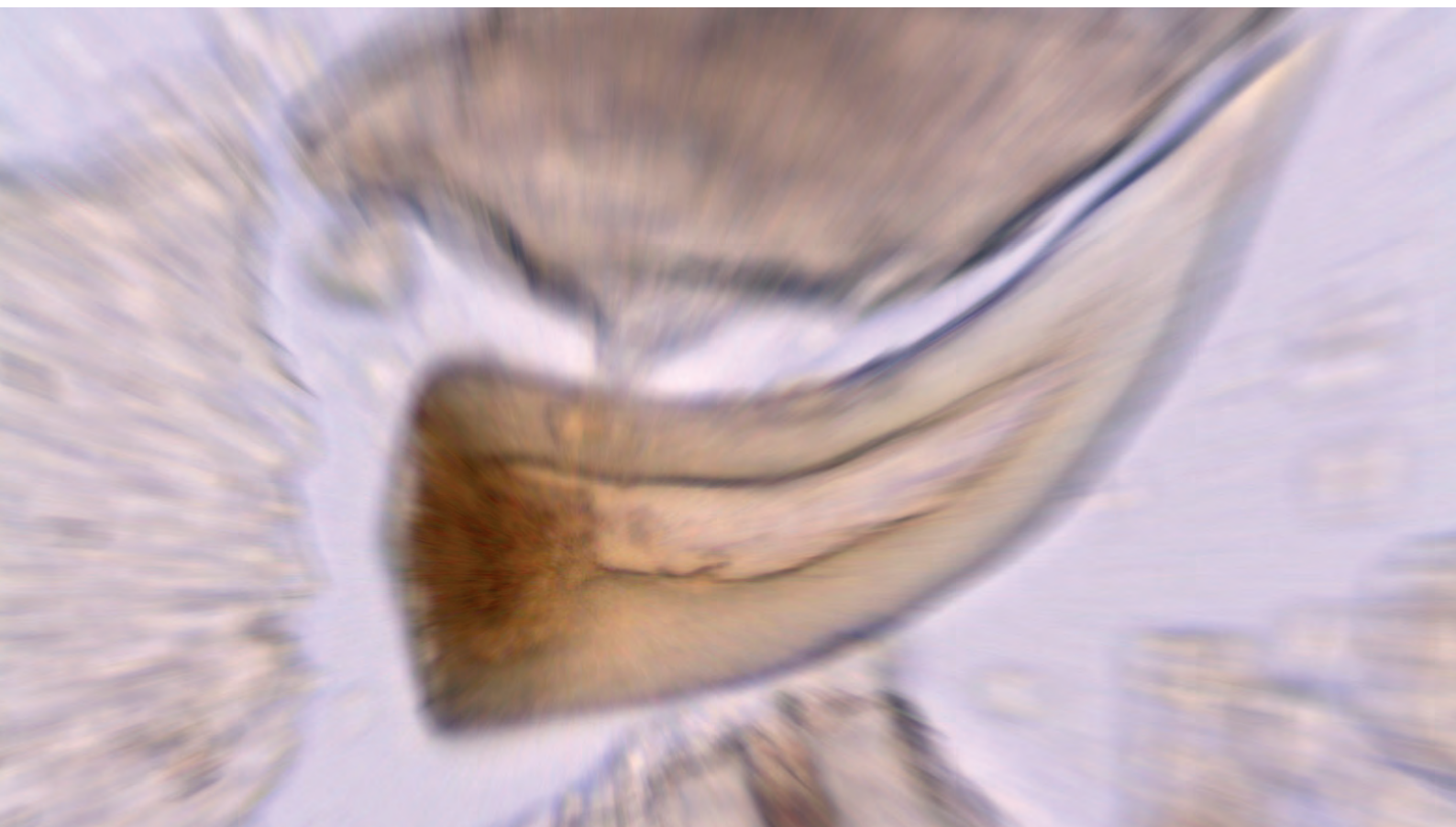




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Animal proteins

Annual Report 2011 of the Dutch National Reference Laboratory

RIKILT Report 2012.013

L.W.D. van Raamsdonk, I.M.J. Scholtens, J.J.M. Vliege and V.G.Z. Pinckaers



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Summary

RIKILT serves as the only official control laboratory for animal proteins in feeds in the Netherlands in the framework of Directive 882/2004/EC.

There has been a long-time desire from society as well as from legislators to lift the extended feed ban, as a whole or partly. Currently two scenarios for lifting parts of the ban are under discussion. A first scenario includes pork material allowed as ingredient in poultry feed, poultry material as ingredient in pig feed, feed, and both sources as ingredient in aquafeed. A second scenario involves only the use of both pork and poultry material in aquafeed. RIKILT developed monitoring strategies for both scenarios. In both cases it is vital to develop tests for identification, that suit the legal needs, and can be applied cost effectively. Good definitions of the targets are necessary for developing suited tests. This is primarily necessary for poultry.

In the framework of the future plans for relaxation of the ban, RIKILT participated, in close cooperation with EURL, in a range of different experiments for developing suitable monitoring methods and in trials for testing the proficiency of these methods. In general, very good results were achieved and the collected data appeared to be useful for method improvement. Only in two occasions one false positive result (misidentification of cereal hairs for fish teeth) and false negative result (unexpected effects of an embedding agent) were reported. Based on the RIKILT results for microscopy more attention was given to the discrimination between certain fish particles and plant material. The PCR study revealed that for several targets, e.g. poultry and ruminant, tests have to be improved. This situation shows that the study revealed valuable information as zero measurement study. Actions plans are already started by EURL and RIKILT.

