Review

A review of the safety of DHA45-oil

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Abstract

Polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), are natural constituents of the human diet; however, dietary intakes of these fatty acids are below recommended values. The main dietary source of DHA is fatty fish, with lesser amounts provided by shellfish, marine mammals, and organ meats. The addition to traditional food products of refined oils produced by marine microalgae represents potential sources of supplemental dietary DHA. DHA45-oil is manufactured through a multi-step fermentation and refining process using a non-toxigenic and non-pathogenic marine protist. Comprising approximately 45\% DHA, and lesser concentrations of palmitic acid and docosapentaenoic acid, DHA45-oil is intended for use in foods as a dietary source of DHA. The safety of DHA45-oil was evaluated in various genotoxicity and acute, subchronic, and reproductive toxicity studies. DHA45-oil produced negative results in genotoxicity assays and demonstrated a low acute oral toxicity in mice and rats. Dietary administration of DHA45-oil to rats in subchronic and one-generation reproductive studies produced results consistent with those observed in oral studies using high concentrations of \(\omega-3\) PUFAs from fish or other microalgal-derived oils. The results of these studies, as well as those of various published metabolic, toxicological, and clinical studies with DHA-containing oils, support the safety of DHA45-oil as a potential dietary source of DHA.

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Keywords: DHA45-oil; \(\omega-3\) Polyunsaturated fatty acid; Docosahexaenoic acid; DHA; Toxicity; Safety; Food ingredient; Marine protist

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Abbreviations: AA; arachidonic acid; ADP; adenosine diphosphate; AI; Adequate Intake; BNF; British Nutrition Foundation; DHA; docosahexaenoic acid; DPA; docosapentaenoic acid; EPA; eicosapentaenoic acid; F\textsubscript{0}; parental generation; FDA; Food and Drug Administration; GLP; good laboratory practice; GRAS; Generally Recognized as Safe; HDL; high-density lipoprotein; IOM; Institute of Medicine; ISSFAL; International Society for the Study of Fatty Acids and Lipids; LDL; low-density lipoprotein; NOAEL; no-observed-adverse-effects level; NOEL; no-observed-effects level; PUFA; Polyunsaturated fatty acid; TFPI; tissue factor pathway inhibitor; USDA; United States Department of Agriculture; VLDL; very low-density lipoprotein.

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1. Introduction

DHA45-oil is a refined, food grade oil of >95% triacylglycerols that is produced through a controlled multiprocess fermentation method and a standard edible oil refining method using a strain of the marine protist, Ulkenia sp. This microalgal species is a member of the non-pathogenic and non-toxigenic family of Thraustochytriaceae, and analytical testing of the oil confirms the absence of known algal toxins.

DHA45-oil contains approximately 45% (w/w) of the \(\omega-3\) fatty acid, docosahexaenoic acid (DHA) (22:6), and is intended for use in food as a source of DHA. The chemical structure of DHA is presented in Fig. 1. The remaining fatty acids of DHA45-oil comprise mainly palmitic acid (16:0) (~35%) and lesser amounts of the \(\omega-6\) fatty acid, docosapentaenoic acid (DPA) (22:5) (~11%). DHA45-oil contains a very low amount of unsaponifiable material (<0.9%) as sterols. To ensure stability, the formulated oil contains a suitable antioxidant (e.g., mixed tocopherols, or alternative), as permitted by the United States Food and Drug Administration (FDA) for use in edible oils. DHA45-oil meets food grade and quality control specifications that are suitable for a refined edible oil intended for use as a food ingredient.

Polyunsaturated fatty acids (PUFAs), including DHA and also DPA and eicosapentaenoic acid (EPA) (20:5), are natural constituents of the human diet. The main dietary source of these PUFAs is fish, particularly fatty fish such as haddock, tuna, salmon, and mackerel, in which concentrations of total \(\omega-3\) fatty acids range from 0.1 to 5.3 g/100 g (Sanders, 1989; Ascherio et al., 1995; Kris-Etherton et al., 2000). Other dietary sources of PUFAs include shellfish, marine mammals, and organ meats (Connor, 1997). Similarly, palmitic acid is a natural component of the diet, occurring mainly in meat, poultry, fish, grain products, and milk and milk products (Jonnalagadda et al., 1995).

In humans, DHA occurs naturally as a cell membrane fatty acid in the brain, retina, testes, and sperm, and has been reported to be essential in the development of these organs and cells (Neuringer et al., 1988; Hsia et al., 1989; Linder, 1991; Linscheer and Vergroesen, 1994; Krummel, 1996). In addition to dietary sources, DHA may be derived endogenously through desaturation and elongation of the dietary precursor essential fatty acid, \(\alpha\)-linolenic acid (18:3) (Linder, 1991; Alvarez et al., 1994).

2. Uses in food

The consumption of \(\omega-3\) fatty acids in the United States is approximately 1.6 g/day, predominantly as \(\alpha\)-linolenic acid, but also including DHA, DPA, and EPA (Dolecek and Grandits, 1991; Kris-Etherton et al., 2000). Of the total amount of \(\omega-3\) fatty acids consumed per person/day in the United States, the intake of DHA was estimated to be 0.1–0.2 g/day (Dolecek and Grandits, 1991; Kris-Etherton et al., 2000). The consumption of DHA and other \(\omega-3\) fatty acids is considerably higher in populations such as Japan, Norway, South Africa, and the Portuguese Island of Madeira, who tend to derive a greater proportion of their diet from marine sources, with reported dietary intakes of DHA in the range of 0.5–0.7 g/day (Bonaa et al., 1992; Schloss et al., 1997; Fluge et al., 1998; Johansson et al., 1998; Sugano and Hirahara, 2000; Torres et al., 2000). Very high intakes of \(\omega-3\) fatty acids occur in Greenland Eskimo populations, who have been reported to consume 5–10 g \(\omega-3\) PUFAs/day from traditional marine-based diets (Bang et al., 1976).

