BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

PROJECT DELIVERABLE

Collaborative project	Large-scale integrating project
Project acronym	BASELINE
Project title	Selection and improving of fit-for-purpose sampling procedures for specific foods and risks
Grant Agreement number	222738
Date of latest version of Annex I	05/07/2010

Del No	Deliverable name	WP no	Lead participant	Nature	Dissemination level	Due delivery date
D 1.3	Final sampling schemes proposed for studied seafood categories	1	NVI	R	PU	43

Delivery Date: 07/05/2013

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Project funded under the Food, Agriculture and Fisheries, and Biotechnology theme (KBBE)

SEVENTH FRAMEWORK PROGRAMME

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SUMMARY

The deliverable describes sampling schemes for fish and shellfish products based on the studies in BASELINE. These studies are built on our previously reported review about current sampling schemes (D1.1) and suggested safety criteria for specific risk agents in seafood (D1.2). Sampling plans described as parts of performance objectives for fresh salmon and cold smoked salmon in D1.2 are briefly described here,

Sampling programs often are carried out with fewer samples per batch than the official sampling plans indicate. This is probably due little awareness of the in-homogenous distribution of risk agents in a batch, and that analysis of many samples is expensive. Two ways to obtain improved and more cost effective sampling may be to 1) make sure that representative samples are selected and 2) to use pooled samples.

The distribution of risk agents in seafood is related to the contamination route. Pathogens like *Listeria monocytogenes* may be absent in the majority of fish samples and present in very low levels in only a few samples immediately after slaughtering and filleting. However, during storage, the levels in the positive samples may have become high. In terms of food safety, it is the few positive samples that are important to detect. *Listeria* is introduced to the fish flesh from processing equipment or from water, fish skin or gills through wounds while the fish is still alive. Unlike fish, shellfish accumulate high amounts of virus, *Vibrio* spp and pollutants from the water if the area is contaminated, because shellfish filter a lot of water. Sampling of a shellfish growing area may therefore be more important than to analyse the shellfish after harvest. Pooled samples of shellfish can be used for mapping areas. Tuna and other top predators accumulate mercury compounds from their diet, i.e. smaller fish which has accumulated the compounds from the plankton in their diet. The mercury levels do not decrease in the fish flesh after catch. Old/large tuna do usually contain more mercury compounds than young/small tuna, but there are differences between species. Also for tuna, pooled samples seem to be a useful way to obtain more cost effective sampling.

Sampling schemes suggested in the present report are developed based the performance objectives for risk agents described in our deliverable 1.2, new experiments and literature reviews about the distribution of risk agents in seafood. In cases where analytical methods are missing (Virus) or the detection levels in established methods are too high for the purpose (*Listeria*), new protocols have been developed and tested

MAIN FINDINGS AND CONCLUSIONS

Listeria in fish and fish products

Performance objective to ensure the food safety objective of fresh salmon intended used raw as it is or as sushi or cold-smoked salmon (defined as "*Listeria* levels not above 100 cfu/g at the last day of shelf life") can be set up at the fresh salmon processing step using a combination of *Listeria* limit values in the range 2-10 cfu/g, the intended use of the salmon, and the expected time-temperature conditions during storage. Performance objectives and sampling plans to identify batches or production companies with the same or higher contamination levels than in the samples analysed in BASELINE have also been developed: for fresh salmon, a high reliability can be obtained by testing 7 samples, using -0.976 log cfu/g as performance objective and rejection criterion. For cold-smoked salmon, absence/25 g in 88.8% samples of lot is a suitable performance objective. The sampling plan at retail (microbiological criteria) to achieve this PO is n=6, absence/25 g. Differentiated sampling plans and rejection criteria can be set up for different analysis according to their detection levels.

The sampling plans above were presented in D1.2 and based on the distributions of *Listeria* in naturally contaminated samples. The studies and conclusions presented in the present deliverable, are focused on systematic variations within a batch of company, analytical methods with improved sensitivity and procedures for sampling of pooled samples.

The prevalence and levels of *L. monocytogenes* in fish sent from the producer vary with the time of the day it has been processed. The levels are very low immediately after processing, but increase during storage. Most of the bacteria seem to remain attached to the positive samples, but a fraction is transferred to the samples in direct contact with the positive samples and the samples below. Water released from the muscle and melting ice seem to be the main contamination route from positive to originally negative samples in a box.

A sampling protocol for harvesting *Listeria* from large samples or pooled subsamples has been developed. Fish samples are washed with a growth medium for *Listeria* and the liquid fraction is collected and analysed. The protocol gives reliable and rapid detection of extremely low levels of *Listeria* in batches even if only 1 of 10 pieces is contaminated as low as 2 cfu/g. The protocol can be adapted to various detection levels and POs suitable for fresh fish, double-frozen fish, other fish species, raw material control for fish intended is used for production of sushi, carpaccio and cold smoking.

Two protocols for detection of low levels of *L. monocytogenes* have been successfully developed. One is an adaptation of the ISO method, giving detection level 2 cfu/g. This method is currently being validated by the European reference laboratory for *Listeria*. The other is a MPN method derived from the ISO method and has detection level 1 cfu/5 g of fish.

Virus in shellfish

Appropriate and user-friendly analytical methods for monitoring viruses are lacking. A PCR method has in-house validated in BASELINE with very good results. Even though the study is not directly related to sampling schemes, we consider this study a valuable contribution to remove a bottleneck for development of sampling schemes for virus in shellfish in the future.

Biotoxins in shellfish

Okadeic acid has been used as model toxin in these studies. Knowledge about the distribution of this toxin has been obtained from reviews of literature and earlier monitoring programs. The impact of

sample size and number of samples has been assessed using the Whitaker method. It is found that the best fit sampling plan is taking two samples of 30-40 samples each.

Mercury compounds in tuna

As for toxins, a lot of data about levels and distributions of mercury compounds are available in the literature. These have been collected and assessed using models. In conclusion, pooled samples from 10 tunas seems to be the best trade-off between reliability and costs for all fresh species except albacore tuna which one pooled sample from 5 tunas is sufficient and skipjack tuna which one pooled sample from 2 tunas is the best sampling plan. For canned tuna, pooled samples with 5 cans seem to be the best trade-off between reliability and costs.

INTRODUCTION AND SCOPE OF THE DELIVERABLE

The deliverable describes sampling schemes for fish and shellfish products developed based on the studies in BASELINE. These studies are built on our previously reported review about current sampling schemes (D1.1) and suggested safety criteria for specific risk agents in seafood (D1.2).

The following seafood matrix-risk agent combinations are studied in BASELINE:

Table 1 Selection of product/risk combinations to study in WP1.

