United States Department of Agriculture
Agricultural Marketing Service | National Organic Program
Document Cover Sheet
https://www.ams.usda.gov/rules-regulations/organic/national-list/petitioned

Document Type:

☒ **National List Petition or Petition Update**

A petition is a request to amend the USDA National Organic Program's National List of Allowed and Prohibited Substances (National List).

Any person may submit a petition to have a substance evaluated by the National Organic Standards Board (7 CFR 205.607(a)).

Guidelines for submitting a petition are available in the NOP Handbook as NOP 3011, National List Petition Guidelines.

Petitions are posted for the public on the NOP website for Petitioned Substances.

☐ **Technical Report**

A technical report is developed in response to a petition to amend the National List. Reports are also developed to assist in the review of substances that are already on the National List.

Technical reports are completed by third-party contractors and are available to the public on the NOP website for Petitioned Substances.

Contractor names and dates completed are available in the report.

March 11, 2020
National List Manager
USDA/AMS/NOP, Standards Division
1400 Independence Ave. SW
Room 2648-So., Ag Stop 0268
Washington DC 20250-0268

To Whom it May Concern:

Enclosed please find a petition requesting that Poly-D-Glucosamine, commonly known as chitosan, be included on the list of materials approved by the National Organic Program. It is being submitted by Tidal Vision, a U.S.-based producer of chitosan, which is processed from fishery byproducts that are otherwise an expensive and potentially environmentally damaging waste stream for the country's fishing industry.

Chitosan is benign and abundant in nature and has many useful properties that can benefit the U.S. organic agriculture industry and provide for environmental improvement by eliminating waste and displacing carbon-intensive and polluting chemical alternatives. It has been extensively tested in the U.S. and abroad and has been found safe in applications as varied as a human dietary supplement, wound care, livestock feed supplement and adjuvant as well as water treatment and textile production. Chitosan has been approved as an adjuvant, applied to crops at a higher rate than this flocculant proposal contemplates.

Chitosan production leaves no harmful environmental impact and if its use as a flocculant is included among substances allowed by the National List of Allowed and Prohibited Substances, it has the potential to preserve value in the organic value chain, benefiting those who use it to treat wastewater as well as farmers who will be given more options for sourcing fertilizer components.

We are very grateful for your time and attention in reviewing our petition.

Sincerely,

Zach Wilkinson
Chief Operating Officer
Tidal Vision
Bellingham, WA 98229
Tidal VisionUSA.com
907-988-8888

ITEM A

1: National List Section

Section 205.601(o): Synthetic substances allowed for use in organic crop production, as production aids.

We are petitioning to add: Chitosan – as a flocculating or coagulating agent to capture material that may be used in the production of fertilizer.

2: OFPA Category

This use of chitosan may fall within the OFPA production aid category. Chitosan is not a synthetic inert ingredient of toxicological concern.

3: Inert ingredients
None

ITEM B

1: Substance Name

Poly-D-glucosamine, commonly called chitosan

2: Petitioner and Manufacturer information

Zach Wilkinson, COO
Tidal Vision Products, LLC
5506 Nielsen Ave., Ste A
Ferndale, WA 98248

3: Intended or Current Use

Chitosan's benign and useful chemical properties along with its abundance have led to its use in applications from wastewater treatment and agriculture to cosmetics, textiles and treating wounds. A form of the substance is sold as a nutritional supplement reputed to improve joint health in humans and animals under the name Glucosamine and has been approved for use as

a non-synthetic substance allowed for use in organic crop production as an adjuvant to help pesticidal treatments to adhere to plants.

Chemically, chitosan is a positively charged polymer of glucosamine and N-acetyl-glucosamine which acts as an effective, natural flocculant. Chitosan's positive charge causes negatively charged particles suspended in water to clump together, making them easier to filter or remove by other methods.

When chitosan is used to treat wastewater from processing organic foods, the suspended solids removed from wastewater streams can retain value as a fertilizer.

For example, a distillery that produces organic whiskey or a manufacturer of organic chips and crackers will produce water laden with grain particles as a byproduct of their manufacturing process. Treated with typical flocculants available in the market, the captured grain is contaminated with petroleum products or heavy metals, while other methods for treating this water such as centrifuge or membranes are expensive, energy-intensive and time consuming.

There is currently no flocculating agent included among approved substances on the National List, leaving food processors without a practical, approved method for retaining the value of post-process food collected from wastewater. If chitosan were included among approved substances on the National List, organic food processors would have a choice allowing them to keep the value of collected food particles within the organic value chain and organic crop producers would have access to a low-cost and nutrient-dense biodegradable fertilizer component.

4: Intended Activities and Application Rate

To treat wastewater, chitosan is first be made into a liquid form. While chitosan is not water soluble, it easily dissolves in mild solutions of organic acids. Acetic acid, the main component of household vinegar, is most commonly used in this process at a ratio of one part chitosan to one part acid or one part chitosan to two parts acid. Due to chitosan's high viscosity at low concentrations, most industrial chitosan water treatment solutions are sold at 1-2% active ingredient. For example, a 1 percent chitosan solution would contain 1-2% acetic acid, and 97-98% water. The chitosan is then further diluted when it is added to wastewater -- in typical use, no more than 0.3 milliliters of 1% chitosan solution is added to a liter of wastewater. The chitosan attracts particles suspended in the wastewater, causing them to clump together and sink or form large enough clusters to easily remove from the water.

Chitosan-treated solids will contain residual chitosan that is bonded to the solids and be included in any subsequent fertilizer or other products made from those solids, in an amount equivalent to the weight of a few insects in several pounds of fertilizer. Chitosan's bonding properties discourage fertilizer runoff, inhibiting a key problem with fertilizer use.

5: Manufacturing process

Chitin and chitosan occur widely in nature, constituting the main substance that forms cell walls for the shells of crabs, shrimp and other crustaceans as well as insect exoskeletons and fungus cell walls. Chitosan is the most abundant naturally occurring polysaccharide after cellulose, the material comprising the cell walls of plants.

Tidal Vision's chitosan is produced by isolating the chitin from crab shells created as waste from the crab fishing industry. Tidal Vision uses a unique, proprietary method for producing chitosan without creating the toxic byproducts that result from the process used by many global manufacturers of the substance.

Separating chitin from the protein, calcium carbonate, lipids and pigment that form the balance of the crustacean shells used can be achieved by fermentation, with enzymes or mechanically, but is currently commercially achieved chemically. A secondary process to deacetylate the chitin into chitosan is also performed chemically on a commercial scale, but methods exist to perform it enzymatically or mechanically. All of the materials used to manufacture chitosan are common industrial chemicals that can be safely neutralized. New, alternative processes for creating chitosan leave little to no waste.

6: Ancillary Substances

Organic acids including acetic acid, citric acid, lactic acid or malic acid, formic acid, and others allow chitosan to dissolve in water. Each of these acids is included among allowed items on the National List and are used in concentrations milder than household vinegar.

7: Previous Reviews

EPA Safer Choice Safer Chemical Ingredients List

2018 Application approved. Addition to the list pending as of June 2019, when Tidal Vision received notification that chitosan passes the EPA's safer choice criteria and EPA is in the process of working with Office of Pesticide Programs to add chitosan to the safer chemical ingredients list.

U.S. EPA FIFRA Minimum Risk Pesticide List 40 CFR 152.25(f)

Petition filed October 2018, under review by the Office of Pesticide Programs as of July 2019.

The U.S. Senate and U.S. House of Representatives 2019 appropriations bills included report language encouraging the EPA to review the petition in a timely matter and to notify the committees when the review has been completed.

Washington State Department of Ecology

Approved chemicals for stormwater chemical treatment facilities, 2004

Oregon Department of Agriculture
The State of Oregon has approved the use of chitosan in unrestricted amounts as a soil amendment

2007 EPA Registration Review Case 6063 EPA-HQ-EPA-2006-0566
In I995, EPA approved an exemption for chitosan from the requirement for tolerance on raw agricultural commodities. Other common applications of poly-D-glucosamine as hypocholesterolemic agents, as dietary fiber in low-calorie diets and as agents to increase the specific loaf volume of bread.

USDA National Organic Standards Board
In 2005, the NOSB recommended that chitosan be added to approved substances on the National List for use as an adjuvant to be used with an organic program-approved plant treatment. The National Organic Program confirmed its allowance without taking further action as it is allowed as an inert ingredient per section 205.601(m) of the National List.

8: Regulatory Authority

The U.S. Environmental Protection Agency
Chitosan is a naturally occurring and biodegradable chemical and in its 1995 decision, the EPA exempted it from the requirement for a tolerance limit when used as a pesticide in the production of any raw agricultural commodity.

According to a Technical Evaluation Report dated February, 2004: “EPA exempted chitosan from the requirement for a tolerance limit due to its low potential for toxicity and abundance in the environment. EPA concluded that chitosan is not expected to harm people, pets, wildlife, or the natural environment, in part because chitosan was found to be nontoxic in acute toxicity studies in mice, rats, and rabbits (EPA 1995).”

USDA National Organic Program
Chitosan is an approved substance on the National List for use as an adjuvant to be used in conjunction with an allowed nonsynthetic or allowed synthetic substance.

A 2004 petitioner noted an application rate of 66mg chitosan per liter of water, amounting to about 5 grams of chitosan per acre when applied to crops. This is a higher rate than the effective concentration of 1 or 2 percent chitosan solution that is diluted when used to treat wastewater, which would then be further diluted when the captured particles are combined with fertilizer components resulting less chitosan being applied per acre in this proposed use.

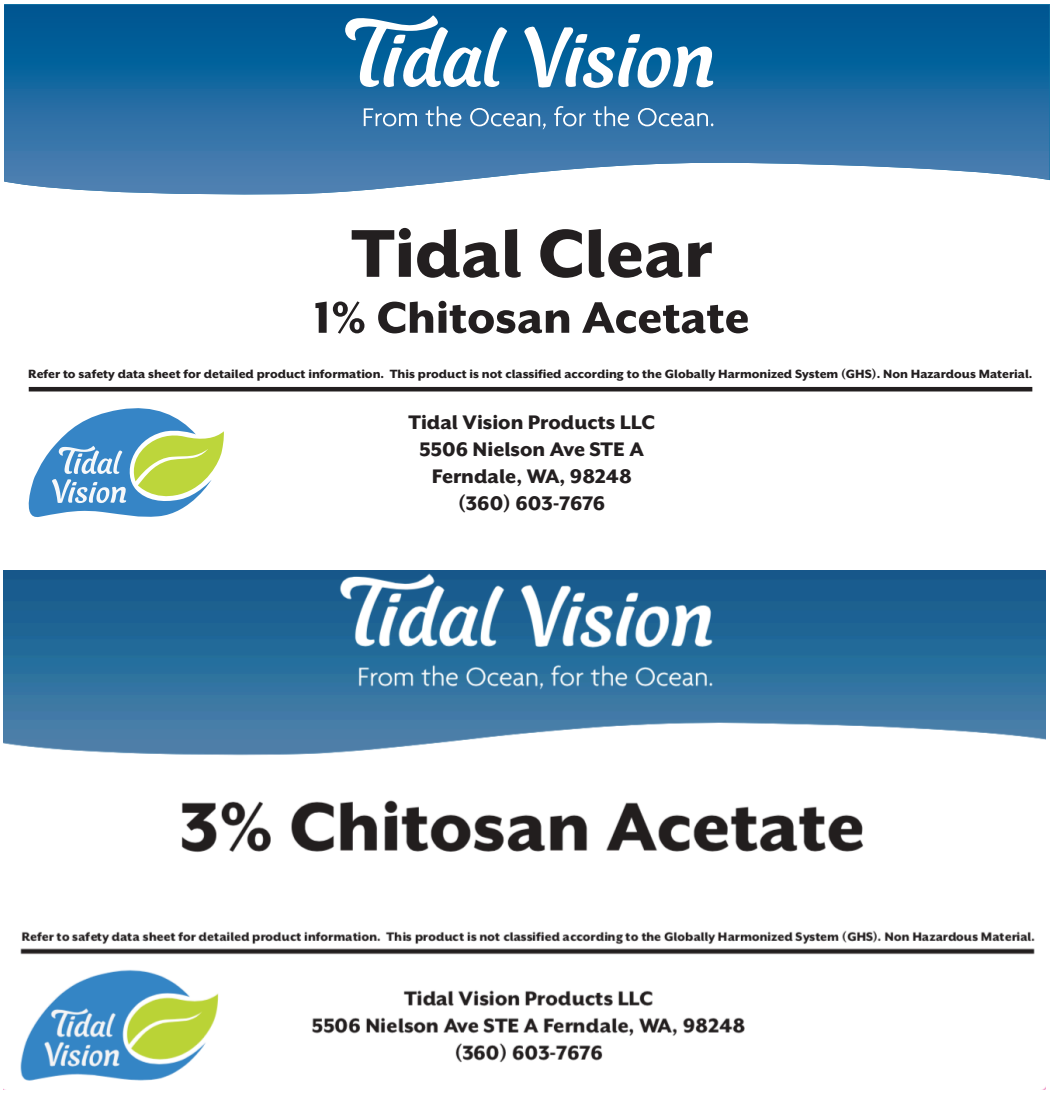
U.S. Food and Drug Administration

The FDA has begun four GRAS evaluations on chitosan, three involving shrimp-derived chitosan, in 2002, 2005 and 2013, and one involving chitosan from *Aspergillus niger* in 2011, which was closed with the FDA having no questions. Each evaluation has been closed without FDA making its own determination regarding the GRAS status of the subject use of chitosan.

U.S. FDA Food Safety Modernization Act Final Rule on Produce Safety
It is expected that chitosan will be used by farms, as defined by the final Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Food rule (PC Human Food).

9: Chemical Abstracts Service number and Product Labels

CAS # 9012-76-4



10: Physical and Chemical Properties

Chitosan is typically distributed as a dry flake or liquid form. While chitosan is not water-soluble, it dissolves in a solution of weak acid about 1/5 as strong as household vinegar. In its liquid form, chitosan is a clear to light yellow transparent, viscous fluid.

a) Chemical interactions with other substances

Chitosan is a positively charged organic polymer. Chitosan's positive charge attracts negatively charged objects making it very effective at causing particles to clump together without otherwise interacting with or affecting the attracted substances.

b) Toxicity and environmental persistence

Chitosan is nontoxic to humans, fish or animals when used at typical rates.

c) Environmental impacts from its use and/or manufacture

Manufacturing chitosan carries the large environmental benefit of diverting waste that occurs as a byproduct of the fishing industry and converting it into a valuable, nontoxic product.

According to the U.S. Environmental Protection Agency, environmental concerns associated with disposal of fish wastes into ocean waters include reduced oxygen levels in the water at the ocean bottom; burial or smothering of living organisms; and introduction of disease or non-native and invasive species to the ecosystem of the sea floor.

Seafood waste piles have been the subject of EPA lawsuits and enforcement actions because they create anoxic, or oxygen-depleted conditions that result in unsuitable habitats for fish and other living organisms and can violate the U.S. Clean Water Act.

Due to chitosan's prevalence in nature, many land- and sea-based organisms readily digest chitosan and consume its sugars for energy by producing enzymes such as chitinase and chitosanase.

d) Effects on human health

Glucosamine, a derivative of chitosan, is marketed as a dietary supplement reputed to improve joint health. In a 2006 study by the U.S. National Institutes of Health, study participants with moderate to severe knee pain from osteoarthritis treated with glucosamine combined with chondroitin sulfate reported statistically significant pain relief compared with a placebo, with 79 percent of subjects reporting 20 percent or greater pain reduction.

The U.S. EPA's fact sheet on chitosan says that no risks to humans are expected when products containing chitosan are used according to label directions. In toxicity tests, the only effect seen was slight skin irritation after chitosan was applied to skin.

e) Effects on soil organisms, crops, or livestock

"Risks to the environment are not expected because chitosan has not shown toxicity in mammals, it is abundant in nature, and it is used in tiny amounts," according to the U.S. EPA's fact sheet on chitosan.

Chitosan is useful as a plant defense booster and growth enhancer, protecting against certain fungal diseases, including early and late blight, downy and powdery mildew, and gray mold when sprayed on leaves. This effect is not expected to be seen in the use of chitosan to capture food particles which are then included in fertilizer applied to soil.

11: Safety Information

MSDS attached as appendix

NTP Technical Report on the Toxicity Study of Chitosan attached as appendix

12: Research Information

A vast body of research on Chitosan exists comprising dozens of books and hundreds of research reports. Below is a selection of summaries and references as examples of research that covers assertions made in this petition.

Chitin in Nature and Technology. A symposium edited by Riccardo Muzzarelli, Charles Jeuniaux, and Graham W. Gooday. Plenum Press, New York 1986.

> The essential background text, devoted chiefly to advanced aspects of pure and applied research.

Rahat Sharif, Muhammad Mujtaba, Mati Ur Rahman, Abdullah Shalmani, Husain Ahmad, Toheed Anwar, Deng Tianchan, and Xiping Wang, 2018. The Multifunctional Role of Chitosan in Horticultural Crops; A Review. Molecules. 23(4): 872. Published online 2018 Apr 10. doi: 10.3390/molecules23040872

The authors describe many uses of chitosan in crop production, including its great efficacy in as a fertilizer component without affecting beneficial soil microbes.

Kendra, D. F., Christian, D., and Hadwiger, L. A. 1998. Chitosan oligomers from Fusarium solani/pea interactions, chitinase/ beta glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. Physiol. Molec. Plant Path. 45:215-230.

The authors describe plants' enzymes that attack fungal cell walls and the chitosan within and the abundance of enzymes released by microbes that have the potential to digest chitin, chitosan and cellulose into simple organic matter.

Hadwiger, L. A. 2015. Anatomy of a nonhost disease resistance response of pea to Fusarium solani: PR gene elicitation via DNase, chitosan and chromatin alterations. Frontiers in Plant Science 6:373-400.

The researchers describe how chitosan boosts plants' disease resistance: chitosan activates defensive Pathogenesis-related genes, while some products of the plant defense are directly antifungal.

Baldrick, P., 2010. The safety of chitosan as a pharmaceutical excipient. Regul Toxicol Pharmacol 56, 290-299.

A review of published research on chitosan and human health describing chitosan's low oral toxicity and broad human oral exposure through the use of chitosan dietary supplements and food additives, medical device and cosmetic applications.

Jull, A.b., Mhurchu, C.N., Bennett, D.A., Dunshea-Mooij, C.A., Rodgers, A., 2008. Chitosan for overweight or obesity. Cochrane Database Syst Rev 16, CD003892.

An example of a study of chitosan as a weight-loss aid. While chitosan has been found ineffective in helping patients lose weight, no damaging side effects are typical.

Barbara Bellich, Ilenia D'Agostino, Sabrina Semeraro, Amelia Gamini and Attilito Cesaro. "The Good, the Bad and the Ugly" of Chitosans. Marine Drugs (2016): 1 - 31. Online Journal. 2018.

The authors present a range of chemical, medical, sustainability and market issues that relate chitosan properties to some basic features and to advanced solutions and applications.

United States Environmental Protection Agency (US EPA) - Office of Pesticide Programs. Chitosan; Poly-D-glucosamine (128930) Fact Sheet. n.d. Online fact sheet. 2018.

13: Petition Justification Statement

By adding chitosan to the National List of allowed products, the USDA NOP can offer manufacturers who process plants, meat, dairy, fish and other food-based agricultural inputs a natural, sustainable product to remove food particles from wastewater, allowing that reclaimed food material to be added to organic fertilizer, offering farmers a natural, biodegradable crop nutrient source while also diverting the food waste from landfills. And this can be done using a safe, natural product which in its own manufacturing removes waste from the fishing industry, forestalling potential environmental damage.

Explain why the substance is necessary for the production or handling of an organic product.

Producers of organic processed foods and organic crop producers are missing the opportunity to retain a high-value fertilizer input in the organic value chain because there is currently no flocculating agent allowed on the USDA NOP's National List of Allowed and Prohibited Substances. Adding chitosan to the National List would give these groups an option for preserving the value of this byproduct and potentially diverting it from landfills by permitting it to be used as fertilizer for organic crop production.

Industrial food processing uses copious amounts of water for transporting food through the production cycle as well as for cleaning equipment. Before being discharged from the factory, the water must be treated to remove the abundant organic particles that are suspended as a consequence. This material can be added to fertilizer to boost its nitrogen content from a natural, biodegradable source.

The wastewater generated by processing organic foods is often treated with polymers derived from petroleum or metals, rendering the waste captured by treatment processes unsuitable to be used for organic farming. Adding chitosan to the National List would give these companies an alternative that comes from a natural, sustainable source that doesn't contribute to fertilizer runoff pollution the way artificial fertilizers do.

Describe any nonsynthetic substance, synthetic substance on the national list, or alternative cultural method that could be used in place of the petitioned synthetic substance

Chemical alternatives:
Metal- and petroleum-based chemicals are commonly used to separate suspended solids from wastewater in food processing as well as other industrial processes such as papermaking and municipal sewage treatment.

Polyacrylamide is a petroleum-based polymer widely used to treat wastewater. Polyacrylamide products also contain acrylamide, which has been found in animal studies to cause neurotoxicity and reproductive system effects after being absorbed through the skin.

Aluminum sulfate, a chemical commonly used as a flocculant in wastewater, is made by adding aluminum hydroxide to sulfuric acid. When combined with water, it creates an acid capable of burning skin and corroding metal. It is harmful if swallowed or inhaled, causing coughing and shortness of breath, irritation, redness, itching and pain. Eating or swallowing aluminum sulfate produces severe irritation to the intestines and stomach, causing vomiting, nausea and diarrhea.

Other chemicals used as flocculants include:
Aluminum chloride
Sodium aluminate
Ferric sulfate
Ferric chloride
Ferric chloride sulfate
Magnesium carbonate

Two chemicals that can be used as flocculants are included on the National List allowed for other purposes:

Hydrated lime is allowed as an external pest control. It is not permitted to cauterize physical alterations or deodorize animal wastes, according to according to the CFR.

Ferrous sulfate is allowed for iron enrichment or fortification of foods when required by regulation or recommended by an independent organization, according to the CFR

Mechanical alternatives

Wastewater can be treated by mechanical means including centrifuge, decantation, settling ponds and filtration. A flocculant is frequently used to increase the effectiveness of other methods, reducing the time, energy and expense needed to clean water employing other methods.

Describe the beneficial effects to the environment, human health or farm ecosystem from use of the synthetic substance that support its use instead of the use of a nonsynthetic substance or alternative cultural method.

Chitosan is one of the most abundant naturally occurring materials on the planet, and is already present virtually everywhere on earth in the exoskeletons of insects, cell walls of fungi and crustacean shells. Decades of research has been performed to verify chitosan's safety. It is biodegradable and has been approved for human consumption as a food additive and dietary supplement as well as being used to treat wounds.

Organic crops must be raised according to the stipulations of the National List. Organic food processors don't currently have a National-List approved chemical treatment option for their waste streams. By including chitosan among substances allowed on the National List, the USDA

NOP can increase the amount and type of acceptable fertilizer components available to organic farmers while also reclaiming the value of two waste streams -- fishing waste which becomes chitosan and food waste that chitosan can treat.

Chitosan has beneficial effects on many plants and animals, boosting growth of plants and helping plants to avoid and react to pests and disease. It has been found to benefit chickens, rabbits and dairy cattle when used as a supplement to normal feed.

When used as a flocculant to treat industrial and municipal wastewater, chitosan displaces petroleum and heavy-metal based chemicals, providing for more sustainable water treatment as well as displacing the alternatives' pollution-rich and energy-intensive supply chains.

Chitosan production leaves no harmful environmental impact, and provides a beneficial, nontoxic alternative to disposing of fishery waste. When used as a flocculant, National-List approved chitosan also has the potential to preserve value in the organic value chain, benefitting growers, food processors and farmers by expanding approved options for each group.

Appendices

Appendix 1

Material Safety Data Sheets

Tidal Vision
From the Ocean, for the Ocean.

SAFETY DATA SHEET

Issuing Date 08-Sep-2017 **Revision Date** 05-Nov-2017 **Revision Number** 1

NGHS / English

1. IDENTIFICATION

Product identifier

Product Name Chitosan Acetate (1%)

Other means of identification

Product Code(s) N/A

Recommended use of the chemical and restrictions on use

Recommended Use Water Treatment

Restrictions on use Not intended for use in fish tanks

Details of the supplier of the safety data sheet

Supplier Identification Tidal Vision Products LLC

Address 5506 Nielsen Ave,
Suite A
Ferndale
WA
98248
US

Telephone (360)603-7676

E-mail ben@tidalvisionusa.com

Emergency telephone number

Company Emergency Phone Number (360)603-7676

2. HAZARDS IDENTIFICATION

Classification

Not classified.

The product contains no substances which at their given concentration, are considered to be hazardous to health

Appearance Clear, amber **Physical state** Liquid **Odor** ..... Vinegar-like

GHS Label elements, including precautionary statements

Hazard statements
Not classified.

Other information
Unknown acute toxicity 0 % of the mixture consists of ingredient(s) of unknown toxicity
0 % of the mixture consists of ingredient(s) of unknown acute oral toxicity
0 % of the mixture consists of ingredient(s) of unknown acute dermal toxicity
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance

Not applicable.

Mixture

The product contains no substances which at their given concentration, are considered to be hazardous to health.

4. FIRST AID MEASURES

First aid measures

Inhalation Remove to fresh air.

Eye contact Rinse thoroughly with plenty of water for at least 15 minutes, lifting lower and upper eyelids. Consult a physician.

Skin contact Wash skin with soap and water.

Ingestion Clean mouth with water and drink afterwards plenty of water.

Most important symptoms and effects, both acute and delayed

Symptoms No information available.

Indication of any immediate medical attention and special treatment needed

Note to physicians Treat symptomatically.

5. FIRE-FIGHTING MEASURES

Suitable Extinguishing Media Use extinguishing measures that are appropriate to local circumstances and the surrounding environment.

Unsuitable extinguishing media CAUTION: Use of water spray when fighting fire may be inefficient.

Specific hazards arising from the chemical No information available.

Hazardous Combustion Products Carbon oxides.

Explosion Data
Sensitivity to Mechanical Impact None.
Sensitivity to Static Discharge None.

Special protective equipment for fire-fighters Firefighters should wear self-contained breathing apparatus and full firefighting turnout gear. Use personal protection equipment.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures

Personal precautions Avoid contact with eyes.

Methods and material for containment and cleaning up

Methods for containment Prevent further leakage or spillage if safe to do so.

Methods for cleaning up Dam up. Soak up with inert absorbent material. Pick up and transfer to properly labeled containers.

7. HANDLING AND STORAGE

Precautions for safe handling

Advice on safe handling Handle in accordance with good industrial hygiene and safety practice.

Conditions for safe storage, including any incompatibilities

Storage Conditions Keep containers tightly closed in a dry, cool and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Control parameters

Exposure Limits This product, as supplied, does not contain any hazardous materials with occupational exposure limits established by the region specific regulatory bodies.

Appropriate engineering controls

Engineering controls Showers
Eyewash stations

Ventilation systems.

Individual protection measures, such as personal protective equipment

Eye/face protection No special protective equipment required.

Skin and body protection No special protective equipment required.

Respiratory protection No protective equipment is needed under normal use conditions. If exposure limits are exceeded or irritation is experienced, ventilation and evacuation may be required.

General hygiene considerations Handle in accordance with good industrial hygiene and safety practice.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical and Chemical Properties

Physical state	Liquid
Appearance	Clear, amber
Odor	Vinegar-like
Color	No information available
Odor Threshold	Not applicable

Property	**Values**	**Remarks Method**
pH	4.0	
Melting / freezing point	No data available	None known
Boiling point / boiling range	99 °C / 211 °F	
Flash Point	No data available	None known
Evaporation Rate	No data available	None known
Flammability (solid, gas)	No data available	None known
Flammability Limit in Air		None known
Upper flammability limit	No data available	
Lower flammability limit	No data available	
Vapor pressure	No data available	None known
Vapor density	No data available	None known
Relative density	1.1	
Water Solubility	Liquid	
Solubility(ies)	No data available	None known
Partition coefficient: n-octanol/water	Not Applicable	
Autoignition temperature	No data available	None known
Decomposition temperature	No data available	None known
Kinematic viscosity	No data available	None known
Dynamic viscosity	25	

Other Information

Explosive properties	No information available
Oxidizing properties	No information available
Softening Point	No information available
Molecular Weight	No information available
VOC Content (%)	No information available
Liquid Density	No information available
Bulk Density	No information available
Particle Size	No information available
Particle Size Distribution	No information available

10. STABILITY AND REACTIVITY

Reactivity No information available.

Chemical stability	Stable under normal conditions.
Possibility of Hazardous Reactions	None under normal processing.
Hazardous Polymerization	Hazardous polymerization does not occur.
Conditions to avoid	None known based on information supplied.
Incompatible materials	None known based on information supplied.
Hazardous Decomposition Products	Carbon oxides.

11. TOXICOLOGICAL INFORMATION

Information on likely routes of exposure

Product Information

Inhalation	Specific test data for the substance or mixture is not available.
Eye contact	Specific test data for the substance or mixture is not available.
Skin contact	Specific test data for the substance or mixture is not available.
Ingestion	Specific test data for the substance or mixture is not available.

Information on toxicological effects

Symptoms No information available.

Numerical measures of toxicity

Acute Toxicity

Unknown acute toxicity 0 % of the mixture consists of ingredient(s) of unknown toxicity
0 % of the mixture consists of ingredient(s) of unknown acute oral toxicity
0 % of the mixture consists of ingredient(s) of unknown acute dermal toxicity
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

Delayed and immediate effects as well as chronic effects from short and long-term exposure

Skin corrosion/irritation	No information available.
Serious eye damage/eye irritation	No information available.
Respiratory or skin sensitization	No information available.
Germ cell mutagenicity	No information available.
Carcinogenicity	No information available.
Reproductive toxicity	No information available.

STOT - single exposure	No information available.
STOT - repeated exposure	No information available.
Aspiration hazard	No information available.

12. ECOLOGICAL INFORMATION

Ecotoxicity	The environmental impact of this product has not been fully investigated.
Persistence and Degradability	No information available.
Bioaccumulation	There is no data for this product.
Mobility	No information available.
Other adverse effects	No information available.

13. DISPOSAL CONSIDERATIONS

Waste treatment methods

Waste from residues/unused products	Dispose of in accordance with local regulations. Dispose of waste in accordance with environmental legislation.
Contaminated packaging	Do not reuse empty containers.

