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Safety assessment of *Euglena gracilis* ATCC 12894 whole cell biomass

Jennifer Symonds[1], Chonggang Zhang[2], Adam Noble[2], and Claire Kruger[1]

Abstract

A safety assessment of the dried whole cell biomass of *Euglena gracilis* ATCC 12894 was performed by the bacterial reverse mutation (Ames) assay, an in vitro micronucleus assay, and a 90-day repeat oral toxicity study in Wistar rats. *E. gracilis* ATCC 12894 whole cell biomass has no added excipients and contains 33.8% protein, 28.8% β-glucans, 19.8% fat, 7.1% ash, and 2.8% moisture. The bacterial reverse mutation assay found no evidence of mutagenicity after exposure to *E. gracilis* ATCC 12894 whole cell biomass, with or without metabolic activity, at levels up to 1581 μg/plate, the limit dose for the assay. Similarly, no evidence of genotoxicity was observed in the micronucleus assay, with or without metabolic activation, up to 320 μg/mL, the limit dose for the assay. The subchronic toxicity study was performed with the following test article dose groups: 0 (control), 1250, 2500, and 5000 mg/kg/day, administered to male and female Wistar rats via oral gavage for 90 days. No test article-related mortalities or adverse events were reported during the study. Histopathological examination revealed some vacuolation in the livers of males in the 5000 mg/kg/day group. This finding was considered adaptive, due to the approximately 20% fat content of whole cell biomass, and was therefore test article-related, but not adverse. No such findings were reported in female rats in the study. The results of the subchronic toxicity study describe a no observed adverse effect level of at least 5000 mg/kg/day.

Keywords

Euglena gracilis, 90-day subchronic oral toxicity, genotoxicity

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Introduction

Euglena gracilis is single-celled microalgae found in fresh water and has historically been used as a model organism. Its utility as a model is primarily due to its ease of culture, ability to thrive in diverse culture conditions and unique cellular biology. *E. gracilis* and most members of the *Euglena* genus contain both chloroplasts and flagella. *E. gracilis* can utilize photosynthesis as a source of energy and also use animal cell-like heterotrophy to metabolize nutrients in the absence of light.[1]

The metabolic plasticity of *E. gracilis* biology makes it an attractive organism in the generation of multiple valuable compounds, including protein, lipids like wax esters, saturated and unsaturated fatty acids, the β-(1-3)-linked polysaccharide paramylon, and provitamins.[1] The culture conditions of *E. gracilis* can be modulated to enhance the production of each of these compounds. For example, heterotrophic, dark culture conditions can be used to induce greater protein production in *E. gracilis*.[2] The nutrients produced by *E. gracilis* have the potential to replace some animal- and plant-based protein sources currently in the food supply. *E. gracilis* biomass has a comparable protein composition by dry weight to baker's yeast, meat, egg, and soy-based foods.[3] The protein produced by *E. gracilis* contains all 20 proteinogenic amino acids, with a similar

[1] Spherix Consulting Group Inc, Rockville, MD, USA
[2] Noblegen, Peterborough, ON, Canada

Corresponding author:
Claire Kruger, Spherix Consulting Group Inc, Rockville 20852, MD, USA.
Email: ckruger@spherixgroup.com

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protein content per gram to other algal whole cell biomass products on the market.[1]

The purpose of the studies described in this report was to assess the toxicity of the whole cell biomass of *E. gracilis* ATCC 12894, cultured in the absence of light to encourage protein production. The resulting biomass is 33.8% protein, 28.8% β-glucans (carbohydrate), 19.8% fat, 7.1% ash, and 2.8% moisture. The majority of the carbohydrates in the test article were measured to be β-glucans. *E. gracilis* will produce the β-glucan paramylon under heterotrophic culture conditions.[4] Paramylon is a polysaccharide consisting of linear glucose chains linked by β-1,3 glycosidic bonds, meaning it is a source of indigestible fiber.

The safety of *E. gracilis* ATCC 12894 whole cell biomass was determined in a bacterial reverse mutation assay, an in vitro micronucleus assay, and a subchronic toxicity study in male and female Wistar rats.

Materials and methods

Test article

E. gracilis ATCC 12894 whole cell biomass is the dried biomass of *E. gracilis* ATCC 12894 cultured under heterotrophic conditions in the absence of light. *E. gracilis* ATCC 12894 whole cell biomass (whole cell biomass) has the following compositional profile: 33.8% protein, 28.8% β-glucans, 19.8% fat, 7.1% ash, and 2.8% moisture.

Bacterial reverse mutation (Ames) test

An Ames test was performed to assess the genotoxic potential of whole cell biomass in compliance with the following principles: Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test," July 21, 1997; EPA Health Effects Test Guidelines, OPPTS 870.5100 "Bacterial Reverse Mutation Test," EPA 712-C-98-247, August 1998; and Commission Regulation (EC) No. 440/2008, B.13/14. "Mutagenicity: Reverse Mutation Test Using Bacteria," May 30, 2008. Bacterial reverse mutation assays were performed by CiToxLAB Hungary Ltd (Szabadságpuszta, Hungary) in compliance with OECD guidelines (OECD No. 471) and the following principles of Good Laboratory Practice: Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17. 4-Nitro-1,2-phenylenediamine (NPD), sodium azide (SAZ), methylmethanesulfonate (MMS), and 2-aminoanthracene (2AA) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). 9-Aminoacridine was obtained from Merck KGaA, Darmstadt. Dimethyl sulfoxide (DMSO) was obtained from VWR (Radnor, Pennsylvania, USA) and distilled water from Hungaro-Gal Kft (Kaposvár, Hungary). *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* WP2 *uvr*A were purchased from Molecular Toxicology Inc. (Boone, North Carolina, USA).

Post-mitochondrial fraction (S9) was isolated according to Ames et al.[5] and Maron and Ames[6] and the S9 fraction was mixed with 0.2 M sodium phosphate buffer and a salt solution (7.66 g/L NADP sodium salt, 3.54 g/L D-glucose 6-phosphate sodium salt, 4.07 g/L magnesium chloride hexahydrate, and 6.15 g/L potassium chloride (KCl)). Nine- to 10-week-old male Wistar rats were treated with phenobarbital and β-naphthoflavone at 80 mg/kg/day by oral gavage for three consecutive days. Rats were given drinking water and feed ad libitum until 12 h before termination. On day 4, the rats were euthanized by carbon dioxide inhalation and the livers were removed aseptically using sterile surgical tools. After excision, the livers were weighed and washed several times in 0.15 M KCl. The washed livers were homogenized and transferred to 3 mL of 0.15 M KCl/g of wet liver. Homogenates were centrifuged for 10 min at 9000 $\times$ *g* and the supernatant fraction was decanted and retained. The freshly prepared S9 fraction was aliquoted into 1–5 mL portions, frozen quickly, and stored at -80 ± 10°C until used.

The mutagenicity of whole cell biomass was determined using both the preincubation and plate incorporation method. All experiments were performed in triplicate with vehicle and strain-specific positive controls. For the plate incorporation method: whole cell biomass was diluted in water at 15.81, 50, 158.1, 500, 1581, and 5000 µg/plate with selective top agar with the tester strains *S. typhimurium* (TA98, TA100, TA1535, and TA1537) or *E. coli* (WP2 *uvr*A), with and without the metabolic activation system (S9 mix). The mixture was overlaid onto solidified agar and the plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies was counted manually. In a confirmatory assay, the tester strains were preincubated with 15.81, 50, 158.1, 500, 1581, and 5000 µg whole cell biomass/plate in the presence or absence of the S9 mix. The plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies was counted manually.

All experiments were performed in triplicate with vehicle and strain-specific positive controls. In the presence of the S9 mix, the positive control for all strains was 2AA. In the absence of the S9 mix, the positive control for strains TA100 and TA1535 was SAZ, WP2 *uvr*A was MMS, TA98 was NPD, and TA1537 was 9AA. Whole cell biomass was considered mutagenic if there was a concentration-related increase in the number of revertants per plate in at least one tester strain over a minimum of two increasing concentrations. For the strains TA98, TA100, and WP2 *uvr*A pKM101, the result was considered positive if the mean number of revertants was equal to or greater than two times the number of revertants obtained with the negative control. For the strains TA1535 and TA1537, the result was

considered positive if the mean number of revertants was equal to or greater than three times the number of revertants obtained with the negative control.

Micronucleus test

The micronucleus test was performed by CiToxLAB Hungary Ltd in compliance with OECD guidelines (OECD No. 487) and the following principles of Good Laboratory Practice: Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17. The human lymphocytes used in the assay were prepared from whole blood samples, collected into heparinized sterile tubes, obtained from young, healthy donors, nonsmoking, and not recently exposed to radiography (consent was obtained from each donor). To prepare each culture, 0.4 mL of heparinized whole blood was added to 8 mL of culture medium (RPMI 1640, with HEPES, 20% fetal calf serum, 2 mM L-glutamine, antibiotic, and antimycotic) containing 0.216 mg/mL phytohemagglutinin. The cultures were then placed at 37°C for 44–48 h.

S9 fraction for metabolic activation was purchased from Moltox (Molecular Toxicology Inc) and obtained from the liver of rats treated with Aroclor 1254 (500 mg/kg) by intraperitoneal route. Each batch of S9 is tested and validated by Moltox for absence of microbiological contamination and its ability to activate benzo(a)pyrene and 2-anthramine (also known as 2-amino anthracene) to mutagenic intermediates. The S9 fraction was preserved in sterile tubes at −80°C until use. The S9 mix was prepared at 5°C immediately before use and maintained at this temperature until added to culture medium.

