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Supporting document 1

Risk and technical assessment – Proposal P1124

Alternative DHA-rich Algal Oil for Infant Formula Products

Executive summary

The Application seeks approval of an additional, replacement or alternative oil source of DHA for use in infant formula products. The alternative DHA-rich oil marine micro-algal oil is derived from a new production strain of *Schizochytrium* sp. The strain is known as American Type Cell Culture (ATCC) (PTA-9655). For ease of reading, throughout this report the oil will be referred to as DHA-B.

FSANZ has previously assessed several marine micro-algal oils for use in infant formula products. DHA-B, as an alternative DHA-rich algal oil is proposed to be added to infant formula products at levels consistent with the current use levels of DHA-rich algal oils and within the maximum level of omega-3 long chain poly unsaturated fatty acids of 1% permitted in Standard 2.9.1 in the *Australia New Zealand Food Standards Code* (the Code).

The composition of the new strain of *Schizochytrium* sp. micro-algae and the oil derived from the new strain of *Schizochytrium* sp. are comparable to other currently permitted sources of DHA. Therefore the objective of this risk and technical assessment is to evaluate:

- the safety of this new production strain of *Schizochytrium* species algae
- the safety and suitability of the DHA-B oil as an additional, replacement or alternative DHA oil source for infant formula products.

The submitted data are considered adequate to define the hazard of DHA-B and support the conclusion that DHA-B is a safe source of DHA for supplementation of infant formula. No evidence was found of risk of genotoxicity, reproductive or developmental toxicity, or toxicity as a consequence of subchronic dietary consumption of either dried *Schizochytrium* or DHA-B by experimental animals. DHA-B was found to be bioequivalent to DHASCO[®], and to have no adverse effects in baby piglets at consumption levels higher than those likely to occur in formula-fed infants.

Limited human studies of *Schizochytrium* sp. or of DHA-B were found. It is noteworthy that *Schizochytrium* species are components of the human diet through consumption of shellfish, and that there are no known toxic compounds synthesised by any thraustochytrid microalgae.

The DHA-rich micro-algal oil products are comparable with respect to presence of LC-PUFA, although there is variation in the DHA amount. The main difference between the products is the ratio of EPA to DHA and the other fatty acids in DHA-B are normal components of edible oils. In general, the fatty acid composition of DHA-B is comparable to that of other oils on the market and the differences are nutritionally insignificant because the maximum amount of omega 3 LC-PUFA that can be added to infant formula products is 1% of the total fatty acids.

An analytical method (AOCS Ce 1b-89) is available for compliance of DHA oils against specifications contained within section S3-21 of the Code. The stability of DHA-B within a food matrix such as infant formula products is assured. The inclusion of the source of DHA-B by reference to its American Type Culture Collection (ATCC) will be added to the Code.

The submitted data are considered appropriate to define the nutritional adequacy of DHA-B. There is no evidence to suggest that absorption, distribution, metabolism and excretion of DHA-B would be different to that of the other marine micro-algal oils. DHA-B was found to be bioequivalent to DHASCO[®] in piglets and infants. FSANZ concludes that the Applicant has provided sufficient technical data to ensure that DHA-B is suitable as an additional, alternative or replacement DHA oil source in infant formula products.

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Abbreviations

Abbreviation	Explanation
AI	Adequate Intake
ALA	α -linolenic acid or Alpha-linolenic acid
AOCS	American Oil Chemists' Society
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
ARA	Arachidonic acid
ARASCO	Arachidonic acid single cell oil
CAERS	CFSAN Adverse Event Reporting System
CAS	Chemical Abstracts Service
CFSAN	Center for Food Safety and Applied Nutrition - FDA
DHA	Docosahexaenoic acid
DHASCO	Docosahexaenoic acid single cell oil
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
DRM	DHA-rich dried microalgae
EAR	Estimated Average Requirement
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FOB	Functional Observational Battery
GLC	Gas Liquid Chromatography
GC	Gas Chromatography
GMO	Genetically modified organism
GMP	Good manufacturing practice
HCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HPBL	Human peripheral blood lymphocytes
IPA	Isopropyl alcohol
LC-PUFA	Long chain polyunsaturated fatty acids
LD	Lactation Day
NHMRC	National Health and Medical Research Council
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
PCE	Polychromatic erythrocytes
RDI	Recommended Dietary Intake
US FDA	United States Food and Drug Administration

1 Background and scope

1.1 Background

The Application has been submitted by DSM Nutritional Products for consideration of the use of an additional, replacement or alternative DHA oil source for infant formula products. The oil is derived from marine microalgae *Schizochytrium* sp.

DHA is an omega-3 (n-3) long chain polyunsaturated fatty acid (LC-PUFA). The n-3 LC-PUFAs, particularly DHA, have been identified as important dietary components and are normal constituents of the human diet. DHA is found in breast milk, but the amount of this and other n-3 LC-PUFAs is largely dependent on maternal intake.

1.1.2 Micro-algal oils as a source of LC-PUFA

In Australia and New Zealand, marine micro-algal oils as a source of DHA and other long chain fatty acids have been permitted to be added to infant formula products since 1998. In 2002 approval was gained for the use of algal oil containing approximately 35% DHA produced by fermentation using *Schizochytrium* sp. micro-algae as a novel food in Australia and New Zealand (ANZFA, 2002). Standard 2.9.1 in the *Australia New Zealand Food Standards Code* (the Code) permits the presence of n-3 LC-PUFA in infant formula products at a maximum of 1% total fatty acid content.

There are a number of micro-algal DHA-rich oils available and used in infant formula products. These oils are derived from several different species including several from different strains of the *Schizochytrium* sp. The fatty acid compositions of these oils are generally similar, with small variations in the amounts of the different LC-PUFAs.

This alternative micro-algal oil is derived from a new production strain of *Schizochytrium* species, identified by its American Type Cell Culture number ATCC PTA-9695. The Applicant has indicated that this oil will be sold under the trade names DHA-B or DHASCO-B. The Applicant advises that this strain is more productive than previous strains from *Schizochytrium* sp. and other micro-algal species.

1.2 Risk Assessment context and scope of assessment

As FSANZ has previously assessed and considered several micro-algal oils for use in infant formula products, the objective of this assessment is to evaluate the suitability of DHA-B as an additional, replacement or alternative DHA oil source for infant formula products.

It is proposed that DHA-B be added to infant formula products at the current use levels, providing n-3 LC-PUFA up to 1% total fatty acid content of infant formula products.

The recent nutrition assessment from the recent Consultation Paper for Proposal P1028 – Infant Formula considered the evidence of supplementation of DHA in infant formula products and concluded that there is no evidence that voluntary DHA addition poses a risk to infant health.

1.3 Risk assessment questions

The following key questions were posed:

- What is the stability of this DHA-rich oil during under recommended storage conditions and shelf life? What are the consequences of improper storage, such that may lead to oxidation and production of radicals and peroxide?
- What are the risks, if any, from the use of *Schizochytrium* sp. (ATCC-PTA-9695) to produce oil?
- Does use of this oil in infant formula at the proposed use levels pose a risk to infant health?
- How does the composition of DHA-B compare to other currently permitted DHA-rich micro-algal oils?
- Is the use of DHA-B in infant formula products as the source of DHA equivalent to the use of other permitted DHA rich oils in infant formula products in relation to:
 - absorption and metabolism as a source of available DHA for infants
 - normal growth and development of infants.

2 Food technology assessment

2.1 Identity of the oil

DHA-B is a free flowing, light yellow-orange oil, comprised of oil extracted from the micro-algae *Schizochytrium* sp. DHA-B is predominantly triglyceride in composition, along with some monoglyceride, diglyceride and non-saponifiable material (essentially identified and unidentified sterols), as is typical for food-grade vegetable oils. The oil contains approximately 40% DHA by weight.

DHA-B is insoluble in water, however is soluble in non-polar organic solvents (e.g. hexane) and partially soluble in polar organic solvents (e.g. ethanol). It has a characteristic 'marine' odour and has a melting point/range of < 10°C.

2.1 Identity of the micro-algal strain

The micro-algal strain *Schizochytrium* sp. (ATCC PTA-9696) from which DHA-B is derived is not a genetically modified organism (GMO). The taxonomy for the source micro-algae for DHA-B is as follows:

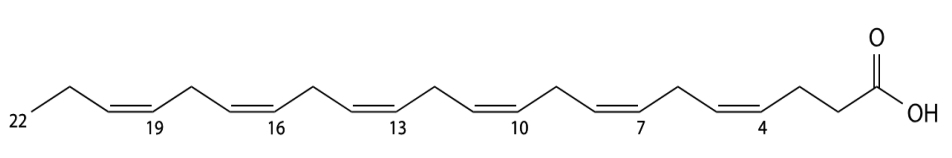
Kingdom – Chromista (Stramenopilia)
Phylum – Heterokonta
Class – Thaumatochytridae
Order – Thaumatochytriales
Family – Thaumatochytridiaceae
Genus – *Schizochytrium*

2.2 Chemical properties of the oil

2.2.1 Chemical name, identification and structure

The chemical name, identification and structure are all listed in Table 2.1 below.

Table 2.1: Chemical properties of the oil

Property	Description
Chemical name:	Docosahexaenoic acid algal oil (from <i>Schizochytrium</i> sp.)
Common names:	DHA algal oil, algal oil, cervonic acid
CAS Registry Number:	The CAS number for fatty acids containing 14-22 carbons (C14-C22), and 16-22 carbons (C16-C22) esterified to glycerol is 68424-59-9 (described in the CAS registry as “glycerides”, C14-C22 and C16-C22-unsatd.)
American Type Culture Collection Number:	PTA-9695
Chemical formula:	C ₂₂ H ₃₂ O ₂
Molecular weight:	328.488 g/mol
Structural formula:	

2.3 Analytical method for detection

The Applicant has cited AOCS Ce 1b-89; “Fatty Acid Composition of Marine Oils by GLC” as the method of choice for identifying and quantifying DHA in oils. This method however, is not applicable to food matrices such as infant formula products.

For fortified foods (food matrices) such as infant formula products, the method of choice is AOAC 996.06, “Fat (Total, Saturated and Unsaturated) in Foods”, which is used to determine DHA potency of a sample.

2.4 Product specification

As specified above, this oil is generally similar in fatty acid composition to other DHA-rich algal oils. Consideration was given as to whether further specifications or adjustment of specifications to those included in section S3—21 were required.

Table 2.2 shows detailed specifications for DHA-B and also that it complies with the specifications in section S3—21. The Applicant provided an analysis of five separate DHA-B lots to indicate that the manufacturing process results in a consistent product (Table 2.1).

The main differences between DHA-B and the specification levels set by FSANZ in section S3—21 are:

- DHA-B contains approximately 40% DHA oil, with its specification stating a minimum of 35% DHA content; compared to the current specification in the Code of a minimum requirement of 32%.
- the level of trans fatty acids listed in the application in DHA-B is 3.5%, however since submitted the Application, the Applicant has advised in writing that the correct figure is <1%. The analytical results for DHA-B show that trans fatty acids were all below 1% (refer to Table 2.2 below).

Table 2.2: Analysis of the oil

Tests		DSM Specification for the oil	Limit of detection	DHA algal oil test results (from DSM)				
				08-6530	08-6586	08-6585	08-6592	08-6643
DHA	(%)	Min. 35.0	N/A	44.35	42.65	42.96	41.23	45.71
EPA	(%)	Max. 10.0	0.10	5.90	6.10	6.43	6.10	6.61
Free fatty acids	(%)	Max. 0.40	0.005	0.07	0.05	0.05	0.07	0.13
Peroxide Value	(meq/kg)	Max. 5.00	0.10	0.40	<0.1	<0.1	<0.1	<0.1
Moisture and volatiles	(%)	Max. 0.02	0.01	<0.1	<0.1	<0.1	<0.1	<0.1
Unsaponifiabiles	(%)	Max. 3.50	0.05	0.97	0.96	0.88	0.78	0.88
Trans fatty acids	(%)	Max. 1.00	1.00	<1.0	<1.0	<1.0	<1.0	<1.0
Arsenic	(mg/kg)	Max. 0.10	0.10	<0.1	<0.1	<0.1	<0.1	<0.1
Cadmium	(mg/kg)	Max. 0.10	0.10	<0.1	<0.1	<0.1	<0.1	<0.1
Copper	(mg/kg)	Max. 0.10	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Iron	(mg/kg)	Max. 0.20	0.02	0.04	0.02	0.05	0.03	0.04
Mercury	(mg/kg)	Max. 0.04	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lead	(mg/kg)	Max. 0.10	0.10	<0.1	<0.1	<0.1	<0.1	<0.1

Paragraph 2.9.1—11(1)(c) specifies the ratio of EPA to DHA must not be less than 1:1. DHA-B meets this requirement, containing not more than 10% EPA and not less than 35.0% DHA; ensuring a ratio of 1:3.5 as a minimum as shown in Table 2.2. An average EPA:DHA ratio of approximately 1:7 is observed across the five samples represented.

The specification for DHA-B is: oil derived from *Schizochytrium* sp. (ATCC PTA-9695) rich in DHA.