In July 2010 a rapid alert was issued by Spain on the presence of blood plasma in an artificial milk feed intended as calve feed. The blood plasma was detected by a staining method with Tetramethyl benzidine (TMB). The results of the experiments, which were finalised early 2011, indicated that the TMB colouring method is currently not applicable for the detection of blood material in a matrix of milk powder, because artificial milk feeds show a slight natural colouring response. The presence of blood plasma in the suspected research sample was not confirmed reliably with any of the applied research methods.

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1 Introduction

In 2006 the European Union appointed a series of European Union Reference Laboratories, one of them dedicated to the field of Detection of animal proteins in feeds. Each member state has appointed a National Reference Laboratory (NRL) in this field. The stakeholders, i.e. the Ministry for Economic Affairs, Agriculture and Innovation as representative of the member state, and the competent authority, need technical and scientific support for their tasks. RIKILT, as appointed NRL in this field, is providing this support by means of technical and strategic advice, method development and participation in international networks of experts.

In order to check the quality and performance of microscopic detection of animal proteins, the EURL annually organises a proficiency test. Also, an interlaboratory study for DNA detection of a series of targets is organised. Further National activities include the support of the competent authorities, participation in the national monitoring program and specific studies in case technical (interpretation) problems occur.

The Dutch NRL gives account of its activities in the framework of collaboration with the EURL and support of the national authorities in this report.

2 Description of work

The tasks of the NRL are laid down in Directive 882/2004/EC. RIKILT serves as the official control laboratory for animal proteins in feeds in the Netherlands. Several of the tasks listed in the Directive do not require activities due to the single laboratory situation. Remaining tasks are:

- Collaboration with the EU-RL, including participation in meetings and workshops, participation in ring trials;
- Communication of information from the EU-RL to the stakeholders;
- Providing technical and scientific support to the stakeholders;
- Performing other specific tasks; RIKILT acts as member of the scientific advisory board of the EURL;
- Support of the national network of official control laboratories. The Netherlands does not maintain a network of official laboratories for detection of animal proteins, although national legislation provides a list of five laboratories that can be involved in monitoring animal feeds in general. RIKILT as NRL identified the desire to support these laboratories in the area of detection of animal proteins.

The performance of all the tasks fits in the additional requirements of Directive 999/2001/EC.

3 Results

3.1 EURL proficiency test microscopy 2010

The regular proficiency test for 2010 consisted of nine samples, blank or contaminated with material of land animals or of fish. The results are published and discussed at the annual meeting in 2011. Therefore these results are presented in the NRL annual report for 2011. The composition of the samples and the overall results are listed in Table 1 (Veys et al., 2011).

Table 1. Results of the proficiency test of 2010. The accuracy indicates the sensitivity in the case of absence of the target, and specificity in the case of the presence of the target. Optimal values are 1.0. Nr: number of reported results.

Sample number	Composition	Nr	AC terrestrial	AC fish
1	Blank I	26	1.00 (0)	0.92 (2)
2	Blank II (pellets)	52	0.98 (1)	0.94 (3)
3	Blank III	26	0.96 (1)	0.96 (1)
4	Blank IV	26	0.96 (1)	0.92 (2)
5	Fish feed I, containing fish	26	0.96 (1)	1.00 (0)
6	Fish feed II, containing fish	26	1.00 (0)	0.96 (1)
7	Fish feed II + 0.1% MBM	26	0.92 (2)	1.00 (0)
8	Blank I + 0.005% MBM	26	0.69 (8)	0.96 (1)
9	Blank II + 0.5% salmon meal	26	0.77 (6)	0.96 (1)

RIKILT found some fish bone fragments in samples 1 and 4 (false positives), and did not find the MBM in samples 7 and 8 (false negatives). The level of contamination in sample 8 was very low and the false negative result is probably related to the low level of contamination. Several other labs (eight) showed the same problem. The other results will be discussed below.

The EURL requested to produce an additional report with information on the background of the RIKILT results and possibilities for improvement. RIKILT made a new set of slides to confirm or falsify the originally reported results.

3.1.1 Fish bone fragments in blank samples

The original set of slides contained seven particles, mainly teeth. The second set of slides revealed some further particles of fish bones. Example images are provided in Figure box 1. The identification of teeth from fish was based partly on images from the picture bank of the EURL. RIKILT realised, based on extensive literature and own experience, that comparable fragments can originate from plant material such as *Lolium* leaf epidermis. Nevertheless, combining all evidence and considering the situation that recent investigations resulted in information on a much larger diversity in fish than previously assumed, RIKILT considered these fragments as fish teeth.