The study consisted of a preliminary cytotoxicity test and a main micronucleus test. As precipitation was observed in the preliminary test, concentrations for the main test were selected based on precipitation, as recommended in the OECD 487 guideline. Vehicle (sterile water for injection) and positive control cultures were included in all test conditions. Concentrations selected for micronucleus analysis in the main test were 15.6, 31.3, and 62.5 µg/mL, the high concentration being the lowest showing precipitate in the culture medium. All conditions for the main micronucleus test were performed in duplicate. Human lymphocytes were exposed to whole cell biomass at those concentrations for 3 h with and without metabolic activation (S9 mix) and for 24 h without metabolic activation as follows:

Without S9 mix	3 h treatment + 24 h recovery
	24 h treatment + 0 h recovery
With S9 mix	3 h treatment + 24 h recovery

At harvest, the cells were collected by centrifugation (300 × *g* for 10 min) and submitted to a hypotonic treatment to induce cells swelling (i.e. incubation of 3 min in 4 mL of KCl 0.075 M pre warmed at 37°C). The cells were then fixed in a methanol/acetic acid mixture (3/1; v/v). Following fixation, the cells were kept at +5°C for at least an overnight period; then, they were centrifuged at 1250 × *g* for 3 min, the supernatant was removed, and cells were resuspended in a methanol/acetic acid mixture (7/1; v/v). After a second centrifugation (3 min at 1250 × *g*) and removal of the supernatant, two drops were spread on glass slides and stained for 7 min with 5% Giemsa.

Micronucleus analysis was performed "blind" under a microscope. Micronuclei were initially analyzed in 1000 binucleated cells per culture (total of 2000 BC per treatment level in all conditions, each culture in duplicate). In order to confirm the reliability of results observed in the main experiment after 3-h treatment without S9 mix, the analysis under those conditions was extended to 1000 additional BC per culture (whenever possible, and in any case 2000 additional BC per concentration) for the vehicle control and all test item concentrations.

The main experiment was considered valid if the following criteria were met: the mean frequency of cells that have undergone mitosis (binucleated + multinucleated cells) in the vehicle control cultures should be at least 50%, the mean background frequency of micronucleated binucleated cells (MNBCs) in the vehicle control cultures should be consistent with the historical vehicle control range, and a statistically significant increase in the mean frequency of MNBC has to be observed in the positive controls over the vehicle control cultures.

A test item is considered to have clastogenic and/or aneugenic potential if, in any of the experimental conditions examined, all the following criteria are met: a statistically significant increase in the frequency of MNBC, in comparison with the corresponding vehicle control, is obtained at one or more dose levels; a dose–response relationship (dose-related increase in the frequency of MNBC) is demonstrated by a statistically significant trend test, for at least one dose level; the frequency of MNBC of each replicate culture is above the corresponding vehicle historical range. A test item is considered clearly negative if none of the criteria for a positive response are met.

Subchronic toxicity study

A 90-day subchronic toxicity study in 7-week old male and female Wistar rats was conducted by CiToxLAB Hungary Ltd in compliance with OECD guidelines (OECD No. 408) and the following principles of Good Laboratory Practice: Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17. The facility is AAALAC accredited. The rats were housed two to three animals per cage during the acclimation period and during the study. Animals were acclimatized for 14–15 days prior to the study. The rats were randomized by body weight (bw) into four groups (n = 10/sex/group). Test

article conformed to established food grade specifications for commercial product and is the dried biomass of *E. gracilis* (whole cell biomass). The treatment groups were as follows: vehicle control (distilled water), 1250, 2500, or 5000 mg whole cell biomass/kg/day, administered via oral gavage.

All animals were observed for clinical signs daily and for mortality and morbidity twice daily. Bw of all animals was recorded at randomization, the first day of treatment, and then weekly, including day 89, and prior to necropsy on day 90, or on the day of death. Animals were fasted on the last day of the study, day 90. Feed and water consumption were recorded for each cage weekly. Toward the end of the treatment period, during week 12/13, all animals were examined in the functional observation battery, including measurements of the landing foot splay, fore/hind grip strength, and motor activity assessment.

Urine collection was conducted over approximately 16 h (days 89–90), during an overnight period of feed deprivation of animals, which were placed in metabolic cages. The following parameters were evaluated in all surviving animals: leukocytes, nitrite, pH, protein, glucose, urobilinogen, ketones, erythrocytes, specific gravity, sediment, volume, and color and clarity. Prior to necropsy, blood samples were collected by heart puncture under pentobarbital anesthesia for hematology, blood clotting time, and serum for clinical chemistry. Blood samples were analyzed using validated internal methods for the following hematological parameters by flow cytometry: red blood cell count, white blood cell count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell volume, platelet count, mean platelet thrombocyte volume, and reticulocyte count. Hemoglobin concentration of the blood was determined by cyanmethemoglobin absorbance. Blood neutrophils, lymphocytes, monocytes, basophils, eosinophils, and large unstained cells were differentiated by myeloperoxidase activity. Activated partial thromboplastin time (APTT) of the blood was determined by the micronized silica method, and prothrombin time of blood was determined by the quick method described by Biggs and MacFarlane.[7]

The following plasma clinical chemistry parameters were analyzed by validated internal methods by colorimetric tests: glucose, total bilirubin, urea, cholesterol, phosphorus, calcium, total protein, albumin, and bile acids. Albumin/globulin ratio was calculated. Creatinine was determined by two-point rate test. Sodium, potassium, and chloride were quantified by potentiometric test. Aspartate aminotransferase activity, alanine aminotransferase activity, and alkaline phosphatase activity were determined by multiple-point rate test.

At necropsy, animals were euthanized by exsanguination under pentobarbital anesthesia.

For thyroid hormone analysis, blood samples were taken from all animals at termination by sublingual venipuncture into tubes containing K3-EDTA as anticoagulant. The levels of thyroid hormone (T3 and T4) and thyroid stimulating hormone (TSH) were determined, respectively, by liquid chromatography with tandem mass spectrometry (LC-MS/MS) or immunoassay (Luminex MAP®; BIO-RAD, Hercules, California, USA). To compute the mean TSH, all results that were below the limit of quantitation were replaced with half the limit of quantitation to conservatively estimate the mean TSH concentrations found in these animals.

After euthanasia, a macroscopic evaluation of all animals was performed. The following organs were trimmed of fat and weighed in all groups: brain, epididymides, heart, kidney, liver, prostate, seminal vesicles with coagulating gland, spleen, testes, thymus, uterus including cervix, adrenal glands, ovaries, thyroid with parathyroid glands, and pituitary glands. Pair organs were weighed together. Absolute organ weights were measured, and relative organ weights to the body and brain weights were calculated.

The eyes with the optic nerves and testes with epididymides were retained in modified Davidson's fixative. The following organs were fixed in 10% buffered formalin solution, and retained in paraffin wax, sectioned at 4–6 μm, and stained with hematoxylin and eosin/phloxine: adrenals, aorta, brain, epididymis, esophagus, eye with optic nerve, femur with marrow, heart, kidney, large intestine, extraorbital lachrymal gland, harderian gland, liver, lungs with bronchi, lymph node, ovary, oviduct, pancreas, pituitary, prostate, salivary glands (including submandibular, sublingual, and parotid glands), sciatic nerve, seminal vesicle with coagulating gland, skin, subcutis with mammary gland (inguinal), skeletal muscle (quadriceps), small intestine, spinal cord, spleen, sternum with marrow, stomach, testis, thymus, thyroid with parathyroid gland, tongue, trachea, urinary bladder, uterus, and vagina. Histopathology was performed on animals in groups 1 (control) and 4 (high dose). In addition, any organs or tissues with macroscopic abnormalities (except minor changes) were subjected to histological examination from all groups.

The normality and heterogeneity of variance between groups were checked by the Shapiro–Wilk and Levene tests using the most appropriate data format (log-transformed when justified). Where both tests showed no significant heterogeneity, an analysis of variance/analysis of covariance test was performed. If the obtained result was positive, Dunnett's (multiple range) test was used to assess the significance of intergroup differences; identifying differences of <0.05 or <0.01 as appropriate. For nonparametric analysis, the Kruskal–Wallis analysis of variance was used after rank transformation. If there was a positive result, the intergroup comparisons were performed using Dunn test; identifying differences of <0.05 or <0.01 as appropriate. For noncontinuous data, the Cochran–Armitage test for trend was applied and the χ^2 test was used for statistical differences relative to control. For pathology data (macroscopic and microscopic data), the Cochran–Armitage test for trend was applied, then if appropriate, the χ^2

Table 1. Whole cell biomass bacterial reverse mutation test plate incorporation method.

