# GRAS Notice for Paramylon Isolate from *Euglena gracilis* (ATCC PTA-123017)

***Prepared for:***

Office of Food Additive Safety (FHS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Drive
College Park, MD
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March 16, 2017

# GRAS Notice for Paramylon Isolate from *Euglena gracilis* (ATCC PTA-123017)

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# GRAS Notice for Paramylon Isolate from *Euglena gracilis* (ATCC PTA-123017)

## Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, Algal Scientific hereby informs the U.S. Food and Drug Administration (FDA) that paramylon isolate from *Euglena gracilis* (ATCC PTA-123017), manufactured by Algal Scientific, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Algal Scientific's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Algal Scientific, Robert Levine hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to Algal Scientific Corporation and pertinent to the evaluation of the safety and GRAS status of the use of paramylon from *Euglena gracilis* ATCC PTA-123017 as an ingredient for addition to food.

Signed,

(b) (6)

3-22-2017

Robert Levine, Ph.D.
CSO
Algal Scientific Corporation
robert.levine@algalscientific.com

Date

### 1.1 Name and Address of Notifier

Algal Scientific Corporation
14925 Galleon Court
Plymouth, MI 48170
USA

### 1.2 Common Name of Notified Substance

Paramylon isolate; *beta*-1,3-glucan

## 1.3 Conditions of Use

Algal Scientific intends to market paramylon isolate as an ingredient in the U.S. for use in conventional food and beverage products across a number of food categories at use levels of up to 250 mg/serving. The individual proposed food-uses and use-levels for paramylon employed in the current intake analysis are summarized in Table 1.3-1. Food codes representative of each proposed food-use were chosen from the 2011-2012 National Health and Nutrition Examination Survey (NHANES) (CDC, 2015; USDA, 2014). Food codes were grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (U.S. FDA, 2016).

**Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for Paramylon Isolate in the U.S.**

| Food Category | Proposed Food Use | RACC* (g or mL) | Paramylon Isolate (mg/serving) | Paramylon Isolate (%)[a] |
|---|---|---|---|---|
| Baked Goods and Baking Mixes | Cookies | 30 | 250 | 0.833 |
| Beverages and Beverage Bases | Fruit-Flavored Drinks | 240 | 250 | 0.104 |
| | Meal Replacement Beverages (not Milk-Based) | 240 | 250 | 0.104 |
| Cereal and Cereal Products | Nutritional Bars[b] (Breakfast, Granola, Protein) | 40 | 250 | 0.625 |
| Dairy Product Analogs | Soy Milk | 240 | 250 | 0.104 |
| | Soy Yogurt | 225 | 250 | 0.111 |
| Milk and Milk Products | Meal Replacement Beverages | 240 | 250 | 0.104 |
| | Probiotic Beverages | 240 | 250 | 0.104 |
| | Yogurt | 225 | 250 | 0.111 |
| | Yogurt Beverages[c] | 240 | 250 | 0.104 |
| Processed Fruits and Fruit Juices | Fruit Juices, Smoothies and Nectars | 240 | 250 | 0.104 |
| Soft Candy | Candy Bars and Chocolates | 40 | 250 | 0.625 |
| Soup and Soup Mixes | Soups | 245 | 250 | 0.102 |

*Serving sizes based on the US FDA Reference Amounts Customarily Consumed per Eating Occasion (RACCs) (21 CFR §101.12 – U.S. FDA, 2016).
[a] Use levels (%) are adjusted according to RACC.
[b] Nutritional bars category captures all breakfast, granola and cereal bars, including soy protein bars.
[c] It should be noted that no yogurt beverages were identified in the NHANES 2011-2012 dataset; therefore, food codes for fermented milks and fruit smoothies were used as surrogate codes.

### 1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a) and (b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2016), paramylon isolate from *Euglena gracilis* (ATCC PTA-123017) manufactured by Algal Scientific, has been concluded to have GRAS status for use as an ingredient for addition to specified conventional food and beverage products, as described in Section 1.3, on the basis of scientific procedures.

### 1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be made available to the United States (U.S.) Food and Drug Administration (FDA) for review and copying upon request during business hours at the offices of:

Algal Scientific Corporation
14925 Galleon Court
Plymouth, MI 48170
USA

In addition, should the FDA have any questions or additional information requests regarding this notification during or after the Agency's review of the notice, Algal Scientific will supply these data and information.

### 1.6 Freedom of Information Act, 5 U.S.C. 552

It is Algal Scientifics' view that all data and information presented in parts 2 through 7 of this notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. 552.

## Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

### 2.1 Description

The ingredient that is the subject of this GRAS determination is paramylon isolate derived from the biomass of *Euglena gracilis* ATCC PTA-123017, a proprietary strain maintained by Algal Scientific and grown under controlled conditions in sterile fermentation vessels. The ingredient is isolated from cultures of *Euglena gracilis* ATCC PTA-123017 propagated under axenic conditions in aerated fermentation vessels using a water-based, low-temperature, organic solvent free extraction process, which yields a dry, cream-colored powder containing >95% paramlyon. Paramylon is a linear, unbranched *beta*-1,3-D-glucan polymer that is produced as a

storage polysaccharide by a number of *Euglena* species. Paramylon is synthesized as a fibrillar high molecular weight polymer (~500 kDa) with a high level of crystallinity in its native state approaching 90%, and is deposited in the cells as small discoid granules between 1 to 2 µm in size (Barsanti *et al.*, 2011). Paramylon granules synthesized by *E. gracilis* are of extremely high purity with NMR spectra corresponding to 100% glucose (Barsanti *et al.*, 2011). [redacted]

The paramylon isolate is a cream-colored powder with a crumble texture, slight floral flavor and tea aroma. The ingredient contains ≥95% *beta*-1,3-glucan on a wet weight basis along with small quantities of protein (≤3.0%), fats (≤3.0%), ash (≤1.0%) and moisture (≤6%), which originate from the cell biomass as carry-over products from the isolation process. At the molecular level, similarities can be drawn between paramylon and curdlan (Figure 2.1-1). Curdlan is a linear *beta*-1,3-glucan produced as granules and capsular polysaccharides by *Rhizobiaceae* sp. and Gram-positive *Cellulomonas falvigena* and *Bacillus* sp. (Stone, 2009). Curdlan is synthesized in bacteria as fibrillar structures and may have upwards of 12,000 glucose units per molecule. Curdlan is insoluble in water but dissolves in dilute bases (0.25 M NaOH) and dimethyl sulfoxide (DMSO) and has a unique property in that the material forms thermo-irreversible gels when heated in aqueous suspensions higher than 80°C (Stone, 2009). NMR data have demonstrated that *beta*-1,3-glucan molecules in curdlan are fully identical in molecular and structural composition to paramylon (Marchessault and Deslandes, 1979). However, curdlan granules display a lower crystalline structure of ~30% and can be hydrated in aqueous solutions forming resilient gels at 55°C. Paramylon is completely insoluble in water, and does not have gelling properties; even immersion of paramylon granules in water for several weeks at room temperature does not result in conversion of the granules to a hydrated form. Hydration of paramylon granules requires annealing in a sealed bomb at 140°C and produced only mild swelling of the granules (Marchessault and Deslandes, 1979).

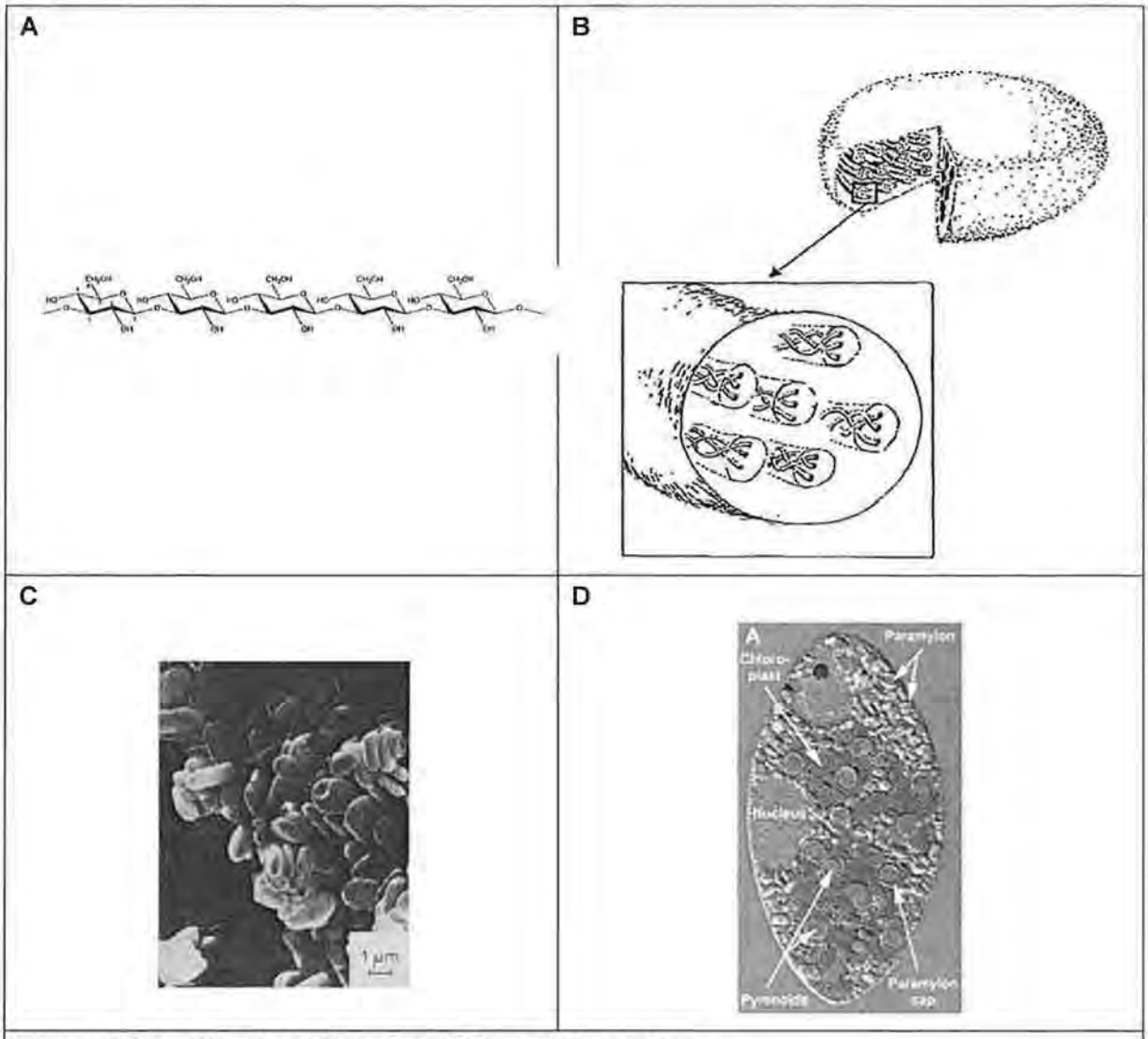



**Figure 2.1-1 Structural Representations of Paramylon**

**A)** Basic molecular composition of paramylon consists of linear *beta*-1,3-linked glucose polymers with an approximate molecular weight of ~500 kDa. **B)** Structural confirmation of *beta*-1,3-glucan polymers. *Beta*-glucan chains form triple helical structures associated by hydrogen bonding and deposited in a lamellar fashion as discoid granules. **C)** Electron micrograph of paramylon granules. Typical granule size from *Euglena gracilis* is 1 to 2 µm in diameter. **D)** Image of *E. gracilis* algae cell with deposition of paramylon storage granules throughout the cell.

## 2.2 Source Organism

### 2.2.1 Phenotypic Identity

*Euglena gracilis* is a microalgae belonging to the *Euglenaceae* family and is a single-celled, spindle-shaped/elongated cell with one nucleus, chloroplasts with pigments (when grown in light) or proplastids (when grown in the dark), a contractile vacuole, an eyespot, and a single flagellum (Figure 2.2.1-1). *Euglena gracilis* occurs widely in nature and is primarily found in freshwater pools, ponds, and lakes. *Euglena gracilis* can adapt to diverse environments under conditions ranging from 1 to 38°C and pH ranging from 2.3 to 11. As such, *E. gracilis* has been identified in a number of differing environments including vegetable and citrus waste-lagoons, water in tree holes, snow, bark of honey locust tree, alkaline marshes, and in acid coal mine water (Buetow, 2005).