Recommendations for daily intakes of \(\omega-3\) PUFAs have been published by several international scientific authorities. Nutrition recommendations published by Health and Welfare Canada provide a recommended daily intake of 1.0–1.8 g \(\omega-3\) PUFAs/day, although differentiation between the individual \(\omega-3\) PUFAs was not

![Fig. 1. Docosahexaenoic acid (DHA) (22:6).](Image)
identified (Health and Welfare Canada, 1990). The International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommended Adequate Intakes (AIs) of a minimum of 0.22 g/day each for DHA and EPA, and 0.65 g/day for DHA and EPA combined (ISSFAL, 1999), while the British Nutrition Foundation (BNF) has recommended a desirable population intake of 1.1 g (females) and 1.4 g (males) of DHA and EPA/day (BNF, 2000). In the United States, the Institute of Medicine (IOM) published a recommended AI of 0.5 g ω-3 PUFAs (including DHA)/day for infants (IOM, 2002). Although no similar AI for total ω-3 PUFAs was identified for adults, the IOM provided recommended AIs of 1.6 and 1.1 g α-linolenic acid/day for men and women, respectively and stated that “up to (10) percent (EPA and DHA) can contribute towards the AI for α-linolenic acid”, equivalent to 0.16 and 0.11 g EPA and DHA/day for men and women, respectively.

Various traditional food products on the market in the United States now incorporate DHA-containing oil ingredients. For example, menhaden oil, a refined DHA-containing oil derived from menhaden fish, was affirmed as Generally Recognized as Safe (GRAS) by the FDA for use as a direct human food ingredient with specific limitations in traditional food products including breads, cereals, fats and oils, condiments, yogurt, and cheese, frozen dairy, meat, egg, nut, and fish products (US FDA, 1989, 1997). In their GRAS affirmation, the FDA concluded that consumption of up to 3 g/day of combined DHA and EPA in menhaden oil is safe (US FDA, 1997). Subsequent to the submission of a GRAS notification in 2000 for the use of DHASCO oil, a microalgal-derived DHA-containing oil, in infant formula products, of which the agency had no questions concerning the notifier’s GRAS determination, two manufacturers of infant food products announced plans to position infant formulas containing DHASCO oil on the US market (US FDA, 2001). Additionally, ω-3 fatty acids, such as EPA and DHA (including DHA derived from marine algae), are sold as dietary supplement products in the United States. The fatty acids present in DHA45-oil are similar to those of fish and microalgal-derived oils already consumed by the human population.

DHA45-oil is intended for use in the United States as a nutritional food ingredient as a dietary source of DHA at levels consistent with reported recommended intakes for ω-3 PUFAs (Health and Welfare Canada, 1990; ISSFAL, 1999; BNF, 2000) in a variety of food products such as baked goods and baking mixes, breakfast cereals, fats and oils, cheeses, frozen dairy desserts, grain and plant protein products, meat products, milk products, processed fruits and fruit juices, snack products, and soups and soup mixes.

Using the United States Department of Agriculture (USDA) 1994–1996 Continuing Survey of Food Intakes by Individuals (USDA CSF I, 1994–1996) and the 1998 Supplemental Children’s Survey (USDA CSF II, 1998) (USDA, 2000), and assuming that DHA45-oil will be used in all anticipated applications at maximum proposed levels of use, the consumption of DHA45-oil from the intended uses in food was estimated by means of accepted FDA methodology. On an all user basis, the mean and 90th percentile intakes of DHA45-oil by the total population from all proposed food uses were estimated to be 1.3 and 3.0 g/person/day, respectively, or 27.3 and 57.1 mg/kg body weight/day, respectively. Considering that DHA45-oil comprises approximately 45% DHA, the mean and 90th percentile intakes of DHA (all-user basis) were estimated to be 0.7 and 1.5 g/person/day, respectively, or 13.6 and 28.6 mg DHA/kg body weight/day, respectively.

3. Metabolic fate of DHA

DHA is present in DHA45-oil in triglyceride form, the predominant form of dietary fats (Linder, 1991). In general, dietary triglycerides undergo enzymatic hydrolysis in the upper intestine to free fatty acids and 2-mono- and triglycerides, which are integrated into bile acid micelles for diffusion into the interior of the intestinal epithelial cells and subsequent incorporation into new or reconstituted triglycerides (Linder, 1991; Linscheer and Vergroesen, 1994; Guyton and Hall, 1996). In the form of chylomicrons, these reconstructed triglycerides enter the lymph for transport to the blood for distribution and incorporation into plasma lipids, erythrocyte membranes, platelets, and adipose tissue. The chylomicron-contained triglycerides are hydrolyzed by lipoprotein lipase during passage through the capillaries of adipose tissue and the liver, thus releasing free fatty acids to the tissues for metabolism or for cellular uptake, with subsequent reesterification into triglycerides and phospholipids for storage as energy or as structural components of cell membranes (Linder, 1991; Krummel, 1996).

The levels of fatty acids in the body are regulated by processes in the liver, with the hydrolysis of triglycerides to fatty acids being stimulated by hormone-sensitive lipase. Free fatty acids and glycerol subsequently released into the bloodstream are transported to tissues for use as a source of energy (Linscheer and Vergroesen, 1994; Krummel, 1996). The metabolism of fatty acids occurs in the mitochondria following their transport across the mitochondrial membrane in the form of acyl carnitine (Linscheer and Vergroesen, 1994). Fatty acids are metabolized predominantly via β-oxidation, a process that involves a shortening of the fatty acid carbon chain and the production of acetic acid and acetyl CoA, which combines with oxaloacetic acid and enters the citric acid cycle for energy production (Krummel, 1996).

The degree of transport of fatty acids across the mitochondrial membrane is contingent upon the length
of the carbon chain, with fatty acids of 20 carbons or more being transported into the mitochondria to a lesser degree than shorter chain fatty acids. Therefore, long chain fatty acids, such as DHA, might not undergo mitochondrial β-oxidation to the same extent, but alternatively may be metabolized via carnitine-independent peroxisomal β-oxidation to shorter-chained fatty acids. The resultant shorter chain fatty acids may undergo further peroxisomal degradation or transport into the mitochondria for oxidative metabolism (Hsia et al., 1994; Pe´richon and Bourre, 1995; Retterstøl et al., 1989; Willumsen et al., 1993; Linscheer and Vergroesen, 1994; Périchon and Bourre, 1995; Retterstøl et al., 2000).

