AIBMR Life Sciences, Inc.

August 25, 2022

Susan Carlson, PhD
Division Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health and Human Services
5001 Campus Drive
College Park, MD 20740

Dear Dr. Carlson:

In accordance with regulation 21 CFR Part 170 Subpart E (Generally Recognized as Safe (GRAS) Notice), on behalf of Triton Algae Innovations (the notifier), the undersigned, Timothy Murbach, submits, for FDA review, the enclosed notice that the dried biomass powder of *Chlamydomonas reinhardtii* algae (TAI114) is GRAS for use in foods.

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

Sincerely,

Timothy Murbach, ND, DABT (agent of the notifier)
Vice President, Scientific & Regulatory Affairs
AIBMR Life Sciences, Inc. ("AIBMR")

Notice to U.S. Food and Drug Administration of the Conclusion that the Dried Biomass Powder of *Chlamydomonas reinhardtii* algae (TAI114) Is Generally Recognized as Safe for Its Intended Use in Foods

Submitted by the Notifier:

Triton Algae Innovations
11760 Sorrento Valley Road, Suite R
San Diego, CA 92121

Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc
1425 Broadway, Suite 458
Seattle WA 98122

August 25, 2022

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Figures and Tables

Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

Triton Algae Innovations (the notifier) is submitting a new GRAS notice in accordance with 21 CFR Part 170, Subpart E, regarding the conclusion that the dried biomass powder of *Chlamydomonas reinhardtii* algae (TAI114) is Generally Recognized as Safe (GRAS) for its intended use, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act.

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

Notifier

David J. Schroeder
Director, Corporate & Regulatory Affairs
Triton Algae Innovations
11760 Sorrento Valley Road, Suite R
San Diego, CA 92121
Tel: (202) 607-3461
dave@tritonai.com

Agent of the Notifier

Timothy Murbach, ND, DABT
Vice President, Scientific & Regulatory Affairs
AIBMR Life Sciences, Inc.
1425 Broadway
Suite 458
Seattle, WA 98122
Tel: (253) 286-2888
tim@aibmr.com

1.3 Name of the Substance

The dried biomass powder of *Chlamydomonas reinhardtii* algae (TAI114)

1.4 Intended Conditions of Use

TAI114 is intended to be used as a flavoring agent and adjuvant (21 CFR 170.3(o)(12)) and nutritive ingredient in meat and seafood/fish analogues at addition levels up to 0.7% w/w of the meat/seafood/fish analogue ingredient fraction of the finished food product. TAI114 is not intended for use in foods where standards of identity would preclude such use, infant formula, or any products that would require additional regulatory review by USDA.

1.5 Statutory Basis for GRAS Conclusion

The conclusion of GRAS status of TAI114 for its intended conditions of use, stated in Part 1.4 of this notice, has been made based on scientific procedures.

1.6 Not Subject to Premarket approval

We have concluded that TAI114 is GRAS for its intended conditions of use, stated in Part 1.4 of this notice, and, therefore, such use of TAI114 is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data and Information Availability Statement

The data and information that serve as the basis for this GRAS conclusion will be available for review and copying during customary business hours at the office of Triton Algae Innovations, 11760 Sorrento Valley Road, Suite R, San Diego, CA 92121, Telephone: (202) 607-3461, email: dave@tritonai.com, or will be sent to FDA upon request.

1.8 Exemption from Disclosure under the Freedom of Information Act

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

1.9 Certification of Completion

We hereby certify that, to the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the dried biomass powder of *Chlamydomonas reinhardtii* algae (TAI114).

[redacted] 8/25/22

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

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

2.1 Identification

2.1.1 Dried biomass powder of *Chlamydomonas reinhardtii* algae (TAI114)

TAI114 is a non-genetically engineered strain of the algae *Chlamydomonas reinhardtii* (wild type), which was previously designated as strain THN6 by the notifier. THN6 was modified to produce a strain (TAI114) rich (3–7% w/w) in protoporphyrin IX (PPIX) at the expense of chlorophyll. Due to this change, TAI114 has a red appearance, rather than the normally green appearance of THN6.

The current taxonomic classification of *C. reinhardtii* is shown in Table 1 below, and the identification and classification of *C. reinhardtii*, as well as the specific genetic confirmation of the identity of THN6, were described in detail in Subpart 2.1, pages 8 and 9, of GRAS notice (GRN) number 773 to U.S. FDA, which is incorporated here by reference.[1] In brief, *C. reinhardtii* is an extensively studied, fully sequenced, single cell organism of approximately 10 micron diameter with two external flagella and a distinctive eye spot near one flagellar root giving the organism an asymmetric appearance; it also has well-defined internal physical characteristics. Further, *C. reinhardtii* can be accurately identified by sequencing of the internal transcribed spacer (ITS) subregion of the nuclear rDNA cistrons. As with the THN6 parent strain, the ITS1 and ITS2 sequences of TAI114 were compared to the standard reference strain using GenBank® and are analyzed with every production lot to validate the identity of the production strain. Additional information was provided as an amendment to GRN 773 and is summarized as follows:

> THN6 is a natural wild-type isolate acquired from the *Chlamydomonas* Resource Center at the University of Minnesota and has not been genetically modified. THN6 is the notifier's internal company name for the wild-type strain described in the scientific literature. THN6 contains some minor genetic variation in the form of single nucleotide polymorphisms (SNP), as is the case with all organisms. The SNP variations in this strain do not impact the safety findings underpinning the conclusion that the intended use of THN6 in food is GRAS, especially given that the toxicological investigations described and cited in Part 6 of GRN 773 were conducted using THN6 specifically.

Table 1. Taxonomic Classification of *Chlamydomonas reinhardtii*

Rank	Scientific Name and Common Name
Empire	Eukaryota—Eukaryotes
Kingdom	Plantae–Plants
Subkingdom	Viridiplantae—Green Plants
Infrakingdom	Chlorophyta infrakingdom—Green algae
Phylum	Chlorophyta—Chlorophytes
Subphylum	Chlorophytina
Class	Chlorophyceae
Order	Chlamydomonadales
Family	Chlamydomonadaceae
Genus	*Chlamydomonas*
Species	*Chlamydomonas reinhardtii* P.A. Dangeard 1888

Reproduced from AlgaeBase Database[2]

The nutritional profile of TAI114 has been evaluated, and in addition to 30–70% complete protein content, the biomass contains complex carbohydrates and fiber, omega 3, 6, and 9 fatty acids, vitamins, and minerals (see also Table 2—Specifications—below).

2.1.2 Protoporphyrin IX

PPIX (InChI Key ZCFFYALKHPIRKJ-UHFFFAOYSA-N; CAS number 553-12-8; IUPAC name 3-[18-(2-carboxyethyl)-8,13-bis(ethenyl)-3,7,12,17-tetramethyl-22,23-dihydroporphyrin-2-yl]propanoic acid) is an endogenous compound in virtually all species.[3, 4] It is the penultimate compound in the biosynthesis of heme (in both humans and *C. reinhardtii*). In *C. reinhardtii* and other photosynthetic organisms, the pathway branches towards chlorophyll or heme following biosynthesis of PPIX making it the last common compound of the tetrapyrrole pathway. In the heme pathway, PPIX is chelated with iron (via the enzyme ferrochelatase) to form heme while in the chlorophyll pathway it is chelated with magnesium (via the enzyme magnesium chelatase) to form magnesium-PPIX, the first committed compound in the chlorophyll pathway. PPIX is a hydrophobic molecule that is poorly soluble in water and has a solubility of 10 mg/mL in dimethylformamide:methanol (1:1).[5, 6] It appears in its pure form as a red to brown to black powder with an absorption spectrum peak at a wavelength of approximately 405 nm and dual fluorescence emission spectra peaks at wavelengths of 635 (dominant peak) and 705 nm (minor peak).[7] PPIX has the molecular formula $C_{34}H_{34}N_4O_4$, a molecular weight of 562.66 g/mol, and the structural formula shown in Figure 1.

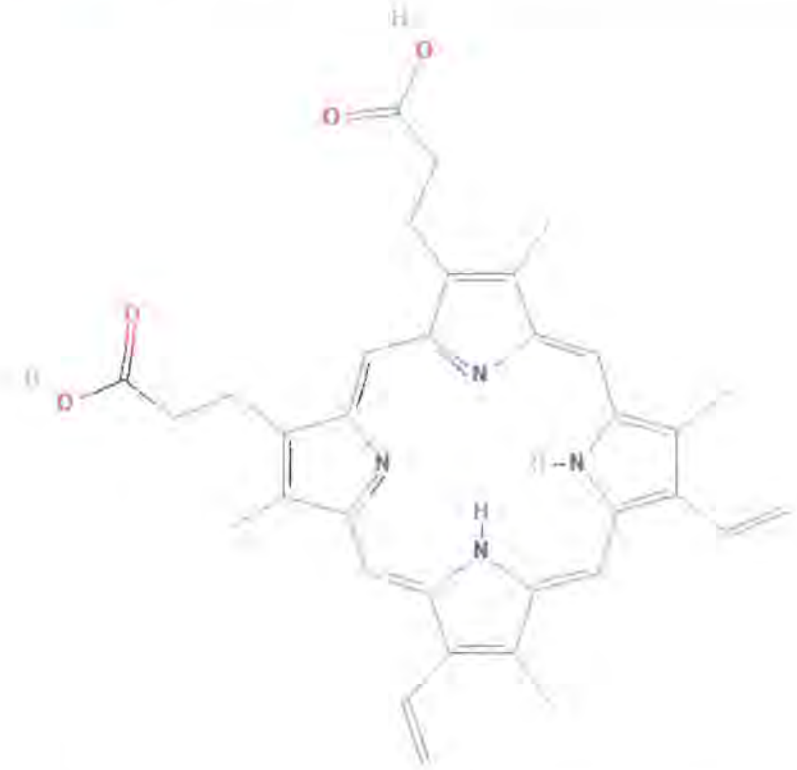

Figure 1. Protoporphyrin IX Structural Formula[8]

