ANNEX B

Laminarin

B.6 Toxicology and metabolism

B.6.1 Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA 5.1)

No specific ADME study has been conducted in mammals.

Laminarin, a linear β D-1, 3-linked glucan was extracted and purified from the brown alga *Laminaria digitata*.
Algae such as *Laminaria*, belonging to the brown seaweed's (*Phaeophyceae*), are commonly found on the beaches, some of them are edible and used unprocessed in food preparations; other are used mainly in livestock feed and occasionally in human food.
Marine algae polymers are mainly non-starch polysaccharides. The majority of them escapes digestion by pancreatic and small-bowel enzymes in the human gut and therefore arrive in the large bowel where their digestive fate is dependent on the depolymerizing and fermentative abilities of the large intestinal microbiota.
Soluble polysaccharides in brown seaweed consist of laminarans, some storage polysaccharides, and fucans, which are cell wand components. Brown algae, unlike the red and green algae, do not synthesise starch-type polysaccharides; instead they store carbohydrate as Laminaran.
Chemically, laminarans are β-1,3 linked glucans containing different proportions of β-1,6 linkages and mannitol residues on the reducing ends. Laminarin is an essentially linear glucan composed of ca. 33 β-1,3-linked Glc residues. These polymers are neutral and soluble in hot water.

For certain animals such as rodents, plant material constitute a major portion of their diets. Microbial digestion of plant material is significant and occurs mainly in the caecum (rats) and large intestine (humans) after prior host digestion in stomach and small intestine. A similar digestion pattern takes place in humans, but only small amounts of plant fibres are degraded. In contrast, with ruminant animals, intake of plant material is extensive, and digestion occurs prior to gastric digestion. The ruminant is dependent on micro-organisms in the forestomach or rumen for the digestion of plant polysaccharides that are consumed. Further digestion of plant materials can occur in the caecum and large intestine, but is considerably less in magnitude.

Overall, plant polysaccharides are degraded to varying extents to soluble forms, mostly oligosaccharides, by the hydrolytic microbial species. Laminarinases that hydrolyse laminarin occur in bacteria, fungi, algae, higher plants, and molluscs (Black and Dewar, 1954).
The small intestine of humans (and animals) does not possess specific β-D-glucans-hydrolases required for hydrolysis of oligosaccharides to their constituent simple sugars. As a result, virtually 100% of ingested polysaccharides reaches the colon unchanged. In the colon, bacteria, in a process called fermentation, mainly degrade them to mono- and oligo-saccharides, and then, these degraded saccharides of lower molecular size are utilised by a variety of intestinal bacteria to give a mixture of short chain fatty acids (acetate, propionate and butyrate), L-lactate, carbon dioxide and hydrogen (Kuda et al.,1992). Unfermented carbohydrates increase faecal bulk.

In France, the Superior Council of Public Hygiene gave a favourable advice for use of *Laminaria digitata* in human food:
For an adult, supply should be $\leq$30 mg (dry product) or 210 mg/day for a fresh product.
Children < 4 year, supply should be below 15 mg dry compound corresponding to 105 mg fresh compound.

B.6.1.1 Mammalian metabolism of laminarin

In the open literature a review article deals specifically with the digestive fate of soluble polysaccharides such as laminarin from marine macro-algae, and on the involvement of the colonic microflora and physiological consequences for the host (Michel and Macfarlane1996):

Non-starch polysaccharides such as laminarin are resistant to hydrolytic enzymes produced by monogastric animals, and their digestive fate is dependent on the depolymerizing and fermentative abilities of the large intestinal microbiota. The monosaccharides are the only units that normally cross the intestinal membrane.

Metabolism: *Bacteroides* species from the human colon ferment a variety of polysaccharides, which are not digested in the stomach or small intestine. Dietary fibre components are an important source of carbohydrate for colon bacteria.

Laminarin is fermented by some species of anaerobic bacteria containing laminarinase activity from the human colon. Laminarinase is an enzyme complex that can involve as many as three types of activity: β-glucosidase, which hydrolyse low molecular weight glucose containing substrates (di- or trisaccharides), an exoglucanase that cleaves single glucose units from the non reducing end of laminarin and an endoglucanase that release laminaribiose, laminaritriose or higher oligomers of glucose from laminarin. Polysaccharide degrading enzymes from several *bacteroides* species have been studied and in most cases the enzyme activities were cell bound rather than extracellular. Laminaran-degrading species mainly belong to the genus Bacteroides and Bifidobacteria (*B. thetaiotaomicron, B.distasonis, Bacteroides 0061-1*, *Bacteroides T4-1*). These organisms are major components of the gut microbiota. The concerted activities of these polymer degrading species, together with other saccharolytic and hydrogenotrophic organisms in the large bowel, gives rise to a range of fermentation products, Short Chain Fatty Acids (SCFAs) (Table B.6.1.1-1). SCFAs are the predominant products of bacterial metabolism from human as well as rat colon. SCFAs are important anions in the colonic lumen, affecting both colonocyte morphology and function. The three main acids (acetate, propionate, and butyrate) stimulate colonic sodium and fluid absorption and exert proliferative effects on the colonocyte (Sheppach, 1994). After uptake by colonic mucosa, they undergo a variety of metabolic fates in host tissues (see table B.6..1.1-1) (Djouzi et al.1995 and Michel and Macfarlane, 1996).

Table B.6.1.1-1: Metabolic fate of the major carbohydrate fermentation products formed by bacteria in the human large intestine (From Michel and Macfarlane, 1996)

Fermentation product	**Metabolic significance**
Short Chain Fatty Acids	- About 85% of total colonic production are absorbed from the large bowel. Stimulate salt and water absorption in the colon - Intestinal motility is increased - Crypt cell formation and mucosal weight increased - Accelerated mucosal healing - Antibacterial effects
Acetate	- Quantitatively most important fermentation product - oxidised in brain, heart, kidney, liver, muscle and peripheral tissues
Propionate	- Mainly cleared by liver, but not a major gluconeogenic substrate - Suppresses cholesterol synthesis
Butyrate	- substrate for membrane lipid synthesis - affects gene expression and induces differentiation in a wide range of human and other mammalian cells - induces apoptosis - Principal fuel for colonic epithelial cells, particularly in distal bowel
Hydrogen	- Electron donor in other biochemical reactions in colonic bacteria - Excreted in breath and flatus - **Excretion:** Converted to H_2S, acetate, or methane by colonic SRB, acetogenic and methanogenic bacteria respectively
Carbon dioxide	- Required in some bacterial fermentation reactions, e.g. methanogenesis, acetogenesis, bacteroides metabolism. Involved in SCFA and sodium absorption in the colon - Excreted in breath and flatus

The involvement of colonic bacteria in laminaran breakdown is illustrated in rats by increases in faecal bacterial excretion following inclusion of laminaran (10%) in the diet (Kuda et al, 1992).

Distribution: SCFAs including acetate, propionate and butyrate are produced in the caecum and colon of nonruminant animals and humans and account for approximately 80% of the colonic anion concentration. They are produced in nearly constant molar ratio 60:25:15. Among their various properties, SCFAs are readily

absorbed by intestinal mucosa, are relatively high in caloric content, are metabolised by oxidation in colonocytes and hepatocytes, stimulate sodium and water absorption in the colon and are trophic to the intestinal mucosa (D'Argesio and Mazzacca 1999,).
Once absorbed, SCFAs are used preferentially as fuel for colonic epithelial cells and have a trophic effect on the epithelium. Butyrate oxidation in colonocytes produced ketone bodies and CO_2 (Cook and Sellin 1998). Beside their action on gut morphology and function, SCFAs influence gastrointestinal motility producing a laxative effect. The increase in faecal output is likely to be due to an increase in biomass (Cummings et al, 2001).

Conclusion:
In human, non-digestible polysaccharides such as laminarin escape enzymatic digestion in the upper gastrointestinal tract; colonic fermentation produces short-chain fatty acids, which are then absorbed. Absorption reaches approximately 90%.
Metabolism is important, involving gut microbiota (colonic fermentation) which degrade the polymers giving rise to SCFAs.
Distribution is large: carbohydrate fermentation products are oxidised in brain, heart, kidney, liver, muscle, peripheral tissues.
Excretion occurs via breath and flatus after conversion of SCFAs into H_2S, CO_2, CH_4, and acetate. Unfermented carbohydrates increase faecal bulk likely as a result of increased biomass.

In contrast, digestible carbohydrates are hydrolysed by alpha-amylases secreted by pancreas and salivary glands and oligosaccharidases responsible for hydrolysing the residual sugars. Active glucose absorption occurs in the small intestine (Szepesi, 1996).

B.6.1.2 Metabolism in plants

Numerous enzymes able to degrade laminarans have been isolated from bacteria, fungi, algae, molluscs and higher plants.
The enzymes that hydrolyse Laminarin are termed laminarinases including β-D-glucanases and β-D-glucosidases. Laminarase occurs in bacteria, fungi, algae, higher plants and molluscs (Black and Dewar, 1954). These enzymes are involved in the intracellular mobilisation of food reserves (not only in algae but also in higher plants and fungi, all of which synthesise β-D-glucans) and are also encountered in the extracellular breakdown of plant debris and in the digestive metabolism of invertebrates. Enzyme preparations have been reported from cereals, potato tubers etc….
The enzyme removes D-glucose units by endwise attack from the nonreducing ends of the chains (Black and Dewar, 1954).

B-Glucan endohydrolases from plants are involved in cell wall degradation. They release oligosaccharides from their substrate and are probably of central importance for the initial solubilization of the (1→ 3, 1→4) β-glucans. The soluble products of the initial hydrolysis are then acted on by glucanglucohydrolases, which preferentially attacks the longer gluco-oligomers (cellotriose) releasing glucose. β-Glucan exohydrolases and β-glucosidases may be important additional enzymes for the conversion of released oligosaccharides to glucose.

B-Glucanases which are widely distributed in plant hydrolyse also polysaccharides that are abundant in fungal cell walls (Hrmova et al., 1997).

In conclusion, in plants, laminarin may undergo degradation by polysaccharide and oligosaccharide hydrolases leading to production of glucose.

B.6.1.3 Metabolism in ruminants

In ruminants, feedstuffs are all initially exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. Dietary polysaccharides are generally degraded by the ruminal micro-organisms into characteristic endproducts. Fermentative production of SCFAs is the principal mechanism of intestinal digestion in ruminants (D'Argenio and Mazzacca, 1999).

Consequently, additional uptake of polysaccharides by animals by way of feeding is of no concern and livestock metabolism studies are not necessary.

