TOXSERVICES
TOXICOLOGY RISK ASSESSMENT CONSULTING

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November 29, 2016

Food and Drug Administration
Division of Animal Feeds (HFV-220)
Office of Surveillance and Compliance,
Center for Veterinary Medicine, Food and Drug Administration,
7519 Standish Place
Rockville, Maryland 20855

Re: CVM GRAS Notification for *Euphausia superba* (krill) meal (Qrill™ Pet)

Dear Sir or Madam,

ToxServices LLC is submitting this Generally Recognized as Safe (GRAS) notice, based on scientific procedures, on behalf of Aker BioMarine Antarctic in accordance with the final rule on animal food ingredients that may be GRAS as specified in 21 CFR 570.220 through 570.255. Qrill™ Pet will be added to dry food for adult dogs as a source of protein and lipid at the maximum inclusion level of 3% by weight.

All aspects of the production of Qrill™ Pet are consistent with good manufacturing practices. Results of studies that have been performed in mink and swine indicate that protein and lipid digestibility of krill meal is high and similar to other ingredients that are used as a source of protein (fish meal) or lipid (soybean). The maximum estimated daily intake (EDI) of Qrill™ Pet is estimated to be 860 mg/kg bw/day in dogs. This intake is below the acceptable daily intake (ADI) of 950 mg/kg bw/day for krill meal with a much higher fluorine content than Qrill™ Pet as established in a series of reproduction, lactation and growth studies in mink and supported by data in dogs and rats.

The use rate of krill meal in adult dog food is limited due to the concentration of fluorine (a known toxicant) in Qrill™ Pet (up to 1,500 ppm). Use of 3% Qrill™ Pet in dog food will result in a maximum fluorine exposure from Qrill™ Pet of 1.3 mg/kg bw/day in dogs. The total daily fluorine intake from Qrill™ Pet, other ingredients in dog food, and drinking water is estimated to be up to 1.6 mg/kg bw/day under very conservative assumptions. This exposure level is below the ADI of 3.5 mg/kg bw/day established based on a NOAEL of 5.2 mg/kg bw/day for fluorine in dogs and supported by studies in mink and rats.

Astaxanthin is another potential component of concern in krill meals. The NOAEL of 158 mg/kg bw/day established in a 52-week study in dogs leads to the establishment of an ADI of 53 mg/kg bw/day. This value is much higher than the EDI of 1.6 mg/kg bw/day both from consumption of Qrill™ Pet at 3% and from other ingredients in food.

The totality of the evidence indicates that use of 3% Qrill™ Pet in dry adult dog food would meet the reasonable certainty of safety standard for a GRAS determination.

This submission is divided into seven parts according to 21 CFR 570.220 through 570.255. The complete data and information cited in the submission that served as the basis of this GRAS notification are available to the Food and Drug Administration upon request. Should you have any questions, please feel free to contact us.

Sincerely,

Margaret H. Whittaker, Ph.D., M.P.H., CBiol., F.R.S.B., E.R.T., D.A.B.T.
Managing Director and Chief Toxicologist
ToxServices LLC

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PART 1:

SIGNED STATEMENTS

AND CERTIFICATION

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 1: Signed Statements and Certification

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

Jennifer G. Fleischer, Ph.D., M.H.S.
Bonnie Ransom Stern, Ph.D., M.P.H.
Raymond York, Ph.D. D.A.B.T, F.A.T.S, E.R.T.

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A. Relevant Regulations
ToxServices LLC is submitting this Generally Recognized as Safe (GRAS) notice on behalf of Aker BioMarine Antarctic (hereinafter referred to as Aker BioMarine) in accordance with the final rule on animal food ingredients that may be GRAS as specified in 21 CFR 570.220 through 570.255.

B. Agent of the Notifier
ToxServices LLC
1367 Connecticut Ave NW #300
Washington, DC 20036

C. Name of the Notified Substance
Euphausia superba (krill) meal (Qrill™ Pet)

D. Conditions of Use
Qrill™ Pet will be added to dry food for adult dogs as a source of protein and lipid at the maximum inclusion level of 3% by weight.

E. Basis for GRAS Conclusion
The GRAS status is determined through scientific procedures in accordance with 21 CFR 570.30(a) and (b).

F. Claim of Exemption from the Requirement for Premarket Approval
ToxServices has determined, on the advice of qualified experts, that *Euphausia superba* (krill) meal (Qrill™ Pet) is GRAS as a source of protein and lipid at the maximum inclusion level of 3% by weight of dry food for adult dogs. Therefore, Qrill™ Pet, under the conditions of its intended use, is exempt from premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

G. Availability of Information
Upon request, all the data and information used as the basis of the GRAS status conclusion are available for FDA to review and copy during customary business hours at:

ToxServices LLC
1367 Connecticut Ave NW #300
Washington, DC 20036

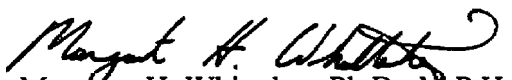
In addition, upon request, ToxServices will provide a complete copy of the data and information that served as the basis of this GRAS status conclusion either in an electronic format accessible for evaluation, or on paper. Please submit requests to mwhittaker@toxservices.com.

H. Data and Information Exempt from Disclosure
None of the information presented in this dossier is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

I. Certification

The undersigned author of the GRAS status notification dossier, for the use of up to 3% Qrill™ Pet in dry adult dog food, hereby certify that, to the best of their knowledge and belief, this document is a complete and unbiased representation of all available information, favorable or unfavorable alike, known by the authors to be relevant to the evaluation of the safety and GRAS status of the substance described herein.

J. Signature



Margaret H. Whittaker, Ph.D., M.P.H., CBiol., F.R.S.B., E.R.T., D.A.B.T.
Managing Director and Chief Toxicologist
ToxServices LLC

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PART 2:

IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECTS

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 2: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

Jennifer G. Fleischer, Ph.D., M.H.S.
Bonnie Ransom Stern, Ph.D., M.P.H.
Raymond York, Ph.D. D.A.B.T, F.A.T.S, E.R.T.

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I. IDENTITY OF THE SUBSTANCE

A general description of Qrill™ Pet is shown in Table 1. Qrill™ Pet is a brownish pink to orange powder that is insoluble in water. It is vacuum-packed and stored at room temperature.

Table 1: General description of Qrill™ Pet

Characteristic	Value
Species	*Euphausia superba*
Synonyms	Antarctic krill meal
Physical description	Brownish pink to orange powder
Solubility	Virtually insoluble in water and most organic solvents
Storage	Room temperature
Conditions of use	Up to 3% in finished dog food as a source of protein and lipid

a. Source

Krill is the common name given to the order *Euphausiacea* of shrimp-like marine crustaceans (ITIS 2016). Krill inhabit oceans throughout the world, predominantly in the Northern (Arctic) and Southern (Antarctic) circumpolar seas. Antarctic krill (*Euphausia superba*) is the most abundant species of krill and represents one of the world's largest single species biomass, estimated to be in the order of 400 – 1,550 million tons (Chen and Jaczynski 2007). Antarctic krill is a vital component of the marine food chain for baleen whales, and is also consumed by seals, penguins, petrels, fish and humans (Sidhu et al. 1970; Yoshitomi 2004). On a body weight basis, krill has been reported to have the greatest amount of protein among all species, with over 60% of dry matter comprised of protein (Storebakken 1988). Krill is also a source of polyunsaturated lipids and the antioxidant astaxanthin (Storebakken 1988).

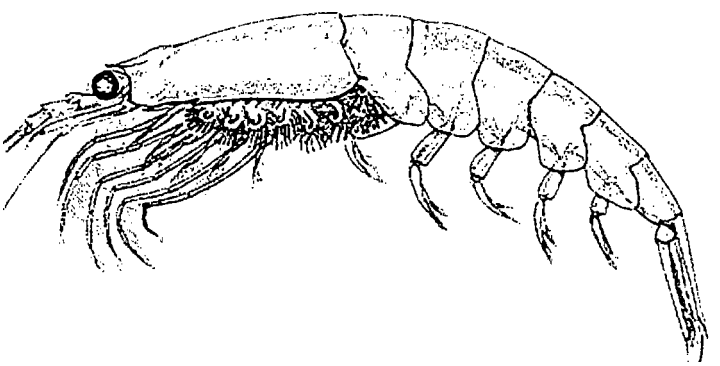

Figure 1: Antarctic krill (*Euphausia superba*) (FAO 2016a)

There are 85 species of krill, varying in length from a few millimeters to 15 centimeters. There are several features that distinguish krill from other crustaceans; the gills are exposed below the carapace, unlike those of most other advanced crustaceans which are sheltered within it; there are luminous organs (photophores) at the base on the swimming lets, as well as pairs of photophores at the genital segment of the cephalothorax, near the mouthparts and in the eyestalks, which

produce a blue light (FAO 1997). The general body plan is, however, similar to many familiar crustaceans (Figure 1). The fused head and trunk (i.e., the cephalothorax) contains most of the internal organs, including the digestive gland, stomach, heart, gonads; the external sensory appendages include two large eyes and two pairs of antennae. The limbs of the cephalothorax are modified into highly specialized feeding appendages; the nine mouthparts are modified for handling and grinding the food and the six to eight pairs of food-collecting limbs trap food particles from the water and move them to the mouth. The muscular abdomen has five pairs of swimming legs (pleopods) (FAO 1997). The primary source of food for krill is phytoplankton (diatoms and cryptophytes) (FAO 2016a).

The Antarctic krill (*Euphausia superba*) is one of the biggest species of krill that can grow to a maximum size of 6.5 cm over approximately 3 – 5 years. The larger adults (40 – 65 mm in length) are the target of commercial fishery (FAO 1997). It resides in the surface layers of the Antarctic waters during the southern summer months and represents the first trophic level in Antarctic food chains, and is one of the main food sources for whales, seals, and penguins (Sidhu et al. 1970).

b. Typical composition of krill meals

The following section describes common features and components of krill and krill meals. A detailed introduction of the composition of Qrill™ Pet can be found in the Specification section (Section III) below.

Krill meal is a brownish-orange powder that is derived from the whole organism of fresh, wild caught *Euphausia superba*. In general, the composition of krill is very similar to that of shrimps, crabs, lobsters and crayfish. On a dry-weight basis, krill contains 60 – 78% crude protein, 7 – 26% crude fat, and 12-17% ash. The wide ranges in composition are due to differences in age, season, location, sex, diet and physiological conditions. Krill is also a potential significant source of vitamins A, D, B-group complexes and astaxanthin (a keto-carotenoid antioxidant) (Savage and Foulds 1987).

As krill meal is rich in protein, fat and the *omega*-3 (ω-3) fatty acids eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Pierce et al. 1969), it is a viable alternative to other protein sources in pet food (e.g., meat, pork, lamb, poultry, fish or soybean meal).

One negative feature of krill meal as food source for humans and terrestrial organisms is the naturally high levels of fluorine in krill, which limits its use as a protein and lipid source for pet food. As fluorine content is relatively low in muscle but high in exoskeleton, carapace and cephalothorax, removal or partial removal of the exoskeleton can reduce the fluorine content of the meal (Hansen et al. 2010; Savage and Foulds 1987).

A summary of Qrill™ Pet's composition and compliance with corresponding regulatory limits is presented in Section III of this dossier.

c. Regulatory status

Dried Antarctic krill has been permitted for use as an animal feed material in Europe since March 26, 2012 under Registration Number 02913-EN (Feed Material Register 2010). Krill meal is currently not listed by the Association of American Feed Control Officials (AAFCO) as a food ingredient for dogs in the United States; however, meals from other marine sources have been employed in animal feed marketed in the U.S. since the 1930's. The AAFCO[1] official list of animal feed ingredients contains definitions for several marine-based meals produced from whole organisms as follows:

> **51.14 Fish meal** is the clean, dried, ground tissue of undecomposed whole fish or fish cuttings, either or both, with or without the extraction of part of the oil. If it contains more than 3% salt (NaCl), the amount of salt must constitute a part of the product name, although in no case must the salt content of this product exceed 7%. The label shall include guarantees for minimum crude protein, minimum crude fat, maximum crude fiber, minimum phosphorus (P) and minimum and maximum calcium (Ca). If it bears a name descriptive of its kind, it must correspond thereto (Adopted 1933, Amended 1984, Amended 2003, 2004) (AAFCO 2014a).
>
> **51.4 Crab meal** is the undecomposed ground dried waste of the crab and contains the shell, viscera and part or all of the flesh. It must contain not less than 25% crude protein. If it contains more than 3% salt (NaCl), the amount of salt must constitute a part of the product name, although in no case must the salt content of this product exceed 7% (Adopted 1933, Amended 2003) (AAFCO 2014b).
>
> **51.5 Shrimp meal** is the undecomposed, ground dried waste of shrimp and contains parts and/or whole shrimp. If it contains more than 3% salt (NaCl), the amount of salt must constitute a part of the product name, although in no case must the salt content of this product exceed 7% (Adopted 1933, Amended 1963, Amended 2003) (AAFCO 2014c).

Pet food companies in the United States formulate different dog food brands and varieties with different raw ingredients, depending on the target human consumer market, and likely because of market trends, typically keep dog food product formulations proprietary. However, the U.S. Food and Drug Administration (U.S. FDA) has established regulations and standards applicable to all animal feeds, including the proper listing of ingredients on feed labels.[2] In addition to U.S. FDA, many states have adopted regulations through the AAFCO Model Bill and Regulations Committee (AAFCO 2014d). Under the AAFCO regulations, an ingredient or a combination of ingredients may form a part of the product name of a pet food or specialty pet food, but only when any of the ingredients constitutes at least 25% of the weight of the product and when a descriptor word is used with the ingredient names, indicating that there are other ingredients in the pet food. The descriptors may include such terms as "dinner", "formula", "recipe", or "entrée". In addition, when a combination of ingredients are included in the product name, each

[1] The U.S. Food and Drug Administration (U.S. FDA) currently recogonizes feed ingredients that have definitions in the Official Publication of AAFCO.

[2] http://www.fda.gov/animalveterinary/resourcesforyou/ucm047113.htm; site last visited April 29, 2016.

of the ingredients must constitute at least 3% of the product weight, excluding water sufficient for processing and appear in predominance of weight in the product.

There are several dog foods sold in the United States that contain the term "fish meal" in the product name, such as Nature's Recipe® "Easy to Digest Fish Meal & Potato Recipe" (Nature's Recipe 2016) and Hi-Tek Ration's "Grain Free Alaskan Fish Formula" (Hi-Tek Rations 2016); the product name "Easy to Digest Fish Meal & Potato Recipe" indicates, according to the AAFCO regulations, that fish meal and potatoes are contained in the formulation at a minimum of 25% of the feed and that fish meal and potatoes each constitute at least 3% of the formulation. The protein content stated in the label for this specific dog food is a minimum of 21%, and because the majority of the protein is coming from fish meal, it is reasonable to assume that fish meal is added to the diet in quantities greater than 3%.

In 2013, AAFCO announced that krill meal was not yet their approved feed ingredient and should not be marketed as shrimp meal. In addition, "Safety concern is over Krill accumulating colors". Accordingly, AAFCO recommended that krill meal undergo the U.S. FDA's Food Additive Petition or Color Additive Petition to obtain approval (AAFCO 2013). In 2015, U.S. FDA determined that at the proposed 3% use level in dry dog food, Qrill™ Pet is not anticipated to change the color of dog food, and is exempt from the requirement to submit a color additive petition (U.S. FDA 2015a).

In 2015, the State of Texas announced their acceptance of krill meal for use as a protein and lipid source in food for adult dogs at levels no greater than 3% in the diet, and required that the label include guarantees for protein, fat, omega 3 fatty acids (EPA and DHA) and maximum fluorine content of 1,650 ppm, as well as salt content under conditions described by AAFCO (above) (Office of the Texas State Chemist 2015).

Aker BioMarine previously filed a GRAS notification (GRN 000371) on krill oil extracted from Antarctic krill meal for use as a food ingredient for humans (in non-alcoholic beverages, breakfast cereals, cheeses, frozen dairy desserts, while and skim milk, processed fruit and fruit juices, and medical foods) at the consumption levels of 0.05 – 0.5 g per serving. The U.S. FDA had no question to this determination (U.S. FDA 2014).

The European Union (EU) currently allows up to 3,000 mg/kg fluorine in feed materials from marine krill and up to 150 mg/kg in complete feeds with a moisture content of 12% (EC 2008). No such limits have been established by the U.S FDA. AAFCO has not established a maximum recommended level of fluorine in dog food. A detailed discussion of ingredients in Qrill™ Pet and their respective regulatory limits is presented in Section III.a.

As for the colorant/antioxidant in krill meal astaxanthin, in 2010 the U.S. FDA had no questions for the GRAS notification (GRN 000294) on the use of *Haematococcus pluvialis* (a freshwater species of Chlorophyta) extract containing astaxanthin esters as a food ingredient for humans (in baked goods, beverages, cereals, chewing gum, coffee and tea, daily product analogs, frozen daily desserts and mixes, hard candy, milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and soft candy) at consumption levels of 0.1 mg preserving (U.S. FDA 2010). In response to a subsequent GRAS notification (GRN 000580) in 2015, the

U.S. FDA had no questions for the GRAS notification of human consumption of astaxanthin esters in *H. pluvialis* extract in baked goods and baking mixes, beverages and beverage bases, energy, sports and isotonic drinks, non-milk based meal replacements, cereals and cereal products, chewing gums, coffee, tea, dairy product analogs, frozen dairy desserts and mixes, hard and soft candy, milk products, processed fruits and fruit juices, and processed vegetables and vegetable juices at a maximum level of 0.15 mg astaxanthin per serving (U.S. FDA 2015b). Currently, Aker BioMarine is in the process of a Color Additive Petition for krill meal in salmonid fish feed with the U.S. FDA as a separate project.

II. METHOD OF MANUFACTURE

Qrill™ Pet is derived from Antarctic krill (*Euphausia superba*). Antarctic krill have a circumpolar distribution with the highest concentrations found in the Southern (Antarctic) Ocean (FAO 1997). Antarctic krill used as the raw material for the production of krill meal is caught exclusively in CCAMLR Area 48 of the Antarctic Ocean (Figure 2) *via* Eco-Harvesting by the vessel Antarctic Sea (Registration Number N-75-VV), which is owned by Aker BioMarine (Figure 3).

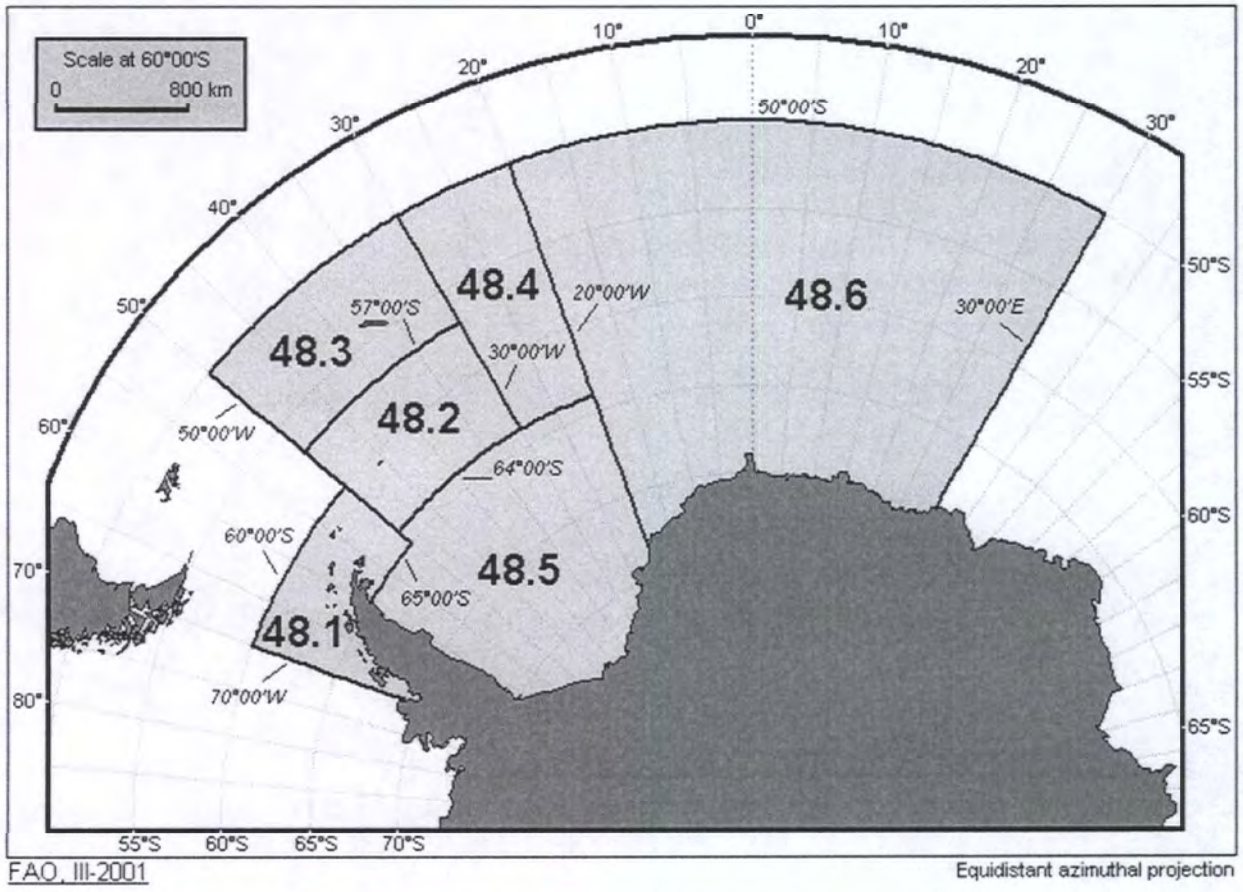

Figure 2: Area 48 of the Antarctic Sea (FAO 2016b)

Figure 3: Aker BioMarine's Fishing Vessel Antarctic Sea

Eco-Harvesting is a new, proprietary fishing method used by Aker BioMarine to secure the quality of the krill catch and at the same time prevent by-catch. Aker BioMarine's Eco-Harvesting fishing system allows the fishing net and equipment to stay underwater during the entire operation. The krill is filtered at the end of the net and flows upwards while continuously staying under water in a flexible hose, with air injection creating the upward lift. This equipment brings the live and fresh krill directly into the factory vessel, which allows for processing of fresh raw material with superior product quality. Independent observers have verified that this method ensures no significant by-catch of species other than krill. The fishery has been certified as sustainable by the Marine Stewardship Council (MSC) (see www.msc.org) (Appendix A, Figure A-1).

All aspects of the production of Qrill™ Pet are consistent with current Good Manufacturing Practice (cGMP). The on board production is approved by the Norwegian Food Safety Authority and is covered by a quality system based on Hazard Analysis and Critical Control Points (HACCP) principles (Appendix A, Figure A-2), the entire process of which has been certified in conformity with Quality Management System Standard(s) ISO 9001:2008 (Appendix A, Figure A-3) and FEMAS (Feed Material Assurance Scheme) (Appendix A, Figure A-4). The use of appropriate in-process controls and analytical testing (as detailed below) to determine the levels of potential impurities or contaminants ensure that possible toxicological, nutritional, or microbiological hazards will be detected and mitigated during manufacture. Aker BioMarine's vessel carries an International Scientific Observer to verify fishing data in accordance with CCAMLR Scheme of International Scientific Observation and Memorandum of Understanding between Norway and the United Kingdom (U.K.), and Eco-Harvesting results in minimal environmental impact.

The manufacturing process employed to produce Qrill™ Pet is described below and summarized in Figure 4. (b) (4)

(b) (4)

(b) (4)

The finished meal is packed in 20 or 25 kg aluminum-coated vacuum bags in modified atmosphere (nitrogen), and labeled or marked with printed labels containing information regarding the product name, lot identification, net quantity, list of ingredients, name and address of manufacturer, origin, and date of minimum durability. An example of the label is shown in Appendix B, Figure B-1. The bagged meal is stored in holding rooms on board the vessel until off-loading. The estimated time for the entire processing, from when the krill leaves the holding tanks to when the meal is bagged, is approximately 2.5 to 3.0 hours. An extra label is added to the packaged krill meal at the warehouse in Montevideo in Uruguay for shipments to the United States, specifying that it is for use at up to 3% in adult dog food only (Aker BioMarine 2016a) (Appendix B, Figure B-2).

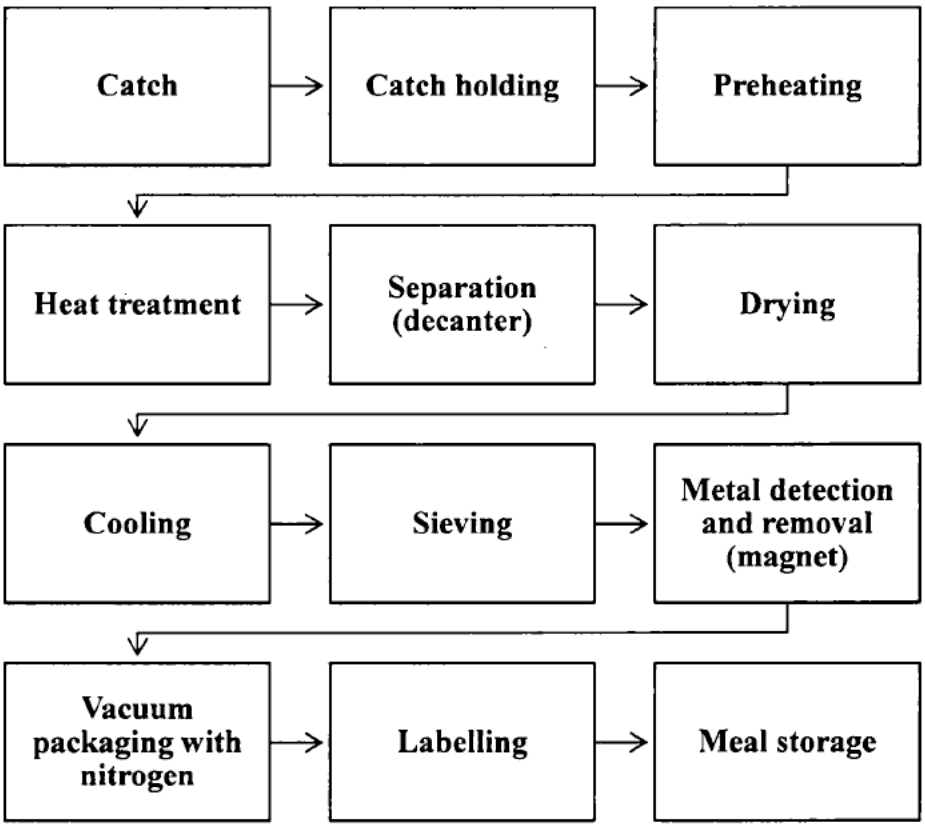

Figure 4: Manufacturing Process for Qrill™ Pet

III. SPECIFICATIONS

a. General

All batches of Qrill™ Pet undergo routine and rigorous testing to ensure compliance with product specifications. The specifications for Qrill™ Pet along with data from five sample batches are summarized in Table 2 and the full specifications sheet is shown in Appendix C.[3] Qrill™ Pet is a good source of protein (≥ 55%) and fat (≥ 20%), and contains *omega*-3-fatty acids (> 17g/100g fat) and astaxanthin (80-160 ppm). Specifications for copper (< 80 ppm) and fluorine (≤ 1,500 ppm) are included because these minerals are known constituents of krill.

Table 2: Specifications of Qrill™ Pet

			Batch Analysis Results ($N = 5$)[a]	
Analysis	**Method**	**Specification**	**Range**	**Average**
Color	Visual	Brownish pink to orange	Meets specification	Meets specification
Total dry matter (g/100g)	ISO 6496	≥ 92	93.2 - 94.0	93.4
Moisture (%)	ISO 6496	6 ± 2	6.8[c]	6.8
Crude Protein (%)	ISO 16634-1	≥ 55	55.3 - 62.9	59.3
Fat (%)	(b) (4) Bligh & Dyer (1959)[b]	≥ 20	18.1 - 27.0	23.5
Ash (%)	ISO 5984	≤ 13	9.7 - 11.6	10.4
Salt (NaCl, %)	AOAC 937.09	≤ 4	2.5 - 3.1	2.8
Total *omega*-3 fatty acids (g/100g fat)	AOCS Ce 1b-89	≥ 17	17.5 - 26.0	20.6
Astaxanthin esters (mg/kg)	(b) (4)	80 - 160	89 - 129	112
Total volatile nitrogen (%)	AOAC 920.03	≤ 0.3	0.02 - 0.03	0.03
Peroxide value (meq peroxide/kg)	AOCS Cd 8b-90	< 10	< 1 - 2.7	< 1.7
Cadaverine (ppm)	(b) (4)	< 10	< 0.10[c]	< 0.10
Histamine (ppm)		< 10	< 0.10[c]	< 0.10
Meat bone meal (%)	2003/126/EU or 2009/152/EC	Not present	Not present[c]	Not present
Minerals				
Phosphorous (%)	ISO 6491 or ICP-SFMS/DIN EN ISO 17294-2 E29	< 2	1.18 - 1.40	1.35
Calcium (%)	ISO/CD 6869 or ICP-SFMS/DIN EN ISO 111885, mod.	< 3	1.58 - 2.00	1.80
Iodine (ppm)	ICP-MS/EN15111	< 50	2.6 - 22	9.1

[3]A specification for domoic acid (a toxin produced by diatoms) is not included. Although diatoms may be consumed by *Euphausia superba*, the level of domoic acid in three lots of Qrill™ Pet is less than the limit of detection (3 ppm). Based on an U.S. FDA action level for domoic acid in seafood of 20 ppm (U.S. FDA 2011), the level of domoic acid in Qrill™ Pet (< 3 ppm) is not considered hazardous.

Copper (ppm)	DIN EN ISO 11885, mod., ICP-OES	< 80	44 - 75	65
Fluorine (ppm)	(b) (4)	≤ 1500	650 – 1300[d]	987[d]
Heavy Metals				
Cadmium (ppm)	EN ISO 11885, mod. or EN 15763:2009	< 1	0.30 - 0.40	0.37
Mercury (ppm)	§ 64 LFGB L00.00-19/4 or EN 15763:2009	< 0.1	0.005[c]	0.005
Lead (ppm)	§ 64 LFGB L00.00-19/3 or EN 15763:2009	< 0.05	< 0.05[c]	< 0.05
Total arsenic (ppm)	EN ISO 11885, mod.	< 8	2.2 - 2.7	2.5
Inorganic arsenic (ppm)	EN ISO 18885, mod. or § 64 LFGB 25.06	0.5	<0.1[c]	<0.1
Microbes				
Total Plate Count (cfu/g)	AFNOR 3M 1/1-9/89	< 20,000	< 250 - 470	305[e]
Enterobacteriaceae (cfu/g)	ISO 21528-2	< 300	< 10[c]	< 10
Salmonella spp. (PCR, %)	NordVal Ref.no. 023	Negative	Negative	Negative
Yeast (CFU/g)	NordVal Ref.no 016	< 100	< 10[c]	< 10
Mold (CFU/g)	NordVal Ref.no 016	< 100	< 10[c]	< 10

§ 64 LFGB (official library for analytical methods according to LFGB); AOAC = Association of Analytical Communities; AOCS = American Oil Chemist's Society; cfu = colony forming unit; DIN = Deutsches Institut für Normung; EN or EU = European Union; ICP-MS = Inductively Coupled Plasma/Mass Spectrometry; ICP-OES = Inductively Coupled Plasma/Optical Emission Spectrometry; ISE = ion sensitive electrode; ISO = International Organization for Standardization; LFGB = Lebensmittel-Bedarfsgegenstände-und Futtermittelgesetzbuch (German equivalent to FDA laws); NaCl = Sodium chloride; ppm = parts *per* million; [a] $n = 4$ for iodine and microbes and $n = 3$ for cadmium, mercury, lead, total arsenic, inorganic arsenic, $n = 55$ for fluorine; [b] Modified by reducing all weights and volumes by a factor of 1:5, and by determining the lipid concentration in a subsample of the chloroform phase; [c] all lots; [d] batches obtained from 3/2/2015 – 6/15/2015; [e] using 250 for < 250.

Qrill™ Pet contains a maximum of 4% salt, which is lower than the maximum amount of salt permitted in fish, shrimp and crab meals for use in animal feed (7%) (AAFCO 2014a, b, c).

The amount of protein in krill meal (≥ 55%) is substantially higher than the minimum amount of protein permitted in crab meal (25%) (AAFCO 2014b).

Qrill™ Pet contains less than 50 ppm iodine, which leads to less than 1.5 ppm[4] iodine in dry dog food when Qrill™ Pet is added at 3%. This level is only 3% of the maximum iodine (≤ 50 ppm) permitted in dry dog food according to AAFCO (2014d). It equals to the minimum iodine level required for adult dog maintenance (AAFCO 2014d). Therefore, adding Qrill™ Pet at 3% in dry dog food ensures that dogs acquire adequate iodine from the diet, and is unlikely to result in the total iodine exceeding the maximum tolerance level.

The Qrill™ Pet specifications allow for up to 80 ppm copper, which would lead to a maximum of 2.4 ppm copper in feed containing 3% Qrill™ Pet[5]. This is approximately one tenth of the European limit of 25 mg/kg copper in complete feeds (EC 2003), and is much lower than the maximum tolerated limit of 250 ppm for dogs, according to AAFCO (2014e). The 2.4 ppm Cu

[4] 50 ppm I x 3% Qrill™ Pet in dry dog food = 1.5 ppm I in dry dog food
[5] 80 ppm Cu x 3% Qrill™ Pet in dry dog food = 2.4 ppm Cu in dry dog food

from 3% Qrill™ Pet is also lower than the level of 7.3 ppm required for adult dog maintenance according to AAFCO. Therefore, Qrill™ Pet at 3% in food is not a significant source of copper.

Analysis of five batches revealed zinc content of 52, 45, 37.2, 39 and 73 ppm in Qrill™ Pet, leading to up to 2.19 ppm zinc in complete feed for dogs[6]. This is much lower than the maximum allowable concentrations of 1,000 and 250 ppm by AAFCO and EU, respectively. The zinc level of 2.19 ppm is also much lower than minimum level of 120 ppm required for adult dog maintenance (AAFCO 2014d). Therefore, Qrill™ Pet at 3% in food is not a significant source of zinc.

According to chemical analysis of three batches of Qrill™ Pet, selenium content was reported to be 2.1, 2.7 and 4.7 mg/kg, with an average of 3.2 mg/kg. Adding Qrill™ Pet to dry dog food at up to 3% can add up to 0.14 ppm selenium to the food[7]. While a regulatory limit for selenium was not specified in the EU or the United States for dog food ingredients, AAFCO specified a limit of 2 ppm Se for complete dog food. As this limit is 14 times higher than the amount contributed by Qrill™ Pet, Qrill™ Pet at 3% in dry dog food is not a significant source of selenium.

Levels of heavy metals (cadmium, arsenic, lead, mercury) in Qrill™ Pet are lower than limits established by AAFCO for heavy metals in complete feed (AAFCO 2014f) as well as limits established by the EU limit for cadmium in feed materials of animal origin, arsenic (total and inorganic) and mercury in feed materials derived from fish or other aquatic animals, and lead in feed materials (EC 2002). A comparison of these parameters with relevant regulatory limits described above is summarized in Table 3.

Table 3: Qrill™ Pet Specifications Compared to Relevant Regulatory Limits

Specification Parameter	Qrill™ Pet	Regulatory Limit for Feed Ingredients[a]	3% Qrill™ Pet[b]	Regulatory Limit for Complete Dog Food
Salt	≤ 4%	≤7% in marine meals, AAFCO	≤ 0.12%	NA
Protein	≥ 55%	≥ 25% in crab meal, AAFCO	≥ 1.65%	NA
Fluorine	≤ 1,500 ppm	≤ 3,000 ppm in feed materials produced from marine krill, EU	≤ 45 ppm	NA
Iodine	< 50 ppm		< 1.5 ppm	≤ 50 ppm, AAFCO
Copper	< 80 mg/kg	NA	< 2.4 mg/kg	< 25 mg/kg, EU < 250 mg/kg, AAFCO
Zinc	73 ppm[c]	NA	2.19 ppm	< 1,000 ppm, AAFCO < 250 ppm, EU
Selenium	3.2 ppm[d]	NA	0.096 ppm	< 2 ppm, AAFCO
Cadmium	< 1 mg/kg	< 2 mg/kg, EU	< 0.03 mg/kg	< 0.5 mg/kg, AAFCO
Mercury	< 0.1 mg/kg	< 0.5 mg/kg, EU	< 0.003 mg/kg	< 2 mg/kg, AAFCO < 0.4 mg/kg, EU

[6] 73 ppm Zn x 3% Qrill™ Pet in dry dog food = 2.19 ppm Zn in dry dog food
[7] 4.7 ppm Se x 3% Qrill™ Pet in dry dog food = 0.14 ppm Se in dry dog food

Lead	< 0.05 mg/kg	< 10 mg/kg, EU	< 0.0015 mg/kg	< 30 mg/kg, AAFCO < 5 mg/kg, EU
Arsenic, inorganic	< 0.5 mg/kg	15 mg/kg (total), EU	< 0.015 mg/kg	< 50 mg/kg (total), AAFCO < 6 mg/kg (total), EU
Arsenic, organic	< 8 mg/kg	15 mg/kg (total), EU	< 0.24 mg/kg	< 50 mg/kg (total), AAFCO < 6 mg/kg (total), EU
Aluminum	90 mg/kg	NA	2.7 mg/kg	200 mg/kg, AAFCO
Chromium	0.9 mg/kg	NA	0.027 mg/kg	1,000 mg/kg, AAFCO

[a] Maximum content relative to a feed with a moisture content of 12%; AAFCO = American Association of Feed Control Officials; EU: European Union

[b] Calculated by multiplying the specifications of Qrill™ Pet by 3%. This represents the amount contributed to complete dog food by Qrill™ Pet when added at 3%.

[c] Zinc is not part of the CoA. The higher value of 73 ppm from analysis of five batches of Qrill™ Pet (52, 45, 37.2, 39 and 73 ppm) is presented.

[d] Selenium is not included in the Specification for Qrill™ Pet and is not routinely tested. However, this is included here as an ingredient of potential toxicological concern. The value of 3.2 mg/kg is the mean of three measured values from three batches of Qrill™ Pet (2.1, 2.7 and 4.7 mg/kg) using the DIN EN ISO 17294-2-E29 method.

The amount of fluorine in Qrill™ Pet (up to 1,500 ppm) is less than the European limit of 3,000 mg/kg in feed materials produced from marine krill (EC 2008). A limit for fluorine has not been established in the U.S. for dog food. The European Union (EC 2008), National Research Council (NRC 2005) and AAFCO (2014g) have established fluorine limits in feed of different animals (Table 4). The National Academy of Sciences (NAS) recommended a fluoride tolerance of 50 ppm based on pathological changes and 100 ppm based on performance for growing dogs (NAS 1974). Both fluoride levels in feed are higher than the level of fluorine originating from use of 3 % Qrill™ Pet in feed (i.e., Qrill™ Pet containing 1,500 ppm fluorine with a use level of 3% would produce a use level of 45 ppm). The amount of fluorine in Qrill™ Pet is considerably less than the limit of fluorine permitted by the European union for feed materials produced from marine crustaceans (3,000 ppm) (EC 2008), and the amount of fluorine in dog food containing 3% Qrill™ Pet (45 ppm) is generally similar to permissible levels of fluorine in livestock feeds, except feed for young and lactating cattle, sheep and goats (Table 4).

Table 4: Recommended or Regulatory Limits for Fluorine in Animal Feed

Feed Category	EC (2008) (mg/kg feed)[a]	NRC MTL (mg/kg feed)[b]	AAFCO (2014g) Adulterants Thresholds (mg/kg feed)[c]
Feed materials produced from marine crustaceans such as marine krill	3,000	-	-
Complete feed for growing dogs	150	NS	NS
Complete feed for fish	350	NS	NS
Complete feed for horses	150	40	NS
Complete feed for pigs	100	150	150
Complete feed for poultry (except chicks)	350	150 (turkeys) 200 (chickens)	300
Complete feed for chicks	250	150	300
Complete feed for cattle in lactation	30	30	40[d]
Complete feed for all other cattle	50	40	90[e]
Complete feed sheep in lactation	30	60	60
Complete feed for all other sheep	50	60	60

Complete feed for lambs	NS	150	100
Complete feed for goats in lactation	30	NS	NS
Complete feed for all other goats	50	NS	NS
Complete feed for rabbits	NS	40	NS
Feed for cattle, sheep, or goats consuming roughage w/out limited amount of grain	NS	NS	50 mg F/100 pounds body weight
All other complete feed	150	NS	NS

[a] Maximum content relative to a feed with a moisture content of 12%; [b] Values are from NRC (2005); [c] Fluorine-bearing ingredients must not increase the total fluorine content of the total ration above this level or else it is considered an adulterant; [d] Level specific to breeding and dairy cattle; [e] Level specific to slaughter cattle; AAFCO = American Association of Feed Control Officials; MTL = maximum tolerable level; NRC = National Research Council; NS = not specified.

b. Amino Acid and Lipid Composition

In addition to ensuring that Qrill™ Pet complies with the established specifications, Aker BioMarine has conducted assessments for amino acid and lipid composition in order to further define the ingredient. Results of the analyses are presented in Table 5 and Table 6, below. Qrill™ Pet is a source of several different amino acids and contains relatively high amounts of the *omega*-3 fatty acids EPA (10.6 g/100 g) and DHA (5.9 g/100 g) compared to other fatty acids.

Table 5: Analysis of the Amino Acid Content of Qrill™ Pet

	Batch Analysis Results ($N = 3$)*	
Analysis (g/100 g)	**Range**	**Average**
Aspartic acid	5.12 - 5.72	5.48
Glutamic acid	6.81 - 7.04	6.93
Hydroxyproline	< 0.01 - 0.03	0.02**
Serine	2.37 - 2.43	2.40
Glycine	2.60 - 2.79	2.71
Histidine	1.31 - 1.35	1.33
Arginine	3.13 - 3.38	3.27
Threonine	2.38 - 2.45	2.41
Alanine	2.82 - 2.90	2.85
Proline	2.01 - 2.11	2.05
Tyrosine	2.01 - 2.14	2.07
Valine	2.86 - 3.03	2.97
Methionine	1.72 - 1.77	1.75
Isoleucine	2.98 - 3.17	3.10
Leucine	4.46 - 4.49	4.47
Phenylalanine	2.64 - 2.75	2.70
Lysine	4.18 - 4.59	4.32
Cysteine/Cystine	0.63	0.63
Tryptophan	0.76	0.76
Taurine	0.18	0.18

NA = not applicable; * $n = 3$ for all analyses; excepting $n = 1$ for cysteine/cystine, tryptophan and taurine; **Assuming < 0.01 = 0.01. All amino acids were analyzed according to Commission Directive 98/64/EC (1998).

Table 6: Analysis of the Lipid Composition and Fatty Acid Profile of Qrill™ Pet

	Batch Analysis Results (N = 4)*	
Analysis (g/100g)	**Range**	**Average**
Triacylglycerol	36 - 40	38
Diacylglycerol	0.9 - 19	1.6
Monoacylglycerol	< 1	< 1
Free fatty acids	3.7 - 6.0	4.4
Cholesterol	1.6 - 3.6	3.0
Cholesterol esters	< 0.5	< 0.5
Phosphatidylethanolamine	1.0 - 2.8	1.5
Phosphatidylinositol	< 1	< 1
Phosphatidylserine	< 1	< 1
Phosphatidylcholine	28 - 30	29
Lyso-phosphatidylcholine	2.2 - 3.9	3.4
Total polar lipids	33.2 - 34.7	34.0
Total neutral lipids	45.4 - 48.7	47.2
Total sum lipids	78.6 - 83.4	81.2
Fatty acid profile		
C 14:0 (Myristic)	7.5 - 9.9	8.8
C 16:0 (Palmitic)	14.9 - 17.6	16.4
C 18:0 (Stearic)	0.8 - 1.0	1.0
C 20:0 (Arachidic)	< 0.1[a]	< 0.1
C 22:0 (Behenic)	< 0.1[a]	< 0.1
C 16: 1 n-7	3.2 - 6.4	4.9
C 18: 1 (n-9) + (n-7) + (n-5)	12.4 - 14.6	13.6
C 20:1 (n-9) + (n-7)	0.5 - 0.7	0.6
C 22:1 (n-11) + (n-9) + (n-7)	0.3 - 0.5	0.4
C 24: 1 n-9	< 0.1 - 0.1	< 0.1
C 16:2 n-4	0.4 - 0.5	0.4
C 16:3 n-4	0.1 - 0.3	0.2
C 18:2 n-6 (Linoleic)	1.1 - 2.1	1.4
C 18:3 n-6	0.1	0.1
C 20:2 n-6	< 0.1 - 0.1	< 0.1
C 20:3 n-6	< 0.1 - 0.1	< 0.1
C 20:4 n-6 (Arachidonic)	0.2[a]	0.2
C 22:4 n-6	< 0.1[a]	< 0.1
C 18:3 n-3	0.3 - 1.5	0.9
C 18:4 n-3	1.0 - 4.2	2.4
C 20:3 n-3	< 0.1 - 0.1	< 0.1
C 20:4 n-3	0.2 - 0.4	0.3
C 20:5 n-3 (EPA)	9.5 - 11.4	10.6
C 21:5 n-3	0.3 - 0.4	0.4
C 22:5 n-3	0.2 - 0.3	0.2
C 22:6 n-3 (DHA)	4.9 - 7.7	5.9
Sum saturated fatty acids	23. 8 - 28.3	26.5
Sum monoenoic fatty acids	16.4 - 22.2	20.0
Sum PUFA (n-6) fatty acids	1.5 - 2.5	1.8
Sum PUFA (n-3) fatty acids	17.5 - 26.0	20.6
Sum total-PUFA fatty acids	19.6 - 29.2	23.0
Sum fatty acids total	64.4 - 70.9	68.7

[a] all lots. All fatty acids were analyzed using method AOCS Ce 1b-89

c. Environmental Pollutants

Each year, three random Qrill™ Pet batches are subjected to measurements for known environmental pollutants (i.e., environmental monitoring), especially persistent organic pollutants. Results of the analyses performed on two product batches are shown in Appendix D. Levels of dioxins, furans, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in Qrill™ Pet are generally under or close to limits of detection. Total levels of dioxins, furans and PCBs are substantially lower than the totals permitted for these substances in animal feed by the European Union (EC 2012). The U.S. FDA has established a temporary tolerance for PCBs in feed of 2 mg/kg (parts *per* million) in animal feed ingredients of animal origin (U.S. FDA 2012), substantially higher than the total sum of PCBs in Qrill™ Pet. To our knowledge, no regulatory limits on polybrominated biphenylethers (PBDEs) or polycyclic aromatic hydrocarbons (PAHs) have been established for animal feed.

Of the 13 PAHs analytically tested, four were detected in Qrill™ Pet (detection limit 0.5 µg/kg), including phenanthrene, fluoranthene, fluorine, and pyrene. Their acceptability was assessed based on relative carcinogenic potencies. EFSA has established a limit of 5 µg/kg benzo[a]pyrene in crustaceans used for human food (EC 2006), which is ten times higher than the detection limit of benzo[a]pyrene found in Qrill™ Pet (0.5 µg/kg). The carcinogenic potencies of phenanthrene, fluorene, fluoranthene, and pyrene (the only PAHs that are present in Qrill™ Pet at > 0.5 µg/kg) are 0.001 times of the potency of benzo[a]pyrene (U.S. EPA 2000). Therefore, the levels of these contaminants detected in Qrill™ Pet (8.7 µg/kg, 14 µg/kg, 0.8 µg/kg, and 0.8 µg/kg, respectively) are also acceptable for crustaceans used for dog food.

d. Composition of Qrill™ Pet Compared to Selected Marine Meals

Krill-based meals contain similar levels of moisture, protein and ash (a measure of total amount of minerals), and a higher amount of fat, compared to fish meals assessed by the FAO (Tacon et al. 2009), but comparable levels of crude protein, crude fat, calcium and phosphorus levels to those in fish protein meals evaluated by Folador *et al.* (2006) for use in dog food (Table 7). Qrill™ Pet has a higher protein and fat content than shrimp and crab meals, with lower ash content as a result. In general, Qrill™ Pet contains a lower amount of calcium and a similar amount of phosphorus than fish, crab or shrimp meal. When pet foods are formulated, they often include meals from various sources, although pet food companies typically maintain percentage formulations as proprietary. The composition of krill meal would allow for the replacement of fish or other marine meals with krill meal as a source of protein and lipids without significant alteration to the nutritional profile of the finished food. Based on AAFCO labeling regulations (summarized above), fish meal is added at levels much greater than 3% and most likely closer to 20% of the diet, meeting product name label requirements. Fish meal and other marine meals have been approved for use in animal feed in the United States since the 1930s, with no known increase in uroliths (possibly due to high protein content) or other known toxicological effects.

Table 7: Proximate Composition of Qrill™ Pet and Marine Meals

Meal	Moisture (%)	Crude Protein (%)	Lipid/Ether Extract (%)	Crude Fat (%)	Ash (%)	Ca (%)	P (%)
Qrill™ Pet	5.4	61.2	-	23.6	10.3	1.80	1.50
Fish Meals							
Anchovy	7.1	65.3	4.1	0.8	14.8	3.75	1.42
Menhaden	3.8	61.1	9.3	0.9	19.0	5.11	2.89
Herring	7.9	72.0	8.4	0.7	10.1	2.04	1.42
Tuna	7.0	59.0	6.9	0.8	17.0	7.86	4.21
Sardine	7.0	59.0	6.7	0.3	14.2	4.44	2.72
Horse/Jack mackerel	4.6	66.6	9.0	-	13.7	-	
White	6.5	62.2	4.2	0.2	18.0	6.84	3.8
Alaskan pollock[1]	3.4	65.2	5.0	-	10.1	2.67	1.7
Cod[1]	8.3	68.6	3.8	-	14.4	3.64	2.35
Alaskan salmon[1]	2.2	69.0	8.8	-	8.0	-	-
Farmed salmon[1]	9.0	60.0	9.5	-	13.0	2.5	2
Trash fish and/or processing waste (Viet Nam)	-	30.0	1.0	0.7	15.8	5	2.2
Shrimp Meals							
Shrimp head	3.2	32.7	1.3	1.5	18.0	6.97	1.15
Shrimp shell	4.0	42.0	0.4	12.0	26.2	7.53	1.37
Sergestid shrimp (*Acetes* sp. whole)	8.2	46.9	3.2	3.6	13.1	-	-
Shrimp (process residue)	7.5	37.2	1.3	14.1	26.8	9.73	1.84
Crab Meals							
Crab (process residue)	4.2	31.7	2.0	10.7	38.4	14.56	1.59
Squat lobster/red crab/langostilla	4.54	39.3	3.6	7.9	12.8	0.97	1.15

Values presented are reported minimums; [1] From processing waste.

e. Quality Assurance

As the production of Qrill™ Pet is a continuous process aboard the fishing vessel, the finished product is divided into production batches according to the date of catch and processing. Each production batch assembled for quality assurance testing contains Qrill™ Pet produced on the ship in one day. In addition to ensuring that Qrill™ Pet is consistently produced according to the same quality standards, the assessment of the batches allows for the examination of variations between batches of Qrill™ Pet produced in different seasonal periods and geographical locations.

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

[8] Accredited laboratory: "a laboratory provided with official credentials as recognized as meeting the essential requirements. Accreditation is a process in which certification of competency, authority, or credibility is presented." (Aker BioMarine 2014a)

[9] Certified laboratory: "A laboratory with official approval from national government (GMP, GCP, GLP, etc.)" (Aker BioMarine 2014a).

(b) (4)

[redacted]

f. Stability

According to the product specification sheet, Qrill™ Pet is vacuum-packed in laminated metalized bags. The recommended storage temperature is < 25°C (Appendix C). As shown in the stability studies below, Qrill™ Pet is stable for at least 18 months when stored under recommended conditions.

A total of two stability studies were conducted on Qrill™ Pet. In the first study, two batches of Qrill™ Pet packaged in July, 2013 were analyzed for microbes, moisture, crude protein, total volatile nitrogen, cadaverine, fat and peroxide value in July, 2013 and in September, 2014 (approximately 13 months later). The results of the analysis are shown in Table 8. The results show that Qrill™ Pet remains within specification for microbes, moisture, crude protein, total volatile nitrogen, cadaverine, fat and peroxide value when stored for one year in original packaging at ambient temperature.

Table 8: Analyses for Qrill™ Pet in Study 1

	Specification	Batch 15-310513		Batch 15-300513	
Analyte		**7/16/2013**	**9/1/2014**	**7/16/2013**	**9/18/2014**
Moisture (%)	≤ 8	(b) (4)			
Crude protein (%)	≥ 55				
Fat (%)	≥ 18				
Total volatile nitrogen (%)	≤ 0.3				
Cadaverine (ppm)	≤ 10				
Peroxide value (meq peroxide/kg)	≤ 10				
Total plate count (cfu/g)	≤ 20,000				
Enterobacteriaceae (cfu/g)	< 300				
Salmonella spp. (*per* 25 g)	Negative				

cfu = colony forming units; NP = not performed; ppm = parts *per* million

In the second study (Aker BioMarine 2016d), more storage conditions and batches were tested. Three batches of Qrill™ Pet manufactured in May of 2014 were divided into 200 g aliquots, vacuum-packed in aluminum foil-coated polyethylene bags, with 80-100 µm thickness and O_2

[10] According to email correspondence with Aker BioMarine dated 8/18/2016.

permeability of < 1.5 g/m^2/day and analyzed after storage at various conditions for different durations. The sample bags were flushed with nitrogen, set under vacuum and heat sealed and stored at 25, 30 or 40°C with relative humidity of 60% or 75%. Samples were/are to be taken for analytical testing at 0, 1, 3, 6, 9, 12, 18, 24, 30 and 36 months (samples stored at 25 and 30°C), or at 0, 1, 3, and 6 months only (samples stored at 40°C). At the time of completion of this report, only samples stored for up to 18 months were analyzed. Preliminary results are summarized below.

The percentage of total fat is slightly decreased for all three batches stored for 18 months at 25°C with 60% humidity from 25.9 -27.9% to 24.9 – 26.7%) and for 12 months at 30°C with 75% humidity from 25.9 -27.9% to 23.8 – 26.3%), and for 6 months at 40°C with 75% humidity from 25.9 -27.9% to 24.5 – 26.6%). However, all the values at the end of the studied periods (23.8-26.7%) were still far greater than 20% listed on the product specification. Percentages of EPA, DHA and total omega-3 fatty acids were unchanged for all three batches stored for 18 months at 25°C with 60% humidity, for 12 months at 30°C with 75% humidity, and for 3 months at 40°C with 75% humidity (samples stored at 40°C for more than 3 months were not analyzed due to expected stability of this parameter). Protein levels slightly reduced in all batches after storage at 25°C with 60% humidity for 18 months (from 57.5 – 58.3% to 56.9 – 57.2%) and at 30°C with 75% humidity for 12 months (from 57.5 – 58.3% to 56.5-57.1%). But these values still meet Qrill™ Pet's specification (≥55%). There were no significant increases in mold or yeast count after 12 months of storage for all three batches at 25°C with 60% humidity and 30°C at 75% humidity. Phospholipid levels slightly decreased in three sample batches after storage at 25°C with humidity of 60% for 18 months (from 11.76 - 12.59% to 11.56 - 12.22%), at 30°C for 12 months with humidity of 75% (from 11.76 - 12.59% to 11.42 - 12.43%), and in two sample batches stored at 40°C with 75% humidity for 6 months (from 11.76 – 11.79% to 10.02 – 11.25%). Levels in the third batch were not measured. However, all the values at the end of the periods studied still meet Aker BioMarine's acceptance criteria. Iodine value remained stable for all storage conditions and sampling points, indicating no oxidation by breaking of double bonds has occurred.

Acid value slightly increased for all three batches after 12 months at 25°C with 60% humidity (mean increase 9.4% from 10.70 – 11.60% to 11.10 – 13.00%) and 30°C with 75% humidity (mean increase 26.4% from 10.70 – 11.60% to 12.30 – 15.10%), and after 3 months at 40°C with 75% humidity (mean increase 13.6% from 10.70 – 11.60% to 11.60 – 13.10%). Acid values reflect fatty acid content of the feed material, which can be generated by hydrolysis of fat (NRA 2008). The fatty acids were monitored for information only, and this parameter is not part of the Qrill™ Pet specification. The slight increase in acid value was considered to be within expected range for the product.

Total volatile nitrogen increased for all three batches after 18 months (but not after 6 months) at 25°C with 60% humidity (from 0.03 – 0.04% to 0.07%), after 12 months 30°C with 75% humidity (from 0.03 – 0.04% to 0.07%), and after 6 months at 40°C with 75% humidity (mean increase 170% from 0.03 – 0.04% to 0.09%). Total volatile nitrogen measures the release of amine-containing bases such as ammonia and trimethylamine during the spoilage of fish (FAO 1989). Aker BioMarine indicates that minor amounts of total volatile nitrogen are expected to form during storage. However, the highest value of 0.09% measured under accelerated

conditions is still less than one third of Qrill™ Pet's specification of ≤0.3%, meeting the specification requirement.

