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nutrinova

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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

United States of America

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NOV 17 2004
OFFICE OF
FOOD ADDITIVE SAFETY

Scientific & Regulatory Affairs

Nutrinova
Nutrition Specialties &
Food Ingredients GmbH
Industriepark Höchst
D-65926 Frankfurt am Main

Date: 11. November 2004
From: Dr. Bernd Haber
Phone: +49 (0)69 305 - 15423
Fax: +49 (0)69 305 - 84682
E-Mail: b.haber@nutrinova.com

Re: GRAS Notification

Dear Sir or Madam,

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the Federal Register (62 FR 18939-18964), I am submitting in triplicate, as the notifier, Nutrinova Nutrition Specialties & Food Ingredients GmbH, Industriepark Höchst, D 706, 65926 Frankfurt, Germany, i) a GRAS notification of DHA-Rich Oil for use as a food ingredient to provide a dietary source of ω-3 polyunsaturated fatty acids, notably docosahexaenoic acid (DHA), ii) a GRAS panel report setting forth the basis for the GRAS determination, and iii) curricula vitae of the members of the GRAS panel for review by the agency.

You received on Monday the 8th of November a notification letter (dated on October 29th, 2004) that contained no further enclosures. That was a human mistake from our consultant. We want to apologize for that. This time enclosed to this letter you will find the necessary documentation in triplicate.

Yours sincerely,

Nutrinova Nutrition Specialties & Food Ingredients GmbH

Dr. Bernd Haber
Head Scientific & Regulatory Affairs

Enclosures

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OFFICE OF
FOOD ADDITIVE SAFETY

I GENERALLY RECOGNIZED AS SAFE (GRAS) EXEMPTION CLAIM FOR DHA-RICH OIL

I.A Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

DHA-rich oil, as defined in the report in Appendix I entitled, "**EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DHA-RICH OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS**", dated September 15, 2004, has been determined to be Generally Recognized As Safe (GRAS), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food, among experts qualified by their relevant national and international experience and scientific training and expertise to evaluate the safety of food ingredients. Therefore, the use of DHA-rich oil in food as described below is exempt from the requirement of premarket approval.

Signed,

October 29, 2004

Dr. Bernd Haber
Head Scientific and Regulatory Affairs
Nutrinova
Nutrition Specialties & Food Ingredients GmbH
Industriepark Höchst, D 706
65926 Frankfurt, Germany

Date

I.B Name and Address of Notifier

Dr. Bernd Haber
Head Scientific and Regulatory Affairs
Nutrinova Nutrition Specialties & Food Ingredients GmbH
Industriepark Höchst, D 706
65926 Frankfurt, Germany

I.C Common Name of the Notified Substance

DHA-rich oil

I.D Conditions of Intended Use in Food

DHA-rich oil is intended for use as a food ingredient to increase the intake of dietary ω-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA), a critical component of most cell membranes and tissues.

The individual intended food use levels of DHA-rich oil in food are consistent with the current GRAS use of menhaden oil specified in 21 CFR §184.1472 and the revised uses outlined in the FDA's tentative final rule regarding GRAS-affirmed food uses for menhaden oil (FDA, 2004a), respectively. The intended food uses and maximum levels for DHA-rich oil are presented in Table 1.

Table 1 Intended Uses and Maximum Use Levels of DHA-rich oil in Food

Food Category	Initial[a] Intended Use Levels (% by weight)	Future[b] Intended Use Levels (% by weight)
Cookies, crackers (1)[1,2]	3.3	-
Breads, rolls (white and dark) (1)[2]	0.7	-
Fruit pies, custard pies (1)[2]	4.7	-
Cakes (1)[2]	6.7	-
Cereals (4)	2.7	2.7
Baked Goods and baking mixes	-	3.3
Fats and oils (12)[3]	13.4	8 0
Yogurt (31)[4]	2.7	-
Milk Products (31)	-	3.3
Cheese products (31)	-	3.3
Frozen dairy products (20)	3.3	3.3
Meat products (29)	6.7	3.3
Egg products (11)	3.3	3.3
Fish products (13)	13.4	3.3
Condiments (8)	3.3	3.3
Soup mixes (40)	2.0	3.3
Snack foods (37)	3.3	3.3
Nut products (32)	3.3	3.3
Gravies and sauces (24)	3.3	3.3
Plant protein products (33)	-	3.3
Processed vegetable juices (36)	-	0.7
Hard candy (25)	-	6.7
Soft candy (38)	-	2 7
Jams and jellies (28)	-	4.7
Dairy product analogs (10)	-	3.3

Docket No. 03–ASO–21." The postcard will be date/time stamped and returned to the commenter. All communications received before the specified closing date for comments will be considered before taking action on the proposed rule. The proposal contained in this notice may be changed in light of the comments received. A report summarizing each substantive public contact with FAA personnel concerned with this rulemaking will be filed in the docket.

Availability of NPRMs

An electronic copy of this document may be downloaded through the Internet at *http://www.dms.dot.gov.* Recently published rulemaking documents can also be accessed through the FAA's web page at *http://www.faa.gov* or the Superintendent of Document's web page at *http://www.access.gpo.gov/nara.* Additionally, any person may obtain a copy of this notice by submitting a request to the Federal Aviation Administration, Office of Air Traffic Airspace Management, ATA–400, 800 Independence Avenue, SW., Washington, DC 20591, or by calling (202) 267–8783. Communications must identify both docket numbers for this notice. Persons interested in being placed on a mailing list for future NPRM's should contact the FAA's Office of Rulemaking, (202) 267–9677, to request a copy of Advisory Circular No. 11–2A, Notice of Proposed Rulemaking Distribution System, which describes the application procedure.

The Proposal

The FAA is considering an amendment to part 71 of the Federal Aviation Regulations (14 CFR part 71) to amend Class E5 airspace at Lexington, TN. Class E airspace designations for airspace areas extending upward from 700 feet or more above the surface of the earth are published in Paragraph 6005 of FAA Order 7400.9L, dated September 2, 2003, and effective September 16, 2003, which is incorporated by reference in 14 CFR 71.1. The Class E airspace designations listed in this document would be published subsequently in the Order.

The FAA has determined that this proposed regulation only involves an established body of technical regulations for which frequent and routine amendments are necessary to keep them operationally current. It, therefore, (1) is not a "significant regulatory action" under Executive Order 12866; (2) is not a "significant rule" under DOT Regulatory Policies and Procedures (44 FR 11034; February 26, 1979); and (3) does not warrant preparation of a Regulatory Evaluation as the anticipated impact is so minimal. Since this is a routine matter that will only affect air traffic procedures and air navigation, it is certified that this rule, when promulgated, will not have a significant economic impact on a substantial number of small entities under the criteria of the Regulatory Flexibility Act.

List of Subjects in 14 CFR Part 71.

Airspace, Incorporation by reference, Navigation (Air).

The Proposed Amendment

In consideration of the foregoing, the Federal Aviation Administration proposes to amend 14 CFR part 71 as follows:

PART 71—DESIGNATION OF CLASS A, CLASS B, CLASS C, CLASS D, AND CLASS E AIRSPACE AREAS; AIRWAYS; ROUTES; AND REPORTING POINTS

1. The authority citation for part 71 continues to read as follows:

Authority: 49 U.S.C. 106(g); 40103, 40113, 40120; E.O. 10854, 24 FR 9565, 3 CFR, 1959–1963 Comp., p. 389.

§ 71.1 [Amended]

2. The incorporation by reference in 14 CFR 71.1 of Federal Aviation Administration Order 7400.9L, Airspace Designations and Reporting Points, dated September 2, 2003, and effective September 16, 2003, is amended as follows: Paragraph 6005 Class E Airspace Areas Extending Upward from 700 feet or More Above the Surface of the Earth.

* * * * *

ASO TN E5 Lexington, TN [Revised]

Lexington, Franklin Wilkins Airport, TN
(Lat. 35°39′05″ N, long. 88°22′44″ W)
Jacks Creek VORTAC
(Lat. 35°35′56″ N, long. 88°21′32″ W)

That airspace extending upward from 700 feet above the surface within a 6.6-mile radius of Franklin Wilkins Airport, and within 8 miles east and 4 miles west of the Jacks Creek VORTAC 166° radial extending from the 6.6-mile radius to 16 miles southeast of the VORTAC.

* * * * *

Issued in College Park, Georgia on January 7, 2004.

Jeffrey U. Vincent,

Acting Manager, Air Traffic Division, Southern Region.

[FR Doc. 04–919 Filed 1–14–04; 8:45 am]

BILLING CODE 4910–13–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

21 CFR Part 184

[Docket No. 1999P–5332]

Substances Affirmed as Generally Recognized as Safe: Menhaden Oil

AGENCY: Food and Drug Administration, HHS.

ACTION: Tentative final rule.

SUMMARY: The Food and Drug Administration (FDA) is issuing a tentative final rule to amend its regulations by reallocating the uses of menhaden oil in food that currently are established in § 184.1472 (21 CFR 184.1472). FDA has tentatively concluded that these uses of menhaden oil are generally recognized as safe (GRAS), but only when the menhaden oil is not used in combination with other added oils that are significant sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because FDA's proposed rule of February 26, 2002, did not include a condition of use for other added oils, FDA is issuing this tentative final rule to give interested persons an opportunity to comment on this use limitation.

DATES: Submit written or electronic comments by March 30, 2004.

ADDRESSES: Submit written comments to the Division of Dockets Management (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Submit electronic comments to *http://www.fda.gov/dockets/ecomments.*

FOR FURTHER INFORMATION CONTACT: Andrew J. Zajac, Center for Food Safety and Applied Nutrition (HFS–265), Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740–3835, 202–418–3095.

SUPPLEMENTARY INFORMATION:

I. Background

Menhaden oil is a refined marine oil that is derived from menhaden fish (*Brevoortia* species). Menhaden oil differs from edible vegetable oils and animal fats in its high proportion of polyunsaturated fatty acids, including omega-3 fatty acids. EPA and DHA are the major source of omega-3 fatty acids from fish oil and together comprise approximately 20 percent by weight of menhaden oil. In response to a petition (GRASP 6G0316) from the National Fish Meal and Oil Association, FDA issued a final rule on June 5, 1997 (62 FR 30751)

(the June 1997 final rule), affirming menhaden oil as GRAS for use as a direct human food ingredient with limitations on the maximum use levels of menhaden oil in specific food categories. FDA concluded that these limitations are necessary to ensure that daily intakes of EPA and DHA from menhaden oil do not exceed 3.0 grams per person per day (g/p/d). As discussed in the following paragraphs, the maximum limit of 3.0 g/p/d on the total daily intake of EPA and DHA is a safeguard against the possible effects of these fatty acids on increased bleeding time (the time taken for bleeding from a standardized skin wound to cease), glycemic control in non-insulin-dependent diabetics, and increased levels of low-density lipoprotein (LDL) cholesterol. The concerns over possible adverse effects of fish oil consumption on bleeding time, glycemic control, and LDL cholesterol were discussed in the June 1997 final rule.

As part of FDA's evaluation of GRASP 6G0316, FDA examined the scientific literature for evidence that consumption of fish oils may contribute to excessive bleeding. In the June 1997 final rule, FDA concluded based on this examination of the scientific literature, including more than 50 reports on fish oils with data on bleeding time, that when consumption of fish oils is limited to 3.0 g/p/d or less of EPA and DHA, there is no significant risk for increased bleeding time beyond the normal range (62 FR 30751 at 30752 to 30753). FDA also concluded that amounts of fish oils providing more than 3.0 g/p/d of EPA and DHA have generally been found to produce increases in bleeding time that are statistically significant, but that there are insufficient data to evaluate the clinical significance of this finding. Therefore, because of the lack of data on clinical significance and because of the potential risk of excessive bleeding in some individuals with intakes at higher levels, FDA concluded that the safety of menhaden oil was generally recognized only at levels that limit intake of EPA and DHA to 3.0 g/p/d.

FDA also concluded in the June 1997 final rule that 3.0 g/p/d of EPA and DHA is a safe level with respect to glycemic control (62 FR 30751 at 30753). This conclusion was based on FDA's review of a series of studies on non-insulin-dependent diabetics. Studies on type-II diabetics that reported increased glucose used higher amounts (4.5 to 8 g/p/d) of omega-3 fatty acids. One study found no change in fasting blood glucose levels among type-II (non-insulin-dependent) diabetics treated with 3.0 g/p/d EPA plus DHA for 2 weeks. Two other studies that used 3.0 g/p/d EPA plus DHA for 6 weeks and 2.7 g/p/d EPA plus DHA for 8 weeks found only transient increases in blood glucose halfway through their respective supplementation periods. Another study that used 3.0 g/p/d EPA plus DHA for 3 weeks found comparable increases in fasting blood glucose when either fish oil or safflower oil was fed, so the increase cannot be attributed specifically to omega-3 fatty acids. A study that compared the effects of fish oil and olive oil fed 3.0 g/p/d of EPA plus DHA did not find a difference in fasting glucose or glycosylated hemoglobin after fish oil supplementation compared to baseline; they did find a significant difference compared to the olive oil treatment, which produced changes in the opposite direction from fish oil. Based on its evaluation of the available information, FDA concluded in the June 1997 final rule that consumption of EPA and DHA in fish oils at 3.0 g/p/d by diabetics has no clinically significant effect on glycemic control, although higher amounts of EPA and DHA (4.5 g/p/d and above) remain of concern.

The June 1997 final rule also considered the reported effects of fish oil on LDL cholesterol levels in healthy persons with normal cholesterol levels, as well as in persons with diabetes mellitus, hypertension, abnormal blood lipid levels, and cardiovascular disease (62 FR 30751 at 30753 to 30754). As a result of its evaluation, FDA found that although reported study reports are variable, there appears to be a trend toward increased LDL cholesterol values with increased fish oil consumption in all population subgroups, with the magnitude of the increase appearing greater and more consistent in populations with abnormal blood lipid levels, hypertension, diabetes, and cardiovascular disease. Based on its evaluation, FDA concluded that 3.0 g/p/d of EPA and DHA is a safe level with respect to LDL cholesterol.

In the **Federal Register** of February 26, 2002 (67 FR 8744), FDA published a proposed rule to amend § 184.1472 by reallocating the uses of menhaden oil in food, while maintaining the total daily intake of EPA and DHA from menhaden oil at a level not exceeding 3.0 g/p/d. The proposal was based on a citizen petition from the National Fish Meal and Oil Association. The maximum limit of 3.0 g/p/d on the total daily intake of EPA and DHA is a safeguard against the possible adverse effects discussed in the June 1997 final rule and the February 2002 proposed rule. The reallocation is performed by the following three actions: (1) Reducing the maximum levels of use of menhaden oil in some of the currently listed food categories; (2) adding additional food categories along with assigning maximum levels of use in these new categories; and (3) eliminating the listing of subcategories, e.g., cookies and crackers, breads and rolls, fruit pies and custard pies, and cakes, and including them under broader food categories, e.g., baked goods and baking mixes.

The purpose of the maximum use levels of menhaden oil in the food categories is to ensure that the total daily intake of EPA and DHA does not exceed 3.0 g/p/d (67 FR 8744 to 8745). When the June 1997 final rule published affirming that menhaden oil is GRAS for use as a direct human food ingredient with specific limitations, FDA considered food sources of EPA and DHA likely to be in the diet at that time, but the agency did not take into account that other sources of EPA and DHA might be developed in the future. The implicit basis for the restrictions in the menhaden oil regulation was that while menhaden oil might be blended with other oils to make a particular food product, the sum of DHA and EPA would not exceed 3.0 g/p/d because other oils were not significant sources of DHA and EPA. However, since publication of the proposed rule, FDA has received notices from three companies that have concluded that fish oils, other than menhaden oil, are GRAS for use in the same food categories as those currently listed in § 184.1472(a)(3) at maximum use levels that are designed to assure that the combined daily intake of EPA and DHA would not exceed 3.0 g/p/d. These oils included small planktivorous pelagic fish body oil (oil derived primarily from sardine and anchovy fish) (Ref. 1), a fish oil concentrate (manufactured from oil extracted from edible marine fish species that normally include anchovy, sardine, jack mackerel, and mackerel) (Ref. 2), and tuna oil (Ref. 3). In each case, the company acknowledged the concerns raised by FDA in the June 1997 final rule and the proposed rule, about consumption of high levels of EPA and DHA. Furthermore, in each case the company stated that its determination of GRAS status related only to the circumstance where its fish oil product is used as the sole added source of EPA and DHA in any given food category and is not combined or augmented with any other EPA/DHA-rich oil.

Because of developing interest in food ingredients that are sources of EPA and DHA, FDA now believes that it is necessary to state explicitly in the regulation that when menhaden oil is added as an ingredient in foods, it may

not be used in combination with any other added oil that is a significant source of EPA and DHA. Without this restriction, the intake of DHA and EPA could exceed 3.0 g/p/d. Because this use restriction was not contained in the proposed rule, FDA is issuing this regulation as a tentative final rule under 21 CFR 10.40(f)(6). FDA will review any comments that are relevant to this condition of use and that are received within the 75-day comment period and will respond accordingly to these comments in the **Federal Register**.

FDA is also making an editorial update to § 184.1472(a)(2)(iii) to reflect that the name for the Office of Premarket Approval has been changed to the Office of Food Additive Safety.

II. Comments on the Proposed Rule

The agency provided 75 days for comments on the proposed rule. At the close of the comment period, the agency had received two comments that expressed concern regarding the environmental impact of the proposed rule. These two comments are addressed separately in section III of this document. The agency also received comments that were submitted from a fish oil company and a trade association that represents the fish oil industry that merely expressed general support for the agency's proposed rule. The other comments were from individual consumers who were opposed to the proposed rule.

Most of the comments FDA received expressing opposition to the proposed rule objected to declaring menhaden oil on food labels by the name "omega-3 fatty acids" or a variation of this name. Many of these comments asserted that "omega-3 fatty acids" is a misleading name for menhaden oil. Some comments were from vegetarians and vegans who stated that listing menhaden oil by the name "omega-3 fatty acids" will make it difficult for them to avoid this animal product in foods. There were also comments that stated that listing menhaden oil by the name "omega-3 fatty acids" will make it difficult for those with fish allergies to avoid this fish oil in foods.

The proposed rule did not address how menhaden oil is to be listed as an ingredient on food labels. Generally, under section 403(i)(2) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 343(i)(2)), a food is misbranded unless its label bears the common or usual name of each ingredient. Although menhaden oil is a significant source of omega-3 fatty acids, FDA knows of no basis for considering omega-3 fatty acids to be its common or usual name. Any consideration of an alternative name for menhaden oil, such as "omega-3 fatty acids," is outside the scope of the proposed rule.

FDA also received comments from consumers asking the agency to consider the use of omega-3 fatty acids from sources other than menhaden fish, such as flax seed. FDA notes that although menhaden oil does contain omega-3 fatty acids (primarily EPA and DHA), omega-3 fatty acids are not the subject of the proposed rule. Therefore, the use of other oils is outside the scope of the proposed rule.

A few comments stated that the menhaden fish is unsuitable for human consumption and, therefore, oil from this fish should not be used as a food ingredient. As stated in the proposed rule, menhaden oil is already affirmed as generally recognized as safe as a direct human food ingredient (§ 184.1472). FDA has not received any new information or comments that would alter its previous determination that menhaden oil that meets the specifications in § 184.1472 is generally recognized as safe for use in food under the conditions specified.

Some of the comments FDA received expressing opposition to the proposed rule were against the addition of menhaden oil to foods because of a concern about the possibility of high levels of contaminants in the menhaden oil due to bioaccumulation of these contaminants in the menhaden fish. Bioaccumulation describes the process that results in an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the environment. FDA has evaluated data on levels of various chemical contaminants, such as pesticides, polychlorinated biphenyls and dioxins in menhaden oil. Based on these data, FDA finds no basis for a safety concern from food uses of menhaden oil due to possible bioaccumulation of lipophilic chemical contaminants in the source fish.

III. Environmental Impact

The agency received two comments expressing concern about the impact that the proposed rule will have on the menhaden fish population. One comment asked whether this action will result in the "near extinction" of menhaden, mackerel, and sardines, and further asked how near extinction, if it results, would effect ocean ecosystems. The other comment asserted that menhaden are being overfished to extinction, and that because of their population decline, larger game fish populations off the Atlantic coast are dropping proportionately. Neither comment cited supporting data or information.

To ensure that the maximum sustainable yield of menhaden is not exceeded and to provide long-term production, the menhaden fisheries are monitored by the Atlantic and Gulf States Marine Fisheries Commissions (which are under the jurisdiction of the National Marine Fisheries Service (NMFS)), as well as by State authorities. If there is a threat to the long-term yield of a fishery, generally, limits will be imposed by these organizations. At present, the Atlantic and Gulf menhaden fisheries are considered to be healthy and not overfished. With regard to the impact that the proposed rule will have on mackerel and sardines, the United Nation's Foreign Agricultural Organization reports that the primary practice used to catch menhaden has one of the lowest discard ratios of any method for general commercial fishing. (Less than 3 percent by weight of the total menhaden catch are other species of fish.) In addition, NMFS reports a numerical bycatch incidence (i.e., fish that are unintentionally caught) of less than 0.1 percent for the menhaden fishing industry. For these reasons, the agency does not believe that the proposed rule would result in overfishing of menhaden or have a significant impact on other species of fish. In summary, the comments do not provide a basis on which to change the conclusions of the environmental analysis that was prepared for the proposed rule, as discussed in the following paragraph.

The agency has previously considered the environmental effects of affirming menhaden oil as GRAS as a direct human food ingredient, provided that the combined daily intake of EPA and DHA from menhaden oil does not exceed 3.0 g/p/d (62 FR 30751 at 30754). The analysis assumed that the maximum use levels would be completely used for each food category and concluded that this action will not have a significant impact on the menhaden population. This rule will reallocate the maximum levels among food categories but will not increase the total maximum allowable level. Therefore, our previous analysis is applicable. No new information or comments have been received that would affect the agency's previous determination that there is no significant impact on the human environment, and that an environmental impact statement is not required.

IV. Analysis of Economic Impacts

A. Final Regulatory Impact Analysis

FDA has examined the economic implications of this tentative final rule as required by Executive Order 12866. Executive Order 12866 directs agencies to assess all costs and benefits of available regulatory alternatives and, when regulation is necessary, to select regulatory approaches that maximize net benefits (including potential economic, environmental, public health and safety, and other advantages; distributive impacts; and equity). Executive Order 12866 classifies a rule as significant if it meets any one of a number of specified conditions, including: having an annual effect on the economy of $100 million, adversely affecting a sector of the economy in a material way, adversely affecting competition, or adversely affecting jobs. A regulation is also considered a significant regulatory action if it raises novel legal or policy issues. FDA has determined that this tentative final rule is not a significant regulatory action as defined by Executive Order 12866.

In the economic analysis of the proposed rule, we stated that the main benefit of this rule would be the expansion of the potential uses of menhaden oil made possible by the new maximum levels. Firms choosing to use menhaden oil will bear labeling and other costs. Because these costs are voluntary, they will be borne only if doing so is anticipated to be advantageous to the firm. Although firms making products that now use menhaden oil at levels below the current maximum but above the new maximum could bear potential compliance costs, we noted in the proposed rule that FDA did not know of any products in that category. We received no comments on this conclusion, or on any other part of the preliminary regulatory impact analysis.

B. Final Regulatory Flexibility Analysis

FDA has examined the economic implications of this tentative final rule as required by the Regulatory Flexibility Act (5 U.S.C. 601–612). If a rule has a significant economic impact on a substantial number of small entities, the Regulatory Flexibility Act requires agencies to analyze regulatory options that would lessen the economic effect of the rule on small entities. FDA finds that this tentative final rule would not have a significant economic impact on a substantial number of small entities.

The use of the menhaden oil by any small business is voluntary and will be undertaken only if doing so is anticipated to be advantageous to the small business. Small businesses would only bear a compliance cost if, as stated previously, they make products that are below the current maximum but above the new maximum.

The agency specifically requested comments from small businesses on its assumption that no small businesses make products that will be affected by reducing the maximum levels of menhaden oil in pies, cakes, fats, oils, fish products, and meat products. We received no comments on that assumption or any other part of the initial regulatory flexibility analysis.

C. Unfunded Mandates

Title II of the Unfunded Mandates Reform Act of 1995 (Public Law 104–4) requires cost-benefit and other analyses before any rulemaking if the rule would include a "Federal mandate that may result in the expenditure by State, local, and tribal governments, in the aggregate, or by the private sector, of $100,000,000 or more (adjusted annually for inflation) in any 1 year." The current inflation-adjusted statutory threshold is $112 million. FDA has determined that this tentative final rule does not constitute a significant rule under the Unfunded Mandates Reform Act.

V. Paperwork Reduction Act

This tentative final rule contains no collections of information. Therefore, clearance by the Office of Management and Budget under the Paperwork Reduction Act of 1995 is not required.

VI. Federalism

FDA has analyzed this tentative final rule in accordance with the principles set forth in Executive Order 13132. FDA has determined that the tentative final rule does not contain policies that have substantial direct effects on the States, on the relationship between the National Government and the States, or on the distribution of power and responsibilities among the various levels of government. Because the agency concludes that this tentative final rule does not contain policies that have federalism implications as defined in the order, a federalism summary impact statement is not required.

VII. Comments

Interested person may submit to the Division of Dockets Management (see **ADDRESSES**) written or electronic comments regarding this document. Submit a single copy of electronic comments or two paper copies of any mailed comments, except that individuals may submit one paper copy. Comments are to be identified with the docket number found in the brackets in the heading of this document. Received comments may be seen in the Division of Dockets Management between 9 a.m. and 4 p.m., Monday through Friday.

VIII. References

The following references have been placed on display in the Division of Dockets Management (see **ADDRESSES**) and may be seen by interested persons between 9 a.m. and 4 p.m., Monday through Friday.

1. GRAS notice GRN 000102, including the response letter to GRN 000102 dated September 3, 2002, from Alan M. Rulis of FDA to Edward Iorio of Jedwards International, available at *http://www.cfsan.fda.gov/~rdb/opa-gras.html.*

2. GRAS notice GRN 000105, including the response letter to GRN 000105 dated October 15, 2002, from Alan M. Rulis of FDA to Nancy L. Schnell of Unilever United States, Inc., available at *http://www.cfsan.fda.gov/~rdb/opa-gras.html.*

3. GRAS notice GRN 000109, including the response letter to GRN 000109 dated December 4, 2002, from Alan M. Rulis of FDA to Anthony Young of Piper Rudnick, LLP, available at *http://www.cfsan.fda.gov/~rdb/opa-gras.html.*

List of Subjects in 21 CFR Part 184

Food additives.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under authority delegated to the Commissioner of Food and Drugs, and redelegated to the Director, Center for Food Safety and Applied Nutrition, it is proposed that 21 CFR part 184 be amended as follows:

PART 184—DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

1. The authority citation for 21 CFR part 184 continues to read as follows:

Authority: 21 U.S.C. 321, 342, 348, 371.

2. Section 184.1472 is amended by revising paragraph (a)(2)(iii) and (a)(3) and adding paragraph (a)(4) to read as follows:

§ 184.1472 Menhaden oil.

(a) * * *

(2)(iii) *Saponification value.* Between 180 and 200 as determined by the American Oil Chemists' Society Official Method Cd 3–25—"Saponification Value" (reapproved 1989), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies of this publication are available from the Office of Food Additive Safety, Center for Food Safety and Applied Nutrition (HFS–200), Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740, or available for inspection at the Center for Food Safety and Applied Nutrition's Library, Food and Drug Administration,

5100 Paint Branch Pkwy., College Park, MD 20740, or at the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

* * * * *

(3) In accordance with § 184.1(b)(2), the ingredient may be used in food only within the following specific limitations to ensure that total intake of eicosapentaenoic acid or docosahexaenoic acid does not exceed 3.0 grams/person/day:

Category of food	Maximum level of use in food (as served)
Baked goods, baking mixes, § 170.3(n)(1) of this chapter.	5.0 percent
Cereals, § 170.3(n)(4) of this chapter.	4.0 percent
Cheese products, § 170.3(n)(5) of this chapter.	5.0 percent
Chewing gum, § 170.3(n)(6) of this chapter.	3.0 percent
Condiments, § 170.3(n)(8) of this chapter.	5.0 percent
Confections, frostings, § 170.3(n)(9) of this chapter.	5.0 percent
Dairy product analogs, § 170.3(n)(10) of this chapter.	5.0 percent
Egg products, § 170.3(n)(11) of this chapter.	5.0 percent
Fats, oils, § 170.3(n)(12) of this chapter, but not in infant formula.	12.0 percent
Fish products, § 170.3(n)(13) of this chapter.	5.0 percent
Frozen dairy desserts, § 170.3(n)(20) of this chapter.	5.0 percent
Gelatins, puddings, § 170.3(n)(22) of this chapter.	1.0 percent
Gravies, sauces, § 170.3(n)(24) of this chapter.	5.0 percent
Hard candy, § 170.3(n)(25) of this chapter.	10.0 percent
Jams, jellies, § 170.3(n)(28) of this chapter.	7.0 percent
Meat products, § 170.3(n)(29) of this chapter.	5.0 percent
Milk products, § 170.3(n)(31) of this chapter.	5.0 percent
Nonalcoholic beverages, § 170.3(n)(3) of this chapter.	0.5 percent
Nut products, § 170.3(n)(32) of this chapter.	5.0 percent
Pastas, § 170.3(n)(23) of this chapter.	2.0 percent
Plant protein products, § 170.3(n)(33) of this chapter.	5.0 percent
Poultry products, § 170.3(n)(34) of this chapter.	3.0 percent
Processed fruit juices, § 170.3(n)(35) of this chapter.	1.0 percent
Processed vegetable juices, § 170.3(n)(36) of this chapter.	1.0 percent
Snack foods, § 170.3(n)(37) of this chapter.	5.0 percent
Soft candy, § 170.3(n)(38) of this chapter.	4.0 percent
Soup mixes, § 170.3(n)(40) of this chapter.	3.0 percent
Sugar substitutes, § 170.3(n)(42) of this chapter.	10.0 percent
Sweet sauces, toppings, syrups, § 170.3(n)(43) of this chapter.	5.0 percent
White granulated sugar, § 170.3(n)(41) of this chapter.	4.0 percent

(4) To ensure safe use of the substance, menhaden oil shall not be used in combination with any other added oil that is a significant source of eicosapentaenoic acid or docosahexaenoic acid.

* * * * *

Dated: January 6, 2004.

L. Robert Lake,

Director, Office of Regulations and Policy, Center for Food Safety and Applied Nutrition.

[FR Doc. 04–811 Filed 1–14–04; 8:45 am]

BILLING CODE 4160–01–S

DEPARTMENT OF THE INTERIOR

Bureau of Indian Affairs

25 CFR Chapter 1

Meeting of the No Child Left Behind Negotiated Rulemaking Committee

AGENCY: Bureau of Indian Affairs, Interior.

ACTION: Announcement of negotiated rulemaking committee meeting.

SUMMARY: The Secretary of the Interior has established an advisory Committee to develop recommendations for proposed rules for Indian education under the No Child Left Behind Act of 2001. As required by the Federal Advisory Committee Act, we are announcing the date and location of the next meeting of the No Child Left Behind Negotiated Rulemaking committee.

DATES: The Committee's next meeting will be held February 2–7, 2004. The meeting will begin at 8:30 pm (PST) on Monday, February 2 and end at 5 pm (PST) on Saturday, February 7, 2004.

ADDRESSES: The meeting will be held at the San Diego Mission Bay Hilton, 901 Camino Del Rio South, San Diego, California 82108, telephone (619) 543–9000.

FOR FURTHER INFORMATION CONTACT: Shawna Smith, No Child Left Behind Negotiated Rulemaking Project Management Office, P.O. Box 1430, Albuquerque, NM 87103–1430; telephone (505) 248–7241/6569; fax (505) 248–7242; email *ssmith@bia.edu*. We will post additional information as it becomes available on the Office of Education Programs Web site under "Negotiated Rulemaking" at *http//www.oiep.bia.edu*.

SUPPLEMENTARY INFORMATION: The Secretary, after consultation with the tribes, has revised the charter of the negotiated rulemaking committee established to negotiate regulations to implement the No Child Left Behind Act of 2001 (Pub. Law 107–110). Under this revised charter, the committee will negotiate new regulations covering Closure or Consolidation of Schools (Section 1121(d)) and National Criteria for Home Living Situations (Section 1122). For more information on negotiated rulemaking under the No Child Left Behind Act, see the **Federal Register** notices published on December 10, 2002 (67 FR 75828) and May 5, 2003 (68 FR 23631) or the Web site at *http//www.oiep.bia.edu* under "Negotiated Rulemaking."

There is no requirement for advance registration for members of the public who wish to attend and observe the Committee meeting or any work group meetings. Members of the public may make written comments on the above-listed items to the Committee by sending them to the NCLB Negotiated Rulemaking Committee, Project Management Office, P.O. Box 1430, Albuquerque, New Mexico 87103. We will provide copies of the comments to the Committee.

The agenda for the February 2–7, 2004, meeting is as follows:

Table 1 Intended Uses and Maximum Use Levels of DHA-rich oil in Food

Food Category	Initial[a] Intended Use Levels (% by weight)	Future[b] Intended Use Levels (% by weight)
Nonalcoholic beverages (3)	-	0.3
Pastas (23)	-	1 3
Poultry products (34)	-	2.0
Processed fruit juices (35)	-	0.7
White grandulated sugar (41)	-	2.7
Sugar substitutes (42)	-	6.7
Chewing gum (6)	-	2.0
Gelatins and puddings (22)	-	0.7
Confections and frosting (9)	-	3.3
Sweet sauces, toppings, and syrups (43)	-	3.3

[a] Based on 21 CFR §184.1472
[b] Based on tentative final rule amending 21 CFR §184.1472 (69 FR 2313; January 15, 2004)
[1] The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n)
[2] Included in "Baked goods and baking mixes"
[3] Not including infant formula
[4] Included in "Milk products"

In a final rule affirming menhaden oil as GRAS for use as a direct human food ingredient (21 CFR §184.1472), the FDA concluded that 3.0 g EPA (eicosapentaenoic acid) and DHA/person/day is a safe intake level with respect to bleeding time, increased bleeding time, glycemic control and LDL cholesterol levels (FDA, 1997). In order to ensure that intake does not exceed 3.0 g/person/day the FDA introduced maximum use levels of menhaden oil in specific food categories. The specific food categories and use limitations for menhaden oil were published recently in a tentative final rule (FDA, 2004a). The use limitations of EPA and DHA were based on the content of EPA and DHA within menhaden oil, approximately 20%. Furthermore, the FDA stated that menhaden oil may not be used in combination with any other added oil that is a significant source of EPA and DHA, as to ensure compliance with the prescribed limitations.

Comparing the composition of menhaden oil (sum of approximately 20% of EPA and DHA) with DHA-rich oil (DHA content of 38 to 50% and no significant EPA content), it can reasonably be concluded that approximately twice as much menhaden oil as DHA-rich oil will have to be consumed for the same intake of ω-3 fatty acids. Inversely, any limitation of use levels from DHA-rich oil will therefore have to be less than 50% of the use levels of menhaden oil to ascertain compliance with the safe intake level. Consequently, the limitation of DHA-rich oil use levels to 40% of the use levels in all food categories considered by the US FDA for the GRAS use of menhaden oil would also ascertain compliance with the safe intake level of 3.0 g/person/day for the sum of EPA and DHA. To ensure safe use levels in the intended food

categories and levels of use, DHA-rich oil shall not be used in combination with any other added oil that is a significant source of EPA or DHA.

DHA is primarily consumed through the ingestion of fatty fish, which contain high amounts of PUFAs with concentrations of ω-3 fatty acids ranging from 0.1 to 5.3 g/100 g (Ascherio *et al.*, 1995; Sanders, 1989). The normal consumption of ω-3 fatty acids in the United States is approximately 1,600 mg/day, primarily as α-linolenic acid (18:3), as well as lesser amounts of EPA, DHA, and DPA. The total amount of DHA consumed per day in the United States has been estimated at 100 to 200 mg/day, while higher consumption of DHA has been reported in other populations such as Japan, Norway, South Africa, and the Portuguese island of Madeira whose diets include many DHA-containing foods with average DHA intakes of approximately 500 to 700 mg DHA/day (Dolecek and Granditis, 1991; Schloss *et al.*, 1997; Fluge *et al.*, 1998; Johansson *et al.*, 1998; Kris-Etherton *et al.*, 2000; Sugano and Hirahara, 2000; Torre *et al.*, 2000). Recommended intakes of ω-3 PUFAs have been published by a number of scientific authorities including Health Canada, which recommends an intake of 1,000 to 1,800 mg ω-3 PUFAs/day in a ratio of 1:4 to 1:10 with ω-6 PUFAs (Health and Welfare Canada, 1990). Furthermore, the International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommended adequate intakes (AI) of a minimum of 220 mg/day for both DHA and EHA separately, while a combined AI for both DHA and EPA was recommended at 650 mg/day. The British Nutrition Foundation also has recommended a desirable intake of DHA and EPA within females and males of the population of approximately 1,100 and 1,400 mg/day, respectively (BNF, 2000).