2.1.3 Modifications to produce TAI114

To create TAI114, an evolution and selection strategy was applied to THN6 (Figure 2A). Initially, THN6 was exposed to ultraviolet (UV) light for 15 seconds and recovered in growth medium (Figure 2B). Following recovery, strains with substantially reduced chlorophyll fluorescence and displaying high fluorescent properties associated with PPIX were selected using fluorescence-activated cell sorting (FACS; Figure 2C–D). Sorted cells can be visualized under a microscope and a visual difference should be noted between high PPIX strains (red) and strains with lower protoporphyrin (clear) content (Figure 2E). After PPIX rich strains of *C. reinhardtii* were isolated by FACS, strains displaying the highest PPIX fluorescence were mated with THN6 to identify progeny that were further enriched for PPIX and that also displayed robust growth in oxidative fermentation conditions (Figure 2F). The strain with the best growth rate in oxidative fermentation conditions was selected and designated TAI114 (Figure 2G).

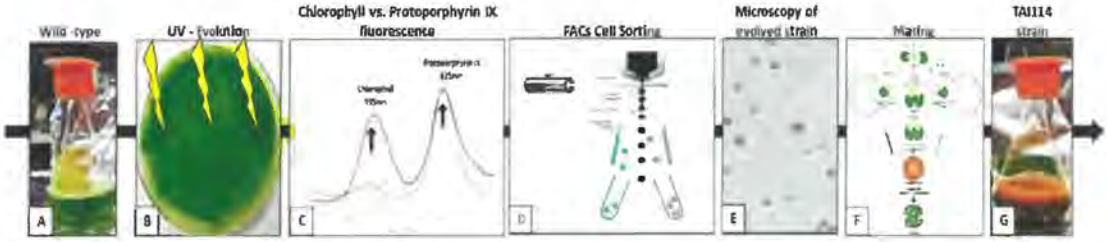

Figure 2. TAI114 Mutagenesis and Selection

Sequence analysis of TAI114 confirmed that a frameshift mutation resulting in PPIX accumulation, and the resulting red color can be found in the magnesium chelatase gene (*ChlH*, H subunit of magnesium chelatase). The mutation that was introduced into the *ChlH* gene introduced a thymine nucleotide into exon 10, at position 3151, that resulted in a non-functional magnesium chelatase gene (see Figure 3). Magnesium chelatase, the first committed compound in the chlorophyll pathway, catalyzes the addition of magnesium to PPIX forming magnesium-PPIX. The absence of the magnesium chelatase H subunit was verified in the proteomics evaluation,[9] as further described in Subpart 6.4.

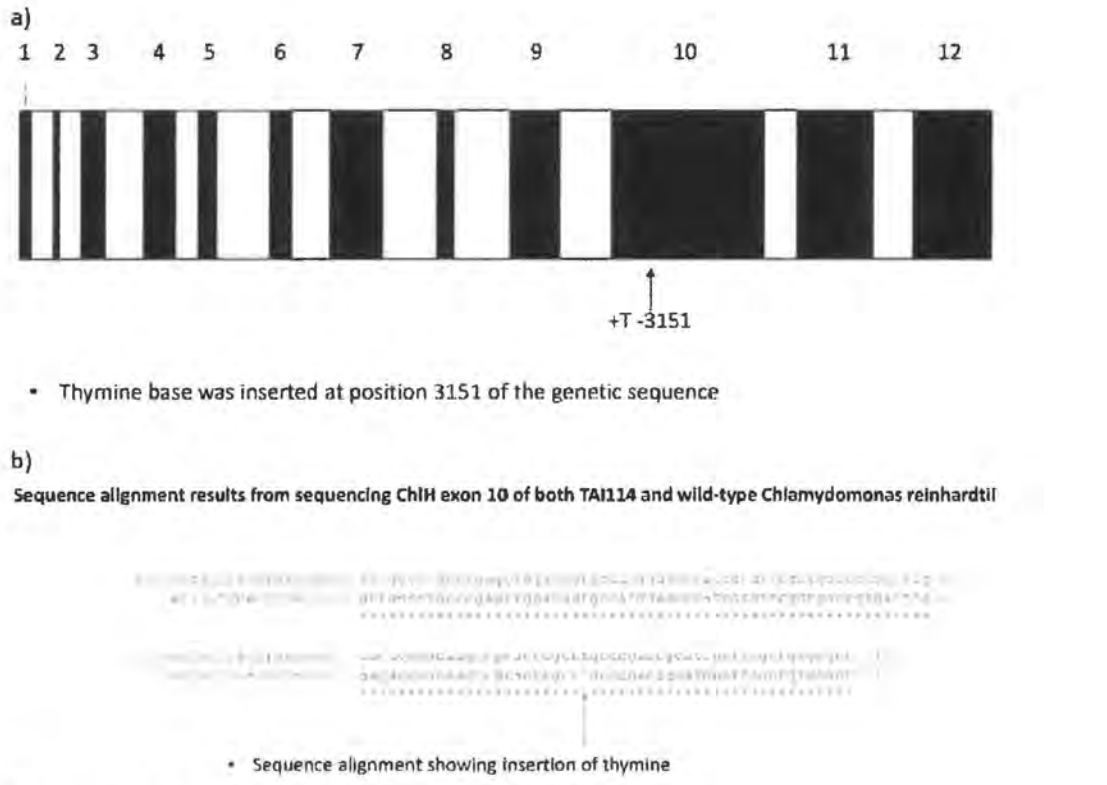

Figure 3. Insertion of Thymine in TAI114 *ChlH* gene

2.2 Manufacturing

2.2.1 Good Manufacturing Practice

TAI114 from Triton Algae Innovations is produced under strict adherence to current good manufacturing practice, hazard analysis, and risk-based preventive controls for human foods set to comply with the U.S. Code of Federal Regulations, 21 CFR part 117.

2.2.2 Raw Materials

Raw materials used in the production of TAI114 are sourced from qualified vendors with certificates of analysis (CoAs) and/or other appropriate documentation, are prepared and handled as food ingredients, and are of suitable quality and purity to the application to produce the final food grade product. American Society for Testing and Materials Type II, Grade B reverse osmosis deionized water is used in the manufacturing process. No material of human or animal origin is used. TAI114 from Triton Algae Innovations has not been genetically engineered.

2.2.3 Manufacturing Narrative and Flowchart

The process of growing and producing TAI114, red *C. reinhardtii*, requires that all ingredient inputs used are food grade chemicals that are food chemical codex compliant (FCC). All materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U. S. Additionally, the algae produced will only be in contact with machines and/or surfaces that are FDA food contact substances (FCS) compliant. This includes the fermenters, spray dryer, and storage containers. The process is diagramed in Figure 3, and briefly described below.