A minor peroxisomal metabolic pathway for DHA (~1.4%) includes retroconversion via DPA (ω-3) to EPA via β-oxidation, the auxiliary enzymes Δ4 enoyl CoA reductase and Δ3, Δ2 enoyl CoA isomerase, and removal of 2 carbon units from the carboxyl end of the fatty acid (Rosenthal et al., 1991; Achard et al., 1995; Brossard et al., 1996). Retroconversion of DHA reportedly occurs primarily in the liver, although some retroconversion has been suggested to occur in the intestinal mucosa during the incorporation of triglycerides into chylomicrons (Brossard et al., 1996).

4. Toxicological studies of DHA45-oil

The safety of DHA45-oil derived from the marine protist, Ulkenia sp., was evaluated in various genotoxicity and acute, subchronic, and reproductive toxicity studies. Additional toxicological studies were performed using the marine protist itself. These studies are summarized below.

4.1. Genotoxicity studies

DHA45-oil was evaluated in several in vitro genetic toxicity assays. Using Salmonella typhimurium strains TA97, TA98, TA100, and TA102, Fuji and Suwa [1998a (unpublished)] investigated the potential mutagenicity of DHA45-oil in the Ames assay at concentrations of 0.5–5 mg DHA45-oil/plate, in the presence or absence of the S9 fraction from the livers of Aroclor-induced rats as a metabolic activation system. Similarly, Bruijntjes-Rozier and van Ommen [2001 (unpublished)] evaluated the potential mutagenicity of DHA45-oil in S. typhimurium strains TA98, TA100, TA1535, and TA1537 and in Escherichia coli WP2 uvrA at concentrations of 0.06–5 mg DHA45-oil/plate, with or without metabolic activation. DHA45-oil produced negative results for all assays.

Additionally, at concentrations of 1.25, 2.5, or 5 mg DHA45-oil/ml, the ability of DHA45-oil to induce chromosomal aberrations was evaluated using Chinese hamster fibroblast cells, with or without metabolic activation (Kashima and Sarwar, 2000, unpublished). DHA45-oil did not induce chromosome aberrations under the conditions of this study.

4.2. Acute toxicity studies

Following a 14-day post-dosing observation period and subsequent necropsy, no adverse effects on clinical parameters, body weight gains, or macroscopic necropsy observations (observed tissues not reported) were reported in 5 ICR male mice administered a single gavage dose of 2000 mg DHA45-oil/kg body weight (Fujii and Suwa, 1998b, unpublished). Under a similar study protocol, no adverse effects were reported in 10 Sprague-Dawley [Crj/CD(SD)IGS] rats (five rats/sex), with the exception of watery diarrhea in two male rats at 6 h post-administration, following single gavage dose administration of 2000 mg DHA45-oil/kg body weight (Neda, 2000a, unpublished).

4.3. Subchronic toxicity study

The subchronic toxicity of DHA45-oil (99.9% purity) was evaluated in relation to a fish oil containing 27% DHA (99.7% purity) or distilled water in a good laboratory practice (GLP)-compliant 90-day rat study with a separate 4-week recovery phase (Neda, 2000b, unpublished). Groups of 30 Sprague-Dawley Crj:CD (SD) IGS rats (15/sex/group) were administered distilled water (control) or various combinations of DHA45-oil/fish oil by daily oral gavage for a period of 90 days. The oils were administered in DHA45-oil/fish oil combinations of 0/2000 (Group 1), 500/1500 (Group 2), 1000/1000 (Group 3) or 2000/0 (Group 4) mg/kg body weight/day. Using the total DHA content of the oils, these dosing combinations provided daily doses of 540, 630, 720, and 900 mg DHA/kg body weight, respectively. A summary of the dosing regimens and corresponding doses of DHA is presented in Table 1.

All animals received the same basal diet of AIN-76A compound feed, containing 55.0% corn starch, 20.0% casein, 10.0% sucrose, 5.0% cellulose powder, 5.0% corn oil, 3.5% AIN-76A mineral mix, 1.0% AIN-76A vitamin mix [% vitamin E not specified], 0.3% DL-methionine, and 0.2% choline bitartrate. Total exposure of controls and treated groups to vitamin E could not be determined from the data presented. The effects of treatment with DHA45-oil on mortality and clinical signs, neurologic responses, body weight gain, food and water consumption, hematology, clinical chemistry, urinalysis, and on the results of ophthalmology and gross pathology and histopathology (on controls and Groups 1 and 4 only) examinations were evaluated. For the 4-week recovery phase, additional groups of 10 rats (5/sex/group) were administered distilled water or 2000 mg DHA45-oil or fish oil/kg body weight/day for a period
of 90 days and were allowed a 4-week period of recovery (see Table 1).

There were no biologically significant differences in the measured parameters between the DHA45-oil- and fish oil-treated groups. Compared with the water control group, treatment with DHA45-oil alone (2000 mg/kg body weight/day) or in combination with fish oil was reported not to produce biologically significant effects on mortality, clinical signs, responses to stimuli, food and water consumption, or ophthalmology examinations. Relative to the water controls, increased or a tendency for increased body weights were reported for males in Groups 1 and 2 and for all oil-treated females during the 90-day treatment period, as well as for both oil-treated groups over the 4-week recovery period.

