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DHA

1. Introduction

DHA-rich algal oil is extracted and refined from the wild-type heterotrophic micro-algae Schizochytrium sp. ONC-T18. This micro-algae is a member of the Thraustochytriaceae family which historically has been comprised of seven genera, Japonochytrium, Schizochytrium, Ulkenia, Althorinia, Diplaphys, Aplanochytrium, and Thraustochytrium (Burgja et al., 2006). It is a mixture of triglycerides containing polyunsaturated fatty acids (PUFA) in which the predominant fatty acid (>35%) is docosahexaenoic acid (DHA).

DHA is an omega-3 fatty acid that has been widely studied for its beneficial effects on human health, particularly brain, eye and heart health. Along with the omega-3 fatty acid arachidonic acid (ARA), it is commonly found in most commercial infant formula, either from algal or tuna oil sources. DHA is accumulated in the retina during gestation, and early infancy and is continually replenished from the plasma (Innis, 2005). Neurodevelopment progresses rapidly during this time and demands for DHA are high to ensure neurite outgrowth and proper brain and retina development (Marszalek and Lodish, 2005). DHA has a prominent role in the development of photoreceptors and synaptic networks (Kurlak and Stephenson, 1999), with as much as two thirds of the fatty acids in the retinal photoreceptor phospholipid membranes being 22 carbon omega-3 fatty acids (Bazan et al., 1986). DHA affects neuronal excitability and transmission by modulating ion channel function and the resulting flow of ions (Assisi et al., 2006).
interact with membrane proteins and affect signal transduction pathways that activate transcription factors such as steroid hormones and glucocorticoids. These transcription factors can affect the expression of a number of different genes, including those for enzymes involved in downstream signal transduction pathways (Assisi et al., 2006). Another possible role for DHA in the brain may be to enhance the activity of catalase and glutathione, thereby providing greater protection from free radicals and oxygen reactive species (Hossain et al., 1999).

*Schizochytrium* and *Ulkenia*-based oils are currently globally marketed as DHA-rich oils. The oil from *Schizochytrium* sp. ONC-T18 has an identical proximate composition and a closely similar lipid profile to that of the presently marketed oil from *Schizochytrium* sp. The nutritional value and metabolism of the oil under study is virtually indistinguishable from the previously authorized and marketed oil from *Schizochytrium* sp. ATCC 20888 produced by Martek Biosciences (now a part of DSM). This is based on an identical proximate composition (both substances being essentially 100% fat) and a closely similar fatty acid profile. At the intended levels of use (60–600 mg DHA-equivalent/100 g of food), the small differences in lipid profiles will have no significance on their relative nutritional value or metabolic impact. Specifications for DHA-rich algal oil ONC-T18 include analysis for omega-3 content, heavy metal and microbial contamination, and other common oil quality measures (see Table 1).

DHA-rich algal oil ONC-T18 is intended for use in an identical manner and same foods as the currently marketed oil. Therefore, it will replace, rather than add to, intake from the currently marketed oils. DHA has been incorporated into a variety of food products with specific limitations including, but not limited to, breads, cereals, fats and oils, condiments, yogurt, cheese, frozen dairy, meat, egg, nut, and fish products (Kroes et al., 2003).

The potential toxicity of various algal oils rich in DHA have been previously studied and its safe use in food evaluated by numerous government agencies and regulatory authorities. Published preclinical studies include subchronic, genotoxicity, and developmental and reproductive toxicity studies (Kroes et al., 2003; Burns et al., 1999; Hempenius et al., 2000; Hammond et al., 2001a, 2001b, 2001c, 2002; U.S. FDA, 2001, 2004a, 2004b, 2010). Numerous clinical trials have been conducted on DHA-containing fish and marine-based oils. The trials have included adults, children, and infants as DHA oil is used commercially in infant formula. Atterburton et al. (2007) stated that algae are the primary producers of DHA in the food chain, and algal sources of DHA are available for fortification of infant formulas and food, and for dietary supplements for adults and pregnant women. The clinical safety of DHA-rich oils has been reviewed by Kroes et al. (2003) and Atterburton et al. (2007) and found to be safe for human use.

The safety of dietary DHA and oils produced from both fish and algal sources is well established in the literature. In affirming the Generally Recognized as Safe (GRAS) status of the fish-based menhaden oil (62 FR 30751; June 5, 1997), FDA concluded that the use of menhaden oil as a direct food ingredient is GRAS, provided that the combined daily intake of DHA and eicosapentaenoic acid (EPA) does not exceed 3 grams/day. The proposed uses of DHA-rich algal oil ONC-T18 comply with this requirement, and when used at the intended levels, the nutritional value and metabolism of the oil from *Schizochytrium* sp. ONC-T18 is indistinguishable from that of the presently authorized and marketed oils as described below.