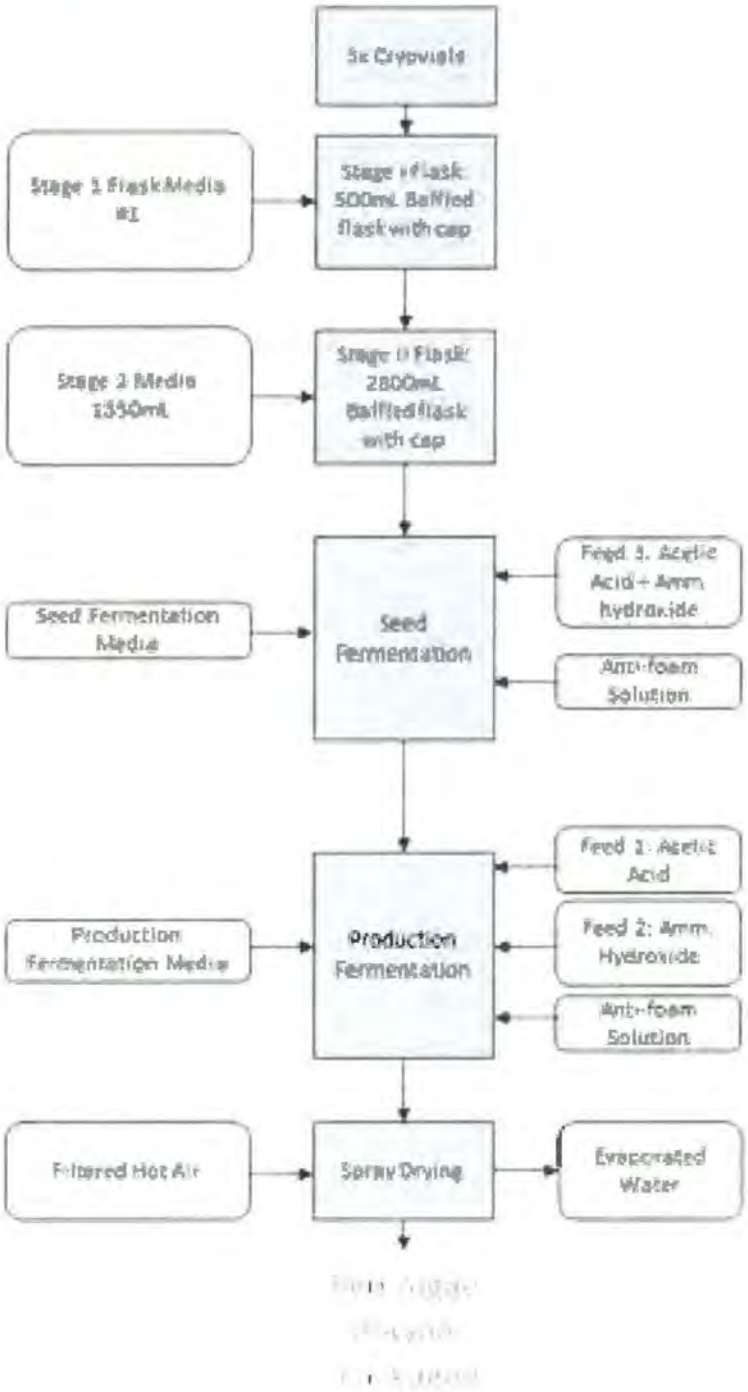

Figure 4. Manufacturing Flowchart

The process of producing TAI114 begins with Cryovials of Triton strain TAI114, which are removed from cryopreservation and thawed. The thawed cultures are then re-suspended in Stage 1 flask media to facilitate the recovery and initial growth of the strains. Following Stage 1 flask growth, the culture is transferred to Stage 2 flask media to facilitate denser growth of the culture.

Once algae in the Stage 2 flask has reached the desired density, it is transferred into seed fermentation vessels. During this fermentation the culture is fed acetic acid (carbon source), ammonium hydroxide (nitrogen source), and anti-foam. Once the culture reaches the desired density, it is then transferred into production fermentation. During production fermentation the culture is also fed acetic acid (carbon source), ammonium hydroxide (nitrogen source), and anti-foam.

Once the culture has reached the desired density in the production fermentation vessel, the culture is transferred to a spray dryer where it is dried before FDA-compliant packaging and shipment to its eventual end use in final product applications.

Critical control points throughout this process are as follows: 1) ITS sequencing for strain validation and identification during Stage 1 of propagation; 2) Sterility checks between tank transfers, which are done by plating and PCR analysis, to ensure sterility between and during fermentations (these checks occur every 24 hours); and 3) Post-harvesting compositional analysis of all compounds represented in the ingredient specifications.

2.3 Specifications

The specifications for the food-grade product TAI114, along with the specification methods, which have been validated for their intended purposes, are listed in Table 2 below.

Table 2. TAI114 Specifications

Tested Parameters	Acceptance Criteria	Methods
Physical Tests		
Appearance	Red powder	Visual
Moisture	≤ 10%	AOAC 925.10
Chemical Tests		
Protoporphyrin IX Assay	3–7%	PPIX prot*
Protein (crude)	30–70%	AOAC 992.15
Fat (crude)	≤ 10%	AOAC 952.06
Fiber (acid detergent)	0–25%	AOCS Ba 6a-05
Starch	≤ 55%	Internal Method†
Ash	≤ 5%	AOAC 923.03
Heavy Metals		
Arsenic	≤ 0.2 ppm	AOAC 993.14 & 2015.06 (ICP-MS)

Cadmium	≤ 0.2 ppm	AOAC 993.14 & 2015.06 (ICP-MS)
Lead	≤ 0.2 ppm	AOAC 993.14 & 2015.06 (ICP-MS)
Mercury	≤ 0.2 ppm	EPA 7473 (TDA/AAS)
Microbiological Tests		
Total Aerobic Microbial	≤ 10,000 CFU/g	AOAC 966.23
Total Yeast & Mold	≤ 1000 CFU/g	BAM Ch. 18
Total Coliforms	≤ 100 CFU/g	AOAC 991.14
Escherichia coli	Negative/10 g	AOAC 991.14
Salmonella spp.	Negative/25 g	AOAC 2013.01
Staphylococcus aureus	≤ 100 CFU/g	AOAC 2003.07

Abbreviations: AOAC, Association of Official Analytical Collaboration; AOCS, American Oil Chemists' Society; BAM, US Food and Drug Administration's Bacteriological Analytical Manual; Ch, chapter; EPA, US Environmental Protection Agency; ICP-MS, inductively coupled plasma-mass spectrometry; TDA/AAS, thermal decomposition, amalgamation/atomic absorption spectrophotometry.
*Triton Algae Innovations internal method: modification of Kang BH, Li N, Liu SG, Li NB, Luo HQ. A Label-free, Highly Sensitive and Selective Detection of Hemin Based on the Competition between Hemin and Protoporphyrin IX Binding to G-Quadruplexes. Anal Sci. 2016;32(8):887-92. doi: 10.2116/analsci.32.887. PMID: 27506716.
†Medallion Labs internal method: modification of AOAC 979.10.

2.3.1 Batch Analysis

Production conformity and consistency of TAI114 are tested in production lots. Batch analyses of three non-consecutive lots, representing approximately 8 months of pilot production, are shown below and are reasonably consistent and met the product specifications.

Table 3. TAI114 Batch Analyses

Tested Parameters	Acceptance Criteria	Lot No./Date of Manufacture		
		Lot# SPRD3 12/09/2020	Lot# SPRD8 02/10/2021	Lot# SPRD12 08/02/2021
Physical Tests				
Appearance	Red powder	Red powder	Red powder	Red powder
Moisture	≤ 10%	7.65%	7.8%	4.72%
Chemical Tests				
Protoporphyrin IX	3–7%	5.21%	5.82%	5.53%
Protein (crude)	30–70%	43.4%	39.5%	40.1%
Fat (crude)	≤ 10%	2.20%	1.47%	1.70%
Fiber (acid detergent)	0–25%	2.0%	1.2%	0.7%
Starch	≤ 55%	27.0%	31.0%	25.9%
Ash	≤ 5%	4.29%	4.9%	4.27%
Heavy Metals				
Arsenic	≤ 0.2 ppm	ND*	ND*	0.02
Cadmium	≤ 0.2 ppm	ND*	ND*	ND*
Lead	≤ 0.2 ppm	ND*	ND*	ND*
Mercury	≤ 0.2 ppm	ND*	ND*	ND*
Microbiological Tests				
Total Aerobic Microbial	≤10,000 CFU/g	3000 CFU/g	2900 CFU/g	2900 CFU/g
Total Yeast & Mold	≤1000 CFU/g	ND*	ND*	ND*

Total Coliforms	≤ 100 CFU/g	ND*	ND*	ND*
Escherichia coli	Negative/10 g	Negative/10 g	Negative/10 g	Negative/10 g
Salmonella spp.	Negative/25 g	Negative/25 g	Negative/25 g	Negative/25 g
Staphylococcus aureus	≤ 100 CFU/g	Negative/1 g	Negative/1 g	Negative/1 g

Abbreviations: ND, not detected.
*Limits of Detection: Arsenic, 10 ppb; Cadmium, 10 ppb; Lead, 10 ppb; Mercury, 4 ppb; Total Yeast and Mold, 10 CFU/g; Total Coliforms, 10 CFU/g; *Staphylococcus aureus*, 10 CFU/g.

2.3.2 Shelf-Life Stability

A one-year shelf-life from the time of manufacture has been recommended as an appropriate expiration period for TAI114. This recommendation is based upon 12-month long-term stability testing of TAI114 lot numbers SPRD3, SPRD8, and SPRD12 (manufactured 9 December 2020, 10 February 2021, and August 2, 2021, respectively). The tests were conducted in an air-conditioned facility at room temperature (approximately 4–25 °C), and TAI114 lots were stored in commercially available fiber drums with plastic liner bags to emulate conditions of commercial packaging. The drums (Uline model https://www.uline.com/Product/Detail/S-10756/Drums/Fiber-Drum-10-Gallon) and liners were made with FDA-compliant materials. At all sampling times, outcome measures included the same physical and chemical tests and test methodologies used for commercial batch analysis (see Table 2). The measures were stable and within specification throughout the test.

2.4 Physical or Technical Effect

TAI114 is not intended to produce any physical or other technical effects that are relevant to the safety of the ingredient.

Part 3: Dietary Exposure

3.1 Intended Use

TAI114 is intended to be used as a flavoring agent and adjuvant (21 CFR 170.3(o)(12)) and nutritive ingredient in meat and seafood/fish analogues at addition levels up to 0.7% w/w of the meat/seafood/fish analogue ingredient fraction of the finished food product. Food codes used as surrogates for the intended use were selected from the National Health and Nutrition Examination Surveys (NHANES) and are shown in Table 4 below along with the maximum addition level used for each code. TAI114 is not intended for use in foods where standards of identity would preclude such use, infant formula, or any products that would require additional regulatory review by USDA.