Products	Type of Product	Biohazards	Partners involved	Chemical Risks	Partners involved
Fresh salmon (farmed)	High volume product	*Listeria monocyotgenes*	NVI, Nordlaks		
Smoked salmon	(minimally) processed food	*Listeria monocyotgenes*	NVI, UN, TEAGASC		
Sushi with salmon	New trend products Minimally processed product	*Listeria monocyotgenes*	NVI		
Minced products – surimi, fresh and frozen	Highly processed products	*Listeria monocyotgenes*	UN		
Double frozen salmon	High volume product	*Listeria monocyotgenes*	NVI		
Sea bass (farmed)	High volume product	*Listeria monocyotgenes*	VETFAC		
Tuna (wild caught)	High volume product (South of Europe) Niche product (North of Europe)			MeHg Mercury	UBO
Shellfish	High volume product Minimally processed product	*Virus*	ISS	Phyco toxins Phyto toxins, limited to algal toxins	NVI UBO
		Vibrio	ISS VETFAC NVI		

The present deliverable describes studies leading to sampling schemes suggested for all combinations except for *Listeria* in smoked salmon and minced products, as the one for smoked salmon was given already in deliverable 1.2 as a part of the development of safety criteria, and an alternative scheme based on HACCP control will be described for surimi in deliverable 1.5. However, the sampling scheme for fresh and cold smoked salmon is given here in order to give a more complete picture. Further, no

sampling scheme for virus and *Vibrio* in shellfish is described, as suitable analytical methods have to be developed and official safety criteria or limit values have to be developed first.

Previous studies in BASELINE WP1 have revealed that current sampling schemes are useful, but also that analysis of a few samples may give a false picture of the real contamination levels. The latter is partly due to that risk agents may be unevenly distributed in a batch. Pathogens like *Listeria monocytogenes* may be absent in the majority of samples in a batch, but present in unacceptably high levels in a few samples. In terms of food safety, it is the few positive samples that are important to identify and detect. The main challenge in sampling is therefore to select representative samples. The challenge is the same for other risk agents that introduced to during processing or transferred from water, skin or gills through wounds while the fish is still alive. Contamination routes like these usually lead to very low levels of risk agent shortly after processing, far below the levels compromising the food safety. Our previous studies of *L. monocytogenes* (D1.2) have indicated that analyses based on detection may lead to a significant loss of fish containing only insignificant traces of Listeria, due to very low detection level in the analytical method. On the other hand, if the same samples are analysed using current methods for quantitative analyses, samples that actually may contain levels compromising food safety at the end of shelf life may actually not be found due to the quantitative methods lack of sensitivity.. Therefore, the detection level of the quantitative method has to be taken into account by selection of sampling schemes, and methods/protocols with appropriate detection levels have to be developed. We report such studies in the present deliverable.

Many risk agents are introduced to the seafood while they are in the sea. Shellfish may accumulate high amounts titres of virus, *Vibrio* spp, toxins and pollutants from the water because they filter a lot of water. A polluted or contaminated area is therefore likely to contain shellfish with high levels of risk agents. The concentrations of risk agents may vary within a shellfish farm, but it is unlikely that the majority of samples are negative while a small fraction is heavily contaminated. Analytical methods based on detection are therefore sufficient in most cases, but there are other challenges. The current quality control of shellfish is based on measured levels of the indicator bacterium *E. coli*, assuming that contamination is related to pollution from humans and animals. *Vibrio*, algae toxins and chemical pollutants, however, may be present in the sea even though there is no pollution from humans or animals in the area. So, as described in D1.2, *E. coli* is not a good indicator for these risk agents. The challenge is that alternative indicators are hard to find, and even worse, that appropriate analytical methods for agents like viruses are missing. For *Vibrio*, there are analytical methods, but the risk assessments needed to set up safety criteria and develop sampling schemes are still ongoing. It is not obvious whether sampling should be based on *Vibrio* spp in general or *Vibrio* possessing pathogenicity markers. In our deliverable 1.2, we suggest that pathogenicity markers are the most useful approach in areas with high prevalence of *Vibrio* in order to avoid large amount of unnecessary waste, while *Vibrio* spp will be a good approach in areas with low prevalence, as the amount of waste in these areas will be minimal anyway. However, such judgments are up to the food authorities. In this deliverable, we do therefore not describe new sampling plans for virus and *Vibrio*, but do report validation of an analytical method for virus.

Toxins in shellfish originate from algae in the water. Different algae may prefer different light and oxygen conditions, and therefore be unevenly distributed within a shellfish farm. In the present deliverable, we report suitable sampling sizes of pooled samples of shellfish. Passive samplers may be an easier way to collect samples from different water depths and water in the surroundings of a shellfish farm than to collect many shellfish samples. Use of passive samples will be reported in our deliverable 1.4.

Tuna and other top predators accumulate mercury compounds from their diet, i.e. smaller fish which have accumulated the compounds from the plankton in their diet. The mercury compounds are inert and do not increase or decrease in the fish flesh after catch. In the present deliverable, the samples sizes of pooled samples have been optimised, both for fresh tuna and canned tuna. As accumulated mercury compounds in tuna are not released from the body, indirect sampling based on fish size and species is also possible. We have considered this aspect in BASELINE, and will report them in deliverable 1.5.

LISTERIA MONOCYTOGENES IN SALMON, SALMON PRODUCTS AND SURIMI (NVI, UN, VETFAC, NORDLAKS)

Introduction

It is well known that fresh salmon occasionally contain *Listeria monocytogenes* and that salmon may be exported, re-exported and bought by several stakeholders and processors before final sale to the consumers (for instance processor-exporter-importer-secondary processor-retailer-consumer). *Listeria* will grow after the fish has left the fresh fish producer, and therefore PO limit value for *L. monocytogenes* at the fresh fish level at 10 cfu/g or higher will not be sufficient to ensure that the maximum limit on the last day of shelf life, 100 cfu/g, is not exceeded. The consumption of raw fish, for instance as sushi, is increasing and therefore the limit value for *Listeria* given for ready-to-eat foods in the Food Law (regulation 2073/2005) has to be considered also for fresh salmon and double-frozen salmon. Our previous studies (see deliverable 1.2), indicates that a levels of *Listeria* in fresh fish by slaughtering and filleting should not exceed 1-8 cfu/g, depending on the intended use.

The detection level in the ISO standard methods and most other methods used for quantitative analyses today is 10 cfu/g. A lower detection level is therefore needed to develop and implement a useful PO. If adaptation of quantitative analytical methods would not be possible, three alternatives could be

- no PO at all,
- the PO would be absence of *L. monocytogenes* in fish (zero tolerance), measured with qualitative analyses with detection level (1 cfu/25 grams), or
- A three-class attribute sampling plan, where some positive samples using the qualitative method (detection level 1 cfu/25 grams) are accepted, and the levels estimated using an MPN approach.

Alternative 1 and 2 are poor alternatives, because it is well known in the fish trade that *L. monocytogenes* will be detected sporadically, even under ideal production and process conditions. No PO at early processing stages is a poor alternative also from the FBOs point of view. The way from the slaughterhouse to the consumer may go through several countries. A PO at an early stage in processing is the only way the FBOs can carry out a risk based quality control for their customers. Alternative 3 is possible with existing methods if a high number of samples is analysed per batch, but as analyses are costly, this is not a desired alternative.

In order to ensure sampling efficiency and to increase the probability of detection of *Listeria* in salmon samples after production, quantitative methods with lower detection levels are needed. Further, selection of representative samples, or better, the samples being likely to contain the highest levels of *Listeria*, is desired. We have addressed these challenges in BASELINE.