S9 activation	Treatment	Dose (μg/plate)	Revertants per plate (mean ± standard deviation)				
			Base-pair substitution type			Frameshift mutation type	
			Salmonella typhimurium		*Escherichia coli*	*Salmonella typhimurium*	
			TA100	TA1535	WP2 *uvr*A	TA98	TA1537
−S9	Untreated control	—	92.3 ± 6.81	11.7 ± 0.58	55.0 ± 1.0	14.3 ± 0.58	7.3 ± 0.58
	DMSO control	—	88.3 ± 0.58	11.7 ± 2.52	52.0 ± 1.73	15.3 ± 0.58	8.3 ± 2.08
	Distilled water control	—	94.7 ± 5.03	13.0 ± 1.73	55.0 ± 1.0	14.0 ± 1.0	8.3 ± 0.58
	Whole cell biomass	15.81	102.0 ± 6.08	11.7 ± 1.53	55.7 ± 1.53	13.0 ± 1.0	7.0 ± 2.0
		50	105.3 ± 3.51	14.0 ± 1.0	53.0 ± 1.73	13.7 ± 0.58	6.7 ± 1.53
		158.1	110.3 ± 6.03	12.0 ± 1.0	52.3 ± 0.58	13.3 ± 0.58	8.3 ± 2.52
		500	108.7 ± 8.14	11.7 ± 1.15	53.0 ± 1.0	14.0 ± 2.0	8.7 ± 1.15
		1581	106.7 ± 5.51	14.7 ± 0.58	54.0 ± 1.0	14.0 ± 1.73	7.3 ± 2.52
		5000[a]	113 ± 8.19	14.7 ± 0.58	54.0 ± 2.0	14.3 ± 2.52	10.0 ± 3.61
	Positive control: 9AA	50	—	—	—	—	439.2 ± 12.06
	Positive control: NPD	4	—	—	—	396.0 ± 8.0	—
	Positive control: SAZ	2	1069.3 ± 14.05	1029.3 ± 16.17	—	—	—
	Positive control: MMS	2 μL	—	—	1098.7 ± 28.10	—	—
+S9	Untreated control	—	103.0 ± 2.65	14.0 ± 3.46	58.3 ± 0.58	16.0 ± 1.73	8.3 ± 4.16
	DMSO control	—	102.0 ± 1.0	12.7 ± 3.51	57.7 ± 0.58	16.3 ± 0.58	12.3 ± 1.53
	Distilled water control	—	108.3 ± 3.79	10.7 ± 2.08	58.0 ± 1.0	16.7 ± 1.53	8.7 ± 2.08
	Whole cell biomass	15.81	122.0 ± 1.0	12.7 ± 0.58	52.3 ± 1.53	15.0 ± 1.73	10.3 ± 1.53
		50	125.3 ± 9.02	12.7 ± 0.58	54.7 ± 1.15	15.0 ± 1.0	8.3 ± 2.52
		158.1	132.3 ± 5.51	13.0 ± 1.0	58.3 ± 0.58	16.7 ± 0.58	7.7 ± 1.53
		500	132.7 ± 9.02	13.0 ± 1.0	57.7 ± 1.53	15.3 ± 1.15	6.7 ± 1.15
		1581	104.3 ± 8.5	14.3 ± 1.53	58.7 ± 0.58	24.7 ± 0.58	11.0 ± 3.61
		5000[a]	136.0 ± 2.65	15.0 ± 1.73	58.3 ± 0.58	28.3 ± 1.53	12.0 ± 2.0
	Positive control: 2AA	2	2466.7 ± 20.53	210.7 ± 5.03	—	2432.0 ± 18.33	212.0 ± 8.72
		50	—	—	254.7 ± 7.02	—	—

NPD: 4-Nitro-1,2-phenylene-diamine; SAZ: sodium azide; MMS: methyl-methanesulfonate; 2AA: 2-aminoanthracene; DMSO: dimethyl sulfoxide.
[a]Slight precipitate formed.

homogeneity test. If significance was plausible based on a user-defined value (0.05), a pairwise test of each treatment group versus the control group was made. If the group size was <5, then Fisher's exact test was used; if the group sizes were >5, then the χ^2 test was used, identifying differences of <0.05, <0.01, or <0.001 as appropriate.

Results

Bacterial reverse mutation (Ames) assay

A slight precipitate was detected in all plates with a high concentration of whole cell biomass (5000 μg/mL). No precipitate was observed in the other concentrations: 15.81, 50, 158.1, 500, or 1581 μg/mL whole cell biomass.

Whole cell biomass was not cytotoxic at any of the doses used compared to the vehicle control. Whole cell biomass did not increase the number of revertant colonies in either the plate incorporation method (Table 1) or the preincubation method (Table 2) or in the presence of S9 metabolic activation. No increase in revertant colonies was observed in the frameshift strains or the base pair substitution strains, while the positive controls for each strain demonstrated a marked increase in revertant colonies, demonstrating the validity of the assay. There were no reproducible dose-related trends and there was no indication of any treatment-related effect.

Micronucleus test

Similar to the bacterial reverse mutation assay, a slight precipitate was detected in all plates with a high concentration of whole cell biomass (>62.5 μg/mL). No precipitate was observed in the other concentrations: 3.91, 7.81, 15.6, or 31.3 μg/mL whole cell biomass. The mean frequency of cells that had undergone mitosis (binucleated and multinucleated cells) was at least 50%, the mean background frequencies of MNBCs for the vehicle control were within historical control range, and positive control cultures induced statistically significant increases in the frequency of MNBC. The study was therefore considered to be valid.

No cytotoxicity was induced at any of the precipitate-free concentrations under all experimental conditions used (Tables 3 and 4).

No statistically significant or dose-related increases in the frequencies of MNBC were observed at any concentration relative to the corresponding vehicle controls

Table 2. Whole cell biomass bacterial reverse mutation test preincubation method.

S9 activation	Treatment	Dose (μg/plate)	Revertants per plate (mean ± standard deviation)				
			Base-pair substitution type			Frameshift mutation type	
			Salmonella typhimurium		*Escherichia coli*	*Salmonella typhimurium*	
			TA100	TA1535	WP2 *uvrA*	TA98	TA1537
−S9	Untreated control	—	103.7 ± 9.29	12.7 ± 1.53	46.7 ± 1.53	16.7 ± 1.53	7.7 ± 1.15
	DMSO control	—	99.7 ± 7.37	11.3 ± 0.58	46.0 ± 4.36	17.0 ± 0.0	8.3 ± 0.93
	Distilled water control	—	102.3 ± 10.69	11.7 ± 2.08	46.0 ± 1.0	18.0 ± 0.0	9.0 ± 1.0
	Whole cell biomass	15.81	99.7 ± 18.77	11.0 ± 2.65	45.7 ± 2.52	20.3 ± 2.08	10.3 ± 2.08
		50	101.3 ± 4.04	9.3 ± 2.08	41.3 ± 5.51	20.7 ± 1.15	8.7 ± 1.53
		158.1	118.3 ± 6.81	11.0 ± 2.0	47.0 ± 1.0	20.3 ± 1.15	10.0 ± 1.0
		500	118.3 ± 13.61	12.0 ± 2.65	59.0 ± 4.58	21.3 ± 1.15	10.3 ± 0.58
		1581	133.0 ± 5.57	11.0 ± 2.65	58.7 ± 3.21	21.3 ± 1.15	11.3 ± 1.15
		5000[a]	161.7 ± 9.07	20.7 ± 4.04	64.0 ± 8.19	22.3 ± 1.15	10.0 ± 2.0
	Positive control: 9AA	50	—	—	—	—	432.0 ± 14.42
	Positive control NPD	4	—	—	—	401.3 ± 12.22	—
	Positive control SAZ	2	1069.3 ± 16.65	1223.0 ± 34.87	—	—	—
	Positive control: MMS	2 μL	—	—	1044.0 ± 18.33	—	—
+S9	Untreated control	—	112.0 ± 1.73	13.3 ± 1.15	50.0 ± 2.0	21.3 ± 1.15	9.3 ± 1.15
	DMSO control	—	106.7 ± 3.06	12.3 ± 0.58	47.3 ± 3.06	21.0 ± 1.0	8.7 ± 1.15
	Distilled water control	—	119.3 ± 1.15	12.7 ± 1.53	49.3 ± 0.58	21.7 ± 1.15	9.3 ± 1.53
	Whole cell biomass	15.81	116.7 ± 9.29	10.0 ± 3.61	49.7 ± 1.53	21.7 ± 0.58	10.3 ± 2.08
		50	134.7 ± 12.50	13.7 ± 1.53	48.0 ± 2.0	20.0 ± 1.73	10.3 ± 1.53
		158.1	137.3 ± 4.04	13.3 ± 4.04	50.3 ± 1.15	21.7 ± 1.53	11.7 ± 2.08
		500	127.0 ± 9.51	11.0 ± 3.0	53.7 ± 2.89	22.0 ± 2.0	10.7 ± 1.53
		1581	142.0 ± 17.09	17.3 ± 3.06	62.3 ± 2.52	31.3 ± 10.97	10.3 ± 2.31
		5000[a]	170.0 ± 23.52	20.3 ± 4.93	73.3 ± 3.21	35.7 ± 1.53	15.0 ± 4.36
	Positive control: 2AA	2	2472.0 ± 12.0	216.3 ± 8.33	—	2408.0 ± 22.27	217.7 ± 6.11
		50	—	—	263.3 ± 5.03	—	—

NPD: 4-Nitro-1,2-phenylene-diamine; SAZ: sodium azide; MMS: methyl-methanesulfonate; 2AA: 2-aminoanthracene; DMSO: dimethyl sulfoxide.
[a]Slight precipitate formed.

following the 3-h treatment with S9 mix or the 24-h treatment without S9 mix, with all values remaining within historical vehicle control ranges.

Following the 3-h treatment without S9 mix, statistically significant increases in the frequency of MNBC were observed at 15.6 and 62.5 μg/mL whole cell biomass without S9 activation (4.0% at both concentrations vs. 0.0% for the vehicle control). However, the mean frequencies of MNBC remained within the historical vehicle control range (1.0–7.0%) and there was no dose–response relationship.

As the statistical significance could have been caused by the particularly low frequency observed with the vehicle control culture (0.0%), complementary readings were undertaken for the test item concentrations and corresponding vehicle controls (a total of 4000 BC per treatment level, data not shown). Following this additional analysis, the same statistically significant increase in the frequency of MNBC was observed at 15.6 μg/mL (4.0% vs. 1.5% for the vehicle control) but this was not observed at the high concentration of 62.5 μg/mL (2.8% vs. 1.5% for the vehicle control). Thus, the mean frequencies of MNBC still remained within the historical vehicle control range (1.0–7.0%) and there was still no dose–response relationship observed.