B.6.1.4 Metabolism in fish

Several reports on (1→3)-β-D-glucans deal with their immunostimulatory effects in fish and indirect evidence that (1→3)-β-D-glucan is absorbed from the posterior intestine was showed in the Atlantic salmon. In fish, it was suggested that Laminaran and aminated (1→3)-β-D-glucans, after i.v. administration, are distributed to tissues rich in immunocompetent cells like the spleen and the anterior kidney. The cellular accumulation of Laminaran may be related to the endocytic function displayed by sinusoidal endothelial cells and macrophages (Dalmo et al, 1995).

Overall conclusion

Laminarins are cell wall components that are degraded by the colonic microflora in monogastric animals. The large bowel fermentation involves bacteria producing laminarinases, and B-glucosidases, which fully degrade the substrate into SCFAs. SCFAs are absorbed and further metabolized before excretion into breath and flatus.
In plants, laminarin may undergo degradation by polysaccharide and oligosaccharide hydrolases leading to production of glucose.
Fermentative production of SCFAs is the principal mechanism of intestinal digestion in ruminants.

B.6.2 Acute toxicity including irritancy and skin sensitization (Annex IIA5.2)

B.6.2.1 Acute oral toxicity (Annex IIA5.2.1)

- Phycarine 96S51, rat, limit test, gavage, 2000 mg/kg bw (Delille, 1998a) (Goemar)

Findings:
Mortality: no mortality occurred during the study.
Body weight: mean body weight and body weight gain in treated animals did not differ significantly from that of control rats.
Necropsy: no macroscopic organ or tissue abnormality was seen at necropsy of control or treated animals.

Conclusion: LD_{50} > 2000 mg/kg bw.

Guidelines: experimental protocol in compliance with test method B.1, dir. 96/54/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods: 5 starved Sprague-Dawley rats/sex received a single dose of Laminarin (batch n°96S51; purity: 91%) at 2000 mg/kg (suspension in water, 10 ml/kg) by gavage. Control rats were dosed with water.
The study is accepted.

B.6.2.2 Acute percutaneous toxicity (Annex IIA5.2.2)

- **Laminarin, rat, limit test, 5000 mg/kg bw (Audeval, 2001d) (Goemar)**

Findings: no mortality occurred. No clinical signs were observed during the course of the study. No dermal reactions were observed during the course of the study. Mean weight gain was normal. No macroscopic organ or tissue abnormalities were seen at necropsy.

Conclusion: LD_{50} > 5000 mg/kg bw

Guidelines: experimental protocol in compliance with test method B.3, dir. 96/54/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods: a single dose of laminarin (batch n°99S24; purity: 94.9%) at 5000 mg/kg bw (solution in water) was applied to the clipped skin of 5 Sprague-Dawley rats/sex using semi-occlusive gauze.
The study is accepted.

Additional study:

- Phycarine 96S51, acute toxicity study, safety test in the Sprague-Dawley rat by the subcutaneous route (Delille, 1998b) (Goemar)

Findings:
Mortality: no mortality occurred during the study.
Clinical signs: no clinical signs were observed during the course of the study in control or treated rats.
At injection site: no abnormality was observed at the injection site of control or treated rats.
Body weight: mean body weight and body weight gain did not differ significantly from the control rats.
Necropsy: a subcutaneous hematoma was seen at the injection site in one control and one treated rat.

Conclusion: subcutaneous administration of Laminarin at doses of 1000 mg/kg bw caused no mortality in rats.

GLP status: study is GLP – certificate of competent authority.

Material and methods: 5 starved Sprague-Dawley rats/sex received a single subcutaneous dose of Laminarin (batch n°96S51; purity: 91%) at 1000 mg/kg (suspension in water, 5 ml/kg). Control rats were dosed with water.
The study is accepted as additional information.

B.6.2.3 Acute inhalation toxicity (Annex IIA5.2.3)

- Acute inhalation toxicity with Laminarin in rats (Müller, 1999)(Goemar)

Findings:
Mortality: no rats died during the course of the study.
Clinical signs: no signs were observed.
Body weight: the slightly decreased weight gain in the female rats in the first week and the statistically significant increased weight gain in the second week in comparison to the control is caused by a slightly increased weight gain in the first week and a slightly decreased weight gain in the second week of control rats.
Necropsy: no findings.

Conclusion: LC_{50} > 1.02 mg/l/4h

Guidelines: experimental protocol in compliance with test method B.2, dir. 96/54/EEC. Limit test.
GLP status: study is GLP – certificate of competent authority.
Material and methods: 5 starved Wistar Crl: WI BR rats/sex were exposed for 4 hours by a head-nose only exposure equipment to a single dose of Laminarin (batch n°96S51; purity: 91%), aerosol. The test article was dissolved in distilled water (10%) and filtered to avoid blockade of the nozzle. A mean concentration of 1.02 mg/l aerosol was reached in the head-nose region of the rats. The concentration was the maximum attainable concentration. 58% of the particles have a diameter $\leq$ 22µm.
The study is accepted.

B.6.2.4 Skin irritation (Annex IIA5.2.4)

- **Phycarine 96S51- cutaneous primary irritation in the rabbit (Baudet, 1998a) (Goemar)**

Findings: the application of the substance induced coloration of the application site. A very slight erythema was noted in one animal.
Score erythema, 24+48+72 h = 0.3
Score oedema 24+48+72 h =0
At day 8, there were no signs of irritation.

Conclusion: Laminarin is not a skin irritant.

Guidelines: experimental protocol in compliance with test method B.4, dir. 96/54/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods: 0.5 g Laminarin moistened with 0.5 ml water (batch n°96S51; purity: 91%) was applied to the skin of 3 New Zealand Albino rabbits using a seem occlusive dressing for 4 hours.
The study is accepted.

B.6.2.5 Eye irritation (Annex IIA5.2.5)

- **Phycarine 96S51- ocular primary irritation in the rabbit (Baudet , 1998b) (Goemar)**

Findings:
Cornea opacity 24+48+72 h = 0
Iris 24+48+72 h = 0
Redness 24+48+72 h = 0
Chemosis 24+48+72 h = 0

Conclusion: Laminarin is non-irritant for the eyes.

Guidelines: experimental protocol in compliance with method B.5 dir.96/54/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
3 white New Zealand SPF rabbits were exposed to 0.1 g Laminarin (batch n°96S51; purity: 91%) in the eye. 24 h later, the substance was washed out with tap water.
The study is accepted.

B.6.2.6 Skin sensitization (Annex IIA5.2.6)

- **Phycarine 96S51- study of cutaneous sensitisation using the Magnusson and Kligman maximisation test in the guinea pig (Baudet, 1998c) (Goemar)**

Findings:
On day 25, the mean body weight of treated males was significantly less than that of control males, at the threshold of 5%.
Under the experimental conditions, Laminarin was not sensitiser.

Conclusion: Laminarin does not have a sensitising effect on the skin under the test conditions.

Guidelines: experimental protocol in compliance with method B.6 dir.96/54/EEC: guinea pig Maximisation test.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
10 male and 10 female guinea pig White, Hartley were exposed to Laminarin (batch n°96S51; purity: 91%); 10 animals were used as control.
A preliminary test was performed for dose selection. The minimal irritant concentration was found to be a 25% solution in water. The 25% preparation caused slight to moderate irritation and was therefore, used for the main test.
The study is accepted.

B.6.2.7 Summary of acute toxicity of laminarin (Annex IIA 5.2)

Laminarin is characterised by a rather low acute toxicity.
According to EU classification, laminarin should not be classified.

Table B.6.2.7-1 Summary of acute toxicity of laminarin (Annex IIA 5.2)

Type of test	Batch n°, purity	Results LD_{50}	Classification	References
Acute oral, rat	batch n°96S51; purity: 91%)	>2000 mg/kg bw	-	**Delille, 1998a**
Acute dermal, rat	batch n°99S24; purity: 94.9%)	>5000 mg/kg bw	-	**Audeval, 2001d**
Acute subcutaneous rat*	batch n°96S51; purity: 91%)	>1000 mg/kg bw	-	**Delille, 1998b**
Acute inhalation rat , aerosol, head-nose	batch n°96S51; purity: 91%)	>1.02 mg/l/4h	-	**Müller, 1999**
Skin irritation, rabbit	batch n°96S51; purity: 91%)	Non irritant	-	**Baudet , 1998a**
Eye irritation, rabbit	batch n°96S51; purity: 91%)	Non irritant	-	**Baudet , 1998b**
Skin sensitisation M & K test	batch n°96S51; purity: 91%)	Not sensitizer	-	**Baudet , 1998c**

*The study is accepted as additional information.

B.6.3 Short-term toxicity (Annex IIA 5.3)

B.6.3.1 Oral 28 day study (Annex II A 5.3.1)

-4 week oral toxicity study in rats, limit test at 1000 mg/kg bw/d (Longobardi, 2000) (Goemar)

Findings:
Mortality: no deaths occurred during the study.
Clinical signs: daily post-dose observations revealed no clinical signs.
Neurotoxicity tests and motor activity measurements performed at the end of treatment did not show changes attributable to treatment.
Body weight: no statistically significant differences were observed between the control and the treated group.
Food consumption: was not affected by treatment.
Haematology: no changes of toxicological significance were seen. The increase in RBCcount in females was considered incidental and was within the range of historical controls.
Clinical chemistry: no treatment related changes and statistically significant variations observed were considered to be incidental and were within the historical controls (table B.6.3-1).
Organ weights:
No biological significant effects observed.
Histopathology: no treatment related effect.

Table B.6.3-1: 4-week toxicity study in rats.

Findings	**0 mg/kg bw/d**		**1000 mg/kg bw/d**	
	M	**F**	**M**	**F**
Bw	349±7.3	231.3±3.6	362.9±22	228.7±12.33
Haematology:				
Red blood cell count	8.75±0.25	8.06±0.15	8.69±0.22	8.28*±0.19
Clinical chemistry:				
K^+	3.998±0.10	3.624±0.176	4.226*±0.134	3.836±0.107
Organ weight (a)(g)				
Heart	1.315±0.02	1.290±0.121	0.925±0.039	0.921±0.036

*P<0.05
Conclusion: NOAEL > 1000 mg/kg bw/d

Guidelines: experimental protocol in compliance with method B.7 dir.96/54/ EEC. Limit test.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
5 male and 5 female Sprague-Dawley rats received by gavage Laminarin (batch n°; purity: 97.6%) at a dose of 1000 mg/kg bw/d. Control group received the vehicle alone (0.5% CMC in distilled water).
Neurotoxicity tests were performed including: removal, handling reactivity, lachrymation, palpebral closure, salivation, piloerection, rearing, clonic movements, tonic movements, gait, mobility impairment, rousal, vocalisation, stereotypes, unusual respiration, bizarre behavior, urination, defecation.
Statistics:
For continuous variables the significance of the differences amongst groups was assessed by analysis of variance. Differences between the treated and the control group were assessed by Dunnett's test using a pooled error variance. The homogeneity of the data was verified by Barlett's test before Dunnett's test. If data were found to be inhomogeneous a Modified t test was applied.
The study is accepted.