Sampling plans for fresh and cold smoked salmon, developed as a part of performance objectives described in D1.2)

Naturally contaminated fresh salmon from a company and cold smoked salmon from retail were analysed and the distributions of *Listeria* estimated Performance objective to ensure the food safety objective of fresh salmon intended used raw as it is or as sushi or cold-smoked salmon (defined as "*Listeria* levels not above 100 cfu/g at the last day of shelf life") can be set up at the fresh salmon processing step using a combination of *Listeria* limit values in the range 2-10 cfu/g, the intended use of the salmon, and the expected time-temperature conditions during storage. Performance objectives and

sampling plans to identify batches or production companies with the same or higher contamination levels than in the samples analysed in BASELINE have also been developed: for fresh salmon, a high reliability can be obtained by testing 7 samples, using -0.976 log cfu/g as performance objective and rejection criterion. The sampling plan can be adapted to various intended uses and time-temperature storage scenarios. For further details, see D1.2.

For cold smoked salmon at retail level, it was found that 6 samples are needed to reject the lot at 95% CL (or to accept it at 5% CL). The sampling plan at retail was formulated as shown in Table 2, for further details, see D1.2.. This proposal suggests include one more sample than the established in the current European legislation (EC Nº 2073/2005). In order to obtain an efficient and cost-effective sampling plan, we have worked on pooling samples, with positive results when 1 out of 6 samples is contaminated with <10 cfu/25 g of *L. monocytogenes*.

Table 2. Suggested sampling plan for *L. monocytogenes* in cold-smoked salmon and trout.

Analysis	Standard/guideline				Assessment		Sampling plan calculations	Sampling stage
	N	c	m	M	Satisfactory	Unsatisfactory		
L. monocytogenes smoked salmon/trout	6	0	0	NA	Not detected	Present	Based on enrichment procedure (25g sample)	at retail

Quantitative analytical methods with improved sensitivity.

Adapted ISO and NMKL method. Enumeration on agar plates of less diluted samples (NVI, UN, NORDLAKS).

The detection level in the established ISO 11290-2 and NMKL 136 methods for quantitative analysis of *Listeria* is 10 cfu/g. This is the limit because the samples are homogenised with 9 parts of diluents, and 0.33 ml is spread on 3 agar plates. The sensitivity can be improved by increasing the number of agar plates, but as *Listeria* medium is very expensive, this is not a cost-effective solution. During discussions between NVI and NORDLAKS (see WP8, Task 8.2), it was clear that the fish industry needs an internationally accepted method to implement POs. Our first approach was therefore to adapt the existing ISO and NMKL methods by homogenising the samples with less diluents.

Salmon, 100 g pieces, were inoculated with 1-5 cfu/g of *L. monocytogenes*, either on the surface or injected inside the fish flesh, and stored at 4°C. After 2 hours, each of the fish pieces was added 100 ml liquid (half Fraser broth or physiological salt water), and homogenised. This dilution procedure gives a theoretical detection level of 2 cfu/g, provided that the homogenised mixture really becomes homogenous and can be pipetted and distributed on agar plates. The results showed that salmon flesh easily homogenized in the diluents, leading to a homogenous and pipettable solution, though with a high viscosity. Analyses on ALOA medium (ISO and NMKL method) gave correct quantification down to 1 cfu/ml, i.e. 2 cfu/g. The method was also tested with good results on naturally-contaminated salmon, double-frozen salmon, and inoculated sushi.

UN carried out further trials with inoculated samples. According to the protocol developed by NVI, UN investigated whether different seafood matrixes (smoked salmon, crabstick, young eels) could be homogenised with a smaller volume of diluents. In addition, some vegetable matrices selected in

BASELINE project were included (in collaboration with WP5: ready-to-eat lettuce and cabbage). All analysed products provided a homogenised mixture that can be pipetted and distributed on agar plates (with the exception of one sample of smoked salmon where the homogenised sample had a high viscosity). Analyses on ALOA medium (ISO method) gave correct quantification down to 2 cfu/g and in the 97% of the inoculated samples (2-3 cfu/g) the method was satisfactory (only 3 samples were negative, result <2 cfu/g).

The results from NVI and UN were presented at the annual workshop for European reference laboratories for *Listeria* at ANSES, France in April in 2012. Our protocol is currently in their validation program, and will possibly be included the next version of the ISO method. Further testing will be carried out in collaboration with reference laboratories in Europe, with the intention to develop a horizontal method, not only a method for seafood.

In addition, this procedure has been proposed in the second edition of the standard ISO 6887-1, which is currently under discussion to replace the current edition (ISO 6887-1:1999. *Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 1: General rules for the preparation of the initial suspension and decimal dilutions)*. In this second edition the following sentence is included: "In other cases where low numbers of microorganisms are sought and the product being tested will produce a suitable liquid initial suspension, it may be desirable to prepare the suspension at lower ratios (e.g. 1:2 or 1:5). However, this approach shall be used with caution and validated on a case-by-case basis (e.g. inhibition of microbial growth by the increased concentration of food components).

Quantification using MPN method (NVI)

The MPN approach is another way to lower the detection level, but it is time consuming and there is the need for additional equipment. In discussions with industry, it came up that they would prefer to have an in-house method for rapid control with a relevant detection level. In-house analysis of *Listeria* by a food producer is not recommended, as the risk of spreading the pathogen is large, and dealing with pathogens also requires special facilities and skills. On the other hand, it should be considered that temperature abuse during transport of samples may lead to overestimation of *Listeria* levels. We tried to combine these factors, and designed a protocol derived from the ISO and NMKL method using a Quantitray™ (IDEXX), which is a tray where a solution is distributed in wells and sealed (Figure 1). If the sample is placed in a Quantitray in the company, growth of *Listeria* during transport to the laboratory will not influence the quantification, because the quantification is based on the number of positive and negative wells.

In the lab, the wells in the quantitray can be analysed for *Listeria* by spotting on specific media and confirmed according to the ISO or NMKL method, or other specifications. The analysis may be time consuming, but can be at least partly automated as the aluminum foil can be easily penetrated by needles. The number of agar plates needed for confirmation is limited, as approximately 25 wells can be tested on one plate.

The protocol has been tested in several experiments using inoculated and naturally-contaminated fish, with good results. The work is still on-going, but it is clear that the detection level can be as low as 1 cfu of *Listeria monocytogenes* per 5 g of fish. The developed MPN protocol is promising both in terms of low detection level, user-friendliness, accurate enumeration, possibility of sampling in industry but incubation and confirmation in external laboratories. Negative samples are correctly identified after 2 days, while presumptive positives require another 1-2 days. Still, this is more rapid than other methods, and no method we are aware of can give accurate enumeration for contamination below 1 cfu/g.