Table 4. TAI114 Intended Uses

NHANES WWEIA Food	NHANES Food Code	Typical Serving Size* (g or mL)	Maximum Concentration (mg/g)*	Maximum Amount per Serving (g)
Bacon strip, meatless, meat substitute	41810200	100	7.0	0.7
Bacon bits, meatless	41810250	100	7.0	0.7
Breakfast link, pattie, or slice, meatless, meat substitute	41810400	100	7.0	0.7
Chicken, meatless, NFS, meat substitute	41810600	100	7.0	0.7
Chicken, meatless, breaded, fried, meat substitute	41810610	100	7.0	0.7
Frankfurter or hot dog, meatless, meat substitute	41811400	100	7.0	0.7
Luncheon slice, meatless-beef, chicken, salami or turkey, meat substitute	41811600	100	7.0	0.7
Meatball, meatless, meat substitute	41811800	100	7.0	0.7
Vegetarian burger or patty, meatless, no bun, meat substitute	41811890	100	7.0	0.7
Sandwich spread, meat substitute type	41812000	100	7.0	0.7
Vegetarian, fillet, meat substitute	41812600	100	7.0	0.7
Meat substitute, cereal- and vegetable protein-based, fried	59003000	100	7.0	0.7
Swiss steak, with gravy, meatless, meat substitute	41811950	1007	4.8	4.8
Vegetarian pot pie, meat substitute	41812400	847	1.3	1.1
Vegetarian chili, made with meat substitute	41812450	1317	1.2	1.6
Vegetarian stroganoff	41812850	1267	2.5	3.2
Soyburger, meatless, with cheese on bun, meat substitute	41901020	144	3.4	0.5
Frankfurter or hot dog sandwich, meatless, plain, on bun, meat substitute	27564420	115	4.3	0.5

Frankfurter or hot dog sandwich, meatless, plain, on bread, meat substitute	27564430	98	5.0	0.5
Frankfurter or hot dog sandwich, meatless, on bun, with meatless chili, meat substitute	27564560	179	2.7	0.5
Frankfurter or hot dog sandwich, meatless, on bread, with meatless chili, meat substitute	27564570	162	3.0	0.5
Broccoli salad with cauliflower, cheese, meatless bacon bits, and dressing	75140500	960	0.4	0.4

*Typical serving sizes and maximum addition concentrations are based on USDA 2017–2018 Food and Nutrient Database for Dietary Studies recipe data for the selected food codes.

3.2 Dietary Exposure Estimates

Exposure to TAI114 from the intended use categories was estimated for the U.S. population using food consumption data from the What We Eat in America (WWEIA) dietary component of NHANES. The most recent data available at the time of this writing (2017–2018) were analyzed using Creme Food Safety software 3.6 (www.cremeglobal.com). These data were obtained from 6639 individuals who underwent two non-consecutive 24-hour dietary recall interviews (the first was collected in-person, the second by phone 3–10 days later). WWEIA food codes that were considered most similar to the intended use categories were utilized in the assessment and were assigned the relevant intended use concentrations.

Creme software is a probabilistic modeling tool that uses high-performance computing to predict intake (including total aggregate exposure) of food groups and/or individual food ingredients. Creme Food Safety performs calculations using large-scale food consumption data sets. It bases the calculated estimates on each individual's body weight from the survey, as opposed to averaged body weights. Calculations also incorporated the NHANES assigned sample weights for each individual in the survey, which measure the number of people in the population represented by that specific subject and help to ensure that the results statistically represent the entire U.S. population. Sample weights for NHANES participants incorporate adjustments for unequal selection probabilities and certain types of non-response, as well as an adjustment to independent estimates of population sizes for specific age, sex, and race/ethnicity categories. The data are shown for "food consumers" (which includes only data from individuals who reported consuming one or more food/beverage categories intended to contain the ingredient over the two-day survey period, as opposed to the whole population). Results are given as both absolute exposure (mg/person(p)/day), as well as exposure relative to body weight (mg/kg bw/day).

The relative standard error (RSE; calculated by dividing the standard error of the estimate by the estimate itself and multiplying by 100) is a statistical criterion that can be used to determine the reliability of estimates as pertains to the population

(the larger the RSE the less reliable the estimate).[10] RSE values greater than 25–30% are often considered reasonable cut-offs by which to consider a value unreliable.[10, 11] For the purpose of this safety assessment, an RSE value of greater than 25% was used to indicate that the estimated value was unreliable with regard to representing the population. RSE values are shown for the 90th percentile values only, as the 90th percentile values are the most pertinent for the exposure estimates. All of the values were considered reasonably reliable using the 25% cut-off.

Data estimated directly from the NHANES short 2-day survey do not necessarily adequately represent individual usual long-term intake due to the large amount of random error. This is because it may not correctly capture infrequent consumers. It assumes that subjects who consumed a product on a survey day consume it every day of the year, and it does not adjust for potential day-to-day variation in intake (i.e., intra-individual variation over time is not accounted for). Thus estimation of "usual" or "lifetime" exposure was also added to the model based on methodologies developed by Nusser et al., 1996, at Iowa State University.[12] These lifetime data are considered the most relevant data, as food/food ingredient exposure estimates should be based on expected regular exposure over the lifespan. The technique of estimating usual/lifetime intakes relies on the ability to transform the input daily average data (from food consumers) into normality, which is tested using the Anderson-Darling test statistic within the Creme Global software.

The TAI114 exposure estimates derived from the Creme assessment based on the intended use categories and concentrations are shown below in Table 5.

Table 5. Exposure to TAI114 by Proposed Use Food Consumers using NHANES 2017–18 data

General Population (Ages 2+)	N (% of total)	TAI114 Estimated Consumption Daily Average				90th% RSE Value	Lifetime 90th% Exposure
		Mean	Mean std err	90th%	90th% std err		
Absolute (mg/p/day)	1.9	122.3	16.2	247.1	6.1	2.5	293.7*
Relative to body weight (mg/kg bw/day)		1.9	0.3	3.9	0.4	11.0	3.5

Creme run #569

*Creme warning: -32, "Fourth moment of Usual intakes less than 3.0": Lifetime data may still be used.

According to the estimates above, approximately 1.9% of the U.S. general population (ages 2 and above) was identified as potential consumers of TAI114 from one or more of the proposed food uses. The lifetime 90th percentile estimated exposure to TAI114 was 293.7 mg/p/day (3.5 mg/kg bw/day).

These estimates are considered very conservative, as they assume that 100% of the intended use food products in the market will contain the maximum intended use

levels of TAI114. Yet there will be cost and market share limitations of adding TAI114 to foods making it unlikely that an individual would consume the ingredient in all of the intended use food categories that are consumed daily. Additionally, because there are a number of food code surrogates within the food categories, it is nearly impossible that an individual will randomly or intentionally consume a product containing TAI114 every single time that he/she consumes that product type daily over a lifetime. While food labels will list TAI114 as an ingredient and may even highlight the ingredient in marketing, it is assumed that many consumers will not always realize that the ingredient is present in the food. In other words, it will likely be an "invisible" ingredient to many consumers, which decreases the chance that only food products that contain the ingredient will be chosen by those consumers.

In order to calculate the exposure to the PPIX constituent from the intended use of TAI114, the TAI114 exposure data from Creme run #569 (shown in Table 5 above) was multiplied by 7%, the maximum concentration of PPIX permitted in TAI114 by the ingredient specification (see Table 2 in Subpart 2.3). The results are shown in Table 6 below.

Table 6. Exposure to PPIX by Proposed Use Food Consumers using NHANES 2017–18 data

General Population (Ages 2+)	N (% of total)	PPIX Estimated Consumption Daily Average				90th% RSE Value	Lifetime 90th% Exposure
		Mean	Mean std err	90th%	90th% std err		
Absolute (mg/p/day)	1.9	8.56	1.14	17.30	0.43	2.5	20.56*
Relative to body weight (mg/kg bw/day)		0.13	0.02	0.27	0.03	11.0	0.24

Creme run #569
*Creme warning: -32, "Fourth moment of Usual intakes less than 3.0": Lifetime data may still be used.

The lifetime 90th percentile estimated exposure to PPIX was 20.56 mg/p/day (0.24 mg/kg bw/day). These estimates are considered very conservative for the same reasons stated above.

Part 4: Self-limiting Levels of Use

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

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

The GRAS conclusion for TAI114 is based on scientific procedures, and thus, experience based on common use in food prior to 1958 is not considered pivotal information. TAI114 was not used in foods prior to 1958.

Part 6: Safety Narrative

6.1 Absorption, distribution, metabolism, and excretion (ADME)

PPIX is the penultimate compound in the endogenous biosynthesis of heme.[3] In photosynthetic organisms (including *C. reinhardtii*), the pathway branches towards chlorophyll or heme following biosynthesis of PPIX making it the last common compound of the tetrapyrrole pathway.[3, 4] In the heme pathway, PPIX is chelated with iron (via the enzyme ferrochelatase) to form heme while in the chlorophyll pathway it is chelated with magnesium (via the enzyme magnesium chelatase) to form magnesium-PPIX, the first committed compound in the chlorophyll pathway.

In mammals, biosynthesis of heme is under homeostatic control, and when adequate levels of heme are present, most remaining PPIX is chelated with zinc (Zn), rather than iron, by the same ferrochelatase enzyme.[3, 13-15] The 1st committed, and rate-limiting, step in heme (and, therefore, PPIX) biosynthesis is the delta-aminolevulinic acid (5-ALA) synthetase catalyzed conversion of glycine and succinyl-CoA to 5-ALA.[3, 14, 15] Feedback inhibition of 5-ALA synthetase by heme and related compounds is the major control point in heme biosynthesis. Ferrochelatase is also a minor target of feedback inhibition by heme. Additionally, heme, at low concentrations, inhibits translation of the housekeeping (present in tissues other than erythroid) 5-ALA synthetase gene while at high concentrations, it blocks translocation (likely in both the housekeeping and erythroid-specific enzymes) of 5-ALA synthetase from the cytosol to the mitochondria, where it is active. Eight reactions, beginning with synthesis of 5-ALA in the mitochondria, are required to produce heme. Following this first committed step of the pathway, 5-ALA is translocated to the cytosol where two molecules are enzymatically condensed and four molecules of the condensation product are combined to form the first tetrapyrrole compound, uroporphyrinogen III, which is then decarboxylated and translocated back to the mitochondria where the final three reactions take place (a decarboxylase reaction followed by a dehydrogenase reaction to produce PPIX followed by the ferrochelatase reaction to form heme). Synthesized heme is incorporated into hemoglobin in erythrocytes.