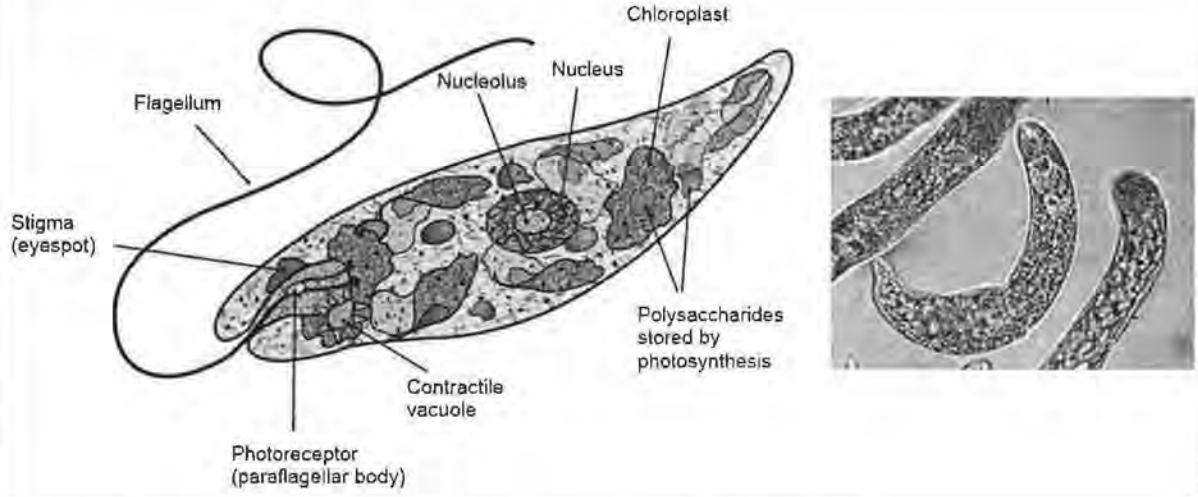



**Figure 2.2.1-1 Structural Characteristics of *Euglena gracilis* Klebs**

*Euglena gracilis* can be cultivated under a variety of conditions including autotrophically with $CO_2$ and light as the sole source of carbon and energy, mixotrophically in light with an organic carbon source, or heterotrophically in the dark with a carbon source (Krajcovic *et al.*, 2015). A characteristic feature of *E. gracilis* is the ability to synthesize large quantities of storage polysaccharides such as paramylon (*beta*-1,3-glucan) and wax esters (20-36 carbon atoms in length comprising C-12 to C-18 chain saturated fatty acids and alcohols with myristyl myristate 14:0-14:0 as the major species) (Krajcovic *et al.*, 2015). Paramylon is the major energy reserve during photosynthetic or heterotrophic growth under aerobic conditions and can comprise up to 90% of the dry cell weight. Under anaerobic growth conditions, wax esters can comprise up to 60% of the total lipids. Transfer of cells from aerobic to anaerobic growth results in conversion

of paramylon to wax esters, whereas transfer from anaerobic to aerobic growth conditions results in the conversion of wax esters to paramylon (Figure 2.2.1-2).

*Euglena gracilis* also are known to synthesize *alpha*-tocopherol, carotenoids, biotin, trehalose, polyunsaturated fatty acids (PUFAs) and amino acids (Fiol and Salerno, 2005). Polyunsaturated fatty acids synthesized by *E. gracilis* include the omega-3 long-chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), with DHA representing about 2% of the total *E. gracilis* fatty acids composition (Krajcovic *et al.*, 2015).

The *Euglena gracilis* ingredient manufactured by Algal Scientific [redacted] while limiting the synthesis of various secondary ingredients such as wax esters and lipids (see Figure 2.2.1-2a below). Analytical data on the nutrient composition of dried algae (*E. gracilis*) is presented in Section 2.4.3.

[redacted]

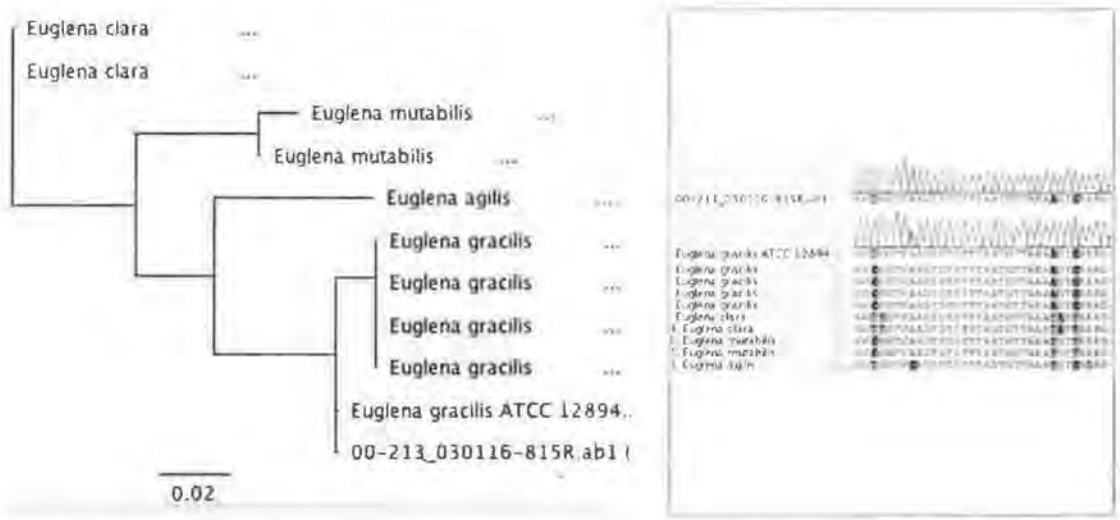
**Figure 2.2.1-2 Biosynthesis of Paramylon and Wax Esters by *Euglena gracilis*** [redacted]

### 2.2.2 Genotypic Identity

#### *2.2.2.1 Species*

The species identity of *E. gracilis* ATCC PTA-123017 was verified by third party experts using an ABI 3130XL Capillary Electrophoresis DNA Sequencer (DNA Species Identification SOP AT-SP-002-5) with 3 separate cultivation lots of the algae. All test samples were analyzed by NSF Authen Technologies' proprietary procedure for DNA species identification. Briefly, a single sample is randomly selected and the DNA is extracted. Then, primers are used to amplify specific gene regions using Polymerase Chain Reaction (PCR). The DNA is sequenced using a Sanger Sequencer. Sequences are compared to a proprietary HERB™ reference DNA sequence database for identification of the species. To ensure the validity of the results, all test samples are run alongside positive and negative controls throughout the process. As illustrated in the branching diagram in Figure 2.2.2.1-1 below, the sample of *E. gracilis* ATCC PTA-123017 submitted by Algal Scientific was identified as *E. gracilis* and divergent from other species of Euglena. The sample was consistent with a single reference sample of *E. gracilis* [strain 1224-5/25 (CCAP 1224/5Z, Pringsheim Z, UTEX 753; ATCC #12894)] that is a distinct lineage from other published DNA sequences of *E. gracilis*. The Certificates of Analysis are provided in Appendix A.

**A. Species Identification Test Results**




**B. Taxonomic Classification**

| | |
|---|---|
| Kingdom | *Eukaryota* |
| Phylum | *Euglenozoa* |
| Class | *Euglenophyceae* |
| Order | *Euglenales* |
| Family | *Euglenaceae* |
| Genus | *Euglena* |
| Species | *Euglena gracilis* Klebs var. *bacillaris* |
| Strain | ATCC PTA-123017 |

**Figure 2.2.2.1-1 Species Identity and Taxonomic Classification**

### *2.2.2.2 Strain*

Algal Scientific's dried algae is derived from pure-culture [redacted] *E. gracilis* Klebs var. *bacillaris* ATCC PTA-123017. This strain is maintained as a master culture in the ATCC Patent Depository.

### *2.2.2.3 Stability of Cell Lines*

The parent cell line is maintained on agar plates stored in cool conditions [redacted] To ensure sterility and viability the algae are transferred to fresh plates once every 2 to 4 weeks and routinely transferred to shake flasks as needed. Microalga, including *Euglena gracilis*, are notoriously recalcitrant to cryogenic preservation (Day *et al.*, 2007), and the most common procedure for conservation of microalgal cultures is perpetual maintenance (*i.e.*, continuous culture) under controlled conditions (Lorenz *et al.*, 2005). The genetic stability of microalgae to continuous sub-culture is reflected in the International Code of Botanical Nomenclature which

does not require microalgal type cultures to be deposited in a publically accessible Biological Resource Center (BRC), and in most cases type cultures have simply been maintained for prolonged periods using serial transfer culture methods. Nevertheless, there have been reports of genetic drift in microalgae (Müller *et al.*, 2007), and Algal Scientific does maintain a master culture collection under liquid nitrogen preservation for use as needed to maintain the performance quality of their strain. This strain has been deposited in the ATTC patent depository. [redacted] Similar observations have been reported for other microalgae such as *Chlorella vulgaris* where Müller *et al.* (2005) have observed no genetic differences between duplicate strains of the same isolate maintained by continuous subculturing over many decades with those stored using cryogenic preservation.

## 2.3 Manufacturing

The production of the paramylon isolate is performed in accordance with current Good Manufacturing Practices (cGMP) for Food as described in 21 CFR Part 110.5) (U.S. FDA, 2016). The manufacturing process of the paramylon isolate is shown in Figure 2.3-1. [redacted] Cells are sterile-transferred to larger shake flasks and then to a glass carboy. [redacted] the contents of the carboy are used to seed a stainless steel production fermenter, [redacted] The same synthetic media is used throughout the culture maintenance, scale-up, and production phases, and is composed of ammonium salts as nitrogen sources, dextrose as a carbon source, and smaller amounts of additional salts and vitamins. A silicon based food-grade anti-foaming agent is used in accordance with 21 CFR §173.340 – Defoaming agents (U.S. FDA, 2016). All media components are food grade and meet Food Chemical Codex (FCC), United States Pharmacopeia (USP), or other equivalent international quality standards. All fermentation ingredients, processing-aids and additives are used in accordance with appropriate federal regulations and/or have been determined to be GRAS for their respective uses.

At the production fermenter scale, the culture is harvested [redacted] In all parts of the process system where broth contact occurs, surfaces are approved for food contact.

To produce the paramylon isolate, the harvested broth is transferred to [redacted] tank and the pH of the slurry is adjusted with food-grade NaOH. The slurry is immediately homogenized [redacted]

The homogenized solution is then allowed to settle [redacted] the settled granules are then washed [redacted] with [redacted] water [redacted] After the final wash, the *beta*-glucan slurry is acidified [redacted] and then centrifuged in a stainless steel bowl [redacted] The centrifuged pellet, [redacted] dried [redacted] until the moisture content is determined to be less than <5% by weight. The dry granules are then milled [redacted] The powder is then packaged in food contact grade Mylar bags and stored in a cool, dry area until shipment.

Schematic diagrams of the production process for paramylon isolate is provided in Figure 2.3-1.

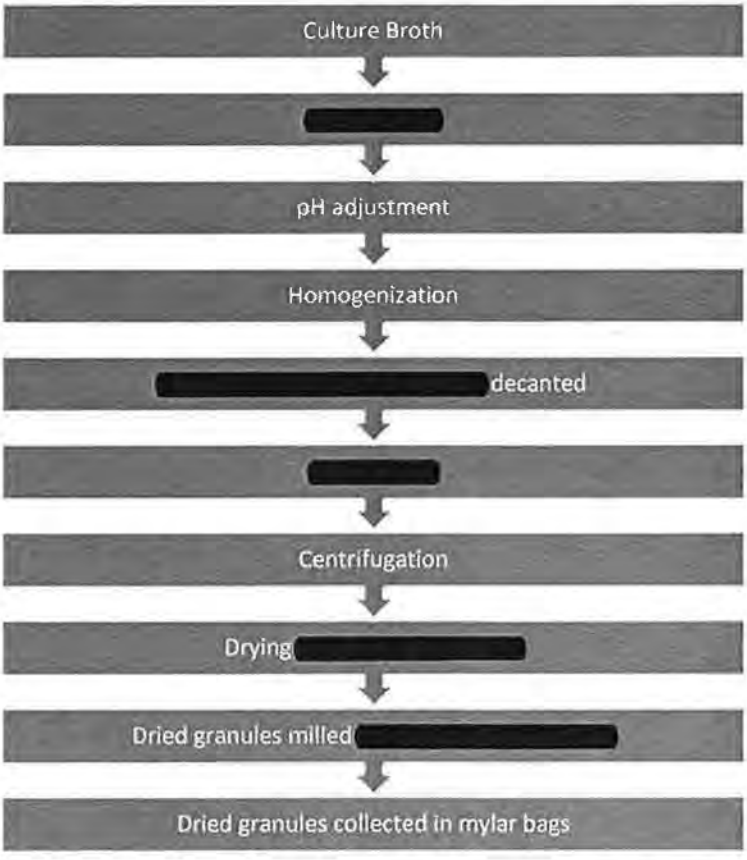



**Figure 2.3-1 Schematic of the Production Process of Paramylon Isolate**

## 2.4 Product Specifications and Batch Analyses

### 2.4.1 Proposed Product Specifications

The proposed product specifications for the paramylon isolate is provided in Table 2.4.1-1.