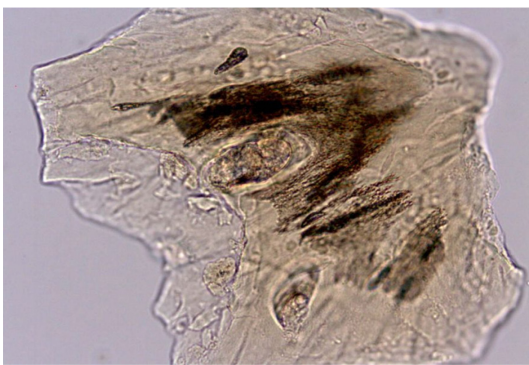
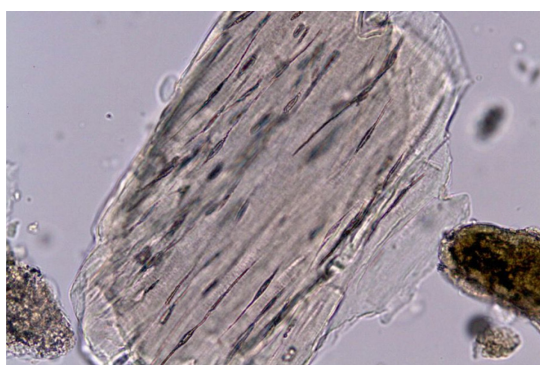


Figure box 1. One image from the original set of slides, as reported officially for the ILS 2010 (top row), and four images from the second set of slides (middle and bottom rows).

3.1.2 Embedding agent

Re-examination of the original set of slides revealed some particles, which can faintly be recognised as originating from terrestrial animals. The second set of slides showed clearly bone particles up to a number that could be designated to a level between 0.01% and 0.1%. Example images are provided in Figure box 2. The first set of slides was embedded in glycerine, the second one in Norland.

There is a clear difference in appearance between the fragments from the first set of slides and this from the second set of slides. This difference could result from the different type of embedding agent used.

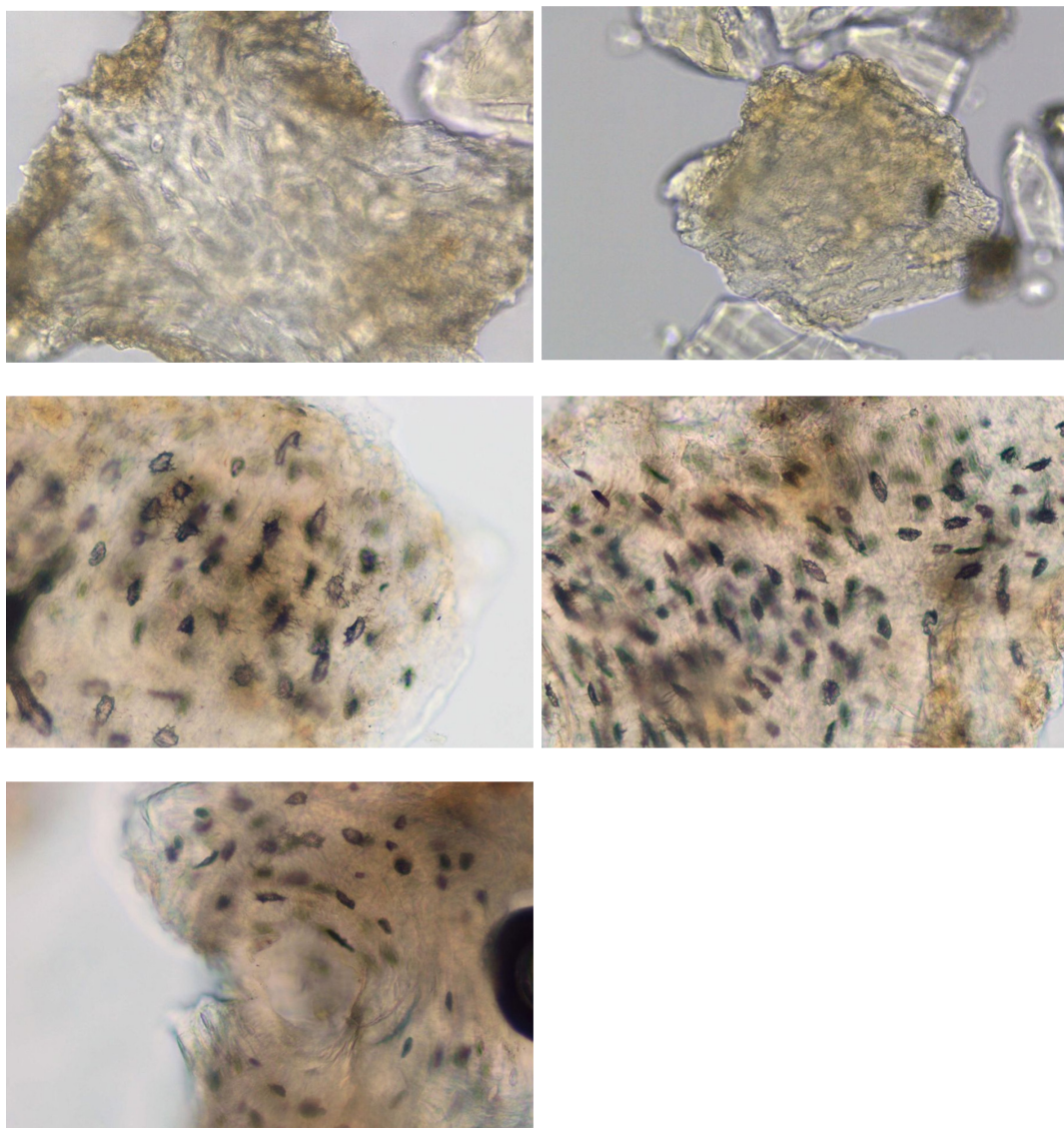


Figure box 2. Two images from the original set of slides (glycerol), as reported officially for the ILS 2010 (top row), and three images from the second set of slides (Paraffin oil, middle and bottom row).