2.5 Manufacturing process

A flow chart of the manufacturing process for the oil is shown in Figure 2.1. Following fermentation, the broth is collected in a centrifuge for further processing and pasteurisation. Enzymatic degradation of the algal cell walls by a food-grade protease is used to facilitate the release of the oil from the algae, followed by sodium chloride addition and centrifugation.

The oil from the centrifuge is collected as the top (light) phase and the spent broth as the lower (heavy) phase. The recovered oil is collected in the oil recovery tank and pumped into a vacuum dryer, where it is dried under maximum vacuum.

The separated algal oil then undergoes the standard food oil industry downstream processing operations of filtration, refining, physical bleaching and deodorization. DHA-B is manufactured under appropriate good manufacturing practice (GMP). The resultant DHA-B is a free flowing, light yellow-orange oil.

The Applicant also maintains material control specifications for all raw materials used in the manufacture of DHA-B. The quality of all raw materials used is verified from the manufacturers' Certificates of Analysis. The Applicant has provided details on all raw materials used in the manufacturing process along with confirmation that they are food grade and are either food additives or processing aids permitted in the Code.

2.6 Product stability

DHA oil typically contains conjugated double bonds which can make it susceptible to decomposition. Therefore its stability must be considered to avoid affecting the final product quality by means of reduced efficacy. Oxidative stability is illustrated by the inclusion of free fatty acid value and peroxide values in Table 2.2, which are well within specification for the analysed samples.

The Applicant has also provided information on the stability of DHA-B, when added to liquid ready-to-feed infant formula, stored for 12 months at ambient temperature. This showed that stability was maintained through a consistently high result for DHA content versus the label claim (Table 2.3).

Furthermore, the Applicant stated that the infant formula company is responsible for assuring that when the oil is combined with approved ingredients that the finished infant formula products is stable throughout its shelf life. Most infant formula products have a shelf life of 12 months and state on the label that the product should be stored in a cool dry place and consumed within four weeks once opened. By following these instructions, the level of the DHA component of infant formula products should be maintained.

Table 2.3: Stability of DHA-B in liquid ready-to-feed infant formula (12 months ambient storage).

Nutrient	Unit	Label Claim (LC)	0 Month		3 Month		6 Month		12 Month	
			Result	%LC	Result	%LC	Result	%LC	Result	%LC
DHA	mg/100 kcal	19	21.6	113	20.4	107	21.8	114	19.5	102

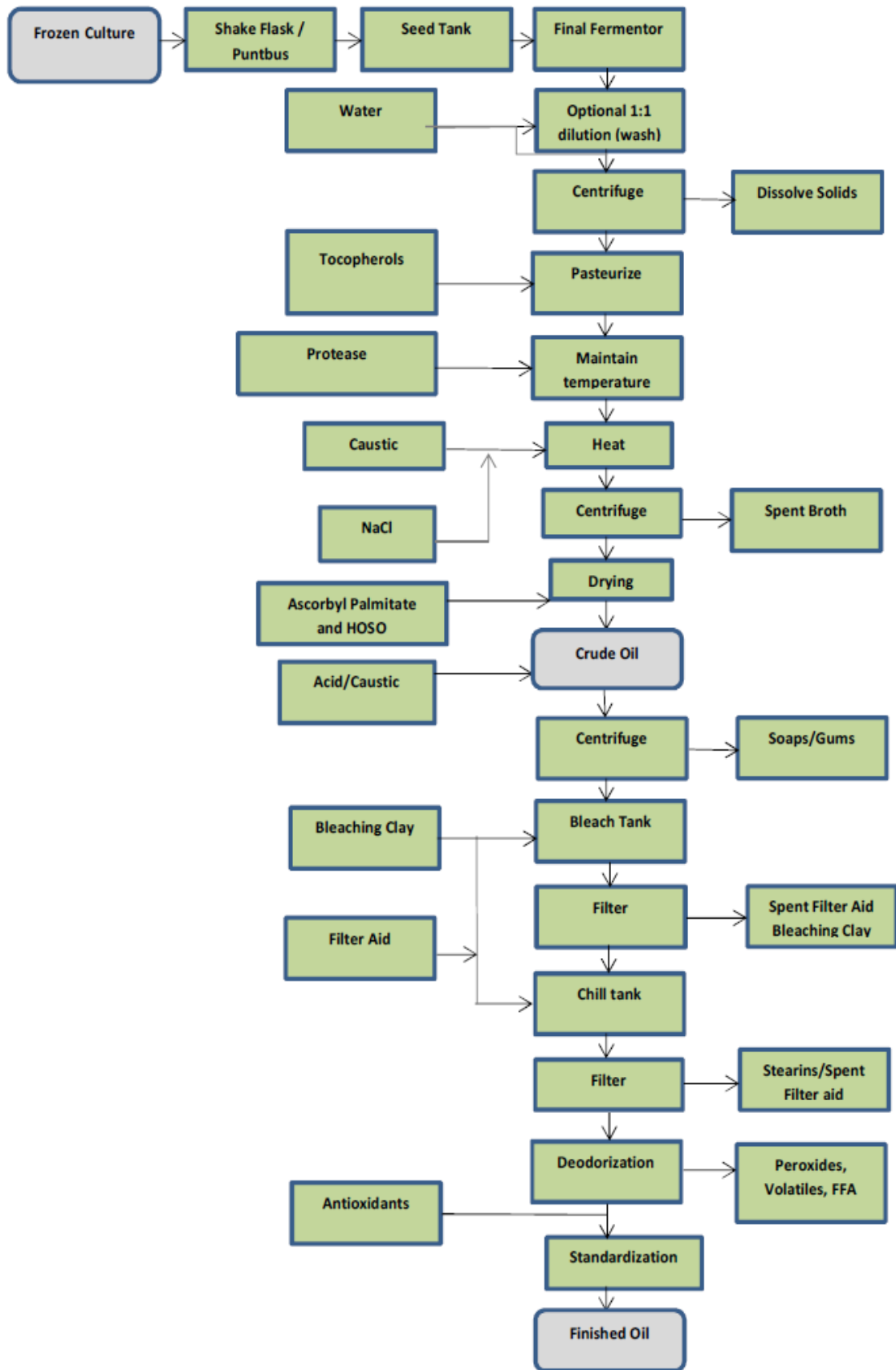


Figure 2.1: DHA-B manufacturing process flow chart

2.7 Food technology conclusion

The Applicant has provided sufficient technical data to ensure that DHA-B is suitable as an additional, alternative or replacement DHA oil source in infant formula products. The stability of DHA-B within a food matrix such as infant formula products is assured. An analytical method (AOCS Ce 1b-89) is available for compliance of DHA oils against specifications contained within section S3—21 of the Code.

A new specification will be added for DHA-rich algal oil from *Schizochytrium sp.* American Type Culture Collection (ATCC) PTA-9695.

3 Hazard assessment

FSANZ has assessed the submitted evidence on the safety of DHA-B, as well as additional information in the scientific literature. The submitted data, together with information from other sources, are considered suitable for hazard assessment of DHA-B.

3.1 Pharmacokinetics

DHA-B[®] is composed primarily of triglycerides. The pharmacokinetics of triglycerides have been extensively described in the scientific literature on nutrition and there is no new information beyond that summarised in the Safety Assessment report for DHASCO[®] prepared for Proposal P93.

3.2 History of human exposure and consumption

3.2.1 Source organism

The source organism of DHA-B is a strain of micro-algal species within the genus *Schizochytrium*. *Schizochytrium* species occur widely in water and are indirect components of the human food chain through the consumption of fish and other marine animals that feed on micro-algae. Direct human consumption also occurs, through consumption of shellfish such as mussels and clams. These micro-algae are thraustochytrids, and there are no known toxic compounds synthesised by any thraustochytrids.

3.2.2 DHA-rich algal oils

Omega-3, or n-3, LC-PUFA, including DHA, EPA, and docosapentaenoic acid (DPA) are normal components of breast milk (Innis 2008; Morse 2012). DHA-rich algal oils have been added to infant formula products sold in Australia and New Zealand since 1998. These oils have also been added to infant formula products in numerous other countries since the 1990s. Since these oils were first permitted in infant formula products, the US FDA has conducted a comprehensive analysis of infant formula adverse event reports in the CFSAN Adverse Event Reporting System (CAERS) database, with particular focus on adverse events related to the gastrointestinal system. The analysis covered the period from 2000, which was before the introduction of the DHA-rich algal oils, to 2009, when practically all infant formulas contained such oils. The US FDA concluded that there were no significant increases in the proportion of adverse gastrointestinal events over the time interval (US FDA, 2011).

3.3 Genotoxicity studies

3.3.1 Source organism

Bacterial reverse mutation assay (Hammond et al. 2002)

For the purpose of this assay, DHA-rich dried microalgae (DRM) were fully lysed by homogenisation. Concentration, stability and homogeneity of this test preparation was confirmed prior to and following the assay. Initial toxicity testing, to define dose levels for mutagenicity testing, was conducted with *Salmonella typhimurium* test strain TA100 with and without S9. Mutagenicity testing was conducted in triplicate using plate incorporation and preincubation assay methods using five *S. typhimurium* test strains (TA98, TA100, TA102, TA1535 and TA1537), with and without S9 fraction. Concurrent assays with positive and negative controls were also conducted. Revertant colonies were counted after incubation for at least 48 hours at 37±1° C.

In initial toxicity testing, no toxicity was observed with DRM levels up to 5000 µg/plate, with or without metabolic activation, but precipitation of DRM at levels higher than 500 µg/plate interfered with the counting of revertant colonies, and therefore the highest DRM treatment level for the mutagenicity assay was set at 500 µg/plate, with additional treatment levels of 5, 15, 50 and 150 µg/plate.

DRM was not mutagenic towards any of the test strains used, with or without the S9 metabolic activation system.

Chinese hamster ovary AS52/XPRT gene mutation assay (Hammond et al. 2002)

The test article for this assay was homogenised, lysed DRM, as for the bacterial reverse mutation assay described above. Positive controls for this assay were benzo[a]pyrene with S9 fraction and actinomycin D without S9 fraction. Following a preliminary cytotoxicity test, DRM was used to treat AS52 Chinese hamster ovary cells for 5 hours. Five dose levels, with and without S9 fraction, were assayed in triplicate.

In the initial cytotoxicity determination, there was no cytotoxicity in the absence of S9 but some cytotoxicity in the presence of S9. Based on these initial screening studies, DRM was tested at 200, 500, 1000, 2000 and 5000 µg/mL without S9, and at 200, 700, 850, 900 and 1000 µg/mL with S9. The positive controls yielded the expected positive responses, but there were no statistically significant increases in mean mutant frequency in response to DRM treatment, with or without S9 fraction. However in the presence of S9, significant cytotoxicity was observed at DRM levels of 900 µg/mL and above. It was concluded that DRM did not cause point mutations (base pair or frameshift) in this assay.

Chromosomal aberration assay in human peripheral lymphocytes (Hammond et al. 2002)

For this assay, dried, unlysed DRM was suspended in water. The test system was human peripheral blood lymphocytes (HPBL). Positive controls were Mitomycin C for use in the test system without S9, and cyclophosphamide in the test system with S9. Water was used as the negative control. In initial dose-range finding, precipitation of DRM was a problem, so the highest dose level demonstrating sufficient unobstructed metaphase cells was selected as the top dose level for the definitive assay. Selected dose levels were 125, 250, 500 and 750 µg/mL. HPBL were inoculated into replicate tubes containing medium, and incubated at 37±1° C for 44-48 h prior to addition of DRM. In the assay without S9, cells were incubated with DRM for 4, 20 or 44 h, whereas for the assay with S9, cells were incubated with DRM for 4 h.

Cells incubated for 4 h (pulse treatment groups) were washed after 4 h, and incubated in fresh medium for a further 16 or 40 h. For all groups, colcemid was added 2 h prior to scheduled collection of metaphase cells for evaluation. Metaphase cells were harvested, processed to slides and evaluated without knowledge of the treatment group. The percentage of cells with numerical or structural aberrations following DRM treatment was not significantly different from control ($P \leq 0.05$), with or without S9.

Mouse micronucleus assay (Hammond et al. 2002)

This assay was conducted using 8- to 10-week old male CD-1 mice, maintained under standard laboratory husbandry conditions. The test article was unlysed DRM, as used in the chromosomal aberration study described above. Preliminary dose-range finding established that DRM was nontoxic to mice at doses up to and including 2000 mg/kg bw. Mice were randomly assigned to groups of at least 10 mice/group, and gavaged with a single dose of 0, 500, 1000 or 2000 mg/kg bw DRM in distilled water at a dose volume of 10 mL/kg.

Mice in a positive control group were dosed with a single gavage dose of cyclophosphamide, at 40 mg/kg bw. Mice were observed for moribundity and mortality until scheduled termination at 24 or 48 hours after treatment. Bodyweights were measured prior to dosing and at scheduled termination. Following termination with CO₂, two slides of femoral bone marrow were prepared from each mouse, and examined without knowledge of the treatment group. The ratio of polychromatic erythrocytes (PCE) to total erythrocytes and the number of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes were calculated.