DHA rich oils from micro-algal sources have been the subject of four authorization decisions and/or notifications under the EU Novel Food Regulation 258/97. The first such measure was EC Commission Decision (2003)2003/427/EC in June of 2003 authorizing the use of DHA-rich oil from the thraustochytrid micro-algae *Schizochytrium* sp. in a range of foodstuffs and establishing a specification for the material. This was followed in December 2003 by a notification under Article 5 of the novel food regulation for placement on the market of a DHA-rich oil derived from a second thraustochytrid micro-algae *Ulkenia* sp. on the grounds of its substantial equivalence with the oil from *Schizochytrium* sp. In 2009 EC Commission Decision, 2009a,b 2009/777/EC and 2009/778/EC authorized extensions to the approved food uses of the oils from *Ulkenia* sp. and *Schizochytrium* sp., respectively. A third DHA-rich oil derived from the micro-algae *Cryptothecodinium cohnii* was already on the EU market before the Novel Food Regulation came into effect and was therefore legally in use without the need for explicit approval. These three DHA rich oils have also been the subject of GRAS notifications to which the FDA had no objections (U.S. FDA GRN Nos. 41, 137, 319).

The present studies were conducted as part of an investigation to examine the developmental and reproductive safety of DHA-rich algal oil. The results reported herein demonstrate a similar toxicity profile as exists for other algal-based oils.

### Table 1

<table>
<thead>
<tr>
<th>Fatty acid composition of DHA-rich algal oils.</th>
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<tr>
<td>Schizochytrium sp. ATCC 20888*</td>
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<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Fatty acid</td>
</tr>
<tr>
<td>Laurate</td>
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<tr>
<td>Myristate</td>
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<tr>
<td>Tetradecatetraenoic acid</td>
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<tr>
<td>Palmitate</td>
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<tr>
<td>Palmitoleate</td>
</tr>
<tr>
<td>Hexadecatetraenoic acid</td>
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<tr>
<td>Stearate</td>
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<tr>
<td>Oleate</td>
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<tr>
<td>Vaccenate</td>
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<tr>
<td>Linoleate</td>
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<tr>
<td>Octadecataetraenoic acid</td>
</tr>
<tr>
<td>Dihomo-gammarlinolenic acid</td>
</tr>
<tr>
<td>Arachidonate</td>
</tr>
<tr>
<td>Eicosatetraenoic acid</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>DHA</td>
</tr>
</tbody>
</table>

* 5 lots; measured and expressed as weight%.
* 3 lots; measured and expressed as area%.
* Schizochytrium sp. ATCC 20888 EU novel food submission designates as co-eluting with Eicosatetraenoic acid 20:4n-7.

### 2. Materials and methods

The test material and methods employed are described for each individual study. In all studies, the stated concentrations or doses reflect the amount of algal oil administered, and appropriate control groups were employed as necessary.

#### 2.1. Prenatal developmental toxicity study

##### 2.1.1. Test material and animals

ONC-T18 DHA-rich algal oil, a frozen liquid (lot no. 22630; approximately 42% DHA; total omega-3 fatty acids, 44%; storage condition, frozen, −10 to −30 °C) was obtained from Ocean Nutrition Canada Limited, Dartmouth, Nova Scotia, Canada. Sexually mature, virgin female Sprague Dawley [Crl:CD(SD)] rats were used as the test system on this study (Charles River Laboratories, Inc., Raleigh, NC). This species and strain of animal is recognized as appropriate for developmental toxicity studies. Upon arrival and until pairing, all rats were individually housed in clean, stainless steel wire-mesh cages suspended above cage-board. The rats were paired for mating in the home cage of the male. Following positive evidence of mating, the females were returned to individual suspended wire-mesh cages; nesting material was not required as the females were euthanized prior to the date of expected parturition. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Test animals were provided continuous access to tap water and PMI Nutrition International, LLC Certified Rodent LabDiet® 5002.
2.1.2. Experimental design
The study was conducted in accordance with U.S. Food and Drug Administration (21 CFR Part 58) and the OECD Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Test. The study was performed in accordance with Animal Welfare Act, 21 Code of Federal Regulations Part 2, 1983 and Guideline 408, Health Effects Test Guidelines, September 1998.

Four groups of male and female Crl:CD(SD) rats (30/group) were offered DHA fish oil or the test article, ONC-T18 Algal Oil (hereafter referred to as algal oil), continuously in the diet with an additional control group (30 rats/sex) that were offered the basal diet (PMI Nutrition International, LLC Certified Rodent LabDiet® 5002). F0 males and females were administered the basal or test diets for at least 70 or 14 consecutive days, respectively, prior to mating. Seventy days covers at least one complete spermatogenic cycle in the male rats and 14 days covers at least two complete estrous cycles in the female rats in order to elicit any adverse effects of spermatogenesis or the estrous cycle. Target test article concentrations were 0, 50,000 ppm DHA fish oil, 10,000/40,000 and 25,000/25,000 ppm algal oil/corn oil (hereafter referred to as 10,000 and 25,000 ppm algal oil, respectively), and 50,000 ppm algal oil for the F0 and F1 generations. F0 males and females were approximately 7 and 12 weeks, respectively, of age at the initiation of basal or test diet administration. The F0 males continued to receive the basal diet or DHA fish oil or algal oil diets throughout mating and continuing through the day of euthanasia. The F0 females continued to receive the basal diet or DHA fish oil or algal oil diets throughout mating, gestation, and lactation, and through the day of euthanasia. Offspring (20/group) from the pairing of the F0 animals were selected on PND 21 to constitute the F1 generation. F0 females were returned to the original dietary regimen. The F0F1 generation offspring (8 pups per litter) were weighed at least once weekly until cohabitation, all F0 offspring were housed individually in clean, stainless steel wire-mesh cages until the scheduled necropsy, and the weaned F1 pups were housed together by litter for 1 week in plastic maternity cages with nesting material. Beginning on PND 28, the F1 offspring were individually housed in suspen-sed wire-mesh cages until the day of euthanasia.

