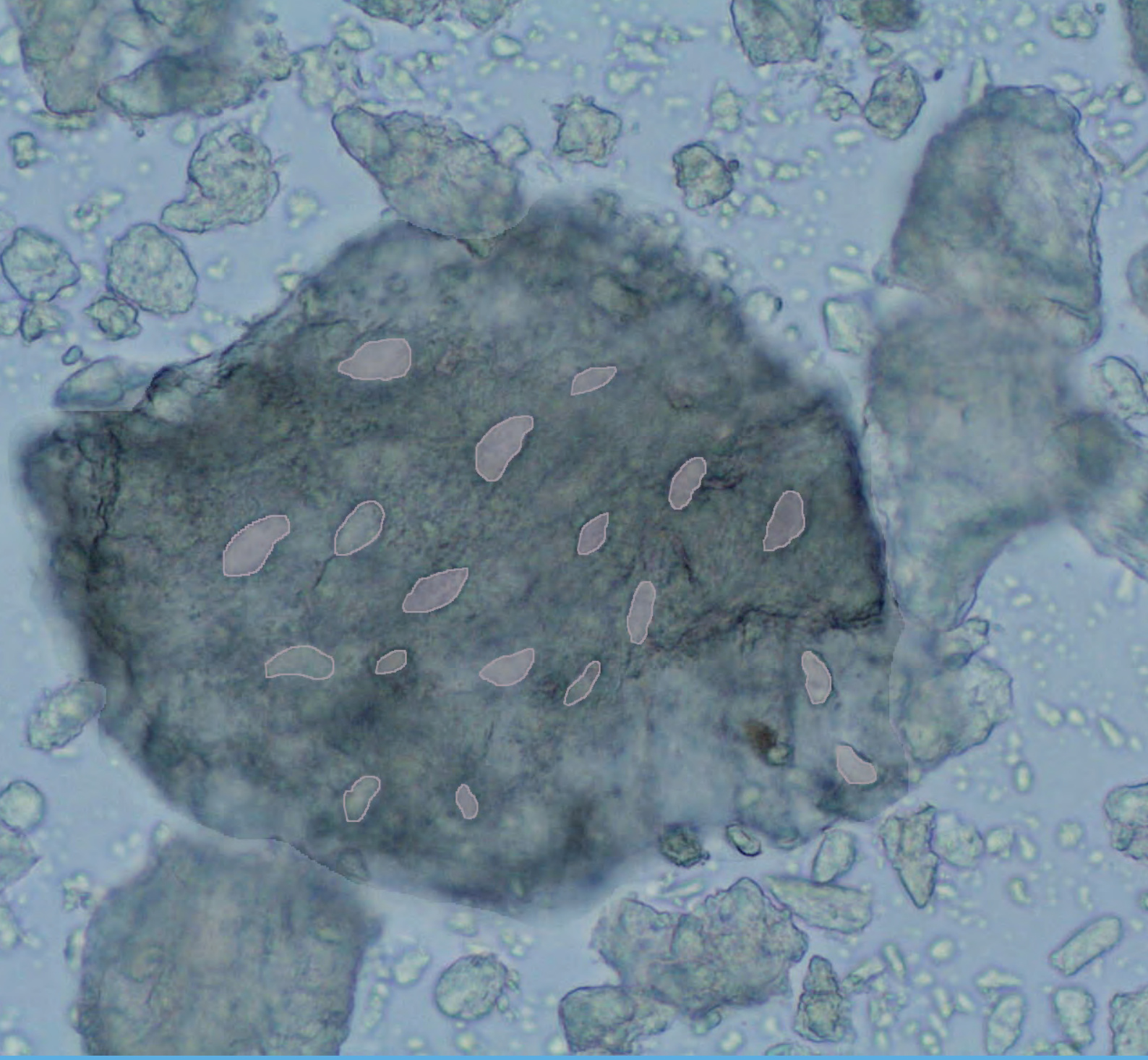

IAG ring test animal proteins 2013

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Contents

Summary

A ring test was organized for the detection of animal proteins in animal feed by microscopy in the framework of the annual ring tests of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy. The organizer of the ring test was RIKILT - Wageningen UR, The Netherlands. The aim of the ring study was to provide the participants information on the performance of the local implementation of the detection method for their local quality systems. A further aim was to gather information about the application of the microscopic method.

All four samples used in the ring test were based on an artificial feed with a formulation comparable to that of an average cattle feed. A mix of minerals was included at a level of 1%. The contaminations were: no animal proteins (blank), 2% of fish meal, 0.05% of land animal material, and 0.1% of tricalcium phosphate (TCP). All participants were requested to determine the presence or absence of land animal and/or fish protein material and to indicate the type of material found. In addition, as requested in the new method for microscopic detection effective from 12 February 2013 (Regulation (EC) 51/2013 amending Annex 6 of Regulation (EC) 152/2009), the participants were asked to examine either the flotate or raw material and to report these results as well. Reporting the number of particles was facultative. Other aspects of the new method were not yet implemented. The participants were also asked to report the amount of sediment found (the fraction containing minerals and bones, if present) and to answer questions on a series of parameters of the microscopic method. Reporting the estimated amount of land animal or fish protein was optional for all participants. All fifty-three participants returned results using the microscopic method. The four feed samples were evaluated as a proficiency test in a strict sense (i.e. no "challenger" sample), although the sample with TCP got special attention in the evaluation of the test.

Incorrect positive results (positive deviations) were expressed in a specificity score and incorrect negative results (negative deviations) were expressed in a sensitivity score. An optimal score is 1.0.

Most of the specificity and sensitivity scores were at good levels. The specificity score for incorrect detection of meat and bone meal (MBM) in the blank is acceptable (0.94). The detection of 0.05% of MBM in feed appeared to be perfect (1.0). The TCP, which is a legal ingredient for non-ruminant feeds, was detected in a vast majority of cases as animal proteins in the sense of the legislation. Four participants reported fish in the presence of TCP. For the first time in the ring test of IAG section Microscopy participants were requested to report on their examination of either the flotate or the raw material of the sample. The results show a rather diverse view. The results for the blank were at a reasonable level (0.91). The detection of animal material (0.05%) in either flotate or raw material needs further improvement (0.34). According to the new procedure the results for examination of sediment and of flotate/raw material need to be combined in one result. Therefore, the sensitivity for the examination of flotate or raw material does not have a direct effect on the performance of the method. The share of the sediment used for examination differed between 2% and 100%. This difference showed to have an effect on the sensitivity of the method.

The PCR results covered a total of ten different targets. The results were generally good.

It can be concluded that the microscopic method and the DNA identification method were generally well implemented among the participants. Several aspects need attention, such as the share of sediment material used, and the examination of the flotate or raw material. The way in which the new method needs to be implemented in the IAG ring test for animal proteins in 2014 needs further discussion.

1 Introduction

Member states of the European Union are requested by EU legislation to maintain an active monitoring program for the safety of feed. The monitoring of the presence of animal proteins in the framework of eradication of mad cow disease is an important part of it. A range of official control methods was in 2009 combined in one Regulation ((EC) 152/2009). With respect to animal proteins, major changes in the protocols and strategy for detection are effective from 12 February 2013 (Regulation (EC) 51/2013 amending Annex 6 of Regulation (EC) 152/2009: EC, 2013a, and its corrigendum EC, 2013b). The changes imply a more detailed procedure for the microscopic detection and the official implementation of DNA identification by means of PCR. The modification of the microscopic method is due to the situation that the reproducibility is insufficient at low contamination levels (e.g. Veys *et al*., 2010). Therefore, a Limit of Detection (LOD) of five particles in a portion (sample for a single analysis) is set. The PCR method is now part of Annex 6 as well. The primary goal is to identify material of ruminant origin, in order to support the lifting of the ban on the use of animal proteins. As of 1 June 2013 non-ruminant material is allowed as ingredient in aquafeed (Regulation (EC) 56/2013 amending Annex 4 of Regulation (EC) 999/2001). Ruminant material remains prohibited, which needs a more specific monitoring in the view of the relaxation of the ban.

The European Commission stimulates testing laboratories to include a lot of procedural details in Standard Operational Procedures (SOPs) instead of a full methodological description in Regulations in order to enhance flexibility. In the area of the monitoring of animal proteins the European Union Reference Laboratory (EURL) is responsible for the development of methods and for the public availability of these SOPs. Intended SOPs will include details of the microscopic and PCR procedures, and the strategy for the combination of these two methods. At the time of writing these SOPs have been published at the website of the EURL Animal Proteins.

