn Inc., Skaneateles Falls, NY, USA).

To collect urine samples, the animals were placed in a metabolism cage on the last day of the 12th week, and urine excreted overnight (18:00-6:00) was collected. Immediately after the urine sample was obtained, its volume (VOL), pH, specific gravity (SG), and urinary protein (UPRO) were measured at Kurume Clinical Laboratories (Fukuoka, Japan).

After the 13-week experimental period, blood from the abdominal aorta was collected under pentobarbital anesthesia following 16-h fasting. Hematological parameters including white blood cell count (WBC), WBC differential counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils, red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets were measured at Kurume Clinical Laboratories (Fukuoka, Japan). Serum biochemical parameters, also measured at Kurume Clinical Laboratories, included total protein (TP), albumin (ALB), albumin/globulin (A/G), lactate dehydrogenase (LDH), aspirate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMY), total bilirubin (TBILI), creatinine (CRE), uric acid (UA), glucose (GLU), total cholesterol (TCHOL), non-esterified fatty acid (NEFA), triglyceride (TRIG), sodium (Na), potassium (K), chloride (Cl), and inorganic phosphorus (IP).

Following termination, necropsy was performed, and the heart, lungs, brain, liver, spleen, kidneys, adrenals, thymus, thyroid, testes, epididymides, seminal vesicle, ovaries, oviducts, and uterus were weighed after dissection. The ratios of each organ to the terminal body weight and brain weight (relative organ weights) were calculated. After the macroscopic examination, histopathological examinations of the liver and kidney were performed at BoZo Research Center Inc. (Tokyo, Japan) on the control and 10% Chlorella Powder groups. All samples were fixed in 10% neutral buffer formalin and stained with hematoxylin and eosin.

Variance in data for body weight, feed intake, water intake, hematology, serum biochemistry and organ weight was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance (ANOVA) was applied. In the heterogeneous cases, the Kruskal-Wallis test was used. When statistically significant differences were found, Dunnett's multiple comparison test was employed for comparison between the control and Chlorella Powder administered groups.

Results. No deaths or adverse clinical signs were observed in any group. There were no differences in the body weights between the control (0%) and Chlorella Powder groups throughout the experimental period (Figure 6). Chlorella Powder feeding also had no effect on feed and water consumption rates (Table 27). Using feed consumption and body weight, the amount of daily CK-22 powder consumption in the 2.5%, 5%, and 10% CK-22 powder groups was calculated as 1.47, 3.03, and 5.94 g/kg body weight/day in males and 1.60, 3.25, and 6.41 g/kg body weight/day in females, respectively.

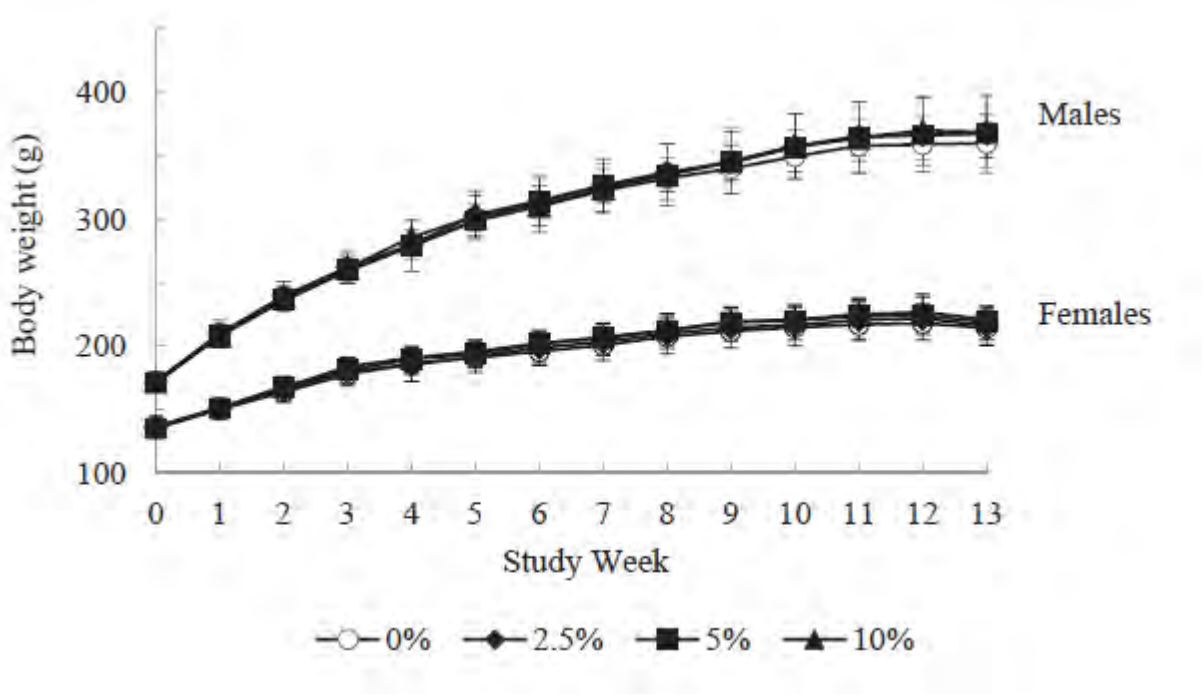

Figure 6. Body Weight Changes in Rats Fed Chlorella Powder During the 90-day Dietary Toxicity Study

Male and female rats are shown, n=10/group. Each point represents the mean ± the standard deviation. There were no differences between the control group and Chlorella Powder fed groups.

Table 27. Final Body Weights and Feed and Water Intake in Wistar Rats Fed Chlorella Powder in the 90-day Dietary Toxicity Study (Himuro et al., 2017)

	Males (n=10/group)			
	% Chlorella Powder in Feed			
	0%	**2.5%**	**5%**	**10%**
Final body weight (g)	350.4 ± 10.5	357.8 ± 31.3	358.8 ± 14.5	362.4 ± 23.4
Feed intake (g/day)	16.3 ± 1.7	16.3 ± 1.8	16.7 ± 1.5	16.5 ± 2.0
Water intake (g/day)	19.4 ± 2.5	18.1 ± 2.3	19.1 ± 2.3	19.7 ± 3.5
	Females (n=10/group)			
	% Chlorella Powder in Feed			
	0%	**2.5%**	**5%**	**10%**
Final body weight (g)	210.7 ± 10.6	210.5 ± 14.2	214.2 ± 9.8	215.5 ± 15.8
Feed intake (g/day)	11.8 ± 1.8	12.0 ± 1.8	12.3 ± 1.6	12.2 ± 1.8
Water intake (g/day)	15.2 ± 3.1	16.2 ± 2.9	15.6 ± 2.3	15.5 ± 3.7
Values are the mean ± S.D.				

The effect of Chlorella Powder in the diet was evaluated for organ weights, urinalysis, hematological parameters, serum biochemical parameters, and histopathology of kidney and liver. Organ weights and relative organ weights after the 13-week Chlorella Powder exposure are shown in Table 28. There were no differences between the control group and Chlorella Powder fed groups in any organ.

No ophthalmological findings or clinical signs were identified in the Chlorella Powder fed animals throughout the experimental period (data not shown).

Table 28. Absolute and Relative Organ Weight Results from the 90-day Dietary Toxicity Study in Wistar Rats. (Himuro et al., 2017)

Organs		Male (n=10/group) % Chlorella Powder in Feed				Female (n=10/group) % Chlorella Powder in Feed			
		0%	2.5%	5%	10%	0%	2.5%	5%	10%
Final body weight (g)		350.4 ± 10.5	357.8 ± 31.3	358.8 ± 14.5	362.4 ± 23.4	210.7 ± 10.6	210.5 ± 14.2	214.2 ± 9.8	215.5 ± 15.8
Heart	(g)	0.8 ± 0.04	0.9 ± 0.06	0.8 ± 0.05	0.9 ± 0.07	0.6 ± 0.03	0.6 ± 0.04	0.6 ± 0.03	0.6 ± 0.03
	(g/100 g BW)	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.01
	(g/100 g brain)	46.8 ± 3.0	48.0 ± 5.3	46.5 ± 3.1	46.3 ± 4.8	34.3 ± 1.8	34.7 ± 2.4	33.6 ± 2.4	34.9 ± 1.6
Lung	(g)	1.0 ± 0.05	1.0 ± 0.06	1.0 ± 0.08	1.0 ± 0.06	0.8 ± 0.04	0.8 ± 0.03	0.8 ± 0.04	0.8 ± 0.04
	(g/100 g BW)	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.01	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.02	0.4 ± 0.02
	(g/100 g brain)	46.8 ± 3.0	48.0 ± 5.3	46.5 ± 3.1	46.3 ± 4.8	47.5 ± 2.6	45.9 ± 1.9	47.0 ± 3.5	46.9 ± 2.3
Brain	(g)	1.8 ± 0.07	1.8 ± 0.1	1.8 ± 0.07	1.9 ± 0.1	1.7 ± 0.04	1.7 ± 0.04	1.7 ± 0.08	1.7 ± 0.05
	(g/100 g BW)	0.5 ± 0.02	0.5 ± 0.06	0.5 ± 0.02	0.5 ± 0.04	0.8 ± 0.05	0.8 ± 0.05	0.8 ± 0.05	0.8 ± 0.06
Liver	(g)	9.2 ± 0.8	9.0 ± 0.8	9.3 ± 1.5	9.0 ± 1.1	5.2 ± 0.5	5.4 ± 0.6	5.5 ± 0.7	5.6 ± 0.6
	(g/100 g BW)	2.6 ± 0.2	2.5 ± 0.09	2.6 ± 0.3	2.5 ± 0.2	2.5 ± 0.3	2.6 ± 0.2	2.5 ± 0.3	2.4 ± 0.2
	(g/100 g brain)	508.6 ± 42.3	505.1 ± 64.2	519.8 ± 66.6	484.4 ± 61.5	302.4 ± 26.9	313.7 ± 29.9	315.1 ± 41.5	305.2 ± 35.8
Spleen	(g)	0.8 ± 0.06	0.8 ± 0.09	0.7 ± 0.07	0.7 ± 0.07	0.5 ± 0.04	0.5 ± 0.04	0.5 ± 0.04	0.5 ± 0.06
	(g/100 g BW)	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.04
	(g/100 g brain)	41.9 ± 4.0	42.6 ± 6.0	41.5 ± 3.1	39.6 ± 4.0	27.8 ± 2.1	27.4 ± 2.5	27.0 ± 2.8	27.9 ± 3.9
Kidneys	(g)	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.08	1.3 ± 0.1	1.3 ± 0.09
	(g/100 g BW)	0.6 ± 0.03	0.6 ± 0.04	0.6 ± 0.02	0.6 ± 0.02	0.6 ± 0.08	0.6 ± 0.04	0.6 ± 0.04	0.6 ± 0.03
	(g/100 g brain)	112.3 ± 6.7	111.8 ± 7.9	113.7 ± 4.8	113.4 ± 7.8	74.4 ± 8.1	69.4 ± 4.0	72.5 ± 7.8	72.9 ± 5.7
Adrenals	(g)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.0	0.06 ± 0.0	0.06 ± 0.01	0.06 ± 0.01
	(g/100 g BW)	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0
	(g/100 g brain)	2.7 ± 0.4	2.8 ± 0.6	3.0 ± 0.3	2.9 ± 0.4	3.5 ± 0.3	3.4 ± 0.2	3.3 ± 0.4	3.5 ± 0.5
Thymus	(g)	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.04	0.2 ± 0.02	0.2 ± 0.05	0.2 ± 0.03	0.2 ± 0.04
	(g/100 g BW)	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.02
	(g/100 g brain)	14.5 ± 2.1	14.8 ± 2.4	14.9 ± 1.6	13.4 ± 1.9	12.4 ± 1.3	11.1 ± 2.6	10.6 ± 1.9	11.6 ± 2.3

Table 28. Absolute and Relative Organ Weight Results from the 90-day Dietary Toxicity Study in Wistar Rats. (Himuro et al., 2017)

Organs		Male (n=10/group) % Chlorella Powder in Feed				Female (n=10/group) % Chlorella Powder in Feed			
		0%	2.5%	5%	10%	0%	2.5%	5%	10%
Thyroid	(g)	0.01 ± 0.0	0.02 ± 0.0	0.01 ± 0.0	0.02 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
	(g/100 g BW)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0	0 ± 0	0 ± 0	0.01 ± 0
	(g/100 g brain)	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
Prostate	(g)	0.4 ± 0.09	0.3 ± 0.1	0.4 ± 0.08	0.4 ± 0.09	-	-	-	-
	(g/100 g BW)	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.03	-	-	-	-
	(g/100 g brain)	22.4 ± 5.5	19.5 ± 7.4	19.4 ± 4.7	19.1 ± 5.0	-	-	-	-
Testes	(g)	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	-	-	-	-
	(g/100 g BW)	0.9 ± 0.04	0.8 ± 0.06	0.8 ± 0.06	0.8 ± 0.09	-	-	-	-
	(g/100 g brain)	164.7 ± 11.4	166.7 ± 13.2	166.5 ± 12.1	162.2 ± 20.3	-	-	-	-
Epididymides	(g)	1.0 ± 0.08	1.0 ± 0.04	1.0 ± 0.07	1.0 ± 0.08	-	-	-	-
	(g/100 g BW)	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.03	-	-	-	-
	(g/100 g brain)	54.8 ± 5.3	54.0 ± 3.9	53.8 ± 3.1	51.7 ± 6.2	-	-	-	-
Seminal vesicle	(g)	1.2 ± 0.2	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	-	-	-	-
	(g/100 g BW)	0.3 ± 0.05	0.3 ± 0.1	0.3 ± 0.07	0.3 ± 0.09	-	-	-	-
	(g/100 g brain)	66.5 ± 11.2	58.7 ± 18.2	62.9 ± 14.9	61.0 ± 18.4	-	-	-	-
Ovaries	(g)	-	-	-	-	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02
	(g/100 g BW)	-	-	-	-	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.0
	(g/100 g brain)	-	-	-	-	6.9 ± 1.0	6.5 ± 0.7	6.9 ± 1.3	7.0 ± 0.9
Uterus/ oviducts	(g)	-	-	-	-	0.09 ± 0.03	0.07 ± 0.02	0.09 ± 0.03	0.06 ± 0.02
	(g/100 g BW)	-	-	-	-	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
	(g/100 g brain)	-	-	-	-	5.1 ± 1.5	4.2 ± 1.4	5.2 ± 1.8	3.5 ± 1.2

Values are the mean ± S.D.
BW: body weight

Urinalysis was performed on urine samples collected at the end of the 12th week of the study to determine the effects of Chlorella Powder exposure on renal function. No Chlorella Powder-induced difference was found in any parameter (Table 29).

Table 29. Urinalysis Results from the 90-day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2017)

Urinalysis Parameter	Male (n = 10/group)			
	% Chlorella Powder in Feed			
	0%	2.5%	5%	10%
Volume (mL)	7.9 ± 4.0	7.3 ± 2.9	8.0 ± 4.4	7.1 ± 5.2
pH	7.5 ± 1.0	8.2 ± 1.2	7.7 ± 1.3	7.4 ± 1.4
Specific gravity	1.03 ± 0.01	1.03 ± 0.01	1.04 ± 0.02	1.04 ± 0.02
Urinary Protein (g/L)	0.7 ± 0.3	0.7 ± 0.6	0.6 ± 0.3	0.6 ± 0.5
	Female (n = 10/group)			
	% Chlorella Powder in Feed			
	0%	2.5%	5%	10%
Volume (mL)	8.3 ± 3.7	9.6 ± 5.3	7.9 ± 3.8	11.2 ± 6.4
pH	6.7 ± 1.0	6.3 ± 0.7	6.1 ± 0.7	6.3 ± 0.9
Specific gravity	1.02 ± 0.01	1.03 ± 0.02	1.03 ± 0.02	1.02 ± 0.02
Urinary Protein (g/L)	0.2 ± 0.08	0.2 ± 0.08	0.2 ± 0.1	0.2 ± 0.2
Values are the mean ± S.D.				

Hematology parameters recorded at the end of the experimental period are presented in Table 30. Slight but significant differences were found in neutrophils, lymphocytes, RBC, hemoglobin, hematocrit, MCV, and MCHC in the Chlorella Powder fed groups compared to the control. A similar increase in neutrophils was also found in the male 10% Chlorella Powder fed group in the 28-day repeated dose toxicity study (Himuro et al., 2014). However, all the alterations observed here were very slight and no dose-dependency was observed. Moreover, since the values remained within the normal ranges previously reported (Himuro et al., 2014), these Chlorella Powder induced alterations were not considered adverse. Although the change in neutrophils was significant, it was within the normal range for Wistar rats, 5.4-37.5% (Traesel et al., 2016).

Table 30. Hematology Results from the 90-day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2017)

Hematology Parameters	Male (n = 10/group)				Female (n = 10/group)			
	% Chlorella Powder in Feed				% Chlorella Powder in Feed			
	0%	2.5%	5%	10%	0%	2.5%	5%	10%
WBC (x 10^3/mL)	3.6 ± 0.9	3.0 ± 1.1	3.2 ± 0.7	2.8 ± 1.0	1.8 ± 0.4	1.9 ± 0.5	2.0 ± 0.5	2.0 ± 0.4
Neutrophils (%)	20.1 ± 3.2	25.0 ± 5.0*	24.2 ± 2.3	26.2 ± 5.0**	23.1 ± 3.1	23.6 ± 5.0	22.6 ± 7.0	26.3 ± 7.0
Lymphocytes (%)	76.5 ± 3.4	71.5 ± 5.3*	72.0 ± 2.9	71.5 ± 5.2*	74.2 ± 3.3	74.1 ± 4.9	73.9 ± 7.5	70.7 ± 6.3
Monocytes (%)	1.6 ± 0.9	1.9 ± 0.9	2.4 ± 1.8	1.0 ± 1.3	1.3 ± 1.3	1.8 ± 1.5	2.6 ± 1.3	1.8 ± 1.9
Eosinophils (%)	1.8 ± 0.7	1.6 ± 0.8	1.3 ± 0.5	1.3 ± 0.7	1.1 ± 0.5	0.8 ± 0.6	0.9 ± 0.5	1.2 ± 0.7
Basophils (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
RBC (x10^6/mL)	9.5 ± 0.2	9.6 ± 0.3	9.6 ± 0.3	9.7 ± 0.3	8.6 ± 0.4	8.9 ± 0.3	9.1 ± 0.3**	8.8 ± 0.8
Hemoglobin (g/dL)	15.9 ± 0.3	15.9 ± 0.4	16.0 ± 0.6	16.2 ± 0.5	14.8 ± 0.8	15.3 ± 0.7	15.9 ± 0.5*	15.4 ± 1.1
Hematocrit (%)	49.6 ± 0.8	49.4 ± 1.4	48.3 ± 1.3*	48.9 ± 1.1	46.4 ± 1.9	47.8 ± 1.9	48.9 ± 2.0*	47.9 ± 2.6
MCV (fL)	52.1 ± 1.5	51.6 ± 1.1	50.6 ± 1.2*	50.6 ± 1.2*	54.2 ± 1.1	53.6 ± 1.1	53.4 ± 1.3	55.0 ± 4.1
MCH (pg)	16.8 ± 0.6	16.7 ± 0.5	16.9 ± 0.3	17.0 ± 0.0	17.1 ± 0.3	17.0 ± 0.0	17.2 ± 0.4	17.5 ± 0.7
MCHC (%)	32.0 ± 0.8	32.2 ± 0.6	33.2 ± 0.6**	33.1 ± 0.7**	31.8 ± 0.9	32.2 ± 0.4	32.6 ± 0.7	32.1 ± 1.4
Platelets (x10^5/mL)	5.3 ± 0.5	5.7 ± 0.5	5.7 ± 0.5	5.7 ± 0.5	4.8 ± 0.6	4.6 ± 0.4	5.0 ± 0.6	5.3 ± 0.5

Abbreviations. WBC: white blood cell count. RBC: red blood cell count.
MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration.
Values are the mean ± S.D.
Significant differences from control values are shown by *($p<0.05$) and **($p<0.01$).

Clinical chemistry parameters in the serum of Chlorella Powder fed rats are presented in Table 31. Chlorella Powder feeding caused a reduction of the total serum cholesterol in a dose-dependent manner in females, though this was not observed in males. The serum triglyceride level demonstrated a dose-dependent decrease in male rats fed Chlorella Powder in the diet. Although the decrease was significantly reduced in the 10% of the Chlorella Powder fed groups, this was not considered an adverse effect, as each value in this group was within a normal range distribution for Wistar rats (Traesel et al., 2016).

Table 31. Serum Biochemistry Results from the 90-day Dietary Toxicity Study in Wistar Rats (Himuro et al., 2017)

% Chlorella Powder in Feed	Male (n=10/group)				Female (n=10/group)			
	0%	2.5%	0%	2.5%	0%	2.5%	0%	10
TP (g/dL)	6.3 ± 0.2	6.3 ± 0.2	6.3 ± 0.2	6.3 ± 0.2	6.1 ± 0.5	6.2 ± 0.6	6.3 ± 0.4	6.3 ± 0.6
ALB (g/dL)	4.6 ± 0.2	4.5 ± 0.2	4.5 ± 0.3	4.5 ± 0.2	4.5 ± 0.4	4.5 ± 0.5	4.5 ± 0.3	4.5 ± 0.5
A/G (ratio)	2.7 ± 0.07	2.7 ± 0.4	2.6 ± 0.4	2.5 ± 0.3	2.7 ± 0.2	2.6 ± 0.6	2.5 ± 0.2*	2.5 ± 0.2
LDH (IU/L)	2584.4 ± 274.5	2741.3 ± 628.3	2769.8 ± 297.4	2937.9 ± 471.4	2251.8 ± 362.7	2269.9 ± 478.7	2204.1 ± 618.8	2464.5 ± 468.3
AST (IU/L)	176.6 ± 37.7	191.3 ± 67.2	171.4 ± 17.4	183.7 ± 25.1	136.2 ± 12.4	146.1 ± 23.2	150.6 ± 23.3	155.6 ± 18.6
ALT (IU/L)	71.4 ± 20.0	74.1 ± 33.4	61.2 ± 20.5	63.6 ± 18.6	37.8 ± 6.8	42.1 ± 7.4	47.1 ± 16.0	43.7 ± 8.0
ALP (IU/L)	333.3 ± 19.1	336.9 ± 30.3	317.9 ± 41.7	312.7 ± 30.3	245.3 ± 47.3	251.2 ± 43.0	252.0 ± 55.5	240.2 ± 52.1
AMY (IU/L)	1631.8 ± 188.7	1628.6 ± 143.0	1597.0 ± 158.3	1645.8 ± 167.2	1033.1 ± 280.6	1334.0 ± 360.8	1255.7 ± 304.5	1284.2 ± 294.9
TBILI (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
CRE (mg/dL)	0.4 ± 0.07	0.4 ± 0.06	0.3 ± 0.06	0.4 ± 0.06	0.4 ± 0.06	0.4 ± 0.05	0.4 ± 0.06	0.4 ± 0.05
UA (mg/dL)	1.8 ± 0.9	1.7 ± 0.7	1.9 ± 0.9	2.3 ± 0.8	2.5 ± 1.3	1.9 ± 0.7	2.1 ± 0.8	2.0 ± 0.7
GLU (mg/dL)	111.1 ± 23.2	120.3 ± 21.7	127.3 ± 20.2	105.6 ± 14.9	66.7 ± 19.6	54.7 ± 15.1	53.0 ± 19.7	54.9 ± 14.6
TCHOL (mg/dL)	89.3 ± 12.0	88.5 ± 11.9	88.3 ± 16.3	75.0 ± 12.5	102.9 ± 12.2	79.2 ± 10.1**	84.1 ± 9.1**	80.1 ± 14.8**
NEFA (mmol/L)	1107.7 ± 358.8	794.9 ± 113.8	826.3 ± 145.8	811.7 ± 214.5	851.5 ± 315.5	649.8 ± 162.7	675.2 ± 169.5	654.2 ± 129.0
TRIG (mg/dL)	151.5 ± 74.7	126.1 ± 49.6	114.6 ± 42.1	73.0 ± 44.3**	26.6 ± 13.5	35.8 ± 22.5	36.5 ± 27.5	26.1 ± 17.9
Na (mmol/L)	144.8 ± 1.5	145.9 ± 1.9	144.5 ± 1.1	145.0 ± 1.3	145.3 ± 1.7	147.0 ± 1.6*	147.1 ± 1.1*	146.7 ± 1.5
K (mmol/L)	4.3 ± 0.2	4.3 ± 0.3	4.4 ± 0.4	4.5 ± 0.4	4.3 ± 1.1	4.0 ± 0.3	4.1 ± 0.3	4.1 ± 0.4
Cl (mmol/L)	97.2 ± 2.2	98.9 ± 2.0	99.7 ± 1.3*	100.2 ± 1.9**	98.1 ± 2.5	99.3 ± 1.8	99.4 ± 1.4	99.5 ± 1.6
IP (mmol/L)	6.0 ± 1.1	6.6 ± 2.2	6.2 ± 0.7	6.0 ± 1.1	6.9 ± 2.2	6.9 ± 2.1	6.4 ± 1.8	6.4 ± 1.3

TP: total protein, ALB: albumin, A/G: albumin/globulin, LDH: lactate dehydrogenase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, AMY: amylase,
TBILI: total bilirubin, CRE: creatinine,
UA: uric acid, GLU: glucose, TCHOL: total cholesterol, NEFA: non-esterified fatty acid, TRIG: triglyceride, Na: sodium, K: potassium, Cl: chloride, IP: inorganic phosphorus.
Values are the mean ± S.D.
Significant differences from control values are shown by *($p<0.05$) and **($p<0.01$).