PPIX is synthesized primarily within erythrocyte progenitor cells in the bone marrow (the site of blood cell production) for production of hemoglobin and to a lesser, but still significant, extent in the liver where heme is needed primarily for production of cytochrome P450 enzymes.[14, 16] Minor amounts are synthesized in other cells, as all cells require heme at some point in their lifecycles for synthesis of a variety of essential hemoproteins (e.g., myoglobin in muscle tissue). While degradation of heme does not proceed through a PPIX intermediate (rather a ring opening oxidase reaction resulting in formation of biliverdin),[3] nonetheless, a small amount of free PPIX enters the blood stream via circulating erythrocytes, binds to plasma proteins (e.g., albumin), and is carried to the liver.[16, 17] PPIX present in the

liver via delivery from plasma proteins or hepatic synthesis is either converted to heme or excreted, unchanged, almost entirely in the bile due to its poor solubility.[16, 18, 19] Experiments using bile acid sequestrants and activated charcoal suggest that some excreted PPIX may be reabsorbed via enterohepatic circulation.[16, 18, 20] Additionally, Ibrahim and Watson administered 3.87 mg of ^{14}C radiolabeled PPIX to a single human male by duodenal intubation.[18] Over the following 12 day period, 76% of the radioactivity was recovered in the subject's stool with PPIX accounting for 20.7% and the remainder was recovered as stercobilin (an end product of heme catabolism that contributes to the brown color of stool), indicating conversion of PPIX to heme followed by catabolism. The remaining, unaccounted for 24% radioactivity was hypothesized to be comprised of PPIX derivatives and other bile pigments not detectable by the pure samples used for analysis, as only negligible amounts of radioactivity were detected in the urine. The authors concluded that early appearance (12h following administration) of radioactivity in stool as both PPIX and stercobilin is evidence that at least some of the administered dose was absorbed, metabolized, and excreted, noting that evidence of conversion of PPIX to heme and then heme catabolites had not been previously observed in gut microbes although radiolabeled stercobilin had been isolated following in vitro incubation of labeled PPIX with normal feces. Nonetheless, peak recovery of PPIX occurred on Day 4 while peak recovery of stercobilin occurred on Day 5, and the authors further noted that unabsorbed PPIX would be expected to be fully excreted within 72h. Thus, it was further concluded that the peaks on Days 4 and 5 and extended excretion throughout the 12 Day study period (although in negligible amounts Days 10–12) provided evidence of enterohepatic circulation.

An unpublished preliminary toxicokinetic (TK) evaluation of TAI114 was conducted in male and female Wistar rats in order to determine parameters for a full TK evaluation for the purpose of determining T_{max} for evaluation of photosensitivity (see discussion regarding photosensitivity in the Subpart 6.2 introductory text) during a 90-day oral toxicity study (see Subpart 6.2.4). TAI114 was administered to 3 rats/sex/group by gavage at 1000 and 4000 mg/kg bw, and 1 animal/sex served as controls, receiving the vehicle only. Animals of each group were dosed immediately following testing for baseline PPIX levels. Due to apparent variation in endogenous PPIX levels in the control and treated animals, no clear dose response could be established and, therefore, a full TK study was not considered feasible; thus, a photosensitive assay was not added to the 90-day study summarized in Subpart 6.2.4 below.

No studies investigating pharmacokinetic parameters of *C. reinhardtii* were located. However, other than the 3–7% PPIX content, TAI114 is considered primarily as a nutritive macro-ingredient in foods and is comprised of at least 30% protein, up to 55% starch and 25% fiber, and up to 10% fat without appreciable amounts of other bioactive compounds or known toxic compounds (see Subparts 2.3 (Table 2) and

6.4). Therefore, the body is expected to act upon it through similar physiological processes of digestion and ADME common to other edible algae, plant-derived foodstuffs, and meats commonly consumed in the human diet.

6.2 Toxicology Studies

Triton Algae Innovations sponsored the published toxicological evaluation of purified PPIX in a battery of genetic toxicity studies and of TAI114 in a 90-day oral toxicity study in rats.[9] Prior to these studies, the unmodified parent strain, THN6, was evaluated in a similar battery of good laboratory practice (GLP) toxicological studies.[21] These studies were summarized in GRN 773, Subpart 6.2, pages 20–27, which are incorporated here by reference,[1] and summarized in brief as follows:

- THN6 failed to induce gene mutations by base pair changes or frameshifts in the genomes of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2 *uvrA*) up to the maximum recommended test concentration of 5000 µg/plate, in the presence and absence of metabolic activation, under the conditions of a bacterial reverse mutation test conducted in accordance with OECD test guideline (TG) 471.
- THN6 was not clastogenic when tested up to the cytotoxic concentrations in short-term (3h treatment) experiments with or without metabolic activation and longer-term (20 h treatment) experiments without metabolic activation in an in vitro mammalian chromosomal aberration test conducted in accordance with OECD TG 473 in V79 male Chinese hamster lung cells.
- THN6 did not cause biologically or statistically significant increases in the frequency of micronucleated polychromatic erythrocytes (MPCEs) in the bone marrow of mice treated up to the limit dose of 2000 mg/kg bw under the conditions of an in vivo micronucleus test in accordance with OECD TG 474.
- In a study conducted in accordance with OECD TG 407, repeated oral exposure to THN6, by gavage, up to the maximum feasible dose (due to the solubility of the test item) of 4000 mg/kg bw/day for 28-consecutive days in male and female rats did not cause any mortality, morbidity, or toxicologically relevant alterations in body weight development, clinical pathology parameters, gross morphology, organ weights, or histology of the various tissues and organs examined.

For all intents and purposes, the only major differences between THN6 and TAI114 are the increased concentration of PPIX and decreased concentration of chlorophyll in the latter strain relative to the former (see Subpart 6.4). As such, the above studies were considered valid as part of the safety assessment of TAI114 in all aspects other than PPIX content. For this reason, a second battery of toxicological evaluations

were conducted to evaluate any potential toxic effects related to TAI114's PPIX content and are summarized in Subparts 6.2.1–6.2.4 below.

For the genetic toxicity tests, in order to achieve PPIX concentrations relevant to the evaluation, 95% pure PPIX was used rather than TAI114 itself, which would have limited PPIX exposures in the test systems due to its PPIX content of only 3–7% w/w. In the oral toxicity study, TAI114 was used as this is the substance intended for ingestion by humans and a margin of exposure (MOE) to the whole biomass powder would also provide a similar MOE (within the range variation permitted by the ingredient specification) with respect to the PPIX content.

PPIX is the blood component that is responsible for toxic effects in two of the rare genetic porphyrias that occur in humans. PPIX abnormally accumulates at the expense of heme in the blood of individuals with erythrocytic protoporphyria (EPP) and X-linked protoporphyria (XLP) due to loss-of-function mutations in the ferrochelatase gene in the former and gain-of-function mutations in the erythroid-specific aminolevulinic acid synthase gene in the latter.[15] In both conditions, this can result in painful photosensitivity, hepatic and biliary toxicity, including liver failure requiring transplantation and/or death, and mild microcytic anemia.[15, 16] PPIX circulating in the superficial vasculature of the skin fluoresces when exposed to sunlight releasing energy that generates reactive oxygen species resulting in skin damage while accumulation of PPIX in the hepatocytes and bile canaliculi can result in damage to liver and biliary cells as well as impaired bile flow.[14-16] The mechanism resulting in anemia remains to be elucidated. Interestingly, there are no recommended dietary restrictions for individuals with EPP or XLP, indicating that normal dietary exposure to PPIX does not present a concern. While the reason for this lack of effect is unknown, it could be that dietary PPIX is not absorbed in the gastrointestinal tract. Nonetheless, based on evidence supporting the occurrence of enterohepatic circulation,[16, 18, 20] it is presumed that some dietary absorption of PPIX occurs, suggesting that levels in foods may be too low to result in significant alterations in blood levels or that, due to its insolubility, PPIX is unable to reach tissue compartments where it would be able to participate in pathology. As PPIX concentrations in meat analogues to which TAI114 is added are expected to be considerably higher than PPIX concentrations naturally occurring in meat, it was important to pay particular interest to findings in the 90-day study of TAI114 (Subpart 6.2.4) that could be indicative of findings in humans with EPP or XLP.

6.2.1 Bacterial Reverse Mutation Test

In order to evaluate the mutagenic potential of 95% pure PPIX, a bacterial reverse mutation test was conducted in compliance with GLP and according to OECD TG 471 (adopted 21 July 1997).[9]

Methods: Four strains of *S. typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *E. coli* (WP2 *uvrA*) were used in the presence and absence of rat liver S9 metabolic activation with appropriate positive and negative controls. Dimethylformamide was used as the vehicle/negative control, and concentrations of PPIX used for the initial mutation test using a plate incorporation procedure and confirmatory mutation test using a pre-incubation procedure were 5000, 1600, 500, 160, 50, 16, and 5 µg/plate. Three replicates were conducted for each test concentration and control (untreated, vehicle, and positive reference).