I.E Basis for the GRAS Determination

The determination that DHA-rich oil, as defined in Appendix I, is GRAS is on the basis of scientific procedures (see Appendix I entitled, "**EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DHA-RICH OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS**").

The safety of DHA-rich oil is based on animal studies using other DHA-containing oils, as well as studies performed specifically with DHA-rich oil (reported as DHA45-oil). The studies performed with DHA-rich oil included mutagenicity/genotoxicity, acute, subchronic, and a single generation reproductive toxicity study. Human clinical trials also are included in support of the safety of DHA-rich oil, whereby a number of toxicologically relevant endpoints are examined including bleeding times and other hemostatic parameters, glycemic control, LDL cholesterol and other plasma lipid fractions, as well as additional clinical studies with endpoints related to the safety of DHA.

I.F Availability of Information

The data and information that serve as the basis for this GRAS determination will be sent to the U.S. Food and Drug Administration upon request or will be available for FDA review and copying at reasonable times at the offices of:

Dr. Bernd Haber
Head of Scientific and Regulatory Affairs
Nutrinova Nutrition Specialties & Food Ingredients GmbH
Industriepark Höchst, D 706
65926 Frankfurt, Germany

Should the FDA have any questions or additional information requests regarding this notification, Nutrinova GmbH will supply these data and information.

II. Detailed Information About the Identity of the Substance

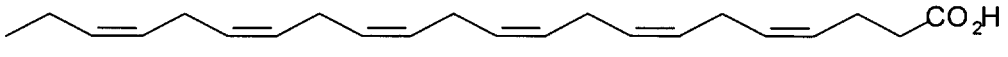
II.A Identity

DHA-rich oil is a refined triacylglycerol oil (>95% triacylglycerols), derived from a marine protist, with a total fatty acid composition of 38 to 50% DHA. The remaining fatty acids of DHA-rich oil are comprised mainly of saturated palmitic acid (16:0) (28 to 37%) and a lesser amount (8 to 14%) of the ω-6 fatty acid, docosapentaenoic acid (DPA) (22:5). In addition, DHA-rich oil contains safe and suitable antioxidants as permitted by the FDA for use in edible oils to ensure stability.

Common or Usual Name:	DHA-rich oil, containing 38 to 50% DHA; DHA oil 45; DHA45-TG; Nutrinova® DHA
Chemical Name:	All-cis-4,7,10,13,16,19-docosahexaenoic acid (22:6) esterified to glycerol
Chemical Abstracts Service	None assigned for DHA-rich oil; however, DHA assigned CAS No. 6217-54-5

The structural formula of DHA is provided below in Figure 1.

Figure 1 All-cis-4,7,10,13,16,19-docosahexaenoic acid (22:6)



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II.B Method of Manufacture

DHA-rich oil is produced in accordance with current good manufacturing practices through a multi-step fermentation and refining process using a genetically stable and pure culture of the marine protist, *Ulkenia sp*. DHA-rich oil is extracted *via* a solvent-based extraction process using food grade solvents and processing aids, and is further refined by processes standard to the edible oil industry, including degumming, deacidification, bleaching and deodorization, using materials appropriate for food processing. A schematic diagram of the proprietary manufacturing process for DHA-rich oil is presented in Figure 3. Product specifications are presented in Table 4.

Figure 3 – Proprietary Manufacturing Process for DHA-rich oil

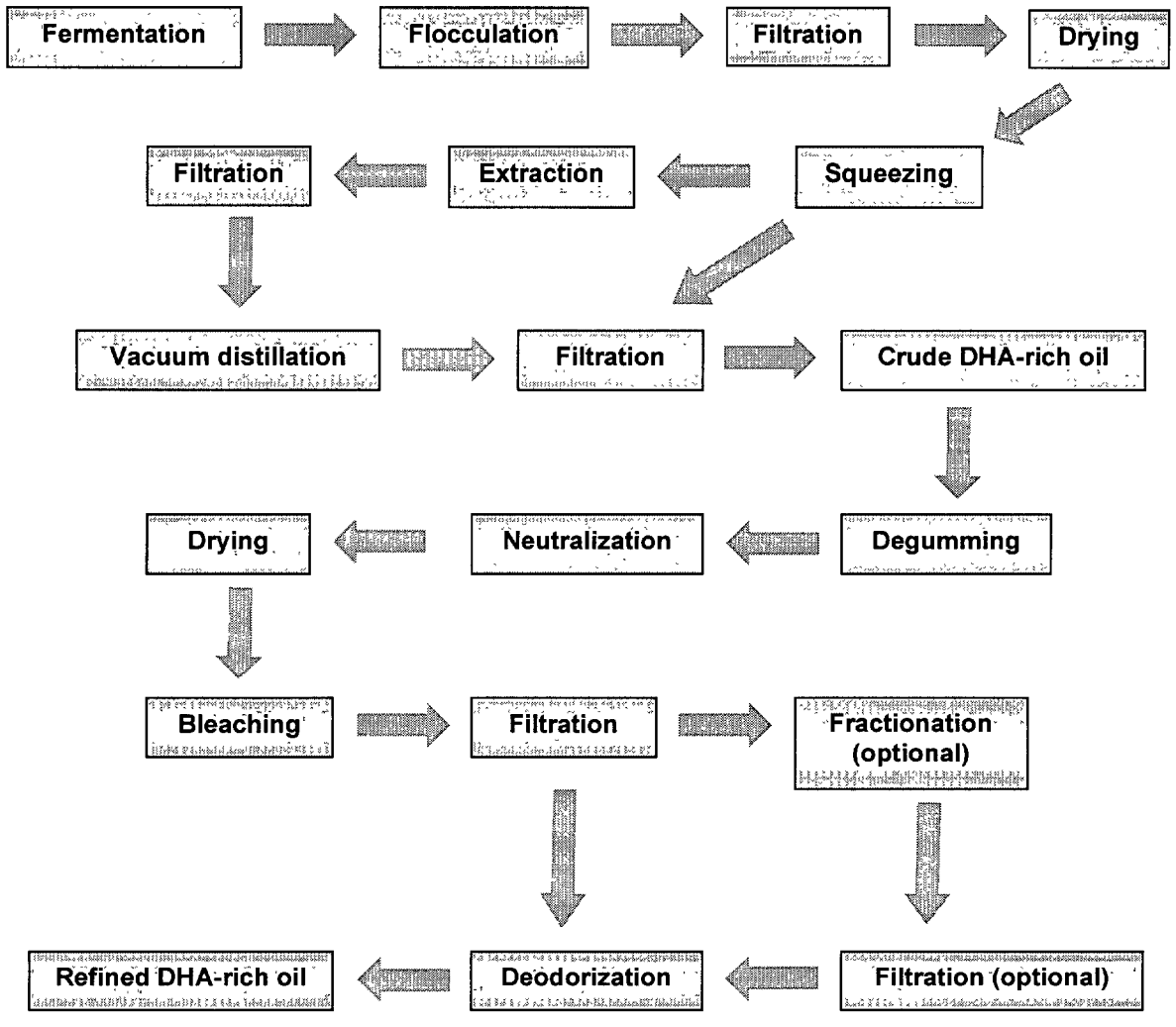

Table 4 Product Specifications for DHA-rich oil	
Parameter	**Specification**
DHA	38 to 50 (% by weight)
Trans Fatty Acids	≤ 2 %
Peroxide value	≤ 5 meq./kg
Acid value	≤ 0.5 mg KOH/g
Moisture and Volatiles	≤ 0.05 %
Unsaponifiables	≤ 4.5 %
Lead	≤ 0.1 ppm
Arsenic	≤ 0.5 ppm
Mercury	≤ 0.5 ppm

III. Basis for GRAS Determination

Pursuant to 21 CFR §170.30, the composition of DHA-rich oil intended for use in foods by Nutrinova GmbH, as defined in Appendix I, has been determined to be GRAS based on scientific procedures. This determination is based on the opinion of experts (the Expert Panel) who are qualified by their scientific training and experience to evaluate the safety of DHA-rich oil as a component of food. Further support for the safety of DHA-rich oil is well established based on the historical consumption of DHA from fatty fish (*e.g.*, haddock, tuna, salmon, mackerel) and other food sources.

The safety of DHA-rich oil is supported by a number of toxicological studies conducted in mice and rats, specifically acute, subchronic and single generation reproduction studies, as well as, with mutagenicity studies conducted using *Salmonella typhimurium*, *Escherichia coli* WP2 *uvrA* (with or without metabolic activation), or using a chromosomal aberration assay in Chinese hamster fibroblast cells (Fujii and Suwa, 1998; Kashima and Sarwar, 2000; Neda, 2000; Bruijntjes-Rozier and van Ommen, 2001; Kuilman and Waalkens-Berendsen, 2001; Kroes *et al.*, 2003). The results of these studies indicate that DHA-rich oil, is not genotoxic or mutagenic, and is of low toxicity. When administered to rats *via* the diet at levels providing up to 2,000 mg/kg body weight/day (equivalent to 900 mg DHA/kg body weight/day) in the subchronic study, and as high as 11,200 mg DHA-rich oil/kg body weight/day (equivalent to 5,040 mg DHA/kg body weight/day) in female rats during gestation and lactation (days 1 to 14), DHA-rich oil did not produce any compound-related adverse effects (aside from effects attributed to the extremely high levels of PUFAs added in the diet of the experimental animals).

The safety of DHA-containing fish and microalgal-derived oils has been studied extensively in toxicological studies, and these studies further support the safety of DHA-rich oil. Overall, the data from these studies indicate that gavage or dietary administration of fish or microalgal-derived oils does not produce significant adverse effects on mortality, body weight gains, food consumption, or clinical observations in 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; Rabbani *et al.,* 1999; Arterburn *et al.,* 2000a,b; Oarada *et al.*, 2000; Hammond *et al.*, 2001a,b,c). Yellow fat disease, which is characterized by accretion of lipofuscin pigment, degeneration of adipose cells (steatosis), and inflammation of adipose tissue (steatitis) (Danse and Steenbergen-Botterweg, 1976; Danse and Verschuren, 1978a; Danse *et al.*, 1979), has been reported to occur naturally in wildlife species and was reported in rats, rabbits, mink, and pigs in various toxicological studies following the consumption of diets rich in ω-3 PUFAs (*i.e.*, ω-3 PUFA-containing oils at dietary concentrations ranging from as high as 12 to 19% for periods of 8 weeks to 12 months in length) in combination with a vitamin E deficient state (Jones *et al.,* 1969; Helgebostad and Ender, 1973; Danse and Steenbergen-Botterweg, 1976, 1978; Danse and Verschuren, 1978a,b; Ruiter *et al.*, 1978; Danse *et al.,* 1979; Charnock *et al.,* 1987; Verschuren et al., 1990; Farwer et al., 1994; Hempenius *et al.*, 1997, 2000). This effect is considered a normal response to a large load of dietary lipids, and may be prevented by concurrently administering vitamin E with high-PUFA diets (Helgebostad and Ender, 1973; Verschuren *et al.,* 1990; Farwer *et al.*, 1994; Muggli, 1994; Ando *et al.*, 2000). Some study authors reported increased liver and spleen weights in mice and rats administered high PUFA diets at daily intakes ranging from 25 to 9,500 mg/kg body weight and varying durations of 4 to 13 weeks (Danse and Verschuren, 1978a,b; Boswell et al., 1996; Hempenius *et al.*, 1997; McGuire *et al.*, 1997; Burns *et al.*, 1999a; Rabbani *et al.*, 1999; Oarada *et al.*, 2000); however, no histopathological effects were observed in these organs and the increased organ weights were reported to be adaptations to accommodate the large lipid load.

The safety of DHA-rich oil is further corroborated by an extensive number of human clinical studies with endpoints relevant to determining the safety and metabolism of DHA (von Schacky *et al.*, 1985; Hostmark *et al.*, 1988; Mehta *et al.*, 1988; Radack *et al.*, 1989; Bønaa *et al.*, 1992; Deslypere, 1992; Haglund *et al.,* 1992, 1998; Kenny *et al.*, 1992; Mori *et al.*, 1992, 1997, 2000; Saynor and Gillott, 1992; Schmidt *et al.*, 1992; Almdahl *et al.*, 1993; Clark *et al.,* 1993; Connor *et al.*, 1993; Hansen *et al.*, 1993a,b, 1998a,b; Harris *et al.,* 1993, 1997; Krokan *et al.*, 1993; Mundal *et al.*, 1993; Nilsen *et al.*, 1993; Swails *et al.*, 1993; Axelrod *et al.*, 1994; Chin and Dart, 1994; Cirillo *et al.*, 1994; de Maat *et al.*, 1994; Eritsland *et al.*, 1994a,b, 1995a,b, 1996; Henderson *et al.*, 1994; Leaf *et al.*, 1994; Lungershausen *et al.*, 1994; Mackness *et al.*, 1994; McVeigh *et al.*, 1994; Mundal *et al.*, 1994; Nordøy *et al.,* 1994, 1998; Prisco *et al.*, 1994, 1995; Turini *et al.*, 1994; Bonnema *et al.*, 1995; Christensen *et al.*, 1995; Luostarinen *et al.*, 1995; Misso and Thompson, 1995; Morgan *et al.*, 1995; Sacks *et al.*, 1995; Toft *et al.*, 1995, 1997; Tremoli *et al.*, 1995; Ågren *et al.*, 1996, 1997; Bagdade *et al.*, 1996; Balestrieri *et al.*, 1996; Conquer and Holub, 1996, 1998; Engström *et al.*, 1996; Fasching *et al.,* 1996; Gray *et al.*, 1996; Hamazaki *et*

al., 1996; Innis and Hansen, 1996; Lenzi *et al.*, 1996; McGrath *et al.*, 1996; McManus *et al.*, 1996; Otto *et al.*, 1996; Palozza *et al.*, 1996; Rivellese *et al.*, 1996; Rossing *et al.*, 1996; Silva *et al.*, 1996; Wigmore *et al.*, 1996; Yosefy *et al.*, 1996; Adler and Holub, 1997; Badalamenti *et al.*, 1997; Brister and Buchanan, 1997; Freese and Mutanen, 1997a,b; Goh *et al.*, 1997; Grimsgaard *et al.*, 1997, 1998; Nelson *et al.*, 1997; Roulet *et al.*, 1997; Sanders *et al.*, 1997; Tsai and Lu, 1997; Gogos *et al.*, 1998; Kelley *et al.*, 1998, 1999; Swahn *et al.*, 1998; Burns *et al.*, 1999b; Conquer *et al.*, 1999; Grundt *et al.*, 1999; Pirich *et al.*, 1999; Seljeflot *et al.*, 1999; Véricel *et al.*, 1999; Wensing *et al.*, 1999; Almallah *et al.*, 2000; Wallace *et al.*, 2000; Yaqoob *et al.*, 2000; Woodman *et al.*, 2003). As reviewed by Kroes *et al.* (2003) and as reported by the Expert Panel, clinical studies include metabolic studies indicating that the absorption, distribution, and metabolism of DHA is similar to other fatty acids, as well as, studies examining the effect of DHA-containing oils on bleeding times and other hemostatic parameters, glycemic control, low density lipoprotein (LDL) cholesterol and other plasma lipid fractions, as well as, additional measured endpoints (*e.g.*, immune function, liver and kidney function) related to the safety of DHA. Overall, the results of these studies provide evidence that DHA, at estimated exposures provided through the intended use of DHA-rich oil, would not have an adverse effect on human health. The available data further indicates other components of DHA-rich oil, namely palmitic acid, docosapentaenoic acid, and sterols, would not have adverse effects on human health (Kroes *et al.*, 2003) under the intended conditions of use of DHA-rich oil.

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% (Mori *et al.*, 1992, 1997; Schmidt *et al.*, 1992; Mundal *et al.*, 1993; Eritsland *et al.*, 1994a; Henderson *et al.*, 1994; Leaf *et al.*, 1994; Turini *et al.*, 1994; Luostarinen *et al.*, 1995; Tremoli *et al.*, 1995; Conquer and Holub, 1996; Engström *et al.*, 1996; Parkinson *et al.*, 1996; Nelson *et al.*, 1997; Conquer and Holub, 1998; Haglund *et al.*, 1998; 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 to 15% of total fatty acids of platelet membranes and tissue phospholipids (Kroes *et al.*, 2003). There is no indication that DHA, at exposures estimated through the proposed uses of DHA-rich oil, 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.*, 1994a; 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).

The safety of DHA-rich oil is further substantiated given that the source organism, *Ulkenia sp.*, a thraustochytrid and a member of the Chromista kingdom (Bahnweg, 1979; Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999; European Register of Marine Species, 2001), has not been reported to produce toxins or to demonstrate pathogenicity (OmegaTech GmbH, 1997; Van Dolah, 2001). Thraustochytrids are known to exhibit worldwide distribution, having been identified in plankton and other marine detritus, as well as comprising a portion of the diet of filter-feeding invertebrates in the marine ecosytem (Ulken *et al.*, 1990; Sathe-Pathak *et al.*, 1993; Azevedo and Corral, 1997; Naganuma *et al.*, 1998). Due to their wide distribution throughout the marine ecosystem, thraustochytrids constitute an indirect component of the human diet through the consumption of marine creatures (*e.g.*, clams or mussels). The source organism has been demonstrated to be nonmutagenic in the Ames assay using *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 with or without metabolic activation (Fujii and Suwa, 1999). Furthermore, the source organism, *Ulkenia sp.* is taxonomically distinct from known toxin-producing organisms and no known species of thraustochytrids have been identified as producers of microalgal toxins (OmegaTech GmbH, 1997; Van Dolah, 2001). The absence of microalgal toxins has been confirmed in lot analyses of DHA-rich oil, thus demonstrating the absence of common algal and cyanobacterial toxins. Furthermore, independent evaluation by scientific experts (Melkonian, 2003; Schaumann, 2003), using data pertinent to safety, has concluded *Ulkenia sp.* to be both nontoxigenic and nonpathogenic to man.

Evaluation of the totality of the scientific data by the Expert Panel pertaining to the safety of DHA-rich oil under the intended conditions of use in foods resulted in the conclusion that DHA-rich oil, "meeting appropriate food grade specifications, and manufactured and used in accordance with current good manufacturing practice, under the conditions of intended use specified herein, is "generally recognized as safe" (GRAS)" (see Appendix I).

The intended intake of DHA through the intended food uses of DHA-rich oil will not exceed the limits assigned by the FDA through the menhaden oil GRAS, and is consistent with other ω-3, DHA-containing oils that have notified the FDA as GRAS for similar uses (FDA, 2002a,b; 2004b,c). Since the DHA level in DHA-rich oil is approximately 2 to 2.5 times higher than the combined levels of DHA and EPA in menhaden oil, the proposed use levels for DHA-rich oil of 40% of those GRAS-affirmed for menhaden oil will ensure an intake within the limits considered safe by the FDA. Furthermore, DHA-rich oil is not intended for use in combination with other oils that are significant sources of ω-3 fatty acids, DHA and EPA.

There are no indications from the published literature, or from the published studies conducted with DHA-rich oil, that under the conditions of intended use in traditional foods, DHA-rich oil would result in any adverse health effects.

IV. References

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APPENDIX I

EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DHA-RICH OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS

EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF DHA-RICH OIL UNDER THE CONDITIONS OF INTENDED USE IN TRADITIONAL FOODS

September 15, 2004

INTRODUCTION

At the request of Nutrinova Nutrition Specialties & Food Ingredients GmbH (Nutrinova), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended use as a nutrient in traditional foods, DHA-rich oil would be "generally recognized as safe" (GRAS), based on scientific procedures. DHA-rich oil is a docosahexaenoic acid (DHA)-rich triacylglycerol oil derived from *Ulkenia sp.* microalgae by Nutrinova. The Panel consisted of the below-signed qualified scientific experts: Robert Kroes, D.V.M., Ph.D. (Utrecht University), Ernst Schaefer, M.D. (Tufts University), and Gary Williams, M.D. (New York Medical College). *Curricula vitae* evidencing the Panel members' qualifications for evaluating the safety of food ingredients are provided in Attachment 1.

The Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data compiled from the literature and other published sources through May 2004. The Panel also evaluated any other data and information, published or unpublished, that the Panel deemed appropriate or pertinent to safety of DHA-rich oil under the conditions of intended use. In addition, the Panel considered data and information provided by Nutrinova, including information pertaining to the method of manufacture and product specifications, analytical data, intended use levels in specified food products, consumption estimates for all intended uses, data on the safety of similar DHA-containing fish and marine oils, and unpublished corroborating toxicological studies conducted on DHA-rich oil and the source microalgae.

Following independent, critical evaluation of such data and information, the Panel unanimously concludes that under the conditions of intended use in traditional foods described herein, DHA-rich oil, meeting appropriate food-grade specifications, and manufactured and used in accordance with current good manufacturing practice, is GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion, excluding confidential data and information, is provided below.

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MANUFACTURING AND COMPOSITION

DHA-rich oil is produced in accordance with current good manufacturing practice following multi-step fermentation of a genetically stable and pure culture of the marine protist, *Ulkenia sp.* DHA-rich oil is extracted from the microalgae *via* a solvent-based extraction process using food grade solvents and processing aids, and is further refined by processes standard to the edible oil industry, including degumming, deacidification, bleaching and deodorization, using materials appropriate for food processing. Specifications for DHA-rich oil are presented in Table 1. Analysis of nonconsecutive representative lots demonstrated compliance with final product chemical, physical, and microbiological specifications, and stability under recommended storage conditions.

Table 1 Product Specifications for DHA-rich oil

Parameter	Specification
DHA	38 to 50 (% by weight)
Trans Fatty Acids	≤2 %
Peroxide value	≤5 meq./kg
Acid value	≤0.5 mg KOH/g
Moisture and Volatiles	≤0.05 %
Unsaponifiables	≤4.5 %
Lead	≤0.1 ppm
Arsenic	≤0.5 ppm
Mercury	≤0.5 ppm

DHA-rich oil is a refined, food grade oil of >95% triacylglycerols, with a total fatty acid composition of 38 to 50% DHA. The remaining fatty acids of DHA-rich oil are comprised mainly of saturated palmitic acid (16:0) (28 to 37%) and a lesser amount (8 to 14%) of the ω-6 fatty acid, docosapentaenoic acid (DPA) (22:5). In addition, the DHA-rich oil product will contain safe and suitable antioxidants permitted by the FDA for use in edible oils to ensure stability.

INTENDED USE AND ESTIMATED INTAKE

The main dietary source of polyunsaturated fatty acids (PUFAs), such as DHA, is fish, particularly fatty fish, in which concentrations of total ω-3 fatty acids range from 0.1 to 5.3 g/ 100 g (1, 2). In the U.S., the usual consumption of ω-3 fatty acids amounts to approximately 1,600 mg/day, predominantly as α-linolenic acid (18:3), but also including eicosapentaenoic acid (EPA), DHA, and DPA. Of the total amount of ω-3 fatty acids consumed per day in the U.S., an estimated 100 to 200 mg is consumed as DHA (3, 4). Other populations with a traditionally higher consumption of DHA-containing foods, such as Japan, Norway, South Africa, and the

Portuguese island of Madeira, have average DHA intakes of approximately 500 to 700 mg DHA/day (5-9).

Several international scientific authorities have published recommendations for daily intakes of ω-3 PUFAs. For example, although the nutrition recommendations of Health Canada do not distinguish the identities of individual ω-3 PUFAs, they provide a recommended daily intake of 1,000 to 1,800 mg ω-3 PUFAs/day, in a ratio of 1:4 to 1:10 with ω-6 PUFAs (10). A workshop convened by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) concluded there was insufficient data to determine a Dietary Reference Intake (DRI) for ω-3 PUFAs; however, Adequate Intakes (AIs) of a minimum 220 mg/day each for DHA and EPA, and 650 mg/day for DHA and EPA combined were recommended (11). In Europe, the British Nutrition Foundation has recommended a desirable population intake of 1,100 mg (females) and 1,400 mg (males) of DHA and EPA/day (12).

Several ω-3 fatty acids, such as EPA (20:5) and DHA (including DHA from marine algae) also are sold as dietary supplements, and the U.S. Food and Drug Administration (FDA) currently allows a qualified health claim for ω-3 fatty acids and a reduced risk of coronary heart disease to appear in labeling for dietary supplement products (13). Additionally, under certain conditions of intended use in foods, similar oils, such as fully hydrogenated, partially hydrogenated, and refined menhaden oils, are affirmed as GRAS by FDA for use in several specified foods (21 CFR §184.1472) (14, 15). The recognized difference between menhaden oil, a refined oil derived from menhaden fish, and edible vegetable oils and animal fats is the higher percentage of PUFAs with 4 to 6 double bonds in menhaden oil, including EPA (13.7%), DHA (6.6%), and DPA (1.9%) (15). Furthermore, other DHA-containing oil ingredients are used in a number of traditional food products, as the FDA has responded with no objections against several GRAS notices submitted for other sources of ω-3 fatty acids for uses identical to those specified as GRAS for menhaden oil (16, 17, 203, 204).

The only notable differentiation regarding DHA-rich oil as compared to other DHA-containing products already on the market is the production source. DHA-rich oil is derived from a fermentation process using a nontoxigenic and nonpathogenic marine protist, which has not previously been used in the production of food products or ingredients; never the less, the fatty acids identified in DHA-rich oil are the same as those of fish and microalgal-derived oils already consumed by the human population.

Nutrinova intends to market DHA-rich oil as a food ingredient to increase the intake of dietary ω-3 PUFAs, particularly DHA, a critical component of most cell membranes and tissues.

In a final rule affirming menhaden oil as GRAS for use as a direct human food ingredient (21 CFR §184.1472) the FDA concluded that 3.0 g/person/day of EPA and DHA is a safe intake level with respect to bleeding time, increased bleeding, glycemic control and LDL cholesterol levels (15). To ascertain menhaden oil intake at a level not exceeding 3.0 g/person/day,

limitations on the maximum use levels in specific food categories were introduced. In a tentative final rule, the FDA published a revised list of food categories and use limitations for menhaden oil in these categories (202). The determination of use limitations was made on the basis of a content of EPA and DHA of approximately 20% in menhaden oil. To ensure compliance with the limitations, FDA stated that menhaden oil may not be used in combination with any other added oil that is a significant source of EPA and DHA.

The DHA content of DHA-rich oil is 38 to 50%, as shown in product specifications and product analyses. DHA-rich oil is a significant source of DHA, but contains only traces of EPA.

The individual intended food uses and use levels for DHA-rich oil are consistent with the current GRAS use of menhaden oil specified in 21 CFR §184.1472 and the revised uses outlined in FDA's tentative final rule regarding GRAS-affirmed food uses for menhaden oil. The food uses and maximum use levels for DHA-rich oil are summarized in Table 2.

Comparing the composition of menhaden oil (sum of approximately 20% of EPA and DHA) with DHA-rich oil (DHA content of 38 to 50% and no significant EPA content), it can reasonably be concluded that approximately twice as much menhaden oil as DHA-rich oil will have to be consumed for the same intake of ω-3 fatty acids. Inversely, any limitation of use levels from DHA-rich oil will therefore have to be less than 50% of the use levels of menhaden oil to ascertain compliance with the safe intake level. Consequently, the limitation of DHA-rich oil use levels to 40% of the use levels in all food categories considered by the US FDA for the GRAS use of menhaden oil would also ascertain compliance with the safe intake level of 3.0 g/person/day for the sum of EPA and DHA. To ensure safe use levels in the intended food categories and levels of use, DHA-rich oil shall not be used in combination with any other added oil that is a significant source of EPA or DHA.

Table 2 Intended Uses and Maximum Use Levels of DHA-rich oil in Food

Food Category	Initial[a] Intended Use Levels (% by weight)	Future[b] Intended Use Levels (% by weight)
Cookies, crackers (n = 1)[1,2]	3.3	-
Breads, rolls (white and dark) (1)[2]	0.7	-
Fruit pies, custard pies (1)[2]	4.7	-
Cakes (1)[2]	6.7	-
Cereals (4)	2.7	2.7
Baked Goods and baking mixes	-	3.3
Fats and oils (12)[3]	13.4	8.0
Yogurt (31)[4]	2.7	-
Milk Products (31)	-	3.3
Cheese products (31)	-	3.3
Frozen dairy products (20)	3.3	3.3

Table 2 Intended Uses and Maximum Use Levels of DHA-rich oil in Food		
Food Category	Initial[a] Intended Use Levels (% by weight)	Future[b] Intended Use Levels (% by weight)
Meat products (29)	6.7	3.3
Egg products (11)	3.3	3.3
Fish products (13)	13.4	3.3
Condiments (8)	3.3	3.3
Soup mixes (40)	2.0	3.3
Snack foods (37)	3.3	3.3
Nut products (32)	3.3	3.3
Gravies and sauces (24)	3.3	3.3
Plant protein products (33)	-	3.3
Processed vegetable juices (36)	-	0.7
Hard candy (25)	-	6.7
Soft candy (38)	-	2.7
Jams and jellies (28)	-	4.7
Dairy product analogs (10)	-	3.3
Nonalcoholic beverages (3)	-	0.3
Pastas (23)	-	1.3
Poultry products (34)	-	2.0
Processed fruit juices (35)	-	0.7
White grandulated sugar (41)	-	2.7
Sugar substitutes (42)	-	6.7
Chewing gum (6)	-	2.0
Gelatins and puddings (22)	-	0.7
Confections and frosting (9)	-	3.3
Sweet sauces, toppings, and syrups (43)	-	3.3

[a] Based on 21 CFR §184.1472
[b] Based on tentative final rule amending 21 CFR §184.1472 (69 FR 2313; January 15, 2004)
[1] The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n)
[2] Included in "Baked goods and baking mixes"
[3] Not including infant formula
[4] Included in "Milk products"

In its existing (21 CFR §184.1472) and tentative revised final rule affirming menhaden oil as generally recognized as safe, the FDA published maximum use levels for menhaden oil to ensure the total daily intake of combined DHA and EPA does not exceed 3 g/person/day. As the DHA level in DHA-rich oil is approximately 2 to 2.5 times higher than the combined levels of DHA and EPA in menhaden oil, the proposed use levels for DHA-rich oil of 40% of those GRAS-affirmed for menhaden oil, ascertain an intake within the limits considered safe by the FDA.

Furthermore, DHA-rich oil is not intended for use in combination with other oils that are significant sources of ω-3 fatty acids, DHA and EPA.

DATA PERTAINING TO SAFETY

Scientific evidence supporting the safety of DHA-rich oil includes a history of ω-3 fatty acid consumption as a result of their natural presence in food, an abundance of published metabolic, clinical, and toxicological data on DHA-containing fish and microalgal-derived oils, and unpublished corroborating toxicological and mutagenicity studies on DHA-rich oil, and mutagenicity and toxicity studies on the microalgal source, *Ulkenia sp.*

Studies of Absorption, Distribution, and Metabolism

DHA is an abundant cell membrane fatty acid (18-22), which is obtained directly in the diet or biosynthetically derived through desaturation and elongation of dietary precursor essential fatty acids (20, 23). The absorption, distribution, and metabolism of DHA are known to be similar to other dietary fatty acids. DHA is present in DHA-rich oil in triglyceride form. Following enzymatic hydrolysis of the triglyceride in the upper intestine, the free fatty acids and 2-monoglycerides are incorporated into bile acid micelles for diffusion into the enterocytes, where they are incorporated into new triglycerides (20, 21, 24). Reconstituted triglycerides enter the lymph in the form of chylomicrons and are transported to the blood for distribution and may rapidly be incorporated into plasma lipid fractions, erythrocyte membranes, platelets, and adipose tissue. In passing through the capillaries of adipose tissue and liver, the chylomicron-contained triglycerides and phospholipids are hydrolyzed with subsequent release of free fatty acids to the tissues for metabolism or reesterification into triglycerides and phospholipids for storage as energy (20, 22). Generally, free fatty acids are transported across the mitochondrial membrane in the form of acyl-carnitine (21), where they then undergo β-oxidation with removal of 2 carbons from the fatty acid chain and production of acetic acid, a shorter-chained fatty acid, and acetyl CoA, which combines with oxaloacetic acid and enters the citric acid cycle for energy production (22). As fatty acids of 20 carbons or more are not easily transported across the mitochondrial membrane, DHA also may be metabolized *via* peroxisomal β-oxidation (19, 21, 25-27). As a minor peroxisomal metabolic pathway, DHA may be retroconverted to EPA, which involves β-oxidation, auxiliary enzymes, and removal of 2 carbon units from the carboxyl end of the fatty acid (28-30).

Clinical Studies Relating to the Safety of DHA-Rich Oil

Human consumption of dietary DHA from traditional sources is considered safe based on the historical consumption of fish, and fish- and marine-based products. In 1993, the FDA identified 3 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 (January 6, 1993)] (31).

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" (15). Subsequently, in a public letter permitting a dietary supplement qualified health claim for ω-3 fatty acids and coronary heart disease, FDA stated that the use of EPA and DHA ω-3 fatty acids, including DHA from marine microalgae, as dietary supplements is safe (13).

Subsequent to FDA's 1997 GRAS affirmation of menhaden oil, a number of additional clinical studies of the potential effects of DHA-containing oils on hemostatic parameters, glycemic control, and LDL-C have been conducted. A comprehensive summary of greater than 90 published clinical trials was reviewed by the Panel (32-135). These clinical studies ranged in duration from 1 week to >1 year, and were conducted with DHA-containing fish and marine oils providing up to 6 g DHA/day, alone or in combination with DPA and/or EPA. The available clinical data do not provide evidence that DHA, at estimated exposures provided through the intended use of DHA-rich oil, would have an adverse effect on human health. The clinical data measured endpoints such as LDL-C levels, glycemic control, bleeding time, platelet aggregation, or other hemostatic parameters, as well as immune function or response, kidney or liver function, or lipid peroxidation. Overall, the results of these studies indicate that DHA, provided in fish or marine-derived oils alone or in combination with DPA and/or EPA, at levels 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.

The Panel noted that 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% (42-45, 48, 56, 58-60, 72, 73, 77, 81, 82, 84, 113, 124, 136). Arachidonic acid (AA), 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 (20, 137, 138). 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 to 15% of total fatty acids of platelet membranes and tissue phospholipids. Additionally, the fatty acid composition of DHA-rich oil comprises up to 1.8% AA. There is no indication that DHA, at exposures estimated through the proposed uses of DHA-rich oil, 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 (42-44, 46, 48, 56, 58-60, 72, 73, 77, 81, 82, 84, 124-128, 136).

When viewed in its entirety, the scientific evidence from published clinical studies with DHA-containing oils indicates that the consumption of DHA-rich oil, under the conditions of intended use, would not be expected to produce adverse effects on human health.

Toxicological Studies

Safety of Other DHA-Containing Oils

The safety of DHA-containing fish and microalgal-derived oils has been studied extensively. Overall, the data from these toxicological studies indicate that gavage or dietary administration of fish or microalgal-derived oils does not produce significant adverse effects on mortality, body weight gains, food consumption, or clinical observations in laboratory animals including mice, rats, and pigs (25, 139-150). Yellow fat disease, which is characterized by accretion of lipofuscin pigment, degeneration of adipose cells (steatosis), and inflammation of adipose tissue (steatitis) (139, 151, 152), has been reported to occur naturally in wildlife species and was reported in rats, rabbits, mink, and pigs in various toxicological studies following the consumption of diets rich in ω-3 PUFAs (*i.e.*, ω-3 PUFA-containing oils at dietary concentrations ranging from as high as 12 to 19% for periods of 8 weeks to 12 months in length) in combination with a vitamin E deficient state (139, 140, 142, 147, 151-159). This effect is considered a normal response to a large load of dietary lipids, and may be prevented by concurrently administering vitamin E with high-PUFA diets (154, 158-161). Some study authors reported increased liver and spleen weights in mice and rats administered high PUFA diets at daily intakes ranging from 25 to 9,500 mg/kg body weight and varying durations of 4 to 13 weeks (139, 141, 142, 144, 147, 148, 162, 163); however, no histopathological effects were observed in these organs and the increased organ weights were reported to be adaptations to accommodate the large lipid load.