Systematic variation of *Listeria* within a batch (NVI, NORDLAKS)

a. Systematic variations in naturally contaminated salmon

We reported in deliverable 1.2 the distribution of *Listeria monocytogenes* in 8 batches of naturally contaminated salmon. *Listeria* population levels and prevalence varied on day 0 and after 7 and 14 days of storage. At day 0, only 1 of 80 samples (10 analysed per batch) contained more than 10 cfu/g, but 28 samples contained detectable levels (1 cfu/25 g) of *L. monocytogenes*, indicating a prevalence of approximately 36 %. On day 7 and 14, on the other hand, 80 new samples were analysed, and 37 and 51 samples contained detectable or quantifiable levels *of L. monocytogenes*, indicating a prevalence of 46 and 64 %, respectively. These results means that while it is possible that some of the results from day 0 were false negative for *L. monocytogenes,* it is much more likely, that the sub-sample did not contain *L. monocytogenes*. The latter is possible, as the samples were 150 g each, while only 25 g was analysed.

Three of the analysed batches were morning batches, while three others were "late day batches" the same day (here called evening batches). Morning batches had in all three cases a greater prevalence of *L. monocytogenes* than evening batches. All *L. monocytogenes* populations in samples from morning batches were higher than in samples from evening batches at day 7 and 14 of storage (results not shown). This observation indicates that the reservoir of *L. monocytogenes* in the factory develops overnight, and further, that evening batches of fish are likely to contain less *Listeria* than morning batches.

It should be noted that we stored the fish under chilled conditions (4 +/-1°C) in our experiments, while the company sell their fish frozen. The *Listeria* levels in the fish reaching the consumer are therefore not as high as we have measured here. Furthermore, the results presented here are from only one company. The systematic variations may be different in other companies as *Listeria* may form other reservoirs in other companies.

b. Spread of Listeria between salmon cubes

The results described above indicate that *Listeria* may be present in very low levels, or unevenly distributed. In order to develop improved sampling schemes for detection of positive samples in batches with unevenly distributed *Listeria*, it was studied how *Listeria* spreads within a batch. The studies were carried out in small scale only.

Salmon cubes inoculated with 100-1000 cfu/g of *L. monocytogenes* were placed in horizontal and vertical rows together with *Listeria*-free salmon cubes, and the *Listeria* levels measured in each of the pieces during storage. In the case of horizontal rows, presence and growth of *Listeria* was observed in the inoculated cubes and in two pieces on each side after 7 days of storage at 4ºC. In columns, on the other hand, presence and growth was observed in all cubes below the inoculated cube, but not above. The released water on the bottom of the column contained detectable amounts of *Listeria* already after a few days of storage. *The results indicate that Listeria spreads downwards, and to some extent to the sides in the batch*. From a practical point of view, the results indicate that samples on the top of a box of salmon are more likely to be negative for *Listeria* than samples near the bottom. Furthermore, it appears that sampling the released water may be a useful way to collect samples from a large amount of fish, and to analyse whether some of the fishes in the box are highly contaminated.

Detection of unevenly distributed contamination (NVI, NORDLAKS)

The results above indicate that local contamination of *Listeria* remains local or the bacterium is transported downwards with released liquid surrounding the fish. Local contamination may grow to high

levels even though most other samples in the lot are negative. If the fish is consumed raw, for instance as sushi, it is beyond doubt that it is such batches that are likely to cause illness This is both because some consumers will be exposed to high levels, and because it is likely that only negative samples are taken out for analysis. Therefore, *ways to collect Listeria from a large part of the batch are needed.* It was tested whether analysis of the liquid on the bottom of fish boxes could be used for this purpose.

Slaughtered fish were stored in a company as usual, and the liquid fraction on the bottom of the box collected for analysis. The results are given in the table below. The contamination levels in the analysed fish were very low and only a few fishes were contaminated. Still, it was possible to detect the bacterium in the bottom liquid fraction after some time of storage.

Table 3. L. monocytogenes in fish fillets and liquid fraction on the bottom of the box.

	Listeria in fish		*Listeria* in bottom liquid fraction	
	<10 cfu/g	>10 cfu/g	<10 cfu/g	>10 cfu/g
Day 0	2/10	0/10	no	no
Day 7, 4C	1/10	0/10	no	no
Day 14, 4 C	0/10	0/10	yes	no
Day 14, 4C+10C	1/10	0/14	yes	no

These initial results indicated that sampling the bottom liquid phase may be a cost effective way of sampling, and that the likelihood of detection would be high if only a few fishes are contaminated.. However, ways to detect the bacterium *earlier* must be found to make the sampling method useful. It was assumed that more rapid detection could be obtained in two ways: increased temperature during storage of the samples, and to lower the detection level of the analysis method.

A sequence of experiments with inoculated fish pieces was carried out. In brief, fish cubes were stacked in flasks, as shown in the figure below. Only one of the pieces were inoculated (levels 1-1000 cfu/g), and the inoculated piece was placed in various positions in the stack. The samples were incubated at temperatures 4-37°C. In the first experiments, liquid samples on the bottom and single fish pieces were analysed. Later, the stack of fish was “washed” with diluents. The washing solution was then collected and analysed.

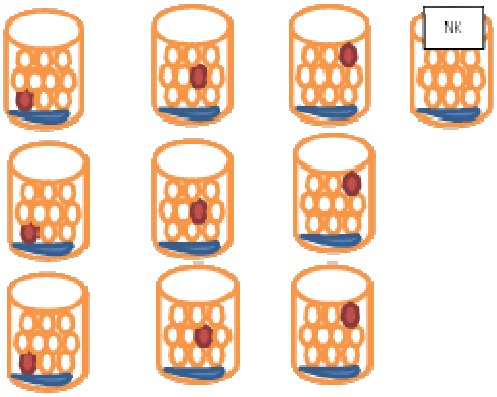

Figure 1. Models of batches with heterogeneous distribution of contamination. The red sample is contaminated with levels 1-1000 cfu/g, while the others are Listeria free

The main findings from these studies were:

Contamination levels above approximately 100 cfu/g were detected in the released water no matter where the contaminated sample was in the batch. For 10 cfu/g, the contamination was detected only if the piece was on the bottom or mid-level. Adding solution to the flask, inverting the flask a couple of times was sufficient to detect all contamination levels tested, no matter where the contaminated piece was in the pile. The *L. monocytogenes* levels measured with the ALOA and MPN method corresponded very well. We assume that the combination of washing many samples in a suitable growth medium, and quantification using the MPN method represents a promising sampling and analysis procedure for detection of *Listeria monocytogenes* from batches with low levels and/or unevenly distributed bacteria.

Conclusions from the *Listeria* studies

Performance objective to ensure the food safety objective of fresh salmon intended used raw as it is or as sushi or cold-smoked salmon (defined as "*Listeria* levels not above 100 cfu/g at the last day of shelf life") can be set up at the fresh salmon processing step using a combination of *Listeria* limit values in the range 2-10 cfu/g, the intended use of the salmon, and the expected time-temperature conditions during storage. Performance objectives and sampling plans to identify batches or production companies with the same or higher contamination levels than in the samples analysed in BASELINE have also been developed: for fresh salmon, a high reliability can be obtained by testing 7 samples, using -0.976 log cfu/g as performance objective and rejection criterion. For cold-smoked salmon, absence/25 g in 88.8% samples of lot is a suitable performance objective. The sampling plan at retail (microbiological criteria) to achieve this PO is n=6, absence/25 g. Differentiated sampling plans and rejection criteria can be set up for different analysis according to their detection levels.