Results: Spontaneous revertant colony numbers of the vehicle control agreed with historical control data, and positive controls induced the expected responses. A strong precipitate interfered with scoring and evaluation of background lawn development at the high concentration of 5000 µg/plate PPIX in the confirmatory mutation test without S9-mix and a slight but unequivocal cytotoxic effect of the test item was observed under some conditions of the confirmatory test with 500 µg/plate considered the lowest concentration showing unequivocal cytotoxicity (in *S. typhimurium* TA1537 without metabolic activation and *E. coli* WP2 *uvrA* with metabolic activation); however, as at least six concentrations remained analyzable under these conditions and there were a minimum of four non-toxic and non-precipitated concentration levels at each tester strain, the validation criteria of the assay were met and the test was considered valid. No biologically-relevant or concentration-related increases were seen in revertant colony numbers of any of the five bacterial strains upon treatment with the test item at any of the concentration levels either in the presence or absence of an S9 activation system. All results were considered unequivocally negative according to the study criteria.

Conclusions: Under the experimental conditions applied, PPIX failed to induce gene mutations by base pair changes or frameshifts in the genome of the strains used at concentrations up to the maximum recommended test concentration of 5000 µg/plate in the initial mutation test and the confirmatory mutation test with S9-mix and up to the maximum analyzable concentration of 1600 µg/plate in the confirmatory mutation test without S9-mix.

6.2.2 In vitro Mammalian Chromosomal Aberration Test

In order to evaluate the clastogenic potential of PPIX, an in vitro mammalian chromosomal aberration assay was conducted in compliance with GLP according to OECD TG 473 (adopted 29 July 2016).[9]

Methods: PPIX was suspended in Dulbecco's Modified Eagle's medium, and four concentrations each were chosen for the short- and long-term experiments on the basis of preliminary cytotoxic investigations. The chromosomal aberration experiments were conducted in duplicate using V79 (Chinese hamster lung) cells. The cells were exposed to the negative control or each test item concentration with

and without metabolic activation using rat liver microsome preparations (S9-mix). Groups of cells were also exposed to the respective positive controls for use with or without S9-mix. Ethyl methanesulfonate was used as positive control without S9-mix based on its known clastogenic activity in the literature and the laboratory's historical database; cyclophosphamide was used as the positive control with S9-mix. Exposure and sampling times were as follows:

- Short-term experiments: 3h treatment with and without S9-mix at PPIX concentrations of 8, 16, 32, and 64 µg/mL and 20h sampling times and, also, a 28h sampling time for treatment without S9-mix only.
- Long-term experiments: 20h treatment without S9-mix at PPIX concentrations of 2, 4, 8, and 16 µg/mL and 20 and 28h sampling times.

Following treatment and sampling, slides were prepared, and aberration frequencies were scored blind and evaluated according to the cited OECD TG.

Results: The numbers of aberrations observed in concurrent negative and positive controls were compatible with the corresponding historical controls and the concurrent positive controls induced the expected statistically significant increases compared to the concurrent negative controls. No statistically significant or concentration-related differences compared to the concurrent negative controls were observed in numbers of cells with structural chromosomal aberrations after the short- or long-term treatments with the different concentrations of PPIX with or without metabolic activation. However, the 95% control limits of the corresponding historical controls were slightly exceeded in the short-term experiment without metabolic activation and a 20h sampling time and the long-term experiment with a 28h sampling time. These findings were not considered to be biologically relevant or to affect the ability to interpret the results as negative because the increases were not concentration related and the values remained within the historical control data (HCD) ranges and were not statistically significant compared to HCD means. No polyploid cells or endoreduplicated metaphases were observed.

Conclusions: TAI114 is not clastogenic in this test system.

6.2.3 In vivo Mammalian Micronucleus Test

In order to evaluate the in vivo genotoxic potential of PPIX, an in vivo mammalian micronucleus assay was conducted in compliance with GLP and according to OECD TG 474 (adopted 29 July 2016) under the permission of the Institutional Animal Care and Use Committee (IACUC) of Toxi-Coop Zrt.[9]

Methods: PPIX was administered twice by gavage at a 24-hour interval to male specific pathogen free (SPF) Win: NMRI mice at doses of 0 (vehicle-control), 500, 1000, and 2000 mg/kg bw. The negative control/vehicle was 1% aqueous methylcellulose. The positive control, cyclophosphamide 60 mg/kg bw, was

administered once by intraperitoneal injection. Each group consisted of five animals, and all treatments were administered at a uniform volume of 10 mL/kg bw. The main micronucleus test was conducted at the doses described above in males only based on the results of a preliminary toxicity test.

All animals were observed immediately following dosing and at regular intervals until sacrifice for mortality, signs of toxicity, or adverse reactions to treatment. Bone marrow smears were prepared in duplicate on standard microscope slides from samples obtained from the femurs of each animal from each dose group immediately following sacrifice 24 hours after the second treatment (24 hours following the single treatment in the positive controls). Scoring and criteria for a positive response were according to the cited OECD TG.

Results: No mortality, clinical signs of toxicity, or adverse reactions to treatment were observed in any animals during the study. MPCE frequencies observed in concurrent negative and positive control groups were compatible with the corresponding HCD and the concurrent positive control induced the expected statistically significant increases compared to the concurrent negative controls. No statistically significant or dose-related increases were observed in frequency of MPCEs in the treated groups compared to the concurrent negative controls, and all results were within the 95% control limits of laboratory's HCD. Exposure of the test item to bone marrow was indicated by a slight, dose-related decrease in the ratio of immature to total erythrocytes in the treated groups compared to the concurrent negative control group.

Conclusions: PPIX, at concentrations up to the limit dose of 2000 mg/kg bw, was negative for producing micronuclei in this in vivo mouse micronucleus test.

6.2.4 Ninety-day Repeated-Dose Oral Toxicity Study

In order to evaluate the potential health hazards, including identification of toxic effects and target organs, of repeated oral exposure to TAI114, and to determine a NOAEL, a 90-day study was conducted in compliance with GLP and in accordance with OECD TG 408 (adopted 25 June 2018) with the exception of the following deviations[9]: analytical control to determine the homogeneity, stability, and concentration of PPIX in the test solutions was not performed; free, rather than total, T4 and T3 were measured, absolute differential leukocyte counts were not evaluated, and male mammary glands were not evaluated histologically. The study was carried out under the permission of the IACUC of Toxi-Coop Zrt and in compliance with the National Research Council Guide for Care and Use of Laboratory Animals[22] and the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) and Government Decree 40/2013 regulating animal protection.

Methods: Ten SPF Han:WIST rats/sex/group were administered TAI114 (containing 5.0% PPIX) dissolved in distilled water (vehicle) at concentrations to provide for uniform administration by gavage of a dose volume of 20 mL/kg bw. In all tested practicable vehicles, the dose formulations became very dense and sticky within a short time at high concentrations and rapidly sedimented at low concentrations. Additionally, storage was not possible because the formulation got dense after some hours. Because of this inability to perform analytical control of the test formulations, the test solutions were formulated freshly each day and administered within 4h. Four groups of rats received doses of 0 (vehicle-control), 1000, 2000, or 4000 mg/kg bw/day for 90 days. Selection of the high-dose was made as the maximum feasible dose on the basis of the solubility of the test item as well as the absence of adverse effects in an unpublished preliminary OECD TG 407 14-day dose-range finding study and the absence of adverse effects in the previous 28-day study using THN6.[21]

Results: No morbidity; altered behavior, neurological deficits, or abnormal reactions to different stimuli; ophthalmological alterations; or toxicologically relevant effects on body weight development or food consumption were observed during the study. Exophthalmos of the right eye was observed in a single male animal of the mid-dose group from Weeks 7–13 and was correlated with a grade 2 (mild) subcapsular pituitary hematoma observed in the same animal during the gross and histopathological examinations; however, no histological alterations were observed in the eye itself. Given their singular occurrence at the mid dose, these were considered individual findings without relation to administration of the test item. No other clinical signs were observed in this or other animals during the study.

No findings of toxicological relevance were observed in hematological or clinical chemistry parameters or thyroid hormone levels. A few statistically significant differences observed in various of these parameters were considered to have occurred spontaneously due to the general lack of dose responses and lack of correlations to other study parameters and, where available, their remaining within the corresponding HCD ranges. At necropsy, in male animals compared to the control group, slight (≤24%), but statistically significant, decreases in absolute (all dose groups) and relative-to-body-weight (low- and high-dose groups) thymus weights were observed without a dose response, and at the high-dose, statistically significant, dose-related, but slight (+7%), increases in liver and kidney weights relative to body weights were observed, while no statistically significant changes in absolute or relative organ weights were observed in the female groups. The statistically significant organ weight changes observed in male animals were all well within the corresponding HCD ranges and were without correlating clinical pathology or histopathology.

No toxicologically relevant findings were observed during the gross or histopathological examinations. All observed findings were common background

lesions occurring with similar frequencies in control and treated animals and/or were individual findings. No histopathological findings were observed in the skin tissue samples of any animals. However, a photosensitivity assay was not performed due to the inability to determine a reliable T_{max} for PPIX in an unpublished preliminary TK study (see Subpart 6.1). Because a photosensitivity assay was not performed, findings indicative of a photosensitivity reaction were not expected in the pathological examinations of this study. For this reason, study parameters that could be indicative of hepatic and/or biliary toxicity were of particular interest.