As all frequencies remained within historical control ranges, there was no dose–response relationship and no increases in frequency at the longer exposure period of 24 h in the absence of S9 mix, the slight increase observed at 15.6 μg/mL was judged to be biologically irrelevant, and the overall results obtained for this test condition were considered to show a negative response. Under the experimental conditions of the study, whole cell biomass was neither clastogenic nor aneugenic in the presence or absence of metabolic activation.

Subchronic toxicity study

Clinical signs. No treatment-related mortality or abnormal clinical observations were noted for any treatment group. One male in the 5000 mg/kg/day group was found dead on day 30 of the study. Enlarged liver, dark red, non-collapsed lungs, and dark red foci in the thymus were observed macroscopically. Histopathological examination confirmed correlating congestion/hemorrhages in liver, lungs, and thymus. The cause of death could not be established, but as this was an isolated instance (there were no other deaths in any of the test item groups), it was considered to be incidental and unrelated to the test item.

Table 3. Short treatment ± S9 mix (3 h treatment + 24 h recovery) cytogenic results.[a]

Test substance	S9	RI	Number of BCs with *n* MN					Total MNBCs		Frequency of MNBCs (%)
			n = 1	*n* = 2	*n* = 3	*n* = 4	*n* = 5	Per culture	Per dose	
Vehicle control	−		0	0	0	0	0	0	0	0
			0	0	0	0	0	0		
	+		0	0	0	0	0	0	3	1.5
			3	0	0	0	0	3		
Whole cell biomass 15.6 μg/mL	−	99	4	0	0	0	0	4	8	4.0[c]
			4	0	0	0	0	4		
	+	103	2	0	0	0	0	2	3	1.5[c]
			0	1	0	0	0	1		
Whole cell biomass 31.3 μg/mL	−	98	2	0	0	0	0	2	2	1.0
			0	0	0	0	0	0		
	+	104	2	0	1	0	0	3	7	3.5
			4	0	0	0	0	4		
Whole cell biomass 62.5 μg/mL[b]	−	91	1	0	0	0	0	1	8	4.0[c]
			6	1	0	0	0	7		
	+	108	2	0	0	0	0	2	4	2.0[c]
			2	0	0	0	0	2		
Positive control	−	78	5	1	0	0	0	6	14	7.0[d]
			7	0	1	0	0	8		
	+	60	12	0	0	0	0	12	19	9.5[d]
			6	1	0	0	0	7		

MNBC: micronucleated binucleated cell; BC: binucleated cell; MN: micronuclei; RI: replication index as mean % of control; Vehicle control: water for injection; Positive control: −S9—0.1 μg/mL colchine and +S9—6 μg/mL cyclophosphamide.
[a]1000 cells counted per culture and two cultures are shown.
[b]Precipitate was noted in the culture medium at the end of treatment.
[c]Statistics: 2 × 2 contingency table—$p < 0.05$.
[d]Statistics: 2 × 2 contingency table—$p < 0.001$.

Table 4. Long treatment without S9 mix (24 h treatment, no recovery) cytogenic results.

	RI	Number of BCs with *n* MN					Total MNBCs		Frequency of MNBCs (%)
		n = 1	*n* = 2	*n* = 3	*n* = 4	*n* = 5	Per culture	Per dose	
Vehicle control		1	0	0	0	0	1	6	2
		4	0	1	0	0	5		
Whole cell biomass 15.6 μg/mL	110	1	1	0	0	0	2	6	3
		4	0	0	0	0	4		
Whole cell biomass 31.3 μg/mL	108	2	0	0	0	0	2	6	3
		3	1	0	0	0	4		
Whole cell biomass 62.5 μg/mL P	102	1	0	0	0	0	1	1	0.5
		0	0	0	0	0	0		
MMC 0.1 μg/mL	66	19	0	0	0	0	19	40	20[a]
		20	1	0	0	0	21		

MNBC: micronucleated binucleated cell; BC: binucleated cell; MN: micronuclei; RI: replication index mean % of control; Vehicle control: water for injection; MMC: mitomycin C; P: precipitate was noted in the culture medium at the end of treatment.
[a]Statistics: 2 × 2 contingency table—$p < 0.001$.

Ophthalmology. No treatment-related ophthalmological changes were observed in any treatment group.

Body weight. No differences were observed in bw (Figure 1) or bw gain in any treated group, in both male and female rats, when compared to the controls.

Feed and water consumption. Feed and water consumption were monitored weekly. No changes were observed in feed or water consumed in males or females administered whole cell biomass at any dose compared to control (Tables 5 and 6).

Functional observational battery. No differences in animal behavior, general physical condition, or reaction to stimuli were observed between the control and any test group in male and female rats. No statistically significant

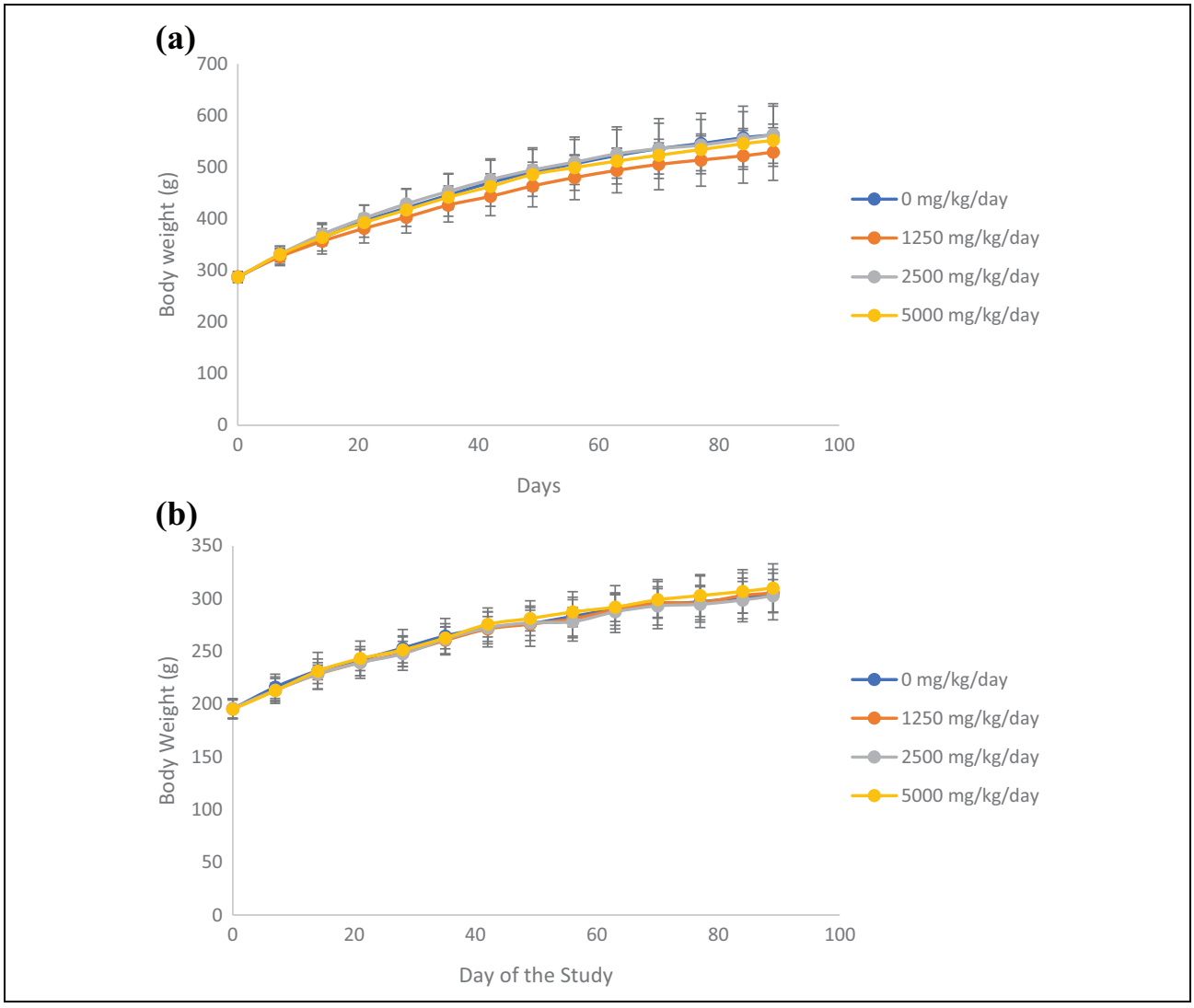

Figure 1. Bws of male and female rats during the subchronic toxicology study. (a) Bws in male rats. (b) Bws in female rats. $N = 10$ for all groups, except for the males administered 5000 mg/kg/day whole cell biomass, as one male died on day 30. Means with standard deviations are plotted. Bw: body weight.

differences were observed in grip strength of the fore and hind limbs in male or female rats administered *E. gracilis* ATCC 12894 whole cell biomass. There was a statistically significant decrease in landing foot splay in the females administered 5000 mg/kg/day compared to the control females. This finding was considered incidental and well within the historical control range. Therefore, it was concluded not to be test article related. Corroborating this conclusion, the effect was not observed in female rats from either of the other dose groups or in any of the male rats from any of the dose groups (data not shown).

All dose groups of males and females had a normal locomotor activity. In all cases, the initial activity was high, with reduced activity in each 5-min period to an approximate plateau by about 25–30 min. There was no statistical significance between the whole cell biomass administered animals and the controls when evaluating the overall distance travelled (0–60 min, cm). A statistically significant change was seen on one occasion at the 10–15 min period in the males in the 2500 mg/kg/day group, but it is considered incidental, as it was only seen at that time period, and not observed in the 5000 mg/kg/day group or in any of the female dose groups (data not shown). Whole cell biomass did not increase or decrease the normal locomotor activity (Figures 2(a) and (b)).