The prevalence and levels of *L. monocytogenes* in fish processed at different times of the day vary in a systematic way. Local contamination grows in the samples during chilled storage, and most of the bacteria seem to remain localised even though some are transported with interstitial fluid. In boxes where one or more samples are contaminated, the bacterium spreads mainly downwards as the released interstitial fluid. and melting ice goes downwards in the box. To obtain a representative sample of the distribution of *Listeria* in a box, samples should be taken both on the bottom and top of the box, no matter if the box contains 5 or 1000 kg of fish. For identification of boxes where *Listeria* is present, fish samples should be taken from the bottom and from the liquid fraction on the bottom. Similarly, systematic differences between morning and late day batches should be taken into account.

A sampling protocol consisting of washing pooled samples from a batch with growth medium for *Listeria* has been developed. The protocol gives reliable and rapid detection of extremely low levels of *Listeria* in batches where only 1 of 10 pieces is contaminated. The protocol can be adapted to various detection levels and POs suitable for fresh fish, double-frozen fish, raw material control for fish intended is used for production of sushi, carpaccio and cold smoking.

Two protocols for detection of low levels of *L. monocytogenes* have been successfully developed. One is an adaptation of the ISO method, giving detection level 2 cfu/g, and has been sent to the European reference laboratory for *Listeria*. The other is a MPN method derived from the ISO method and has detection level 1 cfu/5 g of fish, or 0.2 cfu/g of fish. This detection level is sufficiently low to detect and quantify Listeria even if 9 subsamples are negative and 1 contains only 2 cfu/g.

DETECTION METHODS OF VIRUSES (ISS)

Standardised methods for the detection of enteric viruses (NoV and HAV) in different food are lacking. ISS provided a qualitative method real-time PCR to detect NoV (GI and GII) and HAV in shellfish based on the protocol for molecular detection of viruses in different foods developed by CEN/TC 275/WG6/TAG4 committee. The method has been validated in BASELINE, as described below.

Studies for an in-house *validation* of the method were carried out. The inclusivity and exclusivity of the primers and probes were evaluated using other enteric viruses and bacteria as follows:

HAV: Poliovirus 1, Echovirus 1, 11 and 30, Coxavkievirus A24, Enterovirus 70, Porcine enterovirus 1, Encephalomyocarditis virus, hepatitis E virus, Rotavirus group A, Norovirus, Human Astrovirus type 1 and 40 (Costafreda et al. 2006)

NoV: other enteric viruses (Poliovirus 1; HAV, HEV; Aichivirus, Human Astrovirus, Rotavirus) and bacteria naturally present in shellfish (*E. coli, Shewanella putrefaciens, Cromobacterium violaceum, Aeromonas sobria, V. alginolyticus, V. parahaemolyticus, V. cholerae*) (CEN/TC 275/WG6/TAG4- viruses in food). In addition a *Norovirus Reference Panel,* kindly provided by Rijksinstituut voor Volkgezondheit en Milieu (RIVM, The Netherlands), consisting of desiccated synthetic RNA stabilized with yeast tRNA has been used. The panel included the three NoV genogroups that are found in humans (GI, GII and GIV).

Mengovirus (avirulent clone mutant vMC_0): the results obtained by the studies performed by CEN/TC 275/WG6/TAG4- group experts have been used.

For all three amplification reactions the inclusivity and exclusivity was 100%

Then the method was validated using:

1. Reference materials
2. Ring trial samples
3. Standard synthetic RNA for the calculation of the limit of detection (LOD), the amplification efficiency and linearity
4. Clinical samples and naturally-contaminated shellfish

The results obtained were: Theoretical limit of detection (tLOD) = 10 genomic copies /sample volume (5 µl of nucleic acid suspension)

NoV GI

LOD : 8,6 target copies / reaction

PCR efficiency : 96,7%Linearity (R^2): 0,995

NoV GII

LOD : 3,2 target copies / reaction

PCR efficiency : 96.4%

Linearity (R^2): 0.992

HAV

LOD : 7,1 target copies / reaction

PCR efficiency : 101,8%

Linearity (R^2): 0,999

Mengovirus

LOD : 11 target copies / reaction*

PCR efficiency: 106,7%

Linearity (R^2): 0,992

*dilutions of the titred virus

Conclusion

The developed method has been validated in-house with very good results. Even though the study is not directly related to sampling schemes, we consider this study a valuable contribution to remove a bottleneck for development of sampling schemes for virus in shellfish in the future.

SAMPLING SIZE OF TOXINS IN SHELLFISH (UBO)

The work is published in the proceedings of the ICMSS conference 2011 published by Springer.

INTRODUCTION

Okadaic acid (OA) -group toxins cause Diarrheic Shellfish Poisoning (DSP), which are dose-dependent symptoms of diarrhoea, abdominal cramps and sometimes nausea and vomiting in man. DSP human poisoning is frequently confused with the illness due to enterophathogens*: Vibrio parahaemoliticus* or *Bacillus cereus*, which are both routinely found in bivalve mollusks. This is due to the fact that DSP toxins and enteropathogens have similar symptomatologies. Thus there is no accurate information linked to the annual DSP human poisoning episodes. However, some poisoning events have been reported. Per example in France, in 1984 and 1985, mussels raised in France caused DSP symptoms in 10,000 and 2,000 people respectively. Therefore, accurate sampling plans must be set in order to monitor production areas to check the presence of these biotoxins in shellfish, knowing that a species with the highest contamination rate may be used as an indicator species (Regulation 854/2004/EC). As mussel is the one of the species having the highest accumulation rate, it is considered as the indicator species. A sampling plan validation method, primarily developed by Whitaker and widely applied, is used to compute accept probabilities. These accept probabilities are plotted against mean lot concentrations for various sampling schemes. The curves obtained, called Operating characteristics (OC) curves, enable to quantify consumer and producer risks. Then a best fit sampling plan can be proposed, taking into account the two risk types, as well as the practical feasibility of the sampling plan.

Material and method

The sampling plan validation method developed by Whitaker consists of calculation steps achieved from data of contaminant concentration of samples taken from a lot. The samples concentrations from a lot are adjusted to a theoretical distribution thanks to a goodness of fit test. This operation is made for a few lots. Furthermore, the variability between samples concentrations from a lot is studied in order to predict this variability for any lot mean concentration within a given range of concentrations. Both the theoretical distribution and the prediction of concentrations variability between samples of the same lot are used to calculate the accept probabilities of lots for the sampling plan tested. All these steps are further explained in the following sections and applied to phycotoxins with the evaluation of sampling plans to detect the contaminant okadaic acid in mussels.

Theoretical Distribution

For the application to okadaic acid in mussels, the data used are from Pr. Arne Duinker who has supplied us (personal communication) with raw data on individual mussels contaminated with okadaic acid toxin equivalents obtained during experiments that lead to a publication (Duinker et al. 2007). These data consist of contamination levels in mussels contaminated on collectors of rather high density in a stratified fjord. Four different lots were sampled, and all the samples from a lot were taken at the same sampling point, at the same time. For each lot:

29 or 30 samples were taken, each sample consisting of one mussel. Then each individual sample was submitted to chemical analysis. Given the Regulation 853/2004/EC, the data, expressed in concentrations in steamed mussels, must be converted to concentrations in raw mussels. This conversion is done thanks to the publication of McCarron et al. (2008). Indeed, they published a theoretical conversion value: the concentration level in steamed meat must be divided by 1.2667 to obtain the concentration level in raw meat.