2.2. Experimental design
The study was conducted in accordance with U.S. Food and Drug Administration (21 CFR Part 58) and in general accordance with the OECD Guidelines for Testing of Chemicals, Pre-Natal Developmental Toxicity Test Guideline 414. The study was performed in accordance with Animal Welfare Act, 21 Code of Federal Regulations Part 2, 1983 and Guideline 408, Health Effects Test Guidelines, September 1998.

2.2.1. Test material and animals
ONC T-18 DHA-rich algal oil, a frozen liquid (lot nos. 22629 and 22630; approxi-mately 42% DHA; total omega-3 fatty acids, 44%; storage condition, frozen, –10 to –30 °C) and the DHA fish oil (tuna) control article were produced and received from Ocean Nutrition Canada Limited, Dartmouth, Nova Scotia, Canada. Three lots of the tuna oil control were provided (lot nos. 23516, 24098, and 24921) and all had a DHA concentration of 26–27% and an EPA content of 6–7%. Ascorbyl palmitate and natural tocopherols were added to both the algal oil and tuna oil to prevent oxidation.

2.2.2. Three-month rat dietary toxicity study with an in utero exposure phase

2.2.2.1. Study design
Carcinogenicity studies were conducted using the 2-year rat carcinogenicity test (Wyatt, 1986) for a wide range of compounds. Male and female Sprague-Dawley rats were used as the test article. All test animals were housed individually in clean, stainless steel wire-mesh cages throughout the study. Urinalysis, including microscopic examination, was conducted on all female rats at necropsy. Blood chemistry-serum aspartate aminotransferase (AST), serum alanine transaminase (ALT), gamma glutamyltranspeptidase (GGT), total bilirubin, blood urea nitrogen (BUN), blood creatinine, total cholesterol (CHL), triglycerides (TRIG), glucose (GLUC), total serum protein (TP), albumin (ALB), globulin (GLB), calcium (Ca), phosphorus, sodium (Na), potassium (K), and chloride (Cl). Urinalysis included color, clarity, volume, pH, glucose, specific gravity, protein, ketones, bilirubin, occult blood, leukocytes, urobilinogen, and microscopic urine sediment examination.

A complete necropsy was conducted on all F0 parental and F1 adult animals found dead or that survived to the end of the study. The necropsy included examination of the external surface, all orifices, the cranial cavity, the external surfaces of the brain and spinal cord, and the thoracic, abdominal, and pelvic cavities, including viscera/contents. For F0 females that delivered, the numbers of former implantation sites and attachment site for the F0F2 test article were identified by the identification of the number of unaccounted-for sites was calculated for each female by subtracting the number of pups born from the number of former implantation sites observed. For females...
that failed to deliver, a pregnancy status was determined, and specific emphasis was placed on anatomic or pathologic findings that may have interfered with pregnancy. The following F0 and F1 parental tissues and organs were collected and were placed in 10% neutral-buffered formalin: adrenal glands, brain, bone marrow (femur and sternum), bone marrow smear (from femur), brain, cervix, coagulating gland, epididymis, eyes with optic nerve, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, peyer's patches, heart, kidneys, liver, lungs, lymph nodes (axillary, mesenteric, mandibular), mandibular salivary glands, ovaries and oviducts, pancreas, peripheral nerve (sciatric), pituitary gland, prostate gland, seminal vesicles with coagulating glands, skeletal muscle, skin with mammary gland, spinal cord (cervical, lumbar, thoracic), spleen, testes, thymus glands, thyroid (with parathyroid if present), trachea, urinary bladder, uterus, vagina, vas deferens, and all gross lesions.

The following organs were weighed from all F0 parental and F1 adult animals at the scheduled necropsies: adrenal glands, brain, epididymides (total), kidneys, liver, heart, ovaries, pituitary gland, prostate gland, seminal vesicles with coagulating glands (with accessory fluids), spleen, testes, thyroid gland with parathyroids, thymus gland, and uterus.

Microscopic evaluations were performed on the following tissues for all F0 parental animals from the basel diet control, 50,000 ppm DHA fish oil, and 50,000 ppm algal oil groups, and all adult animals found dead: brain, cervix, coagulating gland, epididymis (caput, corpus and cauda), kidneys, liver, mammary gland (female only), ovaries, pituitary gland, prostate gland, seminal vesicles, testes, uterus, vagina (vaginal epithelium), and all gross (internal) lesions. In addition, reproductive organs of all animals suspected of reduced fertility (e.g., those that failed to mate, conceive, sire, or deliver healthy offspring) were subjected to histopathological evaluation. Microscopic evaluations were performed on all the tissues listed previously for F1 adult animals from the basel diet control, 50,000 ppm DHA fish oil, and 50,000 ppm algal oil groups, and for all adult animals found dead. Gross lesions observed in low- and mid-dose algal oil groups were also examined microscopically.