**Table 2.4.1-1 Proposed Product Specifications – Paramylon**

| Parameter | Specification | Method of Analysis |
|---|---|---|
| ***Proximate Analysis*** | | |
| Moisture | ≤6% | AOAC 950.46 |
| *Beta*-glucan | ≥95% | Total dietary fiber or ASC gravimetric meth. |
| Total Carbohydrates | ≥95% | Calculation |
| Fat | ≤3% | AOAC 922.06 |
| Protein | ≤3% | AOAC 992.15 (mod.) |
| Ash | ≤1% | USP |
| Excipient/Carrier | None | None |
| Extract Solvent | Water | Water |
| Drying Method | Tray Dry | Tray Dry |
| ***Other Testing*** | | |
| Color | Cream | Organoleptic |
| Flavor | Slight Floral | Organoleptic |
| Texture | Powder | Organoleptic |
| Aroma | Slight tea aroma | Organoleptic |
| ***Heavy Metals*** | | |
| Arsenic | ≤0.02 mg/kg | USP<233> |
| Cadmium | ≤0.5 mg/kg | USP<233> |
| Lead | ≤0.5 mg/kg | USP<233> |
| Mercury | ≤0.05 mg/kg | EPA 7471 |
| ***Microbiology*** | | |
| Aerobic plate count (APC) | ≤10,000 CFU/g | AOAC 990.12 |
| Coliforms | ≤100 CFU/g | AOAC 991.14 |
| *Escherichia coli* | Absent/g | AOAC 991.14 |
| *Salmonella* | Absent/25g | AOAC 2003.09 |
| *Staphylococcus aureus* | Absent/g | AOAC 2003.07 |
| Yeast | ≤500 CFU/g | FDA/BAM Chapter 18 |
| Mold | ≤500 CFU/g | FDA/BAM Chapter 18 |
| ***Other Testing*** | | |
| Total aflatoxins | ≤1.0 mg/kg | AOAC 2008.02 (mod) |

AOAC = Association of Official Analytical Chemists; CFU = colony forming units; EPA = U.S. Environmental Protection Agency; FCC = Food Chemical Codex; FDA = Food and Drug Administration; NF = National Formulary; USP = United States Pharmacopeia

### 2.4.2 Batch Analyses

The results of 3 non-consecutive batches of the paramylon isolate demonstrating that the ingredient is manufactured consistent with the proposed product specifications is provided in Table 2.4.2-1 below.

**Table 2.4.2-2 Results of 3 Batch Analyses of Paramylon**

| Parameter | Specification | Manufacturing Lot | | |
|---|---|---|---|---|
| | | 122315-BG-1 | 011916-BG-1 | 021016-BG-1 |
| ***Proximate Analysis*** | | | | |
| Moisture | ≤6% | 1.4 | 1.2 | 1.29 |
| Total Carbohydrates | ≥95% | 96.0 | 97.6 | 96.13 |
| Fat | ≤2% | 0.4 | 0.2 | 2.36 |
| Protein | ≤2% | 2.1 | 1.0 | 0.18 |
| Ash | ≤1% | 0.1 | n.d. | 0.04 |
| ***Heavy Metals*** | | | | |
| Arsenic | ≤0.02 mg/kg | <0.01 | <0.01 | <0.01 |
| Cadmium | ≤0.5 mg/kg | <0.001 | <0.001 | <0.001 |
| Lead | ≤0.5 mg/kg | <0.01 | <0.01 | <0.01 |
| Mercury | ≤0.05 mg/kg | <0.005 | <0.005 | <0.005 |
| ***Microbiology*** | | | | |
| Aerobic plate count | ≤10,000 CFU/g | n.d. | n.d. | 10 |
| Coliforms | ≤100 CFU/g | n.d. | n.d. | n.d. |
| *Escherichia coli* | Absent/g | n.d. | n.d. | n.d. |
| *Salmonella* | Absent/25g | Negative | Negative | Negative |
| *Staphylococcus aureus* | Absent/g | n.d. | n.d. | n.d. |
| Yeast | ≤500 CFU/g | n.d. | n.d. | n.d. |
| Mold | ≤500 CFU/g | n.d. | n.d. | n.d. |
| ***Other Testing***[a] | | | | |
| Aflatoxin B1 (ppb) | NS | <0.6 | <0.6 | <0.6 |
| Aflatoxin B2 (ppb) | NS | <0.6 | <0.6 | <0.6 |
| Aflatoxin G1 (ppb) | NS | <0.6 | <0.6 | <0.6 |
| Aflatoxin G2 (ppb) | NS | <0.6 | <0.6 | <0.6 |
| Total aflatoxins (ppb) | ≤1.0 mg/kg | <0.7 | <0.7 | <0.7 |

CFU = colony forming units; n.d. = not detected; NS = not specified; ppb = parts-per-billion; ppm = parts-per-million
[a] Detection limit = 1 ppb

### 2.4.3 Additional Analytical Information

*2.4.3.1 Beta-Glucan Content*

There are various techniques that can be used to measure the *beta*-glucan content of a material. For the dried algae and paramylon product, 3 methods have been employed to date that all provide very similar estimates of the total *beta*-glucan content. In addition, these estimates of the *beta*-glucan content are also very close to the total carbohydrates value determined by calculation from proximate analysis, indicating that nearly all carbohydrates in the dried algae and paramylon are *beta*-glucan. These methods are described in Table 2.4.3.1-1 below with the data for the 3 batches of paramylon presented in Table 2.4.3.1-2.

**Table 2.4.3.1-1 Method of Analysis for *beta*-Glucan**

| Method Type | Method Basis | Method Description |
|---|---|---|
| Total dietary fiber | AOAC 991.43 (mod) | Samples are dried and defatted (if >10% fat) and then digested with three (3) enzymes (protease, amylase, and amyloglycosidase) to break down starch and protein. Alcohol is used to precipitate soluble fiber and filtration is used to remove insoluble residue. Residues are weighed to determine fiber levels. |
| *Beta*-glucan by FCC method | FCC Monograph "*Beta* Glucan from Baker's Yeast (Saccharomyces cerevisiae)" | Sample is dissolved in cold KOH and then digested with lyticase in a sodium acetate buffer solution. Next, the sample is digested with (1,6)-glucanase enzyme and then with exo-*beta*-glucanase and *beta*-glucosidase enzymes. The digested sample is then mixed with glucose oxidase/peroxidase reagent and the absorbance of the solution is determined at 510 nm. The *beta* glucan content is determined from a standard curve. |
| *Beta*-glucan by ASC method | Internal method developed by Algal Scientific | Sample is hydrated in water for 8 hours, then centrifuged and the water is decanted. The sample is heated in a 2% sodium dodecyl sulfate (SDS) solution for 30 min at 110 °C with stirring. The sample is centrifuged, the supernatant is decanted, and the 2% SDS wash is repeated. The pellet is then washed with 70% iso-propyl alcohol for 15 min followed by 95% ethanol for 5 min. The final pellet is then dried and weighed to determine the *beta* glucan content. |

**Table 2.4.3.1-2 Results of *beta*-Glucan Analyses of Paramylon**

| Parameter | Specification | Manufacturing Lot | | |
|---|---|---|---|---|
| | | 122315-BG-1 | 011916-BG-1 | 021016-BG-1 |
| ***Proximate Analysis*** | | | | |
| Total dietary fiber | ≥95% | 95.58 | 95.25 | 95.43 |
| *beta*-Glucan by FCC method | ≥95% | 97.2 | 104 | 99 |
| *beta*-Glucan by ASC method | ≥95% | 98.5 | 99.3 | 98.9 |
| Total carbohydrates | ≥95% | 95.3 | 95.99 | 96.13 |

### *2.4.3.2 Paramylon – Linkage Analyses*

For glycosyl linkage analysis, samples of paramylon or the dried algae were permethylated, depolymerized, reduced, and acetylated, and the resulting partially methylated alditol acetates (PMAAs) analyzed by gas chromatography-mass spectrometry (GC-MS) as described by York *et al.* (1985). In brief, a dry sample was suspended in ~300 µL of DMSO and placed on a magnetic stirrer for 1 to 2 weeks. The sample was then permethylated by the method of Ciucanu and Kerek (1984) [*i.e.*, treatment with sodium hydroxide (NaOH) and methyl iodide in dry DMSO]. The sample was subjected to the NaOH base for 10 minutes then methyl iodide was added and left for 40 minutes. A second addition of the base was added for 10 minutes and methyl iodide was added for 40 minutes. The second addition of methyl iodide and NaOH base was to ensure complete methylation of the polymer. Following sample workup, the permethylated material was hydrolyzed using 2 M trifluoroacetic acid (2 hours in sealed tube at 121°C), reduced with sodium borodeuteride ($NaBD_4$), and acetylated using acetic anhydride/ trifluoroacetic acid. The resulting PMAAs were analyzed on a Hewlett Packard 5975C GC interfaced to a 7890A MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30m Supelco 2330 bonded phase fused silica capillary column. Linkage results indicate that both samples are mainly composed of 3-linked glucopyranosyl residues (Table 2.4.3.2-1). Minor amounts of 4- and 2,3-linked Glc residues are detected along with negligible to non-detectable levels of 3,6-linked, terminal and 2,3,4-linked Glc.

**Table 2.4.3.2-1 Results of Per-*O*-methylation and Linkage Analysis of Dried Algae and Paramylon**

| Glycosyl Residue | Paramylon | Paramylon | Dried Algae |
|---|---|---|---|
| | Peak Area (%) | | |
| terminally-linked glucopyranosyl residue (t-glc) | 0.34 | 0.3 | 1.5 |
| 3-linked glucopyranosyl residue (3-glc) | 93.03 | 94.1 | 95 |
| 4-linked glucopyranosyl residue (4-glc) | 2.25 | 2.4 | 1.4 |
| 2,3-linked glucopyranosyl residue (2,3-glc) | 3.47 | 2.3 | 1.9 |
| 3,6-linked glucopyranosyl residue (3,6-glc) | 0.36 | 0.8 | 0.3 |
| 2,3,4-linked glucopyranosyl residue (2,3,4-glc) | 0.55 | 0.1 | NR |
| **Total** | **100.00** | **100.00** | **100** |

NR = not reported

### *2.4.3.3 Paramylon – Glycosyl Composition*

Glycosyl composition analysis of a paramylon was performed by combined GC-MS of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. In brief, the sample was placed into a test tube and 20 µg of inositol was added. Methyl glycosides were then prepared from the dry sample by methanolysis in 1 M HCl in methanol at 80°C for 16 hours, followed by re-N-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The sample was then per-O-

trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C for 0.5 hours. These procedures were carried out as previously described in Merkle and Poppe (1994). Analysis of the TMS methyl glycosides was performed by GC-MS on an Agilent 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30 m x 0.25 mm ID). The composition data shows that the sample contains glucose and no other monosaccharides (Table 2.4.3.3-1).

**Table 2.4.3.3-1 Results of Glycosyl Composition Analysis**

| Glycosyl residue | Mol %[a] |
|---|---|
| Ribose (Rib) - | n.d. |
| Arabinose (Ara) - | n.d. |
| Rhamnose (Rha) - | n.d. |
| Fucose (Fuc) - | n.d. |
| Xylose (Xyl) - | n.d. |
| Glucuronic Acid (GlcA) - | n.d. |
| Galacturonic acid (GalA) - | n.d. |
| Mannose (Man) - | n.d. |
| Galactose (Gal) - | n.d. |
| Glucose (Glc) | 100.0 |
| N-Acetyl Galactosamine (GalNAc) - | n.d. |
| N-Acetyl Glucosamine (GlcNAc) - | n.d. |
| N-Acetyl Mannosamine (ManNAc) - | n.d. |

n.d. = not detected
[a] Values are expressed as mole percent of total carbohydrate. The total percentage may not add to exactly 100% due to rounding.

### *2.4.3.4 Minerals and Vitamins*

Analysis of the dried algae (*E. gracilis*) indicates that the ingredient contains small quantities of minerals, vitamins, and pigments (*i.e.*, astaxanthin, canthaxanthin, lutein, and zeaxanthin). As the ingredient is produced in a dark fermenter, the levels of carotenoids are very low (<0.1 mg/g for the pigments). Only trace levels minerals can be detected in the paramylon isolate (Table 2.4.3.4-1). Based estimated intakes of up to 2.5 g of paramylon among heavy consumers, the mineral concentrations are well below their respective safe upper limits established by the Institute of Medicine.

| Table 2.4.3.4-1 Mineral Analysis of 3 Lots of Paramylon | | | | |
|---|---|---|---|---|
| **Mineral (mg/100 g)[a]** | **Lot. 122315-BG-1** | **Lot. 011916-BG-1** | **Lot. 021016-BG-1** | **IOM Upper Limit (mg/day)** |
| Calcium | 9.53 | 4.42 | 3.22 | 2500 |
| Copper | 0.106 | 0.040 | 0.009 | 10 |
| Iron | 1.68 | 1.17 | 2.06 | 45 |
| Magnesium | 2.03 | 1.19 | 2.34 | 350 |
| Manganese | 0.075 | 0.028 | 0.031 | 11 |
| Molybdenum | 0.005 | <0.001 | 0.002 | Not established |
| Phosphorus | 15.3 | 4.75 | 3.31 | 4000 |
| Potassium | 1.06 | 0.45 | 2.00 | Not established |
| Selenium | <0.01 | <0.01 | <0.01 | 0.4 |
| Sodium | 0.82 | 1.41 | 5.83 | 2300 |
| Zinc | 0.278 | 0.076 | 0.082 | 40 |

[a] Data presented for mineral nutrients.

## 2.5 Stability

The paramylon isolate is stable across a broad range of temperature and humidity conditions with recommended storage being within a temperature range of 54 to 90°F (12.2 to 32.2°C) up to 70% RH in sealed containers kept out of direct sunlight. Furthermore, the material is heat stable at temperatures up to 212°F/100°C in boiling water.