3.2 EURL proficiency test microscopy 2011

RIKILT participated in the annual interlaboratory study for microscopy in 2011, which was organised in November. The results will be presented and discussed in 2012.

3.3 Other proficiency tests

In the course of 2011 RIKILT participated in several other regular proficiency tests. These tests focused on either the specific detection of animal proteins, or otherwise on the composition of animal feed in general. In the latter case, animal proteins are always included in the range of possible targets. The results of these tests are in all cases reported in 2011, which makes it possible to review these results in this annual report.

3.3.1 KDLL blind tests on animal proteins

KDLL is a Dutch organisation organising a proficiency test for animal proteins twice a year. Each of these tests consists of five samples of feed. Contents of the test and the RIKILT results are presented in Table 2.

Table 2. Contents of the KDLL proficiency tests with RIKILT results.

Sample	Composition	RIKILT result	
		Fish	Terrestrial
MIK11-1A	0.25 % lamb meal	Absent	Present
MIK11-1B	4.17% fish meal, 0.84% poultry meal, 1.65% meat meal	Present	Present
MIK11-1C	Microscopic examination; no animal proteins	Absent	Absent
MIK11-1D	Label check; no animal proteins	Absent	Absent
MIK11-2A	0.5 % lamb meal	Absent	Present
MIK11-2B	2.08% fish meal, 0.42% poultry meal, 0.82% meat meal	Present	Present
MIK11-2C	Microscopic examination; no animal proteins	Absent	Absent
MIK11-2D	Label check; no animal proteins	Absent	Absent

The RIKILT results were correct in all cases.

3.3.2 IAG blind tests on composition

IAG is a European organisation for supporting microscopic research. One of its activities is to organise several ring tests for composition. The results for two tests on composition are presented in Table 3.

Table 3. Contents of the IAG proficiency tests with RIKILT results.

Sample	Composition	RIKILT result	
		Fish	Terrestrial
Pig feed	No animal proteins	Absent	Absent
Dairy feed	No animal proteins	Absent	Absent

The usual composition of feeds in these proficiency tests does not include animal proteins. The composition in both appeared to exclude animal proteins, which approved the RIKILT results.

The annual IAG ring test for animal proteins is organised by RIKILT (van Raamsdonk et al., 2011a), which means that RIKILT does not participate in this annual test. However, as essential part of any proficiency test, a homogeneity study was carried out of all the samples included in the test. In all repetitions the results were conform the declaration of the samples.

3.4 DNA detection and identification

The EU-RL organised an interlaboratory study for PCR identification early 2011. The objective was to get an overview of potential PCR systems for detection of at least 0.1% MBM of different species. All but one sample were based on a DNA extract of soy bean contaminated at 0.1% or

0.5% with an MBM target. The only exception was DNA extracted from turkey meat, which was diluted 1:1 in a soy bean extract. The process of DNA extraction was not part of the study. The DNA was directly provided by the EU-RL. The final report was issued in June 2011 (Fumière et al., 2011).

The RIKILT results are summarised in Table 4. The RIKILT test for Ruminant PCR was able to detect 0.1% and 0.5% cattle and sheep MBM in all cases. As a subset of ruminant detection, cattle tests and sheep tests were also evaluated. Both the RIKILT Cattle PCR and Sheep PCR could not detect their related targets at 0.1%. There were no samples with 0.5% MBM of these sources. RIKILT Pig PCR could detect the presence of pig at contamination levels of 0.1% and 0.5% in some, but not in all cases. RIKILT Chicken PCR could detect 0.1% and 0.5% chicken MBM in all cases. No false positives were found for any of the tests.

Table 4. RIKILT results in the EURL interlaboratory study for PCR detection of MBMs. Green (+): correct positive results, yellow (+/-): some false negative results, red (-): false negative results, white (-): correct negative results.

Target:	Cattle MBM	Sheep MBM	Pig MBM		Chicken MBM		Turkey meat	Fish MBM	Fish MBM
PCR	0.1% MBM	0.1% MBM	0.1% MBM	0.5% MBM	0.1% MBM	0.5% MBM		0.1% MBM	0.5% MBM
Ruminant	+	+	-	-	-	-	-	-	-
Cattle	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	-	-	-	-	-
Pig	-	-	+/-	+/-	-	-	-	-	-
Chicken	-	-	-	-	+	+	-	-	-
Turkey	-	-	-	-	-	-	+	-	-
Fish	nt	nt	nt	nt	nt	nt	nt	nt	nt

A total of eleven participants submitted results in the interlaboratory study for detection of processed animal proteins by means of PCR, which allows to put the RIKILT results in a broader perspective. The results are summarised in Table 5.

The first issue showing up is the number of participants per target, which is an indication of the number of tests that are implemented at NRLs. Table 5 shows that a minority of the participants were able to run the ruminant and the poultry test, which, together with the pig test, are the basic tools to monitor the foreseen changes in legislation. A higher number of participants submitted results for cattle, sheep and chicken. Furthermore, quite a number of false negative results were reported. It has to be noted that the results of the interlaboratory study reflect the situation of early 2011. An action plan was already started in 2011 by the EURL. This will be discussed further in paragraph 3.5.