No alterations were observed in clinical chemistry parameters that could be indicative of potential hepatic or biliary dysfunction, such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total bilirubin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total protein, albumin, or the albumin/globulin ratio. While there was a dose-related increase in liver weight relative to body weight in male rats that became statistically significant at the high dose, the magnitude of change relative to control at the high dose was only +7% and remained well within the HCD range. Furthermore, as noted above, there were no alterations in clinical chemistry parameters associated with this change nor were there any correlating histopathological findings, with the livers of all control and high-dose animals showing normal architecture with no evidence of damage to the hepatobiliary system, such as cellular degeneration, necrosis, fibrosis, vacuolation, inflammation, or biliary thrombi. Interestingly, there were also slight dose-related increases in liver weight relative to body weight ratios in male rats that were statistically significant at the high-dose (+8% relative to control) in both the unpublished preliminary 14-day range finding study of TAI114 and 28-day study of THN6.[21] Likewise, the slightly increased liver to body weight ratios in these shorter studies were, also, not accompanied by any correlating findings in other study parameters. While liver relative to body weight increases can be predictive of toxicity prior to the occurrence of morphological changes,[23] the lack of progression when rats were dosed with TAI114 for a longer period of time suggest that this finding is not indicative of a hepatotoxic effect related to *C. reinhardtii* in general or TAI114/PPIX specifically, and the very low magnitude and complete lack of related findings suggests the changes observed are simply reflective of normal biological variation or a slight adaptive effect. Finally, no evidence of anemia was observed in the hematological examination or the histological examinations of organs of the hematopoietic system.

Conclusions: Repeated administration by gavage of 1000, 2000, and 4000 mg/kg bw/day of TAI114 for 90 days did not cause adverse effects or signs of toxicity in male or female SPF Han:WIST rats; the NOAEL was determined to be 4000 mg/kg bw/day; the highest dose tested.

6.3 Current Regulatory Status

A thorough search for the current regulatory status of *C. reinhardtii* biomass or PPIX, relevant to their use in food in the United States, was conducted. A summary of the pertinent search results is shown below:

- An FDA GRAS notice (GRN No. 773) was found in the FDA GRAS Notices Inventory database for the dried biomass of *C. reinhardtii* strain THN6. GRN 773 received FDA's no questions letter on March 13, 2019, indicating no current challenge to the safety of the ingredient and its intended use as a source of protein in food at levels equivalent to those currently consumed, replacing other dietary proteins.

6.4 Non-pathogenicity and Non-toxigenicity and Proteomics

Subpart 6.4, page 28, of GRN 773, which is incorporated here by reference, discussed evidence in favor of the non-pathogenicity and non-toxigenicity of THN6.[1] The "searches of the scientific literature and public health-related databases did not find any evidence of pathogenicity or toxigenicity of any members of the class Chlorophyceae," and it was concluded that due to "the extensive body of scientific literature on *C. reinhardtii* as a model laboratory organism and potential recombinant protein production platform, the absence of toxic effects in formal toxicological investigations, and the fact that most closely related toxicogenic organisms diverge at the taxonomic level of subphylum, there is no reason to suspect a pathogenic or toxigenic potential of *C. reinhardtii* (THN6)."

TAI114 was subjected to proteomic evaluation to investigate any differences in the protein content of TAI114 as compared to THN6, the *C. reinhardtii* wild type.[9] Total TAI114 proteins were extracted by resuspending the frozen cell pellet in protein extraction buffer followed by centrifugation to precipitate and collect the extracted proteins. The precipitated pellet was washed in cold acetone, dissolved in 6 M Guanidine solution, boiled, and then cooled at room temperature. The proteins were reprecipitated with methanol and the pellet was suspended in a solution of 100 mM Tris and 8 M urea to which solutions of Tris(2-carboxyethyl) phosphine hydrochloride and chloro-acetamide were added to the desired final concentrations. Three volumes of 50 mM Tris and trypsin were added, and the mixture was incubated at 37 °C for 12h. The trypsin-digested solution was then acidified and desalted, and the digested peptides were analyzed by ultra-high-pressure liquid chromatography coupled with tandem mass spectroscopy using nano-spray ionization. Protein identification[24] and label free quantification were carried out using Peaks Studio 8.5.

The analysis revealed 15,283 peptides, which were used to identify 2,970 proteins. These matched *C. reinhardtii* genome sequences (both uniquely *C. reinhardtii*

proteins as well as housekeeping proteins such as actin, tubulin, and ribosomal protein 18) as presented in the *C. reinhardtii* database.[25] Four proteins were identified as magnesium chelatase I proteins; however, the magnesium chelatase H subunit was not identified. As noted in Subpart 2.1.3, sequence analyses confirmed that the UV mutagenesis process induced a frameshift mutation in the magnesium chelatase gene (*ChlH*, H subunit of magnesium chelatase) (internal data of the notifier), which accounts for the chlorophyll deficiency and PPIX accumulation observed in TAI114. No potentially toxic proteins were detected.

6.5 Allergenicity

TAI114 does not contain or have added, and is manufactured in a facility free of, all nine major allergens (milk, egg, fish, Crustacean shellfish, tree nuts, wheat, peanuts, soybeans, and sesame) identified, and required to be disclosed in labeling, in the Food Allergen Labeling and Consumer Protection Act (FALCPA). Based on the manufacturing process, raw materials used, and the state of the production facility, Triton Algae Innovations is unaware of any reason to suspect the presence of any other allergens (such as those identified by the European Union or the European Food Safety Authority) in TAI114.

THN6, the non-mutated parent strain of TAI114, is a nonpathogenic, nontoxigenic GRAS food ingredient that has a low likelihood of allergenic potential based on an in vitro digestibility test using simulated gastric fluid (SGF).[1] No reports of allergic reactions, or indications of potential allergenicity, to THN6, TAI114, other *C. reinhardtii* strains, or any member of the Chlamydomonadaceae family were found in our searches of the scientific literature and public health-related databases. In addition, Triton Algae Innovations has not received reports of allergic reactions from food use of THN6. TAI114 has undergone proteomic evaluation and was found to contain typical *C. reinhardtii* proteins (see Subpart 6.4).

In addition, TAI114 was also subjected to a digestibility test in SGF.[9] Results of such studies are modestly correlated to food allergy risk,[26, 27] and while there have been claims that these assays are not always predictive, [28, 29] a review of a broad range of representative work found a positive correlation between study results and allergenicity.[26] TAI114 was dissolved in PBS, extracted for 2h, and clarified by centrifugation. Toasted soybean flour (because it also contains a complex mixture of protein, some of which are known allergens that are at least partly stable to digestion in this test system) was used as a positive control and was prepared in the same manner. While SGF assays are normally performed using purified proteins,[30] stable proteins of digested total protein extracts of know allergens have been correctly identified using the same conditions employed in this study.[31] The protein contents of TAI114 and soybean flour were determined by 2-D Quant assay, and the proteins were subjected to digestion in SGF plus porcine pepsin at pH 2, with

timed digestion samples stopped at fixed times from zero to 60 minutes. The digestion samples were evaluated for Coomassie blue stainable protein banding patterns in SDS-PAGE reducing gels. TAI114 was rapidly digested to a smear of indistinct bands below 15 kDa within 30 seconds and further reduced in intensity and apparent molecular size to approximately 5 kDa by 20 minutes under the experimental conditions, suggesting a low likelihood of allergenic potential, while the positive control retained a major band at approximately 48 kDa for 60 minutes.

6.6 History of Consumption

THN6, the non-mutated parent strain of TAI114, is a nonpathogenic, nontoxigenic GRAS food ingredient for nutritive use to replace other dietary proteins although no quantitative or qualitative data related to its consumption since its market introduction in 2018 was located. There is no known history of use of TAI114 as a food or food ingredient.

Robust data on PPIX concentrations in food is lacking; however, one study found only trace amounts (≤3.25 μg/g dry mass) in various meat sources analyzed, with the highest levels in the eight tested meats found in veal and the lowest levels found in beef shoulder.[32] In contrast, the notifier's internal data indicates that ground beef and sirloin steak contain 12 and 9.8 μg PPIX/g dry mass, respectively, and the Impossible burger, produced from the GRAS ingredient (GRN 737) soy leghemoglobin preparation, contains 160 μg PPIX/g dry mass. Note, that Impossible Foods did not report PPIX levels in its soy leghemoglobin preparation ingredient or discuss PPIX (other than diagrammatically depicting its occurrence in a figure (Figure 3 on page 14, Subpart 2.7.3.1 of the notice) showing the biosynthetic pathway of heme) in GRN 737.[33] Nonetheless, based on the notifier's above noted internal data and Impossible Foods' exposure assessment in GRN 737, described in Part 3 of the notice (pages 17–19), which is incorporated here by reference, high end exposure to PPIX by consumers of the Impossible burger can be calculated. A pseudo 90th percentile was calculated by Impossible Foods by doubling the mean calculated based on 100% substitution of ground beef by the Impossible burger. Mean beef consumption data was obtained from a published analysis of 2007-08 NHANES data and multiplied by the percentage of beef sold as ground beef obtained from USDA data. The mean beef consumption (25 g/p/day) was multiplied by the maximum addition percentage of soy leghemoglobin protein of 0.8% to obtain a mean consumption of 200 mg soy leghemoglobin protein/p/day). This was divided by 9% (the concentration of soy leghemoglobin protein in soy leghemoglobin preparation and the result was multiplied by 24% (the concentration of solids in soy leghemoglobin preparation) to obtain a mean consumption of 533 mg/p/day soy leghemoglobin preparation solids. Doubling this mean results in a pseudo 90th percentile soy leghemoglobin preparation solids consumption of 1.067

g/p/day. As this is the fraction of the Impossible burger that contains PPIX, it was multiplied by the notifier's internal data of 160 μgPPIX/g dry weight of the Impossible burger to obtain the pseudo 90th percentile exposure to PPIX from consumption of soy leghemoglobin preparation, and the above calculations were reversed to obtain an exposure of 8 mg PPIX/p/day (0.133 mg PPIX/kg/bw/day for a 60 kg human) from the intended use of soy leghemoglobin preparation (more simply, this can be calculated by doubling the mean consumption to ground beef and multiplying by 160 μg/g). Applying ground beef consumption data presented in Part 3 of GRN 737 to the notifier's internal data on the PPIX concentration in ground beef and assuming an approximate water content of 50% in ground beef results in an estimated exposure of high-end consumers of ground beef to approximately 300 μg PPIX/p/day (5 μg/kg bw/day for a 60 kg human).