Probability density functions of the observed data are drawn. They indicate a possible skewness, orienting towards a theoretical distribution type. Once a theoretical distribution is sensed, its parameters are calculated thanks to the method of moments. Then, the visual comparison of the observed and theoretical cumulative frequency distributions is achieved. Finally, the goodness of fit of the observed data to the theoretical distribution can be tested thanks to the Kolmogorov-Smirnov statistical test, which is a goodness of fit test. This test measures the differences between the theoretical and observed probabilities for each contaminant

concentration within one lot. It involves finding the maximum vertical distance between the cumulative frequency distributions.

The test hypothesis for a Kolmogorov-Smirnov goodness of fit test:

H_0: The observed distribution conforms to the theoretical distribution.

H_1: The observed distribution doesn't conform to the theoretical distribution.

At the desired risk level, H_0 cannot be rejected if the variable tested (D_{calc}) is inferior to a critical value found in a table for the corresponding number of samples in the lot. This means that the adjustment of the observed data to the theoretical distribution test cannot be rejected at the risk level chosen. Furthermore, the *p-value* is the probability of observing the D_{calc} value under H_0.

Variance

Variance and mean concentration data are gathered from the literature. Variance data were compiled from 11 publications from various countries (Sweden, Italy, Germany, Ireland, Hong Kong, Tunisia) and one master thesis (Sweden). However, the literature review involved a much higher number of publications about okadaic acid levels in mussel, but all the publications were not relevant for variance data gathering. Moreover, it was decided to take into account variance data for as much countries as possible in order to ensure that the sampling plan validation would not be country specific, but would on the contrary represent a global validation. This is a good way to check that the variability is not climate specific either.

The variability, more precisely the total variance, between sample concentrations is due to sampling, sub-sampling and analysis. We know that total variance is the sum of variance components, due to the fact that variance components are additive in case they are due to independent sources of random error. So, we assume, according to Whitaker et al., that total variance (S^2_t) is the sum of sampling variance (S^2_s), sub-sampling variance (S^2_{ss}) and analysis variance (S^2_a) in Equation 1:

$$S2t = S2s + S2ss + S2a \qquad (1)$$

In Whitaker's method, S^2_t, S^2_{ss}, and S^2_a are accurately quantified. But, given the fact that, when working on experimental data, Whitaker et al. always found that S^2_{ss}, and S^2_a were negligible in comparison to S^2_t we assume that we will not calculate the negligible variances. This is a slight modification of Whitaker's method that makes the method much easier to achieve. When assuming that S^2_{ss} and S^2_a are negligible, the following approximation, in Equation 2 can be made:

$$S^2_t = S^2_s \qquad (2)$$

This approximation is now used to mention sampling variance instead of total variance.

In order to better comprehend which type of experimental data must be used, it is useful to further define the sampling variance. When two samples haven't got the same mean and when they come from the same population, then the difference between their means is simply due to sampling error. Two factors determine the magnitude of the sampling error: the population variance, and the number of individuals in the sample:

1. The variance of the population: the bigger the population variance, the bigger the sampling error.
2. The number of individuals pooled together in each sample: the bigger the number of individuals, the smaller the sampling error. This principle is called the law of large numbers.

The latest factor requires further explanations:

The variability between samples consisting of pools of individuals is the variability between means. Indeed, we can consider that the concentration of a pool is equal to the mean of the concentrations of the individuals in the

pool. The standard error of the mean is the standard deviation of the sample mean estimate of a population mean. It is usually estimated by the sample estimate of the population standard deviation divided by the square root of the sample size (assuming statistical independence of the values in the sample).

Knowing that the standard deviation is the square root of the variance, we can deduce, in Equation 3 that the sampling variance for pools ($S^2_{s\ for\ pools}$) multiplied by the number n of individuals in each pool is equal to sampling variance (S^2_s).

S2s for pools * n = S2s (3)

Variance experimental data must be plotted against their respective mean concentration levels c (in µg/kg): S^2_s = f(c). Each dot in the graph corresponds to data obtained for one lot with sampling variance and mean concentration calculated from the samples taken from the lot. Then, a regression curve is obtained in order to have an equation of the variance as function of the concentration. This equation is going to be useful to compute the accept probabilities.

The concentration data in hepatopancreas given in a publication had to be transformed in whole flesh concentration data, as the European regulation 853/2004 states that the okadaic acid results must be given per kg of whole flesh. In order to achieve this transformation, the concentration in hepatopancreas is divided by 6 in order to obtain the concentration in whole flesh. This conversion value was calculated from concentration results reported in the publication from Duinker et al. (2007).

Accept Probabilities

For a lot of a given mean concentration, the accept probability is computed as the probability that a sample consisting of a pool of individuals taken from the lot, has got a concentration level inferior or equal to the threshold. This probability is calculated thanks to the theoretical distribution and the total variance equation obtained in the previous sections.

Operating Characteristics (OC) curves principle

Operating Characteristics (OC) curves enable to calculate the probability of mistake in determining the average contaminant concentration level for various sampling schemes (Fig. 2).

They show the risk of:

- accepting lots at a true concentration above the threshold (consumer risk);
- rejecting lots at a true concentration under the threshold (producer risk).

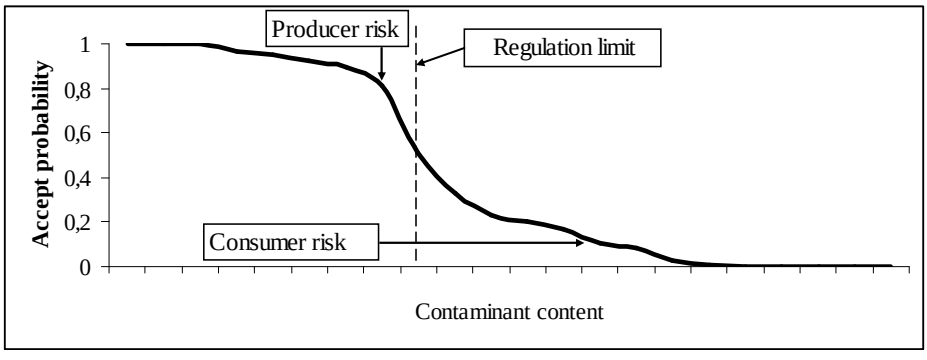

Figure 2: Operating Characteristics (OC) curve principle for a contaminant

The shape of the OC curve, for a specific contaminant and food, is unique for a specific sampling plan design. More concretely, the shape varies along with many criteria stated in the sampling plan:

- the number of samples;
- the sample size and
- the concentration threshold chosen for acceptance or refusal of samples.