Compared with the water control group, increased lymphocyte and decreased neutrophil ratios were reported in males in Groups 1 and 4, and decreased red blood cell counts were reported in females in Groups 1 and 3; however, white blood cell counts were not affected. At the end of the recovery period, prolonged prothrombin times were reported in males in the fish oil group and reduced activated partial thromboplastin times were reported in females in both oil-treated groups compared to the water controls. In addition to alterations in serum lipid profiles in the oil-treated animals compared with water controls, differences in various clinical chemistry parameters (i.e., increased serum alkaline phosphatase, albumin/globulin ratio, and albumin fraction ratio in males of all treatment groups; altered blood urea nitrogen levels in males of Groups 2 and 4; and decreased bilirubin levels in all oil-treated females) were reported; however, white blood cell counts were not affected. At the end of the recovery period, prolonged prothrombin times were reported in males in the fish oil group and reduced activated partial thromboplastin times were reported in females in both oil-treated groups compared to the water controls. In addition to alterations in serum lipid profiles in the oil-treated animals compared with water controls, differences in various clinical chemistry parameters (i.e., increased serum alkaline phosphatase, albumin/globulin ratio, and albumin fraction ratio in males of all treatment groups; altered blood urea nitrogen levels in males of Groups 2 and 4; and decreased bilirubin levels in all oil-treated females) were reported; however, these effects generally disappeared during the treatment period. Following the 90-day treatment period, urinalysis results of oil-treated males revealed decreased sodium (all groups) and potassium (groups 3 and 4) excretion compared with the water controls, with similar results in the DHA45-oil-treated females at the end of the recovery period.

Increased relative liver weights were reported in animals in Groups 3 and 4 at necropsy, which were likely the result of the high exposure to PUFAs. In addition, there were other reported non-dose-related increases in the absolute weights, and in some cases, relative weights, of several organs including the spleen, kidneys, and adrenals. Histopathological lesions of the liver and other organs were not observed, and there were no changes in enzymes indicative of liver toxicity.

### 4.4. One-generation reproductive toxicity study

To investigate the potential in utero toxicity of DHA45-oil, Kuilman and Waalkens-Berendsen (2001, unpublished) conducted a one-generation GLP-compliant study in Wistar (Crl:WI)WU BR) rats. For a period of 10 weeks prior to mating, groups of rats (28/ sex/group) were provided DHA45-oil in the diet at concentrations of 0 (control), 1.5, 3.0, or 7.5%. The control group was administered corn oil at a level of 7.5% in the diet. The vitamin E content of the basal diet (prior to mixing with the test substances) was 133 ppm (w/w), providing a total dose of about 2 mg vitamin E/ day (approximately 5–7 mg/kg body weight/day). The estimated total range of intake of DHA45-oil and corresponding DHA exposures per group throughout the different phases of the study period are presented in Table 2.

A successful mating procedures were allowed to give birth. On postpartum Day 4, all pups were examined for abnormalities, and litters of more than eight pups were culled to four male and four female pups, as feasible. On postnatal Day 21, all pups were weaned, examined for gross external abnormalities and necropsied, and parental (F₀) females were then necropsied, or, in instances where no litters were produced, necropsied after the

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Number animals/group</th>
<th>Dose of DHA45-oil (mg/kg bw/day)</th>
<th>Dose of fish oil (mg/kg bw/day)</th>
<th>Total dose of DHA (mg/kg bw/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90-day dosing period only</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control⁵</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0</td>
<td>2000</td>
<td>540</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>500</td>
<td>1500</td>
<td>630</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>1000</td>
<td>1000</td>
<td>720</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>2000</td>
<td>0</td>
<td>900</td>
</tr>
<tr>
<td>90-day dosing period and additional 4-week recovery period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control⁵</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
<td>2000</td>
<td>540</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>2000</td>
<td>0</td>
<td>900</td>
</tr>
</tbody>
</table>

⁴ Groups comprised 50% male and 50% female rats.

⁵ The total dose of DHA was calculated using the amount of DHA present in both the DHA45-oil (45% DHA) and the fish oil (27% DHA).

⁶ Controls received distilled water.
presumed gestation period had elapsed. Evaluations were made of mortality, clinical signs, body weights, food consumption, fertility and reproductive performance, litter size, malformed pups, pup weights, and gross pathology and histology of dead or stillborn pups and all F0 animals.

Treatment with DHA45-oil did not produce effects on mortality or clinical signs. In the high-dose group (7.5% DHA45-oil), significant increases in body weight gains were reported for both males (from Day 14 onwards) and females (from Day 42 and through gestation and lactation), while sporadic increases in body weights were reported in the low- (1.5% DHA45-oil) and mid-dose (3.0% DHA45-oil) females through gestation and lactation. Increased food consumption was reported in females in the low- and mid-dose groups during pre-mating and during part of the gestation period (Day 14 through 21) and lactation, and may account for the observed sporadic increases in body weights of these groups. Reduced food consumption compared to controls was reported for the high-dose females during gestation Days 14 through 21 and during the lactation period without a decrease in body weights.

With respect to reproductive parameters, treatment with DHA45 oil had no effect on pre-coital time, mating index, fertility indices, fecundity, gestation index and duration, or numbers of stillborn pups and post-implantation losses. Additionally, there were no adverse effects of treatment on the numbers of pups, pups/litter, pup mortality, sex ratio, or pup weight. There was no evidence of a compound-related effect on the incidence of malformations.

At necropsy of the parental animals, absolute liver weights were increased in mid- and high-dose males, and in low- and high-dose females, and relative liver weights were increased in the mid-dose males; however, these increases were not accompanied by gross or histopathological correlates. Absolute spleen weights were dose-dependently increased in both sexes, attaining statistical significance in mid- and high-dose animals of each sex, and relative spleen weights were increased in treated males and in mid- and high-dose females. These increased spleen weights were associated with an increased incidence and/or severity of extramedullary hematopoiesis (see Table 3).