There were no clinical signs of toxicity observed, no significant effects on bodyweight, no unscheduled deaths, no statistically significant decreases in mean ratio of PCE to total erythrocytes and no statistically significant increases in mean micronucleated PCE frequency in any of the DRM-treated groups, compared to negative controls. The expected significant increase in micronucleated PCE was observed in the positive control group. It was concluded that there was no evidence of clastogenic effects or malsegregation of chromosomes attributable to DRM.

3.3.2 DHA-B, aqueous extraction

Bacterial reverse mutation assay (BSL, 2011a)

This study was conducted in compliance with OECD Test Guideline No. 471, Commission Regulation (EC) No. 440/2008, and the EPA Test Guideline OPPTS 870.5100.

Two separate experiments were conducted; a plate incorporation study and a liquid pre-incubation study. In each experiment, mutagenicity testing was conducted in triplicate using *Salmonella typhimurium* test strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* test strain WP2 uvrA., with and without S9 fraction. Concurrent assays with positive and negative controls were also conducted. DHA-B was suspended in DMSO and diluted prior to use. In pre-experiment dose-range finding experiments using test strains T98 and T100, no toxic effects of DHA-B were observed at up to 5000 µg/plate and selected test dose levels were therefore 31.6, 100, 316, 1000, 2500 and 5000 µg/plate. Revertant colonies were counted after incubation for at least 48 hours at 37±1° C.

DHA-B was not mutagenic towards any of the test strains used, with or without the S9 metabolic activation system.

Chromosomal aberration assay in human peripheral lymphocytes (BSL, 2011b).

The study was conducted in compliance with OECD GLP principles. Two separate experiments were conducted, and each experiment was conducted in duplicate. Positive and negative controls were included in each experiment. Positive controls were ethyl methanesulfonate for use in the test system without S9, and cyclophosphamide in the test system with S9. Cell culture medium was the vehicle in which the test item was suspended. In Experiment I, the treatment interval was 4 h with and without metabolic activation. In Experiment II, the treatment interval was 4 h with metabolic activation and 24 h without activation. For the 4 hour exposures, cells were washed and resuspended in fresh medium after 4 hours. All incubations were at 37° C in humidified atmosphere with 5% CO₂. For Experiment I, and for Experiment II without metabolic activation the selected doses of DHA-B were 250, 500, 1000, 2500 and 5000 µg/mL. For Experiment II, the initial doses with metabolic activation were 3000, 4000 and 5000 µg/mL. However, Experiment II with metabolic activation was repeated with the dose levels revised downward to 400, 500, 750 and 1000 µg/mL, because precipitation occurred at concentrations ≥1000 µg/mL.

Colcemid was added to the cultures at least 2 hours before scheduled harvesting of metaphase cells. Metaphase cells were harvested, processed to slides and evaluated without knowledge of the treatment group. No biologically relevant increase in aberration rates was noted in either Experiment I or Experiment II in the absence of metabolic activation. With metabolic activation, there was an increase in aberrant cells at the historical control data range at 500 µg/mL, but not at higher dose levels, in Experiment I. In Experiment II with metabolic activation, there was an increase in aberrant cells above the historical control data range at 4000 and 5000 µg/mL, but no dose-response relationship was evident. When Experiment II was repeated at the lower dose levels, with metabolic activation there was an increase in aberrant cells at 500 µg/mL and above, but no dose-response relationship. It was concluded that DHA-B did not induce structural aberrations in human lymphocytes in the absence of metabolic activation. The increase in aberrant cells in the presence of metabolic activation was considered to be equivocal because the clastogenic effect was moderate and there was no evidence of a dose-response relationship.

Mouse micronucleus assay (BSL, 2011c)

Young adult NMRI mice, 5/sex/dose group, maintained under standard laboratory husbandry conditions, were the test system for this study. The test article was diluted in cottonseed oil and administered as single IP dose of 0 or 2000 mg/kg bw DHA-B at 10 mL/kg bodyweight. Cyclophosphamide, 40 mg/kg bw was administered IP to a positive control group. No clinical evidence of toxicity was observed as a result of treatment. Peripheral blood was collected from the tail vein 44 and 68 h after treatment. Blood cells were fixed and evaluated by flow cytometry. At least 10,000 immature erythrocytes/mouse were scored for the incidence of micronuclei, and the ratio between immature and mature erythrocytes was calculated. DHA-B did not induce structural or numerical chromosomal damage in the immature erythrocytes of the mice, and was therefore was not considered genotoxic under the conditions of the assay.

3.3.3 DHA-B, IPA extraction

Additional studies have been conducted with DHA-B extracted using isopropyl alcohol (IPA) rather than with the extraction method of the product for which the application was made, which uses water rather than IPA. DHA-B extracted with IPA is compositionally similar to DHA-B extracted with water. Therefore, although these studies are not directly relevant to the product for which the application is made, they provide additional information relevant to the hazard assessment. Therefore they are briefly reviewed in this section.

The publication by Federova-Dahms et al. 2011 reports, in brief form, the results of the BSL studies also cited for the following three studies. DHA-B prepared using IPA extraction was referred to as DHA-rich algal oil in these studies.

Bacterial reverse mutation assay (Fedorova-Dahms et al. 2011; BSL 2010a)

This OECD GLP-compliant assay was conducted according to the plate incorporation method using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and *Escherichia coli* strain WP2 uvrA. Two independent experiments were conducted, each with and without S9 for metabolic activation. Each concentration, including negative and positive controls, were tested in triplicate. Concentrations of test item in Experiment I were 31.6, 100, 316, 1000, 2500 and 5000 µg/plate. Concentrations of test item in Experiment II were 50, 158, 500, 1580, 4000 and 5000 µg/plate. The positive control for assays with metabolic activation was 2-aminoanthracene. The positive controls for assays without metabolic activation were sodium azide for *S. typhimurium* strains TA 100 and TA 1535, 4-nitro-o-phenylene diamine for *S. typhimurium* strains TA 98 and TA 1537, and methyl methane sulfonate for *E. coli* WP2 uvrA.

No precipitation or toxic effects of the test article were noted at any dose level, with or without metabolic activation, and there were no biologically relevant increases in revertant colony numbers with any bacterial strain at any dose level, with or without metabolic activation. The positive control mutagens all induced distinct increases in the number of revertant colonies. It was concluded that DHA-rich algal oil did not cause gene mutation by base pair changes or frameshifts in this assay.

Chromosomal aberration assay in human peripheral lymphocytes (Fedorova-Dahms et al. 2011; BSL, 2011b)

Two experiments were conducted as part of this OECD GLP-compliant assay. Experiment I was conducted with and without metabolic activation with a 4 h treatment interval. Experiment II was conducted for 4 h with metabolic activation and 24 h without metabolic activation. Two parallel cultures were used. Positive controls were ethyl methane sulfonate at 400 and 600 µg/mL and cyclophosphamide at 5 µg/mL. Test article concentrations in Experiment I, and in Experiment II without metabolic activation, were 1.0, 2.5 and 5.0 µL/mL. In Experiment II with metabolic activation, test article concentrations were 3.0, 4.0 and 5.0 µL/mL. Some precipitation of the suspended test article occurred at concentrations exceeding 1.25 µL/mL. While the positive control articles induced distinct and biologically relevant increases in in cells with structural chromosomal aberrations, there were no biologically relevant decreases in relevant mitotic index, and no biologically relevant increases in aberration rates or frequencies of polyploid cells at any test article dose level in either experiment, with or without metabolic activation. It was concluded that DHA-rich algal oil was non-clastogenic in this assay.

Mouse micronucleus assay (Fedorova-Dahms et al. 2011; BSL, 2010c)

Young adult NMRI mice, 5/sex/dose group, maintained under standard laboratory husbandry conditions, were the test system for this study. The test article was diluted in corn oil and administered as a single oral dose of 0 or 2000 mg/kg bw DHA-rich algal oil at 10 mL/kg bw. Cyclophosphamide, 40 mg/kg bw was administered IP to a positive control group. No clinical evidence of toxicity was observed as a result of treatment with DHA-rich algal oil. Peripheral blood was collected from the tail vein 44 and 68 h after treatment. Blood cells were fixed and evaluated by flow cytometry. At least 10,000 immature erythrocytes/mouse were scored for the incidence of micronuclei, and the ratio between immature and mature erythrocytes was calculated.

DHA-rich algal oil did not induce structural or numerical chromosomal damage in the immature erythrocytes of the mice, and was therefore considered not to be genotoxic under the conditions of the assay.

3.4 Studies in experimental animals

3.4.1 Source organism

13-week subchronic study in rats (Hammond et al. 2001a)

Powdered *Schizochytrium* sp. (DRM), stabilised against oxidation with Vitamin E acetate, was administered in the diet to Sprague-Dawley derived rats, 20/sex/group, at target dose levels of 0, 400, 1500, and 4000 mg/kg bw/day for at least 13 weeks. An additional group (20/sex) received diet mixed with fish oil at a dose level of 1628 mg/kg bw/day, an equivalent amount of fat as the highest dose (1500 mg/kg bw/day) of DRM. Fresh diets were prepared weekly. Rats were housed under standard laboratory conditions and were 5–6 weeks old at study start. Endpoints measured in all rats on study were survival, clinical signs, bodyweight, food consumption, ophthalmology, gross necropsy, selected organ weights, and histopathology of a complete tissue list.

Samples for clinical pathology (haematology, serum chemistry and urinalysis) were collected from 10 rats/sex/group during week 6-7 and from the same rats at scheduled termination. The parameters measured in clinical pathology samples were standard for GLP-compliant toxicology studies of this duration in this species.

All rats survived to scheduled termination and there were no treatment-related clinical signs observed on cage-side examinations or weekly detailed in-hand examinations. There were no treatment-related effects on ophthalmology, bodyweight gain or food consumption. Actual food consumptions were very close to target dose levels; averaging 392, 1477, and 3905 mg/kg/day in males and 393, 1484, and 3937 mg/kg bw/day in females. There were no treatment-related effects on haematology, urinalysis or urine sediment parameters. Statistically significant decreases in group mean HDL and total cholesterol, relative to those of control groups, were observed in both sexes in the fish oil group, in 4000 mg/kg bw/day DRM females, and in ≥ 1500 mg/kg bw/day DRM males. These effects were not considered to be adverse, have been observed previously in rats supplemented with DHA, and are attributed to the known effects of PUFAs which include increasing hepatic oxidation of fatty acids and decreasing lipogenesis. Periportal hepatocellular accumulation of lipid was observed in the livers of female rats at all dose levels of DRM and also in the fish oil group, but was not considered adverse or unexpected, given that the diets contained a higher total fat content than the basal diet fed to the control females.

A slight increase in the incidence, but not the severity, of subclinical cardiomyopathy was observed only in the 4000 mg/kg bw/day DRM male rats. Cardiomyopathy is a recognised and well documented spontaneous lesion in rats, and it is also well documented that male Sprague-Dawley rats fed diets containing a high level of fat are uniquely sensitive to developing cardiomyopathy. Comparable lesions have not been observed in mice, dogs, pigs or nonhuman primates fed diets high in fat. It is recognized that long-chain PUFAs of the linolenic acid family extensively accumulate in cardiac phospholipids in the rat, but not in other species. Compared to other species, rats have a much lower ability to oxidise C20 and C22 fatty acids, and in the rat these fatty acids cause secondary inhibition of the tricarboxylic acid cycle, an effect not observed in other species. This lesion is therefore not considered to be relevant to human health safety assessment. Myocarditis in the rat is not similar to the most prevalent heart disease in humans, which affects cardiac vasculature rather than cardiac muscle.

The results of this study establish a subchronic dietary No Observed Effect Level (NOEL) of DRM in Sprague-Dawley rats of 4000 mg/kg bw/day.

One-generation reproductive toxicity study in rats (Hammond et al. 2001b).

Sprague-Dawley rats, 30/sex/group, were provided with a diet containing 0, 0.6, 6.0 or 30% DRM w/w, added to the diet as a dry powder. Homogeneity and stability in the diet were established pre-study. These concentrations resulted in mean doses of approximately 0, 400, 3900, and 17,800 mg/kg bw/day for F0 males and 0, 480, 4600, and 20,700 mg/kg bw/day for F0 females, respectively. Rats were maintained under standard husbandry conditions and individually housed, except during mating. F0 males were treated for 10 weeks prior to mating, through mating and to scheduled termination approximately 3 weeks after mating. F0 females were treated for at least 2 weeks prior to mating, through mating, gestation and lactation, through to scheduled termination on postnatal day (PND) 21. Litters were culled to 4/sex/litter on PND 4. Parameters measured for assessment of toxicity included survival, bodyweight, food consumption (except during mating), oestrus cycle duration, mating performance, fertility, gestation length, parturition, gestation index, litter size, pup bodyweights, offspring viability index, vaginal opening of female pups, preputial separation of male pups, and gross necropsy findings of all F0 and F1 rats.