Peroxide values increased for all three batches stored for 9 months at 25°C with 60% humidity and 30°C with 75% humidity, but the values at 9 months (i.e., < 2 to 5.1 meq peroxide/kg oil) were greater than those measured after 12 months and 18 months (i.e., < 2 to 2.6 meq peroxide/kg oil). Peroxide values remained relatively stable for 6 months when stored at 40°C with 75% humidity. Peroxide values reflect the rancidity of fats as they react with oxygen to form hydrogen peroxides during spoilage. Later on, hydroperoxides are oxidized to aldehydes and ketones that can change the odor and flavor of rancid fats. Peroxide values no greater than 4 meq/kg in the complete animal feed do not affect the performance of the feed (NRA 2008). The highest peroxide value of 5.6 meq/kg in this study is below Qrill™ Pet's specification of <10 meq/kg, which translates to 0.3 meq/kg in dog feed when added at the maximum proposed level of 3% in dog food[11]. Therefore, the peroxide values measured for Qrill™ Pet in this study are acceptable.

Astaxanthin content in the three batches is reduced gradually at each sampling points, and the reduction is faster at higher temperatures. At 25°C for 18 months with humidity of 60%, astaxanthin levels decreased from 112 – 115 mg/kg to 91 – 97 mg/kg. At 30°C for 12 months with humidity of 75%, the levels decreased from 112 – 115 mg/kg to 90 – 92 mg/kg. At 40°C with 75% humidity, the levels decreased from 112 – 115 mg/kg to 84 – 88 mg/kg. This is expected as astaxanthin is an antioxidant which is consumed during oxidation. However, astaxanthin levels still meet the specification of 80 – 160 mg/kg for Qrill™ Pet.

In summary, the results of the two stability studies show that Qrill™ Pet remains within specification for microbes, moisture, crude protein, total volatile nitrogen, cadaverine, fat, peroxide value, and/or astaxanthin when stored for one year in original packaging at ambient temperature (25 – 30°C) and for at least 6 months at an elevated temperature of 40°C.

IV. PHYSICAL AND TECHNICAL EFFECTS

Aker BioMarine intends to use Qrill™ Pet as a source of protein and lipid for adult dog food. The intended usage rate is up to 30,000 mg/kg finished food (30,000 ppm[12] or 3%). Qrill™ Pet will be used as a replacement for fish meal or other sources of protein used in pet food. At a 3% inclusion rate, Qrill™ Pet is anticipated to serve as a significant source of protein and lipid for the dogs, but is not anticipated to change the color of dog food (U.S. FDA 2015a). The results of digestibility studies below suggest that krill meal is a highly digestible source of protein and lipid for dogs.

[11] 3% x 10 meq/kg = 0.3 meq/kg

[12] ppm = parts *per* million

a. Historical and Current Use

Antarctic krill have been used mainly for leisure fishing bait and aquaculture feed (e.g., salmon and trout) (Yoshitomi 2004). Freeze dried Antarctic krill is used for the home aquarium market (FAO 1997). Peeled krill may be used for human food (Suzuki and Shibata 1990); however, use is limited mainly to processed products (*e.g.*, paste, sauce, frozen tails, sticks) (Yoshitomi 2004; FAO 2016a). A krill protein concentrate is being developed as a source of protein for human food (Gigliotti et al. 2008).

Krill meal is currently used in Europe in pet food, with use levels ranging from 0.5 to 4.5% of the diet (Meradog 2016; Belcando 2016). European company statements indicate that the volume of pet food sold that contains krill meal is approximately 1,040 tons annually, and no adverse events from dogs consuming the krill meal have been reported (AkerBiomarine 2014b,c).

Products derived from krill (*e.g.*, krill oil and enzymes) have a history of use by humans. Krill oil is used in human food and is a well-known source of *omega*-3 fatty acids for dietary supplements (UMM 2015). An enzyme preparation from krill has been used as a debriding agent for wounds (Westerhof et al. 1990).

b. Utility

i. Nutrient Requirements

Qrill™ Pet will be added to dry adult dog food as a source of protein and lipids. Dogs require daily intakes of protein, amino acids, fat, linoleic acid, minerals and vitamins (AAFCO 2014h, i). As shown in Table 9, at the proposed upper use level of 3%, Qrill™ Pet would provide 9.9% of the protein requirement and 14.0% of the fat requirement for adult dogs. An inclusion level of 3% Qrill™ Pet would provide from 12.5% of the minimum requirement for tryptophan to 24.3% of the minimum requirement for isoleucine in a nutritionally complete dog food. Use of 3% Qrill™ Pet would provide a significant percentage of the daily minimum requirements for sodium and chloride in dogs. Specifications for Qrill™ Pet include routine analysis of NaCl, copper and iodine. At a usage rate of 3%, Qrill™ Pet would be a significant source of vitamin A for dogs.

Table 9: AAFCO Dog Food Nutrient Profiles Based on Dry Matter[a]

Nutrients	Growth and Reproduction Minimum	Adult Maintenance Minimum	Maximum	Qrill™ (3%)[b]	% Minimum (Maintenance) from Qrill™ (3%)[b]
Crude protein (%)	22.0	18.0	-	1.78	9.9
Arginine (%)	0.62	0.51	-	0.10	19.6
Histidine (%)	0.22	0.18	-	0.04	22.2
Isoleucine (%)	0.45	0.37	-	0.09	24.3
Leucine (%)	0.72	0.59	-	0.13	22.0
Lysine (%)	0.77	0.63	-	0.13	20.6
Methionine-cysteine (%)	0.53	0.43	-	0.07	16.3

Phenylalanine-tyrosine (%)	0.89	0.73	-	0.14	19.2
Threonine (%)	0.58	0.48	-	0.07	14.6
Tryptophan (%)	0.20	0.16	-	0.02	12.5
Valine (%)	0.48	0.39	-	0.09	23.1
Crude fat [c] (%)	8.0	5.0	-	0.70	14.0
Linoleic acid (%)	1.0	1.0	-	0.04	4.0
Minerals					
Calcium (%)	1.0	0.6	2.5	0.05	8.3
Phosphorus (%)	0.8	0.5	1.6	0.04	8.0
Ca:P ratio	1:1	1:1	2:1	1.25:1	
Potassium (%)	0.6	0.6	-	-	-
Sodium (%)	0.3	0.06	-	0.03	50.0
Chloride (%)	0.45	0.09	-	0.05	55.6
Magnesium (%)	0.04	0.04	0.3	0.01[d]	25.0
Iron [e] (mg/kg)	80	80	3000	1.30[f]	1.6
Copper [g] (mg/kg)	7.3	7.3	250	1.95	26.7
Manganese (mg/kg)	5.0	5.0	-	-	-
Zinc (mg/kg)	120	120	1000	1.48[h]	1.2
Iodine (mg/kg)	1.5	1.5	50	0.27	18.0
Selenium (mg/kg)	0.11	0.11	.2	-	-
Vitamins and others[i]					
Vitamin A (IU/kg)	5000	5000	250,000	1520	30.4
Vitamin D (IU/kg)	500	500	5000	Negligible	Negligible
Vitamin E (IU/kg)	50	50	1000	2.75	5.5
Thiamine[j] (mg/kg)	1.0	1.0	-	0.05	5.0
Riboflavin (mg/kg)	2.2	2.2	-	0.01	0.5
Pantothenic Acid (mg/kg)	10	10	-	0.14	1.4
Niacin (mg/kg)	11.4	11.4	-	0.91	8.0
Pyridoxine (mg/kg)	1.0	1.0	-	0.03	3.0
Folic Acid (mg/kg)	0.18	0.18	-	0.02	11.1
Vitamin B_{12} (mg/kg)	0.022	0.022	-	0.002	9.1
Choline (mg/kg)	1200	1200	-	-	-

AAFCO = American Association of Feed Control Officials : ppm = parts *per* million

[a] Presumes an energy density of 3500 kcal ME/kg, as determined in accordance with Regulation PF9. Rations greater than 4000 kcal ME/kg should be corrected for energy density; rations less than 3500 kcal ME/kg should not be corrected for energy. Rations of low-energy density should not be considered adequate for growth or reproductive needs based on comparison to the Profiles alone.

[b] Values were calculated using the mean value for each parameter from 5 lots of Qrill™ Pet and a 3% inclusion rate.

[c] Although a true requirement for crude fat *per se* has not been established, the minimum level was based on recognition of crude fat as a source of essential fatty acids, as a carrier of fat-soluble vitamins, to enhance palatability, and to supply an adequate caloric density.

[d] Calculated using magnesium analysis of 0.44% for two lots of Qrill™ Pet

[e] Because of very poor bioavailability, iron from carbonate or oxide sources that are added to the diet should not be considered in determining the minimum nutrient level.

[f] Calculated using an average iron analysis of 43.5 ppm for four lots of Qrill™ Pet (31 ppm, 31 ppm, 41 ppm and 71 ppm).

[g] Because of very poor bioavailability, copper from oxide sources that are added to the diet should not be considered in determining the minimum nutrient level.

[h] Calculated using an average zinc analysis of 49.2 ppm for or five lots of Qrill™ Pet (52, 45, 37.2, 39 and 73 ppm).

[i] Vitamins and others values for krill were obtained from one lot.

[j] Because processing may destroy up to 90% of the thiamine in the diet, allowances in formulation should be made to ensure the minimum nutrient level is met after processing.

ii. Digestibility

It is important evaluate the digestibility of krill meal since Qrill™ Pet will be added to dry adult dog food as a source of protein and lipids. The digestibility of a feed ingredient determines the amount that is actually absorbed by the animal and therefore is a way to evaluate the availability of nutrients for biological processes such as growth or reproduction (FAO 1990). The digestibility of a feed nutrient can be calculated as the difference between nutrient intake and fecal nutrient concentration divided by the amount of nutrient intake. Digestibility values can be expressed as 'apparent' or 'true' digestibility. Apparent digestibility is estimated by subtracting the nutrients contained in the feces from the nutrients contained in the feed intake, but this estimation does not take into account any endogenous losses of nutrients (i.e., nutrients that are lost as methane gas or as metabolic waste products in the feces). However, true digestibility is estimated by correcting for endogenous and microbial amounts of the nutrient that are lost in the feces (FAO 1990; Stein et al. 2007).

Studies in multiple animal species have specifically examined the digestibility of krill meal. Two unpublished tolerance studies investigated the omega-3 index as an indicator of lipid digestibility in dogs when fed 8% of Qrill™ Pet meal in feed (Berge et al. 2014; Hals 2016 – unpublished data). Protein and fat digestibility of krill meal was evaluated in a mink feed study (Krogdahl et al. 2015). The comparison of protein and fat digestibility between krill meal and that of fish meal, a common feed ingredient, was also investigated in the pig (Heinz et al. 1981). The protein quality of krill protein concentrate (KPC) in comparison to casein was evaluated in rats (Gigliotti et al. 2008). These studies provide evidence that krill meal is a highly digestible source of protein and lipid for dogs.

Dog

Studies specifically examining the digestibility of krill meal in dogs were not identified. However, limited data were available from two unpublished in-life tolerance studies in dogs that support the digestibility of the lipid fraction of krill meal. As detailed below, both dog studies reported a significant increase (30 – 40%) in omega-3 index (i.e., the percentage of omega-3 fatty acids EPA + DHA in total phospholipid fatty acids) attributable to the administration of Qrill™ Pet that is rich in omega-3 fatty acids. These studies demonstrated, to a limited extent, that the lipids in Qrill™ Pet are digestible.

The tolerability of an 8% inclusion of Qrill™ Pet meal into feed for dogs was evaluated in a 52-day subchronic feeding study in Husky breed dogs (Berge et al. 2014 - unpublished data). The effect of Qrill™ Pet on the omega-3 index was also investigated. Alaskan Husky dogs (n=30) were included in the study; fourteen dogs were fed control diet and sixteen dogs were fed control diet with 8% added Qrill™ Pet meal. The dogs were randomized into the two groups and stratified for gender and age. All the dogs were being actively trained for use in marathon dog sled races. The dogs were fed once daily a ration of 500 – 700 grams, depending on dog size. The control diet consisted of a mix of Eukanuba kitten (50%) and Eukanuba Dog Working and Endurance (50%), a high protein diet. The study states that "In the Qrill™ Pet diet, 8% of the

feed was Qrill™ Pet (wt/wt[13] dry weight)", indicating that 8% of the control diet was replaced with Qrill™ Pet meal. The diet was prepared daily and the Qrill™ Pet-containing diet was mixed to ensure homogenous distribution. Qrill™ Pet-containing diet had a calculated metabolizable energy (kcal/kg) that was 1% greater than the control diet. The dogs were observed daily for feed intake and to ensure that the dogs were in general good health and behavior. Blood samples were obtained at the start and at the end of the study, and analyzed for clinical chemistry and omega-3 index parameters. Student's t-test was used to calculate significant ($P<0.05$) differences within and between groups. Detailed results of the clinical chemistry findings and the general health of the dogs are described in Part 6 of the dossier. Relevant to the digestibility of krill meal, omega-3 index (i.e., the percentage of omega-3 fatty acids EPA + DHA in red blood cell fatty acids) findings are summarized here. At the end of the study period, the omega-3 index increased by 2.9% ($p = 0.51$) in the control animals, and 41.3% in treated animals ($p < 0.00001$). A statistical test was not performed to determine if there are any statistically significant changes between the Qrill™ Pet and control groups.

Another study was conducted in fewer dogs for a longer period of time (14 weeks) to evaluate telomere length in semen and blood, semen quality parameters, clinical chemistry parameters and omega-3 index (Hals 2016 – unpublished data). As the study is still ongoing at the completion of this GRAS dossier, only an interim report was available for review that only presented results on clinical chemistry parameters and omega-3 index. The same diets as described above were administered to ten adult Alaskan Huskies for 14 weeks, which were randomized into two groups of five animals each (Qrill™ PET and control) and stratified for age. The dogs were observed daily for feed intake and to ensure that the dogs were in general good health and behavior Parameters measured include clinical chemistry and omega-3 index (i.e., EPA+DHA as percentage of total identified fatty acids) in isolated red blood cells and fatty acid methyl esters from red blood cells. Detailed results of the clinical chemistry findings and the general health of the dogs are described in Part 6 of the dossier. Relevant to the digestibility of krill meal, omega-3 index (i.e., the percentage of omega-3 fatty acids EPA + DHA in total phospholipid fatty acids) findings are summarized here. The omega-3 index in the control group increased by 10% from the beginning to the end of the study; this change was not statistically significant. In the Qrill™ PET group, the omega-3 index increased significantly (~30%, $p = 0.011$). While the omega-3 index in the treatment group increased 20% compared to the value in the control group at the end of the study, this change was not statistically significant ($p = 0.087$). The lack of statistical significance between the control and Qrill™ Pet groups may be attributed to the fewer number of animals used in this study as compared to the first study, which decreased the statistical power of the study.

Mink

The mink (a strict carnivore) is a common model animal for evaluation of quality of food ingredients for dogs and cats (Opstvedt et al. 2003; Ahlstrøm et al. 2004; Krogdahl et al. 2004; Hellwing et al. 2005; Tjernsbekk et al. 2014). The results obtained with mink correlate closely with digestibility of nutrients (e.g., protein, fat, amino acids) in dogs and blue foxes (Skrede et al.

[13] wt/wt=weight/weight

1980; Ahlstrøm and Skrede 1998; Tjernsbekk et al. 2014). Mink was therefore used as a model animal in order to demonstrate that Qrill™ Pet can be used as a source of protein and fat in the diet of dogs. As detailed below, Antarctic krill meal has high protein and lipid digestibility and good palatability in a mink feed study.

Four adult male mink of the black genotype (> 6 months) were given diets containing 191g of Antarctic krill meal/kg feed (19.1%) and housed individually in metabolic cages designed for separate collection of feces and urine (Krogdahl et al. 2015). In the experiment, Antarctic krill meal accounted for 100% of the protein to permit calculation of krill meal protein digestibility. Lipid and starch digestibility was also determined, but most of the lipid and starch originated from the other ingredients. Soybean oil was used as the main lipid source, accounting for 82% of the total lipid in the experimental diet. Dietary starch originated from corn only. The mean body weight (BW) ± standard deviation (SD) of the animals was 2.25 ± 0.25 kg. Daily feed allowance (136 g *per* day) was implemented to meet the requirement for metabolizable energy (ME). The experiment lasted for seven days of which the first three days were an adaptation period. During the last four days, feed intake was measured precisely and feces were collected daily for chemical analyses.

Samples of Antarctic krill meal and feces were analyzed for dry matter (heating at 105°C for 16-18 h), ash (combustion at 550°C to constant weight), crude protein (as nitrogen x 6.25) by the semi-micro-Kjeldahl method (Kjeltec-Auto System, Tecator, Sweden), lipid [diethyl ether extraction in a Fosstec analyzer (Tecator, Sweden) after HCl-hydrolysis] and starch [measured as glucose after hydrolysis by *alpha*-amylase (Novo Nordisk A/S, Bagsvaerd, Denmark) and amylo-glucosidase (Bohringer Mannheim GmbH, Mannheim, Germany), followed by glucose determination by the 'Glut-DH method' (Merck, Darmstadt, Germany)]. Amino acid analyses of the Antarctic krill meals were performed according to Commission Directive 98/64/EC (EC 1998).

The intakes of crude protein, fat and starch were calculated based on the chemical analyses of the Antarctic krill meal and the other standard ingredients. Apparent digestibility values for protein, fat and starch were calculated from the four animals fed Antarctic krill meal using the formula:

$$\text{Apparent digestibility (\%)} = \frac{\text{Nutrient consumed} - \text{Nutrient excreted in feces}}{\text{Nutrient consumed}} \times 100$$

The results are summarized in Table 10. Mink consumed most the test diets offered, demonstrating good palatability for Antarctic krill meal. Average protein, lipid and starch digestibility of krill meal was 85.1%, 97.8% and 98.2%, respectively. The protein digestibility of krill (85.1%) was similar to that of low temperature fish meal (87.0%) and the estimated digestibility of the lipid fraction of the Antarctic krill meal diet (97.8%) was similar to that of soybean, assuming (based on previous experiments) a digestibility of 96.0% for soybean oil (Rouvinen-Watt et al. 2005).

Table 10: Diet Composition, Chemical Content and Average Digestibility of Crude Protein, Fat and Starch in Feed Fed to Mink (Krogdahl et al. 2015)

Ingredient (g/kg)	
Antarctic krill meal	191
Pregelatinized corn starch	93
Soybean oil	93
Cellulose powder	15
Vitamins/mineral mixture	0.9
Water	608
Sum	1000
Chemical content (g/kg)	
Dry matter	363
Ash	23
Crude protein	123
Crude fat	113
Carbohydrates (difference)	106
Digestibility (%)	
Protein	85.1 ± 0.6
Fat	97.8 ± 0.6
Starch	98.2 ± 0.3

Digestibility data are presented as mean ± standard deviation.

Pig

There are several similarities between the GI tracts of pigs and dogs and the manner in which they handle nutrients. The stomach of the pig has four distinct regions that include the esophageal, cardiac, fundic, and pyloric regions (Figure 5). The stomach of the pig and the dog is both thick-walled and lined with pepsinogen-secreting chief cells, HCl-secreting parietal cells and mucus-secreting cardiac cells. The pH of the contents of the stomach, small intestine, cecum and colon, and the pancreatic juice secretion rate are similar between dogs and pigs, and GI tract transit time is short in both species (Kararli 1995). Therefore, pigs are good models to estimate the digestibility of food ingredients in dogs.

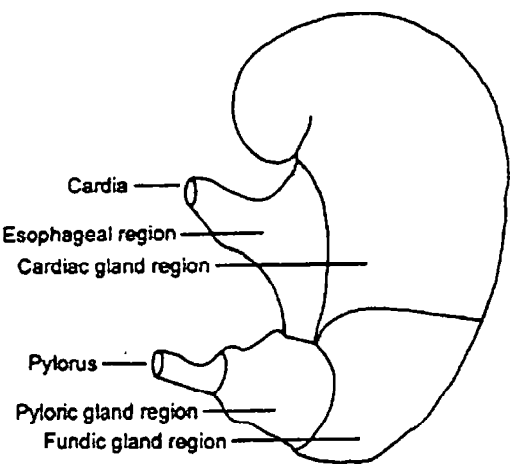

Figure 5: Stomach Regions of the Pig (DeRouchey et al. 2009)

The digestibility of krill meal was examined in twelve male castrated hogs (body weight range of 12-27 kg) (Heinz et al. 1981). Four rations were tested in the same groups of three animals – the base ration consisting of lysine (2.5 g lysine HCl/kg) and vitamin-enriched wheat (Ration I), and rations II-IV contained 74% wheat, 1% vitamin pre-mix, and 25% fish meal (Ration II), krill meal (Ration III) or dried microbial biomass (Ration IV). The results for ration IV were not discussed in the publication. The acclimation and main study periods were each five days long. The animals were housed in metabolic cages from the third day of the acclimation period to the end of the study. The biological values of the studied feeds were measured in accordance with standard methods. The results of the study show that the protein and fat digestibility of the fish meal and krill meal was virtually identical in hogs (Table 11).

Table 11: Digestibility of Fish Meal and Krill Meal in Hogs (Heinz et al. 1981)

Ingredient (g/kg)	Fish meal	Krill meal
Krill meal	250	250
Wheat	740	740
Vitamins/mineral mixture	10	10
Lysine	2.5	2.5
Sum	1002.5	1002.5
Chemical content (g/kg)		
Dry matter	902.7	891.7
Ash	202.3	119.7
Crude protein	655.6	686.3
Crude fat	102.9	109.9
Crude fiber	-	71.9
Digestibility (%)		
Protein	93.8 ± 3.5	95.3 ± 0.6
Fat	85.6 ± 8.4	86.7 ± 4.9
Starch	-	80.0*

Digestibility data are presented as mean ± standard deviation. * The standard deviation was not calculated. The range of values reported was 67-97%.

Rat

Gigliotti et al. (2008) conducted an assessment of the digestibility and safety of a krill protein concentrate (KPC) in 28-day old female Sprague-Dawley rats. The KPC was derived from whole Antarctic krill and isolated using an isoelectric point solubilization/precipitation method (Chen and Jaczynski 2007). Following the 14-day acclimation period, rats (n = 30) were randomly assigned to be fed (*ad libitum*) one of the three diets consisting of: (1) 10% crude protein supplied as KPC for four weeks (n = 10), (2) 10% crude protein supplied as casein for four weeks (n = 10), or (3) 10% casein diet for two weeks followed by a protein-free diet for the final two weeks (n = 10). Diets were formulated to be isocaloric and calcium and phosphorus concentrations were matched among the three diets. Animals were individually housed in metabolic cages during the acclimation and experimental periods. Body weights were measured and urine and fecal samples were collected on a weekly basis.

In order to assess the protein quality of the KPC, the authors determined true digestibility, true biological value, net protein utilization, protein digestion corrected for amino acid score (PDCAAS), and the protein efficiency ratio. The calculations used to derive these values are presented below, where N represents nitrogen.

True Digestibility =	[N Intake - (Fecal N – Endogenous Fecal N)] ÷ N Intake
True Biological Value =	[N Intake - (Fecal N – Endogenous Fecal N) – (Urinary N – Endogenous Urinary Nitrogen)] ÷ [N Intake - (Fecal N – Endogenous Fecal N)]
Net Protein Utilization =	[N Intake - (Fecal N – Endogenous Fecal N) – (Urinary N – Endogenous Urinary Nitrogen)] ÷ N Intake
Protein Efficiency Ratio =	Body Weight ÷ [N Intake X 6.25]
PDCAAS =	[Amount of limiting amino acids in KPC ÷ Amount of limiting amino acids in casein] × True Digestibility

The authors also characterized the lipids contained in KPC as well as the fatty acid oxidation present in the material. At the end of the experimental period the serum cholesterol, triglyceride, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) levels were assessed in blood samples collected prior to euthanasia. Following euthanasia the retroperitoneal and gonadal fat pads were removed and weighed. Additional parameters relating to the safety of KPC administration were examined and these are described in Part 6 of the dossier.

The KPC comprised of 77.7% crude protein (on a dry weight basis), 8.1% total lipids, and 4.4% total ash. The authors stated that the krill that was the source of the KPC contained similar amounts of protein, lipid and ash as had been reported in the literature. There were no significant differences in the food intake, feed efficiency, final body weight, or total body weight gain of the female rats fed the diets containing casein as compared to the female rats fed the diets containing KPC. The absolute weight of the total fat pads as well as the absolute weights of the retroperitoneal and gonadal fat pads was significantly greater in the rats fed the diets containing KPC as compared to those fed the diets containing casein. Although the authors conducted their statistical analysis on the absolute weights of the fat pads, the description of the statistical methods indicates that they actually employed a co-variate analysis to correct for body size.

Following the consumption of the diet containing casein and the diet containing KPC there was no significant difference in the PDCAAS score (both diets had a score of 1), or the protein efficiency ratio (1.57 ± 0.05 for casein *versus* 1.44 ± 0.15 for KPC). These two parameters are the preferred methods for assessing protein quality in foods intended for consumption by humans at all life stages. There were no significant difference between the true digestibility of the protein provided by casein, stated at 93.3% ± 2.0%, and the true digestibility of the protein provided by KPC, at 93.2% ± 1.0%. The biological value and net protein utilization were both significantly lower for the KPC diet as a result of decreased nitrogen retention in the rats. The authors suggested that this might have been the result of the fact that the casein diet was supplemented with DL-methionine (a limiting amino acid in protein synthesis) and the KPC diet

was not. The authors concluded that the KPC was equivalent to casein in protein quality and is suitable as a protein source in food.

Conclusion

Results of studies that have been performed in mink indicate that protein and lipid digestibility of krill meal is high (85.1% and 97.8%, respectively) and similar to other ingredients that are used as a source of protein (fish meal) or lipid (soybean). The protein and fat digestibility of krill in hogs (95.3% and 86.7, respectively) is also high, and similar to that of fish meal. In rats, krill protein concentrate is equivalent to casein in protein quality. This is supported by the significant increase in omega-3 index in dogs receiving Qrill™ Pet at 8% in the diet for up to 14 weeks, providing evidence for the lipid digestibility of krill meal. The results suggest that krill meal is a highly digestible source of protein and lipid for dogs.

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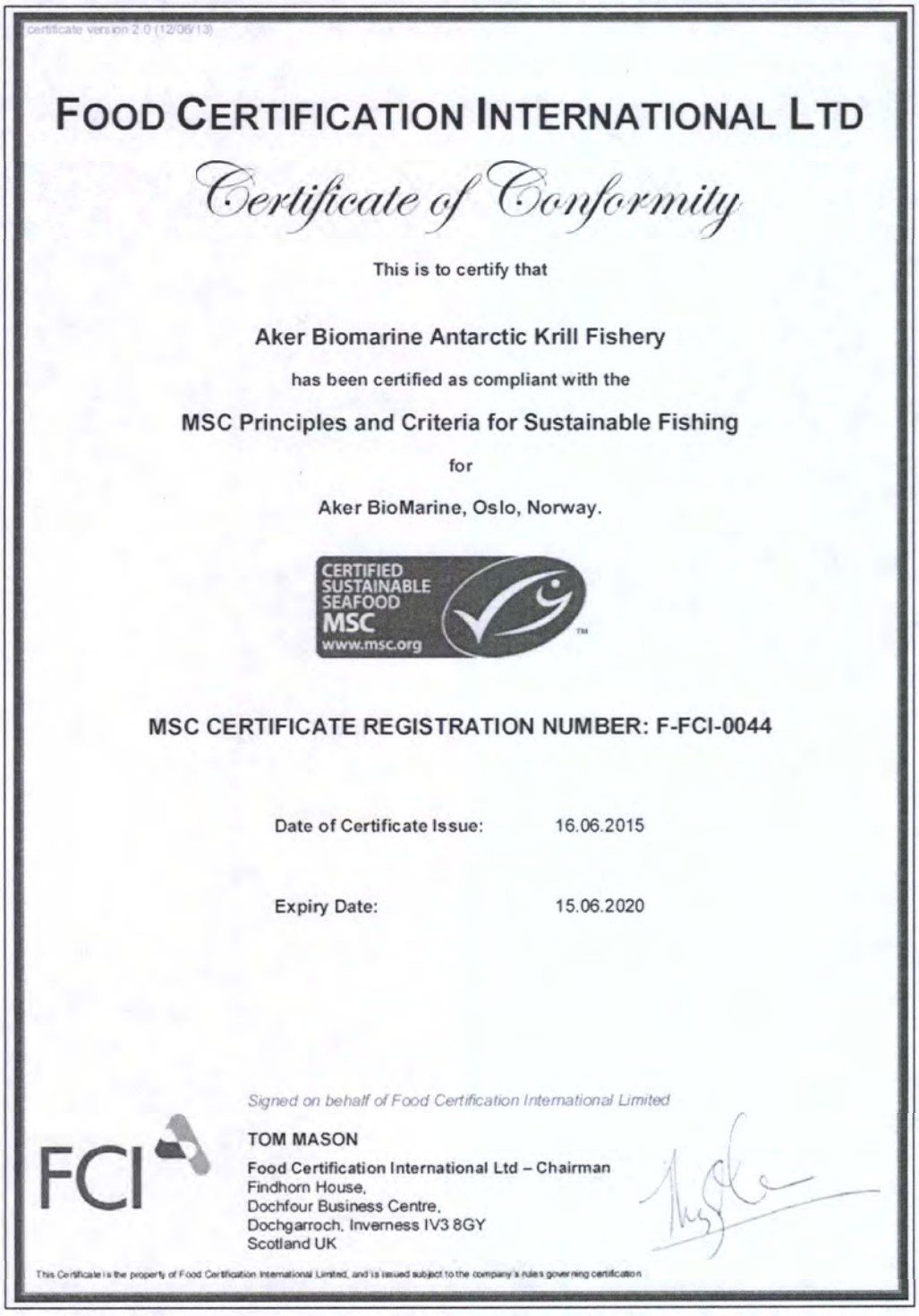
Appendix A: Certificates in the Manufacturing Process of Aker BioMarine's Qrill™ Pet

Figure A-1: Marine Stewardship Council (MSC) Fishery Certificate



certificate version 2.0 (12/06/13)

FOOD CERTIFICATION INTERNATIONAL LTD

Certificate of Conformity

This is to certify that

Aker Biomarine Antarctic Krill Fishery

has been certified as compliant with the

MSC Principles and Criteria for Sustainable Fishing

for

Aker BioMarine, Oslo, Norway.

CERTIFIED SUSTAINABLE SEAFOOD MSC www.msc.org

MSC CERTIFICATE REGISTRATION NUMBER: F-FCI-0044

Date of Certificate Issue: 16.06.2015

Expiry Date: 15.06.2020

Signed on behalf of Food Certification International Limited

FCI

TOM MASON

Food Certification International Ltd – Chairman
Findhorn House,
Dochfour Business Centre,
Dochgarroch, Inverness IV3 8GY
Scotland UK

This Certificate is the property of Food Certification International Limited, and is issued subject to the company's rules governing certification

MSC Sustainable Fishery Certificate - detail

FISHERY NAME:	Aker Biomarine Antarctic Krill Fishery
CLIENT NAME:	Aker Biomarine
MSC CERTIFICATE REGISTRATION NUMBER:	F-FCI- 0044
DATE OF FIRST CERTIFICATION:	16.06.2015
EXPIRY DATE:	15.06.2020

CHAINS OF CUSTODY

The limit of identification of landings from this fishery is to the first point of sale, whereupon fish and fish products may enter chains of custody.

UNIT OF CERTIFICATION

Please note that this is the Unit of Potential Certification for this fishery assessment (ie. that which was assessed). What is actually certified to carry the MSC ecolabel is detailed within the body of the Public Certification Report.

Species:	Antarctic Krill (*Euphausia superba*)
Stock:	Antarctic krill in Area 48
Geographical area:	Area 48, Antarctic Sea
Harvest method:	Pelagic trawl using own patented Eco-Harvesting system
Client Group:	All Aker BioMarine Antarctic vessels targeting Antarctic Krill in the Antarctic Sea area covered in Area 48, using Pelagic trawl using their own patented Eco-Harvesting system.

SCHEDULE OF APPROVED VESSELS

An up to date vessel list can be found by contacting FCI using the details below:

Name	Vessel Reg. No.
Antarctic Sea	LAWR
Saga Sea	LNSK

FCI Fisheries Department

Contact Email: fisheries@foodcertint.com

Contact Tel: +44(0)1463 223 039 (FCI main number)

Figure A-2: Norwegian Food Safety Authority's Approval of Establishments for Fish and Fishery Products

TO WHOM IT MAY CONCERN
Til den det måtte angå

Your ref:
Our ref: 2016/016148
Date: 22.01.2016
Org.no: 985 399 077

Mattilsynet

Statement concerning approval of establishments for fish and fishery products
Erklæring vedrørende godkjenning av tilvirkningsanlegg for fisk og fiskevarer

Name: *Navn:*	Aker Biomarine Antarctic A/S, factory vessel: F/T Antarctic Sea
Address: *Adresse:*	J.M. Johansens vei 99, 8340 Stamsund Norway
Approval number: *Godkjenningsnummer:*	N 2173

This statement represents a continuous certification of the approval of the above mentioned establishment and should as such be renewed yearly.
Denne erklæring er en fortløpende attestering av ovennevnte virksomhets godkjenning og må som sådan fornyes årlig.

The undersigned officials hereby certify that:
De undertegnende tjenestemenn attesterer følgende:

Fish and fishery products originating from this establishment have been handled and prepared or processed under a competent HACCP- and sanitary program consistently implemented and in accordance with U.S. FDA's seafood HACCP regulation 21 CFR 123.
Fisk og fiskevarer som kommer fra ovennevnte virksomhet har blitt håndtert og bearbeidet eller produsert under et fullverdig HACCP- og renholdsprogram, iverksatt i overensstemmelse med FDA's HACCP-forskrift 21 CFR 123 for sjømat i USA.

Norwegian Food Safety Authority
Section Midtre Haalogaland

Issued in Leknes, Norway, date 22. January 2016

Valid to December 31, 2016

Katrine Flostrand
Chief District Officer

Grethe-Kristin Hanssen
Inspector

Norwegian Food Safety Authority
Section Midtre Haalogaland

Official in charge: Grethe-Kristin Hanssen
Phone: +47 48146065
Location:Leknes
E-mail: postmottak@mattilsynet.no
(Remember recipent name)

Postal address: District office for Lofoten
P.O. Box 383
N - 2381 Brumunddal
NORWAY
Telefax: +47 23 21 68 01

AKER BIOMARINE ANTARCTIC AS AVD
STAMSUND
J.M. Johansens vei 99
8340 Stamsund

Deres ref:
Vår ref: 2014/127550
Dato: 28.05.2014
Org nr: 985399077

Statens tilsyn for planter, fisk, dyr og næringsmidler

Mattilsynet

APPENDIX FOR ANTARCTIC SEA APPROVAL NUMBER 1000774

This document applies to:

- Aker Biomarine Antarctics vessel F/T Antarctic Sea, approval number 1000774

Relevant regulations:

- FOR 2007-10-27 nr 1254: Forskrift 27. okt. 2007 nr. 1254 om animalske biprodukter som ikke er beregnet på konsum (forskrift om animalske biprodukter), jf Forordning (EF) nr. 1774/2002

F/T Antarctic Sea has approval for production of krill meal for animal consumption. This approval demands production temperature to exceed 80 degrees C.

F/T Antarctic Sea has implemented HACCP system based on internal process and product control. Aker Biomarine Antarctic A/S has provided the Norwegian Food Authorities with documentation that process temperature exceed 80 degrees Celsius.

All part of the product have been heated to a minimum of

- 90 deg. C for a period of not less than 20 minutes, or
- 80 deg. C for a period of not less than 30 minutes.

Best regard

for
Trond Kroken
Chief Officer

Vanja Jakobsen
1. Inspector.

NORWEGIAN FOOD SAFETY AUTHORITY

Figure A-3: Quality Management System Standard ISO 9001:2008 Certificate

DNV·GL

MANAGEMENT SYSTEM CERTIFICATE

Certificate No:	Initial certification date:	Valid:
110681-2012-AQ-NOR-NA	07 February 2012	07 February 2015 - 07 February 2018

This is to certify that the management system of

Aker Biomarine Antarctic AS

Fjordalléen 16, 0250 Oslo, Norway

has been found to conform to the Quality Management System standard:

ISO 9001:2008

This certificate is valid for the following scope:

Production of krill meal, oil and paste aboard F/T Saga Sea and F/V Antarctic Sea.

Place and date:
Høvik, 05 February 2015

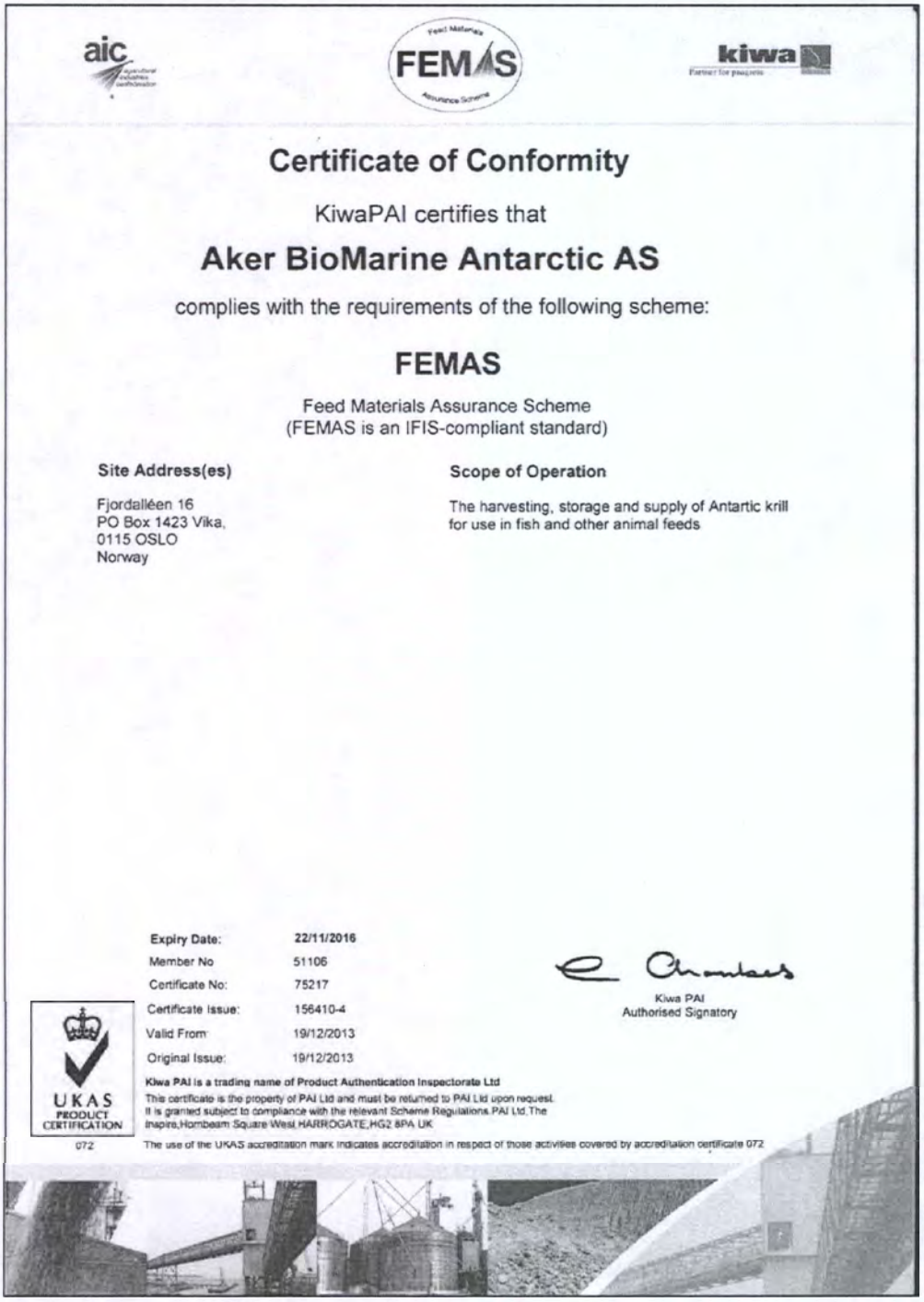
NORWEGIAN ACCREDITATION
MSYS 002

For the issuing office:
DNV GL – Business Assurance, Norway

Jøran Laukholm
Management Representative

Lack of fulfilment of conditions as set out in the Certification Agreement may render this Certificate invalid.
ACCREDITED UNIT: DNV GL Business Assurance Norway AS, Veritasveien 1, 1322 Høvik, Norway. TEL: +47 675 799 00. http://assurance.dnvgl.com

Figure A-4: Feed Materials Assurance Scheme (FEMAS) Conformity Certificate



aic

FEMAS Feed Materials Assurance Scheme

kiwa Partner for progress

Certificate of Conformity

KiwaPAI certifies that

Aker BioMarine Antarctic AS

complies with the requirements of the following scheme:

FEMAS

Feed Materials Assurance Scheme
(FEMAS is an IFIS-compliant standard)

Site Address(es)

Fjordalléen 16
PO Box 1423 Vika,
0115 OSLO
Norway

Scope of Operation

The harvesting, storage and supply of Antartic krill for use in fish and other animal feeds

Expiry Date:	**22/11/2016**
Member No	51106
Certificate No:	75217
Certificate Issue:	156410-4
Valid From:	19/12/2013
Original Issue:	19/12/2013

Kiwa PAI
Authorised Signatory

UKAS PRODUCT CERTIFICATION
072

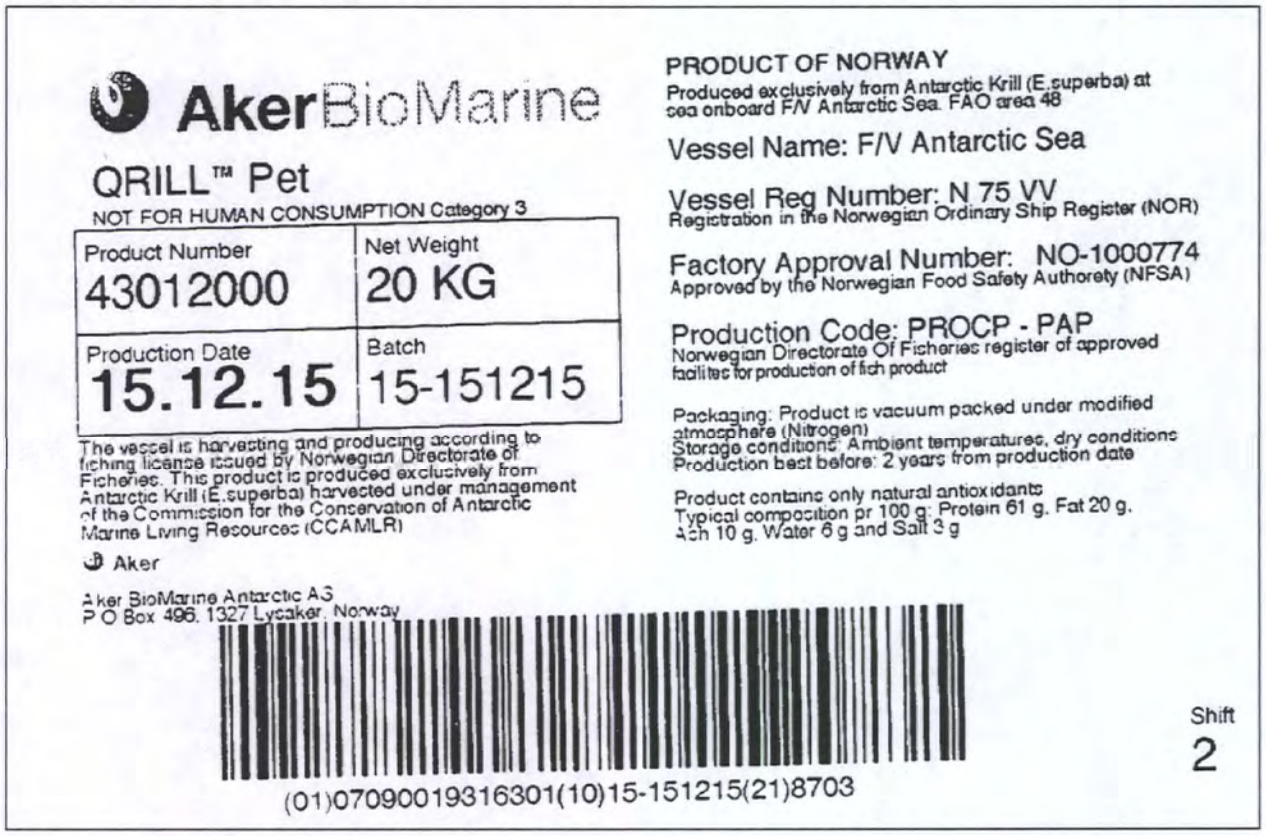
Kiwa PAI is a trading name of Product Authentication Inspectorate Ltd

This certificate is the property of PAI Ltd and must be returned to PAI Ltd upon request. It is granted subject to compliance with the relevant Scheme Regulations.PAI Ltd.The Inspire,Hornbeam Square West HARROGATE,HG2 8PA UK

The use of the UKAS accreditation mark indicates accreditation in respect of those activities covered by accreditation certificate 072

Appendix B: Example Product Labels of Aker BioMarine's QRILL™ Pet

Figure B-1: Aker BioMarine's Label for Qrill™ Pet Onboard the Fishing Vessel



AkerBioMarine

QRILL™ Pet

NOT FOR HUMAN CONSUMPTION Category 3

Product Number	Net Weight
43012000	20 KG
Production Date	**Batch**
15.12.15	15-151215

The vessel is harvesting and producing according to fishing license issued by Norwegian Directorate of Fisheries. This product is produced exclusively from Antarctic Krill (E.superba) harvested under management of the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR)

Aker

Aker BioMarine Antarctic AS
P O Box 496, 1327 Lysaker, Norway

PRODUCT OF NORWAY
Produced exclusively from Antarctic Krill (E.superba) at sea onboard F/V Antarctic Sea. FAO area 48

Vessel Name: F/V Antarctic Sea

Vessel Reg Number: N 75 VV
Registration in the Norwegian Ordinary Ship Register (NOR)

Factory Approval Number: NO-1000774
Approved by the Norwegian Food Safety Authority (NFSA)

Production Code: PROCP - PAP
Norwegian Directorate Of Fisheries register of approved facilites for production of fish product

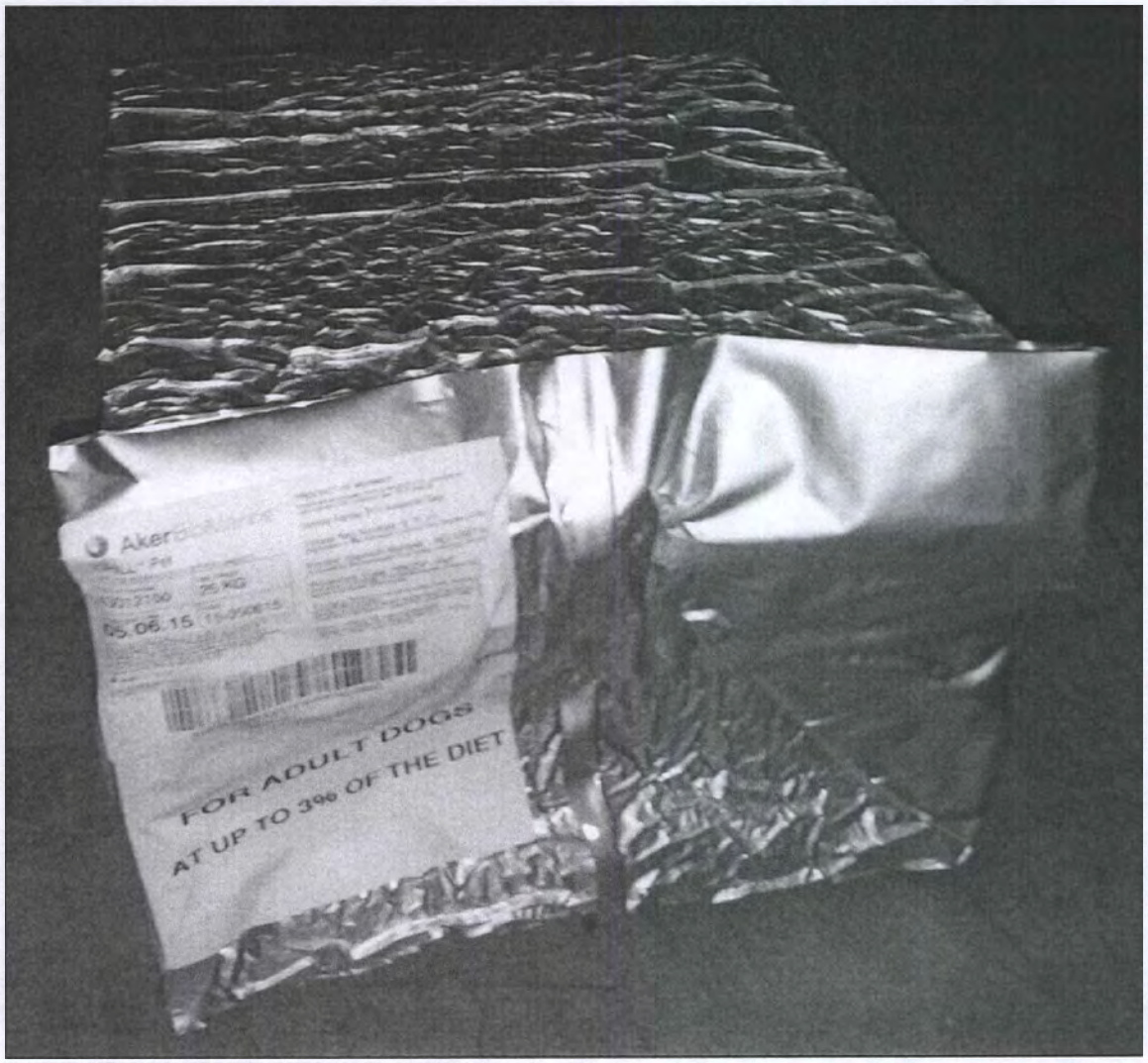
Packaging: Product is vacuum packed under modified atmosphere (Nitrogen)
Storage conditions: Ambient temperatures, dry conditions
Production best before: 2 years from production date

Product contains only natural antioxidants
Typical composition pr 100 g: Protein 61 g, Fat 20 g, Ash 10 g, Water 6 g and Salt 3 g

(01)07090019316301(10)15-151215(21)8703

Shift
2

Figure B-2: Aker BioMarine's Labels for Qrill™ Pet in Warehouse



Appendix C: Qrill™ Pet Product Specification – US

Product specification – US

The following parameters are analyzed for each batch and are a part of the CoA.

Parameter

Composition	Method	Limit	Unit
Fat	Bligh & Dyer (Internal Nofima method)	≥ 20	g/100 g
Crude Protein	ISO 16634-1	≥ 55	g/100 g
Ash	ISO 5984	≤ 13	g/100 g
Salt	AOAC 937.09	≤ 4	g/100 g
Moisture	ISO 6496	6±2	g/100 g
Astaxanthin	(b) (4)	80-160	mg/kg
Microbiology	**Method**	**Limit**	**Unit**
Total plate count	AFNOR 3M 1/1-9/89	<20 000	cfu/g
Salmonella	NordVal 023 (PCR)	Negative	1 sample à 25 g
Enterobacteriacea	ISO 21528-2	<300	cfu/g
Mould & Yeast	NordVal 016	<100	cfu/g
Freshness	**Method**	**Limit**	**Unit**
Total Volatile Nitrogen	AOAC 920.03	≤0.3	g/100 g
Cadaverine	(b) (4)	<10	mg/kg
Histamine	(b) (4)	<10	mg/kg
Peroxide	AOCS Cd 8b-90	<10	mEq peroxide/kg
Meat Bone Meal	2009/152/EC	Not present	
Mineral Level	**Method**	**Limit**	**Unit**
Fluorine	(b) (4)	≤1500	mg/kg

CERTIFIED SUSTAINABLE SEAFOOD MSC www.msc.org MSC-C-52983

The following parameters are analyzed for three random batches each year and are not part of the CoA, but reported on separate document:

Parameter

Typical Minerals Level	Method	Typical content	Unit
Phosphorous	ICP-SFMS/DIN EN ISO 17294-2-E29	<2	g/100 g
Calcium	ICP-SFMS/DIN EN ISO 111885, mod.	<3	g/100 g
Magnesium	ICP-SFMS/NMKL No 139 1991 mod.	<1	g/100 g
Iron	ICP-SFMS/DIN EN ISO 11885, mod.	60	mg/kg
Copper	ICP-SFMS/DIN EN ISO 11885, mod.	<80	mg/kg
Iodine	ICP-MS/EN 15111	<50	mg/kg
Typical Lipid Composition	**Method**	**Typical content**	**Unit**
Total phospholipids	(b) (4)	≥35	g/100 g extracted fat
Triglycerides		≤50	g/100 g extracted fat
FATTY ACID PROFILE			
Total omega-3	AOCS Ce 1b-89	≥17	g/100 g extracted fat
-C 20:5 n-3 (EPA)	AOCS Ce 1b-89	≥10	g/100 g extracted fat
-C 22:6 n-3 (DHA)	AOCS Ce 1b-89	≥5.5	g/100 g extracted fat
Heavy Metals and Dioxines Levels	**Method**	**Limit**	**Unit**
Cadmium	ICP-SFMS/EN 15763:2009	<1	mg/kg
Mercury	ICP-SFMS/§64 LFGB L00.00-19/4	<0.1	mg/kg
Lead	ICP-SFMS/EN 15763:2009	<0.05	mg/kg
Sum of dioxins (WHO-PCDD/F-TEQ)	US EPA 1613/EC Reg 589/2014 (food) and EC Reg709/2014 (feed)	<1.25	ng/kg
Sum of dioxins and dioxin-like PCBS (WHO-PCDD/F-PCB-TEQ)	US EPA 1668/EC Reg 589/2014 (food) and EC Reg709/2014 (feed)	<4.0	ng/kg
Arsenic Levels	**Method**	**Limit**	**Unit**
Arsenic, in-organic	(b) (4)	<0.5	mg/kg
Arsenic, Organic	ICP-SFMS/DIN EN ISO 11885, mod.	<8	mg/kg

CERTIFIED SUSTAINABLE SEAFOOD MSC www.msc.org MSC-C-52985

The following parameters are analyzed in one random batch each year and are not part of the CoA, but reported on separate document:

Parameter

Typical Levels For Miscellaneous Parameters	Method	Typical content	Unit
Chromium	ICP-SFMS/DIN EN ISO 17294-2-E29	0.9	mg/kg
Aluminium	ICP-SFMS/DIN EN ISO 11885, mod.	90	mg/kg
Melamin	Internal NOFIMA/EUROFINS method	<0.1	mg/kg
Typical Amino Acid Composition (only one batch tested each year)	**Method**	**Typical content**	**Unit**
Aspartic Acid	(b) (4)	6	g/100 g
Glutamic Acid		7	g/100 g
Hydroxyproline		0.05	g/100 g
Serine		2	g/100 g
Glycine		3	g/100 g
Histidine		1	g/100 g
Arginine		3	g/100 g
Threonine		2	g/100 g
Alanine		3	g/100 g
Proline		3	g/100 g
Tyrosine		2	g/100 g
Valine		3	g/100 g
Methionine		2	g/100 g
Isoleucine		3	g/100 g
Leucine		4	g/100 g
Phenylalanine		3	g/100 g
Lysine		4	g/100 g
Cysteine/Cystine		2	g/100 g
Tryptophane		1	g/100 g
Taurin		0.3	g/100 g

The following parameters are analyzed in one random batch each year and are not part of the CoA, but reported on separate document:

Parameter

Typical Levels For Vitamins (only one batch tested each year)	Method	Typical content	Unit
A	(b) (4)	1170	µg/100 g
D3		<0.5	µg/100 g
E		9	mg/100 g
K1		1	µg/100 g
C		<0.5	mg/100 g
B1		0.02	mg/100 g
B2		0.03	mg/100 g
B3		3	mg/100 g
B5		0.4	mg/100 g
B6		0.1	mg/100 g
B8		71	µg/100 g
B9		35	µg/100 g
B12		5	µg/100 g

CoA: CoA is issued for each batch. A batch is one production date. The actual values analysed and reported on the CoA will fall within the levels as specified by the tolerances set down in Annex IV of legislation (EC) No 767/2009, independent of batch to batch variations.

Parameters not part of the CoA: The parameters that are tested randomly are reported on a separate report and updated every time new data are available. This can be handed out to customers.

Application: For adult dogs at up to 3% of the finished feed.

Packaging: The product is packed in laminated metalized bags with vacuum.

Storage: The product is best kept at temperatures < 25°C during storage in its original packaging container.