The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy organises annually a ring test for animal proteins in feeds for all their members. RIKILT – Wageningen UR organises this ring test on behalf of the IAG section Microscopy. At the beginning of the organisation of the current ring test (early January 2013), a new version of Regulation (EC) 51/2013 was published. The SOPs, however, were only available in draft. The organisers of the current ring study were involved in the development of these SOPs as external advisers, and, hence, had knowledge of the parameters of the new method. It was, however, not possible to request all the participants to follow the new procedures. Official publication of the SOPs was achieved in late March and early April. Therefore, certain aspects of the new procedures were implemented in this ring test, especially the examination of the flotation or raw sample material, but a full implementation of the method was not required.

In this report the ring test for animal proteins is presented, which was organised by RIKILT in 2013 on behalf of the IAG Section Feeding stuff Microscopy. For this year a sample was designed containing tricalcium phosphate in order to assess whether this material is erroneously recognised as animal proteins.

2 Methods

2.1 Materials

The ring test 2013 was chosen to be based on a compound feed completely produced by RIKILT, in a composition that mimics an average cattle feed. The feed was composed of citrus (20%), wheat (20%), maize (30%), beet pulp (10%), rapeseed (9.5%), palm expeller (9.5%), mineral mix (1%). The ingredients were ground with a mesh size of 2 mm and thoroughly mixed. The mineral mix consisted of limestone, sodium chloride salt, dicalciumphosphate and copper sulphate in equal shares.

Four samples were produced, based on the artificially produced feed.

The composition of the four samples is listed in Table 1.

Table 1
Composition of the samples in the NRL-IAG ring trial 2013.

Label	Content
2013-A	Blank feed
2013-B	Feed with 2% fish meal
2013-C	Feed with 0.05% MBM
2013-D	Feed with 0.1% TCP

The fish meal was composed by mixing several samples from practice which were examined in the RIKILT regular control program and all found to be negative for terrestrial animal material.

The meat and bone meal was produced in Uruguay and collected after export to China. It was declared as ruminant MBM.

The tricalciumphosphate was obtained from a local supplier.

All materials were checked on purity (absence of any contamination) and identity, and were all found to be fit for application.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order:
2013-A - 2013-C - 2013-B - 2013-D. All samples were prepared in a laboratory which is located at a distance from the RIKILT microscopy laboratory.

The production scheme is presented in Figure 1.

Jars for sample 2013-A and for sample 2013-C were filled with 40 grams of the pure feed, closed and set aside. Every jar for sample 2013-C was individually spiked with 20 mg of MBM. The jars of samples 2013-A and 2013-C were wrapped and set aside before the fish meal and the TCP entered the laboratory.

Sample 2013-B was produced by thoroughly mixing 60 g of fish meal in 2.94 kg of feed. This resulted in an concentration of approximately 2% fish meal. The jars of sample 2013-B were set aside and the fish meal was removed before producing sample 2013-D.

Sample 2013-D was produced according to the method of stepwise dilution. 2.8 g of TCP was used to prepare 2.8 kg of contaminated feed as follows. The initial 2.8 g of TCP was mixed in 2.8 g of feed and stirred for one minute. In nine subsequent steps the remaining amount of feed was added stepwise by mixing according to a fixed scheme.

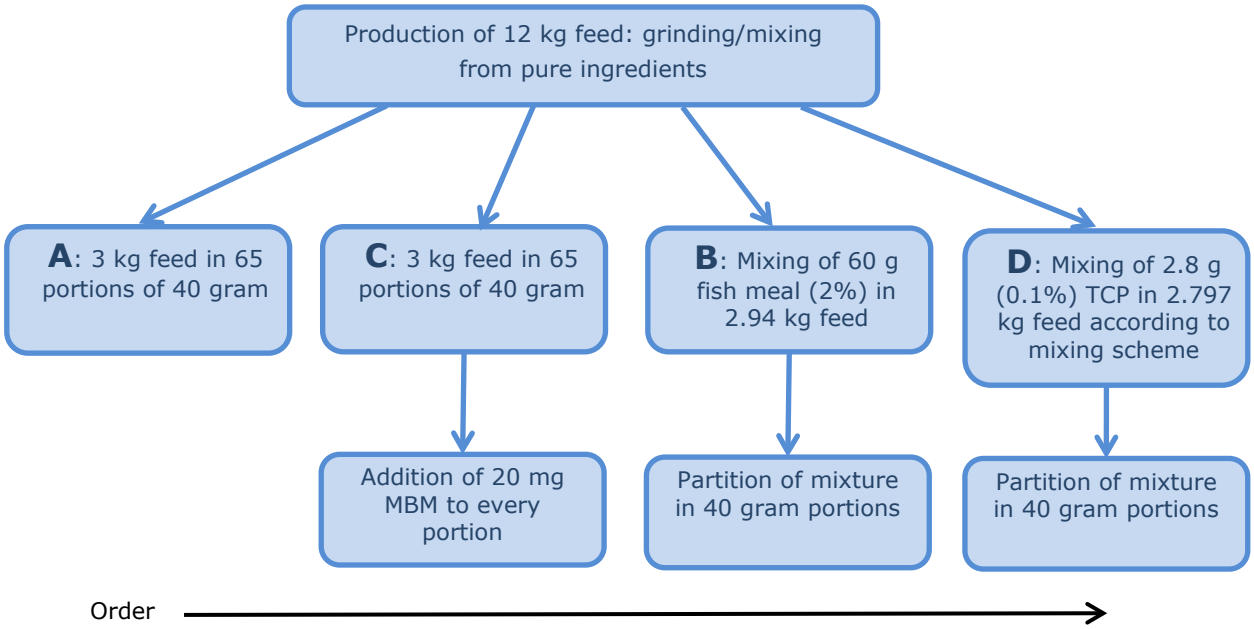

Figure 1 *Overview of the production scheme for the four samples of the IAG ring test animal proteins 2013.*

2.3 Homogeneity study

Two RIKILT microscopists examined independently five jars of all four samples. In all cases a correct result was obtained (Table 2). All materials were also investigated by PCR for three targets: ruminant, pig and fish. The results, as listed in Table 2, are also correct in all cases.

The microscopy research group and the PCR research group of RIKILT did not participate in the further laboratory analysis of this ring trial.

Table 2

*Results of the homogeneity study. Sediment amounts are based on 10 grams. The number of portions is indicated for microscopy. PCR results were based on two repetitions. Green cells indicate the correct positive findings. Rum: ruminant target. *: the positive results for sample 2013-D were related to the presence of TCP.*

Sample			Sediment amount	Microscopy		PCR		
				MBM	Fish	Rum	Pig	Fish
2013-A	blank	(n= 5)	6.9 – 9.5 mg/g	Neg	Neg	Neg	Neg	Neg
2013-B	2% fish meal	(n= 5)	12.1 – 14.8 mg/g	Neg	Pos	Neg	Neg	Pos
2013-C	0.05% MBM	(n= 5)	9.5 – 12.7 mg/g	Pos	Neg	Pos	Neg	Neg
2013-D	0.1% TCP	(n= 5)	10.4 – 12.4 mg/g	Pos *	Neg	Neg	Pos *	Neg

2.4 Organization of the ring trial

All IAG members, all NRLs, and a series of putative interesting laboratories were informed about the ring test for 2013. In all cases an invitation letter, a participation form and an invoice were distributed. Until the beginning of March a total of 53 participants were listed. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on the Tuesday 5th of March 2013. On Wednesday March 6th an E-mail message was sent to all participants, together with a file containing a sheet with instructions (see Annex 2) and the electronic report forms (see Annex 3 and 4), and the request to confirm the receipt of the package.

The closing date for reporting results was fixed at April 5th. Several requests were received to extent the period for analysis with one week. This request was granted and the closing date was set at April 12th. In several cases participants appeared not to be able to submit their results even within the extended period. However, all sets of results were received during April. Since the analysis of the results was carried out early May, all results were considered valid and taken into consideration.

Participants outside Europe were informed to be aware of possible problems with custom regulations. In one occasion the package with samples was kept by customs. Finally a second package arrived safely at the participant's laboratory. In addition to the 53 sets of microscopic results, seven participants reported their results of PCR analysis. The draft report was finalised at May 27th .

The new Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 came into force at February 12th. Officially the new procedures should have been applied by all participants for the analysis of the four samples. However, the supporting Standard Operational Procedures (SOPs) belonging to this Regulation were not officially published at the time of the analysis by the participants (March), but came available in April 2013. Therefore, the choice has been made to follow the basic procedure as laid down in the operational schemes of Regulation (EC) 152/2009, which includes the mandatory examination of both the sediment and either the flotate or the raw material. The report form has been extended accordingly. For the reporting, which now includes a distinction between results based on 1-5 particles or on 6 particles or more, it has been chosen to ask the participants to report "absence" or "presence". The further instructions to the participants send on March 12th and March 18th are included in Annex 5.