B.6.3.2.1 Oral 90 day toxicity (rat) (Annex IIA 5.3.2)

- 90 day, oral, rat study, 1000 mg/kg bw/d by gavage, limit test (Audeval, 2001a)

Findings:
Mortality: No death was recorded during the study.
Clinical signs: No treatment-related clinical signs were recorded during the study.
Body weights of treated rats were similar to those of the controls.
Food consumption was slightly reduced in male and female rats from week 2 up to week 13.
Water consumption was statistically significantly reduced for females from week 2 up to the end of the study. In male rats, water consumption was reduced since week 3 up to the end of the study without reaching statistically significance. In rodents, water consumption is closely associated with food consumption. This was confirmed by the fact that reduced water intake was not associated with a decreased urinary volume, or specific gravity alteration.
Haematology: no biological or statistically significant effects were observed.
Blood chemistry: at the end of treatment period, mean calcium level in females was increased. This difference was mainly due to a high calcium level in one female.
Urinalysis did not reveal any treatment-related effect.
Organ weight: mean absolute and relative heart weight of treated females were statistically significantly greater than that of control females. This effect was not associated to *histopathological* or biochemical findings.

Table B.6.3.2.1-1: 90-day rat study by gavage.

Endpoints/dose	**control**		**1000 mg/kg bw/d**	
	M	**F**	**M**	**F**
Body weight	483±0.12	282.7± 29.5	498±45	284.5±15.6
Food consumption (g)	128.8	95	122.7	89
Water consumption (ml)	155.2	129.4	143	107.7* (↓ 16.7%)
Food/water consumption ratio	0.829/1.2	0.734/1.3	0.858/1.2	0.831/1.2
Haematology			No compound related effect	
Blood chemistry				
calcium	2.60±0.14	2.56±0.13	2.57±0.10	2.72±0.12*
Urinalysis			No compound related effect	
Organ weight				
Heart (absolute)(g)	1.813±0.193	1.078±0.08	1.868±0.169	1.188± 0.13*
Heart (relative % bw)	0.400±0.027	0.414±0.04	0.399±0.030	0.452±0.04 *
Histopathology			No treatment-related effects	

*P<0.05

Comments: Overall, for males as well as for females, a slight body weight increase, not reaching statistically significance, is observed, although food consumption is decreased (not statistically significant). These effects suggest that gavage with Laminarin contributes to the energy content of the diet, the animals have to eat less to meet their caloric requirements.
According to the open literature, addition of soluble forms of fibre to diets often has been found to improve absorption of minerals. Ingestion of resistant sugars resulted in caecal hypertrophy, reduced pH of caecal contents and increased permeability of intracellular junctions to passive absorption of minerals such as calcium (Greger, 1999). A slight increased calcium absorption is not considered as adverse.
Cardiac hypertrophy is defined as an increase in heart muscle. This increase is predominantly due to an increase in the contractile elements and mitochondria. Hypertrophy usually develops as a means of compensation: a thicker muscular wall can generate more power. Abnormal heart weight is usually the result of congenital abnormalities, abnormal heart valve function or systemic hypertension, though it may also arise from long-term chemical influences (thyroid hormone, growth hormone, and catecholamines).
In this case, there is no consistency between sexes, no correlation with clinical observations or with other clinical pathology findings and 2/10 female rats seems to be outside the group. Moreover, this effect was not

observed in the 28-day rat study. So, we conclude that the difference between control and treated female rats is not biologically significant.

Conclusion: NOAEL > 1000 mg/kg bw/d

Guidelines: experimental protocol in compliance with limit test, test method B., annex V, dir.87/302/ EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
10 male and 10 female Sprague-Dawley rats received Laminarin (batch n°. 99S24; 94.9 % purity) by gavage at 1000 mg/kg bw/d for 90 days. Control group received the vehicle (water) only.
Functional and neurobehavioral tests were performed during the 13th week: behaviour profile including awareness, mood, motor activity, motor incoordination; neurological profile including central excitation, muscle tone, body posture and reflexes; autonomic profile.
Statistics:
Food consumption, bw, bw changes and food efficiency: parametric one-way analysis using F-test (Anova) two-sided. If resulting $p<0.05$, a comparison of each dose-group with control group was performed using the Dunnett's test.
Clinical pathology: non-parametric one-way analysis using Kruskal-Wallis test. If resulting $p<0.05$, pair-wise comparison of each dose-group with control group performed using Mann-Whitney U-test (two-sided) for equal medians.
Urinalysis: pair-wise comparison for each dose group with control using Fisher's exact test for the hypothesis of equal proportions.

B.6.3.2.2 Oral 90-day toxicity (dog) (Annex IIA 5.3.2)

- 90 day, diet, dog study, 1000 mg/kg bw/d, limit test (Audeval, 2001b)

Findings:
Mortality: 1 animal in the control group was sacrificed for humane reasons: the female presented convulsions and decreases in spontaneous locomotion activity the day before the sacrifice.
Clinical signs: the main findings reported in control and treated animals were diarrhoea and/or soft stools. These findings are common in young laboratory dogs. However a slight increase in incidence was recorded in treated animals when compared with controls.
Comment: Degradation of polysaccharides in the large bowel gives rise to a range of fermentation products. Fermentation increases bacterial mass in the bowel; undegraded polysaccharide physically dilute gut contents; increased mass in the gut as well as SCFA production stimulates peristalsis and increases gut transit rates. Polysaccharides fermentation is generally a desirable process in the colon and is intimately linked to large bowel physiology (Michel and Macfarlane, 1996)
Haematology: some slight variations, within historical control values were observed, not considered to be treatment –related.
Blood chemistry: no statistically significant differences were found compared with the control group.
Urinalysis, ophthalmology did not reveal any treatment-related effect.
Organ weight: Some small organ weights variations were observed. Since no findings were reported at histopathology examination, these differences were considered to be incidental.

Table B.6.3.2.2-1: 90-day dog study by gavage.

Endpoints/dose	**control**		**1000 mg/kg bw/d**	
	M	**F**	**M**	**F**
Mortality		1 day 31		
Clinical signs				
Diarrhoea	2	1	2	
Soft stool	3	1	1	4
Mucous stool			1	1
Vomiting	1		2	
Body weight (kg)	14.11±0.52	11.78±2.07	14.09±1.17	12.47±1.61
Food consumption (g) week 13	1922±158	1773±205	1904±193	1871±261
Water consumption (ml) week 13	3617±779	2557±1071	3642±643	3640±854
Haematology			No compound related effect	
Blood chemistry			No compound related effect	
Urinalysis			No compound related effect	
Organ weight				
spleen (absolute)(g)	45.32±6.67	29.000±1.92	30.34±3.79*	31.507±5.85
spleen (relative, % bw)	0.330±0.014	0.270±0.052	0.220±0.015*	0.260±0.0268
Adrenal (rel,% bw)	0.0086±0.00042	0.0098±0.0014	0.0097±0.00050 *	0.0103±0.00156
Histopathology			No compound related effect	

*P<0.05

Conclusion: NOAEL > 1000 mg/kg bw/d.

Guidelines: experimental protocol in compliance with test method B., annex V, dir. 87/302/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
4 Beagle dogs/sex/dose received Laminarin by gavage in water (batch n° 99S24; 94.9 % purity) at 1000 mg/kg bw/d for 90 days. The control group received water only.
During the thirteenth week, animals were submitted to a standardised observation battery in order to detect neurobehavioral, neurovegetative or psychotropic signs or neurotoxic effects.

B.6.3.2.3 Oral 1 year toxicity (dog) (Annex IIA 5.3.2)

No data, not necessary.

Additional study from open literature:

-A study of *Laminaria digitata* powder on experimental hyperlipoproteinemia and its hemorrheology (Tang and Shen 1989)

Findings: *Laminaria digitata* had a significant effect of lowering total cholesterol, -lipoprotein, especially triglycerides ($p<0.01$), meanwhile it could increase the level of HDL-c and HDL. Also it could reduce relative blood viscosity and fibrinogen very significantly ($P< 0.01$). However HDL3-c, hematocrit and index of erythrocyte deformability were not much influenced ($p>0.05$).

Material and methods: Twenty rabbits with experimental hyperlipoproteinemia were divided randomly into two groups. The first group was administered *Laminaria digitata* powder (1-gr. daily) for 14 days. Another was given only the routine menu.

B.6.3.3.1 28-day inhalation toxicity (rat) (Annex IIA 5.3.3)

No data, not necessary.

B.6.3.3.2. 90-day inhalation toxicity (rat) (Annex IIA 5.3.3)

No data, not necessary.

B.6.3.3.3 Percutaneous 28-day toxicity (rat) (Annex IIA 5.3.3)

No data, not necessary.

B.6.3. 4 Summary of short-term toxicity of laminarin (Annex IIA 5.3)

Laminarin was given by gavage to rats at 1000 mg/kg bw/d for 28 days and 90 days. A slight body weight increase was observed associated to a slight food consumption reduction as well as water consumption decrease. Laminarin is a polysaccharide and might contribute to increase the energy content of the diet, reducing food intake. In rodents, water consumption is closely associated to food consumption. Therefore, these effects are not considered to be adverse.
In dogs, laminarin was given by gavage at 1000 mg/kg bw/d for 90 days. Slight increased incidence of soft faeces was reported which can be explained by the increased gastrointestinal motility produced by short chain fatty acids (resulting from laminarin fermentation), as well as an increase in biomass which increases the faecal output. Therefore, the observed effects are not considered to be adverse.

Table B.6.3-1: Summary of short-term toxicity of laminarin:

Type of test	Batch n°. purity	Findings	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Ref
28 day feeding rat study	Batch n°: 99S21 97.6%	No effects	>1000 mg/kg bw/d	-	Longobardi, 2000
90 day feeding rat study	Batch n°: 99S24 94.9%	No effects	>1000 mg/kg bw/d	-	Audeval, 2001a
Dog, 90day dog feeding study	Batch n°: 99S24 94.9%	No effects	>1000 mg/kg bw/d	-	Audeval, 2001b

B.6.4 Genotoxicity (Annex IIA 5.4)

B.6.4.1 *In vitro* genotoxicity testing (Annex IIA 5.4.1)

B.6.4.1.1. *In vitro* genotoxicity testing in bacterial cells

- *S.typhimurium* and *E.coli* mammalian microsome mutagenicity test (Marzin, 2000) (Goemar)

Findings both with and without metabolic activation in two independent assays, no biologically significant increase in the number of revertants was noted in four *Salmonella typhimurium* and two *Escherichia coli* strains tested in the presence of Laminarin.

Conclusions: laminarin was not mutagenic in these experimental conditions.