Thyroid hormone analysis. No statistically significant differences were observed in T3, T4, or TSH at any of the dose levels of whole cell biomass in either male or female rats (data not shown). The values reported for TSH were below detection (limit of quantitation was 120 pg/mL) in at least three animals per test article group.

Table 5. Feed consumption in rats.

Sex	Male				Female			
Whole cell biomass (mg/kg/day)	0	1250	2500	5000	0	1250	2500	5000
Observation time (days)	$N = 10$	$N = 10$	$N = 10$	$N = 9$[a]	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0 → 7	28.4 ± 1.6	27.0 ± 1.7	28.4 ± 1.2	27.8 ± 0.1	20.7 ± 1.1	20.3 ± 0.5	20.3 ± 1.2	20.2 ± 1.5
7 → 14	28.4 ± 1.9	26.7 ± 1.2	28.3 ± 1.9	26.7 ± 0.6	21.6 ± 0.7	20.2 ± 0.9	20.2 ± 1.7	20.5 ± 2.3
14 → 21	29.3 ± 2.4	27.1 ± 2.0	28.6 ± 1.7	26.7 ± 0.6	21.8 ± 1.0	20.3 ± 1.3	20.4 ± 2.0	20.2 ± 1.7
21 → 28	29.5 ± 2.6	27.2 ± 1.8	29.3 ± 1.7	26.8 ± 0.8	21.5 ± 1.2	20.2 ± 1.3	20.8 ± 1.6	20.6 ± 2.7
28 → 35	28.9 ± 2.2	27.3 ± 1.8	29.2 ± 2.2	27.1 ± 1.1	21.5 ± 0.9	20.4 ± 0.4	21.0 ± 1.7	20.4 ± 2.7
35 → 42	30.0 ± 1.9	27.3 ± 1.6	29.5 ± 2.1	27.2 ± 1.0	21.8 ± 1.4	21.2 ± 1.0	21.4 ± 1.3	22.3 ± 4.6
42 → 49	30.3 ± 2.2	28.3 ± 1.7	30.1 ± 1.8	28.0 ± 1.7	21.4 ± 1.2	21.1 ± 1.1	20.5 ± 1.4	20.4 ± 3.0
49 → 56	30.7 ± 2.5	28.6 ± 1.8	29.9 ± 1.2	27.4 ± 1.3	21.8 ± 0.9	21.4 ± 1.3	20.8 ± 1.9	20.1 ± 1.9
56 → 63	30.7 ± 2.7	28.2 ± 1.1	30.1 ± 1.2	27.4 ± 1.7	21.9 ± 0.9	21.8 ± 0.9	21.0 ± 2.0	21.5 ± 5.5
63 → 70	29.6 ± 2.4	27.8 ± 1.6	29.1 ± 1.1	26.8 ± 2.6	22.6 ± 0.9	22.6 ± 1.9	20.9 ± 2.3	23.6 ± 9.4
70 → 77	30.1 ± 2.5	27.6 ± 1.7	29.0 ± 0.8	26.9 ± 2.6	22.1 ± 0.7	21.2 ± 1.1	20.0 ± 2.1	19.7 ± 3.0
77 → 84	30.0 ± 2.3	27.2 ± 1.4	29.2 ± 1.1	26.8 ± 2.4	21.8 ± 0.8	21.4 ± 1.0	20.3 ± 1.9	23.8 ± 10.7
84 → 89	29.7 ± 2.3	28.1 ± 1.7	30.4 ± 1.1	27.0 ± 2.4	23.4 ± 1.2	23.4 ± 1.2	22.3 ± 1.2	23.3 ± 7.5
0 → 89	29.7 ± 2.2	27.6 ± 1.5	29.3 ± 1.4	27.1 ± 1.3	21.8 ± 0.9	21.1 ± 0.8	20.7 ± 1.7	21.2 ± 4.3

[a]One 5000 mg/kg/day male died at day 30.

Table 6. Water consumption in rats.

Whole cell biomass (mg/kg/day)	Male				Female			
	0	1250	2500	5000	0	1250	2500	5000
Observation time (days)	$N = 10$	$N = 10$	$N = 10$	$N = 9$[a]	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0 → 7	57.3 ± 14.0	42.6 ± 2.6	52.9 ± 5.5	44.9 ± 6.3	42.5 ± 10.0	43.2 ± 4.7	41.0 ± 4.5	38.8 ± 7.5
7 → 14	61.7 ± 17.7	47.6 ± 6.5	57.6 ± 6.8	49.1 ± 7.6	45.0 ± 12.0	42.1 ± 3.3	42.2 ± 6.6	39.3 ± 5.2
14 → 21	63.5 ± 18.4	46.9 ± 6.3	59.2 ± 7.8	50.2 ± 9.2	45.8 ± 12.9	42.7 ± 4.1	45.4 ± 8.0	39.8 ± 6.5
21 → 28	66.2 ± 20.2	48.1 ± 5.0	62.3 ± 7.8	53.2 ± 10.8	45.8 ± 8.9	45.7 ± 4.4	49.3 ± 9.0	44.2 ± 8.5
28 → 35	65.4 ± 22.4	48.6 ± 4.7	63.1 ± 7.0	53.7 ± 12.1	45.8 ± 8.9	45.8 ± 5.0	50.2 ± 10.3	46.0 ± 9.0
35 → 42	65.2 ± 19.8	49.2 ± 3.9	63.4 ± 7.7	53.0 ± 11.8	47.2 ± 11.6	47.2 ± 5.1	48.2 ± 10.6	46.0 ± 8.1
42 → 49	64.4 ± 23.3	47.4 ± 3.4	63.8 ± 12.7	50.7 ± 10.8	48.2 ± 12.7	47.7 ± 7.2	47.0 ± 12.3	43.5 ± 9.0
49 → 56	64.3 ± 21.2	49.4 ± 4.6	64.8 ± 9.8	53.5 ± 13.8	47.8 ± 11.8	50.6 ± 8.4	49.1 ± 13.5	46.1 ± 9.6
56 → 63	66.6 ± 23.1	50.2 ± 3.4	68.8 ± 11.1	52.6 ± 11.4	49.4 ± 13.8	52.3 ± 7.8	48.65 ± 11.6	45.5 ± 7.9
63 → 70	63.4 ± 20.2	47.7 ± 5.3	65.2 ± 9.9	52.3 ± 14.3	50.5 ± 14.6	52.9 ± 7.4	48.07 ± 12.8	45.1 ± 9.8
70 → 77	62.4 ± 18.7	46.6 ± 3.7	65.1 ± 12.5	54.9 ± 14.4	49.0 ± 13.1	53.0 ± 7.7	48.58 ± 14.7	44.5 ± 8.5
77 → 84	63.1 ± 16.4	47.2 ± 2.5	66.8 ± 13.5	55.1 ± 15.3	48.1 ± 10.5	53.2 ± 6.5	46.55 ± 10.9	46.5 ± 8.9
84 → 89	62.5 ± 18.3	47.2 ± 3.7	66.4 ± 9.4	52.1 ± 15.1	51.7 ± 15.3	53.6 ± 6.6	48.58 ± 13.3	44.5 ± 8.7
0 → 89	63.6 ± 19.5	47.6 ± 3.7	63.0 ± 8.9	51.9 ± 11.5	47.4 ± 11.9	48.4 ± 5.7	47.11 ± 10.5	43.8 ± 7.4

[a]One 5000 mg/kg/day male died at day 30.

Hematology. Male and female rats administered whole cell biomass had no test item-related changes in hematology parameters. No statistically significant changes were observed in the males at any dose level of whole cell biomass administration. Females administered 5000 mg/kg/day had a statistical increase in relative eosinophils compared to controls and 1250 and 2500 mg/kg/day groups (5000 mg/kg/day: 2.5%; 2500 mg/kg/day: 1.85%; 1250 mg/kg/day: 1.77%; control: 1.7%) but these results were within the historical control data range of 0.4–5.2% for the laboratory. Females administered 5000 mg/kg/day had a statistical increase in APTT compared to controls and 1250 and 2500 mg/kg/day groups (5000 mg/kg/day: 12.77 s; 2500 mg/kg/day: 12.39 s; 1250 mg/kg/day: 11.89 s; control: 11.50 s) but these results were within the historical control data range of 10.1–14.9 s. Females administered 5000 and 1250 mg whole cell biomass/kg/day had a statistical decrease in PTT compared to controls and 2500 mg/kg/day groups (5000 mg/kg/day: 9.20 s; 2500 mg/kg/day: 9.28 s; 1250 mg/kg/day: 9.15 s; control: 9.44 s) but these results were within the historical control data range of 8.8–9.9 s for the laboratory. Since each of these reported differences was within the historical control data range, did not demonstrate a dose–response relationship, and was not observed in the