2.2.3. Statistical analyses
Analyses were conducted using two-tailed t-tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test article-exposed group to the 50,000 ppm DHA fish oil group by sex. In addition, the basel diet control group was compared separately to the 50,000 ppm DHA fish oil group.

Parental mating, fertility, copulation, and conception indices were analyzed using the Chi-square test with Yates' correction factor (Hollander and Wolfe, 1999). Mean parental (weekly, gestation, and lactation), offspring, and adult body weights, body weight changes, parental and adult food consumption, and food efficiency data, estrous cycle lengths, pre-coital intervals, gestation lengths, former implantation sites, live litter sizes, unaccounted-for sites, numbers of pups born, clinical pathology values (excluding Gamma glutamyltransferase), balanopreputial separation data (day of attainment and body weight), vaginal patency data (day of attainment and body weight), and continuous FOB, and absolute and relative organ weights, were subjected to a parametric one-way ANOVA to determine intergroup differences (Snedecor and Cochran, 1980). If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunn's test (Dunnett, 1964) or a two-sample t-test (Snedecor and Cochran, 1980), as appropriate, was used to compare the test article-exposed and control groups to the 50,000 ppm DHA fish oil group. Mean litter proportions (percent per litter) of postnatal pup survival and pup sexes at birth (percentage of males per litter) were subjected to the Kruskal Wallis nonparametric ANOVA test (Kruskal and Wallis, 1952) to determine intergroup differences. If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunn's test (Dunn, 1964) was used to compare the test article-exposed groups to the 50,000 ppm DHA fish oil group and the basel diet control group to the 50,000 ppm DHA fish oil group. Gamma glutamyltransferase values under range were assigned a value of 0.


cant at the 0.01 level. Within the framework of the RMANOVA, pair-wise comparisons were made for each individual treated group with the 50,000 ppm DHA fish oil group through linear contrasts. If TRT TIME was significant, the comparisons were conducted for each time interval. If only the TRT effect was significant, the comparisons were conducted across the pooled time intervals of the entire session. These nonmonotonic dose response comparisons were conducted at the 0.01 significance level. In addition, an analysis was conducted to compare to the basel diet control group and the 50,000 ppm DHA fish oil group. A RANOVA was used to include TRT, TIME, and TRT TIME. The SAS® procedure PROC MIXED was used for analysis and the covariance structure across time was selected by comparing AIC. If TRT TIME was significant, the comparison of the 2 groups was conducted for each time interval. If TRT TIME was not significant, the 2 groups were compared through the TRT main effect. All tests were conducted at the 0.05 significance level.

3. Results

3.1. Prenatal developmental toxicity study

3.1.1. Dose formulation analysis
A gas chromatography method using mass spectrometric detection was employed for the determination of test article concentration and homogeneity. The analyzed dosing formulations were within the SOP range for suspensions (85–115%) and were homogeneous based on these results. Test articles were administered to the animals at the test article-exposed and control groups to the 50,000 ppm DHA fish oil group. Mean litter proportions (percent per litter) of postnatal pup survival and pup sexes at birth (percentage of males per litter) were subjected to the Kruskal Wallis nonparametric ANOVA test (Kruskal and Wallis, 1952) to determine intergroup differences. Microscopic evaluations were performed on the following tissues for all F0 parental animals from the basel diet control, 50,000 ppm DHA fish oil, and 50,000 ppm algal oil groups, and all adult animals found dead: brain, cervix, coagulating gland, epididymis (caput, corpus and cauda), kidneys, liver, mammary gland (female only), ovaries, pituitary gland, prostate gland, seminal vesicles, testes, uterus, vagina (vaginal epithelium), and all gross (internal) lesions. In addition, reproductive organs of all animals suspected of reduced fertility (e.g., those that failed to mate, conceive, sire, or deliver healthy offspring) were subjected to histopathological evaluation. Microscopic evaluations were performed on all the tissues listed previously for F1 adult animals from the basel diet control, 50,000 ppm DHA fish oil, and 50,000 ppm algal oil groups, and for all adult animals found dead. Gross lesions observed in low- and mid-dose algal oil groups were also examined microscopically.

3.1.2. Maternal survival and clinical signs
All females in the control, 400, 1000, and 2000 mg/kg/day groups survived to the scheduled necropsy on gestation day 20. Clinical findings noted at the daily examinations and/or 1 h following dose administration, including hair loss or red material on various body surfaces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related.

3.1.3. Maternal study data

Intrauterine growth and survival were unaffected by test article administration at dosage levels of 400, 1000, and 2000 mg/kg/day. Parameters evaluated included postimplantation loss, live litter size, mean fetal body weights, and fetal sex ratios. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups. Differences from the control group were slight and not statistically significant.