GLP status: study is GLP (no certificate of competent authority).
Guidelines: experimental protocol in compliance with test method B.14, dir. 92/69/EEC.
Material and methods: *S.typhimurium* strains TA 1535, TA 100, TA 1537, TA 98 and *E.coli* strain WP2 and WP2 uvrA were used in the standard plate test and preincubation test with and without S9 mix from Arochlor 1254 induced rat liver.
Laminarin (batch n°. 99521, purity > 90%) powder was solubilized in water and tested at 0, 50, 150, 500, 1500, 5000 µg/plate. No bacteriotoxicity was observed. 2 experiments were performed. The second assay with S9 mix was performed using a pre-incubation method. Positive controls were sodium azide, 9 amino-acridine, 2 nitro-fluorene, mitomycin C, K chromate, 2 anthramine and benzoapyrene. Evaluation criteria well defined.
The study is accepted.

B.6.4.1.3 *In vitro* chromosome aberration assay (Annex IIA 5.4.1)

***In vitro* chromosomal aberration assay in CHO cells:**

- From open literature: biological activity in Macrocystis pyrifera from Argentina: sodium alginate, fucoidan and laminaran. II. Genotoxicity (Larripa et al, 1987)

Findings:

Table B.6.4.1.3-1: *in vitro* CA assay with Laminaran.

Compound	Dose	Abnormal anaphases (nb and % abnormal anaphase-telophases observed)				
		Normal	Lagging	Bridges	Tripolars	%
Physiological saline	-	90	3	6	1	10
Laminaran :	1	92	5	3	0	8
	50	90	3	5	2	10
	100	92	4	4	0	8

Conclusion: Laminaran did not induce chromosome aberrations in vitro in CHO cells.

Material and methods: CHO cells were treated with 1, 50 or 100 µg/ml crude laminaran from *Laminara digitata* (product # L 9634) was purchased from Sigma Chemical Co., St Louis, MO, USA. The cultures were incubated at 37°C for 24 hours. The cells were then fixed in ethanol (96%) during 1 hour at room temperature. 100 anaphase cells per culture were analysed. Statistical analysis was carried out using the Chi-square test.

B.6.4.2 *In vivo* genotoxicity testing (somatic cells) (Annex II A 5.4.2)

B.6.4.2.1 *In vivo* mammalian bone-marrow micronucleus test (Annex IIA 5.4.2)

-Mouse micronucleus test, two oral doses of 500, 1000 or 2000 mg/kg bw/d (Haddouk, 2001)

Findings:
Since no observable toxic effects were noted, the top dose level was 2000 mg/kg bw. For both male and females, the mean values of MPE as well as the PE/NE ratio in the groups treated with the test substance were equivalent to those of the vehicle group. Cyclophosphamide induced a highly significant increase in the frequency of MPE, indicating the sensitivity of the test system under these experimental conditions.

Conclusion: Laminarin does not induce micronuclei in mice bone marrow under the experimental conditions.

Guidelines: experimental protocol in compliance with test method B.12, dir. 96/54/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
Laminarin (batch n°. 99S10, 99 %), was solubilized in water and administered orally two times at 24 h interval to 5 male and 5 female Swiss mice Ico: OF1 (IOPS CAW)/dose at 500, 1000 or 2000 mg/kg bw. Control group received the vehicle (water) only. Cyclophosphamide was used as positive control.
Evaluation criteria: ok.

B.6.4.3 Summary of genotoxicity (Annex IIA 5.4)

Laminarin was tested for its mutagenic potential *in vitro* and negative results were observed in the bacterial point mutation assay. *In vivo*, laminarin did not induce micronuclei in mice bone marrow after oral administration.
Negative results were also observed in a chromosomal aberration test *in vitro* in CHO cells, as reported in the public literature.

Table B.6.4.3 Summary of genotoxicity studies with Laminarin.

Study type	Batch n°. purity	Results	Reference
***In vitro* genotoxicity studies**			
Ames test	Batch n°. 99S21 97.6%	negative	Marzin, 2000
***In vivo* genotoxicity studies**			
Mice MN	Batch n°. 99S10 99%	negative	Haddouk, 2001

B.6.5 Long-term toxicity and carcinogenicity (Annex IIA 5.5)

The nature of laminarin, an algae cell wand polysaccharide, rapidly and extensively fermented by intestinal bacteria, gives rise to fermentation products such as, butyrate, propionate, acetate, CO_2, H_2S etc… Such kinds of products are also formed during digestion of vegetables, fruits and legumes. Long term toxicity resulting from a chronic polysaccharide overload as a result of the use of laminarin in plants can be ruled out. The amount of SCFAs, which could be additionally produced in result of the use of laminarin on plants, is not relevant if compared with the amount of polysaccharides in vegetables and legumes, which are, consumed daily for life. No long-term studies are necessary.

B.6.5.1 Long-term (2 years) oral toxicity in the rat (Annex IIA5.5)

Due to the nature of the active substance (natural polysaccharide), no long term oral rat study was conducted.

B.6.5.2 Carcinogenicity study in the rat (Annex IIA 5.5)

Due to the nature of the active substance (natural polysaccharide), no carcinogenicity rat study was conducted.

B.6.5.3 Carcinogenicity study in the mouse (Annex IIA 5.5)

Due to the nature of the active substance (natural polysaccharide), no carcinogenicity mice study was conducted.

B.6.5.4 Summary of long-term toxicity and carcinogenicity (Annex IIA 5.5)

Due to the nature of the active substance, no specific toxic effects are anticipated from long-term exposure. Therefore, no long-term studies were conducted.

B.6.5.4 Mechanism of action and supporting data (Annex IIA5.5)

No data, not necessary.

B.6.6 Reproductive toxicity (Annex IIA 5.6)

B.6.6.1. Two generation reproductive toxicity in the rat (Annex IIA5.6.1)

No data, not necessary.

B.6.6.2.1 teratogenicity test by the oral route in the rat (Annex IIA 5.6.2)

-Prenatal developmental rat study by gavage, at 1000 mg/kg bw/d on day 6 through day 17 (Audeval, 2001c)

Findings:
Laminarin did not induce maternal toxicity. Reproduction parameters were not altered. No embryotoxicity was observed. No developmental toxic effects were reported (Table B.6.6.2.1-1)

Table B.6.6.2.1-1: developmental toxicity of laminarin in rats.

Endpoints/dose	**control**	**1000 mg/kg bw/d**
Pregnant rats (nb)	23	21
Bw at day 20 (g)	375.3±34.1	373.6±35.6
Food consumption (g)	51.7±12.4	50.5±8.3
Water consumption	50.1±9.4	45.6±9.1
Uterus weight	75.4±14	73±16
Nb corpora lutea	17.4±5.6	16.9±3.6
Litter data		
Implantation sites	13.5±2.6	13.1±2.9
% pre-implantation loss	19±14.9	21.4±17.7
% post implantation loss	6.9±6.2	7.6±8.4
Nb live foetuses	12.6±2.6	12.1±2.8
Nb resorptions		
Early	0.9±0.9	1±1
Late	0.0±0.2	0.0±0.2
Foetal parameters:		
Nb foetuses /nb litter examined	140/23	123/21
Nb male/females	65/75	65/58
Caudal-cranial measurement	36.5±1.4	36.8±1.5
Foetus weight	3.93±0.26	3.94±0.27
Placental weight	0.597±0.07	0.596±0.079
External examination		
haematoma	1 fetus/4 dams 2 foetuses /2dams	1fetus/1dam 2fetuses/1dam
Visceral and skeletal examination		
Nb foetuses /nb litter examined	150/23	131/21
	No effects	No effects

Conclusion: Laminarin is not toxic in the rat developmental study. No maternal and no foetal abnormalities were reported.
NOAEL maternal tox >1000 mg/kg bw/d.
NOAEL develop >1000 mg/kg bw/d.

Guidelines: experimental protocol not fully in compliance with test method B. dir. 87/302/EEC.
Deviation: test substance was administered from day 6 to day 17 instead of day 6 to day 15.
GLP status: study is GLP – certificate of competent authority.
Material and methods: 21 pregnant female SPf Sprague-Dawley rats /dose received by gavage Laminarin (batch n°99S24; 94.9%), as an aqueous solution at 1000 mg/kg bw/d on day 6 through day 17 p.c. control group received water.

B.6.6.2.2 teratogenicity test by the oral route in the rabbit (Annex IIA 5.6.2)

-Prenatal developmental rabbit study by gavage, at 1000 mg/kg bw/d on day 6 through day 19 (Audeval, 2001d)

Findings: One female n° 20001576 had a food intake almost nil from the day of delivery to D14. This was correlated with a body weight loss. After that, food intake of this female was quite similar to other females and bodyweight gain was greater than other females (recovery). At terminal necropsy, this female was pregnant but showed a total litter loss. As anorexia and bodyweight loss started before initiating the dosing, involvement of treatment is considered unlikely.

No *mortality* was recorded. During the treatment period, food consumption of treated females was significantly lower than that of control females at the threshold of 5%. Before and after treatment, animals given laminarin at the dose of 1000 mg/kg had similar food consumption to control animals. *Water consumption* was unaltered.
No significant difference was noted between treated and control animals. Number of implantation sites and of live foetuses of treated females was significantly greater than that of control females. Total litter loss in female n° 20001576 was probably not treatment related as anorexia and correlated bodyweight loss was observed before treatment initiation.
Caudo-cranial measurement, weight of foetuses and weight of placenta were not different from control.
External examination of foetuses: forelimb flexure was reported in one foetus of 3 treated females. This minor variation is commonly reported in rabbit foetuses and involvement of treatment is unlikely.
Some slight inter-group differences in ossification parameters were recorded, but there were no consistent associations or trends indicative of any advancement or retardation of foetal ossification related to maternal treatment.
Incompletely ossified thoracic vertebral centra: the applicant considers that the control values for this parameter in the study were in fact quite low. Normally, they expect to see approximately 10% of control foetuses from approximately 50% of litters with incomplete/anomalous ossification of at least one thoracic vertebral centrum. The value recorded for the limit dose group in the study is much more in line with this background control data.
In the limit dose group there appeared to be slight increase in the proportion of foetuses with supernumerary ribs at the thoraco-lumbar border (rib count 13/13), compared with the concurrent control group. Consequently, there was a decrease in the proportion of foetuses with 12/12 ribs. In association with this finding, a number of foetuses with supernumerary ribs had additional (8^{th}) lumbar vertebra. Comparison of the litter incidences of these findings revealed no increase in the number of affected litters in the limit group, indicating that the increase in affected foetuses occurred with litters already containing foetuses with supernumerary ribs/lumbar vertebrae, rather than there being an increase in the number of affected litters.
When the values recorded for the limit dose group were compared with the control values from a study performed in the same lab, same strain of rabbits in September 2000, the distribution of rib numbers were essentially similar in both groups; whilst the proportion of foetuses with an additional lumbar vertebra was lower in the limit dose group. It was considered, therefore, that the apparent shift in rib and lumbar vertebral numbers seen in the limit dose group was of no toxicological significance. (Table B.6.6.2.2-1)

Table B.6.6.2.2-1: developmental toxicity of laminarin in rabbits.