14. TRANSPORT INFORMATION

DOT	NOT REGULATED
Proper Shipping Name	NON-REGULATED
Hazard Class	N/A
TDG	Not regulated
MEX	Not regulated
ICAO	Not regulated
IATA	Not regulated
Proper Shipping Name	NON REGULATED
Hazard Class	N/A
IMDG/IMO	Not regulated
Hazard Class	N/A
RID	Not regulated
ADR	Not regulated
ADN	Not regulated

15. REGULATORY INFORMATION

Safety, health and environmental regulations/legislation specific for the substance or mixture

International Regulations

Ozone-depleting substances (ODS) Not applicable

Persistent Organic Pollutants Not applicable

Export Notification requirements Not applicable

International Inventories

TSCA	Contact supplier for inventory compliance status.
DSL/NDSL	Contact supplier for inventory compliance status.
EINECS/ELINCS	Contact supplier for inventory compliance status.
ENCS	Contact supplier for inventory compliance status.
KECL	Contact supplier for inventory compliance status.
PICCS	Contact supplier for inventory compliance status.
AICS	Contact supplier for inventory compliance status.

Legend

TSCA - United States Toxic Substances Control Act Section 8(b) Inventory
DSL/NDSL - Canadian Domestic Substances List/Non-Domestic Substances List
EINECS/ELINCS - European Inventory of Existing Chemical Substances/European List of Notified Chemical Substances
ENCS - Japan Existing and New Chemical Substances
KECL - Korean Existing and Evaluated Chemical Substances
PICCS - Philippines Inventory of Chemicals and Chemical Substances
AICS - Australian Inventory of Chemical Substances

US Federal Regulations

SARA 313

Section 313 of Title III of the Superfund Amendments and Reauthorization Act of 1986 (SARA). This product does not contain any chemicals which are subject to the reporting requirements of the Act and Title 40 of the Code of Federal Regulations, Part 372

Acute Health Hazard	No
Chronic Health Hazard	No
Fire Hazard	No
Sudden release of pressure hazard	No
Reactive Hazard	No

CWA (Clean Water Act)

This product does not contain any substances regulated as pollutants pursuant to the Clean Water Act (40 CFR 122.21 and 40 CFR 122.42)

CERCLA

This material, as supplied, does not contain any substances regulated as hazardous substances under the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) (40 CFR 302) or the Superfund Amendments and Reauthorization Act (SARA) (40 CFR 355). There may be specific reporting requirements at the local, regional, or state level pertaining to releases of this material

US State Regulations

California Proposition 65

This product does not contain any Proposition 65 chemicals.

U.S. State Right-to-Know Regulations

This product does not contain any substances above threshold limits that are regulated by state right-to-know.

16. OTHER INFORMATION

NFPA	**Health hazards** 1	**Flammability** 0	**Instability** 0	**Physical and Chemical Properties** -
HMIS	**Health hazards** 1	**Flammability** 0	**Physical hazards** 0	**Personal Protection** X

Prepared By

Product Stewardship.
23 British American Blvd.
Latham, NY 12110
1-800-572-6501

Issuing Date 08-Sep-2017

Revision Date 05-Nov-2017

Revision Note Edited for content by Ben Cairns

Disclaimer

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End of Safety Data Sheet

Tidal Vision
From the Ocean, for the Ocean.

SAFETY DATA SHEET

Issuing Date 08-Sep-2017 **Revision Date** 05-Apr-2019 **Revision Number** 1

NGHS / English

1. IDENTIFICATION

Product identifier

Product Name Chitosan Flake

Other means of identification

CAS-No 9012-76-4

Recommended use of the chemical and restrictions on use

Recommended Use Laboratory Chemicals, Manufacture of chemicals

Restrictions on use N/A

Details of the supplier of the safety data sheet

Supplier Identification Tidal Vision Products LLC

Address 5506 Nielsen Ave,
Suite A
Ferndale
WA
98248
US

Telephone .....

E-mail Ben@tidalvisionusa.com

Emergency telephone number

Company Emergency Phone Number 360)603-7676

2. HAZARDS IDENTIFICATION

Classification

Not classified.

The product contains no substances which at their given concentration, are considered to be hazardous to health

Appearance Beige/Light Brown Flake **Physical state** Solid **Odor** None

GHS Label elements, including precautionary statements

Hazard statements
Not classified.

Other information
Unknown acute toxicity 0 % of the mixture consists of ingredient(s) of unknown toxicity
0 % of the mixture consists of ingredient(s) of unknown acute oral toxicity
0 % of the mixture consists of ingredient(s) of unknown acute dermal toxicity
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance

Not applicable.

Mixture

The product contains no substances which at their given concentration, are considered to be hazardous to health.

4. FIRST AID MEASURES

First aid measures

Inhalation Remove to fresh air.

Eye contact Rinse thoroughly with plenty of water for at least 15 minutes, lifting lower and upper eyelids. Consult a physician.

Skin contact Wash skin with soap and water.

Ingestion Clean mouth with water and drink afterwards plenty of water.

Most important symptoms and effects, both acute and delayed

Symptoms No information available.

Indication of any immediate medical attention and special treatment needed

Note to physicians Treat symptomatically.

5. FIRE-FIGHTING MEASURES

Suitable Extinguishing Media Use extinguishing measures that are appropriate to local circumstances and the surrounding environment.

Unsuitable extinguishing media CAUTION: Use of water spray when fighting fire may be inefficient.

Specific hazards arising from the chemical No information available.

Hazardous Combustion Products Carbon oxides.

Explosion Data
Sensitivity to Mechanical Impact None.
Sensitivity to Static Discharge None.

Special protective equipment for fire-fighters Firefighters should wear self-contained breathing apparatus and full firefighting turnout gear. Use personal protection equipment.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures

Personal precautions Avoid contact with eyes.

Methods and material for containment and cleaning up

Methods for containment Prevent further leakage or spillage if safe to do so.

Methods for cleaning up Dam up. Soak up with inert absorbent material. Pick up and transfer to properly labeled containers.

7. HANDLING AND STORAGE

Precautions for safe handling

Advice on safe handling Handle in accordance with good industrial hygiene and safety practice.

Conditions for safe storage, including any incompatibilities

Storage Conditions Keep containers tightly closed in a dry, cool and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Control parameters

Exposure Limits This product, as supplied, does not contain any hazardous materials with occupational exposure limits established by the region specific regulatory bodies.

Appropriate engineering controls

Engineering controls Showers
Eyewash stations

Ventilation systems.

Individual protection measures, such as personal protective equipment

Eye/face protection No special protective equipment required.

Skin and body protection No special protective equipment required.

Respiratory protection No protective equipment is needed under normal use conditions. If exposure limits are exceeded or irritation is experienced, ventilation and evacuation may be required.

General hygiene considerations Handle in accordance with good industrial hygiene and safety practice.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical and Chemical Properties

Physical state	Solid
Appearance	Beige/Light Brown flake
Odor	None
Color	No information available
Odor Threshold	Not applicable

Property	**Values**	**Remarks Method**
pH	No data available	
Melting / freezing point	No data available	None known
Boiling point / boiling range	No data available	
Flash Point	No data available	None known
Evaporation Rate	No data available	None known
Flammability (solid, gas)	No data available	None known
Flammability Limit in Air	No data available	None known
Upper flammability limit	No data available	
Lower flammability limit	No data available	
Vapor pressure	No data available	None known
Vapor density	No data available	None known
Relative density	No data available	
Water Solubility	With Organic Acid	
Solubility(ies)	No data available	None known
Partition coefficient: n-octanol/water	Not Applicable	
Autoignition temperature	No data available	None known
Decomposition temperature	No data available	None known
Kinematic viscosity	No data available	None known
Dynamic viscosity	25	

Other Information

Explosive properties	No information available
Oxidizing properties	No information available
Softening Point	No information available
Molecular Weight	No information available
VOC Content (%)	No information available
Liquid Density	No information available
Bulk Density	No information available
Particle Size	No information available
Particle Size Distribution	No information available

10. STABILITY AND REACTIVITY

Reactivity No information available.

Chemical stability	Stable under normal conditions.
Possibility of Hazardous Reactions	None under normal processing.
Hazardous Polymerization	Hazardous polymerization does not occur.
Conditions to avoid	None known based on information supplied.
Incompatible materials	None known based on information supplied.
Hazardous Decomposition Products	Carbon oxides.

11. TOXICOLOGICAL INFORMATION

Information on likely routes of exposure

Product Information

Inhalation	Specific test data for the substance or mixture is not available.
Eye contact	Specific test data for the substance or mixture is not available.
Skin contact	Specific test data for the substance or mixture is not available.
Ingestion	Specific test data for the substance or mixture is not available.

Information on toxicological effects

Symptoms No information available.

Numerical measures of toxicity

Acute Toxicity

Unknown acute toxicity 0 % of the mixture consists of ingredient(s) of unknown toxicity
0 % of the mixture consists of ingredient(s) of unknown acute oral toxicity
0 % of the mixture consists of ingredient(s) of unknown acute dermal toxicity
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
0 % of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

Delayed and immediate effects as well as chronic effects from short and long-term exposure

Skin corrosion/irritation	No information available.
Serious eye damage/eye irritation	No information available.
Respiratory or skin sensitization	No information available.
Germ cell mutagenicity	No information available.
Carcinogenicity	No information available.
Reproductive toxicity	No information available.

STOT - single exposure	No information available.
STOT - repeated exposure	No information available.
Aspiration hazard	No information available.

12. ECOLOGICAL INFORMATION

Ecotoxicity	The environmental impact of this product has not been fully investigated.
Persistence and Degradability	No information available.
Bioaccumulation	There is no data for this product.
Mobility	No information available.
Other adverse effects	No information available.

13. DISPOSAL CONSIDERATIONS

Waste treatment methods

Waste from residues/unused products	Dispose of in accordance with local regulations. Dispose of waste in accordance with environmental legislation.
Contaminated packaging	Do not reuse empty containers.

14. TRANSPORT INFORMATION

DOT	NOT REGULATED
Proper Shipping Name	NON-REGULATED
Hazard Class	N/A
TDG	Not regulated
MEX	Not regulated
ICAO	Not regulated
IATA	Not regulated
Proper Shipping Name	NON REGULATED
Hazard Class	N/A
IMDG/IMO	Not regulated
Hazard Class	N/A
RID	Not regulated
ADR	Not regulated
ADN	Not regulated

15. REGULATORY INFORMATION

Safety, health and environmental regulations/legislation specific for the substance or mixture

International Regulations

Ozone-depleting substances (ODS) Not applicable

Persistent Organic Pollutants Not applicable

Export Notification requirements Not applicable

International Inventories

TSCA	Contact supplier for inventory compliance status.
DSL/NDSL	Contact supplier for inventory compliance status.
EINECS/ELINCS	Contact supplier for inventory compliance status.
ENCS	Contact supplier for inventory compliance status.
KECL	Contact supplier for inventory compliance status.
PICCS	Contact supplier for inventory compliance status.
AICS	Contact supplier for inventory compliance status.

Legend

TSCA - United States Toxic Substances Control Act Section 8(b) Inventory
DSL/NDSL - Canadian Domestic Substances List/Non-Domestic Substances List
EINECS/ELINCS - European Inventory of Existing Chemical Substances/European List of Notified Chemical Substances
ENCS - Japan Existing and New Chemical Substances
KECL - Korean Existing and Evaluated Chemical Substances
PICCS - Philippines Inventory of Chemicals and Chemical Substances
AICS - Australian Inventory of Chemical Substances

US Federal Regulations

SARA 313

Section 313 of Title III of the Superfund Amendments and Reauthorization Act of 1986 (SARA). This product does not contain any chemicals which are subject to the reporting requirements of the Act and Title 40 of the Code of Federal Regulations, Part 372

Acute Health Hazard	No
Chronic Health Hazard	No
Fire Hazard	No
Sudden release of pressure hazard	No
Reactive Hazard	No

CWA (Clean Water Act)

This product does not contain any substances regulated as pollutants pursuant to the Clean Water Act (40 CFR 122.21 and 40 CFR 122.42)

CERCLA

This material, as supplied, does not contain any substances regulated as hazardous substances under the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) (40 CFR 302) or the Superfund Amendments and Reauthorization Act (SARA) (40 CFR 355). There may be specific reporting requirements at the local, regional, or state level pertaining to releases of this material

US State Regulations

California Proposition 65

This product does not contain any Proposition 65 chemicals.

U.S. State Right-to-Know Regulations

This product does not contain any substances above threshold limits that are regulated by state right-to-know.

16. OTHER INFORMATION

NFPA	**Health hazards** 1	**Flammability** 0	**Instability** 0	**Physical and Chemical Properties** -
HMIS	**Health hazards** 1	**Flammability** 0	**Physical hazards** 0	**Personal Protection** X

Prepared By

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Benjamin Cairns

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End of Safety Data Sheet

Appendix 2

NTP Technical Report on the Toxicity Study of Chitosan

National Toxicology Program
Toxicity Report Series
Number 93

NTP Technical Report on the Toxicity Study of

Chitosan

(CAS No. 9012-76-4)

Administered in Feed to Sprague Dawley [Crl:CD(SD)] Rats

December 2017

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Toxicity Study Report series began in 1991. The studies described in the Toxicity Study Report series are designed and conducted to characterize and evaluate the toxicologic potential of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Toxicity Study Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection *per se* is not an indicator of a substance's toxic potential.

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP Toxicity Study Reports are indexed in the NIH/NLM PubMed database and are available free of charge electronically on the NTP website (http://ntp.niehs.nih.gov).

National Toxicology Program
Toxicity Report Series
Number 93

NTP Technical Report
on the Toxicity Study of

Chitosan

(CAS No. 9012-76-4)

Administered in Feed
to Sprague Dawley [Crl:CD(SD)] Rats

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December 2017

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

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Provided pathology review

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Provided sperm parameter data

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Biotechnical Services, Inc.
Prepared Toxicity Study Report

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PEER REVIEW

The draft report on the toxicity study of chitosan was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of this NTP study are appropriate and ensure that this Toxicity Study Report presents the experimental results and conclusions fully and clearly.

Diane Birt, Ph.D.
Iowa State University (Retired)
Ames, Iowa

Melissa G. Rhodes, Ph.D.
Roivant Sciences, Inc.
Durham, NC

SUMMARY

Background
Chitosan is primarily utilized as a weight loss supplement, although it is also used in hair and skin products, wound dressings, and the wastewater treatment and agriculture industries. We conducted 6-month studies to determine if there were any toxic effects of chitosan in male and female rats. As chitosan is commonly used as a dietary supplement, an oral route of exposure (in feed) was used for these studies.

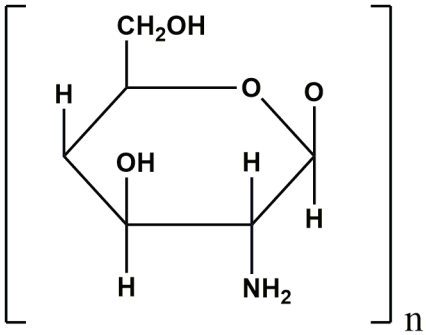
Methods
There were 10 rats in each dose group and the doses were 1%, 3%, and 9% chitosan in feed. These doses corresponded to approximately 450, 1,500, and 5,200 milligrams (mg) of chitosan per kilogram (kg) of body weight per day in male rats and 650, 1,800, and 6,000 mg/kg per day in female rats. A control group received feed free of chitosan. Over the course of the study, samples were collected for fecal analysis, urinalysis, clinical chemistry, and analysis of vitamin levels. At the end of the study, over 40 tissues were assessed, and samples were also collected for reproductive tissue evaluations.

Results
Three male rats (one in the control group and two in the 9% group) and two female rats (one in the 1% group and one in the 3% group) died before the end of the study; the cause of death was undetermined. Serum levels of cholesterol, triglycerides, and phosphorous were significantly decreased in rats exposed to 9% chitosan, as were serum levels of vitamin A and vitamin E; serum levels of $1,25(OH)_2$ vitamin D were increased. Vitamin E concentrations in the livers of exposed rats were significantly lower than those in control rats. Exposure to chitosan also had digestive effects, including increases in fecal weight and moisture, and decreases in percent fat digested. There was a decrease in thymus and liver weights, along with decreased fatty tissue in the livers of exposed rats.

Conclusions
We concluded that dietary exposure to chitosan for 6 months resulted in decreased fat digestion and depletion of some fat-soluble vitamins in male and female rats.

CONTENTS

ABSTRACT



CHITOSAN

CAS No. 9012-76-4

Chemical Formula: $(C_6H_{11}NO_4)_n$

Synonyms: 2-Amino-2-deoxy-beta-D-glucosamine; deacetylated chitin; poliglusam; poly (D-glucosamine)
Trade names: Celox, Chicol, Chitopearl, CTFA 04299, Flonac N, Kytex H, Sea Cure F

Chitosan is a cationic carbohydrate polymer that is commercially derived from the deacetylation of chitin obtained from seafood shells. The most widespread route of human exposure to chitosan is as a dietary supplement for body weight reduction. Chitosan was nominated by the National Cancer Institute for mechanistic studies designed to measure the potential for vitamin E depletion and osteoporosis following ingestion. Male and female Sprague Dawley rats were exposed to chitosan (86.5% deacetylated, with an average molecular weight of approximately 82 kilodaltons and estimated to be approximately 94% pure) in feed for 6 months.

In this 6-month study, groups of 10 male and 10 female core study rats (Group A) were fed control diets (AIN-93M) or diets containing chitosan at concentrations of 1%, 3%, or 9%, for up to 25 weeks. Two additional groups of 10 male and 10 female rats (Groups B and C) were given the same dietary concentrations for up to 26 weeks. All male and female Group A rats survived to the end of the study. Mean body weights and feed consumption of exposed Group A groups were similar to those of the control groups. Dietary concentrations of 1%, 3%, and 9% resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females. There were no treatment-related clinical findings in core study animals.

The 9% male and female rats had significantly decreased cholesterol values (26% to 48%), compared to the controls, at all time points. Triglycerides were significantly decreased in 9% male and female rats, but not at every time point.

Phosphorus levels were significantly decreased in 9% male rats at weeks 13, 19, and 25; a decrease also occurred in 3% males at week 13. Phosphorus levels were significantly decreased in 3% and 9% females at weeks 13 and 25.

Compared to those of the controls, serum vitamin A concentrations were significantly decreased (approximately 30%) at weeks 13, 19, and 26 in 9% males, at weeks 13 and 26 in 3% males (approximately 15%), and at weeks 19 and 26 in 9% females (approximately 20%). Serum vitamin E concentrations were significantly decreased at all time points in 3% (33% to 42%) and 9% (79% to 82%) males, in 1% (17%) males at week 13, and in 9% (62% to 65%) females at all time points. Hepatic vitamin E concentrations were significantly decreased at week 26 in 3% (48%) and 9% (87%) males and 9% (80%) females. Serum concentrations of $1,25(OH)_2$ vitamin D were significantly increased in 9% (105% to 142%) males and (100% to 180%) females at weeks 7, 19, and 26.

Compared to the control groups, percent fat digested was significantly decreased during week 6 in 9% males and females, during week 12 in 3% and 9% males, during week 18 in 9% males and females, and during week 24 in all exposed groups of males and females. Calcium absorption was significantly increased in 9% females during weeks 12 and 24. Fecal weight was significantly increased in 3% and 9% males and females during each collection period, and in 1% females during weeks 12, 18, and 24. Fecal moisture was significantly increased in 9% males (up to 170%) and 9% females at all time points, in 3% males during week 6, and in 3% females during weeks 12 and 18.

Results of this study did not support chitosan as a cause of bone resorption. Significant elevation of parathyroid hormone levels occurred occasionally and inconsistently, while calcium levels remained relatively stable. Bone calcium, bone length, and the histology findings did not indicate calcium loss from the bone following chitosan exposure.

The absolute and relative liver weights of 9% males and females and the absolute and relative thymus weights of 3% males and 9% males and females were significantly less than those of the control groups.

There was a treatment-related decrease in the incidence of periportal fatty change in the liver of 9% females relative to the control group. A decreased incidence of periportal fatty change was observed in the liver of 9% males relative to the control group as well, but this decrease was not significant, and it was the same as that observed in 1% males. The appearance of periportal fatty change was similar in both males and females and in both exposed and control groups.

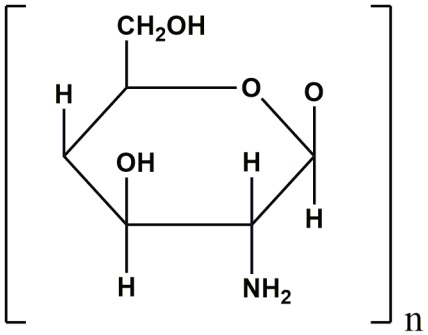
Under the conditions of the 6-month feed study of chitosan, male and female rats fed 3% and 9% chitosan in the diet had significantly decreased levels of serum vitamin A and serum and hepatic vitamin E and increased levels of serum $1,25(OH)_2$ vitamin D. Consumption of high levels of chitosan decreased percentage fat digestion and increased fecal weight and moisture, as well as reduced levels of phosphorous, cholesterol, and triglycerides. Female rats exposed to 9% chitosan also had significant liver weight and histologic changes. Based on the above results, the

lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats.

Summary of Findings Considered to be Toxicologically Relevant in Sprague Dawley Rats Exposed to Chitosan in Feed for 6 Months

	Male Rats	Female Rats
Concentrations in feed	0%, 1%, 3%, 9%	0%, 1%, 3%, 9%
Survival rates	Group A: 10/10, 10/10, 10/10, 10/10 Group B: 9/10, 10/10, 10/10, 8/10 Group C: 10/10, 10/10, 10/10, 10/10	Group A: 10/10, 10/10, 10/10, 10/10 Group B: 10/10, 10/10, 9/10, 10/10 Group C: 10/10, 9/10, 10/10, 10/10
Body weights	Exposed groups similar to the control group	Exposed groups similar to the control group
Clinical findings	None	None
Clinical pathology	↓ Phosphorus ↓ Cholesterol ↓ Triglycerides	↓ Phosphorus ↓ Cholesterol ↓ Triglycerides
Vitamin concentrations	↓ Serum vitamin A ↑ Serum 1,25$(OH)_2$ vitamin D ↓ Serum vitamin E ↓ Hepatic vitamin E	↓ Serum vitamin A ↑ Serum 1,25$(OH)_2$ vitamin D ↓ Serum vitamin E ↓ Hepatic vitamin E
Digestive parameters	↓ Percent fat digested ↑ Fecal weight ↑ Fecal moisture	↓ Percent fat digested ↑ Fecal weight ↑ Fecal moisture ↑ Calcium absorbed
Bone parameters	None	None
Reproductive toxicity	None	Not determined
Organ weights	↓ Absolute and relative liver weights ↓ Absolute and relative thymus weights	↓ Absolute and relative liver weights ↓ Absolute and relative thymus weights
Nonneoplastic effects	None	Liver: periportal, fatty change (7/10, 4/10, 4/10, 0/10)

INTRODUCTION



CHITOSAN

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Chemical Formula: $(C_6H_{11}NO_4)_n$

Synonyms: 2-Amino-2-deoxy-beta-D-glucosamine; deacetylated chitin; poliglusam; poly (D-glucosamine)
Trade names: Celox, Chicol, Chitopearl, CTFA 04299, Flonac N, Kytex H, Sea Cure F

CHEMICAL AND PHYSICAL PROPERTIES

Chitosan is a cationic carbohydrate polymer that is commercially derived from the deacetylation of chitin. The primary unit of the chitosan polymer is D-glucosamine. Chitosan exists in multiple forms that can differ in molecular weight [3 to 3,600 kilodaltons (kDa)] and in the degree of deacetylation (40% to 100%) (Kean and Thanou, 2010). Chitosan is defined as chitin that is sufficiently deacetylated to form soluble amine salts. Solubility in aqueous, acidic media occurs when deacetylation of chitin reaches approximately 50% (Rinaudo, 2006). In addition to the degree of deacetylation, chitosan solubility is also dependent on the molecular weight and the distribution of the remaining acetyl groups on the polymer (Kubota and Eguchi, 1997). Chitosan is insoluble in alkaline solutions at pH levels above 6.5. Chitosan products are highly viscous, resembling natural gums (Peniston and Johnson, 1980).

PRODUCTION, USE, AND HUMAN EXPOSURE

Chitin, from which chitosan is derived, is a naturally occurring carbohydrate polymer second only to cellulose in abundance. Chitin is a structural component found in the exoskeleton of arthropods and in the cell walls of fungi and yeast (Rinaudo, 2006). The primary unit of chitin, *N*-acetyl-D-glucosamine, forms the polymeric structure via 1→4 glycosidic bonds. Discarded crab and shrimp shells from the seafood industry are the primary source material of chitin for the commercial production of chitosan (Hirano, 1996). For chitosan production, seafood shells are deproteinized by treatment with an aqueous 3% to 5% sodium hydroxide (NaOH) solution. The resulting product is

neutralized and calcium is removed by treatment with an aqueous 3% to 5% hydrochloric acid (HCl) solution at room temperature resulting in a white or slightly pink precipitate of chitin. The *N*-deacetylation of chitin is done by treatment with an aqueous 40% to 45% NaOH solution, and the precipitate is washed with water. The precipitate is then dissolved in aqueous 2% acetic acid and the insoluble material is removed. The resulting clear supernatant solution is neutralized with aqueous NaOH solution producing chitosan as a white precipitate.

Chitosan is used in a wide range of products including use as a flocculating agent for water and waste treatment and as a chelating agent for removal of traces of heavy metals from aqueous solutions (Peniston and Johnson, 1980). In agriculture, chitosan is used as a plant growth regulator through foliar application and as an antimicrobial agent and a time-release reservoir for fertilizers in soil amendments.

Chitosan has several current or proposed biomedical applications. Chitosan is considered to be hemostatic due to its cationic nature. As such, wound dressings manufactured from chitosan are available for clinical use (Wedmore *et al.*, 2006). Several drug delivery systems based on chitosan nanoparticles are currently being investigated. Chitosan nanoparticles are capable of permeating the blood brain barrier, and the mucoadhesive properties of chitosan have been shown to enhance drug absorption (Rinaudo, 2006; Songjiang and Lixiang, 2009). Chitosan has also been evaluated for the manufacture of ocular bandage lenses and biodegradable surgical and dental implants (Felt *et al.*, 1998).

In cosmetics, chitosan is used in a variety of hair and skin products, including hair and body washes, coloring shampoos, and agents for skin cleaning and protection (Lang *et al.,* 1985). Chitosan has also been evaluated for use as an additive to toothpaste for prevention of enamel erosion (Carvalho and Lussi, 2014).

As a dietary supplement, chitosan is marketed and sold in weight-loss products, but the mechanism behind chitosan-induced inhibition of fat digestion is not well understood. It has been proposed that chitosan acts as a weak anion exchanger and decreases intestinal cholesterol absorption while also increasing the excretion of bile acids (Ebihara and Schneeman, 1989; Gallaher *et al.*, 2000; Liu *et al.*, 2008). Another possible mechanism is that chitosan traps fat in the intestines by increasing the viscosity of the intestinal contents and preventing the hydrolysis of triglycerides (Ikeda *et al.*, 1993; Kanauchi *et al.*, 1995; Liu *et al.*, 2008). The manufacturer-recommended consumption of chitosan as a weight-loss product in humans typically averages 1,000 mg per day, or approximately 14.3 mg/kg per day (based on a 70 kg adult) (GNC, 2015; Vitamin World, 2015). There are no available dose or prevalence data for human consumption of chitosan as a dietary supplement.

ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION, AND TOXICOKINETICS

The systemic absorption and distribution of chitosan following oral exposure are likely influenced by the molecular weight of the polymer. The effect of molecular weight on chitosan absorption has been evaluated in male Sprague Dawley rats. Oral gavage administration of chitosan with molecular weights of 3.8, 7.5, 13, 22, or 230 kDa resulted

in maximum plasma chitosan concentrations (C_{max}) of 20.23, 9.30, 5.86, 4.32, or less than 0.5 µg/mL, respectively (Chae *et al.*, 2005). The results of this study suggest that the absorption of chitosan from the gastrointestinal tract following oral exposure is inversely related to chitosan molecular weight, as there is likely low bioavalability associated with the higher molecular weight chitosan polymers.

The biodegradation of chitosan influences absorption and distribution because both are dependent on molecular weight. The biodegradation of chitosan *in vivo* is dependent on the degree of deacetylation (Yang *et al.*, 2007). Enzymatic degradation of chitosan depends on the ability to hydrolyze glucosamine-glucosamine, glucosamine-*N*-acetyl-glucosamine and *N*-acetyl-glucosamine-*N*-acetyl-glucosamine linkages (Kean and Thanou, 2010). Degradation of chitosan in vertebrates is thought to occur predominantly by lysozymes and bacterial enzymes in the colon (Kean and Thanou, 2010). While eight human chitinases have been identified with three showing enzymatic activity, their capacity to degrade chitosan has not been investigated (Funkhouser and Aronson, 2007; Kean and Thanou, 2010).

TOXICITY

Experimental Animals

The acute toxicities of chitosan and chitosan oligomers prepared by enzymatic depolymerization of chitosan have been evaluated. Hirano (1996) reported the oral LD_{50} for chitosan as 16 g/kg body weight in mice. No clinical signs of toxicity were observed following a single oral administration of chitosan oligomers up to 10 g/kg in male and female Kunming strain mice (Qin *et al.*, 2006).

No significant differences in weight gain were observed between exposed male Charles River albino rats and the controls in a 4-week study with 1% or 5% dietary chitosan (Vahouny *et al.*, 1983). In male Wistar rats, no significant differences in growth, feed intake, liver weight, or dried fecal weight were observed between control and chitosan-fed (2% or 5%) animals after 21 days (Fukada *et al.*, 1991). In male Sprague Dawley rats fed chitosan in the diet for 8 weeks, no toxicity was observed in animals at concentrations up to 5%, progressive growth reductions and clinical pathology disturbances occurred at 10% and 15%, and enlargement of the liver and kidneys was observed at 15% (Landes and Bough, 1976).

In female BALB/c mice fed a 5% (4.4 ± 0.7 g/day per animal) chitosan diet for 4 weeks, body weight reduction correlated with significantly decreased feed consumption and alterations in normal gut flora (Tanaka *et al.*, 1997).

In a study to evaluate mineral and fat-soluble vitamin status in male Charles River Japan Sprague Dawley rats, exposure to a diet containing 5% chitosan for 2 weeks caused a decrease in mineral absorption and bone mineral content (Deuchi *et al.*, 1995a). Decreased serum vitamin E was observed in rats fed 5% chitosan with ascorbic acid

supplementation in the diet. Serum vitamin E depletion was not observed in rats given glucosamine instead of chitosan.

Depletion of fat-soluble vitamins has been associated with a variety of neurologic and metabolic disorders. Male C57BL/6 mice fed a vitamin E-deficient diet showed signs of cognitive decline after 3 months of exposure and had increased lipid peroxidation products in brain tissue after 6 months of exposure (Fukui *et al.*, 2015). Male rats fed a vitamin A-deficient diet for 3 months had lower levels of serum cholesterol, HDL-cholesterol, and triacylglycerol, as well as decreased synthesis of liver fatty acids (Oliveros *et al.*, 2007).