An accelerated shelf-life conducted using paramylon isolate (Lot No. 021016-BG-1) has been conducted. The samples were stored at 40°C and 75% humidity. Overall, no appreciable changes in the product parameters have been observed to date. The results to date of the accelerated shelf-life study are shown in Table 2.5-1 below.

| Table 2.5-1 Results of an Accelerated Shelf-life Study on Dried Algae (*Euglena gracilis*) (Lot No. 020916-AM-1) and Paramylon (Lot No. 021016-BG-1) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Parameter** | **Paramylon (Lot No. 021016-BG-1)** | | | | | | | |
| | **Day 0** | **Day 14** | **Day 42** | **Day 70** | **Day 98** | **Day 126** | **Day 154** | **Day 168** |
| Moisture (%) | 1.1 | 0.9 | 1.5 | 1.3 | 1 | 1.3 | 1.3 | 0.7 |
| Protein (%) | 0.6 | 0.6 | 0.7 | 0.7 | 0.8 | 0.8 | 0.8 | 0.8 |
| Fat (%) | 0.4 | n.d. | 0.1 | 0.2 | n.d. | 0.1 | n.d. | 0.3 |
| Ash (%) | n.d. | 0.2 | n.d. | n.d. | n.d. | 0.3 | n.d. | n.d. |
| CHO (%) | 97.9 | 98.3 | 97.7 | 97.8 | 98.2 | 97.5 | 97.9 | 98.2 |
| Calories | 398 | 396 | 394 | 396 | 396 | 394 | 395 | 399 |
| Total dietary fiber (%) | 100 | 102 | 102 | 102 | 100 | 100 | 100 | 100 |
| Sensory evaluation of foods | White free flowing powder, airy. No detectable aroma | mild ammonia aroma | No change | No change | No change | No change | Very diminished aroma | No change |

CHO = carbohydrates; n.d. = not detected; N/A = not available; Totox = total oxidation value

# Part 3. §170.235 Dietary Exposure

## 3.1 History of Use in Food

There are no federal regulations, or prior sanctions permitting the use of *E. gracilis* or ingredients derived thereof in food in the U.S. A GRAS Notification for use of the dried biomass of *E. gracilis,* containing *beta*-1,3-glucan from *E. gracilis*, as a "*food ingredient*" was submitted to the offices of the FDA and filed by the agency under GRN 513 (U.S. FDA, 2014). The Notifier subsequently withdrew the Notice on July 10th, 2014 and the Agency therefore ceased their evaluation. As described by the Notifier (Algeon Inc.), the intended food uses of the ingredient were for food supplementation and were expected to provide "*... 125 mg to 650 mg of paramylon (beta-1,3-glucan) per day ... or 250 to 1300 mg of Euglena gracilis biomass per day...*" However, as the Notice was withdrawn, widespread food uses of the ingredient in the U.S. seems unlikely.

General searches conducted on the internet identified dietary supplement type products containing *E. gracilis* (Immune Health, the Synergy Company; Super Euglena Paramylon ARX, Naturally-Plus; *Euglena* Lacris, Synapse Co. Ltd.). However, the most significant market share of these products appears to largely limited to the Japanese and other Asian Marketplaces. Recommended intakes of dried *E. gracilis* from these products appear to be approximately 500 mg per day. *Euglena* ingredients also appear to be marketed in Japan in various conventional food products (*e.g.*, Euglena bar) (Figure 3.2-1). *Euglena* is also being investigated as a potential nutritional supplement for children in developing countries "*... euglena-based protein biscuits, to be distributed for free to school-aged children... Just*

*50 grams of biscuit a day can supplement the child's standard diet with all the protein, and all the necessary minerals, fatty acids and vitamins they need*" (Wall Street Journal - Yang, 2013).

**Euglena supplement**


**Euglena supplement**

**Euglena bar**


**Euglena honey and oat Danish (Euglena farm)**

**Figure 3.2-1 History of Safe Food Use - Examples of Dietary Supplement Products and Food Products Containing *Euglena gracilis***

Euglena pudding with green tea

Grapefruit and strawberry smoothie with Euglena

Euglena Carbonara


Euglena ramen


**Figure 3.2-1 History of Safe Food Use - Examples of Dietary Supplement Products and Food Products Containing *Euglena gracilis***

Overall, there is an apparent history of safe food uses of *E. gracilis*; however, population-wide exposures to *E. gracilis* or ingredients derived from the algae appear to be very limited based on current availability of products. Adjustment of dietary exposures to *E. gracilis* or paramylon from the proposed food uses to account for background dietary intakes was therefore not considered necessary for the U.S. marketplace.

## 3.3 Estimated Consumption of Paramylon Isolate from All Intended Conditions of Use in Food

### 3.3.1 Dietary Intake Survey

Assessments of the anticipated dietary exposure to paramylon isolate as an ingredients in foods and beverages under the proposed conditions of use was conducted using the 2011-2012 NHANES data (CDC, 2015). The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2011-2012. 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). In addition to collecting information on the types and quantities of foods being consumed, NHANES contain socio-economic, 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 were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (USDA, 2014; CDC, 2015). The NHANES data were employed to assess the mean and 90$^{th}$ percentile intake of dried algae for each of the following population groups:

- Infants and Young Children, ages 0 to 3 years;
- Children, ages 4 to 11;
- Female teenagers, ages 12 to 19;
- Male teenagers, ages 12 to 19;
- Female adults, ages 20 and up;
- Male adults, ages 20 and up; and
- Total population (all age and gender groups combined).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of dried algae by the U.S. population. Estimates for the daily intake of paramylon represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2011-2012 data and these individual average amounts comprised the distribution from which mean and percentile intake estimates were generated. Mean and percentile estimates were generated incorporating survey weights in order to provide representative intakes for the entire U.S. population. All-person intake refers to the estimated intake of paramylon averaged over all individuals surveyed, regardless of whether they potentially consumed food products containing paramylon, and therefore includes individuals with "zero" intakes (*i.e.* those who reported no intake of food products containing dried algae during the 2 survey days). All-user intake refers to the estimated intake of paramylon by those individuals who reported consuming food products

containing paramylon, hence the "all-user" designation. Individuals were considered "users" if they consumed 1 or more food products containing paramylon on either Day 1 or Day 2 of the survey.

The NHANES is a short-term (*i.e.*, 2 days of data per cycle) survey; as such, this type of intake methodology is generally considered 'worst case' because of several conservative assumptions made in the consumption estimates. For example, it was assumed that all food products within a food category contain the ingredients at the maximum specified level of use. In addition, it is well-established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, may overestimate the consumption of food products that are consumed relatively infrequently (Anderson, 1988).

### 3.3.2 Estimated Consumption of Paramylon from Proposed Food-Uses

The estimates for the intake of paramylon isolate was generated by combining the maximum proposed use level indicated for each proposed food-use, as presented in Table 1.3-1, with food consumption data from the 2011-2012 NHANES dataset. A summary of the estimated daily intake of paramylon from all proposed food-uses is provided in Table 3.3.2-1 on an absolute basis (g/person/day) and in Table 3.3.2-2 on a body weight basis (mg/kg body weight/day).

Within the NHANES survey data, the percentage of users was high among the total population (82.7%) and all individual age groups evaluated in the current intake assessment. Greater than 77.7% of all population groups consisted of users of those food products in which paramylon isolate is currently proposed for use (see Tables 3.3.2-1). Large user percentages within a population group typically lead to similar results for the all-person and all-user consumption estimates. Consequently, only the all-user intake results will be discussed in detail.

Among the total user population, the mean and 90$^{th}$ percentile all-user intakes of paramylon were determined to be 0.44 and 0.89 g/person/day, respectively (see Table 3.3.2-1). Across the individual age categories, mean intake of dried algae remained relatively similar. Of the population groups, male adults were determined to have the greatest mean all-user intake of dried algae at 0.47 g/person/day; whereas male teenagers were determined to have the highest 90$^{th}$ percentile all-user intakes of 1.07 g/person/day. Female adults were determined to have the lowest mean all-user intakes on an absolute basis at 0.39 g/person/day, whereas female teenagers had the lowest 90$^{th}$ percentile intake at 0.75 g/person/day, respectively.

On a body weight basis, young children were identified as having the highest mean and 90$^{th}$ percentile all-user intakes of 32 and 61 mg/kg body weight/day, respectively. It should be noted that infants (<12 months of age) are not consumers of food products to which paramylon ingredients may be added. Among the other age categories (*i.e.* older than 4 years of age), the highest intake of dried algae was identified in children (aged 4 to 11 years), with mean and 90$^{th}$

percentile intakes of 17 and 35 mg/kg body weight/day, respectively. The lowest mean and 90$^{th}$ percentile all-user intakes of dried algae were identified in both the female and male adult age categories, with mean and 90$^{th}$ percentile intakes of 6 mg/kg body weight/day and 12 mg/kg body weight/day for both age groups, respectively.

Algal Scientific notes that a dried algae ingredient containing paramylon (~50% on wt/wt basis) also has GRAS status for the same food uses described in Table 1.3-1. Foods to which dried algae or paramylon may be added will be substitutional and therefore, there was no need to consider additive exposures to paramylon from both uses.

**Table 3.3.2-1 Summary of the Estimated Daily Intake of Paramylon Isolate from Proposed Food-Uses in the U.S. by Population Group (2011-2012 NHANES Data)**

| Population Group | Age Group (Years) | All-Person Consumption (g/day) | | All-Users Consumption (g/day) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90$^{th}$ Percentile | % Users | n | Mean | 90$^{th}$ Percentile |
| Infants and Young Children | 0 to 3 | 0.34 | 0.79 | 77.7 | 644 | 0.44 | 0.83 |
| Children | 4 to 11 | 0.45 | 0.87 | 96.4 | 1,292 | 0.47 | 0.88 |
| Female Teenagers | 12 to 19 | 0.33 | 0.72 | 78.8 | 458 | 0.42 | 0.75 |
| Male Teenagers | 12 to 19 | 0.37 | 0.95 | 79.7 | 442 | 0.46 | 1.07 |
| Female Adults | 20 and up | 0.33 | 0.73 | 84.0 | 1,847 | 0.39 | 0.79 |
| Male Adults | 20 and up | 0.37 | 0.89 | 78.9 | 1,646 | 0.47 | 0.99 |
| Total Population | All Ages | 0.36 | 0.82 | 82.7 | 6,329 | 0.44 | 0.89 |

NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

**Table 3.3.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Dried Algae from Proposed Food-Uses in the U.S. by Population Group (2011-2012 NHANES Data)**

| Population Group | Age Group (Years) | All-Person Consumption (mg/kg bw/day) | | All-Users Consumption (mg/kg bw/day) | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | 90$^{th}$ Percentile | % | n | Mean | 90$^{th}$ Percentile |
| Infants and Young Children | 0 to 3 | 25 | 58 | 77.7 | 641 | 32 | 61 |
| Children | 4 to 11 | 17 | 33 | 96.4 | 1,292 | 17 | 35 |
| Female Teenagers | 12 to 19 | 6 | 14 | 78.6 | 448 | 7 | 15 |
| Male Teenagers | 12 to 19 | 6 | 15 | 79.7 | 439 | 7 | 18 |
| Female Adults | 20 and up | 5 | 11 | 84.0 | 1,827 | 6 | 12 |
| Male Adults | 20 and up | 4 | 11 | 78.9 | 1,631 | 6 | 12 |
| Total Population | All Ages | 7 | 17 | 82.7 | 6,278 | 9 | 19 |

bw = body weight; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

## Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the notified ingredient.

## Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable

## Part 6. §170.250 Narrative and Safety Information

### 6.1 Introduction

Comprehensive searches of the published literature did not identify relevant publications or other literature characterizing the oral toxicity of *E. gracilis* algae preparations or paramylon. Like all microorganisms, *Euglena* sp. produce a complex array of nutrients and secondary metabolites many of which have unknown toxicity profiles. In the absence of substantial historical evidence establishing a history of safe food use of the species *E. gracilis*, as exists for common microorganisms such as Baker's yeast (*Saccharomyces cerevisiae*) or lactic acid bacteria, hazard characterization of Algal Scientific's proprietary strain (ATCC PTA-123017) was conducted using a short-term and subchronic oral toxicity studies in rodents and using *in vitro* and *in vivo* genotoxicity studies (Simon *et al.*, 2016). These investigations were conducted in accordance with current Good Laboratory Practice (cGLP) and OECD test guidelines for the toxicity testing of chemicals. A summary of the acute and subchronic toxicity of commercial samples of dried *E. gracilis* and paramylon are presented in Section 6.4.1 and 6.4.2. Genotoxicity studies included *in vitro* evaluations of the ingredients using the Ames reverse mutation assay in *Salmonella* and *E. coli* tester strains, and the *in vivo* micronucleus assay (Section 6.4.3).