Endpoints/dose	**control**	**1000 mg/kg bw/d**
Nb pregnant females	16	13
Bw at day 29 (g)	3.762±0.210	3.743±0.432
Food consumption (g)		
D1-D5	556±161	532±206
D6-D19	1883±318	1601±167*
D20-D28	875±106	1017±256
Water consumption D28	252±90	295±117
Uterus weight	464.1±111	507.8±190.4
Nb corpora lutea	11.37±2.73	12.08±2.75
Litter data		
Implantation sites	9.5±2.6	11.5±2.4*
% pre-implantation loss	16.3±15.7	9.1±12.5
% post implantation loss	7.4±10.7	13.9±26.5
Nb live foetuses	8.69±2.36	10±3.85
Nb resorptions		
Early	0.37±0.72	0.46±0.52
Late	0.44±1.03	0.23±0.44
Foetal parameters:		
Caudal-cranial measurement	88.6±3.3	87.8±3.8
Foetus weight	6.75±4.39	6.15±5.25
Placental weight	5.34±0.58	5.19±0.77
External examination		

Forelimb flexure		1 fetus/3 females	
Visceral and skeletal examination			
Incompletely ossified thoracic vertebral centrum : nb litters affected (%litters incidence)	2/16(12.5%)	5/12(41.7%)	
Incompletely ossified thoracic vertebral centrum :actual number foetuses affected	2/139(1.4%)	12/130(9.2%)	
	Control from this study	Laminarin	Control from study 20000153T
Fetuses with supernumerary ribs (rib count 13/13)	42.4%	61.5%	61.1%
Fetuses with 12/12 ribs	40.3%	24.6%	25.8%
Additional lumbar vertebra 8	7.2%	15.4%	22.1%
Unusual morphological changes: nb fetuses affected			
exencephaly	1		
abnormal thoracic vertebrae and ribs	1	2	
abnormal cervical vertebrae		1	
abnormal thoracic vertebrae		2	

Conclusion: Laminarin was well tolerated. The overall picture does not indicate a consistent trend, indicative of either treatment-related retardation or advancement of ossification. No maternal toxicity and and no treatment-related foetal abnormalities were reported.
NOAEL maternal tox >1000 mg/kg bw/d.
NOAEL develop >1000 mg/kg bw/d.

Guidelines: experimental protocol in compliance with test method B. dir. 87/302/EEC.
GLP status: study is GLP – certificate of competent authority.
Material and methods: 13 pregnant albino New-Zealand white (SPF) rabbits /dose received by gavage Laminarin (batch n°99S24; 94.9%), as an aqueous solution at 1000 mg/kg bw/d on day 6 through day 19 p.c. Control group of 16 pregnant rabbits received water.

B.6.6.2.3 Summary of reproductive toxicity and teratogenicity (Annex IIA 5.6)

No reproductive study was performed in rats. A developmental rat and rabbit study showed that laminarin is not toxic for the development. Neither embryotoxicity nor maternal toxicity was observed. The NOAEL in these studies is > 1000 mg/kg bw/d.

B.6.7 Neurotoxicity (Annex IIA 5.7)

Neurotoxicity tests and motor activity measurements were performed at the end of treatment during 28 and 90 day in the rat study and at the end of the 90-day dog study. These studies did not show changes attributable to treatment.

B.6.7.1 Acute neurotoxicity (Annex IIA 5.7)

No data, not necessary.

B.6.7.2 Chronic neurotoxicity (Annex IIA 5.7)

-4 week oral toxicity study in rats (Longobardi, 2000) (Goemar)

Findings:

Neurotoxicity tests were performed including: removal, handling reactivity, lacrimation, palpebral closure, salivation, piloerection, rearing, clonic movements, tonic movements, gait, mobility impairment, arousal, vocalisation, stereotypes, unusual respiration, bizarre behaviour, urination, defecation. No abnormalities were observed.

Conclusion: NOAEL > 1000 mg/kg bw/d

Guidelines: experimental protocol in compliance with method B.7 dir.96/54/ EEC. Limit test.
GLP status: study is GLP – certificate of competent authority.
Material and methods:
5 male and 5 female Sprague-Dawley rats received by gavage Laminarin (batch n°; purity: 97.6%) at a dose of 1000 mg/kg bw/d. Control group received the vehicle alone (0.5% CMC in distilled water).
Neurotoxicity tests were performed including: removal, handling reactivity, lachrymation, palpebral closure, salivation, piloerection, rearing, clonic movements, tonic movements, gait, mobility impairment, arousal, vocalisation, stereotypes, unusual respiration, bizarre behaviour, urination, defecation.
Statistics:
For continuous variables the significance of the differences amongst groups was assessed by analysis of variance. Differences between the treated and the control group were assessed by Dunnett's test using a pooled error variance. The homogeneity of the data was verified by Barlett's test before Dunnett's test. If data were found to be inhomogeneous a Modified t test was applied.
The study is accepted.

B.6.7.3 Summary of neurotoxicity studies (Annex IIA 5.7)

From the different toxicological studies performed in rats and dogs, no symptoms of neurotoxicity were observed. Laminarin is a polysaccharide. No neurotoxic effects are expected for this kind of compound.

B.6.8 Further toxicological studies (Annex IIA 5.8)

B.6.8.1 Toxicity studies on metabolites (Annex IIA 5.8.1)

The batch analysis showed that beside Laminarin, fucans are present, reaching 1-6% of the content. Fucans, which are also cell wand components, are another class of polysaccharides, sulfated polysaccharides, and are considered as impurity co-extracted during extraction of laminarin from the brown alga. Depending on alga species, its stage of development, the type of tissue studied and the harvest season, brown seaweeds may consist of up to 20% fucans on the basis of the dry matter.

Sulphated fucans constitute a heterogeneous group of polysaccharides. Although their fine chemical structure is still under investigation, 3 different types of sulphated fucans were distinguished. The fucoidans are highly branched, highly sulphated polysaccharides mainly composed of fucose polymers: Xylofucoglucuronans (uronic acid skeleton with xylosyl-L-fucose-4-sulphate groups), glucuronofucogalactan β-1,4-galactose skeleton on which L-fucose-3-sulphate or uronic acids occur as branches). They are water-soluble and exhibit very high non-specific cationic exchange capacities.
The colonic fate of sulphated fucans has only been studied *in vitro* using either pure cultures of intestinal bacteria or faecal microorganisms. Sulphated fucans are resistant to fermentation contrasting with the general assumption that all water-soluble polymers are fermentable. It is likely that the primary chemical structure of fucans is responsible for their resistance to fermentation by colonic bacteria, which lack the enzymes needed for its hydrolysis. Fucans are likely to retain their sulphate groups during transit of the colon, thereby manifesting high cationic exchange capacities throughout the digestive tract. This would be of nutritional and physiological significance due to the occurrence of ionic exchange reactions (hypocholesterolemic effects) (Michel and Macfarlane, 1996).

Sulphated polysaccharides exhibit several biological activities including anticoagulant and antiproliferative ones. Data shows that these activities depend on their sulphate content and molecular weight. 20% of sulphate groups is the minimum necessary to obtain a low anticoagulant effect. Biological activity is also improved with increase in their molecular weights (>8000) The heterogeneity and the high molecular weight of these compounds make their action mechanism difficult to understand.
Antithrombin activity was described for fucans extracted from the brown seaweed *Ecklonia kurome* (Nishino et al, 1991) *Pelvetia canaliculate* (Colliec et al, 1991), and from *Ascophyllum nodosum* (Haroun-Bouhedja et al., 1991).
In the dossier, no anticoagulant activity was observed for laminarin.

B.6.9 Medical data and information (Annex IIA 5.9)

B.6.9.1 Medical surveillance on manufacturing plant personnel (Annex IIA 5.9.1)

Due to the low acute and sub-chronic toxicity of Laminarin, no special surveillance on manufacturing plant personnel will be undertaken.

B.6.9.2 Direct observation, e.g.clinical cases and poisoning incidents (Annex IIA 5.9.2)

Laminarin is not produced industrially yet, so no clinical case could have been observed. In the production and handling of the pilot batches, and during the experimental field applications, no incidence occurred.

B.6.9.3 Observations on exposure of the general population and epidemiological studies if appropriate (Annex IIA 5.9.3)

Laminarin being not commercialised yet, no exposure of the general population is possible at the moment.

B.6.9.4 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests (Annex IIA 5.9.4)

In the acute or short-term toxicity studies, no signs of any toxicity have ever been seen and a target organ could not be defined. Therefore, no signs of poisoning can be described.
In case where Laminarin would still be suspected, analytical methods are available for detecting the active substance and its degrades (oligosaccharides, glucose) are available; for this purpose, a method using total hydrolysis would be recommended.

B.6.9.5 Proposed treatment: first aid measures, antidotes, medical treatment (Annex IIA 5.9.5)

In case of accidental ingestion, there is no risk if small quantity is ingested. Wash out mouth with water.
If inhalation, take the patient to get fresh air.
In case of skin contact, take of soiled clothes and wash skin with water.
If eye splashing occurs, wash thoroughly with water. Seek medical advice if irritation develops.
As no sign of poisoning has ever been seen, no antidote can be proposed.

B.6.9.6 Expected effects of poisoning (Annex IIA 5.9.6)

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B.6.10 Summary of mammalian toxicology and proposed ADI, AOEL, ArFD and drinking water limit (Annex IIA 5.10)

B.6.10.1 Summary of mammalian toxicology

Laminarin or phycarin, a linear β D-1, 3-linked glucan was extracted and purified from the brown alga *laminaria digitata*.
Laminarin acts early against cereal diseases inducing natural defence reactions. By inducing systemic resistance on cereals, this allows protection during growth.
Laminarins are cell wall components, which are degraded by the colonic microflora in monogastric animals.
Absorption, distribution, metabolism and excretion:
In human, non-digestible polysaccharides such as laminarin escape enzymatic digestion in the upper gastrointestinal tract. The large bowel fermentation involves bacteria producing laminarases, and B-glucosidases, which fully degrade the substrate into SCFAs, which are then absorbed. Absorption reaches approximately 90%.
Metabolism is important, involving gut microbiota (colonic fermentation) which degrade the polymers giving rise to SCFAs.
Distribution is large: carbohydrate fermentation products are oxidised in brain, heart, kidney, liver, muscle, peripheral tissues.
Excretion occurs via breath and flatus after conversion of SCFAs into H_2S, CO_2, CH_4, and acetate. Unfermented carbohydrates increase faecal bulk likely as a result of increased biomass.