2.5 Participants

The 53 participants originated from 23 countries: 16 member states of the European Union, and five other countries (China, Norway, Peru, Thailand and Switzerland). The list of participants is presented in Annex 6. Five member states have been involved with three or more participating laboratories: Germany (17 labs), Italy (6), Belgium (5), France (3) and the Netherlands (3). These figures are comparable to those of the ring test of last year (van Raamsdonk *et al*., 2012a).

2.6 Analysis of results

For binary results (yes/no, positive/negative, etc.) standard statistics are accuracy, sensitivity and specificity. The accuracy is the fraction of correct results, either positive or negative. The sensitivity is the ability of the method used, to detect the contaminant when it is present, whereas the specificity is the ability to not detect the contaminant when it is absent. The following equations have been used to calculate the statistics:

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics are presented as fractions. Accuracy (specificity or sensitivity) has been calculated for each sample type.

As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

Significance of quantitative results was tested by using Student's t-test statistics; see, for example, Hand (2009). Grubbs' outlier test was used to identify outliers in the data on sediment amounts, which were removed prior to further analysis. It was explicitly asked to report the amount of sediment obtained before any staining was applied.

Differences in the results after applying different parameters were analysed using Fisher's exact test (Fisher, 1945).

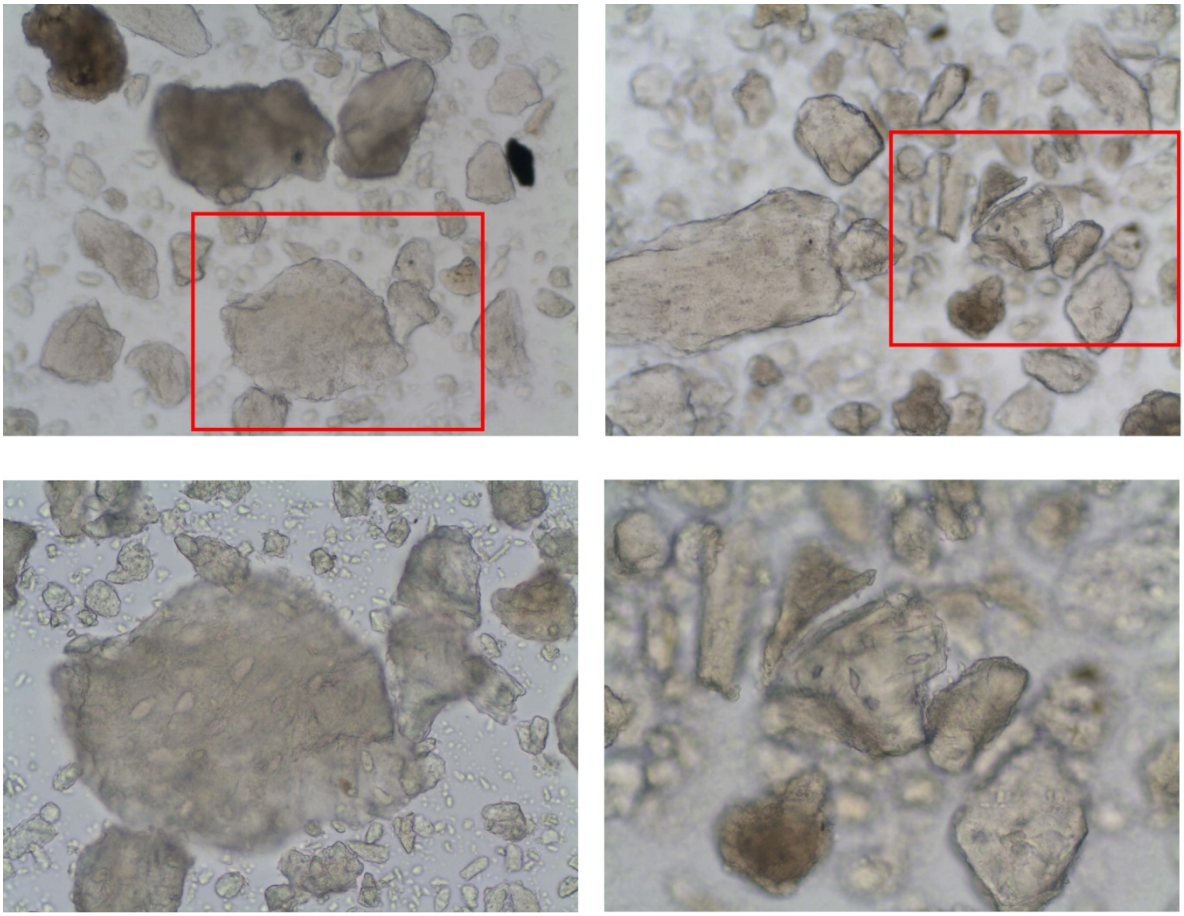

Figure 2 *Top row: two images of TCP particles (100 x). Bottom row: two selections as indicated in the red quadrangles (200 x).*

3 Results

Fifty-three packages with four samples were sent to all participants. The same number of fifty-three participants returned results for the microscopic method, seven sets of results were received for PCR analysis. All results were received by E-mail, and in most cases a FAX message was sent as well. The procedure for FAX handling at RIKILT was changed during the reporting period, which makes it currently impossible to provide a figure for FAX response. Two participants submitted a report sheet with the wrong participants number, which could be corrected based on the name and mail address of the participant. Furthermore one participant did not enter the participants number at all. The link with the original E-mail message and sender could be established beyond doubt in all cases; otherwise these reports would have been omitted. In all those cases that a participant send in several versions of the report sheet the most recent version was used. All reports were included.

The report sheet was produced in Office 2010 as in the previous year. The report sheet was transferred to Office 2003 format before distribution. Errors in using this sheet were not reported by the participants. The message upon saving the sheet of Office version violation did not cause any problem.

The full results are presented in the tables of Annex 6, 7 and 8. Sample 2013-D was a challenge to examine a legal ingredient (TCP) which could be mistaken as animal protein. However, the results for sample 2013-D could be fully evaluated (see Figure 2) since approx. 10% of the particles of the TCP appeared to be recognisable as bone fragments of terrestrial animals. This situation implies an effective contamination with recognisable bone fragments of 0.01% (10% of 0.1%).

3.1 Microscopic detection

Most of the specificity and sensitivity scores were at good levels considering the samples for the proficiency test (Table 3; Annex 8). The specificity score for incorrect detection of MBM in the blank is just below the level of 0.95. The TCP, which is a legal ingredient for non-ruminant feeds, was detected in a vast majority of cases. Three participants reported fish in the presence of TCP.

Table 3

*Sensitivity and specificity scores for the detection of animal proteins in the **sediments** of four samples. Abbreviations: n: number of participants. Capitals A to D: sample indication.*

		Fish				MBM			
		A	B	C	*D*	A	B	C	*D*
n		0%	2%	0%	*0%*	0%	0%	0.05%	*0.1%*
53	specificity	0.96		0.96	*0.94*	0.94	0.98		
	sensitivity		0.98					1.0	*0.94*

For the first time in the ring test of IAG section Microscopy results were requested for the examination of either the flotate or the raw material of the sample. Targets for this examination could be muscle fibres, hair, feather filaments or cartilage. Presence of fish meal or MBM should imply the presence of animal particles in the flotate. Presence of TCP in the sediment should not result in any animal particles in the flotate. However, examination of the raw material could result in the finding of bone particles.

The results show a rather diverse view (Table 4). Especially the presence of muscle fibres or other light particles was not reported by a number of participants, most notable for sample 2013-C containing 0.05% of MBM. More than two-third of the participants reported animal particles in either the flotate or raw material for sample 2013-D, contaminated with TCP.

Table 4

*Sensitivity and specificity scores for the detection of animal proteins in either the **flotates** or the **raw materials** of four samples. Abbreviations: n: number of participants. Capitals A to D: sample indication. *: no material in flotate is to be expected for TCP.*

		Animal materials			
		A	B	C	D
n		no	yes	yes	no*
50	specificity	0.91			
	sensitivity		0.83	0.34	0.68

The results for examination of the flotate or raw material should not be evaluated independently from the results as obtained from examining the sediment. The new procedure of Regulation (EC) 152/2009 combines the results of both examinations in one number of particles. The possible results "absent", "present with 5 or less particles", or "present with 6 or more particles" are based on the total number of all examinations.

3.2 Microscopic procedure

An inventory of ten different parameters was added to the report sheet of the actual results of the four samples. These results are shown in Annex 7 and summarised in Table 5. The main purpose of this inventory was to provide benchmark information for the individual participants for comparison with the general application of the method. Although this has to be considered additional information only, a ring test with a random set of participants provides a good opportunity to collect meta-data on the application of the method. The current results provide the opportunity to discuss some parameters of the microscopic method. The frequencies of application of choices for several method parameters are presented in Table 5.