The only significant gross pathology was the presence of “yellow spots” within the abdominal adipose tissue of a few high-dose males and in many of the high-dose females. This was reported to be compatible with findings associated with “yellow fat disease”, which microscopically is characterized by accretion of lipofuscin pigment (indigestible final product of oxidized unsaturated fatty acids), degeneration of adipose cells (steatosis), and inflammation of adipose tissue (steatitis) (Danse and Steenbergen-Botterweg, 1976; Danse and Verschuren, 1978a; Danse et al., 1979). Yellow fat disease is a condition known to occur in certain species of animals in response to a large load of dietary lipids (i.e., consumption of diets rich in ω-3 PUFA) in combination with a vitamin E deficient state (Green and Bunyan, 1969; Helgebostad and Ender, 1973; Danse and Verschuren, 1978a,b; Danse, 1989; Verschuren et al., 1990; Pollard and Sanders, 1993; Farwer et al., 1994; Muggli, 1994; Ando et al., 2000). Microscopic evaluation of

### Table 2
Estimated daily intake of DHA45-oil and corresponding DHA intake by rats in the one-generation reproduction study

<table>
<thead>
<tr>
<th>Sex (Phase)</th>
<th>Low-dose group (1.5% DHA45-oil)</th>
<th>Mid-dose group (3.0% DHA45-oil)</th>
<th>High-dose group (7.5% DHA45-oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Estimated intake of DHA45-oil (mg/kg bw/day)</td>
<td>Estimated intake of DHA (mg/kg bw/day)</td>
<td></td>
</tr>
<tr>
<td>Females (pre-mating)</td>
<td>800–1000</td>
<td>1500–2000</td>
<td>3400–4700</td>
</tr>
<tr>
<td>Females (gestation and lactation days 1–14)</td>
<td>1800–2200</td>
<td>3400–4300</td>
<td>7900–9700</td>
</tr>
<tr>
<td>Females (gestation and lactation days 1–14)</td>
<td>1800–2700</td>
<td>3700–5300</td>
<td>7800–11,200</td>
</tr>
</tbody>
</table>

### Table 3
Incidence of extramedullary hematopoiesis of the spleen in the one-generation rat reproduction study of DHA45-oil

<table>
<thead>
<tr>
<th>Severity grade</th>
<th>Males (% DHA in the diet)</th>
<th>Females (% DHA in the diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>Very slight</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Slight</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Dosage values for DHA were calculated by multiplying the dosage values for DHA45-oil by 0.45.
these yellow spots yielded the diagnosis of lipogranuloma, and lipogranulomas also were identified in the abdominal adipose tissue of the low- and mid-dose groups upon microscopic evaluation. The incidences of “yellow spots” identified at necropsy and of lipogranuloma diagnosed histopathologically are presented in Table 4. The study authors suggested that the presence of the lipogranulomas, indicative of ongoing inflammatory responses, was associated with the extramedullary hematopoiesis of the spleen, a common response to inflammation (Losco, 1992).

The reproduction no-observed-effects level (NOEL) was identified as 7.5% DHA45-oil in the diet, the highest dose tested. For parental animals, due to the increased severity of extramedullary hematopoiesis and a greater incidence of lipogranuloma at the lowest tested concentration of 1.5% DHA45-oil in the diet, a NOEL was not identified.

4.5. Toxicological studies of the source organism

Using S. typhimurium strains TA97, TA98, TA100, and TA102, Ulkenia sp. at concentrations of 0.5–5 mg/plate was reported to produce negative results in the Ames assay, with or without metabolic activation (Fujii and Suwa, 1999, unpublished).

In a short-term oral toxicity study, Ulkenia sp. suspended in distilled water was administered by gavage to five ICR male mice (SPR) at a dose of 2000 mg Ulkenia sp./kg body weight/day for a period of 14 days (Celenese Ventures, 1999, unpublished). A control group of five mice received gavage administration of distilled water only. No significant differences in clinical signs, body weight gains, or autopic observations were reported for the Ulkenia sp.-treated mice compared to the control group.

5. Toxicological studies of related oils

Several toxicological studies of DHA-containing fish and microalgal-derived oils have been conducted in various species of laboratory animals, including mice, rats, and pigs (Danse and Verschuren, 1978a; Ruiter et al., 1978; Willumsen et al., 1993; Boswell et al., 1996; Hempenius et al., 1997, 2000; Wibert et al., 1997; Rabban et al., 1999; Arterburn et al., 2000a,b; Oarada et al., 2000; Hammond et al., 2001a,b). Overall, the results of these toxicological studies indicate that administration of DHA-containing fish or microalgal-derived oils, by gavage or via the diet, does not produce adverse effects on mortality, body weight gains, food consumption, or clinical observations.

Similar to the effects observed in the one-generation reproductive toxicity study conducted by Kuilman and Waalkens-Berendsen (2001, unpublished) in Wistar rats, yellow fat disease has been reported to occur naturally in wildlife and domestic species as well as in toxicological studies of rats, rabbits, mink, and pigs administered diets rich in ω-3 PUFAs (i.e., ω-3 PUFA-containing oils at dietary concentrations of 12–19%) in combination with a vitamin E deficient state (Jones et al., 1969; Helgebostad and Ender, 1973; Danse and Steenbergen-Botterweg, 1978; Danse and Verschuren, 1978a; Ruiter et al., 1978; Danse et al., 1979; Charnock et al., 1987; Hempenius et al., 1997, 2000; Verschuren et al., 1990; Farwer et al., 1994). While increased liver and spleen weights were reported in some studies in mice and rats administered diets containing levels of PUFAs of 25–9500 mg/kg body weight/day for periods of 4–13 weeks (Danse and Verschuren, 1978a; Boswell et al., 1996; Hempenius et al., 1997, 2000; McGuire et al., 1997; Burns et al., 1999; Rabban et al., 1999; Oarada et al., 2000), no histopathological correlates were observed in these organs, and the increased liver and spleen weights were reported to be adaptations to accommodate the large load of dietary lipids.

The fatty acid composition of DHA45-oil includes a minor amount (<15%) of DPA (ω-6), which, similar to DHA, is a component of fish and microalgal-derived oils. Hence the safety of these DPA isomers is supported by the various existing toxicological studies of these oils. Of these numerous toxicological studies, Hammond et al. (2001a–c) conducted a series of subchronic, reproductive, and developmental toxicity studies in rats and rabbits in which the dietary doses of DPA (as a
component of the administered microalgal-derived oil) were reported to be up to 630 mg/kg body weight/day. The authors reported no compound-related effects on survival, clinical observations, body weight gains, food consumption, urinalysis measurements, hematological parameters, or gross necropsy or histopathological findings (including spleen weights and histopathological appearance of the spleen and adipose tissue), and overall, no effects on reproductive or developmental parameters in either species. In a 6-week feeding study, no effects on final body weight, absolute liver and testes weights, plasma and testes zinc levels, or spermatid content of the testis parenchyma were reported following dietary administration of up to 17 mg DPA/kg body weight/day to male rats (Chanmugam et al., 1984).