Numerous enzymes able to degrade laminarans have been isolated from bacteria, fungi, algae, molluscs and *higher plants*. B-Glucan endohydrolases from plants are involved in cell wall degradation. They release oligosaccharides from their substrate and are probably of central importance for the initial solubilization of the (1→ 3, 1→4) β-glucans. The soluble products of the initial hydrolysis are then acted on by glucanglucohydrolases, which preferentially attacks the longer gluco-oligomers (cellotriose) releasing glucose. β-Glucan exohydrolases and β-glucosidases may be important additional enzymes for the conversion of released oligosaccharides to glucose. Active glucose absorption occurs in the small intestine.

Ruminants: the principal mechanism of intestinal digestion of dietary polysaccharides in ruminants are degradation by the ruminal microorganisms into fermentative production of SCFAs .

Acute toxicity of laminarin

Laminarin is characterised by a rather low acute toxicity.
According to EU classification, laminarin should not be classified.

Short-term toxicity of laminarin

Laminarin was given by gavage to rats at 1000 mg/kg bw/d for 28 days and 90 days. A slight body weight increase was observed associated to a slight food and water intake reduction. Laminarin, as a polysaccharide might contribute to increase the energy content of the diet, reducing food intake. In rodents, water consumption is closely associated to food consumption. Therefore, these effects are not considered to be adverse.
In dogs, laminarin was given by gavage at 1000 mg/kg bw/d for 90 days. Slight increased incidence of soft faeces was reported which can be explained by the increased gastrointestinal motility produced by short chain fatty acids (resulting from caecal fermentation), as well as an increase in biomass which increases the faecal output. Therefore, the observed effects are not considered to be adverse.

Genotoxicity of laminarin

Laminarin was tested for its mutagenic potential *in vitro* and negative results were observed in the bacterial point mutation assay. *In vivo*, laminarin did not induce micronuclei in mice bone marrow after oral administration.
Negative results were also observed in a chromosomal aberration test *in vitro* in CHO cells, as reported in the public literature.

Long-term toxicity studies:

The nature of laminarin, an algae cell wand polysaccharide, rapidly and extensively fermented by intestinal bacteria, gives rise to fermentation products such as, butyrate, propionate, acetate, CO_2, H_2S etc… Such kind of products is also formed during digestion of vegetables, fruits and legumes. Long -term toxicity resulting from a chronic polysaccharide overload as a result of the use of laminarin in plants can be ruled out. The amount of SCFAs, which could be additionally produced in result of the use of laminarin on plants, is not relevant if compared with the amount of polysaccharides in vegetables and legumes, which are, consumed daily for life. No long-term studies are necessary.

Reproductive toxicity and teratogenicity

No reproductive study was performed in rats. A developmental rat and rabbit study showed that laminarin is not toxic for the development. Neither embrytoxicity nor maternal toxicity was observed. The NOAEL in these studies is > 1000 mg/kg bw/d.

Neurotoxicity

Neurotoxicity tests and motor activity measurements were performed at the end of treatment during 28 and 90 day in the rat study and at the end of the 90-day dog study. From the different toxicological studies performed in rats and dogs, no symptoms of neurotoxicity were observed. Laminarin is a polysaccharide. No neurotoxic effects are expected for this kind of compound.

B.6.10.2 Acceptable daily intake (ADI)

Laminarin is a polysaccharide, which is devoid of acute toxicity. No specific effects / target organs were identified from the short-term toxicity studies performed in rat and dog. No developmental toxicity was observed in rats and rabbits. Laminarin is not genotoxic. As laminarin is degraded into glucose by plants, no residue will occur in plants. Since there is no risk for consumers from the use of laminarin as plant protection product, no ADI has been allocated by the rapporteur.

Note: the applicant proposes to use the value of 1000 mg/kg bw/d for setting an ADI. Applying an assessment factor of 100: ADI = 10mg/kg bw/d.

B.6.10.3 Acute reference dose

Not allocated, not necessary.

B.6.10.4 Acceptable operator exposure level (AOEL)

Laminarin is a polysaccharide and is devoid of acute toxicity. No specific effects/target organs were reported from the short-term toxicity studies performed in rat and dog. No developmental toxicity was observed in rats and rabbits. Laminarin is not genotoxic. A significant percutaneous absorption is excluded. The preparation is a solid, non-dusty, non-volatile granule. An inhalation risk is not expected. The allocation of an AOEL is considered not necessary.

Note: the applicant proposes to use the value of 1000 mg/kg bw/d for setting an AOEL. Applying an assessment factor of 100:

AOEL = 10mg/kg bw/d.

B.6.10.5 Drinking water limit

The maximum admissible concentration of an active substance is 0.1 µg/L, as established by the directive 89/778/EEC.

B.6.11 Acute toxicity, including irritancy and skin sensitisation of the preparation PHYLIQ SL (Annex IIIA 7.1)

B.6.11.1 Acute oral toxicity (Annex IIIA 7.1.1)

PHYLIQ: rat, gavage, 2000 mg/kg bw (Audeval, 2000a).

Findings:
Mortality: no mortality was reported.
Body weight gain: expected gain.
Clinical signs: no abnormalities were observed.
Necropsy: no abnormalities were noted.

Conclusion: LD^{50} PHYLIQ >2000 mg/kg bw

GLP status: study is GLP – certificate of competent authority.
Guidelines: experimental protocol in compliance with method B.1 dir.96/54/EEC.
Material and methods:
5 male and /or female Sprague Dawley rats (SPF) received by gavage a single dose of PHYLIQ (batch n°. 9912812) in aqua bidist, at 2000 mg/kg bw.

B.6.11.2. Acute percutaneous toxicity (Annex IIIA 7.1)

PHYLIQ: rat, 2000 mg/kg bw, semi-occluded (Audeval, 2000b)

Findings:
Mortality: no mortality was observed.
Body weight gain: were normal
Local effects: no dermal reactions were observed during the course of the study.
Necropsy: no pathological findings were noted.

Conclusion: LD_{50}dermal PHYLIQ > 2000 mg/kg bw

GLP status: study is GLP – certificate of competent authority.
Guidelines: experimental protocol in compliance with method B.3 dir.96/54/EEC.
Material and methods:
5 male and 5 female Sprague Dawley rats (SPF) received a single application to the clipped epidermis of undiluted PHYLIQ (batch n°. 9912812) at 2000 mg/kg bw for 24 h. followed by covering with a semi-occlusive dressing.

B.6.11.3 Acute inhalation toxicity (Annex IIIA 7.1)

No data, not necessary.

B.6.11.4 Skin irritation (Annex IIIA 7.1)

PHYLIQ: rabbit, 0.5ml, and semi-occlusive (Audeval, 2000c)

Findings:
Score erythema, 24+48+72 h = 0
Score oedema 24+48+72 h = 0

Conclusion: PHYLIQ is not a skin irritant.

GLP status: study is GLP – certificate of competent authority.
Guidelines: experimental protocol in compliance with method B.4 dir.96/54/EEC.
Material and methods:
3 female Rabbit white New Zealand were exposed to 0.5 g solid PHYLIQ (batch n°. 9912812) after clipping of the fur via a test patch of 6 cm^2 for 4 hour under semi-occlusive dressing.

B.6.11.5 Eye irritation (Annex IIIA 7.1)

PHYLIQ: 6 rabbits, 0.1 ml undiluted substance (Audeval, 2000d)

Findings:
Cornea opacity 24+48+72 h=0
Iris 24+48+72 h =0
Redness 24+48+72 h = 0
Chemosis 24+48+72 h = 0

Conclusion: PHYLIQ is not irritating for the eyes.

GLP status: study is GLP – certificate of competent authority.
Guidelines: experimental protocol in compliance with method B.5 dir.96/54/EEC.
Material and methods:
3 male rabbits white New Zealand were exposed to 0.1 ml PHYLIQ (batch n°. 9912812) in the eye.

B.6.11.6 Skin sensitisation (Annex IIIA 7.1)

PHYLIQ, Magnusson and Kligman test (Audeval, 2000e)

Findings: animals were monitored for clinical signs and body weight changes. No effects were observed. No coloration or skin lesion or allergenicity was observed.

Conclusion: PHYLIQ does not have a sensitising effect on the skin under the test conditions.

GLP status: study is GLP – certificate of competent authority.
Guidelines: experimental protocol in compliance with method B.6 dir.96/54/EEC: modified Buehler test
Material and methods:
Male and female guinea pig Hartley albino, were exposed to PHYLIQ (batch n°. 9912812) and 10 control animals. The primary induction, sensitisation and challenge were performed with 100% test substance preparation. A preliminary study was performed.

B.6.11.7 Summary of acute toxicity including irritancy and skin sensitisation of preparation Phyliq (Annex IIIA 7.1)

Table B.6.14-1: Summary of toxicity of Phyliq:

Study type	Results LD_{50}	classification
Acute oral rat	>2000 mg/kg bw	-
Acute dermal rat	>2000 mg/kg bw	-
Acute inhalation, aerosol	-	-
Skin irritation rabbit	Not irritant	-
Eye irritation rabbit	Not irritant	-
Skin sensitisation (M&K)	Non-sensitiser	-

B.6.12 Dermal absorption (Annex IIIA 7.3)

Due to the high molecular weight of the active substance (5000 $g.mol^{-1}$), to its hydrophily and to the absence of solvents in the preparation Phyliq, the dermal absorption of laminarin is probably quite low.
No studies are included in the dossier. A default value of 10% will be used for calculation of operator exposure.

B.6.13 Toxicological data on non active substances (Annex IIIA 7.4)

Besides its active ingredient laminarin, Phyliq SL contains a moisturiser frost proof, a stabiliser, a surfactant, a thickener and 3 preservatives. The toxicological properties of the pure substances are given in table B.6..13-1. The toxicological properties of all coformulants (diluted in the final product) are covered by the toxicological studies submitted for the preparation Phyliq LS.

Table B.6.13-1. Toxicological data relating to the formulants.