Studies of DHA-Rich Oil

In addition to a critical review of the available scientific data on the safety of DHA-containing fish and other microalgal-derived oils, the safety of DHA-rich oil (reported as DHA45-oil) derived from *Ulkenia sp.* was assessed in various genotoxicity and acute, subchronic, and reproductive toxicity studies. These studies were summarized and reported by Kroes *et al.* (201).

DHA-rich oil (reported as DHA45-oil) was not genotoxic in the Ames assay using *Salmonella typhimurium* and *Escherichia coli* WP2 *uvrA*, with or without metabolic activation (164, 165, 201), or in a chromosomal aberration assay in Chinese hamster fibroblast cells (166, 201). DHA-rich oil (reported as DHA45-oil) was reported to have low acute oral toxicity in mice and rats (*i.e.*, LD_{50} values >2,000 mg DHA-rich oil/kg body weight) (167, 168, 201).

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In a subchronic toxicity study of DHA-rich oil (reported as DHA45-oil), groups of 30 Sprague-Dawley Crj:CD (SD) IGS rats (15/sex/group) were administered distilled water or various combinations of DHA-rich oil and DHA27 (an oil containing 27% DHA) by daily oral gavage for a period of 90 days (169, 201). The combinations of DHA-rich oil/DHA27 tested included 0/2,000, 500/1,500, 1,000/1,000, and 2,000/0 mg/kg body weight/day. Based on total DHA oil content, these dosing regiments provided daily doses of 540, 630, 720, and 900 mg DHA/kg body weight/day, respectively. Additional groups of 5 rats/sex/dose receiving distilled water or 2,000 mg/kg body weight/day of DHA-rich oil or DHA27 were allowed a 4-week recovery period following the 90 days of dosing. Effects of treatment on mortality and clinical signs, neurologic responses, body weight gain, food and water consumption, hematology, clinical chemistry, urinalysis, and on the results of ophthalmology, gross pathological, and histopathological examinations were evaluated. There were no treatment-related differences between DHA27 and DHA-rich oil. Compared to the water controls, there were no biologically significant effects of treatment with DHA-rich oil alone or in combination with DHA27. At necropsy, increased relative liver weights were reported in animals fed DHA-rich oil alone or 1,000 mg/kg body weight/day of each oil, which were likely the result of the large lipid load, as reported for rats in several published feeding and gavage studies with fish and marine algal-derived oils providing daily intakes ranging from 25 to 9,500 mg/kg body weight for durations of 4 to 13 weeks. In the absence of histopathological lesions or changes in enzymes indicative of liver toxicity, the increased liver weights were considered to be not toxic in nature. Similarly, reported increases in the absolute weights, and in some cases, relative weights, of several organs including the spleen, kidneys, and adrenals were not considered to be of toxicological significance as there were no histopathological correlates to the organ weight findings and, in all cases, the magnitude of change was small. Thus, 90-day gavage treatment with DHA-rich oil, alone or in combination with DHA27 was reported to have no adverse effect on the findings of the ophthalmology, gross pathology, or histopathology examinations. Additionally, dose-dependent effects were not observed with increasing levels of DHA administration. Given the lack of histopathological effects, the NOAEL in this study was considered to be 2,000 mg DHA-rich oil/kg body weight/day (approximately 900 mg DHA/kg body weight/day), which was the highest dose tested.

The *in utero* toxicity of DHA-rich oil (reported as DHA45-oil) produced from *Ulkenia sp.* was evaluated in a one-generation study in rats (170, 201). Groups of 56 Wistar (Crl:(WI)WU BR) rats (28/sex/group) were administered DHA-rich oil in the diet at concentrations of 0 (control), 1.5, 3.0, or 7.5% for 10 weeks prior to mating. The total intake of test material (as DHA-rich oil) ranged throughout the different phases of the study period and is presented in Table 3. After successful mating, males were sacrificed and necropsied, while females continued to be exposed at their respective concentrations and were allowed to deliver. On post-natal Day (PND) 21, all pups were weaned and sacrificed after gross external evaluation. 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 F_0 animals. There was no effect of treatment with DHA-rich oil on mortality or clinical signs. Sporadic increases in body weight gains were reported for some of the treatment groups compared to corn oil controls; however, differences in food consumption also were detailed, which the authors indicated may have accounted for the observations of weight gains in these groups. With respect to reproductive parameters, treatment with DHA-rich oil was reported to have no effect on precoital time, mating index, fertility indices, fecundity, gestation index and duration, numbers of stillborn pups, or post-implantation losses. Similarly, there were no adverse effects of treatment on the numbers of pups, pups/litter, pup mortality, sex ratio, pup weight, or incidence of malformations. Absolute liver weights were increased in mid- and high-dose males, and in low- and high-dose females; however, these effects were considered by the study authors to be of no toxicological significance, as no gross or histopathological correlates were reported and similar effects have been reported in rats in several feeding and gavage studies of fish and marine algal-derived oils. Increased absolute and relative spleen weights were reported and were associated with an increased incidence and/or severity of extramedullary hematopoiesis observed histologically. The only significant gross pathology reported was the presence of "yellow spots" within the abdominal adipose tissue of the F_0 animals. These yellow spots were reportedly compatible with findings associated with yellow fat disease, and microscopic evaluation of these yellow spots yielded the diagnosis of lipogranuloma by the study authors. To address these reported effects, a panel of internationally recognized expert pathologists (Expert Pathology Panel) was convened to independently and collectively critically evaluate the scientific data of this study, as well as additional relevant data compiled from the published literature and any other data and information deemed by the pathology experts to be pertinent to the evaluation. The Expert Pathology Panel agreed that the reported effects were related to the administration of high levels of DHA-rich oil (reported as DHA45-oil), but, they concluded 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 are relatively low in antioxidants (171). The Expert Pathology Panel unanimously concluded that the steatitis was the "result of a nutritional imbalance... attributable to the extremely high levels of DHA45-oil administered", the increased spleen weights were due to extramedullary hematopoiesis "consistent with a response to steatitis," and 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 findings of the Expert Panel were reported by Kroes *et al.* (201).

Safety of Other Components of DHA-Rich Oil

Palmitic Acid

The total fatty acid content of DHA-rich oil comprises approximately 33% palmitic acid (16:0) of total fatty acids. Palmitic acid is a natural component of the human diet and has been identified in meat, poultry, fish, grain products, and milk and milk products (172). Palmitic acid was

reported to have an estimated daily intake in the U.S. population in the range of 11 to 19 g/day (172).

The exposure to DHA from the intended food uses will be a maximum of 3 g/day under the conditions of intended use. On the basis of DHA-rich oil containing a minimum of 43% DHA of the total fatty acid content, the maximum intake of palmitic acid corresponds to approximately 2.3 g/day. This intake is at least 5 times less than the amount of palmitic acid already consumed as a natural constituent of the diet, and thus the estimated intake of palmitic acid through the intended use of DHA-rich oil would not have an impact on the relative amount of daily dietary palmitic acid. Therefore, the estimated intake of palmitic acid through the proposed uses of DHA-rich oil would not be expected to produce adverse effects on human health.

DPA

The fatty acid composition of DHA-rich oil includes up to 14 and 0.6%, respectively, of the ω-6 and ω-3 isomers of DPA. Similar to DHA, both forms of DPA are cell membrane fatty acids (23, 29, 173-175) which may be derived by biosynthesis through elongation, desaturation, or shortening of endogenous long chain fatty acids (20, 23, 27-29), or by the dietary intake of foods such as fish, seal, or fish or microalgal-derived oils (44, 176, 177). As both DPA isomers are component fatty acids of fish oil, the safety of ω-3 and ω-6 DPA is supported by the numerous non-clinical and clinical studies that have reported no adverse effects following dietary supplementation with fish or marine oil.

In a series of dietary studies in rats and rabbits that evaluated the subchronic, reproductive, and developmental toxicity of microalgal-derived DPA (ω-6)-containing oils (calculated to provide doses of up to 630 mg DPA/kg body weight/day) (149, 150, 178), no compound-related effects on survival, clinical observations, body weight gains, food consumption, urinalysis measurements, or gross necropsy findings, and no effect of treatment on hematological parameters, spleen weight, or on the gross and histopathological appearance of the spleen or adipose tissue were reported. Overall, there were no reported effects of treatment on reproductive performance, duration of gestation, mean litter size, or number of litters with live and/or dead pups, and no effects on postimplantation loss, mean fetal or pup body weight/litter, or morphological developments in either species.

In zinc deficient and non-deficient rats provided a dietary dose of up to 17 mg DPA/kg body weight for a period of 6 weeks, no differences between non-zinc deficient control or treatment groups with respect to final body weight, absolute liver and testes weight, plasma and testes zinc levels, or spermatid content of the testis parenchyma were reported (173).

Additionally, as described previously, subchronic toxicity and one-generation reproduction studies were performed in rats using DHA-rich oil (reported as DHA45-oil) (201). In the 90-day subchronic toxicity study, gavage administration of combinations of DHA-rich oil/DHA27 to

groups of 30 Sprague-Dawley Crj:CD (SD) IGS rats (15/sex/group) provided daily combined ω-3 and ω-6 DPA doses of at least 0, 41, 82, and 163 mg DPA/kg body weight/day (169, 201). As described previously, there were no biologically significant effects of treatment with DHA-rich oil at the highest dose evaluated in the study, 2,000 mg DHA-rich oil/kg body weight/day, providing 163 mg DPA/kg body weight/day. In the one-generation reproductive toxicity study in Wistar (Crl:(WI)WU BR) rats (28/sex/group), DPA intakes ranged from 65 to 913 mg DPA/kg body weight/day (see Table 3), without adverse toxicological effects attributable to DHA-rich oil, or components thereof (170, 201). As discussed above, lipogranuloma of the abdominal adipose tissue and increased absolute and relative spleen weights associated with an increased incidence and/or severity of extramedullary hematopoiesis were reported in some of the treatment animals compared to controls, and it was unanimously concluded by the Expert Pathology Panel that 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" (201).

Table 3 Estimated Daily Intake of DHA-rich oil, DHA, and DPA[1] by Rats in the One-Generation Reproduction Study

	Low-Dose Group (1.5% DHA-rich oil)	Mid-Dose Group (3.0% DHA-rich oil)	High-Dose Group (7.5% DHA-rich oil)
	Estimated Intake of DHA-rich oil (mg/kg body weight/day)		
Males	800 to 1,000	1,500 to 2,000	3,400 to 4,700
Females (pre-mating)	1,800 to 2,200	3,400 to 4,300	7,900 to 9,700
Females (gestation and lactation Days 1 to 14)	1,800 to 2,700	3,700 to 5,300	7,800 to 11,200
	Estimated Intake of DHA (mg/kg body weight/day)		
Males	360 to 450	675 to 900	1,530 to 2,115
Females (pre-mating)	810 to 990	1,530 to 1,935	3,555 to 4,365
Females (gestation and lactation Days 1 to 14)	810 to 1,215	1,665 to 2,385	3,510 to 5,040
	Estimated Intake of DPA (mg/kg body weight/day)		
Males	65 to 82	122 to 163	277 to 383
Females (pre-mating)	147 to 179	277 to 350	644 to 791
Females (gestation and lactation Days 1 to 14)	147 to 220	302 to 432	636 to 913

[1] Estimated DPA values were calculated using a minimum value of 8.15% as the DPA (ω-6 + ω-3) content of DHA-rich oil.

Although no clinical studies were identified that evaluated the possible effects of DPA alone, 6 studies of fish or marine-derived oils with reported DPA contents were reviewed and provide supporting evidence for the safety of dietary DPA. No significant effects on glycemic control or LDL-C 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) (54, 56, 84, 98, 99). 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 and therefore the Panel considered that the overall effect of the fish oil on platelet aggregation is not clear (54). 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 (84). 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.* (84) and Axelrod *et al.* (54) cannot be attributed to a single PUFA, such as DPA. 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-C levels were decreased by 2% and no significant effects on glycemic control and were reported (44). Considering the totality of available evidence, the estimated intake of DPA (<1 g/day) through the proposed uses of DHA-rich oil would not be expected to produce adverse effects in humans.

Sterols

The unsaponifiable fraction of DHA-rich oil is low and consists primarily of sterols at a level around or even below 1%. Representative lot analyses of DHA-rich oil identified the main sterol components as cholesterol, 7-dehydrostigmasterol and 4-methyl-chondrillasterol.

The safety of dietary cholesterol and other sterols is well documented in the scientific literature. Cholesterol is a natural constituent of the human diet, and 4-methyl sterols have been identified in the normal metabolic pathway of cholesterol biosynthesis in man, and in several food sources, including fish, shellfish, and rice bran (180, 181). 7-Dehydrostigmasterol was quantified in phytosterols purified from the unsaponifiable fraction of soybean oil (182). Hence, although quantitatively minor constituents, the sterols in DHA-rich oil would be considered to already occur in the human diet.

With typical sterol levels below 1% in DHA-rich oil, the level of intake of sterols under the intended conditions of use of DHA-rich oil would be minimal. Typical cholesterol levels are around 30% of total sterols. Assuming a maximum intake of DHA-rich oil of 7.9 g/day (based on 38% DHA in DHA-rich oil contributing to 3 g/person/day), a total sterol content of 1% and 30% of sterols being cholesterol, the theoretical maximum daily cholesterol intake from DHA-rich oil would be approximately 24 mg. Similarly, the theoretical maximum intake for phytosterols

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[1]Calculated based on the density value for menhaden fish oil (179)

would be approximately 55 mg/day. The safety of dietary cholesterol and phytosterols is well documented in the scientific literature. Cholesterol and phytosterols are natural constituents of the diet and 4-methyl sterols occur in the endogenous metabolic pathway of cholesterol biosynthesis. These intakes well below the amounts of sterols already consumed as natural constituents of the diet (up to 400 mg/person/day) (183-187), and thus, the estimated intake of sterols through the proposed uses of DHA-rich oil would not have an impact on the relative amount of cholesterol and phytosterols already consumed in the diet. Therefore, the dietary exposure to cholesterol, 7-dehydrostigmasterol, and 4-methyl-chondrillasterol from the intended use of DHA-rich oil would not be expected to produce adverse effects on human health.

Studies Pertaining to the Safety of the Source Organism

The source organism (a thraustochytrid) is a member of the kingdom Chromista (188-191). Although there are no data demonstrating detailed human consumption of the source organism, thraustochytrids have been identified in plankton and other marine detritus and comprise a portion of the diet of filter-feeding invertebrates, and thus constitute an indirect component of the human diet through consumption of fish and other marine animals (192-195). There have been no identified reports of *Ulkenia sp.* producing toxic chemicals or pathogenicity (196, 197). Microalgal toxin analysis of representative lots of DHA-rich oil confirmed the absence of the common algal and cyanobacterial toxins, such as domoic acid, Paralytic Shellfish Poisons (PSP), Diarrhetic Shellfish Poisons (DSP), Neutrotoxic Shellfish Poisons (NSP), pectenotoxins, yessotoxins, azaspiracides, Prymnesium toxins, and microcystins and nodularin. Additionally, independent scientific expert evaluation of the available data pertinent to the safety of the microalgal source concluded *Ulkenia sp.* is both nontoxigenic and nonpathogenic to man (198, 205).

The conclusion that the microalgae is nontoxicogenic is supported by mutagenicity and classical rodent toxicity studies using the DHA-rich oil source organism. The microalgal source was reported to produce negative results in the Ames assay using *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102, with or without metabolic activation (199). In a 14-day feeding study, the microalgal source suspended in distilled water was administered orally to ICR male mice (SPR) at a dose of 2 g source organism/kg body weight/day (200). A control group received distilled water only. No significant differences in clinical signs, body weight gains, or necropsy observations were reported for the microalgal source-treated mice.

Summary

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Overall, when viewed in its entirety, the scientific data summarized above support the safe intake of DHA-rich oil under the conditions of intended use in traditional foods. While the production of DHA-rich oil from the microalgae *Ulkenia sp.* for use as a food ingredient is unique, the source microorganism is nontoxigenic and nonpathogenic, and all components of DHA-rich oil have a history of occurrence in the diet. The safety of DHA-rich oil is established

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. Clinical studies reveal no potential for toxicity of DHA under the conditions of intended use. The epidemiologic and clinical data are supported by animal toxicity studies, which are corroborated by studies on DHA-rich oil (reported as DHA45-oil). The Panel noted the growing body of evidence of cardiovascular risk reduction with the use of ω-3 fatty acids in randomized placebo-controlled clinical trials.

There are no indications from the published literature, or from the published studies conducted with DHA-rich oil, that under the conditions of intended use in traditional foods, DHA-rich oil would result in any adverse health effects.

CONCLUSION

Based on our independent and collective, critical review of the available pertinent scientific evidence, as members of an Expert Panel specially convened for this purpose, we unanimously conclude that DHA-rich oil, meeting appropriate food grade specifications, and manufactured and used in accordance with current good manufacturing practice, under the conditions of intended use specified herein, is "generally recognized as safe" (GRAS), based on scientific procedures.

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000044

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ATTACHMENT 1

CURRICULA VITAE OF EXPERT PANEL MEMBERS

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Submission End

APPENDIX C

Expert Opinions and Microalgal Toxin Analysis

APPENDIX C.1

Expert Pathology Report Concerning the Gross and Histopathological Findings of a One-Generation Rat Reproduction Study of DHA-Rich Oil

EXPERT PATHOLOGY PANEL REPORT CONCERNING THE GROSS AND HISTOPATHOLOGICAL FINDINGS OF A ONE-GENERATION RAT REPRODUCTION STUDY OF DHA45 OIL

August 9, 2001

Introduction

As independent experts qualified by relevant national and international experience and scientific training in pathology, we, the undersigned Robert Squire, D.V.M., Ph.D., and Gary Williams, M.D., were asked by Celanese Ventures GmbH ("Celanese") to evaluate the findings of a one-generation rat reproduction study conducted with DHA45 Oil. *Curricula vitae* evidencing our qualifications in pathology and toxicology are provided in Attachment 1.

We critically and independently examined scientific information and data, provided by Celanese and TNO Food and Nutrition Research (TNO) who conducted the one-generation reproduction study. In addition, we evaluated other information deemed appropriate or necessary, including data and information compiled from the literature and other published sources. The data evaluated by the Panel included information pertaining to the method of manufacture and specifications for DHA45 Oil, the final report of a 90-day subchronic rat study conducted by the Nippon Experimental Medical Research Institute Co., Ltd., the final report and representative microscopic slides from a one-generation rat reproduction study conducted by TNO, and literature studies with other oils, including those containing DHA.

Following independent, critical evaluation of the data and information, we met as an Expert Pathology Panel on August 9, 2001 in Baltimore, MD. A summary of the basis for our conclusion is provided below.

Manufacturing and Specifications

According to manufacture, DHA45 Oil is derived from the marine algae, *Ulkenia sp.*, through a fermentation process. The resultant crude DHA-containing oil is refined through degumming, deacidification, bleaching, and deodorizing processes to produce the final DHA45 Oil product. DHA45 Oil is >95% triacylglycerols, with DHA:DHA:DHA, DHA:DHA:C16, and DHA:C16:C16 present as the main triacylglycerol components. DHA is present as 43.0 to 50.0% of the total fatty acids of DHA45 Oil. The Panel did not independently evaluate or confirm the safety of *Ulkenia sp.*, as the source for DHA45 Oil; however, information from Celanese reported no indications for parasitic or pathogenic concern to human safety.

Toxicology Studies on DHA45 Oil From *Ulkenia sp.*

90-Day Subchronic Rat Study

The subchronic toxicity of DHA45 oil was evaluated in comparison to a commercially available oil containing 27% DHA (DHA27), as well as in comparison to control groups administered water (Neda, 2000). In this GLP-compliant study, groups of 15 male and 15 female Sprague-Dawley Crj:CD (SD) IGS rats were administered distilled water or various combinations of DHA45 oil/DHA27 oil by daily oral gavage, for 90 days. The combination doses of DHA45 oil/DHA27 oil tested included 0/2,000mg/kg body weight/day, 500/1,500 mg/kg body weight/day, 1,000/1,000 mg/kg body weight/day, and 2,000/0 mg/kg body weight/day, respectively. Based on total DHA oil content, these dosing regiments provided for daily DHA doses of 0, 540, 630, 720, and 900 mg DHA/kg body weight/day. Additional groups of 5 rats/sex/dose receiving distilled water or either 2,000 mg/kg body weight/day of DHA45 oil or 2,000 mg/kg body weight/day of DHA27 oil were allowed a 4-week treatment-free period following the 90-days of dosing. The basal diet for all animals was AIN-76A compound feed (corn starch 55.0%, casein 20.0%, sucrose 10.0%, cellulose powder 5.0%, corn oil 5.0%, AIN-76A mineral mix 3.5%, AIN-76A vitamin mix [% vitamin E not specified], DL-methionine 0.3%, and choline bitartrate 0.2%). Total exposure of controls and treated groups to vitamin E could not be determined from the data presented.

The study evaluated the effects of treatment on mortality and clinical signs, neurologic responses, body weight gain, food and water consumption, hematology, clinical chemistry, urinalysis, and on the results of ophthalmology, gross pathology, and histopathology examinations. Gross pathological and histopathological examinations were conducted only on controls and high-dose animals (*i.e.*, groups administered either 2000 mg DHA45 oil/kg body weight/day or 2,000 mg DHA27 oil/kg body weight/day).

There were no biologically significant effects of treatment with DHA45 oil alone (2,000 mg/kg body weight/day) or in combination with DHA27 oil, on mortality, clinical signs, responses to stimuli, and food and water consumption. Hematological analysis revealed increased lymphocyte ratio and decreased neutrophil ratio in groups treated with 2,000 mg/kg body weight/day of either DHA27 or DHA45 oils. Decreased RBC counts were reported in females administered 2,000 mg/kg body weight/day of DHA27oil and in those administered 1,000 mg DHA27 oil and 1,000 mg DHA45 oil/kg body weight/day. Following the 4-week treatment-free period, significantly prolonged prothrombin times were recorded for males administered 2,000 mg DHA27 oil/kg body weight/day. Activated partial thromboplastin times were significantly reduced in females administered 2,000 mg/kg body weight day of either DHA27 oil or DHA45 oil. There were no biologically significant differences on hematological parameters between DHA27 and DHA45 oils.

As expected with ω-3 PUFA treatment, both DHA27 and DHA45 oils, alone and in combination, resulted in decreased total cholesterol, phosphoplipid and free fatty acid levels compared to

water-treated controls. Other changes included: tendency to increased alkaline phosphatase, albumin/globulin ratio, and albumin fraction ratio in males of all treatment groups; significantly decreased BUN in males administered 2,000 mg DHA45 oil/kg body weight/day or 500 mg DHA45 oil and 1,500 mg DHA27 oil/kg body weight/day, and; a general decrease in bilirubin values in females from each treatment group. These changes generally disappeared during the treatment-free period. None of the alterations in clinical chemistry parameters were considered to be of toxicological significance, and, in fact, are known consequences of feeding high levels of ω-3 PUFA.

At the end of the treatment period, urinalysis revealed significantly lower, or a tendency for lower, excretion of sodium in treated males and significantly lower total excretion of potassium in males administered 2,000 mg DHA45 oil/kg body weight day or 1,000 mg DHA45 oil and 1,000 mg DHA27 oil/kg body weight/day. There were no differences in treatment with DHA27 oil or DHA45 oil. Following the 4-week treatment-free period, females administered 2,000 mg DHA45 oil/kg body weight/day were found to have significantly increased total excretion of sodium and potassium in relation to water-treated controls.

At necropsy, a number of absolute and relative organ weight changes were found to be treatment-related. Most notable were increased relative liver weights in treated females and in males fed 2,000 mg DHA45 oil/kg body weight/day or 1000 mg DHA45 oil and 1,000 mg DHA27 oil/kg body weight/day. In addition, there were other reported increases in the absolute weights, and in some cases, relative weights, of several organs including the spleen, kidneys, and adrenals. The organ weight changes were not considered to be of any toxicological significance as there were no histopathological correlates to the organ weight findings and, in all cases, the magnitude of change was small. Increased liver weights were likely a reflection of adaptation to the high exposure to PUFA. The effect was not toxic in nature since there were no histopathological lesions present, and there were no changes in serum GOT and GPT indicative of liver toxicity.

Treatment with DHA45 or DHA 27 oils, alone or in combination, was reported to have no effect on the findings of the ophthalmology, gross pathology, and histopathology examinations. In the high-dose group, there was no report of histological change in adipose tissue or of increased extramedullary hematopoiesis in the spleen.

In this 90-day study (Neda, 2000), no adverse effects were reported that could be attributable to administration of DHA45 oil.

One-Generation Rat Reproduction Study

The toxicity of DHA45 oil was evaluated in a one-generation GLP-study in Wistar rats (Kuilman and Waalkens-Berendsen, 2001). Groups of 28 male and 28 female Wistar (Crl:(WI)WU BR) rats were administered DHA45 oil in the diet at concentrations of 0, 1.5, 3.0, or 7.5% for 10 weeks prior to mating. Controls received 7.5% corn oil in the diet. The basal diet for all animals

was Rat and Mouse No. 3 breeding diet (RM3) (20.2% crude protein, 4.5% crude fibre, 4.1% crude fat, 11.4% moisture, 6.2% ash). The vitamin E content of the basal diet (prior to mixing with the test substances) was stated to be 133 ppm (w/w) providing a total dose of about 2 mg/day (5-7 mg/kg body weight/day).

After successful mating, males were sacrificed and necropsied, and females continued to be exposed at their respective concentrations, and allowed to give birth. Thus parental females were exposed to DHA for a longer period of time then were males. On post-natal Day 21, all pups and F_0 females were sacrificed. 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 F_0 animals.

There was no effect of treatment with DHA45 oil on mortality or clinical signs. The total intake of test material (as DHA45 oil) ranged from 800-1,000, 1,500-2,000, and 3,400-4,700 mg/kg body weight/day in males of the low-, mid-, and high-dose groups, respectively. In females, during pre-mating, intake of DHA45 oil ranged from 1,800-2,200, 3,400-4,300, and 7,900-9,700 mg/kg body weight/day. During gestation and lactation Days 1-14, DHA45 oil intakes in females ranged from 1,800-2,700, 3,700-5,300, and 7,800-11,200 mg/kg body weight/day, respectively. These dosage values can be converted to dosages in terms of mg DHA/kg body weight/day values by multiplying by 0.45.

With respect to reproductive parameters, treatment with DHA45 oil had no effect on precoital time, mating index, fertility indices, fecundity, gestation index and duration, numbers of stillborn pups and post-implantation losses. Similarly, 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 treatment-related effect on the incidence of malformations.

At necropsy of the parental animals, several notable organ weight changes were identified. Absolute liver weights were increased in mid- and high-dose males, and in low- and high-dose females. Relative liver weights were increased in the mid-dose males. Absolute spleen weights were dose-dependently increased in both sexes, attaining statistical significance in mid- and high-dose animals of each sex. Similarly, relative spleen weights were increased in treated males and in mid- and high-dose females.

The only significant reported gross lesions were the presence of yellow spots within the abdominal adipose tissue of a few high-dose males and in many of the high-dose females. These yellow spots were reportedly compatible with findings associated with "yellow fat disease", a condition known to occur in certain species of animals consuming diets rich in ω-3 PUFA and which are in a vitamin E deficient state (Green and Bunyan, 1969; Danse and Verschuren, 1978; Danse, 1989; Verschuren *et al.* 1990; Pollard and Sanders, 1993; Farwer *et al.*, 1994). Microscopic evaluation of these "yellow spots" were reported as lipogranuloma. Also, additional lipogranulomas were reported in the abdominal adipose tissue of the low- and mid-dose groups.

The study authors reported that the presence of the lipogranulomas, indicative of ongoing inflammatory responses, may have been associated with the extramedullary hematopoiesis of the spleen since this is a common response to inflammation (Losco, 1992).

Toxicological Studies with DHA from Other Marine Sources

DHA oil, either alone or in combination with the ω-6 fatty acid ARA, produced from the marine algae *Crypthecodinium cohnii*, was tested in 2 separate 4-week studies (Boswell *et al.*, 1996; Wibert *et al.*, 1997), in a one-generation reproduction study (Burns *et al.*, 1999), and in a 90-day subchronic toxicity study (Arterburn *et al.*, 2000); all in the Sprague-Dawley rat. A microalgae preparation of *Schizotrichium sp* containing DHA at concentrations in the range of 8% was tested in a 90-day subchronic toxicity study in Sprague-Dawley rats (Hammond *et al.*, 2001a), a one-generation reproductive toxicity in Sprague-Dawley rats (Hammond *et al.*, 2001b), and in a development toxicity study in both Sprague-Dawley rats and in New Zealand White rabbits (Hammond *et al.*, 2001c)

No effects on the spleen or on adipose tissue were reported in the 4-week studies, or in the 90-day subchronic toxicity study on DHA oil produced from *Crypthecodinium cohnii* (Arterburn *et al.*, 2000). Only in the one-generation reproduction study, the same type of study in which effects for DHA45 oil were reported (Kuilman and Waalkens-Berendsen, 2001), was DHA produced by *C. cohnii* reported to increase spleen weight (Burns *et al.*, 1999). However, contrary to the study with DHA45 oil, Burns *et al.* (1999) reported that there were no histological changes in the spleen. None of the studies conducted with DHA produced by *C. cohnii* reported an effect of treatment on adipose tissue. The lack of finding of gross or of microscopic evidence of "yellow fat disease", in comparison to one-generation reproduction study with DHA45 oil (Kuilman and Waalkens-Berendsen, 2001), may be related to the shorter duration of exposure in the 4-week studies (Boswell *et al.*, 1996; Wibert *et al.*, 1997), lower rates of either total oil and/or total DHA exposure (at least with respect to doses associated with gross evidence of steatitis as reported for DHA45 oil), and possible differences in the basal diets and degree of vitamin E supplementation.

As with the studies on DHA oil produced from *C. cohnii*, the lack of reports of effects of a DHA-containing microalgae preparation of *Schizotrichium sp.* on the spleen or adipose tissue in a 90-day (Hammond *et al.*, 2001a) and in a one-generation reproduction study (Hammond *et al.* 2001b) can be explained by: a) low maximum doses of total DHA and total ω -3 fatty acids administered in the 90-day study (*i.e.*, 336 and 197 mg DHA/kg body weight/day) and, b) by the lack of recording of spleen weight and histopathology of this organ and of adipose tissue in the one-generation reproduction study (Hammond *et al.*, 2001b). In addition, the dried preparation of *Schizotrichium sp.* contained only about 8% DHA, much less than the DHA concentration of DHA45 oil (45%).

Fish oils, containing various concentrations of DHA and other long-chain PUFAs, have been demonstrated to induce development of "yellow fat disease" in rats (Mason *et al.*, 1946; Danse and Verschuren, 1978a,b; Danse and Steenbergen-Botterweg, 1978; Charnock *et al.*, 1987; Pollard and Sanders, 1993; Farwer *et al.*, 1999; Hempenius *et al.*, 2000). Similarly, other toxicology studies conducted with rats have shown that high dietary concentrations of fish oil increase spleen weight (Danse and Verschuren, 1978; Hempenius *et al.*, 2000; Rabbani *et al.*, 1999).

Conclusions

The Panel affirmed that in the one-generation reproduction study findings related to DHA45 Oil administration were present in the adipose tissue and spleen of the parental generation.

The adipose tissue alteration was concluded by the Panel to be steatitis which is a recognized consequence of peroxidation of administered lipid. This lesion occurs naturally and experimentally in many different species fed diets that are high in unsaturated fatty acids, including DHA and DHA-containing fish oils, and are relatively low in antioxidants, particularly vitamin E and selenium. Thus, the steatitis is a result of a nutritional imbalance, which in the one-generation reproduction study is attributable to the extremely high levels of DHA45 Oil administered. There is little relevance of this finding to humans since this condition has not been documented in humans and the doses used in the study greatly exceeded any anticipated human intakes of DHA45 Oil.

The increased spleen weights appear to be due to increased red pulp with extramedullary hematopoiesis. Extramedullary hematopoiesis is normal in the spleen of rats and increases with active inflammation. The slight degree of extramedullary hematopoiesis observed in this study is consistent with a response to the steatitis and thus is also of little relevance to humans.

In summary, 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.

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APPENDIX C.2

Scientific Expert Opinions Concerning the Safety of *Ulkenia* sp.

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Expert's Report on the Safety of *Ulkenia* (Thraustochytriales) in a Human-biological Context

The aim of this expert report is, based on current knowledge, to examine to what extent the *Ulkenia* genus as well as related genera of the Thraustochytriales order can be described as pathogenic or have a damaging influence on organisms, particularly humans.

The expert report is based on the expert's own knowledge as well as extensive literature research and is split into the following sections:

1. Taxonomy of *Ulkenia* in the systematic environment of the kingdom of Stramenopila
2. Occurrence of toxins in Thraustochytriales and in *Ulkenia* (data from literature)
3. Discussion of toxin analyses in Thraustochytriales and in *Ulkenia*
4. Toxins in algae and Stramenopila: Reference to the Thraustochytriales and to oils extracted from these.

1. Taxonomy of *Ulkenia* in the Systematic Environment of the Kingdom of Stramenopila

Ulkenia is a marine protist, which, together with six other genera (*Althornia, Aplanochytrium, Japonochytrium, Labyrinthuloides, Schizochytrium and Thraustochytrium*) is classed as being a member of the Thraustochytriales order, SPARROW 1973 (with a family Thraustochytriaceae, SPARROW ex CEJP 1959, often the later name of the family, Thraustochytriidae, OLIVE 1975, is used). With the second order, Labyrinthulales DOFLEIN 1901 (with the only family being Labyrinthulaceae CIENKOWSKI 1867; here too the later name of the family, Labyrinthulidae OLIVE 1975 is commonly used) the Thraustochytriales form the Labyrinthulomycota phylum, OLIVE 1975 (= Labyrinthulomorpha LEVINE 1980 = class Labyrinthulea, OLIVE ex CAVALIER-SMITH 1989).

For a long time, there were disputes about the taxonomical status of the Labyrinthulomycota or Labyrinthulea; the two orders were not even regarded as relatives. Most of the time, the Labyrinthulales were classed as amoeba (Rhizopodea) within the protozoa kingdom or slime moulds (Mycetozoa) within the fungi kingdom. The Thraustochytriales, on the other hand, were classed as oomycetes within the fungi kingdom. Due to ultrastructural similarities (biflagellate, heterokont zoospores, formation of the characteristic ectoplasmic net via a special cell organelle sagenogenetosome, tubular, mitochondrial cristae), it was finally

recognised that both orders were closely related and these were united in 1975 by Olive to form a single phylum of the protists, the Labyrinthulomycota. Their ultrastructural features meant that the Labyrinthulomycota were related to the Stramenopila kingdom (Patterson 1989), a large group of organisms which are characterised by the existence of tripartite flagellar hairs on one of the two flagellates of their flagellate cells (often the term Chromista, Cavalier-Smith 1986, is used as a synonym for the Stramenopila, but it should be pointed out that the two terms are not synonymous, as the Stramenopila do not include the Haptophyta and the Cryptophyta, which, according to Cavalier-Smith, are members of the Chromista kingdom).

Molecular-phylogenetic experiments in the 1990s confirmed that the Labyrinthulea can be classed as Stramenopila (Leipe et al. 1994; Cavalier-Smith et al. 1994; Leipe et al. 1996; Honda et al. 1999). In the majority of family trees that are based on sequence comparisons of the nuclear-coded rDNA operon, the Labyrinthulea are recognised as one of the earliest branches of the Stramenopila (van de Peer et al. 1996; Honda et al. 1999; Guillou et al. 1999; van de Peer et al. 2000; Moriya et al. 2002). They represent a monophyletic group, whose members however display considerable sequence diversity and whose relationships with each other must therefore be regarded as largely unknown (Honda et al. 1999; Leander and Porter 2001).

The relationships within the Stramenopila kingdom are presented in a simplified family tree as well as a further, detailed family tree, based on sequence comparisons of the SSU rDNA (Fig. 1, as well as Fig. 2 from the work of Guillou et al. 1999). This illustrates that, in addition to a basal, heterotrophic group of stramenopiles, which includes not only the Labyrinthulea, but also further, colourless protists (Bicoecea, Opalinea, Proteromonadea, *Blastocystis*) and the pseudofungi not listed in the family tree (oomycetes and hypochytriomycetes), there is also a large photoautotrophic group of Stramenopila, which have long been known as Heterokontophyta (= Ochrophyta Cavalier-Smith 1997). The Heterokontophyta include numerous groups of algae that are rich in species and ecologically significant, such as diatoms, brown algae, Chrysophyceae, Xanthophyceae, Raphidophyceae, Pelagophyceae, or other groups. In the illustrated family tree, three dinoflagellates were used as an external group of the Stramenopila in order to provide the tree with roots (the dinoflagellates are members of the Alveolates kingdom, their closest relations are the Apicomplexa, who were formerly known as Sporozoa).