6. Clinical studies related to the safety of DHA45-oil

In 1993, following submission of a petition by the National Fish Meal and Oil Association seeking affirmation of the GRAS status for the use of menhaden oil and partially hydrogenated menhaden oil as direct human food ingredients, the FDA identified three possible adverse effects associated with human consumption of ω-3 PUFAs: (1) reduced platelet aggregation; (2) increased low-density lipoprotein (LDL) cholesterol levels; and (3) reduced glycemic control among diabetics [58 FR 2682 (6 January 1993)] (US FDA, 1993). Subsequently, in 1997, following a thorough evaluation of several published clinical trials, the FDA affirmed that menhaden oil is GRAS under certain specified conditions of intended use (21 CFR §184.1472), and concluded that consumption of up to 3 g DHA + EPA combined/person/day does not pose a “significant risk for increased bleeding time”, has no “clinically significant effect on glycemic control”, and is “safe with respect to the effect on LDL cholesterol” (US FDA, 1997). In affirming the GRAS status of menhaden oil for use in food, the FDA concluded that consumption of up to 3 g/day of combined DHA and EPA in menhaden oil is safe (US FDA, 1997). In addition, the FDA published a letter regarding the dietary supplement qualified health claim for ω-3 fatty acids and heart disease, stating that the use of EPA and DHA ω-3 fatty acids, including DHA from marine algae, as dietary supplements is safe (US FDA, 2000).

Numerous clinical trials, varying in length from 1 week to greater than 1 year, have been conducted on DHA-containing fish and marine oils. The parameters evaluated in these studies included those identified by the FDA as possibly being affected by dietary ω-3 PUFAs (i.e., LDL cholesterol levels, glycemic control, bleeding time, platelet aggregation, and/or other hemostatic parameters). A comprehensive review of relevant clinical trials published up to 2001 indicated that DHA, provided in fish or marine-derived oils alone or in combination with DPA and/or EPA, at intakes up to 6 g DHA/person/day, would not produce significant adverse effects on these parameters. These results are consistent with the earlier conclusions of the FDA that consumption of up to 3 g DHA + EPA/day is safe.

Of the numerous clinical trials with ω-3 PUFAs, several studies were identified that evaluated the possible effects on these parameters following treatment with DHA only. Cirillo et al. (1994) provided 2.12 g DHA (ethyl ester form)/day to 10 healthy subjects (eight males, two females) for a period of 4 weeks with no reported effects on bleeding time or platelet adhesion. Collagen—but not adenosine phosphate (ADP)-induced platelet aggregation was reported to be reduced; however, the clinical significance of these results in the absence of increased bleeding times is uncertain. In 12 vegetarian subjects (six males, six females), 6 weeks of dietary supplementation with DHA oil providing 1.62 g DHA/day was reported not to produce significant effects on platelet aggregation or other hemostatic factors, such as fibrinogen levels or coagulation factor VII activity, however, bleeding times were not evaluated (Conquer and Holub, 1996). In a 90-day clinical trial, dietary supplementation of 6 g marine algal-derived DHA/day was reported not to affect bleeding times or platelet aggregation in six healthy males (Nelson et al., 1997). In a study by Ågren et al. (1997), healthy males (number subjects/group not reported) received either a control diet or a diet containing fish or fish oil for a period of 15 weeks, providing intakes of 0, 1.05, and 2.28 g DHA + EPA/day, respectively. During the same study period, an additional group of healthy males received a diet containing DHA oil, which provided a level of 1.68 g DHA/day. No significant effects on platelet aggregation were reported in males provided the DHA-only diet; however, decreased collagen—but not ADP-induced platelet aggregation was reported for males in the fish and fish oil groups. Subjects receiving the fish diet were reported to have decreased coagulation factor V activity. No significant effects on tissue factor pathway inhibitor (TFPI), prothrombin fragment 1 + 2, fibrinogen levels, or coagulation factor VII activity were reported for any of the diet groups.

In one trial designed to examine the possible effects of DHA on glycemic control, treatment with DHA oil providing 4 g DHA/day was reported to produce an increase in fasting insulin but no effects on blood glucose levels; however, blood glucose levels were measured only at two single time points (Mori et al., 2000).

Omega-3 fatty acids have been reported to contribute to a lowering of serum triglyceride and very low-density lipoprotein (VLDL) levels by decreasing the hepatic synthesis of these lipids (Sanders, 1989; Krummel, 1996). In 26 subjects with combined hyperlipidemia,
Davidson et al. (1997) reported a trend toward greater elevation of LDL cholesterol with increasing dose of DHA. Subjects receiving 2.5 g DHA/day were reported to experience a 13.6% increase in LDL cholesterol levels, while no significant changes in LDL cholesterol from baseline in a placebo group (receiving vegetable oil), or in a treatment group receiving 1.25 g DHA/day were observed. Decreased triglyceride levels and increased high-density lipoprotein (HDL) cholesterol levels were reported in both treatment groups. In mildly hyperlipidemic and obese subjects, 4 g DHA/day was reported to produce an 8% increase in LDL cholesterol levels, accompanied by a decrease in triglyceride and an increase in HDL2 cholesterol levels and adjusted LDL particle size (Mori et al., 2000). The observed increase in LDL cholesterol levels in patients with abnormal lipid levels was suggested to be the result of conversion of small VLDL to LDL with a concomitant decrease in large VLDL. In healthy subjects, dietary supplementation with up to 9.9 g DHA/day for periods of up to 13 weeks was well tolerated and was reported not to produce significant effects on LDL cholesterol levels (Conquer and Holub, 1996, 1998; Hamazaki et al., 1996; Innis and Hansen, 1996; Grimsgaard et al., 1997, 1998; Nelson et al., 1997; Hansen et al., 1998). Interestingly, consumption of DHA-enriched animal products (i.e., milk, eggs, chicken, and pork) was reported not to affect cholesterol levels in 14-year-old boys, and was reported to decrease cholesterol levels, including LDL cholesterol, in 20-year-old women (Horrocks and Yeo, 1999).