A small, but statistically significant increase in sodium (females, low and middle-doses) and chloride (males, middle and high-doses) was observed (Table 31). These increases remained within the range of normal controls for the testing facility and were not considered adverse. All other changes were sporadic, not dose-dependent, or present in both sexes and therefore were not considered treatment-related.

The present 90-day toxicity test was performed based on OECD Guideline 408 (OECD, 1998), with the exception that only liver and kidney from control and high-dose animals of both sexes were subjected to histopathological analysis. In the liver of the 10% Chlorella Powder fed groups, minimal or mild vacuolation in the periportal region was found in both sexes, and females had mineral cell infiltration in the same region of the liver (Table 32). Since these findings were also observed in the control group at the same frequency, they were considered related to aging and not test article-related. In the kidney of Chlorella Powder fed males, minimal chronic progressive nephropathy was observed, but it was considered incidental or spontaneous because this finding was also observed in the control males. In high-dose females, minimal renal mineralization was observed in 2 out of 10 rats. This mineralization was considered unrelated to Chlorella Powder administration because the finding often occurs spontaneously in this species (Peter et al., 1986).

Table 32. Histopathological Results from the 90-day Dietary Toxicity Study in the Liver and Kidney of Wistar Rats (Himuro et al., 2017)

% Chlorella Powder in Feed		Male		Female	
		0%	10%	0%	10%
Organs	Findings				
Liver	Vacuolation, periportal	3/10	3/10	7/10	6/10
	Cell infiltration, periportal	0/10	0/10	3/10	3/10
Kidney	Chronic progressive nephropathy	4/10	2/10	0/10	0/10
	Mineralization	0/10	0/10	0/10	2/10

Discussion. The 90-day toxicity study in Wistar rats was performed with 0, 2.5, 5, and 10% Chlorella Powder mixed into the feed. During the experimental period, no Chlorella Powder treatment-induced differences in general condition, body weight gain, feed and water consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, histopathology, or mortality were observed. The no observed adverse effect levels (NOAELs) were estimated to be at least 5.94 g/kg body weight/day for males and 6.41 g/kg body weight/day for females.

3. Corroborative Animal Studies with *Chlorella* spp.

a. 90-day Toxicity Studies

The safety of *Chlorella* spp. based-food ingredients has been assessed in two 90-day toxicity studies: Szabo et al., 2012 and Szabo et al., 2013. No toxicity was observed in either of these two studies and the NOAELs were estimated to be at least the highest doses tested. These studies are summarized below and in Table 37.

The subject of Szabo et al., 2012 was a high lipid whole algalin flour (WAF) composed of dried, milled *Chlorella protothecoides*. WAF was mixed in the diet at levels of 0, 25000, 50000, or 100000 ppm and fed to male and female HSD:SD rats. No mortalities occurred. No treatment-related effects were identified for general condition, body weight, feed consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, and histopathology. Although statistically significant effects were noted for several endpoints, none was test-substance related. The no observed adverse effect level (NOAEL) for WAF was based on the consumption of the 100000 ppm diet, the highest dietary concentration tested, and was 4807 mg/kg body weight/day in male rats and 5366 mg/kg body weight/day in female rats.

The test article used in Szabo et al. (2013) was the whole algalin protein (WAP) from dried milled *Chlorella protothecoides*. WAP was mixed in the diet at levels of 0, 25000, 50000, or 100000 ppm and fed to male and female HSD:SD rats. No treatment-related mortalities or effects in general condition, body weight, feed consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, and histopathology occurred. Several endpoints exhibited statistically significant effects, but none were dose-related. The NOAEL was based on the highest WAP concentration consumed by the rats and was equivalent to 4805 mg/kg/day in males and 5518 mg/kg/day in female rats.

b. Other Corroborative Animal Studies

The exposure of hamsters, mice, and rats to *Chlorella* spp. in feed has been summarized in the two previous *Chlorella* spp. product GRNs. Corroborative data from GRN 384 (stamped pages 22-37), GRN 469 (stamped pages 22-29) are incorporated by reference. These studies encompass multiple species within the *Chlorella* genus, including *C. protothecoides, C. pyrenoidosa, C. vulgaris, C. stigmatophora, C. sorokiniana, C. regularis,* as well as many studies performed with unspecified *Chlorella* species. Although not all of these studies are explicitly safety studies, none of the studies report test article-related adverse effects associated with the consumption of *Chlorella* spp.-derived products. Studies not available in English were excluded from the summary table below. These studies are summarized below in Table 33.

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Corroborative data from GRN 384 (stamped pages 22-37), GRN 469 (stamped pages 22-29)					
Szabo et al., 2012	High Lipid *C. protothecoides* S106 Flour (HLAF)	HSD:Sprague Dawley rats, n = 10/sex/group	• 0 ppm, control in the diet • 25000 ppm HLAF in the diet (1249 mg/kg/day males, 1413 mg/kg/day females) • 50000 ppm HLAF in the diet (2478 mg/kg/day males, 2739 mg/kg/day females) • 100000 ppm HLAF in the diet (4807 mg/kg/day males, 5366 mg/kg/day females)	90-day toxicity study	• NOAEL of 100000 ppm, the highest dose tested (4807 and 5366 mg/kg/day in male and female rats, respectively).
Szabo et al., 2013	Whole *C. protothecoides* S106 Algal Protein (WAP) in the diet	HSD:Sprague Dawley rats, n = 10/sex/group	• 0 ppm, control in the diet • 25000 ppm WAP in the diet (1177 mg/kg/day males, 1444 mg/kg/day females) • 50000 ppm WAP in the diet (2416 mg/kg/day males, 2700 mg/kg/day females) • 100000 ppm WAP in the diet (4805 mg/kg/day males, 5518 mg/kg/day females)	90-day toxicity study	• NOAEL of 100000 ppm, the highest dose tested (4805 and 5318 mg/kg/day in male and female rats, respectively).

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Khalawan 1980	*C. pyrenoidosa*	Female Harvard rats, n = 3 or 4/group	• Control: basal diet • 7% *C. pyrenoidosa* in the diet (9249 mg/kg/day)	34 days 2 generation reproductive study	• Larger cecum and smaller fat deposits in the abdominal viscera. No change in body weight gain, general appearance, reproduction, or behavior was observed
		Two pairs of weanling Albino mice	• Control: basal diet • 7% *C. pyrenoidosa* in the diet (9249 mg/kg/day)	4 generation reproductive study	• No change in body weight gain, general appearance, reproduction, or behavior was observed
Selah et al., 1985	*C. vulgaris*	Male Sprague Dawley rats (n = 6/group)	• Control: normal feed • 19.6% in the diet (19600 mg/kg/day)	17 days	• Increase in plasma uric acid levels
Tanaka et al., 1990	*C. vulgaris* (later determined to be *C. sorokiniana)*	Female CDF1 mice, n = 7-10/group	• Control: normal feed • 3% in the diet (4500 mg/kg/day) • 10% in the diet (15000 mg/kg/day)	Xenograft (Meth A) tumor study, test article provided 35 days before and 22 days after tumor inoculation	• No adverse events, weight loss, or signs of wasting syndrome reported
Herrero et al., 1993	*C. stigmatophora*	Female Wistar rats, n = 10/group	• Control: standard diet with 12% casein • 12% *C. stigmatophora* (17094 mg/kg/day)	4 weeks	• No statistical difference in food intake or hematological parameters. • The following differences between the control and *C. stigmatophora* fed group were described. The authors did not describe if these differences were adverse: o Rats fed *C. stigmatophora* did not gain as much weight as the casein fed control. o Decreased relative liver and spleen weight o Decreased plasma phosphorus, cholesterol and triglyceride levels

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Singh et al., 1998	*C. vulgaris* strain E-25	Swiss albino rats, n = 6/group	• Oral gavage, Placebo control • 100 mg/kg/day • 300 mg/kg/day • 500 mg/kg/day	First 14 days of gestation or lactation in pregnant and lactating rats	• Significantly increased levels of sulfhydryl (SH) and glutathione S-transferase (GST) were observed in fetal and neonatal livers from doses providing 300 or 500 mg *C. vulgaris*/kg body weight, and significantly decreased hepatic cytochrome B5, cytochrome P450, and malondialdehyde (MDA) levels also were noted in the developing fetuses and neonates whose mothers were administered 500 mg/kg body weight/day. • The dose of 100 mg/kg body weight/day by gavage had no effect on hepatic SH, GST, cytochrome B5, cytochrome P450, or MDA levels. • No other treatment-related effects were reported.
Chovancikova & Simek, 2001	*C. vulgaris*	Male CD1 mice, n = 10/group	• Control: standard diet • 1% (1560 mg/kg/day) *C. vulgaris* + standard chow • High fat diet • 1% (1030 mg/kg/day) *C. vulgaris + high fat diet*	Ten weeks	• No significant differences in body or liver weights or food intake with *C. vulgaris* supplementation • Lipid metabolism was not affected by Chlorella supplementation in the standard diet. • In the high-fat diet groups, *C. vulgaris* supplementation lowered: o Serum triglycerides o Total serum cholesterol o Hepatic triglycerides o Total hepatic cholesterol

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Justo et al., 2001	*C. sorokiniana*	Balb/C mice, n = 8/group	• 0 mg/kg/day placebo gavage • 50 mg/kg/day • 100 mg/kg/day • 200 mg/kg/day	5 days, 6 x 10^6 Ehrlich ascites tumor cells, i.p.	• The authors did not report any adverse events following *C. sorokiniana* administration. • *C. sorokiniana* prevented the tumor driven immunosuppression. • *C. sorokiniana* increases the host survival (from 16 days, untreated to 26 days, treated). • Effects of *C. sorokiniana* independent of dose.
Shibata et al., 2001	*C. regularis*, containing 8.9% lipids	Male Wistar rats, n = 6/group	• Control: normal feed • 12.7% in the diet (12700 mg/kg/day)	14-day oral toxicity study	• Decreased serum cholesterol and liver cholesterol content • No adverse events were reported following *C. vulgaris* supplementation.
Cherng and Shih 2005	*C. pyrenoidosa* (containing 13% lipids)	Male Wistar rats, n=8/group	• Control: cholesterol enriched diet • 0.9% *C. pyrenoidosa* + cholesterol enriched diet (900 mg/kg/day) • 1.8% *C. pyrenoidosa* + cholesterol enriched diet (1800 mg/kg/day) • 7.2% *C. pyrenoidosa* + cholesterol enriched diet (7200 mg/kg/day)	2, 4, or 8 weeks	• Decreased serum triglycerides and total cholesterol following 2, 4, or 8 weeks (≥0.9%) except no significant differences in triglycerides at 2 weeks and total cholesterol at 4 weeks in 1.8% group • Decreased serum low density lipoprotein-cholesterol at 2 weeks (0.9 and 7.2%) at 4 weeks (7.2%), and at 8 weeks (≥ 0.9%) • Increased high density lipoprotein-cholesterol at 4 weeks (7.2%) • Decreased serum total cholesterol to high density lipoprotein-cholesterol ratio at 2, 4, or 8 weeks (≥ 0.9%) • No adverse events were reported following *C. pyrenoidosa* supplementation

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs					
Reference	**Test Article**	**Target Species**	**Test Groups**	**Study Type**	**Safety Endpoints**
		Male Syrian hamsters, n = 8/group	• Control: cholesterol enriched diet • 0.9% *C. pyrenoidosa* + cholesterol enriched diet (1080 mg/kg/day) • 1.8% *C. pyrenoidosa* + cholesterol enriched diet (2160 mg/kg/day) • 7.2% *C. pyrenoidosa* + cholesterol enriched diet (8640 mg/kg/day)	2, 4, or 8 weeks	• Decreased serum triglycerides, total cholesterol, and low density lipoprotein-cholesterol at 2, 4, and 8 weeks (≥0.9%) except no significant differences in 1.8% group at 2 weeks • Increased high density lipoprotein-cholesterol at 2, 4, and 8 weeks (≥ 0.9%) • Decreased serum total cholesterol to high density lipoprotein-cholesterol ratio at 2, 4, and 8 weeks (≥ 0.9%) • No adverse events reported following *C. pyrenoidosa* supplementation
Takekoshi et al., 2005	*C. pyrenoidosa*, in the diet	Male F344/DuCRj rats, n = 15/group	• Control: normal diet • Diethylnitrosamine (DEN) treated rats • DEN + 10% *C. pyrenoidosa* (6960 mg/kg/day) • DEN + 2-amino-3,8-dimethyl-imidazo [4,5f] quino-xaline (MeIQx) • DEN + MeIQx + 10% *C. pyrenoidosa* • MeIQx • MeIQx + 10% *C. pyrenoidos*	8 weeks, single IP injection of 200 mg/kg/day DEN and fed MeIQx, an initiation-promotion carcinogenesis model in the rat liver	• No significant differences in safety parameters • *C. pyrenoidosa* did not affect liver weight.
Janczyk et al., 2006	*C. vulgaris*	Fzt:DU mice	• Control: normal diet • 1.0% spray-dried C. vulgaris in the diet	Three generation reproduction study	• No significant differences in safety, no observed reproductive toxicity

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Lee et al., 2008	*C. vulgaris*	Male Slc:Wistar/ST rats, n = 10/group	• Normal diet • Normal diet + 5% *C. vulgaris* (5000 mg/kg/day) • Normal diet + 10% *C. vulgaris* (10000 mg/kg/day) • High fat diet • High fat diet + 5% *C. vulgaris* (5000 mg/kg/day) • High fat diet + 10% *C. vulgaris* (10000 mg/kg/day)	9 weeks of high fat diet to induce changes in blood glucose as a model of Type II Diabetes	• Decreased liver weight relative to body weight compared to control, but not accompanied by any significant changes in liver enzyme activity or total protein or bilirubin. • Body weight, feed intake, and food efficiency ratio were not affected by chlorella supplementation alone. • Chlorella supplementation in rats receiving normal diet decreased serum leptin levels. The authors did not comment on the safety of this finding.
Bedirli et al., 2009	*Chlorella sp*. (strain not specified) in the diet	15 male Wistar rats/group	• Sham bile duct ligation • Bile duct ligation • Bile duct ligation +50 mg/kg/day *Chlorella* sp via oral gavage • Bile duct ligation +50 mg/kg/day *Spirulina* sp via oral gavage	Experimental jaundice rat model with bile duct ligation, fed Chlorella for 10 days post-surgery	• No safety parameters reported
Day et al., 2009	*C. protothecoides* in the feed	Male and Female Sprague Dawley rats, n = 10/sex/group	• 0% • 2.5% (males 1794 mg/kg/day; females 1867 mg/kg/day) • 5.0% (males 3667 mg/kg/day; females 3918 mg/kg/day) • 10% (males 7557 mg/kg/day; females 8086 mg/kg/day)	28 day repeated oral toxicity study	• No toxicity was observed at the highest dose, 10% algal biomass in the diet. This corresponds with 7557 and 8086 mg/kg/day for males and females, respectively

Table 33. Corroborative *Chlorella* spp. Animal Toxicity Studies Reviewed in Previous GRNs					
Reference	**Test Article**	**Target Species**	**Test Groups**	**Study Type**	**Safety Endpoints**
Shim et al., 2009	*C. vulgaris* mixed in standard diet	14-week old male Sprague Dawley rats, n = 10/group	• Control: standard diet • 3% *C. vulgaris* + standard diet (3000 mg/kg/day) • 5% *C. vulgaris* + standard diet (5000 mg/kg/day) • 160 ppm Cd + standard diet • 160 ppm Cd + 3% *C. vulgaris* + standard diet (3000 mg/kg/day) • 160 ppm Cd + 5% *C. vulgaris* + standard diet (5000 mg/kg/day)	Cadmium toxicity study. $CdCl_2$ administered in drinking water for 10 weeks.	• No significant difference between groups • No other safety parameters reported

c. Corroborative Animal Toxicity Studies Not Discussed in Previous GRNs

The studies described in Table 34 were not discussed in GRNs 384 and 469. These studies were also not explicitly safety toxicity studies but did not report adverse events. The studies in Table 38 all used *Chlorella vulgaris* in the diet or delivered via oral gavage.

Table 34. Corroborative Animal Toxicity Studies Performed using *Chlorella* spp. Products, Not Discussed in GRNs 384 and 469

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
An et al, 2006	Hot water extract *C. vulgaris*, oral gavage	Male CR1 mice	• Control: 0 g/kg/day, distilled water placebo • 0.05 g/kg/day Hot water extract *C. vulgaris* • 0.1 g/kg/day Hot water extract *C. vulgaris* • 0.15 g/kg/day Hot water extract *C. vulgaris*	1 week of daily oral gavage	• *C. vulgaris* supplementation increased the time to fatigue in a forced swim test • *C. vulgaris* did not cause liver damage • 0.15 g/kg/day dose of *C. vulgaris* showed improved renal function and decreased muscle break down following forced exercise • No other safety parameters reported
Morris et al, 2007	Enzymatic protein hydrolysate from *C. vulgaris* (Cv-PH) in standard diet	Female Balb/c mice, n=10/group	• Control: Standard diet • Fasted 3 days, then terminated • Fasted 3 days, re-fed with standard diet • Fasted 3 days, re-fed with standard diet and 0.5 g/kg/day *C. vulgaris*	3-day fasting period followed by Cv-PH supplementation for 8-14 days	• *C. vulgaris* supplementation after fasting increases: o Hematopoiesis o Phagocytic abilities o Antibody response to T-dependent antigen • No other safety parameters reported
Shim et al, 2008	*C. vulgaris* in normal diet	Male, 5-week old Sprague-Dawley rats, n=10/group	• Control, Cadmium (Cd)-free water + normal diet • Water with 10 ppm Cd + normal diet • Water with 10 ppm Cd + 5% *C. vulgaris* in normal diet • Water with 10 ppm Cd + 10% *C. vulgaris* in normal diet	Cadmium-induced toxicity study. Rats given drinking water with 10 ppm Cadmium for 8 weeks	• *C. vulgaris* supplementation increased weight gain compared to the Cd alone treated rats • Concentrations of Cd in liver decreased in *C. vulgaris* rats • Decrease in liver damage (histology) in *C. vulgaris* rats • *C. vulgaris* increased liver metallothionein mRNA

Table 34. Corroborative Animal Toxicity Studies Performed using *Chlorella* spp. Products, Not Discussed in GRNs 384 and 469

Reference	Test Article	Target Species	Test Groups	Study Type	Safety Endpoints
Aizzat et al, 2009	*C. vulgaris* Beijerinck strain 072, oral gavage	Male Sprague-Dawley rats, n=6/group	• Control • 0.15 g/kg/day *C. vulgaris* Beijerinck strain 072 • STZ-induced diabetes • STZ-induced diabetes + 0.15 g/kg/day *C. vulgaris* Beijerinck strain 072	Streptozotocin (STZ) induced diabetes model, *C. vulgaris* oral gavage beginning 2 days after induction of diabetes, administered daily for 4 weeks	• *C. vulgaris* had no effect on glucose levels • *C. vulgaris* supplementation decreased DNA damage and blood MDA in STZ-induced diabetes
Jeong et al, 2009	*C. vulgaris* in normal diet	6-week old Diabetic Goto-katizaki (GK) rats, Wistar rats, n=10/group/rat species	• Control: standard diet • Standard diet + 3% *C. vulgaris* • Standard diet + 5% *C. vulgaris*	Rat model of diabetes, fed *C. vulgaris* for 8 weeks	• No change in feed intake, calorie intake or weight gain • In diabetic rats, *C. vulgaris* decreased liver triglycerides • No changes in blood glucose • Lower fasting plasma glucagon in diabetic rats treated with *C. vulgaris*
Cheng et al., 2017	*C. vulgaris* in normal diet	8-week old male Kunming Mice	• Control: standard diet • Standard diet + cyclophosphamide • Cyclophosphamide + 6% *C. vulgaris* • Cyclophosphamide + 12% *C. vulgaris* • Cyclophosphamide + 24% *C. vulgaris*	6-week cyclophosphamide induced immunosuppression in mice	• No differences observed in growth rates • Cyclophosphamide treated mice fed *C. vulgaris* had decreased expression of cytokines associated with immunosuppression, enhanced natural killer cell cytotoxicity, and ameliorated histological changes in the spleen. • No other safety parameters reported

D. CLINICAL STUDIES

1. Other *Chlorella* Species

The discussion of the clinical studies summarized in Table 35 is incorporated by reference from GRN 384 stamped pages 37-46. The consumption of *Chlorella* spp. by humans was reported to be well-tolerated in a number of studies in which the effects of the algae on the immune system, hypertension, fibromyalgia syndrome, ulcerative colitis, and glioma (primary brain tumors) were investigated, as well as in studies where *Chlorella* spp. replaced dietary protein sources such as fish, egg, and soy as the principal source of nitrogen consumption (Powell et al., 1961; Dam et al., 1965; Lee et al., 1967; Merchant et al., 1990, 2000, 2002; Merchant and Andre, 2001; Halperin et al., 2003). The only adverse effects reported following the consumption of *Chlorella* spp. were feelings of fatigue (Halperin et al., 2003) and symptoms of gastrointestinal distress such as nausea, flatulence, mild abdominal cramping pain, hard bulk stool (Powell et al., 1961; Merchant et al., 1990; Merchant et al., 2000). These adverse events were observed in subjects receiving 200 mg/day (*C. pyrenoidosa* extract) to 100 g/day (autoclaved *Chlorella* and *Scenedesmus*) of Chlorella products.

Clinical studies that were not reviewed in GRN 384 are also summarized in Table 35. These studies were performed on *C. pyrenoidosa*, *C. vulgaris*, and unspecified *Chlorella* species as well as extracts of *C. vulgaris* and *C. pyrenoidosa*. These studies did not report any serious adverse events following the consumption of *Chlorella* spp.-based products in a range of doses from 20 mg/day to 7.65 g/day.