The toxicity of glucosamine oligomers has been evaluated in male and female Charles River Japan F344 rats fed 0%, 0.04%, 0.2%, or 1% oligoglucosamine in the diet for 90 days (Naito *et al.*, 2007). Glucosamine oligomers are prepared by hydrolysis of chitosan and, similar to the chitosan utilized in this 6-month study, are considered low molecular weight chitosan. In the 1% (653.1 mg/kg per day in males, 719.8 mg/kg per day in females) group, erythema and edema in the snout and on the forelimbs and loss of fur on the forelimbs were observed in both male and female rats. Neutrophilic infiltration in the nasal cavity was also observed in both sexes in the 1% group. These findings were considered to be caused by topical exposure to glucosamine oligomers during feeding and grooming. Decreased feed consumption and body weight gain were also observed in animals in the 1% group in this study and were thought to be the result of feeding difficulty due to the snout and forelimb lesions described above. Rats receiving 1% oligoglucosamine also displayed lower weights of the uterus, ovary, seminal vesicles, and testes (with fewer germ cells).

The intravenous administration of chitosan has been investigated due to the development of chitosan formulations for drug delivery. No adverse effects were reported in rabbits up to 60 days following intravenous administration of chitosan oligosaccharides (prepared by oxidative depolymerization of chitosan) at doses up to 8.6 mg/kg daily for 5 consecutive days (Hirano *et al.*, 1991). In this study, increased lysozyme activity was observed in rabbit serum collected the day after the last intravenous injection. Chemical modifications and nanoparticle suspensions of chitosan are currently being investigated for drug delivery (Kean and Thanou, 2010). As such, modifications made to chitosan could alter the toxicity of the unmodified chitosan polymer.

No adverse effects of chitosan were reported in eye or skin irritation tests in rabbits or guinea pigs, respectively (Rao and Sharma, 1997).

Humans

Studies designed to evaluate the effectiveness of chitosan as a weight loss supplement suggest that chitosan is well tolerated in humans. No adverse effects were reported in male (4.5 g chitosan per day) or female (2.5 g per day) volunteers following oral chitosan administration for 12 days (Gades and Stern, 2003, 2005). Additionally, no adverse

effects were reported following oral administration of chitosan at up to 6.75 g per day for 8 weeks in male and female volunteers (Tapola *et al.*, 2008).

CARCINOGENICITY

No 2-year carcinogenicity studies of chitosan were identified in the available literature.

Carcinogenicity and chronic toxicity have been evaluated for *N*-acetyl-D-glucosamine, a monomeric constituent of chitosan. F344 rats administered *N*-acetyl-D-glucosamine at concentrations up to 5% in the diet (1,935 mg/kg per day in males and 2,244 mg/kg per day in females) for 104 weeks had no associated increases in tumor response (Takahashi *et al.*, 2009). In a second study in F344 rats, administration of *N*-acetyl-D-glucosamine in feed at concentrations up to 5% in the diet (2,323 mg/kg per day in males and 2,545 mg/kg per day in females) for 52 weeks did not induce an increase in tumor response (Takahashi *et al.*, 2009).

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY

A limited number of developmental and reproductive toxicity studies were identified in the literature.

In a multigenerational prenatal and postnatal assessment of high molecular weight chitosan (HMWCS), F_0 time-mated ICR mice were administered 0, 125, 500, or 2,000 mg/kg HMWCS via a single intraperitoneal injection on gestational day 6 (GD 6) and subjected to a laparotomy or allowed to litter (Cheng *et al.*, 2013). F_1 offspring (1 mouse/sex per litter) from the same exposure group were mated and females similarly subjected to either a laparotomy or allowed to litter to produce an F_2 generation. F_0 dams in the 2,000 mg/kg group exhibited signs of maternal toxicity (mortality and diarrhea). F_0 dams in the 500 and 2,000 mg/kg groups displayed dose-dependent increases in vaginal bleeding, postimplantation loss, and lower spleen weights. Fetal weights for both generations were lower in the 2,000 mg/kg group. There were no external, visceral, or skeletal malformations attributed to chitosan administration. F_0 dams allowed to litter displayed a dose-related reduction in litter size. F_1 mice exposed *in utero* to 2,000 mg/kg HMWCS and examined on postnatal day 21 (PND 21) exhibited higher uterus, ovary, and thymus weights. Female F_1 mice exposed *in utero* to 2,000 mg/kg HMWCS displayed lower thymus weights on PND 56. F_2 mice exposed *in utero* to 2,000 mg/kg HMWCS displayed lower testis and ovary weights on PNDs 21 and 56.

Chitosan oligomers did not induce morphologic sperm abnormalities in male mice following oral gavage daily for 5 days with up to 5,000 mg/kg (Qin *et al.*, 2006).

The effects of chitosan nanoparticles (spherical; 200 ± 6 nm or 340 ± 10 nm diameter) have been examined in zebrafish (*Danio rerio*) embryos. Embryos exposed 4 to 5 hours after fertilization to 0, 5, 10, 20, 30, or 40 µg/mL (200 nm particles) or 0, 10, 20, or 40 µg/mL (340 nm particles) displayed concentration-dependent decreases in hatching rates and increases in mortality 96 hours after exposure (Hu *et al.*, 2011). Increased rates of cell death and

reactive oxygen species production were observed in all exposure groups. Exposure to 200 nm, but not 340 nm, chitosan nanoparticles induced developmental malformations in embryos, including bent spines, pericardial edema, and opaque yolks.

GENETIC TOXICITY

No *in vitro* or *in vivo* studies evaluating chitosan for mutagenic effects were identified in the available literature.

Chitosan oligomers were negative at concentrations up to 5,000 µg/plate in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 with and without rat liver S9 metabolic activation enzymes, and they were negative for micronucleus induction in mouse bone marrow following oral gavage for 2 days at up to 5,000 mg/kg (Qin *et al.*, 2006).

STUDY RATIONALE

Chitosan was nominated for study by the National Cancer Institute due to widespread human exposure, especially through use as a dietary supplement for body weight reduction, and for concerns regarding potential vitamin E and bone mineral depletion following ingestion. The NTP conducted a 6-month study evaluated the effects of dietary chitosan on the development of osteopenia/osteoporosis, fat and calcium absorption, fat-soluble vitamin depletion, and general toxicity effects in Charles River Sprague Dawley rats.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF CHITOSAN

Chitosan was obtained from Vanson HaloSource, Inc. (Redmond, WA), in one lot (02-ASSF-0715), which was used in the 6-month study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (MRI) (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH) (Appendix F). Reports on analyses performed in support of the chitosan studies are on file at the National Institute of Environmental Health Sciences.

The test article, an off-white powder, was identified using infrared and proton nuclear magnetic resonance (NMR) spectroscopy. The percentage of deacetylation of the test article, determined by proton NMR, ranged from 85.97% to 87.17%, with an average of 86.5%. All spectra were consistent with the literature spectra (Domard and Rinaudo, 1983; Hirai *et al.*, 1991), and with the Sadtler spectral database.

The moisture content for lot 02-ASSF-0715 was determined using weight loss on drying, the inorganic content was determined on the dried test article by ashing, viscosity was determined using a Brookfield viscometer, and the most abundant molecular weight was determined using gel permeation chromatography (GPC) with refractive index (RI) detection.

Moisture content was 4.50% water, the average inorganic content was 2.13%, and viscosity was 81.3 centipoise. GPC/RI indicated one major peak and the determined molecular weight of the bulk chemical ranged from 62,755 to 87,343 daltons (Da). This resulted in an average molecular weight of 81,644 g/mol, or approximately 82 kDa, classifying the test article as a low molecular weight chitosan (LMWCS). A sample of lot 02-ASSF-0715 was submitted to Covance Laboratories, Inc. (Madison, WI), for nutritional and contaminant testing using standard methods. Levels of organochlorine and organophosphorous pesticides, nitrosamines, and aflatoxins were below the detection limits of the analytical methods. The purity of lot 02-ASSF-0715 was estimated to be approximately 94% based on the analysis of moisture and inorganic content. Taken together, these data indicated that the test article was chitosan.

To ensure stability, the test article was stored in sealed amber glass vials at room temperature. Reanalysis of the test article was performed during the study using GPC/RI and no degradation of the test article was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately monthly by mixing chitosan with feed. Dose formulations were stored in lined plastic buckets sealed with lids and stored at –30° C to –15° C for up to 42 days.

Homogeneity studies of approximately 0.5% and 9% formulations (5,046 and 90,049 µg/g, respectively) and stability studies of an approximately 0.5% (5,046 µg/g) formulation were performed by the analytical chemistry laboratory using GPC/RI. Two peaks were attributed to chitosan with retention times of approximately 6.9 minutes and 12.1 minutes, respectively. Chitosan quantitation was based on the larger polymeric components of the first peak only because vehicle components co-eluted with the later oligomeric peak. Homogeneity studies of 1% and 9% (10 and 90 mg/g in feed, respectively) dose formulations were performed by the study laboratory using GPC/RI. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in lined plastic buckets sealed with lids at temperatures up to room temperature and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of chitosan were performed by the study laboratory using GPC/RI. Of the dose formulations analyzed, all nine were within 10% of the target concentrations (Table F3). Animal room samples were also analyzed; all three were within 10% of the target concentrations.

ANIMAL SOURCE

Male and female Sprague Dawley [Crl:CD(SD)] rats were obtained from Charles River Laboratories (Portage, MI) for use in the 6-month study.

ANIMAL WELFARE

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

6-MONTH STUDY

The rats were 5 to 6 weeks old upon receipt. Rats were quarantined for 12 to 15 days and were 7 to 9 weeks old on the first day of the study. Before the study began, five male and five female rats were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix I). A positive test result for parvovirus

occurred in one animal at the 4-week timepoint. Additional testing of serum from this animal and other sentinel animals via other testing methodologies deemed the original positive result to be a false positive. All other test results were negative for rodent pathogens.

The animals in this study were split into three groups, the core group, Group A, and two special study groups, Groups B (vitamin and bone analysis) and C (fat digestion, hematology, clinical chemistry, and urinalysis). Different parameters were evaluated in each group, which allowed for the collection of extensive endpoints (Table 1). Groups of 10 male and 10 female rats were examined per endpoint and there was no crossover of analyses between any of the groups. Group A rats were fed diets containing 0%, 1%, 3%, or 9% chitosan for 25 weeks. Groups B and C rats were fed diets containing the same concentrations for up to 26 weeks. Feed and water were available *ad libitum*. The AIN-93M diet was used for this study instead of the NTP-2000 diet because of the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 diet. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7% to 8%) than the AIN-93M feed (4%) (Reeves *et al.*, 1993; Rao, 1997; Reeves, 1997). One of the primary rationales for this chitosan study was the potential for decreases in fat-soluble vitamin concentrations, and therefore, utilizing a diet with lower levels of preexisting vitamins and a lower fat content was ideal to avoid confounding potential results. Rats were housed individually. Feed consumption was recorded weekly for core study rats. Core study rats were weighed and clinical findings were recorded initially, on day 8, weekly thereafter, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 2.

TABLE 1
Distribution of Evaluated Parameters

	Group		
Parameter	**A**	**B**	**C**
Feed consumption	**X**		
Body weights	**X**		
Clinical findings	**X**		
SMVCE	**X**		
Bone histomorphometry	**X**		
Gross lesions and histopathology	**X**		
Vitamin A (serum and liver)		**X**	
Vitamin E (serum and liver)		**X**	
$1,25(OH)_2$ Vitamin D (serum)		**X**	
Bone calcium, ash, and moisture		**X**	
Hematology			**X**
Clinical chemistry			**X**
Vitamin K_1 (plasma and liver)			**X**
Feed and fecal analysis			**X**
Urinalysis			**X**

On the first day of weeks 7, 13, 19, and 26, blood was collected from all Group B rats via the retroorbital plexus under CO_2/O_2 anesthesia for determination of vitamins A, E, and $1,25(OH)_2$ vitamin D concentrations. Blood was collected into tubes, allowed to clot, and centrifuged. Sera were stored at approximately –70° C until analysis. Blood samples for vitamin K_1 concentrations in Group C rats, collected into tubes containing EDTA at the same time as hematology collections, were centrifuged; the plasma was harvested, snap frozen, and stored at –70° C protected from light. At study termination (week 26), liver samples were collected from surviving Group B and C rats, processed, and stored frozen for determination of vitamins A and E (Group B) or vitamin K_1 (Group C) concentrations. Blood and liver samples were analyzed by high performance liquid chromatography for vitamins A and E (Covance Laboratories, Inc.), by competitive enzyme immunoassay for $1,25(OH)_2$ vitamin D (Antech Diagnostics, Morrisville, NC), or by gas chromatography/mass spectrometry for vitamin K_1 (Analytics, Inc., Gaithersburg, MD). Because most values for vitamin K_1 were below the limit of quantitation, the results are not presented in this Toxicity Study Report.

For 8 days beginning during weeks 6, 12, 18, and 24, Group C rats were placed in metabolism cages (Nalgene Company, Rochester, NY) for fecal and urine collection. During collection periods, rats were allowed control or dosed feed and water *ad libitum*, and feed samples were collected. Feces were collected for a period of 8 days, with each day's collection being combined with previous days' collection and stored at approximately –20° C. Feces were stored at –70° C after each collection period until shipping to Covance Laboratories, Inc., on dry ice for analyses of calcium, fat, and moisture; the feed samples were also sent for analysis. Fat content in feed and feces was determined gravimetrically by Soxhlet extraction. Feed consumption, fat intake [(total feed consumed per interval) × (% fat in feed/100)], and fat excretion [fecal weight × (% fecal fat/100)], were calculated to estimate fat digestion: {[(fat intake – fat excreted in feces)/fat intake] × 100}. Calcium concentrations in feed and feces were determined using inductively coupled plasma emission spectrometry. Moisture was determined by weight loss upon drying. Urine was collected on ice for each Group C rat over a 24-hour period during the last day in the metabolism cage and coincided with the last day of fecal collection. Total urine collected was transferred to centrifuge tubes and the volume was recorded. Urine creatinine was measured using a Hitachi 911™ chemistry analyzer (Roche Diagnostics, Indianapolis, IN), and deoxypyridinoline was measured using a Metra Total DPD Enzyme Immunoassay Kit (Quidel, San Diego, CA).

On the last day in the metabolism cage, at the beginning of weeks 7, 13, 19, and 25, blood was collected from all Group C rats via the retroorbital plexus under CO_2/O_2 anesthesia for hematology (week 25 only) and clinical chemistry. Blood samples for hematology were collected in tubes containing EDTA as an anticoagulant. Hematology parameters were determined using an Advia 120 hematology analyzer (Bayer Diagnostics Division, Tarrytown, NY). Blood for clinical chemistry determinations was collected in tubes without anticoagulant, allowed to clot, and centrifuged and then the serum was harvested. Except as noted, clinical chemistry parameters were determined using a Hitachi 911™ chemistry analyzer (Roche Diagnostics). For osteocalcin and parathyroid hormone, serum was stored frozen at –20° C until analysis. Serum osteocalcin was measured using a Rat-MID™ Osteocalcin ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark). Serum parathyroid hormone was measured using an Intact PTH Enzyme Immunoassay Kit (ALPCO Diagnostics, Salem, NH).

At study termination (week 26), right and left femurs were collected from the Group B rats for determination of calcium, ash, and moisture. Covance Laboratories, Inc., determined bone moisture by measuring weight loss upon drying, calcium by inductively coupled plasma emission spectrometry, and ash gravimetrically.

At the end of the study (week 25), samples were collected for sperm motility and vaginal cytology evaluations on Group A rats. The parameters evaluated are listed in Table 2. Due to inconsistent sample collection and slide staining, an assessment of estrous cyclicity could not be made. Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all Group A animals at study termination (week 25). The heart, right kidney, liver, lung, right ovary, parathyroid gland, right testis, thymus, thyroid gland and parathyroid gland together, and uterus were weighed. Both tibias and both femurs were collected; the lengths of both tibias and the left femur were measured. The right tibia and femur were dehydrated in ethanol (70% to 100%) and infiltrated with glycol methacrylate. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes were first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed by the study laboratory pathologist on 0% and 9% rats. The kidney and liver of males and females and the parathyroid gland and prostate gland of males were examined in all exposure groups. Table 2 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to a NTP Pathology Peer Review (PPR) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologists were resolved by the NTP pathology peer review process. Final diagnoses for reviewed lesions represent a consensus of the PPR or a consensus between the study laboratory pathologist, NTP pathologist, QA pathologist(s), and the PPR coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

TABLE 2
Experimental Design and Materials and Methods in the 6-Month Feed Study of Chitosan

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Strain and Species
Charles River Sprague Dawley [Crl:CD(SD)] rats

Animal Source
Charles River Laboratories (Portage, MI)

Time Held Before Study
Group A (core study): 14 (males) or 15 (females) days
Groups B and C (special studies): 12 (males) or 13 (females) days

Average Age When Study Began
7 to 8 weeks (Group A males and Groups B and C males and females)
8 to 9 weeks (Group A females)

Date of First Exposure
Group A: August 31 (males) or September 1 (females), 2006
Groups B and C: August 29 (males) or 30 (females), 2006

Duration of Exposure
Group A: 25 weeks
Groups B and C: 26 weeks

Date of Last Exposure
Group A: February 15 (males) or 16 (females), 2007
Groups B and C: February 20 (males) or 21 (females), 2007

Necropsy Dates
Group A: February 15 (males) or 16 (females), 2007
Groups B and C: February 20 (males) or 21 (females), 2007

Average Age at Necropsy
32 to 33 weeks (Group A females and Groups B and C males and females)
31 to 32 weeks (Group A males)

Size of Study Groups
10 males and 10 females

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
1

Method of Animal Identification
Tail tattoo

Diet
AIN-93M maintenance purified meal diet (Purina TestDiet, Richmond, IN), available *ad libitum*, changed twice weekly

Water
Tap water (Columbus, OH municipal supply) via automatic watering system (Edstrom Industries, Inc. Waterford, WI), available *ad libitum*

Cages
Polycarbonate solid-bottom (Lab Products, Inc., Seaford, DE), changed weekly, rotated in rack every 2 weeks

TABLE 2
Experimental Design and Materials and Methods in the 6-Month Feed Study of Chitosan

Bedding
Irradiated hardwood bedding chips (P.J. Murphy Forest Products Corporation, Montville, NJ), changed weekly

Rack Filters
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks

Racks
Stainless steel (Lab Products, Inc), changed and rotated every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: 10/hour

Exposure Concentrations
0%, 1%, 3%, and 9% in feed, available *ad libitum*

Type and Frequency of Observation
Observed twice daily; Group A rats were weighed and clinical findings were recorded initially, on day 8, weekly thereafter, and at the end of the study. Feed consumption was recorded weekly for Group A rats and during fecal collection periods for Group C rats.

Method of Euthanasia
100% Carbon dioxide

Necropsy
Necropsies were performed on all Group A rats at the end of the study (week 25). Organs weighed were heart, right kidney, liver, lung, right ovary, parathyroid gland, right testis, thymus, thyroid gland and parathyroid gland together, and uterus. Lengths of both tibias and the left femur were measured.

Clinical Pathology
Blood was collected via the retroorbital plexus from all Group C rats on the first day of weeks 7, 13, 19, and 25 for hematology (week 25 only) and clinical chemistry. Urine was collected from Group C rats for 24 hours beginning the last day of weeks 6, 12, 18, and 24.
Hematology: hematocrit (auto and manual); hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials
Clinical chemistry: urea nitrogen, creatinine, calcium, phosphorous, total protein, albumin, cholesterol, triglycerides, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, bile acids, total osteocalcin, and parathyroid hormone
Urinalysis: creatinine, volume, and deoxypyridinoline

Histopathology
Histopathology was performed on 0% and 9% Group A rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone (left femur and tibia) with marrow, brain, clitoral gland, esophagus, eye, Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung (with mainstem bronchus), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis (with epididymis), thymus, thyroid gland, trachea, urinary bladder, and uterus. The kidney and liver of males and females and the parathyroid gland and prostate gland of males were also examined in the 1% and 3% groups.

Sperm Motility
At the end of the study, sperm samples were collected from male Group A rats for sperm count and motility evaluations. The following parameters were evaluated: spermatid heads per gram testis and per testis, spermatid heads per gram cauda and per cauda, and epididymal spermatozoal motility. The left cauda, left epididymis, and left testis were weighed.

TABLE 2
Experimental Design and Materials and Methods in the 6-Month Feed Study of Chitosan

Digestion Studies Feces were collected from Group C rats for 8 days beginning weeks 6, 12, 18, and 24 and analyzed for calcium, fat, and moisture. Fecal calcium and fat content were compared to that in feed samples collected during the same time period to produce values for fat digested and calcium absorbed.
Serum and Hepatic Vitamins Blood was collected from the retroorbital plexus of Groups B and C rats on the first day of weeks 7, 13, 19, and 25 (Group C), and 26 (Group B). At study termination (week 26), liver samples were collected from Groups B and C rats. Blood and liver samples were analyzed for vitamins A, E, $1,25(OH)_2$ D, and/or K_1.
Bone Analysis At study termination (week 26), right and left femurs were collected from Group B rats, and calcium, ash, and moisture levels were measured.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendix A as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al*., 1979), a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, urinalysis, serum and liver vitamin concentrations, digestive and bone parameters, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis.

QUALITY ASSURANCE METHODS

The 6-month study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 6-month study were submitted to the NTP Archives, this study was audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study

Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Study Report.

RESULTS

6-MONTH STUDY

All male and female Group A rats survived to the end of the study (Table 3); however, five rats from Groups B and C died, often after seizures that occurred near the time of blood collection, with the cause of death undetermined. There were no treatment-related clinical findings in Group A animals, although 13 animals from Groups B and C (10 from the 9% group, one from the 3% group, and two from the 1% group) were observed with seizures either during or after the 18-week blood collections. Seizures were not noted at any other time point. Body weights and feed consumption were measured in Group A rats, and mean body weights of exposed males and females were not significantly different from those of the control groups (Table 3 and Figure 1). Feed consumption by 3% and 9% Group A males was greater than that by the controls, but the increase may not be accurate due to observed food spillage possibly due to poor palatability resulting in feed being wasted (Table G1). Dietary concentrations of 1%, 3%, and 9% resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females, respectively.

TABLE 3
Survival, Body Weights, and Feed Consumption of Group A Rats in the 6-Month Feed Study of Chitosan[a]

Concentration	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 25
Male							
0%	10/10	238 ± 5	669 ± 20	432 ± 18		22.2	21.2
1%	10/10	243 ± 6	702 ± 21	459 ± 17	105	23.8	20.4
3%	10/10	242 ± 6	687 ± 23	445 ± 21	103	23.6	24.7
9%	10/10	243 ± 6	612 ± 17	369 ± 17	91	21.4	27.3
Female							
0%	10/10	175 ± 3	338 ± 11	162 ± 12		17.7	16.4
1%	10/10	173 ± 2	335 ± 13	162 ± 12	99	22.3	20.3
3%	10/10	177 ± 4	328 ± 11	151 ± 9	97	17.3	17.1
9%	10/10	177 ± 2	301 ± 13	124 ± 12	89	16.9	18.8

[a] Weights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.
[b] Number of animals surviving at 25 weeks/number initially in group

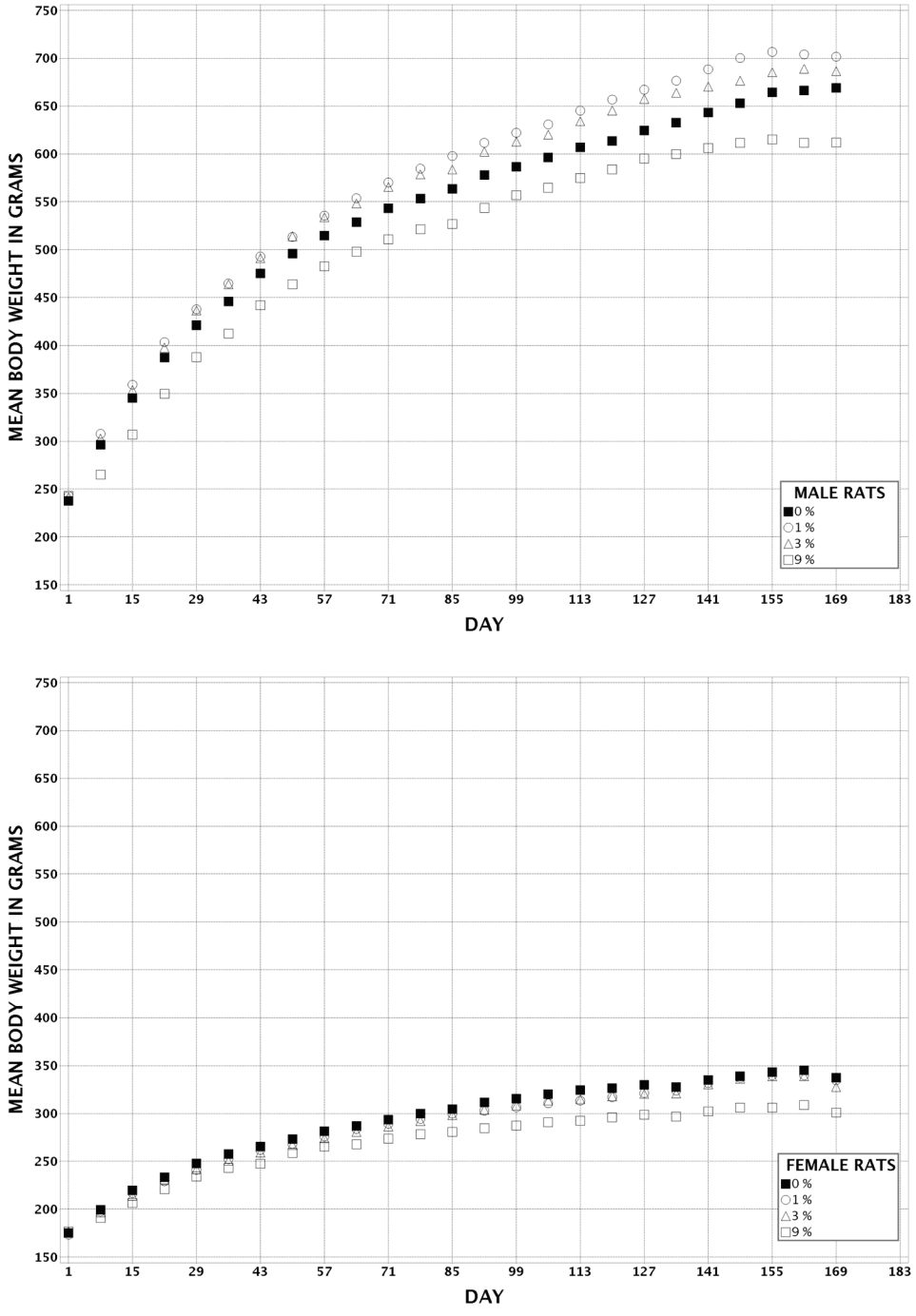

FIGURE 1
Growth Curves for Group A Rats Exposed to Chitosan in Feed for 6 Months

Hematology data for Group C rats are listed in Table B1. Compared to the control group, mild significant increases (4% to 6%) in automated hematocrit, hemoglobin concentration, mean cell volume, and mean cell hemoglobin were observed in 9% males; manual hematocrit and erythrocyte count were similar to those of the controls. These changes may be due to biological variability and are likely not toxicologically relevant. All other differences from control values in the male and female hematology data were mild or sporadic and not considered toxicologically significant.

Clinical chemistry data for Group C rats are listed in Tables 4 and B1. Both the 9% male and female rats had significantly decreased cholesterol values (26% to 48%), compared to the controls, at all time points. Triglycerides values were also significantly decreased in the 9% male (47% to 57%) and female (30%) rats, but not at every time point. Phosphorus levels were significantly decreased in the 9% male rats at weeks 13, 19, and 25 (12% to 18%); a decrease also occurred in the 3% males at week 13 (14%). Similarly, phosphorus levels were significantly decreased in the 3% and 9% females at weeks 13 (20% and 16%, respectively) and 25 (9% and 19%, respectively). A mild, but statistically significant, decrease (4%) in calcium concentration was observed in 9% males at weeks 19 and 25. Alanine aminotransferase (ALT) activity, a marker of hepatocellular injury, was mildly but significantly elevated at week 25 in the 9% male rats (104%) and in the 3% and 9% female rats (28% and 88%, respectively). However, sorbitol dehydrogenase (another marker of hepatocellular injury) was not significantly increased relative to the controls, and hepatocellular changes associated with increases in ALT were not observed microscopically. Thus, the toxicologic significance of the increases in ALT is uncertain. Urea nitrogen was mildly increased in the 9% males (23%) and females (15%) at week 25. Minimal to mild significant alterations were also observed in several other parameters. These alterations were inconsistent or within the range of biological variability.

Total osteocalcin (a marker of bone turnover) and parathyroid hormone levels were analyzed in Group C rats and were occasionally elevated throughout the study. Total osteocalcin was significantly elevated in the 9% males (38%) at week 25, while parathyroid hormone levels were significantly elevated in 9% males (96%) at week 19 and in 9% females (56%) at week 25 (Tables 4 and B1).

Urine deoxypyridinoline/creatinine ratios were calculated at weeks 7, 13, 19, and 25 for both males and females in Group C and were mostly unchanged (Tables 4 and B1). A significant increase, compared to the control group, occurred at week 25 in the 9% males (28%). In females, minimal increases and decreases occurred inconsistently across all time points with a significant increase at week 7 in the 9% group (42%) and significant decreases at weeks 13 (26%) and 19 (20%) in the 1% group compared to controls.

To calculate the deoxypyridinoline/creatinine ratios, urine volume, urine creatinine concentrations, and urine deoxypyridinoline concentrations were measured at weeks 7, 13, 19, and 25. Urine volume was significantly decreased in various male and female exposure groups throughout the study, but most consistently in the 9% chitosan group (approximately 40% to 60%). Increases in urine creatinine concentration tended to parallel the decreases in urine volume indicating proper kidney function.