Table 5

Inventory of parameters for microscopic detection and their application.

Parameter	Parameter state	Number of participants	Amount
amount of material used for sedimentation of feed	5 grams	4	
	10 grams	47	
	other	2	
type of glassware	chemical sedimentation funnel	28	
	conical glass with cock	9	
	champagne glass	7	
	beaker (flat bottom)	5	
	other	2	
sedimentation agent	TCE	51	
	TCE/Petroleumether	0	
	other	2	
use of staining of sediment	no	36	
	yes	16	
use of binocular for examination at lower magnifications	yes	43	
	no	10	
size of cover glass used	small (e.g. 20 x 20 mm)	38	
	medium	5	
	large (e.g. 26 x 50 mm)	9	
share of the total sediment used for examination	minimum		1%
	maximum		100%
embedding agent	glycerine / glycerol	19	
	paraffin oil	16	
	immersion oil	12	
	Norland Adhesive	5	
	other (water, glycerol:water mixture, mineral oil)	1	
Use of ARIES	yes	5	
	no	47	
f-factor for MBM	minimum		25%
	maximum		60%
	none estimated		

The results as presented in Table 5 show generally a good application of the method. Differences with previous years will be presented in the next chapter (Discussion).

Only a very low share of the participants used the knowledge system ARIES (van Raamsdonk *et al.*, 2004, 2010). The information in this system could support the discrimination between confusing particles of land animals and fish.

Correlations between specificity and method parameters are relevant only if some sort of causal relationship exists in order to avoid the analysis of random fluctuations of results. As far as substantial numbers among the participants have applied different parameters of the method (see Table 5), the correlation between results and the application of some parameters have been calculated. These include the use of glassware, and the amount of sediment analysed. A further analysis of the results after stratification for these two parameters will be presented.

3.2.1 Use of glassware

Basically four different types of glassware have been used. These are: chemical sedimentation funnel, conical glass with cock, champagne glass, and beaker (flat bottom). The first two types have a separate way to release the flotate (on top) and the sediment (at the bottom). In this way there is a secure and 100% separated collection of both fractions. The procedure for using the latter two types include the removal of the flotate at first, after which the sediment can be collected from the bottom. Only two participants used a deviating type of glassware: a beaker with a conical bottom, and a mensur. The first one was included in the analyses, the results obtained using the mensur were ignored. The two main categories are indicated by the phrases "release on top" and "release at bottom" , referring to the position of release of the sediment.

The results are shown in Tables 6 and 7. There is no significant difference in terms of accuracy after examination of the sediment obtained by any of the types of glassware. The differences in accuracy based on the examination of the flotate or raw material are larger, although not significant according to Fisher's exact test. A main complication is the circumstance that no question to differentiate the use of either the flotate or the raw material was included in the questionnaire. The difference between the results based on a flotate or on the raw material is especially important for the examination of the addition of TCP, since no animal particles are to be expected in the flotate.

Table 6

*Sensitivity/specificity scores for the detection of animal proteins in the **sediment** of four samples, separate for the use of different types of glassware. The results of one participant were excluded for the use of a deviating type. Abbreviations: n: number of participants per group. Capitals A to D: sample indication. P: probability of a significant difference according to Fisher's exact test.*

Type of glassware	n		Fish				MBM			
			A 0%	B 2%	C 0%	*D 0%*	A 0%	B 0%	C 0.05%	*D 0.1%*
Release on top	13	specificity	1.0		0.92	*0.85*	1.0	1.0		
		sensitivity		1.0					1.0	*0.92*
Release at bottom	39	specificity	0.95		0.97	*0.97*	0.92	0.97		
		sensitivity		0.97					1.0	*0.95*
p			0.559	0.750	0.382	*0.138*	0.414	0.750	1.0	*0.436*

Table 7

*Sensitivity/specificity scores for the detection of animal proteins in the **flotate** or **raw material** of four samples, separated for the use of different types of glassware. Abbreviations: n: number of participants per group. Capitals A to D: sample indication. P: probability of a significant difference according to Fisher's exact test. *: no material of TCP is to be expected in the flotate.*

Type of glassware	n		Animal material A no	B yes	C yes	*D no**
Release on top	11	specificity	1.0		0.18	*0.64*
		sensitivity		0.82		
Release at bottom	38	specificity	0.95		0.39	*0.76*
		sensitivity		0.89		
p			0.598	0.290	0.131	*0.205*

3.2.2 Amount of sediment used

The amount of sediment used for examination differs from 1% to 100%. Since it is to be expected that a lower number of animal particles can be found when using a limited amount of sediment, it seems important to analyse the relation between the parameter Amount of sediment used and the result in terms of accuracy. Thirty-eight out of 53 participants reported their estimation of the amount of sediment used. The results are presented in Table 8.

In those cases that the accuracy expresses the specificity (target absent, indication of false positives), the amount of material examined is not relevant. Absence of material remains the same in all different amounts of material that can possibly be examined. In this respect, the difference between the two classes (less than 50%, 50% or more used) for detection of fish in sample 2013-C (fish absent) is remarkable. The main interesting result is the indication of presence of MBM in sample 2013-D. The indication of 0.1% of TCP in this sample applies to the TCP in general. The share of particles which is still recognisable as bone particles is far less, resulting in an effective contamination level of approx. 0.01%. At such low levels of contamination a difference related to the examined portion is to be expected. The different scores (0.88 vs. 1.0) indicate this dependence although not at an significant level (p= 0.146).

Table 8

*Sensitivity/specificity scores for the detection of animal proteins in the **sediment** of four samples, separate for the amount of sediment used. Abbreviations: n: number of participants per group. Capitals A to D: sample indication. P: probability of a significant difference according to Fisher's exact test.*

Amount of sediment used	n		Fish A 0%	B 2%	C 0%	*D 0%*	MBM A 0%	B 0%	C 0.05%	*D 0.1%*
< 50%	16	specificity	1.0		0.94	*0.88*	0.94	1.0		
		sensitivity		1.0					1.0	*0.88*
>= 50%	25	specificity	0.96		0.96	*0.96*	0.92	1.0		
		sensitivity		1.0					1.0	*1.0*
p			0.610	1.0	0.488	*0.281*	0.450	1.0	1.0	*0.146*

3.3 Quantification of the sediment

The starting amount of material for sedimentation will obviously influence the results of quantification. Contrary to the previous years, the amount of sediment in the current study was calculated per gram of material used. Outliers were identified using the Grubb test, applied two sided (confidence interval 0.025 – 0.975, G = 1.93). The large variation in sediment amounts resulted in rejecting 14 results. The results as expressed in mg/g in Table 9 are based on the results of 34 participants.

For all samples the resulting amount of sediment per gram of raw material is larger than established in the homogeneity study (Table 2). In some cases (samples 2013-A and 2013-C) the difference is significant. The sediment amounts as obtained by glassware with a sediment release at the top is higher for all samples compared to the amounts as obtained by the other glassware (Table 10).

Table 9

Resulting amounts of sediment (in mg/g) for samples A-D. For every result the average (in normal) and standard deviation (in italics) is given. Calculations were based on data after removal of outliers. Five participants did not report sediment quantities.

	n	amount of sediment (mg/g)			
		A	B	C	D
total	34	13.29 *(2.02)*	16.24 *(2.31)*	13.60 *(2.19)*	13.45 *(2.50)*
actual average	5	8.36	14.02	11.20	11.34
t-test		14.23 b	5.60	6.39 a	4.92

a: $p < 0.05$; b: $p < 0.025$.

Table 10

Resulting amounts of sediment (in mg/g) for samples A-D, stratified for the type of glassware used. For every result the average (in normal) and standard deviation (in italics) is given. Calculations were based on data after removal of outliers. Five participants did not report sediment quantities. According to the t statistic results do not differ between the two groups.

Type of glassware	n	amount of sediment (mg/g)			
		A	B	C	D
Release on top	11	13.74 (*2.21*)	16.36 (*2.42*)	14.02 (*3.01*)	13.51 *(2.52)*
Release at bottom	23	13.08 (*1.94*)	16.19 (*2.30*)	13.41 (*1.72*)	13.41 *(2.55)*
t-test		0.606	0.140	0.479	0.075

3.4 Detection by other methods

Participations were invited to perform DNA analysis and to submit their results, separated for every single target. Seven participants submitted results covering a total ten different targets. These targets are classified at three hierarchical levels: class (mammal, avian, fish), order (ruminants), genus/ species (bovine, sheep, pig, chicken, turkey) and a rest group (other). The results are presented in Table 11. In general the results are a good indication of the actual contents of the samples. In three of the four false positives for species (bovine) the higher hierarchical group (ruminant) shows the opposite results. In samples 2013-C (MBM from Uruguay) and 2013-D (TCP) several positive results for avian and chicken were reported. Although the mentioned contaminants are assumed not to contain these sources of DNA, their absence cannot be proved.