Mizoguchi et al. (2008) reported that one subject in their study reported stomach pain following 7.64 g of *C. pyrenoidosa* and dropped from the study. Azocar and Diaz (2013) reported that one subject dropped from their 12-week study due to constipation after the first two days of twice daily treatment of 30 mL of *C. pyrenoidosa* water soluble extract and *C. pyrenoidosa* tablets (3.5 g); the issue resolved upon treatment withdrawal. Panahi et al. (2013) reported two subjects drop out of their study due to unspecified gastrointestinal side effects following consumption of 3.6 mg/day of a *C. vulgaris* extract. One case of nausea and one case of diarrhea were reported by Panahi et al. (2015) following consumption of 1.8 mg/day of a *C. vulgaris* extract, but these events did not cause withdrawal from the study. All the other studies reviewed in Table 35, reported that consumption of *Chlorella* spp. products was well-tolerated, or the authors did not report adverse events.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Studies discussed in GRN 384 (stamped pages 37-46)			
Powell et al., 1961;	Non-randomized, 5 healthy males aged 18-23 years	*Chlorella* and *Scenedesmus* (algae) autoclaved and incorporated into foods according to the following schedule • Control period: 5 days with a diet of 3190 Calories, 91 g protein, 315 g carbohydrates, 167 g fat per day. • Algae added to the diet at 10, then 20, and 50 g/day for successive periods of 6 days each. • 100 and 200 g/day were added for three days and 500 g/day was added for 2 days. Total calorie and protein content were controlled for each group.	• Authors concluded that algae were well-tolerated at levels up to 100 g/day. Difficulty digesting the test item was noted at levels greater than 100 g algae/day. Adverse events observed included: o Abdominal distention, associated with increased eructation and flatulence, early in the study. Increased bowel movements with bulky and dry stools at levels greater than 50 g algae/day. These effects became more severe at levels greater than 200 g algae/day. o Nausea, mild abdominal cramping pain, headache, malaise, and hard bulk stools at level of 500 g algae/day. o 2 subjects dropped out of the study: ▪ 1 due to diffuse lower abdominal cramping pains, increased flatulence, nausea, and persistent vomiting at level of 200 g algae/day. ▪ 1 due to similar effects at a level of 500 g algae/day • All adverse effects disappeared 48 hours after discontinuing algae supplementation. • The subjects lost 1-2 kg in body weight during the study. The authors did not comment on whether this weight loss was adverse. • No abnormalities in physical examinations other than those associated with the gastrointestinal tract. Hematology, urinalysis, and liver function tests were all within normal limits.
Dam et al., 1965	Cross-over study in, healthy adults aged 24-35 years, 4 males, 1 female.	Ethanol extracts of *C. pyrenoidosa* 71105 as a principle source of protein for 20 days. Test article incorporated in pizza and biscuits • 54.2 g • 90.3 g	• 1 subject withdrew from the study (reason not specified). • No complaints of nausea, bloated feeling, or bitter taste were attributed to the test article.
Lee et al., 1967	Healthy adults aged 18-32 years, 3 males, 3 females	57.3 g/day of *C. pyrenoidosa* in the diet for 5 days	• No adverse events were reported by the authors

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Merchant et al., 1990	Adult subjects aged 19 to 69 years, sex not specified, with glioblastoma (n = 15), low-grade astrocytoma (n = 4), anaplastic astrocytoma (n = 1), and high-grade oligo-dendroglioma (n = 1).	• After a one-week escalating dose, the final dose of 20 g tablet and 150 mL extract of *C. pyrenoidosa* was consumed by subjects for at least 1 month, then followed for up to 2 years	• No severe or dose-limiting toxicity was observed in patients that consumed *C. pyrenoidosa* daily for 1 month. • Transient adverse effects reported at the beginning of treatment, which resolved within a few days to a week, and included: o 8/21 subjects (38%) experienced nausea or slight fever o 6/21 subjects (29%) reported irregular bowel movements, intestinal cramping, increased flatus o 3/21 subjects (14%) experienced constipation and nausea • 1 subject withdrew from the study due to aversion to the taste of *Chlorella*, which developed as a result of nausea from radiotherapy. • No adverse effects on hematological and immunological parameters measured were reported. • Adverse changes in clinical status usually correlated with computerized tomography (CT) scan or magnetic resonance imaging (MRI) evidence of tumor recurrence and/or progressive growth and were not attributed to *Chlorella* supplementation.
Merchant et al., 2000	Pilot study in adults aged 18-65 with fibromyalgia, 1 male, 19 females	• 10 g tablet and 100 mL extract of *C. pyrenoidosa* for 2 months	• 2 subjects withdrew from the study: o 1 due to nausea following treatment o 1 did not want to participate in the study • Increased frequency of diarrhea and abdominal cramping reported; symptoms did not require medical intervention and did not limit the activity of subjects. • No effect on serum chemistry or hematology parameters.
Merchant and Andre 2001	98 subjects aged 25-56 years with ulcerative colitis	10 g tablet and 100 mL extract of *C. pyrenoidosa* for 2 months	• 1 subject dropped out of the study (reason not specified). • No significant difference in physical examination results and hematological parameters. • No adverse effects on the symptoms of ulcerative colitis were reported.
	Randomized, double-blind, cross-over, controlled study (1-month washout period before crossover) in subjects with fibromyalgia,	10 g tablet and 100 mL extract of *C. pyrenoidosa* for 3 months (n = 37, 47.1 ± 9.0 years old, 36 females, 1 male)	• No significant difference in physical examination results, and hematological and urinalysis parameters. Adverse events were not reported by the authors.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Merchant et al., 2002	Blinded placebo-controlled trial in 24 adult subjects with mild to moderate hypertension aged 22 to 73 years, 11 males, 13 females. 1-month placebo washout period of antihypertensive medication prior to treatment	10 g tablet and 100 mL extract of *C. pyrenoidosa* for 8 weeks	• 1 subject withdrew after 4 weeks as his mean blood pressure was too high. • No significant differences were observed in physical examination results, body weight, electrocardiogram findings, serum clinical chemistry, hematology, or urinalysis parameters, no significant differences in heart rate, systolic blood pressure, and diastolic blood pressure *vs* placebo period. • Decreased serum total cholesterol and low-density cholesterol vs. baseline and placebo periods. • Decreased high density cholesterol vs. placebo period
Halperin et al., 2003	Randomized, double-blind, controlled trial in healthy male and female adults aged 50-89 years receiving the influenza vaccine, given C. pyrenoidosa daily for 28 days.	Aqueous extract of *C. pyrenoidosa* in capsules, administered prior to a trivalent influenza vaccine • 0 (placebo control, microcrystalline cellulose) n = 42 • 200 mg/day, n = 40 • 400 mg/day, n = 36	• No significant difference in the incidence of fever, rash, headache, body aches, sore joints, abdominal pain, nausea, anorexia, vomiting, and diarrhea between groups. • Increased incidence of fatigue in 200 mg group vs. placebo and 400 mg group. • No significant difference in overall antibody response to influenza vaccination. antibody response in subjects 50 to 55 yrs in the 400 mg group *vs* placebo.
Studies not reviewed in GRN 384			
Nakano et al., 2007	Controlled trial in pregnant Japanese women given *C. pyrenoidosa* for approximately 6 months	• Placebo control, n = 17 • 6 g/day *C. pyrenoidosa* tablet, n = 18	• The stools of subjects receiving *C. pyrenoidosa* were green. No other adverse reactions were observed. • Dioxin levels in breast milk were significantly lower in the breast milk of women taking *C. pyrenoidosa* supplements compared to the control. • Immunoglobulin A (IgA) concentrations in breast milk in the *C. pyrenoidosa* supplemented group was significantly higher than the control group.
Mizoguchi et al., 2008	Non-randomized, non-blinded not controlled observational study *of C. pyrenoidosa* in healthy males and those at high risk of lifestyle-related diseases for 12 weeks with 4-month follow up in	7.64 g/day tablet of *C. pyrenoidosa* (n = 17 for healthy and high-risk groups)	• 1 healthy subject dropped out due to stomach pains. • No subjects reported any complications that were considered harmful side effects during physical examinations. Decreased fasting blood glucose at 8 weeks vs. baseline levels in high-risk subjects. Decreased fasting blood glucose at 12 and 16 weeks vs. baseline in normal subjects. • Decreased total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol at 4, 8, 12, and 16 weeks vs. baseline levels in high-risk subjects.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
			• Decreased total cholesterol and HDL cholesterol at 4 and 8 weeks, • Decreased LDL cholesterol at 4 weeks, vs. baseline in normal subjects.
Shimada et al., 2009	Randomized double-blind placebo-controlled trial of γ-Aminobutyric acid (GABA)-rich Chlorella for 12 weeks in subjects with high to normal blood pressure and borderline hypertension.	• Placebo, green tablet indistinguishable in appearance, size, and color from the test article n = 39 • 20 mg GABA-rich Chlorella (Chlorella species unspecified, cultured with glutamic acid), n = 38	• No adverse events or abnormal laboratory findings were reported during the 12-week study or 4-week follow up observation period. • One subject from the control and one subject from the GABA-rich Chlorella group dropped out of the study due to suspected allergy. • Diarrhea was reported in both the placebo and GABA-rich Chlorella groups. • Systolic blood pressure in subjects receiving GABA-rich Chlorella significantly decreased compared to placebo controls.
Lee et al, 2010	Randomized, double-blinded, placebo-controlled trial in healthy male smokers, given *C. vulgaris* supplementation for 6 weeks	• Placebo (maltodextrin) control, n = 24 • 6.3 g/day *C. vulgaris* tablet, n = 28	• Adverse events were not reported by the authors • 1 drop out – ingestion of Chinese medicinal herb • *C. vulgaris* supplementation increased: o plasma levels of vitamin C and α-tocopherol o antioxidant enzyme (catalase and superoxide dismutase) activity • No changes in blood pressure • No harmful effects on DNA length
Nakano et al., 2010	Controlled trial in Japanese pregnant women ages 18-38 years, given *C. pyrenoidosa* from the 12^{th}-18^{th} week of gestation to delivery	• Placebo control, n = 38 • 6 g/day *C. pyrenoidosa* tablet, n = 32	• *C. pyrenoidosa* supplementation was well tolerated. Although discoloration of stool due to excreted chlorophyll was described, no other adverse reactions were observed. • Red blood cell count, hemoglobin, and hematocrit were significantly increased during the third trimester in *C. pyrenoidosa* supplemented women compared to placebo controls. • Incidence of anemia was decreased in the *C. pyrenoidosa* supplemented group during the second and third trimester compared to placebo controls. • Incidence of leg edema in the third trimester was statistically significantly decreased in *C. pyrenoidosa* supplemented women compared to placebo controls.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Kwak et al, 2012	Randomized, double-blinded, placebo-controlled 8-week study in healthy subjects receiving *C. vulgaris* extract	• Placebo control, n = 21 • 5 g/day *C. vulgaris* extract supplementation, n = 28	• No serious adverse reactions due to *C. vulgaris* extract supplementation were noted, compliance was 85% • Serum levels of interferon-γ (IFNγ) and interleukin (IL)-1β increased with supplementation. • Natural Killer (NK) cell activities increased with supplementation
Panahi et al., 2012a	Randomized, open-label clinical trial in dyslipidemic subjects receiving *C. vulgaris* for 8 weeks	• Control: 20 mg/day atorvastatin, n = 36 • 600 mg/day C. vulgaris + 20 mg/day atorvastatin, n = 27	• The authors did not report any adverse events following *C. vulgaris* supplementation. • No significant change was observed in serum levels of high-density lipoprotein cholesterol, alanine aminotransferase, creatine phosphokinase, creatinine, blood urea nitrogen, and fasting blood glucose • Serum alkaline phosphatase levels were increased in subjects receiving *C. vulgaris* + atorvastatin compared to atorvastatin alone.
Panahi et al, 2012b	Randomized, open label clinical trial in subjects with chronic obstructive pulmonary disease (COPD) or asthma receiving C. vulgaris extract for 8 weeks.	• Control, standard of care for asthma/COPD, n = 29 • 2700 mg/day *C. vulgaris* extract, n = 28	• The authors did not report any adverse events following *C. vulgaris* supplementation. • No significant differences observed in spirometric characteristics following *C. vulgaris* extract supplementation. • Oxidative stress biomarkers (malonedialdehyde, vitamin E, vitamin C, glutathione, glutathione peroxidase, catalase, superoxide dismutase) were increased following *C. vulgaris* extract supplementation. • Rate of improvement in severity and frequency of sputum brought up and wheezing was significantly greater in the *C. vulgaris* extract group compared to the control.
Azocar and Diaz 2013	Observational study of adults with chronic hepatitis C given *C. pyrenoidosa* for 12 weeks	• 30 mL of *C. pyrenoidosa* water soluble extract 2x/day + 3 500 mg *C. pyrenoidosa* tablets twice a day for the first week, then three times a day for the remaining 11 weeks., n = 32	• One subject discontinued treatment due to constipation on the first two days of treatment, resolved upon treatment withdrawal. • Main side effects associated with *C. pyrenoidosa* treatment were mild to moderate constipation and diarrhea but resolved within the first 2 weeks. • A majority (84.61%) of patients had a significant decrease in alanine aminotransferase (ALT) levels after 12 weeks of *C. pyrenoidosa* supplementation compared to baseline.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Panahi et al, 2013	Prospective, open-label clinical trial of 6-week *C. vulgaris* extract supplementation in male and female smokers, aged 17-62 years.	• 3600 mg/day *C. vulgaris* extract, n = 38	• 2 subjects dropped out of the trial due to gastrointestinal side effects. • Marked elevation of all assessed serum antioxidant measures (glutathione, superoxide dismutase, glutathione peroxidase, catalase, vitamin E, vitamin C, total antioxidant capacity) and significant reduction of malondialdehyde.
Ebrahimi-Mameghani et al., 2014	Double-blind, randomized, placebo-controlled trial of *C. vulgaris* in obese, non-alcoholic fatty liver disease (NAFLD) patients for 8 weeks	• Placebo control + 400 mg vitamin E/day n = 30 • 4 300 mg *C. vulgaris* tablets/day + 400 mg vitamin E/day, n = 30	• Adverse events were not reported by the authors. • *C. vulgaris* supplementation decreased body weight, alkaline phosphatase (ALP), and fasting blood sugar compared to baseline and placebo group.
Panahi et al., 2015	Randomized, open-label, controlled trial of 6-week *C. vulgaris* supplementation in male and female patients with major depressive disorder, aged 18-65.	• Standard antidepressant therapy (control), n = 50 • Standard antidepressant therapy + 1800 mg/day *C. vulgaris* extract, n = 42	• *C. vulgaris* extract was well tolerated and no serious adverse events were reported. Reported adverse events were one case of nausea and one case of diarrhea. These adverse events did not cause withdrawal from the trial. • Subjects receiving *C. vulgaris* extract had decreased total Beck Depression Inventory II score and cognitive subscales compared to subjects receiving standard antidepressant therapy. • Total Hospital Anxiety and Depression Scale, individual subscales of depression and anxiety were more reduced in the *C. vulgaris* group than the standard antidepressant therapy group.
Ebrahimi-Mameghani et al., 2017	Randomized, double-blinded, placebo-controlled trial in male and female obese subjects aged 20-50 years with non-alcoholic fatty liver disease (NAFLD), given *C. vulgaris* for 8 weeks	• Placebo control + 400 mg/day vitamin E, n = 26 • 4 300 mg/day *C. vulgaris* + 400 mg/day vitamin E, n = 29	• No adverse effects or symptoms following *C. vulgaris* supplementation were reported by the subjects. • *C. vulgaris* supplemented patients had statistically significant increased weight loss compared to the placebo control. This result was not considered adverse by the authors • Significant decreases in fasting serum glucose and tumor necrosis factor (TNF)-α were observed in *C. vulgaris* supplemented groups compared to control at the end of the study. This result was not considered adverse by the authors.

Table 35. Clinical Trials with *Chlorella* spp.

Reference	Study Design and Population	Test Article Groups	Outcomes and Safety Parameters
Haidari et al., 2018	Double-blind, randomized, placebo-controlled clinical trial in girls with primary dysmenorrhea given Chlorella supplementation for 8 weeks	• Placebo control soft gels (paraffin), n = 23 • 1500 mg/day Chlorella (species not indicated) soft gels, n = 23	• The authors did not report any adverse events. • Chlorella supplementation decreased prostaglandin E2, prostaglandin F2a, high sensitivity C-reactive protein, and malondialdehyde compared to the placebo control and to baseline levels. • Severity and duration of dysmenorrheal pain, as well as systemic symptoms of dysmenorrhea (fatigue, headache, nausea, vomiting, lack of energy) was significantly reduced in the Chlorella group compared to placebo control.

E. ALLERGENICITY

1. *Chlorella* spp. and Allergy in Existing Literature

A search and review of the publicly available literature on September 2nd, 2020 revealed that allergic responses to *C. sorokiniana* are not expected. Published studies of allergy have primarily been limited to C. *vulgaris, C. pyrenoidosa, C. saccharophila* and *C. homosphaera* (Tiberg et al., 1990a; Tiberg et al., 1990b; Ng et al., 1994; Yim et al., 2007; Tiberg et al., 1995). A study in Swedish children found that some individuals have IgE antibodies that recognize *Chlorella* spp. proteins and/or are positive in a skin prick test (Tiberg et al., 1995). One case study documented occupational-related asthma in response to dried *Chlorella* exposure (species not identified). The allergy was confirmed with inhalation and skin prick tests (Ng et al., 1994). A case of acute tubulointerstitial nephritis following three months of daily Chlorella food supplements (200 mg/tablet, 10 tablets/day) was described in an 11-year old boy (Yim et al., 2007). Renal function improved with cessation of the *Chlorella* supplements and a six-month regimen of corticosteroids. A follow-up skin prick test (100 mg and 200 mg *Chlorella* in 5 mL distilled water) six months after steroid therapy failed to produce positive wheal reactions for the 100 mg or 200 mg test amounts.

More recently, the allergenicity of two products (high lipid whole algalin flour, Szabo et al., 2012; whole algalin protein, Szabo et al., 2013) from *C. protothecoides* was assessed with a repeat-insult patch test performed in human subjects. The *C. protothecoides* products used in both studies did not induce allergic contact dermatitis in any of the subjects that completed the study (Szabo et al., 2012; Szabo et al., 2013). None of the reports addressed the potential for ingested *Chlorella* spp. to elicit an allergic response.

Chlorella spp. are widely used as a dietary supplement in Japan, with limited or no allergenic cases reported (Kubota *et al.,* 2012). The low frequency of documented allergic reactions to *Chlorella* spp. products supports that the allergenic potential of Chlorella Powder and Micro Powder is low.

2. Allergen Online Database Assessment for Allergenic Potential of *C. sorokiniana* CK-22 Used to Generate Chlorella Powder

A bioinformatics study was performed to determine if the dried biomass of *C. sorokiniana* is likely to cause allergic reactions in unsuspecting consumers due to potential cross-reactivity with proteins from other organisms (e.g. peanut, tree nuts, soybeans, etc.). To screen for potential allergens, amino acid sequences translated from the genome of *C. sorokiniana* were

compared with a database of known allergens (AllergenOnline.org v.15). This approach is not intended to absolutely identify allergens or cross-reactive proteins, but rather it is designed to identify proteins sufficiently similar to known IgE binding allergens.

a. Methods

The genome of a sample of *C. sorokiniana* from Chlorella Industry Co. was sequenced and translated into 10429 hypothetical protein coding sequences, based on previously published literature on *C. variabilis* (Blanc *et al.*, 2010). The *Chlorella* spp. genome contains introns and exons and consensus sequences for transcription/translation start and stop sites have not yet been characterized, meaning that the expressed protein sequences are hypothetical and based on prediction. The translated, hypothetical protein-coding sequences were compared to a database of known allergen amino acid sequences. The analysis performed here was similar to the E-score method described in Silvanovich et al. (2009). A hit is defined as 35% identity using an 80 mer sliding alignment. The Allergen online database (version 15) was queried with the 10429 hypothetical proteins by FASTA using an E-score cut-off of 10^{-7}. The likely relevance of a given alignment can be further evaluated by examining the percent identity and length of alignment, relative to the length of the *C. sorokiniana* protein and matched allergen. Identities of greater than 50% are generally considered significant and could be potential sources of IgE cross-reactivity, especially if the alignment covers most of the full length of both proteins.

b. Results

One hundred thirty-five hypothetical proteins had an E score of 10^{-7} or lower when compared to the Allergen Online database. These hypothetical protein hits partially aligned with known allergens in plants, molds, fish, and mammals, and one species of bacterium. Upon further inspection, the alignments revealed that 36/135 hits were greater than 50% identical (Table 36), but further analysis had large gaps in the amino acid sequences. These gaps suggest that although the alignments were statistically significant, the length of the aligned amino acid sequences were not likely to trigger an IgE allergy response. Five of the 135 hypothetical proteins had sequence identity with known Celiac disease reactants gliadin and glutenin; however, these proteins did not contain 100% sequence identity to gliadin or glutenin. All hits had gaps in their alignment, suggesting that although there are strings of sequence identity between *C. sorokiniana* and known allergens, none of these sequences are 100% identical and are therefore unlikely to provoke an allergic response.

Table 36. 36 Hypothetical Chlorella Protein Hits to Allergen Online Database with at Least 50% Sequence Identity

NCBI Protein GI (Protein type)	*Species* (common name)	%ID	E-value
373939374 (cyclophilin)	Daucus carota (carrot)	73.5	3.90E-47
1220142 (cyclophilin)	*Catharanthus roseus* (periwinkle)	71.3	1.30E-40
91680605 (cyclophilin)	*Aspergillus fumigatus* (fungus)	70.8	3.20E-48
729764 (HSP Cla h 4)	*Cladosporium herbarum* (fungus)	70.4	3.70E-141
9581744 (enolase)	*Hevea brasiliensis* (rubber tree)	69.7	2.00E-117
11124572 (triosphosphatase isomerase)	*Triticum aestivum* (wheat)	69	4.20E-74
83305635 (60S ribosomal P2)	Aspergillus fumigatus (fungus)	67.9	5.40E-20
253783729 (GAPDH)	*Triticum aestivum* (wheat)	66.8	2.50E-28
253783729 (GAPDH)	*Triticum aestivum* (wheat)	66	1.10E-57
83305621 (60S ribosomal P2)	*Aspergillus fumigatus* (fungus)	64.8	1.40E-108
84029333 (Glutathione)	*Oryza sativa* (rice)	64.2	1.20E-73
11124572 (triosphosphatase isomerase)	*Triticum aestivum* (wheat)	64	5.10E-31
37958141 (cyclophilin)	*Dermatophagoides farina* (house dust mite)	62.8	5.60E-34
373939374 (cyclophilin)	*Daucus carota* (carrot)	62.6	2.40E-39
14585755 (cytochrome c)	*Curvularia lunata* (fungus)	62.1	1.30E-24
4138173 (cyclophilin)	*Malassezia sympodialis* (fungus)	62	8.00E-22
291195949 (Aldolase A)	*Thunnus albacares* (tuna)	59.7	2.90E-71
156938901 (profilin)	*Glycine max* (soybean)	59.2	3.50E-29
208605346 (LMW gluten)	*Triticum aestivum* (wheat)	58.7	7.90E-12
208605348 (LMW gluten)	*Triticum aestivum* (wheat)	57.9	2.10E-24
371537645 (60S ribosomal P2)	*Penicillium crustosum* (fungus)	56.9	5.40E-18
729764 (HSP Cla h 4)	*Cladosporium herbarum* (fungus)	56.1	1.80E-75
73912496 (Omega-5 gliadin)	*Triticum aestivum* (wheat)	56	6.70E-23
4587985 (malate dehydrogenase)	*Malassezia sympodialis* (fungus)	54.7	9.80E-52
21748151 (nuclear transporter)	*Cladosporium herbarum* (fungus)	54.6	3.40E-23
1220142 (cyclophilin)	*Catharanthus roseus* (periwinkle)	54.1	4.50E-35
213511774 (Aldolase A)	*Salmo salar* (salmon)	53.4	5.40E-71
190684059 (periredoxin)	*Triticum aestivum* (wheat)	52.6	3.90E-45
149786150 (Mn superoxide dismutase)	*Pistacia vera* (pistachio)	52.1	7.40E-36
4587985 (malate dehydrogenase)	*Malassezia sympodialis* (fungus)	51.9	1.20E-39
291195949 (Aldolase A)	*Thunnus albacares* (tuna)	51.7	6.50E-48
1168402 (flavodoxin)	*Alternaria alternate* (fungus)	51.3	4.50E-21
76666767 (aldehyde dehydrogenase)	*Alternaria alternate* (fungus)	51.2	2.10E-90
373939374 (cyclophilin)	*Daucus carota* (carrot)	51	1.10E-22
73912496 (Omega-5 gliadin)	*Triticum aestivum* (wheat)	50.6	3.50E-25
8980491 (thioredoxin h)	*Triticum aestivum* (wheat)	50.5	6.40E-16

c. Conclusion

Taken together, the results show that allergic responses to *C. sorokiniana* are not expected, which is corroborated by the lack of allergenic cases reported following the ingestion of *Chlorella* spp.-based products in Japan. Additionally, the risk of developing an allergy to this species of *Chlorella* is expected to be the same as to the other *Chlorella* species that have been previously determined to be GRAS (GRN 469 and GRN 519).

F. REGULATORY APPROVALS ACROSS THE WORLD

Chlorella Powder and Micro Powder are approved for use in foods in Japan.

A related species, *C. protothecoides* S106, is the source organism for three separate GRAS notices that received “no questions” from the FDA: an algal oil (GRN 384), flour with 40-70% lipid (GRN 469), and flour with 40-75% protein (GRN 519).

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

All information included in the following list of references is generally available.

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B. EXPERT PANEL STATEMENT

This GRAS determination for the use of Chlorella Powder for the intended use specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug and Cosmetic Act (FFDCA) as described under 21 CFR §170.30(b). The safety of the intake of Chlorella Powder has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of Chlorella Powder as an ingredient for the intended uses in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. Chlorella Powder is the spray-dried whole cell biomass of *Chlorella sorokiniana* CK-22. Chlorella Micro Powder is jet pulverized Chlorella Powder. The only difference between Chlorella Powder and Chlorella Micro Powder is particle size. The particle size distribution of Chlorella Powder is 19 μm (10 percentile), 60 μm (50 percentile), and 134 μm (90 percentile). The particle distribution of the Chlorella Micro Powder is 4 μm (10 percentile), 12 μm (50 percentile), 22 μm (90 percentile).

2. Manufacturing complies with current Good Manufacturing Practice.

3. Compliance with appropriate specifications and quality control parameters assures the production of a food grade product.

4. All medium ingredients and food contact materials are food grade and comply with the conditions of use and specifications of the United States Code of Federal Regulations, Title 21 and/or Food Chemicals Codex.

5. The major constituents of Chlorella Powder are normal components of the diet and are anticipated to be digested and metabolized in established pathways similar to those occurring with the ingestion of other plants and microalgae.

6. Pivotal toxicology studies demonstrate safety of *C. sorokiniana* CK-22. These studies were performed on the spray-dried biomass, Chlorella Powder.

    a. Ethanol and hot water extracts of Chlorella Powder did not induce DNA damage as assessed by an Umu genotoxicity assay (Himuro et al., 2014).

b. An acute oral toxicity study was performed in male and female Wistar rats fed Chlorella Powder at 0, 1000, 2000, and 5000 mg/kg. No deaths and no differences in body weight were observed. No observable differences were noted between the treatment groups and controls (Himuro et al., 2014).

c. A 28-day repeated oral dose study was performed in male and female Wistar rats fed Chlorella Powder in the diet at 0, 2.5, 5, and 10%. No significant treatment-related adverse effects were observed in any of the parameters evaluated (Himuro et al., 2014).

d. The safety of Chlorella Powder was evaluated in a published 90-day toxicology study performed with 0, 2.5, 5, and 10% Chlorella Powder mixed into the feed in male and female Wistar rats. During the experimental period, no Chlorella Powder treatment-induced differences in general condition, body weight gain, food and water consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, histopathology, or animal death were observed. The no-observed-adverse-effect (NOAEL) was calculated to be 5.94 and 6.41 g/kg body weight/day for male and female rats, respectively (Himuro et al., 2017).

e. Chlorella Powder and Micro Powder differ only in particle size, the safety will be the same between the two forms.

7. Additional studies in *Chlorella* spp. corroborate the pivotal safety studies.

a. A safety assessment of high lipid whole algalin flour from *C. protothecoides* was published in 2012 (Szabo et al., 2012). Whole algalin flour was not mutagenic by bacterial reverse mutation assay (up to 5000 µg/plate) or clastogenic by in vivo chromosome aberration assay (2000 mg/kg in mice). The 90-day toxicity study calculated the NOAEL to be 100000 ppm, the highest dose tested, corresponding to 4807 mg/kg body weight/day in male rats and 5366 mg/kg body weight/day in female rats.

b. A safety evaluation on whole algalin protein from *C. protothecoides* was published in 2013 (Szabo et al., 2013). Whole algalin protein was not mutagenic by bacterial reverse mutation assay (up to 5000 µg/plate) or clastogenic by in vivo chromosome aberration assay (2000 mg/kg in mice). The 90-day toxicity study calculated the NOAEL to be 100000 ppm, the highest dose tested, corresponding to 4805 mg/kg body weight/day in male rats and 5518 mg/kg body weight/day in female rats.