Table 5. Summarised results of the interlaboratory study for detection of processed animal proteins of several targets by means of PCR. Source: Fumière et al., 2011.

	N participants	results
Ruminant	4	Two participants full correct results; two other participants 7 false negative results and 2 false positive results
Cattle	7	Two participants full correct results; five other participants 17 false negative results and 2 false positive results
Sheep	6	One participant full correct results; five other participants 15 false negative results and 1 false positive result
Pig	8	Two participants full correct results; six other participants 20 false negative results
Poultry	2	One participant full correct results; one other participants 1 false negative result and 1 false positive result (turkey not detected)
Chicken	7	Three participants full correct results; four other participants 10 false negative results and 2 false positive results
Turkey	4	Four participants full correct results; tested sample consisted of pure turkey meat, no MBM
Duck	2	No false positive results; duck was only evaluated for aspecificity of other tests

3.5 EURL meetings 2011

The annual meeting of 2011 was organised in Vienna (Austria) in early April 2011. The results of the microscopic and the PCR interlaboratory studies were presented and discussed. The proposal to establish an artificial limit of detection of five bone particles in 10 grams of feed material was accepted. The purpose of this limit is to declare every sample with a lower number of bone fragments as "negative", thus avoiding the lack of reproducibility at these low levels of contamination. The consequence is that a level of detection is set which is higher than the tolerance level (zero). The preliminary results of the interlaboratory study for PCR analysis were presented. An action plan was designed to develop the necessary tests for supporting the lifting of certain parts of the extended feed ban. RIKILT presented the results of the investigations into blood plasma material in milk powder (Van Raamsdonk et al., 2011b).

In order to prepare a new version of Annex VI of Regulation (EC) 152/2009, which describes all methods for feed analysis, the EU-RL organised an expert meeting in Gembloux (Belgium) at the end of November. RIKILT participated in this meeting. Particular attention was given to flow charts describing the procedure of connecting the microscopic and PCR method, and to implement the limit of detection. Furthermore the first draft of a Part B describing the PCR methods was discussed.

At both occasions the definition of "poultry" was discussed. This issue will be further addressed in the next chapter.

3.6 Support of the national authority

On several occasions the Dutch NRL provided information and advice to the Dutch National Authority. A special issue was the lifting of parts of the extended feed ban (Regulation (EC) 999/2001, Annex IV). The views as presented by the Commission and the Council of Ministers were discussed and amended.

A document with scenarios for monitoring the new foreseen legal applications of animal proteins was developed.

The Dutch NRL provided technical assistance for the examination and evaluation of presumed positive samples in the Dutch monitoring program for animal proteins on a series of occasions.

3.6.1 Future scenarios for monitoring

The Dutch NRL developed two different scenarios for monitoring the presence of animal proteins, supporting the future use of certain animal proteins according to amendments to Annex IV of Regulation (EC) 999/2001. These scenarios are:

- Poultry proteins will be allowed as ingredient in pig feed, pig proteins are allowed as ingredient in poultry feed, and both types are allowed as ingredients in aquafeed.
- Exclusively pig proteins and poultry proteins are allowed as ingredients in aquafeed.

The current situation is that the following species or species groups can be detected by means of PCR:

- Cattle; legal limits request the detection of Ruminant proteins.
- Pig.
- Chicken, turkey and duck as representatives of "poultry". This implies that currently the detection of poultry (or a part of this group) can be detected by applying three different tests.

Proposed strategy in the situation of new legislation:

- Microscopy as first method of analysis for all feeds, feed ingredients and fish meal for which no declaration of legal inclusion of a non-ruminant PAP is given. PCR or immunoassays need to be applied only for positive samples (Figure 3). Considering the label requirements of Regulation (EC) 767/2009, Animal proteins should be declared as any other ingredient. If an animal proteins is detected by microscopy, which is not labelled, the feed can be indicated as not fit for the intended use (Figure 3). It could be chosen, however, to identify the material found for purposes of tracking and tracing (bullet "A" in Figure 3).
- PCR or immunoassays are directly applied to samples with a declared inclusion of a non-ruminant PAP, or for analysis of pure non-ruminant PAPs.

The costs are depending on the definition of "poultry", and on the number of PCR tests required to provide a sufficient coverage of that definition.