Contact information:
web: www.akerbiomarine.com / www.qrill.com
e-mail: sales@akerbiomarine.com

CERTIFIED SUSTAINABLE SEAFOOD MSC www.msc.org MSC-C-53985

Appendix D: Measurements of Environmental Pollutants in Qrill™ Pet

Chemical	Results (2 lots)
Dioxins and furans	**ng/kg MC12%**
2,3,7,8-TetraCDD	<0.01
1,2,3,7,8-PentaCDD	<0.02
1,2,3,4,7,8-HexaCDD	<0.02
1,2,3,6,7,8-HexaCDD	<0.03
1,2,3,7,8,9-HexaCDD	<0.03
1,2,3,4,6,7,8-HeptaCDD	<0.05
OctaCDD	<0.36
2,3,7,8-TetraCDF	<0.09
1,2,3,7,8-PentaCDF	<0.09
2,3,4,7,8-PentaCDF	<0.03
1,2,3,4,7,8-HexaCDF	<0.06
1,2,3,6,7,8-HexaCDF	<0.03
1,2,3,7,8,9-HexaCDF	<0.06
2,3,4,6,7,8-HexaCDF	<0.03
1,2,3,4,6,7,8,-HeptaCDF	<0.03
1,2,3,4,7,8,9-HeptaCDF	<0.02
OctaCDF	<0.07
WHO (2005)-PCDD/F TEQ (excluding LOQ)	ND
WHO (2005)-PCDD/F TEQ (including LOQ)	0.078
Polychlorinated biphenyls (dioxin like)	**ng/kg MC12%**
PCB 77	<1.11-2.14
PCB 81	<0.15-0.17
PCB 105	<2.40-3.68
PCB 114	<0.29-0.33
PCB 118	<8.60-11.2
PCB 123	<0.25-0.27
PCB 126	<0.14-0.25
PCB 156	<1.35-<1.36
PCB 157	<0.25-0.26
PCB 167	<0.68
PCB 169	<0.74
PCB 189	<0.25
WHO (2005)-PCB TEQ excluding LOQ	0.000008-0.025
WHO (2005)-PCB TEQ including LOQ	0.037-0.048
WHO-PCDD/F+PCB TEQ	**ng/kg MC12%**
WHO (2005)-PCDD/F TEQ (excluding LOQ)	0.000008-0.025
WHO (2005)-PCDD/F TEQ (including LOQ)	0.115-0.126
Polychlorinated biphenyls (non-dioxin like) (ICES- 6)	**µg/kg MC12%**

PCB 28	0.10-0.20
PCB 52	<0.6-0.6
PCB 101	<0.6
PCB 138	<0.6
PCB 153	<0.6
PCB 180	<0.6
Sum 6 DIN-OCB (excluding LOQ)	0.10-0.26
Sum 6 DIN-OCB (including LOQ)	0.41-0.51
Polycyclic Aromatic Hydrocarbons (EPA 13)	**µg/kg**
Anthracene	<0.5
Benzo[a]anthracene	<0.5
Benzo[a]pyrene	<0.5
Benzo[b]fluoranthene	<0.5
Benzo[ghi]perylene	<0.5
Benzo[k]fluoranthene	<0.5
Dibenz[a,h]anthracene	<0.5
Phenanthrene	<0.5 – 8.7
Fluoranthene	<0.5 – 0.8
Fluorene	<0.5 - 14
Indeno[1,2,3-cd]pyrene	<0.5
Chrysene	<0.5
Pyrene	<0.5 – 0.8
Sum PAH (>=5 ring)	Not present
Sum PAH	Not present - 24
Sum PAH 4	Not present

BDE = brominated diphenyl ether; CDD = chlorinated dibenzodioxins; CDF = chlorinated dibenzofurans; EPA = Environmental Protection Agency; ICE = International Council for the Exploration of the Seas; LOQ = limit of quantitation; PCB = polychlorinated biphenyls; MC 12% = feed containing 12% moisture; PCDD/F = dioxins; TEQ = total dioxin-like toxic equivalents; WHO = World Health Organization

TAB

PART 3:

TARGET ANIMAL AND HUMAN EXPOSURES

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 3: Target Animal and Human Exposures

Prepared for:
Aker BioMarine

November 29, 2016

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I. OVERVIEW

In this part, we calculated the estimated daily intake (EDI) of Qrill™ Pet for adult dogs. In addition, we calculated EDIs for fluorine and astaxanthin from all sources (Qrill™ Pet, other ingredients in food, and/or drinking water), which are components of toxicological concern in krill meal. Based on these calculatinos, the EDIs for Qrill™ Pet, fluoride (all sources) and astaxanthin (all sources) for an adult dog are 0.86g/kg bw/day, 1.6 mg/kg bw/day, and 1.6 mg/kg bw/day, respectively. As dogs are not food-producing animals, human exposures through consumption of human food derived from food-producing animals are not calculated.

II. ESTIMATED DAILY INTAKE OF QRILL™ PET

As part of a food safety risk assessment, consumption of a proposed food ingredient (*i.e.*, Qrill™ Pet) at a proposed use level in a certain food (*i.e.*, dog or cat food) is determined. Pursuant to this end, Aker BioMarine has indicated that the intended use level of Qrill™ Pet is up to 30,000 mg/kg (30,000 ppm or 3%) in adult dog food. The intake level of Qrill™ Pet can be calculated based on the metabolizable energy (ME) requirement of dogs and the ME of Qrill™ Pet.

a. Metabolizable Energy of Qrill™ Pet

According to National Research Council (NRC 2006), the equation to estimate ME for food ingredients for dogs is:

$$ME\ (kcal) = (4 \times protein) + (9 \times fat) + (4 \times NFE)$$

Where:

NFE = Nitrogen free extract, represents starch, sugar and non-starch polysaccharides that get solubilized upon cooking in diluted alkalies and acids).

Based on the mean measured values from five batches (Table 2 of Part 2), Qrill™ Pet contains 59.3% protein (i.e., 0.593 g/g), 23.5% fat (i.e., 0.235 g/g) and negligible NFE. Therefore,

$$ME_{Qrill\ Pet}\ (kcal/g) = (4 \times protein\ (g)) + (9 \times fat\ (g)) + (4 \times NFE\ (g))$$

$$= (4 \times 0.593\ g/g) + (9 \times 0.235\ g/g) + (4 \times 0)$$

$$= 4.487\ (kcal/g)$$

b. Metabolizable Energy of Dog Food

The ME of dog food varies by the composition of nutrients. However, NRC (2006) assumes a caloric density of 4,000 kcal ME/kg dry food (i.e., 4.0 kcal/g) for dog food in the calculation of nutrient requirements. This default caloric density is also used by AAFCO (2014).

c. Metabolizable Energy of Dog Food Containing 3% Qrill™ Pet

Qrill™ Pet is added at up to 3% in dry food for adult dogs. Assuming that Qrill™ Pet is added to a formulated diet instead of replacing any specific existing ingredients intended to provide

protein or fat (such as fish meal and meat), the ME of dog food containing 3% Qrill™ Pet is calculated below.

$ME_{Food} = ME_{Qrill\ Pet} \times 3\% + ME_{Baseline} \times 97\%$

Where:

$ME_{Qrill\ Pet}$ = ME of Qrill™ Pet, calculated as 4.487 kcal/g above;
$ME_{Baseline}$ = ME of dry dog food to which Qrill™ Pet is added, assumed to be 4.0 kcal/g according to NRC (2006).

Therefore,

$ME_{Food} = 4.487\ kcal/g \times 3\% + 4.0\ kcal/g \times 97\% = 4.015\ kcal/g$

d. Daily Metabolizable Energy Requirement of Dogs

According to NRC (2006), the daily ME requirement of dogs differs based on the size, breed, and activity levels. The body weight of dogs can range from 1 kg to more than 90 kg, and energy requirements for dogs are closely related to body weight raised to the power of 0.75 (NRC 2006). Consequently, NRC recommended different equations to calculate daily metabolizable energy requirements for adult dogs at maintenance (Table 15-4 of NRC 2006). Average active pet dogs require 130 kcal × $BW^{0.75}$ per day, while young adult active pet dogs, active pet Great Danes (large breed), and active pet terriers require ME greater than the average. As small dogs with the highest energy levels consume the highest amount of food on a body weight basis, they are expected to be exposed to the highest levels of Qrill™ Pet. Therefore, the equation for laboratory terriers and active pet terriers was selected to derive a conservative EDI (NRC 2006):

$ME_{Required}\ (kcal) = 180 \times BW^{0.75}$

Where:

BW = Body weight of pet terriers. The NRC did not specify what breeds "pet terriers" refer to. A literature search was performed to identify the studies cited by NRC in support of the equation for pet terriers, but most of the studies were published 15 years ago, and not all of them were publically available. However, it appeared that Cairn terrier was the most commonly studied terrier breed among these studies (Burger and Johnson 1991, Kienzle and Rainbird 1991). The ideal weights of adult male and female Cairn terriers are 14 and 13 pounds, respectively, according to American Kennel Club (1938). These correspond to 6.4 and 5.9 kg for males and females, respectively. The smaller weight of 5.9 kg for females was used for subsequent calculations as this will yield a more conservative EDI.

e. EDI of Dog Food Containing 3% Qrill™ Pet

$$EDI_{Food}\ (g/kg\ bw) = \frac{ME_{Required}\ (kcal)}{ME_{Food}\ (kcal) \times BW\ (kg)}$$

$$= \frac{180 \times BW^{0.75}}{ME_{Food} \times BW}$$

$$= \frac{180}{ME_{Food} \times BW^{0.25}}$$

Where:

ME_{Food} = ME of dog food containing 3% Qrill™ Pet, calculated as 4.015 kcal/g previously;
BW: Body weight of pet terriers. As previously discussed, the body weight of 5.9 kg for a female Cairn terrier (American Kennel Club 1938) is used to derive the EDI to obtain a more conservative value.

Therefore,

$$EDI_{Food}\ (g/kg\ bw) = \frac{180}{ME_{Food} \times BW^{0.25}}$$

$$= \frac{180}{4.015 \times 5.9^{0.25}}$$

$$= 28.77\ (g/kg\ bw)$$

f. EDI of Qrill™ Pet

As Qrill™ Pet is added at 3% in dog food based on the above calculations, the estimated daily intake of Qrill™ Pet is calculated below:

$EDI_{Qrill\ Pet} = EDI_{Food} \times 3\% = 28.77 \times 3\% = 0.86$ (g/kg bw/day)

III. ESTIMATED DAILY INTAKE OF FLUORINE

a. Fluorine intake from Qrill™ Pet

The addition of Qrill™ Pet to dog food is limited due to the naturally high level of fluorine, which causes dental and/or skeletal fluorosis at high doses (see Part 6 of the dossier for discussion on fluorine toxicity).

$EDI_{QrillF} = EDI_{Qrill\ Pet} \times$ F Concentration in Qrill™ Pet

Where:

$EDI_{Qrill\ Pet}$ is the estimated daily intake of Qrill™ Pet when added at 3% in the dry food for adult dogs. As previously calculated, the $EDI_{Qrill\ Pet}$ was estimated to be 0.86 g/kg bw;
F Concentration in Qrill™ Pet is the maximum fluoride content in Qrill™ Pet. Qrill™ Pet contains up to 1,500 ppm fluoride *per* specifications.

Therefore,

EDI_{QrillF} = 0.86 g/kg bw × 1,500 × 10^{-6} × 10^{3} mg/g = **1.29 mg/kg bw**

This is the fluoride intake from Qrill™ Pet when added at 3% in dry dog food for an active pet terrier.

b. Fluorine intake from other ingredients in dog food

Other ingredients in incorporated into dog food may also contain fluorine. Very little information is available concerning levels of fluorine in dog food. Two major sources of fluorine in animal feed are ingredients of marine origin (such as fish meal), and local environmental contaminations (such as alumina smelters) (Siebert and Trautner, 1985). In farmed animals, the primary sources of fluoride are phosphorus supplements (containing 2 – 5% fluorine depending on the origin and manufacturing processes, or ≤ 1% for defluorinated phosphorus) and feed ingredients of animal origin (NRC 2005). Another source suggested that the fluorine content come from various animal by-product meals (e.g., chicken meal, lamb meal, beef and bone meal) (EWG 2009).

In 1970-1980s, Shetland Sheepdogs from a kennel in Allegany County of Michigan had increased incidences of perinatal deaths, deformations, mottled teeth, and bony exostoses, and the commercial dog food was found to contain 460 ppm fluorine (Shellenberg et al. 1990). While a later reproductive toxicity study in dogs indicated that the reproductive effects were not attributed to fluorine, bone effects were the result of excess fluorine in the food (Shellenberg et al. 1990). To investigate these adverse effects, Michigan State's Toxic Substance Control Commission conducted tests to determine fluorine levels in commercial dog foods, and found that most dry pet foods contain > 20 ppm fluorine, with one dry dog food sample containing 1,000 ppm fluorine. A bone-meal supplement used as an additive for dog food contained 1,700 ppm fluorine, and the mineral supplement rock phosphate contains up to 2,000 ppm fluorine (most ranged from 500 – 1,500 ppm). The State called for voluntary reduction of fluorine in dog foods (Fluoride Action Network 1981).

An article published in 1986 (Mumma *et al.* 1986) reported the fluorine content of 35 different commercial dog foods averaged 27.7 mg/kg feed (i.e., ppm), which ranged from 2.4 – 116 ppm. Only one sample had the highest fluorine content of 116 ppm, which is made from meat by-products with the major ingredients as lamb and poultry by-products. The next highest fluorine content was 74 ppm for a dog food based on corn, meat, bone meal, and soybean meal.

In previously published dog studies described above, basal diets contain various levels of fluorine (10 ppm in Henrikson et al. 1970 and 50 ppm in Shellenberg et al. 1990).

Siebert and Trautner analyzed fluoride content in eight brands of dog food in Germany on the market in 1985. These brands all indicated the use of osseous material or substances of marine origin like fish on their labels. The reported fluoride content ranged from 2.58 to 101 ppm, with the mean value of 34 ppm. The highest fluorine content of 101 ppm was only found in one product, and the next highest fluorine content is 65.1 ppm (Siebert and Trautner 1985).

In 2009, The Environmental Working Group (EWG), a nonprofit environmental research organization based in Washington, D.C., provided online a summary of a study evaluating the fluorine content of ten major national brands of dry dog food products (EWG 2009). The testing was conducted according to AOAC International method 944.08 (A method that extracts fluoride

with perchloric acid distillation from ashed samples and detected with colorimetry, applicable to determine total fluoride from food) at Covance Laboratories (Madison, WI), with a limit of detection at 0.02 mg/kg fluoride. Fluoride levels ranged between 7 – 11.2 mg fluoride/kg feed in eight of the ten feeds, with an average of 8.9 mg/kg fluoride for the eight major national brands marketed for both puppies and adults. The other two brands (one with vegetarian ingredients only, and one made by a small manufacturer) did not contain any detectable fluoride. The results from this report are summarized in Table 1 below.

Table 1: Fluorine Content of Dog Food Reported by EWG (2009)

Target Dog Population	Fluoride Concentration (mg/kg)	Meat by-product Ingredients
All life stages	< 0.2	None
All life stages	7.44	Chicken meal
All life stages	8.41	Chicken meal, Turkey meal, Lamb meal
All life stages	10.3	Beef and bone meal
Adult	7.56	Poultry by-product meal, Lamb meal
Adult, all breeds	<0.2	None
Adult, active	10	Chicken by-product meal
Adult, large breed	9.02	Chicken meal
Puppy, large breed	7	Chicken meal, Lamb meal
Puppy, large breed	11.2	Chicken by-product meal, Chicken meal

The available information over time indicates that fluorine levels have decreased since the Allegheny incidence in the late 1970s. It also appeared that commercial dry dog food examined by EWG (2009) contains less fluorine (up to 11.2 ppm) than some of the laboratory diets (up to 59 ppm). Therefore, more recent data on fluorine content are more relevant to this assessment. In addition, dog food for pets are considered more relevant than dog food for laboratory dogs as the activity levels, nutrition requirements and energy requirements are different. Therefore, the highest reported fluorine level of 10.3 ppm among the tested dog foods for all life stages and adults from the EWG report (2009), which is the most recent report of fluoride levels in pet dog food identified in the literature, is used to represent the baseline fluoride levels in dog food for EDI calculation.

$EDI_{Baseline\ F} = EDI_{Food} \times$ F Concentration

Where:

EDI_{Food} is the estimated daily consumption of food for an active Cairn terrier, which was previously calculated to be 28.77 g/kg bw;
F Concentration is the concentration of fluoride in dog food, which is 10.3 ppm as discussed above.

Therefore,

$EDI_{Baseline\ F} = 28.77$ g/kg bw $\times 10.3 \times 10^{-6} \times 10^{3}$ mg/g = **0.296 mg/kg bw**

c. Fluorine intake from drinking water

Fluorine is added to community drinking water systems in the United States to attain a recommended level of 0.7 mg/L[1]. A dog typically consumes one ounce of water *per* pound body weight (*i.e.*, 65.13 ml/kg bw/day)[2], which would result in consumption of **0.046 mg/kg bw/day**, which is not a significant intake of fluorine compared to food.

d. Total fluorine intake

Qrill™ Pet would be expected to replace a portion of these animal by-product meals. Under the worst case scenario, if there were no replacement of the current animal by-product meals with Qrill™ Pet, the overall amount of fluorine in the dog food is summed using the equation below.

$EDI_F = EDI_{QrillF} + EDI_{BaselineF} + EDI_{WaterF}$

Where:

EDI_{QrillF} is the estimated daily intake of fluoride from Qrill™ Pet added at 3% to dry food for adult dogs. This was previously calculated to be 1.29 mg/kg bw;
$EDI_{BaselineF}$ is the estimated daily intake of fluoride from other ingredients in dog food. This was previously calculated to be 0.296 mg/kg bw;
EDI_{WaterF} is the estimated daily intake of fluoride from water. This was calculated to be 0.046 mg/kg bw.

Therefore,

EDI_F = 1.29 mg/kg bw + 0.296 mg/kg bw + 0.046 mg/kg bw = **1.6 mg/kg bw (rounded)**

The above fluorine exposure estimations are conservative (i.e., overestimation) for the following reasons: 1) Qrill™ Pet is expected to replace at least portion of other ingredients containing F (e.g., animal by-product ingredients) in the food due to its high protein and lipid content, while the calculation above assumes no replacement; 2) The calculation above assumes the product contains 1,500 ppm fluorine. However, Qrill™ Pet likely contains lower levels of fluorine than required (1,500 ppm) on the product specification. As reported by an independent analytical lab, Qrill™ Pet harvested in 2015 (n = 55 batches) contains 740 – 1,300 ppm fluorine with the mean of 990 ppm (Aker BioMarine 2016). Overestimating total fluorine exposure ensures that even under worst case scenarios, dry food containing 3% Qrill™ Pet would be considered safe for adult dogs.

IV. ESTIMATED DAILY INTAKE OF ASTAXANTHIN

a. Astaxanthin from Qrill™ Pet

According to the specification of Qrill™ Pet, the ingredient contains 80 – 160 ppm astaxanthin. Based on the analysis of five sample batches, the astaxanthin levels ranged between 89 and 129

[1] http://www.cdc.gov/fluoridation/faqs/; site last visited May 13, 2016.
[2] http://pets.webmd.com/dogs/my-dog-always-thirsty; site last visited May 13, 2016.

ppm with the mean of 112 ppm (Table 2 of Part 2). In order to estimate the worse-case (i.e., highest) daily intake, the upper limit of 160 ppm was used. Estimated daily intake of astaxanthin from Qrill™ Pet is calculated using the equation below.

$EDI_{QrillATX} = EDI_{Qrill\ Pet} \times$ ATX Concentration

Where:

$EDI_{Qrill\ Pet}$ is the estimated daily intake of Qrill™ Pet when added at 3% in the dry food for adult dogs. As previously calculated, the $EDI_{Qrill\ Pet}$ was estimated to be 0.86 g/kg bw;
ATX Concentration is the content of astaxanthin in Qrill Pet, which is up to 160 ppm based on the product specification.

Therefore,

$EDI_{QrillATX} = 0.86$ g/kg bw/day $\times 160 \times 10^{-6} \times 10^{3}$ mg/g = **0.14 mg/kg bw/day**

b. Astaxanthin from other ingredients in dog food

Astaxanthin is naturally occurring in seafood (e.g., salmon, red fish, and shell of shrimp, krill and lobster), and is also enriched in *Haematococcus* algae and Phaffia yeast (EAS 2009). Therefore, other ingredients in dog food may also contain astaxanthin. The most common sources are salmon and trout. A search of dog food containing shrimp, lobster, *Haematococcus* algae and Phaffia yeast did not reveal any results, indicating that they are not common ingredients in dog food. According to the literature, various species of salmon contain higher levels of astaxanthin compared to rainbow trout and Arctic charr (EFSA 2005). Commercially farmed Atlantic salmon contains 6 – 8 ppm astaxanthin, and cannot exceed 10 mg/kg flesh, whereas wild salmon caught along the Pacific coast of United States contain on average 13.8 ppm astaxanthin, with the highest level reported as 58 mg/kg flesh in Sockeye salmon (EFSA 2005, Turujman et al. 1997). Limited information was identified regarding the relative amount of astaxanthin in salmon and krill, but one source reports that krill oil contains 2.7 times higher astaxanthin than Wild Alaskan Sockeye Salmon oil (VitalChoice 2016).

No information was located regarding the baseline level of astaxanthin in dog food, as it is not an essential nutrient in food. No information was available describing astaxanthin level in processed salmon meal or the level of salmon meal used in dry dog food. One patent application recommends adding AstaReal 50 F oil, which contains 5% astaxanthin, at 0.1% in dry dog food to achieve the claimed benefits (deodorizing urine and feces, improving sleep, sensibility and visual sense, and preventing, treating and improving diabetes and diabetic complications) in dogs (Honda and Takahashi 2011, Uragami et al. 2012). This leads to a level of 0.005% astaxanthin in dry dog food[3]. Based on the previously estimated EDI of 28.77 g/kg bw/day dog food, this gives rise to an **$EDI_{BaselineATX}$ of 1.44 mg astaxanthin/kg bw/day**[4].

A literature search was also conducted to identify astaxanthin supplementation levels for dogs, but very limited information was available to derive an estimated EDI from these supplements.

[3] 0.1% * 5% = 0.005%
[4] 28.77 g/kg bw/day * 0.005% * 1,000 mg/g = 1.44 mg/kg bw/day

One canine antioxidant supplement Asta Zan 14 K9 currently sold in the U.S. provides 49 mg astaxanthin per jar (3.80 oz), and the recommended daily intake levels are ½ tsp for small dogs (< 20 lbs), 1 tsp for medium dogs (21 – 49 lbs), and 2 tsp for large dogs (> 50 lbs) (BioStar 2016). As the smallest dogs are expected to be exposed to the highest level of astaxanthin, the ½ tsp intake level in combination with the body weight of a female Cairn terrier (5.9 kg, American Kennel Club 1938) was used to derive an EDI of 0.18 mg/kg bw/day[5].

Based on the evaluation above, the higher and hence more conservative EDI of 1.44 mg/kg bw/day derived based on a recommended astaxanthin supplementation level of 0.005% in dry food (Honda and Takahashi 2011) was used to conservatively represent the baseline astaxanthin intake from other sources in food. This is likely an overestimation of astaxanthin in dog food, as most dog foods do not contain any ingredients rich in astaxanthin except those with salmon and trout as ingredients. Even food containing salmon is unlikely to contain astaxanthin as high as 1.44 mg/kg bw/day as derived from the recommendations in the patent application, since astaxanthin supplementation at the recommended level in the patent is considered a novel approach to improve the health of pets raised with traditional pet foods.

c. Total astaxanthin intake

The total intake of astaxanthin is calculated by summing up the intake from Qrill™ Pet and the intake from other ingredients in dry dog food, as shown below.

$EDI_{ATX} = EDI_{Qrill\ ATX} + EDI_{BaselineATX}$

= 0.14 mg/kg bw/day + 1.44 mg/kg bw/day

= 1.6 mg/kg bw/day (rounded)

Where:

EDI_{ATX} is the total daily intake of astaxanthin (ATX);
$EDI_{QrillATX}$ is the amount of astaxanthin ingested from Qrill™ Pet added at 3% to dry food, which was calculated to be 0.14 mg/kg bw/day previously;
$EDI_{BaselineATX}$ is the amount of astaxanthin ingested from other ingredients in dog food, which was conservatively estimated above from a recommended supplementation level to achieve health benefits in dogs.

[5] 49 mg/3.8 oz. * 1/6 oz/tsp. * ½ tsp ÷ 5.9 kg = 0.18 mg/kg bw/day

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TAB

PART 4:

SELF-LIMITING

LEVELS OF USE

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 4: Self-limiting Levels of Use

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

Jennifer G. Fleischer, Ph.D., M.H.S.
Bonnie Ransom Stern, Ph.D., M.P.H.
Raymond York, Ph.D. D.A.B.T, F.A.T.S, E.R.T.

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Part 4: Self-limiting Levels of Use

The use of Qrill™ Pet in dry dog food is not self-limiting by palatability or technical feasibility. However, the use level is limited due to the relatively high levels of fluorine (up to 1,500 ppm according to product specification) in the krill meal, which may be of toxicological concern at high inclusion levels in food. Fluorine levels are closely monitored in Qrill™ Pet to ensure compliance with the product specification. The toxicity of fluorine and demonstration of its safety when Qrill™ Pet is included at 3% by weight in dry food for adult dogs are discussed in detail in Part 6.

TAB

PART 5:

EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 5: Experience Based on Common Use in Food Before 1958

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

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Part 5: Experience Based on Common Use in Food Before 1958

This part is not applicable to the GRAS status determination of Qrill™ Pet, as the GRAS status is determined through scientific procedures in accordance with 21 CFR 570.30(a) and (b), rather than common use in animal food prior to January 1, 1958.

TAB

PART 6:

NARRATIVE

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 6: Narrative

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

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EXECUTIVE SUMMARY

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel),[1] qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, was requested by Aker BioMarine Antarctic (hereinafter referred to as Aker BioMarine) to determine the Generally Recognized As Safe (GRAS) status use of *Euphausia superba* (krill) meal (Qrill™ Pet) as a source of protein and lipid in food for adult dogs, based on scientific procedures.

All aspects of the production of Qrill™ Pet are consistent with good manufacturing practices. Results of studies that have been performed in mink and swine indicate that protein and lipid digestibility of krill meal is high and similar to other ingredients that are used as a source of protein (fish meal) or lipid (soybean).

Qrill™ Pet is to be added to dry adult dog food at up to 30,000 mg/kg food (3%) finished feed, such that the maximum estimated daily intake (EDI) is 860 mg/kg bw/day in dogs. This intake is below the acceptable daily intake (ADI) of 950 mg/kg bw/day for krill meal with a much higher fluorine content than Qrill™ Pet as established in a series of reproduction, lactation and growth studies in mink and supported by data in dogs and rats.

The use rate in adult dog food is limited due to the concentration of fluorine (a known toxicant) in Qrill™ Pet (up to 1,500 ppm). Use of 3% Qrill™ Pet in dog food will result in a maximum fluorine exposure from Qrill™ Pet of 1.3 mg/kg bw/day in dogs. The total daily fluorine intake from Qrill™ Pet, other ingredients in dog food, and drinking water is estimated to be up to 1.6 mg/kg bw/day under very conservative assumptions. This exposure level is below the ADI of 3.5 mg/kg bw/day established based on a NOAEL of 5.2 mg/kg bw/day for fluorine in dogs and supported by studies in mink and rats.

For astaxanthin, the NOAEL of 158 mg/kg bw/day established in a 52-week study in dogs leads to the establishment of an ADI of 53 mg/kg bw/day. This value is much higher than the EDI of 1.6 mg/kg bw/day both from consumption of Qrill™ Pet at 3% and from other ingredients in food.

The totality of the evidence indicates that use of 3% Qrill™ Pet in dry adult dog food would meet the reasonable certainty of safety standard for a GRAS determination.

[1] Modeled after that described in Section 201(s) of the Federal Food, Drug, and Cosmetic Act, as amended.

I. SAFETY DATA ON KRILL MEAL AND ITS COMPONENTS

The safety of Qrill™ Pet for dogs is demonstrated by results of a sequential gestation, lactation and growth study of Antarctic Krill meal in mink, an *in vitro* bacterial mutagenicity study, and an *in vivo* micronucleus study in rats. An acceptable daily intake (ADI) was derived based on the mink studies.

Mink is considered a good model for evaluation of nutritional aspects of feed ingredients for other mammals, including dogs, cats, and foxes (Calabrese et al. 1992; Ahlstrøm and Skrede 1998; Krogdahl et al. 2004; Tjernsbekk et al. 2014). The National Research Council (NRC) states that in the evaluation of suitability of data for assessing animal dietary supplement safety "safety studies in a nontarget species (e.g., wolf for dog, mink for cat, donkey for horse)" is a class of data that may be utilized to assess acceptable and relevant data (NRC 2009). Further, fluoride, iodine, lead, mercury, coumarin, sodium monofluoroacetate, diethylstilbestrol (DES), dioxin, aflatoxin, zearalenane, and nitrosamines all cause their principal toxicities in the same target organs in mink as are reported for other commonly encountered species (Calabrese et al. 1992). A subsection dedicated to justifying mink as an appropriate model for dogs is also included at the end of this section (Section I.d).

Other chemicals of concern in krill meal are fluoride and astaxanthin, due to their abundance in this ingredient. Therefore, a separate evaluation was conducted on each chemical to establish ADIs for dogs.

With the exception of the acute oral toxicity and genotoxicity study on Qrill™ Pet (Wessels 2014 – unpublished data) and two subchronic feeding studies in adult dogs that only examined limited endpoints (Berge et al. 2014; Hals 2016 – unpublished data), all the other studies cited in this report were obtained from publically available sources. The critical studies used to evaluate the safety of krill meal, fluoride and astaxanthin and establish their respective ADIs are all publically available. The three proprietary studies listed above were only used as supportive evidence.

a. Toxicity of Krill Meal

i. Acute toxicity

Results of a range-finding study for a micronucleus study in 6- to 12-week old NMRI mice indicate that the oral lethal dose (LD_0) of Qrill™ Pet is > 2,000 mg/kg bw (when administered in divided doses of 1,000 mg/kg bw two hours apart) (Wessels 2014 - unpublished data). This study was conducted under GLP according to OECD Guidelines 420 and 423. Three animals of each sex received Qrill™ Pet suspended in cottonseed oil via gavage at 2,000 mg/kg bw in split doses of 1,000 mg/kg bw each with an interval of two hours between the administrations, and were observed for 72 hours after the last dose. All animals showed mild toxic effects such as catalepsis[2], ataxia, bradykinesia, piloerection, half eyelid closure and reduction of spontaneous

[2] Muscular rigidity and lack of response to external stimuli

activity within the first hour after application. No toxic symptoms were observed after 24 hours and all animals survived.

ii. Repeated dose toxicity

Several repeated dose toxicity studies were identified for krill meal, including one 15-week feeding study in mink (Krogdahl et al. 2015a), two subchronic in-life tolerance feeding studies in dogs (lasting 52 days and 14 weeks, respectively) (Berge et al. 2014; Hals 2016 - unpublished), and several studies in rats (Pastuszewska et al. 1983; Zaleska-Freljan and Cywińska 1991; Gigliotti et al. 2008; Zhang et al. 2013). These studies support the safety of 3% Qrill™ Pet in adult dog food.

Growth study in mink

The effects of Antarctic krill meal on growing mink were investigated. For the growth study, 64 mink kits (black genotype, 52 – 53 days old, body weight range 338 – 740 g) were allocated into four groups of eight males and eight females *per* group (Krogdahl et al. 2015a). The kits were offspring of females involved in a preceding reproduction trial that is described in detail in the reproductive and developmental toxicity section below (Krogdahl et al. 2015b). The kits were continued in the same groups as they were in the previous trial (control or low, mid or high dose of Antarctic krill meal) for 15 weeks. The animals were fed once daily and daily feed consumption was recorded separately for each gender on a group basis.

Animals were weighed on Days 30, 57, 83 and 102 (the last day of the experiment). On Day 102, blood samples were taken after euthanasia by cardiac puncture, and the kidney, liver, spleen, adrenal glands, heart and gastrointestinal tract (stomach, jejunum, colon and rectum) were dissected, weighed, and grossly examined, fixed in formalin and stained with hematoxylin and eosin (H&E). Liver tissue was stained with periodic acid Schiff (PAS) stain to identify glycogen. All collected organs from control and high dose animals and liver, spleen and kidney samples from all groups were evaluated histologically. Liver and kidney tissue homogenates were analyzed for copper, cadmium, arsenic, and zinc and dried, defatted bone (left femur) was analyzed for fluoride. Blood samples were analyzed for complete blood cell counts (CBC) and clinical chemistry.

Diet Compositions

The diets given to mink were formulated to have a metabolizable energy (ME) content of 5 MJ/kg on a wet weight basis during the first five weeks of the study (first growth period) and 6.5 MJ/kg on a wet weight basis for the remaining ten weeks of the study (second growth period). In the first growth period, the levels of krill were 9.0, 21.0, and 36.2%, based on dry matter content of feed. The krill levels were slightly less in the second period (8.7, 17.5, and 35.3% of dry matter), but not remarkably so. The protein content of the diets was balanced by adding fish meal protein. The diets were labeled K0, K8, K17, and K33, respectively, for both growth periods. Nutrient contents and ME distribution between protein, fat and carbohydrates were similar in the four experimental diets within the two periods (Table 1). Diets containing Antarctic krill meal contained similar amounts of calcium, higher amounts of copper and

fluorine, and lower amounts of arsenic compared to the control diet. Fluorine content was 8.5 times higher in the K33 diet compared to the K0 diet. Fluorine levels in all diets (including the control diet) exceeded some recommended tolerance for fluorine in feeds for growing dogs (50 ppm for bone pathology, 100 ppm for growth/performance (NAS 1974)) and other mammals (see Table 5).

Table 1: Diet Composition, Chemical Content and Energy of Diets in the Growth Period (g/kg)

	K0		K8		K17		K33	
Growth Period	**First**	**Second**	**First**	**Second**	**First**	**Second**	**First**	**Second**
Ingredient (g/kg)								
Krill meal	-	-	34.7	33.5	67.5	65.0	131	126
Fish meal	153.2	148	117.5	113.5	80.5	77.5	12.8	12.0
Precooked carbohydrates	139	134	139	134	136	131.5	127	123
Cod scraps	139	134	139	134	135	130.5	126.5	122
Poultry by-products	139	134	139	134	135	130.5	126.5	122
Lard (pig fat)	13.9	35	13.9	35	13.5	35	12.6	35
Soybean oil	13.9	25	13.9	25	13.5	25	12.6	25
Vitamin/mineral mix[b]	2	2	2	2	2	2	2	2
Water	400	388	401	389	417	403	449	433
Sum	1000	1000	1000	1000	1000	1000	1000	1000
Dry matter, g/kg	370	391	384	384	321	371	362	357
Chemical content (g/kg DM)[c]								
Crude protein	441	414	438	411	424	420	434	406
Crude lipid	173	230	172	234	165	235	174	232
Carbohydrates[d]	272	266	286	259	305	248	295	269
Ash	114	90	104	96	106	97	97	92
ME[c,e] (MJ/kg DM)	16.1	17.5	16.2	17.7	16.0	17.8	16.3	17.6
ME distribution[c]								
Protein	42	37	42	36	41	37	41	36
Lipid	38	47	38	48	32	47	38	47
Carbohydrates	20	16	20	16	27	16	21	17
Mineral content (mg/kg DM)								
Copper		11.5		15.6		20.8		31.3
Fluoride		73		291		419		626
Arsenic		2.7		2.7		2.5		1.8
Calcium		25000		29170		28130		26040

[a] Dates during which diet formulation was fed to mink (month/day).

[b] Content *per* kg: Vitamin A, 2 000 000 IU; vitamin D3, 200 000 IU; vitamin E, 50 000 mg; vitamin B1, 15 000 mg; vitamin B2, 3 000 mg; vitamin B6, 3 000 mg; vitamin B12, 19.5 mg; Ca –D- pantothenic acid, 3332 mg; niacin, 5005 mg; biotin, 30 mg; folic acid, 301 mg; ferrous sulfate, 610 mg; ferrous fumarate, 15280 mg; Fe (chelated), 4110 mg; copper sulfate, 1250 mg; manganese oxide, 7502; zinc oxide, 9998 mg; Ca iodinate, 63.5 mg; Na selenite, 99.9 mg; cobalt carbonate, 60 mg.

[c] Chemical content and energy values are data from one sample *per* diet from the period 6/23- 8/1 and two samples *per* diet from the period 8/2-10/3.

[d] Calculated value (Carbohydrates = DM – Ash - Crude protein – Crude lipid).

[e] Metabolizable energy; content was determined using standard digestibility factors given by the Norwegian Fur Breeders' Association of 82, 90 and 68 % for protein, fat and carbohydrates, respectively, and ME content of 18.8, 39.8 17.6 kJ *per* g digestible protein, fat and carbohydrates, respectively (Enggaard Hansen et al. 1991).

Clinical Observations and Growth

All animals appeared healthy and showed good appetite throughout the experiment, except for one male fed the K33 diet and one male fed the K0 diet, both of which died early on in the study. The early deaths were unrelated to treatment. For each sex, feed consumption was similar among the groups. As expected, feed intake was higher in males (298-310 g/day) than females (210-225 g/day), regardless of krill meal inclusion. There was no effect of krill meal on body weights of male or female mink compared to controls. Based on average feed intake and initial body weights, males in the K8, K17 and K33 groups consumed 16.6, 31.2, and 71.2 g/kg bw/day krill meal (respectively) and females in the K8, K17 and K33 groups consumed 13.3, 26.0, and 63.1 g/kg bw/day krill meal (respectively). Corresponding doses of fluorine from Qrill™ Pet in the K8, K17, and K33 groups are 53.4, 71.9, and 121.1 mg/kg bw/day in males and 43.0, 59.8, and 107.7 mg/kg bw/day in females.

Organ Weights

Relative[3] weights of the stomach (both genders) and rectum (females only) increased with increasing dietary inclusion of krill. The elevations were however, small and statistically significant only for K33 animals. The authors suggested that the increase in gastrointestinal organ weights could possibly be related to the presence of chitin, which is an indigestible polyglucosamine that is a component of the krill shell and exhibits properties similar to that of dietary fiber. Fiber has been shown to increase gastrointestinal tract relative weights in a variety of animal species.

Relative heart weight of K33 females (0.55 ± 0.05 g/100 g body weight) was higher than K8 (0.43 ± 0.07 g/100 g body weight) females ($P < 0.05$), but not significantly different from controls (0.44 ± 0.09 g/100 g body weight). The reason for the increase in relative heart weight of females in the K33 group is unknown, but is not considered toxicologically relevant due to lack of other findings in this organ.

The relative weight of the liver decreased slightly with increasing krill level; however, the decrease was not statistically significant. No relationship was observed between dietary krill level and relative weights of kidneys, spleen, brain, adrenals, or gonads.

Clinical and Anatomical Pathology

There was no clear relationship between dietary krill level and gross pathology, with the exception of joint/bone deformities in the K33 group. During preparation of femur samples for fluoride analysis, several deformities were noted in the animals in the K33 group: 7/8 males and 1/8 females had deformities of the femoral neck or head. No bone deformities were noted in any of the other groups. Significantly higher alkaline phosphatase was observed in K33 males ($P < 0.05$) compared to control males. The authors noted that elevated alkaline phosphatase activity may be associated with an increase in osteoblast activity and therefore indicate increased bone

[3] relative organ weight = organ to body weight ratio

deposition and/or mineralization in the K33 males. However, alkaline phosphatase is not specific for bone since it can also indicate liver injury.

Small focal to multifocal inflammatory lesions were noted in the liver. The frequency of the observation in males and females of the K17 and K33 groups suggests a possible relationship with dietary krill meal level. The etiology and toxicological relevance of the focal inflammatory lesions in the liver are unclear based on the relative lack of changes in aspartate transaminase or alanine transaminase enzyme levels or other histopathological findings in the liver. However, higher alkaline phosphatase levels were observed in K33 males, which is also a clinical indicator of liver injury. Significantly lower plasma bile acids levels was observed in the K33 females ($P < 0.05$) which may possibly indicate inflammation of the bile duct, but the location of the multifocal inflammatory lesions within the liver was not reported. Although aspartate transaminase and alanine transaminase levels were not affected by dietary krill level, liver injury cannot be ruled out in the K17 and K33 groups.

The histological examination of the liver indicated reduced liver glycogen with increasing dietary Antarctic krill meal and staining confirmed this finding. Reduced liver glycogen was found in males and females in the K17 and K33 groups. Glycogen staining had a distinct centrilobular distribution, but also showed higher glycogen deposition around vessels and at the periphery of the liver lobe. Plasma glucose concentrations in all groups were normal, indicating that low liver glycogen did not significantly affect the ability to maintain blood glucose levels. However, significantly higher amylase levels in K33 males were observed ($P < 0.05$). The increase in amylase along with the lower glycogen in the liver suggests that glycogenolysis was stimulated by the krill meal. Free fatty acids were also increased in K17 and K33 males ($P < 0.05$), but not in any female groups. The increase in fatty acids is unclear, but a shift to lipid catabolism is a possibility due to the decrease in stored glycogen observed in the liver. The findings were similar to those of the previous study in adult female mink provided 35% krill meal in the diet during pregnancy and lactation (Krogdahl et al. 2015b). The mechanism for the effect of high doses of Antarctic krill on liver glycogen in mink is unknown, but may be due to lower energy assimilation, increased glycogenolysis, or some combination thereof.

One male in the K8 group and three males in the K17 group exhibited "moderate" active lymphoid follicles in the spleen and seven animals from the K33 group (six males and one female) exhibited "moderate to marked" active lymphoid follicles in the spleen. Splenic nodules were observed during necropsy in two of the K33 males that exhibited active lymphoid follicles.

Crystals (basophilic crystalline material) were observed within tubules in the kidneys of controls as well as in animals ingesting krill meal; however, they appeared more frequently in males and females in the K17 and K33 groups and males in the K8 group (Table 2). There were no pathological changes associated with the crystallization, including inflammation. Further, the fact that urea, creatinine and creatine kinase were not increased in any groups of treated animals indicates that the observed increased frequency of crystalline material in the kidney tubules of treated animals was not pathological in nature. Decreased urea and creatinine levels were observed in K33 males ($P < 0.05$), but this finding may indicate a change in protein intake or metabolism with higher intakes of krill meal. As the finding of crystalline material in the tubules

was a common finding in control animals consuming a fishmeal diet it is concluded that mink may be predisposed to developing crystalline material in the kidneys regardless of diet.

Table 2: Frequency of Crystalline Material within Kidney Tissue

	n	None	Rare	Occasional	Numerous
Males					
K0	7	1	3	3	0
K8	8	1	1	1	5
K17	8	0	0	0	8
K33	8	0	0	3	5
Females					
K0	8	1	5	2	0
K8	8	0	6	0	2
K17	8	0	0	2	6
K33	8	0	1	1	6

n = number of animals.

Upon further study, the composition of the crystals in the kidneys of both control and exposed animals was confirmed to be calcium oxalate in nature (Eurofins 2014). Magnesium was not detected in any of the tissue samples, indicating that the crystals did not consist of struvite. The data are consistent with an ingredient (or ingredients) in the basal feed of the mink supporting formation of calcium oxalate crystals, with exacerbation by an ingredient (or ingredients) in krill meal. A study by Anasuya et al. (1982) indicates that fluorine can promote the formation of calcium oxalate crystals in rats ingesting high calcium diets. Calcium levels in all diets including the control were high in the study (ranged from 25,000 ppm in the control to 29,170 ppm in the K8 group), and fluorine levels of all diets (including the control) were higher than recommended for most species (see Table 4 of Part 2). Therefore, it is likely that the crystals in the kidneys of all groups of animals are a consequence of the dietary formulations containing higher than recommended levels of calcium and fluorine.

There were no test material-related findings in the histopathology of the gastrointestinal tract, adrenal gland or heart. There was no effect of Antarctic krill on red blood cell count, hemoglobin concentration, hematocrit, mean cell volume, or platelet count. Mean cell hemoglobin concentration (MCHC) decreased and red blood cell distribution width (RDW) increased in group K33 animals; however only MCHC of K33 females and RDW of K33 males were significantly different from controls ($P < 0.05$). There were no significant differences in total or differential white blood cell counts between treated animals and controls. The blood biochemistry profiles generally yielded similar results in males and females. There was no effect of Antarctic krill on plasma electrolytes (inorganic P, Ca, Na, K or Cl). There was no significant effect of Antarctic krill meal on cholesterol, triglycerides or glucose in males or females.

Bone Fluoride Concentrations

Bone fluoride showed a clear increase with increasing levels of dietary Antarctic krill meal (Figure 1). The effect was consistent between males and females and fluoride accumulated to very high levels in the K33 group. As described above, femoral bone deformities were observed in the K33 group but not for the other test groups. Palczewska-Komsa *et al.* (2014) found that

dogs consuming typical dog food diets that contain fluorine (*i.e.*, not intentionally provided a fluorine supplemented diet) show an increase in bone fluorine with increasing age. The median fluorine bone content in the dogs evaluated (excluding one-day-old puppies) was 491.3 mg/kg dw[4] bone. No visible toxicity related to fluorine ingestion was noted in these dogs. The authors also stated that concentrations of bone fluorine have been found to exceed 1,700 mg/kg dw bone, but "with no visible signs of intoxication" (Palczewska-Komsa et al. 2014).

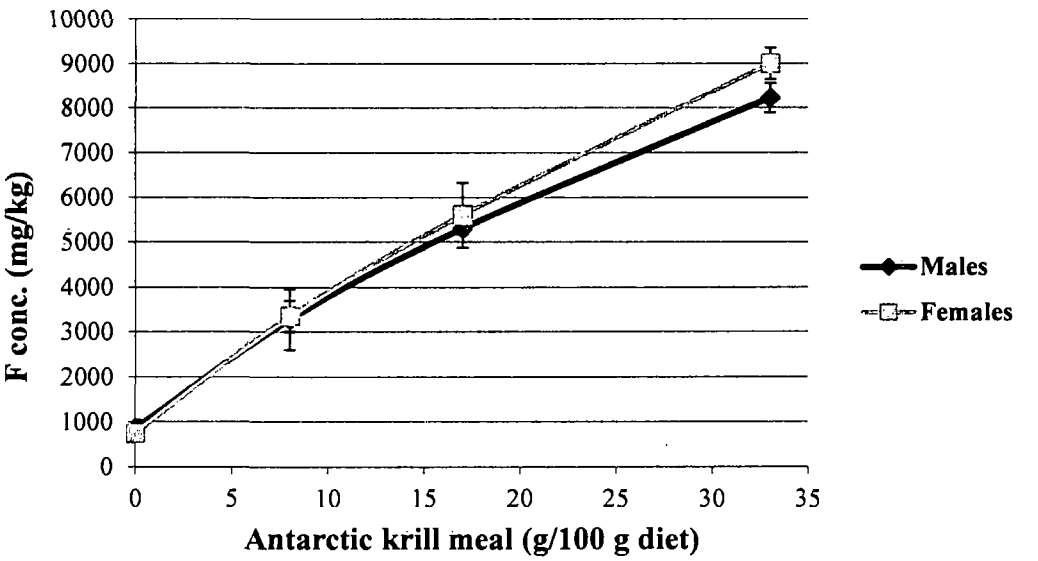

Figure 1: Bone (Left Femur) Fluoride (F) Concentration Showing a Clear Increase with Increasing Dietary Fluorine Concentration.

Data are presented as mean ± standard deviation

Metal Concentrations in Liver and Kidney

Liver cadmium levels increased with increasing dietary krill, with values for K33 males and females significantly different from their respective controls. Arsenic decreased with increasing inclusion of krill meal, but was significantly different from control only for K33 females. There was no effect of administration of krill meal at the 8% or 17% level on liver concentrations of copper, cadmium, or arsenic. While there was a significant increase in zinc content of the liver of K8 males ($P < 0.05$), the increase was slight and inconsequential. There was no such increase in males in the K17 group. There was a positive relationship between krill level and copper and zinc concentrations in the livers of K33 males. Copper concentrations were lower in the kidney compared to liver concentrations (as expected, since liver is the major organ for copper storage) and were not affected by ingestion of krill meal. Kidney cadmium levels increased with increasing dietary krill meal inclusion, and were significantly higher in K33 females than K8 females ($P < 0.05$). Arsenic decreased linearly with increasing krill inclusion and was significantly lower in K33 males and females than their respective controls. There was no effect

[4] dw=Dry weight.

of administration of krill meal at the 8% or 17% level on kidney concentrations of copper, cadmium, arsenic, or zinc.

Conclusion

In conclusion, the protein and energy value of Antarctic krill meal appeared comparable to that of the fish meal when used as a control diet as indicated by feed intake and growth results. Some effects which could be considered adverse were observed when krill meal was included in the diet at the medium and high doses (K17 and K33). Animals in the K17 and K33 groups showed some histological changes in the liver and kidney, as well as a few alterations in some clinical chemistry and hematology values related to nutrient intake or metabolism. K33 animals also exhibited joint/bone deformities and increased weight of the stomach and rectum. At the lowest inclusion level (K8), the only effects noted were an increased level of fluoride in bone without bone deformity and a slight (nonsignificant) increase in the frequency of crystalline material in kidney tubules (which was not associated with microscopic evidence of toxicity to the kidney or changes in urinalysis). Therefore, 8.7% (13.3 g/kg bw/day in females and 16.6 g/kg bw/day in males) is the no observable adverse effect level (NOAEL) of krill meal for growing mink.

52-Day in-life tolerance study in dogs

The tolerability of an 8% inclusion of Qrill™ Pet meal into feed for dogs was evaluated in a 52-day subchronic feeding study in Husky breed dogs (Berge et al. 2014, unpublished data). Alaskan Husky dogs (n=30) were included in the study; fourteen dogs were fed control diet and sixteen dogs were feed control diet with 8% added Qrill™ Pet meal. The dogs were randomized into the two groups and stratified for gender and age. All the dogs were being actively trained for use in marathon dog sled races. The study was conducted in accordance with the Guidelines of the Animal Welfare Act.

The dogs were fed once daily a ration of 500 – 700 grams, depending on dog size. The control diet consisted of a mix of Eukanuba kitten (50%) and Eukanuba Dog Working and Endurance (50%), a high protein diet. The study states that "In the Qrill™ Pet diet, 8% of the feed was Qrill™ Pet (wt/wt[5] dry weight)", indicating that 8% of the control diet was replaced with Qrill™ Pet meal. The diet was prepared daily and the Qrill™ Pet-containing diet was mixed to ensure homogenous distribution. Qrill™ Pet-containing diet had a calculated metabolizable energy (kcal/kg) that was 1% greater than the control diet. The dogs were observed daily for feed intake and to ensure that the dogs were in general good health and behavior. Each dog was checked weekly for abnormalities of the feces or fur. Blood samples were obtained at the start and at the end of the study, and analyzed for clinical chemistry and omega-3 index parameters. The effect on the omega-3 index is discussed in Part 2 of the submission dossier. No necropsy or histopathology was performed for the study. Student's t-test was used to calculate significant (P<0.05) differences within and between groups.

[5] wt/wt=weight/weight

Feed intake was typical for the individual dogs and all food was consumed daily. No dogs showed adverse events throughout the study period and there were no signs that body weights appreciably changed during the study period.[6] For clinical chemistry, there were no significant ($P>0.05$) differences between the groups when baseline values for control and Qrill™ Pet groups were compared , and when end of study values between control and the Qrill™ Pet groups were compared (Table 3). There were significant changes within the groups when comparing clinical chemistry parameters taken at the start of the study to values obtained at the end of the study (Table 3); however most of the changes were within the reference range for healthy canines and are not considered treatment-related or toxicologically significant. Increases in creatinine and BUN levels within the groups could have been due to the dogs' adaptation to the high protein diet utilized in this study for highly active dogs[7], as their previous dietary composition was not stated. Plasma chloride (CL) levels were higher than reference values in both the control and treatment groups, but the study director stated that this might be related to "a high level of chloride in the drink water." No water analysis was provided. The study director concluded that inclusion of 8% Qrill™ Pet was well-tolerated (Berge et al. 2014 - unpublished study). This corroborative study indicates that adult dogs can consume up to 8% Qrill™ Pet meal in the diet (greater than 2.5-times the recommended 3% level to be added to dog food) without adverse effects.

The body weights of the dogs were not reported. Therefore, the ingested doses of krill meal are estimated. Adult male Husky dogs weigh 45 – 60 pounds (20 – 27 kg), and adult female dogs weight 35 – 50 pounds (16 – 23 kg) (Siberian Husky Club of America 2010). The study authors provided 500 – 700 g food to the animals daily based on their body weight. Assuming the smallest dogs (i.e., 35 pounds, or 16 kg) received the lowest amount of food (i.e., 500 g), the daily consumption of krill meal is 2.5 g/kg bw/day[8]. Similarly assuming the largest dog (i.e., 60 pounds, or 27 kg) received the highest ration of 700 g/day, the daily krill meal consumption is 2.1 g/kg bw/day[9]. Therefore, the dogs in the study consumed approximately 2.1 – 2.5g/kg bw/day krill meal.

[6] Body weights were not directly measured in this study, but no visual signs of decreased weight was reported.
[7] All the dogs were being actively trained for use in marathon dog sled races.
[8] 500 g ÷ 16 kg bw/day × 8% = 2.5 g/kg bw/day
[9] 700 g ÷ 27 kg bw/day × 8% = 2.1 g/kg bw/day

Table 3: Clinical Chemistry and Reference Values for Clinical Chemistry Parameters (Berge et al. 2014)

Parameter	Control baseline	8% Qrill Pet Baseline	Control end of study	8% Qrill Pet end of study	Control Delta change	8% Qrill Pet Delta change	Reference value**
AST (U/L)	36.79 ± 43.28	26.75 ± 15.84	24.00 ± 5.82	23.94 ± 4.11	-12.79	-2.81	0-40
ALT (U/L)	42.71 ± 38.91	35.81 ± 9.47	36.07 ± 9.75	42.50 ± 5.83	-6.64	6.69*	0-80
ALKP (U/L)	23.77 ± 8.88	23.67 ± 4.82	23.30 ± 7.06	22.88 ± 7.01	-2.67	-0.53	0-90
BILI (µmol/L)	0.86 ± 0.86	1.00 ± 0.52	1.36 ± 0.84	1.44 ± 0.63	0.50	0.44*	0-7
BUN (mmol/L)	6.49 ± 1.73	6.33 ± 1.71	4.95 ± 0.96	5.21 ± 0.96	-1.54*	-1.12*	3.5-7.2
CREA (µmol/L)	71.29 ± 6.83	71.13 ± 4.18	87.36 ± 7.19	91.94 ± 7.33	16.07*	20.81*	65-110
CHOL (mmol/L)	5.89 ± 0.94	6.39 ± 1.66	5.61 ± 1.04	5.64 ± 1.02	-0.28	-0.76	3.4-10.0
TRIG (mmol/L)	0.63 ± 0.29	0.56 ± 0.12	0.52 ± 0.09	0.51 ± 0.10	-0.11	-0.05	0.2-1.6
GLUC (mmol/L)	5.88 ± 0.45	5.80 ± 0.40	5.56 ± 0.36	5.51 ± 0.35	-0.32	-0.29	3.6-6.6
TP (g/L)	62.07 ± 3.25	60.88 ± 2.06	61.57 ± 3.52	60.69 ± 2.06	-0.50	-0.19	54-75
ALB (g/L)	33.64 ± 2.31	34.13 ± 1.31	32.79 ± 2.33	33.75 ± 1.44	-0.86	-0.38	32-44
GLOB (g/L)	25.36 ± 4.13	23.38 ± 1.54	26.07 ±4.10	23.75 ± 1.81	0.71	0.38	22-31
CALC (mmol/L)	2.57 ± 0.07	2.56 ± 0.07	2.54 ± 0.05	2.55 ± 0.10	-0.04	-0.01	2.2-2.9
IPHS (mmol/L)	1.29 ± 0.22	1.24 ± 0.16	1.14 ± 0.12	1.07 ± 0.18	-0.14*	-0.18*	0.9-2.0
NA (mmol/L)	148.21 ± 0.80	148.13 ± 1.02	149.14 ± 1.29	149.19 ± 1.28	0.93*	1.06*	140-154
K (mmol/L)	4.60 ± 0.20	4.60 ± 0.18	4.68 ± 0.21	4.71 ± 0.30	0.08	0.11	3.7-5.8
CL (mmol/L)	117.29 ± 1.49	117.50 ± 2.16	118.93 ± 2.20	119.31 ± 1.20	1.64*	1.81*	99-115

*$P < 0.05$ (within group significance). All values are means ± S.D. for 14 (Control group) or 16 (Qrill Pet group) dogs, except for ALKP, where 2 values at baseline (1 Control, 1 Qrill pet) and 4 values at End of study (4 Control) are missing due to hemolysis in the serum samples. **All reference values are given by The Central laboratory at Norwegian School of Veterinary Science, Oslo, Norway. ALB = albumin; ALKP = alkaline phosphatase; ALT = alanine amino transferase; AST = aspartate aminotransferase; BILI = bilirubin; BUN = blood urea nitrogen; CALC = calcium; CHOL = cholesterol; CL = chlorine; CREA = creatinine; GLOB = globulin; GLUC = glucose; IPHS = incidence of intraplaque hemorrhages; K = potassium; NA = sodium; TP = Total protein; TRIG = triglycerides.