Table 11

Results for DNA analyses (PCR) for four samples and 10 different targets. Seven participants, indicated by their reference number, submitted results. Red cells: false positive or false negative result. Yellow cells: putative false positive result. Mam.: mammal, rumin.: ruminant.

A	mam.	rumin.	bovine	sheep	pig	avian	chicken	turkey	fish	other
11	no		no		no		no	no	no	
13		no	yes		no					no
15	no		no	no	no	no	no	no	no	no
24		no								
25		no	no	no	no		no			
45		no	no	no	no		no	no		
53		no								

B	mam.	rumin.	bovine	sheep	pig	avian	chicken	turkey	fish	other
11	no		no		no		no	no	yes	
13		no	yes		no					no
15	no		no	no	no	no	no	no	yes	no
24		no								
25		no	no	no	no		no			
45		no	no	no	no		no	no		
53		no								

C	mam.	rumin.	bovine	sheep	pig	avian	chicken	turkey	fish	other
11	yes		yes		no		no	no	no	
13		no	yes		no					no
15	yes		yes	yes	no	yes	yes	no	no	no
24		yes								
25		yes	yes	yes	no		no			
45		yes	yes	yes	no		no	no		
53		yes								

D	mam.	rumin.	bovine	sheep	pig	avian	chicken	turkey	fish	other
11	yes		no		yes		no	no	no	
13		no	no		yes					no
15	yes		no	no	yes	yes	yes	no	no	no
24		no								
25		no	no	no	yes		no			
45		no	no	no	no		yes	no		
53		?								

4 Discussion

4.1 Method performance

The results as obtained in this most recent version of the annual IAG ring tests for microscopic detection of animal proteins in feed is comparable to the historic record of previous years (Table 12). In several occasions the accuracy was comparable to the level that is usually considered as limit (0.95). The sensitivity of the detection at the level of 0.05% MBM or below could be considered as very good (Regulation (EC) 152/2009: detection limit of 0.1%), also in the view of earlier results (Table 12). The 2013 result for 0.1% of land animal material (0.94 overall, or 0.98 for EU member states only) is very good considering the nature of the material (TCP), which in the current case contained approx. 10% of recognisable fragments.

Table 12

*Results for detection of material of terrestrial animals and of fish in feed samples based on sediments of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008-2013) on behalf of the IAG section Microscopy. Results have been communicated in the framework of this Section. Results indicate specificity in the case of the blank, and sensitivity in the case of the other sample types. * TCP used as contaminant for land animal material.*

Detection of :	Land animals						Fish		
Content: fish	0	2-5%	2%	0	2%	0	0	0	0
Year Land animal	0	0	0.1%	0.1%	0.05%	≤0.05%	0	0.1%	≤0.05%
2003 (n=29)	0.86			1.0					
2004 (n=30)	0.93					0.97	0.97		0.93
2005 (n=42)			0.95	0.95				0.76	
2006 (n=43)	0.98		1.0				0.93		
2007 (n=45)		0.89	0.93						
2008 (n=45)	0.93			0.98		0.96	0.98	0.91	0.84
2009 (n=49)	0.96	0.98		1.0			0.96	0.88	
2010 (n=53)	0.96		0.98		0.91		0.98		
2011 (n=56)	1.0					0.98	0.98		0.91
2012 (n=53)	0.94			0.98		0.98	0.94	0.96	0.92
2013 (n=53) current study	0.94	0.98		(0.94)*		1.0	0.96	0.92	0.96

The examination of either the flotate or raw material is mandatory in the new method as published in the amended version of Regulation (EC) 152/2009. The results indicate that an improvement of the effectiveness of examination of flotate or raw material is required (Table 4). Especially for samples 2013-B (2% fish) and 2013-C (0.05% MBM) underperformance can be noted. It is, however, not necessary to have a correct result in all cases for the examination of the flotate or raw material, since the new method in Regulation (EC) 152/2009 is based on the total number of particles counted in at most four slides of the sediment and two slides of the flotate or raw material. If a second or third determination is required, the conclusion is based on the average number of particles counted from a multiple of six slides (i.e. 12 slides after a second and 18 slides after a third determination; EC, 2013a). There are several combinations of absence/presence for animal protein particles in sediment versus flotate/raw material (see Annex 8). Since there is no full overview of the number of particles found by all the participants, and considering the complicating factor that muscle fibres cannot be assigned to one the categories fish vs. terrestrial animals without an assignment model, a full discussion is not feasible. Only some specific situations focussing on the combination of a false negative result for the sediment which can be corrected by a correct positive result for flotate or raw material will be discussed. Three participants reported a false negative for the presence of bone fragments in the sediment of sample 2013-D (0.1% TCP), of which one participant did not report results for the examination of flotate or raw material (part. 50). The other two participants (5 and 6) both reported the correct presence of animal material in the flotate or raw material. If using an assignment

model, at least a part of this material could be assigned to the category terrestrial animals, the final result would be correct positive. The same situation was found for the detection of fish in sample 2013-B. Participant 49 did not report fish particles in this sample, which was combined with the observations of animal material in the flotate or raw material. Assignment to the category fish would have corrected this false negative result for the sediment.

The results for DNA detection were improved in comparison with the result of last year (van Raamsdonk *et al*., 2012a). The results as presented in this study (Table 11) are too erratic for firm conclusions, the total view shows, however, promising results.

4.2 Method parameters

A proficiency test is meant to reveal information on the performance of individual labs. It is not possible to draw conclusions about the validity of the method(s) applied (von Holst *et al*., 2005). In certain occasions a questionnaire is send around with the samples, which can be used to evaluate the way in which the method is implemented. The current and previous ring tests of IAG are examples of those "extended proficiency tests". Although method validation is principally impossible, improvements of method implementation and relationships with the results can be discussed (van Raamsdonk *et al*., 2012b).

As shown in Table 13, a status quo in the shift of method parameters can be found. Still some participants use only 5 grams of material for sedimentation, the use of glassware allowing the release of the sediment at the bottom is slightly increasing, in contrast to the decreasing number of participants that apply staining of the sediment. The use of small cover glasses is increasing, which might indicate that on average a smaller amount of material is mounted on one slide.

The relationship between two parameters and the final results expressed in either specificity or sensitivity is analysed further:

- Type of glassware used: release of the sediment on top (settling beakers, champagne glass) vs. release of sediment at the bottom (sedimentation funnel, special glass with cock). It can be imagined that release of both flotate followed by the sediment could result in mixing a part of the two fractions. Hypothesis: using glassware with "release on top" will result in a larger amount of sediment compared to glassware with "release at bottom" due to remnants of the flotation. An effect can be expected for sensitivity scores only.
  The amount of sediment achieved is larger after using the glassware with "release on top", although there is no significant difference with the result obtained after applying glassware with "release at bottom" for all samples (Table 10). There are no significant differences between the sensitivity scores related to the two types of glassware (Table 6).
  Lower sensitivity scores after using glassware with release of sediment on top were reported in the IAG ring test 2010 (van Raamsdonk *et al*., 2010). Comparable results were achieved in a STRATFEED proficiency test (Figure 5.2 in van Raamsdonk *et al*., 2012b based on data extracted from von Holst *et al*., 2005). In this figure the indication "open" meant a settlement beaker, and the indication "closed" meant a (closed) sedimentation funnel. In a DG-SANCO proficiency test (Boix *et al*., 2004; van Raamsdonk *et al*., 2007) difference was made between an "Austrian method" and the official method according to Directive 2003/126/EC, applied by new member states. In both methods both types of glassware have been used, which prohibits to draw further conclusions.
- The amount of sediment used for examination: less than 50% of the achieved sediment vs. 50% or more sediment material examined. A relationship might exist between the amount of sediment examined and the number of particles found. Hypothesis: a smaller amount of sediment material examined will result in a smaller number of particles found. An effect can be expected for sensitivity scores only, especially for those samples with a low contamination level.
  In the framework of the restriction to consider only the sensitivity scores for samples with low levels of contamination, only the sensitivity for MBM in sample 2013-D applies: the share of recognisable bone fragments in TCP is much below the amount of material used for contamination (0.1%). The difference between using less than 50% of the sediment (0.81) and using 50% or more of the sediment (1.0) is near to being significant ($p=0.146$; Table 8).

Table 13

Comparison between parameters distribution in the IAG ring studies between 2008 and 2013.