*Euglena gracilis* is not known to produce toxins that are harmful to humans or animals; however, there is insufficient published information to establish that the species can be defined as non-toxicogenic. Two related *Euglena* species obtained from the wild, *Euglena sanguinea* and *Euglena granulata*, have been reported to produce secondary metabolites that are toxic to fish (Zimba *et al.*, 2004, 2010). Subsequent investigations using *E. sanguinea* identified a non-protein toxin, euglenophycin, which is structurally similar to the alkaloid present in fire ant venom (Zimba *et al.*, 2010). Analyses conducted on Algal Scientific's proprietary strain have not detected evidence for synthesis of euglenophycin. There is no chemotaxonomic basis to consider that *Euglena* sp. may possess the metabolic capacity to synthesize microcystin, cylindrospermopsin, saxitoxin, brevetoxin, diarrheic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP); however, for available analytical information conducted on *E. gracilis* ATCC PTA-123017 have demonstrated that the strain does not synthesize these algal toxins

(Zimba, 2016)[1]. The results of the analyses are provided in Appendix B. A discussion of the pathogenicity and toxicogenicity of *E. gracilis* is presented in Section 6.7.1.

## 6.2 Literature Search

To obtain the necessary safety information, comprehensive and detailed searches of the published scientific literature were conducted by through March 2016. Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and Toxfile® served as the primary sources of published literature pertinent to the safety of *E. gracilis* and paramylon.

## 6.3 Absorption, Distribution, Metabolism, and Excretion

Paramylon is a highly crystalline granular material (Figure 2.1.2-1), and will be completely insoluble at all physiological conditions throughout the gastrointestinal tract. NMR analyses of paramylon granules obtained from *E. gracilis* sp. have demonstrated that the material consists exclusively of *beta*-linked glucose molecules (Barsanti *et al.*, 2011). With the exception of lactose that is hydrolyzed by pancreatic lactases, *beta*-linked sugars are not digested by mammalian pancreatic, salivary, or small intestinal enzymes (Wisker *et al.*, 1985). Paramylon granules are therefore not absorbed and are transported intact to the large intestine where they are subjected to partial fermentation by the indigenous microbiota. Although paramylon can be considered a form of dietary fiber, the material is not soluble and is not readily hydratable under physiological conditions, and will not have osmotic potential at any point within the intestinal lumen and will therefore not change the viscosity of the intestinal contents.

*In vitro* fermentation studies conducted by Algal Scientific demonstrate that paramylon can provide metabolizable carbon sources for microbial growth in the absence of glucose; however, consistent with paramylon's crystalline structure and corresponding insolubility, and resistance to hydration, microbial fermentation is somewhat limited. These studies are described further in Section 6.3.1.

There is limited published data characterizing the fermentation metabolites of paramylon; however, paramylon can support the growth of human bacteria isolates *in vitro* (Kuda *et al.*, 2009). Paramylon, at its basic molecular level, is comprised of 100% glucose, and therefore microbial hydrolysis products are expected to be fermented by the resident anaerobic microbiota *via* the Embden-Meyerhof-Parnas pathway resulting in the production of short chain fatty acids, $CO_2$ and $H_2$ gas, all of which are common and innocuous dietary metabolites of carbohydrate ingestion (Miller and Wolin, 1996; Suarez et al., 1999; Smiricky-Tjardes *et al.*, 2003). An *in vivo* feeding study in rodents administered curdlan, an insoluble *beta*-1,3-glucan

[1] Personal Communication from P.V. Zimba, 2016

ingredient that shares many physicochemical similarities to paramylon, provides corroborating evidence to suggest that fermentation of *beta*-linked glucose polymers (*i.e.*, paramylon) by the indigenous microbiota of the large intestine would result in the production of short-chain fatty acids (Shimizu *et al.*, 1999). The authors compared the physiological function of curdlan in the digestive tract against cellulose powder (control), and gellan gum. Male Sprague-Dawley rats were fed diets containing 50,000 mg/kg curdlan, cellulose powder, or gellan gum for 4 weeks. At the end of the study, blood, gastrointestinal tract tissues, and feces were collected while the lengths of the small intestine and colon plus rectum were measured. Statistically significant findings relative to the cellulose diet included an increase in cecal weight, a decrease in cecal pH, a decrease in hepatic total cholesterol, a decrease in secondary bile acids, and an increase in volatile fatty acids (*i.e.*, acetic acid, propionic acid, and butyric acid) in the gastrointestinal tract.

### 6.3.1 *In Vitro* Fermentation Studies – Dried *Euglena gracilis* and Paramylon

The ability of various 'probiotic' bacteria to utilize dried *E. gracilis* or paramylon as carbon sources for growth was evaluated *in vitro* (Algal Scientific Corporation, 2016 [unpublished]). A probiotic cocktail containing 22 bacteria and 1 yeast (Nexabiotic; BioProsper Labs) was used as a source of *Lactobacillus* and *Bifidobacterium* sp. Modified thyoglycolate medium (pH 7.2) without sugar was used for culturing *Bifidobacterium* spp. and modified M9 mineral medium (pH 6.5) supplemented with Tween 80 and trace elements was used for culturing *Lactobacillus* spp. The probiotic cocktail was cultured overnight in brain heart infusion (BHI) broth, washed using chilled thioglycolate or modified M9 medium under anaerobic condition for *Bifidobacterium* spp and in a $CO_2$ incubator for *Lactobacillus* spp, and plated on BHI agar plates overnight to determine colony count (CFU). Broth cultures were then diluted in appropriate media to obtain about 100 CFU/mL final count in each culture tube. Culture tubes were filled with additional medium alone, or medium containing dried algae (0.05% final) or paramylon isolate (0.05% final). A minimum amount of glucose (0.05%) was added to one set of culture tubes before initiating the culture and the other set was cultured in the absence of added glucose. The standard glucose concentration used for most bacterial cultures is 2% and, therefore, 0.05% is considered a minimum amount of glucose. Bacterial culture tubes were incubated at 37°C for up to 48 hours in anaerobic condition (to ensure *Bifidobacterium* growth) and in a $CO_2$ incubator (to ensure *Lactobacillus* growth). Sets of triplicate cultures were terminated at different time-points (0, 8, 16, 24, 32, 40, and 48 hours), concentrated by centrifugation or diluted as needed and plated onto complete thioglycolate, blood or BHI agar plates to determine bacterial count (CFU/mL).

As shown in Figure 6.3.1-1 and Figure 6.3.1-2, the incorporation of dried *E. gracilis*, which is approximately 50% *beta*-glucan and 50% non-glucan biomaterial (sugars, proteins, nucleic acid, lipid, *etc.*), to culture medium that is absent of any added sugar promoted considerable growth of both *Lactobacillus* and *Bifidobacterium* spp. Paramylon also promoted growth *Lactobacillus*

and *Bifidobacterium* spp.; however, overall growth was less relative to that promoted by the dried *E. gracilis* preparation. Both ingredients also accelerated sugar-dependent growth of both *Lactobacillus* and *Bifidobacterium* members.

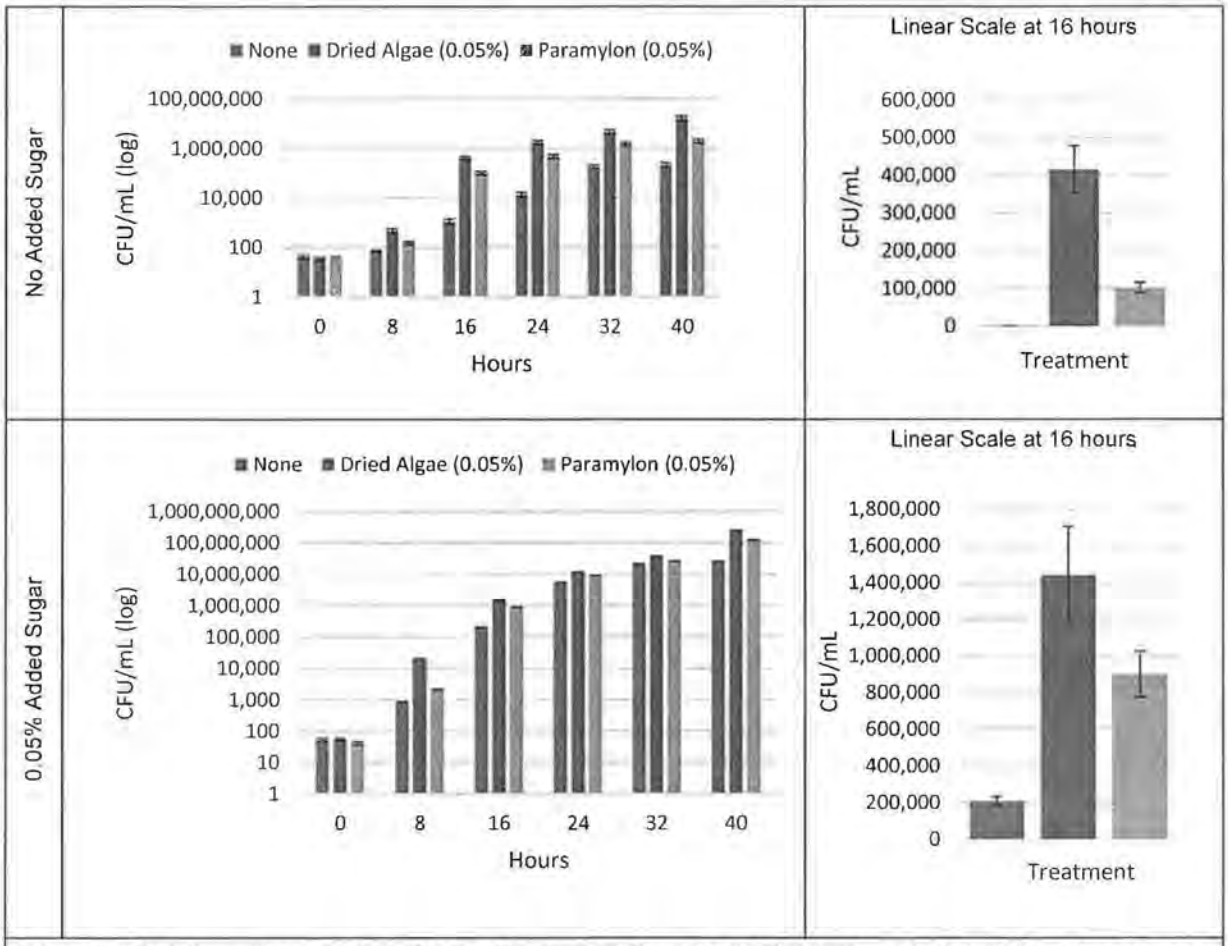



**Figure 6.3.1-1 Bacterial Cultures Grown in Modified M9 Medium (pH 6.5) for Growth of *Bifidobacterium* Spp.**

A commercial mixture of 23 'probiotic' bacterial species (Nexabiotic; BioProsper Labs) was grown in the presence of thioglycolate medium (pH 7.2) without or with minimum sugar (0.05%) and 0.05% dried *Euglena gracilis* or 0.05% paramylon under anaerobic conditions. Mainly *Bifidobacterium* members of the cocktail grow under this culture condition.

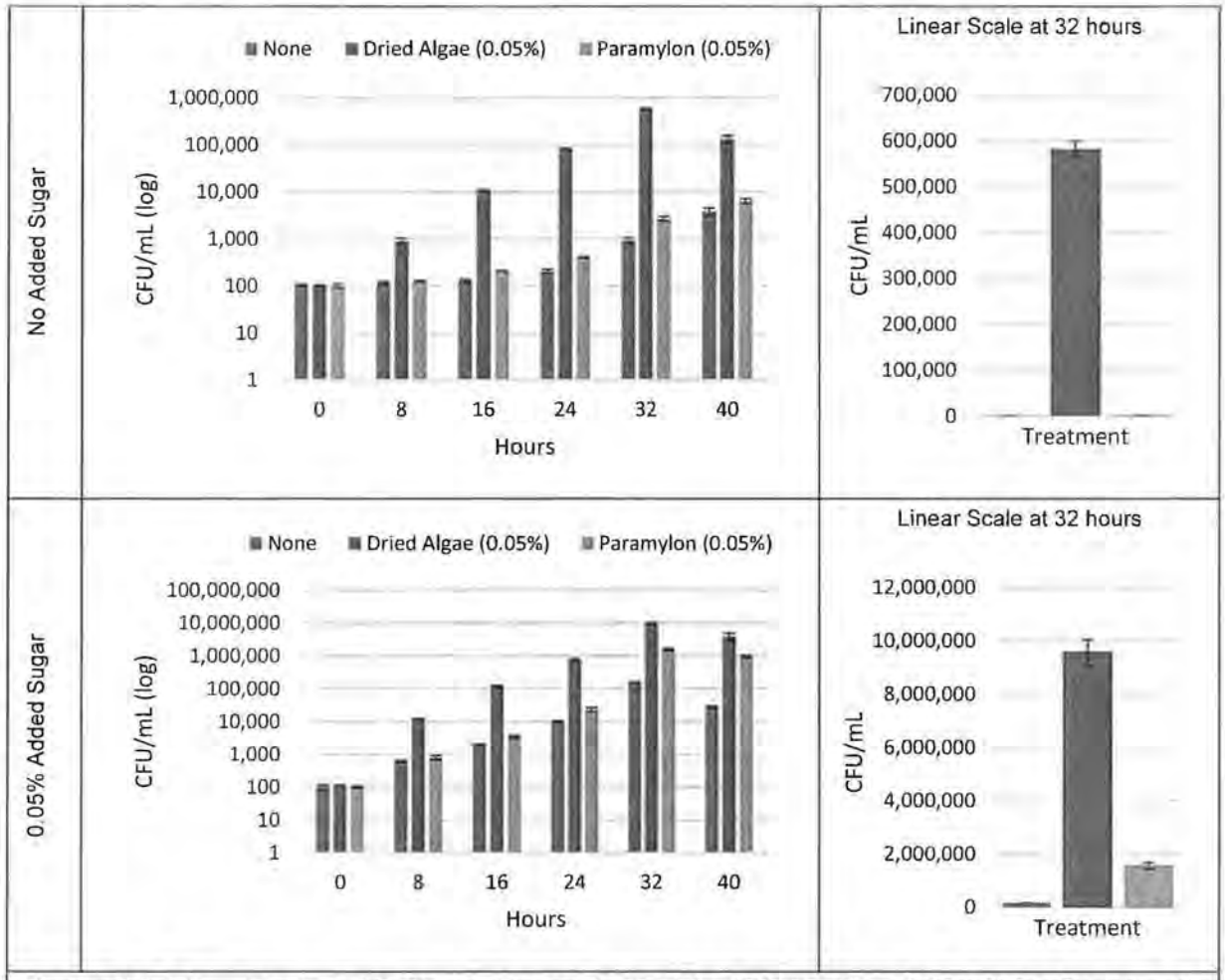



**Figure 6.3.1-2 Bacterial Cultures Grown in Modified M9 Medium (pH 6.5) for Growth of *Lactobacillus* spp.**

A commercial mixture of 23 'probiotic' bacterial species (Nexabiotic; BioProsper Labs) was grown in the presence of modified M9 medium (pH 6.5) without or with minimum sugar (0.05%) and 0.05% dried *Euglena gracilis* or 0.05% paramylon under $CO_2$ incubation. Mainly *Lactobacillus* and *Bacillus* members of the cocktail grow under this culture condition.