14-Week in-life tolerance study in dogs

Another in-life tolerance study was conducted in fewer dogs for a longer period of time (14 weeks) to evaluate telomere length in semen and blood, semen quality parameters, clinical chemistry parameters and omega-3 index (Hals 2016 – unpublished data). As the study is still ongoing at the completion of this GRAS dossier, only an interim report was available for review that only presented results on clinical chemistry parameters and the omega-3 index. The effect on the omega-3 index is discussed in Part 2 of the dossier. The same diets as described above were administered to ten adult Alaskan Huskies for 14 weeks, which were randomized into two groups of five animals each (Qrill™ PET and control) and stratified for age. These dogs were privately owned sled dogs used in marathon races, and were accommodated in the kennel of the owner, located in Alvdal, Norway.

The dogs were observed daily for feed intake and to ensure that the dogs were in general good health and behavior. Each dog was checked weekly for abnormalities of the feces or fur. Parameters measured for clinical chemistry include Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), total protein (Tprot), albumin (Alb), globulin (Glob), albumin/globulin ratio (A/G), urea nitrogen (Urea), creatinine (Creat), total bilirubin (Tbili), total cholesterol (Chol), triglycerides (TG), fasting glucose (Glu), inorganic phosphorus (InorgP), calcium (Ca), sodium (Na), potassium (K), sodium/potassium ratio (Na/K), and chloride (Cl).

For clinical chemistry, there were no significant ($P>0.05$) differences between the groups when baseline values for control and Qrill™ PET groups were compared and, when end of study values between control and the Qrill™ PET groups were compared (Table 4). There were significant changes within the groups when comparing clinical parameters taken at the start of the study to values obtained at the end of the study (Table 4), but these values fell within the reference range for healthy canines and were not considered treatment-related or toxicologically significant. In the few instances where statistically significant differences were observed for the clinical chemistry parameters when baseline values were compared to end of feeding values, the changes were all considered to be incidental and of no biological or toxicological concern as they are within reference ranges.

The body weights of the dogs were not reported. Therefore, the ingested doses of krill meal are estimated. Adult male Husky dogs weigh 45 – 60 pounds (20 – 27 kg), and adult female dogs weight 35 – 50 pounds (16 – 23 kg) (Siberian Husky Club of America 2010). The study authors provided a daily ration of 500 – 700 g based on their body weight. As estimated previously for the Berge et al. (2014) study, the dogs in this study consumed approximately 2.1 – 2.5 g/kg bw/day krill meal.

Table 4: Clinical Chemistry And Reference Values for Clinical Chemistry Parameters (Hals 2016)

Parameter	Control baseline	8% Qrill Pet Baseline	Control end of study	8% Qrill Pet end of study	Control Delta change	8% Qrill Pet Delta change	Reference value**
AST (U/L)	35.6 ± 5.10	33.6 ± 1.69	30.0 ± 3.26	25.8 ± 1.39	-5.6	-7.8*	0-40
ALT (U/L)	47.8 ± 6.38	49.8 ± 3.87	54.4 ± 11.43	45.2 ± 4.72	6.6	-4.6	0-80
ALKP (U/L)	34.8 ± 6.87	37.0 ± 4.99	47.0 ± 11.12	56.2 ± 11.42	12.2	19.2	0-90
BILI (µmol/L)	0.4 ± 0.24	0.2 ± 0.20	0.6 ± 0.24	1.0 ± 0.00	0.2	0.8*	0-7
UREA (mmol/L)	8.16 ± 0.40	9.12 ± 0.74	5.90 ± 1.14	4.86 ± 0.34	-2.26	-4.26*	3.5-7.2
CREA (µmol/L)	64.8 ± 2.60	67.4 ± 3.14	61.0 ± 2.32	61.8 ± 1.28	-3.8	-5.6	65-110
CHOL (mmol/L)	5.34 ± 0.30	6.72 ± 0.43	4.92 ± 0.46	6.16 ± 0.36	-0.42	-0.56	3.4-10.0
TRIG (mmol/L)	0.9 ± 0.14	0.86 ± 0.25	0.9 ± 0.51	0.36 ± 0.04	0	-0.5	0.2-1.6
GLUC (mmol/L)	5.16 ± 0.21	4.78 ± 0.29	5.46 ± 0.10	5.34 ± 0.07	0.3	0.56	3.6-6.6
TP (g/L)	61.0 ± 1.41	63.2 ± 1.36	62.2 ± 1.28	60.8 ± 1.28	1.2	-2.4	54-75
ALB (g/L)	37.0 ± 1.22	39.6 ± 0.68	38.4 ± 0.60	38.8 ± 0.73	1.4	-0.8	32-44
GLOB (g/L)	24.0 ± 1.52	23.6 ± 0.75	23.8 ± 1.77	22 ± 1.05	-0.2	-1.6	22-31
CALC (mmol/L)	2.56 ± 0.05	2.62 ± 0.04	2.60 ± 0.03	2.6 ± 0.04	0.04	-0.02	2.2-2.9
InorgP (mmol/L)	1.48 ± 0.06	1.46 ± 0.09	1.24 ± 0.05	1.26 ± 0.07	-0.24*	-0.2	0.9-2.0
NA (mmol/L)	148.8 ± 0.37	149 ± 0.32	152.0 ± 3.27	149.2 ± 0.49	3.2	0.2	140-154
K (mmol/L)	4.52 ± 0.07	4.74 ± 0.11	4.88 ± 0.15	4.6 ± 0.11	0.36	-0.14	3.7-5.8
CL (mmol/L)	114.2 ± 0.73	113.4 ± 0.40	113.6 ± 1.03	114.6 ± 0.68	-0.6	1.2*	99-115

*$P < 0.05$ (within group significance). All values are means ± S.D. for 14 (Control group) or 16 (Qrill Pet group) dogs, except for ALKP, where 2 values at baseline (1 Control, 1 Qrill pet) and 4 values at End of study (4 Control) are missing due to hemolysis in the serum samples. **All reference values are given by The Central laboratory at Norwegian School of Veterinary Science, Oslo, Norway. ALB = albumin; ALKP = alkaline phosphatase; ALT = alanine amino transferase; AST = aspartate aminotransferase; BILI = bilirubin; CALC = calcium; CHOL = cholesterol; CL = chlorine; CREA = creatinine; GLOB = globulin; GLUC = glucose; InorgP = inorganic phosphate; K = potassium; NA = sodium; TP = Total protein; TRIG = triglycerides.

Subchronic toxicity studies in rats

A number of studies were identified in rats fed krill meal. These studies evaluated the effect of krill meals on soft tissues and hematology. These studies were performed using krill meals that are very different in composition from Qrill™ Pet, such as purified krill chitin meal, krill shell or carapace meal, and krill meals containing much higher levels of fluorine than Qrill™ Pet. One study also evaluated the effect of a krill protein concentrate on the kidney function of rats. Therefore, they are not used in the dose-response assessment of krill meal. Instead, they are evaluated as supportive evidence only.

Pastuszewska et al. (1983) examined the effects of diets supplemented with purified krill chitin and krill (shell) meal on weanling male Wistar rats (non-GLP, non-Guideline). Groups of six rats (23 ± 1 days of age) were administered a control diet (12.6% casein), a diet in which the cellulose component was replaced by purified krill chitin (2% krill chitin), or a diet in which the cellulose and a portion of the starch was replaced with krill (shell) meal (9.2% krill (shell) meal). The authors reported that the composition of the krill (shell) meal was 'within range' of the composition of usual krill meals, as mechanical removal of the shells resulted in a large portion of the krill carapace being left in the shell fraction of the meal. Feed and water were provided *ad libitum* with the exception of a six-day period during the 4th week of the experiment, when 15 g feed/day was provided to each animal and the feces and urine were collected. Feed intake and body weight were measured weekly and the appearance of the teeth was examined at each weighing. At the end of the experimental period the rats were euthanized and the liver, kidney, and testes were excised and weighed. Segments of the intestine (small and large) and testes were fixed, processed, and subjected to histological examinations.

Based on the reported feed intakes and changes in body weight, the diets were calculated to provide an average of 1.1 g krill chitin/kg bw/day or 4.6 g krill meal/kg body weight/day over the course of the experimental period. The fluoride contents of the control, purified krill chitin, and krill (shell) meal diets were 1, 5, and 139 ppm, respectively, which would result in corresponding intakes of 0.053, 0.11, and 6.96 mg fluorine/kg body weight/day based on the calculated feed intakes. A significant decrease in feed intake was observed in the rats consuming diets containing 9.2% krill (shell) meal compared to the rats consuming the casein and purified krill chitin diets ($P < 0.01$). No significant differences in the body weight gains of the rats occurred between any of the groups, suggesting improved feed utilization of the krill (shell) meal diet. The apparent improved feed efficiency of the diet containing the krill (shell) meal may have been due to the higher protein content in the krill (shell) meal diet than the other two diets. However, there was no difference in nitrogen retention of krill (shell) meal-fed rats compared to rats of the control group, despite the higher nitrogen intake. A slight discoloration was observed in the incisors of the rats consuming the diets containing krill (shell) meal. No abnormalities were observed in histological examinations of the intestines.

Rats consuming the diet containing krill (shell) meal had higher relative[10] testes weight ($P < 0.01$) and appeared to have lower relative liver weight than rats consuming the control diet.[11]

[10] Relative organ weight = organ weight to body weight ratio.

While no pathological abnormalities were observed the testes, the authors reported that the development stages of the seminiferous epithelium was delayed in rats fed the purified krill chitin- and krill (shell) meal-containing diets, with the delay appearing to be more severe in the rats consuming the diet containing krill (shell) meal. In the control rats, over 80% of seminiferous tubule sections showed at the 9th to 14th stage of the cycle for seminiferous epithelium development whereas 55-60% and 20-25% of seminiferous tubules showed at the 9th to 14th stage of the cycle in rats given purified krill chitin or krill (shell) meal, respectively. The authors concluded that the effects observed in the animals fed the krill (shell) meal-containing diets were likely due to the fluoride content of the shell, rather than chitin. The dose of fluorine ingested by rats in this study is 3.8 times higher than the amount of fluorine ingested by dogs consuming a diet containing 3% Qrill™ Pet (1.82 mg/kg bw/day).

Zaleska-Freljan and Cywińska (1991) reported effects on hematological parameters in rats fed krill carapace meal, which were attributed to the high fluorine content typically found in the carapace. Two studies were conducted to assess the potential effect of various dietary krill meal formulations on hematological indices in rats (non-GLP, non-Guideline). In the first of these studies, 29 Wistar rats (4 - 5/group; number/ sex/group not specified) of 11 - 12 weeks of age were administered one of six different diets for a period of two months, during which time breeding occurred. The control diet was a standard commercial rats chow and the five experimental diets contained various krill meal formulations (18.1% standardized krill meal, 18.8% krill meal with a lowered chitin content, 12.6% casein supplemented with 0.15% *D,L*-methionine, or casein supplemented with *D,L*-methionine and 9.2% krill carapace meal or 3.0% ash from standardized krill meal). The purpose of including these two latter diets was to establish the potential impact of the minerals contained in krill on the hematological values in rats. The second experiment examined the impact of two diets, one control diet (commercial rat chow) and one test diet containing 18.1% standardized krill meal with low chitin content, on 73 male and female offspring (18 to 19/group/sex) from the animals utilized in the first experiment. The offspring were three months of age at the initiation of the experiment and were administered the diets for a period of two months. In both experiments, blood samples were collected directly from the heart prior to euthanasia. Hematology parameters included hematocrit concentration, hemoglobin levels, erythrocyte and leucocyte counts, the composition of leukocytes, red blood cell size, mean cell hemoglobin (MCH), MCHC, and mean cell volume (MCV). No necropsy or histopathology was conducted for the studies.

The results of the first experiment revealed that overall, the consumption of all but one the test diets had no impact on the hematological parameters examined. In the rats administered the diet containing krill carapace, significant decreases in hematocrit, hemoglobin, mean corpuscular thickness, and MCV were observed in comparison to the values obtained for the rats in the control group ($P < 0.05$). In the second experiment, no significant differences were observed in any of the measured hematological parameters following the administration of a diet comprising 18.8% krill meal with low-chitin content when compared to control diet. The authors suggested that the effects on several of the hematological parameters in rats given the krill carapace meal were related to the fluoride concentration of this portion of the krill and the excess fluoride may

[11] A statistical analysis was not performed due to lack of homogeneity of data

affect the bone marrow and therefore disturb the processes involved in erythropoiesis. Because fluoride concentrations were not measured in any of the diets, one cannot determine the level of fluoride consumption associated with the hematological changes. Based on the default food factor of 98 g/kg bw/day for male and female Wistar rats in a subchronic toxicity study (U.S. EPA 1988), 18.1% in the diet is equivalent to approximately 18,000 mg/kg bw/day[12].

Zhang et al. (2013) evaluated the toxicity of fluorine (F) in Antarctic krill on the soft tissues of Wistar rats (non-GLP, non-Guideline). The toxicity of sodium fluoride (NaF) when given in the diet was also evaluated. Thirty newly weaned Wistar rats (sex not specified) were randomly divided into three groups with ten rats in each group. Rats in the control group were fed with a basal diet obtained from the Shuangshi Laboratory Animal Feed Science Co. Ltd. (Suzhou, China), and the F concentration in the feed was 30.3 ± 1.0 mg/kg dw. Rats in the NaF treatment group and the krill treatment group were fed with feeds prepared by mixing NaF or Antarctic krill powder with the basal feed, respectively. The final concentration of F in both diets was about 150 mg/kg (one tenth of the LD_{50} for F). The rats were kept for 3 months with *ad libitum* access to food and distilled water. Food consumption was recorded daily, and the rats were weighed weekly. After three months, rats were euthanized and tissue samples were collected from the liver, kidney, spleen, testis, and brain. Additionally, three rats were randomly selected from each group and organs were formalin-fixed and stained for histopathological examination.

During the observation period, study animals did not exhibit symptoms of toxicity. No significant differences were observed in food intake, body weight, or relative organ weights (ratio of organ weight to body weight) among the rats in all three groups. Histological analysis of viscera organs revealed pathological changes after NaF and krill treatment. A summary of the observations are presented in Table 5. Although body weights and relative organ weights did not differ significantly between the control group and treatment groups, viscera organs did exhibit pathologic changes related to treatment with 150 mg/kg F for three months in both treatment groups. Liver, kidney, and spleen are commonly affected by fluorine. Pathologic changes were also observed in the brain for both treatment groups, indicating that fluorine from krill may pass through the blood brain barrier. No pathologic changes were observed in the testis for either treatment group. The present study also found that despite equivalent levels of F in Antarctic krill and NaF dietary groups, animals in NaF group exhibited greater F toxicity. The authors proposed that this observation may be related to high levels of selenium and zinc in the krill that increase the activity of antioxidant systems thought to resolve fluorine toxicity. The authors concluded that while fluorine in Antarctic krill was less toxic than an equivalent amount of NaF in the diet, toxicity was still observed in this study from the krill meal. Therefore, fluoride toxicity should be taken into consideration if krill is used as a food source.

The authors did not report how much krill meal was added to the basal diet in this study. However, the fluorine contents of 30 and 2,416 mg/kg dw were measured for basal diet and krill meal used in the study, respectively. To reach a final fluorine content of 150 mg/kg dw, approximately 5% of krill meal is needed[13]. According to the mean food factor of 98 g/kg

[12] 98 g/kg bw/day x 18.1% x 1,000 mg/g = 18,000 mg/kg bw/day

[13] 5% krill meal x 2,416 mg F/kg dw + 95% basal diet x 30 mg/kg dw = 149.3 mg F/kg dw.

bw/day (U.S. EPA 1988) for male and female Wistar rats in a subchronic toxicity study, 5% krill meal in the diet is equivalent to 4,900 mg/kg bw/day[14].

Table 5: Histopathologic Changes In Krill- and NaF-Treated Rats (Zhang et al. 2013)

Organ	NaF	Krill
Liver	Vacuolar degeneration; disruption of epithelium lining,; vacuolization of the cytoplasm	Vacuolar degeneration; disruption of epithelium lining; vacuolization of the cytoplasm
Kidney	Vacuolar degeneration; disintegration of renal tubular epithelium	Vacuolar degeneration; disintegration of renal tubular epithelium
Spleen	Pronounced increased lymphocyte nodules; Pronounced decreased white pulp	Increased lymphocyte nodules; decreased white pulp
Testis	No changes	No changes
Brain	Decreased neurocytes; increased spongiocytes; large areas of vacuolar degeneration	Decreased neurocytes; increased spongiocytes

Gigliotti et al. (2008) conducted an assessment of the digestibility of a krill protein concentrate (KPC) in 28-day old female Sprague-Dawley rats which also included an evaluation of its safety (non-GLP, non-Guideline). The study showed that KPC fed to rats had no effect on clinical measures of kidney function when compared to rats fed casein in the diet. The protein quality of KPC is discussed in Part 2 of the dossier. Following a 14-day acclimation period, rats (n = 30) were randomly assigned to be fed one of the three diets consisting of: (1) 10% crude protein supplied as KPC for four weeks (n = 10), (2) 10% crude protein supplied as casein for four weeks (n = 10), or (3) 10% casein diet for two weeks followed by a protein-free diet for the final two weeks (n = 10). Diets were formulated to be isocaloric and calcium and phosphorus concentrations were matched among the three test diets. Following euthanasia, blood was collected from animals. Serum cholesterol, triglyceride, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were determined. Kidney function was assessed by measuring serum total protein, albumin, blood urea nitrogen (BUN), creatinine, calcium (Ca) and phosphorous (P). Total urinary output was also measured. Major organs (i.e. brain, heart, liver, and kidneys) were weighed as indicators of toxicity and adrenal glands were weighed as an indicator of chronic stress. No histopathology was conducted. Kidney mineral content was also analyzed since studies have shown that different protein sources when fed to animals may influence mineral deposition in the kidneys (Zhang and Beynen, 1992). A summary of the findings are presented in Table 6.

No difference in adrenal weights and other organ weights (data not shown in the publication) were observed between the casein and KPC treatment groups with the exception of the kidney. Absolute and relative kidney weights were statistically lower ($P = 0.003$) in rats fed KPC compared to rats fed casein. The total mineral content of the kidney was higher ($P < 0.001$) in rats fed the casein (0.3 g/g kidney ± 0.04) compared to rats fed KPC (0.07 g/g kidney ± 0.01). Kidney calcium content was higher ($P = 0.002$) in rats fed casein (7.0 mg/g kidney ± 0.9) than rats fed KPC (0.2 mg/g kidney ± 0.05). Similarly, kidney phosphorus content was also higher ($P < 0.001$) in rats fed casein (5.8 mg/g kidney ± 0.6) compared to KPC fed rats (1.0 mg/g kidney ±

[14] 5% x 98 g/kg bw/day x 1,000 mg/g = 4,900 mg/kg bw/day

0.2). Different protein sources fed to animals may influence mineral deposition in the kidneys since one study reported that female rats fed approximately18% casein in the diet had the highest kidney calcium concentration compared to rats fed soybean or cod meal (Zhang and Beynen, 1992). Based on the results, the authors suggest that KPC may protect against kidney calcification and mineralization, but studies of a longer duration may be needed to evaluate whether changes in kidney weight accompanying KPC consumption are protective in animals. Although higher (P = 0.03) urinary output was observed in the rats fed KPC (14.5 ml/day ± 3.0) compared to rats fed casein (6.0 ml/day ± 2.2), there was no difference observed in kidney function as indicated by the absence of significant differences in serum creatinine, blood urea nitrogen, total protein, calcium, and phosphorous in rats fed KPC compared to rats fed casein (see Table 6).

Serum total cholesterol was lower (P = 0.04) in rats fed KPC compared to casein-fed rats, which was also accompanied by a decrease in HDL (P = 0.003). The decrease in total cholesterol and HDL appeared to have no adverse effect in the rats. The authors suggest that sterols present in shellfish such as krill may interfere with the absorption of cholesterol and human studies have shown that consumption fish oils rich in EPA can decrease HDL cholesterol. Overall, despite differences in kidney mineralization and urinary output, there were no differences in kidney function between the rats fed casein or KPC in the diet. The authors conclude that KPC as a protein source is safe for consumption. Based on the default body weight and food consumption of female Sprague-Dawley rats (U.S. EPA 1988), 10% KPC in the diet is equivalent to 9.8 g/kg bw/day, and it is considered the NOAEL of this study.

Table 6: Kidney Weights, Kidney Function, and Serum Lipid Profile of Rats Fed KPC or Casein (Gigliotti et al. 2008)

Measurement[a]	Casein	KPC
Kidney weights		
Absolute kidneys weight (mg)	2.1 ± 0.05	1.9 ± 0.04*
Relative kidneys weight (mg/100g body weight)	897.2 ± 18.2	780.7 ± 20.2*
Kidney function		
Serum total protein (U/L)	6.0 ± 0.7	6.3 ± 1.0
Serum albumin (U/L)	4.2 ± 0.5	4.1 ± 0.9
Serum BUN (U/L)	12.1 ± 1.3	10.1 ± 3.2
Serum creatinine (U/L)	0.5 ± 0.15	0.5 ± 0.1
Serum calcium (U/L)	1.5 ± 1.3	11.4 ± 2.7
Serum phosphorus (U/L)	11.3 ± 0.4	10.7 ± 0.5
Serum lipid profile		
Triglycerides (mg/dl)	218.6 ± 24.6	235.1 ± 19.3
Total cholesterol (mg/dl)	97.9 ± 6.5	67.9 ± 11.0*
VLDL (mg/dl)	43.7 ± 4.9	47.1 ± 3.9
LDL (mg/dl)	38.1 ± 8.7	31.8 ± 5.2

HDL (mg/dl)	95.2 ± 7.3	66.3 ± 3.7*

KPC, krill protein concentrate; BUN, blood urea nitrogen; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.
* Indicates significant difference with $P < 0.05$ by t-test.
a Values are given as mean ± SEM of n = 10.

iii. Reproductive and developmental toxicity

The ability of Antarctic krill meal to cause reproductive and/or developmental toxicity was tested in mink (Krogdahl et al. 2015b) and rats (Tohjo 1980) to support the safety of Qrill™ Pet when added at 3% to adult dog food. The reproductive/developmental toxicity study in mink (Krogdahl et al. 2015b) and the previously described study in their offspring (Krogdahl et al. 2015a), satisfied requirements for animal feed ingredients to be tested in sequential gestation, lactation and growth studies.

Gestation/lactation study in mink

The effects of Antarctic krill meal on the reproductive performance and health of female mink was investigated. The gestation/lactation study utilized standard female mink (black genotype, body weights 955 - 1340 g) that were allocated to four groups of 16 animals each and placed on diets containing 0 (control), 9.4, 18.7, or 38.8% Antarctic krill meal based on dry matter (DM) content of feed for 117 days (Krogdahl et al. 2015b)[15]. The diets are referred to below as K0, K9, K17, and K35. The protein content of the diets was balanced by adding fish meal protein. The diets were formulated to have metabolizable energy (ME) content of 5.0 megajoules (MJ)/kg on a wet weight basis, and percentage proportions of ME from protein, lipid, and carbohydrates of 40%, 45%, and 15%, respectively. The experimental diets were analyzed for nutrients, energy, total volatile nitrogen (TVN), pH, copper, arsenic, calcium, and fluoride. Proximate analysis of samples showed that nutrient and calculated ME requirement content were similar for the four diets. TVN was low for all diets and decreased with increasing inclusion of Antarctic krill meal, indicating that TVN was lower in the Antarctic krill meal than in the fish meal. The pH of the diets increased with increasing content of Antarctic krill meal (K0 = 6.47, K9 = 6.75, K17 = 6.79 and K35 = 7.30). The reason for this is not clear, but the increase in pH was not considered of importance and did not affect palatability of the diets.

The animals were fed once daily. Feed was given on the top wire of the cage and when the kits started to eat at three weeks of age, feed was also provided on the top wire of the nest box. Feed was moderately restricted before mating and during gestation. After birth, feeding was not restricted and individual feed allowance was adjusted according to the number of kits. The 117-day experimental period consisted of a pre-mating period from February 17th until mating started March 7th, the gestation period of approximately 50 days (d), followed by parturition, lactation and early kit growth (lasting 49 d after birth). Females that exhibited poor health prior to mating were replaced by reserve females maintained on the same diet, but no replacements were made after mating. All females were mated twice with untreated males.

[15] Based on information in Tables 2 and 3 of Krogdahl et al. (2015b). For example, for K9, 35.5 g krill meal/kg diet as-is x 1 kg diet as-is/379 g dry matter = 0.094 g krill meal/g dry matter = 9.4%

Feed intake and ME consumption for each treatment were recorded on a group basis. Body weights of females and kits, and litter size were recorded regularly. Maternal animals were euthanized after weaning of kits at 49 days. Blood samples were taken after euthanasia and the kidney, liver, spleen, adrenal glands, heart, and gastrointestinal tract (stomach, jejunum, colon, and rectum) were dissected, weighed, and a gross examination was performed. The stomach, kidney, spleen, adrenal glands, and heart of K0 and K35 animals and the liver, jejunum, colon and rectum of all animals were formalin-fixed and stained with hematoxylin and eosin (H&E) for histopathological examination. Blood samples were analyzed for complete blood cell counts (CBC) and clinical chemistry.

Diet Compositions

Dietary concentrations of copper, fluoride, arsenic, and calcium are shown in Table 7. Concentrations of copper and fluoride were higher in the Antarctic krill meal than in the fish meal, while the opposite was observed for arsenic. The fluoride concentration in the krill meal (1,840 mg/kg) was higher than the maximum amount of fluorine permissible in Qrill™ Pet (1,500 mg/kg). For calcium, the level was similar in the Antarctic krill meal and fish meal. In the diets, the elemental levels reflected to a large extent the inclusion level of Antarctic krill meal and fishmeal. Diet K35 had three times the copper level and four times the fluoride level compared to K0. It should be noted that 2.5 mg/kg $CuSO_2$ was added to all diets (as a component of the mineral supplement). Thus, a much larger proportion of copper originated from the supplement in the K0 diet than in the K35 diet. Arsenic content, which was lower in Antarctic krill meal than in fish meal, was approximately three times higher in the K0 diet than in the K35 diet. The amount of calcium in the K35 diet was approximately 18% lower in the K35 diet than the K0 diet.

Table 7: Mineral Content of Antarctic Krill Meal, Fishmeal and Experimental Diets

Mineral	Antarctic krill meal	Fishmeal	K0	K9	K17	K35
			mg/kg			
Copper	56	3.3	3.7	5.3	6.9	10.1
Fluoride	1840	112	44	91	115	200
Arsenic	2.5	5.7	2.5	2.0	1.7	0.7
Calcium	25000	26000	10750	10150	10590	8070
			mg/kg DM			
Copper	61	3.6	10.0	14.6	19.8	30.2
Fluoride	2004	122	119	251	328	594
Arsenic	2.7	6.2	6.8	5.6	4.8	2.2
Calcium	27230	28410	29170	28130	30210	23960

Clinical Observations

No test diet-related effects were observed on maternal feed consumption or body weights. Fecal consistency was similar and good for all animals. The average body weight of females showed only minor differences among the treatments (Table 9).

Reproductive Performance and Kit Survival

No test diet-related effects were observed on reproductive performance and kit survival. Four animals in the K0 group, two in the K17 group and three in the K35 group did not produce offspring (Table 8). The overall *percentage* of barren females for the study (9/64 or 14%) was slightly higher than usual (10-11%) (Østergaard 2013), but this observation was not considered related to the test diets. Contributing to the relatively high *percentage* of barren females in the control group (25%) was the mating of two of the females with the same male, who was likely sterile because none of his matings had produced offspring. Kits that survived to Day 7 generally survived to study termination, regardless of the test diet. The overall survival rate to weaning was 92.7%, and ranged from 88.6% in the K17 group to 95.7% in the K0 group, which the authors reported to be within normal range.

Table 8: Reproductive Success, Litter Size and Kit Survival of Mink Fed Each of Four Experimental Diets

	K0	K9	K17	K35
Females mated, number	16	16	16	16
Litters born, number	12	16[b]	14	13
Litter size (mean ± SD[a])				
2d post-parturition	5.8 ± 1.4	5.3 ± 1.9	5.0 ± 1.9	6.3 ± 2.3
7d post-parturition	5.6 ± 1.6	5.0 ± 2.2	4.4 ± 1.9	5.8 ± 2.8
21 d post-parturition	5.6 ± 1.6	5.0 ± 2.2	4.4 ± 1.9	5.5 ± 2.8
49 d post-parturition (weaning)	5.5 ± 1.5	4.9 ± 2.1	4.3 ± 1.8	5.1 ± 2.8
Survival until weaning (%)	95.7	93.8	88.6	92.7

[a]SD = standard deviation; [b]One litter with one kit was stillborn.

As shown in Table 8, krill inclusion level did not affect litter size significantly. Body weights of the kits in the K35 group were significantly lower (by approximately 18%) than those of the K0 group at 49 days post-partum. Since the K35 treatment had the highest initial mean litter size, the lower kit body weights may be related to the well-known negative relationship observed between litter size and body weights. Another factor that may have impacted kit body weights was the consistency of the K35 diet, which tended to be dry and crumbly, and this could have contributed to a poorer feed utilization and subsequently affected kit growth. Although feed consumption was generally similar for all groups, the authors observed more frequent unrecorded feed spillage with the K35 diet. Therefore, the apparent effect of the Antarctic krill meal on kit body weight is likely to be an artifact of the study and not directly related to maternal toxicity.

Table 9: Body Weights of Females and Kits from the Start of Reproduction Trial until Weaning

Body weight, g	Diet				*P*-value
	K0	K9	K17	K35	
Females					
BW February 17	1156 ± 106	1151 ± 110	1130 ± 85	1033 ± 413	0.511
BW March 4	1098 ± 121	1030 ± 111	1000 ± 84	1106 ± 112	0.052

U/L) ($P < 0.05$). The increase in amylase along with the smaller size of glycogen vacuoles in the livers of K35 group animals suggest that glycogenolysis was stimulated by the krill meal.

Single focal leukocyte aggregations were observed in the livers of K0 (2/12), K9 (4/16), K17 (1/13), and K35 (0/13) animals. A few more animals in the K17 and K35 groups exhibited small, multifocal (≤ 5) inflammatory foci in the liver compared to the K0 group, namely K0 (0/12), K9 (1/16), K17 (3/13), and K35 (4/13). Liver enzymes (aspartate transaminase and alanine transaminase) and creatine kinase demonstrated large individual variation, most notably in the animals given the K35 diet in which several high values were observed; however, there were no significant difference between the test or control groups for these parameters. The location of the inflammatory foci within the liver was not reported, but given that several high values for liver enzymes were observed in the K35 group, the possibility for liver injury cannot be ruled out for animals given the highest inclusion of krill meal.

Most kidney samples appeared histologically normal. Abnormalities were noted in 3/12 samples from the K0 group, and 4/13 samples from the K35 group. Abnormalities in both groups included the presence of basophilic crystalline material within renal tubules, accompanied by tubular degeneration, which was sometimes but not always accompanied by signs of inflammation. There is no indication that the presence of basophilic crystalline material in the renal tubules is dose-related; however, histopathology of the kidney was conducted only for the K0 and K35 animals.

Overall, spleen samples appeared normal, with the exception of pigment deposition in a greater number of animals in the K35 group than the K0 group (5 *versus* 1) (Table 10). The pigment deposition in the spleen could possibly be related to the astaxanthin that is present in krill. A few animals from both groups had active lymphoid follicles, which did not correlate with peripheral blood lymphocyte counts. Several samples from both the K0 and K35 groups (9/12 samples in the K0 group and 7/13 samples in the K35 group) showed areas of cellular vacuolar degeneration, which was sometimes but not always accompanied by signs of necrosis and mild inflammation in the adrenal cortex. Hematological evaluations were unremarkable with the exception of an increased platelet count in the K35 group compared to the K0 group ($656 \pm 103 \times 10^9$/L) ($P < 0.05$). Mean white blood cell count (WBC) was higher in K35 animals compared to K0 animals; however, these values did not reach statistical significance ($P = 0.0702$). The increases in WBC counts in the K35 group were largely attributed to three animals that exhibited markedly increased total WBC ($20.2 - 20.5 \times 10^9$/L), neutrophil ($14.6 - 17.9 \times 10^9$/L) and monocyte counts ($0.8 - 1.0 \times 10^9$/L). The results indicate the immune system of the animals was stimulated by some component of the K35 diet, which may account for the increase in WBC, platelet counts and spleen weights. The higher spleen weights may possibly be a secondary effect from a turnover in RBCs since K35 animals with lower RBC counts tended to have higher spleen weights. This observation may also be related to the increased platelet count since the animals with the lower RBC tended to have the higher platelet counts.

Total plasma protein was not significantly related to dietary krill meal level, but albumin was lower in the K35 group (34.8 ± 6.1 g/L) than the K0 group (40.4 ± 3.8 g/L) ($P < 0.05$). Consequently, globulin (a calculated value) was higher in the K35 group ($P < 0.05$). The reason for the lower serum albumin is unclear.

Conclusion

Based on average feed consumption and initial body weights, corresponding doses of krill meal for the K9, K17, and K35 groups were 7.2, 14.3, and 31.2 g/kg bw/day[17], respectively. Fluoride intake from diet was 8.9, 18.4, 23.7 and 45.9 mg/kg bw/day for K0, K9, K17 and K35 groups, respectively[18]. The no observable adverse effect level (NOAEL) assigned to the study is 18.7% of dry matter (14.3 g/kg bw/day). Inclusion of 38.8% Antarctic krill meal in diets of pregnant mink, based on dry matter content of the feed (providing a dose of 31.2 g/kg bw/day) was associated with decreased glycogen content of the liver, decreased plasma albumin, increased weights of the stomach, intestine and spleen, intestinal and rectal redness, rectal inflammation and increased platelet counts.. No test diet-related effects were observed for maternal feed consumption, body weights, kit survival or reproductive performance.

Developmental toxicity study in rats

Tohjo (1980) examined the effect of diets containing various forms of krill -supplemented diets on the growth, nitrogen-retention, and offspring of female Sprague-Dawley rats (approximately 240 g weight) during pregnancy and lactation (non-GLP, non-Guideline). The results of the study indicate that various processing methods of krill for a feed ingredient can impact its nutritional quality and therefore possibly affect the development of rats when given in the diet.

Pregnant rats (5/group) were placed on one of four experimental diets. The control diet comprised 16.0% casein, while the test diets contained 22.0% unboiled krill meal, 22.2% boiled krill meal, or 18.6% ethanol-treated krill powder. The various krill meals were processed as follows:

Unboiled Krill Meal:
Within four hours of being caught, the raw krill are broken up in a mixer, put through a continuous centrifugation to separate the cake, and is dried in a rotary dryer at 140-150°C and made into 60-mesh powder.

Boiled Krill Meal:
Immediately boiled in sea water for several minutes and then put into frozen storage. It is then thawed and air-dried at 60-70°C and made into 60-mesh powder.

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[17] Calculated based on K9, K17, and K35 diets containing 35.5, 69.5, and 136 g krill meal/kg feed, respectively, average feed consumption and initial body weights for each group. This takes into account the conversion from an "as dry matter" basis to an "as fed" basis. For example, the K9 group was provided 35.5 g krill meal/kg feed, which consumed on average 233 g feed/day and each mated mink weighed 1.151 kg at initiation: [(35.5 g krill meal/kg feed) * (0.233 kg feed consumed/day)]/(initial mink body weight of 1.151 kg) = 7.2 g/kg bw/day. Similarly, for the K17 group: (69.5 g krill meal/kg feed * 0.233 kg feed consumed/day)/1.130 kg bw = 14.3 g/kg bw/day krill meal; and for the K35 group: (136 g krill meal/kg feed * 0.237 kg feed consumed/day)/1.033 kg bw = 31.2 g/kg bw/day krill meal.

[18] Calculated based on average food consumption (as fed), initial body weight of females, and fluoride content (as fed) reported in the study.

Ethanol-Processed Krill Powder:
Boiled krill powder is extracted three times with triple the amount of ethanol and the residue is air-dried.

The nitrogen levels of the test diets containing the various krill meals were made equal to that of the casein diet. The amount of protein in the various test meals was 83.7% in casein, 61.0% in unboiled krill meal, 60.4% in boiled krill meal and 71.8% in ethanol-processed krill. The amount of crude fat in the test meals was 0.8% in casein, 13.7% in unboiled krill meal, 12.5% in boiled krill meal, and the ethanol-processed krill is reported to have lost nearly all fat and color.

The diets were provided *ad libitum* to the dams on Gestation Days (GD) 1 through 22 and on Lactation Days (LD) 1 through 15. Based on estimated food consumption of 20 g/day[19] and initial body weight (240 g), the dams consumed 13.3 g casein/kg bw/day, 18.3 g unboiled krill meal/kg bw/day, 18.5 g boiled krill meal/kg bw/day, and 15.5 g ethanol-treated krill powder/kg bw/day over the course of the experiment. From the third to the 20th day of pregnancy, nitrogen intake and nitrogen levels in the urine and feces were measured for all test groups. Nitrogen balance and efficiency were calculated using the Kjeldahl method.

There were no significant differences in mean maternal body weights of the control or the various krill-supplemented groups on GD 1 and GD 12. At GD 22, the mean body weight of the dams consuming the diet containing unboiled krill meal (299 ± 9.0 g) was significantly lower than the mean body weight for the group fed the casein diet (381 ± 10.7 g) ($P < 0.01$). There was no difference between the mean body weights of the dams consuming boiled krill meal or ethanol-treated krill meal and the casein diet at GD 22.

The nitrogen intake in dams consuming the unboiled krill meal diet (266 mg/day) for up to 18 days was lower than the casein (402 mg/day), boiled krill meal (390 mg/day) and ethanol treated krill meal (348 mg/day) diets. Whereas the nitrogen retention and nitrogen efficiency ratios of the boiled krill meal and ethanol-treated krill meal groups were similar to those of the casein group, those of the unboiled krill meal group were significantly lower than the casein group throughout pregnancy ($P < 0.01$). There was no statistically significant effect of any of the diets on litter size or weight or growth of offspring compared to the casein group; however, the mean litter size (i.e., pups/litter) was smaller in the unboiled krill meal-fed group (8.4) compared to the casein group (13.0). The litter sizes of the groups given boiled krill meal or casein diet were virtually identical (mean of 12.8 *versus* 13.0, respectively).

The results of the study indicate that there is no effect of boiled krill on the development of rats, and a possible effect of the unboiled krill meal on nitrogen intake in dams and the numbers of offspring in a litter. The authors suggest these effects might be from the loss and alteration of nutritional components in the unboiled krill meal due to dry processing at high temperatures from 140-150°C. However, these effects could also be from the lack of inactivation of endogenous proteases present in the krill since it was not boiled immediately after harvest.

[19] Shirley, B. 1984. The food intake of rats during pregnancy and lactation. *Lab Animal Sci.* 34(2): 169-72 (abstract consulted)

Antarctic krill produce hydrolytic enzymes including proteases, carbohydrases, nucleases and phospholipases, all of which appear to be concentrated in the digestive gland in the cephalothorax (FAO 1997; Chi et al. 2013). The purification and characterization of krill proteases have been reported. For example, krill tryptase is reported to show strong degradative efficiency of up to 60-fold greater than that of bovine pancreatic protease (Chi et al. 2013). The presence of hydrolytic enzymes plays a role in autolysis, which is rapid postmortem and results in the spoilage of Antarctic krill (Kawamura et al. 1981; Gigliotti et al. 2008; Chi et al. 2013). This phenomenon is also observed in the spoilage of fish resulting from enzymatic autolysis and the gutting of fish immediately after its catch is essential to remove the presence of strong proteases found in the digestive tract (Ghaly et al. 2010). The autolysis of frozen intact krill, once thawed, is rapid and is further accelerated upon the homogenization of raw krill. Among the muscle proteins in krill, the myosin heavy chain is degraded extensively during autolysis (Kawamura et al. 1981). The presence of krill proteases have been a major obstacle in its use as a food ingredient and processes have been developed to inactivate its proteolytic enzymes immediately after harvest, including freezing krill to below -40°C to inactivate its enzymes, or heating krill to above 80°C to disable its enzymes (Yoshitomi and Shigematsu 2002; Yoshitomi 2004). In the rat study, the unboiled krill meal was produced within four hours of the krill being caught and the raw krill was broken up in a mixer. Therefore, it is likely that the release of hydrolytic enzymes during the processing may have altered the nutritional quality of proteins in the unboiled krill. The boiled krill meal and the ethanol-treated krill used in the study were both produced from krill immediately boiled for several minutes after harvest. The level of protease in Qrill™ Pet is less than the limit of detection due to processing at elevated temperatures (i.e., cooked by direct steam) after harvest as described for the manufacturing process in Part 2 of the dossier.

The results of the rat study underscore the importance of the proper processing of krill to maintain the quality and functionality of krill proteins and thereby provide adequate nitrogen intake during pregnancy in rats.

iv. Genotoxicity

Bacterial mutagenicity

Qrill™ Pet was nonmutagenic in a bacterial reverse mutation (OECD Guideline 471) assay using a plate incorporation (experiment 1) and a preincubation (experiment 2) method performed under GLP (Schreib 2014). Strains used in the study included *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* (*E. coli*) WP2uvrA. Each assay was conducted in the presence and absence of metabolic activation with S9 mix prepared from the S9 microsomal fraction of the livers of Aroclor 1254-treated adult, male Fischer rats. Preliminary solubility experiments using the solvents water and ethanol showed that the test material was insoluble in each of these solvents. A clear, orange colored stock solution was prepared when a solution that was prepared in DMSO (but not ethanol or water) solvent was homogenized for 1-2 minutes at 37°C using an Ultra Turrax®. The stock solution was diluted in DMSO to the appropriate test concentrations (3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate). The DMSO solvent was compatible with the survival of the bacteria and the S9 enzyme activity.

The test substance did not induce any significant or dose-dependent increases in the numbers of revertant colonies in any strain tested in the absence or presence of S9 mix. Precipitation of the test substance was observed in all strains at concentrations ≥ 31.6 µg/plate with and without metabolic activation. The precipitate did not interfere with the scoring – all concentrations were analyzable. The material did not entirely precipitate out of solution, as wells containing the highest concentrations showed the highest intensity of the color of the test material. No toxic effects of the test substance were noted in any strains, except for strain TA 1537 at concentrations ≥ 2500 µg/plate (pre-incubation test, without metabolic activation). The reference mutagens (sodium azide, 4-nitro-o-phenylene-diamine, and methylmethanesulfonate without S9 and 2-aminoanthracene with S-9) induced a distinct increase in revertant colonies, indicating the validity of the experiment.

Clastogenicity

Qrill™ Pet did not induce chromosomal aberrations in an OECD Guideline 474 study performed under GLP to investigate the potential of Qrill™ Pet to induce micronuclei in polychromatic erythrocytes (PCE) in peripheral blood of 6-12 week old NMRI mice (Wessels 2014 - unpublished data). As mentioned in the acute toxicity section above, results of a range-finding study for the micronucleus study in 6- to 12-week old NMRI mice indicate that the oral lethal dose of Qrill™ Pet is > 2,000 mg/kg bw (when administered in divided doses of 1,000 mg/kg bw two hours apart) (Wessels 2014 - unpublished data).

The test item was suspended in cottonseed oil at a concentration of 100 mg/mL, by using an Ultra Thorax homogenizer for five minutes. The preparation with cottonseed oil resulted in a homogenous suspension (which could not be achieved in an aqueous vehicle). The animals (five/sex/dose group) received (*via* oral intragastric administration) 2000 mg/kg bw as a split dose (the maximum tolerated dose determined in a preliminary study), 1000 mg/kg bw or 500 mg/kg bw test substance or vehicle (cottonseed oil). For all groups, including a positive (40 mg/kg bw cyclophosphamide, *i.p.*)[20] control, blood samples were collected from the tail vein for micronuclei analysis 44 h (all doses) and 68 h after the second split dose application of 2000 mg/kg bw. Blood cells were immediately fixed in ultracold methanol. Before analysis (at least 24 h after fixation), fixed blood cells were washed in Hank's balanced salt solution, centrifuged at 600 x g for five minutes and the supernatant discarded. Blood cell populations were discriminated using specific antibodies against CD71 (expressed only at the surface of immature erythrocytes) and CD61 (expressed at the surface of platelets) and DNA content of micronuclei was determined by the use of a DNA specific stain (propidium iodide, PI). Samples, including those of positive and negative controls, were evaluated using a flow cytometer (FACScan, BD Biosciences).

As shown in Figure 2 and Figure 3, there was no significant effect of Qrill™ Pet on the incidence of micronucleated peripheral blood cells in the mouse, at 44 or 68 hours. Cyclophosphamide induced a statistically significant increase in micronucleus frequency (mean *percentage* of cells with micronuclei was 1.55% for male and 1.23% for female mice). Under

[20] *i.p.* = intraperitoneal

the experimental conditions reported, Qrill™ Pet did not induce structural and/or numerical chromosomal damage in the immature erythrocytes of the mouse.

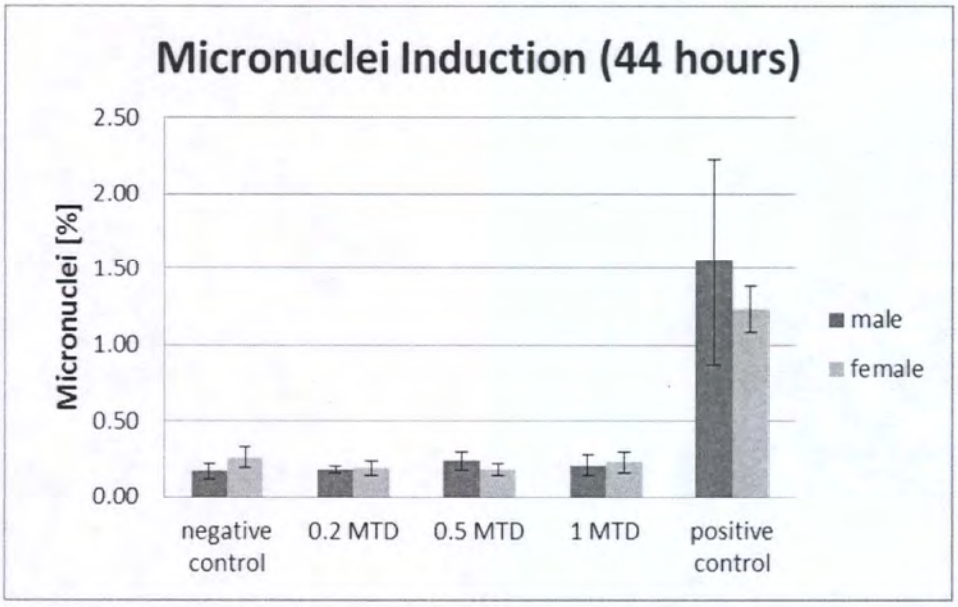

Figure 2: Incidence of Micronucleated Peripheral Blood Cells in Mice Treated With Qrill™ Pet for 44 Hours

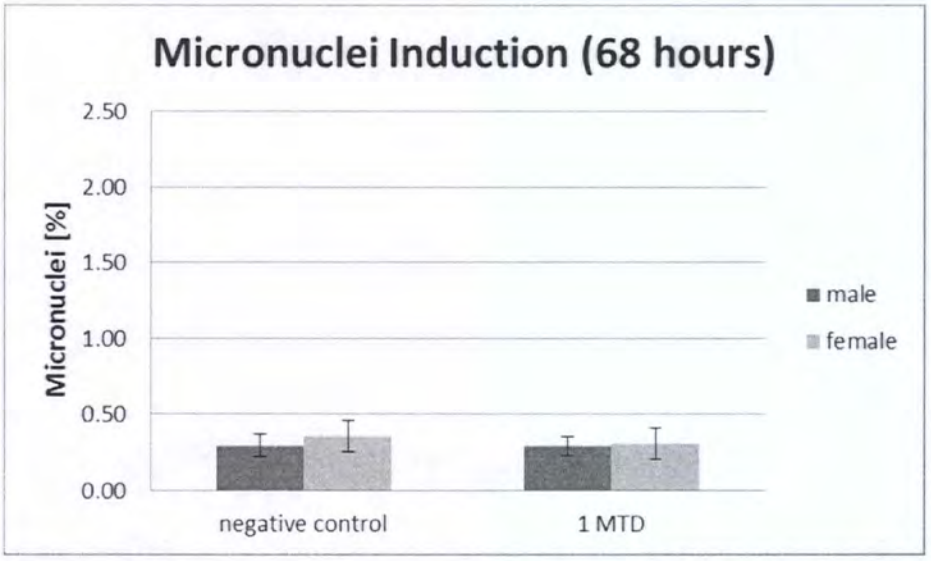

Figure 3: Incidence of Micronucleated Peripheral Blood Cells in Mice Treated With Qrill™ Pet for 68 Hours

v. Summary of krill meal toxicity and ADI derivation

In summary, Qrill™ Pet is not acutely toxic with an LD_{50} of > 2,000 mg/kg bw in mice. It is non-mutagenic in bacteria, and did not induce chromosomal aberrations in an *in vivo* mice

micronucleus assay. Summaries of repeated-dose toxicity studies performed on different compositions of krill meal, including reproductive and developmental toxicity studies in various species are listed in Table 11. In mink, the LOAEL of 31.2 g/kg bw/day (38.8% dw) was determined based on the presence of maternal toxicity, including decreased glycogen content of liver, decreased plasma albumin, increased stomach, intestine and spleen weight, intestinal and rectal redness, rectal inflammation, and increased platelet counts (Krogdahl et al. 2015b). Similar effects were also observed in the 15-week growth study at similar doses in mink (31.2 g/kg bw/day and 26 g/kg bw/day for males and females, respectively, 17.5 – 21.0% dw) (Krogdahl et al. 2015a). Adverse effects were most likely attributed to excess fluorine intake in the krill meal. The increase in gastrointestinal organ weights are likely related to the presence of chitin and the intestinal and rectal redness along with rectal inflammation are likely due to irritation from small shell fragments in the krill meal. The redness may also be due to staining by astaxanthin that is naturally present in krill. In adult Husky dogs, an estimated dose of 2.1 – 2.5 g/kg bw/day (8% dw) did not affect clinical chemistry parameters or cause any apparent signs of toxicity when consumed for up to 14 weeks. Instead, increased omega-3 index (a beneficial effect) was found (Berge et al. 2014; Hal 2016 – unpublished data). In rats, delayed development of seminiferous epithelium was reported at 4.6 g/kg bw/day which was attributed to high fluorine content in the krill meals containing high levels of shell (Pastuszewska *et al.* 1983), and 4.9 g/kg bw/day was associated with histological changes in the liver, kidney, spleen and brain for a krill meal containing 2,416 mg F/kg dw (much higher than 1,500 mg F/kg dw for Qrill™ Pet according to product specification) (Zhang et al. 2013). 18.3 g/kg bw/day unboiled krill meal, but not boiled krill meal or ethanol treated krill powder (with inactivated krill proteolytic enzymes) at similar doses, led to decreased nitrogen intake in dams and a reduced number of offspring per litter in a developmental toxicity study in rats which are likely due to the improper processing of the krill thereby affecting its nutritional quality (Tohjo 1980). 18 g/kg bw/day krill meal did not affect hematological parameters in rats, but krill carapace meal caused decreased hematocrit, hemoglobin, mean corpuscular thickness and MCV, and the study authors attributed the effects to the high fluorine content typically found in the carapace (Zaleska-Freljan and Cywińska 1991). These studies suggest that fluorine, which is most concentrated in the carapace, is the major toxicological concern of krill meal.

As previously discussed, the studies performed in rats used krill meals that are very different in composition from Qrill™ Pet. Although krill meal containing 2,416 ppm fluorine caused soft tissue effects with a LOAEL of 4.9 g/kg bw/day, the NOAEL of a krill meal that is more similar in composition to Qrill™ PET with 1,840 ppm fluorine is 13.3 g/kg bw/day as established in mink. Therefore, the mink studies are selected as critical studies for ADI establishment. The lowest LOAEL among all relevant studies in Table 11 is 26 g/kg bw/day in female mink in the growth study conducted by Krogdahl et al. (2015b). The highest NOAEL lower than this lowest LOAEL is 14.3 g/kg bw/day in female mink in the gestation/lactation study in mink (Krogdahl et al. 2015b). While a higher NOAEL of 16.6 g/kg bw/day is reported in the growth study in male mink (Krogdahl et al. 2015a), this dose level has not been tested in females in any of the studies identified, and **therefore the lower NOAEL of 14.3 g/kg bw/day that has been tested to be protective of both male and female mink is used to derive an ADI for dogs.** Additionally, selection of the lower NOAEL from the gestation/lactation study in mink is also protective of pregnant dogs. This selection is supported by the two tolerance studies in dogs reporting a NOAEL of 2.1 – 2.5 g/kg bw/day, which was the only dose tested.

According to the U.S. FDA (2007), the acceptable daily intake (ADI) is calculated by dividing the dose level of the substance in animal studies that was shown to cause no adverse effects (i.e., NOAEL) with an appropriate safety factor. Typically a safety factor of 100 (10 for intraspecies variation * 10 for interspecies extrapolation) is applied to extrapolate from animal data to humans. A safety factor was not specified to extrapolate between animal species. As a dose in mink is used to establish a safety dose for dogs, interspecies allometric scaling of doses was applied using the method recommended by the Center for Veterinary Medicine of the U.S. FDA for animal drugs (U.S. FDA 2008). The same method is also widely used by veterinarians to extrapolate scale doses from smaller to larger animals to account for pharmacokinetic differences between species (Sharma and McNeill 2009).

$$NOAEL_{Dog} = NOAEL_{Mink} \times (BW_{Mink}/BW_{Dog})^{0.25}$$

Where:

$NOAEL_{Dog}$ is the NOAEL to use to derive an ADI for dogs;
$NOAEL_{Mink}$ is the NOAEL established by the critical study in mink, which is 14.3 g/kg bw/day (Krogdahl et al. 2015b);
BW_{Mink} is the body weight of female mink reported in the critical study before mating, which is 1.156 kg;
BW_{Dog} is the body weight of female Cairn terrier dogs – 5.9 kg (American Kennel Club 1938), a small but active dog breed that has been used in the conservative derivation of estimated daily intake (EDI) in Part 3 of the dossier.

Therefore,

$$NOAEL_{Dog} = 14.3 \text{ g/kg bw/day} \times (1.156/5.9)^{0.25} = 9.5 \text{ g/kg bw/day}$$

A safety factor of 10 is by default used for interspecies extrapolation, which includes 3 for pharmacokinetics differences and 3 for pharmacodynamics differences. As allometric scaling is used to convert mink doses to dog equivalent doses, only a safety factor of 3 is necessary to account for pharmacodynamics differences between species. An additional safety factor of 3 is used to account for intraspecies differences in dogs. This is smaller than the default factor of 10 for intraspecies variation because the body weight and energy requirement data of terrier dogs are used in this calculation as well as in the EDI calculations. Terrier dogs are one of the most active dogs with the highest metabolic rate and energy requirement per kilogram of body weight (NRC 2006a). Therefore, EDIs calculated based on this breed is expected to be reasonably worst case scenarios. However, some uncertainty still exists as limited data are available to evaluate the metabolic rate of the smallest dog breeds (e.g., pocket breeds) that may consume more food on a body weight basis. Therefore, a safety factor of 3 is used to account for this uncertainty. This leads to a composite safety factor of 10 (i.e., 3 for interspecies extrapolation with the use of allometric scaling, and 3 for intraspecies variation).