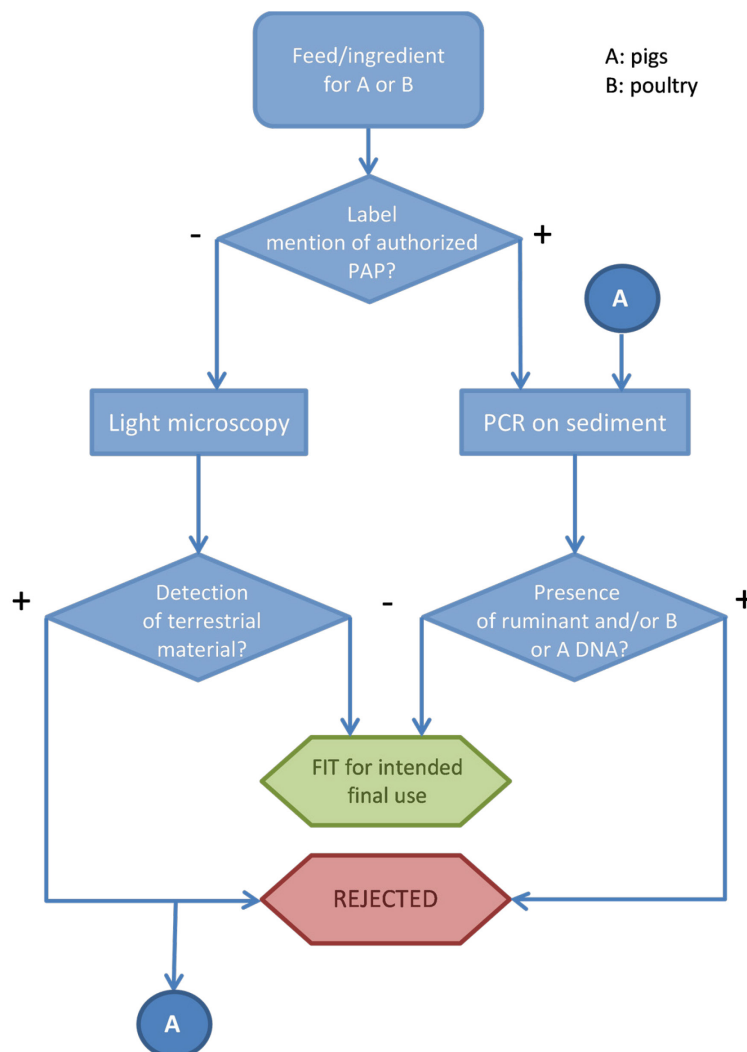


Figure 3. A flow chart of a possible strategy for monitoring the presence of animal proteins of pigs or of poultry in animal feed. See text for further explanation.

The minimal required level of detection could be fixed at 0.1 %, as is now the minimal requirement for microscopic detection (Annex VI in Regulation (EC) 152/2009). This performance requirements applies to feed and feed ingredients. The detection of ruminant processed animal proteins (PAPs) in non-ruminant PAPs, which are planned to be allowed, cannot be carried out by means of microscopy. Detection using PCR or immunoassay methods would imply a detection limit between 0.5% and 1.0%. These levels of detection would fit in the risk assessments as presented by EFSA (2011). It might be possible to lower these limits by applying the PCR method directly on the bone fragments as traced by microscopy.

4 Discussion

4.1 The poultry issue

It is necessary to define a target (i.e. a DNA sequence) for PCR detection carefully in order to detect all entities (species, races etc.) that are included in the definition, and at the same time to exclude all other living plants and animals. In other words, the specificity (avoiding false positives and false negatives) should be assured. In addition, the sensitivity needs also attention, preferably at 0.1%.

The specificity issue in the case of poultry is complicated. This informal group might include:

- Chicken, turkey, guinea fowl a.o.: order Galliformis.
- Duck, goose a.o.: order Anseriformis.

The mentioned orders include a range of game animals as well, such as partridge, pheasant, eider and swan. The 2011 versions of the amended Annex IV of (EC) 999/2001, however, defines poultry as "farmed animals". A reference has been made to Annex I of Regulation (EC) No 853/2004: "Poultry means farmed birds, including birds that are not considered as domestic but which are farmed as domestic animals, with the exception of ratites [ratites]". The EURL sought a more defined solution and proposed to use the definition from (EC) 1260/2010: "Live poultry, that is to say, fowl of the species *Gallus domesticus*, ducks, geese, turkeys and guinea fowls". This definition is also used in the Combined Nomenclature for category 0105 (Regulation (EC) 861/2010). This circumscription is given to be applied in the framework of agricultural product nomenclature and for trade purposes. It provides a list of the five most commonly used birds in trade and export, and might cover a major share of commercially used birds. However, for specificity reasons a "major share" might be insufficient.

The main principle in nature is that the weaker the relationship of two species or groups is, the more differences can be found. If one test needs to be developed for the above mentioned five species, it is more than likely that most or all other species of these two orders will be detected as well, including the game birds.

Several solutions can be found. The definition of "poultry" can be raised to the level of all birds (Aves). The main problem is that most fish meal batches includes DNA of bird species (probably originating from sea birds, e.g. gulls etc.), which renders a general bird test useless, at least in all occasions where fish meal is applied. A second approach is to define poultry as all birds of the orders Galliformis and Anseriformis. This would include a range of game birds, but ratites (e.g. ostrich and relatives) and dove are still excluded, and the costs (for two PCR tests) are still manageable. A third solution, applying five tests for the five species would lead to a high work and financial load for monitoring. The application of a sound and practicable definition needs close cooperation between legislators and scientists.

4.2 Method performance

It is apparently difficult in certain situations of microscopic analysis to identify fragments of animal origin and to discriminate these from fragments of plants or other sources. Certain plant hairs are

apparently difficult to recognise as such, also after comparison with images from the EURL picture bank. There were no samples in the regular monitoring program reported as positive in 2010 solely based on the presence of fish teeth.