8. Application of a 100-fold safety factor to the NOAEL, determined in the pivotal 90-day toxicology study, results in an acceptable daily intake for Chlorella Powder of 59.4 mg/kg/day or 3.56 g/day for a 60 kg human.

9. Clinical studies have reported that other products derived from *Chlorella* spp. are well-tolerated up to 6 g/day for up to 6 months.

10. The addition of Chlorella Powder or Chlorella Micro Powder to the intended foods will result in a mean estimated daily intake (EDI) of 522 mg/day (7.8 mg/kg/day) and a heavy consumer (90^{th} percentile) intake of 870 mg/day (13.0 mg/kg/day).

11. The safety of Chlorella Powder and Chlorella Micro Powder is supported by appropriate documentation of the safety of the source organism, appropriate food grade specifications, a well-controlled production process, and demonstrated safety in pivotal genotoxicity and 90-day rodent bioassays. Ingestion of Chlorella Powder and Chlorella Micro Powder at the proposed EDI is determined to be safe and GRAS.

Therefore, Chlorella Powder is safe and GRAS at the proposed level of addition to food. Chlorella Powder is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: [redacted]

Date: December 17, 2020

A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
Harvard School of Public Health

Signature [redacted]

Date: December 17, 2020

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Principal, Pondview Consulting LLC

Signature: [redacted]

Date: December 17, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signature [redacted]

Date: December 17, 2020

800 pages have been removed in accordance with copyright laws. The removed reference citations can be found in Part VII A. REFERENCES.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000986	Dec 17, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person: Eiichiro Itanami		Position or Title: CEO	
Organization *(if applicable)*: Chlorella Industries Co., Ltd.			
Mailing Address *(number and street)*: 2-4-6, Shiba-daimon, Minato-ku			
City: 105-0012	State or Province: Tokyo	Zip Code/Postal Code: 105-0012	Country: Japan
Telephone Number: 301-775-9476	Fax Number:	E-Mail Address:	

1b. Agent or Attorney *(if applicable)*

Name of Contact Person: Claire Kruger		Position or Title: Managing Partner	
Organization *(if applicable)*: Spherix Consulting Group, Inc.			
Mailing Address *(number and street)*: 11821 Parklawn Drive, Suite 310			
City: Rockville	State or Province: Maryland	Zip Code/Postal Code: 20852	Country: United States of America
Telephone Number: 301-775-9476	Fax Number:	E-Mail Address: ckruger@spherixgroup.com	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Chlorella Powder

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 469

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRN 384

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Chlorella intends to add Chlorella Powder or Chlorella Micro Powder to selected foods in the U.S. food supply. The individual proposed food uses and use levels for Chlorella Powder or Chlorella Micro Powder employed in the current intake analysis are summarized in Table 19 of the GRAS notification.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Chlorella Industries Co., Ltd.
(name of notifier)

has concluded that the intended use*(s)* of Chlorella Powder
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Chlorella Industries Co., Ltd. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

2-4-6, Shiba-daimon, Minato-ku, Tokyo 105-0012, Japan
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Claire L. Kruger, PhD Digitally signed by Claire L. Kruger, PhD Date: 2020.12.17 14:02:16 -05'00'	Claire L. Kruger, PhD, DABT, Managing Partner	12/17/2020

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Chlorella GRAS 12-17-20.pdf	Submission
	References	Submission

From:	Kampmeyer, Christopher
To:	"ckruger@spherixgroup.com"
Subject:	Questions for GRN 000986
Date:	Tuesday, July 20, 2021 3:48:00 PM
Attachments:	image001.png 2021-07-20 GRN 000986 Questions for Notifier.pdf

Dear Dr. Kruger:

During our review of GRAS Notice No. 000986, we noted some questions that need to be addressed and are attached to this email.

We respectfully request a response within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,
Chris Kampmeyer

Chris Kampmeyer, M.S.
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
christopher.kampmeyer@fda.hhs.gov

July 20, 2021

Dear Dr. Kruger:

After reviewing Chlorella Industries Co., Ltd.'s GRAS notice (GRN 000986) for the intended use of *Chlorella* powder and *Chlorella* micro powder, we noted the following questions. We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame or have questions, please contact me to discuss via email at christopher.kampmeyer@fda.hhs.gov.

Chemistry:

1. The notifier describes two products, *Chlorella* powder and *Chlorella* micro powder, with particle size being the difference between the two products. Please clarify if there is any difference in the intended uses (e.g., food categories and use levels) between these two products. In addition, please indicate if these two products are intended to be substitutional for one another or if they will be used in combination in the same foods.
2. The notifier states that a magnetic stirrer is used at two different points in the manufacturing process. We are aware that this is commonly used in the processing of algae. Please clarify the material that comprises the strainer, indicate the function of this step during the processing of your ingredient, and indicate the types of impurities removed during this process.
3. Please specify that all analytical methods are validated for their intended use.
4. The notifier provides an exposure estimate based on food consumption data from the 2015—2016 National Health and Nutrition Examination Survey (NHANES). In order to clarify the intended uses and allow us to verify the exposure estimate, please provide the NHANES food codes used in their exposure estimate.
5. In Tables 3 and 4 (pages 21 and 22), the notifier states that the limit of detection (LOD) for the analysis for chlorophyll b is 0.08 g/100 g but lists the results of the batch analyses as "detectable." Please clarify what is meant by the term "detectable."
6. The notifier provides specifications for chromium and lead of <2 mg/kg and <1 mg/kg, respectively. The results of the batch analyses indicate that chromium is not detected at a LOD of 0.5 mg/kg and lead is not detected at a LOD of 0.2 mg/kg. It is not clear why a specification for chromium of 4 times the results of the batch analyses and a specification for lead of 5 times the batch analyses are

needed. Please consider lowering the specifications for lead and chromium, accordingly.

7. It appears that *Chlorella* powder and *Chlorella* micro powder were not analyzed for the same pesticides. For *Chlorella* powder, Table 5 indicates that 340 pesticides were listed as "not detected." For *Chlorella* micro powder, Table 11 indicates that 354 pesticides were listed as "not detected." There were 3 pesticides listed as "not detected" only in *Chlorella* powder and 17 listed as "not detected" only in *Chlorella* micro powder. Therefore, it is not clear why there were differences in the pesticides in the two products if the only difference in the products is the particle size. Please clarify if similar analyses were conducted for each product and address the discrepancies in the pesticide analyses.

Microbiology:

1. For the administrative record, please provide a detailed description of *Chlorella sorokiniana* strain NITE SD 00247 including genotypic (e.g., pathogenicity and toxigenicity) and phenotypic characteristics (e.g., production of antimicrobials, production of secondary metabolites and toxins, antimicrobial resistance), and whether this poses a safety concern.
2. On page 5, the notifier states, "Although CK-22 was originally identified as *C. vulgaris* CK-22 by morphology, CK-22 has been redesignated as *C. sorokiniana* following 18S rRNA sequencing." Please elaborate and provide a reference (as applicable) for this statement.
3. On page 7, the notifier states, "Comparison of the 18S rRNA sequences of CK-22 with *C. sorokiniana* SAG 211-8k (accession number X62441, Culture Collection of Algae at the University of Göttingen, Germany) and *C. vulgaris* SAG 211-11k (accession number X13688) shows that the CK-22 18S rRNA sequence is 99.8% similar to the *C. sorokiniana*, SAG 211-8k sequence and 99.5% similar to *C. vulgaris* SAG 211-11b." Please discuss whether the full genomic sequence for *C. sorokiniana* strain NITE SD 00247 is publicly available and provide the corresponding accession number.
4. On page 8, the notifier states that the method used to detect yeast and mold is USP 62, which corresponds to Microbiological Examination of Nonsterile Products: Test for Specified Microorganisms. This method does not appear to include a method to detect yeast and mold. For the administrative record, please clarify this discrepancy.
5. On page 9, the notifier describes the manufacturing process and states that *C.*

sorokiniana strain NITE SD 00247 is "... expanded to a shallow, outdoor pool by pipeline and cultured in the presence of ambient light and temperature with agitation." Please discuss whether the outdoor pool is open to the natural environment or whether it is in a closed or controlled setting (i.e., a laboratory or other facility). If the pool is open to the natural environment, please discuss how contamination is controlled.

6. On page 9, the notifier states, "The final slurry is passed through a magnetic strainer, cooled to 2-5 ºC, and stored for up to 24 hours before undergoing heat inactivation for 3 minutes at 100 ºC." Please clarify what "heat inactivation" means and how the notifier ensures that the cells are inactivated.
7. On page 33, the notifier states, "*Chlorella* spp. have not been reported to produce marine toxins." Please provide a reference for this statement.
8. Please state whether any of the raw materials used in the fermentation are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.
9. In reviewing the publicly available literature, we identified published reports of infections in animals and humans from *Chlorella* species (i.e., chlorellosis). Please provide an updated literature search including the date (month and year) the literature search was performed and discuss the reports of infections in animals and humans from *Chlorella* species and whether these reports may be contradictory to a GRAS conclusion.
10. References to "*Salmonella typhimurium*" on pages 34 and 36 should read "*Salmonella* Typhimurium" as serovars are not italicized. Please make a statement that corrects this reference.

Toxicology:

1. On page 23, the notifier states that detectable levels of 2,4,4'-Trichlorobiphenyl were identified in a single lot of *Chlorella* micro powder. Please discuss why the level of 2,4,4'-Trichlorobiphenyl does not present a safety concern.
2. Please clarify if the intended uses of your *Chlorella* powder and *Chlorella* micro powder will be substitutional for other similar *Chlorella*-derived food ingredients or if these uses would be additive. If the intended use would be additive, please provide a cumulative dietary exposure for *Chlorella* ingredients in the diet and discuss the safety information that supports the safe use of *Chlorella* ingredients at the cumulative dietary exposure.

3. On page 33, the notifier states that the safety of the notified material is supported by GRAS notifications for food ingredients derived from *Chlorella* species in GRNs 000384, 000569, and 000519. We believe that the notifier had meant to reference GRN 000469, as stated in a prior section of the notice, and not GRN 000569 (GALACTO-OLIGOSACCHARIDES). Please state if you concur.

4. On page 33, the notifier describes the NOAEL obtained from a 90-day toxicity study of *Chlorella* powder and cites Himuro et al., 2014. Please note that the stated reference does not describe a 90-day toxicity study. We believe that the notifier had meant to reference Himuro et al. (2017), as stated in other sections of the notice. Please state if you concur.

5. On page 33, the notifier states that "The safety of intake is also supported by corroborating published clinical studies of *Chlorella* spp. ingestion with no adverse events reported." However, in the Himuro et al. (2017) manuscript, it states that adverse events related to the *Chlorella* genus have been reported such as erythematopurpuric lesions on sun-exposed areas (Jitsukawa et al., 1984) and occupational asthma (Ng et al., 1994).

    - Please address these reports of adverse effects and describe why such reports should not raise safety concerns related to the intended use of your *Chlorella* powder as a food ingredient.

6. The cited manuscript, Himuro et al. 2017, indicates that *Chlorella* species contain high levels of carotenoids, which is not discussed in the compositional narrative of your notice.

    - Please discuss the level of carotenoids that are present in your *Chlorella* powder, and why such levels do not present a safety concern, particularly for special populations like smokers.

7. The cited 90-day subchronic toxicity study in rodents, Himuro et al. (2017), indicates that histopathological analyses was only conducted in two organs, kidney and liver. Therefore, the study is not compliant with OECD Guideline 408.

    - Please provide a scientific rationale to support the determination of a NOAEL/ADI from a pivotal 90-day toxicity study that did not contain a thorough toxicological assessment of histopathological lesions in all relevant organ systems.

8. The cited 90-day subchronic toxicity study in rodents, Himuro et al. (2017), indicates a consistent effect of increased neutrophil number in male rats in both cited 28-day and 90-day toxicity studies of the subject *Chlorella* powder. The authors cite that this alteration is within the normal range for neutrophils in this strain (5.4-37.5%) and cite a study by Traesel et al. (2016). The Traesel et al. (2016)

study is a subchronic toxicity study of *Caryocar brasiliense* oil extract in Wistar rats obtained from the State University of Maringa. This study does not present any historical control data describing the typical range of neutrophil counts in this strain of rats. Neutrophil counts (up to 31%) were observed in study controls, however, there is no information in the article to support that this level is within the normal biological range observed in this strain of rat. Please note that the "normal biological range" for assessed parameters is based on control data from numerous studies that were conducted under different testing paradigms and animal husbandry/housing conditions. Historical controls may include naïve controls, saline treated controls, DMSO treated controls, cyclodextrin treated controls and the like. Therefore, it is important to compare the treatment group with the concurrent controls and assess the biological significance of the observed changes.

- An increase in neutrophils may be associated with infection, inflammation, injury, exposure to xenobiotics, and given the statements above, please provide additional information to support the notion that the observed increased neutrophil counts in male Wistar rats exposed to *Chlorella* powder is within the historical range for this strain of rat or lacks biological significance.

- A similar rationale is presented with regard to serum triglyceride levels and A/G ratio. The notifier states that Traesel et al. (2016) indicates that the measured values are within the normal distribution for this strain of rat. Given the importance of concurrent controls in an experiment, please discuss further how the cited study provides adequate support/information to support this notion or cite other generally available scientific publications/reports regarding historical ranges for clinical laboratory parameters in the appropriate rat strain. Also, please explain why the observations do not indicate a potential safety concern.

9. The notifier attempts to utilize safety information from other algal sources or *Chlorella* species to corroborate the safety of their subject source organism, *Chlorella sorokiniana* strain NITE SD 00247. The notifiers describe corroborative safety information related to *C. vulgaris*, *C. protothecoides*, *C. pyrenoidosa*, C. *stigmatophora*, *C. regularis*, as well as many studies performed with unspecified *Chlorella* species. However, the provided scientific discussion and rationale is insufficient to establish a case for read-across between these organisms.

    - Please provide additional information and scientific rationale to establish that *C. sorokiniana* strain NITE SD 00247 is sufficiently similar to other *Chlorella* species discussed in the notice. Such rationale might include information

related to composition including any secondary metabolite, anti-nutrient, and potential allergenic or toxic protein profile that addresses the similarity/differences of *C. sorokiniana* strain NITE SD 00247 to other *Chlorella* species. Discussions should clearly address any safety concerns related to differences in pathogenic, toxigenic, and allergenic properties between *C. sorokiniana* strain NITE SD 00247, and other *Chlorella* species with an established safety profile.

10. Information in the notice and in the available literature suggest that the *Chlorella* species/strain and manufacturing and/or culturing methods can significantly impact the composition of the microalgal biomass and derived substances. There may be additional concerns related to levels of toxic contaminants such as heavy metals[1] or cyanotoxins[2] in some preparations. The safety narrative summarizes numerous corroborative animal toxicity and clinical studies with *Chlorella* species derived preparations. However, it is unclear how the compositional profile of these *Chlorella* test materials compares to the subject *Chlorella* powder/micro powder. In the absence of information to establish compositional similarity, the cited studies do not corroborate the safety of your *Chlorella* powder/micro powder.

    - Please provide additional information and scientific rationale that the composition of your *Chlorella* powder/micro powder is sufficiently similar to the *Chlorella*-derived test materials used in corroborative studies. Discussions might consider proximate nutrient content, vitamins/minerals, and the presence/absence of toxic and allergenic components.

11. The generally available literature identifies numerous cyanotoxin contaminants of possible concern identified in products derived from algal biomass, such as microcystins, anatoxin-a, cylindrospermopsin, saxitoxin, and β-methylamino-L-alanine.[3] Notably the risk of unintended contamination by toxic cyanobacteria is increased in uncontained open pond culture systems. In the notice, the subject material is routinely assessed for microcystin and aflatoxin levels to ensure they meet the described specifications.

    - Please address how the limited toxin panel described in the notice adequately addresses safety concerns related to unintended toxin contamination.

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[1] Heussner AH, Mazija L, Fastner J, Dietrich DR. Toxin content and cytotoxicity of algal dietary supplements. Toxicol Appl Pharmacol. 2012 Dec 1;265(2):263-71. doi: 10.1016/j.taap.2012.10.005. Epub 2012 Oct 12. PMID: 23064102.

[2] Afkar, E., Ababna, H., & Fathi, A. A. (2010). Toxicological Response of the Green Alga Chlorella vulgaris, to Some Heavy Metals. American Journal of Environmental Sciences, 6(3). doi:10.3844/ajessp.2010.230.237

[3] Roy-Lachapelle, Audrey et al. “Detection of Cyanotoxins in Algae Dietary Supplements.” Toxins vol. 9,3 76. 25 Feb. 2017, doi:10.3390/toxins9030076

12. Rapid increases in growth/biomass indicates high levels of protein and RNA synthesis. Following consumption, increased RNA breakdown and metabolism could be associated with elevated levels of uric acid and corresponding increased risk of kidney stone formation, or gout. Furthermore, increased plasma uric acid levels have been observed in rats administered *C. vulgaris* in the diet (19.6 g/kg/day) (Selah et al., 1985).

    - Please discuss the RNA content in your subject *Chlorella* powder/micro powder and levels that would be present in food based on your intended use, and address whether this will pose or not pose any safety concern.

13. On page 68, the notifier states, "The studies in Table 38 all used *Chlorella vulgaris* in the diet or delivered via oral gavage"; however, there does not appear to be a Table 38 in the notice. We believe the notifier intended to reference Table 34; please state if you concur.

14. Please note that much of the information presented in the "safety endpoint" columns of Tables 33-35 do not contain information related to safety but summarize efficacy endpoints or possible benefits of *Chlorella* intake reported in the cited studies. Purported efficacy or benefits of *Chlorella* consumption are not relevant for a GRAS conclusion.

15. The allergenicity section needs to discuss step-by-step the entire process based on the following issues that are not clearly described:

    There are two statements made where the contexts are missing and confusing, such as the statement, "...The analysis performed here was similar to the E-score method described in Silvanovich et al. (2009). A hit is defined as 35% identity using an 80 mer sliding alignment..." **and** "The Allergen online database (version 15) was queried with the 10429 hypothetical proteins by FASTA using an E-score cut-off of 10^{-7}."

    It seems that two different paradigms were used: the CODEX recommendation, which is a generally recognized bioinformatic paradigm for allergenicity analysis (with a long history of use) and the paradigm recently proposed by Abdelmoteleb et al. (2021) where the authors proposed the use of an E-score cut-off of 10^{-7}.

    - Please describe the method clearly and in detail step-by-step. Some examples of the steps are mentioned below. Please provide the detail of all the steps of the process as it was performed (do not restrict only to the steps described below; they are just examples), including:

- How the genomic data was harnessed (provide the accession number/RefSeq number if applicable/assembly version, of the genome).
- How all the annotated ORFs were identified emphasizing on the method/any software used **or** whether only the annotated available protein data was downloaded (and in which format). Please describe this step clearly and in great detail and provide links that were used, so the process can be independently replicated.
- How the entire annotated proteome was compared (CODEX method/ Abdelmoteleb et al. method) and distilled into a smaller set of annotated protein. Please clearly describe this method in detail and provide links that were used, so that the process can be independently replicated.

There is no set recommendation of an E-score. Setting an E-score cut-off depends on the size of the database used and the query length. Many experts have proposed different E-score cut-offs. There is a lack of general recognition on the usefulness of $E=10^{-7}$ as the definitive cut-off. Therefore, please address whether this method has undergone a rigorous validation using many examples (i.e., many genomes). When proposing a cut-off E-score as the basis of bioinformatics-driven decision making, the utility of the proposed E-score cut-off should be rigorously validated using many examples.

A clear and step-by-step description of the process (so it can be independently replicated) will help determine the utility of this paradigm.

- Once an E-score cut-off is set, any higher value than the set value is not reported in the output. Please explain why an E-score of 10^{-7} in a small database like AllergenOnline (containing 2200 sequences) will not be too stringent and hence not retrieve many relevant hits.

16. NCBI has discontinued the GI numbers as of September 2016. Please provide the corresponding accession number of all the entries in Table 36.
17. On page 80, the notifier states, “Five of the 135 hypothetical proteins had sequence identity with known Celiac disease reactants gliadin and glutenin; however, these proteins did not contain 100% sequence identity to gliadin or glutenin.” Please identify the accession numbers of these sequences.
18. On page 80, the notifier states, "...but further analysis had large gaps in the amino acid sequences. These gaps suggest that although the alignments were statistically significant, the length of the aligned amino acid sequences were not likely to trigger an IgE allergy response."

- Please provide a relevant scientific citation to support this statement.
- Please state whether these 36 hits show >35% identity over a sliding window of 80 amino acids; also state how many 8-aa epitopes were identified.

Regulatory:

1. On page 7 you state, "All fermentation vessels, food contact materials, raw materials, and processing aids are U.S. food grade." Please state whether all fermentation vessels, food contact materials, raw materials, and processing aids are approved for their intended use.

2. Please clarify whether *Chlorella* powder/micro powder will impart color to food, intentionally or not. If *Chlorella* powder/micro powder is expected to impart color, please clarify whether any imparted color is important. Material that otherwise meets the definition of color additive can be exempt from that definition on the basis that it is used or intended to be used solely for a purpose or purposes other than coloring, as long as the material is used in a way that any color imparted is clearly unimportant insofar as the appearance, value, marketability, or consumer acceptability is concerned.

3. Please specify that *Chlorella* powder/micro powder is not intended to be used in infant formula, or in any products under the jurisdiction of the United States Department of Agriculture.

Sincerely,

Chris Kampmeyer, M.S.
Regulatory Review Scientist
FDA Center for Food Safety and
Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients

Spherix Consulting Group

August 19, 2021

Chris Kampmeyer, M.S.
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
US Food and Drug Administration
5001 Campus Drive, HFS-225
College Park, MD 20740

RE: Questions Regarding GRN 000986

Dear Mr. Kampmeyer:

In response to your email of July 20, 2021, following are our responses to your request for additional information regarding GRN 000986. The FDA questions are shown in *italicized text*. As discussed via email today, a CD containing a copy of this response, references, and all related files will be sent to your attention via UPS for delivery on Monday, August 23, 2021.

Chemistry:

1. *The notifier describes two products, Chlorella powder and Chlorella micro powder, with particle size being the difference between the two products. Please clarify if there is any difference in the intended uses (e.g., food categories and use levels) between these two products. In addition, please indicate if these two products are intended to be substitutional for one another or if they will be used in combination in the same foods.*

   The original proposed food use table combined the food use and estimated intakes for Chlorella Powder and Micro Powder. The individual proposed food uses and estimated intakes for Chlorella Powder and Chlorella Micro Powder are shown below in Tables 1 and 2. The estimated daily intake (EDI) calculation for the combined Chlorella Powder and Chlorella Micro Powder ingredients are not affected by separating out the proposed food uses because these two products are not intended to be substitutional for one another and will not be used in combination in the same food.

Table 1. Summary of Proposed Food Use and Estimated Intake for Chlorella Powder

Food Category	Proposed Food Use	Use Level (mg/g)	Serving Size (g/serving)	Estimated Intake (mg/serving)
Snack Food	Nutrition Bars	5	100	500
Drinks	Protein and nutritional powders	10	50	500

751 Rockville Pike, Unit 30-B
Rockville, MD 20852
301-557-0375; info@spherixgroup.com
http://www.spherixgroup.com

Table 2. Summary of Proposed Food Use and Estimated Intake for Chlorella Micro Powder				
Food Category	**Proposed Food Use**	**Use Level (mg/g)**	**Serving Size (g/serving)**	**Estimated Intake (mg/serving)**
Baked Goods	Yeast Breads	10	50	500
	Doughnuts	5	30	150
Grains	Pasta	10	50	500
	Noodles	10	65	650

2. *The notifier states that a magnetic stirrer is used at two different points in the manufacturing process. We are aware that this is commonly used in the processing of algae. Please clarify the material that comprises the strainer, indicate the function of this step during the processing of your ingredient, and indicate the types of impurities removed during this process.*

   The magnetic strainer consists of a magnet covered in stainless steel. This magnetic strainer is in place to collect any metal impurities.

3. *Please specify that all analytical methods are validated for their intended use.*

   All analytical methods have been validated for their intended use.

4. *The notifier provides an exposure estimate based on food consumption data from the 2015—2016 National Health and Nutrition Examination Survey (NHANES). In order to clarify the intended uses and allow us to verify the exposure estimate, please provide the NHANES food codes used in their exposure estimate.*

   The NHANES food codes used to calculate the exposure estimate for Chlorella Powder and Chlorella Micro Powder are shown in Appendix 1. Each food code is shown with a description to clarify the intended uses.

5. *In Tables 3 and 4 (pages 21 and 22), the notifier states that the limit of detection (LOD) for the analysis for chlorophyll b is 0.08 g/100 g but lists the results of the batch analyses as "detectable." Please clarify what is meant by the term "detectable."*

   A chlorophyll b specification of "detectable" is a requirement for the Japan Health and Nutrition Food Association (JHNFA) as an identity specification for green algae food products. Chlorella Industry Co. has provided the amounts of chlorophyll b present in three lots of Chlorella Powder and three lots of Chlorella Micro Powder.