In addition, sperm count, motility, and morphology were assessed at scheduled termination of F0 males, uteri of F0 females were examined for numbers of implantation sites, and reproductive tracts of both F0 sexes were subject to histopathological examination, including spermatogenic cycle evaluation in the right testis of F0 males. No test article-related adverse effects were observed in any parameter. At the highest test article concentration (30% w/w), food intake was slightly depressed in F0 males and bodyweight gain was slightly increased in F0 females. These effects were attributed to the high caloric content of the diet and not considered to be adverse. At DRM concentrations \geq 6% w/w, females exhibited periportal vacuolation of hepatocytes, but this was attributed to the high lipid content of the diet and not interpreted as an adverse effect.

Dietary study of developmental toxicity in rats (Hammond et al. 2001c).

Female Sprague-Dawley rats, 25/group, were maintained under standard husbandry conditions and provided with *ad libitum* access to diets containing 0, 0.6, 6.0 or 30% DRM w/w, added to the diet as a dry powder, from GD6 through to the morning of GD16. Homogeneity and stability of the test article in the diet were established pre-study. Rats were killed on GD20. Endpoints for assessment of maternal toxicity were survival, clinical signs, bodyweight changes, gross necropsy findings, corpora lutea at necropsy, uterine weight, number and location of all fetuses, early and late resorptions, and total number of implantation sites. Fetuses were killed, weighed, examined, dissected, fixed and examined for skeletal abnormalities using Alizarin Red. Heads were collected from half the fetuses and sectioned for examination of brains.

There were no unscheduled maternal mortalities, and no clinical signs of toxicity. There was no treatment-related effect on bodyweight gain or food consumption at DRM concentrations of \leq 6.0% w/w. The group mean bodyweight gain of the 30% DRM group was greater than that of controls from GD6 to GD15 but significantly less (20% lower) than that of controls from GD16 to GD18. The group mean food consumption of the 30% DRM group was significantly lower (10% lower) than that of the control group from GD6 to GD9 and from GD16 to GD18, but was not significantly to that of controls during other intervals. There was no treatment-related effect on the number of rats in each group that became pregnant.

Maternal consumption of DRM at up to 30% w/w in the diet had no effect on number of corpora lutea, number of implantations, number of live fetuses, percentage of resorptions, percentage of late fetal deaths, mean pup weights, sex ratio of pups, incidence of major malformations, or incidence of minor external or visceral anomalies. A statistically higher incidence ($P < 0.05$) of fetuses (but not litters) with reduced ossification of the ribs was observed in the mid (6%)- and high-dose (30%) DRM groups but in each group, this resulted from a single litter with a number of affected pups. This minor anomaly is known to occur as a background anomaly in this strain of rat, and was not considered to be a treatment-related effect in this study.

The study authors concluded that the No Observed Effect Level (NOEL) was the high-dose of 30% DRM, which was equivalent to 22 g/kg bodyweight/day.

Gavage study of developmental toxicity in rabbits (Hammond et al. 2001c).

Female New Zealand White rabbits were maintained under standard husbandry conditions and synchronised with intravenous human chorionic gonadotrophin (HCG), after which they were artificially inseminated with fresh semen (semen from one male per two females in the same group). Rabbits were gavaged twice daily from GD6 to GD18 with 0, 180, 600 or 1800 mg DRM/kg bw/day, using a vehicle of 0.5% methylcellulose (w/v) and 0.1% polysorbate 80 (v/v).

An additional group was gavaged with fish oil, stabilised with vitamin E, so that the amount of fat administered to the fish oil control rabbits approximated that received by the 1800 mg/kg bw/day DRM rabbits. Maternal endpoints measured during the in-life phase were survival, bodyweight changes, food consumption, and clinical signs. Rabbits were killed on GD29. Post-mortem endpoints determined in the dams were complete gross necropsy, corpora lutea, uterine weight, number and location of all fetuses, early and late resorptions, and total number of implantation sites. Fetuses were euthanased, weighed, examined, dissected, fixed and examined for skeletal abnormalities using Alizarin Red. Kidneys were dissected and renal papilla development was graded.

There were no maternal clinical signs of toxicity. One 1800 mg DRM/kg bw rabbit died as the result of a gavaging accident. One 600 mg DRM/kg bw/day rabbit died for unspecified reasons. One rabbit in the fish oil group spontaneously aborted on GD 23, and two rabbits in the 1800 mg DRM/kg bw/day group spontaneously aborted on GD 25 and 26. There were no DRM-related effects on group mean food consumption, group mean bodyweight, bodyweight change, gravid uterine weight, net body weight (i.e. excluding gravid uterus), or net bodyweight change at doses ≤ 600 mg DRM/kg bw/day. In the 1800 mg DRM/kg bw/day group, bodyweight gain was significantly lower than that of controls over the GD6-19 day treatment interval and from GD 19-24, although it increased during the interval GD 24-29. The fish oil group showed very similar group mean bodyweight changes to the 1800 mg DRM/kg bw/day group. These two groups also showed statistically significant reductions in group mean food consumption between GD 6-19 and between GD 19-24, when compared to the control group, but group mean food consumption of these two groups was comparable to that of controls in the interval GD 24-29. Administration of DRM or fish oil had no effect on number of pregnancies/group. There were no treatment-related effects on mean number of corpora lutea or implantation sites, on live litter size or on post-implantation loss, mean fetal body weights, fetal sex ratios, or prevalence of morphological abnormalities and variations.

The reductions in group mean food consumption and bodyweight gains in the fish oil and 1800 mg DRM/kg bw/day groups, and the possible increase in incidence of spontaneous abortions, were attributed to the high fat diet. It was concluded that, the NOAEL for maternal toxicity of DRM was 600 mg/kg bw/day, and the NOEL for developmental toxicity was 1800 mg/kg bw/day, in New Zealand white rabbits.

3.4.2 DHA-B, aqueous extraction

90-day dietary study in rats, including prenatal phase (Bauter 2013)

Dietary exposure to DHA-B commenced prior to mating of the parental generation in this study in Sprague-Dawley rats. There were five parental groups, comprising 13 males and 26 females in each group. Dietary levels of DHA-B were: 0 (control group), 10,000 ppm, 30,000 ppm, and 50,000 ppm. An additional group was fed diet containing 50,000 ppm tuna oil. Adequate stability and homogeneity of the DHA-B and tuna oil were confirmed during the study. Rats of both sexes were fed *ad libitum* from four weeks prior to mating, through the mating period and through the pregnancies and lactations of the females. Two hundred of the resulting pups (100/sex) were assigned to one of the five dietary groups (20/sex/group) for the 90-day subchronic study, which commenced approximately 22 days after birth. Weaned offspring consumed the same diet as their dam.

Endpoints determined in both sexes in the F0 generation were survival, clinical signs, body weights and body weight changes, and food consumption. Fertility and reproductive performance parameters included mean pre-coital length and fertility indices and, in females, pre-mating and post-weaning oestrus cycles, mean gestation length, gestation index, mean number of implantation sites, corpora lutea, pre-implantation loss percentage, post-implantation loss percentage, live birth percentage, stillborn index, viability index, litter size, birth weight and male:female pup ratio.

Survival, bodyweights and time to developmental indices and sexual maturity of the F1 generation were comparable across all groups. From PND 14 through to weaning, group mean bodyweight gain of pups in the tuna oil group and the 50,000 ppm DHA-B group were significantly lower than that of pups in the control group, and all treated groups had significantly lower group mean bodyweight gains than the control group pups from PND 22. These reductions were attributed to increased caloric load from the high fat diets, and were not considered to be adverse because the pups gained weight within the appropriate range for their age.

Litters were culled on PNDs 4 and 22 and the culled pups were examined for gross abnormalities. No treatment-related abnormalities were found. Non-pregnant females of the F0 generation were culled 25 days after mating, and sires and dams after weaning on PND 22. No treatment-related effects were found on gross necropsies of F0 rats.

Endpoints determined in the 90-day dietary study were survival, clinical observations, ophthalmology, body weights and bodyweight changes, food consumption, food efficiency, motor activity and functional observational battery, clinical pathology (haematology, clinical chemistry, blood clotting, urinalysis, gross necropsy findings, selected organ weights, and histopathology).

Both the 50,000 ppm DHA-B males and the tuna oil males showed an increased incidence of slight to moderate cytoplasmic vacuolation of cortical cells in the adrenal zona fasciculata. This change was considered to be attributable to the high-fat diet and not to be an adverse finding.

Slightly increased incidence of minimal to moderate granulomatous infiltrate in retroperitoneal fat of 50,000 ppm DHA-B males, and mammary fat of 50,000 ppm DHA-B rats of both sexes, was considered to be possibly related to DHA-B consumption. This finding was considered to be non-adverse.

It was concluded that the NOEL for dietary DHA-B in rats was 50,000 ppm, which was equivalent to 3278.9 mg/kg bw/day for males and 3788.4 mg/kg/day in females.

Bioequivalence study in piglets (Federova-Dahms et al., 2014)

The purpose of this 3-week study was to evaluate the bioequivalence of DHA-B to DHASCO®. Piglets were received into the laboratory on Lactation Day (LD) 2, and experimental diets were provided from the day of arrival, designated Study Day (SD) 1. Piglets were randomised into five groups, with 5 piglets/sex/group. The control group was fed milk replacer with no LC-PUFA added in the laboratory, although the milk replacer contained 0.21% arachidonic acid (ARA). There were four treatment groups, representing two different concentrations of either DHA-B to DHASCO, so that DHA from micro-algae represented 0.32% or 0.96% of total fatty acids by weight. Arachidonic acid (ARA) was added to the diets of treated pigs so that the final percentage of total fatty acid that was ARA was twice the percentage that was DHA; that is 0.64% or 1.92%. Diets were prepared fresh daily and piglets were fed 500 mL/kg bw/day. Endpoints assessed during the in-life phase were survival, morbidity, clinical signs, bodyweights and bodyweight gains, and food efficiency. Blood samples were collected on SD 21 prior to necropsy for haematology and clinical chemistry, and urine was collected by cystocentesis during necropsy for urinalysis. Gross necropsies were performed on all piglets. Organ weights were collected for brain, heart, liver, spleen, thymus, adrenal glands, kidneys, ovaries, testes, large intestine and small intestine. The same organs were subject to histopathological examination. Fatty acid analysis was performed on plasma, erythrocytes, cerebral cortex, liver and heart samples.

All piglets survived to scheduled termination and there were no adverse in-life effects. There were no significant differences in bodyweights or bodyweight gains, mean food consumption or feed efficiency. Quantities of DHA consumed were close to target, and there were no differences in DHA consumption between piglets fed DHA-B and piglets fed the corresponding amount of DHASCO®. There were no test article-related effects on haematology, clinical chemistry, urinalysis, gross necropsy findings or histopathology findings. Small increases in group mean liver to bodyweight ratios, relative to that of controls, in males in three of the four group were not dose-dependent, had no histological correlates, remained with normal historical control range and were not considered to be toxicologically significant. Addition of DHA in the diet resulted in dose-dependent increases in DHA in plasma, erythrocytes, liver, heart and cerebral cortex, but there was no significant difference in these increases between DHA-B to DHASCO®.

The two levels of DHA added to the diet were intended to provide 1 and 3 times the expected maximum intake levels for human infants consuming infant formulas. However, because piglets grow much faster than human infants and consume more formula, the calculated mean intakes of DHA from addition of DHA-B to the milk replacer were 92.2 mg/kg bw/day and 294 mg/kg bw/day, corresponding to 1.9 and 5.8-fold the estimated mean DHA intake of formula-fed infants.

3.4.3 DHA-B, IPA extraction

90-day dietary study in rats, including prenatal phase (Federova-Dahms et al., 2011)

Sprague-Dawley rats, 9 to 10 weeks old at study start, were randomised into five groups, each group comprising 13 males and 26 females. Rats were housed under standard laboratory conditions. There were two control groups; a group fed the basal low-fat diet and a group fed the basal diet supplemented with 5% w/w concentrated fish oil. The other three groups were fed the basal diet supplemented with 0.5, 1.5 or 5.0% w/w DHA-rich algal oil (DHA-B extracted with IPA). The low- and mid-dose diets were adjusted with corn oil so that there was a total 5% w/w added fat. Diets were prepared weekly. The rats were fed their assigned diet for 28 days while housed individually prior to mating, through the 14-day mating period while group-housed as one male and two females in each cage, and through the post-mating phase when they were housed individually.

Both male and female rats were fed their assigned diet through the pregnancies of the females, at which time the males were terminated without further assessment. The females were maintained on the diets to PND 22, when they were terminated. From each litter, 1 to 2 pups of each sex were selected on PND 22 to continue on their dams' diets for a further 90 days (20/sex/group), followed by a 30 day recovery period for 10/sex/group.

Endpoints for assessment of toxicity determined on both the F0 and F1 generation were survival, clinical signs, bodyweight gain and food consumption. Additional parameters determined for the F0 females were fertility and reproductive indices, gross necropsies, ovary weights and uterine weights. Pups culled on PND 22 were subject to gross necropsy including examination for hydrocephaly. Additional parameters determined on the F1 generation were ophthalmology, motor activity assessment and Functional Observational Battery (FOB) in the final week of the 90-day interval, and clinical pathology assessments prior to termination, including haematology, clinical chemistry, clotting factors and urinalysis. All rats assigned to the 90-day study were subject to complete gross necropsy, determination of selected organ weights, and fixation of a comprehensive organ list for histopathology. Microscopic examination was conducted on all tissues from both control groups and the 5.0% DHA-rich algal oil group, and on gross lesions noted at necropsy of rats in other groups.