A sampling plan must be selected, considering the technical and economic feasibility, as well as the consumer risk and producer risk levels. It is therefore crucial to assess these two risks in order to select a best fit sampling plan. An ideal sampling plan would lead to the acceptance of all the lots with contaminant content below the allowed limits, and to the rejection of those with contaminant content higher than these limits, thus reducing the risk to consumer and producer to zero. In reality however, these risks cannot be totally eliminated, but only reduced as much as possible, keeping in mind the feasibility. When this is achieved, then a best fit sampling plan is selected.

Results and interpretation

Theoretical Distribution

The probability density functions obtained for each of the four lots show distributions highly skewed to the right, which says that the right tail is longer, the mass of the distribution is concentrated on the left of the figure. An example of a right-skewed distribution obtained is given in Fig. 3.

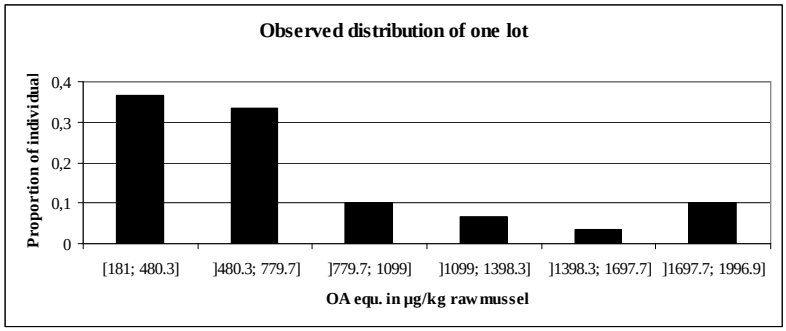

Figure 3: Histogram of observed probability density function for a lot

As it is used for continuous data and can simulate highly positively skewed probability density functions, the theoretical distribution tested is the lognormal distribution. Furthermore, the lognormal distribution parameters can be easily calculated thanks to the method of moments. The comparison of the observed and lognormal cumulative frequency distributions for each lot shows a good visual fit. An illustration of cumulative distribution functions comparisons is given in Fig. 4.

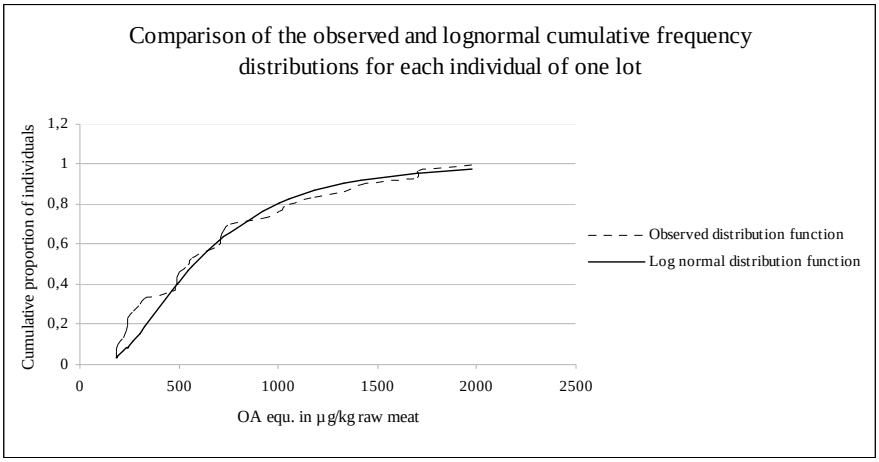

Figure 4: Comparison of theoretical and observed cumulative distribution functions for a lot

The conformity of the observed distribution to the lognormal distribution is further tested thanks to the Kolmogorov-Smirnov goodness of fit statistical tests. The results of these tests are given in Table 4.

Table 4: Goodness of fit test results

	D_{calc}	Critical value	*p-value*
Lot n°1	0.1393	0.2457	57.91%
Lot n°2	0.1829	0.2457	25.39%
Lot n°3	0.2503	0.2417	3.84%
Lot n°4	0.1547	0.2417	42.66%

Critical values and *p-values* are obtained for the risk level of 5%.

For lots n°1, 2 and 4, for both tests, the test statistics (D_{calc}) are inferior to the critical values, which means that the hypothesis H_0 cannot be rejected at a 5% risk level. Moreover, the *p- values* are above 5%, which means too that H_0 is not rejected. So, at a 5% risk level, the lognormal distribution of the population cannot be rejected. There is a discrepancy for lot n°3, as it shows opposite results. However, we consider that three lots validated out of four by the goodness of fit test is enough to consider that when samples from any lot are drawn, their contamination levels fit the lognormal distribution. Moreover, the samples distribution is still considered to be lognormal, even if the samples taken are of a bigger size than one mussel per sample.

Variance

Variances data (Fig. 5) enable the development of a regression equation (Equation 4) to predict S^2_s as function of *c* (in µg/kg).

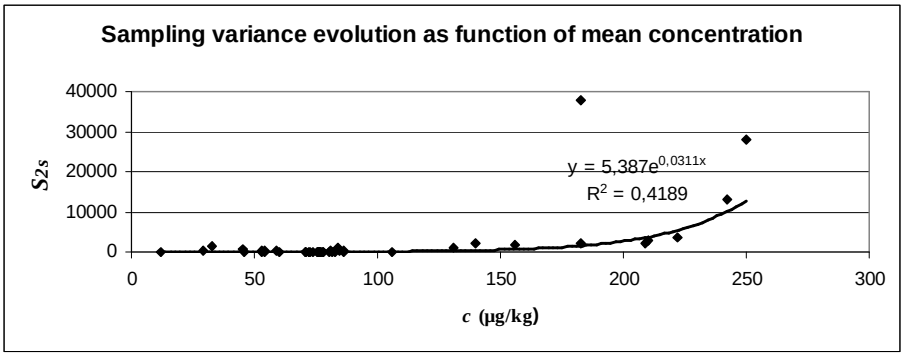

Figure 5: Development of an exponential regression equation

$S^2_s = 5.387 * \exp(0.0311 * c)$ (4)

There is a between mussels variability, even for mussels taken at the same sampling point and at the same time. This variability probably stems from food access variability and variability of response to the toxins. As regards food access, the variability is primarily due to the fact that toxic dinoflagellates are drifted by the currents. Moreover, accumulation and elimination rates of DSP toxins vary within a shellfish species after a contamination event (Duinker et al. 2007). After ingestion, a fraction of the toxins may be transformed by acylation.

Accept Probabilities

The accept probabilities depend on the sample size, and can be calculated for any sample size as shown in Table 5.

Table 5: An example of calculation of the accept probabilities

c (µg/kg)	S^2_s	$S^2_{s\ for\ pools}$ for a pool size of 30 shellfish	*mu*	*sigma*	Accept probability
10	7.35211953	0.24507065	2.301361234	0.04947432	1
20	10.0340935	0.334469783	2.99531436	0.02891064	1
30	13.6944226	0.456480755	3.40094385	0.02251826	1

Here, the variable *c* is an input. S^2_s is calculated with Equation 4. After defining the sample size (the number *n* of individuals in each pooled sample), $S^2_{s\ for\ pools}$ is calculated thanks to Equation 3. The parameters of the lognormal distribution: *mu* and *sigma* are calculated thanks to the method of moments. The accept probability is computed as the ordinate of the lognormal theoretical cumulative frequency distribution at the threshold value of *c,* which is set at 160 µg/kg. These accept probabilities correspond to a sampling plan in which a single sample is taken.