Parameter	Parameter choice	2008	2009	2010	2011	2012	2013
amount of material used for sedimentation	5 grams	16	5	3	3	2	4
	10 grams	26	41	48	50	50	47
	other	3	3	2	3	1	2
type of glassware	chemical sedimentation funnel	22	28	31	33	28	28
	beaker (flat bottom)	11	13	10	9	7	5
	champagne glass	6	5	8	7	7	7
	conical glass with cock	3	1	2	3	6	9
	other	3	2	2	3	4	2
use of staining of sediment	no	31	35	34	33	31	36
	yes	14	14	19	22	22	16
use of binocular for examination at lower magnifications	yes	29	40	45	44	42	43
	no	16	9	8	12	11	10
number of slides used	minimum	1	1	n.d.	n.d.	n.d.	n.d.
	maximum	7	14	n.d.	n.d.	n.d.	n.d.
size of cover glass used	small (e.g. 20 x 20 mm)	34	27	27	36	32	38
	medium	1	9	10	8	7	5
	large (e.g. 26 x 50 mm)	9	13	16	12	13	9
share of the total sediment used for examination	minimum	4%	2%	2%	0.2%	2%	1%
	maximum	100%	100%	100%	100%	100%	100%
embedding agent for sediment	paraffin oil	18	20	23	20	12	16
	immersion oil	8	12	14	12	12	12
	glycerine / glycerol	8	10	12	12	16	19
	Norland Adhesive	0	2	2	6	7	5
	chloral hydrate	3	1	0	0	0	0
	other (e.g. Depar 3000, water)	8	4	2	5	4	1

5 General conclusions and recommendations

5.1 Conclusions

In certain occasions reporting errors were noticed. These problems mainly apply to inconsistent reporting (wrong or missing unique laboratory number: two occasions), and late reporting (several occasions). Some problems with the custom procedures of certain countries were encountered.

The proficiency test showed generally good results. The situation that TCP as legal feed ingredient for non-ruminant feeds still can contain recognisable bone fragments is a matter of concern.

The method as published in Regulation (EC) 152/2009, amended by Regulation(EC) 51/2013, includes several steps for examination additional to the old method, and several repetitions in order to establish the number of particles as accurate as possible. One extra evaluation step involves the examination of either the flotate or raw material. The first results as obtained in the current ring test are in need of improvement. The share of the sediment that is used for examination will influence the number of particles to be found, which will have its effect on reaching the Level of Detection. A certain effect on the sensitivity scores was shown in this study.

5.2 Recommendations

- The examination of the flotate or raw material needs considerable improvement. Training of microscopists remains important.
- Evaluation of the full implementation of the method (e.g. examination of sample or flotate, use of binocular) is desired. In terms of ring test management it is required to include the type of material used (flotate or raw material) in the evaluation of the results.
- It is recommended to evaluate further the effect of several method parameters (e.g. amount of sediment used for examination) because of large variation of application, which violates further harmonization.
- The implementation of the new method in the IAG ring test for animal proteins in 2014 needs further attention.
- The nature of TCP as currently on the market has to be explored further for the possible presence of recognisable bone fragments.

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Annex 1 Invitation letter

Dear colleague, Dear IAG member,

The IAG section Feeding stuff Microscopy organizes annually a ring test for the detection of animal proteins in animal feeds. As in previous years, the presidium of the IAG section Feeding stuff Microscopy and RIKILT have agreed to organize together the 2013 ring test for animal proteins under certain conditions.

On behalf of the IAG section Feeding stuff Microscopy, RIKILT will invite you for participation in this next ring test. The share in the costs of the 2013 ring test as asked from every participant will be a fee of € 200, which is the same as in the previous years.

Three or four samples will be send around late February or early March 2013. Also a questionnaire will be sent by E-mail. A time slot of four weeks is planned for the analyses of the samples by every participants This means that late March or early April all results are expected to be returned to RIKILT. Pooling and evaluation of the results will take place during April and May, and a preliminary report will be presented during the annual IAG meeting in Vienna (Austria) in June. After that, a final report will be made depending on the outcome of the discussions during the meeting. All communications of the evaluation will be fully anonymous.

If you are interested to participate in the ring test 2013 for animal proteins, please return the application form and make a payment of € 200 to RIKILT. For smoothing the administrative procedure, an invoice is already included with this letter. In case of participation, please hand this invoice over to your financial department, and make sure that the reference number, your name and your institute's name is mentioned. This information is necessary to avoid loss of payments that can not be linked to participating institutes.

We are looking forward to have a nice cooperation for the next ring test and to have results which will support your laboratory quality system.

On behalf of the IAG section Microscopy and the RIKILT organizing team,

L.W.D. van Raamsdonk

Annex 2 Basic instructions for the test procedure

IAG ring test 2013 animal proteins

Instructions for the IAG ring test

1 You have received a box with an introduction letter and four vials containing 40 grams of possibly contaminated animal feed. Please report the receipt of your package as soon as possible by E-mail to the address mentioned below.

2 The samples have to be analysed according to Annex 6I of Regulation (EC) 152/2009 from the European Union, modified by (EC) 51/2013. Comparable procedures can be found in the module Methods of the computer program ARIES. Take care to homogenise the content of each vial before taking the amount for analysis.

3 Reporting consists of the following steps:

3a Please fill in the questionnaire on the page "Procedure". Depending on your chosen method, different questions will show up.

Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.

Your unique lab number is mentioned in the introduction letter.

All the fields with a drop-down list have to be completed.

3b Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after your have entered it at the page Procedure. Enter yourself the four unique labels of the vials. There are separate fields for your examination of the sediment and of the flotation/raw material. Select "yes" from the drop-down list if fish or land animal material is detected, or "no" if the respective type of material is absent. You are free to give an estimation of the amount of material found. Please indicate the type of the materials found. More than one indication can apply, e.g. "bone and muscle".

All fields with a drop-down list have to be completed. Please add the exact sediment weight in milligrams, without a decimal sign.

4 After completing the two forms "Procedure" and "Results", they have to be sent to the organisers in two ways:

4a A print out of both forms have to be sent by Fax to RIKILT, Wageningen, the Netherlands. The FAX number will appear in the forms as soon as they are completed.

4b The forms have to be sent by E-mail as well. Save the Excel file by using "Save as …", add your unique lab code to the end of name (just before ".xls") and send the file to leo.vanraamsdonk@wur.nl.

4c Results will be included in the final analyses and report only if both forms are send in by FAX as well as by electronic mail, and after the proper receipt of the requested fee.

5 Direct any questions to leo.vanraamsdonk@wur.nl

6 Closing date is April 5th, 2013.

RIKILT Institute of food safety, Wageningen, the Netherlands

Annex 3 Report form for procedure details

Please complete at least all the cells with a drop down list that apply to your procedure	select your choice from a drop down list	type in your answer if necessary
IAG ring test 2013 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the ring test instructions?	-- select --	
Which detection method do you use?	Microscopy	
Please skip this line		
	-- select --	
Please continue here		
Please indicate your starting amount of material for sedimentation of FEED material	-- select --	
if other, please specify		
Indicate your glassware for sedimentation	-- select --	
if other, please specify		
Describe your sedimentation agent	-- select --	
if other, please specify		
Did you apply staining of the sediment (e.g. alizarin staining) as standard procedure?	-- select --	
Did you examine at lower magnifications (using a binocular)?	-- select --	
Indicate the size of cover glass	-- select --	
Please estimate the amount of sediment you have used for preparing the slide(s) (in %)		
Please describe your embedding agent for the sediment material	-- select --	
if other, please specify		
Did you use the expert system ARIES for identification of particles?	-- select --	

When estimating amounts: please indicate the f-factor used for fish meal please indicate the f-factor used for terrestrial animal meal		

Annex 4 Report form

Please complete at least all the cells with the presence of fish material and land animal material in both sediment and flotation/raw material for every sample

IAG ring test 2013 animal proteins

lab number				
sample number				
weight of sediment (in mg)				
Presence of fish material in sediment	-- select --	-- select --	-- select --	-- select --
If present, estimated amount (in %)				
If present, please indicate type of material (e.g. fish bone, scale, gill, cartilage)				
Presence of material of land animals in sediment	-- select --	-- select --	-- select --	-- select --
If present, estimated amount (in %)				
If present, please indicate type of material (e.g. bone, cartilage)				
Presence of material of vertebrates in flotation or in raw sample material	-- select --	-- select --	-- select --	-- select --
If present, estimated amount (in %)				
If present, please indicate type of material (e.g. muscle fibre, hair, feather, blood)				
Comment, if necessary				

Annex 5 Additional instructions

Mail send on March 12th 2013

Dear participant,

As communicated to you last week, analyses have to be carried out according to Regulation (EC) 152/2009, which is recently amended by Regulation (EC) 51/2013, effective of February 12th 2013. This Regulation can be found at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:020:0033:0043:EN:PDF .