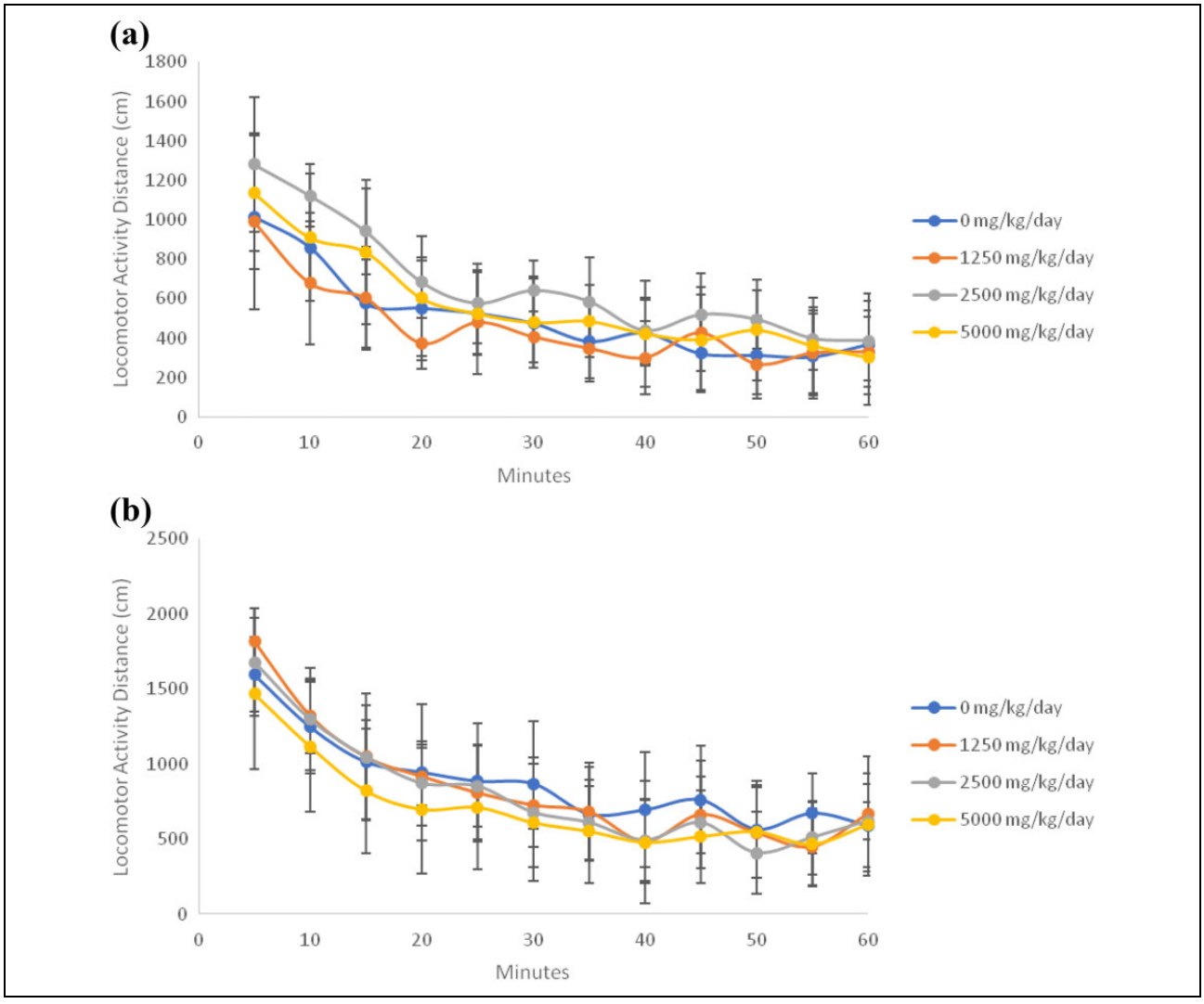

Figure 2. (a) Distance traveled in male rats. (b) Distance traveled in female rats. $N = 10$ for all groups, except for the males administered 5000 mg/kg/day whole cell biomass, as one male died on day 30. Means with standard deviations are plotted.

males, these findings were considered incidental and not adverse (Table 7).

Clinical chemistry. Clinical chemistry results demonstrated some statistically significant changes in urea, total protein, phosphorus, and chloride (Table 8). Significant changes were not observed in both males and females and were inside the historical control data ranges for these parameters. Therefore, these effects were considered to be incidental and not related to test article. Male rats administered 2500 mg *E. gracilis* ATCC 12894 whole cell biomass/kg/day had a statistical increase in the concentration of urea in the serum compared to the control and other dose groups (urea in millimoles per liter, 5000 mg/kg/day: 7.06; 2500 mg/kg/day: 8.8; 1250 mg/kg/day: 7.65; control: 7.43). This finding was within the historical control data range of 4.02–9.42 mmol/L urea for the laboratory. Since this result was not found in other *E. gracilis* ATCC 12894 whole cell biomass administered groups and was not present in females, this finding was considered incidental and not adverse. Male rats administered 5000 mg *E. gracilis* ATCC 12894 whole cell biomass/kg/day had a statistical increase in total protein compared to other test groups and the control (total protein in gram per liter, 5000 mg/kg/day: 57.56; 2500 mg/kg/day: 54.96; 1250 mg/kg/day: 56.39; control: 54.95). This increase was within the historical control data range of 48.9–61.4 g/L total protein, and since no dose–response relationship was observed, it was considered incidental, and not adverse. Furthermore, this effect was not observed in the females at any dose of *E. gracilis* ATCC 12894 whole cell biomass. The female rats administered 5000 mg *E. gracilis* ATCC 12894 whole cell biomass/kg/day had statistically significant differences in phosphorus and chloride compared to the control group. Phosphorus was decreased in the 5000 mg/kg/day females (5000 mg/kg/day: 1.65; 2500 mg/kg/day: 1.94;

Table 7. Hematology results.

	Male				Female			
Whole cell biomass (mg/kg/day)	0	1250	2500	5000	0	1250	2500	5000
Red blood count (M/μL)	8.4 ± 0.3	8.6 ± 0.3	8.5 ± 0.5	8.4 ± 0.4	7.6 ± 0.3	7.6 ± 0.5	7.7 ± 0.4	7.8 ± 0.2
White blood cell count (K/μL)	6.0 ± 1.8	6.3 ± 2.0	6.1 ± 1.3	5.8 ± 1.9	3.6 ± 1.8	4.5 ± 3.1	3.0 ± 1.1	3.4 ± 1.5
Hemoglobin conc. (g/dL)	14.7 ± 0.5	14.8 ± 0.6	14.7 ± 0.9	14.7 ± 0.5	13.8 ± 0.5	14.1 ± 0.7	14.1 ± 0.9	14.0 ± 0.5
Hematocrit (%)	42.8 ± 1.5	43.1 ± 1.3	42.9 ± 2.2	42.8 ± 1.3	39.9 ± 1.5	40.5 ± 2.1	41.1 ± 2.4	40.5 ± 1.1
Mean cell volume (fL)	50.9 ± 1.4	50.1 ± 1.6	50.8 ± 1.2	50.9 ± 1.4	52.5 ± 1.9	53.1 ± 1.4	53.4 ± 2.7	51.8 ± 1.7
Mean cell hemoglobin. (pg)	17.5 ± 0.6	17.2 ± 0.7	17.4 ± 0.5	17.5 ± 0.6	18.2 ± 0.6	18.5 ± 0.6	18.3 ± 0.8	17.9 ± 0.7
MCHC (g/dL)	34.3 ± 1.0	34.3 ± 0.6	34.2 ± 0.8	34.4 ± 0.7	34.6 ± 0.3	34.7 ± 0.4	34.4 ± 0.4	34.6 ± 0.5
Red cell D. width (%)	13.7 ± 0.6	13.5 ± 0.5	13.2 ± 0.6	13.4 ± 0.8	12.3 ± 0.4	12.0 ± 0.4	12.1 ± 0.7	11.8 ± 0.6
Platelet count (K/μL)	963.9 ± 133.9	811.1 ± 89.7	942.0 ± 151.8	857.4 ± 192.0	822.8 ± 151.9	913.8 ± 148.9	862.3 ± 138.3	823.8 ± 239.7
Mean plat. volume (fL)	7.0 ± 0.8	7.0 ± 0.5	6.9 ± 0.5	7.2 ± 0.6	7.0 ± 0.5	7.3 ± 0.9	7.0 ± 1.1	7.2 ± 0.8
Reticulocyte (%)	2.6 ± 0.5	2.4 ± 0.3	2.4 ± 0.8	2.1 ± 0.2	2.2 ± 0.4	2.2 ± 0.4	2.4 ± 0.4	2.2 ± 0.6
Neutorphils (%)	31.2 ± 6.1	34.9 ± 6.2	34.3 ± 7.2	35.4 ± 10.8	35.8 ± 8.4	33.1 ± 10.2	37.2 ± 9.5	38.0 ± 11.0
Lymphocytes (%)	62.3 ± 5.8	58.7 ± 6.4	59.5 ± 7.7	58.7 ± 10.3	59.0 ± 8.0	61.5 ± 10.2	57.7 ± 9.4	56.1 ± 10.4
Monocytes (%)	3.3 ± 0.7	3.7 ± 0.7	3.6 ± 0.6	3.0 ± 0.5	2.9 ± 0.7	3.0 ± 0.7	2.5 ± 0.6	2.9 ± 0.8
Basophils (%)	0.09 ± 0.06	0.07 ± 0.09	0.06 ± 0.07	0.04 ± 0.05	0.05 ± 0.05	0.04 ± 0.05	0.07 ± 0.09	0.03 ± 0.05
Eosinophils (%)	2.3 ± 0.5	1.9 ± 0.6	1.8 ± 0.6	2.2 ± 0.5	1.7 ± 0.5	1.7 ± 0.6	1.9 ± 0.4	2.5 ± 0.9[a]
LUC (%)	0.9 ± 0.4	0.8 ± 0.4	0.7 ± 0.3	0.6 ± 0.4	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.3	0.5 ± 0.2
APTT (s)	12.4 ± 1.6	12.8 ± 1.2	13.1 ± 1.2	13.2 ± 1.0	11.5 ± 1.1	11.9 ± 0.7	12.4 ± 1.1	12.8 ± 1.0[a]
PTT (s)	9.9 ± 0.4	9.8 ± 0.2	9.8 ± 0.2	9.8 ± 0.2	9.4 ± 0.1	9.2 ± 0.3[b]	9.3 ± 0.3	9.2 ± 0.2[b]

LUC: large unclassified cells; APTT: activated partial thromboplastin time.
[a]Dunnet two-sided $p < 0.05$.
[b]Dunnet two-sided $p < 0.05$.