Although no clinical studies were identified that evaluated the possible effects of DPA alone, six studies of fish or marine-derived oils with reported DPA contents were reviewed and provide supporting evidence for the safety of dietary DPA as a component of DHA45-oil. In 12 hyperlipidemic patients (10 males, 2 females) receiving 0.42 or 0.84 ml DPA/day (approximately 0.39 and 0.78 mg, respectively)1 for a period of 4 weeks, LDL cholesterol levels were decreased by 2% and no significant effects on glycemic control were reported (Haglund et al., 1998). No significant effects on glycemic control or LDL cholesterol levels were reported in healthy subjects or diabetic patients receiving supplemental fish oil providing up to 800 mg DPA/day for periods of 6 weeks to 9 months (approximately 13 mg DPA/kg body weight/day for an average 60 kg individual) (Schmidt et al., 1992; Axelrod et al., 1994; McManus et al., 1996; Goh et al., 1997; Conquer et al., 1999). Decreased collagen-induced platelet aggregation was reported in 18 diabetic patients receiving fish oil providing 200 mg DPA/day for 6 weeks; however, ADP-induced aggregation was not affected (Axelrod et al., 1994). Increased bleeding times and decreased von Willebrand factor were reported in 24 healthy subjects (10 males, 14 females) consuming fish oil providing approximately 210 mg DPA/person/day for a period of 9 months (Schmidt et al., 1992). Although bleeding times were increased, fibrinogen levels also were increased and there was no associated reduction in platelet aggregability; thus, the clinical significance of the increased bleeding times is not clear. Additionally, due to the various ω-3 and ω-6 fatty acids comprising the fatty acid content of fish oils, the effects reported by Schmidt et al. (1992) and Axelrod et al. (1994) cannot be attributed to a single PUFA, such as DPA.

7. Discussion

Long-chain ω-3 PUFAs, including DHA, are natural components of the diet in various food products; however, dietary intakes of these fatty acids are generally below recommended values. The safety of dietary DHA is well established in the literature based on the historical consumption of fish and fish- and marine-based foods. In addition to an abundance of DHA-containing dietary supplement products, there exist on the US market various traditional food products that contain DHA-containing oil ingredients. The only notable differentiation regarding DHA45-oil as compared with other DHA-containing products already on the market is the production source. DHA45-oil is derived from a fermentation process using Ulkenia sp., a thraustochytrid microalgae and member of the kingdom Chromista (Bahnweg, 1979; Cavalier-Smith et al., 1994; Honda et al., 1999; European Register of Marine Species, 2001). Although there are no data demonstrating detailed human consumption of Ulkenia sp., thraustochytrids have been identified in plankton and other marine invertebrates and comprise a portion of the diet of filter-feeding invertebrates, thereby constituting an indirect component of the human diet through consumption of fish and other marine animals (Ulken et al., 1990; Sathe-Pathak et al., 1993; Azevedo and Corral, 1997; Naganuma et al., 1998). In addition, the fatty acids components of DHA45-oil are common to those of fish and microagal-derived oils already consumed in the market.

The negative results produced by DHA45-oil in both the Ames and chromosomal aberration assays indicate that this oil lacks potential for genotoxicity (Fujii and Suwa, 1998a, unpublished; Kashima and Sarwar, 2000, unpublished; Bruijnjes-Rozier and van Ommen, 2001, unpublished). DHA45-oil is not acutely toxic and did not produce adverse effects on clinical parameters, body weight gains, or autopic observations in mice or rats (Fujii and Suwa, 1998b, unpublished; Neda, 2000a, unpublished).

The results of the 90-day subchronic study conducted with Sprague–Dawley rats (Neda, 2000b, unpublished)

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1 Calculated based on the density value for menhaden fish oil (Merck, 2001).
and the one-generation reproduction study conducted with Wistar rats (Kuilman and Waalkens-Berendsen, 2001, unpublished) generally were what would be expected of feeding high dietary concentrations of ω-3 PUFA. In the 90-day study, treatment with DHA45-oil, alone (2000 mg/kg body weight/day) or in combination with a fish oil containing 27% DHA, was reported to have no effect on the findings of the ophthalmology, gross pathology, or histopathology examinations. Changes in hematological parameters and decreases in serum concentrations of total cholesterol, phospholipid, and free fatty acid levels occurred compared with water-treated controls. Although there was an increase in absolute and relative liver weights, no histopathological correlates were reported. In the high-dose group, there was no report of histological change in adipose tissue or the spleen. Dose-dependent effects were not observed with increasing levels of DHA administration and, given that none of the effects documented were toxic in nature and there was a lack of histopathological effects, the no-observed-adverse-effects level (NOAEL) in this study was considered to be 2000 mg DHA45-oil/kg body weight/day (approximately 900 mg DHA/kg body weight/day), which was the highest dose tested.