Within the Stramenopila, there are toxin-producing organisms. These, however, are exclusively found in a few genera of the Heterokontophyta (the positions of the toxin-producing Stramenopila are marked red in the family tree), but not in the group of the basal, colourless Stramenopila, of which the Labyrinthulea and thus also *Ulkenia* are members (Fig. 2).

2. Occurrence of Toxins in Thraustochytriales and in *Ulkenia* (Data from Literature)

The available literature makes no reference to the occurrence of toxins in *Ulkenia* itself, even or within the Labyrinthulea phylum (Labyrinthulomycota). In view of the fact that members of the Labyrinthulea phylum have for years been intensively tested for their biotechnical potential (Lewis et al. 1999), the lack of literature data for toxin

formation in *Ulkenia*, as well as in Labyrinthulea as a whole, can only be interpreted by the conclusion that this is highly improbable.
Labyrinthulea is a marine, saprotrophic living protist, which frequently occurs in sediments along with other microbial consorts and decomposes dead algae and plant material by the absorptive intake of dissolved organic substances (Bremer 1976; Raghukumar 2003). Labyrinthulea makes a significant contribution to the mineralization of dead organic substance and are vital components of natural marine food networks (Kimura et al. 2001). The Labyrinthulea per se enter the marine food chain by absorption in filtering organisms (e. g. mussels), and have thus for a long time been indirectly consumed by humans when they eat mussels.
Thus far *Ulkenia* has not been proven to be pathogenic towards other organisms. *Ulkenia visurgensis* has never been found on living cells of diatoms or brown algae, only on the cells of those that had already died (Raghukumar 1986; Sathepathak et al. 1993). This also applies to other Thraustochytriales who, in contrast to the Labyrinthulales, for example (Genus: *Labyrinthulea*, Muehlstein et al. 1988) can be deemed to have a very low potential as parasites of marine plants (Raghukumar 2002). There were individual reports of the pathogenic effects of *Labyrinthulea* on marine molluscs (Alderman and Gras 1969, Polglase 1980, McLean and Porter 1987, Bower 1987, Whyte et al. 1994). The *Schizochytrium, Aplanochytrium* and *Althornia* genera were involved. Although Polglase et al. (1986) also identified *Ulkenia amoeboidea* as parasites on the skin of the *Eledone cirrhosa* curled octopus, McLean and Porter (1982) assume that this case merely involves a secondary infection. For QPX, a member of *Labyrinthulea* that has not been more closely identified and which causes damage in the aquacultures of the hard clam (*Mercenaria mercenaria*) in North America, it is assumed that the virulence originates from heavy excretion of slime by the parasites (Anderson et al. 2003). In spite of intensive studies of the fish pathology, there are no known fish pathogens in Labyrinthulea (Raghukumar 2002).

3. Discussion of the Analyses of Toxins in Thraustochytriales and in *Ulkenia*

The evaluation of relevant literature, as described in 2, gives no hints of the occurrence of toxins in *Ulkenia* per se or in Labyrinthulea as a whole. In view of the fact that discussions are being carried out on using other members of Labyrinthulea (*Schizochytrium sp.*) for biotechnical applications, studies published on the safety of these organisms in a human-pathological context will be briefly addressed. There are currently 4 such publications that were produced by a group of various companies. The first study (Hammond et al. 2001a) involved subchronic feeding studies of rats over a period of 13 weeks (up to 4000 mg/kg/day dried *Schizochytrium sp.*). During this study no clinical effects, and in particular no toxicity (not even in microscopic preparations), were observed whatsoever. The changes noted in the spectrum of the lipoproteins and cholesterol also occurred in tests in which the rats were fed with comparable amounts of fish oil. In a further study (Hammond et al. 2001b), doses of up to 1800 mg/kg/day appeared to have a toxic effect on the embryonic development of neither rats nor rabbits. Equally, no effects of dried *Schizochytrium sp.* in doses of up to 20 g/kg/day were observed on the reproductive behaviour of the F1-generation of rats (Hammond et al. 2001c). Finally, no genotoxic activity of *Schizochytrium sp.* was observed in the CHO AS52/XPRT mutation test (Hammond et al. 2002).
In summary, it can be ascertained that, based on the literature data of all animal experiments, including subchronic feeding studies, no references were made to the presence of toxic components.

4. Toxins in Algae and Stramenopila: With Reference to Thraustochytriales and the Oils extracted from these

As mentioned in 1, there are a few toxin-producing genera in the Stramenopila kingdom (the genetic diversity of which is comparable to the animal, fungi or plant kingdoms).
The following will briefly address the toxins that are produced by algae in general and Stramenopila specifically, with reference to the Thraustochytriales and fat-refinement.

Toxins can occur sporadically in algae and cyanobacteria and have, over the past few years, been characterised extensively as to their occurrence, as well as their structure and effect. With regard to their occurrence, it is worth mentioning that toxins only occur in individual genera (sometimes only in individual species or ecotypes within the genera) and in a few systematic groups. The most significant toxins are formed by the dinoflagellates (Dinozoa) and the cyanobacteria (blue-green algae). There are about 10 genera of dinoflagellates and 8 genera of cyanobacteria in which toxic species/ecotypes are known.

The most significant toxins in the dinoflagellates group are brevetoxin, saxitoxin and okada acid, which trigger toxic effects in invertebrates (PSP = paralytic shellfish poisoning and DSP = diarrhetic shellfish poisoning) (Plumley 1997; Baden et al. 1998). These toxins are mostly heat-resistant cyclic polyethers, which, as liposoluble neurotoxins, block sodium channels in nerves and muscles (Wang and Wang 2003).

The majority of cyanobacteria toxins are cyclic peptides (heptapeptides, pentapeptides) which act as hepatotoxins (Haider et al. 2003). The most important cyanobacteria toxin is the heptapeptide microcystin, which occurs in *Microcystis spp.* as well as in species/phyla of the *Anabaena* genera. The cyclic peptides are polar and highly water-soluble, which is why they can contaminate drinking water reservoirs, especially as the toxic cyanobacteria occur in almost all forms of fresh water (Falconer 1999). No toxins from the group of cyclic peptides have as yet been found outside of cyanobacteria, their synthesis is obviously connected to the prokaryotic metabolism. In addition to hepatotoxins, phyla from *Anabaena, Aphanizomenon, Planktothrix* and *Cylindrospermopsis* also form the neurotoxins anatoxin (an alkaloid) and saxitoxin.

Other than the dinoflagellates and cyanobacteria, toxin-producers from the remaining algae/protists are extremely rare. The formation of toxins of the Haptophyte genus *Prymnesium* (2 species) has been known for a long time. The toxin (Prymnesin) is a polycyclical C90-ether with haemolytic, cytotoxic and neurotoxic properties with regard to mussels and fish (Larsen et al. 1993; Kolderup et al. 1995; Morohashi et al. 2001). Prymnesins are water-soluble and not heat-resistant; the toxin is evidently only formed in light (Dafni et al. 1972). There were recent reports that *Prymnesium parvum* excretes the toxin in order to immobilise food organisms (other flagellates), which can then be phagocyted (Skovgaard and Hansen 2003). This leads one to conclude that *Prymnesium's* toxin production is causally connected to the phagotrophic nutrition of this mixotrophic organism.

A couple of years ago, there were reports on the toxic effects of the marine green alga *Caulerpa taxifolia*, which spread across the whole of the Mediterranean after

accidentally being released from an aquarium (this alga originates from Australia, Wiedenmann et al. 2001). The toxin (caulerpenyne) is a sesquiterpene and has neurotoxic and cytotoxic effects (Brunelli et al. 2000, Barbier et al. 2001). Caulerpenyne has previously only been found in samples of *Caulerpa taxifolia*.

The *Ulkenia* genus and the Thraustochytriales order are members of the Labyrinthulea phylum (see point 1), which, in turn, is a member of the Stramenopila kingdom. The toxin-producing algae/protists that have been discussed up to now are not members of the Stramenopila, but are classed as members of other kingdoms, partly even other domains of life (such as the cyanobacteria, which are members of the Bacteria domain) and are in no way related to the Labyrinthulea.

Within Stramenopila, the specialised literature reports on toxin production in three groups of photosynthetic Heterokontophyta, the diatoms (Bacillariophyceae), Pelagophyceae (only *Aureococcus anophagefferensis*) and Raphidophyceae (*Heterocapsa, Chattonella, Fibrocapsa*).

The potent, water-soluble neurotoxin domoic acid (an amino acid derivative) is produced by species from the *Pseudo-nitzschia* genus (Bacillariophyceae) (Mos 2001). It was first discovered in Canada after food poisoning contracted from eating mussels (Rao et al. 1988) and later connected with mass occurrences of this diatom genus worldwide (Fritz et al. 1992). Domoic acid becomes concentrated as it travels through the food chain and mainly affects "capstone" species in marine ecosystems (e.g. seagulls, sea lions and other creatures; Lefebvre et al. 2002). The worldwide increase in *Pseudo-nitzschia* correlates with the increasing eutrophication of coastal waters over the last decades (Parsons et al. 2002). Domoic acid triggers toxic encephalopathy in humans (Perl et al. 1990) and bonds to kainate receptors (a type of glutamate receptor in the central nervous system), which causes neurons to degenerate. Loss of memory is caused by malfunctioning of the hippocampus, which has many kainate receptors. Domoic acids have not previously been found outside of the *Pseudonitzschia* genus.

For the first time, Sieburth and co-workers described an organism (*Aureococcus anophagefferens*), which is a member of the Pelagophyceae, a further class of photosynthetic Heterokontophyta, which are classed as marine picoplankton. In 1985, this organism formed a "brown tide" in the Narragansett Bay (USA) (Sieburth et al. 1988). Such brown tides were observed during subsequent years over several summer months on the Atlantic coast of the USA (Bricelj and Lonsdale 1997, Gilbert et al. 2001). Toxic effects on sea grasses and mussels were described (Bricelj et al. 2001, Greenfield and Lonsdale 2002). However, up to now it has been impossible to chemically identify a toxin.

Finally, in 1989, during a water blossoming of the *Raphidophyceae Heterosigma akashiwo* in New Zealand, a pathologic effect was observed on salmon (Chang et al. 1990). Neurotoxins from the group of brevetoxins were later isolated from the Raphidophycea *Heterosigma akashiwo, Cattonella marina* and *Fibrocapsa japonica* (Kahn et al. 1996, 1997; Haque and Onoue 2002a, 2002b). At temperatures of >20 °C and low light intensities (<100 $\mu E\ m^{-2}\ s^{-1}$; Ono et al. 2000), the toxicity of *H. akashiwo* was only slight.

The previously known Stramenopila toxins are limited to two classes of photosynthetic Heterokontophyta: Bacillariophycea and Raphidophycea (it has not as yet been chemically proven whether or not the Pelagophyceae *Aureococcus anophagefferens* contains a toxin). Bacillariophycea and Raphidophycea are distantly related to the Labyrinthulea in the Stramenopila family tree (Fig. 2). Domoic acid is water-soluble and has previously only been detected in a few species of a genus of diatoms (there are probably more than 100,000 species of diatom; Norton et al. 1996). Brevetoxins are lipophilic and occur in a few genera of Raphidophycea (as well as in dinoflagellates, cf. above). Domoic acids and brevetoxins are, as far as we know, only found in photosynthetic organisms. At low light intensities (brevetoxin in *H. akashiwo*) or high temperatures, toxin production is greatly reduced.

Judging by the documents with which I have been provided, Nutrinova extracts an oil from the *Ulkenia* biomass which is rich in DHA. Classical processing steps of fat and oil extraction are used on this oil. The refinement measures include desliming, deacidification, bleaching and steaming. Brevetoxins are instable in alkaline media, which means that it should be destroyed when subjected to the usual deacidification conditions (treatment with diluted caustic soda) (Lewis et al. 2000).

5. Summary

1. Production of toxins is known neither in *Ulkenia* nor even in the order of the Thraustochytriales or the phylum of the Labyrinthulea. Extensive literature research produced no work in which possible toxin production of these organisms is discussed.
2. No animal experiments, including subchronic feeding studies which were carried out on members of the Thraustochytriales order, gave any hints as to the presence of toxic components.
3. The toxins known to be present in algae/Stramenopila occur only in photosynthetic or mixotrophic organisms, which are not phylogenetically related to the Labyrinthulea.
4. Domoic acid and brevetoxin are the only currently known toxins in Stramenopila. Their properties (solubility, stability) lead one to conclude that the toxins will not be detectable in an oil extracted from *Ulkenia.*
5. Based on these facts, it can be assumed that oil extracted from *Ulkenia* does not contain algae toxins.

(Signature)

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Gutachten zur Sicherheit von *Ulkenia* (Thraustochytriales) im humanbiologischen Kontext

Im vorliegenden Gutachten soll anhand des gegenwärtigen Wissensstands untersucht werden, inwieweit die Gattung *Ulkenia* sowie verwandte Gattungen der Ordnung Thraustochytriales als pathogen beschrieben wurden bzw. einen schädlichen Einfluß auf Organismen, insbesondere den Menschen, ausüben.
Das Gutachten beruht auf eigenem Wissen sowie einer umfangreichen Literaturrecherche und gliedert sich in folgende Abschnitte:

1. Taxonomie von *Ulkenia* im systematischen Umfeld des Reiches der Stramenopila
2. Vorkommen von Toxinen in den Thraustochytriales und in *Ulkenia* (Literaturdaten)
3. Diskussion der Analysen auf Toxine in den Thraustochytriales und in *Ulkenia*
4. Toxine in Algen und Stramenopila: Bezug zu den Thraustochytriales und zu darausgewonnenen Ölen

1. Taxonomie von *Ulkenia* im systematischen Umfeld des Reiches der Stramenopila

Ulkenia ist ein mariner Protist, der zusammen mit sechs weiteren Gattungen (*Althornia*, *Aplanochytrium*, *Japonochytrium*, *Labyrinthuloides*, *Schizochytrium* und *Thraustochytrium*) in die Ordnung Thraustochytriales SPARROW 1973 (mit einer Familie Thraustochytriaceae SPARROW ex CEJP 1959; oft wird auch der spätere Name der Familie, Thraustochytriidae OLIVE 1975, verwendet) gestellt wird. Mit der zweiten Ordnung Labyrinthulales DOFLEIN 1901 (mit der einzigen Familie Labyrinthulaceae CIENKOWSKI 1867; auch hier ist der spätere Name der Familie, Labyrinthulidae OLIVE 1975, gebräuchlich) bilden die

Thraustochytriales den Stamm Labyrinthulomycota OLIVE 1975 (= Labyrinthulomorpha LEVINE 1980 = Klasse Labyrinthulea OLIVE ex CAVALIER-SMITH 1989).

Der taxonomische Status der Labyrinthulomycota oder Labyrinthulea war lange umstritten, die beiden Ordnungen wurden nicht einmal als miteinander verwandt angesehen. Meist wurden die Labyrinthulales zu den Amöben (Rhizopodea) innerhalb der Protozoen oder zu den Schleimpilzen (Mycetozoa) innerhalb der Pilze gestellt, die Thraustochytriales wurden andererseits bei den Oomyceten innerhalb der Pilze eingereiht. Aufgrund ultrastruktureller Ähnlichkeiten (zweigeißlige, heterokonte Zoosporen; Bildung des charakteristischen, ektoplasmatischen Netzes durch ein besonderes Zellorganell, das Sagenogenetosom; tubuläre, mitochondriale Cristae) wurden schließlich beide Ordnungen als eng miteinander verwandt erkannt, und von Olive 1975 zu einem Stamm der Protisten, den Labyrinthulomycota, vereint. Die ultrastrukturellen Merkmale rückten die Labyrinthulomycota in die Verwandtschaft des Reiches der Stramenopila (Patterson 1989), einer großen Gruppe von Organismen, die sich durch das Vorhandensein dreiteiliger Geißelhaare auf einer der beiden Geißeln ihrer begeißelten Zellen auszeichnen (oft wird für die Stramenopila auch der Begriff Chromista Cavalier-Smith 1986 als Synonym verwendet; es muß jedoch darauf hingewiesen werden, daß die beiden Begriffe nicht synonym sind, da die Stramenopila nicht die Haptophyta und die Cryptophyta umfassen, die jedoch nach Cavalier-Smith zu den Chromista gehören).

Molekular-phylogenetische Untersuchungen in den 1990iger Jahren haben die Zuordnung der Labyrinthulea zu den Stramenopila bestätigt (Leipe et al. 1994; Cavalier-Smith et al. 1994; Leipe et al. 1996; Honda et al. 1999). In den meisten Stammbäumen, die auf Sequenzvergleichen des kernkodierten rDNA-Operons beruhen, werden die Labyrinthulea als eine der frühesten Abzweigungen der Stramenopila aufgelöst (van de Peer et al. 1996; Honda et al. 1999; Guillou et al. 1999; van de Peer et al. 2000; Moriya et al. 2002). Sie stellen eine monophyletische Gruppe dar, deren Vertreter allerdings erhebliche Sequenzdiversität aufweisen und deren Verwandtschaftsbeziehungen untereinander deshalb noch als weitgehend ungeklärt angesehen werden müssen (Honda et al. 1999; Leander und Porter 2001).

Die Verwandtschaftsverhältnisse innerhalb der Stramenopila sind in einem vereinfachten Stammbaum sowie einem weiteren, detaillierten Stammbaum auf der Basis von Sequenzvergleichen der SSU rDNA dargestellt (Abb. 1; sowie Abb. 2 aus der Arbeit von Guillou et al. 1999). Hieraus wird ersichtlich, daß es neben einer basalen, heterotrophen

Gruppe der Stramenopilen, zu denen neben den Labyrinthulea, weitere farblose Protisten (Bicoecea, Opalinea, Proteromonadea, *Blastocystis*) und auch die im Stammbaum nicht dargestellten Pseudofungi (Oomyceten und Hyphochytriomyceten) gehören, eine große, photoautorophe Gruppe von Stramenopila gibt, die man als Heterokontophyta (= Ochrophyta Cavalier-Smith 1997) schon seit langem kennt. Zu den Heterokontophyta gehören zahlreiche artenreiche und ökologisch bedeutsame Algengruppen, wie etwa die Kieselalgen, Braunalgen, Chrysophyceae, Xanthophyceae, Raphidophyceae, Pelagophyceae, u.a. Im abgebildeten Stammbaum wurden drei Dinoflagellaten als Außengruppe zu den Stramenopila verwendet, um den Baum zu wurzeln (die Dinoflagellaten gehören zum Reich der Alveolaten, ihre nächsten Verwandten sind die Apicomplexa, früher als Sporozoen bezeichnet).

Innerhalb der Stramenopila gibt es Organismen, die Toxine produzieren. Diese finden sich jedoch ausschließlich in wenigen Gattungen der Heterokontophyta (im Stammbaum sind die Positionen der toxinbildenden Stramenopila rot markiert), nicht jedoch in der Gruppe der basalen, farblosen Stramenopila, zu denen auch die Labyrinthulea und somit auch *Ulkenia* gehören (Abb. 2).

2. Vorkommen von Toxinen in den Thraustochytriales und in *Ulkenia* (Literaturdaten)

Die vorliegende Literatur ergibt keinerlei Hinweise auf das Vorkommen von Toxinen bei *Ulkenia* selbst oder innerhalb des Stamms der Labyrinthulea (Labyrinthulomycota). In Anbetracht der Tatsache, daß Vertreter der Labyrinthulea seit Jahren intensiv hinsichtlich ihres biotechnischen Potenzials getestet werden (Lewis et al. 1999), kann das Fehlen von Literaturangaben für Toxinbildung bei *Ulkenia* sowie der Labyrinthulea insgesamt, nur so interpretiert werden, daß diese äußerst unwahrscheinlich ist.
Bei den Labyrinthulea handelt es sich um marine, saprotroph lebende Protisten, die sehr häufig in Sedimenten gemeinsam mit anderen mikrobilellen Konsortien vorkommen und abgestorbenes Algen- und Pflanzenmaterial durch absorptive Aufnahme gelöster organischer Substanzen abbauen (Bremer 1976; Raghukumar 2002). Sie tragen wesentlich zur Mineralisierung toter organischer Substanz bei und sind wichtige Komponenten natürlicher mariner Nahrungsnetze (Kimura et al. 2001). Die Labyrinthulea selbst gelangen durch

Aufnahme in filtrierende Organismen (z.B. Muscheln) in die marine Nahrungskette, und werden so indirekt auch vom Menschen durch den Verzehr von Muscheln seit langem konsumiert.

Pathogenizität gegenüber anderen Organismen wurde bislang bei *Ulkenia* nicht nachgewiesen, so konnte *Ulkenia visurgensis* nie auf lebenden Zellen von Kieselalgen oder Braunalgen gefunden werden, nur bei solchen, die bereits abgestorben waren (Raghukumar 1986; Sathepathak et al. 1993). Dies gilt auch für andere Thraustochytriales, deren Potenzial als Parasiten mariner Pflanzen, im Unterschied etwa zu den Labyrinthulales (Gattung *Labyrinthula*; Muehlstein et al. 1988), als sehr gering eingeschätzt wird (Raghukumar 2002). Vereinzelt wurde über pathogene Wirkungen von Labyrinthulea auf marine Mollusken berichtet (Alderman und Gras 1969, Polglase 1980, McLean und Porter 1987, Bower 1987, Whyte et al. 1994). Beteiligt waren die Gattungen *Schizochytrium*, *Aplanochytrium* und *Althornia*. Obwohl Polglase et al. (1986) auch *Ulkenia amoeboidea* als Parasiten auf der Haut des Tintenfisches *Eledone cirrhosa* identifizierten, nehmen McLean und Porter (1982) in diesem Fall nur eine Sekundärinfektion an. Für QPX, einen nicht näher identifizierten Vertreter der Labyrinthulea, der in Aquakulturen der Venusmuschel (*Mercenaria mercenaria*) in Nordamerika Schäden verursacht, wird angenommen, daß die Virulenz auf starke Schleimausscheidung durch den Parasiten zurückzuführen ist (Anderson et al. 2003). Trotz intensiver Untersuchungen zur Fischpathologie sind keine Fischpathogene unter den Labyrinthulea bekannt (Raghukumar 2002).

3. Diskussion der Analysen auf Toxine in den Thraustochytriales und in *Ulkenia*

Die Auswertung der einschlägigen Literatur ergibt, wie unter **2.** beschrieben, keinerlei Hinweise auf das Vorkommen von Toxinen bei *Ulkenia* selbst oder bei den Laburinthulea insgesamt. In Anbetracht der Tatsache, daß andere Vertreter der Labyrinthulea (*Schizochytrium sp.*) für biotechnische Applikationen diskutiert werden, soll kurz auf publizierte Untersuchungen zur Sicherheit dieser Organismen im humanpathologischen Kontext eingegangen werden. Dazu liegen bislang 4 Publikationen vor, die von einem Konsortium verschiedener Unternehmen durchgeführt wurden. In einer ersten Studie (Hammond et al. 2001a) wurden subchronische Fütterungsstudien an Ratten über 13 Wochen durchgeführt (bis zu 4000 mg/kg/Tag getrocknete *Schizochytrium sp.*). Dabei wurden keinerlei klinische Effekte, insbesondere keine Toxizität (auch nicht in mikroksopischen

Präparaten) beobachtet. Die festgestellten Änderungen im Spektrum der Lipoproteine und des Cholesterols traten ebenso in Kontrollen auf, in denen die Ratten mit vergleichbaren Mengen Fischöls gefüttert wurden. In einer weiteren Studie (Hammond et al. 2001b) konnte weder bei Ratten noch Kaninchen ein toxischer Effekt in Dosen bis zu 1800 mg/kg/Tag auf die Entwicklung von Embryonen festgestellt werden. Ebenso konnten keine Effekte von getrockneten *Schizochytrium sp.* in Dosen bis zu 20 g/kg/Tag auf das Fortpflanzungsverhalten sowie die F1-Generation von Ratten beobachtet werden (Hammond et al. 2001c). Schließlich konnte keine genotoxische Aktivität von *Schizochytrium sp.* im CHO AS52/XPRT-Mutationstest festgestellt werden (Hammond et al. 2002).
Zusammengefaßt läßt sich feststellen, daß nach Literaturdaten alle tierexperimentellen Arbeiten einschließlich subchronischer Fütterungsstudien keine Hinweise auf die Anwesenheit toxischer Komponenten.

4. Toxine in Algen und Stramenopila: Bezug zu den Thraustochytriales und zu darausgewonnenen Ölen

Wie unter **1.** erwähnt, gibt es im Reich der Stramenopila (in ihrer genetischen Diversität den Reichen der Tiere, Pilze oder Pflanzen vergleichbar) einige wenige Gattungen, die Toxine bilden.
Im folgenden soll kurz auf die von Algen im allgemeinen und Stramenopila im besonderen gebildeten Toxine in Bezug zu den Thraustochytriales und zur Fettraffination eingegangen werden.

Toxine kommen in Algen und Cyanobakterien sporadisch vor und sind in den vergangenen Jahren eingehend hinsichtlich ihres Vorkommens sowie ihrer Struktur und Wirkungsweise charakterisiert worden. Hinsichtlich ihres Vorkommens ist erwähnenswert, daß Toxine stets nur in einzelnen Gattungen (manchmal nur in einzelnen Arten oder Ökotypen innerhalb der Gattungen) und in wenigen systematischen Gruppen vorkommen. Die wichtigsten Toxine werden von den Dinoflagellaten (Dinozoa) und den Cyanobakterien (Blaualgen) gebildet. Es sind etwa 10 Gattungen der Dinoflagellaten und 8 Gattungen der Cyanobakterien, in denen toxische Arten/Ökotypen bekannt sind.

Die wichtigsten Toxine der Dinoflagellaten sind Brevetoxin, Saxitoxin und Okadasäure, die in Invertebraten toxische Effekte (PSP = paralytic shellfish poisoning und DSP = diarrhetic shellfish poisoning) auslösen (Plumley 1997; Baden et al. 1998). Es handelt sich meist um hitzestabile cyclische Polyäther, die als lipidlösliche Neurotoxine Natrium-Kanäle in Nerven und Muskeln blockieren (Wang und Wang 2003).

Die meisten Toxine der Cyanobakterien sind zyklische Peptide (Heptapeptide, Pentapeptide), die als Hepatotoxine wirken (Haider et al. 2003). Das wichtigste Toxin der Cyanobakterien ist das Heptapeptid Microcystin, das in *Microcystis spp.* aber auch in Arten/Stämmen der Gattungen *Anabaena* vorkommt. Die cyclischen Peptide sind polar und lösen sich leicht in Wasser, weshalb sie Trinkwasser-Reservoirs kontaminieren können, zumal die toxischen Cyanobakterien nahezu ausnahmslos im Süßwasser vorkommen (Falconer 1999). Toxine aus der Gruppe der zyklischen Peptide sind bislang nicht außerhalb der Cyanobakterien gefunden, ihre Synthese ist offensichtlich an den prokaryotischen Stoffwechsel gebunden. Neben Hepatotoxinen bilden Stämme von *Anabaena*, *Aphanizomenon*, *Planktothrix* und *Cylindrospermopsis* auch die Neurotoxine Anatoxin (ein Alkaloid) und Saxitoxin.

Neben den Dinoflagellaten und Cyanobakterien sind Toxinbildner unter den übrigen Algen/Protisten äußerst selten. Schon sehr lange bekannt ist die Bildung von Toxinen bei der Haptophyten-Gattung *Prymnesium* (2 Arten). Das Toxin (Prymnesin) ist ein polycyclischer C90-Äther mit hämolytischen, cytotoxischen und neurotoxischen Eigenschaften hinsichtlich Muscheln und Fischen (Larsen et al. 1993; Kolderup et al. 1995; Morohashi et al. 2001). Prymnesine sind wasserlöslich und nicht hitzestabil; das Toxin wird offensichtlich nur im Licht gebildet (Dafni et al. 1972). Kürzlich wurde berichtet, daß *Prymnesium parvum* das Toxin ausscheidet, um Nahrungsorganismen (andere Flagellaten) zu immobilisieren, die dann phagocytiert werden können (Skovgaard und Hansen 2003). Dies läßt darauf schließen, daß die Toxinbildung bei *Prymnesium* ursächlich mit der phagotrophen Ernährung dieses mixotrophen Organismus in Zusammenhang steht.

Vor einigen Jahren wurde über toxische Wirkungen der marinen Grünalge *Caulerpa taxifolia* berichtet, die sich nach unbeabsichtigter Freisetzung aus einem Aquarium im ganzen Mittelmeerbereich ausgebreitet hatte (der Ursprung dieser Alge ist Australien; Wiedenmann et al. 2001). Das Toxin (Caulerpenyn) ist ein Sesquiterpen und besitzt neurotoxische und

cytotoxische Wirkungen (Brunelli et al. 2000, Barbier et al. 2001). Caulerpenyn wurde bislang nur bei *Caulerpa taxifolia* gefunden.

Die Gattung *Ulkenia* und die Ordnung Thraustochytriales sind Teil des Stamms der Labyrinthulea (s. **1.**), der wiederum zum Reich der Stramenopila gehört. Die bislang vorgestellten, toxinbildenden Algen/Protisten gehören nicht zu den Stramenopila, sondern sind anderen Reichen, teilweise sogar anderen Domänen des Lebens (wie etwa die Cyanobakterien, die zur Domäne Bacteria gehören) zugeordnet und in keiner Weise mit den Labyrinthulea verwandt.

Innerhalb der Stramenopila wird in der Fachliteratur über Toxinbildung in drei Gruppen von photosynthetischen Heterokontophyta berichtet, den Kieselalgen (Bacillariophyceae), den Pelagophyceae (nur bei *Aureococcus anophagefferensis*) und den Raphidophyceae (*Heterocapsa, Chattonella*, *Fibrocapsa*).

Das potente, wasserlösliche Neurotoxin Domoinsäure (ein Aminosäurederivat) wird von Arten der Gattung *Pseudo-nitzschia* (Bacillariophyceae) gebildet (Mos 2001). Es wurde zuerst in Kanada nach Vergiftungen durch Muschel-Verzehr entdeckt (Rao et al. 1988) und später weltweit mit Massenvorkommen dieser Kieselalgen-Gattung in Zusammenhang gebracht (Fritz et al. 1992). Domoinsäure wird in der Nahrungskette angereichert und wirkt im wesentlichen auf sogenannte Schlußstein-Arten in marinen Ökosystemen (z.B. Seevögel, Seelöwen u.a.; Lefebvre et al. 2002). Die weltweite Zunahme von *Pseudo-nitzschia* korreliert mit der zunehmenden Eutrophierung der Küstengewässer in den vergangenen Jahrzehnten (Parsons et al. 2002). Domoinsäure löst beim Menschen toxische Encephalopathie aus (Perl et al. 1990) und bindet an Kainat-Rezeptoren (ein Typ von Glutamat-Rezeptoren im Zentralnervensystem) wodurch Neurone degenerieren. Ein Gedächtnisverlust wird durch Störungen im Hippocampus, der reich an Kainat-Rezeptoren ist, verursacht. Domoinsäure wurde bislang nicht außerhalb der Gattung *Pseudonitzschia* gefunden.

Bei den Pelagophyceae, einer weiteren Klasse photosynthetischer Heterokontophyta, die zum marinen Picoplankton gehören, wurde von Sieburth und Mitarbeitern erstmals ein Organismus beschrieben (*Aureococcus anophagefferens*), der 1985 eine „braune Tide" in der Narragansett Bay (USA) bildete (Sieburth et al. 1988). Solche braunen Tiden wurden in den folgenden Jahren über mehrere Sommermonate an der Atlantikküste der USA beobachtet (Bricelj und

Lonsdale 1997, Glibert et al. 2001). Dabei wurden toxische Effekte auf Seegräser und Muscheln beschrieben (Bricelj et al. 2001, Greenfield und Lonsdale 2002). Ein Toxin konnte allerdings bislang chemisch nicht identifiziert werden.

Schließlich wurde 1989 während einer Wasserblüte der Raphidophyceae *Heterosigma akashiwo* in Neuseeland ein pathologischer Effekt auf Lachse beobachtet (Chang et al. 1990). Aus den Raphidophyceen *Heterosigma akashiwo*, *Cattonella marina* und *Fibrocapsa japonica* wurden später Neurotoxine isoliert, die zur Gruppe der Brevetoxine gehören (Kahn et al. 1996, 1997; Haque und Onoue 2002a, 2002b). Die Toxizität von *H. akashiwo* war bei Temperaturen >20 °C nur gering ausgeprägt, ebenso bei niedrigen Lichtintensitäten (< 100 $\mu E\ m^{-2}\ s^{-1}$; Ono et al. 2000).

Die bislang bei den Stramenopila bekannten Toxine beschränken sich auf zwei Klassen von photosynthetischen Heterokontophyta, den Bacillariophyceen und Raphidophyceen (bei der Pelagophyceae *Aureococcus anophagefferens* ist ein Toxin bislang chemisch nicht nachgewiesen). Bacillariophyceen und Raphidophyceen stehen im Stammbaum der Stramenopila weit von den Labyrinthulea entfernt (Abb. 2). Domoinsäure ist wasserlöslich und bislang nur bei wenigen Arten einer Gattung der Kieselalgen bekannt (die Kieselalgen umfassen wahrscheinlich mehr als 100 000 Arten; Norton et al. 1996). Brevetoxine sind lipophil und kommen bei einigen Gattungen der Raphidophyceen vor (außerdem bei den Dinoflagellaten, s. oben). Domoinsäure und Brevetoxine sind, soweit bekannt, auf photosynthetische Organismen beschränkt. Die Toxinbildung ist bei niedrigen Lichtintensitäten (Brevetoxin bei *H. akashiwo*) oder höheren Temperaturen stark reduziert.

Nach den mir vorliegenden Unterlagen gewinnt Nutrinova aus der *Ulkenia*-Biomasse ein DHA-reiches Öl, bei dem klassische Verarbeitungsschritte der Fett- und Ölgewinnung angewendet werden. Die Raffinationsmaßnahmen beinhalten u.a. Entschleimung, Entsäuerung, Bleichung und Dämpfung. Brevetoxine sind in alkalischen Medien nicht stabil, so daß es unter den üblichen Bedingungen der Entsäuerung (Behandlung mit verdünnter Natronlauge) zerstört werden sollte (Lewis et al. 2000).

5. Zusammenfassung

(1) Weder für *Ulkenia* selbst noch für die Ordnung der Thraustochytriales oder den Stamm der Labyrinthulea ist die Bildung von Toxinen bekannt. Umfangreiche Literaturrecherchen haben keine Arbeiten ergeben, in denen die Möglichkeit einer Toxinbildung durch diese Organismen diskutiert wird.

(2) Alle tierexperimentellen Arbeiten inklusive subchronischer Fütterungsstudien, die an Vertretern der Thraustochytriales durchgeführt wurden, ergaben keine Hinweise auf die Anwesenheit toxischer Komponenten.

(3) Die bei Algen/Stramenopila bekannten Toxine treten nur in photosynthetischen bzw. mixotrophen Organismen auf, die phylogenetisch keine Verwandtschaftsbeziehungen zu den Labyrinthulea aufweisen.

(4) Domoinsäure und Brevetoxin sind die einzigen bislang bekannten Toxine in den Stramenopila. Ihre Eigenschaften (Löslichkeit, Stabilität) lassen den Schluß zu, daß sie in einem aus *Ulkenia* extrahierten Öl nicht nachweisbar sein werden.

(5) Aufgrund dieses Sachverhalts muß nicht mit der Anwesenheit von Algentoxinen im Öl aus *Ulkenia* gerechnet werden.