## 6.4 Toxicology Studies

### 6.4.1 Acute Toxicity

#### *6.4.1.1 Studies in Rats Administered Dried Algae (E. gracilis)*

The acute oral toxicity of the dried algae (*E. gracilis*) was evaluated in nulliparous and non-pregnant female Sprague-Dawley rats (3/group; aged 9 to 12 weeks; 161 to 189 g body weight) (Simon *et al.*, 2016). The study was conducted in compliance with FDA Good Laboratory Practice (GLP) requirements defined under Title 21 CFR Part 58 and in accordance with OECD Test Guideline No. 402 (OECD, 1987). Animals were housed singly in suspended stainless

steel cages and were allowed to acclimate to the laboratory conditions for 9 or 21 days. Food and water were provided *ad libitum*. Animals were fasted overnight and then orally administered dried *E. gracilis* preparation (Lot #: 040715-AM-1) as a 40% w/w mixture in distilled water at a dose of 5,000 mg/kg body weight. All animals were observed for mortality, signs of gross toxicity, and behavioral changes immediately after dose administration, during the first several hours after dosing, and at least once daily thereafter for 14 days. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. At the end of the observation period, all animals were euthanized by carbon dioxide inhalation. Gross necropsies were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined.

All animals survived test substance administration, gained body weight, and appeared active and healthy during the study. There were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. Under the conditions of this study, the acute oral median lethal dose ($LD_{50}$) of the dried *E. gracilis* preparation is greater than 5,000 mg/kg of body weight in female rats.

#### *6.4.1.2 Studies in Rats Administered Paramylon*

The acute oral toxicity of paramylon also was evaluated in female Sprague-Dawley rats 9 to 12 weeks of age (Simon *et al.*, 2016). The study was conducted in compliance with FDA GLP requirements defined under Title 21 CFR Part 58 and in accordance with OECD Test Guideline No. 402 (OECD, 1987). Paramylon isolate (Lot #: 052015-BG-1; purity = 99% *beta*-1,3-glucan) was administered at a dose of 5,000 mg/kg body weight as a 40% w/w mixture in distilled water to the stomach using a stainless steel ball-tipped gavage needle attached to an appropriate syringe. The study was performed as previously described above for dried *E. gracilis* powder. All animals survived test substance administration, gained body weight, and appeared active and healthy during the study. There were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. Under the conditions of this study, the acute oral $LD_{50}$ of paramylon is greater than 5,000 mg/kg of body weight in female rats.

### 6.4.2 Repeat-Dose Toxicity

#### *6.4.2.1 Short-Term Studies in Rats*

A 14-day dietary toxicity study was conducted in Crl:SD® CD® IGS rats to evaluate the palatability and general toxicity of dried algae in rats following at least 14 days of dietary administration (Product Safety Labs, 2015 [unpublished]). This study was used, along with

additional relevant data, to select dietary levels for the 90-day subchronic study. This study was not performed in full compliance with GLP standards, but was conducted in a GLP-compliant facility. The study design was based on the following guidelines: OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407) (OECD, 2008):Health Effects, *Repeated Dose 28-Day Oral Toxicity Study in Rodents* (2008); and US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. (2007) (U.S. FDA, 2003). Fifty-six CRL Sprague-Dawley CD® IGS rats 6 to 7 weeks of age (28 males and 28 females) were used for the study. After acclimatization rats were randomized to 1 of 5 groups provided dried *E. gracilis* powder (Lot #: 040715-AM-1) or paramylon (Lot #: 052015-BG-1) in the diet as described in Table 6.4.2.1-1. All diets were prepared weekly and stored under refrigerated conditions. The test articles were demonstrated to be stable for at least 10 days in the dietary matrix. Samples were prepared in standard feed jars with followers and retaining rings, and were stored at ambient temperature in the animal room. Samples from each dietary concentration were evaluated for homogeneity and concentration verification conducted from samples obtained on Days 4, 7, and 10.

**Table 6.4.2.1-1 Treatment Allocation and Mean Intake of Test Articles**

| Group | No. Animals Per Group (Male/Female) | Dietary Concentration (ppm)[a] | % in Diet | Mean (Days 0-14) Daily Intake (mg/kg/day) (Male/Female) |
|---|---|---|---|---|
| 1 | 5/5 | Basal diet control (0) | 0 | 0 |
| 2 | 5/5 | Paramylon High Dose Comparator *(50,000 ppm)* | 5.0 | 5211.6/5152.8 |
| 3 | 5/5 | Low-dose *Euglena gracilis* powder *(12,500 ppm)* | 1.25 | 1284.0/1296.2 |
| 4 | 5/5 | Intermediate-dose *E. gracilis* powder *(25,000 ppm)* | 2.5 | 2434.2/2542.9 |
| 5 | 5/5 | High-dose *E. gracilis* powder *(50,000 ppm)* | 5.0 | 4844.6/5327.3 |

[a] Concentration of dried algae or paramylon, as mixed in the diet.

All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study. Individual body weights were recorded during acclimation and on Days 0, and on test Days 3, 7, 10, and 14. Individual food consumption was measured and recorded to coincide with all body weight measurements. At terminal sacrifice, all animals were euthanized by carbon dioxide asphyxiation. All animals in the study were subjected to a gross necropsy, which included examination of the external surface of the body, all orifices, musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities, with their associated organs and tissues. No mortalities were observed during this study. There were no clinical observations attributable to the administration of dried algae or paramylon. All males from Groups 1 to 5 appeared active and healthy during the 15-day observation period with the exception of 1/5 Group 2 males that exhibited moderate alopecia on the head and 1/5

Group 3 males that had superficial eschar on the back. One detailed clinical observation corresponding to the daily findings included: hair loss in 1/5 Group 2 males. All females from Groups 1-5 appeared active and healthy during the 15-day observation period. There were no findings in daily or detailed clinical observations. There were no body weight or body weight gain findings considered attributable to dried algae or paramylon administration. Mean body weights and mean daily body weight gain for the treated male rats in Group 2 to 5 were comparable to the control group values throughout the study. Mean body weights for the treated female rats in Groups 2 to 5 were comparable to the control group values throughout the study. Mean daily body weight gain for the treated female rats in Group 2 to 5 were generally comparable to the control group values throughout the study with the exception of Group 2 females had significantly increased body weight gain on Days 0 to 3. This finding was considered incidental and unrelated to paramylon administration. There were no food consumption or food efficiency findings considered attributable to dried algae or paramylon administration. Mean daily food consumption and food efficiency for the treated male rats in Group 2 to 5 were comparable to the control group values throughout the study.

Mean daily food consumption for the treated female rats in Group 2 to 5 was comparable to the control group values throughout the study. Mean food efficiency for the treated female rats in Group 2 to 5 were generally comparable to the control group vales throughout the study with the exception of Group 2 females had significantly increased food efficiency on Days 0 to 3. This finding was considered incidental and unrelated to paramylon administration. The statistically significant increase in food efficiency for Group 2 females correlated with a similar change in body weight gain and was considered non-adverse and toxicologically insignificant.

There were no macroscopic findings attributable to dried algae or paramylon administration. Macroscopic findings of fluid-filled uterus, observed for 2/5 Group 2, 4/5 Group 3, 2/5 Group 4, and 1/5 Group 5 females, were attributable to normal variation in the estrous cycle for this age and strain of rat and were not associated with test substance administration.

*6.4.2.2 Subchronic Studies in Rats*

A 90-day oral toxicity study was conducted in rats provided the dried algae (*E. gracilis*) or paramylon in the diet (Simon *et al.*, 2016). The study was conducted in accordance with FDA GLP requirements defined under Title 21 CFR Part 58 and in accordance with OECD Test Guideline No. 408 (OECD, 1998). Groups of male and female CRL Sprague-Dawley CD IGS rats (10/sex/group; aged 7 to 8 weeks) were provided the dried algae in the diet for 90 days at concentrations of 0 (control), 12,500 (low-dose), 25,000 (mid-dose), or 50,000 (high-dose) ppm, resulting in dietary intakes of 0, 838, 1,660, or 3,318 mg/kg body weight/day, respectively, for males and 0, 971, 1,966, or 3,961 mg/kg body weight/day, respectively, for females. The control group was given a basal control diet. An additional group was provided paramylon at a concentration of 50,000 ppm (producing intakes of 3,450 and 3,877 mg/kg body weight/day for males and females, respectively). Animals were observed twice daily for general condition and

clinical signs of behavior, and measurements of body weight and food consumption were obtained on a weekly basis. An ophthalmologic exam was conducted on all animals prior to study initiation and on all control and high-dose animals at the end of the study period. A complete hematology, serum biochemistry, and urinalysis assessment was conducted, and included evaluation of the following parameters: erythrocyte count (RBC), hematocrit, hemoglobin concentration, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), absolute reticulocyte count, total white blood cell (WBC) and differential leukocyte count, red cell distribution width (RDW), platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), sorbitol dehydrogenase, total bilirubin, blood urea nitrogen (BUN), creatinine, triglycerides, total serum protein, total cholesterol, fasting glucose, albumin, globulin, and inorganic phosphorus, potassium, calcium, sodium and chloride concentrations, urine quality, color, clarity, volume, pH, glucose, specific gravity, protein, ketone, bilirubin, blood, and urobilinogen. At the end of the study period, all animals were killed and subjected to a gross necropsy of the following tissues and organs were extracted and weighed: adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries with oviducts, spleen, testes, thymus, and uterus. Bilateral organs were measured together. Histopathological examination was conducted on all organs and tissues [accessory genital organs (prostate and seminal vesicles), adrenals, aorta, bone (femur), bone marrow (from femur and sternum), brain (medulla/pons, cerebellar, and cerebral cortex), cecum, cervix, colon, duodenum, esophagus, Harderian gland, heart, ileum with Peyer's patches, jejunum, kidneys, larynx, liver, lungs, lymph node (mandibular and mesenteric), mammary gland, nasal turbinates, nose, ovaries, oviducts, pancreas, parathyroid, peripheral nerve (sciatic), pharynx, pituitary gland, rectum, salivary glands (sublingual, submandicular and parotid), skeletal muscle, skin, spinal cord (cervical, mid-thoracic and lumbar), spleen, sternum, stomach, thymus, thyroid, trachea, urinary bladder, uterus, vagina and all gross lesions] of control and high-dose animals.

All animals survived to the end of the study period. No significant changes were observed in clinical signs, body weight, food consumption, ophthalmology, hematology, serum biochemistry, or urinalysis. Significant decreases in hematocrit and MCV values were observed in male animals of the mid-dose *E. gracilis* group and paramylon group, respectively. A significant decrease in AST levels was observed in males of the high-dose group. These changes were within the expected biological variation, and therefore were not considered treatment-related.

A number of effects were noted in the macroscopic examination; however, these changes were sporadic and incidental in nature, and were not considered treatment-related.