In the one-generation reproduction study, increases in absolute and/or relative liver weights also were reported, however, without histological correlates indicative of a toxic action. Similar effects have been reported in rats in several feeding and gavage studies of fish and marine algal-derived oils at daily intakes ranging from 4 to 13 weeks (Boswell et al., 1996; Hempenius et al., 1997, 2000; McGuire et al., 1997; Wibert et al., 1997; Burns et al., 1999; Rabbani et al., 1999). At gross necropsy, yellow spots (fatty deposits) were observed in abdominal adipose tissue of some high-dose rats, and increased absolute and relative spleen weights were recorded in many of the treated groups of parental animals of both sexes. At histopathological examination, the gross necropsy findings were correlated to findings of lipogranuloma (microscopic in the low- and mid-dose groups in which no gross necropsy observations of yellow spots were recorded), while increased spleen weights were likely related to increased severity (males) and incidence (females) of extramedullary hematopoiesis. Similar effects on spleen weight were reported by Burns et al. (1999) in a 90-day feeding study that included an in utero and lactation exposure phase in which male and female Charles River Sprague–Dawley rats were administered a DHA/arachidonic acid oil blend at dietary concentrations up to 12% for at least 4 weeks prior to mating. In addition, similar findings of lipogranulomas were reported in rats provided fish oil in the diet at levels ranging from 12 to 15% for varying periods of length (i.e., 8 weeks to 12 months) (Danse and Verschuren, 1978a,b; Charnock et al., 1987). It must be noted that these effects were not observed in similar studies of marine algal-derived oils in rats at doses less than 1/3 the highest level used in this study (i.e., up to 645 mg DHA/kg body weight/day) (Arterburn et al., 2000a,b; Hammond et al., 2001a), suggesting the findings of Kuilman and Waalkens-Berendsen (2001, unpublished) were the result of the very high doses used in this study. The lack of finding of histopathological effects of DHA45 oil on adipose tissue or the spleen in the 90-day study (Neda, 2000b, unpublished) may be related to the different strains of rats used (Sprague–Dawley versus Wistar) or, more likely, due to higher relative dosages in the one-generation study. There may also have been differences in the vitamin E contents of the diets provided in the two studies, although the data provided were not sufficient to make an accurate determination. Independent and collective critical expert evaluation of the scientific and pathology data of this study, and any other available data and information deemed pertinent, concurs with Kuilman and Waalkens-Berendsen (2001, unpublished) in that the reported effects were related to the administration of high levels of DHA45-oil. Expert review of the pathology indicated that the adipose tissue alteration was in fact steatitis, which occurs naturally and experimentally in many different species fed diets that are high in PUFAs (including DHA-containing oils) and relatively low in antioxidants. It can be concluded that the steatitis was the result of a nutritional imbalance attributable to the extremely high levels of DHA45-oil administered, and that the increased spleen weights were due to extramedullary hematopoiesis, consistent with a response to steatitis. The findings reported in the one-generation reproduction study are those expected with high exposure to PUFAs and raise no concern for human safety of DHA45-oil under appropriate conditions of use.

The safety of DHA45-oil is further supported by a wealth of historical information on populations consuming higher levels of DHA than those that would result from the conservative estimate of intake under the conditions of intended use. Additionally, clinical studies reveal no potential for toxicity of DHA under the conditions of intended use. While increased intakes of DHA have been associated with reduced platelet aggregation and elevations in LDL cholesterol levels (US FDA, 1993, 1997), the results of clinical studies utilizing dietary DHA alone at levels of up to 9.9 g/day indicate that, overall, DHA does not produce adverse effects on hemostasis or have an adverse effect on plasma LDL-cholesterol lipids (Cirillo et al., 1994; Conquer and Holub, 1996; 1998; Hamazaki et al., 1996; Innis and Hansen, 1996; Agren et al., 1997; Grimsaard et al., 1997, 1998; Nelson et al., 1997).

It is noted that the consumption of DHA-containing oils providing up to 6 g DHA/day, alone or in combination with DPA and/or EPA, has been reported to result
in alterations of platelet and total serum phospholipid and nonesterified fatty acid compositions, with increases in the levels of ω-3 PUFAs, including DHA, and concomitant decreases in levels of arachidonic acid (AA) (20:4; ω-6) of up to 26% (Haglund et al., 1992, 1998; Mori et al., 1992, 1997; Schmidt et al., 1992; Eritsland et al., 1994; Henderson et al., 1994; Leaf et al., 1994; Parkinson et al., 1994; Turini et al., 1994; Luostarinen et al., 1995; Tremoli et al., 1995; Conquer and Holub, 1996, 1998;Engström et al., 1996; Nelson et al., 1997; Conquer et al., 1999; Yaqoob et al., 2000). Arachidonic acid, either obtained in the diet or as a biosynthetic product of linoleic acid (18:2; ω-6), occurs as a fatty acid component of platelet membranes and most tissue phospholipids functioning as the main precursor of eicosanoids, which are involved in mediation of hemostatic parameters and immune cell function and response (Linder, 1991; Foegh et al., 1998; Kelley and Rudolph, 2000). Critical review of the scientific literature indicates that while AA levels may decrease following consumption of ω-3 PUFAs, the concentration of AA generally remains within normal physiological concentrations of 5–15% of total fatty acids of platelet membranes and tissue phospholipids. Additionally, the fatty acid composition of DHA45-oil comprises up to 1.8% AA. There is no indication that DHA, at exposures estimated through the proposed uses of DHA45-oil (total population all-user mean and 90th percentile intakes of 0.7 and 1.5 g DHA/person/day, or 13.6 and 28.6 mg DHA/kg body weight/day, respectively), would adversely affect hemostatic parameters or immune function or response as a result of possible concomitant decreases in platelet and total serum phospholipid and nonesterified fatty acid levels of AA (Haglund et al., 1992, 1998; Mori et al., 1992, 1997; Schmidt et al., 1992; Eritsland et al., 1994; Henderson et al., 1994; Leaf et al., 1994; Parkinson et al., 1994; Turini et al., 1994; Luostarinen et al., 1995; Tremoli et al., 1995; Conquer and Holub, 1996; Engström et al., 1996; Nelson et al., 1997; Gogos et al., 1998; Kelley et al., 1998, 1999; Conquer et al., 1999; Almallah et al., 2000; Yaqoob et al., 2000).

When viewed in its entirety, the scientific evidence from the toxicological studies of DHA45-oil, as well as the various published metabolic, toxicological, and clinical studies with DHA-containing oils indicates that the consumption of DHA45-oil, under the conditions of intended use in foods, would not be expected to produce adverse effects on human health. Based on the available data, it is concluded that other evaluated food safety experts would concur that under the conditions of intended use as a food ingredient in traditional foods, DHA45-oil, meeting appropriate food-grade specifications, and manufactured and used in accordance with current acceptable manufacturing practices standard to the edible oil industry, is generally recognized as safe.

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