Therefore,

$$ADI_{Qrill\ Pet} = \frac{NOAEL_{Dog}}{Safety\ Factor} = \frac{9.5\ g/kg\ bw/day}{10} = 0.95\ g/kg\ bw/day$$

Table 11: Summary of Studies Conducted to Assess The Effect of Krill Meal in Dogs, Mink and Rodents

Species	Dose	Duration	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Critical Effect	Reference
Female mink (black genotype, 16/group)	0 (control), 9.4, 18.7, or 38.8% dw (0, 7.2, 14.3 or 31.2 g/kg bw/day)	117 days, including a pre-mating period of 17 days, a gestation period of ~50 days, and 49 days after birth	14,300 (18.7% dw)	31,200 (38.8% dw)	Decreased glycogen content of liver, decreased plasma albumin, increased stomach and intestine weight, intestinal and rectal redness, rectal inflammation and increased platelet counts.	Krogdahl et al. 2015b
Growing mink (black genotype, 52-53 days old, 8/sex/group, offspring of mink exposed during reproduction)	0, 9.1, 21.0 and 36.2% dw (1st growth period); 0, 8.7, 17.5 and 35.3% dw (2nd growth period), equivalent to 0, 16.6/13/3, 31.2/26.0, and 71.2/63.1 g/kg bw/day for M/F, respectively	15 weeks	16,600/13,300 (M/F) (8.7 – 9.1% dw)	31,200/26,000 (M/F) (17.5 – 21.0% dw)	Histological changes in the liver and kidney, alterations in clinical chemistry and hematology at two highest doses, and joint/bone deformities and increased stomach and rectum weights at the highest dose.	Krogdahl et al. 2015a
Adult Alaskan Husky dogs (14 control, 16 treated, 13 M, 17F)	0 or 8% wt/wt dw (0 or 2.1 – 2.5 g/kg bw/day)[a]	52 days	2,100 – 2,500 (M & F) (8% dw)	NA	No adverse effects on clinical chemistry	Berge et al. (2014) – unpublished
Adult Alaskan Husky dogs (14 control, 16 treated, 13 M, 17F)	0 or 8% wt/wt dw (0 or 2.1 – 2.5 g/kg bw/day)[b]	14 weeks	2,100 – 2,500 (M & F) (8% dw)	NA	No adverse effects on clinical chemistry	Hal (2016) - unpublished
Weanling male Wistar rats (6/group)	0, 2% krill chitin, or 9.2% krill shell meal (0, 1.1 g krill chitin/kg bw /day, or 4.6 g krill meal/kg bw/day)	8 weeks	NA	1,100 for krill chitin (2% dw) 4,600 for krill meal (9.2% dw)	Delayed development of seminiferous epithelium in animals treated with krill chitin and krill meal, which the authors attributed to the toxicity of fluorine	Pastuszewska et al. (1983)

Wistar rats (11-12 weeks old, n = 29, 4 – 5/group, sex not specified)	0, 18.1% standardized krill meal, 18.8% krill meal with a lowered chitin content, 12.6% casein supplemented with 0.15% D,L-methionine, or casein supplemented with D,L-methionine and 9.2% krill carapace meal or 3.0% ash from standardized krill meal	2 months	18,000 (18.1% dw)[c]	NA	Decreased hematocrit, hemoglobin, mean corpuscular thickness and MCV only in the group consuming krill carapace meal. Adverse hematological effects observed with krill carapace were attributed to fluorine	Zaleska-Freljan and Cywińska (1991)
Wistar rats (3 months old, n = 73, male and female offspring from the study above)	0 or 18.1% standardized krill meal with low chitin content	2 months	18,000 (18.1% dw)[c]	NA	No effects on hematological indices	Zaleska-Freljan and Cywińska (1991)
Newly weaned Wistar rats (10/group, sex not specified)	0, or 150 mg F/kg bw/day (Antarctic krill or NaF)	3 months	NA	4,900 (5% dw)[c]	Fluorine effects in liver, kidney, spleen and brain. Effects less severe in rats treated with krill meal compared to those treated with equivalent NaF	Zhang et al. (2013)
Female SD rats	16.0% casein (control), 22.0% unboiled krill meal, 22.2% boiled krill meal, or 18.6% ethanol-treated krill powder (13.3 g casein/kg bw/day, 18.3 g unboiled krill meal/kg bw/day, 18.5 g boiled krill meal/kg bw/day, and 15.5 g ethanol treated krill powder/kg bw/day)	GD 1 – 22 and LD 1 - 15	18,500 (boiled krill meal) 15,500 (ethanol-treated krill powder)	18,300 (unboiled krill meal)	Decreased nitrogen intake and reduced number of offspring in a litter in rats receiving unboiled krill meal. No adverse effects in other test groups	Tohjo (1980)

Female SD rats	10% KPC, 10% casein, 10% casein for two-weeks followed by a protein0free diet for another two weeks	28 days	9,800 (KPC)	NA	No effects on clinical chemistry, kidney function, urine output, and organ weights	Gigliotti et al. (2008)

bw = body weight; dw= dry weight; F = females; F = fluorine; GD = gestation day; LD = lactation day; M = males; NaF = sodium fluoride; NOAEL = No observed adverse effect level; LOAEL = Lowest observed adverse effect level; ppm = parts *per* million; SD = Sprague-Dawley; [a] doses estimated based on the standard body weight of Husky dogs (Siberian Husky Club of America 2010) and food consumption data provided by Berge et al. (2014); [b]doses estimated based on the standard body weight of Husky dogs (Siberian Husky Club of America 2010) and food consumption data provided by Hal (2014); [c]estimated using default values for Wistar rats in subchronic toxicity studies (U.S. EPA 1988)

b. Toxicity of Fluorine

Fluorine is present at relatively high concentrations in Qrill™ Pet; therefore, it is important to determine whether fluorine toxicity could occur in dogs ingesting food containing up to 3% Qrill™ Pet. In humans, fluoride is considered an important dietary element to prevent dental caries. The Institute of Medicine (IOM) concluded that daily intake of 0.01 – 4 mg/day (approximately 0.0014 mg/kg bw/day for infants 0 – 6 months old and 0.05 mg/kg bw/day for older populations) is adequate to decrease the occurrence of dental caries. The World Health Organization stated that fluoride is "essential" because the resistance to dental caries is a physiologically important function (ATSDR 2003).

Dietary fluorine is well absorbed, distributed primarily to bone and teeth, and eliminated in urine (NAS 1974; Bagga et al. 1979). Toxicokinetic studies indicate that absorption and excretion of ingested fluoride do not significantly differ by age. However, bone uptake of fluoride is strongly age-dependent – a higher percentage of ingested fluoride is sequestered in bone in the young compared to adults (ATSDR 2003). In bone and teeth, fluorine substitutes for the hydroxyl group in calcium hydroxyapatite crystals, forming fluorapatite (NAS 1974; Romanus 1974). The fluoroapatite crystal is smaller than hydroxyapatite, stabilizes the unit cell, and decreases the solubility of bone, enamel, and dentin (NAS 1974; Romanus 1974). Fluoroapatite is also more resistant to acid attack than hydroxyapatite, helping to prevent dental caries. Once fluoride is incorporated into the apatite of bone, it cannot be removed without resorption of the unit crystalline structure of the mineral phase (NAS 1974). However, fluoride concentrations in teeth decrease after fluorine exposure is withdrawn in dogs (Saunders and Weidmann 1969).

An individual on a long-term, relatively constant fluoride intake reaches an equilibrium between intake and retention, at which time fluoride uptake by bone is reduced and the concentration of fluoride in urine increases (NAS 1974; Bagga et al. 1979). However, skeletal fluorosis may occur when fluorine is consumed in excess. Skeletal fluorosis is characterized by an initial thickening and slight increase in density of cortical bone, followed by irregular periosteal and enosteal thickening, reduced dimensions of the medullary canal, and calcification of the attachment of ligaments and tendons (Romanus 1974). Stiffness, lameness, osteoporosis, osteosclerosis, osteomalacia, hyperostosis, and/or osteophytosis have been observed in animals ingesting excessive levels of fluoride for long periods of time (NAS 1974). Dental fluorosis may also occur in developing or calcifying teeth, but not in mature teeth (NAS 1974). Teeth affected by dental fluorosis are often discolored (creamy yellow to brown or black) and subject to more rapid attrition, and in some cases, show erosion of enamel (NAS 1974). Other symptoms of fluorine toxicity include restlessness, anorexia, excessive salivation, nausea, vomiting, incontinence, clonic convulsions, necrosis of the digestive tract mucosa, weakness, severe depression, and cardiac failure (NAS 1974). Fluorine does not affect reproduction at doses that are not maternally toxic (NAS 1974).

A literature search was conducted to locate information about the effects of fluorine in dogs. A total of seven studies were identified in dogs, all of which have limitations, such as the test of only one dose and the use of diets low in magnesium or calcium. Therefore, data on mink were also presented as supportive evidence. Numerous studies were identified in rats, mice and rabbits, which have been extensively reviewed by ATSDR (2003). The ATSDR summary table

is attached in Appendix E. Summaries of the studies by oral route of exposure in dogs and mink are reported in Table 12, along with additional studies in rodents we identified that had not reviewed by ATSDR. As discussed below, rodents appear to be more sensitive to fluorine toxicity compared to dogs and mink, and therefore may not serve as good models for dogs. Therefore, an ADI for fluorine was derived primarily based on data in dogs and mink.

i. Studies in dogs

A total of seven studies were identified in dogs. However, only two can be used for dose-response analysis. They were described as the first two studies below. Four of the studies used modified diets to induce diseases in the dogs, and one study focused on findings of uncertain toxicological significance. These studies were also described following the key studies below.

Greenwood et al. (1946) conducted a chronic feeding study in dogs (non-GLP, non-Guideline) to investigate the effects of different forms for fluoride on the health of dogs. In this study, young dogs of unknown origin or age weighing 10 kg on average received 0 (21 dogs) or 5 mg added F/kg bw/day in the form of bone meal powder (36 dogs, up to 518 days), Defluorophos (rock phosphate) (22 dogs, up to 249 days) or sodium fluoride (20 dogs, up to 429 days). The basal ration contributed approximately 0.2 mg F/kg bw/day. The basal ration consisted of prepared dehydrated dog food (Pard), fresh beef lung (from U. S. inspected plant), evaporated milk (Carnation), cod liver oil, and Chicago city water ad libitum. Different fluorine supplements (bone meal powder, Defluorophos or sodium fluoride) were mixed in the basal ration and the supplements were fed at a level to provide 5 mg F/kg bw/day in dogs. These dogs went on to produce 21 litters that appeared to have received the same diets as their mothers. Body weight of puppies was measured every week to determine growth. Blood was taken from mothers and their puppies (during calcification and eruption of teeth) to evaluate coagulation time, hemoglobin, serum calcium, phosphorus and fluorine, X-rays of the femurs and tibias of representative dogs were taken starting from the age of 3 months and then every three months. X-rays were also taken on the jaws and teeth of representative dogs that were sacrificed for histology at the end of the study. Histopathological examinations were performed on the bones (rib, sternum and femur). In addition, physical measurements and breaking strength tests were made on the femurs and tibias of representative sacrificed dogs fed bone meal powder and defluorinated phosphate for approximately one year. Early in the experiment, several young dogs died due to reasons unrelated to the treatment (i.e., respiratory infection and canine distemper), which were subsequently controlled by vaccines and serums. There were no significant changes in serum parameters in any group. Puppies in the sodium fluoride group developed dental fluorosis and excess fluorine deposition in the bones while puppies in the other groups were normal. Dogs fed the bone meal and rock phosphate had superior teeth than those receiving fluorine as sodium fluoride or controls. Bone development in all groups was normal as demonstrated by breaking-strength tests, X-rays and histopathology. *NAS estimated a fluoride intake of about 50 ppm in the diet for this study and considered this dose a NOAEL due to lack of effects on growth). The NAS concluded that 50 ppm fluorine had no effect on growth and established 50 ppm as the permissible level of fluorine in feed of growing dogs. According to the reported doses, this NOAEL is equivalent to 5.2 mg/kg bw/day (5 mg added F + 0.2 mg F in basal ration). While only one dose was tested in this study, critical endpoints such as bone and teeth development has been evaluated in this chronic study. ToxServices identified a NOAEL of*

5.2 mg F/kg/day for this study for adult dogs for all three forms of fluorine (NaF, bone meal powder, and rock phosphate).

A reproductive toxicity study (non-GLP, non-Guideline) was conducted by Shellenberg et al. (1990) in dogs prompted by the report of increased incidence of perinatal deaths, mottled teeth in the surviving pups, and exostoses on bones in two breeding facilities over the course of 10 years in dogs consuming a particular brand of commercial dog food containing 460 ppm fluoride from rock phosphate added as a mineral source. The authors conducted this study to investigate the causes of the reproductive effects observed previously. In this study, twenty purebred Shetland Sheepdogs of proven fertility were divided into four groups of four females and one male each, and received high fluoride food (460 ppm) with well water (Group A), high fluoride food with distilled water (Group B), low fluoride food (55 pm) with well water (Group C) and low fluoride food with distilled water for two years. Animals were weighed weekly and examined for signs of estrous activity. Clinical chemistry, hematology and urinalysis and levels of serum T3, T4, progesterone, estradiol and F were examined approximately every 4 months. Thyrotropin (i.e., TSH) was determined in month 20 and at the end of the study. Animals were examined for bony exostoses every 4 months. Body weight of pups were measured daily for the first week and then weekly for 6 weeks. Necropsies were performed on pups that died, and 8 carcasses were examined for histopathology, bacterial cultures, and virology. In addition, to study factors contributing to perinatal deaths, 8 litters whelped away from the study kennel during their first week of life were studied for another 4 months at the end of the 2-year study. They remained at the study kennel until near term, moved to a host kennel for whelping and one week after whelping, and then returned to the study kennel with their litters. The host kennel was the place reporting a cluster of deformities prior to the study. The dams in this group received low F feed. The same parameters were examined as those in the main study. Study animals generally remained healthy during the study. Based on the reproductive histories of tested dogs, the authors expected at females would show estrus at least once a year, with pregnancy rate of 70%. However, only Group C produced as many pups as expected. The missed pregnancy rate (i.e., had estrous but did not mate, or mated but had no breedings) in the high F dogs (69% in Group A+B) was higher than that in the low F dogs (21% in Group C+D). However, missed pregnancy rate was also higher in the distilled water groups (56% in Groups B+D) than that in the well water groups (39% in Group B+D). A statistical test of these data was not possible due to some animals represented more than once in these counts. Reproductive failure of two high F males was attributed to heartworm treatment. Litters gestated at the study kennel but whelped at a host kennel had lower perinatal death rate (31%) compared to litters kept at the study kennel throughout gestation and whelping (50%). Examination of carcasses of pups did not find obvious causes of the perinatal deaths. The overall missed pregnancy rate of 44% and perinatal death rate of 50% in the 2-year study were consistent with reported values by the breeding facility. The authors noted that the generally poor reproductive performance in all groups except Group C was consistent with the poor performance in the same breed of animals grown in the study kennel, and that it was not due to F toxicity. Animals introduced to the study kennel had reduced estrous activity, which paralleled the problems with resident dogs in the kennel. Four dogs treated with 460 ppm fluoride had bony exostoses. The authors concluded that the high missed pregnancy rate and perinatal death rate likely occurred by chance, and that reproductive and developmental toxicity previously reported by breeders did not appear to be caused by high fluoride levels of 460 ppm in the diet, water sources, foliage, genetic factors or infectious

diseases. *Therefore, the NOAEL for reproductive and developmental toxicity was 460 ppm, which is equivalent to 11.5 mg/kg bw/day based on the conversion factors published by U.S. FDA (PAFA 1993). The NOAEL and LOAEL for systemic toxicity of adult animals are 55 and 460 ppm, respectively, based on bony exostoses development, which correspond to 1.4 and 11.5 mg/kg bw/day, respectively.*

Bunce et al. (1962) conducted a series of dietary experiments (non-GLP, non-Guideline) to investigate the impact of certain dietary factors (including fluorine) on the magnesium deficiency syndrome in dogs. For the fluoride study, weanling Beagle or Shepherd-Collie and Chow dogs (4 – 6 per group, sex, weight and age not specified) received a semi-purified low magnesium basal ration, the basal diet with 100 ppm added magnesium, or the basal diet with 250 ppm added fluorine as sodium fluoride for 11 weeks. For the first three weeks of study, animals supplemented with fluorine had 50 – 75% reduction in weight gain compared with animals fed the basal ration only. Both groups stopped gaining weight afterwards while the magnesium-supplemented dogs continued to gain weight. Fluorine-treated animals also had convulsions, muscular weakness, and reduced serum magnesium levels, but no aortic lesions were found, and calcium, phosphorus and total ash content of the aortas were normal. *ToxServices identified a LOAEL of 250 ppm based on decreased body weight gain, convulsions, and muscular weakness. Based on an estimated body weight of 2 kg and 1 cup/day (100 g/day) feeding schedule for Purina Healthy Puppy Formula (purinaone.com), the LOAEL of 250 ppm is equivalent to 12.5 mg/kg bw/day. As this study used a magnesium deficient diet, the findings may not be relevant to fluorine toxicity in the presence of normal diets.*

Chiemchaisri and Philips (1965), the same group of authors that conducted the Bunce et al. (1962) study above, conducted another series of studies in dogs and rats to investigate the impact of fluoride (as sodium fluoride) on magnesium calcinosis (non-GLP, non-Guideline). In the first experiment, groups of 2 – 4 dogs received magnesium-deficient diet (Lot 1), magnesium adequate diet (Lot 2), magnesium-deficient diet with 200 ppm fluoride (Lot 3), or pair-fed magnesium-deficient diet receiving the same amount of magnesium-adequate diets consumed by animals in Lot 3 (Lot 4). The duration, sex or age of dogs was not clearly stated, but it seems that the study lasted at least 6 weeks. Calcification of aortas, heart values and kidneys was observed in Lots 1 and 4, but not in groups receiving fluoride or adequate magnesium. Cumulative body weight gains were reduced for animals in Lots 3 and 4 (2.62, 2.77, 2.06 and 2.07 in Lots 1, 2, 3 and 4, respectively). The authors concluded that low magnesium calcinosis was not the direct result of reduced feed intake or the reduction of weight gain caused by fluoride. In the second experiment, dogs (4 – 6 per group, age and sex not specified) received the same magnesium-deficient diet (Lot 1), magnesium adequate diet (Lot 2), or the magnesium-deficient diet supplemented with 25, 50, 100 or 200 ppm fluoride (Lots 3 – 6) for 6 weeks. While magnesium deficiency symptoms were not observed in groups supplemented with fluoride and magnesium, a reduction in cumulative weight gain was found when dogs received 100 ppm and 200 ppm fluoride. Fluoride retention was evident in all groups fed sodium fluoride. The authors concluded that 25 ppm fluoride protected dogs from low magnesium calcinosis. The third study was conducted in Holtzman male rats (8/group) which received diets containing 30 (deficient), 200, or 400 ppm magnesium in the presence or absence of 400 ppm fluoride for four weeks. Rats fed the magnesium deficient diet (i.e., 30 ppm) had reduced cumulative weight gain with and without fluoride supplementation. In addition, high dietary fluoride increased calcium

concentration in the kidney at each dietary magnesium level. The fourth experiment did not involve fluoride and was therefore not described in this report. The fifth study was conducted in dogs. Animals (3-5 per group) received the magnesium-deficient diet for 6 weeks with or without 200 ppm fluoride supplementation followed by a 25-day repletion period. Fluoride supplementation slightly decreased growth rates in both the depletion and repletion periods, but prevented low magnesium-induced calcinosis of soft tissues. *A NOAEL of 50 ppm and a LOAEL of 100 ppm can be established for this series of studies based on growth retardation, which is equivalent to 2.5 mg/kg bw/day and 5 mg/kg bw /day, respectively, according to an estimated body weight of 2 kg and 1 cup/day (100 g/day) feeding schedule for Purina Healthy Puppy Formula (purinaone.com). As this study used a magnesium deficient diet, the findings may not be relevant to fluorine toxicity in the presence of normal diets.*

Henrikson *et al.* (1970) conducted a study to investigate the impact of dietary fluoride on bones in nutritionally induced hyperparathyroidism in adult dogs (non-GLP, non-Guideline). Ten beagle dogs (2/group, 3 males and 7 females) were fed a purified diet (550 g/two dogs/day) deficient in calcium and excess in phosphorus, which has been proven to induce nutritional secondary hyperparathyroidism in previous studies conducted by the same authors. This diet contained low fluoride but the actual level was not measured. Four groups of dogs received fluoride as sodium fluoride at 1, 3, 9, or 27 ppm for 287 days. The study authors estimated daily fluoride intake of 0, 0.026, 0.085-0.088, 0.295, and 0.825 – 1.125 mg/kg bw/day for each dose group. Parameters examined included dental radiographic examinations, densitometry and specific gravity of bones, chemical analysis of bone ash, and biomechanical examination of the bones. The results indicated that fluoride supplementation did not have any effect on nutritional osteoporosis. *Therefore, a NOAEL of 1.125 mg/kg bw/day can be established for fluoride in this study. As this study used a diet containing insufficient calcium and excess phosphorus, the findings may not be relevant to fluorine toxicity in the presence of normal diets.*

Snow and Anderson (1986) conducted a drinking water pilot study to examine the alterations in trabecular bone remodeling activity in response to sodium fluoride exposure (non-GLP, non-Guideline). Eight spayed 4-year-old Beagle dams were provided with regular tap drinking water (2 dogs) or tap water containing 11.6 ppm sodium fluoride (6 dogs) for 6 months. The authors calculated that the average NaF intake was 0.7 mg/kg bw/day (equivalent to 0.3 mg F/kg bw/day[21]). At the end of the study all dogs were euthanized to perform histomorphometric analysis of trabecular bone. The results indicated that fluoride activated trabecular bone remodeling activity but interfered with bone cell differentiation, the functional efficiency and/or life-span of individual osteoclasts and osteoblasts. The early onset cellular toxic effects of NaF suggested that the early increase in bone mass may be negated later on by prolonged exposure to the substance. However, long term exposure will probably lead to the preservation of bone mass because of decreased bone cell number, their functional efficiencies and individual life-spans. *As it was not clear if the observed effects are adverse, a NOAEL could not be identified for this study.*

[21] 0.7 mg NaF x MW (F)/MW (NaF) = 0.7 mg x 19/42 = 0.3 mg F

ii. Studies in mink

Two chronic feeding studies were identified in mink to support the safety evaluation of fluorine in dogs. They are described below.

Shupe et al. (1987) conducted a dietary study in nursing kits and adult male mink (non-GLP, non-Guideline). Kits and adult male mink (6/group) were fed diets containing 25.5 (basal diet), 46.0, 111.5, and 287.0 ppm fluorine (adult male mink only) as sodium fluoride on a wet weight basis (equivalent to 64.0, 125.0, 307.0, or 759.5 ppm fluorine on a dry weight basis), which the authors calculated to be equivalent to fluorine doses of 2.48, 4.75, 11.93 and 30.75 mg F/kg bw/day in adult mink. These diets are composed of scrap fish, poultry offal, whole poultry, liver, frozen egg, frozen fat, liquid fat, poultry meal, cereal, potato flakes, vitamin E, aureomycin, and salt. The ingested fluorine doses for kits were not calculated. The adults were on these diets for approximately 8 months while the kits were fed for approximately 7 months (kits were fed diets only up to 111.5 ppm fluorine). The nursing kits received relatively little fluorine as the fluorine content of the milk is low. At the end of the study, all mink were sacrificed and pelts were evaluated. Tissues and organs (including bones) underwent gross, radiographic and histopathologic evaluations and chemical analyses. The results indicated that fluorine did not cause any detectable effects on pelt quality, but bone fluorine levels are increased dose-dependently with dietary intake. Kits accumulated fluorine faster than adults. Visible and microscopic changes in bones were found in adult animals at the two highest doses. Microscopic bone changes in the femur and humeri were more pronounced in adults and were slight in kits given the highest dose of fluorine. Dental lesions were observed in kits at the highest dose of fluorine, but no visible lesions of the teeth were observed in adults. No detectable gross, radiographic or microscopic changes were found at lower doses. The study authors recommended a fluorine tolerance level of 50 ppm (on a wet weight basis) in feed for breeding stock and 100 ppm (on a wet weight basis) for mink being raised only for pelts, based on slight skeletal system effects such as on teeth and bones observed at 100 ppm. The authors also noted that this level was intended for very soluble fluoride, such as sodium fluoride, as tolerance levels differ according to the solubility and bioavailability of fluorine. *Therefore, the NOAEL for fluorine toxicity in mink adults is 4.75 mg/kg bw/day (125 ppm by dry weight), and the LOAEL is 11.93 mg/kg bw/day (307 ppm by dry weight) based on visible and microscopic changes in the one. For kits, based on body weight and food consumption of mink up to 7 months of age (NRC 1968), the NOAEL and LOAEL are estimated to be 6.0 and 14.7 mg/kg bw/day, respectively*[22].

In a chronic dietary toxicity study (non-GLP, non-Guideline) in mink (Aulerich *et al.* 1987), seventy-two 3-month-old pastel mink received diets containing added fluoride at 0, 33, 60, 108, 194 or 350 ppm as sodium fluoride for 382 days. The diet was prepared by dissolving NaF in

[22] For males, time-weighted average body weight is [0.69 kg (at week 7) + 2.02 kg (at week 28)]/2 = 1.40 kg. Time-weighted daily mean food (dry) consumption is [37 g (at week 7) + 78 g (at week 28)]/2 = 56 g/day. For females, time-weighted average body weight is [0.56 kg (at week 7) + 1.13 kg (at week 28)]/2 = 0.85 kg. Time-weighted daily mean food (dry) consumption is [32 g (at week 7) + 64 g (at week 28)]/2 = 48 g/day. The mean food factor of males and females are calculated as (56 g/1.4 kg + 48 g/0.85 kg)/2 = 48 g/kg bw/day. Therefore, 125 mg/kg food x 48 g food/kg bw/day x 10^{-3} kg/g = 6 mg/kg bw/day, and 307 mg/kg food x 48 g food/kg bw/day x 10^{-3} kg/g = 14.7 mg/kg bw/day

water and then mixing it with the basal feed (consisted of commercial mink cereal, fish trimmings, beef liver, ground chicken, lungs and trimmings of beef, and water). Analysis of the diets containing 0, 33 and 350 ppm added F reported F content of 35, 75 and 360 ppm as fed. Mink in the same dose group mated during the study to examine fluoride's impact on breeding, gestation and early kit growth. Body weight of mink was recorded every two weeks for approximately 4 months. Then all but one male in each group were pelted. The remaining animals were kept on their respective diets for approximately three additional months, and then females were mated with the male on the same diet. When a successful mating was achieved, the females mated again 8 days afterwards, or on the next day if the initial mating occurred late in the breeding season (lasting a total of 18 days). This procedure is consistent with customary commercial mink breeding practices. Kits whelped by the females were counted and weighed upon birth and when reaching 3 and 6 weeks of age. Kits were given solid feed containing various level of F from approximately 3 weeks old to 12 weeks old, and were weaned at approximately 6 weeks old. Teeth of kits were examined at the age of 12 weeks. Approximately one year after the start of the experiment, urine was collected from adult females to determine urinary F. At the end of the study, blood samples were taken from adult female survivors to determine hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, hematocrit, red, white and differential blood cell counts, serum calcium, serum alkaline phosphatase activity and F. Upon necropsy, brain, heart, liver, kidney, spleen, thyroid and adrenal gland were weighed and F from a femur bone was determined. No histopathology was performed.

Survival of adult female mink fed 350 ppm was reduced to 16.7% compared to 100% for controls with a mean survival time of 316 days. No effects were observed regarding body weight gain, fur quality, breeding, gestation, whelping, lactation, hematology, or serum calcium levels. Some males exposed for four months at the high dose had weakened frontal, parietal and femoral bones that fractured during pelting. Also at this dose, only 14% of the kits whelped survived to three weeks of age. Kits also exhibited dark mottling of teeth, particularly the canine teeth, when fed 194 and 350 ppm, and some kits had broken canines and exostotic lesions. Serum alkaline phosphatase activities and fluoride concentrations were statistically significantly increased at the two highest doses, and femoral ash contents at these doses were reduced compared to the control ($p < 0.05$). Urine and femoral fluorine concentrations in all treated groups were statistically significantly increased compared to controls. Increased adrenal weight ($P < 0.05$) as a percentage of brain weight was observed at 194 ppm and increased heart weight relative to brain weight ($P < 0.05$) was observed at 108 ppm (25%) and 194 ppm (17%). The relative heart weight at the highest dose was not calculated as only one animal survived this dose level. However, the absolute heart weight was not statistically significantly changed at any dose. No data were available on the effects on brain weight by the treatment, but body weight was not affected by treatment as described above. The authors stated that the significance of increased heart weight is unknown and may reflect subtle secondary effect of fluoride toxicosis although no primary lesions in organs or soft tissue have been consistently reported for chronic fluorosis. No histopathology was conducted in this study. Increased skeletal and dental lesions were found in immature mink at the two highest doses. *A NOAEL of 108 ppm and a LOAEL of 194 ppm can be established based on adverse effects on the bone and teeth. As the basal diet contains 35 ppm fluoride, the total dietary fluoride levels are calculated to be 143 and 229 ppm, respectively. Although body weight was recorded for this study, food consumption data were not. Therefore,*

the F doses in terms of mg/kg bw/day were calculated below. According to the mean food consumption of 89 and 54 g wet food/day for adult male and female mink, respectively, and the mean body weight of 1.7 and 1.0 kg for adult male and female mink, respectively (U.S. EPA 1988), 143 ppm F is equivalent to 7.5 and 7.7 mg F/kg bw/day for males and females, respectively[23]. Similarly, 229 ppm is equivalent to 12.0 and 12.4 mg/kg bw/day for males and females, respectively.

iii. Studies in other species

The toxicity of fluoride in all species evaluated has been extensively reviewed by various regulatory agencies in order to establish/evaluate action levels of fluoride in drinking water/food for humans (ATSDR 2003, Health Canada 1993, U.S. EPA 1987, NRC 2006b). Among these evaluations, ATSDR conducted the most comprehensive review of toxicity studies in animals. The summary table for oral studies in rats, mice, rabbits, and mink (Table 3-4 in the ATSDR report) can be found in Appendix A. While no oral dog studies and only one mink study were reviewed by ATSDR, numerous studies in rats, mice and rabbits were listed. One more recent study published after the review (Zhang et al. 2013), and one study not included in the ATSDR table (Heindel et al. 1996) were summarized in Table 12, below. There are a few inconsistencies between the doses calculated in the unit of mg/kg bw/day in this report and the doses calculated by ATSDR in the dog and mink studies. The calculations in this report are described in detail in each study description above and in Table 12, while ATSDR did not explain how their values were derived. Rodents appear to be more sensitive to the toxicity of fluoride compared to dogs and mink.

Soluble fluoride was not carcinogenic in female rats or male and female mice in two chronic oral bioassays conducted by the National Toxicology Program (NTP 1990). Equivocal evidence of carcinogenicity was found in male rats due to the occurrence of a rare tumor (osteosarcoma) with a weakly significant dose-response (NTP 1990). No evidence of carcinogenicity was observed in another chronic bioassay in rats (Maurer et al. 1990), but this study has several limitations such as low fluoride diet insufficient for normal growth and development, the suspected presence of a virus, and the insufficient examination at gross necropsy and histopathology (ATSDR 2003). Nevertheless, fluoride has not been classified as a carcinogen by any regulatory agencies.

No reproductive toxicity was observed in a 3-generation study in rats at up to 10.7 mg/kg bw/day in drinking water (Collins et al. 2001), but reduced fertility, decreased sperm mobility and/or counts, decreased seminiferous tubule diameter, and/or reduced testosterone levels and Leydig cell diameter were found with the lowest LOAEL of 2.3 mg/kg bw/day in two rat studies (Araibi et al. 1989, Chinoy and Sequeira 1992). No developmental toxicities were reported in the majority of the rat studies, while increased number of fetus with 3+ skeletal variations was found at 11.4 mg/kg bw/day in the presence of maternal toxicity (reduced water consumption) (Collins et al. 1995).

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[23] 95 ppm: 89 g/day ÷ 1.7 kg bw × 1,000 mg/g × 95 ÷ 1,000,000 = 5.0 mg/kg bw/day for males; 54 g/day ÷ 1.0 kg bw × 1,000 mg/g × 95 ÷ 1,000,000 = 5.1 mg/kg bw/day for females

The primary target organ/tissue for fluorine is the bone, as consistently observed across all studies in all species evaluated. The lowest LOAEL is 0.5 mg/kg bw/day based on decreased vertebral strength and bone mineralization in male rats in a drinking water study lasting up to 48 weeks (Turner et al. 2001). The LOAEL of 0.5 mg/kg bw/day was also established in a 2-month drinking water study conducted by Bobek *et al.* (1976) based on endocrine effects (decreased thyroxine levels and increased T3- resin uptake ratio). Increased rate of bone formation and slight decrease in bone calcium were observed in a 4-week drinking water study in mice (Marie and Hott 1986). Hepatic pathological changes were observed at 0.95 mg/kg bw/day in a 280-day drinking water study in mice (Greenberg 1986). The highest NOAEL lower than the lowest LOAEL of 0.5 mg/kg bw/day is 0.15 mg/kg bw/day identified in the rat study conducted by Turner et al. (2001). The same NOAEL was also identified in a human study (Li et al. 2001). This study was used as the critical study in ATSDR's derivation of the chronic-duration oral minimal risk level (MRL) of 0.05 mg/kg bw/day for humans.

iv. Summary of fluorine toxicity and ADI derivation

Rodents appear to be more sensitive to the toxicity of fluorine, with the lowest LOAEL of 0.5 mg/kg bw/day established in two subchronic drinking water studies in rats (Bobek *et al.* 1976, Turner *et al.* 2001) based on decreased vertebral strength and bone mineralization and altered thyroid hormone levels. The lowest NOAEL below this LOAEL is 0.15 mg/kg bw/day identified in the Turner et al. (2001) study. Rodents may not be a good model for fluoride toxicity in dogs. In the reproductive toxicity study conducted by Marks et al. (1984), reproductive effects observed in dogs at a relative high dietary level of 460 ppm were not duplicated in rats, and the authors concluded that rats were not good models for dogs for this study. Chavassieux (1990) reviewed fluoride toxicity to the bone in rats, mice, rabbits, cats, pigs, sheep, lambs ewe and dogs, and found that fluoride increased periosteal formation and decreased endosteal formation in rats, increased cancellous osteoid and osteoblastic perimeters in the mice, and decreased osteoid parameters in dogs. Fluoride decreased bone mineralization in rats, but did not affect this parameter in dogs. Rats and mice had nearly no ability to remodel their bones (Chavassieux 1990), while the dog is a good model to study bone remodeling (Huja *et al.* 2006, Gomes and Fernandes 2011) (mink have bone resorption and remodeling capacities as well (Lerner 2006)). Although the very limited information available precludes a comprehensive inter-species comparison of fluoride toxicity, it is more appropriate to use data on dogs and mink to establish a safety level of fluorine in dogs. Data on rodents are presented as supportive evidence only.

Gardner *et al.* (1959) evaluated fluoride deposition in dogs and found that bone fluoride concentrations increase with age in dogs, but do not seem to affect overall health, when comparing a young dog (less than one year of age) to 3 – 6 year-old healthy dogs. NAS (1974) concluded that based on the available data, young pups can tolerate 100 ppm fluoride with no adverse effects on growth. NAS also noted that inadequate data were available to determine if this level of fluoride ingestion would have an adverse effect on the teeth of mature dogs. Among all the dog studies identified in Table 12, the lowest LOAEL is 5 mg/kg bw/day based on a reduction of cumulative weight gain in young dogs in a series of studies conducted by Chiemchaisri and Philips (1965). Fluoride was administered in the form of sodium fluoride in this study. As Qrill™ Pet is targeting adult dogs only, this LOAEL is not relevant to the current assessment. Similarly the LOAELs of 12.5 mg/kg bw/day and 10 mg/kg bw/day from Bunce et

al. (1962) and Chiemchaisri and Philips (1965) were based on effects on growth in young dogs as well. In addition, these two studies used diets deficient in magnesium. Therefore, these two studies are not included in the dose-response assessment of fluoride for adult dogs, either. Caruso and Hodge (1965) reported that a single dose of 15 mg/kg bw/day led to a hypotensive response in dogs, but since only a single dose was studied, and it was not clear if these effects are toxicologically significant or reversible upon repeated exposure. Therefore, this study is not appropriate for use in the dose-response analysis, either. Snow and Anderson reported activation of bone remodeling at a fluoride level of 0.7 mg/kg bw/day in adult dogs, but it was not reported if this effect is adverse. The lowest LOAEL relevant to this GRAS evaluation is 11.5 mg/kg bw/day derived from the study by Shellenberg et al. (1990), based on large, palpable bony exostoses on the skull of adult Shetland Sheepdogs exposed to F from rock phosphate for 2 years. The highest NOAEL among all the relevant dog studies below the lowest LOAEL is 5.2 mg/kg bw/day identified in the Greenwood et al. (1946) study. In this study, fluoride was provided in the form of bone meal, rock phosphate or sodium fluoride at the same dose, but adverse effects (dental fluorosis) were only observed with sodium fluoride and were observed only in puppies. As Qrill™ Pet is a mixture of protein and minerals rather than pure sodium fluoride, the bioavailability of fluoride in Qrill™ Pet is expected to be lower than that from sodium fluoride. In addition, rats receiving sodium fluoride exhibited higher fluoride toxicity compared to those receiving Antarctic krill at equivalent levels of fluoride (Zhang et al. 2013). **Therefore, the NOAEL of 5.2 mg/kg bw/day in this study is conservatively used to derive the ADI for F in Qrill™ Pet.**

Data from the two studies in mink (Shupe et al. 1987; Aulerich et al. 1987), which identified NOAELs of 4.75 – 7.7 mg F/kg bw/day and LOAELs of 11.93 – 14.7 mg F/kg bw/day, support the safety of approximately 5.2 mg F/kg bw/day established in dog studies. The lowest LOAEL of 11.93 mg/kg bw/day was identified by Shupe et al. (1987) in adult male mink exposed to fluoride in the diet for eight months based on visible and microscopic changes in the bones. This LOAEL is almost identical to the LOAEL of 12.0 mg/kg/day for male mink identified in the other mink study (Aulerich et al. 1987). The highest NOAEL lower than this LOAEL for adult mink is 7.5 (males) and 7.7 (females) mg/kg bw/day in the chronic dietary study by Aulerich et al. (1987). Effects on bone and teeth were observed in both mink studies, and are relevant to those observed in adult dogs upon repeated exposure to fluoride. In addition, fluoride was administered in the diet, which is a relevant route for Qrill™ Pet's expected route of exposure as well.

Soluble fluoride from NaF is rapidly absorbed with reported absorption rates of up to 99% in the fasted state (Krogdahl et al. 2015a). The bioavailability of fluoride (i.e., the fraction of fluoride ingested that are systemically available) from krill appears to be lower than that from soluble NaF, although reported values vary. The bioavailability of fluoride from exoskeleton of krill was reported at 80% in rats, and another group of authors reported absorption rates of 93 – 100% relative to NaF in rats (Tenuta-Filho and Alvarenga (1999). These may at least partially explain the higher sensitivity of rats to the toxicity of fluoride. Some researchers suggested that fluoride from krill acts as a hardener in the exoskeleton and is in the form of fluorapatite, which is low in solubility and digestibility. On the other hand, other researchers suggested that fluoride in krill is in a water-soluble form (Hansen et al. 2011). Other dietary elements can reduce the bioavailability of fluoride. The presence of food reduces fluoride absorption to 50 – 80%

(Cerklewski 1997), and calcium can form insoluble complexes with fluoride, thereby reducing its absorption from krill (Tenuta-Filho and Alvarenga 1999). Bone fluoride concentrations correlate well with dietary fluoride levels. In the mink study performed by Shupe et al. (1987), fluoride was given in the form of sodium fluoride in the diet, and a fluoride level of 4,716 ppm was reported in dried, fat-free femur for adult male mink receiving 30.75 mg F/kg bw/day for 8 months. In the mink growth study on krill meal (Krogdahl et al. 2015a), a dietary level of 71.9 mg F/kg bw/day (~17% krill meal) over 15 weeks led to a fluoride level of approximately 5,200 ppm in dried, defatted femur. Based on the bone fluoride content versus dietary krill meal level chart reported in the publication (Figure 1 in this report), a bone fluoride level of 4,700 ppm corresponds to a dietary krill meal level of approximately 15% for males, corresponding to approximately 62 mg F/kg bw/day. The most rapid uptake of fluoride into bones occurs during the period of rapid growth, and the uptake is much slower in adults (Cerklewski 1997). A comparison of femoral fluoride in the unit of ppm in ash in the Shupe et al. (1987) study indicates that fluoride from the adult bone after dietary exposure to fluoride for over a year (3,511 ppm at 4.05 mg F/kg bw/day) (Aulerich et al. 1987) did not increase over time, as the level reported in adult mink after an 8-month exposure at a higher dose level did not result in higher bone fluoride (3,675 ppm, 4.75 mg F/kg/day). Therefore, while the Shupe et al. (1987) study used a longer exposure period of approximately 8 months compared to the Krogdahl et al. (2015) study that used a 15-week exposure, the difference in bone fluoride is unlikely significantly affected by the difference in exposure time, as both studies were conducted in adult mink. The difference in bone fluoride is most likely attributed to the difference in the bioavailability of fluoride from NaF and krill meal. As 30.75 mg F/kg bw/day from NaF and 62 mg F/kg bw/day from krill meal led to similar femoral bone levels (Krogdahl et al. 2015a, Shupe et al. 1987), **a bioavailability factor of 2 (i.e., 62/30.75) is applied to the NOAEL of 5.2 mg/kg bw/day in the critical dog study to account for the decreased fluoride bioavailability from krill meal compared to NaF used in the critical study.**

According to the U.S. FDA (2007), the acceptable daily intake (ADI) is calculated by dividing the dose level of the substance in animal studies that was shown to cause no adverse effects (i.e., NOAEL) with an appropriate safety factor. Typically a safety factor of 100 (10 for intraspecies variation * 10 for interspecies extrapolation) is applied to extrapolate from animal data to humans. As the critical study is performed in dogs, and the species of concern is dog, a safety factor of 1 was used for inter-species extrapolation. An additional safety factor of 3 is used to account for intraspecies differences in dogs. This is smaller than the default factor of 10 for intraspecies variation because the body weight and energy requirement data of terrier dogs are used in this calculation as well as in the EDI calculations. Terrier dogs are one of the most active dogs with the highest metabolic rate and energy requirement per kilogram of body weight (NRC 2006a). Therefore, EDIs calculated based on this breed is expected to be reasonably worst case scenarios. However, some uncertainty still exists as limited data are available to evaluate the metabolic rate of the smallest dog breeds (e.g., pocket breeds) that may consume more food on a body weight basis. Therefore, a safety factor of 3 is used to account for this uncertainty. Consequently, a composite safety factor of 3 is used in the ADI derivation, which consists of 3 for intraspecies variation and a safety factor of 1 for interspecies extrapolation.

$$ADI_F = \frac{NOAEL_F * \text{Bioavailability Factor}}{\text{Safety Factor}} = \frac{5.2 \text{ mg/kg bw/day} * 2}{3} = 3.5 \text{ mg/kg bw/day}$$

Summary of Studies Conducted to Assess The Effect of Fluorine in Dogs, Mink and Rodents

	Duration	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Critical Effect	Reference
ppm F (12.5 v/day) as NaF Mg diet[a]	11 weeks	NA	12.5 (250 ppm presumably dry weight)	Decreased weight gain. Aortic lesions were observed in the animals provided the low Mg diet but not the low Mg diet including F. Muscular weakness and convulsions were observed in both low Mg and low Mg + F groups.	Bunce *et al.* (1962)
5, 10, 15, 23 or kg bw/day stomach tube. ; was given one	35-60 minutes depending on dose	10	15	A hypotensive response was noted in dogs given ≥ 15 mg/kg bw.	Caruso and Hodge (1965)
ppm F (10 v/day) as NaF deficient or Mg- t diet[a]	7 weeks	NA	10 (200 ppm presumably dry weight)	Decreased growth in Mg-deficient dogs. Magnesium-decalcinosis was observed in the animals provided the low Mg diet but not the low Mg diet including F.	Chiemchaisri and Phillips (1965) (experiments 1 and 5)
, 100, or 200 , 1.25, 2.5, 5.0 ıg/kg bw/day) ı a low Mg diet[a]	7 weeks	2.5 (50 ppm)	5.0 (100 ppm presumably dry weight)	Decreased weight gain at doses above 50 ppm. Diets containing at least 25 ppm F were protective against magnesium-deficiency induced calcinosis. Retention of F in bone was evident in all NaF groups.	Chiemchaisri and Phillips (1965) (experiment 2)
g bw/day as ıe meal, or rock e + 0.2 mg F/kg n basal ration.	Up to 437 days	5.2 (50 ppm dry weight according to NAS 1974)	NA	No significant effect of diet on growth, hemoglobin, serum Ca and P, coagulation time, breaking strength tests, or growth of bones and teeth. Puppies treated with NaF (but not other forms of F) developed dental fluorosis.	Greenwood et al. (1946)

Adult Beagle dogs (at least 1 years of age) with osteoporosis from consumption of a calcium-deficient diet (2/group)	0, 1, 3, 9, and 27 ppm (approx. 0.026, 0.85, 0.295, and 1.125 mg/kg bw/day) in calcium-deficient diets	287 days	1.125 (27 ppm dry weight)	NA	No significant effects of any dose of dietary F on osteoporosis induced by calcium deficiency.	Henrikson et al. (1970)
Twenty adult Shetland Sheepdogs of proven fertility (4F, 1M *per* group)	A: high F (460 ppm, 11.5 mg/kg bw/day[b]) dog food, well water. B: high F dog food, distilled water. C: low F (55 ppm, 1.4 mg/kg bw/day[b]) dog food, well water. D: low F dog food, distilled water.	2 years	11.5 (460 ppm dry weight) (developmental) 1.4 (55 ppm dry weight) (maternal)	NA (developmental) 11.5 (460 ppm dry weight) (maternal)	No effect of high dose F on reproduction or malformations. Four high F dogs, but no low F dogs developed large, palpable bony exostoses on the skull.	Shellenberg et al. (1990)
Adult beagle dogs, 4 years of age (2 control, 6 F)	Tap water or water containing 11.6 ppm NaF (0.7 mg NaF/kg bw/day)	6 months	NA	0.7 (LOEL) (11.6 ppm in water)	Activation of bone remodeling activity. No demonstration that the effect was adverse.	Snow and Anderson (1986)
Mink, Adult male	25.5, 46.0, 111.5 or 287 ppm (2.48, 4.75, 11.93, or 30.75 mg/kg bw/day) F as NaF	8 months	4.75 (46 ppm wet weight)	11.93 (111.5 ppm wet weight)	Visible and microscopic changes in bones in the highest two dose groups.	Shupe *et al.* (1987)
Mink, kits	25.5, 46.0, or 111.5 ppm F as NaF	7 months	6.0 (46 ppm wet weight)[c]	14.7[c] (111.5 ppm wet weight)	Dental lesions in the high dose group.	Shupe *et al.* (1987)

Mink, adult pastel and pastel kits	Diets providing 0, 33, 60, 108, 194, or 350 ppm F in addition to 35 ppm F in basal diet and in combination with normal fluoridated drinking water containing 0.345 ppm F.	382 days	7.5 (M), 7.7 (F) (108 ppm + 35 ppm = 143 ppm wet weight)[d]	12.0 (M), 12.4 (F) (194 ppm + 35 ppm = 229 ppm wet weight)[d]	Survivability of kits and adults affected by 350 ppm. Adverse effects on bone and teeth at 194 and 350 ppm.	Aulerich *et al.* (1987)
Rats, Wistar (10/group, sex unspecified)	30 (control), 150 (Antarctic krill) or 150 (NaF) ppm (5.8, 29.1 and 29.1 mg/kg bw/day)[c]	3 months	5.8 (30 ppm dry weight)[e]	29.1 (150 ppm dry weight)[e]	Pathological changes (fluorosis) in the liver, kidney, spleen and brain	Zhang et al. (2013)
Rat, SD (26 F, group)	0, 50, 150 or 300 ppm in drinking water	Gestation Days 6-15	18 (150 ppm) (maternal)	27 (300 ppm) (maternal)	Decreased water consumption at 300 ppm; no developmental effects	Heindel et al. (1996)
			27 (300 ppm) (developmental)	NA (developmental)		

bw = body weight; Ca = Calcium; F = females; F = fluorine; F0, F1, F2 = parental, first and second generation; M = males; Mg = magnesium; NAF = sodium fluoride; NAS = National Academy of Sciences; NOAEL = No observed adverse effect level; P = Phosphorus; ppm = parts *per* million; SD = Sprague-Dawley; [a] doses in ppm converted to mg/kg bw using an estimated 2 kg bw and 1 cup/day (100 g/day) feeding schedule for Purina Healthy Puppy Formula (purinaone.com); [b]doses in ppm are converted to mg/kg bw using conversion data supplied by FDA (PAFA 1993); [c]estimated from Table 3 presented in NRC 1968; [d]estimated using default body weight and moist food consumption of adult mink recommended U.S. EPA (1988), mean values for males and females were presented in the table; [e]estimated using default values for Wistar rats in subchronic toxicity studies (U.S. EPA 1988)

c. Toxicity of Astaxanthin

Astaxanthin, also known as (3S,3'S)-3,3'-dihydroxy-β,β-carotene-4,4'dione, is an antioxidant that belongs to the oxygenated carotenoids class (Figure 4). Unlike β-carotene, astaxanthin is not converted to vitamin A in the body, and is ten times more potent as an antioxidant compared to other carotenoids. Astaxanthin is naturally occurring in seafood (e.g., salmon, red fish, and shell of shrimp, krill and lobster), and is also enriched in *Haematococcus* algae and Phaffia yeast. Therefore, it is consumed by humans in food (EAS 2009).

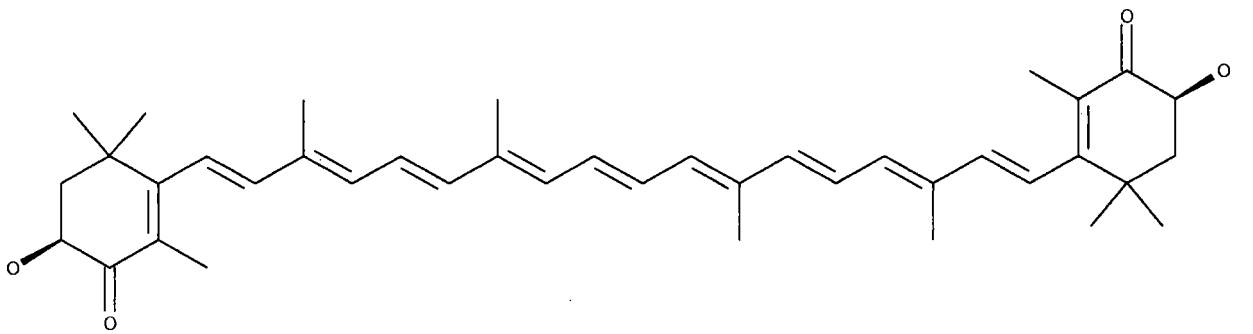

Figure 4: Structure of Astaxanthin (CAS #472-61-7)

Astaxanthin along with dried cells of *H.* pluvialis algae and *Phaffia* rhodozyma yeast that are rich in astaxanthin are U.S. FDA-approved color additives when added at up to 80 mg/kg feed for salmonid fish to enhance the pink to orange-red color of the flesh (21 CFR 73.35). Astaxanthin is also marketed as a dietary supplement for humans for its antioxidant properties, as it has been shown to exert protective effects against oxidative damage to the cell membrane, mitochondria membrane, and ocular tissues. In addition, astaxanthin exhibits antitumor, anti-*Helicobacter pylori*, and cardioprotective effects (EAS 2009). Due to its high susceptibility to oxidation, astaxanthin levels diminish over time if not stabilized (EFSA 2014a). The U.S. FDA had no questions on the GRAS notifications of *Haematococcus* extract and concluded that the extract is GRAS as a food ingredient in baked goods, beverages, cereals, chewing gum, coffee and tea, diary product analogs, frozen daily desserts and mixes, hard candy, milk products, processed fruits and fruit juices, processed vegetables and vegetable juices and soft candy when used to provide 0.15 mg astaxanthin per serving (U.S. FDA 2010, 2015).

i. Toxicokinetics

Ingested astaxanthin is absorbed with relatively low bioavailability based on the low plasma concentrations. However, absorption is enhanced in the presence of lipids, and esterified astaxanthin has higher absorption as well. Astaxanthin was absorbed at a rate of approximately 13-20% following direct administration into the duodenum at a concentration of 5 to 20 μmol/L in rats. None of the other studies in animals reported the extent of oral absorption, but humans have higher oral absorption of astaxanthin compared to dogs and cats based on comparison of plasma concentrations after ingestion of similar doses (EFSA 2014a,b, EAS 2009).

Astaxanthin has a high affinity for organs and tissues via the oral route, as supported by tissue/plasma ratios of greater than 1 at 8 and 24 hours after administration in rats. Upon oral administration, free astaxanthin was found in serum, liver, heart, kidneys, spleen, adrenals,

gastrocnemius muscle, skin, and to a less extent in the brain in rats and mice. In dogs, astaxanthin was transported in the blood mainly by HDL and was taken up into all subcellular organelles of blood leukocytes. Peak plasma concentrations were reached 3 to 6 h postprandially in dogs after a single dose, whereas continued increase was observed when astaxanthin was given in the diet for 15 or 16 days (Park et al. 2010). While a steady state was not reached in this study, a subsequent 16-week study by the same group of authors reported that steady state concentration was reached by week 8 when the dogs received 20 mg astaxanthin per day (Park et al. 2013, EFSA 2014a,b, EAS 2009).

Astaxanthin esters are rapidly hydrolyzed after ingestion to release free astaxanthin. Metabolism of astaxanthin is rapid ad extensive. Upon intravenous and oral administration in rats, only a very low amount of astaxanthin remained unchanged in the 24-hour urine samples. Biotransformation studies in primary rat and human hepatocytes showed the cleavage of the polyene chain at the C9, C9' positions and subsequent stepwise reduction. Metabolites include (rac)-3-hydroxy-4-oxo-β-ionone and (rac)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone, as detected in *in vitro* metabolism studies in rat hepatocytes. These metabolites are further transformed mainly via glucuronidation and then eliminated. No saturation of the metabolic pathway appears to occur via oral administration in rats (EFSA 2014a,b, EAS 2009).

Short-term and subchronic studies in rodents, cats and dogs indicate that astaxanthin is rapidly eliminated or metabolized. After 48 hours, astaxanthin and its metabolites are mainly excreted in the feces (53%) and urine (34%) in rats. Petri and Lundebye (2007) reported that astaxanthin accumulated in the rat eyes dose-dependently when exposed for two weeks to 215 - 2,000 mg/kg bw/day. However, there were large variations in the reported data, with standard deviations greater than mean values. In addition, there appeared to be a decrease of astaxanthin accumulation in the eye from day 7 to day 14. These data as well as the relatively constant plasma level of astaxanthin in subchronic dietary studies in rats suggest that longer exposure did not lead to tissue accumulation of astaxanthin over time. The elimination half-life was 9 – 18 hours in dogs, which is similar to the reported half-life of 8 – 9 hours in rats, but much shorter than 11 – 32 hours reported in humans (Park et al. 2010, EFSA 2014a,b, EAS 2009).

ii. Toxicity studies

Astaxanthin have low acute oral toxicities with LD_{50} values of > 2,000 mg/kg in rats (Roche 1987). It was not genotoxic when tested *in vitro* (bacteria) and *in vivo* (mice), and no adverse effects were found in reproductive and developmental toxicity studies in rats and rabbits (EAS 2009). Repeated exposure toxicity studies revealed that astaxanthin is not carcinogenic in rats, but liver is the most sensitive organ to its toxicity upon chronic exposure in rats. Kidney weight changes and increased prothrombin time were also observed in rats. While the toxicokinetic study conducted by Petri and Lundebye (2007) suggests that astaxanthin may accumulate in the eye of rats, no adverse effects on the eye were observed in the studies identified. Repeated exposure studies, including reproductive and developmental toxicity studies performed in animals (mammals) are described in detail below and summarized in Table 13. Most of these studies, if not all, were summarized by peer-reviewed secondary sources that are publically available, and the original study reports were not available for review.

Studies in Dogs

A total of three studies were identified in dogs. Although full study reports were not available for review, they were summarized in publically available secondary sources. The first two studies below have been reviewed and summarized by a peer-reviewed EFSA document (EFSA 2007, 2014c), and were determined to be of acceptable quality.

A 52-week oral study was conducted in Beagle dogs (4/sex/dose) (GLP compliant, broadly conformed to OECD Test Guideline 452, but the number of animals/dose is lower than the recommended number of 20). These animals received gelatin capsules providing 0, 6, 24 or 96 mg astaxanthin/kg bw/day for 371 days. Animals at the highest dose group received 200 mg/kg bw/day from the sixth month onwards. Based on a later EFSA review (2014c), these doses were slightly different: 0, 6.4, 24 and 104/218 mg/kg/day. ToxServices adopted the lower reported dosages in the subsequent analyses as a conservative approach. The administered astaxanthin was prepared from an 8% preparation of synthetic astaxanthin. There were no effects observed regarding clinical signs, hematology, clinical chemistry, urinalysis, organ weight, gross pathology and histopathology (EFSA 2007, 2014c). There was a small decrease in feed intake (extent and statistical significance not reported) at the highest dose with corresponding decrease of food intake in this group. However, EFSA did not consider this an adverse effect. Therefore, the highest dose was identified as the NOAEL. The time-weighted dose for this group is 158 mg/kg/day[24].

In a tolerance study (GLP status not reported, group size lower than recommended in OECD Guideline 409 for 90-day studies in non-rodents), male and female Beagle dogs (n=3/sex/dose) received astaxanthin administered as beadlets in feed. The beadlets were given at 6.1% w/w in the feed, and astaxanthin were added at concentrations of 0, 2.5, 5.0, or 10.0% in the beadlets. These astaxanthin doses were determined to be equivalent to 0, 41, 76, and 162 mg/kg bw/day according to Roche (1987). EFSA (2014c) reported slightly different astaxanthin doses of 0, 40, 75 and 165 mg/kg/day in males and 0, 42, 77 and 158 mg/kg/day in females. EFSA (2007) reported the doses to be 0, 40, 80 and 160 mg/kg/day. ToxServices adopted the lower reported dosages in the subsequent analyses as a conservative approach. Animals were evaluated for mortality, feed intake and clinical signs, and body weights were recorded throughout the study. Additionally, hematological, clinical chemistry, urinalysis and ophthalmoscopy gross pathology, organ weights and histopathological changes were assessed. A slight decrease in body weight (statistical significance or extent of decrease not reported) was found in all groups except the low dose group. Due to the lack of dose-response, it was not considered a treatment-related effect. No adverse effects were observed at any dose (Roche 1987, EFSA 2007, 2014c). EFSA identified a NOAEL of 158 mg/kg/day for this study.