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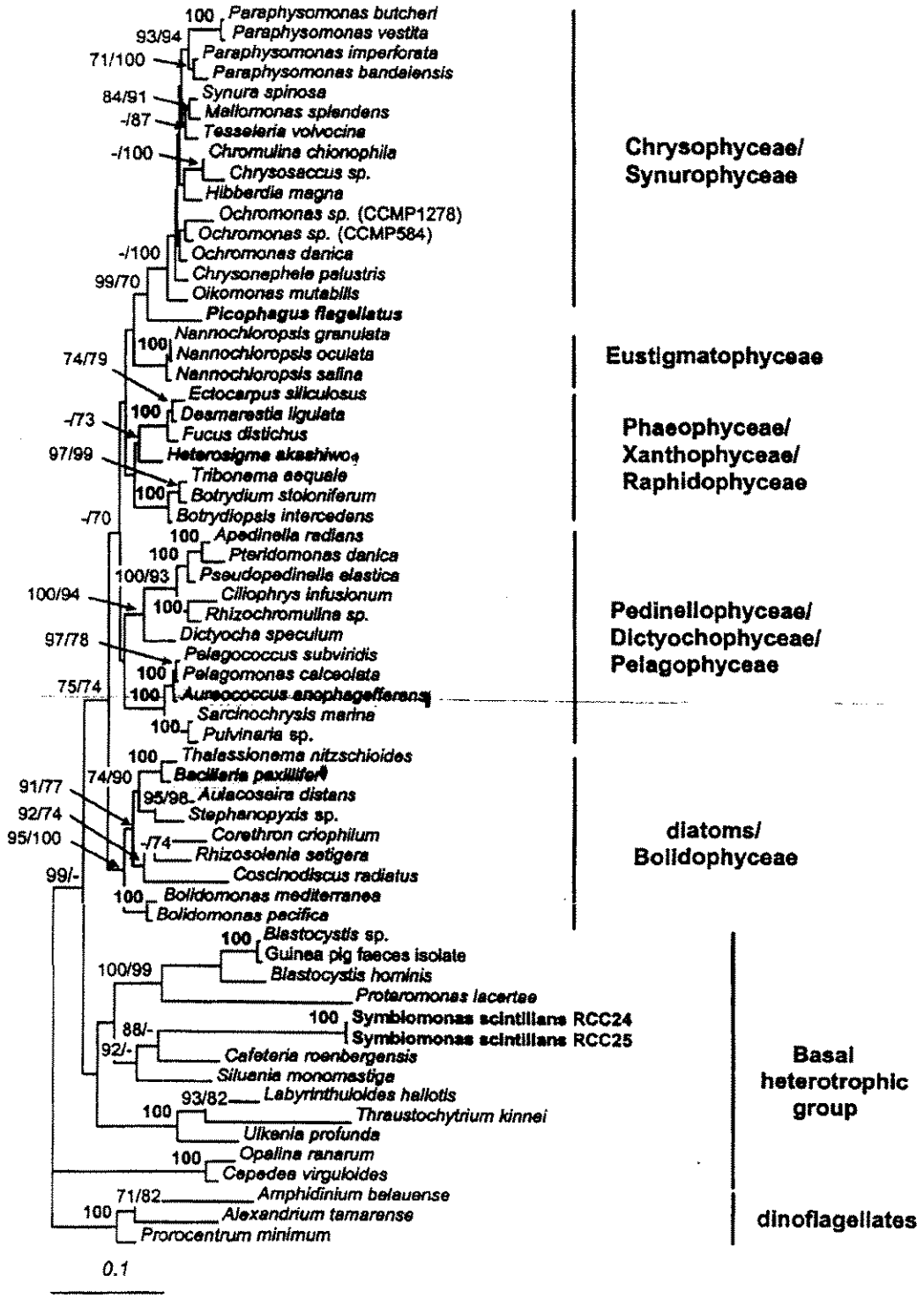
Abb. 2 Stammbaum der Stramenopila auf der Basis von Sequenzvergleichen der SSU rDNA (Distanzanalyse; komplexes Evolutionsmodell) aus: Guillou et al. (1999) Protist 150: 383-398

toxinbildende Taxa sind farbig markiert



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08.09.2003

Extended Expert's Report on the "Safety" of *Ulkenia* sp., [redacted]

Re: Previous Expert's Report by Dr. Karsten Schaumann, Alfred-Wegener-Institute for Polar and Marine Research, Bremerhaven, to Dr. Thomas Kiy, Celanese Ventures GmbH, Frankfurt, of 14.11.2001, including Appendices (cf. Appendix I-III)

The following statement on the "safety" of *Ulkenia* is based on my own experience in scientific dealings with *Ulkenia* and on findings of taxonomy and phylogeny, as well as biology, ecology, physiology and toxicology of the genus *Ulkenia* and related taxa taken from the specialist literature. Here the entire spectrum of the organisms generally described as "microalgae" will be included and evaluated, i.e.: Cyanobacteria (blue-green algae), Chlorophyta (green algae), Rhodophyta (red algae), Euglenophyta, Dinophyta (dinoflagellates), Bacillariophycea (diatoms), Chrysophycea (golden-brown algae), Eustigmatophycea, Oomycetidae (oomycetes), Thraustochytridae (thraustochytrides), Xanthophyceae (yellow-green algae), Cryptophyta (cryptomonades), Prymnesiophyta (haptophytes).

Taxonomic-Phylogenetic Characterisation

The production phylum of *Ulkenia* sp. being discussed is systematically and phylogenetically characterised as follows:

Domain:	Eukaryota
Kingdom:	Chromista (Stramenopila)
Subkingdom:	Heterokonta
Division:	Labyrinthulomycota (Labyrinthulomorpha)
Class:	Labyrinthulomycetes
Subclass:	Thraustochytridae
Order:	Thraustochytriales
Family:	Thraustochytriaceae
Genus:	Ulkenia
Species:	"Species"
[redacted]	[redacted]

Today, we know of six different species within the well-defined genus *Ulkenia* (cf. Fig. 1). In addition to *Ulkenia*, members of the Thraustochytriales order include the further genera: *Althornia* (1 sp.), *Aplanochytrium* (1 sp.), *Japonochytrium* (1 sp.), *Labyrinthuloides* (5 spp.), *Schizochytrium* (5 spp.), *Thraustochytrium* (15 spp.), as well as five uncertain genera: *Corallochytrium* (1 sp.), *Diplophrys* (2 spp.) *Elina* (2 spp.), *Sorodiplophrys* (1 sp.), *Rostafinscia* (1 sp.). Together with the Labyrinthulales, these make up the class of the Labyrinthulomycetes, which, in turn, are members of the Labyrinthulomycota phylum (Labyrinthulomorpha) and the subkingdom Heterokonta, kingdom Chromista (cf. Fig. 2 - 4).

From a historical viewpoint, after their discovery by Sparrow (1936), thraustochytrides were first classed as Saprolegniales (Oomycota, Fungi). Modern molecular-genetic analyses, however, show that they neither belong in the fungi kingdom, nor in the so-called fungi-like protists (Protozoa), but in the Chromista (Stramenopila) kingdom (Cavalier-Smith et al., 1994; Patterson, 1989; Patterson et Larsen, 1992; Corliss, 1994; Alexopoulos et al., 1996) (cf. Fig. 2 - 4).

Biology, Ecology and Physiology

As typical marine microorganisms, the thraustochytrides can be found in all the world's oceans and epeiric seas, as well as estuaries. They can be found from the Arctic to temperate, subtropical and tropical through to Antarctic latitudes. They live both freely in plankton (mycoplankton) (Sieburth, Smetacek et Lenz, 1978) as well as in solid plant and animal substrata, which they use either parasitically or saprophytically, while releasing soluble organic compounds and forming detritus. There have rarely been reports of phagotrophic nutrition of thraustochytrides (Raghukumar, 1992). The findings of parasitism on marine plants and animals are more numerous; they were compiled by Alderman (1976), Polglase (1980), McLean et Porter (1982), Polglase, Alderman et Richards (1986), Alderman et Polglase (1986), Porter (1986).

The only examples of parasitism of *Ulkenia* on marine animals (or even on man) concern those of the *Eledone cirrhosa* curled octopus, where progressive formation of ulcers, swelling and dermal decomposition were observed. This was often accompanied by infections with *Labyrinthula* sp. (Polglase, 1980, 1981). Jones et O'Dor (1983) proved thraustochytrides to be connected to gill diseases of squid and McLean and Porter (1982) identified thraustochytrides as the cause of yellow spot disease in a sea slug (*Tritonia diomedea*). Finally, Bower (1987) succeeded in proving the existence of Thraustochytriceae *Labyrinthuloides haliotidis* as a pathogenic parasite on the Northern abalone (*Haliotis kamtschatkana*) and the red abalone (*Haliotis rufescens*).

The qualitative and quantitative detection of thraustochytrides in situ is carried out by using direct and indirect techniques, in particular the pollen-bait method, the dilution plate method and various dye procedures, as well as electron-microscopic techniques. Cell densities of 10^5 to 10^8 gills per litre sediment were determined. (Gaertner, 1967; Riemann et Schrage, 1983). Cell densities are also very high on detritus and detritus accumulation, up to 8×10^7 (Gaertner et Raghukumar, 1980; Raghukumar, 1987; Riemann et Schaumann, 1973). In free-flowing waters,

particularly in the open ocean, cell densities are considerably lower, sometimes less than 1 cell per litre seawater.

There are no known reports of the direct human consumption of *Ulkenia* sp. or other thraustochytrides. Merely the incidental consumption in connection with eating other marine feeding organisms, such as mussels, crabs and fish, which, in turn, feed on typical invertebrates, particularly filterers, is general knowledge.

By way of example, Fig. 5 features an illustration of the food chains and biotic interrelations in a mangrove habitat and features man as the top consumer.

Toxicology

Extensive literature research provides no indications of toxin production by *Ulkenia* or other thraustochytrides. Feeding freeze-dried or fresh cells from Thraustochytriaceae, e. g. from *Schizochytrium* sp., to marine microalgae for the purpose of producing aquaculture feed for copepods and fry showed no negative consequences whatsoever. Quite the opposite: The aquaculture organisms that had been fed with thraustochytrides grew more quickly and with a higher content of unsaturated fatty acids than those feeding organisms that had been fed conventionally.

However, a few groups of microalgae are known to be potent toxin-producers. They were marked by a point (.) in the phylogram (cf. Fig. 2 - 4). Below, in particular the following toxins, also with regard to possible occurrence of oils from microalgae, will be briefly discussed:
The water-soluble domoic acid is produced by a few Bacillariophyta from the Pennales order, above all several species of the *Pseudonitzschia* genus. These have a neurotoxic effect on humans. An occurrence in oils extracted from microalgae can be ruled out due to the solubility.
The water-soluble Prymnesin is produced by a few Haptophyta (Prymnesiophyta), specifically *Prymnesium parvum* and *P. patelliferum*, and probably also *Chrysochromulina polylepsis*. It also has a neurotoxic effect and, due to its solubility in water, passage to oil is impossible.
The lipophilic brevetoxin is synthesised by the dinoflagellate *Phytodiscus brevis*. It has a neurotoxic effect on humans. This toxin is instable in alkaline media. Passage to an oil extracted from algae is potentially conceivable, but the toxin should be destroyed when subjected to the usual conditions of fat-refinement. Numerous other toxins are produced by dinoflagellates. They possess neurotoxic or diarrhetic properties.

None of the aforementioned toxic components has previously been detected in *Ulkenia* or in DHA oil.

<u>In summary</u>, it can be ascertained that, based on the existing information on the production and properties of toxins in Chromista, the occurrence of algae toxins is not to be expected in DHA oil extracted from *Ulkenia*. Furthermore, all animal feeding experiments (cf. P. 2/3 above), including subchronic studies, gave no indication as to the existence of possible toxic components. In conclusion, descriptions or evidence of possible toxin production exist neither for *Ulkenia* nor for other thraustochytrides. This is not least confirmed by the non-detection of relevant algae toxins in DHA oil extracted from *Ulkenia*.

All in all, it can be said that *Ulkenia* sp., the [redacted] phylum and the products extracted from these, such as DHA oil, pose no dangers for human health – providing that these are subjected to the normal production and application conditions.

08.09.2003

Dr. Karsten Schaumann
Research Group for Marine Mycology

Date

Enc:

List of Appendices and Illustrations

Appendix 1: Previous Expert's Report dated 14.11.2001
Appendix 2: Curriculum vitae
Appendix 3: Selection of relevant publications

Figure 1: corresponds to Fig. 7 and Tab. 1 in Gaertner, A. (1977), publication of the Bremerhaven Institute for Marine Research, Volume 16: 139-157
Figure 2: Phylogram based on the 18S rRNA gene sequences according to Honda, Fig. 1 (personal)
Figure 3: Phylogram of Chromista (Stramenopila) according to Honda, Fig. 2 (personal)
Figure 4: Phylogram of the Thraustochytriales according to Honda, Fig. 3 (personal)
Figure 5: Cf. key on the illustration

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Erweitertes Gutachten zur „Sicherheit" von *Ulkenia* sp., [redacted]

Bezug: Vorhergehendes Gutachten von Dr. Karsten Schaumann, Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven an Herrn Dr. Thomas Kiy, Celanese Ventures GmbH, Frankfurt, vom 14.11.2001 nebst Anlagen (siehe Anlage I - III)

Die nachfolgende Stellungnahme zur „Sicherheit" von *Ulkenia* basiert auf eigenen Erfahrungen im wissenschaftlichen Umgang mit *Ulkenia* und auf Erkenntnissen zur Taxonomie und Phylogenie, aber auch der Biologie, Ökologie, Physiologie und Toxikologie der Gattung *Ulkenia* und verwandter Taxa, die der Fachliteratur entnommen wurden. Dabei soll das gesamte Spektrum der gemeinhin als „Mikroalgen" bezeichneten Organismen, also: Cyanobacteria (Blaugrüne Algen), Chlorophyta (Grünalgen), Rhodophyta (Rotalgen), Euglenophyta, Dinophyta (Dinoflagellaten), Bacillariophycea (Diatomeen), Chrysophycea (Goldalgen), Eustigmatophycea, Oomycetidae (Oomyceten), Thraustochytridae (Thraustochytriden), Xanthophyceae (Gelbgrüne Algen), Cryptophyta (Cryptomonaden), Prymnesiophyta (Haptophyten) in die Betrachtung einbezogen und bewertet werden.

Taxonomisch-phylogenetische Charakterisierung

Der zur Diskussion stehende Produktionsstamm von *Ulkenia* sp. ist systematisch und phylogenetisch folgendermaßen charakterisiert:

Domäne:	Eukaryota
Reich:	Chromista (Stramenopila)
Unterreich:	Heterokonta
Phylum/Division:	Labyrinthulomycota (Labyrinthulomorpha)
Klasse:	Labyrinthulomycetes
Unterklasse:	Thraustochytridae
Ordnung:	Thraustochytriales
Familie:	Thraustochytriaceae
Gattung:	Ulkenia
Art:	„Species"
[redacted]	[redacted]

Heute kennen wir sechs verschiedene Species innerhalb der gut definierten Gattung *Ulkenia* (siehe Abb.1). Zur Ordnung der Thraustochytriales gehören - neben *Ulkenia* – die weiteren Gattungen: *Althornia* (1 sp.), *Aplanochytrium* (1 sp.), *Japonochytrium* (1 sp.), *Labyrinthuloides* (5 spp.), *Schizochytrium* (5 spp.), *Thraustochytrium* (15 spp.) sowie fünf unsichere Gattungen: *Corallochytrium* (1 sp.), *Diplophrys* (2 spp.), *Elina* (2 spp.), *Sorodiplophrys* (1 sp.), *Rostafinscia* (1 sp.). Diese bilden zusammen mit den Labyrinthulales die Klasse der Labyrinthulomycetes, die wiederum dem Phylum Labyrinthulomycota (Labyrinthulomorpha) und dem Unterreich Heterokonta, Reich Chromista angehören (siehe Abb. 2 - 4).

Historisch betrachtet wurden die Thraustochytriden nach ihrer Entdeckung durch Sparrow (1936) zunächst zu den Saprolegniales (Oomycota, Fungi) gestellt. Moderne molekulargenetische Analysen zeigten aber, dass sie weder in das Reich der Pilze (Fungi), noch der sogenannten pilzähnlichen Protisten (Protozoa), sondern in das Reich der Chromista (Stramenopila) zu stellen sind (Cavalier-Smith et al., 1994; Patterson, 1989; Patterson et Larsen, 1992; Corliss, 1994; Alexopoulos et al., 1996) (siehe Abb. 2 - 4).

Biologie, Ökologie und Physiologie

Die Thraustochytriden sind als typisch marine Mikroorganismen weltweit in allen Ozeanen und ihren Randmeeren sowie Ästuaren verbreitet. Sie kommen vor von der Arktis über gemäßigte, subtropische und tropische bis hin zu antarktischen Breiten. Sie finden sich sowohl freilebend im Plankton (Mycoplankton) (Sieburth, Smetacek et Lenz, 1978) als auch auf festen pflanzlichen und tierischen Substraten, die sie entweder parasitisch oder saprophytisch unter Freisetzung von löslichen organischen Verbindungen und unter Bildung von Detritus nutzen. Selten wurde auch über eine phagotrophe Ernährung bei Thraustochytriden berichtet (Raghukumar, 1992). Zahlreicher sind die Befunde über das parasitische Vorkommen auf marinen Pflanzen und Tieren; sie wurden von Alderman (1976), Polglase (1980), McLean et Porter (1982), Polglase, Alderman et Richards (1986), Alderman et Polglase (1986), Porter (1986) zusammengestellt.

Beispiele über das parasitische Vorkommen von *Ulkenia* auf marinen Tieren (oder gar dem Menschen) finden sich lediglich bei der Krake *Eledone cirrhosa*, wo eine fortschreitende Geschwürbildung, Schwellung und Zersetzung der Haut beobachtet wurde. Oft kamen gleichzeitig Infektionen mit *Labyrinthula* sp. vor (Polglase, 1980, 1981). Jones et O'Dor (1983) wiesen Thraustochytriden im Zusammenhang mit Kiemenerkrankungen bei Tintenfischen nach, und McLean et Porter (1982) konnten Thraustochytriden als Ursache der Gelbfleckenkrankheit einer Nacktschnecke (*Tritonia diomedea*) identifizieren. Schließlich konnte Bower (1987) die Thraustochytriceae *Labyrinthuloides haliotidis* als pathogenen Parasiten auf dem Seeohr (*Haliotis kamtschatkana* und *H. rufescens*) nachweisen.

Der qualitative und quantitative Nachweis von Thraustochytriden in situ erfolgt mit Hilfe direkter und indirekter Techniken, insbesondere der Pollen-Ködermethode, der Verdünnungsplattenmethode und verschiedenen Färbeverfahren sowie elektronenmikroskopischen Techniken. Es wurden Zelldichten von 10^5 bis 10^8 Keimen pro Liter Sediment festgestellt (Gaertner, 1967; Riemann et Schrage, 1983). Auch auf Detritus und Detritusansammlungen sind die Zelldichten sehr hoch, bis zu 8×10^7 (Gaertner et Raghukumar, 1980; Raghukumar, 1987; Riemann et Schaumann, 1973). Im freien Wasser,

insbesondere des offenen Ozeans, sind die Zelldichten wesentlich niedriger, bisweilen unter 1 Zelle pro Liter Meerwasser.

Es sind keinerlei Mitteilungen über die direkte Aufnahme von *Ulkenia* sp. oder anderen Thraustochytriden durch den Menschen bekannt. Lediglich die zufällige Aufnahme im Zusammenhang mit dem Verzehr anderer mariner Nahrungsorganismen, wie z.B. Muscheln, Krebsen und Fischen, die sich wiederum von typischen Invertebraten, insbesondere Filtrierern ernähren, ist allgemein bekannt.

Eine Illustration der Nahrungsketten und biotischen Wechselbeziehungen in einem Mangrove-Habitat ist beispielhaft und unter Einbeziehung des Menschen als Topkonsumenten in Abb. 5 wiedergegeben.

Toxikologie

Eingehende Literaturrecherchen haben keinerlei Anhaltspunkte für Toxinbildung durch *Ulkenia* oder andere Thraustochytriden ergeben. Auch die Verfütterung von gefriergetrockneten oder frischen Zellen der Thraustochytriaceae z.B. von *Schizochytrium* sp. an marine Mikroalgen zum Zwecke der Erzeugung von Aquakulturfutter für Kleinkrebse und Fischbrut zeigte keinerlei negative Folgen. Im Gegenteil: Die mit Thraustochytriden gefütterten Aquakulturorganismen wuchsen schneller und mit einem höheren Gehalt an ungesättigten Fettsäuren heran als konventionell ernährte Futterorganismen.

Dennoch sind einige Mikroalgengruppen als potente Toxinproduzenten bekannt. Sie wurden im Phylogramm (s. Abb. 2 - 4) durch einen Punkt (•) markiert. Nachstehend sollen insbesondere die folgenden Toxine, auch im Hinblick auf ein mögliches Auftreten in Ölen aus Mikrolagen, kurz diskutiert werden:
Die wasserlösliche Domoinsäure wird von einigen Bacillariophyta aus der Ordnung der Pennales, namentlich mehreren Arten der Gattung *Pseudonitzschia*, produziert. Ihre Wirkung auf den Menschen ist neurotoxisch. Ein Auftreten in aus Mikroalgen gewonnenen Ölen ist auf Grund der Löslichkeit auszuschließen.
Das wasserlösliche Prymnesin wird von einigen Haptophyta (Prymnesiophyta), namentlich *Prymnesium parvum* und *P. patelliferum* sowie vermutlich auch *Chrysochromulina polylepsis* gebildet. Seine Wirkung ist ebenfalls neurotoxisch und auf Grund der Wasserlöslichkeit ist ein Übergang in ein Öl auszuschließen.
Das lipophile Brevetoxin wird von dem Dinoflagellaten *Phytodiscus brevis* synthetisiert. Es hat eine neurotoxische Wirkung für den Menschen. Dieses Toxin ist in alkalischen Medien nicht stabil. Ein Übergang in ein aus Algen gewonnenes Öl ist potentiell denkbar, allerdings sollte es unter den üblichen Bedingungen der Fettraffination zerstört werden. Zahlreiche weitere Toxine werden von Dinoflagellaten produziert. Sie besitzen neurotoxische oder diarrhotische Eigenschaften.

Keine der vorstehend genannten toxischen Komponenten ist bisher weder bei *Ulkenia* noch im DHA-Öl nachweisbar gewesen.

<u>Zusammenfassend</u> lässt sich feststellen, dass auf Grund der vorliegenden Informationen, die Bildung und Eigenschaften von Toxinen bei Chromista betreffend, nicht mit dem Auftreten von Algentoxinen im DHA-Öl aus *Ulkenia* zu rechnen ist. Auch ergaben alle

tierexperimentelle Füttererungsversuche (s. S. 2/3 oben), einschließlich subchronischer Studien, keinerlei Hinweise auf das Vorhandensein eventueller toxischer Komponenten. Schließlich ist weder für *Ulkenia* noch für andere Thraustochytriden die Möglichkeit zur Toxinbildung beschrieben oder angedeutet worden. Das wird nicht zuletzt auch durch die Nicht-Nachweisbarkeit einschlägiger Algen-Toxine im DHA-Öl aus *Ulkenia* bestätigt.

Alles in allem ist festzustellen, dass von *Ulkenia* sp. [redacted] und den daraus gewonnenen Produkten, wie z.B. DHA-Öl, keine Gefahren für die menschliche Gesundheit ausgehen – normale Produktions- und Anwendungsbedingungen vorausgesetzt.

08. Sept. 2003

Dr. Karsten Schaumann
Forschungsgruppe Marine Mykologie

Datum

Anlagen

Liste der Anlagen und Abbildungen

Anlage 1: Vorhergehendes Gutachten vom 14.11.2001

Anlage 2: Curriculum vitae

Anlage 3: Auswahl relevanter Publikationen

Abbildung 1: entspricht Fig. 7 und Tab. 1 in Gaertner, A. (1977), Veröff. Inst. Meeresforsch. Bremerh., Bd. 16: 139-157

Abbildung 2: Phylogramm auf der Basis der 18S rRNA Gensequenzen nach Honda, Fig. 1 (persönlich)

Abbildung 3: Phylogramm der Chromista (Straminopila) nach Honda, Fig. 2 (persönlich)

Abbildung 4: Phylogramm der Thraustochytriales nach Honda, Fig. 3 (persönlich)

Abbildung 5: siehe Legende auf der Abbildung

Attachment 2

Selection of pertinent publications

Beakes, G.W. (1998) In: Coombs, G.H.; Vickerman, K.; Sleigh, M.A. and Warren, A. (eds.) Evolutionary Relationships Among Protozoa. Chapman & Hall, London, 1998, pp. 352-373

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November 14, 2001

SCIENTIFIC EXPERT OPINION CONCERNING ULKENIA SP.[redacted]

As requested by Celanese Ventures GmbH, I herewith submit the following scientific expert opinion concerning Ulkenia sp. strain[redacted]. I understand that the strain [redacted] will be used in the professional commercial production of certain polyunsaturated fatty acids ("PUFAs") for use as food ingredients.

My internationally recognized scientific expertise in the field of marine fungi and fungoid protists is evidenced in the attached curriculum vitae.

I have critically evaluated the currently available data and information pertinent to the safety of Ulkenia sp.. To the best of my knowledge, it is my opinion that the strain[redacted], identified as a member of the marine fungoid protist genus Ulkenia, i.e. as Ulkenia sp., is both non-toxigenic and non-pathogenic to man. The genus Ulkenia belongs to the family Thraustochytriaceae, order Thraustochytriales, class Labyrinthulomycetes, phylum Labyrinthulomycota, kingdom Protista. However recently, on the basis of molecular genetic analysis (16S- or 18S-rRNA gene sequencing), the phylum Labyrinthulomycota has been classified within the subkingdom Heterokonta, kingdom Stramenopila/Chromista. I am not aware of any facts, which would jeopardize the safety of Ulkenia sp..

14. 11. 2001
Date

Dr. Karsten Schaumann
Head, Marine Mycology Research Group

Attachments 1 - Curriculum Vitae,
2 - Selection of pertinent literature

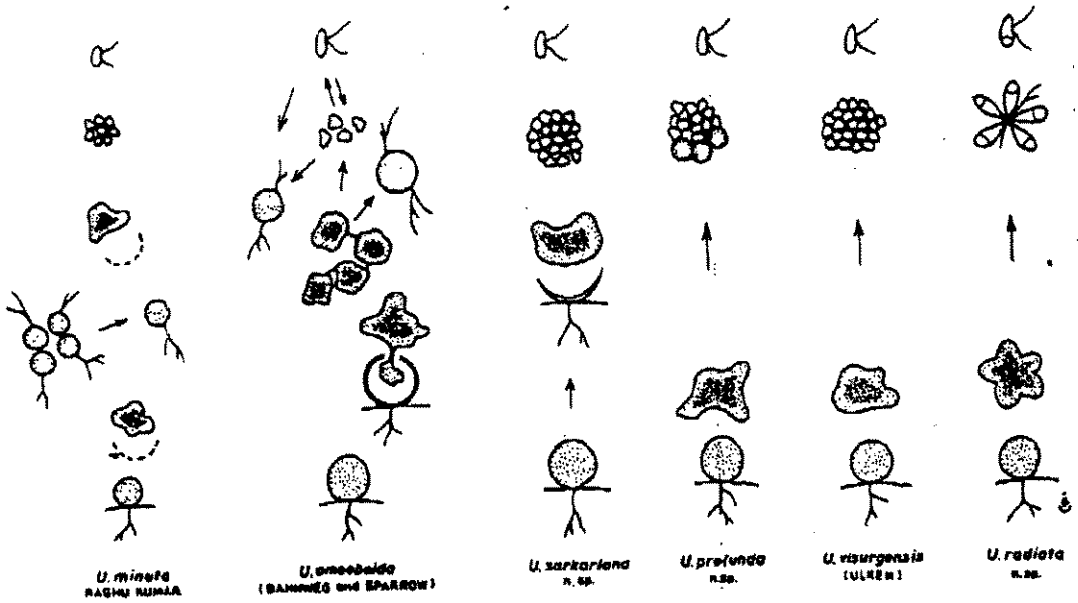

Fig. 7: Species of *Ulkenia* nov. gen. known till now, with their main characters.

Aus: Gaertner, A. (1977) Veröff.Inst.Meeresforsch.Brh. 16:139-157

Table 1: Identifying characters of the members of the genus *Ulkenia*.

Species	Sporangium grown on		Zoospore size µm	Length of unequal flagella µm	Protoplast					Prolifer-ating bodies	Successive bipart-itioning
					amoeboid liberation			cleavage			
	pine pollen µm	brine shrimp µm			pore form-ation	wall dissolution: par-tial	wall dissolution: tot-al	not reg-ular	centri-petal		
U. visurgensis	15.0-20.0		3.0x4.3	5.0+6.0(?)			+	+		–	–
U. amoeboida	–	20-25	3.0x5.8	10.0+5.0(?)	+			+		–	+
U. minuta	6.2-10.5		1.2x2.0	4.0+1.3			+	+		–	+
U. sarkariana	6.0-28.0		2.9x4.2	6.3+5.9		+		+		–	–
U. profunda	16.0-22.0		3.1x4.1	6.7+6.6			+	+		1-5	–
U. radiata	8.0-18.0		3.4x7.3	6.6+6.3			+		+	–	–

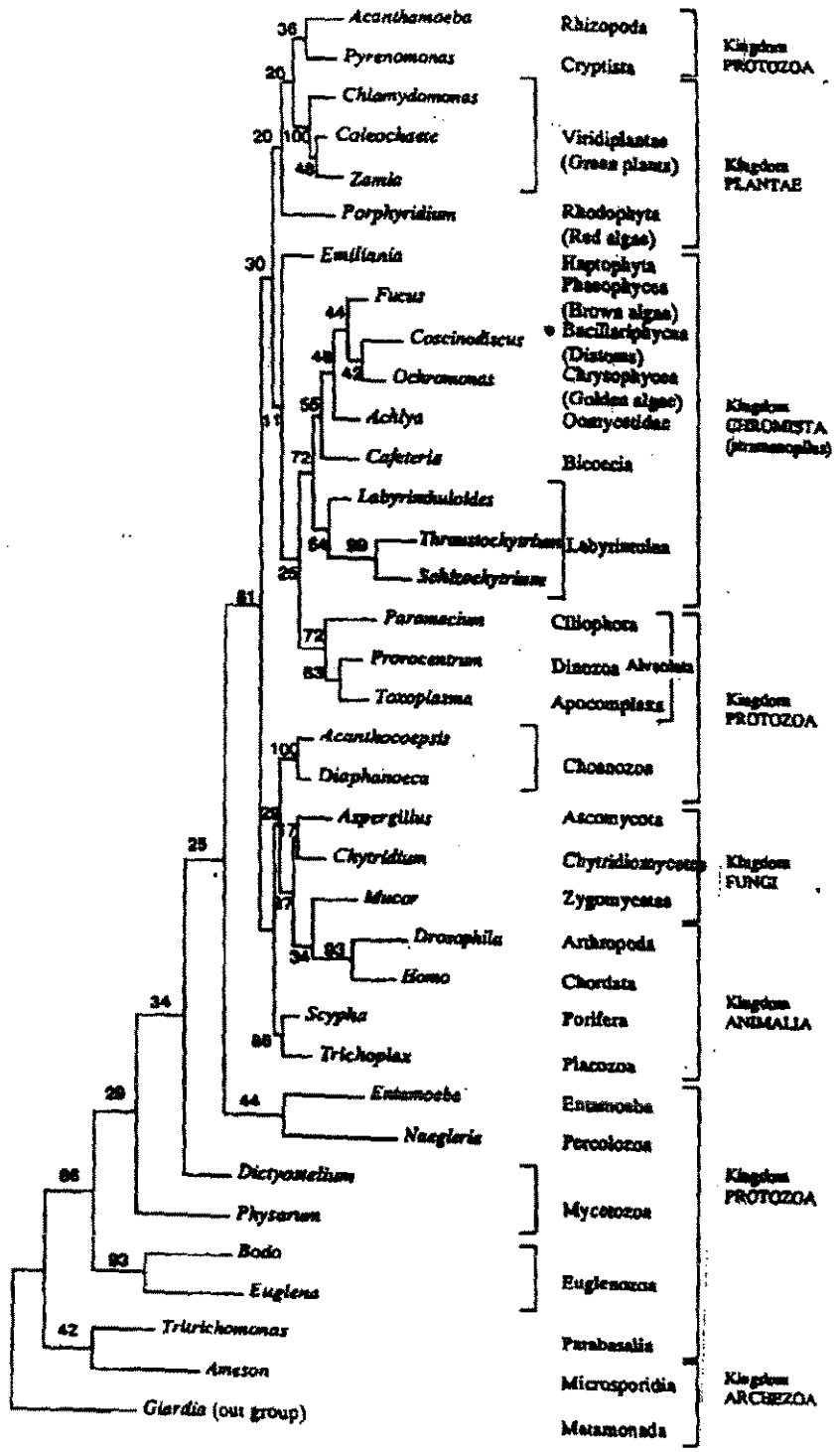

Fig. 1. Phylogenetic analysis of 18S rRNA gene sequences. Tree was constructed with the maximum parsimony method (PAUP, Swofford 1993). The percentage of 100 bootstrap resamplings from the aligned data set supports that the topological elements is indicated at the left of each node. Outgroup species was *Giardia*.

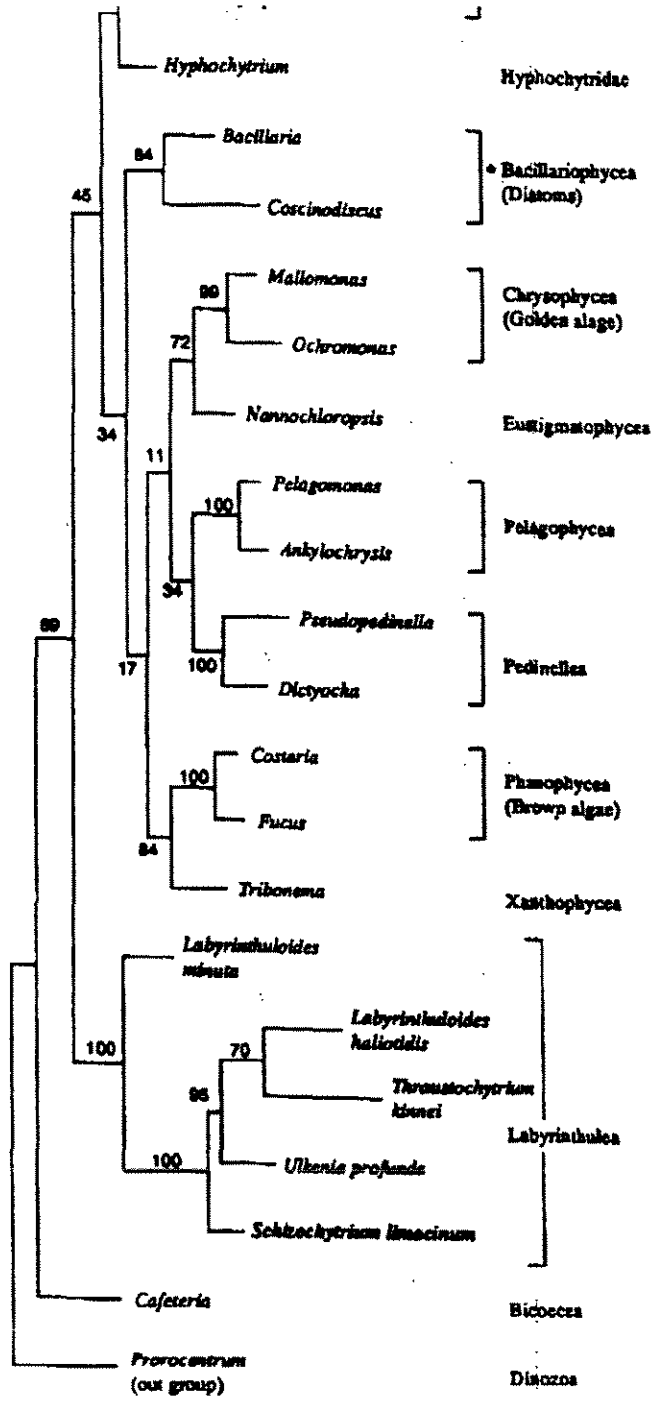

Fig. 2. Phylogenetic analysis of 18S rRNA gene sequences. Tree was constructed with the maximum parsimony method (PAUP, Swofford 1993). The percentage of 100 bootstrap resamplings from the aligned data set supports that the topological elements is indicated at the left of each node. Outgroup species was *Prorocentrum* (dinoflagellate).