Stability and homogeneity of the test and control articles was confirmed during the study. All F0 rats survived to scheduled termination, with no adverse clinical observations, and no effects on body weight, bodyweight gain, food consumption or food efficiency attributable to DHA-rich algal oil. Both the 5% DHA-rich algal oil dams and the 5% fish oil dams had statistically significant decreases in group mean food consumption during lactation when compared to dams on the basal diet, but not when compared to each other. Consumption of DHA-rich algal oil had no effect on any fertility or reproductive performance parameters, including mean pre-coital length, male and female mating, fertility index, mean gestation length, mean number of implantation sites, number of corpora lutea, pre- and post-implantation loss percentage, litter size, stillbirth index, sex ratio, gestation index, pup viability, pup survival or mean pup weight during lactation. Similarly, DHA-rich algal oil had no effect on developmental landmarks in the pups, such as hair growth, pinna detachment, incisor eruption and eye opening. No macroscopic findings were noted on gross necropsy of the dams or the pups culled on PND 22. Dams in the 5% fish oil group and dams in the 5% DHA-rich algal oil group had statistically significant reductions in group mean ovarian and uterine weights when compared to dams on the basal diet, but the changes were small and remained within historical control ranges, so were considered to be non-adverse.

There were no test article-related mortalities in rats in the F1 generation assigned to the 90-day study. Consumption of DHA-rich algal oil had no effect on clinical observations, ophthalmology findings, motor activity or performance on the FOB. DHA-rich algal oil had no effect on the group mean bodyweights of males, however bodyweight gain was significantly reduced over the course of the study in males consuming fish oil. Females consuming \geq 1.5% w/w DHA-rich algal oil gained more weight over the course of the study than females consuming the basal diet, and this effect persisted through the 30-day recovery period. Male controls consuming the basal diet tended to have higher mean daily food consumption than males in the treatment groups and the fish oil-supplemented group, but food consumption was not affected by oil supplementation in females.

Male rats that consumed 5% w/w DHA-rich algal oil had slightly lower mean erythrocyte counts (4.75% decrease) and haematocrit (3.67% decrease) than controls at the end of the 90-day treatment phase, with some resolution during the recovery phase. These decreases were not of clinical significance, and the authors noted that such haematological changes in male rats have been reported in other dietary studies of LC-PUFA oils.

At 90 days, alkaline phosphatase (AP) activity was significantly increased, and mean cholesterol level decreased, in both sexes in the 5% w/w DHA-rich algal oil group and in the fish oil group, compared to sex-matched controls. The AP activity increase persisted through the recovery period, while the cholesterol level returned to a level comparable with that of controls. Similar elevation in AP activity has been reported in other studies of high LC-PUFA consumption in rats. The reduction in mean serum cholesterol was likewise an expected effect of LC-PUFA intake. Both the fish oil rats and the 5% w/w DHA-rich algal oil rats exhibited higher protein levels in urine than the control rats, but there were no clinical or pathological correlates and therefore the urine protein findings were considered to be non-adverse.

There were no treatment-related findings on gross necropsy, and no organ weight changes in DHA-rich algal oil-treated rats that were not also found in the fish oil rats. Both the fish oil rats and the 5% w/w DHA-rich algal oil had increased cytoplasmic vacuolation of the cortical cells in the zona fasciculata of the adrenal glands. Therefore this change was considered to be a non-adverse physiological adaptation to high LC-PUFA intake.

It was concluded that the NOAEL for DHA-rich algal oil was 5% w/w in the diet. This dose corresponded to 4122 and 4399 mg/kg bw/day, for male and female rats, respectively, with the average for both genders 4260 mg/kg bw/day. The average intake of DHA-rich algal oil by a human infant consuming formula would be 19–51 mg/kg bw/day. Therefore, the NOAEL from this study, 4260 mg/kg bw/day, represents a margin of exposure for infants of 84–224.

3.5 Studies in humans

No human studies specifically addressing the safety of the source organism were found. As previously noted, there have been no significant increases in the proportion of adverse gastrointestinal events associated with use of infant formula reported in the CAERS database over the time interval since before DHA supplementation of formula was introduced to 2011, when addition of DHA to infant formula became commonplace (US FDA, 2011).

3.6 Hazard assessment discussion

No evidence of genotoxicity of dried *Schizochytrium* was found using a bacterial reverse mutation assay (Ames test), gene mutation assay in Chinese hamster ovary cells, chromosomal aberration assay in human peripheral lymphocytes, or the mouse micronucleus assay. Subchronic dietary administration of dried *Schizochytrium* to Sprague-Dawley rats for 13 weeks did not produce any adverse effects of relevance to humans at dosages up to 4000 mg/kg bw/day. There was no evidence of reproductive toxicity in rats, or developmental toxicity in rats or rabbits.

Genotoxicity assays conducted using DHA-B included a bacterial reverse mutation assay, chromosomal aberration assay in human peripheral lymphocytes and mouse micronucleus assay. DHA-B (extracted without isopropyl) was negative *in vitro* in a bacterial reverse mutation assay and an equivocal result was reported in a chromosome aberration test. The test substance was negative in the *in vivo* mouse micronucleus model. Overall the weight of evidence indicates that DHA-B is unlikely to be genotoxic. No evidence of genotoxicity was found in any of the assays in which DHA-B extracted using isopropyl alcohol was the test article.

There was no evidence of adverse subchronic effects, or of reproductive or developmental toxicity in a 90-day dietary study in which rats were administered DHA-B (extracted with water) prior to mating and throughout gestation and lactation. It was concluded that the NOAEL for dietary DHA-B in rats was 50,000 ppm, or equivalent to 3279 mg/kg bw/day for males and 3788 mg/kg bw/day in females.

In a similar study conducted with DHA-B extracted with isopropyl alcohol, the average (male and female) NOAEL was 4260 mg/kg bw/day. Assuming an average intake of a human infant of 19–51 mg/kg bw/day this represents a margin of exposure for infants of 84–224.

DHA-B, extracted with water, was confirmed to be bioequivalent to DHASCO® in piglets in a three-week study, and no adverse effects of consumption were found. The calculated mean intakes of DHA from DHA-B corresponded to between 1.9 and 5.8-fold the estimated mean DHA intake of formula-fed human infants.

No carcinogenicity studies of *Schizochytrium*, or of DHA-B, in experimental animals are available. A carcinogenicity study is not considered to be necessary because there was a lack of evidence of genotoxicity, and because no dose-related proliferative lesions were found in subchronic repeat-dose studies.

It is noteworthy that *Schizochytrium* species are components of the human diet through consumption of shellfish, and that there are no known toxic compounds synthesised by any thraustochytrid micro-algae.

The US FDA has observed no increases in the incidence of adverse gastrointestinal events in infants over the time during which DHA supplementation of infant formula has become commonplace.

3.7 Hazard assessment conclusions

The submitted data are considered adequate to define the hazard of DHA-B. No evidence was found of risk of genotoxicity, reproductive or developmental toxicity, or toxicity as a consequence of subchronic dietary consumption of either dried *Schizochytrium* or DHA-B by experimental animals. DHA-B was found to be bioequivalent to DHASCO® and to have no adverse effects in baby piglets at consumption levels higher than those likely to occur in formula-fed infants.

The submitted data support the conclusion that DHA-B does not pose a risk to infant health and safety when used as a source of DHA for supplementation of infant formula products.

4 Nutrition assessment

4.1 Essential fatty acids

Linoleic acid (LA) and α -linolenic acid (ALA) are essential fatty acids that are metabolised, respectively, to n-6 and n-3 LC-PUFAs (Koletzko et al. 2008; Agostoni 2008). Humans cannot interconvert n-6 and n-3 fatty acids (including LC-PUFA), and so a dietary imbalance in these fatty acids can potentially result in a state of nutritional insufficiency. Amounts of LA and ALA must be balanced since the metabolic conversions of LA to arachidonic acid (ARA), an n-6 LC-PUFA, and ALA to DHA utilise the same enzymatic pathways.

The biosynthesis of n-3 and n-6 LC-PUFA from their precursors is shown in Figure 4.1. The rate determining first step uses Δ -6-desaturase enzyme to insert a double bond between carbons 6 and 7 for linoleic and α -linolenic acid. The desaturation is followed by the addition of two carbon atoms to form 20:4n-3 and 20:3n-6 fatty acids respectively and then these fatty acids are desaturated at carbon 5 by the Δ -5- desaturase enzyme, forming arachidonic acid and EPA respectively. These fatty acids are then sequentially elongated to generate 24:5n-3 and 24:4n-6 long chain fatty acids. The action of Δ -6-desaturase forms 24:6n-3 and 24:5n-6 fatty acids which undergo direct desaturation and elongation or the initial stages of β -oxidation to form DHA and 22:5n-6 (Lauritzen et al. 2001).

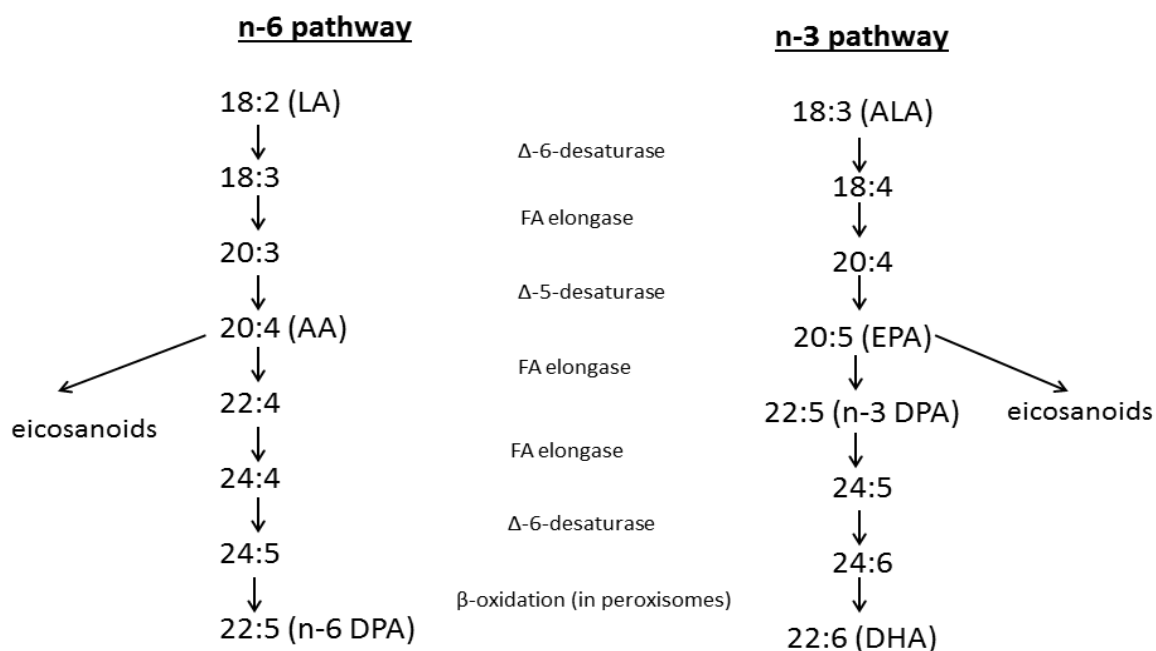


Figure 4.1: Major pathways for the synthesis of LC-PUFA from linoleic and α -linolenic acids

Studies suggest that excessive LA compared to the ALA amount can affect n-3 LC-PUFA (DHA and EPA) synthesis (Makrides et al. 2000a; Wood et al. 2015). The imbalance is avoided by specifying the minimum and maximum ratios of LA: ALA or with addition of DHA. Standard 2.9.1 prescribes minimum amounts for LA and ALA.

4.2 Long chain polyunsaturated fatty acids (LC-PUFA)

DHA and ARA are the biologically active LC-PUFAs which may be present in infant formula. EPA, an n-3 LC-PUFA which may also be present in infant formula, is a precursor for cell signalling compounds called eicosanoids that control inflammatory, immunological, and other cellular responses. ARA (C20) is present in cell membranes and is also a precursor to eicosanoids that are required for normal cell functions. ARA concentrations in breast milk range from 0.24% to 1.0% of total fatty acids (FAs) (Brenna et al. 2007) (FSANZ 2007b) and vary to a lesser extent than DHA.

DHA has been shown to be preferentially inserted into brain membranes over other LC-PUFA (Makrides et al. 1994). Therefore, if DHA is added to infant formula, then endogenous EPA and AA also need to be present in amounts that allow balanced incorporation of these PUFAs into cells. Humans have some capacity to synthesise EPA and DHA from precursor dietary fatty acids. Research using stable isotopes has demonstrated the ability of preterm infants to synthesise LC-PUFA from LA and ALA precursors at one month of age (Carnielli, Wattimena et al. 1996). Another study in breastfed term human infants found evidence of n-6 LC-PUFA synthesis from ^{13}C -labelled LA in the first week of life (Szitanyi, Koletzko et al. 1999). A study of preterm infants found those born as early as 26 weeks gestation were able to form ARA and DHA from deuterated LA and ALA (Rojas, Greiner et al. 2002).