A third dog study (GLP status not reported) was described in a patent application. In this study, 29 dogs (sex and strain not reported) weighing 2.2 – 27 kg were first fed commercial dog food for a month and then received tablets containing 1 mg free astaxanthin twice per day with the

[24] [96 mg/kg/day * 5 months * 30 days/month + 200 mg/kg/day * (371 days – 5 months * 30 days/month)]/371 days = 158 mg/kg/day

same meal for another month. Therefore, the astaxanthin doses ranged from 0.07 and 0.91 mg/kg bw/day[25]. The animals were examined for the degree of deep sleep during night, sensibility to external stimulation, and visual sense before and after astaxanthin administration. "Improved sleep" was defined as showing a quick response to abnormal sound during sleep. "Improved sensibility" was defined as running around aggressively showing expressive face and having better hair gloss. "Improved visual sense" was defined as a "change in attitude", such as focusing the eyes at the owner or an object, with reduced eye mucus. It was reported that 8/29, 12/29 and 10/29 dogs showed improved deep sleep, sensibility and visual sense, respectively, and the remaining animals did not show any changes. No dogs had deterioration in any of the parameters measured. No statistical analysis was performed (Honda and Takahashi 2011). *This study is limited by testing only one dose, and focusing on limited endpoints. Therefore, this study only served as supportive evidence.*

A few other dog studies were also described in the above patent to test the benefit of astaxanthin supplementation to reduce feces odor and improve diabetic conditions. However, these studies were conducted in very few animals (1 and 4), and were not relevant to support the safety of astaxanthin in dog food. Therefore, they were not described in this report.

Studies in Rats

Wistar rats (n=50/sex/dose) received astaxanthin as part of a beadlet formulation diet. The doses administered were equivalent to 0 (untreated control), 0 (placebo control), 40, 200, or 1,000 mg/kg bw/day astaxanthin for two years. Additionally, a satellite group of 10 animals of each sex received treatment for one year with a recovery period (untreated) of one year. Animals treated for two years had survival rates of 76 to 88% in males and 56 to 82% in females. In all animals receiving the beadlet formulation feed (both treated and placebo control), body weight was reduced compared to untreated controls. In females receiving astaxanthin, body weights were lower than controls and this was statistically significant in the 200 and 1,000 mg/kg bw/day groups. However, body weight recovery was observed in the satellite groups during the recovery year (i.e., the second year of the study). No clinical signs were observed in any dose group. Reduced erythrocyte counts, reduced packed cell volume, increased mean corpuscular hemoglobin, and increased mean corpuscular hemoglobin concentration were observed in the groups receiving 200 and 1,000 mg/kg bw/day for two years; however, these effects were considered to be minor. In females receiving 1,000 mg/kg bw/day and sporadically in females receiving 200 mg/kg bw/day, increases were observed in plasma cholesterol levels, bilirubin, alkaline phosphatase, alanine transaminase, and aspartate aminotransferase. In the satellite groups, no hematological or biochemical changes were observed during the recovery phase. Heart, brain, and spleen organ weights were increased in the placebo control and astaxanthin treated groups; however, this was considered to be due to low body weight rather than toxicity of the test substance. Histopathological changes were only observed in the liver after two years of treatment, and these effects were almost exclusively observed in females. Specifically, increased incidences of hepatocellular vacuolation, hepatocellular hypertrophy, and multinuclear hepatocytes were observed in all dose groups. Additionally, in the mid and high dose females,

[25] 2 mg astaxanthin/day ÷ 2.2 kg bw= 0.91 mg/kg bw/day; 2 mg astaxanthin/day ÷ 27 kg bw= 0.07 mg/kg bw/day

there was a statistically significantly increased incidence of hepatocellular adenomas. An increase incidence of centrilobular vacuolation of hepatocytes was observed in the 200 and 1,000 mg/kg bw/day males; it was not stated if these effects were significant. Malignant tumors were not observed in any dose group for either sex. In female control, 40, 250, and 1,000 mg/kg bw/day astaxanthin dose groups, 13, 23, 29, and 41 animals, respectively, had micronucleated hepatocytes; however, this was considered to be an response to increased hepatic cell injury and cell death as supported by increased single-cell necrosis in high dose females as well as inflammatory foci in the mid and high dose groups. No treatment related effects were observed in the satellite group after a one year recovery period. Based on histopathological observations in the liver in all dosed females, a NOAEL could not be established.

In order to assess the incidence of liver adenoma and liver hypertrophy in dosed females and derive a point of departure for risk assessments, EFSA employed benchmark dose modeling. The placebo control and three dose groups were evaluated using this approach. Two valid models were identified: a $BMDL_{10}$ of 15.7 mg/kg bw/day was determined using the continuous H4 model, and a $BMDL_{10}$ of 22.1 mg/kg bw/day was determined using the continuous E4 model. As the 15.7 mg/kg bw/day $BMDL_{10}$ was the most conservative value, it was selected as the $BMDL_{10}$ for liver hypertrophy in females. Additionally, two valid models were identified for liver adenomas: a $BMDL_{10}$ of 10.0 mg/kg bw/day was determined using the continuous E4 model, and a $BMDL_{10}$ of 3.4 mg/kg bw/day was determined using the continuous H4 model. The 10.0 value shows slightly higher certainty, as supported by a BMD/BMDL ratio of 1.4 versus a BMD/BMDL ratio of 2.0 for the 3.4 value. However, as the certainty values are relatively close, the 3.4 mg/kg bw/day value was selected as the $BMDL_{10}$ for liver adenomas in order to be more conservative and, thus, protective of health effects (EFSA 2007, 2014a). *This study was selected as the key study by EFSA in the evaluation of the safety of astaxanthin for humans, and the $BMDL_{10}$ of 3.4 was selected to derive the ADI (Allowable Daily Intake) of 0.034 mg/kg bw/day (EFSA 2014a).*

In a 52-week study, Wistar rats (number not specified) was exposed to 8% synthetic astaxanthin preparations in the diet providing 0, 125, 250, 500 or 1,000 mg/kg bw/day astaxanthin. Satellite groups were included to study the reversibility of the observed effects after 51 weeks of recovery. Animals were examined for clinical observation, mortality, body weight, food and water consumption, ophthalmology, hematology, clinical chemistry, urinalysis, organ weights, and histopathology. The only clinical abnormality observed was discoloration of the feces in higher (unspecified) dose groups. Decreased body weight gain was found in all groups, but a difference was also found between control and females receiving 250 mg/kg bw/day and higher doses (statistical significance not reported). Animals in all groups had decreased food consumption, but relative food consumption decreased when test group is compared to the control group. There were slight variations in hematology parameters without dose-response, and all the changes were within physiological ranges. Serum cholesterol increased in all astaxanthin-treated groups in both sexes, and bilirubin increased in males ($\geq$ 500 mg/kg bw/day) and females ($\geq$ 125 mg/kg bw/day). In females, increased levels were found regarding ALAT and ALP ($\geq$ 250 mg/kg bw/day) and ASAT ($\geq$ 500 mg/kg bw/day). ALAT decreased in males at the highest dose. Increased specific gravity was reported in females at 250 mg/kg bw/day and higher doses upon urinalysis. Decreased relative organ weights (spleen, adrenals, ovaries, liver, brain and kidney) were found at even the lowest dose, and the effects were more pronounced in

females (except kidney). However, histopathological changes were only found in the liver (brownish pigmentation of hepatocytes and macrophages in all dose groups, centrilobular hypertrophy at ≥250 mg/kg bw/day, and inflammatory cell foci and multinucleated hepatocytes at 1,000 mg/kg bw/day), which was more pronounced in females. EFSA identified the lowest dose of 125 mg/kg bw/day as the LOAEL, and no NOAEL could be established (EFSA 2007).

In a third study in rats, Sprague-Dawley rats (n=10/sex/dose; 6 weeks old) received astaxanthin via gavage at doses of 0, 2, 10, and 50 mg/kg bw/day for 90 days (Takahashi et al. 2004; Yoshihiko et al. 2004). The test substance was derived from *H. pluvialis* biomass via solvent extraction (solvent not identified) with a trade name of AstaReal Oil 50F, and it was administered as an astaxanthin-rich oil at doses of 0, 37.0, 185.2, and 925.9 mg/kg bw/day in a maize oil vehicle. Animals were evaluated for clinical signs, and body weight and feed intake were evaluated throughout the study. Hematology, coagulation, clinical chemistry, and urinalysis were performed at the end of the study, and organ weights, macroscopic, and microscopic evaluations were performed at necropsy. No mortality or clinical signs were observed. In all high doses animals and some mid-dose animals, orange-colored stool was observed. Body weight and food intake were not affected by treatment. In high dose males, a statistically significant increase in prothrombin time and active partial thromboplastin time was observed. No statistically significant difference in hematological parameters was observed. In two high dose animals and one mid dose animal (sex not specified), relatively high values for alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase were observed. Of these animals, focal necrosis in the liver was observed in one high dose and one mid dose animal. No statistically significant differences were observed during clinical chemistry analysis or urinalysis; however, urinalysis was only carried out on five animals per group. Orange coloration of the forestomach mucosal surface and cecum content was observed in all mid and high dose groups. Heart, lung, spleen, liver, and kidney weights were comparable to controls in all dose groups, and no toxicologically relevant findings were observed at histological examination of these organs. EFSA (2014a) established a NOAEL of 10 mg/kg bw/day based on prolonged prothrombin time and active partial thromboplastin time in high dose males. However, EAS (2009) did not consider the increases in prothrombin time and activated partial thromboplastin time to be toxicologically significant as the values were within historical control ranges. As a result, EAS (2009) established a NOAEL of 50 mg/kg bw/day.

A fourth rat study also evaluated the toxicity associated with astaxanthin extracted from the biomass of *H. pluvialis* (identified as "algal meal HPP", 3% astaxanthin) (Stewart et al. 2001, 2008). This study was performed according to OECD Guideline 408. Male and female Wistar rats (n=10/sex/dose) were administered astaxanthin via a diet containing *H. pluvialis* (astaxanthin source) at concentrations of 0, 10,000, 50,000, or 200,000 ppm for 13 weeks. The authors established a dose equivalent of 14,161 mg/kg bw/day and 17,076 mg/kg bw/day *H. pluvialis*, respectively, in males and females, and 465 and 557 mg/kg bw/day astaxanthin, respectively, in males and females. Animals were observed for mortality and clinical signs, and body weight and food consumption were recorded throughout the study. In control and high dose animals, ophthalmoscopic evaluations were performed before dosing and at week 12. Urinalysis was also performed at week 12. Hematology, clinical chemistry, and organ weight analyses were performed at week 13. Histology was performed in the following manner: select tissues from all animals in all dose groups, and a wider selection of tissues from control and high dose animals.

In the high dose groups, plasma concentrations ranged from 39.5 to 162.5 μg/L in males and 54.8 to 190.2 μg/L in females as measured on days 2, 8, 31, and 91. Survival was not affected by treatment. Fur and feces were orange in all dosed animals. No difference was observed between dosed and control groups with regard to body weights and body weight gains. A marginal decrease in food consumption was observed in high dose females; however, in general, feed consumption and feed efficiency were not affected by treatment. In high dose males, a statistically significantly lower count of absolute neutrophils was recorded. In both high dose males and females, mean platelet counts were lower than controls. In females, this effect was statistically significant, and, in males, a significant dose-response was observed. In high dose males, a slightly statistically significant increase in prothrombin time was reported. A trend was observed with regard to active partial thromboplastin time; however, no information regarding the significance of active partial thromboplastin time was provided. A statistically significantly lower potassium concentration was observed in high dose males. Additionally, alkaline phosphatase activity was elevated in a dose dependent matter. In mid and high dose animals, an increase in plasma cholesterol concentration was recorded; however, this was determined to be associated with the high fat content of the test material. Urine volume and urine pH were both statistically significantly lower in high dose males. Other minor urinary composition changes were noted, but no details were provided on these changes. Orange/red coloration of the stomach's mucosal surface was observed in a number of animals in the mid and high dose groups. Additionally, coloration of the duodenum and cecum was observed in individual animals, but no information was provided regarding the doses or if there was a dose-response for this effect. Microscopically, no changes were observed in these organs. In 5 of the 10 high dose females, orange coloration of the kidneys was observed; however, this was noted to be minor. Relative mean kidney weights were significantly increased in high dose males and females. Based on the coloration of the kidneys as well as the increase in relative mean kidney weights, EFSA (2014a) established a NOAEL of 122 and 144 mg/kg bw/day astaxanthin for males and females, respectively.

Limited details were available for additional subchronic studies. In a 13-week oral study in F344 rats, phaffia color was administered at concentrations of 0, 0.2, 0.6, 17, or 5.0% for 90 days. Phaffia color contains astaxanthin; however, no information regarding the amount of astaxanthin in phaffia color was provided. Mortality was not observed, and no effects were observed on body weight gain, hematological analysis, or biochemistry values. No effects were observed following histopathology. Based on the results of this study, the study investigators established a NOAEL of 5% phaffia color, as no effects were observed at any dose. This was determined to be equivalent to approximately 20 mg/kg bw/day astaxanthin (Onodera et al. 1997). No additional details were provided.

In another 13-week study, male and female rats (number, species not specified) received astaxanthin in the form of gelatin beadlets in the diet at 0, 6.25, 12.5, or 25.0%. This was stated by the authors to be equivalent to 0, 310, 620, or 1,240 mg/kg bw/day astaxanthin. Survival and body weights were not affected by treatment. Red coloration of the feces was observed in all animals, and adipose tissue was yellow at time of necropsy. Ophthalmoscopic evaluation revealed no effects following administration of astaxanthin. In the mid and high dose groups, kidney, ovary, adrenal, uterus, and spleen weights were decreased. In mid and high dose males,

decreased total serum protein levels were observed. Additionally, occasional increases in liver enzymes in several animals were observed; however, in general, the hematology and blood chemistry parameters were comparable to historical values. In all treated groups, plasma cholesterol was slightly increased; however, it was not statistically significant. As a result, the authors established a NOAEL of 1,240 mg/kg bw/day, the highest dose tested, as no significant toxicological effects were observed (Roche 1987).

Nishikawa et al. (1997) studied the repeated-dose toxicity and reproductive toxicity of β-carotene and astaxanthin in rats. For the repeated dose toxicity study, male Wistar rats (6/dose) received astaxanthin at 0.04% in the diet for 41 days. There were no adverse effects regarding growth, clinical observations, organ weights and levels of liver enzymes in the plasma. For the reproductive toxicity study, Wistar rats (15/sex/dose) received diets containing 0.02% astaxanthin for 14 days before paring until delivery or for 42 days. No effects were observed on fertility, rates of pregnancy and delivery, litter size, sex, pup size, and gross pathology of offspring. No further details were available. Based on the default subchronic food factor of 92 g/kg bw/day and 103 g/kg bw/day for male and females Wistar rats, respectively (U.S. EPA 1998), the NOAEL for the repeated dose toxicity study is 36.8 mg/kg bw/day[26], and the NOAEL for reproductive toxicity is 18.4 mg/kg bw/day in males and 20.6 mg/kg bw/day in females[27].

In a multi-generation toxicity study conducted according to U.S. FDA and UK CSM guidelines, astaxanthin (96% pure) was administered to rats (strain unspecified, 32/sex/dose) at 0, 25, 100 or 400 mg/kg bw/day by gavage 70 days prior to mating until sacrifice for males, and 14 days before mating, through gestation until sacrifice or weaning. Half of the mated females were terminated on gestation day 14, and the remaining animals were allowed to litter. Selected F1 litters were examined for developmental indices during lactation and selected weanlings underwent learning and memory testing or reproductive capability on lactation day 23. No adverse effects were observed in the parental generation regarding mortality, body weight gain, percentage of males mated, ratio of mated to pregnant females, and median precoital time. No adverse effects were observed in the F1 generation regarding body weight gain, time of onset of developmental landmarks, learning and memory ability, and gross examination of weanlings. Neonatal mortality of the F1 generation was at the upper limit of biological range at the highest dose; this was not statistically significant and not considered to be adverse. Isolated anomalies were observed upon macroscopic and soft tissue examination of pups that died during lactation, but these were not considered to be treatment-related. The fertility of F1 animals was not adversely affected by treatment. While the number of F2 pups that died or were cannibalized between lactation days 1 and 4 was "unusually high", this occurred at all doses including controls. Therefore, this was not considered to be treatment-related. It was concluded that the NOAEL for reproductive and developmental toxicity was 400 mg/kg bw/day, the highest dose tested (Roche 1987).

[26] 0.04% * 92 g/kg bw/day * 1,000 mg/g = 36.8 mg/kg bw/day

[27] Males: 0.02% * 92 g/kg bw/day * 1,000 mg/g = 18.4 mg/kg bw/day; Females: 0.02% * 103 g/kg bw/day * 1,000 mg/g = 20.6 mg/kg bw/day

Study in Mice

In an 80-week carcinogenicity study in mice, NMRI MORO mice (number not specified) received preparations of synthetic 8% astaxanthin in the diet providing astaxanthin at 0, 0 + carrier, 14, 300, 650 or 1,400 mg/kg bw/day. Mortality of 38-50% was reported for this study, although there were no differences among groups. Fecal discoloration was the only treatment-related clinical abnormality, which was expected. Decreased body weight (statistical significance or extent of change not reported) was found at the three highest doses during the last six months. Animals in the highest dose group had increased cholesterol (statistical significance or extent of change not reported). No treatment-related effects on non-neoplastic or neoplastic lesions were found upon necropsy and histopathology, except for discoloration of adipose tissue in some animals at the highest dose. EFSA identified a NOAEL and LOAEL of 14 and 300 mg/kg bw/day, presumably based on decreased body weight. No further details were available (EFSA 2007).

Study in Rabbits

A developmental toxicity study was conducted according to the guidelines established by U.S. FDA and UK CSM. Pregnant rabbits (strain and number not reported) received astaxanthin by gavage at 0, 100, 200 or 400 mg/kg bw/day during gestation days 7 to 19, and sacrificed on gestation day 30. Fetuses were obtained by ovariohysterectomy and examined for viability (24 hours) and macroscopic, skeletal, visceral and soft tissue anomalies. Astaxanthin was well tolerated and no adverse effects were observed regarding maternal sensitivity to treatment or body weight changes. Reproductive and litter parameters and course and outcome of pregnancy at the low and mid doses were more favorable compared to controls. A nominal increase in the incidence of resorptions (37.7%) was found at the highest dose, which was not statistically significant. No effects were observed in any other parameters examined. It was concluded that astaxanthin is unlikely to cause embryotoxic or teratogenic effects, and a NOAEL of 400 mg/kg bw/day was identified (Roche 1987).

iii. Summary of astaxanthin toxicity and ADI derivation

Most of the available studies on astaxanthin were conducted in rats. Only three dog studies were identified from the literature, and limited details were available for evaluation. However, it appears that rats are more sensitive to the toxicity of astaxanthin compared to dogs, as toxicities (decreased food consumption, increased serum cholesterol and bilirubin, decreased organ weights and liver histopathology) were observed at 125 mg/kg bw/day in rats after being exposed to astaxanthin in the diet for 52 weeks (EFSA 2007), while no effects regarding body weight, feed intake, hematology, clinical chemistry, urinalysis, organ weight, and gross- and histopathology were observed in dogs after being exposed for the same period (i.e., 52 weeks) at much higher doses of up to 200 mg/kg bw/day (EFSA 2007). In addition, a subchronic study in Sprague-Dawley rats found increased prothrombin time at the dose of 50 mg/kg bw/day (Takahashi et al. 2004, Yoshihiko et al. 2004), while no effects were observed in the subchronic toxicity study in dogs regarding hematology and clinical chemistry when tested at up to 158 mg/kg bw/day (Roche 1987), although the toxicological significance of increased prothrombin time is uncertain. Therefore, to establish an ADI for dogs, NOAELs derived from dog studies

are most appropriate. Among the three dog studies available, both the 52-week feeding study and the 13-week study reported the same NOAEL of 158 mg/kg/day based on lack of adverse effects. The third study is limited due to the testing of only one low dose and focusing on limited endpoints. The 52-week study was considered reliable as it was conducted under GLP and generally conformed to OECD guideline 452, although fewer number of animals was used compared to the guideline's recommendation. **Therefore, the 52-week dog study was selected as the key study, and the NOAEL of 158 mg/kg bw/day was used as the point of departure for subsequent risk assessment on astaxanthin.**

According to the U.S. FDA (2007), the acceptable daily intake (ADI) is calculated by dividing the dose level of the substance in animal studies that was shown to cause no adverse effects (i.e., NOAEL) with an appropriate safety factor. Typically a safety factor of 100 (10 for intraspecies variation * 10 for interspecies extrapolation) is applied to extrapolate from animal data to humans. A safety factor was not specified to extrapolate between animal species. As the critical study was performed in dogs, the species of interest, a safety factor of 1 is used for inter-species extrapolation. An additional safety factor of 3 is used to account for intraspecies differences in dogs. This is smaller than the default factor of 10 for intraspecies variation because the body weight and energy requirement data of terrier dogs are used in this calculation as well as in the EDI calculations. Terrier dogs are one of the most active dogs with the highest metabolic rate and energy requirement per kilogram of body weight (NRC 2006a). Therefore, EDIs calculated based on this breed is expected to be reasonably worst case scenarios. However, some uncertainty still exists as limited data are available to evaluate the metabolic rate of the smallest dog breeds (e.g., pocket breeds) that may consume more food on a body weight basis. Therefore, a safety factor of 3 is used to account for this uncertainty. Consequently, a composite safety factor of 3 is used (i.e., 1 for interspecies extrapolation * 3 for intraspecies variation).

$$ADI_{ATX} = \frac{NOAEL_{ATX}}{\text{Safety Factor}} = \frac{158 \text{ mg/kg bw/day}}{3} = 53 \text{ mg/kg bw/day}$$

EFSA (2014a,c) selected the 2-year study in rats as the critical study in their derivation of an ADI for astaxanthin in humans. EFSA performed benchmark dose modeling on liver hypertrophy in female rats in this study and derived a $BMDL_{10}$ of 3.4 mg/kg/day. The ADI of 0.034 mg/kg/day was calculated by dividing the $BMDL_{10}$ by an uncertainty factor of 100 (10 for extrapolation from rats to humans and 10 for inter-individual variation). For a 70-kg adult, this equals to 2.4 mg/day[28]. In the United States, an ADI has not been established by any regulatory bodies for astaxanthin, although U.S. FDA had no questions to the GRAS notifications on astaxanthin added to food and beverages resulting in up to 0.15 mg per serving (U.S. FDA 2015). However, astaxanthin has been used safely in humans for 12 weeks to 12 months at doses of 4 – 40 mg/day (Natural Medicines 2016). Therefore, EFSA's ADI is a very conservative value.

The dog ADI of 53 mg/kg/day derived in this report is based on a critical study of good quality in dogs, and dogs appear to be less sensitive to astaxanthin toxicity than rodents for which the EFSA human ADI was based on. In addition, due to the use of a critical study in the same

[28] 0.034 mg/kg/day * 70 kg = 2.4 mg/day

species, we were able to reduce the interspecies extrapolation safety factor by 10. In addition, by using a conservative dog breed (Cairn terrier) in subsequent exposure calculations, we were able to reduce the intra-species variation safety factory by 3. Therefore, while the ADI of 53 mg/kg/day for dogs is much higher than the conservative human ADI derived by EFSA, it is our belief that this value is still protective of dog health.

Table 13: Summary of Oral Repeated Exposure Toxicity Studies Conducted to Assess The Effect of Astaxanthin

Species	Dose	Duration	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Critical Effect	Reference
Beagle dogs (3/sex/group)	0, 41, 76, or 162 mg/kg bw/day (administered as beadlets in feed; derived from concentrations of 0, 2.5, 5.0, or 10.0% in feed)	13 weeks	158	NA	No critical effects observed	Roche 1987, EFSA 2007, 2014c
Beagle dogs (4/sex/group)	0, 6, 24 or 96 (200 after 6 months) (by gelatin capsules)	52 weeks	158 (Time weighted)	NA	No critical effects observed	EFSA 2007, 2014c
Dogs (strain and sex not reported)	2 mg/day, leading to 0.07 – 0.91 mg/kg bw/day depending on bw	1 month	0.91	NA	Improved deep sleep, sensibility to external stimulation and visual sense in 8 to 12 animals.	Honda and Takahashi 2011
Rats (species not specified) (50/sex/dose)	0 (untreated control); 0 (placebo control), 40, 200, or 1,000 mg/kg bw/day (administered as a beadlet feed formulation containing 8% astaxanthin)	2 years (satellite groups of 10/sex were treated for only one year)	$BMDL_{10}$: (liver hypertrophy in females): 3.4 $BMDL_{10}$ (liver adenomas in females): 15.7	40	Males: Increased incidence of centrilobular vacuolation of hepatocytes at 200 and 1,000 mg/kg/day. Females: Non-neoplastic hepatic changes in the liver at all doses, including increased incidences of hepatocellular vacuolation, hepatocellular hypertrophy, and multinuclear hepatocytes. Significantly increased incidence of hepatocellular adenomas in 200 and 1,000 mg/kg/day females.	EFSA 2014a

Rats, Wistar (number not specified)	0, 125, 250, 500 and 1,000 mg/kg bw/day in the diet	52 weeks	NA	125	Decreased food consumption, increased serum cholesterol in both sexes, increased bilirubin in females, Decreased organ weights, liver histopathology (brownish pigmentation of hepatocytes and macrophages)	EFSA 2007
Rats; Sprague-Dawley; (10/sex/dose; 6 weeks of age at beginning of study)	0, 2, 10, and 50 mg/kg bw/day (administered via gavage as 0, 37.0, 185.2, and 925.9 mg/kg astaxanthin rich oil)	90 days	10 (EFSA 2014b); 50 (EAS 2009)	50 (EFSA 2014b; NA (EAS 2009)	Males: Statistically significant increase in prothrombin time and active partial thromboplastin time in high dose group. Females: No significant effects observed.	Takahashi et al. 2004; Yoshihiko et al. 2004
Rats; Wistar; (10/sex/dose)	Males: 0, 10,000, 50,000, or 200,000 ppm biomass in diet (equivalent to 0, unknown, 122, or 465 mg/kg/day, as derived by the study authors; no dose equivalent was provided for the 10,000 ppm dose group) Females: 0, 10,000, 50,000, or 200,000 ppm biomass in diet (equivalent to 0, unknown, 144, or 557 mg/kg/day, as derived by the study authors; no dose equivalent was provided for the 10,000 ppm dose group)	13 weeks	Male: 122 (equivalent to 50,000 ppm biomass in diet) Female: 144 (equivalent to 50,000 ppm biomass in diet)	Male: 465 (equivalent to 200,000 ppm biomass in diet) Female: 557 (equivalent to 200,000 ppm biomass in diet)	Males: Statistically significant increase in mean kidney weights in high dose group. Slightly statistically significant increase in prothrombin time and a trend was observed with regard to active partial thromboplastin time. Females: Statistically significant increase in mean kidney weights in high dose group and orange coloration of kidneys.	Stewart et al. 2001; Stewart et al. 2008

Rats; F344; (10/sex/dose)	0, 0.2, 0.6, 1.7, or 5.0% phaffia color (in the diet, no dose equivalents of astaxanthin provided except for high dose, which was 20 mg/kg bw/day astaxanthin)	13 weeks	20	NA	No critical effects observed	Onodera et al. 1997
Rats; Species, number not specified	0, 310, 620, or 1,240 mg/kg bw/day (administered as beadlets in feed; derived from concentrations of 0, 6.25, 12.5, or, 25.0% in feed)	13 weeks	1,240	NA	No critical effects observed	Roche 1987
Rats; Wistar (6 males/dose)	0 and 0.04% in the diet (equivalent to 0 and 36.8 mg/kg bw/day)	41 days	36.8	NA	No critical effects observed	Nishikawa et al. 1997
Rats, Wistar (15/sex/dose)	0 and 0.02% in the diet (equivalent to 0 and 18.4 mg/kg bw/day in males and 0 and 20.3 mg/kg bw/day in females)	~42 days, including 14 days prior to mating until delivery	Male: 18.4; Female: 20.3	NA	No critical effects observed	Nishikawa et al. 1997
Rats (strain not specified, 32/sex/dose)	0, 25, 100 or 400 mg/kg bw/day by gavage	Unspecified, at least 70 days, multi-generation study	400	NA	No critical effects observed	Roche 1987
Mice (NMRI MORO, number not specified)	0, 0+carrier, 14, 300, 650 and 1,400 mg/kg bw/day in the diet	80 weeks	14	300	Decreased body weight	EFSA 2007
Rabbits (strain and number not specified)	0, 100, 200 or 400 mg/kg bw/day by gavage	Gestation days 7 - 19	400	NA	No critical effects observed	Roche 1987

ppm = parts *per* million

d. Mink as a Model for Dogs

Mink is used as a surrogate model for dogs in multiple studies on krill meal and fluoride in Section I.a and Section I.b. A detailed discussion of mink as an appropriate model to study the digestibility and toxicity of krill meal in dogs is included below.

i. Background on mink

The mink (*Mustela vison*) is a carnivorous mammal and lives near streams or marshes. The mink is part of the family Mustelidae, which is comprised of carnivores, including mink, weasels, ferrets, martens, sables, wolverines, badgers, skunks, and otters. The genus Mustela includes the weasel and ferret in addition to the mink (Calabrese et al. 1992).

Published reviews of mink biology have addressed the question of whether the mink is an applicable and appropriate animal model in toxicology (Calabrese et al. 1992, NRC 2000). A review by Calabrese et al. (1992) specifically considered the applicability of mink as a toxicity model for assessing risk. The National Research Council (NRC) Committee on Toxicology also evaluated the use of mink as a predictive model in toxicology (NRC 2000). Specifically, the NRC reviewed the literature on mink to assess the differences reported between mink and other laboratory animal species. This evaluation was performed to determine the use of mink as an appropriate model, and whether basic mink biology or physiology provided any reason to exclude mink for assessing data to set a human drinking-water guideline for diisopropyl methylphosphonate. The NRC compared basic biological parameters of mink, rat and dog as described in Table 14 below:

Table 14: Several Biologic Parameters of Mink, Rats, and Dogs (adapted from NRC 2000)

Parameter	Mink	Rat	Dog
Body weight, kilograms	1-1.5	0.5	10
Age at maturity, months	8-12	3	6+
Life span, years	6-11	3	12-18
First litter, months	8-12	2	6-12

The NRC discusses notable differences in mink compared to rats and dogs, which include: 1) Minks having been bred in captivity for lesser duration compared to dog and rat; 2) Minks are semi-aquatic animals with higher basal metabolic lability when held in farms; and 3) Minks are strictly seasonal breeders and exhibit delayed implantation (NRC 2000).

In light of the differences noted for mink compared to other animal models, the husbandry, life history, and biology of mink are well understood, and this knowledge has permitted controlled experiments to be conducted using mink reared in captivity (Basu et al. 2007). Sundqvist et al. (1989) conducted an extensive literature review of mink reproductive biology and concluded that mink do not differ greatly from other mammals. Unlike rats and dogs, however, mink are seasonal breeders and exhibit delayed implantation. In mink, developing blastocysts can remain in the upper region of the uterine horns for days prior to implantation, which accounts for the wide variation in the gestation period of 40 to 75 days that is observed (Calabrese et al. 1992).

These characteristics can make husbandry and breeding more challenging, but are not reasons to exclude reproductive experimental results from mink (NRC 2000). Calabrese et al. (1992) noted the practical reasons mink are not widely used in toxicology, which include the expensive cost of mink, long life span, seasonal reproductive cycle, difficulty in handling, and objectionable musk odor; however, these factors are not directly related to the capacity of mink to be used as a predictive model for risk assessment (Calabrese et al. 1992, NRC 2000).

The NRC noted that an extensive review of the literature has addressed metabolism and biochemistry, body morphometrics, physiologic functions and rates, and allometric relationships for mink. In these reviews, researchers have concluded that mink was typical of other mammals and that toxicologic results can be extrapolated to other animal species and humans (NRC 2000). Mink is considered a good model for the evaluation of nutritional and toxicological aspects of feed ingredients for other mammals, including dogs, cats, and foxes (Calabrese et al. 1992, Krogdahl et al. 2015a,b). The NRC states that in the evaluation of suitability of data for assessing safety of animal dietary supplements, "safety studies in a nontarget species (e.g., wolf for dog, mink for cat, donkey for horse)" is a class of data that may be utilized to assess acceptable and relevant data (NRC 2009). The utility of mink as a sentinel species in environmental health has also been recognized by many organizations, including Environment Canada, the United States Environmental Protection Agency (U.S. EPA), the United States National Academy of Sciences, and the Swedish Environmental Protection Agency (Basu et al. 2007, 2009). The NRC found nothing regarding the basic biology or physiology of mink to preclude it from being used as a predictive model of toxicity and concluded that mink can be used for quantitative human health risk assessments (NRC 2000).

Data on the response of mink to several dozen toxic agents reveal that mink respond in a manner qualitatively and quantitatively similar to that of other common animal models as well as humans (Calabrese et al. 1992). Similar target organ toxicity is reported in both mink and in other commonly encountered animal species exposed to fluorine, iodine, lead, mercury, coumarin, sodium monofluoroacetate, diethylstilbestrol (DES), dioxin, aflatoxin, zearalenone, and nitrosamines (Calabrese et al. 1992, Krogdahl et al. 2015a). In regards to relative susceptibility, mink are more susceptible to the acute toxicity of polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs) and iodine compared to other models, but mink are less susceptible to other agents such as fluoride (a more thorough discussion of sensitivity to fluoride toxicity in mink and dogs is included below), copper, sodium hypochlorite and sodium monofluoroacetate. However, Calabrese et al. (1992) reported that mink are not uniquely susceptible to toxic agents in general, nor do mink routinely respond with an unusual and/or enhanced susceptibility.

ii. Mink as a model for fluorine toxicity

It is important to determine whether fluorine toxicity could occur in dogs ingesting dog food containing up to 3% Qrill™ Pet since fluorine is present at concentrations up to 1,500 ppm in Qrill™ Pet. For the purposes of this GRAS assessment, mink studies are considered in the risk assessment of fluoride insofar they support the dog studies investigating the toxic effects of fluorine. Mink is a suitable and complementary model to dogs since chronic studies in mink have shown the most biologically meaningful effects of fluorine were related to bone changes.

Bone effects from fluorine are demonstrated in more than one animal species and Calabrese et al. (1992) stated that "exposure of mink to fluorine causes skeletal and dental alterations comparable to those of other species" (Calabrese et al. 1992). Available studies in mink and dogs have shown similar responses to fluorine as indicated by dental effects observed in kits and puppies and bone effects (such as exostotic anomalies) observed in adult mink and dogs. Chronic studies in both mink and dog show that the bioavailability of fluorine can impact its effects (Aulerich et al. 1987, Shupe at al. 1987, Greenwood et al. 1946, Krogdahl et al. 2015a). Furthermore, both mink and dog are reported to be highly tolerant of fluorine (Ranjan and Ranjan 2015) and allometric scaling of NOAEL and LOAEL values from chronic fluorine data in mink did not result in significantly different values compared to chronic dog data, indicating that mink and dog may have similar sensitivity to fluoride toxicity.

iii. Fluorine effects in mink and dogs

Fluorine-induced hyperostosis (excessive bone growth) and dental lesions are commonly observed signs of fluorosis reported in other species (Aulerich et al. 1987, EFSA 2004, Ranjan and Ranjan 2015). In addition, bone alkaline phosphatase is reported to increase when dietary fluorine is increased as observed in dairy heifers and cows (NAS 1974). These effects were observed in mink exposed to fluorine in two chronic dietary studies (Aulerich et al. 1987, Shupe et al. 1987). The chronic studies used sodium fluoride mixed in the diet and were fed to mink on a wet weight basis. Skeletal lesions and significantly elevated serum alkaline phosphatase activity were observed in 3-month old mink fed the two highest doses of supplemental sodium fluoride (194 and 350 ppm F) for up to 382 days, but no bone related effects were seen at 108 ppm (NOAEL is 7.5 mg F/kg/day in males and 7.7 mg F/kg/day in females and the LOAEL is 12.0mg F/kg/day in males and 12.4mg F/kg/day in females) (Aulerich et al., 1987). Adult mink had parietal, frontal and femoral bone fragility at the high dose (350 ppm F) and exhibited exostotic anomalies of the sagittal crests and jaw bones at 194 and 350 ppm F. No signs of dental lesions were observed in adult mink, but kits whelped and nursed by females fed the two highest doses (194 and 350 ppm F) and fed the same dietary doses post-weaning until 12 weeks of age showed dental lesions in the form of dark mottled teeth, particularly for the canine teeth, and several of the kits had broken canines and exostotic lesions. In the other chronic dietary study (Shupe at al. 1987) in nursing kits and adult males (adult age not specified), dental lesions were observed only in kits given the high dose of 111.5 ppm F after 7 months and bone changes were observed in adult male mink given the two highest doses (111.5 and 287 ppm F) after 8 months (NOAEL and LOAEL are 6.0 and 14.7 mg F/kg bw/day, respectively, in kits, and the NOAEL and LOAEL are 4.75 and 11.93 mg F/kg bw/day, respectively, in adult male mink). Dental lesions consisted of dull pale cream color of teeth with decreased translucency and small focal areas of chalky white discoloration. Visible changes in the mandibles and skull in adult mink included slight to moderate periosteal proliferation and thickening of the mandibles, thickened zygomatic arch, enlarged external sagittal crest, and ridging and feathering of the periosteal surface of the crania. The gross osteofluorotic changes were more pronounced in the mandible and skull compared to the leg bone and the changes were dose related. Dose-related microscopic bone changes were detectable in the femurs and humeri of adult mink given the two highest doses of fluorine (111.5 and 287 ppm F) and less pronounced microscopic changes were observed in kits given 111.5 ppm F. The changes consisted of stratified layers of periosteal new bone, excessive resorption cavities, irregular distribution and clumping of osteocytes, and zones

of incomplete mineralization in some osteones. The authors of the study reported that these microscopic changes observed in mink are characteristic of changes seen in other species with osteofluorosis. Additionally, bone fluorine levels increased dose-dependently with dietary intake of sodium fluoride, and kits accumulated fluorine at a faster rate than adults. This age-related response for fluorine accumulation in bone has been observed in other species in which rapid uptake of fluoride into bones occurs during a period of rapid growth (Shupe at al. 1987, Cerklewski 1997). In a 15-week growth study in mink kits evaluating Antarctic krill meal (fed on a dry weight basis), bone deformities of the femoral neck or head in the high-dose group (7/8 males and 1/8 females) and higher plasma alkaline phosphatase activity in the high-dose males were observed (NOAEL is 16.6 g/kg krill meal in males and 13.3 g/kg krill meal in females, providing 53.4 mg F/kg/day in males and 43.0 mg F/kg/day in females, respectively) (Krogdahl et al. 2015a). In comparison, dietary studies have shown dental effects in puppies and bone effects in adult dogs. Puppies given 5 mg F/kg/day (amounting to 50 ppm F in the diet per NAS 1974) as sodium fluoride developed dental fluorosis and had excess fluorine deposition in the bones, but no bone effects were observed in female mongrel dogs (age not specified) given the same dose (NOAEL is 5.2 mg F/kg bw/day) (Greenwood et al. 1946, Greenwood 1956, Ranjan and Ranjan 2015). In a 2-year study evaluating fluoride on reproduction and development, adult female Shetland dogs had developed large, palpable bony exostoses on the skull when given the highest dose of fluorine (11.5 mg F/kg/day obtained from powdered rock phosphate) mixed in dog food (460 ppm F on a wet weight basis), but no effects on reproduction were observed (maternal NOAEL and LOAEL are 1.4 and 11.5 mg F/kg/day, respectively) (Shellenberg et al. 1990).

The dietary fluorine studies indicate that mink may exhibit bone resorption and remodeling capacity although no extensive evaluation for this process have been conducted in mink. Bone remodeling is a tightly regulated process that involves repair of micro-damage (i.e., targeted remodeling) and the replacement of old bone with new bone through sequential osteoclastic resorption and osteoblastic bone formation (Eriksen 2010). There is evidence that fluoride at mitogenic, micromolar doses can stimulate several mature osteoblast activities, including alkaline phosphatase expression, collagen synthesis, and osteocalcin synthesis in bone cell cultures (Lau and Baylink 1998). *In vivo* data in lambs suggest that the presence of osteocalcin, the major vitamin K-dependent protein of the bone, may be important for the maintenance of a normal bone mass and remodeling of trabecular bone (Pastoureau et al. 1993). Serum alkaline phosphatase activity and osteocalcin content may be considered as reference indicators of bone metabolism changes in fluoride exposures (Song et al. 2011). The authors of one of chronic fluorine studies noted that bone effects (exostotic anomalies) in adult mink may be attributable to increased osteoblastic activity from fluorine exposures, thus leading to abnormal bone mineralization (Aulerich et al., 1987). The authors of mink growth study for krill meal noted that elevated alkaline phosphatase activity observed in high-dose males that had bone deformities may be associated with an increase in osteoblast activity and therefore indicate increased bone deposition and/or mineralization (Krogdahl et al. 2015a). Alkaline phosphatase is not specific for bone since it can also indicate liver injury; however, there were no other adverse clinical chemistry findings indicating hepatic dysfunction in the study (i.e., no abnormal aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) (Krogdahl et al. 2015a). Unfortunately, none of studies in mink have evaluated effects on osteocalcin levels. In comparison, the dog is noted as a good model for evaluating bone remodeling (Huja et al. 2006,

Gomes and Fernandes 2011). In adult beagle dogs given tap water providing a dose of 0.7 mg F/kg/day for 6 months, activation of bone remodeling was observed, but it was not demonstrated if this effect was adverse (Snow and Anderson 1986). Bone remodeling have also been evaluated in ewes and lamb following sodium fluoride exposures (Chavassieux et al. 1991a,b). In contrast, rodents may not be a good model for the fluoride toxicity in dogs since rodents are reported to have limited capacity for bone remodeling (Chavassieux 1990).

Changes in heart and adrenal weights of unknown significance were reported in 3-month old female mink fed sodium fluoride in the diet for 382 days (Aulerich et al. 1987). Increased heart weight ($P < 0.05$) as a percentage of brain weight was observed at 108 ppm F and above and an increased adrenal gland weight ($P < 0.05$) as a percentage of brain weight was observed at 194 ppm F. The authors stated that the significance of the increased heart and adrenal gland weight is unknown and may reflect subtle secondary effect of fluoride toxicosis, although no primary lesions in organs or soft tissue have been consistently reported for chronic fluorosis. Kidneys are noted to contain more fluorine than other soft tissues or organs, which is likely due to the kidney serving as the major route of elimination for fluorine. Soft tissues usually contain small amounts of fluorine (<2.5 ppm) in multiple species including dogs (Shupe et al. 1987). No histopathology was conducted in the mink study to verify if the increase in heart or adrenal weight relative to brain weight is adverse. Nevertheless, the authors concluded that the tolerance of chronic fluorine in mink may be estimated at approximately 100 ppm F based on increased heart weight. The other chronic study in male mink fed sodium fluoride in the diet for up to 8 months reported no effects on the heart or adrenal gland (Shupe at al. 1987). In the 15-week growth study in mink kits evaluating Antarctic krill meal, no effects on adrenal gland weight and histopathology were reported in mink; however, the relative heart weight of high-dose females (0.55 ± 0.05 g/100 g body weight) was higher than low-dose females (0.43 ± 0.07 g/100 g body weight), but was not significantly different from controls (0.44 ± 0.09 g/100 g body weight). The reason for the increase in relative heart weight of females in the high-dose group is unknown and this finding was not observed in the males. Furthermore, there were no histopathology findings in the heart so the authors concluded that increased relative heart weight in high-dose females is not toxicologically relevant. No heart and adrenal gland effects were reported in available dog studies for dietary fluorine. Electrocardiogram changes such as sinus bradycardia along with a decrease in heartbeat were reported in eight dogs with chronic fluorosis compared to healthy dogs; however, these animals were street dogs obtained from an area in which chronic fluorosis is endemic and in which fluorosis was determined after clinical examinations (i.e., dental fluorosis and exostoses of jaw and long bones) (Kilicalp et al. 2004). Therefore, the amount and duration of fluorine exposure in the street dogs with fluorosis are unknown along with other factors, such as the general level of nutrition.

iv. Bioavailability of fluorine in mink and dogs

Chronic studies in both mink and dog indicate that the bioavailability of the fluorine is dependent on the source or form of the fluoride compound and this impacts its toxicity (NAS 1974). The tolerance levels for fluorine-containing compounds are noted to depend on the solubility and bioavailability of the fluorine (Shupe et al. 1987). Greenwood et al. (1946) evaluated 5 mg F/kg/day (50 ppm F) as sodium fluoride, bone meal, or rock phosphate in the diet given to mongrel dogs and puppies, and dental fluorosis was observed in puppies only for the sodium

fluoride form. Compared to NOAEL values of 4.75 mg F/kg/day in males (Shupe at al. 1987) and 5.0 mg F/kg/day in males and 5.1 mg F/kg/day in females (Aulerich et al., 1987) established from chronic dietary studies in mink using sodium fluoride, the higher NOAEL values of 53.4 mg F/kg/day in males and 43.0 mg F/kg/day in females established from the mink growth study evaluating Antarctic krill meal are likely due to reduced bioavailability of fluorine in krill meal. Sodium fluoride is readily soluble in the fasted state (Krogdahl et al. 2015a) and its relative absorption rate is reported at 100% compared to other fluorine sources such as raw rock phosphate (69%), dicalcium phosphate (52%) or defuorinated phosphate (20%) (EFSA 2004). The toxicity of a fluoride compound appears to generally increase with its water solubility and fluorine in sodium fluoride is reported to be as twice as toxic compared to other sources such as cyrolite and rock phosphate (NAS 1974).

v. Tolerance of fluorine in mink and dogs

Both mink and dog are reported to be highly tolerant to the effects of fluorine (Ranjan and Ranjan 2015). The chronic dietary studies conducted in minks used sodium fluoride mixed in the diet (Aulerich et al. 1987, Shupe at al. 1987), and the authors for one of the chronic mink studies (Shupe at al. 1987) reported that fluoride doses given in mink feed were based on data pertaining to fluorine tolerance of dogs in chronic studies conducted by Greenwood et al. (1946). Fluoride tolerance appears high in dogs as there are limited reports on the occurrence of dental and bone lesions in dogs reared in fluorosis-endemic areas (Ranjan and Ranjan 2015). The relative tolerance levels of fluoride in feed (on a dry matter basis) and water in different animal species were reported by Ranjan and Ranjan 2015, as summarized below.

Table 15: Tolerance Levels of Fluoride in Feed (on a Dry Matter [DM] basis) and Water for Different Animals (adapted from Ranjan and Ranjan 2015)

Animal Species	Fluoride in feed (ppm)	Fluoride in water (ppm)
Beef and dairy heifers	30	2.5-4
Mature dairy cattle	30	3-6
Mature beef cattle	40	4-8
Sheep	50	12-15
Horse	60	4-8
Swine	70-100	5-8
Poultry	100	10-13
Dog	100	-

It should be noted for the table above that the 100 ppm tolerance was set for young pups based on a review by the National Research Council (NRC) (NAS 1974) in which the endpoint was an adverse effect on growth. The NRC describes no effects on growth in young dogs and puppies fed 5 mg F/kg/day (which amounted to 50 ppm F in the diet per NAS 1974) as sodium fluoride (Greenwood et al. 1946), but other studies in weanling Beagles or mixed pups given sodium fluoride in the diet shown an adverse growth rate at 250 ppm F and a slight growth depression at 200 ppm F (Bunce et al. 1962, Chiemchaisri and Philips 1965, NAS 1974). Notably, the dog studies conducted by Bunce et al. (1962); Chiemchaisri and Philips (1965) were conducted in magnesium-deficient dogs, and this condition may have impacted the toxic response to fluorine.

The NAS determined a tolerance level of 100 ppm fluorine with no further discussion of how they arrived at this specific concentration, but they noted that the available data are inadequate to determine if 100 ppm fluorine will have any effects on mature dogs (NAS 1974). Although one dose level of fluorine was evaluated in puppies and their mothers, female mongrel dogs tolerated 50 ppm F as sodium fluoride in the diet (5 mg F/kg/day) in addition to 0.2 mg F/kg/day that was present in the basal diet ration (total of 5.2 mg F/kg/day) for up to 429 days (Greenwood et al. 1946, Greenwood 1956). A chronic dose of 5.2 mg F/kg/day is therefore considered reasonably tolerable in adult dogs; however, limitations of the chronic study are that one dose level of fluorine was evaluated and the origin and age of the female mongrel dogs were not specified.

In comparison, adult male mink are reported to tolerate up to 111.5 ppm fluoride in feed on a wet weight basis as fed, which is equivalent to 307.0 ppm on a dry weight basis (fluorine contents of wet and dry diets are provided from Shupe et al. 1987) without any adverse effects on pelt quality and growth rate and on bone or teeth (Shupe et al. 1987, Ranjan and Ranjan 2015). For adult male mink given 111.5 ppm fluoride, the corresponding dose is 11.93 mg F/kg/day, which is considered the LOAEL, and the NOAEL is 4.75 mg F/kg/day (46 ppm F) for the absence of bone effects in adult male mink. In the chronic mink study by Aulerich et al. (1987), 3-month old mink tolerated up to 194 ppm of supplemental fluorine from sodium fluoride in the diet on a wet weight basis in addition to 35 ppm of fluorine present in the basal diet (194 ppm F supplemental + 35 ppm F in basal diet = 229 ppm F total) for 382 days. The corresponding dose is 12.0 mg F/kg/day in males and 12.4 mg F/kg/day in females, which are the LOAELs, and the NOAELs are 7.5mg F/kg/day in males and 7.7 mg F/kg/day in females (108 ppm F supplemental + 35 ppm F in basal diet = 143 ppm F total) in the absence of bone effects.

The chronic studies indicate that mink do not exhibit unusual susceptibility or tolerance to chronic fluorine compared to dogs. The chronic studies in mink and dog evaluated fluorine as sodium fluoride when mixed in the basal diet. However, available chronic data for fluorine in adult dogs are limited (i.e., one dose level of 5.2 mg F/kg/day was evaluated). In addition, data for the pharmacokinetics of fluorine in dogs and mink are lacking (ATSDR 2003). Therefore, to account for these data limitations, interspecies dose extrapolation using allometric scaling (adjusting for body surface area) is used to determine dog equivalent doses from the mink chronic toxicity data. The dog equivalent doses estimated from the mink data are used to further examine if the mink exhibit unusual susceptibility or tolerance to chronic fluorine toxicity compared to dog below.

vi. Interspecies dose extrapolation

Allometric scaling is performed to extrapolate a dose between species and is based on the normalization of dose to body surface area (Nair and Jacob 2016). Interspecies dose scaling is required for three main situations: 1) for the selection of the recommended starting dose in humans for Phase 1 clinical trials; 2) for the selection of a safe and effective dose in veterinary practice; 3) for dose selection for experimental purposes (Sharma and McNeill 2009). Allometric scaling accounts for possible differences in pharmacokinetics and physiological time among species, and the approach assumes unique species characteristics are present for anatomical, physiological, and biochemical processes (Nair and Jacob 2016). The U.S. FDA uses this approach in which the exponent of 0.67 (2/3) for body surface is used to scale doses

between species to estimate the first-in-human dose for Phase 1 clinical trials (U.S. FDA 2005, Nair and Jacob 2016). The exponent of 0.75 (3/4) for body weight (to account for body surface area difference) is used to scale doses between species, which is the method currently used in veterinary practice and by the U.S. FDA for new animal drugs (U.S. FDA 2008, Nair and Jacob 2016). It has been debated in the literature whether the exponent of 0.75 is more appropriate to account for metabolic rate and physiological time among species, and better scaling of doses have been demonstrated in published literature (Sharma and McNeill 2009). Therefore, in this report, the body weight exponent of 0.75 is used to be consistent with current veterinary practices.

Mink are noted to display allometrically predictable biochemical and physiological parameters of both pharmacokinetic and toxicological significance. A review by Calabrese et al. (1992) reports that numerous physiological parameters in mink are predictable from those of other mammalian species and can be used to predict such parameters in other mammals. This information is important for extrapolating critical toxicological effects from one species to another. Therefore, in regards to allometric relationships, mink displays a consistency and commonality with other mammals (Calabrese et al. 1992).

To address limitations in the chronic fluorine data for mink and dog and to facilitate a more accurate comparison of critical doses in relevant studies between mink and dogs, allometric scaling is applied to the NOAEL and LOAEL values obtained from the mink chronic studies for fluorine to determine dog equivalent doses. The exponent of 0.75 (3/4) is chosen to scale doses between the species. The equivalent doses in dog are determined from chronic mink NOAEL and LOAEL values using the following formula:

$DED_N = NOAELm \times (BWm/BWd)^{1/4}$

Or

$DED_L = LOAELm \times (BWm/BWd)^{1/4}$

Where:

DED_N or DED_L = dog equivalent dose from the critical effect dose level in mink (i.e., NOAELm, LOAELm);
NOAELm = No Observed Adverse Effect Level established in chronic mink studies;
LOAELm = Lowest Observed Adverse Effect Level established in chronic mink studies;
BWm = mink body weight (Mean animal body weight, which is either reported in the animal study or the default value specified by the U.S. EPA (1988);
BWd = dog body weight (using the adult body weight of the Cairn terrier of 6.4 and 5.9 kg for males and females, respectively. See Part 3 of the dossier).

Using allometric scaling, dog equivalent doses of 3.69, 5.48 and 5.0 mg F/kg/day[29] are estimated from the mink NOAEL values and dog equivalent doses of 9.27, 8.78 and 8.02 mg F/kg/day[30] are estimated from the mink LOAEL values.

Mink do not appear to exhibit unusual susceptibility or tolerance to chronic fluorine compared to dogs when provided as sodium fluoride in the diet since allometric scaling of NOAELs from chronic mink data did not result in significantly different values from the NOAEL in the chronic dog study conducted by Greenwood et al. (1946). The dog equivalent doses determined from chronic mink NOAELs are slightly below the NOAEL of 5.2 mg F/kg/day in the chronic dog study with the exception of 5.48 mg F/kg/day determined from male mink in the chronic study by Aulerich et al. (1987). In addition, the dog equivalent doses determined from mink LOAEL values are higher than the dog NOAEL. The LOAEL values in the mink studies were based on the adverse effects on bone. As shown in Table 16 below, dog equivalent doses of LOAELs in the chronic mink studies are lower than LOAELs in dog studies. This indicates that mink is as sensitive as, if not more sensitive than, dogs in the toxicity response to chronic dietary consumption of fluoride.

Table 16: Comparison of Critical Doses in Chronic Dietary Studies in Adult Dogs and Mink

Species	Duration	NOAEL (mg/kg bw/day)[a]	LOAEL (mg/kg bw/day)[a]	Critical Effect	Reference
Female mongrel dogs	Up to 437 days	5.2	NA	No significant effect of diet on growth, hemoglobin, serum Ca and P, coagulation time, breaking strength tests, or growth of bones and teeth. F given as NaF, Bone meal or rock phosphate	Greenwood et al. (1946)
Adult female Shetland sheepdogs	2 years	1.4	11.5	No effect of high dose F on reproduction or malformations. Four high F dose dogs developed large, palpable bony exostoses on the skull. Two fluoride doses in combination with well-water and distilled water were tested. F given as rock phosphate	Shellenberg et al. (1990)
Adult male mink	8 months	3.69	9.27	Visible and microscopic changes in bones in animals in the two highest dose groups. F given as NaF	Shupe *et al.* (1987)

[29] DED_N = NOAELm x $(BWm/BWd)^{1/4}$: 4.75 mg/kg/day x $(2.338 kg/6.4 kg)^{1/4}$ = 3.69 mg/kg/day; 7.5 mg/kg/day x (1.838 kg/6.4 kg)1/4 = 5.48 mg/kg/day (males) and 7.7 mg/kg/day x (1.042 kg/5.9 kg)1/4 = 5.0 mg/kg/day (females).

[30] DED_L = LOAELm x $(BWm/BWd)^{1/4}$: 11.93 mg/kg/day x $(2.338 kg/6.4 kg)^{1/4}$ = 9.27 mg/kg/day; 12.0 mg/kg/day x $(1.838 kg/6.4 kg)^{1/4}$ = 8.78 mg/kg/day (males) and 12.4 mg/kg/day x $(1.042 kg/5.9 kg)^{1/4}$ = 8.02 mg/kg/day (females).