Coformulants	**Acute toxicity**	**Skin irritation**	**Eye irritation**	**Other effects**
Moisturiser frost proof	LD_{50} oral, rat = 12600 mg/kg bw			
Stabiliser	LD_{50} oral, rat = 2000 mg/kg bw	Non-irritant (rabbit)	Non-irritant (rabbit)	4 week oral rat NOAEL > 1000 mg/kg bw/d; not genotoxic
Surfactant			Xi, R41	
Thickener	LD_{50} oral, rat > 5000 mg/kg bw			
Acidifier preservative	LD_{50} oral, rat > 3500 mg/kg bw LD_{50} dermal , rat > 7940 mg/kg bw	Severe irritant C, R34	Severe irritant	
Preservative	LD_{50} oral, rat = 4200 mg/kg bw	irritant	Xi, R36	
Preservative	LD_{50} oral, mice = 2000 mg/kg bw Xn, R22	irritant	Irritant, R41	Repeated dust contact may cause sensitisation to the skin

B.6.14 Exposure data (Annex IIIA 7.2)

B.6.. 4.1 Estimation of operator exposure (Annex IIIA 7.2)

According to the notifier, Phyliq SL (37 g/L) is intended to be used on cereals (wheat and barley), 1 litre per hectare. Phyliq must be sprayed one time between full tailoring stage and 1 cm ear stage.
Phyliq is sprayed after dilution in 50L to 500 L water, and applied using a vehicle mounted field crop sprayer with hydraulic boom and nozzles. On certain occasions it might also be applied at 50 L/ha by aerial application.
Operator exposure estimates were calculated according to Uniform Principles for Safeguarding the Health of Applicators of PPP, German model as well as according to the UK POEM model
The following assumptions have been made in calculating operator exposure:
Area treated: 20 ha/day for field crops/tractor mounted (BBA model)
Area treated: 50 ha/day for field crops/tractor mounted (UK POEM model)
Application rat: 37-g a.s. /ha
The applicant proposes to use the value of 1000 mg/kg bw/d for setting an AOEL. Applying an assessment factor of 100: AOEL = 10mg/kg bw/d. this value will be used for the estimation of operator exposure, as no AOEL is proposed by the RMS.

German model:

Use information			
Product	Phyliq SL	Active substance	laminarin
Formulation type	Liquid	a.s. concentration	37 mg/ml
Method of use	Tractor field crops	Dose(product)	1 litre product/ha
Work rate	20 ha/day	Dose (a.s.)	0.037 kg a.s./ha
		Amount handled	0.74 kg a.s./day
Exposures-mix/loading			
	Specific exposures	Estimated exposures	
Inhalation	0.0006 mg/kg a.s.handled	0.000444 mg a.s./day	
Dermal-hands	2.4 mg/kg a.s.handled	1.776 mg a.s./day	
Exposure -application			
	Specific exposures	Estimated exposures	
Inhalation	0.001 mg/kg a.s.handled	0.00074 mg a.s./day	
Dermal-head	0.06 mg/kg a.s.handled	0.0444 mg a.s./day	
Dermal-hands	0.38 mg/kg a.s.handled	0.2812 mg a.s./day	
Dermal-body	1.6 mg/kg a.s.handled	1.184 mg a.s./day	
Total exposures		Estimated exposures	Percent absorbed
Total potential inhalation		0.001184 mg a.s./day	100%
Total dermal-mix		1.776 mg a.s./day	10%
Total dermal-application		1.5096 mg a.s./day	10%
Total absorbed dose		0.3297 mg a.s./day	
Body weight		70 kg	
Mg/kg bw/day		**0.0047106 mg/kg bw/d**	**0.047% of AOEL**

UK POEM: tractor mounted hydraulic boom and nozzles model

Product data			
Product	Phyliq SL		
Active substance	Laminarin		
Concentration	37 mg/ml		
Formulation type	SC		
Maximum in use a.s.concentration	0.074 mg/ml		
Exposure during mixing and loading			
Container size	5 L		
Hand contamination/operation	0.01 ml		
Application dose	1 L product/ha		
Work rate	50 ha/day		
Number of operations	10 day		
Hand contamination	0.1 ml/day		
Protective clothing	None		
Transmission to skin	100%		
Dermal exposure to formulation	0.1 ml/day		
Exposure during spray application			
Application technique-tractor, hydraulic boom and nozzles			
Application volume	500 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	trunk	Leggs
	65	10	25%
Clothing	none	Permeable	Permeable
	100	5	15%
Dermal exposure	B.B.6.5	0.05	0.375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41.55 ml/day		
Absorbed dose	Mix/load	Application	
Dermal exposure	0.1 ml/day	41.55 ml/day	
Concentration of a.s.	37 mg/ml	0.074 mg/ml	
Dermal exposure to a.s.	3.7 mg/day	3.07 mg/day	
Percent absorbed	10%	10%	
Absorbed dose	0.37 mg/day	0.307 mg/day	
Inhalation exposure during spraying			
Inhalation exposure	0.01 ml/h		
Duration of exposure	6 h		
Concentration of a.s.	0.074 mg/ml		
Inhalation exposure to a.s.	0.00444 mg/day		
Percent absorbed	100%		
Absorbed dose	0.00444 mg/day		
Predicted exposure			
Total absorbed dose	0.681 mg/day		
Operator body weight	60 kg		
Operator exposure	**0.01136 mg/kg bw/d**	**0.113% of AOEL**	

Conclusion: the results of the calculation according to both models show that without PPE, exposure is ≤ 0.1% of AOEL.

B.6.14.2 Estimation of bystander exposure (Annex IIIA 7.2)

Bystanders may be exposed persons that accidentally walk trough a treated crop or stand or live in the proximity of an area being treated. If good plant protection practices are adopted during application of the pesticides,

accidental exposure is not anticipated and must not take place. If exposure takes place accidentally, the anticipated exposure pattern would be of an acute type.
According to the acute toxicity studies performed with the formulation, no acute risk is anticipated.
Bystanders may also be residential bystanders, that is persons who permanently live close to crops being treated. These persons may be exposed through drift via inhalation and/or dermal absorption.
It is assumed that ordinary clothing is worn; the total uncovered area amounts to 0.4225 m^2.
The expected air concentration as a function of the distance from the spray (high tractor mounted as a worst case) are as follows (Ganzelmeier et al.1995):
14.1% at 7.5 m
10.6% at 10 m
6.2 % at 15 m
4.2% at 20 m
2.0% at 30 m.

Calculation of dermal exposure of bystander:

D= 100% deposition x drift deposition x exposed area

Calculation of 100% deposition: 0.037-kg a.s. /ha = 3.7 mg/m^2
Drift depot at 7.5 m = 14.1%
Total uncovered area amounts to 0.4225 m^2

D= 3.7 x 0.141 x 0.4225 = 0.220 mg a.s. /person/day

Assuming a dermal absorption of 10%, the absorbed dose = 0.022 mg/person/day, which represents 0.0036% of AOEL

The inhalation exposure of bystander is calculated as for the operator (German model):

$I = I^*_{A\ (tractor\ mounted)}$ x WR x AR

WR = work rate (field crop): 20 ha/day
AR = application rate: 0.037 kg/ha

I = 0.018 x 20 x 0.037 = 0.0133 mg a.s. /person/day
But adapted to 1 hour instead of 6 hours for an operator:

I = 0.0022 mg a.s. /person/hour

Representing 0.00036% of AOEL.

Conclusion:
Under practical conditions of use, the potential exposure of a bystander, even standing for a 1 hour period nearby sprayer, represents 0.0036% of the AOEL. The risk resulting from this exposure is negligible.

B.6.14.3 Estimation of worker exposure (Annex IIIA 7.2)

Phyliq SL is applied to cereals once between full tillering stage and 1 cm ear stage, which do not require manual operations. Operations involving humans in such crops are essentially performed mechanically (e.g. spraying and harvest). This provides only limited opportunity for workers to enter treated areas and be exposed to this non-volatile, low toxicity product.
Because of these considerations, workers exposure was not estimated.

B.6. 15 reference relied on:

Table B.6.15-1 : Toxicology of the active substance

Author(s)	*Annex* **Point / Reference number**	**Year**	**Title** **Testing facility,** **Report n°,** **GLP or GEP** **Status published or not**	**Data Protection Claimed** **Y/N**	Owner
Anonymous (CSHP)	IIA, 5.5/01	1998	Avis du Conseil Supérieur d'Hygiène Publique de France sur l'emploi en alimentation humaine d'algues du genre Laminaria. B.I.D. N°2 Non-GLP, Published	N	-
AUDEVAL GERARD C.	IIA, 5.2/07	2001d	H11 (Batch 99S24) – Acute dermal toxicity study in the rat CERB - Study N° 20000698 ST GLP, unpublished	Y	GOË MAR
AUDEVAL GERARD C.	IIA, 5.3/02	2001a	H11 - 90-Day repeated dose oral toxicity study in the rat. CERB - Study N° 20000389 T GLP, unpublished	Y	GOË MAR
AUDEVAL GERARD C.	IIA, 5.3/03	2001b	H11 - 90-Day repeated dose oral toxicity study in the dog. CERB - Study N° 20000390 T GLP, unpublished	Y	GOË MAR
AUDEVAL GERARD C.	IIA, 5.6/01	2001c	H11 - Study for the effects on embryo-fœtal development in the rat by the oral route CERB - Study N° 20000387T GLP, unpublished	Y	GOË MAR
AUDEVAL GERARD C.	IIA, 5.6/02	2001e	H11 - Study for the effects on embryo-fœtal development in the rabbit by the oral route CERB - Study N° 20000388T GLP, unpublished	Y	GOË MAR
BAUDET L.	IIA, 5.2/04	1998a	Phycarine® 96S51- Cutaneous primary irritation in the rabbit CERB - Study N° 970349 ST GLP, unpublished	Y	GOË MAR
BAUDET L.	IIA, 5.2/05	1998b	Phycarine® 96S51- Ocular primary irritation in the rabbit CERB - Study N° 970350 ST GLP, unpublished	Y	GOË MAR
BAUDET L.	IIA, 5.2/06	1998c	Phycarine® 96S51- Study of cutaneous sensitisation using the Magnusson and Kligman maximisation test in the guinea pig CERB - Study N° 970351 ST GLP, unpublished	Y	GOË MAR
BLACK W.A.P., DEWAR E.T.	IIA, 5.1/04	1954	Laminaran J.Sci. Food Agri., 5, 137-145	N	-