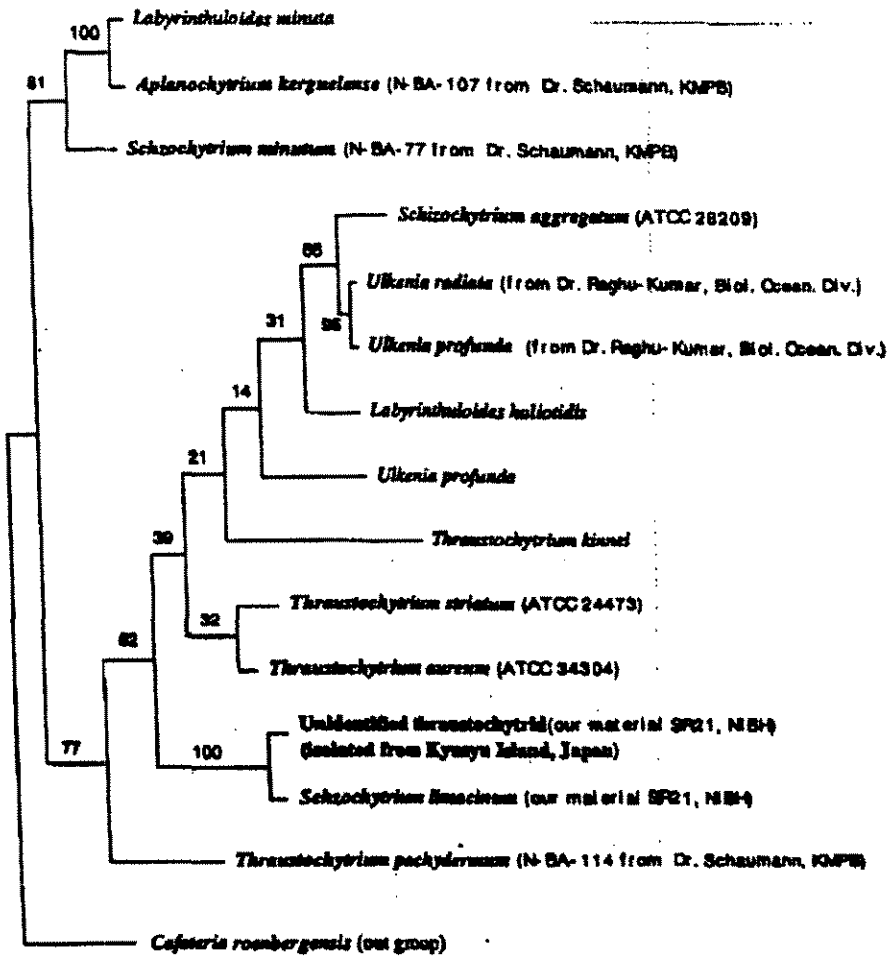

Fig. 3. Phylogenetic analysis of partial 18S rRNA gene sequences. Tree was constructed with the maximum parsimony method (PAUP, Swofford 1993). The percentage of 100 bootstrap resamplings from the aligned data set supports that the topological elements is indicated at the left of each node. Outgroup species was *Cafeteria* (Bicoecea). The examined species in the present study are represented by bold letters.

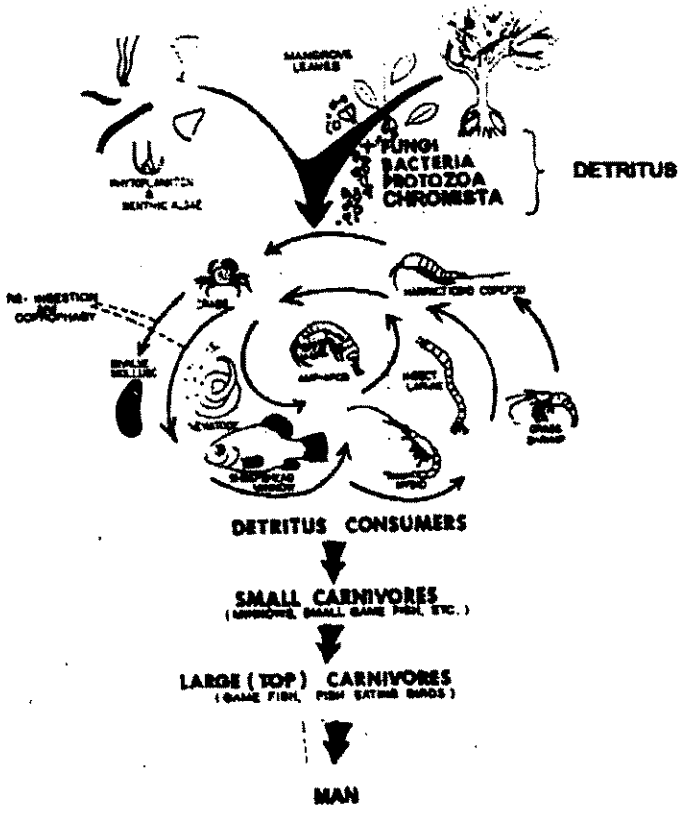

Abb. : Illustration der Rolle von Thraustochytriden (einschließlich *Ulkenia*) innerhalb der Nahrungskette in einem Mangrove-Ökosystem. Nach einer Abbildung von Odum, W.E. (1970) – mit Ergänzungen durch Schaumann, K. (2003).

APPENDIX C.3

Certificate of Microalgal Toxin Analysis and Analytical Report

MASTER

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Jena, 30.10. 2001

Untersuchungen auf Algentoxine

Proben:

Nr.	Proben-Nr.	Labor-Nr.	Eingangsdatum
PUFA-Öle			
01.	Lot. 990222	45 1001-1	29.10.01
02.	Lot. 110708	45 1001-2	29.10.01
03.	Lot. 120306	45 1001-3	29.10.01

ASP (Wasserlösliche Domoinsäure, Gehalte in µg/kg*):

01.	n. n.
02.	n. n.
03.	n. n.

*) Grenzwert lt. §16 der Fisch-HV v. 08.06.2000:
20 mg Domoinsäure/kg
n. n. (nicht nachweisbar, Nachweisgrenze LC/MS: 100 µg/kg

DSP (Fettlösliche Algentoxine, Gehalte in µg/kg*):

Nr:	OA**	DTX 1 ***	DTX 2****
01.	n. n.	n. n.	n. n.
02.	n. n.	n. n.	n. n.
03.	n. n.	n. n.	n. n.

*) Grenzwert lt. §16 in Verb. mit Anl. 3 der Fisch-HV v. 08.06.2000:
400 µg DSP/kg Hepatopankreas
n. n. (nicht nachweisbar, Nachweisgrenze LC/MS: 50 µg/kg)
**) Okadasäure
***) Dinophysistoxin 1
****) Dinophysistoxin 2

PSP (Wasserlösliche Algentoxine, Gehalte in µg/kg*):

Nr:	dc-STX	STX	sonst. PSP**	PSP-Gehalt in STX Äquiv.**
01.	n. n.	n. n.	n. n.	n. n.
02.	n. n.	n. n.	n. n.	n. n.
03.	n. n.	n. n.	n. n.	n. n.

*) Grenzwert lt. §16 in Verb. mit Anl. 3 der Fisch-HV v. 08.06.2000:
800 µg STX-Äquivalente/kg
n.n. nicht nachweisbar (Nachweisgrenze HPLC: 10 µg PSP-Toxin/kg)
**) unter Berücksichtigung der Äquivalenzfaktoren (TEFs) zur Berechnung der Gesamttoxizität als STX Äquiv. (der Äquivalenzfaktor von STX beträgt 1,0)

Microcystine (Microcystin-RR, -LR, -YR, -LA, -LW, -LF) und Nodularin

Nr:	MC-RR	MC-LR	MC-YR	MC-LA	MC-LW	MC-LF
01.	n. n.	n. n.	n. n.	n. n.	n. n.	n. n.
02.	n. n.	n. n.	n. n.	n. n.	n. n.	n. n.
03.	n. n.	n. n.	n. n.	n. n.	n. n.	n. n.

Nr:	Nodularin
01.	n. n.
02.	n. n.
03.	n. n.

n. n.: nicht nachweisbar

(Nachweisgrenze HPLC: 100 µg MC-RR, -YR, -LR, -LA, -LW, -LF, Nodularin/kg)

Beurteilung:

Die eingesandten Proben PUFA-Öle wurden chemisch (HPLC/FLD bzw. LC/MS-Bestimmungen) auf das Vorhandensein von ASP-, DSP-und PSP-Toxinen sowie Nodularin und Microcystinen untersucht.

In den Proben lag die Konzentration an ASP-, DSP-, PSP-Toxinen und Microcystinen unter den gesetzlich festgelegten Höchstmengen.

Damit entspricht Ware gleicher Art und Beschaffenheit bzgl. ihrer Kontamination mit Algentoxinen den für Lebensmittel festgesetzten Anforderungen und ist verkehrsfähig.

Summary:

The PUFA-oils LOT 990222, 110708, 120306 were analysed on a potential contamination with algal and cyanobacterial toxins. Our analysis covered ASP, DSP, and PSP toxins regulated by national food control legislation. In addition, the investigation included the hepatotoxic microcystins and nodularin formed by cyanobacteria.
The absence of these toxins in all samples was proven by application of LC-MS.

Prof. Dr. B. Luckas

Staatl.-gepr. Lebensmittelchemiker

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Protos-Biotech

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D-65926 Frankfurt am Main

Jena, den 12.Sept. 2003

On the possible occurrence of algal toxins in microalgal oil (supplied as DHA45-oil) and dry algal biomass

I have been working on analysis of algal toxins since 1986, i.e. more than 15 years. In the course of my work I developed sensitive analytical methods for the detection and quantitation of algal toxins and studied occurrence of algal toxins in seafood and food products.
Integral part of development of analytical methods were investigations in the stability of analytes under conditions of sample preparation and analysis to ascertain reliability of the methods developed.
On the basis of this experience and work I am able to conclude on the possible occurrence of algal toxins in microalgal oil and dry algal biomass obtained from Nutrinova GmbH.

Analytical results for DHA45-oil and dry algal biomass

I was asked to analyse refined microalgal oil (supplied as DHA45-oil) and dry algal biomass for the presence of the following toxins:

Amnesic shellfish poisoning (ASP) toxins
= Domoic acid
Diarrhetic shellfish-poisoning (DSP) toxins
= Okadaic acid and Dinophysistoxins, Pectenotoxins, Yessotoxins, Azaspiracides
Paralytic shellfish-poisoning (PSP) toxins
= Saxitoxin, Neosaxitoxin, Decarbamoylsaxitoxin, Gonyautoxins
Neurotoxic shellfish-poisoning (NSP) toxins
= Brevetoxins
Cyanobacterial toxins (hepatotoxic cyclic peptides)
= Microcystins, Nodularin
Substances with haemolytic activity (Ichthyotoxins)
= Prymnesium toxins

The following batches were analyzed for the content of the listed toxins:

DHA45-oil:
Batch 990222,110708,120306,
AA00107-1, AA00108-1
Dry algal biomass:
Batch DIP 28M, 30M, 31M, 32M, 33M

According to the information of the supplier the oils and biomass batches originated from different fermentation batches all using the same production strain.

The information provided with the oil indicated that all batches underwent refining including alkali treatment, bleaching with absorbing agents and deodorising by steam injection.

The same samples were also sent to Prof. Dr. Gerd Liebezeit (Forschungszentrum Terramare, Wilhelmshaven), a laboratory experienced in a.o. biotests as well as tests for haemolytic activity as I do not have the necessary testing facilities in my own laboratories.

These analyses include both tests for haemolytic activity and measuring the effects of harmful substances as the ichthyotoxins Prymnesin 1 and 2 on larvae of *Artemia salina*.

Results

The control of samples for algal and cyanobacterial toxins carried out by application of physical chemical methods as HPLC with selective detection and LC-MS results in clear evidence of absence of algal or cyanobacterial toxins.
Sample extracts in high concentrations revealed haemolytic activity. However, PUFAs in high concentrations cause haemolysis, too. Therefore, the same extracts were tested regarding their toxicity against larvae of *Artemia salina*.
The results of the *Artemia salina* assay were negative, i.e. no effects on the larvae were observed. Consequently, ichthyotoxins as Prymnesin 1 and 2 were also not present in the samples.

Summing up it can be stated that none of the samples supplied by Nutrinova GmbH contained detectable amounts of ASP, DSP, PSP, NSP, microcystins, nodularin or prymnesium toxins.

The samples received, methods of analysis and results are described in detail in the attached analytical reports (Annex 1 and 2).

Stability considerations and characteristics of the toxins

Published information on the occurrence of the toxins in question and on their characteristics renders their presence in refined oils highly unlikely. Consequently, the analytical data are as expected and do not show presence of these toxins.

Although none of the listed toxins were detectable in DHA45-oil and dry algal biomass my consideration is that it is very unlikely that such toxins might contaminate DHA45-oil because of their chemistry, solubility and because of the used downstream process of the algal biomass (e.g. refining).

PSP toxins, domoic acid, microcystins, nodularin, and prymnesium toxins are highly water-soluble and not soluble in organic solvents.

Regarding the solubility and chemistry it is unlikely to believe that the very high polar cationic substances as PSP toxins or the non-lipophilic toxins domoic acid, the cyclic peptides, and the prymnesium toxins will remain in DHA45-oil after extraction of the biomass and further refining.

Furthermore, PSP toxins will be inactivated and oxidized by alkaline exposure (e.g. neutralization) and also removed by the bleaching step during refining.

Domoic acid is a hydrophilic, water-soluble substance containing three carboxyl groups. Therefore, it would be removed by alkali treatment applied for DHA 45-oil to remove free fatty acids.

Microcystins and nodularin are cyclic peptides and will be removed during bleaching. Prymnesium toxins are acidic polar phosphor-proteolipids and are not heat stable. Therefore, they will be degraded during deodorization.

Although some groups of the algal toxins are more lipophilic(e.g. the substances belong to the DSP complex such as okadaic acid and the dinophysistoxins, yessotoxins, pectenotoxins, azaspiracids and the NSP toxins brevetoxins) it is highly unlikely to assume that they will be found in DHA45-oil after complete refining.

The substances of the DSP complex are soluble in polar organic solvents as methanol, acetone or dichloromethane. However, they are not soluble in non-polar organic solvents as n-hexane. In addition, DSP toxins will be degraded in acid or base (e.g. degumming and neutralization).

Brevetoxins are not stable in solutions of pH beyond 2 or 10 and would therefore be degraded during alkali treatment (e.g. neutralisation) to remove free fatty acids.

Conclusions

It has been proven that biotoxins such as those from microalgae pose a serious hazard to human health when present in food or special diet. Therefore, it is necessary to control particularly algal based components foreseen for production of functional food or nutraceuticals regarding their content of algal and cyanobacterial toxins.

The analytical results regarding these harmful substances in samples of DHA45-oil and dry algal biomass obtained from Nutrinova GmbH showed the absence of algal based biotoxins. These results reflect my experience with stability and chemistry of the toxins under investigation and renders their presence in refined oils highly unlikely.

The analyses covered all groups of algal and cyanobacterial toxins published in the literature and mentioned in international food regulations. If no maximum level for a toxin in food is established the analysis was directed to exclude also very low concentrations of a certain toxin using both LC/MS methods and bioassays.

I do, therefore, not anticipate any risk of presence of microalgal toxins known to occur in DHA45-oil.

Prof. Dr. Bernd Luckas
Chemist and licensed Food Chemist

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Annex 1

Analyses regarding algal toxins

Samples:

No.	Sample-No.	Labor-No.	Date:
1:	Biomass DIP28M	48 0803-1	06.08.03
2:	Biomass DIP30M	48 0803-2	06.08.03
3:	Biomass DIP31M	48 0803-3	06.08.03
4:	Biomass DIP32M	48 0803-4	06.08.03
5:	Biomass DIP33M	48 0803-5	06.08.03
6:	Oil LotAA00107-1	48 0803-6	06.08.03
7:	Oil LotAA00108-1	48 0803-7	06.08.03

Amnesic Shellfish Poisoning (ASP) Toxins
Domoic acid: DA (Content in µg/kg*)

No.	DA
01.	n. d.
02.	n. d.
03.	n. d.
04.	n. d.
05.	n. d.
06.	n. d.
07.	n. d.

n. d.: not detectable Limit of detection LC/MS: 100 µg/kg

*) Maximum level of ASP according to §16 of the German Fisch-Hygiene-Verordnung of 08.June2000: 20 mg Domoic acid/kg

Diarrhetic Shellfish Poisoning (DSP) Toxins
Okadaic acid and Dinophysistoxins: OA and DTXs (Content in µg/kg*)

No:	OA	DTX 1	DTX 2	DTX 3
01.	n. d.	n. d.	n. d.	n. d.
02.	n. d.	n. d.	n. d.	n. d.
03.	n. d.	n. d.	n. d.	n. d.
04.	n. d.	n. d.	n. d.	n. d.
05.	n. d.	n. d.	n. d.	n. d.
06.	n. d.	n. d.	n. d.	n. d.
07.	n. d.	n. d.	n. d.	n. d.

n. d.: not detectable Limit of detection LC/MS: 50 µg/kg

*) Maximum level of DSP according to §16 of the German Fisch-Hygiene-Verordnung of 8.June2000: 400 µg OA/kg hepatopancreas

*) Maximum level of OA, DTXs and Pectenotoxins according to Official Journal of the European Communities of 15. March 2002 (L 75/62-64): 160 mg OA equivalents/kg

Pectenotoxins: PTX-1 and PTX-2 (Content in µg/kg*)

No:	PTX-1	PTX-2
01.	n. d.	n. d.
02.	n. d.	n. d.
03.	n. d.	n. d.
04.	n. d.	n. d.
05.	n. d.	n. d.
06.	n. d.	n. d.
07.	n. d.	n. d.

n. d.: not detectable Limit of detection LC/MS: 50 µg/kg

*) Maximum level of Pectenotoxins according to Official Journal of the European Communities of 15. March 2002 (L 75/62-64): 160 µg OA equivalents/kg

Yessotoxins: YTX, OH-YTX, Homo-YTX and OH-Homo-YTX (Content in µg/kg*)

No:	YTX-1	OH-YTX	Homo-YTX	OH-Homo-YTX
01.	n. n.	n. n.	n. d.	n. d.
02.	n. n.	n. n.	n. d.	n. d.
03.	n. n.	n. n.	n. d.	n. d.
04.	n. n.	n. n.	n. d.	n. d.
05.	n. n.	n. n.	n. d.	n. d.
06.	n. n.	n. n.	n. d.	n. d.
07.	n. n.	n. n.	n. d.	n. d.

n. d.: not detectable Limit of detection LC/MS: 50 µg/kg

*) Maximum level of Yessotoxins according to Official Journal of the European Communities of 15. March 2002 (L 75/62-64): 1 mg/kg

Azaspiracides: AZA-1, AZA-2 and AZA-3 (Content in µg/kg*)

No:	AZA-1	AZA-2	AZA-3
01.	n. d.	n. d.	n. d.
02.	n. d.	n. d.	n. d.
03.	n. d.	n. d.	n. d.
04.	n. d.	n. d.	n. d.
05.	n. d.	n. d.	n. d.
06.	n. d.	n. d.	n. d.
07.	n. d.	n. d.	n. d.

n. d.: not detectable Limit of detection LC/MS: 50 µg/kg

*) Maximum level of Azaspirazids according to Official Journal of the European Communities of 15. March 2002 (L 75/62-64): 160 µg/kg

Paralytic Shellfish Poisoning (PSP) Toxins

Saxitoxin (STX), Neosaxitoxin (Neo), Decarbamoylsaxitoxin (dcSTX), Gonyautoxins (GTXs) (Content in µg/kg*)

No:	STX	Neo	dcSTX	GTXs	PSP content (STX equiv.**)
01.	n. d.	n. d.	n. d.	n. d.	n. d.
02.	n. d.	n. d.	n. d.	n. d.	n. d.
03.	n. d.	n. d.	n. d.	n. d.	n. d.
04.	n. d.	n. d.	n. d.	n. d.	n. d.
05.	n. d.	n. d.	n. d.	n. d.	n. d.
06.	n. d.	n. d.	n. d.	n. d.	n. d.
07.	n. d.	n. d.	n. d.	n. d.	n. d.

n. d.: not detectable Limit of detection HPLC: 10 µg PSP/kg

*) Maximum level of PSP toxins according to §16 of the German Fisch-Hygiene-Verordnung of 8.June2000: 800 µg STX equivalents/kg

**) with regards to the PSP equivalent factors (TEFs) for calculation of total PSP toxicity as STX equivalents (equivalent factor for STX = 1.0)

Neurotoxic Shellfish Poisoning (NSP)-Toxine

Brevetoxins PbTx-2 and PbTx-3 (Content in µg/kg)

No:	PbTx-2	PbTx-3
01.	n. d.	n. d.
02.	n. d.	n. d.
03.	n. d.	n. d.
04.	n. d.	n. d.
05.	n. d.	n. d.
06.	n. d.	n. d.
07.	n. d.	n. d.

n. d.: not detectable Limit of detection LC/MS: 50 µg/kg

Cyanobakterial Toxins (Hepatotoxins)

Microcystine MC-RR, -LR, -YR, -LA, -LW, -LF and Nodularin (Content in µg/kg)

No:	MC-RR	MC-LR	MC-YR	MC-LA	MC-LW	MC-LF
01.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
02.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
03.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
04.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
05.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
06.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
07.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

No:	Nodularin
01.	n. d.
02.	n. d.
03.	n. d.
04.	n. d.
05.	n. d.
06.	n. d.
07.	n. d.

n. d.: not detectable Limit of detection LC/MS: 100 µg/kg

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Annex 2

Bestimmung der hämolytischen Aktivität von Biomasse- und Ölproben

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Einleitung

Nach Angaben des Auftraggebers Prof. Dr. Luckas enthalten die untersuchten Proben mehrfach ungesättigte langkettige Fettsäuren (PUFAs). Neben deren Wirkung in den eingesetzten Testsystemen war die Anwesenheit von Prymnesinen zu untersuchen.

Von PUFAs ist bekannt, daß sie auf Ionenkanäle wirken (Vreugdenhil et al., 1996; Perez et al., 1997; Xiao et al., 1997, 1998). Buffington (1987) berichtet, daß hohe Dosen von n-3 Fettsäuren die Funktion der Blutplättchen soweit verändern können, daß die Haemostasis gestört wird. Signifikante Erhöhungen der Blutungszeiten werden in anderen Studien über die Effekte von Fischölzusätzen zur Nahrung von Katzen und Hunden erwähnt (Landhmore et al., 1986). Die Food and Drug Administration der USA (FDA) gibt an, daß der Konsum von <3 g/d EPA plus DHA Blutungszeiten nicht verlängert (Fed Reg 62:30751). Andere Nebeneffekte bei hohen Dosen können Lethargie, Pruritis, Diarrhoea und Urticaria sein (Scott & Buerger, 1988).

Andererseits werden positive Effekte beschrieben, wenn diese Fettsäuren als Nahrungsmittelzusatz eingesetzt werden (z.B. Wood et al., 1999; Koletzko & Rodriguez-Palmero, 1999). Langkettige PUFAs können Herzarythmie durch Modulation der spannungsabhängigen Inaktivierung von Natrium- (I_{Na}) und Calciumströme (I_{Ca}) verhindern (Vreugdenhil et al. 1996). Zudem zeigen eine Vielzahl klinischer Studien die Vorteile der PUFA-Zugabe zu Nahrungsmitteln bei Entzündungen und Autoimmunerkrankungen (Borlak & Welch, 1994; Simopoulos, 2002).

Bislang nicht bekannt ist die hämolytische Aktivität dieser Verbindungen. Für die aus der Mikroalge *Fibrocapsa japonica* isolierten Fettsäuren 6,9,12,15-Octadecatetraensäure, (C18:4ω3), 5,8,11,14,17-Eicosapentaensäure (EPA, C20:5ω3) und 5,8,11,14-Eicosatetraensäure (Arachidonsäure, C20:4ω6) konnte hämolytische Aktivät nachgewiesen werden (Fu, 2003). Docosahexaensäure konnte in *F. japonica* und anderen Phytoplanktonspezies bislang nur als Nebenprodukt nachgewiesen werden (Marshall et al., 2002).

Ebenfalls bekannt ist, daß *Prymnesium*-Spezies hämolytisch wirkende Toxine bilden (z.B. Meldahl et al., 1994; Igararashi et al., 1996). Prymnesine wirken im Artemien-Biotest (Meldahl et al., 1994), da sie wasserextrahierbar sind bzw. als wasserlösliche Komponenten in das Kulturmedium ausgeschieden werden (Yariv & Hestrin, 1961). Durch eine geeignete Kombination von Tests sollte die An- bzw. Abwesenheit dieser Toxine in den untersuchten Proben festgestellt werden.

Methoden

Biomasseextraktion

200 mg der getrockneten Proben 1 bis 5 wurden mit 1,5 mL Methanol für 2 Minuten unltraschallbehandelt (Soniprep 150) und dann bei 13.000 UPM für 2 Minuten zentrifugiert.

Der Überstand wurde für die photometrischen Untersuchungen verwendet.

Vorbehandlung der Ölproben

Die Proben 6 und 7 wurden in Aceton gelöst und entsprechend Tabelle 1 eingesetzt.

10 µL der Proben 6 und 7 wurden zu 1 mL ELA-Puffer gegeben. Nach 10-minütiger Ultraschallbehandlung wurde die erhaltene Suspension über Nacht bei Raumtemperatur zur Phasentrennung stehengelassen. Die wäßrige Phase wurde untersucht.

Tabelle 1 Zusammensetzung der Reaktionsansätze im Erythrocytenlysistest (Ölproben)

	Probe [µL]	Aceton [µL]	Erythrocytenlösung [mL]
Negative Kontrolle	-	110	1,9
6	10	100	1,9
6	20	90	1,9
7	10	100	1,9
7	20	90	1,9
Positive Kontrolle		110	1,9 lysierte Erythrocyten

Probe 6 wurde zusätzlich in einer Verdünnungsreihe untersucht (Tabelle 2).

Tabelle 2 Zusammensetzung der verdünnten Reaktionsansätze im Erythrocytenlysistest (Ölprobe 6)

	Probe [µL]	Aceton [µL]	Erythrocytenlösung [mL]
Negative Kontrolle	-	110	1,9
6	110*	-	1,9
6	80**	30	1,9
6	60	50	1,9
6	40	70	
6	20	90	1,9
positive Kontrolle	-	110	1,9 lysierte Erythrocyten

* 10 µL Probe 6 + 100 µL Aceton, ** 80, 60, 40, 20 µL des mit * gekennzeichneten Ansatzes

Verdünnungsreihe im Artemientest

Für die notwendige Ermittlung der Aktivitätsabnahme mit abnehmendem Extraktvolumen wurden Analysen entsprechend Tabelle 3 durchgeführt.

Tabelle 3 Zusammensetzung der Reaktionsansätze im Erythrocytenlysistest (Biomasse-Verdünnungsreihe)

	Rohextrakt [µL]	Methanol [µL]	Erythrocytenlösung [mL]
negative Kontrolle	-	100	1,9
4	20	80	1,9
4	40	60	1,9
4	60	40	1,9
4	80	20	1,9
4	100		
positive Kontrolle	-	100	1,9 lysierte Erythrocyten

Erythrocytenlysisuntersuchungen (nach Yariv & Hestrin, 1961)

Jeweils 100 µL des Biomasserohextrakts, 110 µL des Öl/Acetongemisches bzw. 110 µL der wäßrigen Phase wurden mit 1,9 mL Erythrocytenlösung (5 x 10^6 Zellen/mL) in 12 mL Zentrifugenröhrchen versetzt. Als negative Kontrollen dienten 100 µL Methanol/1,9 mL Erythrocytenlösung, als positive Kontrollen 100 µL Methanol/1,9 mL lysierte Erythrocytenlösung bzw. 110µL Aceton/1,9 mL Erythrocytenlösung und 110 µL Aceton/1,9 mL lysierte Erythrocytenlösung.

Nach Inkubation für 24 h bei Raumtemperatur wurden die Reaktionsansätze bei 4.500 UPM für 5 Minuten bei 10 °C zentrifugiert. Danach wurden1,2 mL des Überstandes bei 414 nm photometrisch vermessen.

Alle Proben wurden zweifach bestimmt. Dabei ergab sich eine mittlere relative Standardabweichung von 5,5 %. Bei den untersuchten wäßrigen Phasen betrugen diese Werte als Folge der sehr niedrigen Konzentrationen der Öle 12,4 bzw. 44,9 %.

Die Blindwerte der reinen Lösungsmittel Aceton bzw. Methanol betrugen 0,118 E und 0,133 E: Alle Meßwerte wurden für diese Blindwerte korrigiert.

Erythrocytenlysistest

Der Test basiert auf der photometrischen Bestimmung des bei der Lysis von Erythrocyten freigesetzten Hämoglobins bei 414 nm (Abb. 1).

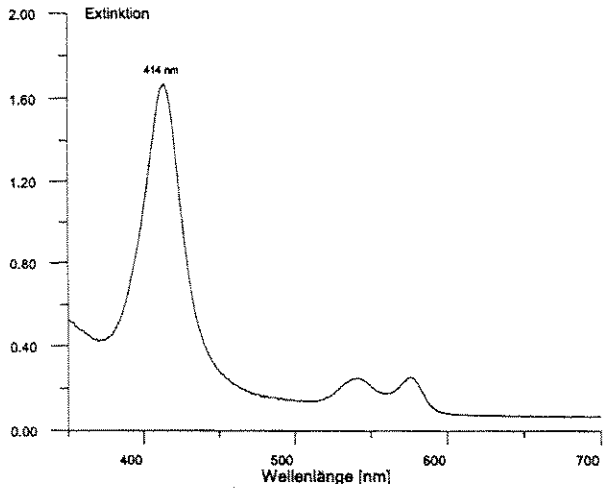

Abb. 1 Absorptionsspektrum vollständig lysierter Humanerythrocyten (7 x 10^6 Zellen/mL)

Die Untersuchung ist über den Bereich 10^5 bis 7 x 10^6 Zellen/mL linear (Abbildung 2).

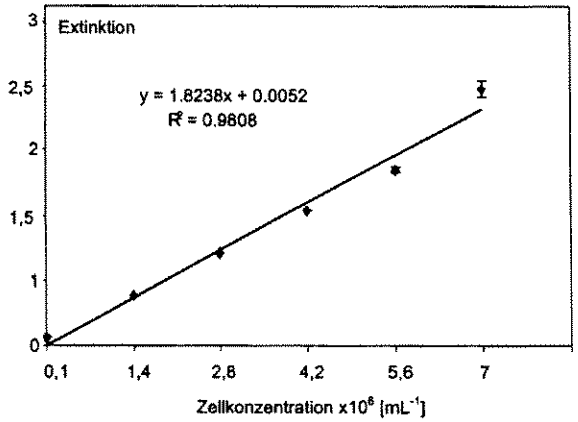

Abb. 2 Linearität des Erythrocytenlysistests

Humanerythrocyten der Blutgruppe A wurden mit ELA-Pufferlösung versetzt. Die Pufferlösung bestand aus 150 mM NaCl, 3,2 mM KCl, 1,25 mM $MgSO_4$-Heptahydrat, 3,75 mM $CaCl_2$ und 12,2 mM Tris-Base, die mit HCl auf pH 7,4 eingestellt war. Die Erythrocycten wurden mit ELA-Puffer gewaschen, 5 Minuten bei 4.500 UPM bei 10 °C zentrifugiert und dann resuspendiert. Zellzahlen wurden mit Erythrocytometrie bestimmt.

Artemientest

Der Artemientest beruht auf der Wirkung toxischer Stoffe auf Larven des Salinenkrebses *Artemia salina*. Er ist ein unspezifischer Test mit relativ geringer Empfindlichkeit.

Artemia-Larven wurden vor den Untersuchungen frisch in filtriertem Meerwasser erzeugt. Sie wurden innerhalb von 24 h nach dem Schlüpfen eingesetzt.

Jeweils 100 µL Rohextrakt wurden mit 3,9 mL filtriertem Meerwasser in 10 mL Bechergläsern vermischt. Als Kontrollen wurden 100 µL Methanol verwendet. Für die beiden Ölproben 6 und 7 wurde entsprechend Tabelle 4 verfahren. Zu jedem Ansatz wurden 12 Artemia-Larven zugegeben. Da die Larven phototrop sind, wurden die Ansätze für 24 h bei Raumtemperatur belichtet. Die Auswertung erfolgte unter einem Binokular.

Tabelle 4 Zusammensetzung der Proben für den Artemientest

	Probe [µL]	Aceton [µL]	Seewasser [mL]
Kontrolle	-	100	3,9
Probe 6	5	95	3,9
Probe 6	10	90	3,9
Probe 7	5	95	3,9
Probe 7	10	90	3,9

Ergebnisse

Hämolytische Effekte der Ölproben

In reiner Form zeigten die beiden Ölproben hohe Aktivitätswerte (Abb. 3). Bei 20 µL Ölvolumen wurden 93,3 % (Probe 6) bzw. 92,4 % (Probe 7) Lysis beobachtet, bei 10 µL Ölvolumen reduzierten sich die Werte auf 76,7 % (Probe 6) und 75,8 % (Probe 7).

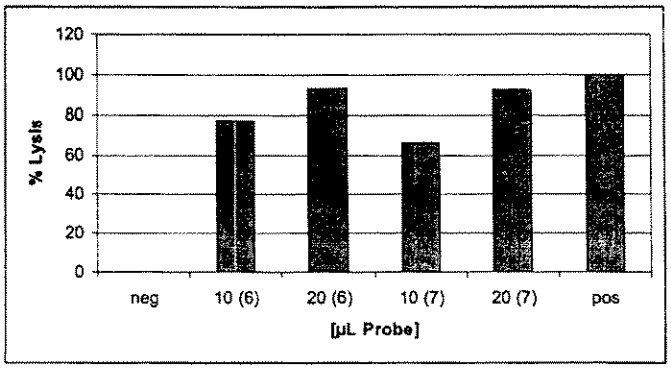

Abb. 3 Hämolytische Aktivität der Ölproben 6 und 7 (Erythrocytenlösung 5 x 10^6 Zellen mL^{-1})

Es wurde daher zusätzlich eine Verdünnungsreihe der Probe 7 untersucht (Abbildung 4).

Dabei zeigte sich eine Reduktion um 46 % bei 5-facher Verdünnung. Extrapoliert ergibt dies eine Nullaktivität des Öles bei 0,47 µL.

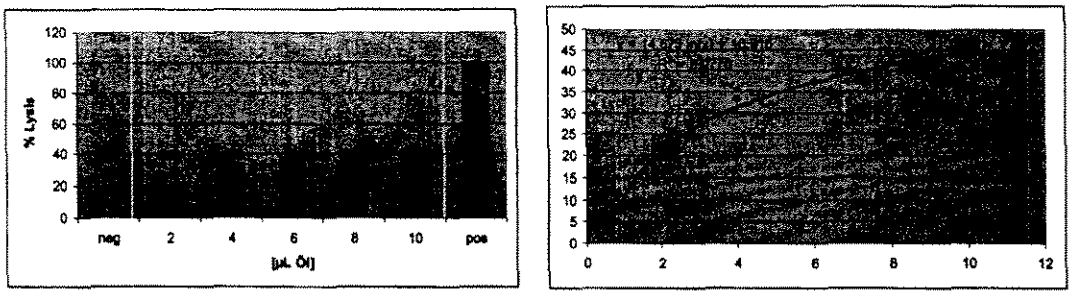

Abb. 4 Verdünnungsreihe der Probe 7 (Erythrocytenlösung 1,4 x 10^6 Zellen mL^{-1})

Die wäßrige Phase des Gemisches Öl/ELA-Puffer zeigte vernachlässigbare hämolytische Aktivität von 1,9 % (Probe 6) bzw. 6,8 % (Probe 7; Abb. 5).

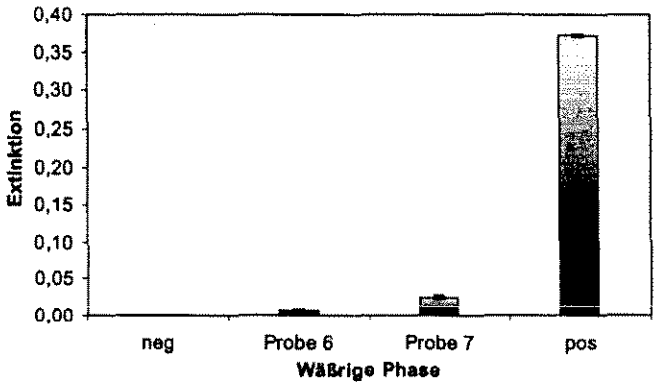

Abb. 5 Hämolytische Effekte der wäßrigen Phase von Öl/Wassergemischen (Erythrocytenlösung 1,4 x 10^6 Zellen mL^{-1})

Hämolytische Effekte der Biomasseproben

Die Rohextrakte der Proben 1 bis 5 zeigten Extinktionswerte um 1 entsprechend einer 100-%igen Lysis (Abb. 6).