Significant decreases in the absolute weights of the adrenal glands and epididymides were observed in males of the paramylon group and low-dose dried algae group, respectively. In addition, a significant decrease in the relative weights of the adrenal glands and epididymides were observed in the low-dose dried algae males. These effects were not considered to be

toxicologically relevant as they did not occur in a dose-dependent manner and were not accompanied by relevant pathological changes. In male animals of the high-dose group, slight increases were observed in the incidence of minimal or mild mononuclear cell infiltration in the heart. Minimal hepatocellular vacuolation and alveolar macrophage aggregates was observed in the lungs of high-dose females. The incidence of minimal or mild mononuclear cell infiltration in the heart was within the expected incidence rate and severity range for the strain and age of animals, and was consistent with the background cardiomyopathy in Sprague-Dawley rats. Therefore, it was not considered a treatment-related effect. In female rats, the vacuolation was randomly distributed and was considered to be spontaneous and unrelated to test article administration. With respect to the alveolar macrophage aggregates, all aggregates were focal in distribution with the exception of two females. In addition, the aggregates were distributed randomly in the alveolar spaces and were consistent with normal tissue adaptations that maintain lung function, and were therefore unrelated to test article administration.

Based on the results of this study, the authors determined the no-observed-adverse-effect level (NOAEL) of dried algae (*E. gracilis*) and paramylon to be 50,000 ppm, equivalent to 3,318 and 3,961 mg dried algae/kg body weight/day, the highest dose administered, in male and female rats, and 3,450 and 3,877 mg/kg body weight/day for males and females, respectively, for paramylon.

### 6.4.3 Genotoxicity Studies

#### *6.4.3.1 In Vitro Studies*

A reverse bacterial mutation assay was conducted with *Salmonella* Typhimurium TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2*uvr*A using the plate incorporation method (Simon *et al.*, 2016). This study was conducted in accordance with the following test guidelines: US EPA Health Effects Test Guidelines, OPPTS 870-5100; FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.1.a (U.S. FDA, 2000); OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 471) (OECD, 1997); ICH S2(R1) "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use"; and Commission Regulation (EU) No 440/2008 B.13/14. Dried *E. gracilis* was tested in triplicate at concentrations of 0, 1.58, 5.0, 15.8, 50, 158, 500, 1,580, and 5,000 µg/plate in the presence and absence of metabolic activation. The negative control consisted of the vehicle, sterile water, whereas the positive controls included: sodium azide ($NaN_3$), ICR 191 acridine, daunomycin, and methylmethanesulfonate (MMS) in the absence of metabolic activation, and 2-aminoanthracene (2-AA) in the presence of metabolic activation. The main test was performed with the plate incorporation method, while the confirmatory test was performed with the pre-incubation method. The number of revertant colonies per plate was counted.

The number of revertant colonies did not significantly change at concentrations of dried cell *E. gracilis* up to 5,000 µg/plate in the presence or absence of metabolic activation compared to the negative control. In addition, cytotoxicity and precipitation of the test article were not observed in any strain at any test concentration. Based on the results of this study, the authors concluded that dried cell *E. gracilis* is not mutagenic under the conditions of this study.

*6.4.3.2 In Vivo Studies*

A mammalian erythrocyte micronucleus test was conducted in male and female Swiss albino (ICR) mice (3/sex/group) (Simon *et al.*, 2016). Animals were housed in plastic solid bottom cages and allowed to acclimate to the laboratory conditions for 5 to 18 days. Food and water were provided *ad libitum*. In the preliminary test, dried *E. gracilis* was administered by oral gavage at doses of 0, 500, or 2,000 mg/kg body weight/day for 2 consecutive days. Animals were observed twice daily for clinical signs and mortality, and cage-side observations of all animals were performed daily throughout the study. Surviving animals were killed 44 to 48 hours following administration of the second dose. All animals survived at the end of the study period and the highest dose tested was used in the main study. In the main test, Swiss albino (ICR) mice (5/sex/group) were administered dried cell *E. gracilis* at doses of 0 or 2,000 mg/kg body weight. The positive control, cyclophosphamide monohydrate, was dissolved in water for injection and was administered at a dose of 40 mg/kg body weight/day to a group of 5 male and female mice. Animals were observed similar to the preliminary test. Blood samples were collected by cardiac puncture and prepared in duplicate for analysis of the proportions of immature erythrocytes and total erythrocytes and micronucleated erythrocytes.

All animals survived test article administration. No significant effects on reticulocyte fraction, micronucleus frequency in normochromatic erythrocytes and frequency of micronucleated reticulocytes were observed. Based on the results of this study, the authors concluded that dried cell *E. gracilis* was not genotoxic or cytotoxic in mice.

### 6.4.4 Anti-Carcinogenicity Studies

The antitumor activity of paramylon from *Euglena* on pre-neoplastic colonic aberrant crypt foci was investigated in mice (Watanabe *et al.*, 2013). In this study, 60 weaning male Jcl:ICR mice were randomly assigned to 8 treatment groups and received a semi-purified diet (control) or a diet containing *Euglena*, paramylon, or amorphous paramylon at a concentration of 2% in the diet (20,000 mg/kg diet) for 11 weeks. The *Euglena* contained about 34% carbohydrates and approximately 70 to 80% of carbohydrates in *Euglena* were assumed to be paramylon. Following consumption of the control or test diet for 8 days, cell proliferation in the colonic mucosa was induced in 30 animals by intraperitoneal injection of 1,2-dimethylhydrazine (DMH) at a dose of 20 mg/kg body weight/week for 6 weeks. All animals were killed at the end of the study period. Blood was collected from the descending aorta, and organs (liver, spleen, cecum, small and large intestine) were extracted and weighed. The presence of aberrant crypt foci in

the large intestine was evaluated in all animals. The concentration of *N*-(ε)-hexanoyl lysine (HEL), a biomarker of oxidative stress, in the small intestine and urine was measured.

No significant effects on body weight, dietary intake, HEL concentration, or organ weight were observed in any group. The cecum weight of animals of the amorphous paramylon group was significantly decreased compared to the *Euglena* group. There were no significant differences in the number of aberrant crypt foci in the groups that were not administered DMH. Conversely, the number of aberrant crypt foci was significantly increased in animals administered DMH. Animals provided *Euglena*, paramylon, and amorphous paramylon showed a significant decrease in aberrant crypt foci formation compared to the control group. Overall, no significant adverse effects were observed in this study that could be attributed to treatment.

## 6.5 Other Published Studies

Comprehensive searches of the literature identified various studies evaluating *E. gracilis* algal preparations experimental feeding studies in rats and growth studies in poultry related to the use of *E. gracilis* as a feed additive were identified. A discussion of these studies as they apply to the GRAS status of algal meal from *E. gracilis* ATCC PTA-123017 is presented below.

In one study, untreated and dried (*i.e.*, lyophilized) *E. gracilis* cells were grown either heterotrophically or autotrophically and fed to rats (5/group; strain not reported) to evaluate the nutritive value, *in vivo* digestibility, biological value, protein efficiency ratio and net protein utilization (Hosotani and Kitaoka, 1977). Groups of rats received a protein-free diet for 7 days followed by a diet containing 11.8% casein, 24.0% lyophilized green cells grown in the light (GL cells), 18.3% ethanol-extracted GL cells, 19.9% paramylon-removed GL cells, or 16.3% lyophilized cells of a colorless strain of *Euglena* rich in paramylon for 10 days. Body weight gain, protein efficiency ratio, biological value, and net protein utilization were evaluated in rats and comparisons were made between lyophilized GL cells and the other diets. Percent digestibility was greatest for casein (96.9 ± 0.6%) and was statistically significantly different from lyophilized GL cells (93.1 ± 0.6%). No other differences were observed between casein and lyophilized GL cells and the actual difference in digestibility were not likely to be of biological significance. Percent digestibility was similar between other *E. gracilis*-containing diets with the exception of the lyophilized colorless cell-containing diet which had a digestibility of 95.1 ± 0.4%. Ethanol-extracted GL cells had the lowest biological value, net protein uptake, and protein efficiency ratio; rats fed this diet also had the lowest body weight gain. These values were significantly different from the lyophilized GL cell diet with P-values of <0.01 for body weight gain, protein efficiency ratio, and biological value; net protein utilization was significantly different with a P-value of <0.05. The study authors noted that the ethanol extraction may have removed an essential amino acid which would affect growth performance. Overall, lyophilized *E. gracilis* cells had a similar profile to casein with only slightly lower body weight gains

observed in rats fed these diets. This study demonstrates the tolerability of lyophilized *E. gracilis* and the lack of any short-term adverse effects when fed as part of the diet to rats.

Nakano *et al.* (1995) evaluated the nutritional value of *E. gracilis* in Wistar rats. The *Euglena* cells were lyophilized and fed to rats for 10 days at a concentration of 10% as protein and compared to milk casein to evaluate weight gain, protein efficiency ratio, digestibility, biological value and net protein utilization. The study authors noted that the endpoints evaluated were similar between the *Euglena* diet and the milk casein diet. These results indicate that the *Euglena* cells were well-tolerated when fed to rats.

Kuda *et al.* (2009) evaluated the effects of the storage proteins *beta*-1,3-glucan, and laminarin from *Eicenia bicyclis* and *Euglena gracilis* on cecal environment and plasma lipid levels in rats. Eighteen Sprague-Dawley rats 3 weeks of age were randomized to 1 of 3 groups provided a control diet containing no dietary fiber, or the control diet containing 1% laminarin, or 1% paramylon for 14 days. Measures of body weight gain, liver weights, cecal parameters (pigmentation, cecal weight, cecal microbial counts and cecal organic acid), and plasma levels of lipids, glucose and albumin were obtained on day 14. The authors also conducted an *in vitro* experiment evaluating the fermentation of *beta*-1,3-glucans (*i.e.*, paramylon, laminarin and curdlan) by human fecal microflora. The authors reported that laminarin and paramylon supported the growth of fecal microflora resulting in time-dependent increases in OD counts and reduction of the media pH. In contrast curdlan did not affect OD counts or media pH suggesting that curdlan is a poor substrate for the microflora tested in the study. The data from the *in vitro* incubation studies indicate that paramylon can be fermented by human microbiota; however, paramylon was appreciably less effective that the control media or laminarin in this regard. In the in feeding study no statistically significant differences in body weight or body weight gain was observed between the groups. Significant fecal bulking effects were reported in the paramylon group; cumulative fecal weights were increased by 53% in this group relative to controls. Corresponding increases in cecal weights (+16% relative to controls; $p>0.05$) were reported, a change that is consistent with the fecal bulking effect of paramylon. Absolute and relative liver weights were reduced in the paramylon group by 17% ($p<0.05$) and 14% ($p<0.01$) respectively. No major differences in cecal microbe counts were noted; however, the ratio of lactobacilli to Total Viable Counts (TVC) were increased in the paramylon group *vs.* controls. The authors also reported that cecal contents in the paramylon group were dark green in coloration which differed from the cecal coloration in the laminarin group, which was yellowish. The authors suggested that the green discoloration could be due to changes in the cecal contents of biliary pigments bilirubin (yellow) *vs.* biliverdin (green), and further suggest that accumulation of green pigments may be attributed to liver injury. The green discoloration of the cecal contents is more likely attributed to the fecal bulking effects of undigested paramylon that were reported by the authors, an effect that would be associated with increased fecal transit times and corresponding reduced metabolic conversion of bilverdin (green) to bilirubin (yellow) by the cecal microflora (Mayo Clinic, 2016). No differences in the plasma levels of

phospholipids, total cholesterol, glucose or albumin were reported by the authors; however, plasma levels of triacylglycerols were increased in the paramylon group (201 ± 44 mg/100 mL) relative to controls (114 ± 11 mg/100 mL). The authors stated that this effect was further evidence of adverse effects of paramylon on lipid metabolism and hepatocyte function. An explanation for the change in liver triglycerides is unclear; however, hypertriglyceridemia is not a validated endpoint for liver toxicity. Paramylon is an insoluble dietary fiber that is not absorbed, therefore exposure of the liver to intact paramylon will not occur. In addition, paramylon at its basic molecular level consists of 100% glucose molecules, and will be fermented to short-chain fatty acids in a similar manner to other dietary fibers; no toxic microbial metabolites will be produced. An explanation for the hypertriglyceridemia reported by Kuda *et al.* (2009) is unclear, and may be a transient effect of the particular diet used by the authors, which appears to have some amino acid imbalances relative to the NRC recommendations for purified diets used in rodent studies (NRC, 1995). The findings by Kuda *et al.* (2009) also are inconsistent with reports by Sugiyama *et al.* (2009) where paramylon produced a hepatoprotective effect in Wistar rats treated with carbon tetrachloride prior to administration of paramylon at doses of 0, 500, 1,000, or 2,000 mg/kg body weight. Furthermore, in a recent study by Shimada *et al.* (2016), the authors reported that administration of 2% paramylon in the diets of Long-Evans Tokushima Otsuka (OGTT) rats (type 2 diabetes model) for 10 weeks did not affect liver weight or serum triglycerides, and tended to lower liver triglyceride concentrations relative to control rats. Finally, as no evidence of liver toxicity or hypertriglyceridemia has been reported in other experimental studies, including subchronic toxicity studies in Sprague-Dawley rats administered paramylon at concentrations up to 5% in the diet (Simon *et al.*, 2016), the effects of paramylon reported by Kuda *et al.* (2009) were not of toxicological concern.

### 6.5.1 Poultry

Two tolerability and utility feed studies in poultry were identified in the literature in which broiler chicks were fed diets containing up to 1.0% *E. gracilis* for up to 5 weeks (Choi *et al.*, 2004a,b). Broiler chicks are rapidly growing animals and can be sensitive to dietary manipulations. The absence of overt toxic effects on growth reported in these studies corroborates the conclusion that *E. gracilis* is non-toxic and not nutritionally disadvantageous in this animal model. These studies were published in Korean, therefore further details are presented in brief in Table 6.5.1-1 below.