TABLE 4
Selected Clinical Chemistry and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
Clinical Chemistry				
n	10	10	10	10
Calcium (mg/dL)				
Week 13	12.6 ± 0.1	12.5 ± 0.1	12.3 ± 0.2	12.4 ± 0.2
Week 19	12.5 ± 0.1	12.3 ± 0.2	12.3 ± 0.1	12.0 ± 0.1*
Week 25	12.1 ± 0.1	12.1 ± 0.2	12.0 ± 0.1	11.6 ± 0.1*
Phosphorus (mg/dL)				
Week 13	8.4 ± 0.3	8.1 ± 0.3	7.2 ± 0.3**	7.4 ± 0.4*
Week 19	8.2 ± 0.4	7.7 ± 0.2	7.4 ± 0.3	6.7 ± 0.2**
Week 25	6.9 ± 0.3	6.8 ± 0.2	6.7 ± 0.1	5.8 ± 0.3**
Cholesterol (mg/dL)				
Week 7	82 ± 5	75 ± 8	80 ± 6	53 ± 3**
Week 13	95 ± 7	84 ± 8	90 ± 7	53 ± 2**
Week 19	101 ± 6	87 ± 10	94 ± 8	59 ± 4**
Week 25	95 ± 6	81 ± 8	90 ± 6	49 ± 4**
Triglycerides (mg/dL)				
Week 7	202 ± 28	234 ± 43	226 ± 30	88 ± 15*
Week 13	198 ± 33	202 ± 38	195 ± 24	86 ± 8**
Week 19	180 ± 26	218 ± 43	210 ± 29	95 ± 13*
Week 25	173 ± 18	207 ± 30	218 ± 24	109 ± 13
Alanine aminotransferase (IU/L)				
Week 25	28 ± 3	29 ± 2	29 ± 1	57 ± 2**
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	15 ± 1	14 ± 1
Total osteocalcin (ng/mL)				
Week 7	445.7 ± 17.2	439.8 ± 15.8	441.8 ± 18.2	520.4 ± 22.6
Week 13	306.2 ± 13.0	289.7 ± 28.6	245.4 ± 37.9	372.6 ± 23.4
Week 19	239.4 ± 12.4	225.7 ± 10.6	181.6 ± 26.8	269.2 ± 20.9
Week 25	158.3 ± 10.0	168.1 ± 11.6	145.9 ± 22.7	218.3 ± 14.6*
Parathyroid hormone (ng/mL)				
Week 7	1.882 ± 0.137	1.643 ± 0.449	1.838 ± 0.348	1.521 ± 0.368
Week 13	2.343 ± 0.350	2.763 ± 0.479	3.215 ± 0.537	2.433 ± 0.222
Week 19	1.879 ± 0.186	3.101 ± 0.475	2.710 ± 0.365	3.679 ± 0.361**
Week 25	2.668 ± 0.475	2.924 ± 0.276	3.981 ± 0.349	2.848 ± 0.506
Urinalysis				
n				
Week 7	10	9	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	10	10	10
Volume (mL)				
Week 7	8.3 ± 0.8	7.5 ± 1.5	6.4 ± 0.8	5.0 ± 1.1*
Week 13	7.9 ± 1.0	4.6 ± 0.3**	5.1 ± 0.4*	4.5 ± 0.5**
Week 19	10.7 ± 1.6	4.0 ± 0.4**	5.3 ± 0.7*	5.6 ± 0.6
Week 25	8.6 ± 1.2	5.4 ± 0.6*	6.1 ± 0.8*	5.1 ± 0.6**
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.810 ± 0.135	1.889 ± 0.148	1.810 ± 0.159	1.920 ± 0.160
Week 13	0.910 ± 0.035	0.890 ± 0.031	0.930 ± 0.040	0.960 ± 0.078
Week 19	0.530 ± 0.050	0.550 ± 0.034	0.570 ± 0.042	0.660 ± 0.048
Week 25	0.430 ± 0.030	0.470 ± 0.030	0.480 ± 0.020	0.550 ± 0.027**

TABLE 4
Selected Clinical Chemistry and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female				
Clinical Chemistry				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	10
Calcium (mg/dL)				
Week 13	12.9 ± 0.2	13.1 ± 0.1	12.7 ± 0.2	12.5 ± 0.2
Week 19	12.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.1	12.5 ± 0.1
Week 25	12.7 ± 0.3	12.8 ± 0.1	12.7 ± 0.2	12.3 ± 0.2
Phosphorus (mg/dL)				
Week 13	8.1 ± 0.5	7.4 ± 0.4	6.5 ± 0.4**	6.8 ± 0.3*
Week 19	8.4 ± 0.4	8.2 ± 0.5	8.1 ± 0.3	7.4 ± 0.5
Week 25	6.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.3*	5.5 ± 0.3**
Cholesterol (mg/dL)				
Week 7	80 ± 6	81 ± 8	67 ± 4	59 ± 4**
Week 13	92 ± 8	86 ± 7	73 ± 5	58 ± 4**
Week 19	107 ± 7	105 ± 9	91 ± 8	67 ± 5**
Week 25	94 ± 7	108 ± 5	96 ± 8	63 ± 4**
Triglycerides (mg/dL)				
Week 7	88 ± 12	130 ± 48	81 ± 8	86 ± 14
Week 13	125 ± 10	163 ± 30	140 ± 23	88 ± 23*
Week 19	143 ± 15	181 ± 32	137 ± 18	90 ± 13
Week 25	188 ± 31	231 ± 44	245 ± 31	158 ± 35
Alanine aminotransferase (IU/L)				
Week 25	25 ± 3	28 ± 3	32 ± 2**	47 ± 4**
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	19 ± 2	16 ± 1
Total osteocalcin (ng/mL)				
Week 7	293.6 ± 19.4	287.5 ± 21.2	282.1 ± 34.7	316.7 ± 23.5
Week 13	197.9 ± 22.6	202.3 ± 15.4	184.4 ± 19.4	234.2 ± 14.5
Week 19	158.1 ± 18.3	184.8 ± 13.2	166.7 ± 24.7	210.1 ± 16.0
Week 25	107.9 ± 18.6	97.1 ± 7.1	96.0 ± 16.2	148.8 ± 15.1
Parathyroid hormone (ng/mL)				
Week 7	0.995 ± 0.150[b]	1.156 ± 0.176	1.092 ± 0.182	1.023 ± 0.146
Week 13	1.506 ± 0.203	1.734 ± 0.194	1.925 ± 0.306	1.767 ± 0.212
Week 19	1.406 ± 0.232	1.994 ± 0.353	1.845 ± 0.418	1.673 ± 0.223
Week 25	1.471 ± 0.189[b]	1.628 ± 0.220	1.818 ± 0.224	2.301 ± 0.212*

TABLE 4
Selected Clinical Chemistry and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female (continued)				
Urinalysis				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	9
Volume (mL)				
Week 7	8.2 ± 1.2	8.5 ± 1.0	5.4 ± 0.8	3.4 ± 0.3**
Week 13	6.4 ± 0.7	6.5 ± 0.6	4.1 ± 0.8*	2.9 ± 0.5**
Week 19	7.7 ± 1.2	8.1 ± 1.4	5.0 ± 0.8	3.4 ± 0.5**
Week 25	8.2 ± 1.5	9.1 ± 1.5	5.8 ± 0.9	3.7 ± 0.5**
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.620 ± 0.128	1.240 ± 0.129	1.940 ± 0.229	2.300 ± 0.182*
Week 13	0.580 ± 0.039	0.430 ± 0.037**	0.540 ± 0.034	0.570 ± 0.042
Week 19	0.450 ± 0.017	0.360 ± 0.016*	0.440 ± 0.016	0.520 ± 0.020
Week 25	0.340 ± 0.043	0.222 ± 0.022	0.340 ± 0.027	0.411 ± 0.026

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9

Serum and hepatic vitamin concentrations were measured in Group B rats (Tables 5 and C1). Exposure concentration-dependent decreases were observed in serum vitamin A concentrations starting at week 13 in the male rats. The decreases reached statistical significance at weeks 13 (27%), 19 (26%), and 26 (29%) in 9% males and at weeks 13 (15%) and 26 (16%) in 3% males. Females were less affected with significant decreases observed in the 9% group at weeks 19 (18%) and 26 (21%). Exposure concentration-dependent decreases were also observed in serum vitamin E concentrations in male rats at all time points. The decreases were statistically significant at all time points in 3% (33% to 42%) and 9% males (79% to 82%) and in 1% males at week 13 (17%), with the 9% group measuring between 18% to 21% that of control values throughout the study. Females were less affected with significant decreases in serum vitamin E levels observed in the 9% group (approximately 60%) only (all time points). Hepatic vitamin E concentrations were significantly decreased at week 26 in 3% and 9% males (48% and 87%, respectively) and 9% females (80%). In the 9% group, levels of hepatic vitamin E measured only 13% and 20% of control values in the males and females, respectively. Serum concentrations of $1,25(OH)_2$ vitamin D were significantly increased in 9% males (105% to 142%) and females (100% to 180%) at weeks 7, 19, and 26 compared to the control groups. Results of plasma hepatic vitamin K concentrations in Group C rats are not discussed or presented, as many samples were below the level of quantification.

TABLE 5
Serum and Hepatic Vitamin Concentration Data for Group B Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Week 7	9	10	10	10
Week 13	9	10	10	10
Week 19	9	10	10	10
Week 26	9	10	10	8
Serum vitamin A (µg/mL)				
Week 7	0.532 ± 0.021	0.506 ± 0.033	0.513 ± 0.026	0.453 ± 0.018
Week 13	0.561 ± 0.024	0.499 ± 0.019	0.476 ± 0.022*	0.410 ± 0.009**
Week 19	0.533 ± 0.028	0.506 ± 0.031	0.475 ± 0.019	0.392 ± 0.014**
Week 26	0.476 ± 0.019	0.444 ± 0.024	0.398 ± 0.017**	0.336 ± 0.026**
Serum 1,25$(OH)_2$ vitamin D (pg/mL)				
Week 7	124.4 ± 19.6	163.3 ± 21.7	183.2 ± 26.9	297.4 ± 41.0**
Week 13	70.1 ± 7.3	57.4 ± 5.3	77.3 ± 4.4	86.1 ± 8.5
Week 19	20.6 ± 2.8	21.7 ± 6.1	22.9 ± 2.2	42.3 ± 3.1**[b]
Week 26	27.7 ± 3.4[c]	28.0 ± 4.3	36.1 ± 4.6[b]	66.9 ± 11.9**
Serum vitamin E (µg/mL)				
Week 7	19.33 ± 1.43	15.38 ± 1.29	12.92 ± 0.48**	4.14 ± 0.23**
Week 13	21.08 ± 1.61	17.45 ± 1.06*	12.27 ± 0.86**	4.33 ± 0.27**
Week 19	20.59 ± 1.61	16.19 ± 0.96	12.86 ± 0.42**	4.07 ± 0.32**
Week 26	19.66 ± 1.66	17.35 ± 1.37	12.35 ± 0.61**	3.59 ± 0.65**
Liver vitamin E (µg/g)				
Week 26	66.8 ± 16.2	55.0 ± 6.8	34.6 ± 2.2**	8.5 ± 0.8**
Female				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 26	10	10	9	10
Serum vitamin A (µg/mL)				
Week 7	0.272 ± 0.011	0.253 ± 0.007	0.260 ± 0.012	0.266 ± 0.012
Week 13	0.308 ± 0.020	0.295 ± 0.011	0.309 ± 0.019	0.281 ± 0.018
Week 19	0.283 ± 0.014	0.271 ± 0.015	0.291 ± 0.012	0.231 ± 0.010*
Week 26	0.316 ± 0.015	0.302 ± 0.014	0.294 ± 0.018	0.249 ± 0.010**
Serum 1,25$(OH)_2$ vitamin D (pg/mL)				
Week 7	104.0 ± 15.1	96.7 ± 10.9	111.0 ± 8.7	208.1 ± 18.2**
Week 13	60.6 ± 7.5	60.7 ± 7.9	69.3 ± 11.0	110.1 ± 16.9
Week 19	11.6 ± 1.6	12.6 ± 1.7	15.8 ± 1.4	31.4 ± 3.2**
Week 26	19.2 ± 2.2	20.7 ± 4.2	28.6 ± 6.5	53.7 ± 5.8**
Serum vitamin E (µg/mL)				
Week 7	18.65 ± 0.71	20.08 ± 0.87	18.38 ± 0.85	6.99 ± 0.58**
Week 13	19.81 ± 1.41	20.85 ± 1.06	20.19 ± 1.20	7.48 ± 0.38**
Week 19	21.02 ± 1.76	19.74 ± 1.75	19.86 ± 1.08	7.37 ± 0.57**
Week 26	20.94 ± 1.56	23.43 ± 1.66	22.23 ± 1.75	7.28 ± 0.64**
Liver vitamin E (µg/g)				
Week 26	84.5 ± 8.9	97.1 ± 10.1	82.0 ± 11.8	17.2 ± 3.2**

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=7

Digestive parameters were calculated for Group C rats and are listed in Table 6. Compared to the control groups, percent fat digested was significantly decreased at week 6 in 9% males (28%) and females (14%), during week 12 in 3% and 9% males (8% and 33%, respectively), during week 18 in 9% males (20%) and females (10%), and during week 24 in all exposed groups of males and females (up to 32%). Calcium absorption was significantly increased in 9% females during weeks 12 (55%) and 24 (154%). Fecal weight was significantly increased in 3% and 9% males (up to 170%) and females (up to 126%) during each collection period and in 1% females during weeks 12, 18, and 24 (18% to 29%). Fecal moisture was significantly increased in 9% males and females at all time points (10% to 15%), in 3% males (4%) at week 6, and in 3% females (7%) at weeks 12 and 18.

Male rats did not display any changes in testis or epididymis weights or sperm parameters, indicating that chitosan did not exhibit the potential to be a reproductive toxicant in male rats (Table E1).

TABLE 6
Digestive Data for Group C Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Weeks 6-7	10	10	10	10
Weeks 12-13	10	10	10	10
Weeks 18-19	10	10	10	9
Weeks 24-25	10	10	10	10
Fat digested (%)				
Weeks 6-7	97.04 ± 0.40	97.55 ± 0.22	94.37 ± 0.84	69.55 ± 3.01**
Weeks 12-13	94.79 ± 0.46	93.36 ± 0.83	87.08 ± 0.68**	63.50 ± 2.40**
Weeks 18-19	97.56 ± 0.58	98.48 ± 0.19	95.87 ± 0.70	77.59 ± 1.83**
Weeks 24-25	97.01 ± 0.19	95.61 ± 0.32**	92.14 ± 0.87**	66.18 ± 3.24**
Calcium absorbed (%)				
Weeks 6-7	31.69 ± 1.84	34.57 ± 4.05	27.54 ± 1.83	33.01 ± 1.59
Weeks 12-13	19.81 ± 3.36	14.73 ± 0.76	18.42 ± 3.25	28.01 ± 2.69
Weeks 18-19	13.33 ± 4.33	18.42 ± 5.43	3.64 ± 2.62	11.11 ± 1.35
Weeks 24-25	2.93 ± 1.54	5.14 ± 1.08	0.70 ± 1.57	9.46 ± 1.88
Fecal weight (g)				
Weeks 6-7	21.42 ± 0.68	21.01 ± 1.93	31.33 ± 0.90**	52.39 ± 2.85**
Weeks 12-13	24.32 ± 1.68	27.70 ± 1.37	32.84 ± 1.73**	47.59 ± 4.30**
Weeks 18-19	23.11 ± 1.25	22.67 ± 1.85	33.30 ± 1.72**	62.38 ± 3.67**[b]
Weeks 24-25	26.43 ± 1.12	25.75 ± 0.73	37.17 ± 1.11**	56.35 ± 3.45**
Fecal moisture (%)				
Weeks 6-7	45.0 ± 0.5	42.0 ± 1.6	46.8 ± 0.4*	51.0 ± 0.8**
Weeks 12-13	46.8 ± 2.0	49.0 ± 0.8	48.8 ± 0.6	53.6 ± 0.8**
Weeks 18-19	47.7 ± 1.1	45.3 ± 1.8	49.1 ± 0.7	54.8 ± 1.5**[b]
Weeks 24-25	47.2 ± 0.6	45.7 ± 0.5	49.3 ± 0.7	53.1 ± 0.8**

TABLE 6
Digestive Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female				
n				
Weeks 6-7	10	10	10	9
Weeks 12-13	10	10	10	10
Weeks 18-19	10	10	10	10
Weeks 24-25	8	9	10	10
Fat digested (%)				
Weeks 6-7	96.47 ± 0.49	95.53 ± 1.30	95.46 ± 0.66	83.23 ± 2.69**
Weeks 12-13	97.12 ± 1.58	98.54 ± 0.99	97.27 ± 1.16	91.95 ± 2.70
Weeks 18-19	99.17 ± 0.18	97.52 ± 0.50	97.15 ± 1.24	89.61 ± 2.53**
Weeks 24-25	98.66 ± 0.08	97.68 ± 0.39**	96.79 ± 0.49**	86.73 ± 1.55**
Calcium absorbed (%)				
Weeks 6-7	31.44 ± 2.35	24.42 ± 2.54	24.36 ± 2.50	32.29 ± 1.69
Weeks 12-13	14.84 ± 1.76	17.03 ± 3.11	17.96 ± 1.22	23.02 ± 2.39*
Weeks 18-19	8.96 ± 3.00	9.78 ± 1.98	0.47 ± 3.37	13.07 ± 1.65
Weeks 24-25	5.65 ± 2.84	9.23 ± 2.74	8.25 ± 1.59	14.50 ± 1.40*
Fecal weight (g)				
Weeks 6-7	14.37 ± 0.91	15.76 ± 0.60	19.85 ± 1.64**	32.61 ± 1.67**[b]
Weeks 12-13	15.37 ± 0.60	18.41 ± 1.28*	21.11 ± 1.07**	30.83 ± 2.78**
Weeks 18-19	16.30 ± 0.86	19.23 ± 0.97*	25.21 ± 1.42**	36.58 ± 2.41**
Weeks 24-25	16.01 ± 0.92[b]	20.66 ± 1.14**	24.85 ± 1.19**	35.78 ± 2.27**
Fecal moisture (%)				
Weeks 6-7	45.3 ± 1.1	45.3 ± 0.4	47.3 ± 0.8	50.0 ± 0.9**[b]
Weeks 12-13	45.9 ± 0.7	47.5 ± 1.0	49.3 ± 0.5**	52.7 ± 1.0**
Weeks 18-19	46.1 ± 1.1	47.2 ± 0.4	49.5 ± 0.9**	53.0 ± 0.7**
Weeks 24-25	47.2 ± 0.6[b]	48.4 ± 1.4	49.2 ± 0.6	52.6 ± 0.9**

* Significantly different (P≤0.05) from the control group by Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=10

Bone parameters in Groups A and B rats were generally unaffected by chitosan exposure (Table C2). Bone moisture was significantly increased, relative to the control group, in 9% females (7%).

The absolute and relative liver weights of Group A 9% males and females were significantly less (22% and 21% lower, respectively) than those of the respective control groups (Tables 7 and D1). The absolute and relative thymus weights of Group A 3% and 9% males and 9% females were significantly less than those of the controls (Table D1).

There was a significant decrease in the incidence of periportal fatty change of the liver in Group A female rats in the 9% group compared to the control group and decreases in 1% and 3% females that resulted in a negative trend (Tables 7 and A2). In male rats, there were decreases in the incidences of periportal fatty change in the 1% and 9% groups, and the severities were decreased in the 3% and 9% groups (Tables 7 and A1). Fatty change was characterized by hepatocytes with clear vacuoles (lipid), mostly located within the periportal region of the liver (zone 1) (Plate 1).

TABLE 7
Liver Parameter Data for Group A Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Male				
n[a]	10	10	10	10
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
Liver weight[b]				
Absolute	25.19 ± 0.87	24.87 ± 1.35	23.74 ± 1.51	19.53 ± 0.71##
Relative	37.662 ± 0.731	35.321 ± 1.179	34.345 ± 1.411#	31.933 ± 0.817##
Periportal, Fatty Change[c]	6 (1.7)[d]	3 (1.7)	6 (1.3)	3 (1.0)
Female				
n	10	10	10	10
Necropsy body wt	338 ± 11	335 ± 13	328 ± 11	301 ± 13
Liver weight				
Absolute	12.54 ± 0.82	12.47 ± 0.39	11.85 ± 0.29	9.85 ± 0.20##
Relative	36.900 ± 1.502	37.341 ± 0.444	36.346 ± 0.904	33.036 ± 0.910#
Periportal, Fatty Change	7 (1.1)	4 (1.0)	4 (1.0)	0**

Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
Significantly different (P≤0.01) from the control group by Williams' test
** Significantly different (P≤0.01) from the control group by the Fisher exact test
[a] Number of animals with liver weighed and with liver examined microscopically
[b] Liver weights (absolute weights) and body weights are given in grams; Liver-weight-to-body-weight ratios (relative weights) are given as mg liver weight/g body weight (mean ± standard error).
[c] Number of animals with lesion
[d] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Hepatocytes contained large, well-defined, single round vacuoles (macrovesicular) within each cell that displaced the nuclei and cytoplasm to the cell periphery (Plate 2) and can be compared with a liver lacking fatty change (Plate 3).

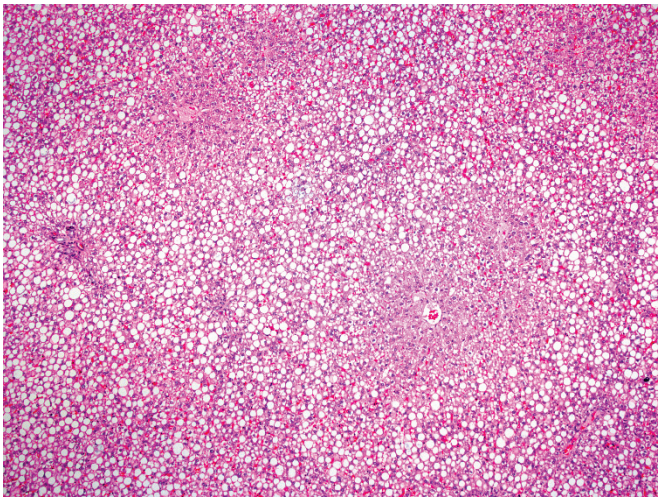

PLATE 1
Section of the liver from a control male Sprague Dawley rat from the 6-month feed study of chitosan with a moderate degree of fatty change. There is a predominant periportal distribution of affected hepatocytes. H&E

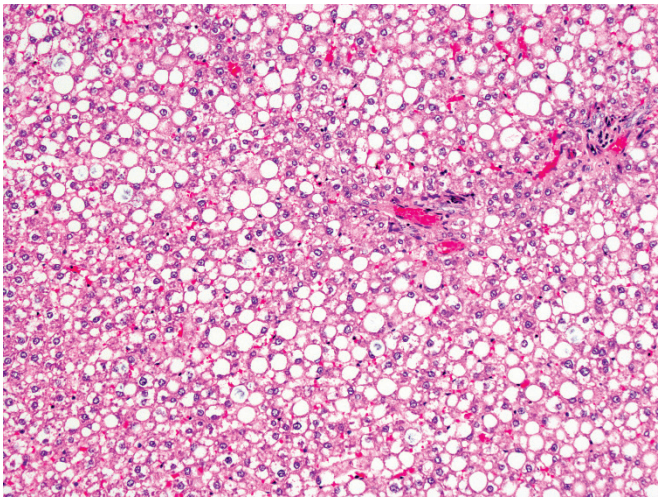

PLATE 2
Higher magnification of Plate 1. The fatty change is characterized by round, discrete vacuoles within hepatocytes that displace the nuclei and cytoplasm to the periphery. H&E

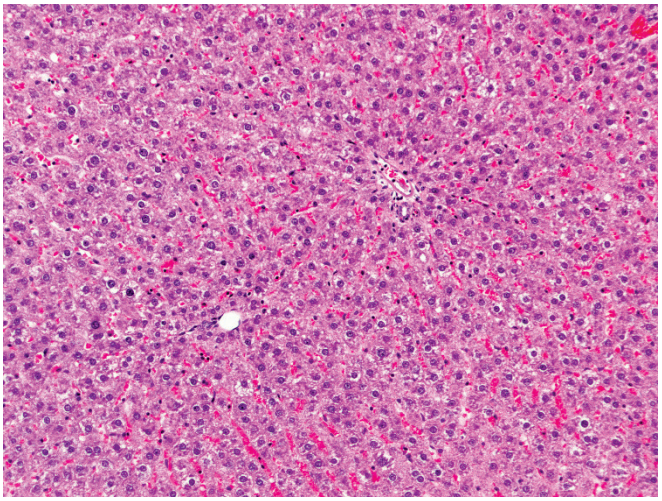

PLATE 3
Section of the liver with a lack of fatty change from a male Sprague Dawley rat exposed to 9% chitosan in feed for 6 months. There is some minimal vacuolization within many hepatocytes. The vacuoles lack distinct round borders and the nuclei are centrally located, consistent with glycogen accumulation. H&E

DISCUSSION

Human exposure to chitosan occurs primarily through consumption of dietary supplements, as chitosan is marketed as a fiber-like supplement to increase satiation and promote weight loss through inhibition of fat absorption (GNC, 2015). The acute toxicity of chitosan has previously been examined in human studies (12 days or up to 8 weeks) evaluating the effectiveness of chitosan as a weight loss supplement, and the results from these studies demonstrated no observable toxicity following oral administration of chitosan (Gades and Stern, 2003, 2005; Tapola *et al.*, 2008). However, there is indication of serum vitamin and bone mineral depletion following consumption of chitosan in rats (Deuchi *et al.*, 1995a). Therefore, the NTP conducted 6-month feed studies to evaluate the effects of dietary chitosan on bone metabolism, fat-soluble vitamin levels, and dietary fat and calcium absorption, as well as general toxicity in Charles River Sprague Dawley rats.

Feed concentrations of 1%, 3%, and 9% chitosan, which resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females, were selected based on existing data from animal studies (Landes and Bough, 1976; Deuchi *et al.*, 1995a). The 9% concentration is higher than the typical 5% NTP concentration limit, but the 9% diet was considered to be nutritionally adequate. The AIN-93M feed was selected for this study over the NTP-2000 feed based on the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 feed. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7% to 8%) than the AIN-93M feed (4%) (Rao, 1997; Reeves, 1997). One of the primary rationales for this chitosan study was the potential for decreases in fat-soluble vitamin concentrations, and therefore utilizing a diet with lower levels of pre-existing vitamins and a lower fat content was ideal to avoid confounding potential results.

The animals used in this study were split into three groups, the core group, Group A, and two special study groups, Groups B and C. Different parameters were evaluated in each group, which, while allowing for the collection of extensive endpoints, meant that only 10 animals were examined per endpoint instead of 30, as there was no crossover of analyses between the groups.

Multiple endpoints were evaluated at multiple time points (6, 12, 18, and 24 weeks) in Group C rats to determine effects on fat absorption. Treatment-related decreases in percentage fat digestion of 20% to 33% in males and 5% to 14% in females relative to control, were consistently observed in the 9% group with effects also noted in males in the 3% group (decreases of 2% to 8%). Stronger responses were observed in males relative to females. Additionally, fecal weight was significantly increased in 1% females at weeks 12, 18, and 24 (19%, 18%, and 29%, respectively), and in 3% (35% to 56%) and 9% (96% to 170%) males and females relative to controls at all time points. These data

suggest that consumption of chitosan reduced the absorption of fat in the feed, resulting in increased fecal weight due to fat being excreted. Similar results have been observed in other studies. Deuchi *et al.* (1995b) reported that rats fed deacetylated chitosan had decreased fat digestion; as the degree of deacetylation increased, fat digestibility decreased. The chitosan used by Deuchi *et al.* (1995b) was 70% to 90% deacetylated, which is a level very similar to the chitosan (86.5% deacetylated) used in the current study. Gallaher *et al.* (2000) demonstrated that male Wistar rats exposed to 10% chitosan in AIN-93 feed had increased fecal fat excretion and dry fecal weight and decreased cholesterol absorption relative to control rats, similar to what was observed in the current study.

Due to the high percentage of chitosan in the feed of the 9% group, it is possible that the observed decreases in percentage fat digested were due to bulk chitosan in the feces confounding the amount of fat actually being excreted. Misrepresented fecal weights would alter the calculated amount of fat excreted in the feces, which would subsequently affect the calculation of percentage fat digested. The observed increases in fecal weight could also be attributed to an increase in the percentage fecal moisture, which was significantly increased in both males and females in the 3% and 9% groups. In Group A, there were decreases, albeit not significant, in mean body weights of 9% males and females (decreases of 9% and 11%, respectively), but overall there were no significant changes in the body weights of rats exposed to chitosan; the mean body weights of exposed animals were similar to those of control animals. Considering the large decrease in percent fat digested, combined with the significant increase in fecal weight observed in 9% males and females, it would be expected that mean body weights would significantly decrease due to more fat being excreted than digested. The slight mean body weight decrease observed in this study could be due in part to excretion of bulk chitosan, but regardless, the magnitude of increase in fecal fat excretion as well as the decrease in hepatic periportal fatty change still indicates a treatment-related response.

Consistent significant decreases in cholesterol levels were observed in 9% male and female rats; triglycerides levels were also affected but not as consistently as cholesterol. Decreases in cholesterol were consistent with many other studies and not an unexpected finding, as chitosan is well known to have a cholesterol lowering effect in rats (Sugano *et al*., 1978, 1980; Ikeda *et al*., 1993; Chiang *et al*., 2000; Hossain *et al*., 2007). The mechanism by which chitosan lowers cholesterol is still controversial, but recent studies indicate that chitosan, acting as a weak anion exchange resin, reduces cholesterol by causing a decrease in its absorption in the small intestine and by inducing increases in bile acid excretion (Ebihara and Schneeman, 1989; Gallaher *et al*., 2000; Liu *et al*., 2008). With bile acid excretion, plasma or liver cholesterol is utilized to maintain the bile acid pool (Gallaher *et al*., 2000). Alternatively, the cholesterol lowering effects of chitosan may be related to an increase in viscosity of intestinal contents, which entrap fat and prevent lipolysis, or this mechanism may be in addition to chitosan's ability to bind bile acids (Ikeda *et al*., 1993; Kanauchi *et al*., 1995; Liu *et al*., 2008).

Along with an inhibition in dietary fat absorption and decreases in serum lipids there were also treatment-related decreases in the levels of fat-soluble vitamins A and E. Serum and liver vitamin E levels were substantially affected, being 62% to 87% lower in the 9% males and females. These findings are similar to those of Deuchi *et al.* (1995a)

where decreases in serum and liver vitamin E levels were observed after 14 days of consuming a 5% chitosan feed. In this same study, liver vitamin A levels were decreased, but vitamin A serum levels were unchanged. Bile and lipids are needed for the absorption of dietary vitamins A and E, as both must be incorporated into intestinal micelles for their absorption (Rucker *et al.*, 2008). Thus, it is highly plausible that the decrease in dietary fat absorption, including cholesterol, led to the decreases in serum and liver concentrations of these vitamins. It is also possible that, by some unknown mechanism, chitosan may enhance vitamin A or E requirements in the peripheral tissues.