Table 3. Chlorophyll b Content in Chlorella Powder and Chlorella Micro Powder			
Chlorella Powder Lot Number	**#170725**	**#180926**	**#190805**
Chlorophyll b (g/100 g)	0.7	0.7	0.7
Chlorella Micro Powder Lot Number	**#170728**	**#180914**	**#190805**
Chlorophyll b (g/100 g)	0.8	0.7	0.7
Method: Alkaline pyridine method, Analytical Biochemistry 57,255-267(1974)			

6. *The notifier provides specifications for chromium and lead of <2 mg/kg and <1 mg/kg, respectively. The results of the batch analyses indicate that chromium is not detected at a LOD of 0.5 mg/kg and lead is not detected at a LOD of 0.2 mg/kg. It is not clear why a specification for chromium of 4 times the results of the batch analyses and a specification for lead of 5 times the batch analyses are needed. Please consider lowering the specifications for lead and chromium, accordingly.*

   Chlorella Industry Co. will lower the specification for lead to <0.5 mg/kg and the specification for chromium to <1 mg/kg. These specifications will be used for all future batches of both Chlorella Powder and Chlorella Micro Powder.

7. *It appears that Chlorella powder and Chlorella micro powder were not analyzed for the same pesticides. For Chlorella powder, Table 5 indicates that 340 pesticides were listed as "not detected." For Chlorella micro powder, Table 11 indicates that 354 pesticides were listed as "not detected." There were 3 pesticides listed as "not detected" only in Chlorella powder and 17 listed as "not detected" only in Chlorella micro powder. Therefore, it is not clear why there were differences in the pesticides in the two products if the only difference in the products is the particle size. Please clarify if similar analyses were conducted for each product and address the discrepancies in the pesticide analyses.*

   Three lots of Chlorella Powder (lot numbers 180704, 170725, and 160705) and Chlorella Micro Powder (lot numbers 180705, 170728, 160708) were screened for pesticides by Vision Bio. Vision Bio used two different pesticide screens for this analysis, one standard screen and one designed for pesticides in vegetables. One lot of Chlorella Powder (Lot No. 180704) and two lots of Chlorella Micro Powder (Lot numbers 180705 and 160708) were assessed with the standard screen. Two lots of Chlorella Powder (Lot numbers 170725 and 160705) and one lot of Chlorella Micro Powder (Lot 170728) were assessed with the vegetable pesticide screen. 345 pesticides are included in both screens provided by Vision Bio. The results of the 345 pesticides from the combined screens is shown for three lots of Chlorella Powder and three lots of Chlorella Micro Powder in Appendix 2.

Microbiology:

1. *For the administrative record, please provide a detailed description of Chlorella sorokiniana strain NITE SD 00247 including genotypic (e.g., pathogenicity and toxigenicity) and phenotypic characteristics (e.g., production of antimicrobials, production of secondary metabolites and toxins, antimicrobial resistance), and whether this poses a safety concern.*

   As described in the GRAS notice on page 7, a comparison of the *18S* rRNA sequences of CK-22 with *C. sorokiniana* SAG 211-8k and *C. vulgaris* SAG 211-11k shows that the CK-22 *18S* rRNA sequence is 99.8% similar to the *C. sorokiniana* SAG 211-8k sequence and 99.5% similar to *C. vulgaris* SAG 211-11b. The pathogenicity is addressed in Toxicology: Question 9.

In order to address the question of phenotypic characteristics of *Chlorella sorokiniana* including secondary metabolites, toxins, antimicrobials, or antimicrobial resistance, a search of the literature using both GoogleScholar and PubMed was performed in August of 2021.

A hit was considered for further review if it met the following inclusion criteria: the hit was in English, published in a peer-reviewed journal/publication, and described a study performed using *C. sorokiniana*. Hits were excluded from further review if the test article was an uncharacterized extract derived from *C. sorokiniana*, or if *C. sorokiniana* was cultured or treated with an agent that is not reasonably expected to be present during Chlorella Industry Co.'s described production process (such as co-culture experiments, use of *C. sorokiniana* as a bioremediator of toxins or heavy metals, or studies of culturing *C. sorokiniana* in wastewater). Co-culture experiments were excluded from further review due to the monoculture utilized by Chlorella Industry Co. This search was limited to hits since 1999 due to the genotypic definition of *C. sorokiniana* (Huss et al., 1999).

None of the studies reviewed described production of toxins by *C. sorokiniana*. For completeness, the search terms were expanded to include all species within the *Chlorella* genus, with no studies describing the production of toxins. A recent book chapter confirms the results of this search, stating that no microalgae from the Chlorellaceae family have been reported to produce toxins (Markou, Chentir, and Tzovenis, 2021). It is important to note that the methodologies used, and the results obtained from co-culture experiments do not yield relevant information for the risk assessment of *C. sorokiniana* for human ingestion. For example, *C. sorokiniana* is frequently used in co-culture experiments with toxin producing cyanobacteria since it is a known competitor for resources (Schmidt et al., 2020), and may produce small molecular weight peptides and glycosides that are toxic to predators of *Chlorella* spp. such as the bacteria *Vampirovibrio chlorellavorus*, although the identity of these antibacterial compounds remains to be elucidated (Bagwell et al., 2016). Under stress conditions, *Chlorella* spp. are known to produce long chain fatty acids and polysaccharides that may be toxic to microcystin producing cyanobacteria and fecal coliforms in wastewater, but the mechanism and discrete compounds that exert this effect have not been determined (Dar, Sharma, and Kaur, 2019).

Similarly, none of the retrieved studies identified secondary metabolites that would pose a safety concern following the consumption of Chlorella Powder- or Chlorella Micro Powder-containing products in humans. Although *C. sorokiniana* has been reported to produce a variety of biomedically-important secondary metabolites in response to altered culture conditions and treatments, including antioxidants and flavonoids (Ansilago et al., 2021), as well as the carotenoids lutein, astaxanthin, β-carotene (Cordero et al., 2011; Azaman et al., 2020; Khalili et al., 2019; Matsukawa et al., 2000) and α-tocopherol (Matsukawa et al., 2000), none of these studies reported secondary metabolites that would pose a safety concern. Additionally, an expanded search of secondary metabolites produced by other species within the *Chlorella* genus identified studies that describe the production of multiple

metabolites, including carotenoids, amino acids, fatty acids, polysaccharides, vitamins, and antioxidants. None of these additional studies reported secondary metabolites that would pose a safety concern (Gautam et al., 2019; Yusof et al., 2011; Vello et al., 2018; de Morais et al., 2015).

The antimicrobial activity of *Chlorella* spp. has been characterized in co-culture with cyanobacteria, in the treatment of wastewater, and in the mitigation of fecal coliforms. It is important to note that the antimicrobial activity has been studied in living *Chlorella* spp. cells in aquatic environments, in response to the presence of predatory bacteria through the production of peptides, polysaccharides, and fatty acids (Dar, Sharma, and Kaur, 2019; Rojas et al., 2020; Alsenani et al., 2020). In an in vitro model, pepsin protein hydrolysates of *C. sorokiniana* have been reported to have activity against *Staphylococcus aureus* and *Escherichia coli* (Tejano et al. 2019). The identity of these antibacterial compounds was not characterized. No other studies performed in *C. sorokiniana* monoculture reported this effect. Additionally, transfer of antimicrobial resistance in the human gastrointestinal tract relies on live organisms and occurs between bacteria in the microbiome (McInnes et al., 2020). Because the subject of GRN 986 does not contain live cells and gene transfer of antimicrobial resistance has not been observed in eukaryotes, there is reasonable certainty that Chlorella Powder and Chlorella Micro Powder do not possess antimicrobial activity based on the publicly-available literature.

In conclusion, the results of the literature search did not find any reports of *C. sorokiniana* producing secondary metabolites of concern, toxins, or antimicrobial activity; therefore, it is reasonable to conclude that the strain used in the production of the subject of GRN 986 is safe.

2. *On page 5, the notifier states, "Although CK-22 was originally identified as C. vulgaris CK-22 by morphology, CK-22 has been redesignated as C. sorokiniana following 18S rRNA sequencing." Please elaborate and provide a reference (as applicable) for this statement.*

   Chlorella Industry Co. concluded that their organism was *C. sorokiniana* based on the sequence alignment described on page 7, Section 2. Genotypic Description of CK-22. Furthermore, multiple organisms originally identified as *C. vulgaris* have been reassigned to *C. sorokiniana* (Champenois, Marfaing, and Pierre, 2015).

3. *On page 7, the notifier states, "Comparison of the 18S rRNA sequences of CK-22 with C. sorokiniana SAG 211-8k (accession number X62441, Culture Collection of Algae at the University of Göttingen, Germany) and C. vulgaris SAG 211-11k (accession number X13688) shows that the CK-22 18S rRNA sequence is 99.8% similar to the C. sorokiniana, SAG 211-8k sequence and 99.5% similar to C. vulgaris SAG 211-11b." Please discuss whether the full genomic sequence for C. sorokiniana strain NITE SD 00247 is publicly available and provide the corresponding accession number.*

   The full genomic sequence for this strain is proprietary.

4. *On page 8, the notifier states that the method used to detect yeast and mold is USP 62, which corresponds to Microbiological Examination of Nonsterile Products: Test for Specified Microorganisms. This method does not appear to include a method to detect yeast and mold. For the administrative record, please clarify this discrepancy.*

   The method used to detect yeast and mold in Chlorella Powder and Chlorella Micro Powder is USP 61. USP 62 was a typographical error.

5. *On page 9, the notifier describes the manufacturing process and states that C. sorokiniana strain NITE SD 00247 is "… expanded to a shallow, outdoor pool by pipeline and cultured in the presence of ambient light and temperature with agitation." Please discuss whether the outdoor pool is open to the natural environment or whether it is in a closed or controlled setting (i.e., a laboratory or other facility). If the pool is open to the natural environment, please discuss how contamination is controlled.*

   The final step in culture occurs in an outdoor concrete pool that is open to the air. Since the pool is open to the natural environment, the final culture is washed multiple times to remove any potential environmental contaminants. The subject of GRN 986 is routinely screened for the following environmental contaminants: microbials and heavy metals (product specifications, every batch tested), pesticides (tested yearly), polyaromatic hydrocarbons (PAHs, tested yearly), polychlorinated biphenyls (PCBs, tested yearly), and radioactive isotopes (tested every 6 months). In ten years of production, Chlorella Industry Co. has not detected microbials, heavy metals, pesticides, PAHs, or radioactive isotopes in any batches of Chlorella Powder or Chlorella Micro Powder. PCBs have been tested in the last three years of production, with one PCB residue detected at the level of detection in one batch of Chlorella Micro Powder (see the response to Toxicology: Question 1). Based on these data, Chlorella Industry Co. concludes that although the last step in the culture is open to the natural environment, the final product does not contain environmental contaminants, and culturing the algae in the outdoor pool does not affect the safety of the finished product.

6. *On page 9, the notifier states, "The final slurry is passed through a magnetic strainer, cooled to 2-5ºC, and stored for up to 24 hours before undergoing heat inactivation for 3 minutes at 100ºC." Please clarify what "heat inactivation" means and how the notifier ensures that the cells are inactivated.*

   The heat inactivation step refers to a sterilization step. Chlorella Industry Co. has also confirmed that no living cells are present in the finished Chlorella Powder or Chlorella Micro Powder as assessed by culturing samples of both finished Chlorella Powder and Chlorella Micro Powder on agar plates and in liquid culture. No growth was observed in either agar plates or liquid culture after 2 weeks of culture at 30℃ with illumination of 3000 lux by fluorescent lamp.

7. On page 33, the notifier states, "Chlorella spp. have not been reported to produce marine toxins." Please provide a reference for this statement.

A search of the literature performed in August of 2021 did not find any reports of marine toxin production by *Chlorella* spp. (see the response to Microbiology: Question 1). Specifically, two recent extensive reviews of toxin producing algae do not describe toxin production by any member of the *Chlorella* genus, and a recent book chapter also reported that no member of the Chlorellaceae family, to which the genus *Chlorella* belongs, have been reported to produce toxins (Gärtner, Stoyneva-Gärtner, and Uzunov, 2021; Hofbauer 2021; Markou, Chentir, and Tzovenis, 2021). Thus, Chlorella Industry Co. concludes that marine toxins are not produced by *Chlorella* spp or *C. sorokiniana* CK-22.

8. Please state whether any of the raw materials used in the fermentation are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.

Chlorella Industry Co. confirms that the raw materials are free of major allergens and that none of the raw materials are derived from major allergens.

9. In reviewing the publicly available literature, we identified published reports of infections in animals and humans from Chlorella species (i.e., chlorellosis). Please provide an updated literature search including the date (month and year) the literature search was performed and discuss the reports of infections in animals and humans from Chlorella species and whether these reports may be contradictory to a GRAS conclusion.

Diagnosis of chlorellosis depends on both the morphological and ultrastructural characteristics of the lesions and the invading algal cells (Riet-correa, Silva do Carmo, and Uzal, 2021). A literature search was performed on August 16, 2021 using the search term "chlorellosis" in both PubMed and GoogleScholar. Only studies that were peer-reviewed and published in English were considered for further review. Although case studies of opportunistic infections of green algae has been described in livestock (Cordy, 1973; Le Net et al., 1993; Hafner, Brown, and Zhang, 2012; Riet-correa, Silva do Carmo, and Uzal, 2021), one gazelle (Haenichen et al. 2002), one dog (Quigley, Knowles, and Johnson, 2009), and twice in humans (Jones et al. 1983; Hart et al. 2014), none of the case studies identified *Chlorella* spp. as the cause of chlorellosis using genotypic techniques. In one of the studies, genotypic techniques were used; however, when the algal ITS sequences from the masses in cows were analyzed, it was determined that these sequences had the greatest homology to the *Scenedesmus* sp., a related green algae (Hafner, Brown, and Zhang, 2012). In humans only two cases of chlorellosis have been described following exposure of an injury to fresh water (Hart et al., 2014; Jones et al., 1983). Both cases relied on only morphology to identify the infection as being caused by *Chlorella* sp. Additionally, all studies noted that the cause of the green algae infection was likely from the consumption of stagnant water or exposure of a

wound to stagnant water that is particularly rich in green algae. Chlorella Powder and Chlorella Micro Powder consist of the spray-dried biomass of *C. sorokiniana* CK-22 and the manufacturing process includes a heating step that ensures there are no live *C. sorokiniana* CK-22 cells present in the finished product. Therefore, these reports are not contradictory to a GRAS conclusion and there is reasonable certainty that the ingestion of Chlorella Powder and Chlorella Micro Powder will not pose a risk of developing chlorellosis in consumers.

10. *References to "Salmonella typhimurium" on pages 34 and 36 should read "Salmonella Typhimurium" as serovars are not italicized. Please make a statement that corrects this reference.*

    We confirm that this was a typographical error, and the text should read "*Salmonella* Typhimurium."

Toxicology:

1. *On page 23, the notifier states that detectable levels of 2,4,4'-Trichlorobiphenyl were identified in a single lot of Chlorella micro powder. Please discuss why the level of 2,4,4'-Trichlorobiphenyl does not present a safety concern.*

    2,4,4'-Trichlorobiphenyl was detected at the level of detection, 0.01 ng/g, in one lot of Chlorella Micro Powder. To estimate the exposure to 2,4,4'-trichlorobiphenyl from the consumption of products containing Chlorella Micro Powder, the 90th percentile Estimated Daily Intake (EDI) of 870 mg Chlorella Micro Powder/day was multiplied by 0.01 ng 2,4,4'-trichlorobiphenyl/g Chlorella Micro Powder, resulting in an exposure of 0.0087 ng 2,4,4'-trichlorbiphenyl/day. The no significant risk level (NSRL) of PCBs is 0.09 µg/day, as described by Proposition 65 of the California Office of Environmental Health Hazard Assessment (https://oehha.ca.gov/proposition-65/chemicals/polychlorinated-biphenyls). Furthermore, a no observed adverse effect level (NOAEL) for 2,4,4'-trichlorobiphenyl was determined to be 36 µg/kg body weight in a 90 day subchronic toxicity study in Sprague Dawley rats (Chu et al. 1996). Assuming a 60 kg person and a 100-fold safety factor, the acceptable daily intake (ADI) from this NOAEL would be 21.6 µg/day. The exposure to 2,4,4'-trichlorobiphenyl from Chlorella Micro Powder is approximately 10,000-fold below the NSRL, and 2,500,000 below the ADI; therefore, there is a reasonable certainty of no harm from the consumption of Chlorella Micro Powder with 2,4,4'-trichlorobiphenyl at the limit of detection for the assay.

2. *Please clarify if the intended uses of your Chlorella powder and Chlorella micro powder will be substitutional for other similar Chlorella-derived food ingredients or if these uses would be additive. If the intended use would be additive, please provide a cumulative dietary exposure for Chlorella ingredients in the diet and discuss the safety information that supports the safe use of Chlorella ingredients at the cumulative dietary exposure.*

Chlorella Powder and Chlorella Micro Powder are intended to be substitutional for other Chlorella sp.-containing products on the market. Accordingly, a calculation of a cumulative estimated daily intake of *Chlorella* sp.-derived food ingredients is not needed.

3. *On page 33, the notifier states that the safety of the notified material is supported by GRAS notifications for food ingredients derived from Chlorella species in GRNs 000384, 000569, and 000519. We believe that the notifier had meant to reference GRN 000469, as stated in a prior section of the notice, and not GRN 000569 (GALACTO-OLIGOSACCHARIDES). Please state if you concur.*

   We concur.

4. *On page 33, the notifier describes the NOAEL obtained from a 90-day toxicity study of Chlorella powder and cites Himuro et al., 2014. Please note that the stated reference does not describe a 90-day toxicity study. We believe that the notifier had meant to reference Himuro et al. (2017), as stated in other sections of the notice. Please state if you concur.*

   We concur.

5. *On page 33, the notifier states that "The safety of intake is also supported by corroborating published clinical studies of Chlorella spp. ingestion with no adverse events reported." However, in the Himuro et al. (2017) manuscript, it states that adverse events related to the Chlorella genus have been reported such as erythematopurpuric lesions on sun-exposed areas (Jitsukawa et al., 1984) and occupational asthma (Ng et al., 1994). Please address these reports of adverse effects and describe why such reports should not raise safety concerns related to the intended use of your Chlorella powder as a food ingredient.*

   Ng, Tan, and Lee, 1994 reported a case of occupational asthma in a phamacist who had worked in a pharmaceutical factory for two years and was experiencing frequent episodes of rhinitis, coughm, phlegm, shortness of breath, and wheezing when the factory was manufacturing *Chlorella* sp.-containing tablets. Histamine inhalation challenge, specific inhalation, and skin prick testing confirmed that the subject was reactive to the *Chlorella* sp. ingredient. Importantly, whether exposure to *Chlorella* sp. or another related antigen sensitized the subject to having allergic reactions to *Chlorella* sp. or whether the subject would develop an allergic reaction following the ingestion of *Chlorella* sp. is not known. Moreover, as described in the GRAS notice, Chapter VI, Section E. Allergenicity, a search of the literature did not find any studies of asthma or food-allergic reactions following the ingestion of *Chlorella* sp.-containing products (also see Toxicology: Question 9). Therefore, the case report by Ng, Tan, and Lee, 1994 does not raise a safety concern for the ingestion of finished products in which Chlorella Powder or Chlorella Micro Powder are ingredients.

   The erythematopurpuric lesions on sun exposed areas was caused by pheophorbide, a chlorophyll degradant, that was present in tablets containing *Chlorella* sp.

Following this report in the 1980s, supplements and food ingredients in Japan containing chlorophyll typically have set a product specification for pheophorbide to mitigate the risk posed by the consumption of these products. Accordingly, Chlorella Industry Co. has a product specification for pheophorbide that limits the amount of pheophorbide in the finished Chlorella Powder and Chlorella Micro Powder.

6. *The cited manuscript, Himuro et al. 2017, indicates that Chlorella species contain high levels of carotenoids, which is not discussed in the compositional narrative of your notice. Please discuss the level of carotenoids that are present in your Chlorella powder, and why such levels do not present a safety concern, particularly for special populations like smokers.*

   The negative association of β-carotene supplementation was originally described in smokers and those exposed to asbestos receiving either 20 mg/day β-carotene for five to eight years (The Alpha-Tocopherol, 1994) or 30 mg/day β-carotene in combination with 25,000 IU retinol for 4 years (Omenn et al., 1996).

   Except for Vitamin B2, Chlorella Industry Co. has not quantified the vitamin content of Chlorella Powder and Chlorella Micro Powder. *Chlorella* spp. are noted for the production of the carotene lutein, but production of β-carotene has also been reported (Guedes, Amaro, and Malcata 2011; Cordero et al. 2011; Matsukawa et al. 2000). *C. sorokiniana* has been reported to contain 0.6 mg β-carotene/g dry weight (Matsukawa et al. 2000). A conservative estimate of β-carotene exposure from the ingestion of Chlorella Powder and Chlorella Micro Powder can be calculated from the 90th percentile consumer's Estimated Daily Intake (EDI). The 90th percentile EDI was calculated to be 0.87 g/day. Assuming there is 0.6 g β-carotene/g dried *C. sorokiniana* CK-22 biomass, the exposure to β-carotene would be 0.522 mg β-carotene/day for the 90th percentile consumer.

   For reference, one cup of mashed, boiled sweet potato contains 31 mg β-carotene (https://ods.od.nih.gov/pubs/usdandb/VitA-betaCarotene-Content.pdf). Comparing to the levels of β-carotene in one cup of mashed, boiled sweet potatoes, consumers will be exposed to 60-fold less β-carotene through the ingestion of Chlorella Powder and Chlorella Micro Powder. The subject of GRN 519, the dried biomass of another Chlorella sp., would also be expected to contain similar concentrations of beta carotene, and the FDA had no questions about the safety of the consumption of this product. Thus, there is reasonable certainty that the ingestion of carotenoids from the ingestion of Chlorella Powder and Chlorella Micro Powder will not pose a safety concern for special populations, such as smokers.

7. *The cited 90-day subchronic toxicity study in rodents, Himuro et al. (2017), indicates that histopathological analyses was only conducted in two organs, kidney and liver. Therefore, the study is not compliant with OECD Guideline 408. Please provide a scientific rationale to support the determination of a NOAEL/ADI from a pivotal 90-day toxicity study that did not contain a thorough toxicological assessment of histopathological lesions in all relevant organ systems.*

The 90-day subchronic toxicity study in rodents was performed following the completion of the acute oral toxicity study and 28-day oral toxicity study, both of which were OECD-compliant. The results of both the acute oral and 28-day oral toxicity studies did not report any treatment related adverse effects following the consumption of up to 5 grams of Chlorella Powder in the acute study and up to 10% of the diet in the 28-day study which corresponds to 8570 mg/kg/day for males and 8620 mg/kg/day for females. Because the consumption of Chlorella Powder in these two studies did not result in adverse effects, and previously published 90-day subchronic rat toxicity studies of *Chlorella* spp. products reported NOAELs at the highest doses tested, (100,000 ppm in the diet, corresponding to 4807 mg/kg/day for males and 5366 mg/kg/day for females (Szabo et al., 2012) and 4805 mg/kg/day for males and 5518 mg/kg/day for females (Szabo, Matulka, and Chan, 2013)), only the kidneys and livers were subjected to histopathological analysis. These studies corroborate the lack of target organ toxicity and the derivation of a NOAEL from the 90-day subchronic study in rodents published by Himuro et al. (2017) of 5940 mg/kg/day in males and 6410 mg/kg/day for females.

8. *The cited 90-day subchronic toxicity study in rodents, Himuro et al. (2017), indicates a consistent effect of increased neutrophil number in male rats in both cited 28-day and 90-day toxicity studies of the subject Chlorella powder. The authors cite that this alteration is within the normal range for neutrophils in this strain (5.4-37.5%) and cite a study by Traesel et al. (2016). The Traesel et al. (2016) study is a subchronic toxicity study of Caryocar brasiliense oil extract in Wistar rats obtained from the State University of Maringa. This study does not present any historical control data describing the typical range of neutrophil counts in this strain of rats. Neutrophil counts (up to 31%) were observed in study controls, however, there is no information in the article to support that this level is within the normal biological range observed in this strain of rat. Please note that the "normal biological range" for assessed parameters is based on control data from numerous studies that were conducted under different testing paradigms and animal husbandry/housing conditions. Historical controls may include naïve controls, saline treated controls, DMSO treated controls, cyclodextrin treated controls and the like. Therefore, it is important to compare the treatment group with the concurrent controls and assess the biological significance of the observed changes.*

   - *An increase in neutrophils may be associated with infection, inflammation, injury, exposure to xenobiotics, and given the statements above, please provide additional information to support the notion that the observed increased neutrophil counts in male Wistar rats exposed to Chlorella powder is within the historical range for this strain of rat or lacks biological significance.*

   - *A similar rationale is presented with regard to serum triglyceride levels and A/G ratio. The notifier states that Traesel et al. (2016) indicates that the measured values are within the normal distribution for this strain of rat. Given the importance of concurrent controls in an experiment, please discuss further*

how the cited study provides adequate support/information to support this notion or cite other generally available scientific publications/reports regarding historical ranges for clinical laboratory parameters in the appropriate rat strain. Also, please explain why the observations do not indicate a potential safety concern.

We agree with the comment concerning the use of historical controls. It is important to note that Himuro et al. (2017) is a published, peer-reviewed paper and documents scientific consensus of the interpretation of results as presented by the authors. Increased neutrophil counts are associated with responses to stress or excitement after routine handling in rats (Dhabhar et al., 2012), or in response to a bacterial infection (Kobayashi et al., 2018). This finding was not a treatment-related biologically significant change because the increases noted in neutrophils were not dose-related, nor did they occur in both sexes. Additionally, although neutrophils were also statistically significantly increased in high dose males in the 28-day study, the study complied with OECD protocol 407, which includes histopathologic examination of bone marrow. No correlating adverse histopathology was noted in the high dose males. Thus, the findings for neutrophil increases in the 90-day study were accepted by peer review to be attributed to background variability in this parameter.