Tests and selection of the best fit sampling plan thanks to OC curves

The accept probabilities that have been calculated, correspond to a sampling plan with a single sample taken and compute the probability that this sample is inferior or equal to the threshold concentration, given the lot mean concentration. In order to obtain an OC curve, the accept probabilities, for a given sample size, must be plotted against *c*. In the following, various sampling strategies are tested in order to observe their effect on the OC curve shapes.

Single sample sampling plans: effect of sample size

When testing single sample sampling plans, it can be observed that increasing sample size reduces uncertainty (Fig. 6). As sample size increases, the OC curves become steeper around the threshold concentration. As a result, consumer and producer risks become smaller. So, when increasing the sample size, the sampling plans become more efficient.

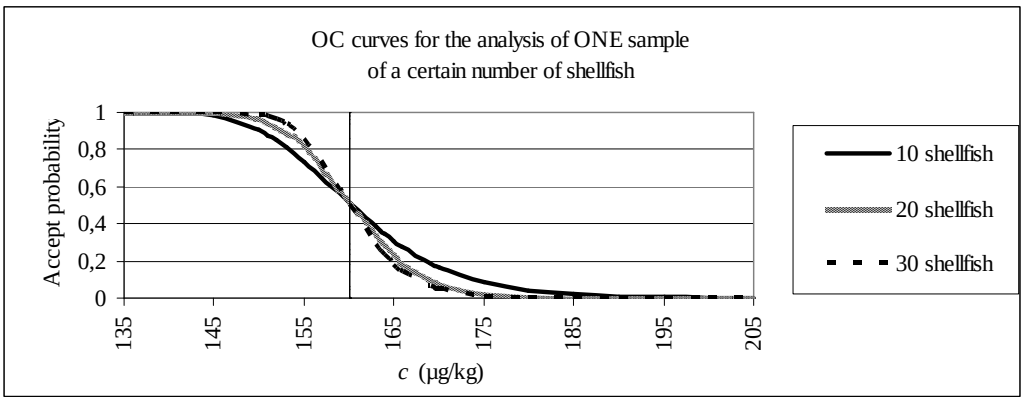

Figure 6: OC curves for sampling plans with one sample taken of a sample size of 10, 20, or 30 shellfish

In Fig. 6, the sampling plan with the lowest consumer and producer risks is the one with a 30 shellfish sample. For this sampling plan, the accept probability is of 95% for a concentration of 152 µg/kg. This means that the producer risk is at 5% for this concentration. Moreover, the accept probability amounts to 5% for a concentration of 169 µg/kg, so, the consumer risk is at 5% for 169 µg/kg.

Multiple Samples Sampling Plans

The other strategy tested is multiple samples sampling plans. When the sampling plan consists of taking a number *q* of samples, and if the lot is accepted only if each of the *q* samples tests under the threshold concentration, then:

For a given sample size, the accept probabilities of the multiple samples sampling plan, correspond to the accept probabilities of a single sample sampling plan raised to the power of *q*. The comparison of the OC curves for a single sample sampling plan, and a double sample sampling plan is given in Fig. 7 for a 30 shellfish sample size.

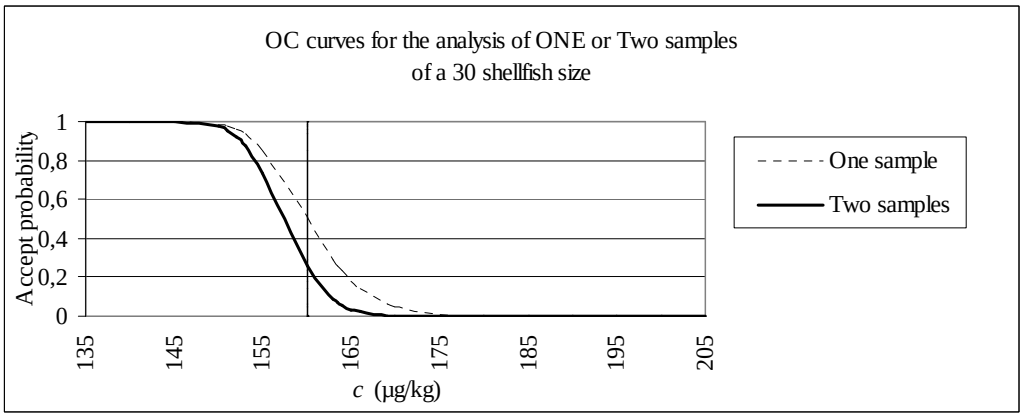

Figure 7: OC curves for sampling plans with one sample taken, or two samples taken

This comparison enables to notice that the two samples sampling plan enables to decrease the consumer risk (Accept probability at 5% for a concentration of 164 µg/kg) but increases slightly the producer risk (Accept probability at 95% for a concentration of 151 µg/kg).

Finally, for food safety reasons, the best fit sampling plan that can be proposed is a two samples sampling plan with a sample size of 30 individual mussels.

CONCLUSIONS

We can conclude that we have shown that Whitaker's method is applicable to phycotoxins in shellfish. To our knowledge, this is the first time that a probabilistic evaluation of sampling plan designs for phycotoxins in shellfish is achieved. Sampling plan designs are of utmost importance to determine, in a trustworthy way, the status of a shellfish growing area. This method has been validated on variance data that we had at our disposal, but it would worth being validated on more data. It would be a good point if a Food Sanitary Agency, involved in phycotoxin monitoring, validated this method on a bigger data set.

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MERCURY IN TUNA (UBO)

The content of mercury compounds in tuna is to a large extent related to fish size and tuna species. The acceptable levels have already been set up in EU regulation. Because mercury compounds are inert, the acceptable levels serve both as a food safety objective and a performance objective. The challenge is to optimise the sampling plans to obtain as reliable and cost-effective analysis as possible. A lot of data are available from literature and survey programs the distributions of mercury compounds in five fresh tuna species and three types of canned tuna have been studied using these data. Further, sampling plans for analyses have been developed and validated.

The sampling plan established by Whitaker's parametric method was applied in this study (Whitaker, et al., 2007). Indeed, mercury levels of tunas are similar and incidence of mercury contamination within a tuna lot is high, thus allowing the use of this method.

Raw data collecting: mercury level and variance of each lot

A Fresh tuna

The native habitat of yellowfin tuna is the Atlantic Ocean, the Pacific Ocean and the Indian Ocean. However, the initial source is not a determinant factor because this migratory species moves from one ocean to the other.

For yellowfin tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Adams (2004), Al-Busaidi *et al.* (2011), Aldrin *et al.* (1973), Boush & Thieleke (1983), Burger & Gochfeld (2011), Cai *et al.* (2007), Chang (2009), Food and Drug Administration (2010), García-Hernández *et al.* (2007), Greig & Krzynowek (1979), Hisashimi *et al.* (2010), Kaneko & Ralston (2007), Kojadinovic *et al.* (2006), Krapiel, *et al.* (2003), Kumar *et al.* (2003), Lowenstein *et al.* (2010), Matthews (1983), Menasveta & