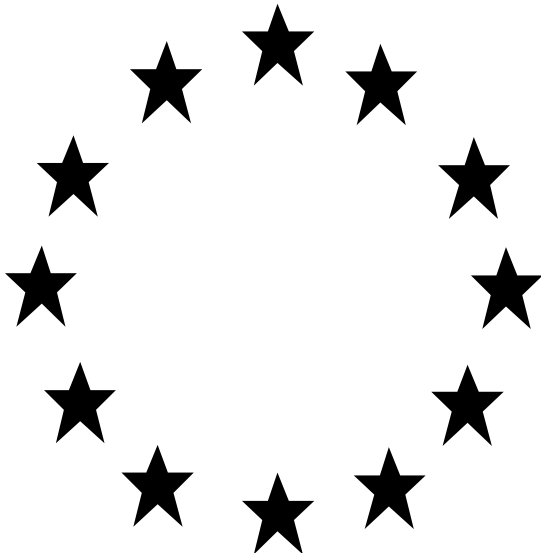

Draft Assessment Report (DAR)

- public version -

Initial risk assessment provided by the rapporteur Member State Estonia for the existing active substance

PHLEBIOPSIS GIGANTEA

of the fourth stage of the review programme referred to in Article 8(2) of Council Directive 91/414/EEC

Volume 3, Annex B, part 2, B.6

September 2008

Draft Assessment Report

Phlebiopsis gigantea

Volume 3

Annex B.6

Effects on Human Health

Rapporteur Member State: Estonia

April 2007

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B.6 Effects on human health (Annex IIB 5)

B.6.1 Tier I – the active micro-organism

B.6.1.1 Basic information (Annex IIB 5.1)

Phlebiopsis gigantea (synonyms *Phlebia gigantea*, *Peniophora gigantea*, *Phanerochaete gigantea* (Holdenrieder & Greig, 1998)) is currently the most commonly used name for this fungus, although work on the precise taxonomic position of *P. gigantea* is on-going. However, until this question is finally resolved and the outcome generally accepted, the name *Phlebiopsis gigantea* is considered valid.

P. gigantea is a common and widely distributed saprophytic wood-decay fungus in the coniferous forests of the Northern Hemisphere. *P. gigantea* is a saprophytic wood-rotting fungus, causing a typical white rot of coniferous timber. The fungus is a primary coloniser of wood, and requires high moisture content for its growth. *P. gigantea* produces two spore types: sexual basidiospores, and asexual arthroconidia (oidia). The former arise naturally from sporocarps, and are airborne. Oidia arise from fragmenting mycelium, and grow easily in laboratory cultures. Their mode of spread in nature is unclear and they are sensitive structures which rapidly die if mishandled (Korhonen & Kauppila, 1988; Holdenrieder & Greig, 1998). PG Suspension and Rotstop are formed solely of oidia and mycelial fragments. Because of these different spore types, the allergenic potential of both need to be considered separately, in terms of the product (oidia) and the natural environment, which is dominated by basidiospores.

P. gigantea is able to prevent colonisation of stumps by *H. annosum* through competition for resources. Although there may be a degree of hyphal interference of *H. annosum* by *P. gigantea* (Ikediugwu et al. 1970)

Metabolites production by various fungi in liquid culture was investigated, Lup-19(22)-ene and Lup-15,19(22)-diene were found in the neutral fraction of chloroform extract, and 2',3',5'-trimethoxy-p-terphenyl was detected in the neutral fraction of an ethyl acetate extract of the mycelium (Briggs *et al*. 1975). According to Hütterman (1997), this compound is a typical secondary fungal metabolite, and substances like this have been found in almost all wood-inhabiting fungi that have been analysed for the presence of this kind of compounds. This particular compound definitely has a lower toxicity than many other secondary metabolites which have been isolated from fungi against which *P. gigantea* is antagonistic. It is not considered to pose any special harm in the following scenarios: (i) acute toxicity during application, (ii) toxicity on the treated stump, (iii) accumulation in the wood and on the forest floor. Lup-19(22)-ene and Lupa-15,19(22)-diene belong to a class of substances which are widely distributed in nature, e.g. in the bark of trees, in leaves and stems of annual plants, or in seeds. No high toxicity can be expected from these compounds in the case of stump treatment.

There are no other records of metabolites produced by P. gigantea that would be of concern for human health and/or the environment (Holdenrieder & Greig, 1998). Studies conducted by Ikediugwu et al. (1970) and Capretti & Mugnai (1989) indicate *P. gigantea* does not depend on the production of toxins for its ability to combat *H. annosum*, but acts through competition for the wood resource. In conclusion, there is no evidence in the available literature that *P.gigantea* controls *H.annosum* by antibiotic or toxic means.

P.gigantea is not thermophilic fungi and has an optimal growth temperature of 28^{o}C, it was found a cessation of growth at 35^{o}C, but this temperature is not lethal limit as samples re-incubated at lower temperature recovered (Niemi, M.1992), and *P.gigantea* spore counts decrease or is killed at temperature exceeding 38^{o}C, and is not capable of colonising or invading humans or animals, as verified by animal tests (Niemi, M.,1992).

B.6.1.1.1 Medical data (Annex IIB 5.1.1)

B.6.1.1.2 Medical surveillance on manufacturing plant personnel (Annex IIB 5.1.2)

See point 6.1.1.3.

B.6.1.1.3 Sensitisation/allergenicity observations, if appropriate (Annex IIB 5.1.3)

The following publications were submitted by the notifier. These articles describe health effects that were caused by *Phlebiopsis gigantea*.

- The Forestry Commission, UK, 1992 reported that in 20 years of production and use there has not been suggestion of any allergic effects to users wearing the gloves and protective clothing required for chainsaw operators. No specific studies conducted.

- Kemira Agro Oy, Finland, 1994, reported that *P. gigantea* was on the market in Finland first time in 1978-1983 and between then no indications of sensitisation were noted in personnel working with the original formulation. A new development project started in 1991 to create a new formulation of the product, and during the three years of research, development and manufacturing of Rotstop, no allergic reactions in personnel has been observed so far.

- Forestry Commission, UK, 1995: a review of employment records of 21 out of 75 chainsaw operators working between 1945 and 1995 revealed no evidence of occupational health referrals relating to chest complaints or incidences of sick absence associated with the use of *P. gigantea*.

- Forestry Commission, UK, 1996 reported that man volunteered for skin patch test performed to suspension of *Peniophora gigantea*. The result of the test was negative.

- Kuopio Regional Occupational Health Institute (KATTL), Finland, 1997 reported a monitoring study which was performed in an area where Rotstop was extensively used, exposure and health effects were monitored in harvest machine operators, forestry students and their teacher. Exposure was investigated by measuring concentrations of the micro-organism in the breathing zone, in wipe samples taken from the cab and by measuring antibodies in blood. Symptoms questionnaires were used to assess health effects. The students also kept a symptoms diary and measured their pulmonary peak expiry flow (PEF) three times per day. No *P. gigantea* spores were found in samples taken from cab air or in wipe samples and there was no rise in antibodies in blood. A range of symptoms, including skin and respiratory effects, were reported but none of the operators (who had been working in forestry for 6-27 years) associated symptoms with the use of Rotstop. Amongst students, only two applied the fungicide and they experienced the most symptoms including variation in PEF. Occupational hygiene and antibody measurements show that there was no exposure to *P. gigantea* spores and, although in students exposure to Rotstop may have been associated with a higher occurrence of respiratory symptoms, this was not the case with the harvest machine operators. Due to the low numbers of subjects involved it is not possible to conclude whether the normal uses of Rotstop have any adverse health effects.

- Kemira Oy, Finland, 2000: Medical director stated that no clinically significant findings were found in workers associated with the occupational exposure of Rotstop (*Phlebiopsis gigantea*). Workers were subjected to regular examinations including respiratory function tests.

- Finnish Forest Research Institute (METLA), 2000 reported that during the use of *Phlebiopsis gigantea* for the past 20 years, in field and laboratory conditions, there have been no allergic reactions or sensitisation reported by personnel, who were surveyed.

- Kuopio Regional Occupational Health Institute (KATTL), Finland, 2002: The study compared the incidence of dermal and respiratory symptoms in harvester operators exposed to a range of biological fungicides, log marking pigments and oils with the incidence in a control group consisting of timber truck drivers and forestry technicians. Exposure was also measured using air samples taken from the breathing zone and harvester cab filters and compared with samples taken from outdoor air.
  Harvester operators had higher rates of asthma and other respiratory symptoms compared with control subjects even though concentrations of viable spores in the breathing zone and outdoor air were comparable. Incorrect mixing of fungicide spray (Rotstop) can expose the operator to an irritating dust and there was some correlation between incorrect mixing and the incidence of respiratory symptoms and asthma. The correlation was not statistically significant Mild skin conditions (eczema and dermatitis) were also higher in the operators compared with controls and correlated with both handling hydraulic hoses and the use of the biological fungicide.

- Kuopio Regional Occupational Health Institute (KATTL), Finland 2004: Respiratory exposure to *P. gigantea*, other microorganisms and endotoxins was assessed using air samples from the workers breathing zone. Dermal exposure was assessed using a fluorescent tracer method. Systemic exposure was also investigated by antibody determinations in serum samples.
  Respiratory and dermal exposure to *P. gigantea*, log colour-coding agents, hydraulic and chainsaw oils was insignificant during normal harvester operations; exposure may occur during maintenance work but can be controlled by the use of appropriate personal protective equipment.

- Forest Research, UK, 2005: Questionnaires were carried out among 7 forestry workers who used PG Suspension and 2 personnel involved in manufacture of the product. Four of those surveyed had been using the product daily for more than 5 years. Four of these had some hand/arm contact with the formulation but had not experienced any adverse effects. Two others who had asthma or eczema had also not experienced any adverse effects. Only one of the workers, who was exposed less than once per week, recorded any effects (skin dermatitis) but considered that this was unrelated to working with PG Suspension.

- Verdera Oy, Finland, 2005. Following information was included to the medical certificate: based on medical examination and clinical follow-up studies of all workers who may have been exposed to Rotstop dust, it was stated that there were no significant findings related to occupational exposure. No one had acute or chronic symptoms, and there were no details on occurrence of hypersensitivity or chronic sensitisation.

RMS comments:

Forestry Commission, UK, 1996: the document should not be taken into account due to the absence of original study document, additionally it is not possible reach to conclusion bases on the one person test.

Kuopio Regional Occupational Health Institute (KATTL), Finland, 1997: it is not clear were the respiratory effects caused by *Phlebiopsis gigantea* or by another factors. However, the manufacturer´s protections must be complied with while working within the safety area.

Kuopio Regional Occupational Health Institute (KATTL), Finland, 2002: it is not clear were the respiratory effects caused by *Phlebiopsis gigantea* or by another factors.

Forest Research, UK, 2005: no statistical analyses could be drawn due to lack of information.

B.6.1.1.4 Direct observation, e.g. clinical cases

B.6.1.2 Basic studies (Annex IIB 5.2)

B.6.1.2.1 Sensitisation (Annex IIB 5.2.1)

Reference:	S. Allen (1996) Rotstop skin sensitisation in the guinea pig. Huntingdon Life Sciences. Unpublished report No.: HLS RKY 131/960116/SS.
Guideline / GLP:	OECD nr. 406/Yes
Deviation:	No information on homogeneity or stability of the test compound is given in the study report.
Acceptability:	**Not acceptable**
Test substance / purity:	Rotstop/purity not stated in the report
Vehicle:	Sterile phosphate buffered saline
Species / Strain:	Dunkin Hartley guinea pigs

Doses / No. of animals	$1.07x10^7$ cfu/g / 10 control and 20 test animals
Exposure time	6 h

Protocol:

Test design:
Twenty test and ten control male Dunkin Hartley guinea pigs (bodyweight range: 306 – 350 g, 6-7 weeks old) were use in the study. The method used was a modification of Buehler-test.
Induction
On day 1 of the study, a 20 x 20 mm patch of surgical gauze was saturated with approximately 0.5 ml of Rotstop, as supplied in saline ($1.07x10^7$ cfu/g). The patch was placed on the skin (on the left shoulder) and covered with an occlusive dressing. The patch remained in contact with the skin for 6 hours. After that the skin was assessed for dermal reactions 24 hours later. The induction process was repeated on days 8 and 15. Control animals were treated in the same manner; the gauze patch was saturated with saline only.
Challenge

Control and test animals were challenged topically two weeks after the final induction procedure using Rotstop, as supplied in saline. Animals were clipped free of hair on the right flank. A 20 x 20 mm gauze patch was saturated with approximately 0.5 ml of the test substance in a method similar to that used for induction. The patch was covered with an occlusive dressing and the patch remained in contact with the skin for 6 hours. The challenge sites were examined for skin reactions 24 and 48 hours after removal of the patches.

Results:

No clinical signs of toxicity, bodyweight increases for all guinea pigs over the study period.

Induction
There were no dermal reactions seen in any of the test animals after the first two inductions. However, no assessments could be made after the third induction because there was red staining on the dose site which interfered with scoring.

Challenge
Red staining was seen on the test site of all the animals. No dermal reactions were seen in any of the control or test animals. The red staining was due to the test material. The test material is a red powder and the dye used to produce the colour of the powder stained the test animals skin.

Conclusion:

Rotstop did not produce evidence of skin sensitisation. However it was difficult at times to assess the skin for erythema due to staining by the red dye from test material.

RMS comments: Sensitisation studies are considered not suitable for micro-organisms, also, negative results in a Buehler test are not acceptable, since this test is considered less sensitive. According to the directive 91/141/EEC all micro-organisms should be regarded as potential sensitiser.

B.6.1.2.2 Acute toxicity, pathogenicity and infectiveness (Annex IIB 5.2.2)

B.6.1.2.2.1 Acute oral toxicity, pathogenicity and infectiveness (Annex IIB 5.2.2.1)

STUDY TYPE: Acute Oral Infectivity and Toxicity - rat
U.S. EPA OPPTS 885.3050

TEST MATERIAL (PURITY): Rotstop, 1.00 x 10^7 colony forming units (cfu) of *Phlebiopsis gigantea* per gram

SYNONYMS: *Peniophora gigantea, Phlebia gigantea,Phanerochaete gigantea*

CITATION: Author: McRae, Lewis A. (1996). Acute oral toxicity and pathonicity to the rat. Huntingdon Life sciences, Huntingdon, England. RKY 132/953004/AC, 02 February 1996. MRID. Unpublished.

SPONSOR: Kemira Agro Oy, PO Box 330, Helsinki, Finland (which former Biocontrol Unit is now Verdera Oy).

EXECUTIVE SUMMARY: In an acute oral toxicity study, groups of male and female rats of strain (Hsd/Ola:Sprague-Dawley (CD)), aged 8-12 weeks and fasted overnight prior to dosing, were given a single oral dose of Rotstop WP, containing 1.00 x 10^7 colony forming units (cfu) of *Phlebiopsis gigantea* per gram, suspended in sterile physiological saline, at doses of 4.26 x 10^7 cfu of *Phlebiopsis gigantea*/ kg bw. The animals were then observed for a period of up to 22 days with interim scheduled sacrifices on Days 2, 4 8 and 15. Control groups were autoclaved Rotstop, untreated shelf-control and untreated non-shelf control.

There were no deaths following a single oral administration of Rotstop to groups of six rats (three males and three females) at a dose of 20 ml (4.26 x 10^7 cfu of *P. gigantea*)/kg bodyweight.

Based on the results of this study, Rotstop is of **LOW Toxicity** and *Phlebiopsis gigantea* is not infective or pathogenic in the rat.

There were no treatment related clinical signs, necropsy findings or changes in body weight. Recovery was complete in all instances by Day 5.

This acute oral study is classified acceptable.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS:

1.	**Test Material:**	Rotstop
	Description:	Dry formulation (WP) of *Phlebiopsis gigantea* spores in amporphous silica as a carrier. Red powder due to a dye added to the cream-white powder.
	Lot/Batch #:	2/1997
	Purity:	Not applicable
	CAS #:	Not applicable
	Storage conditions:	Stored at 2-8 ^oC in the dark. The fungal spores stay viable for up to 12 months at this temperature.
	Microbiology:	Viable count of the test substance was determined to be 1.00 x 10^7 cfu of *P. gigantea*/gram

2. Sample Preparation:

The test substance was quantified using a procedure based on that supplied by the sponsor. A 0.15 g sample of Rotstop was uniformly suspended in sterile distilled water (SDW) to give a final volume of 150 ml. The suspension was then homogenised using an Ultra Turrax 25 homogeniser for 45 seconds at full speed. Serial dilutions to 10^{-5} to 10^{-7} were then prepared using 9 ml SPBS (Oxoid BR14a) and 1 ml pour plates prepared in triplicate using Sabouraud Dextrose Agar (SDA - Oxoid CM41). The plates were incubated at 24 $\pm$ 1^oC for 7 days.

The test substance dosing suspension was prepared by suspending 70 g Rotstop in 280 ml sterile physiological saline and homogenising for 45 seconds at full speed using an Ultra Turrax T25 homogeniser to provide the viable dosing suspension. Approximately 55 ml of this suspension was transferred into a separate glass container and then sterilised by autoclaving at 121 ^oC for 15 min. to provide the autoclaved

dosing suspension. The viable dosing suspension was quantified as described above and the plates incubated at 28 ± 1°C for 5-7 days.

3. Controls:
Groups A-E: treatment and sacrification at Day 2, 4, 8, 15 or 22
Group F: Autoclaved Rotstop (see above)
Group G: Untreated shelf control
Group H: Untreated non-shelf control

4.	**Test animals:**		
	Species:	Rat	
	Strain:	(Hsd/Ola:Sprague-Dawley (CD)	
	Age/weight at dosing:	8-12 weeks, 219-296 g	
	Source:	Harlan Olac Ltd, Bicester, Oxon, England	
	Housing:	Housed in groups of three or five rats of the same sex in metal cages with wire mesh floors in either Building R14 Room 6 (group H) or Building R17 Room 15 (all other treatment groups)	
	Diet:	Standard pelleted rodent diet (SDS LAD 1) *ad libitum*	
	Water:	Drinking water *ad libitum*	
	Environmental conditions:	**Temperature:**	17 – 20 °C
		Humidity:	34-91 %
		Air changes:	10-15 air changes/hr
		Photoperiod:	12 hr dark / 12 hr light
	Acclimation period:	13 days	

B. STUDY DESIGN and METHODS:

1. In Life Dates: Start: 15 November 1995 End: 6 December 1995

2. Preliminary Challenge Assay: -

3. Animal Assignment and Treatment: Animals were assigned to the test groups noted in Table 1. Following an overnight fast, rats were given a single dose of Rotstop by gavage.

TABLE 1. Doses, mortality/animals treated

Test Group	Test Substance	Dose Level	Males	Females	Combined
A-E	Rotstop	20 ml (4.26×10^7 cfu of *P. gigantea*)/kg bodyweight	0/15	0/15	0/30
F	Autoclaved Rotstop	20 ml /kg bodyweight	0/3	0/3	0/6
G		0	0/3	0/3	0/6
H		0	0/5	0/5	0/10

4. Clinical Observations and Body Weights: Cage-side recordings of the nature, severity, approximate time of onset and duration of each sign of toxicity were made shortly after dosing and at frequent intervals for the reminder of the experimental day. On subsequent days the animals were observed once in the morning and once at the end of the day. Body weights of all rats were measured on Day 1 (prior to dosing). The bodyweights of rats of Groups A and B and those animals of Group H scheduled for interim kills were also recorded immediately prior to their sacrifice on days 2 and 4, respectively. In addition, bodyweights

were recorded for all remaining rats on days 8, 15 and 22. Faeces were collected

5. **Feed Consumption:** Feed consumption was not reported.

6. **Necropsy and Organ Weight Determination:** Interim sacrifices (3 male and 3 female rats per group) were performed on days 2, 4, 8 and 15. The test animals were not fasted overnight prior to sacrifice. On the day of scheduled sacrifice, animals were weighed and anaesthetized by ether inhalation prior to blood collection from the orbital sinus, and sacrificed by ether asphyxiation. On each scheduled termination day the animals were sacrificed and autopsied in the order of non-shelf then shelf control (Day 22 only) followed by treated groups. The necropsy included a post mortem examination by opening the cranial, abdominal and thoracic cavities. The appearance of the body organs were recorded and relevant organs aseptically removed for subsequent microbiological examination. Organs were removed in the following order: brain, kidney, spleen, liver, heart, lungs, mesenteric lymph nodes, stomach, seventh loop (small intestine) and caecum.

7. **Microbial Enumeration:** Quantification of viable test organisms in blood, organs, intestinal contents and faeces was carried out from aseptically taken samples, using SDA pour plates and incubating at 28 ± 1 ^{o}C for 5-7 days. The organs were only touched with sterile forceps and scissors, and care was taken to avoid any contact with potentially contaminated surfaces. Organs were finely divided by snipping with sterile scissors and when sufficiently rendered down, approximately 1 g samples were aseptically transferred to appropriately labelled, pre-weighed Universal containers of 9 ml SPBS diluent, re-weighed to determine the weight of the samples. Using sterile instruments each intestinal sample was gently squeezed to extrude its contents, samples of which were taken with a sterile loop, and approximately 1 g transferred to a Universal container of 9 ml SPBS diluent.

   The samples were then Vortex- mixed for 15 seconds to suspend the material, serial ten-fold dilutions were prepared to give a range of dilutions determined by the expected numbers of organisms in the sample, with the objective of using appropriate dilutions to produce count plates with, ideally, 30-300 colonies on each of the triplicate plates. Where very low numbers of organisms were expected, the initial suspension of the sample was used to prepare the count plate.

8. **Sensitivity of Detection:** Recovery of viable *P. gigantea* from spiked blood, organs, caecal contents and faeces was ~100 %.

9. **Statistics:** No statistical analysis of the results was done.

II. RESULTS

A. **MORTALITY**: there was no mortality in any treatment group.

B. **CLINICAL OBSERVATIONS:** Clinical signs of reaction to treatment were confined to faecal disturbances (characterised by soft to liquid red stained faeces) and pink staining on the cage litter tray paper. Signs were evident for rats treated with viable test substance and in those treated with autoclaved test substance. Recovery of rats was complete in all instances by Day 5. Necropsy examination revealed no abnormal findings for any of the test animals.

C. **BODY WEIGHT:** There was no evidence of treatment related bodyweight changes observed throughout the study.

D. **FEED CONSUMPTION:** Treatment related effects on feeding consumption were not reported.

E. **NECROPSY:** No macroscopic abnormalities were revealed in any instance.

F. **ORGAN WEIGHTS:** -

G. **MICROBIAL ENUMERATION:** Viable *P. gigantea* was not recovered from any organ, blood, intestinal contents or faecal sample from treated, autoclaved control or untreated control rats during the study.

III. CONCLUSION

There was a rapid loss of viability of the test organism following oral dosing, with no evidence of toxicity or infectivity/pathogenicity to rats following a single oral administration of 4.26 x 10^7 cfu/kg bodyweight of viable *P. gigantea*.

RMS comment: According to OPPTS 885.3050 one dose level of at least 10^8 units of the MPCA per test animal should be used. The maximum permissible dose volume is 20 ml/kg bodyweight. The study was performed with formulation, such that each animal received a maximum practical dose of 4.26 x 10^7 cfu *of P.gigantea*/kg bw.

DEFICIENCIES: No deficiencies.

B.6.1.2.2.2 Acute inhalation toxicity, pathogenicity and infectiveness (Annex IIB 5.2.2.2)

Reference:	L. McRae (1996b) Rotstop acute pulmonary toxicity and pathogenicity to the rat. Huntingdon Life Sciences Ltd. Unpublished report No.: RKY 133/953005/AC
Guideline / GLP:	152A-12/Yes
Deviation:	Increasing of humidity (beyond 30-70% RH); a miniature laryngoscope was not used during the study as was described in protocol.
Acceptability:	Acceptable
Test substance / purity:	Rotstop (viable count is 1.00x10^7 CFU)/ purity is not stated
Vehicle:	Sterile physiological saline
Species / Strain:	Sprague Dawley rats
Doses / No. of animals	1.12x10^6 CFU *P. gigantea*/kg bodyweight. Three rats per sex
Exposure time	21

Protocol:

Test design

Test material: Rotstop (Batch No.: 2/1997, viable count determined to be 1.00 x 10^7 cfu of *P. gigantea* per gram). Male and female Sprague Dawley rats (bodyweight range: 232-340g, 8 –12 weeks old) were allocated to one of the following test groups according to the treatment regime below:

Group	Treatment	No of rats	Rat numbers		Sacrifice Day
			M	F	
A	Rotstop	3 M 3 F	1-3	4-6	1
B	Rotstop	3 M 3 F	7-9	10-12	2
C	Rotstop	3 M 3 F	13-15	16-18	4
D	Rotstop	3 M 3 F	19-21	22-24	8

E	Rotstop	3 M 3 F	25-27	28-30	15
F	Rotstop	3 M 3 F	31-33	34-36	22
G	Autoclaved Rotstop	3 M 3 F	37-39	40-42	22
H	Untreated shelf control	3 M 3 F	43-45	46-48	22
I	Untreated non-shelf control	5 M 5 F	49-53	54-58	*

*One animal from group I was sacrificed concurrently with each of the treated groups, the remaining animals from Group I and all animals from groups F, G and H were sacrificed on Day 22.

The appropriate dose volume (1.12 x 10^6 cfu/kg bw) of the test substance was instilled into each rat under ether anasthesia using an intratracheal tube. Animals were observed daily for mortalities and signs of toxicity. Individual bodyweights were recorded prior to dosing and on days 2, 4, 8, 15 and 22 or at death. Individual body temperatures were recorded (using a rectal probe) prior to dosing and at 2, 4, 24 and 48 hours after dosing. Faeces samples were collected and subjected to microbiological analysis.
At scheduled termination rats were sacrificed and subjected to necropsy examinations. Organs were removed for microbiological assay. Quantification of viable test organisms in blood, organs, intestinal contents and faeces was carried out from aseptically taken samples, using SDA pour plates and incubating at 28 $\pm$ 1 ^oC for 5-7 days. The organs were only touched with sterile forceps and scissors, and care was taken to avoid any contact with potentially contaminated surfaces. Organs were finely divided by snipping with sterile scissors and when sufficiently rendered down, approximately 1 g samples were aseptically transferred to appropriately labelled, pre-weighed Universal containers of 9 ml SPBS diluent, re-weighed to determine the weight of the samples. Using sterile instruments each intestinal sample was gently squeezed to extrude its contents, samples of which were taken with a sterile loop, and approximately 1 g transferred to a Universal container of 9 ml SPBS diluent. The sample was Vortex mixed for 15 seconds to suspend the material for the counting procedure.

Results

Two male rats from group C, one male and one female rat of Group D and one female of group G died during the study. All deaths occurred within 24 hours of dosing. Macroscopic examination revealed no abnormalities. Clinical signs included piloerection, hunched posture, waddling gait, lethargy, decreased respiratory rate, partially closed eyelids, gasping/noisy respiration, unsteadiness, increased lacrimation, hyperactivity, increased respiratory rate, protruding eyes, bluish colour to skin/extremities, cold body surfaces and staining around nose and mouth. Most of the clinical signs were noted soon after dosing and were persistent throughout day, all signs had resolved within 24 hours of dosing. Minor bodyweight changes were recorded among treated animals. There were no notable effects on body temperatures for rats treated with Rotstop when compared to the controls.

Low to moderate numbers of *P. gigantea* were recovered from the lungs of all group A treated rats one hour after dosing. Similar levels of *P. gigantea* were recovered from the lungs of 3 out of 4 rats that died during day 1 of the study. Viable *P. gigantea* was not recovered from any of the other samples during the study, indicating a rapid loss of viability of the test organism following dosing into rats. Recovery of viable test organisms from the blood and organs are shown in Table 6.1.2.2.2-01.

No abnormalities were recorded at the macroscopic examinations.

Table 6.1.2.2.2.-01 Recovery of viable *P. gigantea* from blood and organs of treated rats

Group	Rat No.	Time after dosing	Colony forming units per gram							
			Brain	Kidney	Spleen	Liver	Heart	Lungs #	MLN	Blood
A	1 M	1 hour	<10	<10	<10	<10	<10	7.40 x 10^2	<10	<10
	2 M		<10	<10	<10	<10	<10	1.05 x 10^4	<10	<10
	3 M		<10	<10	<10	<10	<10	2.46 x 10^3	<10	<10
	4 F		<10	<10	<10	<10	<10	9.67 x 10^2	<10	<10
	5 F		<10	<10	<10	<10	<10	8.96 x 10^3	<10	<10

	6 F		<10	<10	<10	<10	<10	1.40 x 10^3	<10	<10
B	7 M	1 day	<10	<10	<10	<10	<10	<10	<10	<10
	8 M		<10	<10	<10	<10	<10	<10	<10	<10
	9 M		<10	<10	<10	<10	<10	<10	<10	<10
	10 F		<10	<10	<10	<10	<10	<10	<10	<10
	11 F		<10	<10	<10	<10	<10	<10	<10	<10
	12 F		<10	<10	<10	<10	<10	<10	<10	<10
C	13 M	3 days	NS	NS	NS	NS	NS	*	NS	NS
	14 M		NS	NS	NS	NS	NS	*	NS	NS
	15 M		<10	<10	<10	<10	<10	<10	<10	<10
	16 F		<10	<10	<10	<10	<10	<10	<10	<10
	17 F		<10	<10	<10	<10	<10	<10	<10	<10
	18 F		<10	<10	<10	<10	<10	<10	<10	<10
D	19 M	7 days	NS	NS	NS	NS	NS	*	NS	NS
	20 M		<10	<10	<10	<10	<10	<10	<10	<10
	21 M		<10	<10	<10	<10	<10	<10	<10	<10
	22 F		<10	<10	<10	<10	<10	*	NS	NS
	23 F		<10	<10	<10	<10	<10	<10	<10	<10
	24 F		<10	<10	<10	<10	<10	<10	<10	<10
E	25 M	14 days	<10	<10	<10	<10	<10	<10	<10	<10
	26 M		<10	<10	<10	<10	<10	<10	<10	<10
	27 M		<10	<10	<10	<10	<10	<10	<10	<10
	28 F		<10	<10	<10	<10	<10	<10	<10	<10
	29 F		<10	<10	<10	<10	<10	<10	<10	<10
	30 F		<10	<10	<10	<10	<10	<10	<10	<10
F	31 M	21 days	<10	<10	<10	<10	<10	<10	<10	<10
	32 M		<10	<10	<10	<10	<10	<10	<10	<10
	33 M		<10	<10	<10	<10	<10	<10	<10	<10
	34 F		<10	<10	<10	<10	<10	<10	<10	<10
	35 F		<10	<10	<10	<10	<10	<10	<10	<10
	36 F		<10	<10	<10	<10	<10	<10	<10	<10
G	37 M	21 days	<10	<10	<10	<10	<10	<10	<10	<10
	38 M		<10	<10	<10	<10	<10	<10	<10	<10
	39 M		<10	<10	<10	<10	<10	<10	<10	<10
	40 F		NS	NS	NS	NS	NS	NS	NS	NS
	41 F		<10	<10	<10	<10	<10	<10	<10	<10
	42 F		<10	<10	<10	<10	<10	<10	<10	<10
H	43 M	21 days	<10	<10	<10	<10	<10	<10	<10	<10
	44 M		<10	<10	<10	<10	<10	<10	<10	<10
	45 M		<10	<10	<10	<10	<10	<10	<10	<10
	46 F		<10	<10	<10	<10	<10	<10	<10	<10
	47 F		<10	<10	<10	<10	<10	<10	<10	<10
	48 F		<10	<10	<10	<10	<10	<10	<10	<10
I	49 M	1 hour	<10	<10	<10	<10	<10	<10	<10	<10
	54 F	1 day	<10	<10	<10	<10	<10	<10	<10	<10
	50 M	3	<10	<10	<10	<10	<10	<10	<10	<10
	55 F	7	<10	<10	<10	<10	<10	<10	<10	<10
	51 M	14	<10	<10	<10	<10	<10	<10	<10	<10
	52 M	21	<10	<10	<10	<10	<10	<10	<10	<10
	53 M	21	<10	<10	<10	<10	<10	<10	<10	<10
	56 F	21	<10	<10	<10	<10	<10	<10	<10	<10
	57 F	21	<10	<10	<10	<10	<10	<10	<10	<10
	58 F	21	<10	<10	<10	<10	<10	<10	<10	<10

colony forming units per pair of lungs
MLN: mesenteric lymph nodes
NS: not sampled rat died
*13 M lungs – 1.24 x 10^3 viable *P. gigantea* (day 1)
14 M lungs – 4.43 x 10^3 viable *P. gigantea* (day 1)
19 M lungs – <10 viable *P. gigantea* (day 1)
22 M lungs – 2.15 x 10^3 viable *P. gigantea* (day 1)

Cnonclusion

It could be assumed that deaths of the animals and clinical signs could be attributed to the effects of anaesthesia and the trauma of the dosing procedure based on that all signs had resolved within 24 hours of dosing. There was no evidence of treatment related trends in bodyweight changes, therefore, the changes were considered not to be related directly to treatment with Rotstop. Under the conditions of the study there was a rapid loss of

viability of the test organism following intratracheal dosing and no overt evidence of toxicity, infectivity/pathogenicity to rats following a single pulmonary administration of 1.12 x 10^6 cfu/kg bodyweight of viable *P. gigantea.*

RMS comments: According to OPPTS 885.3550 one dose level of at least 10^8 units of the MPCA per test animal should be used. Respectively to the explanation that the notifier has provided 10% solution of the product was quite thick suspension due to the carrier. In order to have a dose of min. 10^8 spores per animal, more than 100 ml of the concentrated solution should have been administered into the lungs of each rat. Clearly this was impossible.

B.6.1.2.2.3 Intraperitoneal/subcutaneous single dose (Annex IIB 5.2.2.3)

Reference:	E. Blanchard (2002) Rotstop: Acute intraperitoneal toxicity and pathogenicity to the rat. Huntingdon Life Sciences Ltd. Unpublished report No.: HLS KEY 008/023033/AC
Guideline / GLP:	US EPA OPPTS Guidelines 885.3200/Yes
Deviation:	The test substance was suspended in sterile phosphate buffered saline instead of sterile physiological saline
Acceptability:	The study is considered to be acceptable
Test substance / purity:	Rotstop
Buch number:	01151
Vehicle:	Sterile phosphate buffered saline
Species / Strain:	CD rats of Sprague-Dawley origin (Hsd: Sprague-Dawley (CD))
Doses / No. of animals	$9.31x10^4$-$1.27x10^5$cfu/rat
Exposure time	21 day

Protocol:

Test design:

Test material: Rotstop (Batch No.: 01151, potency: 10^6- 10^7 living oidias of *Phlebiopsis gigantea* / g).
Male and female Sprague Dawley rats (bodyweight range: 191.1 – 256.1 g, approximately 6 – 8 weeks of age) were dosed with test material were each given 4.73 x 10^5 CFU/ml at a dose volume of 1 ml/kg, which resulted in doses ranging from 9.31 x 10^4 – 1.27 x 10^5 CFU/rat. Suspension was mixed during dosing using magnetic stirrer. All animals were dosed by injection into the intraperitoneal cavity using a 1 ml syringe, see test regime below.

Test group	Numbers of rats	Further information
Treated groups (all received same treatment)		
Group 4A	3 male, 3 female	Sacrifice day 4
Group 4B	3 male, 3 female	Sacrifice day 8
Group 4C	3 male, 3 female	Sacrifice day 15

Group 4D	3 male, 3 female	Sacrifice day 22
Control groups		
Group 5	3 male, 3 female	Treated with autoclaved test material
Group 6	3 male, 3 female	Untreated control group. Held in a separate room

All animals were observed daily for mortalities and clinical signs of toxicity throughout the study period.
The body temperature of each rat was recorded using a rectal probe prior to dosing and approximately 2, 4 and 24 hours after dosing. Body weights were recorded prior to dosing and on days 4, 8, 15 and 22.
Faeces samples were collected from group 4D rats on days 4, 8, 15 and 22. Each rat was placed overnight in a single housing unit and the faeces were collected. Faeces samples were subjected to microbiological analysis.
All animals were sacrificed and subjected to *post mortem* examinations. Blood and organs were collected for microbiological analysis.

A preliminary study was performed to determine the highest suitable dose volume for administration in the main study. In the preliminary study, three groups of two male and two female rats were dosed with the test substance, at a concentration of 1.04-5.68x10^5 cfu/rat (4.75 x10^5 cfu/ml). See test regime and clinical signs below:

Group	Dose	Clinical signs
Group 1	1 ml/kg	No deaths. Piloerection, reduced body tone (abdomen), hunched posture, and dark faeces in all animals. Additionally, abnormal gate and lethargy (both males and a female), flat posture, lacrimation and loose faeces (one male), brown staining around the nasal area and increased body temperature (one male and both females) and urine staining (one femail). Clinical signs were first evident from approximatly 15 minutes after dosing and had resolved compleatly by day 9. Macroscopic examination at study termination on day 22 revealed some fusing of the intestines to the liver for all animals and white noudles on small intestine of one female
Group 2	2 ml/kg	All animals were sacrificed on human grounds on either Day 2 or 3. Piloerection, abnormal gate, hunched posture, and brown staining in all animals. In addition, flat posture, aggressive behavior and reduced body temperature (one female), urine staining, increased body temperature and dehydration (one male and both females), lacrimation and vocalisation (one male and one female), discharge of blood from penis, firm abdomen and dark yellow/green urine (one male), reduced body tone (both females). Clinical signs were first evident from approximatly 15 minutes after dosing. A loss in bodyweight was observed for all decedents. Macroscopic examination revealed fluid in peritoneal cavity or stomach in one male and both females, pallor of the liver and kidneys in one male, white noudles on the liver in one female and some fusing of the stomach to the liver in one male.
Group 3	5 ml/kg	All animals were sacrificed on human grounds on either Day 2 or 3. Piloerection, reduced body tone, hunched posture, and brown staining in all animals. In addition, abnormal gate educed body temperature (both males and one female), discharge of blood from penis and lethargy (both males), pallor of the skin, lacrimation and firm abdomen (one male), vocalisation, urine staining, dehydration, fast respiration, increased body temperature and loose faeces (one female). Clinical signs were first evident from approximatly 15 minutes after dosing and had resolved compleatly in survival female by day 6. A loss in bodyweight was observed for all decedents. Macroscopic examination revealed fluid in the peritoneal cavity or stomach of all animals, pallor of the spleen and kidneys in both males and in the liver of one male, white noudles on the liver and spleen of both males and liver of the female and congestion in the caecum of the female. No macroscopic abnormalities were observed in the female that survived treatment.

Results
Main study, dose: 9,31x10 ^{4} - 1,27x10 ^{5} cfu per rat

Clinical signs: piloerection in all treated animals, hunched posture (12/24). These signs were seen less than one hour after dosing and had ceased by two hours after dosing. Muscle reaction in ventral abdomen (13/24 and in 2 rats in autoclaved group), deformity prominent (4/24), vocalization (2/12 treated and 1 autoclaved females), excreta (1/24), brown staining (19/24 and in 6 rats in autoclaved group) were seen during the study. There were no other clinical signs, which were considered to be associated with the test organism.
Body weights: The majority of animals treated with viable or autoclaved test substance lost weight between days 1 and 4. After day 4, the majority of treated animals showed bodyweight increases, which were similar to that of the untreated controls.
There were no changes in body temperatures in animals treated with either activated or autoclaved test organism.
Macroscopic examination: Macroscopic examination revealed white nodules on the organs (liver, spleen, kidneys and intestines) of all animals in groups 4A and 4B and one male in group 4D. The nodules were not present in any of the animals in the autoclaved or untreated control groups. In addition, fusion of the intestines to the spleen and liver or liver and caecum was noted in 5/6 rats in group 4B and 1/6 rats in group 4C and 4D. This sign was also noted in 1/6 rats treated with autoclaved test organism. A pus filled lump under the skin was found in one female treated with autoclaved test substance. No further abnormalities were observed in any of the test animals. Viable organisms were not recovered from the organs or blood of any rats treated with viable or autoclaved test substance.

Conclusion:
Under the conditions of the study Rotstop showed evidence of toxicity to rats following a single intraperitoneal injection at a dose volume of 1 ml/kg and dose of 9.31 x 10^4 – 1.27 x 10^5 cfu/ rat.

Additional literature
The notifier provided the articles below.

Reference: Salfelder, K., Schwarz, J. (1976): Basidomycetes as infectious agents. Mykosen Vol. 19 (10), pp. 373-382. Not GLP; Published

Summary: Fifteen species of basidiomycetes (e.g. *Peniophora gigantea*) inoculated intraperitoneally, intratesticularly, subcutaneously and into the foot pad of hamsters elicited frequently granulomatous tissue lesions and other types of inflammatory tissue reactions (with fungus cells present in tissue) demonstrable as long as four months after inoculation. In mice, only non-specific lesions without fungus cells were observed in the peritoneum five weeks after intraperitoneal inoculation. Recovery by culture was achieved only from one hamster and two mice after five weeks. Dissemination of the infection in animals was not observed.

Reference: Salfelder, K., De Roman, A.R., De Mendelovici, M., Silberborg, S.B. (1975.) Inoculation of basidiomycetes into mice: Tissue reaction and survival of fungi in tissues
Mykosen Vol. 18 (10), pp. 417-424

Summary: Not GLP; Published After administration of suspensions of ten species of *Basidiomycetes* intravenously, intraperitoneally and subcutaneously to mice lesions were observed microscopically in lungs, omentum and skin respectively 1 and 2 months after treatment. Fungi were detected in lung, liver and spleen after intravenous and intraperitoneal administration but there was no correlation between the presence of fungi and tissue lesions. This is an old study and experimental details, such as the doses used, are lacking. There was also a high mortality and the effects of different strains are not reported. Under these circumstances the results cannot be considered a reliable indicator of the toxicity or infectivity of *P. gigantea.*

B.6.1.2.3 Genotoxicity testing (Annex IIB 5.2.3)

B.6.1.2.3.1 *In vitro* studies

No study submitted because there are no metabolites of toxicological concern.

B.6.1.2.4 Cell culture study (Annex IIB 5.2.4)

No study has been performed, and is not considered to be necessary because the active substance is not a virus or viroid, it does not thermophile fungi.

B.6.1.2.5 Information on short-term toxicity and pathogenicity (Annex IIB 5.2.5)

According to commission directive 2001/36/EC the short-term toxicity (minimum 28 days) of the micro-organisms must be reported.
The micro-organism does not show any evidence of persistence toxicity or pathogenisity/enfectivity via oral and intratracheal administration, so the short-term toxicity and pathogenisity study is not required.

B.6.1.2.5.1 Health effects after repeated inhalatory exposure

The study is not submitted.

RMS comments: According to commission directive 2001/36/EC the study of health effects after repeated inhalatory exposure (minimum 28 days) of the micro-organisms must be reported.
However, there was a rapid loss of viability of the test organism following intratracheal dosing and additional studies are not required.

B.6.1.2.6 Proposed treatment: first aid measures, medical treatment

Inhalation: Remove from exposure, to fresh air.

Skin contact: Wash off immediately with soap and plenty of water. If skin irritation persists, call a physician.

Eye contact: Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

Ingestion: Immediately give plenty of water. Never give anything by mouth to an unconscious person. Induce vomiting and call a physician.

B.6.1.3 Summary and conclusions of Tier I studies

All acute toxicity studies were performed with preparation

Study	Vehicle	Dose levels	Results
Rotstop skin sensitisation in the guinea pig	Saline	$1.07x10^{7}$ cfu/g	Rotstop did not produce evidence of skin sensitisation.
Acute oral toxicity, pathogenicity and infectiveness	Sterile physiological saline	4.26×10^{7} cfu of Phlebiopsis gigantea/ kg bw	Soft to liquid red stained faeces
Rotstop acute pulmonary toxicity and pathogenicity to the rat	Sterile physiological saline	1.12×10^{6} cfu/kg bw	Animal's deaths. Clinical signs included piloerection, hunched posture, waddling gait, lethargy, decreased respiratory rate, partially closed eyelids, gasping/noisy respiration, unsteadiness, increased lacrimation, hyperactivity, increased respiratory rate, protruding eyes, bluish colour to skin/extremities, cold body surfaces and staining around nose and mouth. Minor bodyweight changes
Acute intraperitoneal toxicity and pathogenicity to the rat	Sterile physiological saline	$9.31x10^{4}$ – $1.27x10^{5}$ cfu per rat	White nodules on the organs (liver, spleen, kidneys and intestines), fusion of the intestines to the spleen and liver or liver and caecum was noted; a pus filled lump under the skin was found in one female treated with autoclaved test substance.

Genotoxicity testing	Not submitted	Not submitted	Not submitted
Cell culture study	Not submitted	Not submitted	Not submitted
Short-term toxicity and pathogenicity	Not submitted	Not submitted	Not submitted
Repeated inhalatory exposure	Not submitted	Not submitted	Not submitted

B.6.2 Tier II – the active micro-organism

B.6.2.1 Specific toxicity, pathogenicity and infectiveness studies (Annex IIB 5.3)

No studies provided

B.6.2.2 *In vivo* studies in somatic cells (Annex IIB 5.4)

No studies provided.

B.6.2.3 Genotoxicity – *In vivo* studies in germ cells (Annex IIB 5.5)

No studies provided.

B.6.2.4 Summary and conclusions of Tier II studies

Study	Vehicle	Dose levels	Results
In vivo studies in somatic cells	-	-	No studies provided
In vivo studies in germ cells	-	-	No studies provided
Specific toxicity, pathogenicity and infectiveness	-	-	No studies provided

B.6.3 Summary of mammalian toxicity, pathogenicity and effectiveness and overall evaluation of the active micro-organism (Annex IIB 5.6)

Phlebiopsis gigantea is the saprophytic wood-decay fungus that naturally occure in the coniferous forests of the Northern Hemisphere, and has been used as a pest control agent for many years. The fungus causes a typical white rot of coniferous timber, especially pine. *P. gigantea* is able to prevent colonisation of stumps by *H. annosum* through competition for resources. There may be although a degree of hyphal interference of *H. annosum* by *P. gigantea*.

A complete set of acute toxicity studies with *P. gigantea* using the different application routes has been performed. Thus, with regard to the acute toxicity investigations as included in the "Tier I - Basic studies", the respective requirements under Annex IIB, are fulfilled.
Subsequently, the individual studies are described in detail.

The results of acute studies indicated that *Phlebiopsis gigantea* showed slight signs of toxicity in the intraperitoneal route, and no evidence of toxicity or pathogenicity when administered to rats by other routes. Viable organisms were recovered from the lungs of rats 1 hour after treatment by intratracheal installation but not at later times. *Phlebiopsis gigantea* was not detected in tissues or faeces of rats that received micro-organisms by oral or intraperitoneal administration showing that there is a rapid loss of viability. On the basis of

submitted studies it could be concluded that micro-organism does not provide any indication of the possible infection after oral, intraperitoneal and intratracheal exposure to rats.

The acute oral LD_{50} of *Phlebiopsis gigantea* in rats is > $4.26x10^7$ cfu/kg bw. There were no mortalities, no macroscopic abnormalities or treatment related effects on body weight. Treatment-related clinical signs included faecal disturbances, characterised by soft to liquid red stained faeces and pink staining on the cage litter tray paper.

The acute intratrahheal LC_{50} of *Phlebiopsis gigantea* in rats was >$1.12x10^6$ cfu/kg bodyweight of *P.gigantea*. Five animals died during the study, all deaths occurred within 24 hours of dosing. Macroscopic examination revealed no abnormalities. Clinical signs included piloerection, hunched posture, waddling gait, lethargy, decreased respiratory rate, partially closed eyelids, gasping/noisy respiration, unsteadiness, increased lacrimation, hyperactivity, increased respiratory rate, protruding eyes, bluish colour to skin/extremities, cold body surfaces and staining around nose and mouth. Most of the clinical signs were noted soon after dosing and were persistent throughout day, all signs had resolved within 24 hours of dosing. Minor bodyweight changes were recorded among treated animals. There were no notable effects on body temperatures for rats treated with Rotstop when compared to the controls. Under the conditions of the study there was a rapid loss of viability of the test organism

The dose of *Phlebiopsis gigantea* in the acute intraperitoneal study in rats was $9.31x10^4$ -$1.27x10^5$ cfu/kg bw. Clinical signs: piloerection in all treated animals, hunched posture (12/24). These signs were seen less than one hour after dosing and had ceased by two hours after dosing. Muscle reaction in ventral abdomen (13/24 and in 2 rats in autoclaved group), deformity prominent are (4/24), vocalization (2/12 treated and 1 autoclaved females), excreta (1/24), brown staining (19/24 and in 6 rats in autoclaved group) were seen during the study. There were no other clinical signs, which were considered to be associated with the test organism. Macroscopic examination revealed white nodules on the organs (liver, spleen, kidneys and intestines) of all treated animals with micro-organism, fusion of the intestines to the spleen and liver or liver and caecum was noted in 5/6 rats in group 4B and 1/6 rats in group 4C and 4D. Decrease of the bodyweight.

Rely on the studies above it could be concluded that under the proposed conditions of use (use protective clothing and shoes, rubber or plastic (e.g. nitrile) gloves and a cap when handling the product, use also a half mask with dust filter P2 when preparing the working solution) micro-organism dose not have any harmful effects on human health.

Based on Directive 2001/36/EC all micro-organisms are considered sensitising agents, due to lack of suitable test for micro-organisms and need to be classified as R43.

Metabolites production by *P.gigantea* was investigated, Lup-19(22)-ene, Lup-15,19(22)-diene and 2',3',5'-trimethoxy-p-terphenyl were found. There are no other records of metabolites produced by *P. gigantea* that would be of concern for human health and/or the environment. It was proved by the notifier based on literature that named metabolites are not hazardous to humans.

The sensitivity of *Phlebiopsis gigantea* to typical antibiotics used against dermatophytes was investigated and as the result was found that *P. gigantea* was highly sensitive to chlotrimazol and pimaricin at all concentrations, while nystatin inhibited colony formation completely at the two higher concentrations and reduced viability at the lowest concentration. The fungus showed the lowest sensitivity to amphotericin B, which completely inhibited fungal growth only at the highest concentration. At the lower concentrations the growth rate of the fungus was reduced, but the number of colonies was not affected (Pulkkanen, 1996).

B.6.4 Effects on human health – the preparation (Annnx IIIB 7)

Depending on the type of formulation, the concentration of micro-organism in the formulated product is $2x10^6$-10^7 cfu of *P. gigantea*/g for a wettable powder WP (nominal 5 x 10^6 cfu/g) and 3.5 $x10^6$-10^7 cfu of *P. gigantea*/ml for a suspension concentrate (SC). The representative formulation Rotstop contains 8-12 % w/w of *P. gigantea* (nominal 10 % w/w). The alternative formulation PG Suspension contains <0.5 % w/w of *P. gigantea*.

B.6.4.1 Basic acute toxicity studies – the preparation (Annex IIIB 7.1.)

B.6.4.1.1 Acute oral toxicity (Annex IIIB 7.1.1.)

A study with Rotstop is evaluated under paragraph B 6.1.2.2.1

B.6.4.1.2 Acute inhalation toxicity (Annex IIIB 7.1.2.)

A study with Rotsop is evaluated under paragraph B 6.1.2.2.2

B.6.4.1.3 Acute percutaneous toxicity (Annex IIIB 7.1.3.)

Reference:	Emma L. Blanchard (2002) Rotstop: Acute Dermal toxicity/pathology to the rat. Huntingdon Life Sciences Ltd. Unpublished report No.: KEY 009/022717/AC
Guideline / GLP:	US EPA OPPTS Guidelines 885.3100/Yes
Deviation:	Chemical analysis of formulated test articles for determination of stability, homogeneity and concentration was not undertaken for this study
Acceptability:	
Test substance / purity:	Rotstop (Batch No.: 01151)/ 10^6-10^7 living oidias of *Phlebiopsis gigantea*/gram
Vehicle:	Physiological saline
Species / Strain:	CD rats of Sprague-Dawley origin (Hsd: Sprague-Dawley (CD))
Doses / No. of animals	2000 mg/kg bw/10 rats
Exposure time	24h

Protocol:
Test design:
Five male and five female CD rats of Sprague-Daweley origin (bodyweight range: 207-261 kg, approximately 8 – 11 weeks old) were selected for the study. Twenty-four hours prior to dosing, animals were clipped free of hair on the dorso-lumbar region. The maximum practical dose used in the study was 40% w/v in physiological saline. The viable count of the test material was measured as $5.57x10^6$ cfu/g. Rotstop was formulated at a maximum practical concentration of 40% w/v in physiological saline and administered at a dose volume of 5 ml/kg bodyweight. The absorption of the test substance was not determined, homogeneity, stability and purity of the test substance was not undertaken in this study. The rats were treated at 2000 mg/kg bodyweight. The testing substance was applied by spreading it evenly over the prepared skin. The treatment area as covered with porous gauze held in place with a non-irritating dressing, and further covered by a waterproof dressing. At the end of the exposure period, the dressings were removed and the test site was washed with water to remove any residual test material. No control animals were included in this study. All animals were observed for 14 days after dosing.
Results:
There were no deaths at a dose level 2000mg/kg bw. Clinical signs include lethargy, seen in all animals on Day 2. A loss in bodyweight and low bodyweight gains were recorded for four animals (2f+2m).
Very slight dermal irritation was observed in four females from Day 3, resolving completely by either Day 4,7 or 10. Additionally, desquamation (characterised by sloughing and/or scaling) was recorded in one female from Day 6, resolving by Day 11. No abnormalities were recorded at the macroscopic examination at study termination on Day 15. Individual dermal scores are shown in Table 6.4.1.3.-01

Table 6.4.1.3.-01 Individual animal dermal scores

Rat No.sex	Dermal response	Day									
		2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11 to15**
1 M	Erythema Oedema	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
2 M	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
3 M	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
4 M	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
5 M	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
6 F	E O	0 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
7 F	E O	0 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
8 F	E O	0 0	1 0	1 0	1 0	1a 0	1a 0	1a 0	1a 0	0a 0	0 0
9 F	E O	0 0	1 0	1 0	1 0	1 0	0 0	0 0	0 0	0 0	0 0
10 F	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

a-Desquamation (characterised by sloughing and/or scaling of the skin)

Conclusion:
Under the conditions of the study the acute lethal dermal dose to rats of Rotstop was to be greater than 2000 mg/kg bodyweight

RMS comments:
The recommended vehicle for the end-use product usually is the same material in which the MPCA will be mixed, suspended, or diluted for application.

B.6.4.2 Additional acute toxicity studies – the preparation (Annex IIIB 7.2)

B.6.4.2.1 Skin irritation (Annex IIIB 7.2.1.)

Reference: Brenda I. Parcell (1996) Rotstop skin irritation to the rabbit. Huntingdon Life Sciences Ltd. Unpublished report No.: RKY 130/960291/SE

Guideline / GLP: OECD Guideline 404/Yes

Deviation: -

Acceptability: The study is considered to be acceptable

Test substance / purity: Rotstop (Batch No.:2, viable count 1.07 x 10^7 CFU *P. gigantea* per gram)

Vehicle: -

Species / Strain: New Zealand white rabbits

Doses / No. of animals	0.5 g of Rotstop/animal
Exposure time	4h

Protocol:

Test design:

Three New Zealand white rabbits (bodyweight range: 2.5 – 3.6 kg, approximately 11 to 15 weeks of age) were selected for the study. Twenty-four hours prior to dosing, animals were clipped free of hair on the dorso-lumbar region. 0.5 g of the test material was applied under a 25 mm x 25 mm gauze pad, which was moistened with 0.5 ml of distilled water. The treatment site was then covered with a semi-occlusive dressing for four hours. At the end of the four hour exposure period, the dressings and gauze pad were removed and the treatment site was washed with warm water (30-40^{o}C) to remove any residual test material. All test animals were observed daily for mortalities and clinical signs of toxicity. Dermal responses to treatment were examined 1, 24, 48 and 72 hours after patch removal. The test sites were scored for erythema, eschar and oedema formation.
The absorption, homogeneity, stability and purity of the test substance was not determined in this study.

Results:

There were no mortalities or clinical signs of toxicity noted throughout the study. No dermal responses to treatment were noted for any of the test animals during the study.

Conclusion:

Under the conditions of the study a dermal application of Rotstop to rabbit skin for four hours elicited no dermal irritation response.

B.6.4.2.2 Eye irritation (Annex IIIB 7.2.2.)

Reference:	B. Parcell (1996b) Rotstop: Eye irritation to the rabbit. Huntingdon Life Sciences, Ltd. Unpublished report No.: RKY 138/960815/SE
Guideline / GLP:	OECD Guideline 405/Yes
Deviation:	-
Acceptability:	The study is considered acceptable
Test substance / purity:	Rotstop (Batch No.: 2, viable count: 4.20 x 10^5 CFU *P. gigantea* per gram)
Vehicle:	-
Species / Strain:	Rabbits of the New Zealand White strain
Doses / No. of animals	40 mg dose/four animals

Protocol:

Test design

Four New Zealand white rabbits (2.9 – 3.8 kg, approximately 13 to 15 weeks of age) were selected for the study. One animal was treated prior to the others to check the response to treatment. A 40 mg dose of the test material was instilled into the lower lid of one eye of each animal. The eyelids were gently held together for one second before releasing. The remaining eye was left untreated and served as a control. All animals were observed daily for signs of ill health or toxicity. The eyes were examined 1, 24, 48 and 72 hours after dosing, further examinations were performed 4, 7 and 14 days after instillation.

Results:

There were no signs of toxicity or ill health noted for any rabbit during the study.
The screen animal (rinsed eye) showed no corneal damage or iridal inflammation. A temporary mild conjunctival reaction was seen, however a full recovery was observed two days after dose application. In the main study, dulling of the cornea was seen in two animals one hour after instillation. Corneal opacification developed in both of these animals. A diffuse crimson colouration of the conjunctivae was recorded in two

animals and was accompanied in one by swelling with partial eversion of the eyelids. The pilot animal showed mild temporary conjunctival irritation. All eyes had returned to normal seven and fourteen days after instillation. Individual animal ocular scores are shown in Table 6.4.2.2-01 below.

Table 6.4.2.2.-01 Ocular reactions observed after instillation of Rotstop

Rabbit No. Sex	Region of eye		1 hour after dosing	Day after instillation					
				1	2	3	4	7	14
2242 M*	Cornea	Density	0	0	0	0	0	7	-
		Area	0	0	0	0	0	0	-
	Iris		0	0	0	0	0	0	-
	Conjunctiva	Redness	1	1	0	0	0	0	-
		Chemosis	1	0	0	0	0	0	-
2243 M#	Cornea	Density	0	0	0	0	0	0	0
		Area	0	0	0	0	0	0	0
	Iris		0	0	0	0	0	0	0
	Conjunctiva	Redness	1	1	1	1	1	1	0
		Chemosis	1	1	0	0	0	0	0
2244 M	Cornea	Density	D	1	0	0	0	0	0
		Area	4	1	0	0	0	0	0
	Iris		0	0	0	0	0	0	0
	Conjunctiva	Redness	1	2	1	1	1	0	0
		Chemosis	1	1	1	0	0	0	0
2258 M	Cornea	Density	D	2	1	0	0	0	0
		Area	2	1	1	0	0	0	0
	Iris		0	0	0	0	0	0	0
	Conjunctiva	Redness	1	2	2	1	1	0	0
		Chemosis	1	2	1	0	0	0	0

- First screen animal (rinsed eye)
  # Pilot animal
  D Dulling of the cornea

Conclusion:
Under the conditions of the study instillation of Rotstop into the rabbit eye elicited corneal opacification and well-defined conjunctival irritation.

Comments:
The test substance does not need to be classified for eye irritation, but should be labelled with S25 phrase (avoid contact with eyes) because of slight eye irritation possibility.

B.6.4.2.3 Skin sensitisation (Annex IIIB 7.2.3.)

See evaluation point 6.1.2.1.

B.6.4.3 Data on exposure – the preparation (Annex IIIB 7.3)

Rotstop WP is a water dispersible powder formulation containing 2 x 10^6 – 10^7 cfu/g and the representative use supported in this DAR is for the control of root and butt rot on stumps of spruce and pine. The formulation concentrate is dispersed with water before being applied using either a mechanical spraying device mounted on the harvester or alternatively using a knapsack or hand held sprayer.

Applications are made at a rate of 2.0 L working solution per m^2 stump surface using water volumes of 25 L per 25 g package (working solution equivalent to 1 g/L). One package of Rotstop is sufficient for stump treatment in an area equivalent to 37-75 m^3 harvested timber (1/6 – 1 ha).

Exposure of the operator, workers and bystander to the colony forming units of *Phlebiopsis gigantea* during application of the different formulations, or the workers during re-entry can occur.
Micro-organisms are living organisms capable of infecting a suitable host and able to replicate. There are no suitable exposure models that could estimate exposure of micro-organisms considering all aspects.

The typical routes of exposure are from dermal absorption, inhalation and ingestion. For both types of application, operators would consider exposure from handling the product and working solution during mixing and loading activities. The formulation is described as a fine powder and therefore there could be the potential for inhalation from dust.

Operators performing hand held applications, particularly under protected conditions, would have greater potential for exposure from spray drift and therefore suitable protection should be considered to minimise contamination.

Due to the potential of all micro-organisms being sensitisers suitable protective clothing and equipment must be considered. Besides, according to the toxicity studies Rotstop WP is considered as a mild eye irritant therefore it would be recommended to wear suitable eye protection such as goggles or face shield when handling the product and working solution or when performing hand held applications. In order to minimise exposure, further protective clothing (gloves and dust mask) could potentially be considered when handling the product and working solution or when performing hand held applications. To sum up, it would be recommended to wear suitable protective clothing (protective suit, gloves, boots, hat), eye protection (goggles or face shield) and respiratory equipment (effective dust mask (P2) is recommended by the notifier) when handling the concentrate or when performing hand held or open cabin applications. This is consistent with the current PPE recommendations.

Workers entering treated areas, whose exposure is typically related to dermal exposure and is likely to be significantly less than that of an operator, would not be at risk from exposure to Rotstop WP. Based on the recommendations indicated for operators, with a potential for eye irritancy, it is considered reasonable that re-entry into treated areas should not occur until the application has finished.

The potential routes of exposure for bystander are via dermal and inhalation exposure to the spray solution. Bystander exposure will result primarily from the drift. Bystanders, whose exposure is assumed significantly less than that of an operator, would not be at risk from incidental exposure to Rotstop WP when applied to tree stumps. For such applications it is considered that bystanders would normally be prevented from entering areas where the harvesting of trees was being performed and therefore the opportunity for incidental exposure would be removed. If the exposure of a bystander is proportional to the level of airborne material, it is unlikely that exposure of bystanders outside the treatment area will be concern; it seems to be unlikely that any such exposure would be greater than the natural background deposition rate of spores of *P.gigantea.*

B.6.4.4 Available toxicological data relating to non-active substances – the preparation (Annex IIIB 7.4)

Confidential information. See Volume 4

B.6.4.5 Supplementary studies for combinations of plant protection products (Annex IIIB 7.5)

Not applicable. Not required.

B.6.5 Summary and evaluation of health effects – the preparation (Annex IIIB 7.6)

Type of study	Test material	Species	Result	Reference
Acute oral	Rotstop	Rat, CD	Dose: 4.26 x 10^7 cfu of *Phlebiopsis gigantea*/ kg bw No mortalities. Soft to liquid red stained faeces	McRae, Lewis A. (1996)

Acute dermal	Rotstop	Rat, CD	Dose: 2000mg/kg bw No mortalities. Clinical signs: lethargy, loss in bodyweight, very slight dermal reaction	Emma L. Blanchard (2002)
Inhalation (intratracheal)	Rotstop	Rat, CD	Dose: 1.12 x 10^6 cfu/kg bw Animal's deaths. Clinical signs included piloerection, hunched posture, waddling gait, lethargy, decreased respiratory rate, partially closed eyelids, gasping/noisy respiration, unsteadiness, increased lacrimation, hyperactivity, increased respiratory rate, protruding eyes, bluish colour to skin/extremities, cold body surfaces and staining around nose and mouth. Minor bodyweight changes	L. McRae (1996b)
Acute intraperitoneal toxicity and pathogenicity to the rat	Rotstop	Rat, CD	Dose: 9.31x10^{4} – 1.27x10^{5} cfu per rat White nodules on the organs (liver, spleen, kidneys and intestines), fusion of the intestines to the spleen and liver or liver and caecum was noted; a pus filled lump under the skin was found in one female treated with autoclaved test substance.	Blanchard, E. (2002)
Dermal irritation	Rotstop	Rabbit	Non-irritant	Blanchard, E. (2002)
Eye irritation	Rotstop	Rabbit	Mild irritant	B. Parcell (1996b)
Skin sensitisation	Rotstop	Guinea pig	Non - sensitiser	S. Allen (1996)

Rotstop shows a low acute toxicity via the oral and dermal routes. The acute lethal dermal dose to rats of Rotstop was to be greater than 2000 mg/kg bw and the acute lethal oral dose to rats was greater than 4.26 x 10^7 cfu of *Phlebiopsis gigantea/* kg bw. In an intratracheal study with rats, no infection hazard was noted after exposure, LC_{50} was to be greater than 1.12 x 10^6 cfu/kg bw.
Based on the severity of the clinical signs observed in the animals treated at high dose via intraperitoneal administration, it was concluded that lower (9,31x10^4-1,27x10^5 cfu/animal) dose was a suitable for the main acute intraperitoneal toxicity study and as the results were found the following signs of toxicity: piloerection in all treated animals, hunched posture, muscle reaction in ventral abdomen, deformity prominent, vocalization, excreta. Macroscopic examination revealed white nodules on the organs (liver, spleen, kidneys and intestines) of all animals in groups 4A and 4B and one male in group 4D. The nodules were not present in any of the animals in the autoclaved or untreated control groups. In addition, fusion of the intestines to the spleen and liver or liver and caecum was noted in 5/6 rats in group 4B and 1/6 rats in group 4C and 4D.
The irritating and skin sensitizing potential of this biological fungicide was also examined. Skin irritation and skin sensitisation studies with rabbits resulted in Rotstop being classified as a non-irritant and non-sensitiser, however, according to the directive 2001/36/EEC all micro-organisms should be regarded as potential sensitiser. Besides this, studies of the practical application of Rotstop showed a prevalence of skin symptoms, however it is unclear did the skin symptoms and eczema related to using of Rotstop or another factors.
The eye irritation study showed a mild irritating potential of Rotstop.
The information on occupational health of workers indicates possible causes for concern with respect to possible allergic response from the use of *Phlebiopsis gigantea* in plant protection products.
On the basis of the available literature and toxicological studies that have been made, the product is not considered to cause health risks, when it used with personal protective equipment: suitable protective clothing (protective suit, gloves, boots, hat), eye protection (goggles or face shield) and respiratory equipment (effective dust mask (P2)).

Overall, it is concluded that Rotstop WP formulation can be used in a manner consistent with personnel protection equipment without potential health risks to operators, workers or bystanders.

B.6.6 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex II Data and Information					
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IIB 5.1	Korhonen, K., Kauppila, P.	1988	The sexuality of *Phlebiopsis gigantea* Karstenia. Vol. 27, pp. 23 – 30. Not GLP. Published.	N	-
IIB 5.1	Ikediugwu, F.E.O., Dennis, C., Webster, J.	1970	Hyphal interference by *Peniophora gigantea* and *Heterobasidion annosum*. Trans. Br. Mycol. Soc., Vol. 54 (2), pp. 307 – 309. Not GLP. Published.	N	-
IIB 5.1	Briggs, L.H., Cambie, R.C., Dean, I.C., Dromgoole, S.H., Fergus, B.J., Ingram, K.G., Lewis, K.G., Small, C.W., Thomas, R. & Walker, D.A.	1975	Chemistry of fungi 10. Metabolites of some fungal species. N. Z. J. Sci. Vol. 18, pp. 565 – 576. Not GLP. Published.	N	-
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IIB 5.1	Jennison, M.W., Richberg, C.G. Krikszens, A.E.	1957	Physiology of wood-rotting basidiomycetes. II. Nutritive composition of mycelium grown in submerged culture. Appl. Microbiol. Vol. 5, pp. 87 – 95. Not GLP. Published.	N	-

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
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IIB 5.1	Niemi, M.	1992b	Effect of high temperature on the viability of the spores of *Phlebiopsis gigantea.* Test report 9252, 1 p. Not GLP. Unpublished.	Y	VRA
IIB 5.1.3	Greig, B.J.W	1992	*Peniophora gigantea.* Letter to Pekka Seiskari from B.J.W Greig with Extracts from *Peniophora* data sheet. Ref: PAT.175 Not GLP; Unpublished	Y	FOC
IIB 5.1.3	Seiskari, P.	1994	Sensitization of *Phlebia gigantea* Statement 23.6.1994 Not GLP; Unpublished	Y	VRA
IIB 5.1.3	Nelson, S.M	1995	Application for clearance to use *Peniophora gigantea:* Search of chainsawyers personal health records. Letter to Mr J. Pratt from Mrs S.M. Nelson Ref: SMN/AT/E19/8/1 Not GLP; Unpublished	Y	FOC
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IIB 5.1.3	Korhonen, K., Lipponen, K.	2000	Sensitization of *Phlebiopsis gigantea* Memo dated 6 July 2000 Not GLP; Unpublished	Y	METLA
IIB 5.1.3	Kallunki, H., Kangas, J., Laitinen, S., Mäkinen, M., Susitaival, K.O.J.P	2002	Exposure to and health effects of chemical and biological agents in mechanical wood harvesting. Final report (In Finnish, English summary and relevant translated sections submitted). Not GLP; Published	N	KATTL
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IIB 5.2.1	Allen, S.	1996	Rotstop skin sensitisation in the guinea pig. Huntingdon Life Sciences Ltd. Company report No.: HLS RKY 131/960116/SS. GLP; Unpublished	Y	VRA
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IIB 5.2.2.2	McRae, L.	1996b	Rotstop acute pulmonary toxicity and pathogenicity to the rat. Huntingdon Life Sciences Ltd. Company report No.:HLS RKY 133/953005/AC GLP; Unpublished	Y	VRA
IIB 5.2.2.3	Blanchard, E.	2002	Rotstop: Acute intraperitoneal toxicity and pathogenicity to the rat. Huntingdon Life Sciences Ltd Company report No.: HLS KEY 008/023033/AC GLP; Unpublished	Y	VRA
IIB 5.2.2.3	Salfelder, K., Schwarz, J.	1976	Basidomycetes as infectious agents Mykosen Vol. 19 (10), pp. 373-382 Not GLP; Published	N	-
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IIB 5.2.5		2001	Commision Directive 2001/36		
IIB 5.6	Pulkkanen, H.	1996	Antibiotic sensitivity of *Phlebiopsis gigantea.* Kemira Agro Oy, Espoo Research Centre. Test report 186/96. 3 pp. Not GLP. Unpublished.	Y	VRA
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IIIB 7.2.2.	Paracell, B	1996b	Rotstop: Eye irritation to the rabbit. Huntingdon Life Sciences Ltd. Company report No.: RKY 138/960815/SE. GLP; Unpublished	Y	VRA

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

***: Owners' code identifications and names (Code identification: VRA, Name: Verdera*