Similarly, A/G ratio was not dose-related, and the decrease was significant only in mid dose females and not in males. Triglycerides were very variable among groups and although the high dose males were statistically significantly reduced compared to control, this is not considered to be an adverse effect and is considered likely due to natural variability in the levels. There were no reductions in triglycerides in females. These observations described in Himuro et al. (2017) do not indicate a safety concern for the consumption of Chlorella Powder.

9. *The notifier attempts to utilize safety information from other algal sources or Chlorella species to corroborate the safety of their subject source organism, Chlorella sorokiniana strain NITE SD 00247. The notifiers describe corroborative safety information related to C. vulgaris, C. protothecoides, C. pyrenoidosa, C. stigmatophora, C. regularis, as well as many studies performed with unspecified Chlorella species. However, the provided scientific discussion and rationale is insufficient to establish a case for read-across between these organisms.*

- *Please provide additional information and scientific rationale to establish that C. sorokiniana strain NITE SD 00247 is sufficiently similar to other Chlorella species discussed in the notice. Such rationale might include information related to composition including any secondary metabolite, anti-nutrient, and potential allergenic or toxic protein profile that addresses the similarity/differences of C. sorokiniana strain NITE SD 00247 to other Chlorella species. Discussions should clearly address any safety concerns related to differences in pathogenic, toxigenic, and allergenic properties between C. sorokiniana strain NITE SD 00247, and other Chlorella species with an established safety profile.*

The genus *Chlorella* is considered to have low species diversity (Huss et al., 1999), and many of the organisms originally classified as a species of *Chlorella* by morphology and intracellular structures have been reassigned or even classified to different genera. In particular, many strains originally identified as *C. pyrenoidosa* and *C. vulgaris* are now assigned to *C. sorokiniana* (Champenois, Marfaing, and Pierre 2015). As established and accepted with no questions by the FDA in GRNs 384, 469, and 519, corroborative data from other species in the *Chlorella* genus may be considered relevant to support safety of a different species of *Chlorella*.

Literature searches for toxins, secondary metabolites, and allergy in *Chlorella* spp. did not yield any reports of hazardous compounds produced by *Chlorella* spp., see Microbiology: Question 1 and recent reviews by (Hofbauer, 2021) and (Gärtner, Stoyneva-Gärtner, and Uzunov, 2021). Additionally, animal studies performed with *C. vulgaris, C. sorokiniana, C. protothecoides, C. pyrenoidosa, C. stigmatophora,* and *C. regularis* performed in the last thirty years did not report adverse effects. Together, this provides a basis of comparison across the *Chlorella* genus with regards to the absence of pathogenic, toxigenic, or allergenic properties.

Chlorella Industry Co. intends to add Chlorella Powder and Chlorella Micro Powder to conventional foods as a source of macronutrients. Similarly, the subject of GRN 469 and 519 are intended to be added to conventional foods as sources of fats and proteins, respectively. Although the proportion of fat, protein, and carbohydrates produced by *Chlorella* spp. may vary depending on the culture conditions (Sharma et al., 2016), no toxicity or safety concerns have been reported from the ingestion of macronutrients derived from *Chlorella* spp.

Due to the similarity of the cultured organisms used as the test article as a source of macronutrients, studies performed using *C. vulgaris, C. protothecoides, C. pyrenoidosa, C. stigmatophora, C. regularis,* and other *Chlorella* spp. support the safe use of *C. sorokiniana* as a source of macronutrients in the human diet.

10. *Information in the notice and in the available literature suggest that the Chlorella species/strain and manufacturing and/or culturing methods can significantly impact the composition of the microalgal biomass and derived substances. There may be additional concerns related to levels of toxic contaminants such as heavy metals(Afkar, Ababna, and Fathi 2010) or cyanotoxins (Heussner et al. 2012) in some preparations. The safety narrative summarizes numerous corroborative animal toxicity and clinical studies with Chlorella species derived preparations. However, it is unclear how the compositional profile of these Chlorella test materials compares to the subject Chlorella powder/micro powder. In the absence of information to establish compositional similarity, the cited studies do not corroborate the safety of your Chlorella powder/micro powder. Please provide additional information and scientific rationale that the composition of your Chlorella powder/micro powder is sufficiently similar to the Chlorella-derived test materials used in corroborative studies. Discussions might consider proximate nutrient content, vitamins/minerals, and the presence/absence of toxic and allergenic components.*

The macronutrient content and heavy metal specifications of the subject of GRN 519, an algal flour high in protein are similar to the subject of GRN 986 (Table 4). Averages of three lots are shown demonstrating the similarity in product specifications and observed values. Averages of three lots is not shown for heavy metals, as no heavy metals were detected above the limit of detection for each specification for either the subject of GRN 986 or GRN 519.

Although *Chlorella* spp. may accumulate heavy metals from the environment, as described in bioremediation studies and in Afkar et al. (2010), the amount of heavy metal contaminants in the subject of GRN 986 are controlled through the manufacturing process and compliance is established by the product specifications. A recent book chapter states that no microalgae from the Chlorellaceae family have been reported to produce toxins (Markou, Chentir, and Tzovenis, 2021). The contamination with cyanotoxins described in Heussner et al. (2012) occurred in finished products that were not the dried biomass of a *Chlorella* sp. monoculture. The subject of GRN 986 consists of the dried biomass of a *Chlorella* sp. monoculture and will not be blended with other algae; therefore toxic algae will not be present in the finished product. Furthermore, literature searches for toxins, secondary metabolites, and allergy in *Chlorella* spp. did not yield any descriptions of hazardous compounds produced by any *Chlorella* sp., see the response to Microbiology, Question 1 and recent reviews by Hofbauer (2021) and Gärtner, Stoyneva-Gärtner, and Uzunov (2021).

Table 4. Macronutrient Content and Heavy Metal Specifications of the Subjects of GRN 986 and GRN 519

Macronutrient	**GRN 986: dried biomass of *C. sorokiniana* CK-22 (Chlorella Powder)**		**GRN 519: dried biomass of *C. protothecoides* S106**	
	Specification	**Average of 3 lots**	**Specification**	**Average of 3 lots**
Protein (%)	≥55.0	64.2%	40-75	52.4%
Fat (%)	≥8.8	12.0%	5-25	15.8%
Carbohydrate (%)	≥7.0	11.7%	Fiber: 5-25%	Fiber: 16.3%
Heavy Metals	**Specification**		**Specification**	
Arsenic (ppm)	<1.0		<0.2	
Lead (ppm)*	<0.5		<0.5	
Cadmium (ppm)	<0.2		<0.1	
Mercury (ppm)	<0.1		<0.1	
Chromium (ppm)*	<1.0		<2	
*These specifications have been updated according to the response to Chemistry: Question 6. Macronutrient information from GRN 519 stamped page 17.				

11. *The generally available literature identifies numerous cyanotoxin contaminants of possible concern identified in products derived from algal biomass, such as microcystins, anatoxin-a, cylindrospermopsin, saxitoxin, and β-methylamino-L-alanine (Roy-Lachapelle et al. 2017). Notably the risk of unintended contamination by toxic cyanobacteria is increased in uncontained open pond culture systems. In the notice, the subject material is routinely assessed for microcystin and aflatoxin levels to ensure they meet the described specifications. Please address how the limited toxin panel described in the notice adequately addresses safety concerns related to unintended toxin contamination.*

    The final outdoor culture step for the subject of GRN 986 occurs in an artificial pond, where contamination with other potential toxic organisms is unlikely (Heussner et al., 2012). Co-culture experiments have described that the presence of *C. sorokiniana* creates an inhospitable environment for cyanobacteria, as *Chlorella* spp. compete for resources and produce compounds that may be toxic to cyanobacteria (Dar, Sharma, and Kaur, 2019; Schmidt et al., 2020).

    Chlorella Industry Co. also monitors the outdoor culture for the presence of microbial contamination. In the event of a microbial contamination, the entire culture is discarded. The control of the production process, the artificial pond environment, and the inhospitable environment for cyanobacteria created by *Chlorella* spp. together support that there are no safety concerns related to unintended toxin contamination in Chlorella Powder and Chlorella Micro Powder.

12. *Rapid increases in growth/biomass indicates high levels of protein and RNA synthesis. Following consumption, increased RNA breakdown and metabolism could be associated with elevated levels of uric acid and corresponding increased risk of kidney stone formation, or gout. Furthermore, increased plasma uric acid levels have been observed in rats administered C. vulgaris in the diet (19.6 g/kg/day) (Saleh et al., 1985). Please discuss the RNA content in your subject Chlorella powder/micro powder and levels that would be present in food based on your intended use, and address whether this will pose or not pose any safety concern.*

    In Saleh et al. (1985), the increase in uric acid was observed in rats administered the dried biomass of *C. vulgaris* at 19.6 g/kg body weight/day. The total nucleic acid content of the test articles in this study was 4%. For a human to consume the same amount of *C. vulgaris* as the rats in this study, assuming a 60 kg adult, the person would consume 1776 g *C. vulgaris*/day, 71.04 g of which would consist of nucleic acids. The Estimated Daily Intake (EDI) of the subject of GRN 986 is 0.87 g/day for the 90th percentile consumer. Although RNA was not measured in the subject of GRN 986, the total nucleic acid content of microalgae is typically between 4-6% (Markou, Chentir, and Tzovenis, 2021). Therefore, assuming that nucleic acids account for 6% of the dry weight of dried *C. sorokiniana* CK-22 biomass, the 90th percentile consumer will be exposed to 0.0522 g nucleic acid/day, which is approximately 1000-fold less than the amount of nucleic acid present in the test articles in the studies performed by Saleh et al. (1985). The RNA content of the subject of GRN 986 therefore does not pose a safety concern.

Additionally, the FDA had no questions regarding the safety of the consumption of a similar product in GRN 519, the dried whole cell biomass from *C. protothecoides* S106. The mean EDI for the subject of GRN 519 is 2319 mg/day, with the 90th percentile user consuming 5562 mg/day. Comparing the EDIs of the subjects of GRN 519 and GRN 986, the EDIs described in GRN 519 are approximately four-fold higher than the EDIs calculated for the subject of GRN 986.

13. *On page 68, the notifier states, "The studies in Table 38 all used Chlorella vulgaris in the diet or delivered via oral gavage"; however, there does not appear to be a Table 38 in the notice. We believe the notifier intended to reference Table 34; please state if you concur.*

    We concur.

14. *Please note that much of the information presented in the "safety endpoint" columns of Tables 33-35 do not contain information related to safety but summarize efficacy endpoints or possible benefits of Chlorella intake reported in the cited studies. Purported efficacy or benefits of Chlorella consumption are not relevant for a GRAS conclusion.*

    We agree. This section was not intended to demonstrate efficacy or benefits, but was included to provide context for the studies.

15. *The allergenicity section needs to discuss step-by-step the entire process based on the following issues that are not clearly described:*

    There are two statements made where the contexts are missing and confusing, such as the statement, "…The analysis performed here was similar to the E-score method described in Silvanovich et al. (2009). A hit is defined as 35% identity using an 80 mer sliding alignment…" ***and*** *"The Allergen online database (version 15) was queried with the 10429 hypothetical proteins by FASTA using an E-score cut-off of 10-7."*

    It seems that two different paradigms were used: the CODEX recommendation, which is a generally recognized bioinformatic paradigm for allergenicity analysis (with a long history of use) and the paradigm recently proposed by Abdelmoteleb et al. (2021) where the authors proposed the use of an E-score cut-off of 10-7.

    - *Please describe the method clearly and in detail step-by-step. Some examples of the steps are mentioned below. Please provide the detail of all the steps of the process as it was performed (do not restrict only to the steps described below; they are just examples), including:*
        - *How the genomic data was harnessed (provide the accession number/RefSeq number if applicable/assembly version, of the genome).*

- *How all the annotated ORFs were identified emphasizing on the method/any software used **or** whether only the annotated available protein data was downloaded (and in which format). Please describe this step clearly and in great detail and provide links that were used, so the process can be independently replicated.*

- *How the entire annotated proteome was compared (CODEX method/ Abdelmoteleb et al. method) and distilled into a smaller set of annotated protein. Please clearly describe this method in detail and provide links that were used, so that the process can be independently replicated.*

There is no set recommendation of an E-score. Setting an E-score cut-off depends on the size of the database used and the query length. Many experts have proposed different E-score cut-offs. There is a lack of general recognition on the usefulness of E=10-7 as the definitive cut-off. Therefore, please address whether this method has undergone a rigorous validation using many examples (i.e., many genomes). When proposing a cut-off E-score as the basis of bioinformatics-driven decision making, the utility of the proposed E-score cut-off should be rigorously validated using many examples.

A clear and step-by-step description of the process (so it can be independently replicated) will help determine the utility of this paradigm.

- *Once an E-score cut-off is set, any higher value than the set value is not reported in the output. Please explain why an E-score of 10-7 in a small database like AllergenOnline (containing 2200 sequences) will not be too stringent and hence not retrieve many relevant hits.*

The genomic sequence of *C. sorokiniana* CK-22, which is used to produce the Chlorella Powder and Chlorella Micro Powder that are the subject of this GRAS Notice, was determined by ACGT, Inc. using the Illumina MiSeq and NextSeq 500 platforms, and is proprietary. A copy of the analytical report that describes the details of sequencing is attached (Appendix 3).

Briefly, total DNA was extracted and sheared, and pair-end and mate-pair libraries were prepared. The sequencing data were demultiplexed into raw FASTQ reads using bcl2fastq version 2.16 and the adapter and low-quality sequences were trimmed and discarded. The genome was then assembled using SOAPdenovo version 2.04 and ABySS version 1.9.0. GapFiller version 1.10 and SSPACE version 3.0 were used for gap closure and scaffolding to generate the final genome assembly. The genes were *ab initio* predicted using AUGUSTUS version 3.1 and annotated with BlastKOALA using the KEGG Orthology (KO) system. The protein sequences were then queried against the NCBI nr database and the InterPro collection of protein signature databases using Blast2GO version 3.13, yielding 10,429 “hypothetical” protein sequences. A subsequent and confidential analysis of the genomic sequence conducted by the University of Nebraska confirmed that the

assembled genome sequences were reliable by comparing the N50 scores and the total genome size of *C. sorokiniana* CK-22 and the type strain of a related *Chlorella* species, *Chlorella variabilis* NC64A. The N50 scores and total genome size were comparable for the two strains.

To determine if the 10,429 hypothetical proteins potentially encoded by the *C. sorokiniana* CK-22 genome were similar to known allergenic proteins, the hypothetical proteins of *C. sorokiniana* were compared by FASTA against v15 of the Allergen Online (AOL v15) database using a standard of >35% identity over alignments of 80 or more amino acid, which meets the recommendation of the Codex Alimentarius Commission (2003). Using an E-score cut-off of 0.001, three hundred and fifty (350) query proteins matched at least one of the AOL v15 proteins. Most matched two or more homologous proteins, for a total of 2140 alignments and many of the alignments were of low sequence identity and primarily partial alignments.

To prevent manually comparing the alignments to sort out probable irrelevant alignments, the analysis was redone with smaller E scores (10^{-7}) to reduce irrelevant matches as suggested by Silvanovich, Bannon, and McClain (2009). An E-score cut off of at least 10^{-7} is a recommended strategy for allergenicity risk assessments using the AllergenOnline database, as recently demonstrated in a study of three novel sources: *Chlorella variabilis, Galdieria suphurarira,* and *Fusarium* strain flavolapis (Abdelmoteleb et al., 2021).

The number of unique protein matches with E-scores of less than 10^{-7} was 135 hypothetical *C. sorokiniana* CK-22 protein hits. This list of 135 hits was further analyzed for hits that had a >50% identity with alignments, as these hits are considered more significant (Aalberse 2000; Hileman et al. 2002). Of the 135 original hits, 36 met the criteria of having greater than 50% identity. Alignments that cover most of the full length of the query and hit sequences are likely to have the same confirmational folding and could be potential sources of IgE cross-reactivity (Aalberse, 2000). A comparison of the length of alignments between the full-length hypothetical 36 *C. sorokiniana* CK-22 proteins and the known allergens show that there were large gaps and mismatches in the alignments (Table 5). These results indicate that the 36 hypothetical proteins identified in *C. sorokiniana* CK-22 are unlikely to provoke an IgE-mediated allergic response.

Table 5. 36 Hypothetical Chlorella Protein Hits to Allergen Online Database with at Least 50% Sequence Identity

NCBI Protein Accession Number	NCBI Protein GI (Protein type)	*Species* (common name)	%ID	E-value	Length of hypothetical C. sorokiniana CK-22 protein query	Length of Known allergen hit
AEY79726	373939374 (cyclophilin)	Daucus carota (carrot)	73.5	3.90E-47	496	171
CAA59468	1220142 (cyclophilin)	*Catharanthus roseus* (periwinkle)	71.3	1.30E-40	526	172
CAI78448	91680605 (cyclophilin)	*Aspergillus fumigatus* (fungus)	70.8	3.20E-48	236	163
P40918	729764 (HSP Cla h 4)	*Cladosporium herbarum* (fungus)	70.4	3.70E-141	1258	643
CAC00532	9581744 (enolase)	*Hevea brasiliensis* (rubber tree)	69.7	2.00E-117	477	445
CAC14917	11124572 (triosphosphatase isomerase)	*Triticum aestivum* (wheat)	69	4.20E-74	767	253
Q9UUZ6	83305635 (60S ribosomal P2)	Aspergillus fumigatus (fungus)	67.9	5.40E-20	456	111
CAZ76054	253783729 (GAPDH)	*Triticum aestivum* (wheat)	66.8	2.50E-28	2274	337
CAZ76054	253783729 (GAPDH)	*Triticum aestivum* (wheat)	66	1.10E-57	746	337
Q8NKF4	83305621 (60S ribosomal P2)	*Aspergillus fumigatus* (fungus)	64.8	1.40E-108	770	392
Q948T6	84029333 (Glutathione)	*Oryza sativa* (rice)	64.2	1.20E-73	745	291
CAC14917	11124572 (triosphosphatase isomerase)	*Triticum aestivum* (wheat)	64	5.10E-31	1239	253
AAP35065	37958141 (cyclophilin)	*Dermatophagoides farina* (house dust mite)	62.8	5.60E-34	223	164
AEY79726	373939374 (cyclophilin)	*Daucus carota* (carrot)	62.6	2.40E-39	274	171
AAK67492	14585755 (cytochrome c)	*Curvularia lunata* (fungus)	62.1	1.30E-24	173	108
CAA09884	4138173 (cyclophilin)	*Malassezia sympodialis* (fungus)	62	8.00E-22	717	162
CAX62602	291195949 (Aldolase A)	*Thunnus albacares* (tuna)	59.7	2.90E-71	815	364
ABU97472	156938901 (profilin)	*Glycine max* (soybean)	59.2	3.50E-29	131	131
CAR82266	208605346 (LMW gluten)	*Triticum aestivum* (wheat)	58.7	7.90E-12	677	272
CAR82267	208605348 (LMW gluten)	*Triticum aestivum* (wheat)	57.9	2.10E-24	363	346
AEX34122	371537645 (60S ribosomal P2)	*Penicillium crustosum* (fungus)	56.9	5.40E-18	106	107
P40918	729764 (HSP Cla h 4)	*Cladosporium herbarum* (fungus)	56.1	1.80E-75	668	643
BAE20328	73912496 (Omega-5 gliadin)	*Triticum aestivum* (wheat)	56	6.70E-23	919	439
AAD25927	4587985 (malate dehydrogenase)	*Malassezia sympodialis* (fungus)	54.7	9.80E-52	593	342
CAD38166	21748151 (nuclear transporter)	*Cladosporium herbarum* (fungus)	54.6	3.40E-23	163	125
CAA59468	1220142 (cyclophilin)	*Catharanthus roseus* (periwinkle)	54.1	4.50E-35	219	172
NP_001133181	213511774 (Aldolase A)	*Salmo salar* (salmon)	53.4	5.40E-71	387	363
ACE82290	190684059 (periredoxin)	*Triticum aestivum* (wheat)	52.6	3.90E-45	587	218
ABR29644	149786150 (Mn superoxide dismutase)	*Pistacia vera* (pistachio)	52.1	7.40E-36	193	230

Table 5. 36 Hypothetical Chlorella Protein Hits to Allergen Online Database with at Least 50% Sequence Identity						
NCBI Protein Accession Number	**NCBI Protein GI (Protein type)**	***Species* (common name)**	**%ID**	**E-value**	**Length of hypothetical C. sorokiniana CK-22 protein query**	**Length of Known allergen hit**
AAD25927	4587985 (malate dehydrogenase)	*Malassezia sympodialis* (fungus)	51.9	1.20E-39	1167	342
CAX62602	291195949 (Aldolase A)	*Thunnus albacares* (tuna)	51.7	6.50E-48	650	364
P42058	1168402 (flavodoxin)	*Alternaria alternate* (fungus)	51.3	4.50E-21	721	204
CAA55071	76666767 (aldehyde dehydrogenase)	*Alternaria alternate* (fungus)	51.2	2.10E-90	1121	497
AEY79726	373939374 (cyclophilin)	*Daucus carota* (carrot)	51	1.10E-22	236	171
BAE20328	73912496 (Omega-5 gliadin)	*Triticum aestivum* (wheat)	50.6	3.50E-25	757	439
CAB96931	8980491 (thioredoxin h)	*Triticum aestivum* (wheat)	50.5	6.40E-16	494	125

Importantly, the sequencing and analysis of the *C. sorokiniana* CK-22 genome and hypothetical protein sequences was conducted following the precedent that was established in GRN 469 and GRN 519 for products derived from *C. pyrenoidosa* S106, which received "no questions" letters in 2013 and 2014, respectively. Specific details regarding the sequencing, assembly, annotation, and analysis of the *C. pyrenoidosa* S106 genome and the resulting hypothetical proteins were not provided in the GRAS Notices. Specifically, in the analyses described in GRN 469 and GRN 519, hypothetical proteins were compared to the Structural Database of Allergenic Proteins (SDAP) from the University of Texas Medical Branch and described 1635 hits to known allergens. Conversely, the analysis conducted by Chlorella Industry Co. following the Codex Alimentarius Commission (2003) methodology identified 135 hits in the AllergenOnline database, which is an order of magnitude fewer hits than those described in GRNs 469 and 519.

Additionally, it is not known if the genes identified by ACTG, Inc. encode legitimate proteins, if the resulting proteins are expressed in *C. sorokiniana* CK-22, or if the resulting proteins are expressed at levels that would sensitize and/or provoke an allergic response in consumers are not known. Allergic reactions are secondary immune responses that require the presence and crosslinking of IgE antibodies bound to Fc receptors expressed on the surface of mast cells and basophils that are either specific to a particular protein or bind similar proteins. Antigen-mediated IgE-crosslinking in turn induces mast cell and basophil degranulation, releasing histamine and leukotrienes into the extracellular matrix, leading to capillary venule dilation, endothelium activation, and increased vascular permeability, causing redness and swelling. Importantly, IgE antibodies develop during an abnormal primary immune response to a protein or antigen that would otherwise be tolerated. Also, not all allergens are created equally. Some are capable of inducing the primary immune response, which may result in the production of the IgE antibodies that make people sensitive, whereas others only engage preformed IgE antibodies and provoke secondary/hypersensitivity responses (Aalberse 2000). Thus, the identification of "hypothetical" protein sequences that have some similarity with known allergens using bioinformatic techniques does not indicate that protein-containing ingredients manufactured from *C. sorokiniana* CK-22 will sensitize or provoke allergic responses in consumers.

As discussed in the Chapter 6, Section E.1, a review of the publicly available literature shows that allergic reactions via inhalation, not through exposure via consumption, have been limited to *C. vulgaris, C. pyrenoidosa, C. saccharophila* and *C. homosphaera,* and an updated literature search found no discussion of allergic reaction to *C. sorokiniana* ingestion (literature search performed on August 16, 2021). Additionally, these allergic reactions did not occur following the ingestion of *Chlorella* sp. or *Chlorella* sp.-containing products. All of the reported allergic reactions have resulted from occupational exposure. Furthermore, a recent published study describing the potential allergenicity of multiple organisms including the related species *C. variabilis*, concluded that *C. variabilis* "does not represent a significant risk of food allergy to the general population as matches to similar proteins from many diverse species are very common" (Abdelmoteleb et al. 2021).

16. *NCBI has discontinued the GI numbers as of September 2016. Please provide the corresponding accession number of all the entries in Table 36.*

    Although the GI numbers were discontinued, GI numbers are still available in the NCBI Protein database. For ease of review, we have provided the corresponding accession numbers to Table 5.

17. *On page 80, the notifier states, "Five of the 135 hypothetical proteins had sequence identity with known Celiac disease reactants gliadin and glutenin; however, these proteins did not contain 100% sequence identity to gliadin or glutenin." Please identify the accession numbers of these sequences.*

    Please see Table 5 for corresponding accession numbers for all 135 sequences.

18. *On page 80, the notifier states, "…but further analysis had large gaps in the amino acid sequences. These gaps suggest that although the alignments were statistically significant, the length of the aligned amino acid sequences were not likely to trigger an IgE allergy response."*

    Please provide a relevant scientific citation to support this statement.

    Please state whether these 36 hits show >35% identity over a sliding window of 80 amino acids; also state how many 8-aa epitopes were identified.