The current procedure is renewed and more detailed than the previous version. Especially the application of a limit of detection (5 particles) is a new element. The implications for our new daily routine are yet not completely clear. In Chapter 1 of Annex 6 of Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 reference is made to Standard Operational Procedures. These are only available in draft at this moment.

It is, however, clear that every (positive) result should be reported to the competent authority. Chapter 2.1.5 of the mentioned Annex provides texts for reporting the different amounts of particles found in a sample. There are separate texts for the situation that 1-5 particles, or more than 5 particles are found, distinctive for fish and terrestrial animals. Since RIKILT can be considered to be the "competent authority" for the samples of this ring test, we ask you to report any particle found. In order to avoid any confusion, the report form asks about the "presence" or "absence" instead of the judgment "positive" or "negative".

You are free to indicate the number of particles found in the free cells of the report form. Be aware that when using less than 100% of the sediment the chance to found particles of animal origin will DEcrease accordingly. It could be considered to discuss these issues further during the IAG annual meeting in Vienna, if implications are expected for our daily work.

If any question arise please do not hesitate to contact me.

All the best with the analyses.

Kind regards,

Leo van Raamsdonk

Mail send on March 18th 2013

Dear participant,

As communicated to you previously, analyses have to be carried out according to Regulation (EC) 152/2009, which is recently amended by Regulation (EC) 51/2013, effective of February 12th 2013. This Regulation can be found at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:020:0033:0043:EN:PDF .

The existence of two Regulations (152/2009 and 51/2013) could be complicated. Reference to 152/2009 could suggest an indication to the old method. The official situation is that reference to Regulation (EC) 51/2013 is not correct, since this Regulation is only an amendment to Annex 6 of Regulation (EC) 152/2009 and not a replacement of the entire regulation. A replacement is not possible in this way, since 152/2009 consists of much more than only Annex 6. This means that the "old version" of 152/2009 does not formally exist anymore after February 12th. Reference to 152/2009 is a reference to the new procedure. This is extremely important for both legal as well as practical reasons. The Commission publishes Consolidated versions of Regulations that are amended regularly. The new Consolidated version of (EC) 152/2009 has yet to come.

So, there is only one procedure: the new one. **This new procedure has to be followed in the examination of the IAG samples, EXCEPT FOR THE REPORTING. The organisers of the IAG ring test want to know any amount of particles: one particle is "present". As already stated, you are requested to indicate the number of particles and the nature of it in the comment cells of the report sheet.**

We realise that the procedure is new to you and might be complicated. So, we grant you an extra week for the examinations. **The final date for submission of your results is therefore April 12, 2013.**
If any question arise please do not hesitate to contact me.

All the best with the analyses.

Kind regards,

Leo van Raamsdonk

Annex 6 List of participants

Institute	Country
Austrian Agency for Health and Food Safety-AGES	Austria
CRA-W	Belgium
FLVVT	Belgium
LFSAL	Belgium
Oleotest N.V.	Belgium
Laboratorium ECCA nv	Belgium
China Agricultural University	China
Croatian Veterinary Institute	Croatia
Central Institute for Supervising and Testing in Agriculture	Czech Republic
Danish Veterinary and Food Administration	Denmark
S.C.L. Laboratoire de Rennes	France
IDAC	France
IPL Atlantique	France
WESSLING GmbH	Germany
Agri Q-service GmbH	Germany
Universität Hohenheim, LA Chemie (710)	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
CVUA-RRW	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	Germany
LTZ Augustenberg	Germany
LUFA-Speyer	Germany
Thüringer Landesanstalt für Landwirtschaft	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
Landesuntersuchungsamt für Chemie, Hygiene und Veterinärmedizin	Germany
LUFA Rostock	Germany
Landeslabor Berlin-Brandenburg	Germany
SGS Germany GmbH	Germany
Futtermittelinstitut Stade (LAVES)	Germany
LUFA Nord-West	Germany
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Equine Centre	Ireland
Inst. Zooprofilattico Sperimentale delle Venezie	Italy
Inst. Zooprofilattico Sperimentale della Sardegna	Italy
Istituto Zooprofilattico Sperimentale Abruzzo & Molise "G. Caporale"	Italy
Ist. Zooprofilattico Sperimentale delle Lombardia e dell'Emilia Romagna	Italy
Istituto Zooprofilattico della Sicilia	Italy
IZS PLV Torino - CReAA	Italy
CCL - Nutricontrol	Netherlands
Eurofins Food Testing	Netherlands
MasterlabBV	Netherlands
Nofima Ingredients	Norway
International Analytical Services SAC	Peru
Cargill Poland	Poland
Instytut Zootechniki PIB, Pracownia w Szczecinie	Poland
Lab. Regional de Veterinária	Portugal
Laboratório Nacional de Investigação Veterinária INRB, IP	Portugal
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
Trouw nutrition Espana	Spain
Dirección General de Produccion Agropecuaria, Laboratorio Agrario Regional	Spain
SVA	Sweden
Agroscope (ALP), Swiss Research Station	Switzerland
CPF(Thailand) Public Company Limited	Thailand

Annex 7 Details of procedures applied, microscopic method

Lab nr	Amount Feed	Glassware	Type	Agent	Staining	Binocular	Size	Sed. used	Embedding	ARIES	F-factor
3	10	conical glass with cock	top/bottom	TCE	no	yes	small	50%	paraffin oil		
5	5	conical glass with cock	top/bottom	chloroform	no	no		-		no	
6	10	chem.sed.funnel	top/bottom	TCE	no	yes	large	1%	paraffin oil	no	
7	10	conical glass with cock	top/bottom	TCE	no	yes	small	50%	glycerin	no	
8	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	25%	immersion oil	no	40%
9	10	conical champagne glass	top	TCE	no	yes	small	60-80%	glycerin	no	
10	10	beaker (flat bottom)	top	TCE	no	yes	small	100%	immersion oil	no	
11	10	conical glass with cock	top/bottom	TCE	no	no	medium	60%	immersion oil	no	
12	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	50%	immersion oil	no	60%
13	10	conical glass with cock	top/bottom	TCE	no	no	small	25%	immersion oil	yes	
14	10	chem.sed.funnel	top/bottom	TCE	yes	no	small	15%	Norland	no	
15	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	10%	paraffin oil	no	
16	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	50%	paraffin oil	no	60%
17	10	chem.sed.funnel	top/bottom	TCE	yes	no	small	-	glycerin	no	40%
18	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	50%	Norland	no	
19	10	chem.sed.funnel	top/bottom	TCE	no	yes	large	50%	glycerin	no	
20	10	conical champagne glass	top	TCE	no	yes	small	-	glycerin	no	
21	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	50%	glycerin	no	
22	10	conical champagne glass	top	TCE	no	yes	small	30%	paraffin oil	no	
23	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	-	glycerin	yes	
24	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	60%	Norland	no	
25	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	60%	glycerin	no	
26	10	conical glass with cock	top/bottom	TCE	yes	yes	small	20%	immersion oil	no	25%
27	10	chem.sed.funnel	top/bottom	TCE	yes	no	small	15%	Norland	no	50%
28	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	90%	glycerin	no	
29	10	beaker (flat bottom)	top	TCE	no	no	small	100%	glycerin	no	50%
30	10	conical glass with cock	top/bottom	TCE	no	no	small	100%	paraffin oil	no	
31	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	-	glycerin	no	
32	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	-	immersion oil	yes	40%
33	10	chem.sed.funnel	top/bottom	TCE	no	yes	medium	25%	paraffin oil	no	60%
34	10	conical champagne glass	top	TCE	yes	yes	small	100%	glycerin	no	
35	10	conical champagne glass	top	TCE	yes	yes	small	10%	glycerin	no	
36	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	50%	paraffin oil	no	