**Table 6.5.1-1 Studies Conducted Using *Euglena gracilis* Pertinent to the Safety and Utility for Poultry**

| Study Description | Utility or Safety-Related Results and Remarks |
|---|---|
| *Choi et al., 2004a*<br><br>**Objective**<br>Effect of dietary supplementation of *Euglena gracilis* Z on the performance and fatty acid composition of breast muscle of broiler chickens<br><br>**Birds**<br>Study 1: 210 1-day old Ross broiler chicks (3 replicates/ treatment; 10 birds/replicate)<br>Study 2: 250 1-day old Ross broiler chicks (50 birds/treatment)<br><br>**Dietary treatments**<br>*Study 1:*<br>0 (control), 0.25, 0.5, 1.0% *E. gracilis* Z<br>0 (control), 0.5, 1.0 or 2.0% E. gracilis Z (bleached and enriched with DHA)<br><br>*Study 2:*<br>0 (control), 0.25, 0.5 or 1.0% *E. gracilis*<br>0 (control), 0.5, 1.0 or 2.0% E. gracilis (bleached and enriched with DHA)<br><br>**Duration**<br>*Study 1:* 5 weeks<br>*Study 2:* not available<br>**Endpoints**<br>Weight gain, feed intake/gain, and mortality; nutrient availability of diets; fatty acid composition of breast muscle | **Results**<br>No treatment-related adverse effects were reported<br><br>Fatty acid composition of breast tissue was influenced by the fatty acid profile of the test diet<br><br>**Remarks**<br>Provides corroborative evidence of the utility and tolerability of *E. gracilis* species as nutrient sources in feed when provided at significant dietary inclusion rates in broiler diets.<br><br>Clinical chemistry and hematology safety measures not available |
| *Choi et al., 2004b*<br><br>**Objective**<br>Effect of dietary supplementation of *Euglena gracilis* Z. (EG) on the performance, and egg quality and fatty acid composition of the yolk in laying hens<br><br>**Birds**<br>Study 1: 250 32-wk ISA Brown layers (4 replicates/treatment; 10 birds/replicate)<br>Study 2: 200 84-wk ISA Brown layers (5 replicates/treatment; 12 birds/replicate)<br><br>**Dietary treatments**<br>*Study 1:*<br>0 (control), 0.25, 0.5, 1.0% *E. gracilis* Z<br>0 (control), 0.5, 1.0 or 2.0% *E. gracilis* Z (bleached and enriched with DHA)<br><br>*Study 2:*<br>0 (control), or 0.5 *E. gracilis* (bleached and enriched with DHA)<br>0 (control), or 0.5, 1.0 or 2.0% *E. gracilis* (enriched with DHA)<br><br>**Duration**<br>Study 1: 4 weeks<br>Study 2: 4 weeks | **Results**<br>Study 1&2: *Euglena* supplementation did not significantly affect egg production but increased egg weight and feed intake<br><br>Egg yolk color was positively influenced (increased score) and the fatty acid composition reflected any increased DHA in the feed<br><br>**Remarks**<br>Provides corroborative evidence of the utility and tolerability of *E. gracilis* species as nutrient sources in feed when provided at significant dietary inclusion rates of up to 2.0%.<br><br>Clinical chemistry and hematology safety measures not available |

| Table 6.5.1-1 Studies Conducted Using *Euglena gracilis* Pertinent to the Safety and Utility for Poultry | |
|---|---|
| **Study Description** | **Utility or Safety-Related Results and Remarks** |
| <u>Endpoints</u><br>Layer performance and eggshell quality, egg fatty acid composition, feed intake | |

## 6.6 Toxicity Studies and Safety of Related *beta*-1,3-Glucans

While there are numerous studies in the published literature characterizing the toxicity of soluble and insoluble *beta*-1,3-glucans from various sources, many of these glucans may be considered structurally distinct from the *beta*-1,3-glucan produced by *E. gracilis*. Although all *beta*-1,3-glucans share a basic structural motif comprised of glucose, *beta*-glucans vary in terms of physico-chemical parameters (*e.g.*, solubility, susceptibility to hydrolysis, fermentability), which arise from differences in primary and tertiary structures, variances in polysaccharide chain length, extent of branching, linkage type, *etc.* (Barsanti *et al.*, 2011). Differences in the source organisms and manufacturing also result in differences in impurity profiles between various *beta*-glucan sources, which limit comparisons between various *beta*-glucan preparations. Although various qualitative and quantitative differences in *beta*-glucan structure and identity limit direct extrapolation of NOAEL values that have been reported for various *beta*-glucans to paramylon, it is worthwhile noting that there have been no reported toxic effects attributed to insoluble *beta*-1,3-glucan ingredients in animal toxicity studies (Feletti *et al.*, 1992; Spicer *et al.*, 1999; Babicek *et al.*, 2007). In all cases, the NOAEL has been the highest dose administered to the animals.

## 6.7 Safety of Source Organism

### 6.7.1 Pathogenicity and Toxicogenicity

There are no documented case-reports of any member of the *Euglena* sp. being pathogenic to humans or animals. To identify evidence for potential pathogenicity, comprehensive and detailed searches of the published scientific literature were conducted through February 2016 using an online search engine and the following databases: MEDLINE®, AGRICOLA, ToxFile, Biosis Previews®, Biosis Toxline®, Food Sci. & Tech. Abstracts, CAB Abstracts, FOODLINE®, NTIS and EMBASE. The dinoflagellates, diatoms, pelagiophytes and prymnesiophytes represent the only divisions of microalgae that are known to produce toxins (*e.g.*, domoic acid, saxitoxin, brevetoxin) (Wehr and Sheath, 2003). Photosynthetic cyanobacteria also are known to produce at least 3 classes of toxins (*e.g.*, anatoxins, microcystins, and nodularins) (Chorus, 2001). Although toxin producing microalgae are typically associated with taxa that inhabit marine or estuary environments, the production of toxins by freshwater *Euglenophyceae* has been reported (Zimba *et al.*, 2004). To date, evidence for the production of toxic secondary metabolites in freshwater algae has only been reported for 2 species of *Euglena* (*E. sanguinea*

and *E. granulata*) obtained from the wild (Zimba *et al.*, 2004, 2010). Implication of *Euglena* sp. as a potential toxin producer was first suggested following a series of case investigations of complete fish mortality in a commercial aquaculture facility (Zimba *et al.*, 2004). Water chemistry was evaluated and determined to not be the cause of mortality. A microscopic evaluation of the water revealed that the water contained >99% *Euglena* by numeric counts. It was determined through serial filtration that both the dissolved water and algal fractions obtained from the commercial ponds were toxic to fish. The toxin was determined to be a water-soluble substance that was not a protein. The *Euglena* species was identified to be *E. sanguinea via* microscopic analysis. The study authors also evaluated a strain of *Euglena* from a collection at the University of Texas (Strain LB2345) which also elicited a toxic response in catfish and sheepshead. This species was later determined to be *E. granulata*. These were considered by the study authors to be the first freshwater algae to produce toxins as confirmed by fish bioassays.

In a subsequent study by Zimba and colleagues, further characterization of the toxin using high-performance liquid chromatography/mass spectrometry, tandem mass spectrometry, and nuclear magnetic resonance imaging analyses of a clonal isolate of *E. sanguinea* grown in batch culture was conducted (Zimba *et al.*, 2010). In addition to *E. sanguinea*, several additional isolates were cultured (*Euglena viridis*, *E. granulata*, *Euglena splendens*) and assayed to determine if production of the toxin was common among other Euglena isolates. The toxin produced by *E. sanguinea* was identified as euglenophycin and was determined to be structurally similar to an alkaloid present in fire ant venom. Studies in fish confirmed that euglenophycin was ichthyotoxic. Results in other *Euglena* sp. were not discussed; however, it was noted that toxin production was observed in "at least" 2 *Euglena* sp. (*E. sanguinea and E. granulata*).

To date, the synthesis of euglenophycin or other algal toxins have not been reported for *E. gracilis*. Algal Scientific has conducted an analysis of *E. gracilis* used in the production of dried algae *(E. gracilis* ATCC PTA-123017*)* for the presence of toxins including euglenophycin, microcystin, cylindrospermopsin, saxitoxin, brevetoxin, DSP, and ASP (Zimba, 2016[2]). The results of the analyses are provided in Appendix B. The lack of toxin production by *E. gracilis* also is strongly corroborated by its history of safe use as an aquaculture and animal feed ingredient and as a human food ingredient. Unpublished studies demonstrating that *E. gracilis* ATCC PTA-123017 as manufactured by Algal Scientific, is well-tolerated in marine animals (fish, prawns), chickens, and pigs have been conducted; these studies were not considered pivotal to the safety of the dried algae and therefore are not discussed further.

[2] Personal Communication from P.V. Zimba, 2016

## 6.8 Expert Panel Evaluation

Algal Scientific has concluded that paramylon isolate from *E. gracilis* ATCC PTA-123017 manufactured by Algal Scientific meeting appropriate food grade specifications and manufactured consistent with current Good Manufacturing Practices, is GRAS for use as an ingredient in specified conventional food and beverage products, as described in Part 1.3, on the basis of scientific procedures.

This GRAS determination is based on data generally available in the public domain pertaining to the safety of *E. gracilis* ATCC PTA-123017 and on a unanimous opinion among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Michael W. Pariza (University of Wisconsin-Madison), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, convened by Algal Scientific, independently and critically evaluated all data and information presented herein, and concluded that dried *E. gracilis* from strain ATCC PTA-123017 was GRAS for use in food as described in Section 1.3 based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of paramylon from *E. gracilis* ATCC PTA-123017 is presented in Appendix C.

## 6.9 Conclusions

Based on data and information presented herein Algal Scientific has concluded that paramylon isolate from *Euglena gracilis* ATCC PTA-123017, meeting appropriate food-grade specifications and manufactured according to cGMP, is safe for use in specified conventional food and beverage products as presented in Table 1.3-1. Algal Scientific also has further concluded that pivotal data and information relevant to the safety of paramylon from *E. gracilis* ATCC PTA-123017, are publicly available and therefore the proposed uses of dried *E. gracilis* algae and paramylon can be determined to be Generally Recognized as Safe (GRAS) on the basis of scientific procedures.

## Part 7. §170.255 List of Supporting Data and Information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|---|---|---|
| **Part** | **Section §** | **Section Title** |
| 58—Good laboratory practice for nonclinical laboratory studies | All | All |
| 101—Food labeling | 101.12 | Reference amounts customarily consumed per eating occasion |
| 110— Current good manufacturing practice in manufacturing, packing, or holding human food | 110.5 | Current good manufacturing practice |

| CFR Sections Referenced (Title 21—Food and Drugs) | | |
|---|---|---|
| **Part** | **Section §** | **Section Title** |
| 170— Food additives | 170.3 | Definitions |
| | 170.30 | Eligibility for classification as generally recognized as safe (GRAS) |
| 173—Secondary direct food additives permitted in food for human consumption | 173.340 | Defoaming agents |


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| | |
|---|---|
| **From:** | Robert.Levine@kemin.com |
| **To:** | Perrier, Judith |
| **Cc:** | Gaynor, Paulette M; Kristi.Krafka@kemin.com |
| **Subject:** | Updated contact information for recent GRAS filing by Algal Scientific |
| **Date:** | Thursday, April 13, 2017 4:03:47 PM |

Dear Ms. Perrier,

Algal Scientific recently filed two GRAS notices for Dried Algae Euglena Gracilis and Paramylon. The company was recently acquired by Kemin and my contact info has changed.

Please use this email (robert.levine@kemin.com) to reach me or my cell: (b) (6) - (b) (6)

I would also like to list a regulatory expert at Kemin as a secondary contact for these notices: Kristi Krafka at Kristi.Krafka@kemin.com and cell (b)) (6)

Please confirm receipt.

Sincerely,

Robert Levine

**Kemin Foods, L.C.**
2100 Maury Street
Des Moines, IA 50317
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tel: 515.248.4000
toll free: 888.248.5040
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April 20, 2017

Center for Food Safety and Applied Nutrition (CFSAN)
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
U.S.A.

**Re: Updated contact information related to GRN 697 and GRN 698**

Dear Dr. Perrier:

Kemin Foods, L.C. (Kemin) hereby submits updated contact information related to the two Generally Recognized As Safe (GRAS) notices, GRN 697 and GRN 698. Since acquiring Algal Scientific in March, Kemin would also like to update the primary contact for these GRAS notices.

Updated Name and Address of Notifier:

Joanne Lasrado, Ph.D.
Director, Regulatory Affairs and Quality Assurance

Kemin Foods, L.C.
2100 Maury St.
Des Moines, IA 50317
Telephone: 515-559-5429
e-mail address: joannelasrado@kemin.com

Should you have any questions, please do not hesitate to contact us.

Kind Regards,

(b) (6)

Joanne Lasrado, Ph.D.
Director, Regulatory Affairs and Quality Assurance
Kemin

cc: Robert Levine, Ph.D.