There were no histologic changes associated with the observed decreases in vitamin levels; however, the decreases were significant enough to suggest nutritional inadequacies. The long-term effects of vitamin A and vitamin E deficiencies are well-known (Rucker *et al.*, 2008; Sommer, 2008; Traber, 2014; Wiseman, *et al.*, 2017), and it is unknown what deficiency-related effects would have been observed had these decreased levels been maintained for a longer period of time. When circulating levels of vitamin E, specifically α-tocopherol, are depleted, tissue damage can occur. Vitamin E depletion in humans has subsequently been correlated with anemia, disruption of normal growth, decreased responses to infection, and pregnancy concerns (Traber, 2014). Vitamin A is essential in numerous biological processes and pathways, including growth, vision development, immune function, and metabolism. Severe vitamin A deficiency (VAD) results in disruption of normal tissue function and is associated with childhood blindness, anemia, and depressed responses to infection; VAD during a severe infection may result in death (Sommer, 2006; Traber, 2014; Wiseman *et al.*, 2017). While the long-term effects of vitamin deficiency in rodents are not as well-understood, the available literature on human deficiencies suggests that the decreases in vitamin A and E observed in this study may be detrimental over time.

In contrast to decreases in vitamins A and E, $1,25(OH)_2$ vitamin D (bioactive vitamin D) levels were significantly elevated in 9% male and female rats. Vitamin D's main function is to help maintain normal calcium and phosphorus levels by regulating the intestinal absorption of these minerals from the diet. In addition to the increased $1,25(OH)_2$ vitamin D levels, significant decreases in serum phosphorus were also seen in male and female rats. Although intestinal absorption of phosphorus was not measured in this study, chitosan has been observed by others to cause a significant reduction in intestinal phosphorus absorption (Yang *et al.*, 2002). Low phosphorus concentrations stimulate $1,25(OH)_2$ vitamin D production by the kidney, therefore the increased levels of $1,25(OH)_2$ vitamin D observed in this study may be the result of the low phosphorus levels. Increased levels of $1,25(OH)_2$ vitamin D can cause an increase in intestinal absorption of calcium regardless of serum calcium levels. Significant elevation in intestinal absorption of calcium was observed sporadically in the female rats, but serum calcium levels were relatively stable. This effect is most likely due to a loss of calcium through the urine, which has been observed in other chitosan feed studies (Wada *et al.*, 1997; Yang *et al.*, 2002) and is known to occur in cases of hypophosphatemia-induced elevations in $1,25(OH)_2$ vitamin D due to Fanconi's syndrome (Tieder *et al.*, 1988). The reported urinary calcium loss in chitosan feed studies may be compensatory or directly induced by the chitosan.

Significant decreases in urine volume were observed in various male and female groups, but most consistently in the 9% group where decreases of 40% to 58% of the control group volume were observed. As the urine volumes decreased, urine creatinine concentrations were seen to increase significantly. This is consistent with proper renal function. The most likely cause of the decrease in urine volume is decreased consumption of water, although water consumption was not measured, so this cannot be certain. However, the mild increases in urea nitrogen in the 9% male and female rats at 25 weeks (the only time point measured) supports decreased water consumption (i.e., mild dehydration). Water retention in the intestine may have contributed to the decreases in urine volume, as fecal moisture was mildly increased in some of the treatment groups, although it is highly unlikely this would be the primary cause and no diarrhea was observed.

There was a significant decrease in the occurrence of periportal fatty change, or lipid accumulation, in the livers of 9% females relative to the controls, and this negative trend was maintained in both 1% and 3% females, although not significantly. In male rats, the incidences of periportal fatty change were decreased in both 1% and 9% groups and the severities were decreased in both the 3% and 9% groups. The decrease in lipid accumulation was inconsistent between male and female rats in the 9% exposure groups, as a more severe decrease was observed in the 9% female rats (100% lower) compared to the 9% male rats (50% lower) relative to the respective controls. The morphologic features observed during this study (periportal hepatocytes with large, single, well-defined intracytoplasmic vacuoles displacing the nucleus), were consistent with the intracytoplasmic lipid accumulation that is associated with fatty change (Thoolen *et al.,* 2010). During normal function, fatty acids circulate between the liver and adipose tissue, which maintains a balance of triglycerides between the two locations. When this balance becomes skewed, hepatic fatty acids can accumulate as small vacuoles in the hepatocytes and progress over time into larger globules (Thoolen *et al.*, 2010; Hassan *et al.*, 2014).

Lipid accumulation in the liver can occur via multiple mechanisms, including 1) increased synthesis of fatty acids, 2) increased uptake of fatty acids from adipose tissue and/or the diet, 3) improper removal of fatty acids from the liver, or 4) decreased oxidation of fatty acids (Sozio *et al.*, 2010). Diet and nutritional status can also influence lipid accumulation (Greaves, 2007; Hassan *et al.*, 2014). Singh *et al.* (1969) demonstrated that albino rats administered vitamin A orally for 2 days had increased hepatic lipid accumulation. In the present study, there were treatment-related decreases in hepatic vitamin A and E in both male and female rats, which could have contributed to the loss of periportal lipid accumulation observed in the animals fed 9% chitosan. Lipid accumulation in the liver can also occur due to imbalanced uptake of lipids from the blood and secretion of lipoproteins from the hepatocytes (Kucera and Cervinkova, 2014). In this chitosan study, the fatty change (lipid accumulation) observed was periportal, or in Zone 1. Zone 1 is closest to the incoming vasculature and receives the majority of oxygenated blood, and Zone 1 hepatocytes are generally resistant to the effects of nutritional deficiencies (Jungermann and Katz, 1989). Therefore, the decrease in fatty change observed in rats fed 9% chitosan could be an adaptive response to the vitamin and mineral depletion noted in this study.

The incidences and severities of fatty change in both male and female control animals was particularly high (6/10, males; 7/10, females; average severity 1.7 and 1.1, respectively), suggesting that the Charles River Sprague Dawley rats used in this study may have a normally high level of hepatic periportal lipid accumulation. Plates 1, 2, and 3, included in this report, are well representative of the observations made in this study, as the increased severity of periportal fatty change in control animals was a strong response.

Absolute and relative liver weights of male and female rats were significantly decreased in animals fed 9% chitosan relative to control animals. As described above, there were decreases in the incidence of periportal fatty change in all exposed animals, particularly in the female rats fed 9% chitosan. The decrease in liver weights observed in the 9% animals could be due to the loss of fat accumulation in the livers, which would alter the weight of the organs.

The absolute and relative thymus weights of 3% and 9% males and 9% females were also significantly decreased relative to those of control groups. The thymus is extremely sensitive to toxic compounds and similar stressors, and alterations in thymus weight can be an indicator of apoptosis and organ atrophy in response to a toxic insult. Nutritional status can cause a decrease in thymus weight, in particular vitamin, mineral, and fatty acid deficiencies (Pearse, 2006). In the current study, male and female rats fed 9% chitosan had depleted levels of serum vitamin A and E, liver vitamin E, and serum cholesterol and triglycerides, indicating nutritional inadequacies. The observations from this chitosan study, combined with what is known about the thymus, suggest that exposure to chitosan may have induced reductions in thymus weight secondary to nutritional deficiencies.

Results of this study did not support chitosan as a cause of bone resorption. Significant elevation of parathyroid hormone levels occurred occasionally and inconsistently, while calcium levels were relatively stable. Calcium was mildly, but significantly, decreased at only two time points in male groups by no more than 4%. Additionally, serum total osteocalcin and urinary deoxypyridinoline level, both biomarkers of bone turnover, while occasionally significantly elevated, lacked any consistent increases over time or between sexes. In fact, deoxypyridinoline was significantly decreased at some time points. Lastly, bone calcium, bone length, and the histology findings of this study did not support calcium loss from the bone.

Although bone parameters were unaffected by chitosan exposure, a limitation of this study may be that the time frame of the study was not extensive enough to adequately evaluate bone loss. Rats are generally not considered skeletally mature until 10 months of age, and the long bones in rats can continue to grow until 30 months of age, making it difficult to observe any loss of bone before that point (Lelovas *et al.*, 2008). In a study of female Charles River Sprague Dawley rats, Wronski *et al.* (1989) observed closed growth plates in the tibias of 15-month-old animals. In a separate study, Fukuda and Iida (2004) noted that natural decreases in bone mineral density did not begin until 15 months of age in female Wistar rats. Also, standard osteoporosis studies using rat models commonly utilize ovariectomized animals, which mimics the conditions of menopause and generally increases rates of bone remodeling and bone loss. Ovariectomized SHRSP rats fed 10% chitosan alongside a low calcium diet exhibited decreased bone

mineral density and increased femur stiffness (Yang *et al.*, 2002). Following ovariectomy, bone loss in the femurs, specifically the femoral neck, is still not observed until a minimum of 30 days postprocedure (Lelovas *et al.*, 2008). Therefore, given the time frame of the study there was reduced likelihood of observing any osteologic changes possibly induced by chitosan exposure.

There were no treatment-related clinical findings in the core, Group A animals, but there were instances of seizures in Groups B and C animals. Thirteen animals from Groups B and C (two 1%, one 3%, and ten 9%) were observed with seizures either during or after the 18-week blood collection. Seizures were not noted at any other time point. Similarly, there was no treatment-related mortality in the Group A animals, but five animals from Groups B and C died, often after seizures, near the time of blood collection. Cause of death was undetermined for these animals. While there was no clear connection between chitosan treatment and the incidence of seizures, there was an exposure concentration-related increase in the occurrence of seizures. Therefore, it is possible that chitosan exposure may have induced the increased rate of seizures observed in this study.

Under the conditions of the 6-month feed study of chitosan, male and female rats fed 3% and 9% chitosan in the diet had significantly decreased levels of serum vitamin A and serum and hepatic vitamin E and increased levels of serum $1,25(OH)_2$ vitamin D. Consumption of high levels of chitosan decreased percentage fat digestion and increased fecal weight and moisture, as well as reduced levels of phosphorous, cholesterol, and triglycerides. Female rats exposed to 9% chitosan also had significant liver weight and histologic changes. Based on the above results, the lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats.

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APPENDIX A
SUMMARY OF LESIONS IN RATS IN THE 6-MONTH FEED STUDY OF CHITOSAN

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Group A Male Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Disposition Summary				
Animals initially in study	10	10	10	10
Survivors				
Terminal euthanasia	10	10	10	10
Animals examined microscopically	10	10	10	10
Alimentary System				
Liver	(10)	(10)	(10)	(10)
Degeneration, cystic	0	0	0	1 (10%)
Hematopoietic cell proliferation	2 (20%)	3 (30%)	3 (30%)	6 (60%)
Inflammation, chronic active	10 (100%)	10 (100%)	10 (100%)	9 (90%)
Periportal, fatty change	6 (60%)	3 (30%)	6 (60%)	3 (30%)
Pancreas	(10)	(0)	(0)	(10)
Basophilic focus	1 (10%)			0
Inflammation	2 (20%)			1 (10%)
Stomach, forestomach	(10)	(0)	(0)	(10)
Epithelium, hyperplasia	3 (30%)			1 (10%)
Cardiovascular System				
Blood vessel	(10)	(0)	(0)	(10)
Inflammation	0			1 (10%)
Heart	(10)	(0)	(0)	(10)
Cardiomyopathy	5 (50%)			3 (30%)
Mineralization	0			1 (10%)
Endocrine System				
Adrenal cortex	(10)	(1)	(0)	(10)
Vacuolization cytoplasmic	0	0		1 (10%)
Parathyroid gland	(10)	(10)	(10)	(10)
Hyperplasia	1 (10%)	0	0	0
Pituitary gland	(10)	(0)	(0)	(10)
Cyst	1 (10%)			0
Thyroid gland	(10)	(0)	(0)	(10)
C-cell, hyperplasia	0			1 (10%)
General Body System				
None				
Genital System				
Preputial gland	(10)	(0)	(0)	(10)
Inflammation	0			1 (10%)
Inflammation, chronic active	0			2 (20%)
Prostate	(10)	(10)	(10)	(10)
Inflammation	8 (80%)	9 (90%)	10 (100%)	10 (100%)
Testes	(10)	(0)	(0)	(10)
Mineralization	0			1 (10%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Group A Male Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Hematopoietic System				
Lymph node, mandibular	(10)	(0)	(0)	(10)
Infiltration cellular, plasma cell	1 (10%)			2 (20%)
Spleen	(10)	(0)	(0)	(10)
Hematopoietic cell proliferation	5 (50%)			2 (20%)
Thymus	(10)	(0)	(0)	(10)
Atrophy	1 (10%)			0
Integumentary System				
Skin	(10)	(0)	(0)	(10)
Hemorrhage	0			1 (10%)
Mineralization	0			1 (10%)
Ulcer	0			1 (10%)
Musculoskeletal System				
Skeletal muscle	(0)	(0)	(0)	(1)
Inflammation, granulomatous				1 (100%)
Nervous System				
None				
Respiratory System				
Lung	(10)	(0)	(0)	(10)
Hemorrhage	2 (20%)			0
Inflammation, chronic active	2 (20%)			4 (40%)
Nose	(10)	(0)	(0)	(10)
Inflammation	1 (10%)			0
Goblet cell, hyperplasia	0			1 (10%)
Special Senses System				
Eye	(10)	(1)	(0)	(10)
Choroid, fibrosis	0	1 (100%)		0
Lens, cataract	0	1 (100%)		0
Harderian gland	(10)	(0)	(0)	(10)
Hyperplasia	0			1 (10%)
Infiltration cellular, lymphocyte	2 (20%)			1 (10%)
Urinary System				
Kidney	(10)	(10)	(10)	(10)
Infarct	0	0	1 (10%)	0
Mineralization	2 (20%)	4 (40%)	3 (30%)	5 (50%)
Nephropathy	9 (90%)	9 (90%)	9 (90%)	9 (90%)
Cortex, cyst	1 (10%)	0	0	0
Pelvis, dilatation	2 (20%)	0	1 (10%)	0
Pelvis, inflammation	1 (10%)	0	0	0
Urinary bladder	(10)	(0)	(0)	(10)
Transitional epithelium, hyperplasia	0			1 (10%)

TABLE A2
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Group A Female Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Disposition Summary				
Animals initially in study	10	10	10	10
Survivors				
Terminal euthanasia	10	10	10	10
Animals examined microscopically	10	10	10	10
Alimentary System				
Liver	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation	1 (10%)	1 (10%)	2 (20%)	1 (10%)
Inflammation, chronic active	9 (90%)	9 (90%)	9 (90%)	10 (100%)
Periportal, fatty change	7 (70%)	4 (40%)	4 (40%)	0
Pancreas	(10)	(0)	(0)	(10)
Atrophy	0			1 (10%)
Inflammation	1 (10%)			0
Inflammation, chronic active	0			1 (10%)
Cardiovascular System				
Heart	(10)	(0)	(0)	(10)
Cardiomyopathy	1 (10%)			0
Endocrine System				
Pituitary gland	(10)	(0)	(0)	(10)
Rathke's cleft, hyperplasia	1 (10%)			0
General Body System				
None				
Genital System				
Clitoral gland	(10)	(0)	(0)	(10)
Inflammation, chronic active	2 (20%)			0
Hematopoietic System				
Spleen	(10)	(0)	(0)	(10)
Hematopoietic cell proliferation	1 (10%)			0
Thymus	(10)	(0)	(0)	(10)
Atrophy	1 (10%)			0
Integumentary System				
Mammary gland	(10)	(0)	(0)	(10)
Adenoma	0			1 (10%)
Musculoskeletal System				
None				

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Group A Female Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Nervous System				
Brain	(10)	(0)	(0)	(10)
Developmental malformation	1 (10%)			0
Respiratory System				
Lung	(10)	(0)	(0)	(10)
Mineralization	0			1 (10%)
Alveolar epithelium, hyperplasia	0			1 (10%)
Alveolus, infiltration cellular, histiocyte	2 (20%)			0
Artery, mineralization	1 (10%)			1 (10%)
Nose	(10)	(0)	(0)	(10)
Goblet cell, hyperplasia	1 (10%)			0
Special Senses System				
Harderian gland	(10)	(0)	(0)	(10)
Infiltration cellular, lymphocyte	1 (10%)			1 (10%)
Urinary System				
Kidney	(10)	(10)	(10)	(10)
Mineralization	8 (80%)	8 (80%)	5 (50%)	6 (60%)
Nephropathy	5 (50%)	6 (60%)	5 (50%)	0

APPENDIX B
CLINICAL PATHOLOGY RESULTS

TABLE B1
Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
Hematology				
n	10	10	10	10
Hematocrit (auto) (%)				
Week 25	45.5 ± 0.4	47.1 ± 0.5	46.3 ± 0.4	47.4 ± 0.6*
Hematocrit (manual) (%)				
Week 25	47.2 ± 0.5[b]	48.2 ± 0.5	47.6 ± 0.5	48.9 ± 0.6
Hemoglobin (g/dL)				
Week 25	14.9 ± 0.2	15.4 ± 0.2	15.2 ± 0.1	15.7 ± 0.2**
Erythrocytes (10^6/μL)				
Week 25	8.44 ± 0.08	8.59 ± 0.12	8.43 ± 0.08	8.45 ± 0.12
Reticulocytes (10^3/μL)				
Week 25	186.0 ± 14.3	138.4 ± 6.3**	157.7 ± 7.2	139.3 ± 9.1**
Mean cell volume (fL)				
Week 25	53.9 ± 0.4	54.9 ± 0.6	54.9 ± 0.2	56.1 ± 0.6*
Mean cell hemoglobin (pg)				
Week 25	17.6 ± 0.2	18.0 ± 0.2	18.0 ± 0.1	18.6 ± 0.2**
Mean cell hemoglobin concentration (g/dL)				
Week 25	32.7 ± 0.2	32.7 ± 0.1	32.9 ± 0.2	33.2 ± 0.2
Platelets (10^3/μL)				
Week 25	916 ± 52	824 ± 26	921 ± 19	973 ± 36
Leukocytes (10^3/μL)				
Week 25	10.62 ± 0.98	9.39 ± 0.94	7.38 ± 0.69	9.54 ± 0.91
Segmented neutrophils (10^3/μL)				
Week 25	2.04 ± 0.38	1.48 ± 0.24	1.06 ± 0.12	1.76 ± 0.39
Lymphocytes (10^3/μL)				
Week 25	8.02 ± 0.64	7.42 ± 0.74	6.01 ± 0.62	7.44 ± 0.65
Monocytes (10^3/μL)				
Week 25	0.31 ± 0.05	0.32 ± 0.05	0.20 ± 0.02	0.19 ± 0.03
Basophils (10^3/μL)				
Week 25	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
Eosinophils (10^3/μL)				
Week 25	0.21 ± 0.05	0.15 ± 0.03	0.09 ± 0.01*	0.11 ± 0.03
Clinical Chemistry				
n	10	10	10	10
Urea nitrogen (mg/dL)				
Week 25	12.4 ± 0.6	12.1 ± 0.5	12.7 ± 0.5	15.3 ± 0.9**
Creatinine (mg/dL)				
Week 25	0.62 ± 0.01	0.64 ± 0.02	0.62 ± 0.01	0.64 ± 0.02
Calcium (mg/dL)				
Week 13	12.6 ± 0.1	12.5 ± 0.1	12.3 ± 0.2	12.4 ± 0.2
Week 19	12.5 ± 0.1	12.3 ± 0.2	12.3 ± 0.1	12.0 ± 0.1*
Week 25	12.1 ± 0.1	12.1 ± 0.2	12.0 ± 0.1	11.6 ± 0.1*
Phosphorus (mg/dL)				
Week 13	8.4 ± 0.3	8.1 ± 0.3	7.2 ± 0.3**	7.4 ± 0.4*
Week 19	8.2 ± 0.4	7.7 ± 0.2	7.4 ± 0.3	6.7 ± 0.2**
Week 25	6.9 ± 0.3	6.8 ± 0.2	6.7 ± 0.1	5.8 ± 0.3**
Total protein (g/dL)				
Week 25	7.4 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	6.9 ± 0.1*

TABLE B1
Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Male (continued)				
Clinical Chemistry (continued)				
n	10	10	10	10
Albumin (g/dL)				
Week 19	4.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.5 ± 0.0*
Week 25	4.8 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.0
Cholesterol (mg/dL)				
Week 7	82 ± 5	75 ± 8	80 ± 6	53 ± 3**
Week 13	95 ± 7	84 ± 8	90 ± 7	53 ± 2**
Week 19	101 ± 6	87 ± 10	94 ± 8	59 ± 4**
Week 25	95 ± 6	81 ± 8	90 ± 6	49 ± 4**
Triglycerides (mg/dL)				
Week 7	202 ± 28	234 ± 43	226 ± 30	88 ± 15*
Week 13	198 ± 33	202 ± 38	195 ± 24	86 ± 8**
Week 19	180 ± 26	218 ± 43	210 ± 29	95 ± 13*
Week 25	173 ± 18	207 ± 30	218 ± 24	109 ± 13
Alanine aminotransferase (IU/L)				
Week 25	28 ± 3	29 ± 2	29 ± 1	57 ± 2**
Alkaline phosphatase (IU/L)				
Week 7	134 ± 7	134 ± 7	138 ± 8	137 ± 16
Week 13	100 ± 6	95 ± 6	102 ± 6	82 ± 5
Week 19	91 ± 11	87 ± 7	84 ± 4	72 ± 7
Week 25	85 ± 7	83 ± 7	82 ± 5	64 ± 5*
Creatine kinase (IU/L)				
Week 25	192 ± 29	205 ± 27	233 ± 23	245 ± 20
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	15 ± 1	14 ± 1
Bile acids (µmol/L)				
Week 25	9.6 ± 2.3	6.4 ± 2.7	2.4 ± 0.2**	4.3 ± 0.8
Total osteocalcin (ng/mL)				
Week 7	445.7 ± 17.2	439.8 ± 15.8	441.8 ± 18.2	520.4 ± 22.6
Week 13	306.2 ± 13.0	289.7 ± 28.6	245.4 ± 37.9	372.6 ± 23.4
Week 19	239.4 ± 12.4	225.7 ± 10.6	181.6 ± 26.8	269.2 ± 20.9
Week 25	158.3 ± 10.0	168.1 ± 11.6	145.9 ± 22.7	218.3 ± 14.6*
Parathyroid hormone (ng/mL)				
Week 7	1.882 ± 0.137	1.643 ± 0.449	1.838 ± 0.348	1.521 ± 0.368
Week 13	2.343 ± 0.350	2.763 ± 0.479	3.215 ± 0.537	2.433 ± 0.222
Week 19	1.879 ± 0.186	3.101 ± 0.475	2.710 ± 0.365	3.679 ± 0.361**
Week 25	2.668 ± 0.475	2.924 ± 0.276	3.981 ± 0.349	2.848 ± 0.506
Urinalysis				
n				
Week 7	10	9	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	10	10	10
Creatinine (mg/dL)				
Week 7	192.5 ± 15.1	227.2 ± 30.7	269.4 ± 33.6	254.9 ± 37.2
Week 13	249.4 ± 25.1	360.7 ± 19.5*	350.3 ± 22.5*	334.0 ± 35.8
Week 19	204.3 ± 20.4	394.2 ± 32.5**	345.1 ± 26.0**	302.5 ± 26.6
Week 25	254.1 ± 27.4	374.8 ± 25.6*	345.9 ± 27.1	325.4 ± 36.0

TABLE B1
Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Male (continued)				
Urinalysis (continued)				
n				
Week 7	10	9	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	10	10	10
Volume (mL)				
Week 7	8.3 ± 0.8	7.5 ± 1.5	6.4 ± 0.8	5.0 ± 1.1*
Week 13	7.9 ± 1.0	4.6 ± 0.3**	5.1 ± 0.4*	4.5 ± 0.5**
Week 19	10.7 ± 1.6	4.0 ± 0.4**	5.3 ± 0.7*	5.6 ± 0.6
Week 25	8.6 ± 1.2	5.4 ± 0.6*	6.1 ± 0.8*	5.1 ± 0.6**
Deoxypyridinoline (nmol/L)				
Week 7	3,396.0 ± 268.0	4,210.0 ± 643.0	4,917.0 ± 826.0	4,754.0 ± 761.0
Week 13	2,185.1 ± 188.9	3,197.3 ± 148.3*	3,233.8 ± 218.0*	3,129.1 ± 296.5*
Week 19	1,084.9 ± 158.9	2,209.6 ± 246.3**	1,963.0 ± 200.5*	1,994.9 ± 214.3*
Week 25	1,083.5 ± 145.9	1,699.3 ± 139.6*	1,658.3 ± 136.7*	1,750.8 ± 167.6*
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.810 ± 0.135	1.889 ± 0.148	1.810 ± 0.159	1.920 ± 0.160
Week 13	0.910 ± 0.035	0.890 ± 0.031	0.930 ± 0.040	0.960 ± 0.078
Week 19	0.530 ± 0.050	0.550 ± 0.034	0.570 ± 0.042	0.660 ± 0.048
Week 25	0.430 ± 0.030	0.470 ± 0.030	0.480 ± 0.020	0.550 ± 0.027**
Female				
Hematology				
n	10	9	10	10
Hematocrit (auto) (%)				
Week 25	45.5 ± 1.0	44.9 ± 0.9	44.5 ± 0.8	45.2 ± 0.9
Hematocrit (manual) (%)				
Week 25	47.4 ± 1.1	46.9 ± 1.0	46.5 ± 0.8	46.6 ± 0.9
Hemoglobin (g/dL)				
Week 25	15.2 ± 0.4	15.0 ± 0.3	15.0 ± 0.3	15.1 ± 0.3
Erythrocytes (10^6/μL)				
Week 25	8.16 ± 0.19	8.17 ± 0.18	8.01 ± 0.11	8.10 ± 0.15
Reticulocytes (10^3/μL)				
Week 25	135.2 ± 14.6	109.2 ± 6.1	109.6 ± 7.7	129.5 ± 14.6
Mean cell volume (fL)				
Week 25	55.8 ± 0.6	55.0 ± 0.3	55.6 ± 0.3	55.8 ± 0.8
Mean cell hemoglobin (pg)				
Week 25	18.7 ± 0.2	18.4 ± 0.1	18.7 ± 0.1	18.7 ± 0.3
Mean cell hemoglobin concentration (g/dL)				
Week 25	33.5 ± 0.1	33.4 ± 0.2	33.6 ± 0.2	33.4 ± 0.2
Platelets (10^3/μL)				
Week 25	791 ± 43	798 ± 40	848 ± 38	1,024 ± 51**
Leukocytes (10^3/μL)				
Week 25	6.62 ± 0.92	3.66 ± 0.49*	5.72 ± 0.87	4.92 ± 0.58
Segmented neutrophils (10^3/μL)				
Week 25	1.15 ± 0.24	0.53 ± 0.10*	0.67 ± 0.11	0.67 ± 0.11
Lymphocytes (10^3/μL)				
Week 25	5.09 ± 0.79	2.93 ± 0.41	4.78 ± 0.77	4.06 ± 0.49
Monocytes (10^3/μL)				
Week 25	0.24 ± 0.04	0.13 ± 0.02	0.18 ± 0.03	0.11 ± 0.02*

TABLE B1
Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female (continued)				
Hematology (continued)				
n	10	9	10	10
Basophils (10^3/μL)				
Week 25	0.02 ± 0.00	0.01 ± 0.00*	0.02 ± 0.01	0.01 ± 0.00
Eosinophils (10^3/μL)				
Week 25	0.12 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
Clinical Chemistry				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	10
Urea nitrogen (mg/dL)				
Week 25	14.2 ± 1.4	14.1 ± 0.7	15.2 ± 0.7	16.3 ± 0.7*
Creatinine (mg/dL)				
Week 25	0.65 ± 0.02	0.68 ± 0.01	0.70 ± 0.01	0.69 ± 0.02
Calcium (mg/dL)				
Week 13	12.9 ± 0.2	13.1 ± 0.1	12.7 ± 0.2	12.5 ± 0.2
Week 19	12.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.1	12.5 ± 0.1
Week 25	12.7 ± 0.3	12.8 ± 0.1	12.7 ± 0.2	12.3 ± 0.2
Phosphorus (mg/dL)				
Week 13	8.1 ± 0.5	7.4 ± 0.4	6.5 ± 0.4**	6.8 ± 0.3*
Week 19	8.4 ± 0.4	8.2 ± 0.5	8.1 ± 0.3	7.4 ± 0.5
Week 25	6.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.3*	5.5 ± 0.3**
Total protein (g/dL)				
Week 25	8.2 ± 0.2	9.0 ± 0.1**	8.6 ± 0.2	8.4 ± 0.2
Albumin (g/dL)				
Week 19	5.9 ± 0.2	6.2 ± 0.1	5.9 ± 0.2	5.7 ± 0.1
Week 25	5.8 ± 0.2	6.5 ± 0.2*	6.2 ± 0.1	6.2 ± 0.1
Cholesterol (mg/dL)				
Week 7	80 ± 6	81 ± 8	67 ± 4	59 ± 4**
Week 13	92 ± 8	86 ± 7	73 ± 5	58 ± 4**
Week 19	107 ± 7	105 ± 9	91 ± 8	67 ± 5**
Week 25	94 ± 7	108 ± 5	96 ± 8	63 ± 4**
Triglycerides (mg/dL)				
Week 7	88 ± 12	130 ± 48	81 ± 8	86 ± 14
Week 13	125 ± 10	163 ± 30	140 ± 23	88 ± 23*
Week 19	143 ± 15	181 ± 32	137 ± 18	90 ± 13
Week 25	188 ± 31	231 ± 44	245 ± 31	158 ± 35
Alanine aminotransferase (IU/L)				
Week 25	25 ± 3	28 ± 3	32 ± 2**	47 ± 4**
Alkaline phosphatase (IU/L)				
Week 7	102 ± 7	99 ± 5	99 ± 7	95 ± 10
Week 13	57 ± 4	63 ± 4	71 ± 7	59 ± 5
Week 19	49 ± 5	53 ± 3	55 ± 6	46 ± 6
Week 25	46 ± 4	44 ± 2	51 ± 6	44 ± 7
Creatine kinase (IU/L)				
Week 25	258 ± 44	193 ± 46	210 ± 50	225 ± 26
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	19 ± 2	16 ± 1