Lab nr	Amount Feed	Glassware	Type	Agent	Staining	Binocular	Size	Sed. used	Embedding	ARIES	F-factor
37	10	chem.sed.funnel	top/bottom	TCE	yes	yes	small	5%	glycerin	no	
38	10	conical champagne glass	top	TCE	no	yes	large	50-100%	paraffin oil	no	50%
39	10	chem.sed.funnel	top/bottom	TCE	no	yes	large	50%	glycerin	yes	
40	10	conical champagne glass	top	TCE	no	yes	large	10%	immersion oil	no	
41	5	conical glass with cock	top/bottom	TCE	yes	yes	large	100%	paraffin oil	no	
42	10	chem.sed.funnel	top/bottom	TCE	yes	yes	medium	100%	immersion oil	no	
43	5	beaker (flat bottom)	top	TCE	no	yes	small	30%	immersion oil	no	
44	10	mensur		TCE	no	yes	large	100%	mineral oil	no	
45	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	9-38%	paraffin oil	no	
46	10	chem.sed.funnel	top/bottom	TCE		yes	large	100%	paraffin oil	yes	
47	25	beaker (flat bottom)	top	TCE	no	yes	medium	50%	paraffin oil	no	55%
48	10	beaker (conical bottom)	top	TCE	no	no	small	30.0%	paraffin oil	no	40%
49	40	chem.sed.funnel	top/bottom	TCE	no	yes	small	-	paraffin oil	no	
50	5	beaker (flat bottom)	top	aceton	no	yes	medium	15%	paraffin oil	no	
51	10	conical glass with cock	top/bottom	TCE	no	yes	small	-	glycerin	no	
52	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	-	glycerin	no	
53	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	-	immersion oil	no	
54	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	-	glycerin	no	
55	10	chem.sed.funnel	top/bottom	TCE	yes	no	large	90%	glycerin	no	40%
56	10	chem.sed.funnel	top/bottom	TCE	no	yes	small	90%	immersion oil	no	

Annex 8 Results: presence of animal proteins in sediment and in flotate or raw material, microscopic detection

Lab nr	Sample numbers				Fish				MBM				flotation			
					A	B	C	D	A	B	C	D	A	B	C	D
3	51	97	203	04	no	yes	no	no	no	no	yes	yes	no	yes	no	no
5	141	222	88	299	no	yes	no	no	no	no	yes	no	yes	yes	yes	yes
6	91	137	208	39	no	yes	no	no	no	no	yes	no	yes	yes	yes	yes
7	296	282	163	254	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
8	46	142	33	249	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
9	216	27	248	104	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
10	241	177	43	259	no	yes	no	no	no	no	yes	yes	no	yes	no	no
11	56	32	218	74	yes	yes	no	no	yes	no	yes	yes	no	yes	no	no
12	11	17	243	24	no	yes	no	no	no	no	yes	yes	no	yes	no	no
13	266	92	128	174	no	yes	no	no	no	no	yes	yes				
14	291	07	258	34	no	yes	no	no	no	no	yes	yes	no	yes	no	no
15	191	12	23	239	no	yes	yes	no	no	no	yes	yes	no	yes	yes	no
16	221	37	283	179	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
17	01	57	253	29	no	yes	no	no	no	no	yes	yes	no	yes	no	no
18	161	172	118	224	no	yes	no	no	no	no	yes	yes	no	yes	no	no
19	166	62	28	269	no	yes	no	no	no	no	yes	yes	no	no	no	no
20	131	202	78	289	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
21	86	147	93	119	no	yes	no	no	no	no	yes	yes	no	yes	no	no
22	16	192	08	194	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
23	206	102	263	19	no	yes	no	no	no	yes	yes	yes	no	yes	no	no
24	236	22	68	124	no	yes	no	no	no	no	yes	yes	no	yes	no	no
25	36	247	73	209	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
26	196	127	288	169	no	yes	no	no	yes	no	yes	yes	no	no	no	no
27	246	132	213	264	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
28	201	167	18	59	no	yes	no	no	yes	no	yes	yes	no	no	no	no
29	26	252	198	14	no	yes	no	no	no	no	yes	yes	no	yes	no	no
30	31	272	158	54	no	yes	no	no	no	no	yes	yes	no	yes	yes	yes
31	156	67	153	149	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
32	96	257	143	09	no	yes	no	no	no	no	yes	yes	no	yes	yes	yes
33	81	47	123	279	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
34	76	112	108	84	no	yes	yes	no	no	no	yes	yes	no	yes	no	no
35	171	107	223	164	no	yes	no	no	no	no	yes	yes	no	no	no	no
36	176	152	98	199	no	yes	no	no	no	no	yes	yes	no	yes	no	no
37	41	277	13	144	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
38	126	52	113	139	no	yes	no	no	no	no	yes	yes	no	yes	no	no
39	116	292	83	189	no	yes	no	no	no	no	yes	yes	no	yes	yes	yes
40	211	227	38	109	no	yes	no	yes	no	no	yes	yes	no	no	no	yes
41	151	42	228	294	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
42	71	02	188	214	no	yes	no	no	no	no	yes	yes	no	yes	yes	no
43	06	197	63	154	no	yes	no	no	no	no	yes	yes	no	yes	no	no
44	101	287	58	99	no	yes	no	no	no	no	yes	yes	no	yes	yes	yes
45	286	72	273	69	no	yes	no	no	no	no	yes	yes	no	yes	no	no
46	66	162	183	244	no	yes	no	no	no	no	yes	yes	no	no	no	no
47	261	87	233	129	no	yes	no	no	no	no	yes	yes				
48	21	187	193	79	no	yes	no	no	no	no	yes	yes	no	yes	yes	yes
49	281	157	138	114	yes	no	no	no	no	no	yes	yes	no	yes	yes	no
50	111	297	48	159	no	yes	no	yes	no	no	yes	no				
51	276	77	53	204	no	yes	no	no	no	no	yes	yes	no	yes	no	no
52	106	122	173	184	no	yes	no	no	no	no	yes	yes	no	yes	no	no
53	146	117	03	64	no	yes	no	no	no	no	yes	yes	no	yes	no	no
54	271	82	148	49	no	yes	no	no	no	no	yes	yes	no	yes	no	yes
55	121	217	103	229	no	yes	no	no	no	no	yes	yes	no	yes	no	no
56	256	212	168	134	no	yes	no	yes	no	no	yes	yes	no	yes	yes	no

Annex 9 Results: sediment and quantification

Lab nr	Amount of sediment (mg)				Sediment (mg/g)			
	A	B	C	D	A	B	C	D
3	127	170	148	156	12.7	17.0	14.8	15.6
5								
6	114	163	122	124	11.4	16.3	12.2	12.4
7	82	127	119	120	8.2	12.7	11.9	12.0
8	193	224	136	170	19.3	22.4	13.6	17.0
9	106	160	118	122	10.6	16.0	11.8	12.2
10	70	110	110	90	7.0	11.0	11.0	9.0
11	127	146	142	130	12.7	14.6	14.2	13.0
12	149	173	141	155	14.9	17.3	14.1	15.5
13	150	160	130	130	15.0	16.0	13.0	13.0
14	131	191	142	147	13.1	19.1	14.2	14.7
15	577	577	577	577	57.7	57.7	57.7	57.7
16	106	141	106	115	10.6	14.1	10.6	11.5
17	73	107	57	75	7.3	10.7	5.7	7.5
18	134	173	133	142	13.4	17.3	13.3	14.2
19	185	209	186	202	18.5	20.9	18.6	20.2
20	148	193	156	155	14.8	19.3	15.6	15.5
21								
22	130	150	150	120	13.0	15.0	15.0	12.0
23	81	96	92	79	8.1	9.6	9.2	7.9
24	163	189	134	138	16.3	18.9	13.4	13.8
25	188		197		18.8	0.0	19.7	0.0
26	99	100	87	83	9.9	10.0	8.7	8.3
27	117	154	150	144	11.7	15.4	15.0	14.4
28	113	159	116	125	11.3	15.9	11.6	12.5
29	160	170	130	140	16.0	17.0	13.0	14.0
30	85	67	87	66	8.5	6.7	8.7	6.6
31								
32	170	180	140	190	17.0	18.0	14.0	19.0
33	131	182	152	139	13.1	18.2	15.2	13.9
34	140	160	160	140	14.0	16.0	16.0	14.0
35	113	124	93	98	11.3	12.4	9.3	9.8
36	100	126	125	96	10.0	12.6	12.5	9.6
37	206	225	194	225	20.6	22.5	19.4	22.5
38	140	193	195	189	14.0	19.3	19.5	18.9
39	135	191	114	150	13.5	19.1	11.4	15.0
40	152	161	154	135	15.2	16.1	15.4	13.5
41	140	176	168	172	28.0	35.2	33.6	34.4
42	131	162	142	115	13.1	16.2	14.2	11.5
43	76	101	81	76	15.2	20.2	16.2	15.2
44	261	402	385	352	26.1	40.2	38.5	35.2
45	242	289	271	273	24.2	28.9	27.1	27.3
46	112	167	155	104	11.2	16.7	15.5	10.4
47	255	351	254	326	10.2	14.0	10.2	13.0
48	168	146	122	105	16.8	14.6	12.2	10.5
49	580	500	549	537	14.5	12.5	13.7	13.4
50								
51	142	184	161	124	14.2	18.4	16.1	12.4
52	124	146	126	117	12.4	14.6	12.6	11.7
53	160	170	130	135	16.0	17.0	13.0	13.5
54	48	94	115	173	4.8	9.4	11.5	17.3
55	129	171	150	192	12.9	17.1	15.0	19.2
56								

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