The circumstances in which the method is applied are important. Application of a method in the framework of a proficiency test should be identical to the implementation of that method in the daily practice. For the current method some differences exist. RIKILT implemented several years ago the strategy for every positive sample to repeat the full standard analysis four times using four different portions of 10 grams. The five results are reported to the competent authority, but in those cases that the four repetitions appear to be negative no further actions are undertaken. This strategy cannot be used in proficiency tests, since the amount of material is too limited to repeat the analysis. Furthermore, the finding of one or two particles that could be subject to misidentification is more likely to be rejected in practice. Although it is vital to harmonise the microscopic method as much as possible, some degrees of freedom might be helpful in those few cases that a modification could match the locally available experience, e.g. type of embedding agent. Good documentation is necessary in the form of illustrations and descriptions, in databanks or expert systems. These problems deserve further attention at the annual meetings of the NRL network.

The wrongly reported results of RIKILT in the EURL proficiency test as caused by a misidentification of cereal hairs for fish teeth, and unexpected effects of an embedding agent, need to be viewed in the broader perspective of the correct results in other proficiency tests and in the background examination (homogeneity testing) of the annual IAG ring test for animal proteins (van Raamsdonk et al., 2011a). RIKILT is at the edge of scientific research for examination of animal proteins (van Raamsdonk et al., 2010; 2011c), and will continuously seek for possibilities to improve the microscopic detection.

The inventory of PCR tests available among the NRLs can be considered as a zero measurement. It is a description of the actual (IST) situation, which is useful to plan the required goals (SOLL situation). The EURL action plan provides the planning of further experiments to optimise and validate a method for detection of ruminant DNA, and in a second phase of pig and poultry DNA. The final method descriptions are planned to be published in 2012. RIKILT will participate in this action plan. Besides that, RIKILT will continue to carry out additional experiments for the development of final tests for species identification.

4.3 Future developments

The establishment of a ban on animal proteins which combines a minimum risk and a maximum but safe application, and which will be supported by a sound monitoring system, needs a carefully designed concept. The basic requirements are set out in the TSE Roadmap 2 (EU, 2010): dedicated production lines and availability of monitoring tools. A total ban for ruminants (except weaning animals) and for cannibalism are not envisaged. A tolerance level higher than zero in those cases that a very low amount of PAPs is detected, could be considered. Further requirements such as definition issues, specificity and sensitivity could be added in order to establish a sufficient monitoring system. The possibility to quantify the amount of PAPs remains problematic (Veys et al., 2008; Raamsdonk et al., 2009), but is still important in those cases that a tolerance level higher than zero is established.

5 Recommendations

The confusion in the detection of fish meal especially in the exclusive presence of land animal material, needs further attention.

Proper tests for less obvious products such as blood meal need attention.

Any future legislation would benefit from two prerequisites: a sufficient monitoring system and established management of production chains.