TABLE B1
Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female (continued)				
Clinical Chemistry (continued)				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	10
Bile acids (µmol/L)				
Week 25	10.7 ± 1.8	8.2 ± 1.1	32.0 ± 14.1	10.8 ± 1.1
Total osteocalcin (ng/mL)				
Week 7	293.6 ± 19.4	287.5 ± 21.2	282.1 ± 34.7	316.7 ± 23.5
Week 13	197.9 ± 22.6	202.3 ± 15.4	184.4 ± 19.4	234.2 ± 14.5
Week 19	158.1 ± 18.3	184.8 ± 13.2	166.7 ± 24.7	210.1 ± 16.0
Week 25	107.9 ± 18.6	97.1 ± 7.1	96.0 ± 16.2	148.8 ± 15.1
Parathyroid hormone (ng/mL)				
Week 7	0.995 ± 0.150[b]	1.156 ± 0.176	1.092 ± 0.182	1.023 ± 0.146
Week 13	1.506 ± 0.203	1.734 ± 0.194	1.925 ± 0.306	1.767 ± 0.212
Week 19	1.406 ± 0.232	1.994 ± 0.353	1.845 ± 0.418	1.673 ± 0.223
Week 25	1.471 ± 0.189[b]	1.628 ± 0.220	1.818 ± 0.224	2.301 ± 0.212*
Urinalysis				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	9
Creatinine (mg/dL)				
Week 7	98.2 ± 14.7	107.1 ± 12.3	206.3 ± 55.2*	192.0 ± 8.3**
Week 13	144.7 ± 14.8	139.5 ± 15.9	247.5 ± 30.9*	241.6 ± 29.0**
Week 19	142.0 ± 19.7	137.7 ± 19.4	196.3 ± 23.6	230.3 ± 19.2**
Week 25	179.8 ± 59.7	120.3 ± 24.1	184.5 ± 20.5	217.9 ± 23.4*
Volume (mL)				
Week 7	8.2 ± 1.2	8.5 ± 1.0	5.4 ± 0.8	3.4 ± 0.3**
Week 13	6.4 ± 0.7	6.5 ± 0.6	4.1 ± 0.8*	2.9 ± 0.5**
Week 19	7.7 ± 1.2	8.1 ± 1.4	5.0 ± 0.8	3.4 ± 0.5**
Week 25	8.2 ± 1.5	9.1 ± 1.5	5.8 ± 0.9	3.7 ± 0.5**
Deoxypyridinoline (nmol/L)				
Week 7	1,622.0 ± 328.0	1,378.0 ± 295.0	4,130.0 ± 1,109.0*	4,423.0 ± 355.0**
Week 13	875.5 ± 129.6	587.0 ± 68.1	1,364.0 ± 215.9	1,421.6 ± 267.0
Week 19	666.3 ± 106.9	487.7 ± 68.5	894.9 ± 122.1	1,212.3 ± 107.4**
Week 25	565.7 ± 178.2	250.4 ± 47.1	625.1 ± 83.7	891.5 ± 114.1*
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.620 ± 0.128	1.240 ± 0.129	1.940 ± 0.229	2.300 ± 0.182*
Week 13	0.580 ± 0.039	0.430 ± 0.037**	0.540 ± 0.034	0.570 ± 0.042
Week 19	0.450 ± 0.017	0.360 ± 0.016*	0.440 ± 0.016	0.520 ± 0.020
Week 25	0.340 ± 0.043	0.222 ± 0.022	0.340 ± 0.027	0.411 ± 0.026

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9

APPENDIX C
VITAMIN CONCENTRATION AND BONE PARAMETER RESULTS

TABLE C1
Serum and Hepatic Vitamin Concentration Data for Group B Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Week 7	9	10	10	10
Week 13	9	10	10	10
Week 19	9	10	10	10
Week 26	9	10	10	8
Serum vitamin A (µg/mL)				
Week 7	0.532 ± 0.021	0.506 ± 0.033	0.513 ± 0.026	0.453 ± 0.018
Week 13	0.561 ± 0.024	0.499 ± 0.019	0.476 ± 0.022*	0.410 ± 0.009**
Week 19	0.533 ± 0.028	0.506 ± 0.031	0.475 ± 0.019	0.392 ± 0.014**
Week 26	0.476 ± 0.019	0.444 ± 0.024	0.398 ± 0.017**	0.336 ± 0.026**
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	124.4 ± 19.6	163.3 ± 21.7	183.2 ± 26.9	297.4 ± 41.0**
Week 13	70.1 ± 7.3	57.4 ± 5.3	77.3 ± 4.4	86.1 ± 8.5
Week 19	20.6 ± 2.8	21.7 ± 6.1	22.9 ± 2.2	42.3 ± 3.1**[b]
Week 26	27.7 ± 3.4[c]	28.0 ± 4.3	36.1 ± 4.6[b]	66.9 ± 11.9**
Serum vitamin E (µg/mL)				
Week 7	19.33 ± 1.43	15.38 ± 1.29	12.92 ± 0.48**	4.14 ± 0.23**
Week 13	21.08 ± 1.61	17.45 ± 1.06*	12.27 ± 0.86**	4.33 ± 0.27**
Week 19	20.59 ± 1.61	16.19 ± 0.96	12.86 ± 0.42**	4.07 ± 0.32**
Week 26	19.66 ± 1.66	17.35 ± 1.37	12.35 ± 0.61**	3.59 ± 0.65**
Liver vitamin A (µg/g)				
Week 26	57.4 ± 17.6	29.9 ± 2.5	39.6 ± 3.1	31.4 ± 3.7
Liver vitamin E (µg/g)				
Week 26	66.8 ± 16.2	55.0 ± 6.8	34.6 ± 2.2**	8.5 ± 0.8**

TABLE C1
Serum and Hepatic Vitamin Concentration Data for Group B Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
Female				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 26	10	10	9	10
Serum vitamin A (µg/mL)				
Week 7	0.272 ± 0.011	0.253 ± 0.007	0.260 ± 0.012	0.266 ± 0.012
Week 13	0.308 ± 0.020	0.295 ± 0.011	0.309 ± 0.019	0.281 ± 0.018
Week 19	0.283 ± 0.014	0.271 ± 0.015	0.291 ± 0.012	0.231 ± 0.010*
Week 26	0.316 ± 0.015	0.302 ± 0.014	0.294 ± 0.018	0.249 ± 0.010**
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	104.0 ± 15.1	96.7 ± 10.9	111.0 ± 8.7	208.1 ± 18.2**
Week 13	60.6 ± 7.5	60.7 ± 7.9	69.3 ± 11.0	110.1 ± 16.9
Week 19	11.6 ± 1.6	12.6 ± 1.7	15.8 ± 1.4	31.4 ± 3.2**
Week 26	19.2 ± 2.2	20.7 ± 4.2	28.6 ± 6.5	53.7 ± 5.8**
Serum vitamin E (µg/mL)				
Week 7	18.65 ± 0.71	20.08 ± 0.87	18.38 ± 0.85	6.99 ± 0.58**
Week 13	19.81 ± 1.41	20.85 ± 1.06	20.19 ± 1.20	7.48 ± 0.38**
Week 19	21.02 ± 1.76	19.74 ± 1.75	19.86 ± 1.08	7.37 ± 0.57**
Week 26	20.94 ± 1.56	23.43 ± 1.66	22.23 ± 1.75	7.28 ± 0.64**
Liver vitamin A (µg/g)				
Week 26	65.2 ± 5.4	58.9 ± 5.0	62.3 ± 6.3	60.3 ± 4.8
Liver vitamin E (µg/g)				
Week 26	84.5 ± 8.9	97.1 ± 10.1	82.0 ± 11.8	17.2 ± 3.2**

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=7

TABLE C2
Bone Data for Groups A and B Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Male				
Bone calcium (%)	23.79 ± 0.21[b]	23.95 ± 0.22	23.92 ± 0.30	23.74 ± 0.11[c]
Bone ash (%)	45.33 ± 0.79[b]	45.24 ± 0.67	45.83 ± 0.52	43.46 ± 0.62[c]
Bone moisture (%)	29.90 ± 0.49[b]	30.30 ± 0.44	29.72 ± 0.36	31.79 ± 0.62[c]
Left femur length (mm)	43.96 ± 0.34	44.33 ± 0.30	44.10 ± 0.30	43.42 ± 0.37
Left tibia length (mm)	48.00 ± 0.37	48.27 ± 0.36	47.95 ± 0.37	47.57 ± 0.41
Right tibia length (mm)	48.06 ± 0.32	48.41 ± 0.41	47.95 ± 0.33	47.57 ± 0.43
Female				
Bone calcium (%)	24.65 ± 0.17	24.96 ± 0.20	24.77 ± 0.23[b]	24.84 ± 0.12
Bone ash (%)	47.07 ± 0.58	47.14 ± 0.57	47.44 ± 0.46[b]	45.87 ± 0.44
Bone moisture (%)	28.40 ± 0.54	28.45 ± 0.45	28.53 ± 0.49[b]	30.37 ± 0.37**
Left femur length (mm)	36.65 ± 0.21	36.75 ± 0.17	36.73 ± 0.28	36.37 ± 0.26
Left tibia length (mm)	40.56 ± 0.28	40.25 ± 0.23	40.62 ± 0.40	40.10 ± 0.24
Right tibia length (mm)	40.53 ± 0.30	40.42 ± 0.24	40.74 ± 0.42	40.12 ± 0.21

** Significantly different ($P \leq 0.01$) from the control group by Shirley's test

[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data. Bone content data are from Group B rats at week 26 and bone lengths are from Group A rats at week 25.

[b] n=9

[c] n=8

APPENDIX D
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Group A Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Male				
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
Heart				
Absolute	1.82 ± 0.07	1.81 ± 0.06	1.86 ± 0.08	1.77 ± 0.06
Relative	2.723 ± 0.089	2.589 ± 0.070	2.710 ± 0.091	2.904 ± 0.085
R. Kidney				
Absolute	2.04 ± 0.04	2.04 ± 0.04	2.11 ± 0.06	1.88 ± 0.04*
Relative	3.068 ± 0.088	2.920 ± 0.047	3.093 ± 0.088	3.093 ± 0.094
Liver				
Absolute	25.19 ± 0.87	24.87 ± 1.35	23.74 ± 1.51	19.53 ± 0.71**
Relative	37.662 ± 0.731	35.321 ± 1.179	34.345 ± 1.411*	31.933 ± 0.817**
Lung				
Absolute	2.49 ± 0.11	2.77 ± 0.09	2.62 ± 0.08	2.53 ± 0.14
Relative	3.738 ± 0.163	3.949 ± 0.095	3.841 ± 0.138	4.120 ± 0.160
R. Testis				
Absolute	1.696 ± 0.054	1.778 ± 0.046	1.726 ± 0.062	1.750 ± 0.028
Relative	2.555 ± 0.108	2.546 ± 0.078	2.534 ± 0.107	2.883 ± 0.104
Thymus				
Absolute	0.763 ± 0.045	0.727 ± 0.065	0.606 ± 0.063*	0.489 ± 0.032**
Relative	1.147 ± 0.071	1.030 ± 0.077	0.888 ± 0.091*	0.797 ± 0.045**
Thyroid gland and parathyroid gland				
Absolute	0.033 ± 0.003	0.034 ± 0.002	0.034 ± 0.002	0.031 ± 0.002
Relative	0.049 ± 0.004	0.048 ± 0.003	0.050 ± 0.003	0.051 ± 0.003
Parathyroid gland				
Absolute	0.0012 ± 0.0001	0.0010 ± 0.0001	0.0011 ± 0.0001	0.0011 ± 0.0001
Relative	0.002 ± 0.000	0.001 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Female				
Necropsy body wt	338 ± 11	335 ± 13	328 ± 11	301 ± 13
Heart				
Absolute	1.14 ± 0.03	1.09 ± 0.02	1.15 ± 0.03	1.03 ± 0.02**
Relative	3.393 ± 0.121	3.295 ± 0.094	3.515 ± 0.100	3.473 ± 0.134
R. Kidney				
Absolute	1.12 ± 0.04	1.10 ± 0.02	1.13 ± 0.03	1.01 ± 0.03
Relative	3.311 ± 0.085	3.311 ± 0.095	3.465 ± 0.108	3.399 ± 0.104
Liver				
Absolute	12.54 ± 0.82	12.47 ± 0.39	11.85 ± 0.29	9.85 ± 0.20**
Relative	36.900 ± 1.502	37.341 ± 0.444	36.346 ± 0.904	33.036 ± 0.910*
Lung				
Absolute	1.83 ± 0.06	1.80 ± 0.08	1.81 ± 0.05	1.65 ± 0.05
Relative	5.463 ± 0.181	5.396 ± 0.170	5.552 ± 0.202	5.557 ± 0.281
R. Ovary				
Absolute	0.054 ± 0.005	0.049 ± 0.005	0.057 ± 0.005	0.056 ± 0.007
Relative	0.161 ± 0.015	0.147 ± 0.015	0.179 ± 0.021	0.190 ± 0.026
Thymus				
Absolute	0.436 ± 0.033	0.400 ± 0.036	0.383 ± 0.023	0.302 ± 0.021**
Relative	1.284 ± 0.081	1.188 ± 0.083	1.169 ± 0.062	1.000 ± 0.047**
Thyroid gland and parathyroid gland				
Absolute	0.028 ± 0.002	0.027 ± 0.002	0.035 ± 0.002	0.031 ± 0.002
Relative	0.084 ± 0.005	0.082 ± 0.007	0.106 ± 0.007	0.104 ± 0.008

TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Group A Rats in the 6-Month Feed Study of Chitosan

	0%	1%	3%	9%
n	10	10	10	10
Female				
Necropsy body wt	338 ± 11	335 ± 13	328 ± 11	301 ± 13
Parathyroid gland				
Absolute	0.0007 ± 0.0001	0.0009 ± 0.0001	0.0008 ± 0.0001	0.0008 ± 0.0001
Relative	0.002 ± 0.000	0.003 ± 0.000*	0.002 ± 0.000	0.003 ± 0.000*
Uterus				
Absolute	0.657 ± 0.052	0.744 ± 0.060	0.714 ± 0.038	0.789 ± 0.096
Relative	1.980 ± 0.186	2.252 ± 0.191	2.184 ± 0.104	2.650 ± 0.329

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX E
REPRODUCTIVE TISSUE EVALUATIONS

TABLE E1
Summary of Reproductive Tissue Evaluations for Group A Male Rats in the 6-Month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Weights (g)				
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
L. Cauda epididymis	0.2013 ± 0.0073	0.2134 ± 0.0079	0.2281 ± 0.0167	0.2072 ± 0.0103
L. Epididymis	0.6874 ± 0.0184	0.7047 ± 0.0274	0.7398 ± 0.0175	0.6402 ± 0.0165
L. Testis	1.7349 ± 0.0423	1.8209 ± 0.0478	1.7922 ± 0.0619	1.7900 ± 0.0333
Spermatid measurements				
Spermatid heads (10^6/testis)	207.79 ± 18.44	183.39 ± 9.19	238.70 ± 20.45	175.57 ± 8.43
Spermatid heads (10^6/g testis)	120.38 ± 11.23	101.50 ± 5.84	135.54 ± 14.11	98.05 ± 4.29
Epididymal spermatozoal measurements				
Sperm motility (%)	86.0 ± 0.37	86.1 ± 0.46	85.9 ± 0.46	85.8 ± 0.47
Sperm (10^6/cauda epididymis)	169.25 ± 14.82	182.38 ± 8.81	160.75 ± 12.63	157.63 ± 12.41
Sperm (10^6/g cauda epididymis)	833 ± 52	856 ± 33	711 ± 33	760 ± 46

[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body and tissue weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

APPENDIX F
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF CHITOSAN

Chitosan was obtained from Vanson HaloSource, Inc. (Redmond, WA), in one lot (02-ASSF-0715), which was used in the 6-month study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (MRI) (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH). Reports on analyses performed in support of the chitosan studies are on file at the National Institute of Environmental Health Sciences.

The test article, an off-white powder, was identified as chitosan by the analytical chemistry laboratory using infrared (IR) and proton nuclear magnetic resonance (NMR) spectroscopy and by the study laboratory using IR spectroscopy. The percentage of deacetylation of the test article, determined by proton NMR, ranged from 85.97% to 87.17%, with an average of 86.5%. All spectra were consistent with the literature spectra (Domard and Rinaudo, 1983; Hirai *et al.,* 1991), and with the Sadtler spectral database. Representative IR and NMR spectra are presented in Figures F1 and F2, respectively.

The moisture content for lot 02-ASSF-0715 was determined by the analytical chemistry laboratory using weight loss on drying in a 110° C oven for 24 hours; the inorganic content was determined on the dried test article by ashing at 500° C for 4 hours. Viscosity was determined at approximately 22.5° C using a Brookfield viscometer fitted with an SC4-18/R13 spindle at a speed of 30 rpm. Lot 02-ASSF-0715 was characterized by the analytical chemistry laboratory using gel permeation chromatography (GPC) with refractive index (RI) detection using system A (Table F1) to find the most abundant molecular weight. Samples were prepared by transferring approximately 75 mg of the test article into a vial, and adding a 25 mL aliquot of diluent; vials were sealed with Teflon®-lined septa and crimp caps, allowed to stand for 2 hours at ambient temperature, swirled by hand, and placed on a rotary shaker for at least 1 hour. Standards containing a total of six molecular weight dextran markers with known peak molecular weights (Mp) (4,400, 21,400, 43,500, 196,000, 277,000, and 3,900,000 Mp) were prepared; approximately 10 mg of each marker (3 mg of 3,900,000 Mp marker) and 10 mL of diluent were pipetted into vials, sealed with Teflon®-lined septa and crimp caps, allowed to stand for a least 2 hours (the 3,900,000 marker was allowed to stand overnight) at ambient temperature to dissolve the standards, then swirled to mix prior to analysis.

For lot 02-ASSF-0715, weight loss on drying indicated 4.50% water, the average inorganic content by ashing was determined to be 2.13%, and viscosity was 81.3 centipoise. GPC/RI indicated one major peak and the determined molecular weight of the bulk chemical ranged from 62,755 to 87,343 daltons (Da). This resulted in an average molecular weight of 81,644 g/mol, or approximately 82 kDa, classifying the test article as a low molecular weight chitosan (LMWCS). A sample of chitosan was submitted to Covance Laboratories, Inc. (Madison, WI), for nutritional and contaminant testing using standard methods. For lot 02-ASSF-0715, levels of organochlorine and organophosphorous pesticides, nitrosamines, and aflatoxins were below the detection limits of the analytical methods. The purity of lot 02-ASSF-0715 was estimated to be approximately 94% based on the analysis of moisture and inorganic content. Taken together, these data indicated that the test article was chitosan.

To ensure stability, the test article was stored in sealed amber glass vials at room temperature. Reanalysis of the test article was performed during the study by the study laboratory using GPC/RI by system B, and no degradation of the test article was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately monthly by mixing chitosan with feed (Table F2). Dose formulations were stored in lined plastic buckets sealed with lids and stored at –30° C to –15° C for up to 42 days.

Homogeneity studies of approximately 0.5% and 9% formulations (5,046 and 90,049 µg/g, respectively) and stability studies of an approximately 0.5% (5,046 µg/g) formulation were performed by the analytical chemistry

laboratory using GPC/RI by system C (Table F1). Two peaks were attributed to chitosan with retention times of approximately 6.9 minutes and 12.1 minutes, respectively. Chitosan quantitation was based on the larger polymeric components of the first peak only because vehicle components co-eluted with the later oligomeric peak. Homogeneity studies of 1% and 9% dose formulations (10 mg/g and 90 mg/g in feed, respectively) were performed by the study laboratory using GPC/RI by system B. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in lined plastic buckets sealed with lids at temperatures up to room temperature and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of chitosan were performed by the study laboratory using GPC/RI by system B. Of the dose formulations analyzed, all nine were within 10% of the target concentrations (Table F3). Animal room samples of dose formulations were also analyzed; all three were within 10% of the target concentrations.

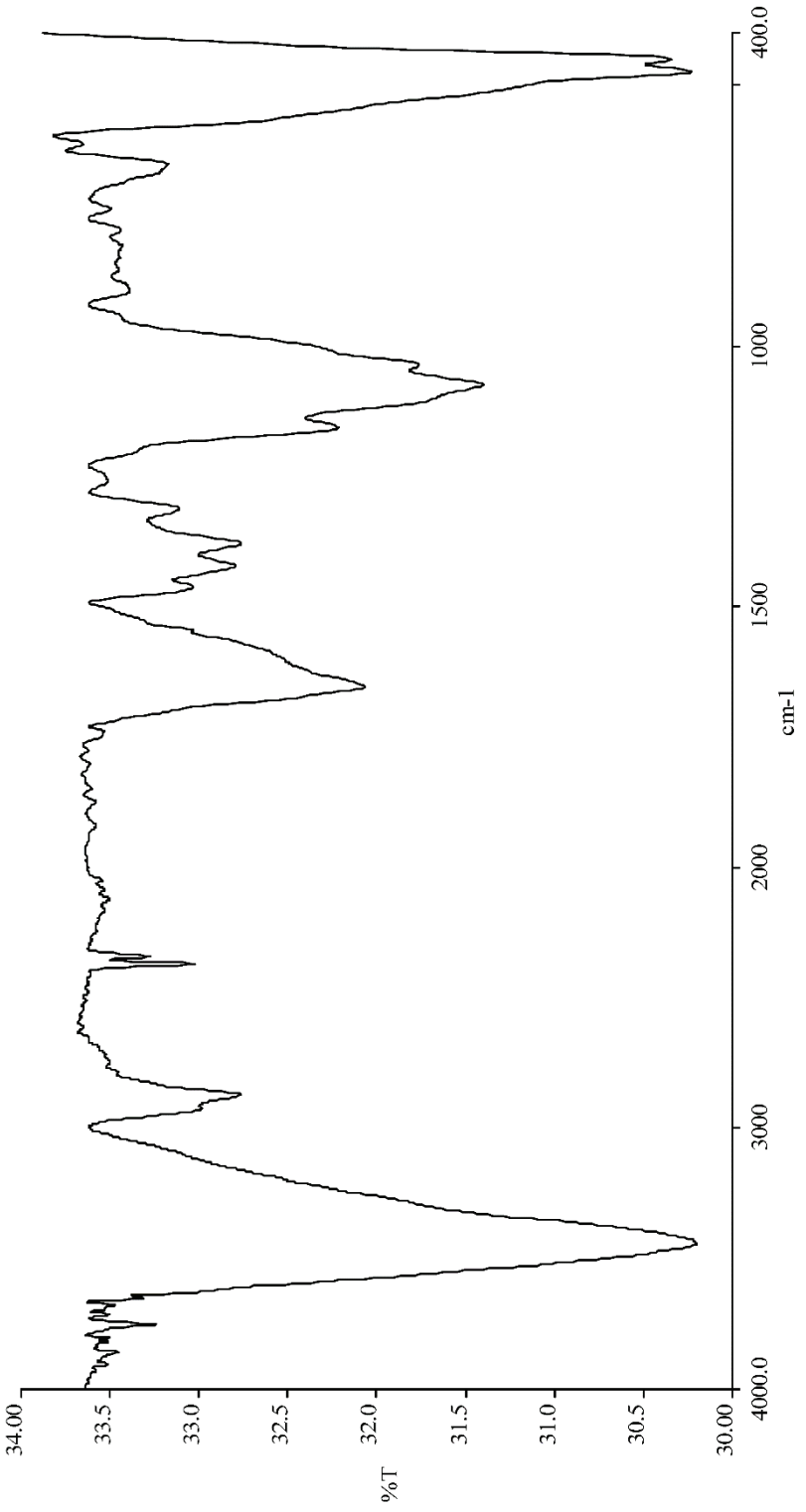

FIGURE F1
Infrared Absorption Spectrum of Chitosan

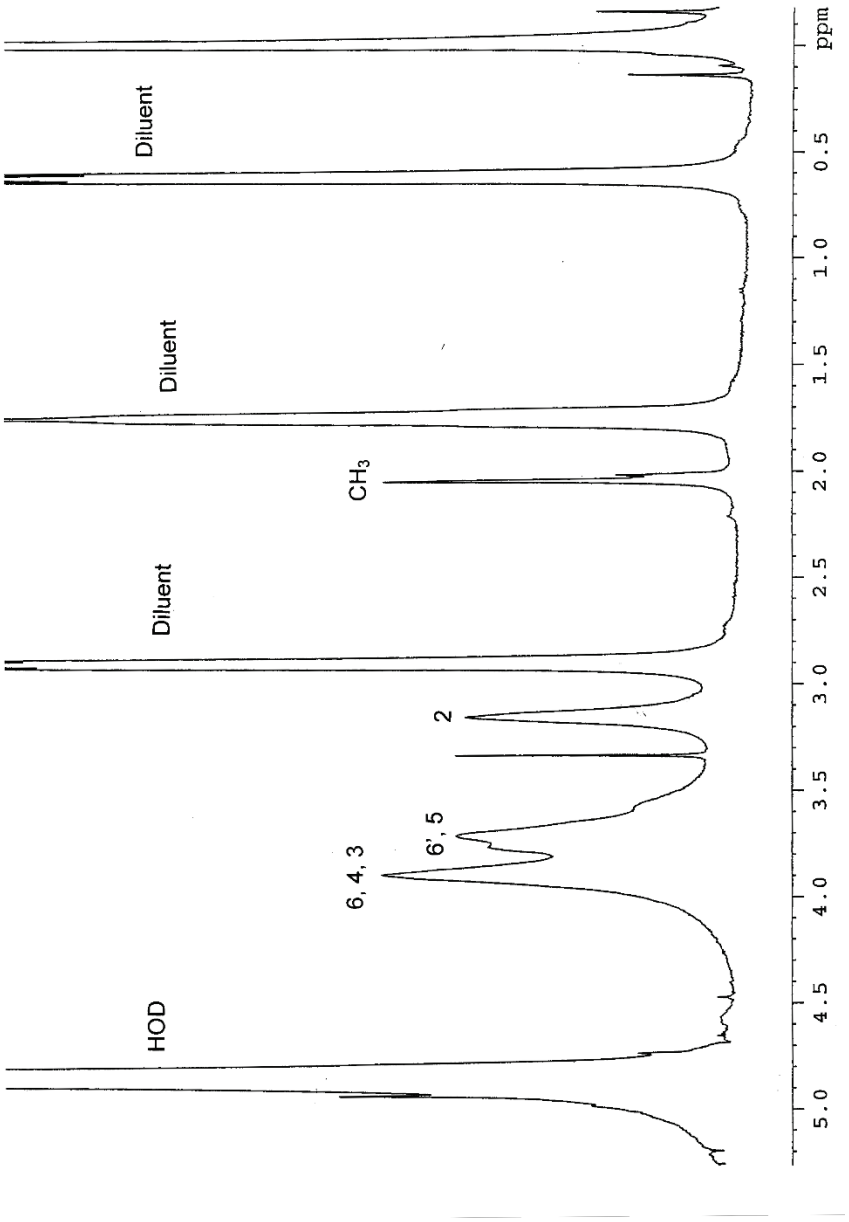

FIGURE F2
Proton Nuclear Magnetic Resonance Spectrum of Chitosan

TABLE F1
Gel Permeation Chromatography Systems Used in the 6-Month Feed Study of Chitosan[a]

Detection System	Column	Solvent System
System A		
Refractive index	In series: NOVEMA 10,000 Å, 300 mm × 8 mm, 10 µm and NOVEMA 3,000 Å, 50 mm × 8 mm (guard) and NOVEMA 3,000 Å, 300 mm × 8 mm, 10 µm (Polymer Standards Service GmbH, Mainz, Germany)	0.25% Trifluoroacetic acid, isocratic, flow rate 1.0 mL/minute
System B		
Refractive index	In series: BioSep-SEC-S2000 145 Å, 300 mm × 4.6 mm, 5 µm and BioSep-SEC-S3000 290 Å, 300 mm × 4.6 mm, 5 µm (Phenomenex, Torrance, CA)	1% Trifluoroacetic acid, isocratic, flow rate 0.35 mL/minute
System C		
Refractive index	In series: Alltech® Macrosphere 100 Å, 250 mm × 4.6 mm, 7 µm and Alltech® Macrosphere 300 Å, 250 mm × 4.6 mm, 7 µm (Grace, Columbia, MD)	1% Trifluoroacetic acid, isocratic, flow rate 0.5 mL/minute

[a] The liquid chromatographs were manufactured by Waters Corporation (Milford, MA) (System A), Agilent (Palo Alto, CA) (System B), or Perkin Elmer (Boston, MA) (System C).

TABLE F2
Preparation and Storage of Dose Formulations in the 6-Month Feed Study of Chitosan

Preparation
The appropriate amounts of chitosan and AIN-93M feed (87 kg for 1% and 3% formulations and 79 kg for the 9% formulation) were weighed in tared stainless steel buckets and layered into a Patterson-Kelly twin-shell blender. The chitosan beaker was rinsed twice with portions of the blank feed, added to the blender, and the formulation was mixed for 15 minutes. The dose formulations were prepared approximately monthly.