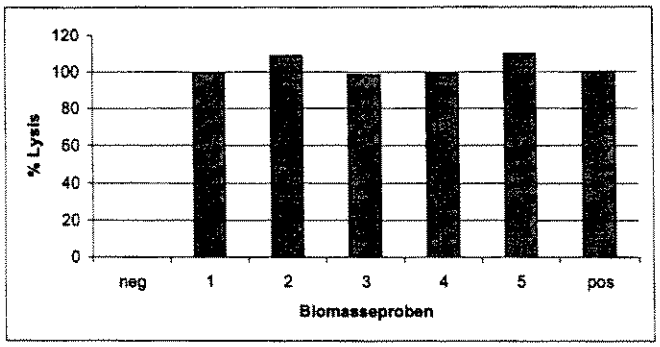

Abb. 6 Hämolytische Effekte der Proben 1 bis 5 (Erythrocytenlösung 5 x 10^6 Zellen mL^{-1})

Von Probe 4 wurde daher eine Verdünnungsreihe entsprechend Tabelle 3 untersucht. Hier zeigte sich eine Reduktion um 31 % bei einer 5-fachen Verdünnung (Abb. 7).

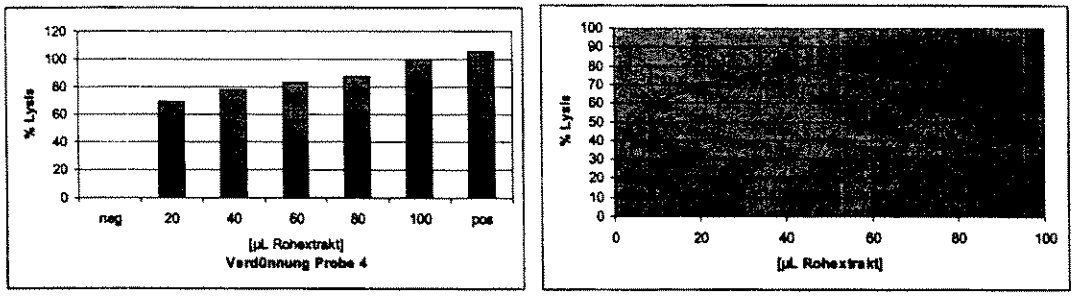

Abb. 7 Hämolytische Effekte des Rohextrakts der Probe 4 in verschiedenen Verdünnungen (Erythrocytenlösung 1,4 x 10^6 Zellen mL^{-1})

Artemientest

Bei allen Proben konnte nach 24 h Exposition keine Aktivitätsänderung bei den Artemien-Larven festgestellt werden, d.h. es wurde weder eine akute noch eine chronische Wirkung festgestellt.

Allgemeine Bewertung

Die hämolytisch wirkenden Stoffe sind nur in sehr geringem Umfang löslich in Wasser und hydrophilen organischen Lösungsmitteln. Dies wird durch die geringen Effekte der Wasser/Ölgemische im Hämolysetest und die nicht nachweisbare Aktivität im Artemientest belegt.

Damit kann die Anwesenheit wasserlöslicher Prymnesine oder anderer wasserlöslicher Toxine ausgeschlossen werden.

Die hämolytische Wirkung der untersuchten Öle ist mit hoher Wahrscheinlichkeit auf die Anwesenheit mehrfach ungesättigter Fettsäuren zurückzuführen, die in reiner Form hämolytische Aktivität zeigen.

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1964-1970 studies of chemistry at the Ernst-Moritz-Arndt-University Greifswald;

1970 Department of toxicology/toxic residues at the hygiene institute Rostock;

1978 Ph. D. graduation at the University of Greifswald, Germany in analytical chemistry;

1979 Head of the analytical department of the faculty for Biology at the University of Rostock;

1980 escape from the GDR into the Federal Republic of Germany,

1981-1989 director of the governmental food control laboratory of the country of Schleswig-Holstein in Flensburg, Germany;

1981-1984 studies of food chemistry an the University of Hamburg parallel to the employment in Flensburg;

1989 habilitation (Dr. habil) at the University of Hamburg in food chemistry;

1989 Professor (C3) for food chemistry at the Institute of food technology at the University of Stuttgart-Hohenheim, Germany;

since 1993 full Professor (C4) for food chemistry at the Friedrich-Schiller-University of Jena, Germany

Appendix 2

Definition of Haemolytic Activity of Biomass and Oil Samples

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Introduction

According to data provided by the client Prof. Luckas, the tested samples contain long-chain polyunsaturated fatty acids (PUFAs). Tests are to be carried out on both their effect in the applied test systems and the presence of prymnesins.

PUFAs are known to have an effect on ion channels (Vreugdenhil et al., 1996; Perez et al., 1997; Xiao et al., 1997, 1998). Buffington (1987) reports that high doses of n-3 fatty acids can change the function of blood platelets so that haemostasis is inhibited. Significant increases in bleeding times are mentioned in other studies on the effects of fish oil supplements in cat and dog food (Landhmore et al., 1986). The USA Food and Drug Association (FDA) states that the consumption of <3 g/d EPA plus DHA does not extend bleeding times (Fed Reg 62:30751). Other side effects of high doses can be lethargy, pruritus, diarrhoea and urticaria (Scott & Buerger, 1988).

On the other hand, positive effects are described when these fatty acids are used as a dietary supplement (e.g. Wood et al., 1999; Koletzko & Rodriguez-Palmero, 1999). Long-chain PUFAs can prevent cardiac arrhythmia via modulation of the voltage-dependent inactivation of sodium (I_{Na}) and calcium (I_{Ca}) currents (Vreugdenhil et al. 1996). Furthermore, many clinical studies show the advantages of PUFA supplements in food for inflammations and autoimmune illnesses (Borlak & Welch, 1994, Simopoulos, 2002).

The haemolytic activity of these compounds is as yet unknown. Haemolytic activity was shown for the following fatty acids isolated from the microalga *Fibrocapsa japonica*: 6, 9, 12, 15-octadeca-tetraenoic acid (C18:4⌉3), 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA, C20:5⌉3) and 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid, C20:4⌉6) (Fu, 2003). Docosahexaenoic acid was up to now only detected as a byproduct in *F. japonica* and other species of phytoplankton (Marshall et al., 2002).

It is also known that *Prymnesium* species produce toxins with haemolytic effects (e.g. Meldahl et al., 1994; Igarashi et al., 1996). Prymnesins have an effect in the Artemia biotest (Meldahl et al., 1994) as they are hydroextractable or are deposited as water-soluble components in the culture medium (Yariv & Hestrin, 1961). The presence or absence of these toxins was to be determined in the samples by a suitable combination of tests.

Methods

Biomass Extraction

200 mg of the dried samples 1 to 5 were subjected to ultrasonic treatment with 1.5 ml methanol for 2 minutes (Soniprep 150) and then centrifuged at 13,000 r.p.m. for 2 minutes. The supernatant was used for photometric tests.

Pre-treatment of Oil Samples

Samples 6 and 7 were dissolved in acetone and used according to Table 1. 10µl of samples 6 and 7 were added to the 1 ml ELA buffer. After a ten-minute ultrasonic treatment, the obtained suspension was left to stand over night at room temperature for the purpose of phase separation. The aqueous phase was examined.

Table 1 Composition of Reaction Preparations in Erythrocytolytic Test (Oil Samples)

	Sample (µl)	Acetone (µl)	Erythrocyte Solution (ml)
Negative test	-	110	1.9
6	10	100	1.9
6	20	90	1.9
7	10	100	1.9
7	20	90	1.9
Positive test		110	1.9 lytic erythrocytes

Sample 6 was additionally tested in a dilution series (Table 2).

Table 2 Composition of the Diluted Reaction Preparations in Erythrocytolytic Test (Oil Sample 6)

	Sample (µl)	Acetone (µl)	Erythrocyte Solution (ml)
Negative test	-	110	1.9
6	110*	-	1.9
6	80**	30	1.9
6	60	50	1.9
6	40	70	
6	20	90	1.9
Positive test	-	110	1.9 lytic erythrocytes

* 10 µl sample 6 + 100 µl acetone, **80, 60, 40, 20 µl of the preparation marked with *.

Dilution Series in the Artemia Test

Analyses were performed in correspondence with Table 3 for the necessary determination of the reduction in activity with a reduction in extraction volume.

Table 3 Composition of the Reaction Preparations in Erythrocytolytic Test (Biomass Dilution Series)

	Raw Extract (µl)	Methanol (µl)	Erythrocyte Solution (ml)
Negative test	-	100	1.9
4	20	80	1.9
4	40	60	1.9
4	60	40	1.9
4	80	20	1.9
4	100		
Positive test	-	100	1.9 lytic erythrocytes

Erythrocytolytic Tests (according to Yariv & Hestrin, 1961)

100 µl of the biomass raw extract, 110 µl of the oil/acetone mixture or 110 µl of the aqueous phase were mixed with 1.9 ml erythrocyte solution (5 x 10^6 cells/ml) in 12 ml centrifugal tubes. 100 µl methanol/1.9 ml erythrocyte solution were used as a negative test. 100 µl methanol/1.9 ml lytic erythrocyte solution or 110 µl acetone/1.9 ml erythrocyte solution and 110 µl acetone/1.9 ml lytic erythrocyte solution were used as a positive test.

After incubating for 24 hours at room temperature, the formulations were centrifuged at 4,500 r.p.m. for 5 minutes at 10 °C. Then, 1.2 ml of the supernatant was photometrically measured at 414 nm.

All samples were measured twice. This resulted in an average standard deviation of 5.5 %. Due to the very low concentration of the oils, these values for the aqueous phases tested were 12.4 % or 44.9 %.

The blank values of the pure solvents acetone or methanol were 0.118 E and 0.133 E: All measured values were corrected for these blank values.

Erythrocytolytic Test

The test is based on the photometric determination of the haemoglobin released at 414 nm when the erythrocytes are subjected to lysis (Fig. 1).

Extinction
Wavelength (nm)

Fig 1. Absorption Spectrum of Human Erythrocytes subjected to Complete Lysis (7 x10^6 cells/ml). The test is linear within the range of 10^5 to 7 x 10^6 cells/ml (Fig. 2).

Extinction
Cell concentration x 10^6 (ml^{-1})

Fig. 2 Linearity of the Erythrocytolytic Test

Human erythrocytes from blood group A are mixed with the ELA-buffer solution. The buffer solution consisted of 150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$-heptahydrate, 3.75 mM $CaCl_2$ and 12.2 mM Tris-base, which was set to pH 7.4 with HCl. The erythrocytes were washed with ELA-buffer solution, centrifuged for 5 minutes at 4,500 r.p.m. at 10 °C and then resuspended. Cell numbers were defined by means of erythrocytometry.

Artemia Test

The Artemia test is based on the effect of toxic agents on the larvae of the saltwater brine crab *Artemia salina*. It is an unspecific test with a relatively low level of sensitivity.

Artemia larvae were freshly produced in filtered seawater prior to testing. They were used within 24 hours of hatching.

100 µl raw extract were mixed with 3.9 ml filtered seawater in 10 ml beakers. 100 µl methanol were used for testing. For both oil samples 6 and 7, tests proceeded in accordance with Table 4. 12 Artemia larvae were added to each formulation. As the larvae are phototrophic, the formulations were lighted for 24 hours at room temperature. The evaluation was carried out by viewing with a binocular microscope.

Table 4 Composition of the Samples for the Artemia Test

	Sample (µl)	Acetone (µl)	Seawater (ml)
Test	-	100	3.9
Sample 6	5	95	3.9
Sample 6	10	90	3.9
Sample 7	5	95	3.9
Sample 7	10	90	3.9

Results

Haemolytic Effects of Oil Samples

In their pure form, the two oil samples displayed high activity values (Fig. 3). At an oil volume of 20 µl, a lysis of 93.3 % (sample 6) or 92.4 % (sample 7) was observed; at an oil volume of 10 µl, these values dropped to 76.7 % (sample 6) and 75.8 % (sample 7).

% Lysis
µl Sample

Fig. 3 Haemolytic Activity of Oil Samples 6 and 7 (Erythrocyte Solution 5 x 10^6 cells ml^{-1}).

Thus, a dilution series of sample 7 was additionally tested (Fig. 4).

This showed a 46 % reduction at 5-fold dilution. Extrapolated, this equals zero activity of the oil at 0.47 µl.

% Lysis
µl Oil

Fig. 4 Dilution Series of Sample 7 (Erythrocyte Solution 1.4 x 10^6 cells ml^{-1}).

The aqueous phase of the oil/ELA-buffer mixture showed negligible haemolytic activity of 1.9 % (sample 6) or 6.8 % (sample 7; Fig. 5).

Extinction
Aqueous phase
Neg Sample 6 Sample 7 Pos

Fig. 5 Haemolytic Effects of the Aqueous Phase of Oil/Water Mixtures (Erythrocyte Solution 1.4 x 10^6 cells ml^{-1}).

Haemolytic Effects of the Biomass Samples

The raw extracts of the samples 1 to 5 displayed absorption values of around 1 in accordance with 100 % lysis (Fig. 6).

% Lysis
Biomass Samples

Fig. 6 Haemolytic Effects of Samples 1 to 5 (Erythrocyte Solution 5 x 10[illegible] cells ml^{-1}).

Thus, a dilution series was tested for sample 4 in accordance with Table 3. This displayed a 31 % reduction for a 5-fold dilution (Fig. 7).

% Lysis
[µl Raw Extract]
Dilution, Sample 4

% Lysis
[µl Raw Extract]

Fig. 7 Haemolytic Effects of the Raw Extract of Sample 4 in Various Dilutions (Erythrocyte Solution 1.4 x 10^6 cells ml^{-1}).

Artemia Test

For all samples, after 24 hours of exposure, no change in activity was determined for

Artemia larvae, i.e. neither an acute nor a chronic effect was observed.

General Evaluation

The substances with haemolytic effects are only to a very slight extent soluble in water and hydrophilic organic solvents. This is proven by the slight effects of the water/oil mixtures in the haemolysis test and the non-detectable activity in the Artemia test.

Thus the presence of water-soluble prymnesins or other water-soluble toxins can be ruled out.

The haemolytic effect of the tested oils is highly probable due to the presence of polyunsaturated fatty acids, which, in their pure form, display haemolytic activity.

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FOOD ADDITIVE SAFETY

Scientific & Regulatory Affairs

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Additional information for GRN 000160 / email dated 6th of January 2005

Dear Dr. Gaynor,

Following the preliminary review of the Generally Recognized As Safe (GRAS) notification for DHA-rich oil, GRN 000160, the U.S. Food and Drug Administration (FDA) requested additional information from Nutrinova regarding the questions below.

1. The notice does not describe the identity of the source organism for the micro-algal oil (*Ulkenia sp.*) in detail. Please describe the taxonomy and source of the strain used for the production of the micro-algal oil in detail and provide copies of all the references cited in this regard. In addition, please provide a copy of the following references listed in Appendix I of the notice: reference numbers 188, 189, and 191 through 195[1]. Please provide an English translation for any reference that is not in English.

2. The notice does not provide a comprehensive discussion of the data and information to establish the non-pathogenic and non-toxicogenic nature of the source organism, and the information appears to be mainly from unpublished sources. Please describe the data and information to establish the non-pathogenic and non-toxicogenic nature of the source organism in detail and provide copies of all the references cited in this regard. In addition, please provide a copy of the following references listed in Appendix I of the notice: references numbers 196 (we are unable to access the information from this Internet site), 198, 199, 200, and 205. Please provide an English translation for any reference that is not in English.

3. The notice does not fully characterize the composition of the micro-algal oil (*Ulkenia sp.*). Please provide a complete characterization of the micro-algal oil, including the fatty acid profile of the oil and the sterol composition of the oil. If batch analy-

[1] All numbered references requested by the FDA are contained in Appendix A.

ses have been performed, please include these data in summary tables. Specifications for the ingredient, based on these batch analyses, should be sufficient to show that the article in commerce is similar to the ingredient that was used in toxicological studies cited in the notice.

4. The notice mentions that the oil contains a metabolite of chondrillasterol, but the notice gives no description of this sterol, nor does the notice give a basis for concluding that it is safe to consume. From our review of the literature, it appears to be a novel sterol. Please provide a comprehensive discussion of the properties of the sterol and it prevalence in the diet, including known dietary sources.

5. The notice cites several references to algal oil (*Schizochytrium* sp.) to support Nutrinova Nutrition Specialities & Food Ingredients GmbH's GRAS determination for micro-algal oil (*Ulkenia sp.*). However, the notice has not provided an adequate comparison of the composition of the micro-algal oil (*Ulkenia sp.*) to the algal oil (*Schizochytrium sp.*). The toxicological studies conducted with micro-algal oil (*Ulkenia sp.*) are largely unpublished, with the exception of their mention in a summary/review article. Please provide an adequate comparison of the composition of the micro-algal oil (*Ulkenia sp.*) to the algal oil (*Schizochytrium sp.*).

6. According to the notice, the intended use of the micro-algal oil (*Ulkenia sp.*) does not exceed 40% of the levels specified in 21 CFR 184.1472(a)(3) for menhaden oil. The notice states that the micro-algal oil (*Ulkenia sp.*) contains up to 50% DHA, while menhaden oil contains approximately 20% EPA plus DHA. However, the intended use levels (both initial and future) listed in Tables 1 and 2 of GRN 000160 are approximately 67% of the respective levels specified for menhaden oil (in 21 CFR 184.1472(a)(3) and the tentative final rule amending this regulation). Please correct Tables 1 and 2 to match the intended use as stated in the notice.

7. The notice states that "analysis of non-consecutive representative lots demonstrated compliance with final product chemical, physical, and microbiological specifications..." but does not list the microbiological and physical specifications. Please provide the microbiological and physical specifications.

8. The notice uses the term "proprietary" to describe the manufacturing process for micro-algal oil (*Ulkenia sp.*). While it is not clear to us whether the notice equates the term "proprietary" with confidential, the information provided in a GRAS notice should not be considered confidential. Please provide sufficient non-confidential detail about the method of manufacture to ensure that a safe product is consistently produced.

9. The notice states that a "solvent based extraction process" is used in the method of manufacture. What solvent is used? Please provide a specification for any "solvent" in the final product.

In response to your request for clarification of a number of issues, please find following, the requested supplementary information to clarify any queries regarding the safety of DHA-rich oil (see Attachment 1). The name of the microalgae strain used in the manufacture of DHA-rich oil has been provided and is enclosed separately in Attachment 2. Details of the strain are however considered proprietary information and should remain confidential. In addition, the list of requested references can be found in Appendix A, while all references, including those used in this response are available in Appendix B. Furthermore, the expert opinions related to the safety of *Ulkenia sp.* used in the production of DHA-rich oil and batch analysis certificates cited in this document can be found within Appendix C.

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I trust this detailed response provides the necessary clarification to your request for additional information. All of the information provided in this response was made available to the Expert Panel during the GRAS evaluation of the DHA-rich oil. If I can be of any further assistance please do not hesitate to contact me.

Yours sincerely,

Nutrinova Nutrition Specialties & Food Ingredients GmbH

Dr. Bernd Haber
Head Scientific & Regulatory Affairs

Encl.: Attachment 1 "Additional information for GRN 000160"
Attachment 2 "Statement on the strain used to produce DHA-rich oil – CONFIDENTIAL INFORMATION"
Appendices A, B and C
CD-ROM with Letter, Attachment 1, Appendices A, B and C

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Attachment 1

"Additional information for GRN 000160"

Question 1

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Answer 1

DHA-rich oil is produced through a fermentation and refining process using *Ulkenia sp.* as the source of DHA. *Ulkenia sp*. is a marine protist that was discovered by A. Gaertner and S. Raghu Kumar in 1977 (European Register of Marine Species, 2001). *Ulkenia sp.* belongs to the Thraustochytriaceae family, also referred to as thraustochytrids, which includes the following 6 genera: Aplanochytrium, Japonochytrium, Althornia, Thraustochytrium, Schizochytrium, and *Ulkenia* (Bahnweg, 1979a; Honda et al., 1999; European Register of Marine Species, 2001). Initially, a separate genus, Labyrinthuloides, was identified as belonging to the Thraustochytriaceae family; however, Leander and Porter (2000), determined that this genus was synonymous with Aplanochytrium, and therefore transferred 5 species of Labyrinthuloides and 1 species of Labyrinthula to the genus Aplanochytrium. *Ulkenia* genus may be further subdivided into the following species: U. profunda, U. radiata, U. sarkariana, U. minuta, and U. visurgensis (Honda et al., 1999; European Register of Marine Species, 2001).

The taxonomy of the current Thraustochytriaceae family has undergone various modifications since its original classification as a fungus (Cavalier-Smith et al., 1994). Thraustochytrids were originally recognized as a fungus of the order Chytridiales, but due to their structure, were reassigned to the Oomycete order, Saprolegniales (Cavalier-Smith et al., 1994; Honda et al., 1999). The addition of new genera prompted segregation of the thraustochytrids into their own order, Thraustochytriales, and based on their similarity to the labyrinthulids and recent discoveries made through 18S-rRNA-gene sequencing, the thraustochytrids were subsequently removed from the Oomycetes class and classified with the labyrinthulids in the class Labyrinthulea or Labyrinthulomycetes (Cavalier-Smith et al., 1994; Honda et al., 1999). Labyrinthulea were further grouped under Heterokonta within the Chromista kingdom. Thraustochytrids have also been further classified under a separate protist phylum, Labyrinthulomycota; however, it is not clear whether or not this name has been validated under the Botanical Code (Cavalier-Smith et al., 1994; Honda et al., 1999). A summary of the current taxonomic assignment (based on the 8 kingdom classification scheme) of *Ulkenia sp*. is presented in Table 1.

The Chromista kingdom, also referred to as Stramenophiles, incorporates a divergent evolutionary line from the same ancestor as plants, fungi, and animals (Waggoner and Speer, 2001). Chromista are tubulocristate protists defined by a distinct subset of tripartite tubular hairs, which usually occur on the flagella, and include diatoms, water molds, kelp, coccolithophorids, bicoeceans, slime nets, silicoflagellates, and golden, brown, and yellow-green algae (Sogin and Patterson, 1995; Waggoner and Speer, 2001).

[1] All numbered references requested by the FDA are contained in Appendix A.

Table 1 Summary of the Taxonomic Assignment of *Ulkenia sp.*	
Taxonomy	**Taxonomic Assignment**
Kingdom:	Chromista
Subkingdom	Heterokonta
Phylum:	Labyrinthulomycota
Class:	Labyrinthulea (Labyrinthulomycetes)
Order:	Thraustochytriales
Family:	Thraustochytriaceae
Genus:	*Ulkenia*
Species	*Ulkenia sp.*

Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999

Thraustochytrids are present in marine and estuarine environments and have been isolated from coastal plankton and marine macrophytic detritus (Ulken et al., 1990; Sathe-Pathak et al., 1993; Naganuma et al., 1998). *Ulkenia sp*. has been identified and enumerated in decomposing algal tissues of the brown alga Sargassum cinereum J. Ag., and due to their saprotrophic manner, it has been proposed that thraustochytrids play a functional role in marine detrial dynamics, utilizing ectoplasmic net elements to penetrate a wide range of organic particles for vital nutrients, such as carbohydrates, phenols, proteins, reducing sugars, and cellulose (Bahnweg, 1979a,b; Sathe-Pathak et al., 1993; Raghukumar et al., 1995). Additionally, *Ulkenia sp*. might play an important role in marine ecology as they were suggested to induce the settlement of barnacle larvae to surfaces of glass, aluminum, mild steel, and fiberglass panels immersed in seawater for periods of 1 to 4 days (Raghukumar et al., 2000; Huang et al., 2001).

Question 2

The notice does not provide a comprehensive discussion of the data and information to establish the non-pathogenic and non-toxicogenic nature of the source organism, and the information appears to be mainly from unpublished sources. Please describe the data and information to establish the non-pathogenic and non-toxicogenic nature of the source organism in detail and provide copies of all the references cited in this regard. In addition, please provide a copy of the following references listed in Appendix I of the notice: references numbers 196 (we are unable to access the information from this Internet site), 198, 199, 200, and 205. Please provide an English translation for any reference that is not in English.

Answer 2

The safety of *Ulkenia sp*. used in the production of DHA-rich oil is based on the expert opinions of Dr. Karsten Schaumann and Prof Dr. M. Melkonian (Appendix C). The expert opinion of Schaumann (2003), “Extended Expert’s Report on the Safety of *Ulkenia sp.*”, was based on experience in scientific dealings with *Ulkenia*, findings on taxonomy and phylogeny, as well as biology, ecology, physiology, and toxicology of the genus *Ulkenia* and related taxa in the published literature. Schaumann (2003) concluded that algal toxins are not expected to occur in docosahexaenoic acid (DHA) obtained from *Ulkenia sp.* and that products extracted from *Ulkenia sp.*, such as DHA oil, pose no dangers to human health. Similarly, Melkonian (2003) based his expert report, “Expert’s Report on the Safety of *Ulkenia* (Thraustochytriales) in a Human-biological Context”, on his own knowledge, as well as an

extensive literature review of the occurrence of toxins in the Thraustochytriales and in *Ulkenia*. Prof Dr. M. Melkonian concluded that the oil extracted from *Ulkenia* does not contain algae toxins based on the extensive literature demonstrating the absence of the production of toxins in *Ulkenia*, the order Thraustochytriales gave no indication as to the presence of any toxic components (Melkonian, 2003). Furthermore, toxins known to be present in algae/Stramenopila occur only in photosynthetic or mixotrophic organisms, which are not phylogenetically related to *Ulkenia*, or its phylum Labyrinthulea. Both Schaumann (2003) and Melkonian (2003) concluded that *Ulkenia sp.* used in the production of DHA-rich oil is non-pathogenic and non-toxicogenic.

To further characterize the safety of *Ulkenia sp.* used in the production of DHA-rich oil, 3 lots of *Ulkenia sp*.-derived DHA-rich oil (reported as DHA45-oil[2]) were analyzed for algal and cyanobacterial toxins using liquid chromatography-mass spectrometry (LC-MS) by Prof. Dr. Bernd Luckas communicated in a report titled "On the possible occurrence of algal toxins in microalgal oil (supplied as DHA45-oil) and dry algal biomass" (Appendix C). Domoic acid, Paralytic Shellfish Poisons (PSP), Diarrhetic Shellfish Poisons (DSP), and microcystins/nodularin were not detected at detection limits of 100, 50, 10, and 100 µg/kg, respectively.

In addition to the expert opinion reports on the safety of *Ulkenia sp*. and the microalgal toxin analysis, an expert pathology panel reviewed the results of a one-generation rat reproduction study with DHA-rich oil (reported as DHA45-oil) thereby providing additional support for the safety of *Ulkenia sp*. in a mammalian context. The expert panel consisted of Robert Squire, D.V.M., Ph.D., and Gary Williams, M.D., the authors of the report titled, "Expert Pathology Panel Report Concerning the Gross and Histopathological Findings of a One-Generation Rat Reproduction Study of DHA45 Oil" (Squire and Williams, 2001). The expert pathological panel concluded that the alterations in the adipose and spleen tissue of the parental generation in the one-generation reproduction study was consistent with changes expected with high exposure to polyunsaturated fatty acids (PUFAs) and raise no concern for human safety of DHA-rich oil (reported as DHA45-oil) under the appropriate conditions of use.

Question 3

The notice does not fully characterize the composition of the micro-algal oil (*Ulkenia sp.*). Please provide a complete characterization of the micro-algal oil, including the fatty acid profile of the oil and the sterol composition of the oil. If batch analyses have been performed, please include these data in summary tables. Specifications for the ingredient, based on these batch analyses, should be sufficient to show that the article in commerce is similar to the ingredient that was used in toxicological studies cited in the notice.

Answer 3

The chemical and physical specifications of DHA-rich oil are demonstrated to be in compliance in Table 2 with the specifications outlined in GRN 000160. An analysis of 3 non-consecutive, representative lots of DHA-rich oil (Table 2) demonstrates compliance with final product chemical and physical specifications.

[2] DHA-rich oil and DHA45-oil are produced by the same manufacturing process.

Table 2 Chemical and Physical Specifications plus Analyses of DHA-Rich Oil				
Test	**Specification**	**Lot 990222**	**Lot 110708**	**Lot 120306**
DHA [%] in the oil	38 to 50	38.1	42.0	38.2
Trans Fatty Acids (%)	≤ 2	< 1	< 1	< 1
Peroxide value [meq./kg]	≤ 5	1.8	0.5	3.6
Acid value [mgKOH/g]	≤ 0.5	0.08	0.03	0.03
Unsaponifiables [%]	≤ 4.5	0.3	0.5	0.4
Arsenic (ppm)	≤ 0.5	< 0.1	< 0.1	< 0.1
Lead (ppm)	≤ 0.1	< 0.1	< 0.1	< 0.1
Mercury (ppm)	≤ 0.5	< 0.004	< 0.004	< 0.004
Hexane (ppm)	< 1	< 1	< 1	< 1

The characterization, with regard to the fatty acid profile, and sterol composition, as well as the compliance of the trans fatty acid content of DHA-rich oil with the chemical and physical specifications listed in Table 2, are outlined in Table's 3 through 8.

Table 3 The Fatty Acid Composition of DHA-Rich Oil Intended for Use as a Food Ingredient			
Fatty acid content (% of total fatty acids)	**Lot 990222**	**Lot 110708**	**Lot 120306**
Tetradecanoic acid (Myristic acid) (14:0)	2.4	2.9	2.7
Pentadecanoic acid (15:0)	1.4	1.6	1.2
Hexadecanoic acid (Palmitic acid) (16:0)	32.2	33.5	33.1
Heptadecanoic acid (17:0)	0.5	0.5	0.4
Octadecanoic acid (18:0)	1.1	1.0	1.1
Eicosatetraenoic acid (20:4, ω-6)	1.1	1.2	0.9
Eicosatetraenoic acid (20:4, ω-3)	0.8	0.7	0.8
Docosapentaenoic acid (22:5, ω-6)	11.6	10.5	11.4
Docosapentaenoic acid (22:5, ω-3)	0.3	0.3	0.3
Docosahexaenoic acid (22:6, ω-3)	46.2	45.3	45.3
Others[a]	2.7	2.7	3.0
Sum	100.0	100.0	100.0

[a] Other fatty acids identified by GC-MS as minor components of DHA-rich oil include: 10:0, 10:1, 11:0, 12:0, 13:0, 14:1, 14:2, 16:1, 16:2, 16:3, i-17:0, 18:2, 18:3, 18:4, 19:0, 20:0, 20:3, 20:5, 22:0, 24:0, and 26:0. No erucic acid (22:1, n-9) is present and trans-fatty acids were not detected

Lipid-analysis of DHA-rich oil is conducted in accordance with validated and approved official methods, as recommended by the German Government, the American Oil Chemists Society (AOCS) or the Deutsche Gesellschaft für Fettwissenschaft (DGF) (German Society for Fat Science). Similarly, the fatty acid composition of DHA-rich oil (Table 3) is determined through validated and approved official methods, as recommended by the German Government, the AOCS (AOCS Official Method Ce 1e-91; 1993), or the DGF.

The analysis of triacylglycerol species of DHA-rich oil is conducted by HPLC (AOCS Official Method Ce 5b-89; 1993). To determine the distribution of fatty acids of DHA-rich oil (Lot.

120306) in the glycerol backbone, ^{13}C-NMR spectrometry analyses were performed according to Haraldsson *et al*. (2000) (Table 4).

Table 4 Analyses of Isolated Triacylglycerols of DHA-Rich Oil by ^{13}C-NMR Spectrometry

HPLC Peak No.	Triacylglycerol Species	NMR Sample Identifier	Triacylglycerol Species Confirmed
2	DHA:DPA:DHA	FY124-2	Yes
4	DHA:DHA:C16	FY124-3	Yes
5	DHA:DPA:C16 DHA:DHA:C16	FY124-4	No
6	C16:DHA:C16	FY124-5	Yes
7	C16:DHA:C16 C16:DPA:C16	FY124-6	Yes
Standard[a]	DHA:DHA:DHA	FY124-1	-
Standard[a]	C16:C16:C16	FY124-7	-

[a] Available from Sigma-Aldrich®

The quantification of triacylglycerol species of DHA-rich oil also is conducted by HPLC (AOCS Official Method Ce 5b-89; 1993). The main triacylglycerols of DHA-rich oil, by percent composition, are provided in Table 5.

Table 5 The Main Triacylglycerol Species of DHA-Rich Oil

Triacylglycerol species (% of total triacylglycerols)	Lot 990222	Lot 110708	Lot 120306	Range
DHA:DHA:C16[a]	26.1	24.5	26.3	20.0-30.0
C16:DHA:C16	20.0	17.4	16.5	14.0-23.0
DHA:DPA:C16	14.0	12.0	12.7	8.0-16.0
DHA:DHA:DHA	6.8	6.8	7.4	5.0-10.0
DHA:DPA:DHA	4.4	5.0	4.8	3.0-8.0
C16:DPA:C16	5.2	4.3	5.2	1.5-8.0
DHA:C14:DHA[a]	3.0	3.5	3.3	1.0-5.0
C16:C16:C16	1.7	2.8	2.9	0.8-4.0
Others	18.8	23.7	20.9	15-30

[a] Positions of the fatty acids not determined

The unsaponifiable fraction of DHA-rich oil is generally below 1.4% and is made up primarily of sterols, which are, however, present at levels below 1%. These sterols have been identified as sterines, 4-methylsterines, and triterpenes by GC-MS and NMR-spectroscopy. The total sterol content of 3 non-consecutive representative lots of DHA-rich oil intended for use as a food ingredient are provided in Table 6.

Table 6 Sterol Content of DHA-Rich Oil

	Method	Lot 990222	Lot 110708	Lot 120306
Sterol content (mg/100g)	ISO/FDIS 12228:1999(E)	179	216	306

In addition to cholesterol, several 4-methyl sterols were identified in DHA-rich oil. The main sterols of DHA-rich oil, isolated by silver column chromatography and identified by GC-MS and NMR spectroscopy are presented in Table 7. The three main sterols were identified as cholesterol, 24-ethyl-cholesta-5,7,22-trien-3-ol (7-dehydrostigmasterol) and 4-methyl-24-ethyl-cholesta-7,22-dien-3-ol (4-methyl-chondrillasterol).

Table 7 The Main Sterols of DHA-Rich Oil

Sterol	Molecular weight (g/mol)	Molecular weight acetates (g/mol)	Molecular weight TMS (g/mol)	Content % (of total sterol, TMS-derivatives)		
				Lot 990222	Lot 110708	Lot 120306
Cholest-5-en-3-ol (Cholesterol)	386	428	458	19.4	19.1	30.7
24-Ethyl-cholesta-5,7,22-trien-3-ol (7-Dehydrostigmasterol)	410	452	482	33.5	28.4	29.3
4-Methyl-24-ethyl-cholesta-7,22-dien-3-ol (4-Methyl-chondrillasterol)	426	468	498	14.5	18.5	14.2
Others				32.6	34.0	25.8

The formation of trans-fatty acids is known to result from exposure of unsaturated fatty acids to high temperatures (*e.g.*, in the deodorization step). As the presence of trans-fatty acids is undesirable from a nutritional point of view, refining conditions, especially conditions of deodorization were optimized in the course of product development of DHA-rich oil. Table 8 shows results of analyses for trans-fatty acids and demonstrates that the trans fatty acid content of DHA-rich oil is in compliance with the specifications outline in Table 2. The values are reported on the basis of total fatty acids, while the limit in the specifications applies for the oil itself. These values for trans-fatty acids were determined by indirect calculation using GC of fatty acids following the AOCS Official Method Cd 14c-94.

Table 8 Trans Fatty Acids in DHA-Rich Oil

Value	Method	Lot 990222	Lot 110708	Lot 120306
Trans Fatty Acids (%)	SOP: TRANSGC.S based on AOCS Official Method Cd 14c-94	< 1	< 1	< 1

The analysis of 3 non-consecutive, representative lots of DHA-rich oil demonstrates compliance with final product chemical and physical specifications, as well as provides compositional information with regard to the fatty acid composition, identify of the isolated triacylglycerol species, main triacylglycerol species, sterol content, main sterols, and trans fatty acids present in DHA-rich oil.