3.1.4. Gestation day 20 laparohysterectomy data
Intrauterine growth and survival were unaffected by test article administration at dosage levels of 400, 1000, and 2000 mg/kg/day. Parameters evaluated included postimplantation loss, live litter size, mean fetal body weights, and fetal sex ratios. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups. Differences from the control group were slight and not statistically significant.

3.1.5. Fetal data

The numbers of fetuses (litters) available for morphological evaluation were 360(24), 372(25), 361(24), and 378(25) in the control, 400, 1000, and 2000 mg/kg/day groups, respectively. The only malformation observed was for a single fetus in the 2000 mg/kg/day group and was considered spontaneous in origin. No external malformations or developmental variations were noted in fetuses at any dosage level. There were no soft tissue malformations noted in fetuses at any dosage level.

A soft tissue developmental variation of distended ureter(s) was noted for 3/2 fetuses (litters) in the 2000 mg/kg/day group. Two fetuses from 1 litter in the 400 mg/kg/day group were noted with accessory liver lobule(s) and 1 fetus in this group had an extra papillary muscle in the right ventricle of the heart. Because the aforementioned findings occurred in single litters, in a manner that was
not dose-related, and/or the mean litter proportions were not statistically significantly different compared to the concurrent control group and were within range of values in the historical control data, these findings were not considered to be test article-related. A major blood vessel variation (right carotid and right subclavian arteries arose independently from the aortic arch, no brachiocephalic trunk) was noted in a single control group fetus. The skeletal malformation sternoschisis (sternal band Nos. 5 and 6 not joined) was noted for 1 fetus in the 2000 mg/kg/day group. Because this malformation was noted in a single fetus and the mean litter proportion was within the range of values in the Wil Research Laboratories historical control database, the finding was not considered test article-related. No skeletal malformations were noted in the 400 and 1000 mg/kg/day groups. Skeletal developmental variations, consisting of 14th rudimentary rib(s), 27 presacral vertebrae, cervical centrum No. 1 ossified, sternebra(e) Nos. 5 and/or 6 unossified, 7th cervical rib(s), hyoid unossified, 14th full rib(s), sternebra(e) Nos. 1, 2, 3, and/or 4 unossified, reduced ossification of the 13th rib(s), reduced ossification of the skull, pubis unossified, 25 presacral vertebrae, sternebra(e) slightly or moderately malaligned, reduced ossification of the vertebral arches, bent rib(s), entire sternum unossified, and extra site of ossification anterior to sternebra No. 1 were noted similarly in the test article-treated and concurrent control groups and/or the mean litter proportions were not statistically significant from the concurrent control group and/or were within the WIL historical control data ranges. Therefore, these skeletal developmental variations were not considered to be test article-related.

3.2. Three-month rat dietary toxicity study with an in utero exposure phase

3.2.1. Dose formulation analysis

A gas chromatography method using mass spectrometric detection was validated and employed in the study for confirmation of concentration, homogeneity, and stability of the test and control articles. Diet admix formulations prepared at target algal oil concentrations ranging from 10,000 to 50,000 ppm or DHA fish oil at a concentration of 50,000 ppm met the requirement for test substance concentration and homogeneity and, following up to 15 days of room temperature storage, stability with a few exceptions. The few out-of-specification test diet homogeneity and concentration results had no impact on the study integrity, as the test diets were increased in concentration; therefore, the animals were not unexposed to the test diet at any time during the study and the NOAEL was the highest exposure level tested, 50,000 ppm. In addition, each of the test diets was only fed for one week duration throughout the entire study. Algal oil and DHA fish oil were not detected in the basal diet provided to the control group.

3.2.2. Clinical observations and survival

There was no test article-related mortality in the F₀ and F₁ generations at any exposure level. No exposure-related clinical findings were noted for the F₀ and F₁ animals at the daily examinations.

3.2.3. Body weight and food consumption

No test article-related effects on mean F₀ body weights and body weight gains were noted in the 10,000, 25,000, and 50,000 ppm algal oil groups throughout the study. Mean food consumption in the 50,000 ppm algal oil group males was similar to the DHA fish oil group during study days 0–28; however, mean food consumption for the 50,000 ppm algal oil group males was statistically slightly higher (p < 0.05) than the DHA fish oil group beginning on study days 28 and generally continuing for the remainder of the study. The differences were considered to be a result of algal oil exposure but not adverse as there was no effect on mean food efficiency or mean body weight gain for these males. Mean food consumption and food efficiency in the 10,000 and 25,000 ppm algal oil group males and 10,000, 25,000, and 50,000 ppm algal oil group females was unaffected by test article exposure during the study.

Mean body weight gain for the 50,000 ppm algal oil group females was similar to the DHA fish oil group during PND 21–35. However, slightly higher mean body weight gain was noted for females in this group beginning on PND 35 and generally continued throughout the remainder of the study; the difference was significant (p < 0.05) during PND 77–84 only. As a result, mean body weight gain in the 50,000 ppm algal oil group females was 32 g higher than the DHA fish oil group when the entire generation (PND 21–112) was evaluated and higher mean body weight during PND 70–112 (significant; p < 0.05 on PND 84 only). These increases were attributed to algal oil exposure. Mean food consumption in the 50,000 ppm algal oil group females was generally higher than the DHA fish oil throughout the entire generation (PND 21–112); the differences were often significant (p < 0.05 or p < 0.01). These increases corresponded to the effects on mean body weights observed in this group and therefore, were attributed to test article exposure.