Adult male and female mink	382 days	5.48 (M) 5.0 (F)	8.78 (M) 8.02 (F)	Adverse effects on bone observed in animals in the two highest dose groups. F given as NaF	Aulerich *et al.* (1987)

[a]: Critical doses in mink studies are presented as dog equivalent doses calculated using BW to the exponent of 0.75

vii. Summary

Overall, mink is considered a suitable surrogate model for assessing the safety of krill meal (Qrill™ Pet) as an ingredient intended for adult dog food based on the literature supporting the use of mink as a predictive model of toxicity. Available mink studies for fluorine and krill meal show that mink respond to fluorine in a similar manner as other animal species and it is not apparent that mink exhibit unusual susceptibility to chronic fluorine toxicity compared to dogs. However, chronic data for fluorine in adult dogs are limited, and data for the pharmacokinetics of fluorine in dogs and mink are lacking. To account for these limitations, interspecies dose extrapolation using allometric scaling (adjusting for body surface area) is used to determine dog equivalent doses from mink chronic toxicity data. The determined dog equivalent doses from the chronic mink data for fluorine indicate that mink are not unusually susceptible or tolerant of fluorine compared to dogs when given as sodium fluoride in the diet. As fluoride is the critical component of concern for krill meal toxicity, mink is an appropriate model for dogs for krill meal as well.

II. GRAS EVALUATION

Qrill™ Pet is meal derived from fresh, wild caught, whole Antarctic krill (*Euphausia superba*) intended for use as a replacement for fish meal or other sources of protein and lipids used in adult dog food. All aspects of the production of Qrill™ Pet are consistent with good manufacturing practices. The intended usage rate is up to 30,000 mg/kg food (30,000 ppm or 3%).

Qrill™ Pet is a good source of protein (≥ 55%) and fat (≥ 18%), and contains *omega*-3-fatty acids (> 17g/100g fat) and astaxanthin (80-160 ppm). It is not a significant source of iodine, copper, zinc, or selenium at the maximum proposed use level in dry dog food. Qrill™ Pet remains within specification for microbes, moisture, crude protein, total volatile nitrogen, cadaverine, fat and peroxide value when stored for 18 months in original packaging at ambient temperature.

Dried Antarctic krill is permitted for use in animal feed in Europe and meals from other marine sources (fish, crab and shrimp) are permitted for use in the United States. The proximate composition of Qrill™ Pet is similar to fish, crab and shrimp meal, except that Qrill™ Pet has a higher protein and fat content and lower ash, NaCl and calcium content. Results of studies that have been performed in mink and swine indicate that protein and lipid digestibility of krill meal is high and similar to other ingredients that are used as a source of protein (fish meal) or lipid (soybean). This is supported by the significant increase in omega-3 index in dogs receiving Qrill™ Pet at 8% in the diet for up to 14 weeks.

The safety of Qrill™ Pet for dogs is demonstrated by results of an acute oral toxicity in mice reporting an LD_{50} of > 2,000 mg/kg bw, a sequential gestation, lactation and growth study of Antarctic krill meal in mink, a bacterial mutagenicity study and a micronucleus study in rats. The mink is an appropriate surrogate model for evaluation of the safety of food ingredients for dogs. A published reproductive study and a growth study in mink were identified as the critical studies. At the LOAEL of 31.2 and 26 g/kg/day, adult males and females had minimal effects such as decreased liver glycogen, alterations in clinical chemistry and hematology, increased intestinal and stomach weight attributed to chitin content (a form of fiber), and rectal inflammation. The NOAEL was established at 14.3 g/kg/day in these studies. In adult Husky dogs, an estimated dose of 2.1 – 2.5 g/kg bw/day (8%) did not affect clinical chemistry parameters or cause any apparent signs of toxicity when consumed for up to 14 weeks. Instead, increased omega-3 index (a beneficial effect) was found. Rats appear to be more sensitive to the toxicity of fluoride than dogs and mink. Studies in rats suggest that fluorine, which is most concentrated in the carapace, is the major toxicological concern of krill meal, while protease may decrease the protein quality of the meal if not deactivated. The lowest NOAEL of krill meal with much higher fluorine content than Qrill™ Pet is 14.3 g/kg bw/day as established in mink and supported by data in dogs and rats. This is allometrically scaled to a dog equivalent dose of 9.5 g/kg bw/day. An ADI of 0.95 g/kg bw/day was established based on this dog equivalent NOAEL for krill meal and a composite safety factor of 10.

Qrill™ Pet is heated to above (b) (4) in the manufacturing process. The protease activity of the product was less than the limit of detection (<0.1 Sigma units). Therefore, protease is unlikely to be a concern in Qrill™ Pet. In addition, the shell fraction (carapace) of the harvested Antarctic krill is largely removed during the manufacturing process, and each batch of Qrill™ Pet is tested for fluorine content twice to ensure the fluorine content meets the specification of ≤ 1,500 ppm. The use of Qrill™ Pet at 30,000 ppm in dry food would result in a maximum EDI of 0.86 g/kg bw/day Qrill™ Pet in a small and active Cairn terrier dog. As the EDI is smaller than the ADI, 3% Qrill™ Pet in dry food is safe for adult dogs.

The usage rate of Qrill™ Pet is limited due to the high concentration of fluorine, which causes dental and/or skeletal fluorosis at high doses. The fluorine level in Qrill™ Pet is tightly controlled and closely monitored to ensure that each batch produced meet the product specification of ≤ 1,500 ppm. The permissible amount of fluorine in Qrill™ Pet (up to 1,500 ppm) is less than the European limit of 3,000 mg/kg fluorine in feed materials. Although a regulatory limit is not established for fluorine content in dog food in the United States, NAS established tolerances of 50 ppm (pathology) and 100 ppm (performance) for growing dogs in 1974. Quite a number of studies of fluorine toxicity in dogs and other species were published since the NAS evaluation, and commercial and laboratory dog food frequently contain fluorine levels greater than 50 ppm, and sometimes even 100 ppm. A literature review of fluorine toxicity in dogs identified a NOAEL of 5.2 mg F/kg bw/day, which is supported by data in mink. An ADI of 3.5 mg F/kg bw/day was established based on this NOAEL, a factor of 2 to account for reduced bioavailability of F from krill meal, and a composite safety factor of 3.

Dry dog food containing 3% Qrill™ Pet that meet the ingredient specification of ≤1,500 ppm fluorine will contain a maximum of 45 ppm fluorine from Qrill™ Pet. Maximum doses of fluorine in dogs provided food containing 3% Qrill™ Pet is estimated at 1.29 mg/kg bw/day.

Other ingredients in dog food may also contain fluorine, especially mineral supplements and animal by-products. In addition, drinking water contains 0.7 mg/L fluorine. The total EDI for an adult dog from all exposure sources is conservatively estimated to be up to 1.6 mg F/kg bw/day. This EDI is lower than the ADI for fluoride, indicating that including Qrill™ Pet at 3% in dry dog food is safe for adult dogs.

For astaxanthin, the NOAEL of 158 mg/kg bw/day established in a 52-week study in dogs leads to the establishment of an ADI of 53 mg/kg bw/day. This value is much higher than the EDI of 1.6 mg/kg bw/day both from consumption of Qrill™ Pet at 3% and from other ingredients in food. While one rat study (Petri and Lundabye 2007) reported a dose-dependent accumulation of astaxanthin in the eyes from approximately 200 to > 2,000 mg/kg bw/day, large variations exist in the study and the levels measured after a 14-day exposure appeared to decrease from those measured after 7 days. In addition, ophthalmoscopic examination was conducted in a 13-week tolerance study in dogs, which did not identify any adverse effects up to the highest tested dose of 162 mg/kg bw/day. Additionally, a study in dogs reported beneficial effects to the eyes (i.e., increased visual sense) in 10/29 animals after consuming up to 0.91 mg/kg bw/day astaxanthin for a month. In addition, rats appeared to be more sensitive to the effects of astaxanthin than dogs. Further, the estimated EDI for astaxanthin was very conservative, as described previously. Therefore, it is unlikely that the intake of astaxanthin from 3% Qrill™ Pet will lead to significant accumulation of astaxanthin or adverse health effects in dogs.

Table 17: Comparison of ADI and EDI for Qrill™ Pet and its Components of Concern

Substance	ADI (Acceptable Daily Intake) mg/kg bw/day	EDI (Estimated Daily Intake) mg/kg bw/day
Qrill™ Pet	950	860
Fluorine	3.5	1.6
Astaxanthin	53	1.6

The lack of robust studies in the target species (i.e.., dogs) on the safety of krill meal is a limitation of the available toxicological dataset. This was overcome by using two robust studies in mink. We demonstrated that mink is an appropriate surrogate model for dogs. We applied allometric scaling to the NOAELs identified in the mink studies to account for toxicokinetic differences between the two species and a safety factor of 3 to account for toxicodynamic differences in the derivation of the ADI. Further, the krill meal used in the critical studies in mink contained higher levels of fluoride than Qrill™ Pet. As the critical studies investigated the safety of females during pregnancy, the ADI established using these studies is expected to be protective of pregnant dogs as well. The ADI of 0.95 g/kg bw/day is lower than the NOAEL of 2.0 – 2.5 g/kg/day established in the two tolerance studies in adult Husky dogs, demonstrating the validity and conservativeness of this ADI. In addition, separate hazard were conducted on ingredients of concern in krill meal – fluorine and astaxanthin. The ADIs for these ingredients were both established based on robust studies in dogs.

The EDIs for Qrill™ Pet, fluorine and astaxanthin are also calculated with conservative assumptions, such as using energy requirement equations and body weights of active pet terriers, assuming Qrill™ Pet will not replace other ingredients containing fluoride, and using a fluoride

level of 1,500 ppm based on product specification instead of the highest level reported in analytical testing of batches produced in the last two year.

The EDIs and ADIs of Qrill™ Pet and its components of concern are summarized in Table 17 above. The ADIs for Qrill™ Pet, fluorine and astaxanthin are higher than their respective EDIs, all of which were established with conservative assumptions. The totality of the evidence indicates that use of 3% Qrill™ Pet in dry adult dog food would meet the reasonable certainty of safety standard for a GRAS determination.

III. CONCLUSION AND OVERALL ASSESSMENT OF SAFETY OF QRILL™ PET

Following a critical evaluation of the information available (favorable or unfavorable alike) regardless of whether these data are generally available, we have determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that *Euphausia superba* (krill) meal (Qrill™ Pet), produced in accordance with current Good Manufacturing Practice (cGMP), is safe under the intended conditions of use, and is Generally Recognized As Safe (GRAS) by scientific procedures when used as a source of protein and lipid in dry dog food at 3% level of inclusion in finished food for adult dogs.

With the exception of the acute oral toxicity and genotoxicity study on Qrill™ Pet (Wessels 2014 – unpublished data) and two subchronic feeding studies in adult dogs that only examined limited endpoints (Berge et al. 2014; Hals 2016 – unpublished data), all the other studies cited in this report were obtained from publically available sources. The critical studies used to evaluate the safety of krill meal, fluoride and astaxanthin and establish their respective ADIs are all publically available. The three proprietary studies listed above were only used as supportive evidence.

A signed GRAS Panel conclusion letter from an independent panel of recognized experts is attached as Appendix B. These experts are qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients. Their curriculum vitae are attached as Appendix C.

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Appendix A: ATSDR (2003) Summary Table for Toxicological Studies on Fluoride – Oral

Table 3-4 Levels of Significant Exposure to Fluoride - Oral

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
	ACUTE EXPOSURE						
	Death						
1	Human	1d 1x/d (C)				16 (1 child)	Eichler et al. 1982 sodium fluoride
2	Rat (Sprague-Dawley)	1 d 1x/d (GW)				52 (LD50 for 150g rats) 54 (LD50 for 80g rats) 31[b] (LD50 for 250g rats)	DeLopez et al. 1976 sodium fluoride
3	Rat (Rochester)	1 d 1x/d (GW)				51 6 (LD50)	Lim et al. 1978 sodium fluoride
4	Rat (Sprague-Dawley)	1 d 1x/d (GW)				101.3 (LD50)	Skare et al. 1986 sodium fluoride
5	Rat (Sprague-Dawley)	once (GW)				126.3 M (LD50)	Whitford et al. 1990 sodium fluoride
6	Rat (Sprague-Dawley)	once (GW)				85.5 M (LD50)	Whitford et al. 1990 sodium fluoride
7	Rat (Sprague-Dawley)	once (GW)				146.3 M (LD50)	Whitford et al. 1990 Monofluorophosphate
8	Rat (Sprague-Dawley)	once (GW)				84.3 M (LD50)	Whitford et al. 1990 Monofluorophosphate

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
9	Mouse (Swiss)	1 d 1x/d				44.3 (LD50)	Lim et al. 1978 sodium fluoride
	Systemic						
10	Rat	2wk (W)	Musc/skel		9.5 (decreased modulus of elasticity)		Guggenheim et al. 1976 sodium fluoride
	Reproductive						
11	Mouse	5d 1x/d (G)		32			Li et al. 1987a sodium fluoride
	Developmental						
12	Rat (Wistar)	GD 6-19 (GW)			18 F (increased percentage of skeletal and visceral abnormalities)		Guna Sherlin and Verma 2001 sodium fluoride
13	Rat (Sprague-Dawley)	Gd 6-15 daily (W)		13.21			Heindel et al. 1996 sodium fluoride
14	Rabbit (New Zealand)	Gd 6-19 daily (W)		13.72			Heindel et al. 1996 sodium fluoride
	INTERMEDIATE EXPOSURE						
	Death						
15	Mouse (B6C3F1)	6 mo daily (W)				67 (increased mortality)	NTP 1990 sodium fluoride
16	Mouse	6mo ad lib (W)				300[c] M (increased mortality) 600 F (increased mortality)	NTP 1990 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
	Systemic						
17	Rat	2 mo 7d/wk 24hr/d (W)	Endocr		0.5 (decreased thyroxine levels; increased T3- resin uptake ratio)		Bobek et al. 1976 sodium fluoride
18	Rat (CD)	daily 16-19 weeks (W)	Musc/skel	8.25 F	10.7 F (prominent growth lines on upper incisors)		Collins et al. 2001a sodium fluoride
19	Rat (Sprague-Dawley)	7d/wk 24hr/d (W)	Musc/skel		10.5 (decr mineral content and incr proline in tooth enamel matrix)		DenBesten and Crenshaw 1984 sodium fluoride
20	Rat (Wistar)	5 wk (W)	Musc/skel	13	19 (histological fluorosis; decr bone growth)		Harrison et al. 1984 sodium fluoride
21	Rat (Fischer- 344)	6 mo daily (W)	Gastro		7 (hyperplasia of glandular stomach)		NTP 1990 sodium fluoride
			Hepatic	20			
			Renal	20			
22	Rat (Sprague-Dawley)	daily 16 or 48 weeks (W)	Musc/skel	0.15 M	0.5 M (decreased vertebral strength and bone mineralization)		Turner et al. 2001 sodium fluoride
23	Rat	30d (W)	Musc/skel		14 (delayed healing of broken bones)		Uslu 1983 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)		LOAEL Serious (mg/kg/day)		Reference Chemical Form
24	Mouse	280 d daily (W)	Hepatic		0.95	(pale, granular hepatocytes with fatty vacuoles)			Greenberg 1982a sodium fluoride
25	Mouse	280d (W)	Renal		1.9	(nephron degeneration)			Greenberg 1986 sodium fluoride
26	Mouse	4 wk 7d/wk daily (W)	Musc/skel		0.8	(incr bone formation rate; slight decr bone calcium)			Marie and Hott 1986 sodium fluoride
27	Mouse (B6C3F1)	6 mo daily (W)	Cardio				67	(multifocal mineralization and degeneration of the myocardium)	NTP 1990 sodium fluoride
			Musc/skel		5.6 M	(increased osteoid in femur and tibia)			
			Hepatic		67	(megaolocytosis and syncytial alteration)			
			Renal				67	(multifocal nephrosis)	
			Bd Wt		67	(20% decr bw gain)			
28	Mouse	35 d 1x/d (GW)	Hemato		5.2	(decr RBC and hemoglobin, incr WBC)			Pillai et al. 1988 sodium fluoride
			Bd Wt		5.2	(decr body weight)			

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
29	Mouse (Kunmin)	daily 100-150 days (W)	Musc/skel	0.06 M	3.2 M (incisor fluorosis)		Zhao et al. 1998 sodium fluoride
			Endocr	0.06 M	3.2 M (decreased radiolabelled iodine uptake)		
			Bd Wt	3.2 M			
30	Rabbit (NS)	6 mo daily (F)	Resp		4.5 (congestion, edema fluid, desquamation of respiratory epithelium in lungs)		Purohit et al. 1999 sodium fluoride
	Neurological						
31	Rat (Sprague-Dawley)	6 wk daily (W)			6 F (altered spontaneous behavior)		Mullenix et al. 1995 sodium fluoride
32	Rat (Sprague-Dawley)	6 wk daily (W)		5.5 F	7.5 F (altered spontaneous behavior)		Mullenix et al. 1995 sodium fluoride
33	Rat (Wistar)	60 d daily (GW)			9 (decr spontaneous activity)		Paul et al. 1998 sodium fluoride
	Reproductive						
34	Rat (Sprague-Dawley)	daily 30 days (W)				10.21 F (decreased number of viable fetuses, increased resorptions)	Al-Hiyasat et al. 2000 sodium fluoride
35	Rat (CD)	60 d 7d/wk (F)			2.3 (decr seminiferous tubule diameter)	4.5 (50% reduction in fertility, decr in percentage of seminiferous tubules containing spermatozoa and decr testosterone levels)	Araibi et al. 1989 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
36	Rat (NS)	daily 30 d (GW)				2.3 (decreased fertility and sperm counts)	Chinoy et al. 1992 sodium fluoride
37	Rat (Charles Foster)	30 or 50 days d (F)				4.5 (decreased sperm motility and count)	Chinoy et al. 1995 sodium fluoride
38	Rat (CD)	daily 16-19 weeks (W)		10.7 F			Collins et al. 2001a sodium fluoride
39	Rat (Wistar)	daily 6 wk (W)		21			Krasowska and Wlostowski 1992 sodium fluoride
40	Rat (Wistar)	daily 16 wk (W)			7.5 (seminiferous tubule atrophy)		Krasowska and Wlostowski 1992 sodium fluoride
41	Rat	3 mo 7d/wk (F)		23			Marks et al. 1984 sodium fluoride
42	Rat Charles Foster	daily 50 d (GW)			4.5 (decr testosterone levels and Leydig cell diameter)		Narayana and Chinoy 1994 sodium fluoride
43	Rat (Sprague-Dawley)	daily (W)		16			Sprando et al. 1997 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
44	Rat (Sprague-Dawley)	daily (W)		16			Sprando et al. 1998 sodium fluoride
45	Mouse (Swiss)	30 d daily (F)				4.5 (decr sperm motility and count and infertility)	Chinoy and Sequeira 1992 sodium fluoride
46	Mouse (Swiss-Webster)	25 wks (W)		9.5		19 (nearly complete infertility)	Messer et al. 1973 sodium fluoride
47	Mouse	35 d 1x/d (GW)		5.2			Pillai et al. 1988 sodium fluoride
48	Gn Pig (NS)	30 d daily (GW)				4.5 (decr sperm motility and viability)	Chinoy et al. 1997 sodium fluoride
	Developmental						
49	Rat (CD)	Gd 1-20 daily (W)		11.2	11.4 (incr in average number of fetuses per litter with 3+ skeletal variations)		Collins et al. 1995 sodium fluoride
50	Rat (CD)	daily 16-19 weeks (W)		12.2 F			Collins et al. 2001b sodium fluoride
51	Rat (Sprague-Dawley)	28 wk 7d/wk 24hr/d (W)		21			Ream et al. 1983 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
	CHRONIC EXPOSURE						
	Systemic						
52	Human	daily (W)	Musc/skel	0.04			Hillier et al. 2000 sodium fluoride
53	Human	Daily (W)	Musc/skel	0.15[d]	0.25 (Increased prevalence of bone fractures)		Li et al. 2001 sodium fluoride
54	Human	4 yr (C)	Musc/skel		0.56 (increased fracture rate)		Riggs et al. 1990 sodium fluoride
55	Rat (Fischer- 344)	103 wk (W)	Resp	3.9			NTP 1990 sodium fluoride
			Cardio	3.9			
			Gastro	3.9			
			Hemato	3.9			
			Musc/skel	2.5	4.3 (osteoscleosis)		
			Hepatic	3.9			
			Renal	3.9			
			Bd Wt	3.9			

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)		LOAEL Serious (mg/kg/day)		Reference Chemical Form
56	Mouse (B6C3F1)	103 wk (W)	Resp	7.6					NTP 1990 sodium fluoride
			Cardio	7.6					
			Gastro	7.6					
			Hemato	7.6					
			Musc/skel	4.3 M	7.6 M	(dentine dysplasia)			
			Hepatic	7.6					
			Renal	7.6					
			Bd Wt	7.6					
57	Rabbit	24 mo 1x/d (GW)	Gastro		5	(roughened duodena mucosa)			Susheela and Das 1988 sodium fluoride
58	Rabbit	7-12 mo 1x/d (G)	Hemato		4.52	(decr leukocyte and hemoglobin levels)			Susheela and Jain 1983 sodium fluoride
59	Mink	382 d 24hr/d (F)	Musc/skel		5	(mottled and brittle kit teeth)	9.1	(sagittal crests deformed, 3/6 adults)	Aulerich et al. 1987 sodium fluoride
	Immuno/ Lymphoret								
60	Rabbit (albino)	18 mo 1x/d (G)			4.5	(decr primary and secondary antibody titers)			Jain and Susheela 1987 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference Chemical Form
	Reproductive						
61	Mouse	3 gen (F)		13			Tao and Suttie 1976 sodium fluoride
62	Rabbit (NS)	daily 18 mo (GW)				4.5 M (structural damage of the spermatid and epididymal spermatozoa)	Kumar and Susheela 1994 sodium fluoride
63	Rabbit (NS)	daily 20 or 23 mo (GW)				4.5 M (structural damage of the spermatid and epididymal spermatozoa)	Kumar and Susheela 1995 sodium fluoride
64	Rabbit (NS)	daily 18 or 29 mo (GW)				4.5 (complete cessation of spermatogenesis)	Susheela and Kumar 1991 sodium fluoride
65	Rabbit (New Zealand)	daily 18 or 23 mo (GW)			4.5 (Leydig cell damage)		Susheela and Kumar 1997 sodium fluoride
66	Mink	382 d daily (F)		9.1			Aulerich et al. 1987 sodium fluoride

Table 3-4 Levels of Significant Exposure to Fluoride - Oral (continued)

Key to figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL		Reference Chemical Form
					Less Serious (mg/kg/day)	Serious (mg/kg/day)	
	Cancer						
67	Rat (Fischer- 344)	103 wk (W)				2.4 M (osteosarcoma of bone)	NTP 1990 sodium fluoride

a The number corresponds to entries in Figure 3-4.

b Only this dose level, for the most sensitive group, is plotted in Figure 3-4.

c Differences in levels of health effects and cancer effects between males and females are not indicated in Figure 3-4. Where such differences exist, only the levels of effect for the most sensitive gender are presented.

d Used to derive a chronic-duration oral minimal risk level (MRL) of 0.05 mg fluoride/kg/day; the dose was divided by an uncertainty factor of 3 to account for human variability.

ad lib = ad libitum; Bd Wt = body weight; (C) = capsule); d = day(s); decr = decrease; Endocr = endocrine; (F) = feed; F = female(s); (G) = gavage; Gastro = gastrointestinal; Gd = gestational day; gen = generation(s); (GW) = gavage in water; Hemato = hematological; hr = hour(s); incr = increase; LD50 = lethal dose, 50% kill; LOAEL = lowest-observed-adverse-effect level; M = males; mg/kg/day = milligram per kilogram per day; mo = month(s); Musc/skel = muscular/skeletal; NOAEL = no-observed-adverse-effect level; RBC = red blood cell(s); Resp = respiratory; T3 = triiodothyronine; (W) = water; WBC = white blood cell(s); wk = week(s); x = time

Appendix B: Signed GRAS Panel Conclusion Letter

TOXSERVICES
TOXICOLOGY RISK ASSESSMENT CONSULTING

November 18, 2016

GRAS Panel Conclusion

We, the members of the Expert Panel, have individually and collectively, critically evaluated data and information presented in this GRAS report and conclude that based on scientific procedures, *Euphausia superba* (krill) meal (Qrill™ Pet), produced under good manufacturing practices, is generally recognized as safe (GRAS) when used as a source of protein and lipid at up to 3% (by weight) in dry food for adult dogs.

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

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11/18/16
Date

Appendix C: Qualifications of Expert Panel Members

Jennifer G. Fleischer, Ph.D., M.H.S.

Bonnie Ransom Stern, Ph.D., M.P.H.

Raymond G. York, Ph.D., D.A.B.T, F.A.T.S, E.R.T.

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SUMMARY OF QUALIFICATIONS

Dr. Jennifer G. Fleischer is a Senior Toxicologist, Risk Assessor, and Project Manager who earned her Ph.D. in Toxicology and M.H.S. in Environmental Health Sciences from Johns Hopkins University. Dr. Fleischer prepares, reviews, and manages quantitative human health risk assessments, exposure assessments, product and ingredient safety assessments, and regulatory compliance evaluations for a diverse range of substances within a variety of national and international regulatory contexts, including food allergens, food additives, food contact materials, medical devices, consumer/household products, personal care products/cosmetics, pharmaceuticals, and dietary supplements, among others. Recent work has focused on quantitative exposure assessments, particularly for Proposition 65-listed substances, nanomaterials, and impurities, often in response to actual or potential litigation. Dr. Fleischer advises clients on key technical issues, such as clinical and nonclinical testing strategies, study design and monitoring, and data interpretation; the scientific merit of test data provided by claimants or other parties; regulatory compliance requirements for U.S., EU, and other markets; and comprehensive interpretation of test data and literature. She provides critical scientific support to inform her clients' risk management decisions (e.g., product recalls, product liability claims) in a transparent and scientifically-sound manner. Dr. Fleischer additionally prepares Safety Data Sheets (SDS), alternatives assessments, and comparative hazard assessments; compiles and reviews toxicological evaluations of chemicals considered for regulation under EPA's Safe Drinking Water Act; and performs hazard assessments and modeling of chemicals under EPA's ECOSAR, EPI Suite, and Benchmark Dose Software and OECD Toolbox software. Prior to joining ToxServices, Dr. Fleischer served as a consulting toxicologist for a private firm and as a project manager, toxicologist, and study director in *in vivo* or *in vitro* contract toxicology/pharmacology laboratories.

PROFESSIONAL EXPERIENCE

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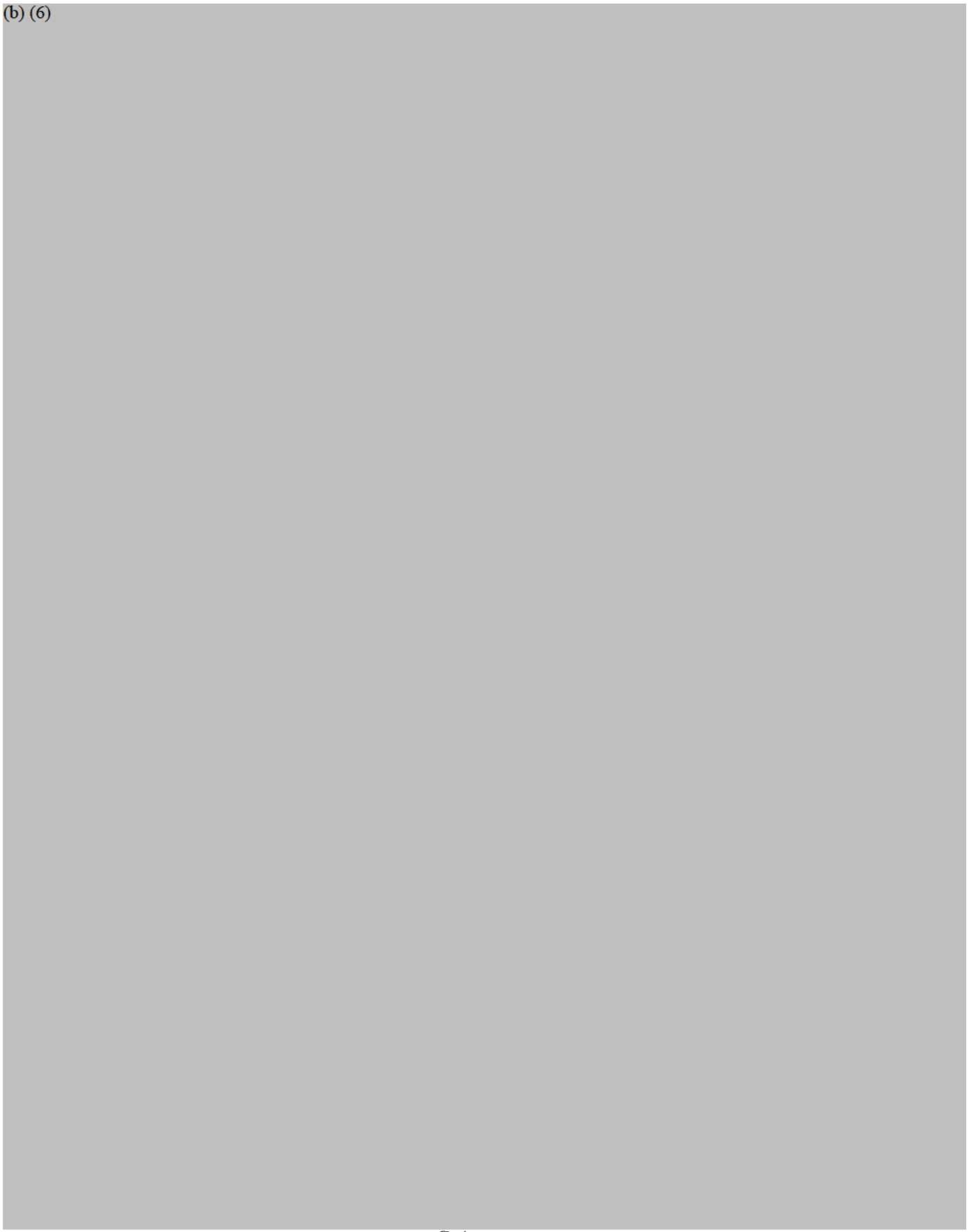

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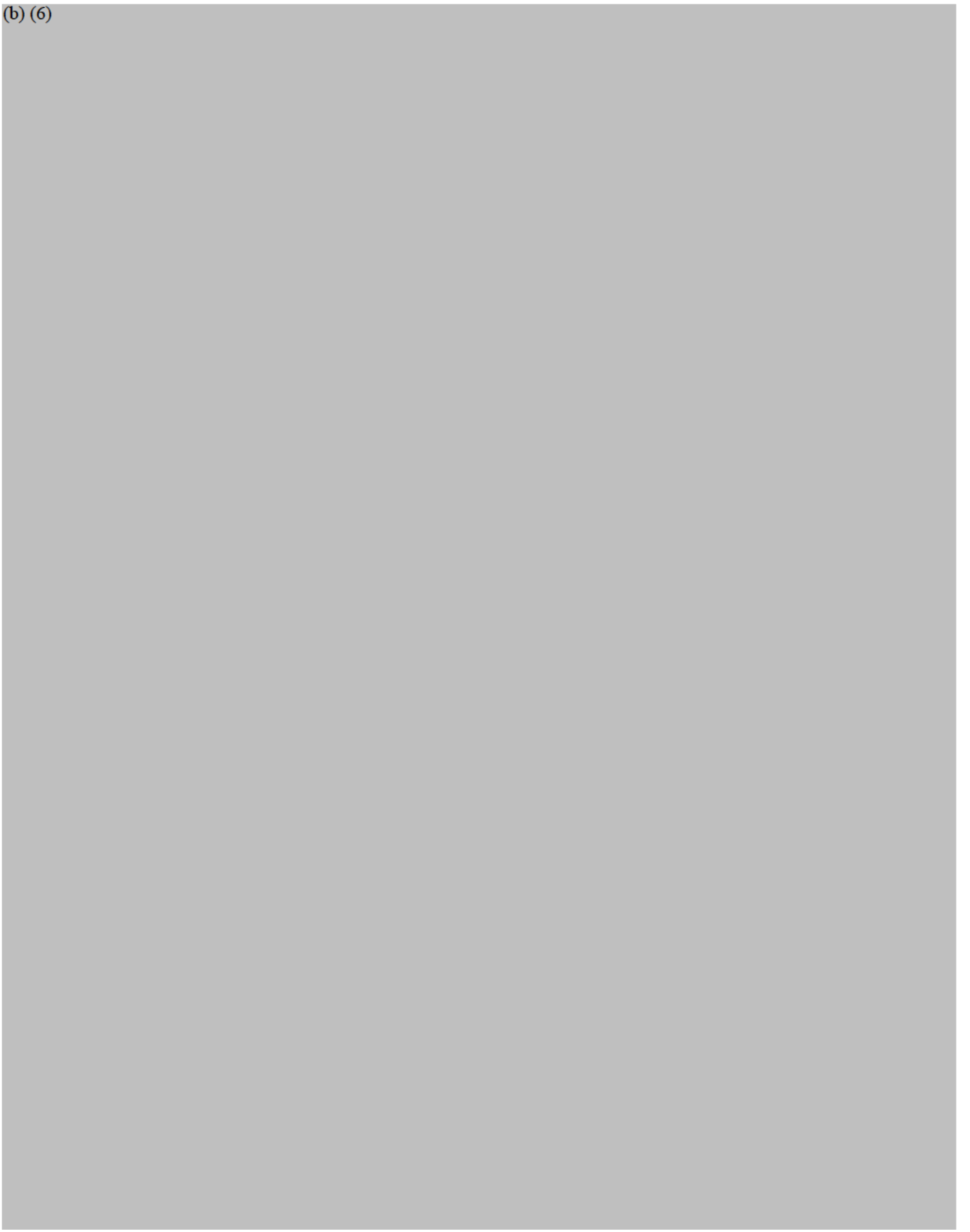

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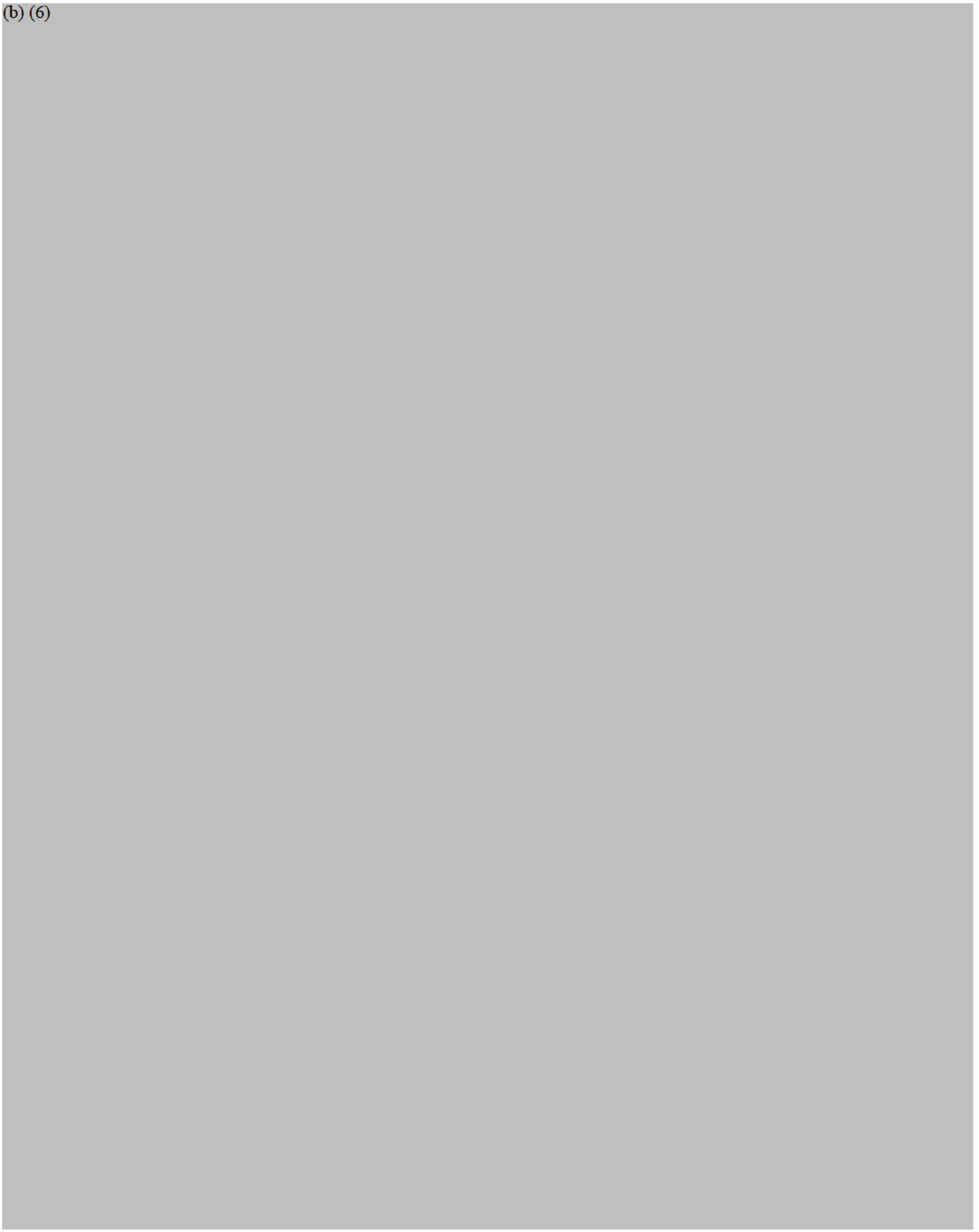

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SUMMARY OF QUALIFICATIONS

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PROFESSIONAL EXPERIENCE

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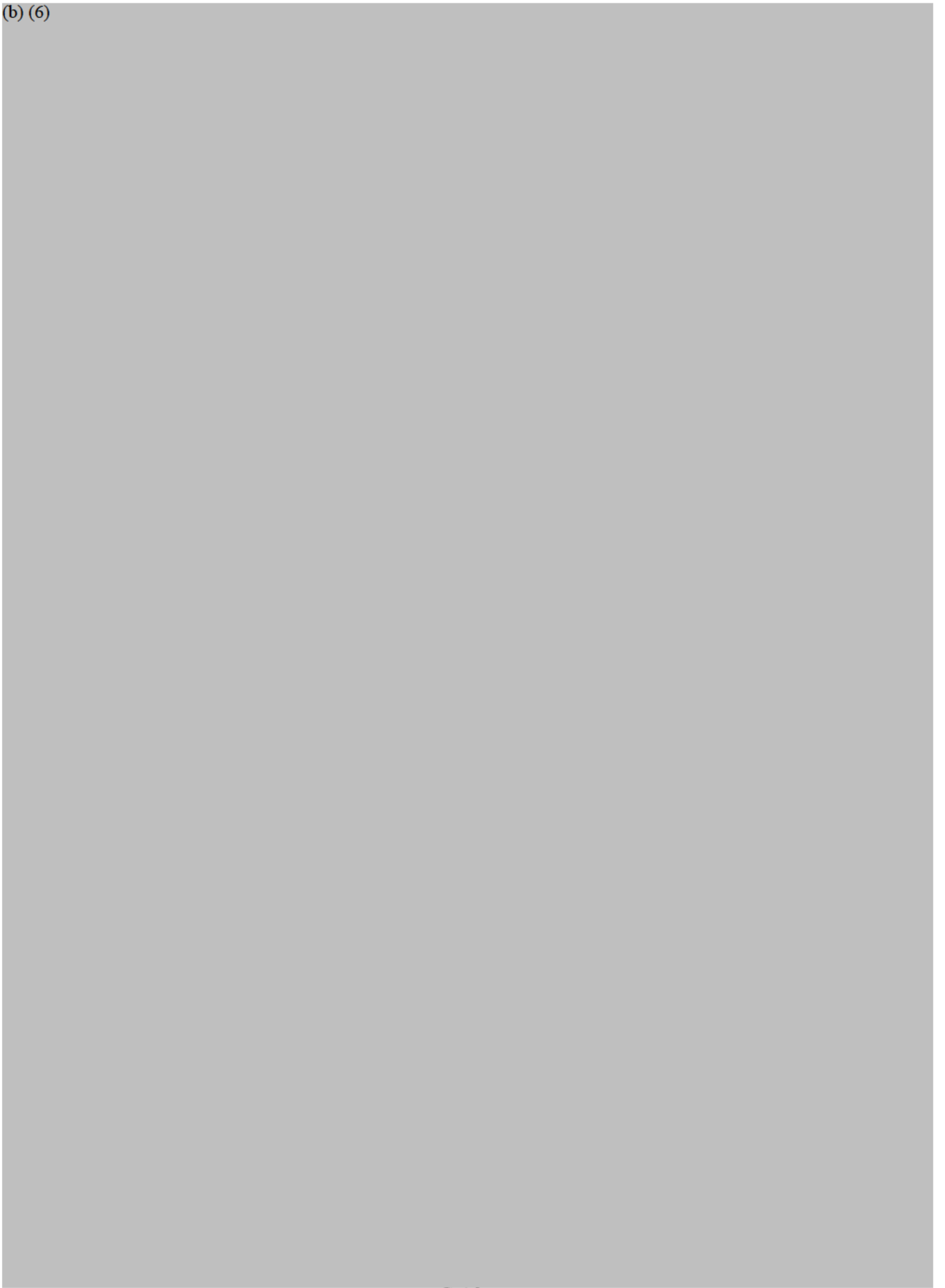

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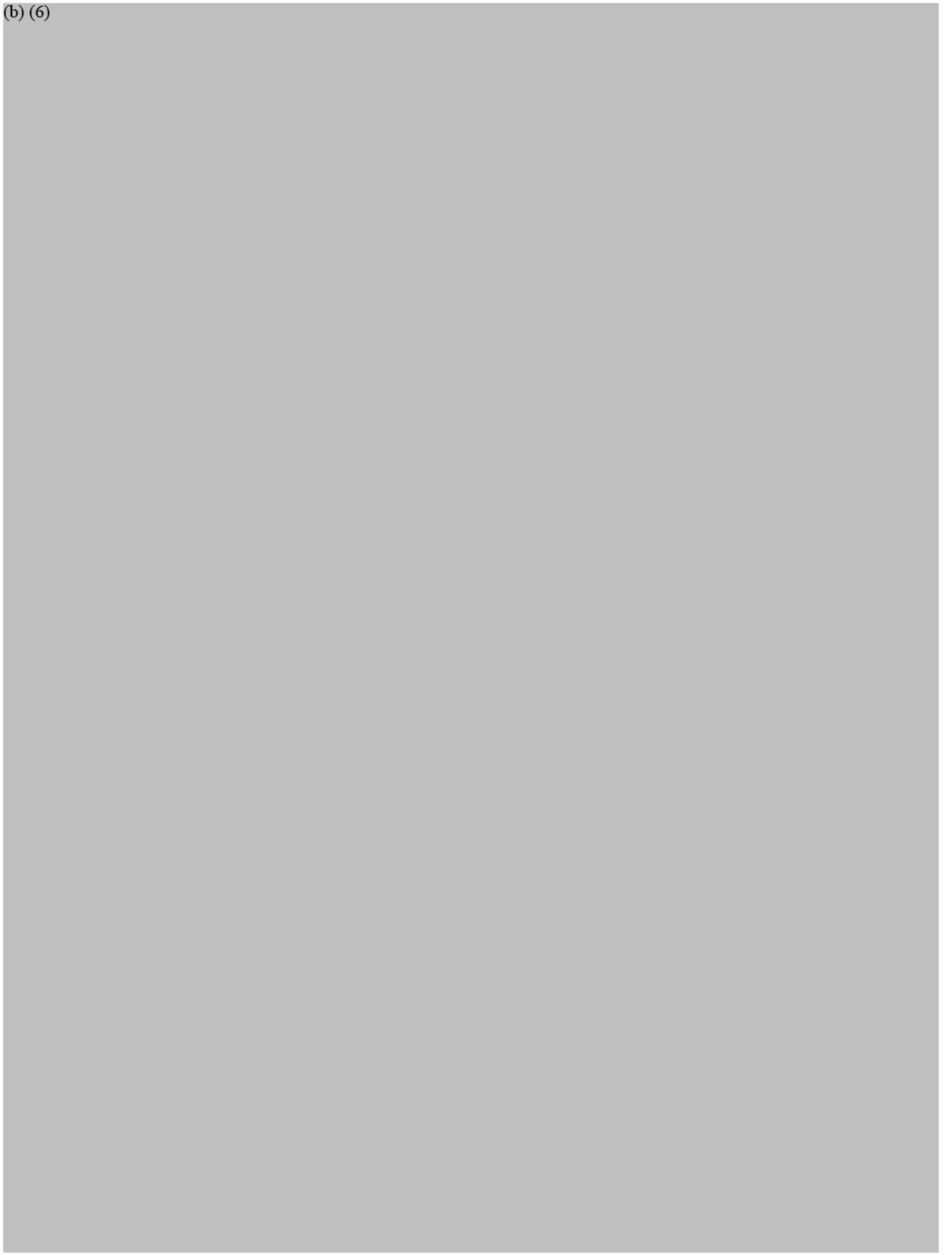

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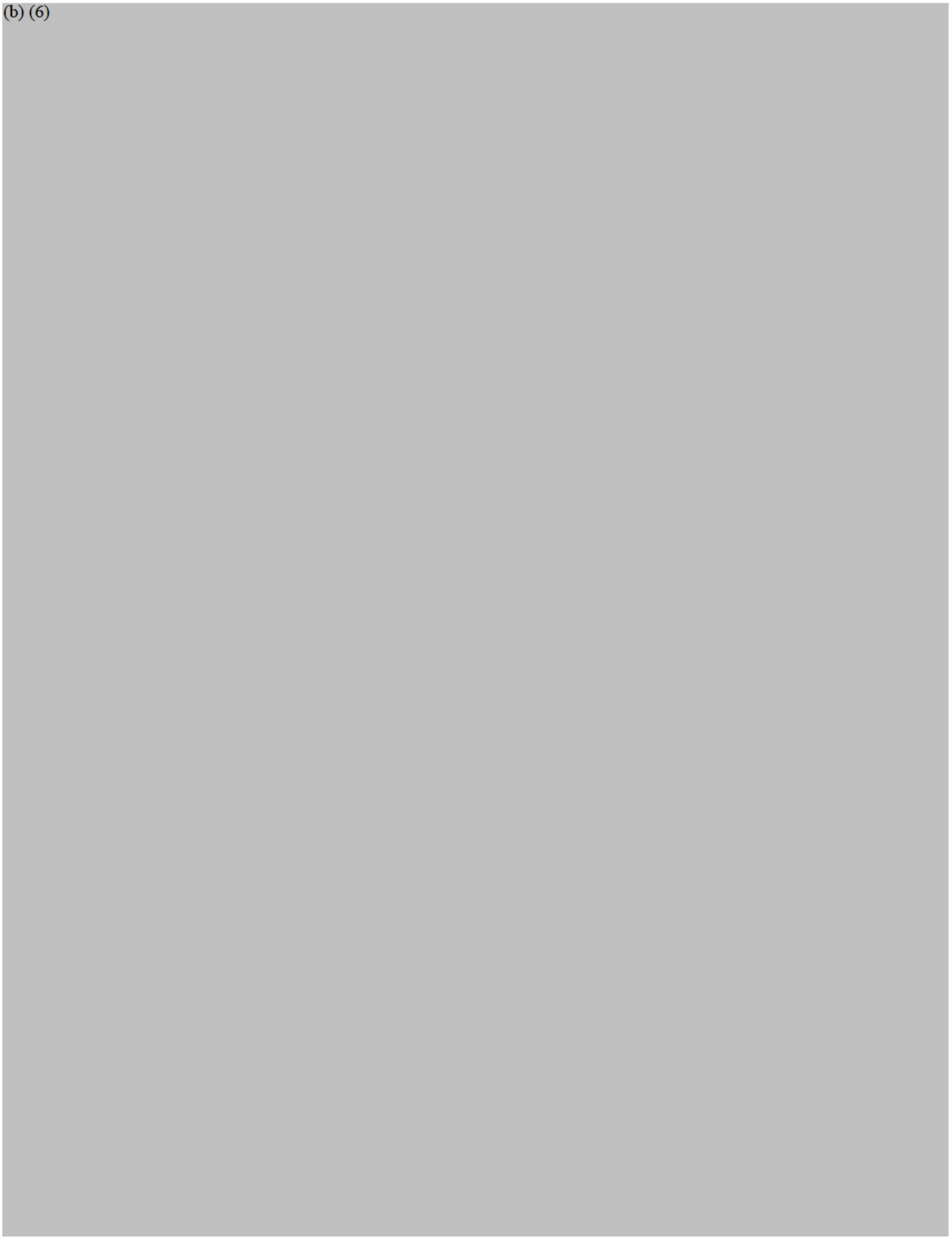

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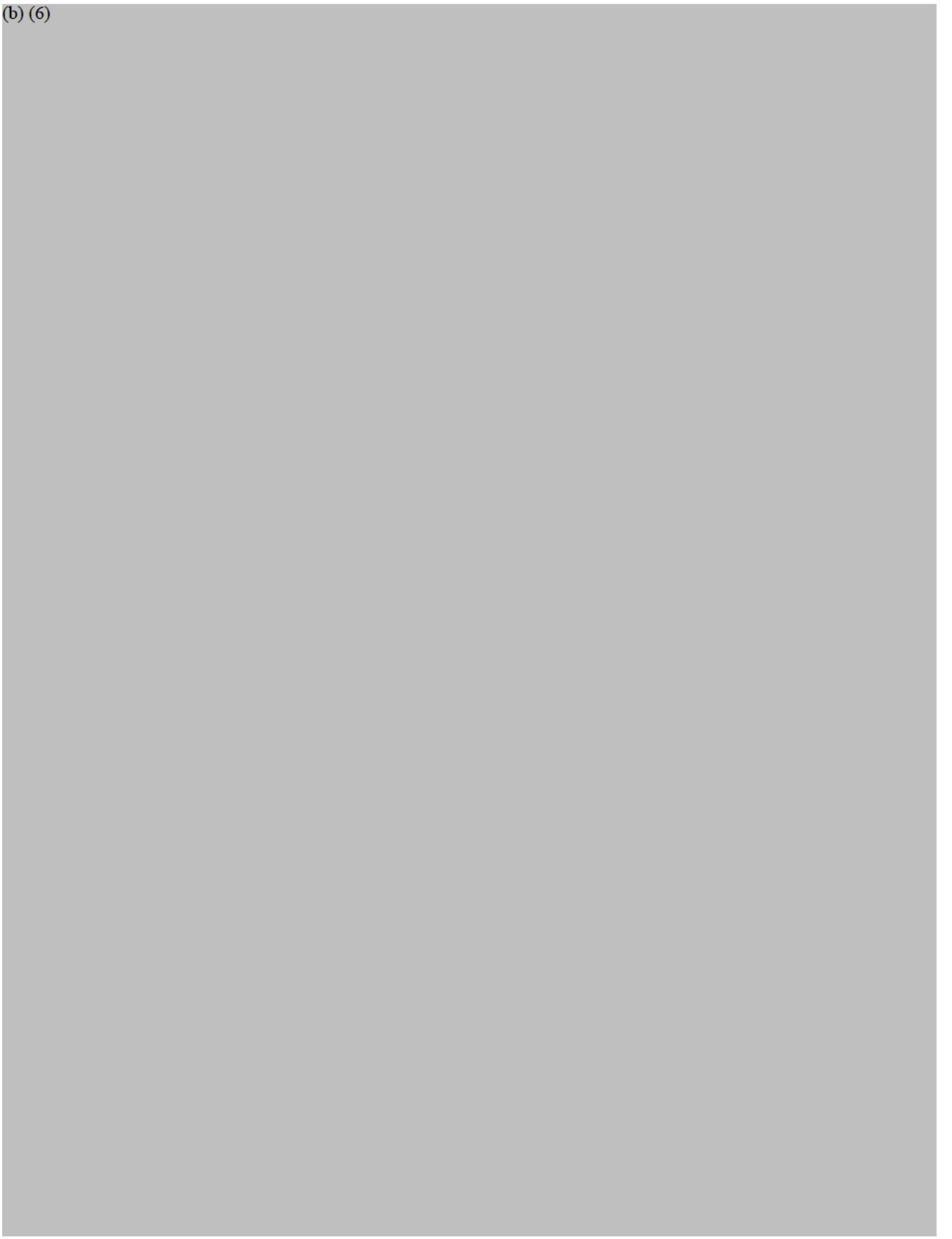

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SUMMARY OF QUALIFICATIONS:

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EDUCATION:

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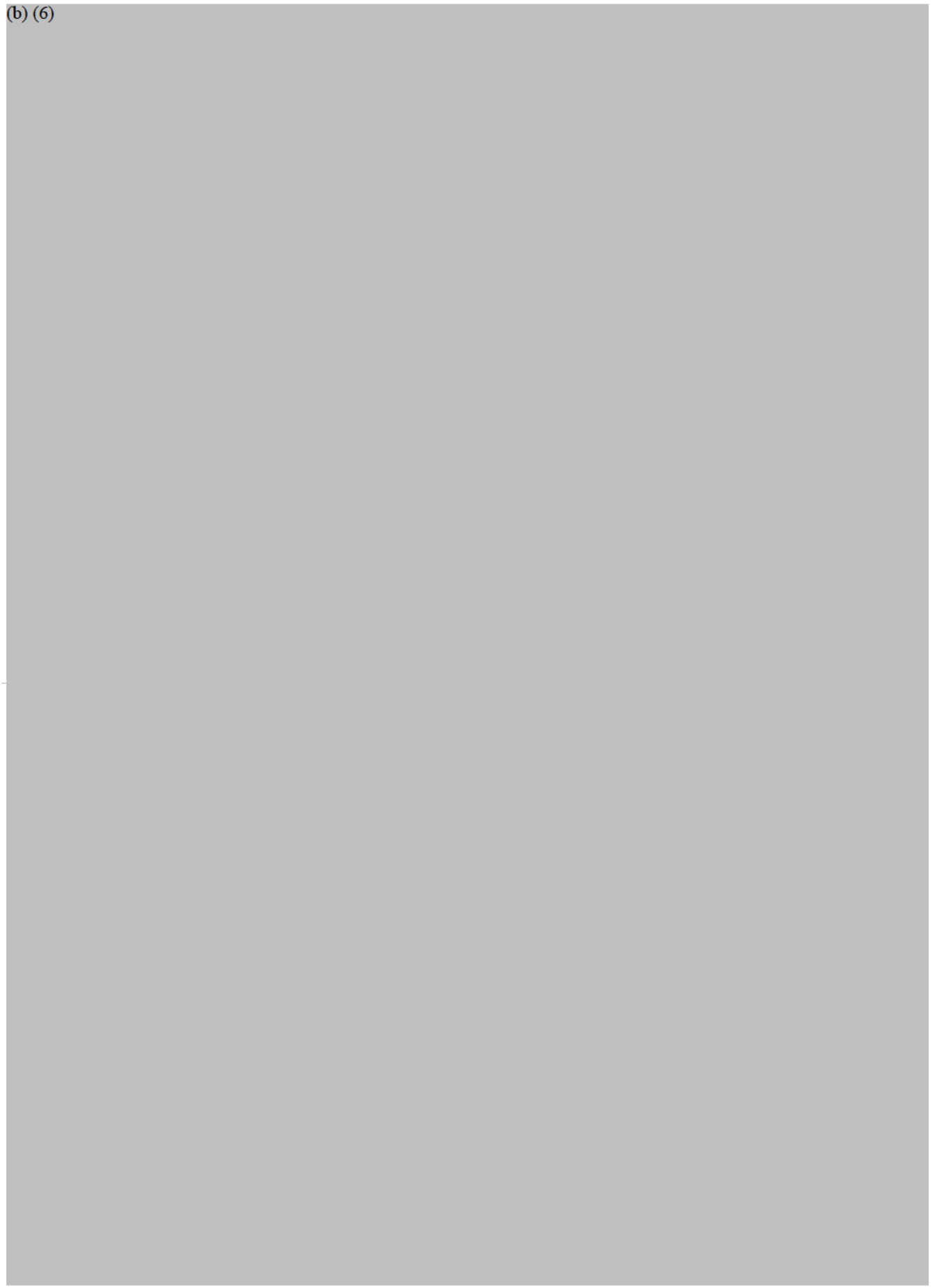