Question 4

The notice mentions that the oil contains a metabolite of chondrillasterol, but the notice gives no description of this sterol, nor does the notice give a basis for concluding that it is safe to consume. From our review of the literature, it appears to be a novel sterol. Please provide a comprehensive discussion of the properties of the sterol and it prevalence in the diet, including known dietary sources.

Answer 4

The unsaponifiable fraction of DHA-rich oil is low, typically <1.4%, and consists primarily of sterols. Sterol components that are structurally similar to cholesterol, the primary sterol of animals, but are derived from non-animal sources, *i.e.*, plants, are collectively termed phytosterols (Kritchevsky, 1997). Such phytosterols have also been described in marine invertebrates (Piretti and Viviani, 1989). Phytosterols are common, naturally occurring constituents of the diet, with estimated daily intakes in the U.S. population ranging from 78 to 344 mg/day (Grundy and Mok, 1975; Nair et al., 1984; Ling and Jones, 1995). Ling and Jones (1995) indicated an average phytosterol intake of 250 mg/day that could be doubled in vegetarian consumers. However, estimating total phytosterol intake is dependent upon additional diet considerations, as Nair et al. (1984) reported daily phytosterol intakes of pure vegetarian Seventh Day Adventists was 89 mg/day and comparable to that of the non-vegetarian general population (78 mg/day). Phytosterol intakes were considerably higher in lacto-ovo vegetarians (344 mg/day) and non-vegetarian Seventh Day Adventists (231 mg/day). Nair et al. (1984) considered the dietary groups were better distinguished by the ratio of phytosterol intake to that of cholesterol, with higher ratios reported for both vegetarian groups compared with the non-vegetarian groups. In summarizing the reported intakes of phytosterols from various populations, Jones et al. (1997) indicated consumers of Western diets had intakes of 200 to 400 mg total phytosterols/day. Following comparison of 1957 and 1982 national diet survey data from Japan, Hirai et al. (1986) concluded daily per capita consumption of phytosterols has remained historically constant in Japanese diets, and reported intakes of 373 mg phytosterols/day for both survey years.

The predominant phytosterols consumed in the diet occur in various plant and seed oils, as β sitosterol, campesterol, stigmasterol, brassicasterol, and ergosterol, although greater than 40 plant sterols have been identified (Linscheer and Vergroesen, 1994; Kritchevsky, 1997). Phytosterols are poorly absorbed compared to cholesterol and the safety of dietary phytosterols has been well documented (Grundy and Mok, 1975; Linscheer and Vergroesen, 1994; Ling and Jones, 1995; Ayesh et al., 1999; Baker et al., 1999; Hepburn et al., 1999; Waalkens-Berendsen et al., 1999; Westrate et al., 1999; Sanders et al., 2000).

Representative lot analyses of DHA-rich oil (Table 8) identified the main sterol components as cholesterol (approximately 20 to 30%), 24-ethyl-cholesta-5,7,22-trien-3-ol (7 dehydrostigmasterol) (approximately 28 to 34%), and 4-methyl-24-ethyl-cholesta-7,22-dien-3 ol (4-methyl-chondrillasterol) (approximately 14 to 19%). Exposure to the sterol components of DHA-rich oil would not be considered novel as 4-methyl sterols are found in the normal metabolic pathway of cholesterol biosynthesis in man and they have been identified in several other food sources, including fish and shellfish (Piretti and Vivani, 1989). 4-Methyl-24-ethyl-cholesta-7,22-dien-3-ol was previously described by Řezanka et al. (1986) in green algae and by Narumi and Takatsuto (2000) in rice bran. 24-Ethyl-stigmasta-5,7,22-trien-3-ol has been previously described as 7-dehydrostigmasterol by Smith and Korn (1968) in amoebae, and identified by Wright (1981) in algae, Dunaliella tertiolecta. Yates et al. (1992) indicated 24-ethyl-stigmasta-5,7,22-trien-3-ol had been reported in sponges, Axinella cannabina, and identified this sterol in celery cells as an intermediate in the synthesis of plant sterols. Furthermore, Pelletier et al. (1995) quantified levels of 492 mg 7-dehydrostigmasterol/100 g fat in phytosterols purified from the unsaponifiable fraction of soybean oil. Hence, the phytosterols in DHA-rich oil would be considered to already occur in the diet, although quantitatively minor constituents of the consumed diet.

With typical sterol levels around or even below 1% in DHA-rich oil, the level of exposure to sterols under the intended conditions of use of DHA-rich oil would be minimal. Typical cholesterol levels are around 30 % of total sterols. Assuming a maximum intake of DHA-rich oil of 7.9 g/day (based on 38% DHA in DHA-rich oil contributing to 3 g/person/day), a total sterol content of 1% and 30% of sterols being cholesterol, the theoretical maximum daily cholesterol intake from DHA-rich oil would be approximately 24 mg. Similarly, the theoretical

maximum intake for phytosterols would be approximately 55 mg/day. The safety of dietary cholesterol and phytosterols is well documented in the scientific literature. Cholesterol and phytosterols are natural constituents of the diet and 4-methyl sterols occur in the endogenous metabolic pathway of cholesterol biosynthesis. These intakes are well below the amounts of sterols already consumed as natural constituents of the diet (up to 400 mg/person/day), and thus, the estimated intake of sterols through the proposed uses of DHA-rich oil would not have an impact on the relative amount of cholesterol and phytosterols already consumed in the diet. Therefore, the dietary exposure to cholesterol, 7 dehydrostigmasterol, and 4-methyl-chondrillasterol from the intended use of DHA-rich oil would not be expected to produce adverse effects on human health.

Question 5

The notice cites several references to algal oil (*Schizochytrium* sp.) to support Nutrinova Nutrition Specialities & Food Ingredients GmbH's GRAS determination for micro-algal oil (*Ulkenia sp*.). However, the notice has not provided an adequate comparison of the composition of the micro-algal oil (*Ulkenia sp*.) to the algal oil (*Schizochytrium sp*.). The toxicological studies conducted with micro-algal oil (*Ulkenia sp.*) are largely unpublished, with the exception of their mention in a summary/review article. Please provide an adequate comparison of the composition of the micro-algal oil (*Ulkenia sp.*) to the algal oil (*Schizochytrium sp*.).

Answer 5

The comparison of the composition of micro-algal oil from *Ulkenia sp*. to algal oil from *Schizochytrium sp.* has to be made on data that is public available on *Schizochytrium* oil. Data used here are from a published dossier of the European novel food application for DHA-rich oil from *Schizochytrium sp*. made by OmegaTech GmbH (OmegaTech GmbH, 2001).

Table 9 Comparison of Specifications of DHA-rich oil (*Ulkenia sp.*) and DHA-rich oil (Schizochytrium sp.)

Oil Value	DHA-rich oil (*Ulkenia sp.*)	DHA-rich oil (Schizochytrium sp.)
DHA [%]	38 to 50	Not less than 32
Trans fatty acids [%]	≤ 2	Not more than 2
Peroxide value [meq./kg]	≤ 5	Not more than 5
Moisture and volatiles [%]	≤ 0.05	Not more than 0.05
Unsaponifiables [%]	≤ 4.5	Not more than 4.5
Acid value [mg KOH/g]	≤ 0.5	Not more than 0.5
Lead [ppm]	≤ 0.1	Not more than 0.2
Arsenic [ppm]	≤ 0.5	Not more than 0.2
Copper [ppm]	n.s. (mean of 3 batches < 0.2 ppm)	Not more than 0.05
Iron [ppm]	n.s. (mean of 3 batches = 0.7 ppm)	Not more than 0.2
Mercury [ppm]	≤ 0.5	Not more than 0.2

n.s.: not specified (n.s. values in brackets: not specified but information elsewhere in this application)

Table 10 Comparison of analytical data for representative batches of DHA-rich oil (*Ulkenia sp.*) and DHA-rich oil (*Schizochytrium sp.*)		
Oil value	DHA-rich oil (*Ulkenia sp.*)	DHA-rich oil (*Schizochytrium sp.*)
	Mean values (n=3)	Mean values (n=5)
DHA	45.6 % of fatty acids	36.6 % of f. a. methyl esters
DHA [% in oil]	39.4	n.a.
Peroxide value [meq./kg]	2.0	2.5
Acid value [mgKOH/g]	0.05	0.14
Iodine value	234.2	n.a.
Saponification value [mgKOH/g]	194.2	n.a.
Unsaponifiables [%]	0.4	2.5
Trans fatty acids [%]	< 1	1.4
Colour	pale yellow to colourless	pale yellow to colourless
Appearance (at room temperature)	fluid/waxy	fluid/waxy
Lead [ppm]	< 0.1	< 0.2
Arsenic [ppm]	< 0.1	< 0.2
Mercury [ppm]	< 0.004	< 0.2
Proteins [%]	< 0.1	< 0.1
Hexane [ppm]	< 1	< 3 *

n.a.: no data available
* hexane level of one lot of 5 lots was 13 ppm, 4 of 5 lots were below 3 ppm

Comparison of both oils show that there are only minor differences in the different parameters. Nutritionally potentially relevant items are discussed more specifically below in comparison to the values for DHA-rich oil from *Schizochytrium sp.*.

<u>*DHA*</u>

Differences

The DHA values vary from 45.3 % to 46.2 % of the fatty acids (i.e. < 1 % abs.) for *Ulkenia* oil and from 34.2 to 39.3 % of fatty acid methyl esters (i.e. up to 5.1 % abs.) for *Schizochytrium* oil.
Comment

The DHA values are likely to result from better process stability for DHA-rich oil from *Ulkenia sp.*.

<u>*Unsaponifiables*</u>

Differences

The values for unsaponifiables vary from 0.3 % to 0.5 % for *Ulkenia* oil and from 2.1 % to 4.1 % for DHA Gold.
Comment

The values for unsaponifiables are likely to result from better process stability for *Ulkenia* oil. The unsponifiable fraction of *Ulkenia* oil is generally below 1.4 % and is made up primarily of sterols, which are, however, present at levels below 1%.

Table 11 Comparison of fatty acid composition for representative batches of DHA-rich oil (*Ulkenia sp.*) and DHA-rich oil (*Schizochytrium sp.*)

Fatty acid	DHA-rich oil (*Ulkenia sp.*) Mean values (n=3)	DHA-rich oil (*Schizochytrium sp.*) Mean values or ranges (n=5)
Dodecanoic acid (12:0)	traces	0.4
Tetradecanoic acid (14:0)	2.7	10.1
Tetradecatrienoic acid (14:3)	n.d.	traces-0.5
Pentadecanoic acid (15:0)	1.4	n.a.
Hexadecanoic acid (16:0)	32.9	23.7
Hexadecaenoic acid (16:1)	traces	1.8
Hexadecatrienoic acid (16:3)	n.a.	traces-0.5
Heptadecanoic acid (17:0)	0.5	n.a.
Octadecanoic acid (18:0)	1.1	0.5
Octadecaenoic acid (18:1), trans 11	n.a.	traces-1.4
Octadecatetraenoic acid (18:4)	n.a.	traces-0.9
All-cis-4,7,10,13-eicosatetraenoic acid (20:4,n7)	1.1	2.2*
All-cis-4,7,10,13-eicosatetraenoic acid (20:4, n6)	n.a.	0.9
All-cis-8,11,14,17-eicosatetraenoic acid (20:4, n3)	0.8	0.9
All-cis-4,7,10,13,16-eicosapentaenoic acid (20:5, n3)	traces	2.6
All-cis-4,7,10,13-docosatetraenoic acid (22:4, n9)	n.a.	0.5
All-cis-4,7,10,13,16-docosapentaenoic acid (22:5, n6)	11.6	13.5
All-cis-7,10,13,16,19-docosapentaenoic acid (22:5, n3)	0.3	n.a.
All-cis-4,7,10,13,16,19-docosahexaenoic acid (22:6, n3)	45.6	35.0
Others	rest	rest

n.d. not detectable, n.a. no data available
* All-cis-7,10,13-eicosatrienoic acid (20:3, n6) and all-cis-4,7,10,13-eicosatetraenoic acid (20:4, n7)

The major fatty acids are listed as % of the total fatty acids for DHA-rich oil from *Ulkenia sp.* and as mg of fatty acid methyl esters per gram of oil for DHA-rich oil from *Schizochytrium sp.*. The difference in molecular weight between three fatty acid methyl esters and a triglyceride of the same fatty acids is minute. Fatty acids make up approximately 95 % of the total weight of DHA-rich oil from *Ulkenia sp.*. Therefore, direct comparison of the compositional data of the dossiers can be made as a simple approximation to identify major differences.
The fatty acid composition is similar, although some differences are found:
Differences for numerical values in the dossiers (+ higher, - lower in *Ulkenia* oil)

Differences in fatty acids > 5 %

C 14:0	- 7.4 %
C 16:0	+ 9.2 %
C 22:6 n3 (DHA)	+ 10.6 %

Differences in fatty acids > 1 %

C 15:0		+ 1.4 %
C 16:1	below	- 1.8 %
C 18:0		+ 1.1 %
C 18:1 trans 11	below	- 1.4 %
C 20:4 n7	below	- 1.1 %
C 20:5 n3 (EPA)	below	- 2.6 %
C 22:5 n6		- 1.9 %

Comment

DHA
DHA is the nutritionally relevant component. The level in *Ulkenia* oil is substantially higher but addition levels to food will be based on intended or permissible DHA levels. Therefore this difference is of no nutritional or toxicological significance.
EPA
Ulkenia oil contains only traces of this nutritionally meaningful fatty acid, while typical levels present in Schizochytrium oil are in the range of 5 % of the DHA level. Accordingly, the EPA intake resulting from intake of *Schizochytrium* oil cannot be considered nutritionally meaningful when used at typical DHA enrichment levels.
C 22:5 n-6
This fatty acid is discussed as a potential precursor of arachidonic acid (C20:4, n-6). While levels in *Ulkenia* oil are lower with an average of 11.2 % compared to an average of 13.5 % in *Schizochytrium* oil, they do not seem to constitute a nutritionally meaningful difference.
C 14 and C 16
Both fatty acids are common constituents of many fats and are normally present at rather high levels in the diet. Intake from both *Ulkenia* oil and Schizochytrium oil will be insignificant compared to intake from normal food consumption.
C 15 and C 17
The levels of these two fatty acids in *Ulkenia* oil are in the range of their presence in some conventional fats and oils, e. g., according to “Leitsätze für Speisefette and Speiseöle” dated 17. 04. 1997:

Fat	**C 15 (% of fatty acids)**	**C 17 (% of fatty acids)**
Ulkenia oil	1.4	0.5
Cocoa butter		- 0.3
Olive oil		- 0.3
Rapeseed oil		- 0.3
Lard	< 0.2	< 1.0
Tallow	- 1.0	- 2.0

Compared to potential intake from conventional fats the intake of these two fatty acids from *Ulkenia* oil are therefore not considered nutritionally meaningful when used at typical enrichment levels.
Trans fatty acids
The level of C 18:1 trans 11 in Schizochytrium oil is higher than the levels of the identified trans fatty acids (in total less than 2 % and normally even below 1 %) in *Ulkenia* oil. No single trans fatty acid coming close to 1 % has been found in *Ulkenia* oil. The intake from expected addition levels of DHA can be considered insignificant for both *Ulkenia* and Schizochytrium oil.
Others
Considering the rather low levels of other fatty acids in both *Ulkenia* and *Schizochytrium* oil and the limited differences in concentrations no nutritionally or toxicologically relevant differences must be assumed.

Table 12 Comparison of analyses of (heavy) metals in DHA-rich oil (*Ulkenia sp.*) and DHA-rich oil (*Schizochytrium sp.*)		
(Heavy) metals (ppm)	**DHA-rich oil (*Ulkenia sp.*) Mean values (n=3)**	**DHA-rich oil (*Schizochytrium sp.*) Mean values (n=5)**
Arsenic	< 0.1	< 0.5
Cadmium	< 0.02	< 0.04
Copper	< 0.5	< 0.13
Iron	0.7	< 0.5
Lead	< 0.1	< 0.05
Mercury	< 0.005	< 0.025
Nickel	0.29	n.a.

n.a. no data available

Differences

The values for *Ulkenia* oil include nickel beyond the elements analysed for Schizochytrium oil.

Comments

Values for both products conform to specifications and indicate a consistent manufacturing process.

Table 13 Comparison of unsaponifiable content and sterols in typical batches of DHA-rich oil (*Ulkenia sp.*) and DHA-rich oil (*Schizochytrium sp.*)		
	DHA-rich oil (*Ulkenia sp.*) Mean values (n=3)	**DHA-rich oil (*Schizochytrium sp.*) Mean values or ranges (n=5)**
Unsaponifiables (g/100g)	0.4	3.1
Sterol content (mg/100g)	234	2 500*
- Cholesterol (% of total sterols)	23.1	25
- 7-Dehydro stigmasterol (% of total sterols)	30.4	n.a.
- 4-Methyl chondrillasterol (% of total sterols)	15.7	n.a.
- Brassicasterol (% of total sterols)	n.a.	15
- Stigmasterol (% of total sterols)	n.a.	19
- Stigmasta-5,23-dien-3-ol (% of total sterols)	n.a.	8
- others (% of total sterols)	30.8	Traces-13

n.a. no data available, * sum of cholesterol and phytosterol

Differences

The values for *Ulkenia* oil are substantially lower than for *Schizochytrium* oil. The unsponifiable fraction of *Ulkenia* oil is generally below 1.4 % and is made up primarily of sterols, which are, however, present at levels below 1%.

Based on the two to three times higher levels of unsaponifiables in *Schizochytrium* oil a sterol content at least twice as high as in *Ulkenia* oil can be anticipated, and according to dossier on DHA-rich oil from *Schizochytrium sp.* the sum of cholesterol and phytosterols is approximately 2.5 %.

Main sterols of *Ulkenia* oil are 7-dehydro stigmasterol (average value 30.4 % of sterols) cholesterol (average value 23.1 % of sterols) and 4-methyl chondrillasterol (average value 15.7 % of sterols). Others are present at an average value of 30.8 % of sterols. These are almost exclusively cholesten-3-ol, cholestadien-3-ol and cholestatrien-3-ol derivatives.
Six main sterols were identified in *Schizochytrium* oil, cholesterol (average value 25 %), stigmasterol (average value 19 %), brassicasterol (average value 25 %), stigmasta-5,23-dien-3-ol (average value 8 %) and two ergostadienols (average values ca. 5 %, all as % of peak areas).

Comment
Generally, the lower sterol levels in *Ulkenia* oil may be considered beneficial, provided no uncommon sterols are present in relevant concentrations (see also answer to question 4). The intake of sterols from addition of rather small amounts of *Ulkenia* oil to foods will be incremental only. Equivalent DHA fortification levels will result in a lower sterol intake from *Ulkenia* oil compared to *Schizochytrium* oil. Not only is the sterol content in *Ulkenia* oil lower, but the higher DHA content of *Ulkenia* oil will result in a sterol intake value one-third of that compared to the addition of *Schizochytrium* oil for the same DHA fortification level.

The sterol levels as analysed in the representative batches of *Ulkenia* oil are within the range of sterol levels of some commonly used fats and oils. Average sterol levels in some common fats and oils are listed in composition tables like: Souci/Fachmann/Kraut, Food Composition Tables 1981/82, Stuttgart, 1981:

Fat / Oil	**(sum of sterols specified as numerical levels in %)**
Palm oil	0.04
Palm kernel oil	0.08
Coconut oil	0.10
Olive oil	0.11
Cocoa butter	0.20
Peanut oil	0.24
Rapeseed oil	0.25
Cottonseed oil	0.33
Soybean oil	0.34
Sunflower oil	0.35
Linseed oil	0.43
Safflower oil	0.44
Wheat germ oil	0.55
Corn oil	0.85
Sesame seed oil	0.87
Cod liver oil	0.85

Among the main sterols of *Ulkenia* oil is cholesterol the common sterol in animal fats. Furthermore, 7-dehydro stigmasterol occurs in soy oil at a level of ca. 0.5 g / 100 g of oil, and was 4-methyl chondrillasterol has been identified in rice bran.

Accordingly, presence of sterols in *Ulkenia* oil and differences to Schizochytrium oil can be considered to be of no nutritional and toxicological relevance.

Question 6

According to the notice, the intended use of the micro-algal oil (*Ulkenia sp.*) does not exceed 40% of the levels specified in 21 CFR 184.1472(a)(3) for menhaden oil. The notice states that the micro-algal oil (*Ulkenia sp.*) contains up to 50% DHA, while menhaden oil contains approximately 20% EPA plus DHA. However, the intended use levels (both initial and future) listed in Tables 1 and 2 of GRN 000160 are approximately 67% of the respective levels specified for menhaden oil (in 21 CFR 184.1472(a)(3) and the tentative final rule amending this regulation). Please correct Tables 1 and 2 to match the intended use as stated in the notice.

Answer 6

The individual intended food uses and use levels for DHA-rich oil, consistent with the current GRAS use of menhaden oil specified in 21 CFR §184.1472 and the revised uses outlined in FDA's tentative final rule regarding GRAS-affirmed food uses for menhaden oil, are summarized in Table 14. To ensure safe use levels in the intended food categories and levels of use, DHA rich oil shall not be used in combination with any other added oil that is a significant source of EPA or DHA. The intended use levels as listed in Table 1 and 2 of GRN 000160 have been amended as proposed in your email from 6th of January 2005. The amended levels can be found in Table 14.

Table 14 Intended Uses and Maximum Use Levels of DHA-Rich Oil in Food

Food Category	Initial[a] Intended Use Levels (% by weight)	Future[b] Intended Use Levels (% by weight)
Cookies, crackers (n = 1)[1,2]	2.0	-
Breads, rolls (white and dark) (1)[2]	0.4	-
Fruit pies, custard pies (1)[2]	2.8	-
Cakes (1)[2]	4.0	-
Cereals (4)	1.6	1.6
Baked Goods and baking mixes	-	2.0
Fats and oils (12)[3]	8.0	4.8
Yogurt (31)[4]	1.6	-
Milk Products (31)	-	2.0
Cheese products (31)	-	2.0
Frozen dairy products (20)	2.0	2.0
Meat products (29)	4.0	2.0
Egg products (11)	2.0	2.0
Fish products (13)	8.0	2.0
Condiments (8)	2.0	2.0
Soup mixes (40)	1.2	1.2
Snack foods (37)	2.0	2.0
Nut products (32)	2.0	2.0
Gravies and sauces (24)	2.0	2.0
Plant protein products (33)	-	2.0
Processed vegetable juices (36)	-	0.4
Hard candy (25)	-	4.0

Table 14 Intended Uses and Maximum Use Levels of DHA-Rich Oil in Food		
Food Category	**Initial[a] Intended Use Levels (% by weight)**	**Future[b] Intended Use Levels (% by weight)**
Soft candy (38)	-	1.6
Jams and jellies (28)	-	2.8
Dairy product analogs (10)	-	2.0
Nonalcoholic beverages (3)	-	0.02
Pastas (23)	-	0.8
Poultry products (34)	-	1.2
Processed fruit juices (35)	-	0.4
White granulated sugar (41)	-	1.6
Sugar substitutes (42)	-	4.0
Chewing gum (6)	-	1.2
Gelatins and puddings (22)	-	0.4
Confections and frosting (9)	-	2.0
Sweet sauces, toppings, and syrups (43)	-	2.0

[a] Based on 21 CFR §184.1472
[b] Based on tentative final rule amending 21 CFR §184.1472 (69 FR 2313; January 15, 2004)
[1] The number in parenthesis following each food category refers to the paragraph listing of the particular food category in 21 CFR 170.3(n).
[2] Included in “Baked goods and baking mixes”.
[3] Not including infant formula.
[4] Included in “Milk products”.

Question 7

The notice states that "analysis of non-consecutive representative lots demonstrated compliance with final product chemical, physical, and microbiological specifications..." but does not list the microbiological and physical specifications. Please provide the microbiological and physical specifications.

Answer 7

The analysis of 3 non-consecutive, representative lots of DHA-rich oil (Table 15) demonstrates the compliance of DHA- rich oil with microbiological quality control parameters for the final product.

Table 15 Microbiological Analyses of DHA-Rich Oil				
Test	**Limit**	**Lot 990222**	**Lot 110708**	**Lot 120306**
Total viable counts (cfu[a]/g)	<1,000	<1,000	<1,000	<1,000
Coliforms (cfu/g)	<10	<10	<10	<10

[a] Colony forming units

The chemical and physical characteristics of DHA-rich oil are presented in Table 16. The chemical and physical specifications for DHA-rich oil are listed in Table 2.

Table 16 Chemical and Physical Characteristics of DHA-Rich Oil	
Characteristic	**Description**
Consistency	slightly waxy to liquid
Color	pale yellow
Molecular weight	1,020 g/mol (tripalmitate to tridocosahexaenoate)
Melting point	40 to 45°C
Hygroscopy	absent
pH value	7 (in ethanol)
Volatile	no
Purity	>95% triacylglycerols
Relative Density (25 °C)	0.95 g/mL
Solubility in water	< 0.1 mg/L in water

Furthermore, additional metal analyses beyond the metals specified in the product specifications for DHA-rich oil (Table 2) demonstrate very low levels of cadmium, copper, and nickel in the final product (Table 17)

Table 17 Additional Metal Analyses of DHA-Rich Oil			
Test	**Lot 990222**	**Lot 110708**	**Lot 120306**
Cadmium (ppm)	< 0.1	< 0.1	< 0.1
Copper (ppm)	< 0.2	< 0.2	< 0.2
Nickel (ppm)	< 0.2	< 0.2	< 0.2

Question 8

The notice uses the term "proprietary" to describe the manufacturing process for micro-algal oil (*Ulkenia sp.*). While it is not clear to us whether the notice equates the term "proprietary" with confidential, the information provided in a GRAS notice should not be considered confidential. Please provide sufficient non-confidential detail about the method of manufacture to ensure that a safe product is consistently produced.

Answer 8

METHOD OF MANUFACTURE

A Raw Material Specifications

a *Ulkenia sp.*

DHA-rich oil is produced through a multi-step fermentation and refining process using a marine microalgae, *Ulkenia sp.*. This strain of *Ulkenia* was originally isolated from the Inland Sea in Japan.

b Culture Medium

Production medium used in the manufacturing process for DHA-rich oil is a glucose corn steep medium (GC). It contains the following ingredients: dextrose monohydrate or glucose syrup, potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), calcium chloride ($CaCl_2 \cdot 2H_2O$), corn steep liquor, ammonium sulfate

$(NH4)_2SO_4$, and an antifoaming agent. In addition, phosphoric acid (H_3PO_4) and sodium hydroxide (NaOH) are used in the GC medium as pH adjusting agents just prior to sterilization and during fermentation. All components of the culture medium meet food grade specifications or are of adequate purity for food fermentation processes. A safe and suitable antifoaming agent, meeting appropriate specifications, is used during fermentation steps in the production of DHA-rich oil.

c Flocculating Agents

A safe and suitable flocculating agent may be used during the extraction phase of the manufacturing of DHA-rich oil.

d Hexane

Hexane may be used as solvent for crude oil extraction from *Ulkenia sp.* biomass and as processing aid during refining of the oil. Hexane, meeting appropriate food grade specifications, is approved for food additive use in the production of spice oleoresins and hops extract in accordance with 21 CFR §173.270. It is also listed as a permissible residue in various food ingredients under certain conditions (*e.g.*, fish protein isolate, cocoa butter substitute) (21 CFR §172.340, 184.1259).

e Degumming Acids

Aqueous solutions of food acids, meeting appropriate food grade specifications, especially acetic, phosphoric or citric acid, are used as degumming agents in the manufacturing process of DHA-rich oil.

f Neutralizing Agent

Dilute aqueous solutions of sodium or potassium hydroxide, meeting appropriate food grade specifications, are used to remove any free fatty acids in the manufacturing process of DHA-rich oil.

g Bleaching Agents

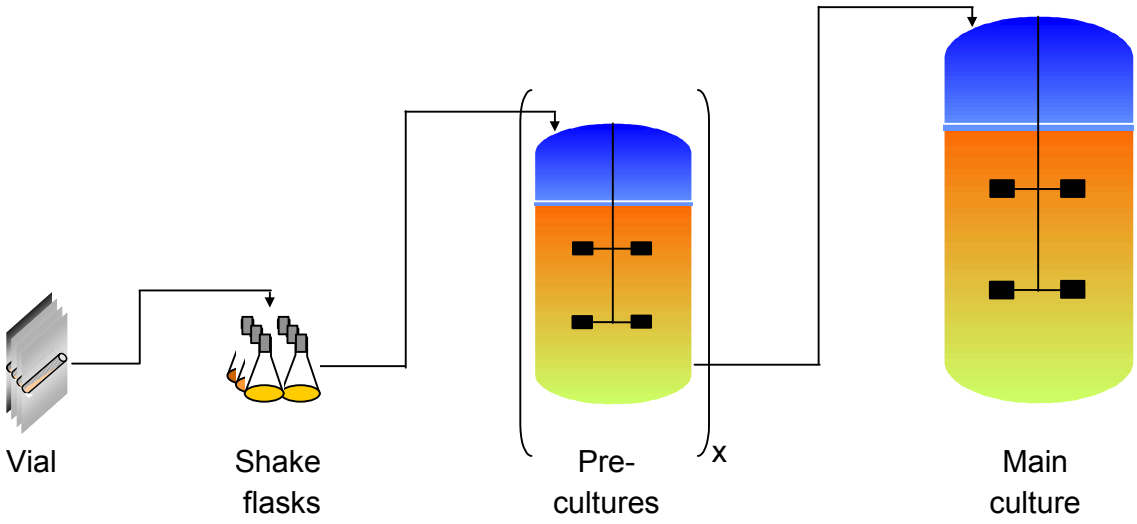
Bleaching clay and activated carbon, of appropriate food-grade specification, are used as the bleaching agents during the refining of crude DHA-rich oil.

B Manufacturing Process for DHA-Rich Oil

a Fermentation

The production of DHA-rich oil commences with large-scale fermentation of *Ulkenia sp*., as presented in Figure 1. Pre-culture flasks are shaken and inoculated with one vial of *Ulkenia sp.*. Nutrinova maintains the genetic stability, cultural purity, sterility, and integrity of the microalgae, *Ulkenia sp*. Before and after each fermentation step, samples of the pure strain, and samples of shake flask culture, pre-culture, and main culture, are checked on Petri dishes with nutrient agar for the detection of microbial contamination.
The culture is transferred to the first seed fermentor (pre-cultures). This culture is subsequently transferred to additional seed fermentors until the volume is sufficient for inoculation of the main fermentor (main culture).

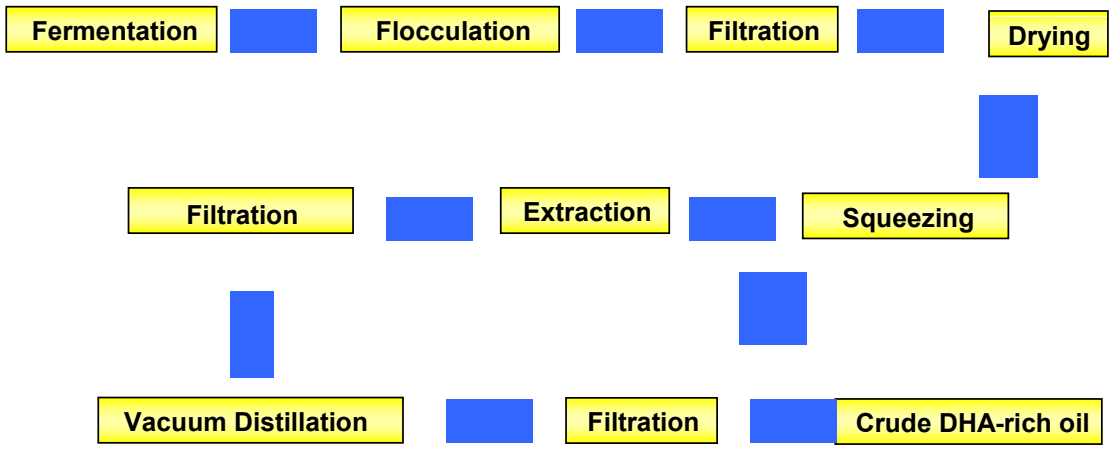
Figure 1 Schematic Overview of the Fermentation Process for DHA-Rich Oil



b Extraction and Refining

Following fermentation, DHA-rich oil is extracted from the biomass as follows in Figure 2. The fermentation broth is cooled and flocculated by adding suitable flocculating agents. The flocculated broth is then filtered and the filter cake is dried. The dry biomass can be squeezed to obtain crude oil directly (press oil). The squeezed biomass may additionally be extracted using hexane to obtain the residual oil (sep oil). Both fractions could be mixed or held separate. After filtration any solvents used are removed from the miscella using vacuum distillation. The retained oil is then filtered to obtain crude DHA-rich oil.

Figure 2 Production of DHA-Rich Oil from Fermentation Biomass

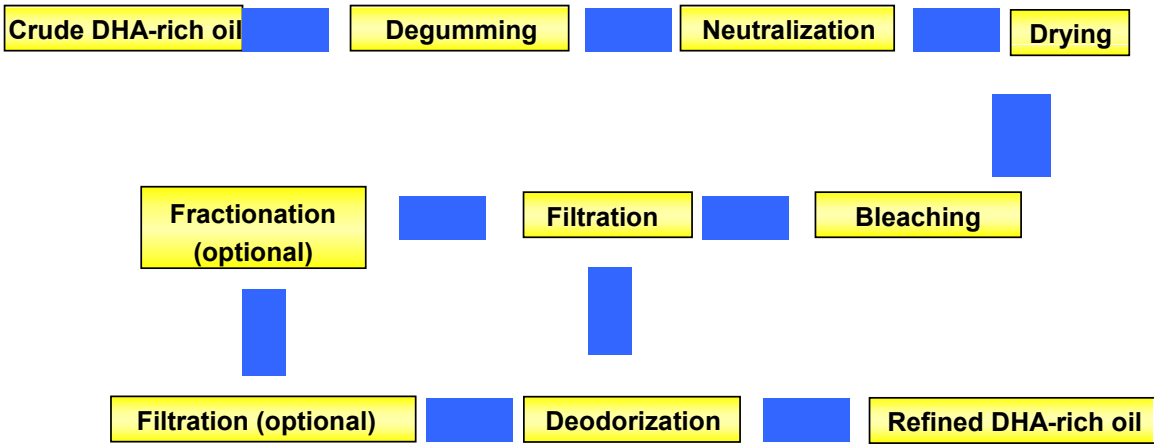


The crude DHA-rich oil is refined *via* a standardized food-oil refining process to a final food-grade product, as shown in the following Figure 3. The crude DHA-rich oil is degummed by adding aqueous food acid to remove traces of phospholipids. The organic and inorganic phases are separated by centrifugation and the organic phase is neutralized. The degummed neutralized oil is bleached using clay. The suspension is filtered to remove residual clay. The oil might be fractionated (*e.g.* winterized) to remove higher melting fats by filtration. The

bleached oil is deodorized by hot water steam that is driven through the oil. The final product is refined DHA-rich oil, which is stabilized by addition of safe and suitable antioxidants.

Refined DHA-rich oil is stored and transported in vessels suitable for food storage under nitrogen at ≤5°C.

Figure 3 Refining Process for DHA-Rich Oil



Question 9

The notice states that a "solvent based extraction process" is used in the method of manufacture. What solvent is used? Please provide a specification for any "solvent" in the final product.

Answer 9

As outlined in the manufacturing process (response to question 8), a portion of crude DHA-rich oil is extracted from the fermentation biomass using hexane, which is subsequently removed by vacuum distillation. Hexane is further used in the refining process, prior to deodorization of DHA-rich oil final product, and removed by evaporation. No traces of hexane (<1 ppm) were detected in 3 non-consecutive lots of DHA-rich oil using GC headspace analysis. The results of analyses of 3 non-consecutive representative lots of DHA-rich oil for hexane are presented in Table 18

Table 18 Hexane Residue in DHA-Rich Oil

Residue	Method	Lot 990222	Lot 110708	Lot 120306
Hexane (ppm)	DIN EN ISO 9832	<1	<1	<1

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Attachment 2

Statement on the strain used to produce DHA-rich oil

CONFIDENTIAL INFORMATION

Appendix A – List of References Requested by FDA

The FDA requested references 188, 189, 191 through 195, 196, 198, 199, 200, and 205. Appendix A contains these references. For simplicity of use the requested numbered references are listed below with their corresponding papers.

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