    Please see our response to question 15 regarding the bioinformatics analysis conducted by Chlorella Industry Co. to identify potential allergens in the Chlorella Powder and Chlorella Micro Powder. Although a sequence of 8 amino acids may be sufficient to provoke an allergy response, the IgE-mediated allergic response requires at least two IgE binding epitopes; therefore, the use of a single contiguous 8 amino acid sequence may identify false positive potential allergens (Hileman et al., 2002). Accordingly, a screen for 8 contiguous amino acid sequences was not performed in the analysis conducted by Chlorella Industry Co. because it is considered to be of little additional value.

Regulatory:

1. *On page 7 you state, "All fermentation vessels, food contact materials, raw materials, and processing aids are U.S. food grade." Please state whether all fermentation vessels, food contact materials, raw materials, and processing aids are approved for their intended use.*

    All fermentation vessels, food contact materials, raw materials, and processing aids are approved for their intended use.

2. *Please clarify whether Chlorella powder/micro powder will impart color to food, intentionally or not. If Chlorella powder/micro powder is expected to impart color, please clarify whether any imparted color is important. Material that otherwise meets the definition of color additive can be exempt from that definition on the basis that it is used or intended to be used solely for a purpose or purposes other than coloring, as long as the material is used in a way that any color imparted is clearly unimportant insofar as the appearance, value, marketability, or consumer acceptability is concerned.*

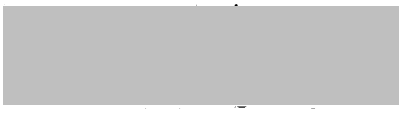
   The subject of GRN 986 is intended to be used solely for purposes other than coloring. Any color imparted is unimportant insofar as the appearance, value, marketability, or consumer acceptability is concerned.

3. *Please specify that Chlorella powder/micro powder is not intended to be used in infant formula, or in any products under the jurisdiction of the United States Department of Agriculture.*

   The subject of GRN 986 is not intended to be used in infant formula or in any products under the jurisdiction of the United States Department of Agriculture.

Should you need additional information, please feel free to contact me at 301-775-9476 or ckruger@spherixgroup.com.

Sincerely,



Claire L. Kruger, Ph.D. D.A.B.T.
Managing Partner

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Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
4202: Yeast breads	
51000100	Bread, NS as to major flour
51000110	Bread, NS as to major flour, toasted
51000180	Bread, made from home recipe or purchased at a bakery, NS as to major flour
51000190	Bread, made from home recipe or purchased at a bakery, toasted, NS as to major flour
51101000	Bread, white
51101010	Bread, white, toasted
51101050	Bread, white, made from home recipe or purchased at a bakery
51101060	Bread, white, made from home recipe or purchased at a bakery, toasted
51102010	Bread, white with whole wheat swirl
51102020	Bread, white with whole wheat swirl, toasted
51105010	Bread, Cuban
51105040	Bread, Cuban, toasted
51106010	Bread, native, water, Puerto Rican style
51106020	Bread, native, water, toasted, Puerto Rican style
51106200	Bread, lard, Puerto Rican style
51106210	Bread, lard, toasted, Puerto Rican style
51106300	Bread, caressed, Puerto Rican style
51106310	Bread, caressed, toasted, Puerto Rican style
51107010	Bread, French or Vienna
51107040	Bread, French or Vienna, toasted
51108010	Focaccia, Italian flatbread, plain
51108100	Naan, Indian flatbread
51109010	Bread, Italian, Grecian, Armenian
51109040	Bread, Italian, Grecian, Armenian, toasted
51109100	Bread, pita
51109110	Bread, pita, toasted
51109150	Bread, pita with fruit
51109200	Bread, pita with fruit, toasted
51111010	Bread, cheese
51111040	Bread, cheese, toasted
51113010	Bread, cinnamon
51113100	Bread, cinnamon, toasted
51115010	Bread, cornmeal and molasses
51115020	Bread, cornmeal and molasses, toasted
51119010	Bread, egg, Challah
51119040	Bread, egg, Challah, toasted
51121015	Garlic bread, NFS
51121025	Garlic bread, from fast food / restaurant
51121035	Garlic bread, from frozen
51121045	Garlic bread, with parmesan cheese, from fast food / restaurant
51121055	Garlic bread, with parmesan cheese, from frozen
51121065	Garlic bread, with melted cheese, from fast food / restaurant
51121075	Garlic bread, with melted cheese, from frozen
51121110	Bread, onion
51121120	Bread, onion, toasted
51122000	Bread, reduced calorie and/or high fiber, white or NFS
51122010	Bread, reduced calorie and/or high fiber, white or NFS, toasted
51122100	Bread, reduced calorie and/or high fiber, white or NFS, with fruit and/or nuts
51122110	Bread, reduced calorie and/or high fiber, white or NFS, with fruit and/or nuts, toasted
51122300	Bread, white, special formula, added fiber

Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
51122310	Bread, white, special formula, added fiber, toasted
51123010	Bread, high protein
51123020	Bread, high protein, toasted
51127010	Bread, potato
51127020	Bread, potato, toasted
51129010	Bread, raisin
51129020	Bread, raisin, toasted
51130510	Bread, white, low sodium or no salt
51130520	Bread, white, low sodium or no salt, toasted
51133010	Bread, sour dough
51133020	Bread, sour dough, toasted
51134000	Bread, sweet potato
51134010	Bread, sweet potato, toasted
51135000	Bread, vegetable
51135010	Bread, vegetable, toasted
51140100	Bread, dough, fried
51300050	Bread, whole grain white
51300060	Bread, whole grain white, toasted
51300110	Bread, whole wheat
51300120	Bread, whole wheat, toasted
51300140	Bread, whole wheat, made from home recipe or purchased at bakery
51300150	Bread, whole wheat, made from home recipe or purchased at bakery, toasted
51300175	Bread, chappatti or roti, wheat
51300180	Bread, puri, wheat
51300185	Bread, paratha, wheat
51300210	Bread, whole wheat, with raisins
51300220	Bread, whole wheat, with raisins, toasted
51300300	Bread, sprouted wheat
51300310	Bread, sprouted wheat, toasted
51301010	Bread, wheat or cracked wheat
51301020	Bread, wheat or cracked wheat, toasted
51301040	Bread, wheat or cracked wheat, made from home recipe or purchased at bakery
51301050	Bread, wheat or cracked wheat, made from home recipe or purchased at bakery, toasted
51301120	Bread, wheat or cracked wheat, with raisins
51301130	Bread, wheat or cracked wheat, with raisins, toasted
51301510	Bread, wheat or cracked wheat, reduced calorie and/or high fiber
51301520	Bread, wheat or cracked wheat, reduced calorie and/or high fiber, toasted
51301540	Bread, French or Vienna, whole wheat
51301550	Bread, French or Vienna, whole wheat, toasted
51301600	Bread, pita, whole wheat
51301610	Bread, pita, whole wheat, toasted
51301620	Bread, pita, wheat or cracked wheat
51301630	Bread, pita, wheat or cracked wheat, toasted
51401010	Bread, rye
51401020	Bread, rye, toasted
51401030	Bread, marble rye and pumpernickel
51401040	Bread, marble rye and pumpernickel, toasted
51401060	Bread, rye, reduced calorie and/or high fiber
51401070	Bread, rye, reduced calorie and/or high fiber, toasted
51404010	Bread, pumpernickel
51404020	Bread, pumpernickel, toasted

Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
51407010	Bread, black
51407020	Bread, black, toasted
51501010	Bread, oatmeal
51501020	Bread, oatmeal, toasted
51501040	Bread, oat bran
51501050	Bread, oat bran, toasted
51501060	Bread, oat bran, reduced calorie and/or high fiber
51501070	Bread, oat bran, reduced calorie and/or high fiber, toasted
51601010	Bread, multigrain, toasted
51601020	Bread, multigrain
51601210	Bread, multigrain, with raisins
51601220	Bread, multigrain, with raisins, toasted
51602010	Bread, multigrain, reduced calorie and/or high fiber
51602020	Bread, multigrain, reduced calorie and/or high fiber, toasted
51801010	Bread, barley
51801020	Bread, barley, toasted
51804010	Bread, soy
51804020	Bread, soy, toasted
51805010	Bread, sunflower meal
51805020	Bread, sunflower meal, toasted
51806010	Bread, rice
51806020	Bread, rice, toasted
51807000	Injera, Ethiopian bread
51808000	Bread, gluten free
51808010	Bread, gluten free, toasted
5402: Cereal bars	
53710400	Cereal or granola bar (General Mills Fiber One Chewy Bar)
53710500	Cereal or granola bar (Kellogg's Nutri-Grain Cereal Bar)
53710502	Cereal or granola bar (Kellogg's Nutri-Grain Yogurt Bar)
53710504	Cereal or granola bar (Kellogg's Nutri-Grain Fruit and Nut Bar)
53710600	Milk 'n Cereal bar
53710700	Cereal or granola bar (Kellogg's Special K bar)
53710900	Cereal or granola bar (General Mills Nature Valley Chewy Trail Mix)
53710902	Cereal or granola bar, with yogurt coating (General Mills Nature Valley Chewy Granola Bar)
53710904	Cereal or granola bar (General Mills Nature Valley Sweet and Salty Granola Bar)
53710906	Cereal or granola bar (General Mills Nature Valley Crunchy Granola Bar)
53711000	Cereal or granola bar (Quaker Chewy Granola Bar)
53711002	Cereal or granola bar (Quaker Chewy 90 Calorie Granola Bar)
53711004	Cereal or granola bar (Quaker Chewy 25% Less Sugar Granola Bar)
53711006	Cereal or granola bar (Quaker Chewy Dipps Granola Bar)
53711100	Cereal or granola bar (Quaker Granola Bites)
53712000	Snack bar, oatmeal
53712100	Cereal or Granola bar, NFS
53712200	Cereal or granola bar, lowfat, NFS
53712210	Cereal or granola bar, nonfat
53713000	Cereal or granola bar, reduced sugar, NFS
53713100	Cereal or granola bar, peanuts , oats, sugar, wheat germ
53714200	Cereal or granola bar, chocolate coated, NFS
53714210	Cereal or granola bar, with coconut, chocolate coated
53714220	Cereal or granola bar with nuts, chocolate coated
53714230	Cereal or granola bar, oats, nuts, coated with non-chocolate coating

Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
53714250	Cereal or granola bar, coated with non-chocolate coating
53714300	Cereal or granola bar, high fiber, coated with non-chocolate yogurt coating
53714400	Cereal or granola bar, with rice cereal
53714500	Breakfast bar, NFS
53714510	Breakfast bar, date, with yogurt coating
53714520	Breakfast bar, cereal crust with fruit filling, lowfat
5404: Nutrition bars	
53710800	Cereal or granola bar (Kashi Chewy)
53710802	Cereal or granola bar (Kashi Crunchy)
53720100	Nutrition bar (Balance Original Bar)
53720200	Nutrition bar (Clif Bar)
53720210	Nutrition bar (Clif Kids Organic Zbar)
53720300	Nutrition bar (PowerBar)
53720400	Nutrition bar (Slim Fast Original Meal Bar)
53720500	Nutrition bar (Snickers Marathon Protein Bar)
53720600	Nutrition bar (South Beach Living Meal Bar)
53720610	Nutrition bar (South Beach Living High Protein Bar)
53720700	Nutrition bar (Tiger's Milk)
53720800	Nutrition bar (Zone Perfect Classic Crunch)
53729000	Nutrition bar or meal replacement bar, NFS
5506: Doughnuts, sweet rolls, pastries	
51160000	Roll, sweet, no frosting
51160100	Roll, sweet, cinnamon bun, no frosting
51160110	Roll, sweet, cinnamon bun, frosted
51161000	Pan Dulce, with fruit, no frosting
51161020	Roll, sweet, with fruit, frosted
51161030	Roll, sweet, with fruit, frosted, diet
51161050	Roll, sweet, frosted
51161250	Pan Dulce, no topping
51161270	Pan Dulce, with sugar topping
51161280	Pan Dulce, with raisins and icing
51165000	Coffee cake, yeast type
51166000	Croissant
51166100	Croissant, cheese
51166200	Croissant, chocolate
51166500	Croissant, fruit
51167000	Brioche
51168000	Bread, Spanish coffee
51188100	Pannetone
53415120	Fritter, apple
53415200	Fritter, banana
53415220	Fritter, berry
53420000	Cream puff, eclair, custard or cream filled, NS as to icing
53420100	Cream puff, eclair, custard or cream filled, not iced
53420200	Cream puff, eclair, custard or cream filled, iced
53420210	Cream puff, eclair, custard or cream filled, iced, reduced fat
53420250	Cream puff, no filling or icing
53420300	Air filled fritter or fried puff, without syrup, Puerto Rican style
53420310	Wheat flour fritter, without syrup
53420400	Sopaipilla, without syrup or honey
53420410	Sopaipilla with syrup or honey

Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
53430700	Tamale, sweet
53430750	Tamale, sweet, with fruit
53452100	Pastry, fruit-filled
53452120	Pastry, made with bean or lotus seed paste filling, baked
53452130	Pastry, made with bean paste and salted egg yolk filling, baked
53452150	Pastry, Chinese, made with rice flour
53452170	Pastry, cookie type, fried
53452200	Pastry, Italian, with cheese
53452400	Pastry, puff
53452420	Pastry, puff, custard or cream filled, iced or not iced
53452450	Cheese pastry puffs
53452500	Pastry, mainly flour and water, fried
53500100	Breakfast pastry, NFS
53510000	Danish pastry, plain or spice
53510100	Danish pastry, with fruit
53511000	Danish pastry, with cheese
53520000	Doughnut, NS as to cake or yeast
53520110	Doughnut, cake type
53520120	Doughnut, chocolate, cake type
53520140	Doughnut, cake type, chocolate covered
53520150	Doughnut, cake type, chocolate covered, dipped in peanuts
53520160	Doughnut, chocolate, cake type, with chocolate icing
53520200	Churros
53520500	Doughnut, Asian
53520600	Cruller, NFS
53520700	French cruller
53521100	Doughnut, chocolate, raised or yeast, with chocolate icing
53521110	Doughnut, raised or yeast
53521120	Doughnut, chocolate, raised or yeast
53521130	Doughnut, raised or yeast, chocolate covered
53521140	Doughnut, jelly
53521210	Doughnut, custard-filled
53521220	Doughnut, chocolate cream-filled
53521230	Doughnut, custard-filled, with icing
53530000	Breakfast tart
53530010	Breakfast tart, lowfat
53610100	Coffee cake, crumb or quick-bread type
53610170	Coffee cake, crumb or quick-bread type, with fruit
53610200	Coffee cake, crumb or quick-bread type, cheese-filled
55801000	Funnel cake with sugar
55801010	Funnel cake with sugar and fruit
58123120	Sweet bread dough, filled with bean paste, meatless, steamed
58124210	Pastry, cheese-filled
9802: Protein and nutritional powders	
95201000	Nutritional powder mix (Carnation Instant Breakfast)
95201010	Nutritional powder mix, sugar free (Carnation Instant Breakfast)
95201200	Nutritional powder mix (EAS Whey Protein Powder)
95201300	Nutritional powder mix (EAS Soy Protein Powder)
95201500	Nutritional powder mix, high protein (Herbalife)
95201600	Nutritional powder mix (Isopure)
95201700	Nutritional powder mix (Kellogg's Special K20 Protein Water)

Appendix 1. Food Codes used in the Exposure Estimate for Chlorella Powder and Chlorella Micro Powder	
Food Code	**Description**
95202000	Nutritional powder mix (Muscle Milk)
95202010	Nutritional powder mix, light (Muscle Milk)
95210000	Nutritional powder mix (Slim Fast)
95210010	Nutritional powder mix, sugar free (Slim Fast)
95210020	Nutritional powder mix, high protein (Slim Fast)
95220000	Nutritional powder mix, NFS
95220010	Nutritional powder mix, high protein, NFS
95230000	Nutritional powder mix, whey based, NFS
95230010	Nutritional powder mix, protein, soy based, NFS
95230020	Nutritional powder mix, protein, light, NFS
95230030	Nutritional powder mix, protein, NFS
4004: Pasta, noodles, cooked grains	
41425010	Vermicelli, made from soybeans
56104000	Pasta, vegetable, cooked
56112000	Noodles, cooked
56113000	Noodles, whole grain, cooked
56113990	Noodles, vegetable, cooked
56116990	Long rice noodles, made from mung beans, cooked
56117090	Rice noodles, cooked
56130000	Pasta, cooked
56132990	Pasta, whole grain, cooked
56140100	Pasta, gluten free
56200390	Barley, NS as to fat added in cooking
56200400	Barley, fat not added in cooking
56200410	Barley, fat added in cooking
56200490	Buckwheat groats, NS as to fat added in cooking
56200500	Buckwheat groats, fat not added in cooking
56200510	Buckwheat groats, fat added in cooking
56201990	Millet, NS as to fat added in cooking
56202000	Millet, fat not added in cooking
56202100	Millet, fat added in cooking
56204000	Quinoa, NS as to fat added in cooking
56204005	Quinoa, fat not added in cooking
56204010	Quinoa, fat added in cooking
56207110	Bulgur, fat not added in cooking
56207120	Bulgur, fat added in cooking
56207130	Bulgur, NS as to fat added in cooking
56207160	Couscous, plain, cooked