4.3 Fats and fatty acids in different milk sources

Fats provide approximately 50 to 60% of the energy in breast milk (Saurewald, Hachey et al. 1996). Milk fat is a complex substance, containing approximately 400 fatty acids (Mansson 2008).

In the case of bovine milk fat, fatty acids are derived almost equally from two sources, the feed, and the microbial activity in the rumen of the cow. Mansson's (2008) study showed that almost 70% of the fat in milk of Swedish cows was saturated. Around 11% of the fat is short chain fatty acids, with approximately half of that as butyric acid. Approximately 25% of the fatty acids were monounsaturated, 2.3% polyunsaturated with an n-6 to n-3 ratio around 2.3 and a trans fatty acid concentration of 2.7% (Mansson 2008).

DHA is found in both triglyceride and phospholipids forms in human breast milk. Approximately 98% of breast milk fat is triglyceride with only about 1% phospholipid and 1% unsaponifiable fats such as cholesterol and phytosterols (Jensen, 1996).

In the case of humans, milk fatty acid composition is variable depending on maternal diet. Koletzko et al. (1992) reviewed studies from European and African countries on fatty acid composition in mature breast milk. These researchers found fatty acid composition in breast milk to be surprisingly consistent, given the differences in dietary intake across the study groups and concluded that metabolic processes seem to be important in regulating breast milk LC-PUFA.

Reported averages for DHA in breast milk of Australian mothers consuming typical (not supplemented) diets range from 0.14 to 0.28% of total fatty acids (Makrides et al. 1996; Yuhas et al. 2006). Worldwide DHA concentration in breast milk averages approximately 0.34%, with a range of 0.4 to 0.67% for arachidonic acid (AA) (Nettleson 1995; Saurewald, Hachey et al. 1996). Not surprisingly, the highest breast milk DHA levels of 1.33% of total fatty acids are found in Japanese women with high fish intake whereas urban women in the US have 0.06% DHA per cent of total fatty acids (Saurewald, Hachey et al. 1996).

4.4 Composition of DHA-B compared to other DHA-rich oils

The compositional equivalence of this oil to other micro-algal oils was examined. DHA-B is predominately triglyceride in composition, and also contains some monoglyceride, diglyceride and non-saponifiable material, which is typical for edible vegetable oils. Triglycerides represent the main source of dietary fat in the human diet and in this respect DHA-B is no different to other types of dietary fat. The DHA in DHA-rich oil derived from *Schizochytrium* sp is found predominantly in the triglyceride fraction, which is also true for other micro-algal oils (e.g. *Cryptocodinium cohnii*) and for DHA present in fish oils. The other fatty acids present in DHA-B are all components of other edible oils.

Analytical data submitted by the Applicant shows that DHA-B contains approximately 40% DHA (based on GC peak area) as well as saturated and unsaturated fatty acids. The provided information shows no new fatty acid methyl ester peaks (≥ 4 mg/g) were identified in any of the tested lots of the DHA algal oil. The fatty acid profile was consistent and reproducible in all lots. The major fatty acids ($\geq 1\%$) were DHA (22:6n-3), myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), linoleic acid (18:2n-6), EPA (20:5n-3) and DPA (22:5n-6). Oleic, EPA, DHA, palmitic and stearic acids account for approximately 90% of the total peak area of the gas chromatographic fatty acid profile of DHA-B. All of these fatty acids are well known and are present in the diet from a variety of vegetable and animal sources. The Applicant also provided evidence to show less than one percent trans fatty acid in all five tested lots.

There are a number of micro-algal DHA- rich oils available in the market, known by a variety of common and trade names. These oils are produced from three species: *Cryptocodinium cohnii*, *Schizochytrium* sp. and *Ulkenia* sp. The fatty acid composition of DHA-rich oils produced by these microalgae is shown in Table 4.1. The different oil products show similarities as well as differences in the fatty acid make up. When DHA-B is compared to other DHA-rich oils produced by *Schizochytrium* sp. similarities are seen with DHA and stearic acid (18:0). More variation is seen in the majority of fatty acids for the oils produced from *Cryptocodinium cohnii* and *Ulkenia* sp. As shown in Table 4.1, the differences are particularly relevant to the DHA, EPA and DPA content. DHA-B contains approximately 6% EPA, one oil from *Schizochytrium* sp. had low amounts of EPA and another negligible amounts, as did the oil produced from *Cryptocodinium cohnii*. Oil from *Ulkenia* sp. contains approximately 20% DPA compared to 3% DPA from another oil from *Schizochytrium* sp.

However, generally the fatty acid composition of DHA-B is comparable to that of other oils on the market and the differences are nutritionally insignificant because the maximum amount of n-3 LC-PUFA that can be added to infant formula products is less than 500 mg/L¹.

The Applicant provided information which shows that the major sterols (≥ 10 wt% total sterols) of DHA-B include stigmasterol, β -sitosterol and cholesterol, all of which are found in breast milk and commercially available infant formula products. The minor sterols and trace sterols and stanols are also found in breast milk, infant formula products and common foods and dietary oils.

¹ Calculation based on maximum allowable n-3 LC-PUFA content of 1% of total fatty acids in infant formula products and a maximum allowable fat concentration of 1.5 g/100kJ. Given that the fatty acid content of fat is approximately 95% wt/wt, the maximum allowable fatty acid content is 1.4 g/100 kJ, and thus the maximum allowable n-3 LC-PUFA is 0.014 g/100kJ. A maximum energy content of 3150 kJ/L in infant formula products means a maximum of 450 mg n-3 LC-PUFA and 360 mg per 800 mL intake, which is the average intake of breast milk for 0–6 month infants (Nutrient Reference Values for Australia and New Zealand, NH&MRC, 2005).

Table 4.1: Comparison of fatty acid profiles of different micro-algal oils

Fatty acid	Formula	DHA-B oil ¹	DHA oil <i>Schizochytrium</i> sp ²	DHA oil <i>Cyrtpto-</i> <i>thecodimium</i> <i>cohnii</i> ³	DHA oil <i>Ulkenia</i> sp ⁴	Breast milk ⁵
Lauric acid	12:0	<0.1	0.4	4.4	-	-
Myristic acid	14:0	1.18	10.1	12.7	2.7	-
Palmitic acid	16:0	14.49	23.7	9.7	32.9	-
Palmitoleic acid	16:1n-7	-	1.8	-	-	-
Stearic acid	18:0	1.52	0.5	1.1	1.1	-
Vaccenic acid	18:1n-7	0.26	0.7	-	-	-
Linoleic acid	18:2n-6	1.82	-	1.2	-	-
Linolenic acid	18:3n-3,n-6	<0.1	-	-	-	-
Octadecatetraenoic acid	18:4n-3	-	0.6	-	-	-
Dihomogammalinolenic & eicosatetraenoic n-7	20:3n-6, 20:4n-7	<0.1	2.2	-	-	-
Arachidonic acid	20:4n-6	0.69	1.8	-	-	0.47 ⁶
EPA	20:5n-3	6.23	2.6	-	-	0.09 ⁷
Docosatetraenoic acid	22:4n-9	<0.1	0.6	-	-	-
Docosapentaenoic acid (DPA)	22:5n-6	2.53	13.6	-	11.2	0.19/0.06 ⁸
Docohexanoic acid (DHA)	22:6n-3	43.38	35.0	40.0	45.6	0.32 ⁹

Notes to table:

1 Oil derived from *Schizochytrium* sp (ATCC PTA-9695) oil composition data from DSM Nutritional products

2 Oil derived from *Schizochytrium* sp.; oil composition data from Application A428 (ANZFA, 2001)

3 Oil derived from *Cyrtptothecondimium cohnii*; oil composition data from Application A522 (FSANZ, 2004)

4 Oil derived from *Ulkenia* sp oil composition information from Application A522

5 Brenna et al. *Am J Clin Nutr* 2007;85:1457-64

6 Yuhas et al. *Lipids* 2006; 41:851-858 (excluding Japan)

7 DPA n-3/n-6 mean (% total fatty acids) Yuhas et al. *Lipids* 2006; 41:851-858.

9 Brenna et al *Am J Clin Nutr* 2007;85:1457-64

4.5 Nutritional safety, tolerance and efficacy of DHA-B

Triglycerides represent the main source of dietary fat in the human diet and in this respect DHA-B is no different to other types of dietary fat. The general physiological processes for digestion, absorption, distribution, metabolism and excretion of lipids and fatty acids are well described in the general literature and summarised in the dossier provided by the Applicant.

The intestinal absorption of LC-PUFA from similar marine algal oils has been assessed by FSANZ previously (FSANZ 2002), and it was concluded that intestinal absorption of LC-PUFAs from the supplemented infant formula is similar to that from breast milk. There is no evidence to suggest, or reason to believe, that absorption, distribution, metabolism and excretion of DHA-B would be different.

4.5.1 Specific studies with DHA-B in animals and humans

4.5.1.1 Bioequivalence in animals

Preclinical studies with animals have been used to evaluate the safety and suitability of the newly developed DHA-B compared with another DHA-rich oil source DHASCO®.

As discussed in section 3.5.2, the neonatal pig model was used in a study by Federova-Dahms (2014) to evaluate the bioequivalence and safety of DHA-B relative to DHASCO® when administered in a blend with ARASCO (ARA Single cell oil). DHA-B and DHASCO were added to infant formula at 0.32 and 0.96 per cent DHA (% total fatty acids). Dietary administration was well tolerated by the pre-weaning piglets during the 3-week experimental period right after birth. Based on food consumption and body weight data there were no differences in calculated compound consumption between the corresponding doses of DHA-B and DHASCO. No test article-related changes were noted in clinical observations and all piglets grew and developed as expected for their age without any differences in mean body weight between test diets or the control diet (Federova-Dahms et al. 2014).

Bioequivalence of DHA-B and DHASCO® was indicated by piglet growth and tolerance data, as well as DHA accretion in tissues. In plasma and red blood cells dose-dependent increases in DHA levels were seen in the DHA-B and DHASCO® treated animals.

4.5.1.2 Growth and tolerance studies in infants

FSANZ found only one human study which directly compared DHA-B in infant formula products to DHASCO® in infant formula products. Patterson et al. (2016) studied the growth and tolerance in term infants of a routine infant formula containing DHA-B compared to the marketed formula with DHASCO® and reported results in an abstract published in the *FASEB Journal*. In this multi-centre double blind randomised controlled parallel group prospective study infants received either DHASCO® or DHA-B and growth rates from 14 to 120 days were compared, as well as DHA and AA concentrations in red blood cells. No significant group differences were observed for weight, length, or head circumference growth rates by gender from 14 to 120 days of age. No statistically significant differences in total red blood cell DHA or AA concentrations or per cent of total fatty acids (w/w) were detected. The authors concluded that the results of the study demonstrated that DHA-B provided adequate growth and dietary equivalence to DHASCO® when fed to healthy term infants.

A large scale clinical trial supported by DSM comparing DHASCO® and DHA-B in terms of infant growth and development is currently under way.

The primary objective of this study is to determine if the weight gain of healthy term infants fed a commercially available term infant formula supplemented with DHASCO[®] is similar to that of infants fed the same formula supplemented with DHA-B. Results are expected to be published late in 2016.

In addition, the Applicant provided details of six moderate to high quality studies conducted in over 100 infant participants to demonstrate the maintenance of normal growth by DHA (combined with ARA) levels ranging from 0.12 to 0.35% of total fatty acids. Studies were only included if supplementation started within the first month of life and continued for at least three months. All were double blind, randomised controlled trials and included a reference group consuming breast milk. Both preterm infants (Vanderhoof 1999; O'Connor et al. 2001; Clandinin et al. 2005) and term infants (Auestad, Montalto, Hall et al. 1997; Auestad, Halter, Hall et al. 2001; Auestad, Scott, Janowsky et al. 2003; Makrides et al. 1999) were studied. The supplemental DHA (and ARA) was sourced from algal oil, fish oil and phospholipids from eggs. All studies maintained at least 20 infants per group throughout the duration of investigation. Studies of term infants reported no differences between DHA (and ARA) supplemented groups and control or breast milk fed groups. Enhanced growth outcomes were reported in the studies of preterm infants in response to DHA (and ARA) supplementation when compared to control and breastfed infants. These results are summarised in Tables A.1 and A.2 in Appendix 1.