			Non-GLP, published		
DALMO R.A., INGEBRIGSTEN K., BOGWALD J., HORSBERG T.E., SELJELID R.	IIA, 5.1/02	1995	Accumulation of immunomodulatory laminaran [β1-3-D-glucan] in the spleen and kidney of Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases, 18, 545-553 Non-GLP, published	N	-
DALMO R.A., INGEBRIGSTEN K., SVEINBJORNSSON B., SELJELID R.	IIA, 5.1/03	1996	Accumulation of immunomodulatory laminaran [β1-3-D-glucan] in the heart,spleen and kidney of Atlantic cod, *Gadus morhua* L. Journal of Fish Diseases, 19, 129-136 Non-GLP, published	N	-
DELILLE M.	IIA, 5.2/01	1998a	Phycarine® 96S51- Acute toxicity study- Safety test in the rat by the oral route CERB - Study N° 970352 ST GLP, unpublished	Y	GOË MAR
DELILLE M.	IIA, 5.2/02	1998b	Phycarine® 96S51- Acute toxicity study- Safety test in the rat by the subcutaneous route CERB - Study N° 970353 ST GLP, unpublished	Y	GOË MAR
ERASMUS J.H., COOK P.A., COYNE V.E.	IIA, 5.1/07	1997	The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae.* Aquaculture, 155, 377-386 Non-GLP, published	N	-
FUJII T., KUDA T., SAHEKI K., OKUZUMI M.	IIA, 5.1/12	1992	Fermentation of water-soluble polysaccharides of brown algae by human intestinal bacteria *in vitro*. Nippon Suisan Gakkaishi 58(1), 147-152 Non-GLP. Published	N	-
HADDOUK H.	IIA, 5.4/03	2001	Laminarin: Bone Marrow Micronucleus Test by Oral Route in Mice CIT - Study N° 21149 MAS GLP, unpublished	Y	GOË MAR
IBARROLA I., NAVARRO E., IGLESIAS J.I.P., URRUTIA M.B.	IIA, 5.1/05	1999	Time-course of digestive-enzyme acclimation in the cockle *Cerastoderma edule*. Marine Biology, 135, 47-56 Non-GLP, published	N	-
INGEBRIGSTEN K., HORSBERG T.E., DALMO R., SELJELID R.	IIA, 5.1/01	1993	Tissue distribution of the immunomodulator aminated β1-3 polyglucose in Atlantic salmon (*Salmo salar*) after intravenous, intraperitoneal and peroral administration. Aquaculture, 117, 29-35 Non-GLP, published	N	-
KUDA T., FUJII T., HASEGAWA A., OKUZUMI M.	IIA, 5.1/09	1992	Effect of degraded products of laminaran by *Clostridium ramosum* on the growth of intestinal bacteria. Nippon Suisan Gakkaishi, 58 (7), 1307-1311 Non-GLP, published	N	-
KUDA T.,	IIA,	1992	Effects of brown algae on faecal flora of rats.	N	-

FUJII T., SAHEKI K., HASEGAWA A., OKUZUMI M.	5.1/10		Nippon Suisan Gakkaishi 58(2), 307-314 Non-GLP. Published		
LARRIPA I.B., MUDRY DE PARGAMENT M., LABAL DE VINUESA M., MAYER A.M.S.	IIA, 5.4/02	1987	Biological activity in *Macrocystis pyrifera* from Argentina : sodium alginate, fucoïdan and laminaran. II. Genotoxicity. Hydrobiologia 151/152, 491-496 Non-GLP, Published	N	-
LONGOBARDI C.	IIA, 5.3/01	2000	4-week oral toxicity study in rats RTC - Study N° 7286 GLP, unpublished	Y	GOË MAR
MARZIN D.	IIA, 5.4/01	2000	Mutagenicity test on bacteria (*Salmonella typhimurium* his and *Escherichia coli* trp) using B.N. Ames's technique with H11 Institut Pasteur de Lille Study N° IPL–R 991011/H11 GLP, unpublished	Y	GOË MAR
MICHEL C., BENARD C., LAHAYE M., FORMAGLIO D., KAEFFER B., QUEMENER B., BEROT S., YVIN J.C., BLOTTIERE H.M., CHERBUT C.	IIA, 5.1/10	1999	Les oligosides algaux comme aliments fonctionnels : étude *in vitro* de leurs effets cellulaires et fermentaires. Sciences des Aliments, 19, 311-332 Non-GLP, published	N	-
MÜLLER W.	IIA, 5.2/03	1999	Evaluation of acute inhalation toxicity with Phycarine® in rats CERB - Study N° 980001 EX GLP, unpublished	Y	GOË MAR
SABOROWSKI R., BUCHHOLZ F.	IIA, 5.1/06	1999	A laboratory study on digestive processes in the Antarctic krill, *Euphausia superba*, with special regard to chitinolytic enzymes. Polar Biol., 21, 295-304 Non-GLP, published	N	-
SELJELID R.	IIA, 5.5/02	1986	A water-soluble aminated β1-3D-Glucan derivative causes regression of solid tumors in mice. Bioscience Reports, Vol 6, N°9, 845-851 Non-GLP, Published	N	-
STURMBAUER C.	IIA, 5.1/08	1991	Different enzymes for laminarine digestion in *Chondrostoma nasus* (Cyprinidae) and *Oreochromis* sp. (Cichlidae). Comp. Biochem. Physiol ,Vol.100A, N°1, 199-202 Non-GLP, published	N	-

Table B.6.15-2 : Toxicology of the formulation

Author(s)	*Annex* **Point /Reference Number**	**Year**	**Title** **Testing facility,** **Report No.,** **GLP or GEP** **Status published or not**	**Data Protection Claimed** **Y/N**	***Owner***
AUDEVAL-GERARD C.	IIIA, 7.1/01	2000a	Phyliq - Acute oral toxicity study in the rat. CERB Report No. 990762 ST GLP, unpublished	Y	GOË MAR
AUDEVAL-GERARD C.	IIIA, 7.1/02	2000b	Phyliq - Acute dermal toxicity in the rat. CERB Report No. 990763 ST GLP, unpublished	Y	GOË MAR
AUDEVAL-GERARD C.	IIIA, 7.1/03	2000c	Phyliq - Acute skin irritation study in the rabbit. CERB Report No. 990759 ST GLP, unpublished	Y	GOË MAR
AUDEVAL-GERARD C.	IIIA, 7.1/04	2000d	Phyliq - Acute eye irritation study in the rabbit. CERB Report No. 990760 ST GLP, unpublished	Y	GOË MAR
AUDEVAL-GERARD C.	IIIA, 7.1/05	2000e	Phyliq - Skin sensitisation study in the guinea pig (Magnusson-Kligman maximisation) CERB Report No. 990761 ST GLP, unpublished	Y	GOË MAR

Table B.6.15-3 : Open literature

Author(s)	**Annex point/ reference number**	**year**	**Title** **Source** **Company, report no** **GLP or GEP** **Published or not**	**Data protection claimed**	**Owner**
Black and Dewar	IIA,5.1/01 and IIA,5.1/02	1954	Laminaran. J. Sci. Food Agric., 5, 137-145 Not GLP, published	N	-
Colliec, S., Fischer, A.M., Tapon-Bretaudière, J., Boisson, C., Duran D, P and Jozefonvicz, J.	IIA,5.8.1/01	1991	Anticoagulant properties of fucoidan fraction Tromb. Res,64 (2):143-154 Not GLP, published	N	-

Cook S.I. and Sellin, J.H.	IIA,5.1/01	1998	Review article: short-chain fatty acids in health and disease. Aliment Pharmacol Ther. , 12(6); 499-507. Not GLP, published	N	-
Cummings, J.H., Macfarlane, G.T., and Englyst, H.N.	IIA,5.1/01	2001	Prebiotic digestion and fermentation Am.J. Clin. Nutr. 73 (suppl): 415S-420S Not GLP, published	N	-
Dalmo R.A., Ingebrigsten, K., Sveinbjornsson, B., and Seljelid, R.	IIA,5.1/04	1995	Accumulation of immunomodulatory laminaran [β(1,3)-D-glucan] in the spleen and kidney of atlantic salmon *Salmo salar* L. J.Fish Dis., 18, 545-553 Not GLP, published	N	-
D'Argenio, G., and Mazzacca, G.	IIA,5.1/01 and IIA,5.1/03	1999	Short-chain fatty acids in the human colon. Reaction to inflammatory bowel disease and colon cancer. Adv. Exp. Med. Biol, 472, 149-158. Not GLP, published	N	-
Djouzi et al.,	IIA,5.1/01	1995	Degradation and fermentation of gluco-oligosaccharides by bacterial strains from human colon: *in vitro* and *in vivo* studies in gnotobiotic rats; J.Applied Bacteriol, 79, 117-127 Not GLP, published	N	-
Greger, J.L.	IIA,5.3.2/02	1999	Nondigestible carbohydrates and mineral bioavailability. J.Nutr., 129, 1434S-1435S Not GLP, published	N	-
Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., Boisson-Vidal, C.	IIA,5.8.1/01	2000	Relationship between sulfate groups and biological activities of fucans. Tromb. Res.100, 453-459 Not GLP, published	N	-
Hrmova, M., Banik, M., Harvey A.J., Garrett, T.P., Varghese, J.N., Hoj, P.B., Osmond, G.	IIA,5.1/02	1997	Polysaccharide hydrolases in germinated barley and their role in the depolymerization of plant and fungal cell walls. Int. J; Biol. Macromol, 21, 67-72 Not GLP, published	N	-
Kuda ,T., Fujii, T., Hasegawa, A. and Okuzumi, M.	IIA,5.1/01	1992	Effect of degraded products of laminaran by *Clostridium ramosum* on the growth of intestinal bacteria. Nippon Suisan Gakkaishi, 58,1307-1311 Not GLP, published	N	-
Larripa et al	IIA,5.4.1/03	1987	Biological activity in *Macrocystis oyrifera* from Argentina: sodium alginate, fucoidan and laminaran. II. Genotoxicity. Hydrobiologia 151/152: 491-49B.B.6. Not GLP, published	N	-
Michel, C., and Macfarlane, G.T.	IIA,5.1/01 and IIA,5.3/02 and IIA,5.8.1/01	1996	A review: digestive fate of soluble polysaccharides from marine macroalgae: involvement of the colonic microflora and physiological consequences for the host. J.Applied Bacteriol.,80, 349-369. Not GLP, published	N	-

Nishino, T., Aizu, Y., Nagumo, T.	IIA,5.8.1/01	1991	The influence of sulfate and molecular weight of a fucan sulfate from the brown seaweed *Ecklonia kurome* on its antithrombine activity. Tromb. Res,64 (6):723-731 Not GLP, published	N	-
Scheppach, W.	IIA,5.1/01	1994	Effects of short chain fatty acids on gut morphology and function. Gut, 35(1): S35-S38. Not GLP, published	N	-
Sturmbauer, Ch.	IIA,5.1/02	1991	Different enzymes for Laminarin digestion in *Chondrostoma nasus* (*Cyprinidae*) and *Oreochromis* SP (*Cichlidae*) Comp.Biochem.Physiol., 100A, 199-202. Not GLP, published	N	-
Szepesi, B.	IIA,5.1/01	1996	Present knowledge in nutrition. 7th edition, ILSI Press, Washington, DC. In Ziegler, E., Filer, L.J. Eds. Chapter 5, pp 33-43 : Carbohydrates Not GLP, published	N	-
Tang, Z.L., and Shen, S.F.	IIA,5.3.2/03	1989	A study of *Laminaria digitata* powder on experimental hyperlipoproteinemia and its hemorheology Zhong Xi Yi Jie He Za Zhi, 9 (4) , 223-225 Not GLP, published	N	-