No test article-related effects on mean F₁ body weights, body weight gains, and food consumption were noted in the 10,000 and 25,000 ppm algal oil group males and females and 50,000 ppm algal oil group males throughout the study.

In the DHA fish oil group, mean F₁ male body weight gain was generally higher than the basal diet control group throughout the entire treatment period and resulted in higher mean body weights for the F₀ males in the DHA fish oil group during study days 21–88; however, mean F₀ female body weight and body weight gain were generally similar to the basal diet control group throughout the study. Lower mean F₀ food consumption and correspondingly higher mean food efficiency were noted for the F₀ males and females in the DHA group compared to the basal diet control group during the study. Mean F₁ male body weight gain in the DHA fish oil group was generally higher than the basal diet control group throughout the entire treatment period and resulted in slightly higher mean F₁ male body weight beginning PND 49 and generally continuing throughout the remainder of the generation. Mean F₁ female body weight and body weight gain in the DHA fish oil group were generally similar to the basal diet control group throughout the entire generation. Lower mean food consumption and correspondingly slightly higher mean food efficiency were noted in the DHA fish oil group F₁ males and females compared to the basal diet control group during PND 28–112.

3.2.4. Reproductive performance

F₀ reproductive performance values (fertility, mating, conception/copulation, and number of days between pairing and coitus) and estrous cycle length in the 10,000, 25,000, and 50,000 ppm algal oil groups were similar to the DHA fish oil group. No DHA fish oil-related effects on estrous cycle length, F₀ reproductive performance, and number of days between pairing and coitus were observed compared to the basal diet control group. No test article-related effects were noted on mean gestation lengths or the process of parturition at any algal oil exposure concentration or in the DHA fish oil group. The number of former implantation sites and the number of unaccounted-for sites were unaffected by algal oil or DHA fish oil exposure (see Table 2).

3.2.5. F₀ generation; anatomic pathology

When compared to the basal diet control group, higher liver weight parameters were noted in the DHA fish oil group males and females (absolute, relative to body and brain weight) that were
considered to be a result of the high lipid content of the diet. No differences were noted between DHA fish oil and algal oil groups.

Two females in the 10,000 ppm algal oil group were found dead or euthanized in extremis during the study. One animal was found dead on lactation day 12 with centrilobular congestion and moderate hepatocellular degeneration that was not considered to be related to algal oil. The other female rat was euthanized in extremis on study day 126 (after weaning). Malignant lymphoma was observed in the adrenal glands, liver, spleen, and mediastinal lymph nodes which corresponded to the enlargement of these organs grossly and was not considered algal oil related. All other F0 animals survived to the scheduled necropsies.

Oil-related microscopic findings in the F0 generation were noted in the mammary gland of the DHA fish oil and 10,000, 25,000, and 50,000 ppm algal oil group females. The ducts of mammary glands were minimally to moderately dilated and filled with an inflammatory exudate consisting of neutrophils, macrophages, and cell debris or increased secretion in the ducts. One 50,000 ppm algal oil group female had pyogranulomatous inflammation associated with fibrosis that was a diluted duct that had ruptured. This rat also had adjacent ducts that were dilated and filled with inflammatory exudate (see Table 3).

In the F0 males, there was a higher incidence of chronic progressive nephropathy in 50,000 ppm algal oil treated males (25/30) when compared to DHA fish oil treated males (17/30). This incidence was slightly higher than that observed in rats given the basal diet (19/30). This was not considered test article-related as the higher incidence was not observed in the F1 generation rats and chronic progressive nephropathy is considered a background lesion of rats that has no relevance to other species (Hard and Khan, 2004).

### 3.2.6. Postnatal survival and developmental parameters

The mean number of pups born, live litter size, postnatal survival, and percentage of males at birth were unaffected by algal oil exposure. The mean number of pups born, live litter size, and postnatal survival in the basal diet control group were similar to the DHA fish oil group.

Pup clinical observations, body weights, and body weight gains were unaffected by DHA fish oil or algal oil exposure. Mean ages of attainment of balanopreputial separation and vaginal patency and mean body weights at the age of attainment were unaffected by algal oil or DHA fish oil exposure.

No algal oil or DHA fish oil-related effects were noted on FOB parameters, including home cage, handling, open field, sensory, neuromuscular, and physiological observations, in the DHA fish oil group and the 10,000, 25,000, and 50,000 ppm algal oil group F1 males and females. Locomotor activity in these groups was not affected by algal oil or DHA fish oil administration. In addition, no ophthalmic lesions indicative of toxicity were observed in the algal oil or DHA fish oil group F1 males and females.