The Applicant also provided details of eight moderate to high quality studies to demonstrate no risk of nutritional imbalances as a result of infants fed infant formula containing DHA (along with ARA). Studies were only included if LC-PUFA supplementation started within one month of age and supplementation continued for a minimum of 3 to 4 months, and also included a breast milk fed control group. Seven of the studies were double blinded randomised controlled trials. Over 1700 infants, of both term and preterm status participated. No evidence was found to suggest that a risk of nutrient imbalance exists as a result of feeding infants infant formula products containing DHA (along with ARA). The Applicant noted that no studies have reported reduced ARA blood levels when DHA and ARA are provided at levels consistent with current commercial practice, and feeding the two LC-PUFA together supports blood levels similar to those of breastfed infants. Supplementation of DHA alone typically reduces ARA tissue levels. However the recent scientific opinion from the EFSA (2014) concluded that concomitant supply of ARA was unnecessary when DHA was added to infant formula products. However, Koletzko et al. 2015 noted that addition of DHA without AA is not supported by clinical trials. The current composition restrictions on fatty acids in infant formula products in Standard 2.9.1 include prescribed maximum amounts of n-6 and n-3 PUFAs and the ratio $n-6:n-3 \geq 1$. This restriction is intended to apply to maximum amounts and ratios of ARA and DHA based on a FSANZ assessment for an application to change the Code which sought to amend the n-6:n-3 ratio from 2:1 to at least 1:1 (or $n-6 \geq n-3$) (FSANZ 2016).

4.6 Dietary Intake

DHA-B is proposed to be added to infant formula products at similar levels to the current use of DHA-rich oils in infant formula products. This is consistent with Standard 2.9.1, which allows n-3 LC-PUFA up to 1% of total fatty acids in the infant formula product. Thus a dietary intake assessment was not required for this application. Previous assessments reported DHA-rich oils were being added to infant formula products at levels that provide up to 0.5% of formula fat (FSANZ, 2002).

The NHMRC has not set an Estimated Average Requirement (EAR), Recommended Dietary Intake (RDI) or Adequate Intake (AI) for total fat as it is the type of fats consumed that relate to nutritional essentiality and to many of the physiological and health outcomes.

In infancy, as fat is the major source of energy in breast milk, an AI recommendation for total fat has been made based on breast milk composition. Recommendations for fatty acids in infancy are also based on total n-6 or n-3, derived from the composition of breast milk (NHMRC 2006).

The AI for infants 0–6 months was set based on the average intake of breast milk (0.78 L/day) and the average concentration of total, omega-6, and omega-3 fats in breast milk (40, 5.6 and 0.63 g/L respectively) from nine studies reviewed by the Food and Nutrition Board, Institute of Medicine (FNB:IOM) (2002) and rounding. The AI for 7 months was similarly set by multiplying together the average intake of breast milk (0.6 L/day) and the average concentration of total, n-6 and n-3 in breast milk and adding the median intake of fats from complementary foods (5.7, 1.2 and 0.11 g/day respectively) from the US CSFII data for 1994–1996) (Table 1) (FNB:IOM 2002). It is estimated that 100 mg DHA is the daily reference value for infants and young children aged 7 to 24 months (EFSA 2010).

Table 4.2: Total fat and fatty acid recommendations for infants

Infant age (months)	Type of fat	Adequate Intake (g/day)
0–6	Total fat	31
	n-6 PUFA	4.4
	n-3 PUFA	0.5
7–12	Total fat	30
	n-6 PUFA	4.6
	n-3 PUFA	0.5

From NHMRC & MoH 2006.

4.7 Nutrition assessment conclusion

The submitted data are considered appropriate to define the nutritional adequacy of DHA-B. The main difference between the products is the ratio of EPA to DHA and the other fatty acids in DHA-B are normal components of edible oils. In general, the fatty acid composition of DHA-B is comparable to that of other oils on the market and the differences are nutritionally insignificant because the maximum amount of n-3 LC-PUFA that can be added to infant formula products is less than 14 mg/100 kJ. The DHA rich micro-algal oil products are comparable with respect to DHA.

There is no evidence to suggest that absorption, distribution, metabolism and excretion of DHA-B would be different to that of the other marine micro-algae oils. DHA-B was found to be bioequivalent to other DHA rich oils in piglets and human infants.

5 Summary

FSANZ has assessed the submitted evidence, as well as additional information in the scientific literature, to consider the equivalence of DHA-B as an additional, replacement or alternative DHA oil source for infant formula products compared to other DHA-rich oils currently used. The data from the available studies, taken together with the composition data do not indicate any risk to infants when used in infant formula products within the current use levels.

6 References

Advisory Committee on Novel Foods (ACNF) (2012). Reply from Leigh Henderson to Dr Simon Brooke-Taylor. 31 October 2012.

Agostoni C (2008) Role of long-chain polyunsaturated fatty acids in the first year of life. *J Pediatr Gastroenterol Nutr* 47 Suppl 2:S41–S44

Anthony M (2012) Is Algae DHA as healthy as fish oil DHA? available at: <http://www.foodprocessing.com/articles/2012/algae-dha-healthy-as-fish-oil/>

Australia New Zealand Food Authority (ANZFA) (2001) Draft Assessment Report Application A428 – Marine Microalgae as a Novel Food, available at: <http://www.foodstandards.gov.au/code/applications/documents/A428DA.pdf>

ANZFA (2002) Supplementary Final Assessment Proposal P93 – Review of Infant Formula. available at: http://www.foodstandards.gov.au/code/proposals/Documents/P93_SuppFAR.pdf

ANZFA (1999) Proposal P93 – Review of Infant Formula, Inquiry Report. Report prepared by the Australia and New Zealand Food Authority, Canberra. <http://www.foodstandards.gov.au/code/proposals/Pages/proposalp93reviewofinfantformula/Default.aspx>

Auestad N, Montalto MB, Hall RT (1997) Visual acuity, erythrocyte fatty acid composition and growth in term fed infants fed formulas with long chain polyunsaturated fatty acids for one year. *Ross Pediatric Lipid Study. Pediatr Res.* 41(1):1–10

Auestad N, Halter R, Hall RT, Blatter M, Bogle ML, Burks W, Wesley E, Erickson JR, Fitzgerald KM, Dobson V, Innis SM, Singer LT, Montalto MB, Jacobs JR, Qiu W, Bornstein, Marc H (2001) Growth and Development in infants fed long-chain polyunsaturated fatty acids: a double-masked, randomized, parallel, prospective, multivariate study. *Pediatrics* 108:372–381.

Auestad N, Scott DT, Janowsky JS (2003) Visual, cognitive and language assessments at 39 months: a follow-up study of children fed formulas containing long-chain polyunsaturated fatty acids to 1 year of age. *Paediatrics.* 112(3):e177–183.

Bauter MR, *et al.* (2013) DHASCO-B: A 90-Day Dietary Study in rats preceded by an *in utero* exposure phase. Product Safety Labs Study Number 33810/Sponsor Study Number 2012-9038. Unpublished. February 15, 2013.

Ben Y ZX, Zhao W, Yu W. (2004). Growth and development of term infants fed with milk with long-chain polyunsaturated fatty acid supplementation. *Chinese Medical Journal.* 117(8):1268-1270

Bioservice Scientific Laboratories GmbH. (2010a) Reverse Mutation Assay Using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) With DHA algal oil: (BSL Bioservice Study No: 101021). Prepared by Bioservice Scientific Laboratories GmbH, Planegg, Germany for Martek Biosciences Corporation, Columbia, MD; [unpublished].

Bioservice Scientific Laboratories GmbH. (2010b) In vitro Mammalian Chromosome Aberration Test in Human Lymphocytes With algal oil Rich in DHA from *Schizochytrium* sp: (BSL Bioservice Study No: 101022). Prepared by Bioservice Scientific Laboratories GmbH, Planegg, Germany for Martek Biosciences Corporation, Columbia, MD; 2010b; [unpublished].

Bioservice Scientific Laboratories GmbH. (2010c) Mammalian Micronucleus Test of Murine Peripheral Blood Cells With DHA Algal Oil: (BSL Bioservice Study No: 101023). Prepared by

Bioservice Scientific Laboratories GmbH, Planegg, Germany for Martek Biosciences Corporation, Columbia, MD; [unpublished].

Bioservice Scientific Laboratories GmbH. (2011a) Reverse mutation assay using bacteria (*Salmonella typhimurium* and *Escherichia coli*) with DHASCO-B. BSL Bioservice Study No.: 105886. Unpublished. February 07, 2011.

Bioservice Scientific Laboratories GmbH. (2011b) *In vitro* Mammalian chromosome aberration test in human lymphocytes with DHASCO-B. BSL Bioservice Study No.: 105887. Unpublished. May 30, 2011.

Bioservice Scientific Laboratories GmbH. (2011c) Mammalian Micronucleus test of murine peripheral blood cells. BSL Bioservice Study No.: 105888. Unpublished. March 07, 2011.

Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM (2007) Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. *Am J Clin Nutr* 85(6):1457–1464.

Clandinin MT, Van Aerde JE, Merkel KL, Harris CL, Springer MA, Hansen JW, Diersen-Schade DA (2005) Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. *J Pediatr*. 146(4):461–468.

Carnielli VP, Wattimena DJ, Luijendijk IH, Boerlage A, Degenhart HJ, Sauer PJ (1996) The very low birth weight premature infant is capable of synthesising arachidonic and docosahexaenoic acids from linoleic and linolenic acids. *Pediatr Res* 40(1):169–174.

Calder PC (2014) Very long chain omega-3 (n-3) fatty acids and human health. *European Journal of Lipid Science and Technology* 116(10): 1280-3000

Carlson SE (2009a) Docosahexaenoic acid supplementation in pregnancy and lactation. *American Journal of Clinical Nutrition* 89(suppl.): 678S-84S

Carlson SE (2009b) Early determinants of development: a lipid perspective. *American Journal of Clinical Nutrition* 89(suppl.): 1523S-9S

Cohen JT, Bellinger DC, Connor We and Shaywitz BA (2005) A quantitative analysis of prenatal intake of n-3 polyunsaturated fatty acids and cognitive development. *American Journal of Preventative Medicine* 29(4): 366-374

EFSA (2012) (EFSA Panel on Dietetic Products, Nutrition and Allergies) Scientific Opinion on the Tolerable Upper Intake Level of EPA, DHA and docosapentaenoic acid (DPA). *EFSA Journal* 10(7):2815–2863.

EFSA (2014) (EFSA Panel on Dietetic Products, Nutrition, and Allergies) Scientific opinion on the essential composition of infant and follow-on formula. *EFSA Journal* 12(7):3760–3866
<http://www.efsa.europa.eu/en/efsajournal/pub/3760>

EC SCF (2003) Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. European Commission Health and Consumer Protection Directorate- General, Brussels
http://ec.europa.eu/food/safety/docs/labelling_nutrition-special_groups_food-children-out199_en.pdf

Food Standards Australia New Zealand (FSANZ), (2003) DHASCO and ARASCO OILS as sources of long-chain polyunsaturated fatty acids in infant formula. A Safety Assessment. Technical Report Series No. 22, available at
<http://www.foodstandards.gov.au/publications/Documents/DHASCO%20and%20ARASCO%20in%20infant%20formula.doc>

FSANZ (2004) Draft Assessment Report Application A522 – DHA-rich Micro-algal Oil from *Ulkenia* sp. as a Novel Food, available at:
<http://www.foodstandards.gov.au/code/applications/Pages/applicationa522dhari2348.aspx>

FSANZ (2005) [Final assessment report. Application A522, DHA-rich micro-algal oil from *ulkenia* sp. as a novel food.](#) Food Standards Australia New Zealand, available at <http://www.foodstandards.gov.au/code/applications/Documents/A522%20DHA-rich%20oil%20FAR1.doc>

FSANZ (2007b) Application A532 Final Assessment Report, Ratio of Long Chain Polyunsaturated Fatty Acids in INFANT FORMULA PRODUCTS. Report prepared by Food Standards Australia New Zealand, Canberra
<http://www.foodstandards.gov.au/code/applications/Documents/A532%20LCPUFAs%20FAR%20FINAL.pdf>

FSANZ (2015) [Systematic review of the evidence for a relationship between docosahexaenoic acid \(DHA\) and maintenance of normal brain function and normal vision.](#) Food Standards Australia New Zealand, available at <http://www.foodstandards.gov.au/industry/labelling/Documents/DHA%20systematic%20review.docx>

FSANZ (2016) SD1 Attachment A1.1 – Nutrition Assessment Proposal P1028 – Infant Formula Food Standards Australia New Zealand, available at <http://www.foodstandards.gov.au/code/proposals/Documents/P1028-Consult-SD1-Attachment-A1.pdf>

Fedorova-Dahms I, Marone PA, Bauter M and Ryan AS (2011) Safety evaluation of DHA-rich algal oil from *Schizochytrium* sp. Food and Chemical Toxicology 49: 3310-3318

Federova-Dahms I, Thorsrud BA, Bailey E, and Salem Jr., N (2014) A 3-week dietary bioequivalence study in preweaned farm piglets of two sources of docosahexaenoic acid produced from two different organisms. Food and Chemical Toxicology 65: 43-51

Guesnet P and Alessandri J-M (2011) DHA and the developing central nervous system (CNS) – Implications for dietary recommendations. Biochimie 93: 7-12

Hadders-Algra M (2011) Prenatal and early postnatal supplementation with long-chain polyunsaturated fatty acids: neurodevelopmental considerations. American Journal of Clinical Nutrition 94(suppl): 1874S-9S

Hammond BG, Mayhew DA, Naylor MW, Reucker FA, Mast RW and Sander WJ (2001a) Safety Assessment of DHA-Rich Microalgae from *Schizochytrium* sp. I. Subchronic Rat Feeding Study. Regulatory Toxicology and Pharmacology 33: 192–204

Hammond BG, Mayhew DA, Holson JF, Nemas MD, Mast RW, Sander WJ. (2001b). Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. Part III. Single generation rat reproduction study. Regulatory Toxicology and Pharmacology 33: 356-362.