Table 8. Clinical chemistry.

Whole cell biomass (mg/kg/day)	Male				Female			
	0	1250	2500	5000	0	1250	2500	5000
Clinical chemistry parameters								
Glucose (mmol/L)	8.3 ± 0.8	8.7 ± 0.7	8.1 ± 0.3	8.2 ± 0.4	8.0 ± 0.9	8.1 ± 0.9	8.1 ± 1.2	8.5 ± 1.0
Total bilirubin (μmol/L)	3.6 ± 0.9	3.6 ± 1.1	3.4 ± 0.7	3.8 ± 0.7	3.9 ± 0.7	4.0 ± 1.2	4.0 ± 0.7	4.1 ± 1.2
Urea (mmol/L)	7.4 ± 1.2	7.7 ± 0.8	8.8 ± 1.2[a]	7.1 ± 0.7	9.6 ± 0.9	10.0 ± 1.7	9.6 ± 1.6	9.5 ± 1.2
Cholesterol (mmol/L)	1.5 ± 0.4	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.3	1.6 ± 0.3	1.6 ± 0.38	1.6 ± 0.4	1.5 ± 0.4
Creatinine (μmol/L)	47.3 ± 4.0	48.8 ± 6.2	49.0 ± 5.0	49.1 ± 3.4	52.6 ± 6.6	53.6 ± 7.6	51.8 ± 3.3	48.2 ± 4.4
Phosphorus (mmol/L)	2.5 ± 0.3	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	1.7 ± 0.2[b]
Sodium (mmol/L)	143.4 ± 1.3	144.0 ± 0.9	143.9 ± 1.1	144.1 ± 1.6	143.9 ± 0.9	144.4 ± 1.0	144.7 ± 0.8	144.8 ± 1.4
Potassium (mmol/L)	5.0 ± 0.4	4.9 ± 0.3	5.0 ± 0.3	5.2 ± 0.4	4.7 ± 0.4	4.6 ± 0.3	4.7 ± 0.4	5.0 ± 0.4
Calcium (mmol/L)	2.6 ± 0.1	2.6 ± 0.07	2.6 ± 0.08	2.6 ± 0.08	2.6 ± 0.07	2.6 ± 0.07	2.6 ± 0.09	2.6 ± 0.07
Chloride (mmol/L)	96.3 ± 1.0	96.7 ± 1.3	97.6 ± 1.5	97.2 ± 1.1	98.1 ± 1.1	98.9 ± 1.5	99.2 ± 1.7	100.3 ± 1.6[b]
Total protein (g/L)	55.0 ± 2.0	56.4 ± 2.5	55.0 ± 1.7	57.6 ± 2.1[a]	54.4 ± 1.5	56.1 ± 2.8	56.1 ± 3.4	55.4 ± 3.6
Albumin (g/L)	29.0 ± 1.7	29.9 ± 1.6	29.0 ± 1.3	30.5 ± 1.5	29.8 ± 1.0	30.8 ± 1.4	31.0 ± 2.1	30.9 ± 3.2
A/G ratio	1.1 ± 0.05	1.1 ± 0.04	1.1 ± 0.05	1.14 ± 0.05	1.2 ± 0.06	1.2 ± 0.06	1.2 ± 0.07	1.3 ± 0.2
AST/GOT (U/L)	192.8 ± 59.2	187.8 ± 87.9	164.2 ± 75.9	149.6 ± 57	151.2 ± 67.1	156.2 ± 123.5	140.7 ± 54.9	168.4 ± 160.9
ALT/GPT (U/L)	63.3 ± 26.7	69.7 ± 38.8	61.6 ± 38.0	52.4 ± 27.4	59.4 ± 45.6	54.0 ± 29.0	62.1 ± 36.7	73.9 ± 88.4
ALKP (U/L)	69.6 ± 12.5	62.7 ± 8.8	64.7 ± 12.0	63.3 ± 11.7	40.8 ± 10.3	40.5 ± 8.8	42.2 ± 16.1	36.0 ± 13.7
GGT (U/L)	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
Triglycerides (mmol/L)	0.9 ± 0.3	0.9 ± 0.4	0.7 ± 0.4	0.7 ± 0.2	0.4 ± 0.07	0.4 ± 0.05	0.4 ± 0.06	0.4 ± 0.2
Bile acid (μmol/L)	8.0 ± 1.2	9.3 ± 2.3	8.7 ± 1.8	9.5 ± 1.7	7.4 ± 1.4	7.02 ± 2.0	7.7 ± 1.2	9.1 ± 4.2

A/G: albumin/globulin; AST/GOT: aspartate aminotransferase; ALT/GPT: alanine aminotransferase; ALKP: alkaline phosphatase; GGT: gamma glutamyltransferase.
[a]Dunnett two-sided $p < 0.05$.
[b]Dunnett two-sided $p < 0.01$.

1250 mg/kg/day: 1.98; control: 2.15) but was within the historical control data range of 1.47–2.82 mmol/L phosphorus. Chloride was increased in the 5000 mg/kg/day females (5000 mg/kg/day: 100.25; 2500 mg/kg/day: 99.24; 1250 mg/kg/day: 98.92; control: 98.08) but was also within the historical control data range of 93.7–107.8 mmol/L chloride.

Urinalysis. Statistically significant differences observed in urinalysis parameters were either not associated with a dose response (decreased urinary nitrite and increased urine crystals for males given 5000 mg/kg bw/day) or were inconsistent between the sexes (increased urinary leucocytes for males given 5000 mg/kg bw/day; decreased urinary bacteria for females given 5000 mg/kg bw/day); thus, they were considered to be unrelated to administration of the test item and not adverse (data not shown).

Organ weights. In males, no treatment-related effect was seen on absolute organ weights in any dose group. The relative liver weights (relative to bw) were statistically significantly higher (12%) in the high-dose group compared to the relative liver weights in the control dose group (Table 9). No effect was seen on this parameter in the low- and mid-dose groups. The finding in relative liver weight was correlated with an increase in minimal hepatocellular vacuolation of the liver (typically macrovesicular at the

Table 9. Organ weights relative to bw.

	Sex	Males				Females			
% Organ/bw	Whole cell biomass (mg/kg/day)	0	1250	2500	5000	0	1250	2500	5000
Bw (g)		532.8 ± 57.2	499.7 ± 52.6	534.5 ± 53.1	522.8 ± 25.3	282.4 ± 21.3	285.1 ± 14.4	283.9 ± 16.7	290 ± 21.4
Brain		0.42 ± 0.041	0.45 ± 0.049	0.43 ± 0.042	0.42 ± 0.028	0.73 ± 0.046	0.74 ± 0.046	0.74 ± 0.043	0.71 ± 0.067
Adrenals		0.014 ± 0.0021	0.015 ± 0.0021	0.014 ± 0.0022	0.014 ± 0.001	0.034 ± 0.0059	0.033 ± 0.0025	0.032 ± 0.0031	0.031 ± 0.0042
Heart		0.27 ± 0.018	0.29 ± 0.027	0.28 ± 0.029	0.28 ± 0.019	0.34 ± 0.026	0.33 ± 0.021	0.32 ± 0.026	0.33 ± 0.019
Kidneys		0.59 ± 0.048	0.64 ± 0.056	0.61 ± 0.04	0.62 ± 0.035	0.63 ± 0.053	0.64 ± 0.061	0.66 ± 0.053	0.65 ± 0.06
Liver		2.79 ± 0.19	2.98 ± 0.18	2.94 ± 0.17	3.12 ± 0.24[a]	2.86 ± 0.30	2.86 ± 0.22	2.94 ± 0.50	2.94 ± 0.28
Spleen		0.18 ± 0.026	0.18 ± 0.02	0.17 ± 0.015	0.17 ± 0.017	0.22 ± 0.026	0.22 ± 0.025	0.23 ± 0.031	0.23 ± 0.045
Thymus		0.079 ± 0.014	0.074 ± 0.02	0.065 ± 0.012	0.08 ± 0.02	0.12 ± 0.018	0.10 ± 0.035	0.11 ± 0.017	0.11 ± 0.018
Thyroid + parathyroid		0.0054 ± 0.00082	0.0059 ± 0.0011	0.0050 ± 0.00066	0.0057 ± 0.0012	0.0067 ± 0.0022	0.0082 ± 0.0013	0.0076 ± 0.0012	0.0073 ± 0.0011
Epididymides		0.30 ± 0.034	0.31 ± 0.039	0.31 ± 0.041	0.30 ± 0.029	—	—	—	—
Prostate		0.20 ± 0.049	0.18 ± 0.029	0.19 ± 0.043	0.17 ± 0.027	—	—	—	—
Testes		0.80 ± 0.14	0.81 ± 0.086	0.75 ± 0.062	0.76 ± 0.071	—	—	—	—
Seminal vesicle		0.61 ± 0.12	0.62 ± 0.091	0.58 ± 0.10	0.57 ± 0.059	—	—	—	—
Pituitary gland		0.0024 ± 0.0003	0.0026 ± 0.0003	0.0026 ± 0.0004	0.0027 ± 0.0004	0.0053 ± 0.0009	0.0055 ± 0.0004	0.0055 ± 0.0009	0.0056 ± 0.001
Ovaries						0.040 ± 0.0072	0.046 ± 0.0068	0.044 ± 0.0055	0.038 ± 0.0043
Uterus including cervix						0.23 ± 0.039	0.27 ± 0.11	0.25 ± 0.11	0.23 ± 0.068

Bw: body weight; —: organs not collected.
[a]Dunnett two-sided $p < 0.01$.

Table 10. Lesions and abnormalities found in necropsy.