Chemical Lot Number
02-ASSF-0715

Maximum Storage Time
42 days

Storage Conditions
Stored in plastic-lined 5 gallon plastic buckets sealed with lids at –30° to –15° C

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE F3
Results of Analyses of Dose Formulations Administered to Rats in the 6-Month Feed Study of Chitosan

Date Prepared	Date Analyzed	Target Concentration[a] (mg/g)	Determined Concentration[b] (mg/g)	Difference from Target (%)
August 15, 2006	August 17-18, 2006	10	9.1	–10
		30	27.3	–9
		90	83.5	–7
	October 2-3, 2006[c]	10	9.99	0
		30	30.3	+1
		90	92.4	+3
October 10, 2006	October 11-12, 2006	10	9.5	–5
		30	27.0	–10
		90	94.2	+5
January 2, 2007	January 2-3, 2007	10	10.6	+6
		30	29.5	–2
		90	94.3	+5

[a] 10, 30, and 90 mg/g are equivalent to 1%, 3%, and 9% chitosan concentrations, respectively.
[b] Results of duplicate analyses
[c] Animal room samples

APPENDIX G
FEED AND COMPOUND CONSUMPTION IN THE 6-MONTH FEED STUDY OF CHITOSAN

TABLE G1
Feed and Compound Consumption by Group A Male Rats in the 6-Month Feed Study of Chitosan

	0%		1%			3%			9%		
Week	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	22.2	238	23.8	243	980	23.6	242	2,929	21.4	243	7,931
2	21.6	297	23.1	308	750	23.0	303	2,278	26.9	265	9,137
3	22.8	346	24.0	359	668	25.4	354	2,156	27.3	307	8,002
4	22.6	388	23.5	404	582	24.9	398	1,877	26.7	350	6,872
5	22.3	421	23.5	438	537	25.8	436	1,774	27.4	388	6,355
6	21.5	446	21.7	465	467	24.6	464	1,591	26.4	413	5,759
7	23.1	475	23.5	493	477	25.7	491	1,570	27.8	442	5,662
8	22.3	496	24.1	513	470	25.2	514	1,471	27.1	464	5,259
9	22.3	514	24.1	535	450	25.1	534	1,411	26.7	483	4,980
10	21.8	529	23.2	554	419	25.1	548	1,373	26.2	498	4,735
11	21.8	543	23.3	570	409	25.5	566	1,353	25.5	511	4,493
12	21.6	554	23.1	585	395	24.5	579	1,270	26.4	521	4,557
13	22.3	563	22.6	598	378	24.8	584	1,274	26.9	527	4,595
14	21.5	578	22.9	612	374	25.7	602	1,280	26.4	544	4,371
15	21.7	587	22.7	622	365	25.5	613	1,249	26.7	557	4,315
16	22.2	597	22.7	631	360	27.0	620	1,306	28.5	565	4,542
17	23.4	607	23.7	645	367	28.0	634	1,325	29.2	575	4,569
18	23.6	614	24.6	657	375	28.4	646	1,320	29.0	584	4,468
19	22.3	624	23.0	667	345	26.6	657	1,214	27.8	595	4,202
20	23.6	633	23.2	677	343	25.4	664	1,148	27.4	600	4,112
21	23.8	643	23.4	689	340	24.8	670	1,110	28.5	606	4,230
22	24.1	653	23.1	700	330	25.7	677	1,139	26.9	612	3,959
23	22.6	665	21.3	707	301	25.7	686	1,125	25.4	615	3,715
24	21.5	666	19.6	704	278	24.9	689	1,084	25.4	612	3,738
25	21.2		20.4			24.7			27.3		
Mean for Weeks											
1-13	22.2	447	23.3	466	537	24.9	462	1,717	26.4	416	6,026
14-24	22.8	624	22.7	665	343	26.2	651	1,209	27.4	588	4,202

[a] Grams of feed consumed per animal per day
[b] Milligrams of chitosan consumed per kilogram body weight per day

TABLE G2
Feed and Compound Consumption by Group A Female Rats in the 6-Month Feed Study of Chitosan

	0%		1%			3%			9%		
Week	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	17.7	175	22.3	173	1,286	17.3	177	2,940	16.9	177	8,606
2	15.1	199	15.4	198	779	15.6	197	2,381	17.6	191	8,298
3	15.8	220	16.2	217	747	15.0	214	2,102	16.9	206	7,371
4	16.2	233	16.7	229	728	15.6	231	2,023	17.3	221	7,044
5	16.0	248	17.4	241	722	15.9	242	1,974	17.3	234	6,649
6	14.7	258	17.5	252	694	14.7	251	1,759	16.4	243	6,067
7	15.9	266	17.9	262	683	15.2	259	1,759	16.7	248	6,069
8	15.9	274	17.3	268	645	15.5	267	1,739	16.7	259	5,810
9	15.9	281	17.7	276	641	15.9	274	1,740	16.7	266	5,656
10	15.9	287	18.2	284	641	15.7	281	1,678	16.6	268	5,577
11	15.2	294	17.4	289	601	15.5	286	1,624	16.2	274	5,329
12	15.8	300	17.5	295	594	15.9	292	1,632	15.7	279	5,071
13	15.3	305	16.1	300	537	16.9	298	1,699	16.2	281	5,192
14	15.3	312	16.8	303	555	16.3	304	1,609	16.5	285	5,218
15	14.7	316	16.2	307	528	16.7	309	1,623	16.8	288	5,258
16	16.2	320	19.1	311	615	17.7	314	1,694	19.2	291	5,940
17	15.9	325	18.2	314	581	16.9	315	1,611	18.0	293	5,537
18	16.4	327	19.5	317	615	17.8	318	1,679	19.1	296	5,803
19	17.8	330	19.6	321	610	18.9	321	1,768	18.6	299	5,607
20	17.3	328	21.7	324	670	18.8	321	1,757	19.2	297	5,819
21	18.2	335	21.1	332	636	19.0	330	1,725	19.1	302	5,688
22	18.5	339	19.7	337	584	19.2	336	1,712	18.9	306	5,554
23	17.4	343	17.5	340	515	17.3	339	1,532	17.7	306	5,201
24	16.1	345	17.2	340	506	15.6	339	1,381	16.7	309	4,863
25	16.4		20.3			17.1			18.8		
Mean for Weeks											
1-13	15.8	257	17.5	253	715	15.7	251	1,927	16.7	242	6,364
14-24	16.7	329	18.8	322	583	17.7	322	1,645	18.2	297	5,499

[a] Grams of feed consumed per animal per day
[b] Milligrams of chitosan consumed per kilogram body weight per day

APPENDIX H
INGREDIENTS AND NUTRIENT COMPOSITION IN AIN-93M MAINTENANCE PURIFIED DIET

TABLE H1
Ingredients of AIN-93M Maintenance Purified Rodent Diet

Ingredients	Percent by Weight
Corn starch	46.5692
Dextrin	15.5000
Casein (vitamin free)	14.0000
Sucrose	10.0000
Powdered cellulose	5.0000
Soybean oil	4.0000
AIN-93M mineral mix	3.5000
AIN-93M vitamin mix	1.0000
Choline bitartrate	0.2500
L-Cystine	0.1800
t-Butylhydroquinone	0.0008

TABLE H2
Vitamins, Minerals, and Nutrient Composition of AIN-93M Maintenance Purified Rodent Diet

	Amount
Vitamins	
A	4.00 IU/g
D_3 (added)	1.00 IU/g
E	78.80 IU/g
K (as menadione)	0.75 ppm
Thiamine hydrochloride	6.00 ppm
Riboflavin	6.50 ppm
Niacin	30.00 ppm
Pantothenic acid	16.00 ppm
Folic acid	2.10 ppm
Pyridoxine	5.80 ppm
Biotin	0.20 ppm
B_{12}	28.00 mcg/kg
Choline chloride	1,250.00 ppm
Ascorbic acid	0.00 ppm
Minerals	
Calcium	0.50 %
Phosphorus	0.31 %
Potassium	0.36 %
Magnesium	0.05 %
Sodium	0.13 %
Chlorine	0.20 %
Fluorine	1.00 ppm
Iron	39.00 ppm
Zinc	35.00 ppm
Manganese	11.00 ppm
Copper	6.00 ppm
Cobalt	0.00 ppm
Iodine	0.21 ppm
Chromium	1.00 ppm
Molybdenum	0.14 ppm
Selenium	0.22 ppm
Typical Analysis	
Protein	13.06 %
Fat	4.00 %
Fiber	5.00 %
Carbohydrate	73.80 %
Metabolizable energy	3.83 %

APPENDIX I
SENTINEL ANIMAL PROGRAM

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera from extra (sentinel) animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

Blood samples were collected from each rat and allowed to clot and the serum was separated. All samples were processed appropriately and tested for the presence of pathogens at BioReliance Corporation (Rockville, MD) or the Research Animal Diagnostic Laboratory (RADIL), University of Missouri, Columbia, MO. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five rats per sex per time point, except at study termination when blood was collected from four males and five females.

Method and Test	Time of Collection
ELISA	
Kilham rat virus (KRV)	4 weeks
Pneumonia virus of mice (PVM)	End of quarantine, 4 weeks, study termination
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	End of quarantine, 4 weeks, study termination
Rat parvovirus (RPV)	4 weeks
Sendai	End of quarantine, 4 weeks, study termination
Toolan's H-1 virus (H-1)	4 weeks
Immunofluorescence Assay	
H-1	4 weeks
KRV	4 weeks
Parvovirus	End of quarantine, 4 weeks, 6 weeks, study termination
RCV/SDA	End of quarantine
RPV	4 weeks
Multiplex Fluorescent Immunoassay	
H-1	6 weeks
KRV	6 weeks
Parvo NS-1	6 weeks
Rat minute virus	6 weeks
RPV	6 weeks

RESULTS

A positive test result for parvovirus occurred in one animal at the 4-week timepoint; additional testing of serum from this animal and other sentinel animals via other testing methodologies deemed the original positive result to be a false positive. All other test results were negative for rodent pathogens.

Appendix 3

AAFCO Chitosan definition
Source: 2019 Official Publication

Floculants (73.221–240)

73.221 (old 87.16) Chitosan is a cationic carbohydrate polymer intended for use as a precipitating agent of proteinaceous material from food processing plants. It is chemically derived by deacetylation of the naturally occurring chitin in crab and shrimp shells. It may be used in an amount not to exceed that necessary to accomplish its intended effect. Chitosan when fed as a component of feed to livestock shall be present at no more than 0.1% of the feed. Proteinaceous material coagulated with chitosan must have safety and efficacy data approved before it can be registered or offered for sale. (Proposed 1984, Adopted 1985)

IFN 8-17-730 Chitosan

Appendix 4

Washington State Department of Ecology
Approved Chemicals for Stormwater Chemical Treatment Facilities
C-TAPE

Approved Chemicals for Stormwater Chemical Treatment facilities

C-TAPE

Tidal Clear, produced by Tidal Vision, USA

The Washington State Department of Ecology (Ecology) evaluates stormwater Chemical Treatment Facilities through the Chemical Technical Assessment Protocol – Ecology (C-TAPE) program. Ecology has approved several Chitosan Enhanced Sand Filtration (CESF) devices through this program. Each of the approved devices specifies a single type of Chitosan as the flocculent chemical. Varieties of previously approved Chitosan and Sand Filter combinations include:

- FlocClear
- 1.0% ChitoVan^{tm}
- 1.5% ChitoVan^{tm}
- StormKlear®LiquiFloc® 1% solution
- StormKlear®LiquiFloc® Maximum Strength 3% solution

Ecology received requests for approval of chemicals as an alternative to the approved chemicals. Previously approved chemicals for use as a substitute for ChitoSan in a CESF treatment device include:

- BHR-P50
- HaloSource Dual Polymer
- Poly Aluminum Chloride
- SoundFloc

Ecology created a Whole Effluent Testing protocol for applicants to follow if they want Ecology to evaluate them for approval. The protocol is located in Appendix G of the *Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria* (Publication W-R-95-80). You can obtain a copy of this document at: https://fortress.wa.gov/ecy/publications/documents/9580.pdf

Ecology approved Tidal Clear, produced by Tidal Vision, USA following completion of the toxicity testing procedures. You may use this chemical in a CESF system as a replacement of one of the five Chitosan products or the previously approved chemicals listed above. You must follow the requirements in the General Use Level Designation document for the remainder of the CESF system including sand filter, monitoring, backwash, and maximum flow rate.

Use of this chemical in CESF operation requires submittal of the Request for Chemical Treatment form (https://fortress.wa.gov/ecy/publications/summarypages/ecy070258.html). The applicant must list the alternative chemical in the space for "Other" in the "Check Chemical Being Requested" item.

Refer to the attached Intended Use Plan. A copy of the Aquatic Toxicology Report is available from Ecology upon request to Douglas C. Howie, P.E. (360) 407-6444 or douglas.howie@ecy.wa.gov .

Tidal Clear™

Intended Use Plan

Prepared by:

Frank Kneib, CPESC
C4E Environmental, LLC on behalf of Tidal Vision USA
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602-334-3474

Table of Contents

1.0 Purpose

This document will explain how Tidal Clear 1% is intended to be used on construction sites, as well as provide toxicity testing data and explain the system's operational and inherent safety factors.

Chitosan enhanced sand filtration (CESF) using 1% chitosan acetate was approved for use in Washington State in 2004. Tidal Clear 1% is designed to be used in a chitosan enhanced sand filtration.

Tidal Clear System CESF Components

Reference is made herein to chitosan acetate 1% solutions Intended Use Plans (IUPs) previously approved by Washington State Department of Ecology in 2004. This Tidal Clear CESF System IUP is an used & designed in similar proven BMP technology.

Chitosan Enhanced Sand Filtration systems have a number of required equipment and system components. The most simplistic system for Tidal Clear 1% incorporates the use of in a Chitosan Enhanced Sand Filtration (CESF). A schematic process flow diagram for a typical CESF system is shown in Figure 2.1 and is cross referenced to the list of system components described below.

1. **Stormwater Storage Structure**: The storage structure is used for flow equalization, pre-settling, and for recirculation of off-specification stormwater. This may consist of mobile storage tanks, a temporary or permanent pond, in- or above-ground, or any combination of the two. The storage sizing should per the most current version of the Washington State Department of Ecology Storm Water Manual for Western Washington BMP C250.
2. **Influent Pump:** This may be a submersible or centrifugal pump powered by electricity, gas, or diesel fuel. Pump sizing is determined by the desired flow rate through the sand filters. A minimum of 30 psi through the sand filter is usually required (refer to sand filter specification for operating ranges). The intake from the influent pump is positioned so as to collect water from near the top of the detention structure, to avoid suction of settled sediment. A floating platform can be used to support most pumps and/or suction hose ends and should be anchored such a way as to prevent contact with the bottom and sides of detention structure. Influent turbidity is not to exceed 600 ntu. If in excess to 600 ntu, a pretreatment is required.
3. **Water Quality Monitoring:** Inline pH and turbidity meters or probes are to be used to monitor both influent and effluent water quality. Influent water quality monitoring equipment will assist with system optimization – pre-dose rates and pH adjustment. Grab samples are to be collected to verify these readings. The system must include an audio or visual alarm to indicate if effluent water quality is outside the allowed

discharge ranges[1]. The site technician will be responsible for monitoring the probe readings and making necessary adjustments including shutting down the system or diverting water that does not meet the required specifications to the detention structure. The data are to be recorded and displayed. Programmable data controllers may be programmed to control the flow valves for recirculation/discharge.

4. **Influent Flow Meter:** An influent flow meter must be used to determine flow rates for Tidal Clear 1% dose-rate calibration.

5. **pH Adjustment:** Tidal Clear 1% are most effective within a specific pH range of 6.5-11.0 standard units. Should the stormwater pH fall outside of this range, pH adjustment may be necessary. A CO_2 injection or other chemical neutralization system may be required to adjust the pH.

6. **Tidal Clear primary:** A bulk container of Tidal Clear 1% is to be located nearby the injection system. Bulk containers require secondary containment to prevent releases of leaks and spills to the ground. The system should not be allowed to freeze and engineering precautions should be employed to prevent this (e.g. heat blankets or equivalent). The production date is marked on each bulk storage container and they should be used in order of their relative production dates and in a timely manner.

7. **Tidal Clear Injection System:** A high viscosity metering pump is to be used to pump Tidal Clear from the bulk storage container into the influent line prior to the injection system. The injection point should be located in an operation shed. The pump(s) should have the ability to pump 10 gph at 80 psi. In some cases it may be beneficial to incorporate variable flow rate technology to ensure accurate dosing during variable pressure and flow situations. The pump should be calibrated to inject at a pre-determined dose rate based upon the flow and influent water characteristics and requires regular calibration during operations and maintenance activities. Polypropylene tubing may be used from the chemical metering pump to the injection point and must be protected to prevent abrasion or puncture as significant vibration can occur.

8. **Static Mixer:** Static mixers will be located in-line after the injection points for Tidal Clear 1%.

9. **Optional: Tidal Clear 1%:** A bulk container of Tidal Clear 1% is to be located nearby the injection system. Bulk containers require secondary containment to prevent releases of leaks and spills to the ground. The system should not be allowed to freeze and engineering precautions should be employed to prevent this (e.g. heat blankets or equivalent). The production date is marked on each bulk storage container and they should be used in order of their relative production dates and in a timely manner.

10. **Optional: Tidal Clear Pretreatment Injection System:** A metering pump is to be

[1] In accordance with Federal, State, and local regulations.

used to pump Tidal Clear 1% from the bulk storage container into the influent line prior to the sand filters. The injection point should be located in an operation shed. The pump(s) should have the ability to pump 10 gph at 80 psi. In some cases it may be beneficial to incorporate variable flow rate technology to ensure accurate dosing during variable pressure and flow situations. The pump should be calibrated to inject at a pre-determined dose rate based upon the flow and influent water characteristics and requires regular calibration during operations and maintenance activities. Polypropylene tubing may be used from the chemical metering pump to the injection point and must be protected to prevent abrasion or puncture as significant vibration can occur. Some systems will have a second Tidal Clear 1% metering pump for pre-dose applications.

11. **Conveyance Lines:** Conveyance piping between the influent pump, the rest of the system components, and the discharge point can be flex or Schedule 40 PVC piping rated for the pressure of the system and sized for the expected flow rate. Hydraulic calculations based on friction, head pressure and desired flow rates should be made and considered for the final system design. Typically 4" piping is standard for flow rates up to 350 gpm, 6" for 350-750 gpm, and 8" for 750 gpm and above. Flow control valves for recirculation/discharge are used to direct flow through the system and can be manual or automated. (Recirculation is any water passing through the system that is diverted back to the source – discharge is any water passing through the system and released to the final outfall.) Manual or automated valves set according to the effluent pH and turbidity reading are used to direct any water that is out of pre-set values back to the source preventing discharge violations.

12. **Sand Filtration Unit:** Size and capacity of pressure sand filters will depend on desired flow rate. Use ¾" to 5/8" triple-washed crushed rock for base and #30 silica sand (or equivalent) as filtration media, see sand filter specifications manual for fill quantities. A minimum three-pond sand filter is required for adequate backwash and should be operated at a flow rate not to exceed 15 gpm per square foot of sand filtration bed.

13. **Backwash Return:** The backwash line discharge should not be more than 50 feet from the filters for best performance and the outfall must be at or below the sand filter discharge elevation. Backwash must be discharged to separate cell in or near the detention structure and overflow can be allowed to enter the detention structure. Five to ten percent of the detention capacity should be reserved to accommodate backwash volumes; retain suitable freeboard in the detention structure.

14. **Effluent flow totalizer** must be used to determine discharge volumes and totals for recording and reporting purposes.

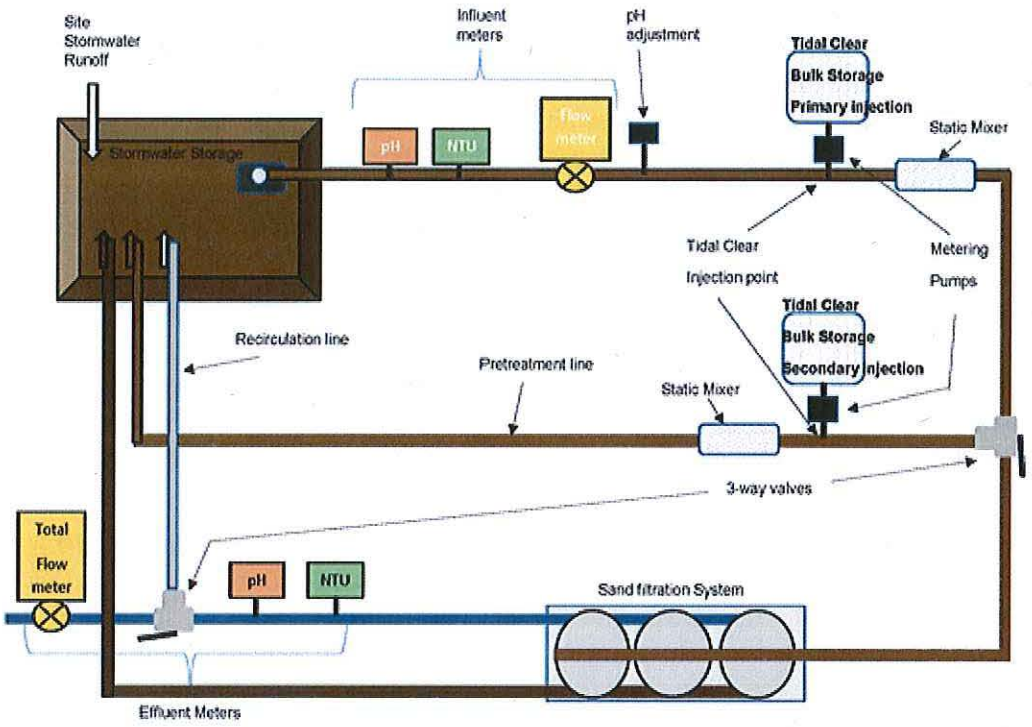
2.0 Tidal Clear System Dose Rates

Tidal Clear 1% Dose Rates

Only Tidal Clear 1% solution will be used as primary chemical. No more than 300 mg/L of Tidal Clear 1% will be dosed to a construction stormwater waste stream. Treatment using Tidal Clear 1% between 300 mg/L and 500 mg/L will only be used when approved on a case-by-case basis.

1. Bench/jar testing should always be performed with site stormwater to confirm dose rate. For accurate chemical amounts, refer to the Tidal Clear Dose Calculator.

Figure 2.1



3.0 Toxicity Testing

The following toxicity studies have been conducted using Tidal Clear 1%:

96-hour Acute Adult Fathead Minnow Survival Bioassay Protocol EPA-821-R-02-012 (2002)

96-hour Acute Rainbow Trout Survival Bioassay Protocol EPA-821-R-00-012 (2002)

7-day Survival and Growth of Rainbow Trout (*Oncorhynchus mykiss*). Lazorchak, JM and ME Smith. 2007

48-hour Acute *Ceriodaphnia dubia Survival Bioassay*

Chronic *Ceriodaphnia dubia* Survival Bioassay Protocol EPA-821-R-02-013 (2002)

A copy of each report is provided in Appendix A of this document.

4.0 Intended Discharge Concentration

The intended discharge concentration is less than 0.2 mg/L chitosan acetate. Tidal Clear's Residual Chitosan test kit is used to perform routine residual test when operating a Tidal Clear 1% System per Washington State Department of Ecology approved field methods. If chitosan acetate remains in a neutral to basic solution the cationic charge of the polymer is neutralized which renders the polymer insoluble. The insoluble particles are removed by a standard sand filter media. All of these characteristics are in addition to the nearly two-fold safety margin discussed above.

5.0 System Safety Measures

Physical Safety Measures

- Secondary containment for the Tidal Clear 1% totes and metering pump will be sized to contain at minimum the volume of the tote.
- Tidal Clear 1% will be stored at least 50 feet away from all-natural drainages, conveyances and storm drain inlets or a one foot high earthen berm will be constructed and maintained down-gradient as additional spill control.
- Spill adsorbent materials will be available and put to immediate use to mitigate any spill of Tidal Clear 1% during transport or tote refill.
- Tidal Clear 1% metering pump shall be positive displacement and come equipped with an anti-siphon valve, which shall be inspected and documented at the beginning of each shift.

Operational Safety Measures

- All inspections, calibrations, tests, measurements, dose rate changes, and equipment adjustments shall be recorded in a daily operating log which must be kept onsite and available for at least the duration of the treatment project.
- Jar tests will be conducted at startup to determine the dosage level of chitosan acetate solution. Additional jar tests will be conducted when influent turbidity changes by 20% or greater. Jar test results must be recorded in the daily operating log. If the results jar test indicates that the dose needs to be adjusted, the jar testing results and the indicated dose rate change shall be documented in the daily operating log.
- Only Tidal Clear 1% solution will be used at the dose rates given above, with the maximum dose being 1 mg/L chitosan acetate prior to treatment. The volume of chitosan in the tote will be recorded at the beginning and end of the treatment period. The volume used will be determined and compared to the volume of water treated to further validate the dose rate.
- The Tidal Clear 1% metering pumps shall be calibrated using a calibration tube at the beginning of each treatment shift and any time the dose rate changes. The treatment operator will record the calibration at the beginning of each shift and at any time the calibration is repeated. Stroke frequency will be set at max and the stroke speed (or length) adjusted to provide the correct dose rate.
- Influent and effluent pH and turbidity as well as the flow rate will be measured and recorded continuously.
- All stormwater treated with Tidal Clear 1% will be passed through the sand filters, no bypass allowed.
- The Residual Chitosan Field Screening Test will be performed as specified by Washington State Department of Ecology's Use Level Designation including 30 minutes after start-up, 2 hours afterwards to confirm free residual chitosan levels are below 0.2 mg/L and should

be repeated any time there is significant change in dose rate, turbidity or flow rate (20% or greater). Regional field offices can specify more test if they deem necessary. A positive residual chitosan test greater than or equal to 0.2 mg/L will initiate immediate response, all discharge will stop and the system will be thoroughly examined for malfunctions.

- All operators will be thoroughly familiar with requirements of Construction Stormwater General Permit, general water quality parameters and their measurement, calibration, CESF system components & operation.

Overdose Prevention Measures

If the operational measures discussed above & in the O&M Manual protocol are employed, an overdose of chemical is highly unlikely. However mechanical malfunction and human error can occur. Should the metering pumps fail, overdose is avoided because the anti-siphon valves will prevent the Tidal Clear 1% from being siphoned into the system.

The only way to overdose is to incorrectly calibrate the metering pumps. If this should happen and the stormwater stream is overdosed, and the water does not coagulate well enough to be clarified by the sand filter, this would trigger the effluent turbidimeter to prevent discharge by activating the three-way valve returning the effluent to the detention structure signaling the operator. The operator will immediately perform the Residual Chitosan Field Screening Test and record the results in the operating log.

Appendix A

Tidal Clear Toxicity Reports

eurofins | Environment Testing TestAmerica

AQUATIC TOXICOLOGY REPORT

Project Name: TIDAL VISION USA

Location: FERNDALE, WASHINGTON

Prepared by: Eurofins TestAmerica - Corvallis

1100 NE Circle Boulevard, Suite 310
Corvallis, Oregon 97330
541-243-6137

Oregon Environmental Laboratory Accreditation Program #OR100022 (NELAP)
State of Washington DOE Environmental Laboratory Accreditation Program, Lab ID C556
California State Environmental Laboratory Accreditation Program, Certificate No.: 1726

Report Date: June 17, 2019 Released by: Michelle Bennett

Eurofins TestAmerica – Corvallis Lab I.D. No. B4345

Tidal Clear 1% Intended Use Plan

CONTENTS

LABORATORY CONTACT: Michelle Bennett, Aquatic Toxicity Department Supervisor
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INTRODUCTION

Eurofins TestAmerica – Corvallis (ET-C) Aquatic Toxicology Laboratory conducted toxicity testing on sample(s) from the Tidal Vision USA, Ferndale, Washington.

Testing was initiated on: May 20, 2019

The test(s) were conducted using:

- the water flea (*Ceriodaphnia dubia*)
- the fathead minnow (*Pimephales promelas*)
- the rainbow trout (*Oncorhynchus mykiss*)

SUMMARY OF TEST RESULTS

Exhibits 1 and 2 provide a summary of the final test results.

EXHIBIT 1
Summary of Acute Test Results

Species	NOEC (mg/L)	LOEC (mg/L)	LC_{50} (mg/L)	MATC (mg/L)
C. dubia	100	200	262	150
P. promelas	400	> 400	> 400	> 400
O. mykiss	400	> 400	> 400	> 400

Note: acronyms are as defined below.

More detailed information is provided in the Results and Discussion section.

EXHIBIT 2
Summary of Chronic Test Results

Species	NOEC (mg/L)	LOEC (mg/L)	IC_{25} (mg/L)	MATC (mg/L)
C. dubia	25	50	33.7	37.5
O. mykiss	400	> 400	> 400	> 400

Note: acronyms are as defined below.

More detailed information is provided in the Results and Discussion section.

ACRONYM DEFINITIONS (from EPA guidance):

NOEC = No Observed Effect Concentration: The highest test concentration that causes no observable adverse effects on the test organisms (i.e. no statistically significant reduction from the control).
LOEC = Low Observed Effect Concentration: The lowest test concentration that does cause an observable adverse effect on the test organisms (i.e. is statistically significant reduction from the control).
LC_{50} = Lethal Concentration (50%): A point estimate of the test concentration that would cause death in 50 percent of the test population.
IC_{25} = Inhibition Concentration (25%): A point estimate of the test concentration that would cause a 25 percent reduction of a non-quantal biological measurement (i.e. growth, reproduction, etc.) for the test population.
MATC = Maximum Allowable Threshold Concentration (the allowable concentration of residual, or dissolved, coagulant/flocculant in effluent): Defined by California Construction General Permit as equal to the geometric mean of the NOEC and the LOEC values.

SAMPLE INFORMATION

EXHIBIT 3
Sample Conditions on Receipt

Sample ID	**400 mg/L**
ET-C SDG	B4345-01
Collection - Date and Time	5/21/2019 09:35
Receipt - Date and Time	5/21/2019 09:35
Temperature (°C)	21.6
Dissolved Oxygen (mg/L)	7.5
pH	4.9
Conductivity (µS/cm)	-
Total Residual Chlorine (mg/L)	0.02
Ammonia (mg/L as NH_3-N)	< 0.10
Total Hardness (mg/L as $CaCO_3$)	100

METHODS AND MATERIALS

TEST METHODS

The acute test methods were performed according to: *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms,* USEPA Office of Water (2002), EPA-821-R-02-012.

The chronic test methods were performed according to: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms,* Fourth Edition, (2002), EPA-821-R-02-013.

(AECT 2007) – *Rainbow Trout (Oncorhynchus mykiss) and Brook Trout (Salvelinus fontinalis) 7-Day Survival and Growth Test Method,* Arch Environ Contam Toxicol 53, 397-405 (2007), Lazorchak and Smith.

Additional guidance was provided by:

- *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program,* (EPA June 2000), EPA 833-R-00-003.
- *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing* (40 CFR Part 136), (EPA July 2000), EPA 821-B-00-004.
- *California Construction General Permit,* 2009-0009-DWQ amended by 2010-0014-DWQ & 2012–2006-DWQ, Attachment F. Provided by email from Frank Kneib of C4E Environmental, LLC.
- *Whole Effluent Toxicity Testing Guidance and Test Review Criteria,* Washington State Department of Ecology (revised Jun 2016) Pub# WQ-R-95-80.

DEVIATIONS FROM PROTOCOLS

Deviations from required procedures in the test methods:

- The *O. mykiss* chronic test did not meet the AECT 53 TAC for a minimum growth of 1.5 times the average initial dry weight. See the results and Discussion section for further information.

Deviations from recommended procedures in the test methods:

- The dissolved oxygen levels concentration in the *O. mykiss* chronic test dropped below the recommended minimum of 6.0 mg/L at 24 hours in all sample concentrations. Aeration was begun at that point. See the results and Discussion section for further information.

TEST DESIGN

The following summarizes the conditions used for both overall testing and the specifics for each test (observations and notations can be found on the datasheets in Appendix A):

Overall Test Design:

- Acute tests: 25, 50, 100, 200, and 400 mg/L sample + dilution water for the control.
- Chronic tests: 25, 50, 100, 200, and 400 mg/L sample + dilution water for the control.

Test Organism Conditions:

- All organisms tested were fed and maintained during culturing, acclimation, and testing as prescribed by the EPA (2002).
- The test organisms appeared vigorous and in good condition prior to testing.