Hammond BG, Mayhew DA, Holson JF, Nemas MD, Mast RW, Sander WJ. (2001c) Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. Part II. Developmental toxicology evaluation in rats and rabbits. Regulatory Toxicology and Pharmacology 33:205-217.

Hammond BG, Mayhew DA, Kier LD, Mast RW and Sander WJ (2002) Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. IV. Mutagenicity studies. Regulatory Toxicology and Pharmacology 35: 255-265

Hoffman DR, Birch EE, Birch DG, et al. (2000). Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. J Pediatr Gastroenterol Nutr. 31(5):540-553.

Innis SM (2008) Dietary omega 3 fatty acids and the developing brain. Brain Research 1237: 35-43

OM (2002) Dietary Reference Intakes for energy, carbohydrates, fiber, fat, fatty acids, cholesterol, protein and amino acids. Food and Nutrition Board: Institute of Medicine National Academy Press, Washington D.C.

Janssen CIF and Kiliaan AJ (2014) Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging and neurodegeneration. *Progress in Lipid Research* 53: 1-17

Jensen RG (1996) The lipids in human milk. *Prog Lipid Res* 35(1):53–92.

Koletzko B, Lien E, Agostoni C, Bohles H, Campoy C, Cetin I, Decsi T, Dudenhausen JW, Dupont C, Forsyth S, Hoesli I, Holzgreve W, Lapillonne A, Putet G, Secher NJ, Symonds M, Szajewska H, Willatts P, Uauy R (2008) The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. *J Perinat Med* 36(1):5–14

Lauritzen L, Hansen HS, Jorgensen MH, Michaelson KE (2001) The essentiality of long-chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lip Res* 40(1-2):1–94.

Lauritzen L, Carlson SE (2011) Maternal fatty acid status during pregnancy and lactation and relation to newborn and infant status. *Matern Child Nutr* (7) sup 2:41–58.

Leipe et al., 1994. The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. *Phycologia* 33: 369-377.

Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA (1994) Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. *Am J Clin Nutr* 60(2):189–194

Makrides M, Neumann MA, Gibson RA (1996) Effect of maternal DHA supplementation on breast milk composition. *Eur J Clin Nutr* 50:352–357.

Makrides M, Nuemann MA, Simmer K, Gibson RA (1999) Dietary long-chain polyunsaturated fatty acids do not influence growth of term infants: A randomized clinical trial. *Paediatrics*. 104(3):468–475.

Makrides M, Neumann MA, Jeffrey B, Lien EL, Gibson RA (2000) A randomized trial of different ratios of linoleic to alpha-linolenic acid in the diet of term infants: effects on visual function and growth. *Am J Clin Nutr* 71(1):120–129

Mansson HL (2008) Fatty acids in bovine milk fat. *Food and Nutrition Research*. DOI 10.3402/fnr.v52i0.1821

Morse NL (2012) Benefits of docosahexaenoic acid, folic acid, vitamin D and iodine on foetal and infant brain development and function following maternal supplementation during pregnancy and lactation. *Nutrients* 4: 799-840

National Health and Medical Research Council (NHMRC) and Ministry of Health (MoH) (2006). Nutrient reference values for Australia and New Zealand including recommended dietary intakes. Canberra, National Health and Medical Research Council. <https://www.nrv.gov.au/home>

O'Conner DL, Hall R, Adamkin D (2001) Growth and development in preterm infants fed long-chain polyunsaturated fatty acids: a prospective, randomized controlled trial. *Pediatrics*. 108(2):359–371.

Patterson AC, Maditz KH, Wampler J, Kirchoff A, Zissman A, Berseth CL (2016) Growth and tolerance of a routine infant formula with an alternative DHA source fed to term infants. Abstract from the Experimental Biology 2016 Meeting. *The FASEB Journal* 30(1) sup. 671.1

Pelliccia F, Marazzi G, Greco C, Franzoni F, Speciale G and Gaudio C (2013). Current evidence and future perspectives on n-3 PUFAs. *International Journal of Cardiology* **170** S3-S7

Rodrigues R Al F, Rodrigues M V N, Oliveira T I V, Bueno C Z, de Oliveira SOUZA I M, Sartoratto A, Foglio M A (2011) Docosahexaenoic acid ethyl ester (DHAEE) microcapsule production by spray-drying: optimization by experimental design. [Food Science and Technology \(Campinas\)](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-20612011000300007), available at http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-20612011000300007

Rogers LK, Valentine CJ and Keim SA (2013) DHA supplementation: Current implications in pregnancy and childhood. *Pharmacological Research* 70(1): 13-19

Rojas CV, Greiner RS, Fuenzalida LC, Martinez JI, Salem N, Uauy R (2002) Long term n-3 FA deficiency modifies peroxisome proliferator-activated receptor beta mRNA abundance in rat ocular tissue. *Lipids* 37(4):367–374.

Simmer K, Patole SK, Rao SC (2011) Long-chain polyunsaturated fatty acid supplementation in infants born at term. *Cochrane Database Syst Rev*(12):CD000376

Saurewald TDL, Hachey CL, Jensen CL, Chen H, Anderson RE, Heird WC (1996) Effect of dietary α -linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants. *Lipids*. 31:S131–S135.

Sun H, Como PG, Downey LC, Murphy D, Ariagno RL, Rodriguez W (2015) Infant formula and neurocognitive outcomes: impact of study end-point selection. *J Perinatol* 35(10):867–74

Szitaryi P, Koletzko B, Mydlilova A, Demmelmair H (1999) Metabolism of ¹³C-labelled linoleic acid in newborn infants during the first week of life. *Pediatr Res* 45(5):253–262.

Takumi Kobayashi et.al. (2011) [Increase of Eicosapentaenoic Acid in Thraustochytrids through Thraustochytrid Ubiquitin Promoter-Driven Expression of a Fatty Acid \$\Delta\$ 5 Desaturase Gene](#), *Appl. Environ. Microbiol.*, 77(11): 3870-3876. Accessed 28 June 2016.

U.S. FDA. (2011) Response Letter to Will Fantle. Docket No. FDA-2008-P-0074. September 23, 2011.

Vanderhoof J, Gross S, Hegyi T, Clandinin T, Porcelli P, DeCristofaro J, Rhodes T, Tsang R, Shattuck K, Cowett R (1999) Evaluation of a long-chain polyunsaturated fatty acid supplemented formula on growth, tolerance and plasma lipids in preterm infants up to 48 weeks postconceptional age. *J Pediatr Gastroenterol Nutr* 29(3):318–326.

Wood KE, Mantzioris E, Gibson RA, Ramsden CE, Muhlhausler BS (2015) The effect of modifying dietary LA and ALA intakes on omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) status in human adults: a systematic review and commentary. *Prostaglandins Leukot Essent Fatty Acids* 95:47–55

Yuhas R, Pramuk K, Lien EL (2006) Human milk fatty acid composition from nine countries varies most in DHA. *Lipids* 41:88–845.

Appendix 1

Table A1: Randomised Controlled Trials with DHA from various sources (and ARA) Reporting Growth Outcomes

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Age at Start/Duration of Supplementation	Outcome Summary	Study Quality and Limitations
Auestad <i>et al.</i> (1997) (2003) DB RCT	0.43	0.12	63/45/46	~ 7 days/12 mo	No difference in growth outcomes (weight, length, head circumference) between groups No difference in growth between groups 27 mo post-supplementation	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured. Limitations – high drop-out rate (23%); limited follow-up (80%) at 27 mo post supplementation; sufficient statistical power not verified (i.e. no power calculation provided) Overall – moderate quality
Auestad <i>et al.</i> (2001) DB RCT	0.45	0.14	82/77/162	~9 days/12 mo	No difference in growth (weight, length, head circumference) between groups	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study population. Limitations – high drop-out rate (27%) Overall – moderate-high quality

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Age at Start/Duration of Supplementation	Outcome Summary	Study Quality and Limitations
Clandinin <i>et al.</i> (2005)* DB RCT	0.64	0.32	76/62/117	14 days/~12 mo	Feeding formulas with DHA and ARA from algal and fungal oils resulted in enhanced growth, i.e. weight and length significantly (P<.05) greater in response to DHA+ARA formula.	Quality - selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study population. Limitations – Groups differed somewhat at baseline; Drop-out rate 14% Overall – moderate quality
Makrides <i>et al.</i> (1999) DB RCT	0.34	0.34	33/21/21	5-7 days/12 mo	No difference in growth (weight, length, head circumference) between groups	Quality - selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured. Limitations – Groups differed somewhat at baseline; Drop-out rate 18%; statistical power limited, i.e. n=25 per group required, n=21 completed. Overall – limited quality
O'Connor <i>et al.</i> (2001)* DB RCT	0.41-0.43	0.15-.0.27	43/91/180	~3 days/12 mo	No difference in growth (weight, length, head circumference) between groups	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study population. Limitations – drop-out rate (20%) Overall – moderate-high quality

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Age at Start/Duration of Supplementation	Outcome Summary	Study Quality and Limitations
Vanderhoof et al (1999)* DB RCT	0.50	0.35	133/78/77	~21 days/6 mo	DHA and ARA from algal and fungal oils promoted length and wt at 40 wk PCA vs. BF (P<.05).	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study Limitations – none Overall – high quality

Source: DSM Dossier: Application for the Approval of DHA Algal Oil from Schizochytrium sp. To Infant Formula Products (Including Pre-Term Infant Formula, Term Infant Formula and Follow On Formula. October 2015

Abbreviations:

DB RCT: double-blind, randomized controlled trial

DHA: docosahexaenoic acid

ARA: arachidonic acid

Mo: months

%FA: percent of fatty acids

BF: breast-fed

C: control

*Preterm infant study

Table A.2: Randomised Controlled Trials with DHA+ARA Intervention Reporting Blood Fatty Acid Status

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Duration of Supplementation (months)	Outcome Summary	Study Quality and Limitations
Auestad <i>et al.</i> (2001) DB RCT	0.45	0.14	82/77/162	12	DHA+ARA formula maintained DHA and ARA blood levels similar to BF and higher than C (P<.01).	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study population. Limitations – high drop-out rate (27%) Overall – moderate-high quality
Ben <i>et al.</i> (2004) RCT	NR	NR	26/52/69	6	DHA+ARA formula group had plasma DHA & ARA levels that were: – higher than control at 3 and 6 mo. – similar to BF at 3 mo and higher than BF at 6 mo	Quality – Groups comparable at baseline; appropriate outcomes, outcomes reliably measured. Limitations – Limited description of selection and allocation procedures described; blood measures not main outcome of study; disposition of subjects not provided; no power calculation provided. Overall – limited quality

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Duration of Supplementation (months)	Outcome Summary	Study Quality and Limitations
Hoffman <i>et al.</i> (2000) DB RCT	0.72	0.36	20/20/18	4	ARA levels supported. DHA+ARA maintained ARA levels comparable to BF infants and significantly higher than C (P<.05).	Quality – Appropriate outcomes, outcomes reliably measured. Limitations – Limited description of selection and allocation procedures described; disposition of subjects not provided; no power calculation provided. Overall – limited quality
Makrides <i>et al.</i> (1999) DB RCT	0.34	0.34	33/21/21	12	ARA levels supported. At 34 and 52 wk DHA+ARA same as BF and significantly higher ARA than C or DHA-alone (P<.01).	Quality - selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured. Limitations – Groups differed somewhat at baseline; Dropout rate 18%; statistical power limited, i.e. n=25 per group required, n=21 completed. Overall – limited quality

Study/Study Design	ARA (%FA)	DHA (%FA)	Number of Subjects at longest duration (BF/C/DHA)	Duration of Supplementation (months)	Outcome Summary	Study Quality and Limitations
O'Connor <i>et al.</i> (2001) DB RCT	0.42	0.26	40/120/253	12	ARA levels supported. ARA levels were similar to BF and higher or the same as C.	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study population. Limitations – high drop-out rate (20%) Overall – moderate-high quality
Vanderhoof <i>et al.</i> (1999)" DB RCT	0.50	0.35	133/78/77	6	ARA levels supported. ARA levels similar to BF and significantly higher than C (P<.05).	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured; adequate statistical power, large study Limitations – none Overall – high quality

Source: DSM Dossier: Application for the Approval of DHA Algal Oil from Schizochytrium sp. To Infant Formula Products (Including Pre-Term Infant Formula, Term Infant Formula and Follow On Formula. October 2015

Abbreviations:

DB RCT: double-blind, randomized controlled trial

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Mo: months

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*Preterm infant study