### 3.2.7. F1 generation; clinical pathology-hematology and clinical chemistry

There were no adverse alterations in hematology, coagulation and urinalysis parameters that were considered related to treatment with the algal oil test article. However, some significant \( p < 0.05 \) differences were observed when the basal diet control and DHA fish oil groups were compared and were considered to be an effect of high oil administration. There were lower hemoglobin and mean corpuscular hemoglobin concentration values in DHA fish oil males that were similar to the values observed in the algal oil groups. In the DHA fish oil females, there were lower hematocrit and mean platelet values that were similar to or slightly lower than values in 50,000 ppm algal oil females.

There were no algal oil-related adverse changes in serum chemistry when compared to the DHA fish oil group. Lower cholesterol values were noted in the DHA fish oil male and female groups and lower triglyceride values were noted in DHA fish oil females when compared to the basal diet control group. This finding was considered related to the presence of n-3 long chain polyunsaturated fatty acids in the fish oil added to the diet and was not associated with hepatocellular changes. There were no other test article-related effects observed on serum chemistry parameters.

### 3.2.8. F1 generation; anatomic pathology

Higher final body weights and liver and kidney weight parameters (absolute, relative to body and brain weight) were observed in the DHA fish oil and all algal oil group males and females resulting from high lipid content in the diet when compared to the basal diet group. However, mean values, percent differences from the DHA fish oil group and statistical significance when compared to the DHA fish oil group were variable in all algal oil groups and were dependent on final body weight which varied in algal oil groups and did not show a clear dose response.

Some organ weight differences were statistically significant \( p < 0.05 \) or \( p < 0.01 \) when compared to the basal diet control and/or DHA fish oil group but were considered to be a result of a high lipid effects on final body weight. These included: lower brain weight parameters in DHA fish oil group males and females and all algal oil group males and females, lower right epididymis weights relative to body weight in DHA fish oil and all algal oil male groups, lower left and right testis weights relative to body weight in the DHA fish oil and all algal oil group males, higher heart and splenic weight parameters in the DHA fish oil and all algal oil males and groups, higher prostate weight parameters in the DHA fish oil and all algal oil group males and 50,000 ppm algal oil group females and higher prostate weight parameters in the DHA fish oil and 50,000 ppm algal oil group males.

### Table 2

F0 reproductive performance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exposure level (ppm)</th>
<th>HC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50,000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male mating index (%)</td>
<td>93.3</td>
<td>96.7</td>
</tr>
<tr>
<td>Female mating index (%)</td>
<td>93.3</td>
<td>96.7</td>
</tr>
<tr>
<td>Male fertility index (%)</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Female fertility index (%)</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Male copulation index (%)</td>
<td>96.4</td>
<td>93.1</td>
</tr>
<tr>
<td>Female conception index (%)</td>
<td>96.4</td>
<td>93.1</td>
</tr>
<tr>
<td>Estrous cycle length (days)</td>
<td>4.3</td>
<td>4.4</td>
</tr>
<tr>
<td>Pre-coital interval (days)</td>
<td>2.9</td>
<td>3.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> DHA fish oil group.

<sup>b</sup> Algal oil groups.

<sup>c</sup> WIL Research Laboratories historical control data; data presented as mean (range).
In the 10,000 ppm algal oil group, one male was found dead on PND 117 with a ductal adenocarcinoma in the ventral neck that was adjacent to the mandibular salivary gland. There was mild hemorrhage and pigmented macrophages in the alveolar lumens of the lung. The cause of death was a ductal adenocarcinoma of unknown origin and was not considered algal oil-related. A male in the basal diet control group was found dead on PND 117 with moderate hemorrhage and fibrinoid exudate at the base of the heart and surrounding the aorta. There was moderate hemorrhage in the alveolar lumens of the lung and minimal hemorrhage in the thymus. Hemorrhage in the heart and lungs corresponded to the red fluid in the thoracic cavity. The appearance of blood at the base of the heart and surrounding the aorta was suggestive of trauma.

Findings related to the presence of oil (DHA fish or algal derived) in the diet of the F1 generation were noted in the adrenal cortex of the DHA fish oil and 50,000 ppm algal oil group males, in the liver of the DHA fish oil and 10,000, 25,000, and 50,000 ppm algal oil group females, in the kidney of the DHA fish oil males and 50,000 ppm algal oil group males and females, and in the lungs of the DHA fish oil and 50,000 ppm algal oil group males and females (see Table 4).

There was a higher incidence and severity of adrenal cortical vacuolation in the DHA fish oil (incidence of 13/20 with 8 minimal and 5 mild) and 50,000 ppm algal oil (incidence of 16/20 with 12 minimal and 4 mild) group males when compared to the basal diet control group (incidence of 9/19, all minimal). The algal oil group had a slightly higher incidence than the DHA oil group. The cells in the adrenal cortex (zona fasciculata) of males were minimally to mildly vacuolated with well-delineated cytoplasmic vacuoles of varying sizes. This finding was only observed in one 50,000 ppm algal oil group female.

In the liver, there was an increase of minimal to mild cytoplasmic vacuolation of hepatocytes in the periportal region of the DHA fish oil and 50,000 ppm algal oil group females. The vacuoles were well-delineated and varied in size. Algal oil and DHA fish oil group males had a similar incidence to basal diet control group males. There was a higher incidence in DHA fish oil and algal oil group females when compared to basal diet control group females but the incidences were similar when DHA fish oil and algal oil group females were compared.