	Males				Females			
Whole cell biomass (mg/kg/day)	0	1250	2500	5000	0	1250	2500	5000
Animals/group	10	10	10	9	10	10	10	10
Coagulating gland								
No visible lesions	6	7	8	3	—	—	—	—
Not present at necropsy: bilateral	1	1	1	1	—	—	—	—
Not present at necropsy: left	0	0	0	2	—	—	—	—
Not present at necropsy: right	3	2	1	3	—	—	—	—
No visible lesions	0	0	0	0	0	0	0	0
Abnormal, bilateral	0	0	0	0	0	0	0	1
Fur								
No visible lesions	0	0	0	0	0	0	0	0
Alopecia, bilateral, flank	0	0	0	1	0	0	0	0
Thin, bilateral, thoracic	0	0	0	0	1	0	0	0
Thin, right, forelimb	0	0	0	0	1	0	0	0
Hindpaw								
No visible lesions	0	0	0	0	0	0	0	0
Abnormal, bilateral	0	0	0	0	0	0	0	1
Lungs								
No visible lesions	10	10	10	9	9	10	10	10
Discoloration, red, left lobe, focal	0	0	0	0	1	0	0	0
Axillary lymph node								
No visible lesions	0	0	0	0	0	0	0	0
Enlargement, bilateral	0	0	0	0	0	0	0	1
Lung-associated lymph node								
No visible lesions	0	0	0	0	0	0	0	0
Enlargement	0	0	0	0	0	0	0	1
Stomach								
No visible lesions	10	10	10	9	9	10	10	9
Discoloration, red, glandular, mucosa, focal	0	0	0	0	0	0	0	1
Raised area, non-glandular region, mucosa	0	0	0	0	1	0	0	0
Testis								
No visible lesions	9	10	10	9	—	—	—	—
Enlargement, left	1	0	0	0	—	—	—	—
Thymus								
No visible lesions	10	10	10	9	10	9	10	10
Small	0	0	0	0	0	1	0	0
Uterine cervix body and horn								
No visible lesions	—	—	—	—	10	8	9	9
Dilatation, body, horn	—	—	—	—	0	2	1	1

periportal region) in the high-dose group. There were no statistically significant differences in the absolute or relative weights of other organs in the test groups compared to controls.

In females, the ovary weights (absolute and relative to brain weight) were statistically significantly higher in the low-dose group compared to control. In low-dose females, absolute ovary weights were larger by 17.1% and the organ weights related to brain weights were 15.3% greater than control. There was no histopathological correlate for the weight changes in the ovaries in low-dose females and there was no dose response. Therefore, this difference was considered to be incidental and not related to treatment. There were no other statistically significant differences in the absolute or relative weights of organs from females in treated groups compared to controls.

Gross pathology. No test article-related changes were observed in gross pathology for the following organs in all treatment groups in both male and female rats: abdominal aorta, adrenal gland, thoracic aorta, bone marrow smear, brain, cecum, colon, duodenum, epididymis, esophagus, eye, femur marrow, Harderian gland, heart, ileum, jejunum, kidney, lacrimal gland, liver, lymph node, mandibular, mesenteric lymph node, inguinal mammary gland area, optic nerve, ovary, oviduct, pancreas, Peyer's patch, pituitary, prostate, rectum, mandibular salivary gland, parotid salivary gland, sublingual salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, inguinal skin and subcutis, spinal cord, spleen, sternum and marrow, tongue, thyroid and parathyroid gland, trachea, urinary bladder, and vagina (Table 10). All observed changes were considered incidental or consistent with background incidence.

Histopathology. Test item-related findings were observed in the liver of high-dose males; there were no histological changes in livers from the low- and mid-dose male animals or in livers of females from any dose group. There were also no histological changes in any other examined organs/tissues that were considered to be related to treatment. In males, the incidence of minimal hepatocellular vacuolation of the liver (typically macrovesicular at the periportal region) in controls, low-, mid-, and high-dose animals was 2/10, 1/10, 0/10, and 7/9, respectively. The increased incidence of hepatocellular vacuolation in high-dose males was correlated with a minimal (12% increase compared to control) increase of the relative organ weight of the liver in this group. The incidence of minimal hepatocellular vacuolation of the liver in females was 2/10, 1/10, 0/10, and 1/10 in control, low-, mid-, and high-dose groups, respectively.

Minimal cortical vacuolation was noted in the adrenals of 3/10 control males, 1/10 low dose, 4/10 mid dose, and 4/9 high dose. The incidence was consistent with incidence in historical controls (frequency range 1/10-9/10) and considered to be unrelated to test item. Cortical vacuolation was not noted in the adrenals from the female test groups or controls.

All other changes, such as purulent inflammation in the subcutis, focal degeneration/necrosis of cardiomyocytes, tubular basophilia, eosinophil casts, pyelonephritis and pelvic dilatation in the kidneys, harderian metaplasia in the lacrimal gland, autophagic vacuoles in the pancreas, dilatation tubule in the testis, congestion/hemorrhages and involution in the thymus, inflammatory cell infiltrate in the urinary bladder, increased cellularity in the lymph nodes, and signs of estrus in the uterus, were seen in control and/or treated animals, or without meaningful differences in severity and incidence. They were regarded as incidental and consistent with background incidence.

Discussion

The purpose of these studies was to assess the toxicity of *E. gracilis* ATCC 12894 whole cell biomass. *E. gracilis* is a member of the euglenid group of photosynthetic flagellates. Given the ease of culture and relatively low carbon footprint compared to other protein sources, *E. gracilis* protein is an attractive alternative to animal protein as a source of protein in the food supply. Algal biomass products have been consumed by humans for thousands of years, as evident of Spirulina consumption in Aztec Mexico.[3] There are several generally recognized as safe notices that received "no questions" letters from the US FDA describing food products produced from algae, including *Arthrospira platensis* (also called Spirulina, GRNs 127, 394, 417),[8–10] *Chlorella protothecoides* (GRNs 469 and 519),[11,12] *Dunaliella bardawii* (GRN 351),[13] and *Chlamydomonas reinhardtii* (GRN 773).[14]

E. gracilis ATCC 12894 whole cell biomass safety was assessed in genotoxicity and subchronic rodent bioassays. The whole cell biomass was not genotoxic in an Ames assay or in an in vitro micronucleus assay.

Daily administration of *E. gracilis* ATCC 12894 whole cell biomass by oral gavage to Wistar rats for 90 days at dose levels of 0, 1250, 2500, or 5000 mg/kg bw/day, under the conditions of this study, did not result in test item-related mortality or adverse clinical signs. There was no effect on the bws, feed consumption, water consumption, or neurological assessment (by functional observation battery) in any of the dose groups. No treatment-related alterations were found during the ophthalmoscopic examination. Clinical pathology did not reveal any test item-related changes, when compared with controls. The thyroid hormone analysis did not show any test item-related changes.

There were no test item-related macroscopic changes seen at necropsy, except the enlargement of the livers (compared to the bw) in the high-dose group males. Test item-related, increased incidence of hepatocellular vacuolation (typically macrovesicular at the periportal region) was observed in the high-dose male livers and associated with a 12% relative liver weight increase. This change can be correlated with the high (approximately 20%) lipid content of the test item. Macrovesicular lipidosis is a reaction to a wide variety of injuries and can also be regarded as a physiological adaptation demonstrated as an imbalance between uptake of lipids from blood and secretion of lipoproteins by the hepatocyte.[15] Rats in the high-dose group were gavaged with test material at 5000 mg/kg, resulting in an exposure to 2500 mg per day of test material (assuming a bw of approximately 500 g). The test material is approximately 20% fat which means that these animals were given 500 mg fat per day from the test article. Rat chow contains 3.3% fat; male rats consume on average 27 g feed per day or 891 mg fat per day from diet. The addition of 500 mg fat from test article contributes significantly to the fat intake and is likely responsible for the hepatic vacuolation seen in the males from this group. These results were not observed in high-dose females, as the females received less total test article per day, because their bws were lower than male rats (female rats from all groups weighed an average of 285 g at the end of the study, compared to 522 g for males). Macrovesicular fatty changes are the most common form of liver fatty changes in aging rats and are generally considered benign changes when they are attributed to nutritional imbalances[15–17]; these changes were therefore considered not an adverse test item-related toxicity but a consequence of nutritional imbalance. This is further supported by the lack of significant hepatic changes in low- and mid-dose males or in high-dose females (low- and mid-dose females were not examined because effects were not seen in the histopathology of the high-dose females).

Minimal cortical vacuolation was observed in adrenals of 3/10 control, 1/10 low dose, 4/10 mid dose, and 4/9 high dose males. It is noted that cortical vacuolation is regarded as a spontaneous, age-related change.[18] Adrenal cortical vacuolation of low incidence is not considered to be an

adverse finding in the context of a subchronic study; it is sometimes seen as secondary to stress from various causes. This change was not present in females at any dose level.

In conclusion, under the conditions of this study, the no observed adverse effect level (NOAEL) for oral administration of *E. gracilis* ATCC 12894 whole cell biomass was at least 5000 mg/kg bw/day. The results reported here are similar to those reported in safety assessment for other *E. gracilis* biomasses. *E. gracilis* ATCC PTA-123017 was determined to have an NOAEL of 50,000 ppm (3318 and 3961 mg/kg/day for males and females, respectively) and *E. gracilis* eu029 had an NOAEL of 1000 mg/kg/day.[19,20] The safety assessment described in this report supports the safe consumption of *E. gracilis* biomass as a source of protein, fats, and carbohydrates.

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Authors' contribution

The Noblegen team contributed by reviewing and evaluating the studies and results as well as performing review of the manuscript. Jennifer Symonds and Claire Kruger contributed equally to the preparation of this manuscript.

Declaration of conflicting interests

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