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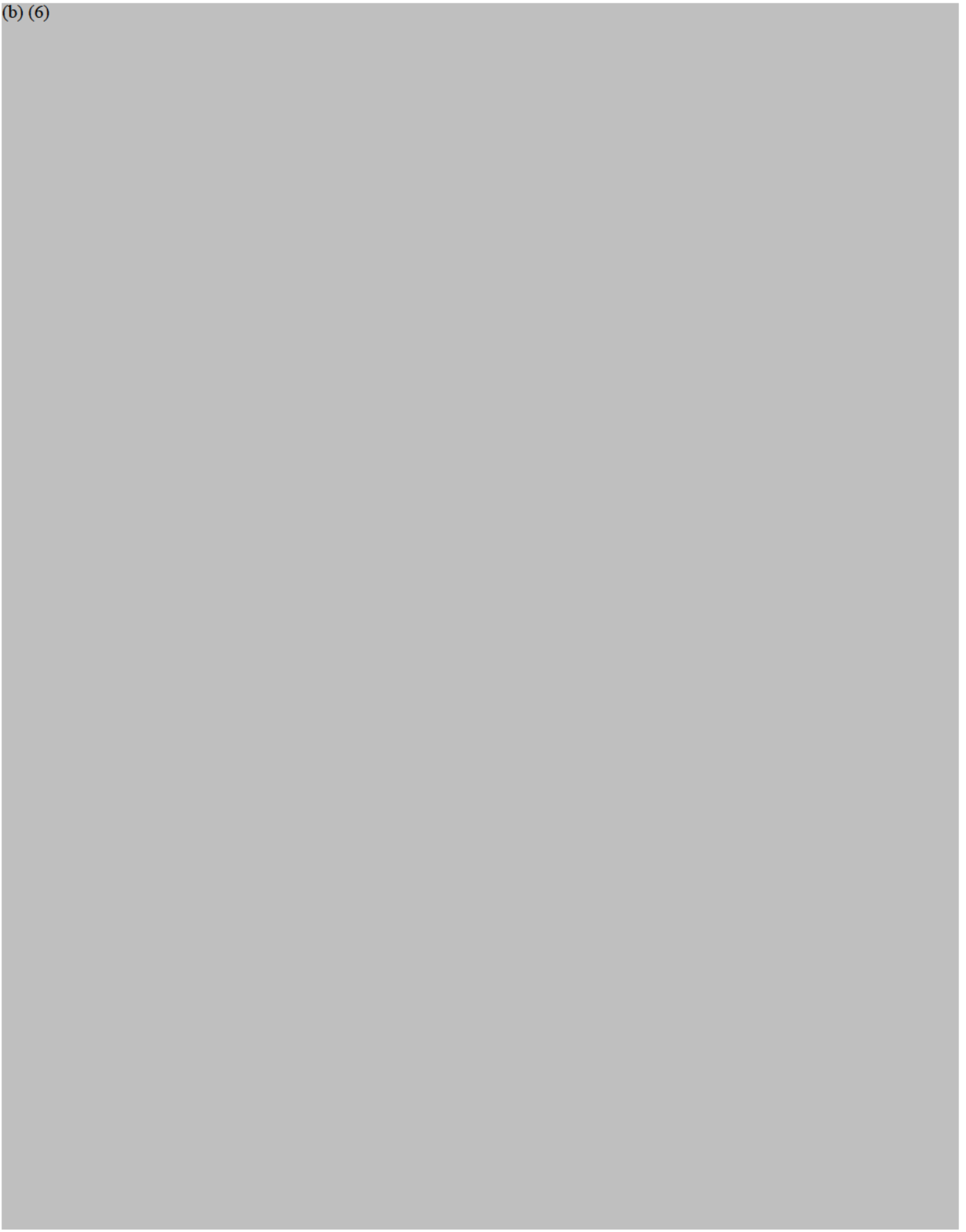

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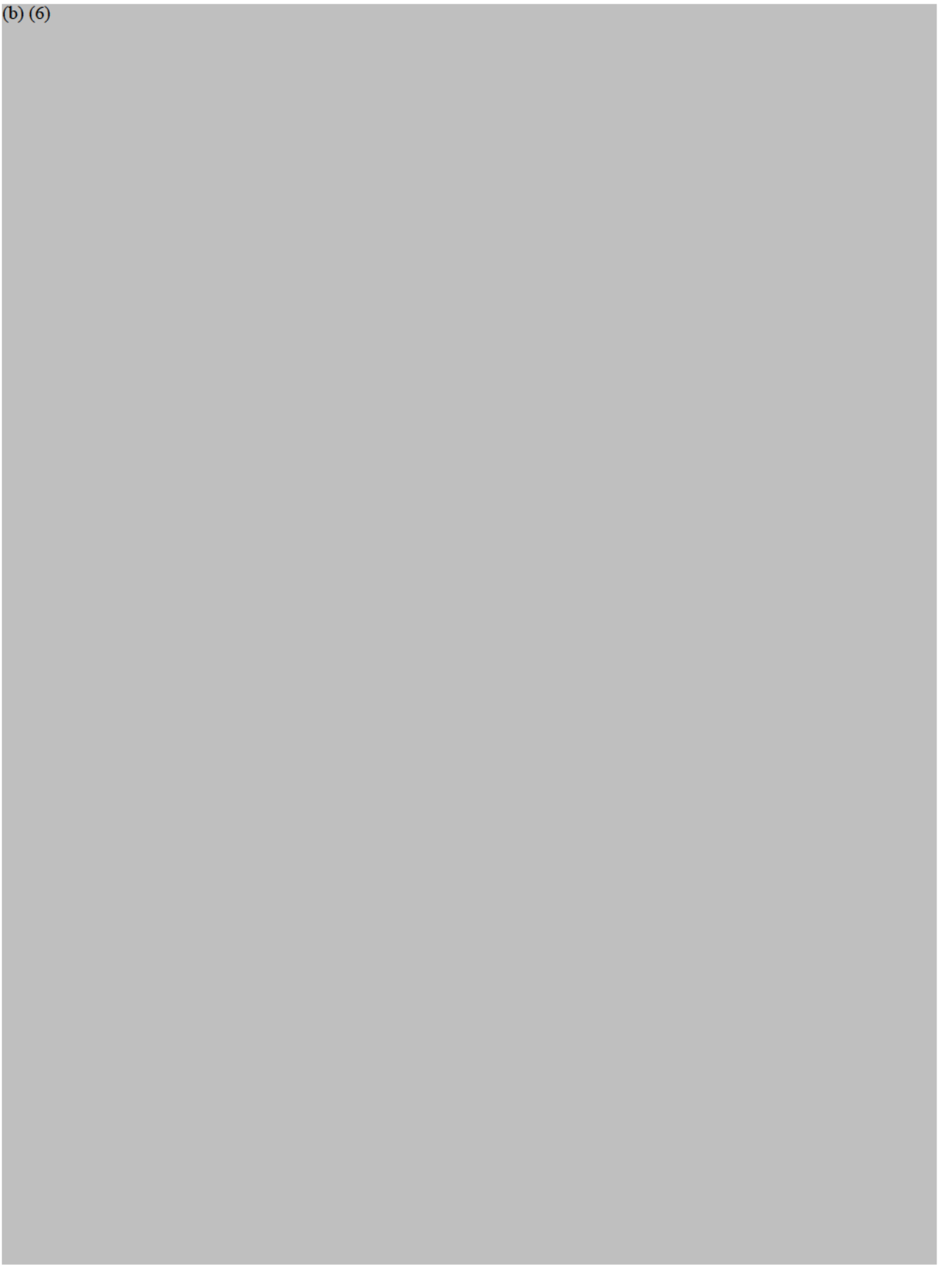

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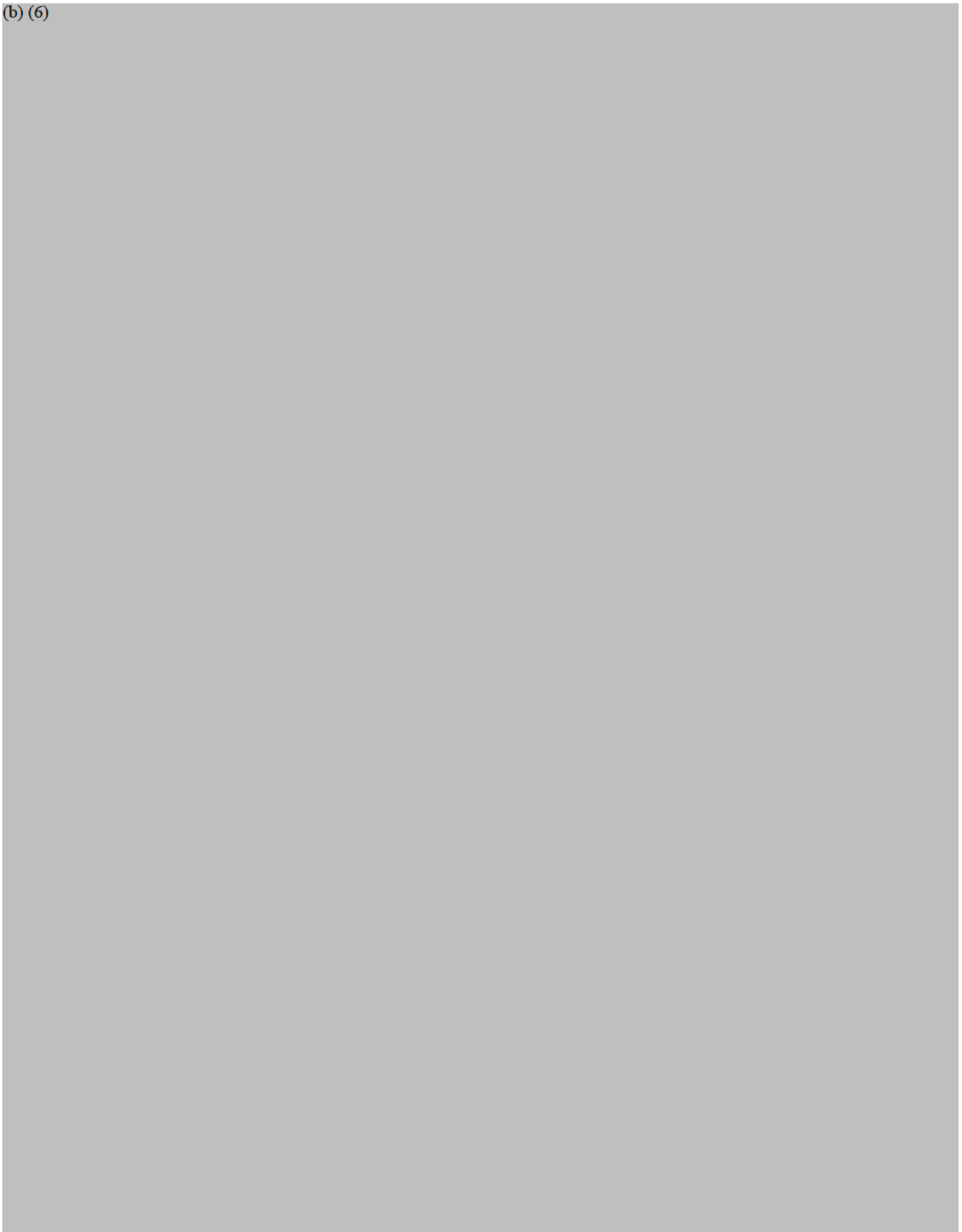

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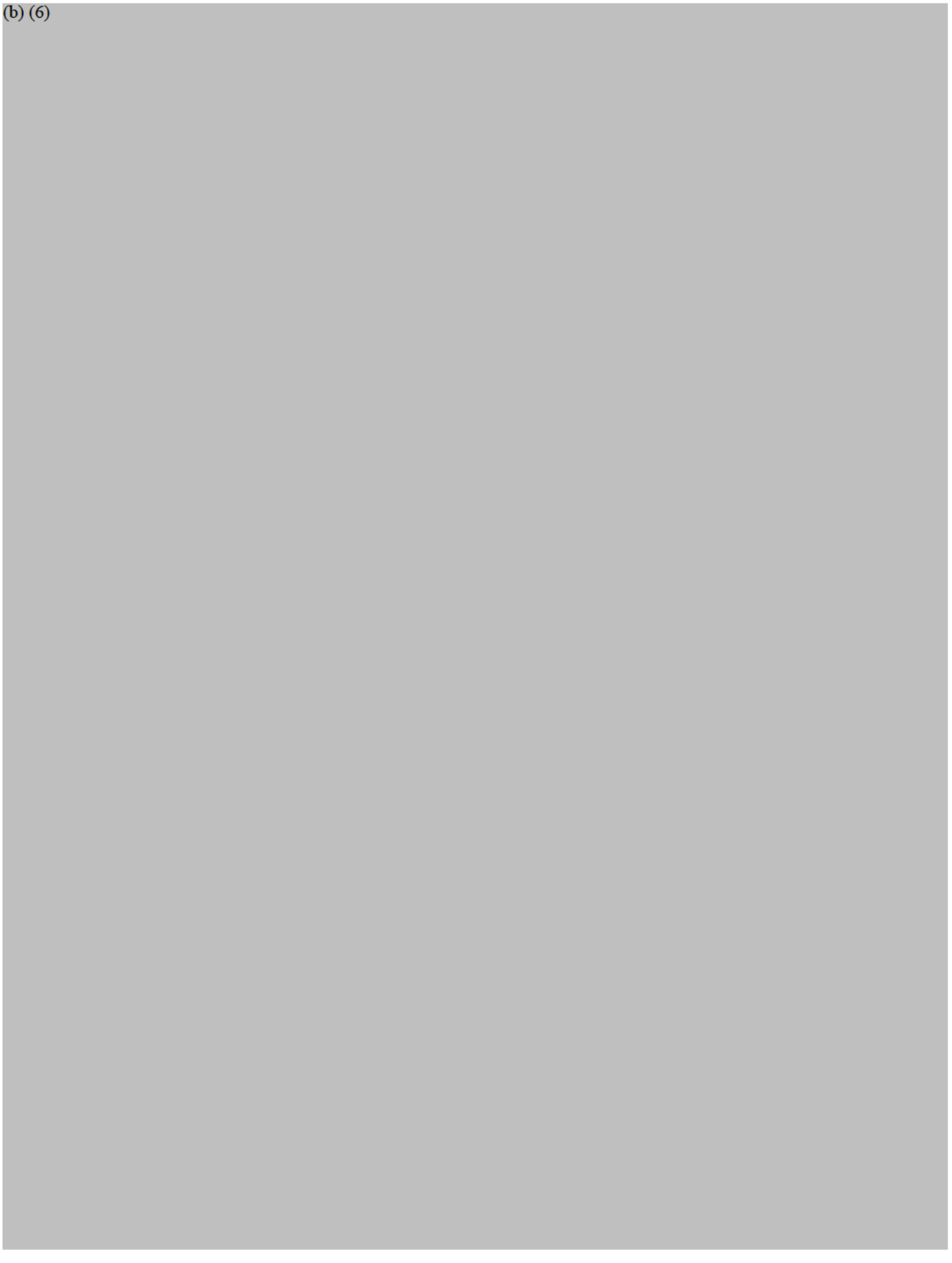

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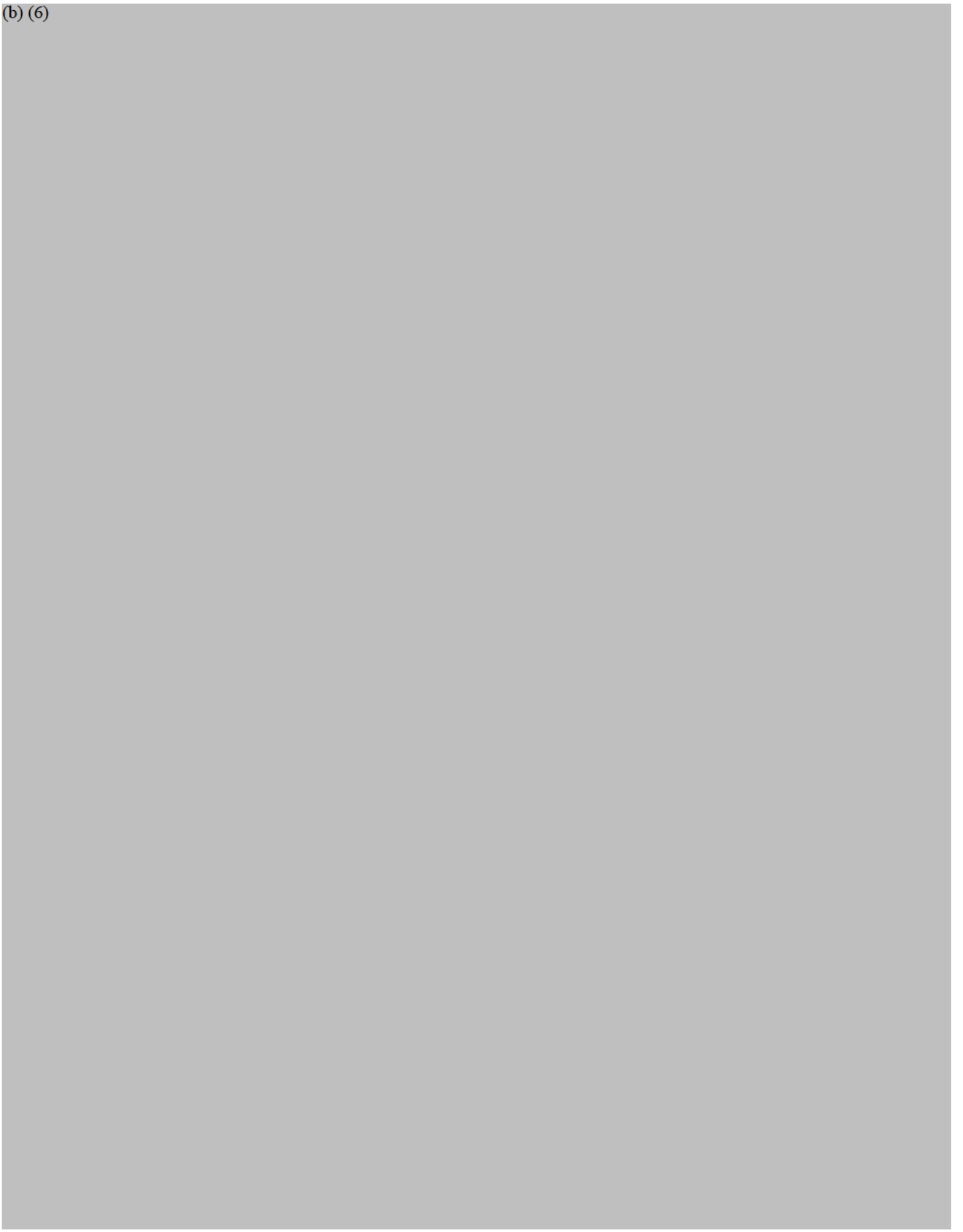

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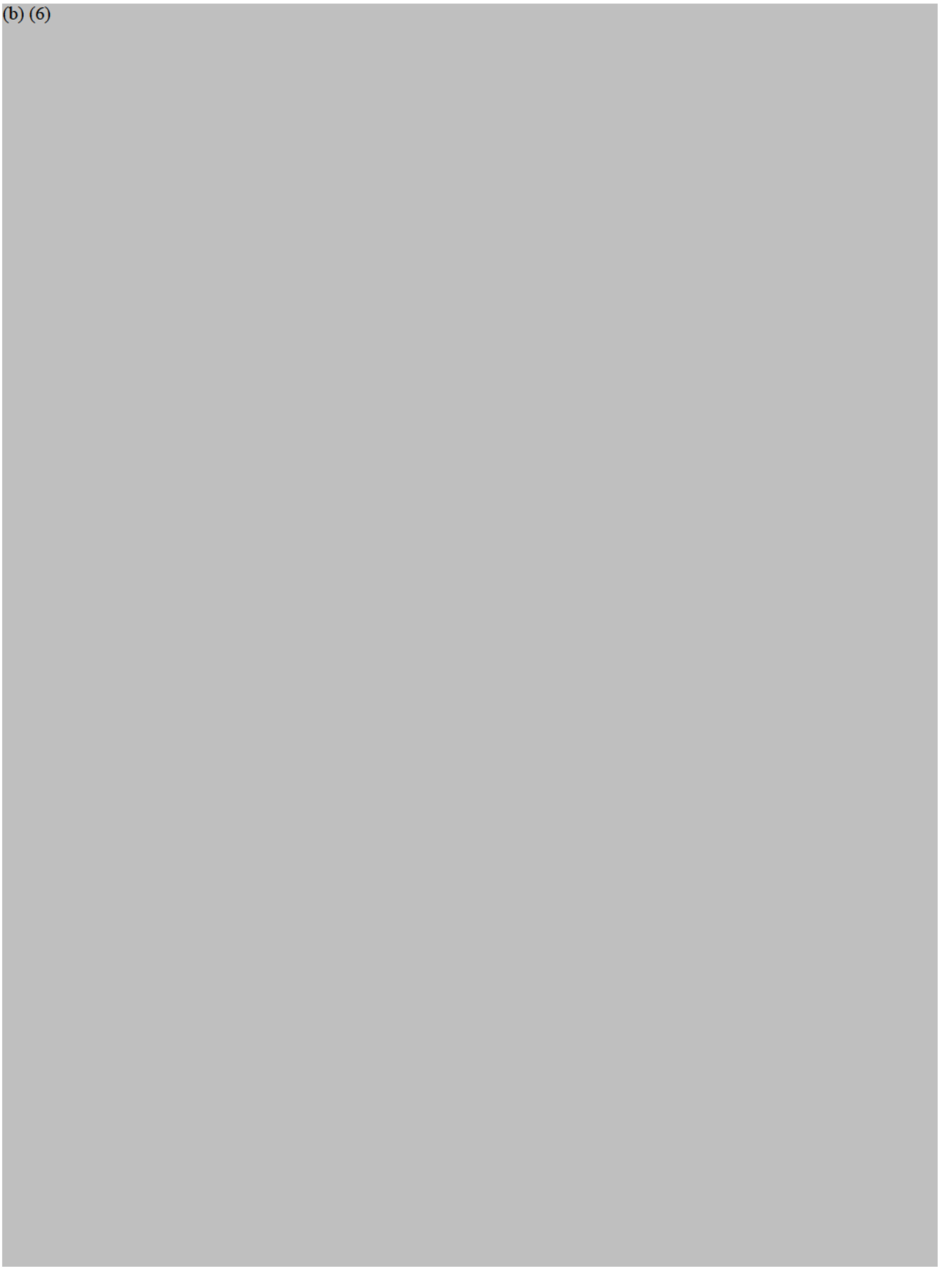

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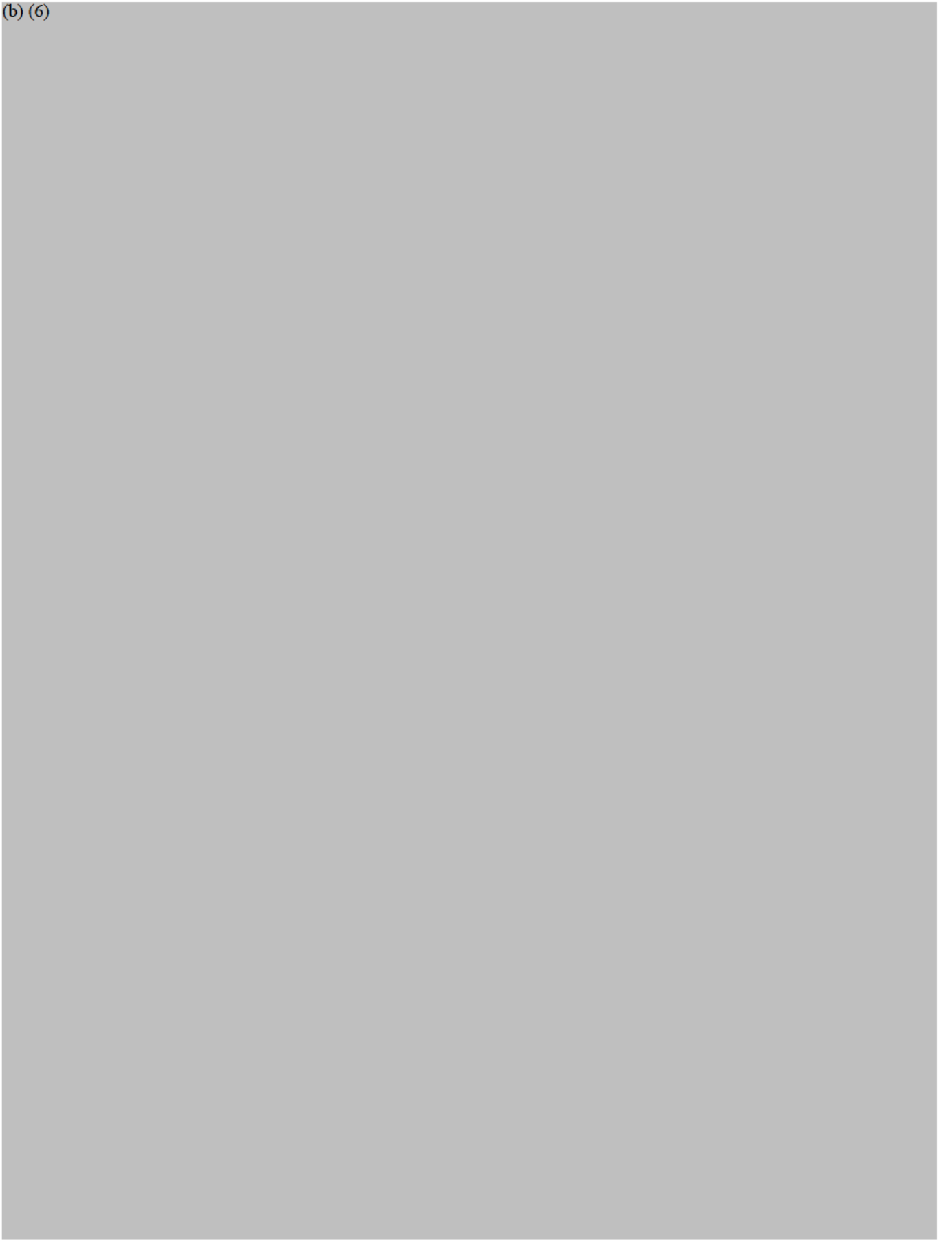

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TAB

PART 7:

LIST OF SUPPORTING

DATA AND INFORMATION

EUPHAUSIA SUPERBA (KRILL) MEAL (QRILL™ PET) AS A SOURCE OF PROTEIN AND LIPID IN FOOD FOR ADULT DOGS: GRAS NOTIFICATION

Part 7: List of Supporting Data and Information

Prepared for:
Aker BioMarine

November 29, 2016

Panel Members:

Jennifer G. Fleischer, Ph.D., M.H.S.
Bonnie Ransom Stern, Ph.D., M.P.H.
Raymond York, Ph.D. D.A.B.T, F.A.T.S, E.R.T.

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Part 7: List of Supporting Data and Information

Per regulation 21 CFR 570.255, a list of all the data and information is included below that are discussed in Part 6 of the dossier as the basis for the GRAS status of Qrill™ Pet at 3% inclusion level in dry food for adult dogs. Generally available data and unpublished data are presented in two sections below.

Please note that ToxServices does not have access to two of the unpublished studies listed under Section II (Stewart et al. 2001 and Yoshihikko et al. 2004), and a number of unpublished studies cited in secondary sources. All these studies are related to astaxanthin toxicity, and the evaluation of these studies was solely based on publically available information provided in these sources. These secondary sources include the peer-reviewed and publically available technical documents authored by the European Food Safety Authority (EFSA 2007, 2014a,c), the color additive petition prepared by Roch (1987), and the GRAS notification prepared by EAS consulting group (EAS 2009). In 2010, the U.S. FDA had no questions for the GRAS notification (GRN 000294) on the use of *Haematococcus pluvialis* (a freshwater species of Chlorophyta) extract containing astaxanthin esters as a food ingredient for humans (in baked goods, beverages, cereals, chewing gum, coffee and tea, daily product analogs, frozen daily desserts and mixes, hard candy, milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and soft candy) at consumption levels of 0.1 mg preserving (U.S. FDA 2010). In response to a subsequent GRAS notification (GRN 000580) in 2015, the U.S. FDA had no questions for the GRAS notification of human consumption of astaxanthin esters in *H. pluvialis* extract in baked goods and baking mixes, beverages and beverage bases, energy, sports and isotonic drinks, non-milk based meal replacements, cereals and cereal products, chewing gums, coffee, tea, dairy product analogs, frozen dairy desserts and mixes, hard and soft candy, milk products, processed fruits and fruit juices, and processed vegetables and vegetable juices at a maximum level of 0.15 mg astaxanthin per serving. Collectively, these secondary sources provide adequate information on the studies performed on astaxanthin, and this information was used as corroborative evidence to support the conclusion of the GRAS status of Qrill™ Pet.

The GRAS expert panel has access to all the published and unpublished data cited in the GRAS notification. For studies cited by seoncary sources that ToxServices has no access to, full secondary sources were provided to the panel for evaluation.

All the unpublished studies (i.e. acute toxicity study, genotoxicity studies, and tolerance studies in dogs) are used as corroborative evidence in this GRAS notification. The key studies on the safety of krill meal (Krogdahl et al. 2015a, b) are published in peer-reviewed journals. As krill meal used in these studies contain considerable amount of fluorine and astaxanthin, the safety of these two ingredients of concern is addressed in the evaluation of the key studies. Additional evaluation of fluorine and astaxanthin as single chemicals was performed to provide further assurance of the safety of Qrill™ Pet at the recommended use level. Qualified experts without access to the unpublished data would still be able to reach the same conclusion on the GRAS status of Qrill™ Pet based on publicaly available data on krill meal, fluorine and astaxanthin cited in this GRAS notification.

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II. UNPUBLISHED DATA

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Parameter	Method	File
Moisture	ISO 6496	01 - ISO 6496 Moisture
Crude protein	ISO 16634-1	02 - ISO 16634-1_2008 Crude protein
Fat	Bligh & Dyer (A56)	03 - E. G. Bligh and W. J. Dyer-Can J. of Biochem and Phys-1959-37-8-911-917
Ash	ISO 5984	04 - ISO 5984 Crude ash
Salt	AOAC 937 09	05 - AOAC 937.09
Total Omega 3	AOCS Ce 1b-89	06 - AOCS Ce 1b-89 Fatty Acids in Marine Oils
Astaxanthin esters	(b) (4)	07 - (b) (4)
		07b - (b) (4)
Total volatile nitrogen	AOAC 920 03	08 - AOAC 920.03
Peroxide value	AOCS Cd 8b-90	09 - AOCS Cd 8b-90
Cadaverine	(b) (4)	10 - (b) (4) Biogenic amines methodology
Histamine		
		10b - (b) (4) Validation report
Meat bone meal	2003/126/EU	11 - 2003_126_EC
	2009/152/EU	12 - 2009_152_EC
Phosphorous	ISO 6491 or ICP-SFMS	13 - ISO 6491
	DIN EN ISO 17294-2 E29	14 - ISO 17294-2
Calcium	ISO / CD 6869 or ICP-SFMS	15- ISO 6869
	DIN EN ISO 11885, mod	16 - EN ISO 11885
Iodine	ICP-MS/EN15111	17- EN 15111
Fluorine	DIN EN 16279	18 - BS EN 16279_2012
	(b) (4)	19 - (b) - Fluoride method
Cadmium	EN ISO 11885, mod	16 - EN ISO 11885
	EN 15763:2009	20 - NS EN 15763 2009
Mercury	§ 64 LFGB L 00.00 -19/4	21 - NS EN 13806 2002 (§ 64 LFGB L 00.00 -19/4 is the german version of EN 13806:2002)
	EN 15763:2009	19 - NS EN 15763 2009
Lead	§ 64 LFGB L 00.00 -19/3	22 - NS EN 14083 2003 (§ 64 LFGB L 00.00 -19/3 is the german version of EN 14083:2003)
	EN 15763:2009	19 - NS EN 15763 2009
Total arsenic	EN ISO 11885, mod	16 - EN ISO 11885
Inorganic arsenic	EN ISO 11885, mod	16 - EN ISO 11885
	64 LFGB 25.06	23 - NS EN 15517 2008 (§ 64 LFGB 25.06 is the german version of EN 15517:2008)
Total plate count	AFNOR 3M 1/1 -9/89	24 - AFNOR 3M-01-01-09-89_en
Enterobacteriaceae	ISO 21528-2	25 - ISO-21528-2-Enterobacteriacea count
Salmonella	NordVal 023	26 - NordVal 023 Salmonella_2015
Yeast	NordVal 016	27 - NordVal0162015
Mold		
Magnesium	ICP-SFMS/NMKL No. 139 1991 mod. (for magnesium)	28 - NMKL 139 1991
Mercury	ICP-SFMS/ § 64 LFGB L00.00-19/4 (for mercury)	29 - NS EN 13806 2002
Total phospholipids and triglycerides	(b) (4) methods for total phospholipids and triglycerides	30a - (b) (4) Lipid classes, polar and neutral
		30b - Working_paper_Lipid_class_analyses_(b) (4) _validation report
		30c - AOCS Ce 1b-89 Fatty Acids in Marine Oils
Dioxins	US EPA 1613	31- epa_method_1613b_1994
	EC Reg 589/2014 (food)	32 - EC Reg 589_2014 dioxines and PCB
	EC Reg 7091 2014 (food) (for the dioxins)	33 - EC Reg 709 2014
Melamine	(b) (4) for melamin	34 - Method Description SFIDZ Melamine
Amino Acids	(b) (4) method for amino acids	35a - (b) (4) Total amino acids
		35b - Stanford Moore, J. Biol. Chem. 1963, 238235-237
		35c - E.L. Miller, J. Sci. Fd Agric., 1967, Vol. 18, September
		35d - Validation report amino acids
Vitamin A	Vitamin A - EN 12823-1 2014	36 - NS EN 12823-1 2014
Vitamin D3	Vitamin D3 - EN 12821 2009	37 - NS EN 12821 2009
Vitamin E	Vitamin E - EN 12822:2014	38 - NS EN 12822 2014
Vitamin K1	Vitamin K1 - EN 14148:2003	39 - NS EN 14148 2003
Vitamin C	Vitamin C - Food Chemistry, 94 626-631	40 - Food Chemistry 94 626-631
Vitamin B1	Vitamin B1 - EN 14122:2006, mod.	41 - NS EN 14122 2003
Bitamin B2	Vitamin B2 - EN 14152:2006, mod.	42 - NS EN 14152 2003
Vitamin B3	Vitamin B3 - EN 15652:2009	43 - NS EN 15652 2009
Bitamin B5	Vitamin B5 - AOAC 2012.16	44 - AOAC 2012.16
Vitamin B6	Vitamin B6 - EN 14164:2008	45 - NS EN 14164 2008
Bitamin B8	Vitamin B8 - Biacore	46 - Biacore - Eurofins
Vitamin B9	Vitamin B9 - AOAC 2004.05	47 - NMKL 111-1985
Vitamin B12	Vitamin B12 - J AOAC 2008 vol 91 no 4	48 - J AOAC 2008 vol 91 no 4

T-1

Das, Manisha

From: Bingxuan Wang <bwang@toxservices.com>
Sent: Tuesday, December 27, 2016 10:05 AM
To: Das, Manisha
Cc: Margaret Whittaker; Line Johnsen (line.johnsen@akerbiomarine.com)
Subject: Name and address for the GRAS notification on Krill Meal

Dear Manisha,

Nice talking to you just now. As requested, here is the additional information required for the submitted GRAS notification on krill meal:

Company Name: Aker BioMarine Antarctic
Address: Oksenøyveien 10, NO-1366, Lysaker, Norway
Person of contact at Aker BioMarine: Dr. Line Johnson

Please feel free to let me know if there is anything else I can do to assist the process.

Sincerely,
Bingxuan

Bingxuan Wang, Ph.D., D.A.B.T.,
Toxicologist,
ToxServices LLC
1367 Connecticut Ave NW,
Washington, D.C. 20036

T-4

TOXSERVICES
TOXICOLOGY RISK ASSESSMENT CONSULTING

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June 28, 2017

Dr. David Edwards
Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance,
Center for Veterinary Medicine, Food and Drug Administration,
7519 Standish Place
Rockville, Maryland 20855

Re: Supporting Documentation for CVM GRAS Notification for *Euphausia superba* (krill) meal (Qrill™ Pet)

Dear Dr. Edwards,

On November 29, 2016, ToxServices LLC had submitted a Generally Recognized as Safe (GRAS) notice to the Center for Veterinary Medicine (CVM), Food and Drug Administration (FDA), on behalf of Aker BioMarine Antarctic for Qrill™ Pet to be added to dry food for adult dogs as a source of protein and lipid at the maximum inclusion level of 3% by weight. On June 13, 2017, ToxServices LLC was contacted by Manisha Das, Ph.D., from the Division of Animal Feeds via e-mail to discuss the GRAS notification. On the same date, ToxServices held a teleconference with Dr. Das and Mr. Geoffrey Wong, M.S. of the Division of Animal Feeds. Dr. Das and Mr. Wong requested additional information about the GRAS notification, specifically the items listed as follows to be provided on a CD and mailed to CVM:

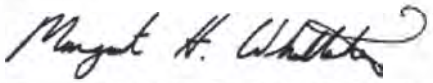
1) All references in full text in Part 7: "List of Supporting Data and Information" of the GRAS notification;
2) All analytical methods mentioned in the product specification sheet including those methods listed as "internal" and "modified";
3) Individual product specification data from 3 – 5 batches of krill samples to be provided in tabulated form.
4) Individual stability data from 3 – 5 batches of krill samples described in the second stability study in the GRAS notification, which was tested at 25 °C, to be provided in tabulated in form.

All requested documents are provided in the enclosed CD. ToxServices has ensured that the documents on the CD met the recommendations of CVM Document Control Unit as follows: 1)

Individual files are < 100 MB; 2) Any directory structure has no more than 2 levels; and 3) Files are submitted in only .pdf, .xml, or .xpt formats.

Any further data and information cited in the submission that served as the basis of this GRAS notification are available to the Food and Drug Administration upon request. Should you have any questions, please feel free to contact us.

Sincerely,



Margaret H. Whittaker, Ph.D., M.P.H., CBiol., F.R.S.B., E.R.T., D.A.B.T.
Managing Director and Chief Toxicologist
ToxServices LLC

Data from Stability Test (Aker BioMarine 2016d) - Samples kept at 25 deg C

Total Fat (g/100 g) at storage condition 25 °C, 60 % Relative humidity (RH)

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Total omega-3 (g/100 g extracted fat) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

EPA (g/100 g extracted fat) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

DHA (g/100 g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

Phospholipids (g/100 g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

Acid Value (%) at storage condition 25 °C, 60 % Relative humidity (RH)

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		

9 month stability pull	(b) (4)
12 month stability pull	

Iodine Value at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

Total Volatile Nitrogen (g/100 g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

Astaxanthin Content (mg/kg) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Peroxide Value meq peroxide/kg oil) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Total Protein (g/100 g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
12 month stability pull			
18 month stability pull			

Total Plate Count (CFU/g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
12 month stability pull			

Mold (CFU/g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
12 month stability pull			

Yeast (CFU/g) at storage condition 25 °C, 60 % RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
12 month stability pull			

As mentioned in the report, analytical tests are performed by different labs and the results are provided in different reports. Therefore, data presented below came from different reports and different batches based on reports' availability at the time of the GRAS report completion

General Parameters	Unit	Batch Date					Product Specification
		2/9/2012	5/27/2013	5/29/2013	3/19/2014	5/31/2103	
Moisture	%	(b) (4)					6 ± 2
Crude protein	%						≥ 55
Fat (B&D)	%						≥ 20
Ash	%						≤ 13
Salt	%						≤ 4
Astaxanthin esters	mg/kg						80 - 160
Total volatile nitrogen	%						≤ 0.3
Peroxide value							< 10
Cadaverine	g/kg						< 10
Histamine	g/kg						< 10
Meat bone meal							Not present

Microbiology Parameters	Unit	Batch Date				Product Specification
		2/9/2012	5/27/2013	5/29/2013	3/19/2014	
Total plate count	CFU/g	(b) (4)				< 20,000
Enterobacteriaceae	CFU/g					< 300
Salmonella						Negative

Microbiology Parameters	Unit	Batch Date				Product Specification
		3/8/2013	3/9/2013	3/12/2013	3/19/2014	
Yeast	CFU/g	(b) (4)				< 100
Mold	CFU/g					< 100

Omega-3	Unit	Batch Date					Product Specification
		2/9/2012	5/29/2013	3/19/2014	3/22/2014	5/31/2013	
Total Omega-3 fatty acids	g/100g fat	(b) (4)					≥ 17

Iodine	Unit	Batch Date			Typical Content
		3/8/2013	3/9/2013	3/12/2013	
Iodine	ppm	(b) (4)			< 50

Minerals	Unit	Batch Date					Typical Content
		5/29/2013	3/19/2014	3/20/2014	3/22/2014	5/31/2013	
Phosphorous	%	(b) (4)					< 2
Calcium	%						< 3
Copper	mg/kg						< 80

Selenium	Unit	Batch Date			Not part of COA
		5/11/2015	3/18/2016	3/22/2016	

Selenium	mg/kg	(b) (4)	No limits speicifed

Zinc	Unit	Batch Date					Not part of COA
		Composite 1/12/2013 - 2/6/2013	Composite 4/20/2013 - 5/6/2013	2/6/2014 + 5/6/2014 + 6/6/2014	5/11/2015	3/18/2016	
Zinc	mg/kg	(b) (4)					No limits speicifed

Heavy Metals	Unit	Batch Date			Limit
		3/19/2014	3/20/2014	3/22/2014	
Cadmium	ppm	(b) (4)			< 1
Mercury	ppm				< 0.1
Lead	ppm				< 0.05
Total arsenic	ppm				< 8
Inorganic arsenic	ppm				0.5

Fluorine	Specification : ≤ 1500 mg/kg
Batch date	F (mg/kg)
3/2/2015	(b) (4)
3/3/2015	
3/4/2015	
3/5/2015	
3/6/2015	
3/7/2015	
3/8/2015	
3/9/2015	
3/10/2015	
3/28/2015	
3/29/2015	
3/30/2015	
3/31/2015	
4/1/2015	
4/2/2015	
4/3/2015	
4/4/2015	
4/13/2015	
4/14/2015	
4/15/2015	
4/16/2015	
4/17/2015	
4/18/2015	
5/3/2015	
5/4/2015	
5/5/2015	

5/6/2015	(b) (4)	
5/7/2015		
5/8/2015		
5/9/2015		
5/10/2015		
5/11/2015		
5/12/2015		
5/13/2015		
5/14/2015		
5/15/2015		
5/16/2015		
5/17/2015		
5/18/2015		
5/19/2015		
5/22/2015		
5/23/2015		
5/24/2015		
5/25/2015		
5/26/2015		
6/5/2015		
6/6/2015		
6/8/2015		
6/9/2015		
6/10/2015		
6/11/2015		
6/12/2015		
6/13/2015		
6/14/2015		
6/15/2015		

Accelerated and long-term stability of low-fluoride krill meal (Qrill Pet) in modified atmosphere

Approvals	Printed Name	Signature	Position in Company	Date
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1. Introduction

1.1 Purpose

The purpose of this study is to identify and conclude on the stability (accelerated and long-term) of low-fluoride krill meal (Qrill Pet), which is intended for use as an ingredient in pet food, in modified atmosphere (vacuum).

The study will provide data to establish shelf life for the product, recommended storage and packing material, and transport conditions. The study is based on VICH GL3(R) (VICH, 2007).

1.2 Scope

This report is written based on analytical data compiled from 18 months storage at recommended storage conditions (<25°C/ 60% RH), 12 months at intermediate storage conditions (30 °C/ 75% RH) and 6 months at accelerated storage conditions (40 °C/ 75% RH).

2. Study setup

The study was performed on samples from three non-consecutive production batches manufactured using full-scale equipment on board the vessel Factory Trawler (F/V) Antarctic Sea and are representative of the material intended for commercialization.

2.1 Batches tested

Table 2.1: Batch information

Batch no.	Manufacturing date	Batch size	Repacking date	Start stability studies*
15-010514	01.05.14	20 kg	06.08.14	09.02.15
15-050514	05.05.14	20 kg	06.08.14	09.02.15
15-070514	07.05.14	20 kg	06.08.14	09.02.15

*Storage between production, sub-dispensing, and start of stability studies: Below -18°C at AKBM warehouse in Montevideo and (b) (4)

2.2 Sample techniques and transport of test materials

At F/V Antarctic Sea, the samples were packed in aluminium coated 20kg bags, flushed with nitrogen and heat sealed before sending to storage in warehouse in Montevideo, Uruguay. The bags were repacked into samples of 200 g using material specified in section 2.3, before being shipped for labelling and storage at (b) (4) The bags were subtracted from storage according to the storage plan and shipped to (b) (4) (b) (4) for analytical testing. The Sample Monitoring system at (b) (4) is compliant with GMP/GLP/FDA (21 CFR Part 11), JCAHO, GAMP, ISO 19005 and USP 1079.

All transportation and storage between production and study start was done at temperatures below -18°C. Under these conditions, the product is considered to be stable.

2.3 Packaging material

Samples were packed in aluminium foil coated PE bags with 80-100 µm thickness, and O_2 permeability of <1.5 g/m^2/day. The sample bags were flushed with nitrogen, set under vacuum and heat sealed.

2.4 Study timelines and pull points

The batches were tested at the defined sampling points (Table 2.2).

Table 2.2: Overview of stability study

Batch number	°C (±2)	O_2 permeability cc/m2/day	Atmosphere	Relative Humidity (% ± 5)	Sampling points (months) [1]
15-010514	25	<1.5	Modified	60	0-1-3-6-9-12-18-24
	30	<1.5	Modified	75	0-1-3-6-9-12
	40	<1.5	Modified	75	0-1-3-6
15-050514	25	<1.5	Modified	60	0-1-3-6-9-12-18-24
	30	<1.5	Modified	75	0-1-3-6-9-12
	40	<1.5	Modified	75	0-1-3-6
15-070514	25	<1.5	Modified	60	0-1-3-6-9-12-18-24
	30	<1.5	Modified	75	0-1-3-6-9-12
	40	<1.5	Modified	75	0-1-3-6

[1] Not all parameters are measured at all sampling points. See Protocol; Accelerated and long-term stability of low-fluoride krill meal in modified atmosphere, document no: Q-2015-01 for complete test schedule.

2.5Analytical testing

All analysis were performed according to "Protocol; Accelerated and long-term stability of low-fluoride krill meal in modified atmosphere, document no: Q-2015-01 (Table 2.3). Testing took place at (b) (4) (b) (4) is accredited and complies with the requirements in NS-EN ISO/IEC 17025 (2005). (b) (4) is an independent private Laboratory and is GMP- and GLP-certified and is approved by the US Food and Drug Administration (FDA), FEI-Nr. (b) (4).

Table 2.3: Analytical tests and acceptance criteria

Test	Method reference	Acceptance criteria	Test Site
Fatty Acid profile	AOCS Ce 1b-89	Product specification QRILL™ PET	(b) (4)
Phospholipids*	Spectralservice 31P-NMR	> 7 g/100g[1]	(b) (4)
Acid Value	AOCS CA 5A-40	Monitored for information only	(b) (4)
Iodine Value	AOCS Cd 1d-92	Monitored for information only	
Astaxanthin content	Roche Method A 23	Product specification QRILL™ PET	
Peroxide Value	AOCS Cd 8b-90	Product specification QRILL™ PET	
Total Plate Count	AFNOR 3M 1/1-9/89	Product specification QRILL™ PET	
Yeast & Mould	NordVal 016	Product specification QRILL™ PET	
Total volatile Nitrogen	AOAC 920.03	Product specification QRILL™ PET	
Total Protein	ISO 16634-1	Product specification QRILL™ PET	

[1] This value is in this report given as g/100g and not g/100 extracted fat and therefore differ from the value on the product specification of Qrill Pet.

3. Analytical results

The results of analytical testing are reported in this chapter.

3.1 Total fat

Table 3.1.1: Reported total fat at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g.

Table 3.1.2: Reported total fat at accelerated storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			

Values reported as g/100g.

Table 3.1.3: Reported total fat at accelerated storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			
6 month stability pull			

Values reported as g/100g.

A slight decrease in the percentage of total fat is observed during the period at all storage conditions. The results are within acceptance criteria.

3.2 Total omega-3

Table 3.2.1: Reported omega-3 at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g extracted fat.

Table 3.2.2: Reported omega-3 at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
9 month stability pull			
12 month stability pull			

Values reported as g/100g extracted fat.

Table 3.2.3: Reported omega-3 at storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
3 month stability pull			

Values reported as g/100g extracted fat.

Total omega-3 is stable after 18 months of storage at 25°C/60% RH.
Also at the intermediate and accelerated storage conditions, the levels of total omega-3 remain stable. Measurements of total omega-3 at the accelerated storage condition (40°C/75% RH) were not conducted at the 6 months pull point.

3.3 EPA

Table 3.3.1: Reported EPA at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g extracted fat.

Table 3.3.2: Reported EPA at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
9 month stability pull			
12 month stability pull			

Values reported as g/100g extracted fat.

Table 3.3.3: Reported EPA at storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			

Values reported as g/100g extracted fat.

EPA levels are stable after 18 months of storage at 25°C/60% RH. Also at the intermediate and accelerated storage conditions, the levels of total EPA remain stable. Measurements of total EPA at the accelerated storage condition (40°C/75% RH) were not conducted at the 6 months pull point.

3.4 DHA

Table 3.4.1: Reported DHA at storage condition 25°C/60% RH

Timepoint/ Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g.

Table 3.4.2: Reported DHA at storage condition 30°C/75% RH

Timepoint/Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
9 month stability pull			
12 month stability pull			

Values reported as g/100g extracted fat.

Table 3.4.3: Reported DHA at storage condition 40°C/75% RH

Timepoint/ Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			

Values reported as g/100g extracted fat.

DHA levels are stable after 18 months of storage at 25°C/60% RH.
Also at the intermediate and accelerated storage conditions, the levels of total DHA remain stable. Measurements of total DHA at the accelerated storage condition (40°C/75% RH) were not conducted at the 6 months pull point.

3.5 Phospholipids

Table 3.5.1: Reported phospholipids at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
6 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g.

Table 3.5.2: Reported phospholipids at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
6 month stability pull			
12 month stability pull			

Values reported as g/100g.

Table 3.5.3: Reported phospholipids at storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start	(b) (4)		
1 month stability pull			
6 month stability pull			

Values reported as g/100g.

The levels of phospholipids are stable after 18 months of storage at 25°C/60% RH and after 12 months storage at 30°C/75% RH. A slight reduction in the level of phospholipids was observed in one of the batches after 6 months storage at 40°C/75% RH but the results are within the acceptance criteria.

3.6 Acid value

Table 3.6.1: Reported acid value at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
3 month stability pull			
12 month stability pull			

Values reported as %.

Table 3.6.2: Reported acid value at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
3 month stability pull			
12 month stability pull			

Values reported as %.

Table 3.6.3: Reported acid value at storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
3 month stability pull			

Values reported as %.

The acid values increased slightly during the period of storage at all storage conditions. Measurements of acid value at the accelerated storage condition (40°C/75% RH) were not conducted at the 6 months pull point.

3.7 Iodine value

Table 3.7.1: Reported iodine value at storage condition 25°C/60% RH

Timepoint/ Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
3 month stability pull			
6 month stability pull			
12 month stability pull			

Table 3.7.2: Reported iodine value at storage condition 30°C/75% RH

Timepoint/ Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
3 month stability pull			
6 month stability pull			
12 month stability pull			

Table 3.7.3: Reported iodine value at storage condition 40°C/75% RH

Timepoint/ Batch number	**15-010514**	**15-050514**	**15-070514**
Stability study start		(b) (4)	
3 month stability pull			
6 month stability pull			

Iodine values are stable after 18 months of storage at 25°C/60% RH. Also at the intermediate and accelerated storage conditions, the iodine values are stable.

3.8 Total volatile nitrogen

Table 3.8.1: Reported total volatile nitrogen at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
6 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as g/100g.

Table 3.8.2: Reported total volatile nitrogen at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
6 month stability pull			
12 month stability pull			

Values reported as g/100g.

Table 3.8.3: Reported total volatile nitrogen at storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
6 month stability pull			

Values reported as g/100g.

Total volatile nitrogen increase slightly during the period of storage at all storage conditions. The results are within acceptance criteria.

3.9 Astaxanthin content

Table 3.9.1: Reported astaxanthin at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as mg/kg.

Table 3.9.2: Reported astaxanthin at accelerated storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			

Values reported as mg/kg.

Table 3.9.3: Reported astaxanthin at accelerated storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			

Values reported as mg/kg.

Astaxanthin levels decrease during the period of storage. Downturn in astaxanthin levels is significantly increased with higher temperatures.

3.10 Peroxide value

Table 3.10.1: Reported peroxide value at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			
18 month stability pull			

Values reported as meq peroxide/kg oil.

Table 3.10.2: Reported peroxide value at accelerated storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			
9 month stability pull			
12 month stability pull			

Values reported as meq peroxide/kg oil.

Table 3.10.3: Reported peroxide value at accelerated storage condition 40°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
1 month stability pull			
3 month stability pull			
6 month stability pull			

Values reported as meq peroxide/kg oil.

An increase in the peroxide levels are seen after 9 months of storage for all three batches at 25°C/60% RH and 30°C/75% RH. After 12 and 18 months storage at 25°C /60% RH, a decrease in the values is observed.
Peroxide value is stable after 6 months of storage at 40°C/75% RH.

3.11 Total protein

Table 3.11.1: Reported total protein at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			
18 month stability pull			

Values reported as g/100g.

Table 3.11.2: Reported total protein at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as g/100g.

The levels of total protein are stable for all three batches after 18 months of storage at 25°C/60% RH and after 12 months storage at 30°C/75% RH.

3.14 Total plate count

Table 3.14.1: Reported total plate count at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

Table 3.14.2: Reported total plate count at storage condition 30°C/75% RH

Timepoint/Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

There is no significant growth of aerobic microorganisms in any of the three batches after 12 months of storage at 25°C/60% RH and 30°C/75% RH.

Total plate count will be measured again at the 24 months pull point

3.15 Mould

Table 3.15.1: Reported mould at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

Table 3.15.2: Reported mould at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

There is no significant growth of mould in any of the three batches after 12 months of storage at 25°C/60% RH and 30°C/75% RH.

Mould will be measured again at the 24 months pull point.

3.16 Yeast

Table 3.16.1: Reported yeast at storage condition 25°C/60% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

Table 3.16.2: Reported yeast at storage condition 30°C/75% RH

Timepoint/ Batch number	15-010514	15-050514	15-070514
Stability study start		(b) (4)	
12 month stability pull			

Values reported as CFU/g.

There is no significant growth of yeast in any of the three batches after 12 months of storage at 25°C/60% RH and 30°C/75% RH.

Yeast will be measured again at the 24 months pull point

4. Evaluation of results

4.1 Total fat

There was observed some variation in the reported values for fat. This variation is within the expected analytical variation of the Bligh & Dyer analytical method. Fat content is therefore regarded as stable after 6, 12 and 18 months storage for all storage conditions.

4.2 Total Omega-3

Some minor variations was observed in the reported total omega-3 values. The variation is within the expected analytical variation for the method. Omega-3 is regarded as stable after 3 and 18 months storage for all storage conditions.

4.3 EPA

Some minor variations was observed in the reported total EPA values . The variation is within the expected analytical variation for the method. EPA is regarded as stable after 3 and 18 months storage for all storage conditions.

4.4 DHA

Some minor variations was observed in the reported total DHA values The variation is within the expected analytical variation for the method. DHA is regarded as stable after 3 and 18 months storage for all storage conditions.

4.5 Phospholipids

Phospholipids are stable or within the acceptance criteria for all storage conditions. Batch 15-070514 was not measured after 6 months.
Some variations in the reported results was observed. The variation is within the expected analytical variation for the method

4.6 Acid value

Some increase in acid value was observed. The acid values are monitored for information only and not part of the Qrill Pet specification. The acid value is estimated to be low and within the expected range for the product.

4.7 Iodine value

The iodine values are stable for all storage conditions. The iodine value can be used to idenfity the level of unsaturated fatty acids in the product (by being a measurement for the number of double bonds present). A stable iodine value indicates that no oxidation (breaking of double bonds) is taking place.

4.8 Total volatile nitrogen

The level of volatile nitrogen increased slightly for all batches after 6, 12 and 18 months storage. It is expected that minor amounts of TVN is formed during storage. The level is regarded as low and indicates a good quality of product.

4.9 Astaxanthin content

A reduction in astaxanthin was observed for all batches after 6, 12 and 18 months storage. This is an expected result as astaxanthin is an antioxidant, which protects the product from oxidation. As expected astaxanthin is decreasing more rapidly at higher temperatures. However, the product is still within specification at all storage conditions.

4.10 Peroxide

A slight increase in the peroxide values was observed after 9 months storage, while lower levels are reported after 12 and 18 months storage. The values are within product specification and the variation in peroxide values can be due to analytical variation. This parameter will be monitored throughout the study.

4.11 Total protein

Protein levels are stable for all storage conditions.

4.12 Total plate count, mould and yeast

There is no growth of aerobic, microorganisms, mould and yeast after 12 months of storage.

Total plate count, mould and yeast will be measured again at the 24 months pull point.

5. Conclusion

All the parameters in the tested batches are stable or within product specification. Based on the data presented in this report from storage at recommended, intermediate and accelerated storage conditions, the recommended shelf life for the product is set to 24 months.

Next pull point is after 24 months storage.

6. References

- "Protocol; Accelerated and long-term stability of low-fluoride krill meal in modified atmosphere" Document no: Q-2015-01

- VICH, 2007. Guidance for Industry: Stability Testing Of New Veterinary Drug Substances (Revision) VICH GL3(R). International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), Brussels, Belgium, January 2007.

- Product Specification Qrill Pet