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Method: GC-MS/MS, Limit of Quantitation: 0.01 ppm						
1,1-Dichloro-2,2-Bis(4-ethylphenyl)ethane	ND	ND	ND	ND	ND	ND
2-(1-Naphthyl)acetamide	ND	ND	ND	ND	ND	ND
2,4-DB	ND	ND	ND	ND	ND	ND
2-phenylphenol	ND	ND	ND	ND	ND	ND
Acetochlor	ND	ND	ND	ND	ND	ND
Acrinathrin	ND	ND	ND	ND	ND	ND
Alachlor	ND	ND	ND	ND	ND	ND
Aldrin/dieldrin	ND	ND	ND	ND	ND	ND
Ametryn	ND	ND	ND	ND	ND	ND
Atrazine	ND	ND	ND	ND	ND	ND
Azaconazole	ND	ND	ND	ND	ND	ND
Azinphos-methyl	ND	ND	ND	ND	ND	ND
Benalaxyl	ND	ND	ND	ND	ND	ND
Bendiocarb	ND	ND	ND	ND	ND	ND
Benfluralin	ND	ND	ND	ND	ND	ND
Benoxacor	ND	ND	ND	ND	ND	ND
Bifenox	ND	ND	ND	ND	ND	ND
Bifenthrin	ND	ND	ND	ND	ND	ND
Bioresmethrin	ND	ND	ND	ND	ND	ND
Bitertanol	ND	ND	ND	ND	ND	ND
Bromacil	ND	ND	ND	ND	ND	ND
Bromobutide	ND	ND	ND	ND	ND	ND
Bromophos ethyl	ND	ND	ND	ND	ND	ND
Bromopropylate	ND	ND	ND	ND	ND	ND
Bupirimate	ND	ND	ND	ND	ND	ND
Butafenacil	ND	ND	ND	ND	ND	ND
Butamifos	ND	ND	ND	ND	ND	ND
Cadusafos	ND	ND	ND	ND	ND	ND
Cafenstrole	ND	ND	ND	ND	ND	ND
Captan	ND	ND	ND	ND	ND	ND
Chinomethionat	ND	ND	ND	ND	ND	ND
Chlorbenside	ND	ND	ND	ND	ND	ND
Chlorbufam	ND	ND	ND	ND	ND	ND
Chlordane	ND	ND	ND	ND	ND	ND
Chlorethoxyphos	ND	ND	ND	ND	ND	ND
Chlorfenapyr	ND	ND	ND	ND	ND	ND
Chlorfenson	ND	ND	ND	ND	ND	ND
Chlorfenvinphos	ND	ND	ND	ND	ND	ND
Chlorobenzilate	ND	ND	ND	ND	ND	ND
Chloroneb	ND	ND	ND	ND	ND	ND
Chlorpropham	ND	ND	ND	ND	ND	ND
Chlorpyrifos	ND	ND	ND	ND	ND	ND
Chlorpyrifos methyl	ND	ND	ND	ND	ND	ND
Chlorthal-dimethyl	ND	ND	ND	ND	ND	ND
Chlozolinate	ND	ND	ND	ND	ND	ND
Cinidon-ethyl	ND	ND	ND	ND	ND	ND
Clodinafop-propargyl	ND	ND	ND	ND	ND	ND
Clomazone	ND	ND	ND	ND	ND	ND
Clomeprop	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Cloquintocet-mexyl	ND	ND	ND	ND	ND	ND
Cyanazine	ND	ND	ND	ND	ND	ND
Cyanophos	ND	ND	ND	ND	ND	ND
Cychloxydim	ND	ND	ND	ND	ND	ND
Cyclosulfamuron	ND	ND	ND	ND	ND	ND
Cyfluthrin	ND	ND	ND	ND	ND	ND
Cyhalofop-butyl	ND	ND	ND	ND	ND	ND
Cyhalothrin	ND	ND	ND	ND	ND	ND
Cypermethrin	ND	ND	ND	ND	ND	ND
Cyproconazole	ND	ND	ND	ND	ND	ND
DCIP	ND	ND	ND	ND	ND	ND
Deltamethrin/tralomethrin	ND	ND	ND	ND	ND	ND
Demeton-s-methyl	ND	ND	ND	ND	ND	ND
Desmedipham	ND	ND	ND	ND	ND	ND
Diazinon	ND	ND	ND	ND	ND	ND
Dichlobenil	ND	ND	ND	ND	ND	ND
Dichlofenthion	ND	ND	ND	ND	ND	ND
Dichlofluanid	ND	ND	ND	ND	ND	ND
Diclocymet	ND	ND	ND	ND	ND	ND
Diclofop-methyl	ND	ND	ND	ND	ND	ND
Diclomezine	ND	ND	ND	ND	ND	ND
Dicloran	ND	ND	ND	ND	ND	ND
Dicofol	ND	ND	ND	ND	ND	ND
Dicrotophos	ND	ND	ND	ND	ND	ND
Diethofencarb	ND	ND	ND	ND	ND	ND
Difenoconazole	ND	ND	ND	ND	ND	ND
Difenzoquat	ND	ND	ND	ND	ND	ND
Diflufenican	ND	ND	ND	ND	ND	ND
Dimepiperate	ND	ND	ND	ND	ND	ND
Dimethametryn	ND	ND	ND	ND	ND	ND
Dimethenamid	ND	ND	ND	ND	ND	ND
Dimethipin	ND	ND	ND	ND	ND	ND
Dimethoate	ND	ND	ND	ND	ND	ND
Diniconazole	ND	ND	ND	ND	ND	ND
Dioxathion	ND	ND	ND	ND	ND	ND
Disulfoton	ND	ND	ND	ND	ND	ND
Endosulfan	ND	ND	ND	ND	ND	ND
Endrin	ND	ND	ND	ND	ND	ND
EPN	ND	ND	ND	ND	ND	ND
EPTC	ND	ND	ND	ND	ND	ND
Esprocarb	ND	ND	ND	ND	ND	ND
Ethalfluralin	ND	ND	ND	ND	ND	ND
Ethiofencarb	ND	ND	ND	ND	ND	ND
Ethion	ND	ND	ND	ND	ND	ND
Ethofumesate	ND	ND	ND	ND	ND	ND
Ethoprophos	ND	ND	ND	ND	ND	ND
Etobenzanid	ND	ND	ND	ND	ND	ND
Etofenprox	ND	ND	ND	ND	ND	ND
Etoxazole	ND	ND	ND	ND	ND	ND
Etridiazole	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Etrimfos	ND	ND	ND	ND	ND	ND
Fenamidone	ND	ND	ND	ND	ND	ND
Fenamiphos	ND	ND	ND	ND	ND	ND
Fenarimol	ND	ND	ND	ND	ND	ND
Fenbuconazole	ND	ND	ND	ND	ND	ND
Fenchlorphos	ND	ND	ND	ND	ND	ND
Fenitrothion	ND	ND	ND	ND	ND	ND
Fenoxycarb	ND	ND	ND	ND	ND	ND
Fenpropathrin	ND	ND	ND	ND	ND	ND
Fenpropimorph	ND	ND	ND	ND	ND	ND
Fensulfothion	ND	ND	ND	ND	ND	ND
Fenvalerate	ND	ND	ND	ND	ND	ND
Flamprop-methyl	ND	ND	ND	ND	ND	ND
Fluacrypyrim	ND	ND	ND	ND	ND	ND
Flucythrinate	ND	ND	ND	ND	ND	ND
Fludioxonil	ND	ND	ND	ND	ND	ND
Flufenpyr-ethyl	ND	ND	ND	ND	ND	ND
Flumioxazin	ND	ND	ND	ND	ND	ND
Fluquinconazole	ND	ND	ND	ND	ND	ND
Fluridone	ND	ND	ND	ND	ND	ND
Flusilazole	ND	ND	ND	ND	ND	ND
Flutolanil	ND	ND	ND	ND	ND	ND
Fluvalinate	ND	ND	ND	ND	ND	ND
Folpet	ND	ND	ND	ND	ND	ND
Fthalide	ND	ND	ND	ND	ND	ND
Halfenprox	ND	ND	ND	ND	ND	ND
Heptachlor	ND	ND	ND	ND	ND	ND
Hexachlorobenzene	ND	ND	ND	ND	ND	ND
Hexaconazole	ND	ND	ND	ND	ND	ND
Hexazinone	ND	ND	ND	ND	ND	ND
Imazamethaben methyl ester	ND	ND	ND	ND	ND	ND
Imazaquin	ND	ND	ND	ND	ND	ND
Imibenconazole	ND	ND	ND	ND	ND	ND
Isazophos	ND	ND	ND	ND	ND	ND
Isocarbophos	ND	ND	ND	ND	ND	ND
Isofenphos	ND	ND	ND	ND	ND	ND
Isoprothiolane	ND	ND	ND	ND	ND	ND
Isoxathion	ND	ND	ND	ND	ND	ND
Kresoxim-methyl	ND	ND	ND	ND	ND	ND
Lactofen	ND	ND	ND	ND	ND	ND
Lenacil	ND	ND	ND	ND	ND	ND
Malathion	ND	ND	ND	ND	ND	ND
Mecarbam	ND	ND	ND	ND	ND	ND
Mefenpyr-diethyl	ND	ND	ND	ND	ND	ND
Mepronil	ND	ND	ND	ND	ND	ND
Metalaxyl-mefenoxam	ND	ND	ND	ND	ND	ND
Metconazole	ND	ND	ND	ND	ND	ND
Methabenzthiazuron	ND	ND	ND	ND	ND	ND
Methacrifos	ND	ND	ND	ND	ND	ND
Methidathion	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Methoprene	ND	ND	ND	ND	ND	ND
Methoxychlor	ND	ND	ND	ND	ND	ND
Metolachlor	ND	ND	ND	ND	ND	ND
Metominostrobin	ND	ND	ND	ND	ND	ND
Metribuzin	ND	ND	ND	ND	ND	ND
Mevinphos	ND	ND	ND	ND	ND	ND
Molinate	ND	ND	ND	ND	ND	ND
Monocrotophos	ND	ND	ND	ND	ND	ND
Monolinuron	ND	ND	ND	ND	ND	ND
Myclobutanil	ND	ND	ND	ND	ND	ND
Napropamide	ND	ND	ND	ND	ND	ND
Nicotine	ND	ND	ND	ND	ND	ND
Norflurazon	ND	ND	ND	ND	ND	ND
Oxadixyl	ND	ND	ND	ND	ND	ND
Oxpoconazole fumarate	ND	ND	ND	ND	ND	ND
Oxyfluorfen	ND	ND	ND	ND	ND	ND
Paclobutrazol	ND	ND	ND	ND	ND	ND
Parathion	ND	ND	ND	ND	ND	ND
Parathion-methyl	ND	ND	ND	ND	ND	ND
Penconazole	ND	ND	ND	ND	ND	ND
Pendimethalin	ND	ND	ND	ND	ND	ND
Pentoxazone	ND	ND	ND	ND	ND	ND
Permethrin	ND	ND	ND	ND	ND	ND
Phenothrin	ND	ND	ND	ND	ND	ND
Phenthoate	ND	ND	ND	ND	ND	ND
Phorate	ND	ND	ND	ND	ND	ND
Phosalone	ND	ND	ND	ND	ND	ND
Phosphamidon	ND	ND	ND	ND	ND	ND
Picolinafen	ND	ND	ND	ND	ND	ND
Piperonyl butoxide	ND	ND	ND	ND	ND	ND
Pirimiphos-methyl	ND	ND	ND	ND	ND	ND
Pretilachlor	ND	ND	ND	ND	ND	ND
Prochloraz	ND	ND	ND	ND	ND	ND
Procymidone	ND	ND	ND	ND	ND	ND
Profenofos	ND	ND	ND	ND	ND	ND
Prohydrojasmon	ND	ND	ND	ND	ND	ND
Prometryn	ND	ND	ND	ND	ND	ND
Propachlor	ND	ND	ND	ND	ND	ND
Propahos	ND	ND	ND	ND	ND	ND
Propanil	ND	ND	ND	ND	ND	ND
Propargite	ND	ND	ND	ND	ND	ND
Propazine	ND	ND	ND	ND	ND	ND
Propoxur	ND	ND	ND	ND	ND	ND
Prothiofos	ND	ND	ND	ND	ND	ND
Pyraclofos	ND	ND	ND	ND	ND	ND
Pyraflufen ethyl	ND	ND	ND	ND	ND	ND
Pyrazophos	ND	ND	ND	ND	ND	ND
Pyrethrins	ND	ND	ND	ND	ND	ND
Pyridaben	ND	ND	ND	ND	ND	ND
Pyridafenthion	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Pyrifenox	ND	ND	ND	ND	ND	ND
Pyrimethanil	ND	ND	ND	ND	ND	ND
Pyrimidifen	ND	ND	ND	ND	ND	ND
Pyriproxyfen	ND	ND	ND	ND	ND	ND
Quinalphos	ND	ND	ND	ND	ND	ND
Quinoclamine	ND	ND	ND	ND	ND	ND
Quinoxyfen	ND	ND	ND	ND	ND	ND
Quintozene	ND	ND	ND	ND	ND	ND
Resmethrin	ND	ND	ND	ND	ND	ND
Simazine	ND	ND	ND	ND	ND	ND
Simetryn	ND	ND	ND	ND	ND	ND
Spirodiclofen	ND	ND	ND	ND	ND	ND
Tebuconazole	ND	ND	ND	ND	ND	ND
Tebufenpyrad	ND	ND	ND	ND	ND	ND
Tecnazene	ND	ND	ND	ND	ND	ND
Tefluthrin	ND	ND	ND	ND	ND	ND
Tepraloxydim	ND	ND	ND	ND	ND	ND
Terbacil	ND	ND	ND	ND	ND	ND
Terbufos	ND	ND	ND	ND	ND	ND
Terbutryn	ND	ND	ND	ND	ND	ND
Tetrachlorvinphos	ND	ND	ND	ND	ND	ND
Tetraconazole	ND	ND	ND	ND	ND	ND
Tetradifon	ND	ND	ND	ND	ND	ND
Thifluzamide	ND	ND	ND	ND	ND	ND
Thiobencarb	ND	ND	ND	ND	ND	ND
Thiometon	ND	ND	ND	ND	ND	ND
Tiadinil	ND	ND	ND	ND	ND	ND
Tolclofos-methyl	ND	ND	ND	ND	ND	ND
Tolfenpyrad	ND	ND	ND	ND	ND	ND
Tolylfluanid	ND	ND	ND	ND	ND	ND
Triadimefon	ND	ND	ND	ND	ND	ND
Triadimenol	ND	ND	ND	ND	ND	ND
Triallate	ND	ND	ND	ND	ND	ND
Triazophos	ND	ND	ND	ND	ND	ND
Tribuphos	ND	ND	ND	ND	ND	ND
Trichlamide	ND	ND	ND	ND	ND	ND
Trifluralin	ND	ND	ND	ND	ND	ND
Uniconazole p	ND	ND	ND	ND	ND	ND
Vamidothion	ND	ND	ND	ND	ND	ND
Vinclozolin	ND	ND	ND	ND	ND	ND
XMC	ND	ND	ND	ND	ND	ND
Zoxamide	ND	ND	ND	ND	ND	ND
Method: HPLC-MS/MS, Limit of Quantitation: 0.01 ppm						
Acetamiprid	ND	ND	ND	ND	ND	ND
Alanycarb	ND	ND	ND	ND	ND	ND
Anilazine	ND	ND	ND	ND	ND	ND
Anilofos	ND	ND	ND	ND	ND	ND
Aramite	ND	ND	ND	ND	ND	ND
Azafenidin	ND	ND	ND	ND	ND	ND
Azimsulfuron	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Azoxystrobin	ND	ND	ND	ND	ND	ND
Bensulfuron methyl	ND	ND	ND	ND	ND	ND
Bensulide	ND	ND	ND	ND	ND	ND
Benthiavalicarb isopropyl	ND	ND	ND	ND	ND	ND
Bispyribac sodium	ND	ND	ND	ND	ND	ND
Boscalid	ND	ND	ND	ND	ND	ND
Buprofezin	ND	ND	ND	ND	ND	ND
Carbaryl	ND	ND	ND	ND	ND	ND
Carpropamid	ND	ND	ND	ND	ND	ND
Chlorantraniliprole	ND	ND	ND	ND	ND	ND
Chloridazon	ND	ND	ND	ND	ND	ND
Chlorimuron ethyl	ND	ND	ND	ND	ND	ND
Chlorsulfuron	ND	ND	ND	ND	ND	ND
Chromafenozide	ND	ND	ND	ND	ND	ND
Cinosulfuron	ND	ND	ND	ND	ND	ND
Clofencet	ND	ND	ND	ND	ND	ND
Clofentezine	ND	ND	ND	ND	ND	ND
Cloransulam-methyl	ND	ND	ND	ND	ND	ND
Clothianidin	ND	ND	ND	ND	ND	ND
Cumyluron	ND	ND	ND	ND	ND	ND
Cyazofamid	ND	ND	ND	ND	ND	ND
Cycloate	ND	ND	ND	ND	ND	ND
Cycloprothrin	ND	ND	ND	ND	ND	ND
Cyenopyrafen	ND	ND	ND	ND	ND	ND
Cyflufenamid	ND	ND	ND	ND	ND	ND
Cyprodinil	ND	ND	ND	ND	ND	ND
Diallate	ND	ND	ND	ND	ND	ND
Diflubenzuron	ND	ND	ND	ND	ND	ND
Dimethirimol	ND	ND	ND	ND	ND	ND
Dimethomorph	ND	ND	ND	ND	ND	ND
Diuron	ND	ND	ND	ND	ND	ND
Epoxiconazole	ND	ND	ND	ND	ND	ND
Ethametsulfuron-methyl	ND	ND	ND	ND	ND	ND
Ethoxysulfuron	ND	ND	ND	ND	ND	ND
Ethychlozate	ND	ND	ND	ND	ND	ND
Famoxadone	ND	ND	ND	ND	ND	ND
Fenhexamid	ND	ND	ND	ND	ND	ND
Fenobucarb	ND	ND	ND	ND	ND	ND
Fenpyroximate	ND	ND	ND	ND	ND	ND
Flazasulfuron	ND	ND	ND	ND	ND	ND
Florasulam	ND	ND	ND	ND	ND	ND
Flufenoxuron	ND	ND	ND	ND	ND	ND
Flumetsulam	ND	ND	ND	ND	ND	ND
Fluometuron	ND	ND	ND	ND	ND	ND
Fluopicolide	ND	ND	ND	ND	ND	ND
Flutriafol	ND	ND	ND	ND	ND	ND
Fosthiazate	ND	ND	ND	ND	ND	ND
Halosulfuron methyl	ND	ND	ND	ND	ND	ND
Hexythiazox	ND	ND	ND	ND	ND	ND
Imazosulfuron	ND	ND	ND	ND	ND	ND

Appendix 2. 345 Pesticides Not Detected in Chlorella Powder and Chlorella Micro Powder						
	Chlorella Powder Lot No.			Chlorella Micro Powder Lot No.		
Pesticide	**180704**	**170725**	**160705**	**180705**	**170728**	**160708**
Indoxacarb	ND	ND	ND	ND	ND	ND
Iososulfuron methyl	ND	ND	ND	ND	ND	ND
Iprovalicarb	ND	ND	ND	ND	ND	ND
Isoprocarb	ND	ND	ND	ND	ND	ND
Isouron	ND	ND	ND	ND	ND	ND
Linuron	ND	ND	ND	ND	ND	ND
Mandipropamid	ND	ND	ND	ND	ND	ND
Mesosulfuron-methyl	ND	ND	ND	ND	ND	ND
Metamitron	ND	ND	ND	ND	ND	ND
Methoxyfenozide	ND	ND	ND	ND	ND	ND
Metosulam	ND	ND	ND	ND	ND	ND
Metsufuron-methyl	ND	ND	ND	ND	ND	ND
Naptalam	ND	ND	ND	ND	ND	ND
Nicosulfuron	ND	ND	ND	ND	ND	ND
Oxamyl	ND	ND	ND	ND	ND	ND
Oxaziclomefone	ND	ND	ND	ND	ND	ND
Oxycarboxin	ND	ND	ND	ND	ND	ND
Oxydemeton-methyl	ND	ND	ND	ND	ND	ND
Pencycuron	ND	ND	ND	ND	ND	ND
Penoxsulam	ND	ND	ND	ND	ND	ND
Phenmedipham	ND	ND	ND	ND	ND	ND
Phosmet	ND	ND	ND	ND	ND	ND
Phoxim	ND	ND	ND	ND	ND	ND
Pirimicarb	ND	ND	ND	ND	ND	ND
Propaquizafop	ND	ND	ND	ND	ND	ND
Pyraclonil	ND	ND	ND	ND	ND	ND
Pyraclostrobin	ND	ND	ND	ND	ND	ND
Pyrazolynate	ND	ND	ND	ND	ND	ND
Pyrazoxyfen	ND	ND	ND	ND	ND	ND
Pyriftalid	ND	ND	ND	ND	ND	ND
Rimsulfruon	ND	ND	ND	ND	ND	ND
Silafluofen	ND	ND	ND	ND	ND	ND
Simeconazole	ND	ND	ND	ND	ND	ND
Sulfosulfuron	ND	ND	ND	ND	ND	ND
Sulfotep	ND	ND	ND	ND	ND	ND
Sulprofos	ND	ND	ND	ND	ND	ND
Tebufenozide	ND	ND	ND	ND	ND	ND
Tebupirimfos	ND	ND	ND	ND	ND	ND
Tebuthiuron	ND	ND	ND	ND	ND	ND
Thiacloprid	ND	ND	ND	ND	ND	ND
Thiamethoxam	ND	ND	ND	ND	ND	ND
Thifensulfuron-methyl	ND	ND	ND	ND	ND	ND
Tolyfloxysulfuron	ND	ND	ND	ND	ND	ND
Tralkoxydim	ND	ND	ND	ND	ND	ND
Tribenuron-methyl	ND	ND	ND	ND	ND	ND
Tricyclazole	ND	ND	ND	ND	ND	ND
Triflumuron	ND	ND	ND	ND	ND	ND

ACGT, INC.

Project Report

Study Title	*De novo* Whole Genome Sequencing of one algal genome
Study Number	688781
Sponsor	Toshihiro Kanno, Ph.D. Assistant General Manager toshihiro_kanno@chlorella.co.jp +81-3-3437-0901
Sponsor Organization	CHLORELLA INDUSTRY CO., LTD. No.18-16, Hamamatsucho 1-Chome, Minato-Ku Tokyo, 105-0013, Japan
Study Manager	Hargeet Brar, Ph.D. hargeet_brar@acgtinc.com 800.557.2248 ext 107
Submitted	October 8, 2015

Objective

The objective of this study was the whole genome sequencing and *de novo* assembly of one algal genome. Two libraries, one standard paired-end and one mate-pair, were constructed. Sequencing was performed on the Illumina MiSeq and NextSeq 500 platforms. Both paired-end and mate-pair libraries was used for the assembly. Genes were *ab initio* predicted from the best genome assembly and annotated with various methods.

Library preparation and sequencing

Total DNA was extracted from the algal cell pellet provided by the Sponsor with the ZR Fungal/Bacterial DNA MicroPrepÊ Kit as per manufacturerõs instructions. The quality and quantity of extracted genomic DNA was evaluated with NanoDrop spectroscopy (Table1) and agarose gel electrophoresis (Figure 1). The extracted DNA was found to be of sufficient quantity and quality to proceed with library preparations.

Paired-End Library Preparation

For paired-end sequencing, 2µg of extracted genomic DNA was sheared by focused-ultrasonication to an average 600 bp target fragment size, and used for constructing a sequencing library using the Illumina TruSeq DNA PCR-Free Sample Preparation Kit as per manufacturer's instructions. Appropriate quality control analysis was performed at every step, and the libraries were evaluated and quantified with Qubit 2.0 fluorometer (Table 2) and Agilent 2100 Bioanalyzer (Figure 2). Library was then size-selected on BluePippin platform for 750 to 950 bp fragment size range. Final size selected library was evaluated and quantified with Qubit 2.0 fluorometer (Table 3) and Agilent 2100 Bioanalyzer (Figure 3).

Mate-Pair Library Preparation

Four (4) εg of genomic DNA was used to prepare the Nextera Mate Pair Library. The Gel-Plus Manufacturerõs protocol was followed closely. Initial size selection was done using the BluePippin 0.75% agarose cassette system at 4 to 10 kb. The circularized DNA was sheared using a Covaris M220 with an average 600 bp target fragment size. The library was visualized on the Bioanalyzer (Figure 4). A final size selection step using the BluePippin was performed. The Bioanalyzer and Qubit concentration are in Figure 5 and Table 4, respectively.

Bioinformatics

Sequencing data were demultiplexed into raw FASTQ reads using bcl2fastq version 2.16. About 69 millions (2x150 bp) paired-end reads (CK-22_PE.R[12].raw.fastq.gz) and 34 millions (2x75 bp) mate-pair reads (CK-22_MP.R[12].raw.fastq.gz) were generated in total.

For the paired-end reads, the adapter and low quality sequences (<Q20) were trimmed, short reads (<2x50 bp) were filtered out using Trim Galore version 0.3.7 (CK-22_PE.R[12].trimmed.fastq.gz). The trimmed, paired-end reads were then error-corrected using Musket (CK-22_PE.R[12].corrected.fastq.gz). For the mate-pair reads, the adapter and low quality sequences (<Q20) were trimmed, short reads (<2x35 bp) were discarded using cutadapt version 1.8.1(CK-22_MP.R[12].trimmed.fastq.gz).

To optimize the genome assembly, the error-corrected, paired-end reads and the trimmed, mate-pair reads were *de novo* assembled by running SOAPdenovo version 2.04 and ABySS version 1.9.0 using different Kmer values (41, 51, 55, 61, 65, 71, 73, 75, 77, 79, 81, 83, 85). Based on the N50 size, total number of contigs/scaffolds and the size of the longest contigs/scaffolds, the best assembly was achieved by running ABySS with the Kmer value of 75. Sequences less than

500 bp were removed from the assembly. Then GapFiller version 1.10 and SSPACE version 3.0 were iteratively used for gap closure and scaffolding to generate the final genome assembly (Chlorella_sorokiniana_CK-22.de_novo_genome_assembly.fa).

Genes were *ab initio* predicted from the final genome assembly using AUGUSTUS version 3.1 (Chlorella_sorokiniana_CK-22.gtf) and corresponding coding sequences and protein sequences were generated (Chlorella_sorokiniana_CK-22.CDS.fa, Chlorella_sorokiniana_CK-22.AA.fa). The predicted protein sequences were annotated with BlastKOALA (http://www.kegg.jp/blastkoala/, last accessed on September 4, 2015) using the KEGG Orthology (KO) system (Chlorella_sorokiniana_CK-22.KEGG_annotation.txt and Chlorella_sorokiniana_CK-22.KEGG_annotation.html). In addition, the protein sequences were queried against NCBI nr database and scanned against InterPro collection of protein signature databases using Blast2GO version 3.13 (results under the Blast2GO directory). Gene Ontology (GO) terms and Enzyme Codes (ECs) were also mapped to each sequence (results under the Blast2GO directory).

Deliverables

RUN QC REPORT (pdf files)

QC_report.run1.pdf
QC_report.run2.pdf
QC_report.run3.pdf
QC_report.run4.pdf
QC_report.run5.pdf
QC_report.run6.pdf

RAW FASTQ FILES (compressed fastq files)

CK-22_PE.R1.raw.fastq.gz
CK-22_PE.R2.raw.fastq.gz
CK-22_MP.R1.raw.fastq.gz
CK-22_MP.R2.raw.fastq.gz

TRIMMED FASTQ FILES (compressed fastq files)

CK-22_PE.R1.trimmed.fastq.gz
CK-22_PE.R2.trimmed.fastq.gz
CK-22_PE.R1.corrected.fastq.gz
CK-22_PE.R2.corrected.fastq.gz
CK-22_MP.R1.trimmed.fastq.gz
CK-22_MP.R2.trimmed.fastq.gz

***DE NOVO* ASSEMBLY** (fasta file)

Chlorella_sorokiniana_CK-22.de_novo_genome_assembly.fa
Chlorella_sorokiniana_CK-22.de_novo_genome_assembly.statistics.txt

GENE PREDICTION AND ANNOTATION

Chlorella_sorokiniana_CK-22.gtf
Chlorella_sorokiniana_CK-22.CDS.fa

Chlorella_sorokiniana_CK-22.AA.fa
Chlorella_sorokiniana_CK-22.KEGG_annotation.html
Chlorella_sorokiniana_CK-22.KEGG_annotation.txt
Blast2GO (a directory includes all Blast2GO analysis results, chlorella_sorokiniana_ck_22_aa.b2g can be opened by Blast2GO)

Table 1. NanoDrop Spectroscopy analysis of extracted DNA

Sample ID	Conc.	Units	A260	A280	260/280	260/230	Total Vol	Total DNA	Units
CK-22	64.46	ng/ul	1.289	0.643	2.01	1.95	123	7.9	µg

Table 2. Qubit fluorometry analysis of TruSeq DNA PCR-free Library

Library ID	Assay Conc.	Units	Stock Conc.	Units	Total Vol.	Total DNA	Units
CK-22 TSP1	22	ng/ml	4.4	ng/µl	20	88	ng

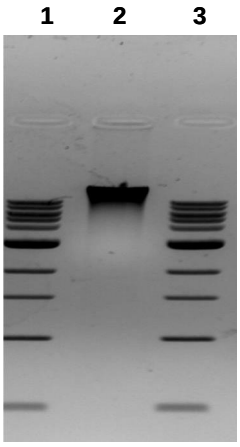
Table 3. Qubit fluorometry analysis of final size-selected TruSeq DNA PCR-free Library

Library ID	Assay Conc.	Units	Stock Conc.	Units	Total Vol.	Total DNA	Units
CK-22 TSP1 Pippin	1.67	ng/ml	0.334	ng/µl	38	12.7	ng

Table 4. Qubit concentration of Nextera Mate Pair Final Library after size selection

	Qubit HS Assay	Unit	Adaptor
CK-22 MP Pippin	0.382	ng/ul	AD018

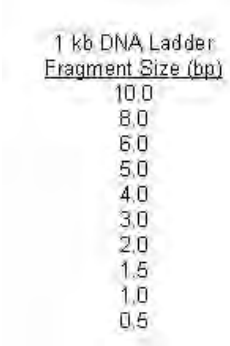
Figure 1. Agarose Gel Electrophoresis of extracted DNA



Lane 1: 1Kb DNA Ladder

Lane 2: CK-22

Lane 3: 1Kb DNA Ladder



4 µl of extracted DNA was loaded on 0.8% agarose gel prepared using TBE buffer

Figure 2. Analysis of TruSeq DNA PCR-free Library on Agilent 2100 Bioanalyzer

CK-22 TSP1

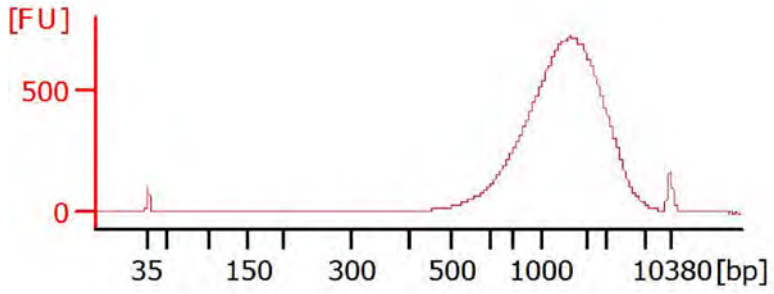


Figure 3. Analysis of Final Size-Selected Library on Agilent 2100 Bioanalyzer

CK-22 TSP1 Pippin

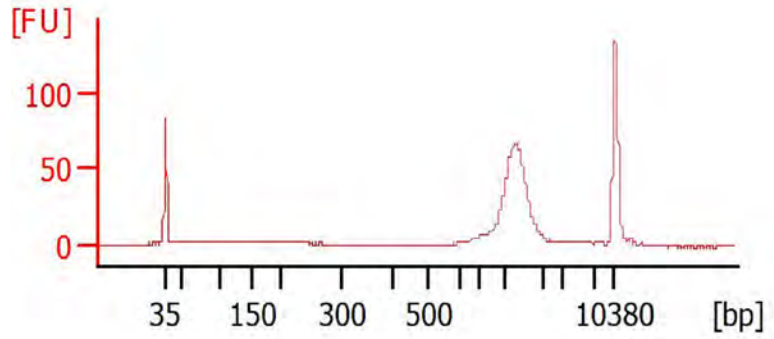


Figure 4. Nextera Mate Pair Library before final size selection

CK-22 MP

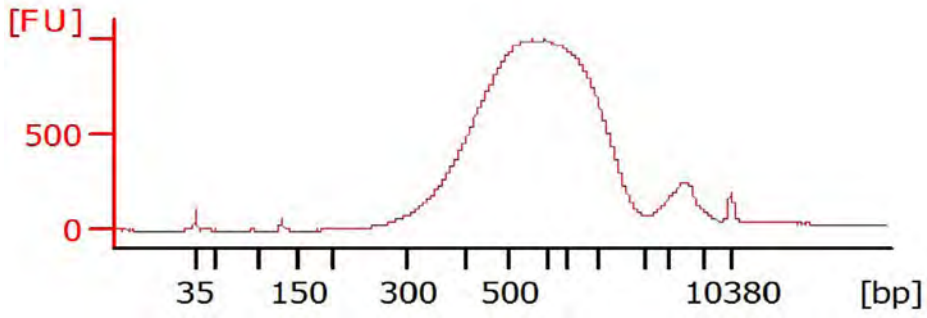


Figure 5. Nextera Mate Pair Final Library after final size selection

CK-22 MP Pippin