C. dubia acute test: (non-renewal)

- Source: ET-C's in-house cultures
- Age: Less than 24 hours old
- Design: Four test vessels per concentration, five organisms per vessel
- Test Solution Renewal: None (i.e. static test)
- Monitoring:
    - Test Initiation and Termination:
        - DO and pH; all concentrations
        - Conductivity; control and highest concentration
    - Daily: Survival and temperature; all concentrations.
    - Daily: DO and pH; those concentrations where survival = 0%.
- Termination: 48 hours.
- Endpoints: Survival (at termination)

P. promelas acute test (renewal):

- Source: Aquatox Inc., Hot Springs, Arkansas
- Age: 1 to 14 days old, within a 24 hour age range
- Design: Four test vessels per concentration, Ten organisms per vessel
- Test Solution Renewal: Once @ 48 hours (i.e. static-renewal test)

- Monitoring:
  - Daily: Survival, DO, pH, and temperature; all concentrations.
  - Pre and Post Renewal solutions: DO and pH, all concentrations.
  - Test Initiation, with each new sample use, and Termination:
    - Conductivity, control and highest concentration (EPA)
- Termination: 96 hours.
- Endpoints: Survival (at termination)

O. mykiss acute test:

- Source: Thomas Fish Company, Anderson, California
- Age: 15 to 30 days old (After Swim Up), within a 24 hour age range
- Design: Four test vessels per concentration, Ten organisms per vessel
- Test Solution Renewal: Once @ 48 hours (i.e. static-renewal test)
- Monitoring:
  - Daily: Survival, DO, pH, and temperature; all concentrations.
  - Pre and Post Renewal solutions: DO and pH, all concentrations.
  - Test Initiation, with each new sample use, and Termination: Conductivity, all concentrations
- Termination: 96 hours.
- Endpoints: Survival (at termination)

C. dubia chronic test:

- Source: ET-C's in-house cultures
- Age: Less than 24 hours old and within an 8-hour age range, with blocking by known parentage
- Design: Ten test vessels per concentration, one organism per vessel
- Test Solution Renewal: Daily
- Monitoring:
  - Daily: Survival and neonate production (with brood determination)
  - Daily: DO and pH in pre and post-renewal solutions, all concentrations
  - Daily: Temperature in pre-renewal solutions, all concentrations
  - With each new sample: Conductivity in post-renewal solutions, control and highest sample concentration
- Termination: When 60%+ of surviving control organisms produce a 3rd brood.
  - Survival: @ after 7 days.
  - Reproduction: When 60%+ of surviving control organisms produce a 3rd brood.
- Endpoints: Survival (at termination) and Reproduction (through first 3 broods)

O. mykiss chronic test:

- Source: Thomas Fish Company, Anderson, California
- Age: 2 to 6 days old (After Swim Up), within a 24 hour age range
- Design: Four test vessels per concentration, ten organisms per vessel
- Test Solution Renewal: Daily
- Monitoring:
  - Daily: Survival
  - Daily: DO and pH in pre and post-renewal solutions, all concentrations
  - Daily: Temperature in pre-renewal solutions, all concentrations

    - With each new sample: Conductivity in post-renewal solutions, control and highest sample concentration
- Termination: 7 days after test initiation.
- Endpoints: Survival and Growth (average dry weight per organism added @ initiation)

DILUTION WATER

The dilution water used was the standard culture water used by ET-C:

- Reconstituted, moderately hard water (as per EPA protocol) with a total hardness of 75 to 105 mg/L as $CaCO_3$ and an alkalinity of 50 to 75 mg/L as $CaCO_3$.

SAMPLE COLLECTION AND STORAGE

Samples were collected by Tidal Vision USA personnel. The samples were accepted as scheduled by ET-C. Chain of Custody and Sample Receipt Records are provided in Appendix C.

- The sample was received at ambient temperature (21.6 °C).
- Following receipt, the samples were stored ambient temperature (~ 21 °) until test solutions were prepared and tested.

SAMPLE PREPARATION

Samples used during these tests were:

- Test solutions were made by diluting the sample into dilution water.
- Temperature adjusted prior to test initiation and each daily renewal.

DATA ANALYSIS

The statistical analyses performed for the acute tests were those outlined in *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, USEPA Office of Water, Fifth Edition (2002), EPA-821-R-02-012, using CETIS.

The statistical analyses performed for the chronic tests were those outlined in *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, USEPA Office of Water, Fourth Edition (EPA 2002), EPA-821-R-02-013, using CETIS.

- The specific statistical analysis and CETIS version used for each endpoint evaluation is listed with the statistical outputs included with each test in Appendix A.
- The calculations for MATC (= (NOEC + LOEC) / 2) were performed by hand calculator and included on the CETIS printouts.

Additional guidance was provided by:

- *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program*, (EPA June 2000), EPA 833-R-00-003.
- *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing* (40 CFR Part 136), (EPA July 2000), EPA 821-B-00-004.
- *Whole Effluent Toxicity Testing Guidance and Test Review Criteria*, Washington State Department of Ecology (revised Jun 2016) Pub# WQ-R-95-80.

RESULTS AND DISCUSSION

The raw data sheets for all tests are presented in Appendix A.

ACUTE BIOASSAYS

Table 1 summarizes the survival data for the *C. dubia* acute test.

Table 1
Summary of Acute Results
C. dubia

Sample Concentration (mg/L)	Percent Survival (at Test Termination)
Control	100
25	100
50	100
100	65
200	40 [a]
400	55 [a]

[a] Indicates a statistically significant difference from the control at alpha = 0.05.

Statistical analysis in accordance with the EPA and WDOE guidance protocol results in:

- NOEC = 100 mg/L
- LOEC = 200 mg/L
- LC_{50} = 262 mg/L
- MATC = 150 mg/L

Dissolved oxygen concentrations remained at 4.0 mg/L or greater throughout the test period. Test temperatures remained in the range of 20±1 °C.

The *C. dubia* acute test meets Test Acceptability Criteria (TAC) of a minimum 90 percent control survival. Unless referenced above, the tests proceeded without any noted deviations or interruptions that could have affected test results. The testing should be considered "valid".

Table 2 summarizes the survival data for the *P. promelas* acute test.

Table 2 **Summary of Acute Results** ***P. promelas***	
Sample Concentration (mg/L)	**Percent Survival (at Test Termination)**
Control	100
25	97.5
50	100
100	100
200	97.5
400	100

Statistical analysis in accordance with the EPA and WDOE guidance protocol results in:

- NOEC = 400 mg/L
- LOEC > 400 mg/L
- LC_{50} > 400 mg/L
- MATC > 400 mg/L

Dissolved oxygen concentrations remained at 4.0 mg/L or greater throughout the test period. Test temperatures remained in the range of 20±1 °C.

The *P. promelas* acute test meets Test Acceptability Criteria (TAC) of a minimum 90 percent control survival. Unless referenced above, the tests proceeded without any noted deviations or interruptions that could have affected test results. The testing should be considered "valid".

Table 3 summarizes the survival data for the *O. mykiss* acute test.

Table 3 **Summary of Acute Results** ***O. mykiss***	
Sample Concentration (mg/L)	**Percent Survival (at Test Termination)**
Control	100
25	100
50	100
100	100
200	100
400	100

Statistical analysis in accordance with the EPA and WDOE guidance protocol results in:

- NOEC = 400 mg/L
- LOEC > 400 mg/L
- LC_{50} > 400 mg/L
- MATC > 400 mg/L

Dissolved oxygen concentrations remained at 4.0 mg/L or greater throughout the test period. Test temperatures remained in the range of 20±1 °C.

The *O. mykiss* acute test meets Test Acceptability Criteria (TAC) of a minimum 90 percent control survival. Unless referenced above, the tests proceeded without any noted deviations or interruptions that could have affected test results. The testing should be considered "valid".

CHRONIC BIOASSAYS

Table 4 summarizes the survival and reproduction data for the *C. dubia* chronic test.

Table 4
Summary of Chronic Results
C. dubia

Sample Concentration (mg/L)	Percent Survival	Mean Number of Young Per Adult
Control	80	22.0
25	80	19.7
50	70	12.2 [a]
100	90	5.7 [a]
200	100	1.3 [a]
400	40	0 [a]

[a] Indicates a statistically significant difference from the control at alpha = 0.05.

Statistical analysis in accordance with the EPA and WDOE guidance protocol results in:

- NOEC = 25 mg/L
- LOEC = 50 mg/L
- IC_{25} = 33.7 mg/L
- MATC > 37.5 mg/L

Dissolved oxygen concentrations remained at 4.0 mg/L or greater throughout the test period. Test temperatures remained at 25±1 °C.

The *C. dubia* test meets Test Acceptability Criteria (TAC) for a minimum 80 percent control survival and a minimum 15 young produced per surviving control adult. Unless referenced above, the tests proceeded without any noted deviations or interruptions that could have affected test results. The testing should be considered "valid".

Table 5 summarizes the survival and growth data for the *O. mykiss* chronic test.

Table 5 Summary of Chronic Results *O. mykiss*		
Sample Concentration (mg/L)	**Percent Survival**	**Mean Dry Weight Per Organism Added (mg)**
Control	100	36.3
25	100	35.9
50	100	36.8
100	100	36.4
200	100	36.6
400	100	36.0

Statistical analysis in accordance with the AECT 53, 397-405 and WDOE guidance protocol results in:

- NOEC = 400 mg/L
- LOEC > 400 mg/L
- LC_{50} > 400 mg/L
- MATC > 400 mg/L

Note: The dissolved oxygen (DO) levels concentration in the *O. mykiss* chronic test dropped below the recommended minimum of 6.0 mg/L at 24 hours in all of the sample concentrations. However, it should be noted that there was no statistically significant effect observed in the effected sample concentration. As such, the low DO levels did not appear to have significantly affected test results.

Unless noted above, the dissolved oxygen concentrations remained at 6.0 mg/L or greater throughout the test period. Test temperatures remained at 15±1°C.

The *O. mykiss* test meets Test Acceptability Criteria (TAC) for a minimum 90 percent control survival. The test did not meet a minimum growth of 1.5 times the average initial dry weight. This TAC has been deemed difficult to achieve by the source method. However, these results are not part of compliance testing and should be considered "valid".
Unless referenced above, the tests proceeded without any noted deviations or interruptions that could have affected test results.

REFERENCE TOXICANT TESTS

Reference toxicant (reftox) testing is performed to document both initial and ongoing laboratory performance of the test method(s). While the health of the test organisms is primarily evaluated by the performance of the laboratory control, reftox test results also may be used to assess the health and sensitivity of the test organisms. Reftox test results within their respective cumulative summary (Cusum) chart limits are indicative of consistent laboratory performance and normal test organism sensitivity.

The results of the reftox tests indicate that the test organisms were within their respective cusum chart limits based on EPA guidelines. This demonstrates ongoing laboratory proficiency of the test methods and suggests normal test organism sensitivity in the associated client testing.

The *O. mykiss* reftox test was conducted using potassium chloride. The *P. promelas* acute reftox test was conducted using sodium chloride. The *C. dubia* reftox tests were conducted using sodium chloride.

The data sheets for the reference toxicant tests are provided in Appendix B.

Table 6 and 7 summarizes the reference toxicant test results and Cusum chart limits.

Table 6
Acute Reference Toxicant Tests (g/L)

Species	LC_{50}	Cusum Chart Limits
C. dubia	1.92	1.75 to 2.62
P. promelas	7.7	5.9 to 8.9
O. mykiss	2.30	0.76 to 2.64

Table 7
Chronic Reference Toxicant Tests (g/L)

Species	IC_{25}	Cusum Chart Limits
C. dubia (survival)	1.26	0.99 to 2.03
C. dubia (reproduction)	1.14	0.20 to 1.17
O. mykiss (survival)	2.19	-0.03 to 2.80
O. mykiss (growth)	2.16	-0.01 to 2.75

Appendix 5

Chitosan TR EPA Factsheet

Poly-D-Glucosamine (Chitosan)

Crops

Identification of Petitioned Substance

Chemical Names:
poly-D-glucosamine

CAS Numbers:
9012-76-4

Other Name:
Chitosan

Other Codes:
128930 (EPA/OPP Chemical Code)

Trade Names:
Chito-stik

Characterization of Petitioned Substance

Composition of the Substance:
Chitosan (poly-D-glucosamine) is a polymer[1] of glucosamine sugars, specifically glucosamine and N-acetyl-glucosamine (Hadwiger 2004). Its structure and composition are similar to both cellulose (i.e., the primary structural component of plant fiber) and chitin. Like chitin, chitosan is found naturally in the shells of all crustaceans and insects, as well as certain other organisms such as many fungi, algae, and yeast. Chitosan is one of the most common polymers found in nature (EPA 2003).

Properties of the Substance:
Chitosan is a chemically stable, white to pale yellow powder or flake (Polysciences 2003). Chitosan has a strong positive charge, which is the basis of its use as a "sticking" agent (i.e., an adhesive adjuvant). The positively charged molecules adhere to negatively charged pesticides and plant surfaces.

Chitosan is not soluble in water. It can be made soluble in water, however, by treating it with an acid to form soluble chitosan ions (Rabea et al. 2003). See Evaluation Question #1 form more information the production of chitosan.

Specific Uses of the Substance:
The petitioned use of the substance is as an adhesive adjuvant for use in organic crop production (Hadwiger 2004). As an adhesive adjuvant, the substance would be used to make a pesticide or fungicide stick to plant surfaces. Specifically, the petitioner seeks approval to test chitosan as a sticking agent for the fungicide copper sulfate pentahydrate for the control of potato late blight.

Approved Legal Uses of the Substance:
Chitosan is a registered pesticide (OPP No. 128930) that is used in crop production as a plant growth enhancer and plant defense booster (EPA 2003). In these uses, chitosan is applied to treat field crops, ornamentals, turf, home gardens, and nurseries. Target pests include early and late blight, downy and powdery mildew, and gray mold. Proposed application rates for the petitioned use as a sticking agent are much lower than the application rates for use as a pesticide/fungicide. Chitosan is exempt from the requirement for a pesticide tolerance (EPA 1995). See Evaluation Question #6 for more information on chitosan application rates and Evaluation Question #8 for more information on the modes of action for approved legal uses as a biopesticide.

According to the petition, chitosan is listed as an animal feed component in the Official Publication of the Association of American Feed Control Officials (Hadwiger 2004).

[1] A polymer is a large molecule that is a chain of linked, identical or similar molecular units called monomers.

Chitosan is used as a human dietary supplement for weight loss and cholesterol reduction (Rabea et al. 2004).

Chitosan is also used as a flocculating (i.e., settling) agent in wastewater treatment systems, a hydrating agent in cosmetics, a pharmaceutical agent in biomedicine, and an antimicrobial food wrap (Rabea et al. 2003). The State of Oregon has approved the use of chitosan in unrestricted amounts as a soil amendment (fertilizer). This use is not regulated by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act (EPA 1995).

Status

Action of the Substance:

Chitosan has a positive chemical charge, which causes it to attract negatively charged materials. This property is the mode of action for the petitioned use as an adhesive adjuvant. Specifically, chitosan would be used to adhere to negatively charged copper sulfate particles and plant surfaces.

International

Chitosan is not specifically listed for the petitioned use or other uses in the following international organic standards:

- Canadian General Standards Board
- CODEX Alimentarius Commission
- European Economic Community (EEC) Council Regulation 2092/91
- International Federation of Organic Agriculture Movements
- Japan Agricultural Standard for Organic Production

Evaluation Questions for Substances to be used in Organic Crop or Livestock Production

Evaluation Question #1*:* Is the petitioned substance formulated or manufactured by a chemical process? (From 7 U.S.C. § 6502 (21))

According to the petition, the raw material for chitosan is crab shell waste byproduct (Hadwiger 2004). Other potential raw material sources for commercially produced chitosan include shrimp shells (e.g., FDA 2002), lobster shells (EPA 1995), and cultured fungi (Rabea et al. 2003).

The process used to formulate chitosan is shown in Figure 1. The process begins with chitin obtained from seafood byproducts. Non-chitinous components of the seafood byproduct are stripped with a hydrochloric acid (not shown in Figure 1). Next, sodium hydroxide (NaOH), which is a base, and heat are used to remove residual meat attached to the shell material. Next, a stronger sodium hydroxide solution is used (Step 1 in Figure 1), in a step called deacetalation, to convert some N-acetyl glucosamine (the primary component of chitin) to glucosamine (the primary component of chitosan) (Rabea et al. 2003).

Following deacetalation, the chitosan is rinsed with water to remove remaining sodium hydroxide and impurities (Step 2 in Figure 1). A mild organic acid, such as lactic or acetic acid, is then applied (Step 3 in Figure 1) to adjust the pH of the chitosan below neutral (i.e. from basic to acidic). This step is required to make the chitosan soluble in water (Rabea et al. 2003). In the last manufacturing step, the chitosan is dried.

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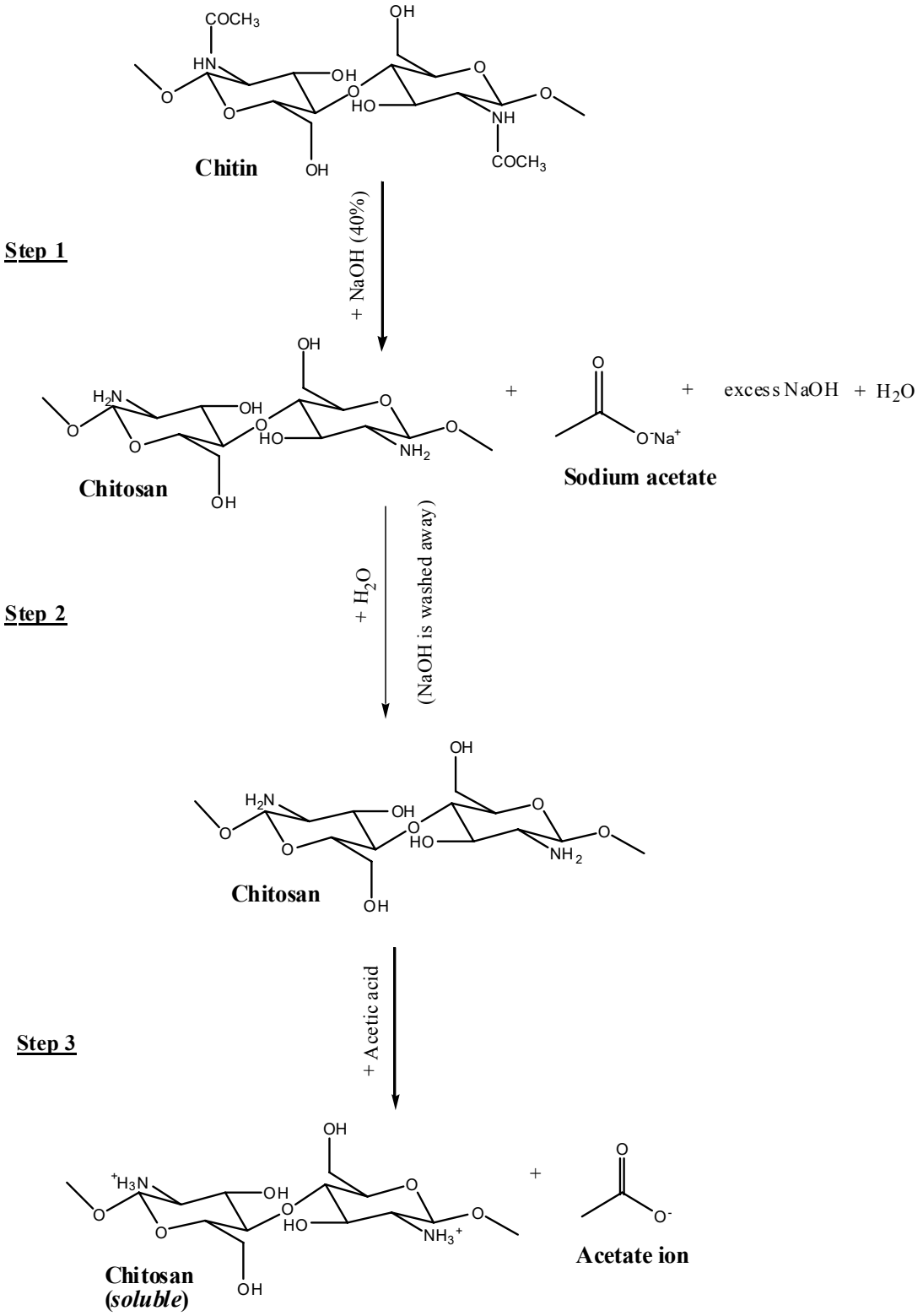

Figure 1. Formulation of Chitosan

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Evaluation Question #2: Is the petitioned substance formulated or manufactured by a process that chemically changes the substance extracted from naturally occurring plant, animal, or mineral sources? (From 7 U.S.C. § 6502 (21).)

In the chitosan manufacturing process, the primary component of chitin (i.e., N-acetyl glucosamine) is chemically changed to the primary component of chitosan (glucosamine). Both the starting and ending chemicals in this process are natural components of both chitin and chitosan and are present in the natural animal byproduct source (e.g., crab shells). The proportion of the two chemicals determines whether a mixture is chitin or chitosan. According to the petitioner, chitosan is approximately 80 percent glucosamine and 20 percent N-acetyl glucosamine (Hadwiger 2004). There are no precise definitions, however, of chitin and chitosan based on the percentage composition of glucosamine and N-acetyl glucosamine (Rabea et al. 2003). In both chitin and chitosan, these two chemicals are linked together in chains, called polymers, of as many as 5,000 glucosamine and N-acetyl glucosamine molecules (i.e., monomers).

Although N-acetyl glucosamine is converted to glucosamine in nature, the conversion does not occur by the controlled process used for commercial production (Figure 1).

Evaluation Question #3: Is the petitioned substance created by naturally occurring biological processes? (From 7 U.S.C. § 6502 (21).)

In nature, N-acetyl glucosamine may be deacetylated to glucosamine. The natural deacetalation process, however, does not occur as a result of the specific process (i.e., application of NaOH) used for commercial manufacturing.

Evaluation Question #4: Is there environmental contamination during the petitioned substance's manufacture, use, misuse, or disposal? (From 7 U.S.C. § 6518 (m) (3).)

There is no information available from EPA or FDA to suggest that environmental contamination results from the manufacture, use, misuse, or disposal. Chitosan is a registered pesticide, which implies a potential for misuse or improper disposal. It is a naturally occurring and biodegradable chemical, however, and EPA exempted it from the requirement for a tolerance limit when used as a pesticide in the production of any raw agricultural commodity (EPA 1995). In exempting chitosan from the requirement for a tolerance limit, EPA cited the following considerations:

> "Chitosan (1) is not toxic, as demonstrated in acute toxicity studies in mice, rats, and rabbits; (2) is naturally occurring in the environment in large concentrations; (3) has been exempted from the requirement of a tolerance in or on barley, beans, oats, peas, and wheat (40 CFR 180.1072) when used as a seed treatment at an application rate of 4 oz./100 lbs. seed; (4) has been approved by the State of Oregon for use in unrestricted amounts as a soil amendment (fertilizer), a use not regulated by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act." (EPA 1995)

In addition, according to EPA's pesticide fact sheet for chitosan, it is not expected to harm people, pets, wildlife, or the environment when used according to label directions due to its low potential for toxicity and abundance in the natural environment (EPA 2003).

The petitioner notes that manufacturing chitosan from crab shell waste reduces the potential for environmental contamination associated with disposal of the wastes (Hadwiger 2004).

Evaluation Question #5: Is the petitioned substance harmful to the environment? (From 7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i).)

Chitosan is a naturally occurring chemical and is one of the most common polymers found in nature (EPA 2003). EPA exempted chitosan from the requirement for a tolerance limit due to its low potential for toxicity and abundance in the environment. EPA concluded that chitosan is not expected to harm people,

pets, wildlife, or the natural environment, in part because chitosan was found to be nontoxic in acute toxicity studies in mice, rats, and rabbits (EPA 1995).

Evaluation Question #6: Is there potential for the petitioned substance to cause detrimental chemical interaction with other substances used in organic crop or livestock production? (From 7 U.S.C. § 6518 (m) (1).)

The adhesive property of chitosan that is the basis of its petitioned use could cause negatively charged particles other than the co-applied organic pesticide to stick to plant surfaces. Potential examples of other particles that may be attracted to the chitosan adhesive adjuvant include other agricultural products or fine soil particles. No information sources reviewed for this report described or evaluated potential adverse impacts of this nature.

Biochemically, however, chitosan is unlikely to cause detrimental chemical interaction with other substances used in organic crop or livestock production. As a component of the shells of insects and crustaceans, as well as certain other organisms such as many fungi, algae, and yeast (EPA 2003), chitosan is naturally present in agroecosystems. In addition, plants and microbes (e.g., in soil) have enzymes called chitinases and chitosanases that can break chitosan down to utilizable carbohydrates (Hadwiger 2004; Brzezinski and Neugebauer 2004).

Evaluation Question #7: Are there adverse biological or chemical interactions in the agro-ecosystem by using the petitioned substance? (From 7 U.S.C. § 6518 (m) (5).)

For the petitioned use, chitosan is unlikely to cause adverse biological or chemical interactions in the agroecosystem. Chitosan is found naturally in agroecosystems, and it may be broken down and utilized by plants and microbes (Hadwiger 2004; Brzezinski and Neugebauer 2004).

Although chitosan attracts positively charged particles, it is not highly reactive and it is not known to be toxic (e.g., EPA 1995). EPA has approved the use of chitosan as a pesticide and plant growth promoter at much higher application rates than proposed for its use as an adhesive adjuvant (see Evaluation Question #6).

Evaluation Question #8: Are there detrimental physiological effects on soil organisms, crops, or livestock by using the petitioned substance? (From 7 U.S.C. § 6518 (m) (5).)

Chitosan has documented physiological effects on plants and soil organisms, including plant growth enhancement, and antimicrobial ability. These effects, which are regarded as beneficial to crop production, are not involved in the petitioned use of chitosan as an adhesive adjuvant. The rate at which chitosan would be applied for the petitioned use (i.e., 5 to 10 grams per acre) is well below the recommended application rates for these other uses (e.g., 180 to 1,080 grams per acre [EPA 2001]). The known physiological effects of chitosan are described further below.

Chitosan has been shown to have antimicrobial, antifungal, and antiviral effects, and it is also known to be a plant growth enhancer (Rabea et al. 2003). The antimicrobial and antifungal effects are influenced by the length and composition of the chitosan polymers, environmental conditions, and other factors (Rabea et al. 2003). For example, very short chitosan polymers have the strongest antimicrobial and antifungal effects. Although Rabea et al. (2003) summarized several hypotheses about chitosan's mode of antimicrobial action, the exact mode of action is still unknown.

As a plant growth enhancer, the mode of action is believed to be that chitosan is taken up by plant cells where it enters the cell nucleus and stimulates messenger RNA and enzyme production. This action stimulates the plant to produce more lignin in the stems, resulting in stronger stems (EPA 1995, Rabea et al. 2003).

At the proposed application rates, chitosan is unlikely to create unacceptable changes in soil temperature, water availability, pH levels, nutrient availability, or salt concentration.

Evaluation Question #9: Is there a toxic or other adverse action of the petitioned substance or its breakdown products? (From 7 U.S.C. § 6518 (m) (2).)

Breakdown products of chitosan include smaller chitin and chitosan polymers (i.e., shorter chains of glucosamine and N-acetyl-glucosamine monomers), unlinked monomers, and other glucose-related molecules. These breakdown products are all nontoxic. Glucose is a sugar that can be utilized by many organisms.

Evaluation Question #10: Is there undesirable persistence or concentration of the petitioned substance or its breakdown products in the environment? (From 7 U.S.C. § 6518 (m) (2).)

Chitosan and its breakdown products are not persistent in the environment, and significant environmental accumulation of chitosan and its breakdown products would not result from repeated use of chitosan at the proposed application rate. Chitosan is biodegradable. For example, plants and microbes (e.g., in soil) have enzymes called chitinases and chitosanases that can break chitosan down to utilizable carbohydrates (Hardwiger 2004).

Evaluation Question #11: Is there any harmful effect on human health by using the petitioned substance? (From 7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i)) and 7 U.S.C. § 6518 (m) (4).)

Chitosan is not known to be toxic to humans. Chitosan is marketed as a human dietary supplement for control of obesity and high cholesterol. The scientific evidence of these benefits is questionable, however, and FDA sent a warning letter concerning unsubstantiated claims to the maker of one chitosan supplement (FDA 2004).

In 2001, Primex Ingredients, ASA, submitted a Generally Regarded as Safe (GRAS) notification to FDA for chitosan produced from shrimp. Primex subsequently withdrew the GRAS notification (FDA 2002), and chitosan is not currently GRAS.

Evaluation Question #12: Is there a wholly natural product that could be substituted for the petitioned substance? (From 7 U.S.C. § 6517 (c) (1) (A) (ii).)

The availability of alternative products was investigated by consulting organic industry resources, researching sources cited by the poly-D-glucosamine petition, and conducting Internet searches. This investigation identified one adhesive adjuvant product formulated with the functional agents lactose, bentonite, and casein. These ingredients are recognized as natural by organic industry sources (e.g., OMRI, 2005). The investigation also identified two similar adjuvant products formulated with pine-based functional agents (i.e., di-1-P-menthene, poly-1-P-menthene). However, it is unknown whether these products are synthetic or not.

ATTRA (Kuepper and Sullivan 2004) published a guide to organic alternatives for late blight control in potatoes. Although this source does not discuss adhesive adjuvants, it does describe alternative late blight control practices (e.g., application of compost tea) that do not involve fungicides and thus would not require an adhesive adjuvant.

Evaluation Question #13: Are there other already allowed substances that could be substituted for the petitioned substance? (From 7 U.S.C. § 6518 (m) (6).)

Based on a review of organic industry resources, there are at least three products marketed as organic adhesive adjuvants. As described in Evaluation Question #12, one of the products contains the functional agents bentonite, lactose, and casein. The two other products are pine-based. However, it is unknown whether the pine-based functional agents of these closely-related products are synthetic. No information

about the manufacturing process for these products was found and National List petitions have not been submitted for any uses of the functional agents.

Evaluation Question #14: Are there alternative practices that would make the use of the petitioned substance unnecessary? (From 7 U.S.C. § 6518 (m) (6).)

The petition proposes the use of chitosan as an adhesive adjuvant for use with an approved organic fungicide, such as copper sulfate (7 CFR 205.601(i)(2)). The petitioned use would enhance the ability of the fungicide to stick to plant surfaces, thereby improving effectiveness and reducing fungicide application rates. Potential alterative practices include application of the organic fungicide without the adhesive adjuvant or use of an alternative organic adhesive adjuvant (see Evaluation Questions #12 and #13).

Although the petitioner states that there is no effective control currently available for potato late blight, a publication by ATTRA (Kuepper and Sullivan 2004) provides guidance on organic control of late blight in potatoes. The ATTRA guidelines include cultural controls, such as:

- Field scouting and inspection of tubers going into storage to catch outbreaks in their earliest stages;
- Avoiding piling and leaving culls;
- Using certified seed and mixing seed lots;
- Using an AireCup® planter;
- Carefully monitoring seed planting depth and hilling operations;
- Using organic contact herbicides to kill infected plants;
- Managing irrigation to regulate leaf wetness;
- Destroying green vines;
- Harvesting tubers two weeks after destroying green vines; and
- Minimizing damage to tubers and keeping regulated air flow through storage piles.

In addition, ATTRA identified foliar feeding (e.g., with products made from kelp or horsetail) and application of compost tea as a method of enhancing disease resistance (Kuepper and Sullivan 2004).

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