In addition to the above described PPIX concentrations in meat, humans are also exposed to additional amounts of PPIX, following ingestion of meat, via metabolism of heme to PPIX within the gut lumen.[34] Levels of PPIX and its intestinal metabolites measured in stool of healthy volunteers following ingestion of red meat or blood can be equivalent to concentrations found in the stool of individuals with porphyrias.

6.7 Reported Adverse Events

No products containing TAI114 have yet been introduced into the food supply. However, since the time of the independent conclusion of GRAS status in March 2022, cooked meat analogue sample products—such as tuna, meatballs, and pork dumplings—containing TAI114 have been consumed by many people at three widely attended conference events. A conservative estimate of the number of people who have consumed these products would be in the range of 3,000 to 4,000. The notifier is not aware of any adverse events as the result of this consumption.

No FDA letters, recalls, market withdrawals, or safety alerts regarding safety concerns related to products containing any *C. reinhardtii*- or PPIX-derived ingredients were located. A search of FDA's Center for Food Safety and Applied Nutrition Adverse Event Reporting System did not uncover any mention of *C. reinhardtii*- or PPIX-derived ingredient containing products. All databases were accessed on August 22, 2022.

6.8 Basis for Conclusion of Safety

Triton Algae Innovations' TAI114 has been the subject of a thorough safety assessment as described above. The totality of evidence supporting safety is comprised of data and information that establish the safety of TAI114 under the conditions of its intended use and data and information that is corroborative of

safety. The general availability and general acceptance, throughout the scientific community of qualified experts, of the data and information that establish the safety of TAI114 under its intended conditions of use establish the general recognition of this data and information. Together, the establishment of safety based on scientific procedures and its general recognition form the basis for Triton Algae Innovations' conclusion of GRAS status of TAI114 for its intended use.

6.8.1 Data and Information that Establish Safety

The scientific data, information, and methods forming the basis of this conclusion are:

- The establishment of identity by comparing ITS sequences to the standard reference strain using GenBank® and the proteomic data demonstrating that no other significant alterations were induced in the organism by the evolution and selection strategy used to develop the TAI114 strain containing 3–7% PPIX from a GRAS pure wild-type strain of *C. reinhardtii* of known origin that is established as, non-pathogenic, and non-toxigenic;
- The method of manufacture and food grade specifications, demonstrating the robust quality control standards and production processes that yield TAI114;
- The bacterial reverse mutation test, in vitro mammalian chromosomal aberration test, and in vivo mammalian micronucleus test, establishing the lack of genotoxic potential of 95% pure PPIX;
- The digestibility study demonstrating the proteins in TAI114 are rapidly digested and unlikely to present a significant risk of food allergy; and
- The ninety-day repeated-dose oral toxicity study in rats and highly conservative dietary exposure estimate, establishing the lack of adverse health effects and or target organs of repeated exposure to TAI114 in rats, and establishing an adequate MOE for the intended conditions of use by humans of TAI114 as food.

In the ninety-day study, the NOAEL was 4000 mg/kg bw/day TAI114 (equivalent to 200 mg/kg bw/day PPIX) in male and female Han:WIST rats; the highest level tested. Based on the intended use of the ingredient as a flavoring agent and adjuvant (21 CFR 170.3(o)(12)) and nutritive ingredient in meat and seafood/fish analogues at addition levels of up to 0.7% w/w, the NOAEL allows for an adequate MOE (NOAEL/Exposure; 4000 mg/kg/3.5 mg/kg) of 1144-fold when compared to the estimated lifetime human exposure level at the 90th percentile of consumers in the U.S. general population. This also equates to an MOE to PPIX of 817-fold under the worst-case scenario in which PPIX were present in the TAI114 biomass at the maximum concentration of 7% w/w permitted by the ingredient specification (note, the best-case scenario of the specification range for PPIX (3%) would result in an

MOE of 1907-fold for PPIX; based on the CoAs reviewed for this report as well as the CoA for the toxicological study test item, typical PPIX concentrations appear to range between 5 and 6%, which would equate to an MOE range of 954–1144-fold). Together, the above data and information support a conclusion that the intended use of TAI114 is reasonably certain to be safe.

6.8.2 Data and Information that are Corroborative of Safety

The safety of TAI114 is corroborated by a fourteen-day repeated-dose oral toxicity study in rats, a battery of published toxicity studies on the wild-type strain and its GRAS status as a source of protein in food at levels of protein currently consumed, the lack of reported adverse events associated with consumption of *C. reinhardtii*, and a history of consumption of PPIX from common food stuffs, particularly meats, both as primary and secondary digestion products, without any recommended dietary restrictions in people with the inherited porphyrias EPP and XLP.

6.8.3 General Recognition

The scientific data, information, and methods herein reported, that provide the basis of this GRAS conclusion by scientific procedures are published and available in the public domain. Part 7 of this GRAS notice contains the citations for the published studies. These publicly available data and information fulfill the requirement of the GRAS standard for general availability of the scientific data, information, and methods relied on to establish the safety of TAI114 for its intended conditions of use. The peer review of the published studies and lack of letters to the editor or other dissenting opinions provide ample evidence of general recognition among qualified experts that there is reasonable certainty that consumption of TAI114 for its intended use is not harmful. The general availability and acceptance of these scientific data, information, and methods satisfy the criterion of the GRAS standard that general recognition of safety requires common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food that there is reasonable certainty that the substance is not harmful under the conditions of its intended use.

6.8.4 Data and Information that are Inconsistent with the GRAS Conclusion

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

6.8.5 Information that is Exempt from Disclosure under FOIA

There are no data or information in this report that are considered trade secret or commercial or financial information that is privileged or confidential.

Part 7: Supporting Data and Information

Initial literature searches for the safety narrative described in Part 6 of this GRAS notice were conducted from June 2019 through July 2019. Additional literature searches were conducted during the time courses spanning January 2020 through July 2020 and August 2021 through February 2022 and again during August 2022. Searched databases included PubMed; PubChem; toxplanet (https://toxplanet.com/, including its indexed databases, such as the former TOXNET databases); Google Scholar National Toxicology Program; websites of U.S. FDA, EFSA, WHO, and FAO; medical libraries of University of Washington and University of Arizona; and AIBMR's internal library.

Search parameters included *Chlamydomonas reinhardtii*, TAI114, protoporphyrin IX, and various derivatives, synonyms, and identifiers of the aforementioned; algal toxins; toxicity; toxicology; toxicity tests—acute, subacute, subchronic, chronic, developmental and reproductive toxicity, mutagenicity, genetic toxicity, clastogenicity, carcinogenicity, and various derivatives of the aforementioned; safety, no observed adverse effect level, no observed effect level, lowest observed adverse effect level, lowest observed effect level, and various derivatives of the aforementioned; chromosome aberrations, micronucleus, bacterial reverse mutations, comet, pharmacokinetics, absorption, distribution, metabolism, excretion, elimination, bioavailability, and various derivatives of the aforementioned. The searches included MESH terms associated with these terms when and where applicable. Initial search results for the name of the GRAS substance and its major constituent returning less than or equal to 200 results were screened individually without application of addition search terms to narrow results. When narrowing of results was necessary, the terms were put together in various Boolean search strings used when and where applicable. On some search occasions databases were searched more specifically, such as for a specific paper (for example, a reference cited in another article). Some databases were searched using primarily key words related to the name of the substance rather than Boolean strings (e.g., toxplanet, FDA's Food Ingredient and Packaging Inventories).

7.1 Data and Information that are *not* Generally Available

Some of the corroborative data and information described in Part 6 of this GRAS notice are unpublished and, therefore, are not generally available, as follows:

- Toxi-Coop Zrt. Preliminary toxicokinetic study of PPIX in rats; 2020 (see Subpart 6.1).
- Toxi-Coop Zrt. OECD 407 14-day dose-range-finding study of TAI114 in rats; 2020 (see Subpart 6.2.4).

- Triton Algae Innovations. Chlamydomonas and its derivatives in food. Presentation to U.S. FDA; November 5, 2020 (see Subpart 6.6).

The data and information cited above strengthen the weight of evidence and, thereby, corroborate the data and information that establish the safety of TAI114 under the conditions of its intended use. We believe the GRAS conclusion can still be made even if qualified experts throughout the scientific community do not generally have access to this information.

7.2 References that *are* Generally Available

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