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Annex I

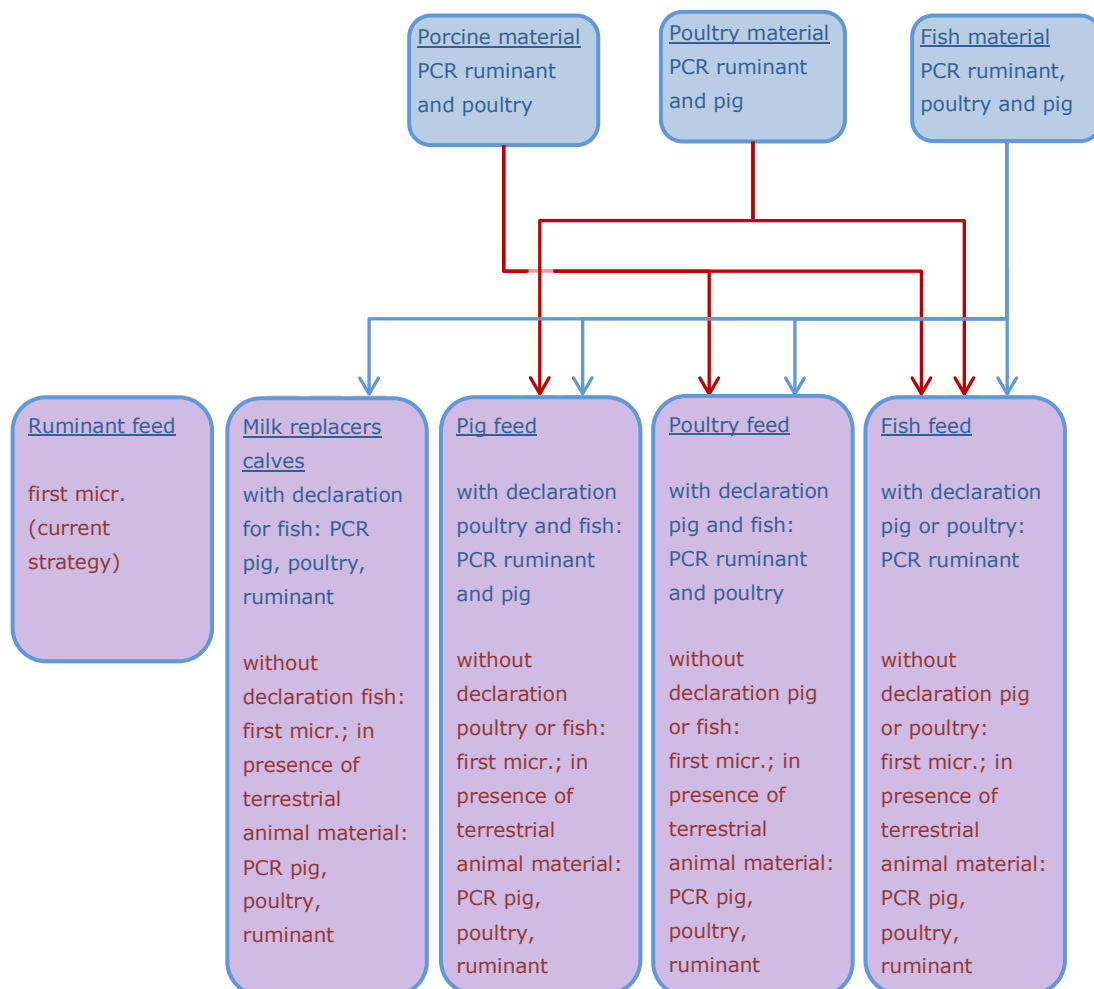
Monitoring scenario's

Flow charts for monitoring scenario's after implementation of new legislation. Explanation: red arrows: new application; blue text: PCR as primary method; red text: microscopy as primary method (see Figure 3).

Scenario 1:

Ban is lifted for:

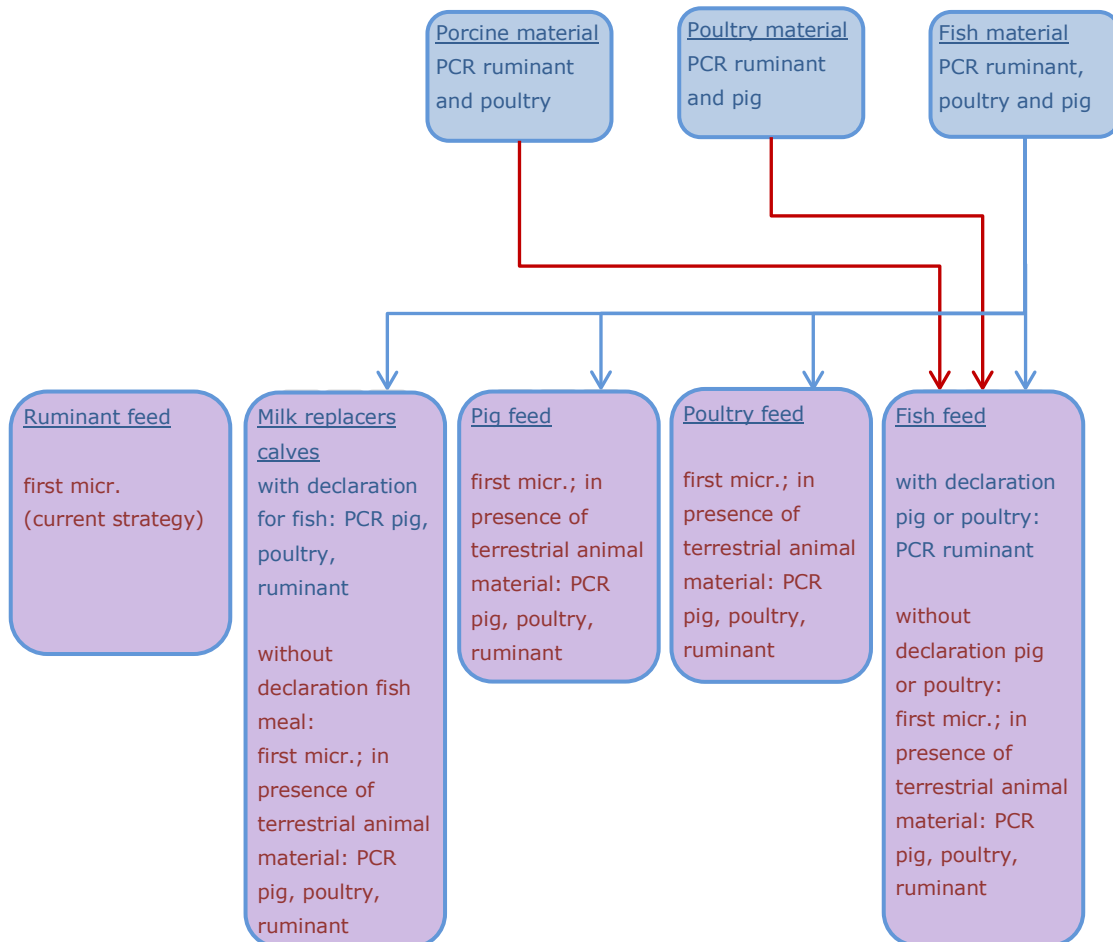
- Poultry (bird-) proteins for pig feed;
- Pig proteins for poultry (bird-) feed;
- Poultry (bird-) and pig proteins for fish feed.



Scenario 2:

Ban is only lifted for:

- Poultry (bird-) and pig proteins for fish feed.



RIKILT - Institute of Food Safety is part of the international knowledge organisation Wageningen UR (University & Research centre). RIKILT conducts independent research into the safety and quality of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

RIKILT advises national and international governments on establishing standards and methods of analysis. RIKILT is available 24 hours a day and seven days a week in cases of incidents and food crises.

The research institute in Wageningen is the National Reference Laboratory (NRL) for milk, genetically modified organisms, and nearly all chemical substances, and is also the European Union Reference Laboratory (EU-RL) for substances with hormonal effects.

RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

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