In the kidney, there was a slightly higher incidence of lesions consistent with chronic progressive nephropathy (basophilic tubules with or without basement membrane thickening, fibrosis and chronic inflammation) in the DHA fish oil group males (incidence of 17/20) and 50,000 ppm algal oil group males (incidence of 16/20) and females (incidence of 11/20) when compared to basal diet control groups (incidence of 12/19 males and 6/20 females). The incidences were similar when DHA fish oil algal oil groups were compared.

There was minimal to moderate fibroplasia of pancreatic islets in the basal diet control group, DHA fish oil group males, and 50,000 ppm group algal oil males and females. Islet fibroplasia was more prevalent and severe in males than in females and had a slightly higher incidence in the DHA fish oil (males) and/or algal oil groups (males and females).

4. Discussion

The studies were conducted to assess the developmental and reproductive safety of DHA-rich algal oil when fed to rats during pregnancy, lactation, and for 90 days post-weaning. The results of the studies indicate that administration of DHA-rich algal oil to rats either by gavage or in the diet did not produce evidence of developmental or reproductive toxicity under the conditions of the studies.

In the developmental toxicity study in rats, all animals in the control, 400, 1000, and 2000 mg/kg/day groups survived to the scheduled necropsy on gestation day 20. There were no test article-related clinical findings noted at any dosage level at the daily examinations or one hour following the daily dose. Mean maternal body weights, body weight gains, net body weights, net body weight gains, gravid uterine weights, and food consumption in all test article groups were similar to those in the control group. At the scheduled necropsy, no remarkable maternal macroscopic findings were noted at any dosage level. Intrauterine growth and survival were unaffected by maternal test article administration at any dosage level tested. Additionally, there were no test article-related external, visceral, and skeletal malformations or developmental variations observed for fetuses in the 400, 1000, or 2000 mg/kg/day groups. Based on the absence of maternal or developmental toxicity at any dosage level, a dosage level of 2000 mg/kg/day was considered to be the NOAEL for maternal toxicity and embryo/fetal development when DHA-rich algal oil was administered orally by gavage to pregnant Crl:CD(SD) rats once daily during gestation days 6–19. Hammond et al. (2001b) described a similar lack of maternal and developmental toxicity following administration of dried Schizochytrium sp. algae to rats at doses up to 22000 mg/kg/day (30%) in the diet during organogenesis.

In the 3-month rat dietary toxicity study with an in utero exposure phase, there was no F0 and F1 test article-related mortality at any exposure level. No adverse algal oil-related effects on mean F0 male and female and F1 male body weights, body weight gains, food consumption, and food efficiency were noted at any exposure level. In addition, no adverse algal oil-related effects on macroscopic, microscopic, and organ weights were noted for the F0 and F1 males and females and clinical pathology parameters for the F1 males and females at any exposure level. Therefore, the no-observed-adverse-effect level (NOAEL) for F0 male and female and F1 male systemic toxicity of algal oil was considered to be 50,000 ppm. Higher mean body weight, body weight gain, and food consumption were noted for the 50,000 ppm algal oil group F1 females throughout the generation and were considered to be a result of algal oil exposure. Therefore, the NOAEL for F1 female systemic toxicity of algal oil was considered to be 25,000 ppm. F0 reproductive performance values, estrous cycle length, gestation length, or the process of parturition, and the numbers of former implantation sites and unaccounted-for sites were unaffected by algal oil exposure. Based on these results, an exposure level of...
50,000 ppm was considered to be the NOAEL for F₀ reproductive toxicity of algal oil when administered continuously in the diet to Crl:CD(SD) rats. Postnatal survival and developmental parameters in the F₁ generation were unaffected by algal oil exposure at all dietary concentrations. Based on these results, an exposure concentration of 50,000 ppm was considered to be the NOAEL for neonatal toxicity of algal oil. There were no neurotoxic effects noted at any algal oil exposure level. Based on these results, an exposure level of 50,000 ppm was considered to be the NOAEL for F₁ developmental toxicity and neurotoxicity of algal oil when administered continuously in the diet to Crl:CD(SD) rats. The 50,000 ppm exposure level was equivalent to 3421 and 2339 mg/kg/day for F₀ males during pre-mating and after mating, respectively; 3558, 3117, and 7464 mg/kg/day for F₀ females during pre-mating, gestation, and lactation, respectively; and to 3526 and 4138 mg/kg/day for F₁ males and females, respectively. Burns et al. (1999) and Hammond et al. (2001c) describe a similar lack of developmental and reproductive toxicity following administration of algal-based test articles (dried algae and oils) from Schizochytrium sp. or C. cohnii.

The aforementioned toxicity studies were conducted as part of an investigation to examine the safety of DHA-rich algal oil ONC-T18 and confirm that it possesses a similar toxicity profile as exists for other currently marketed algal oils. The results obtained from these studies support the safety of DHA-rich algal oil for its proposed use in food.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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References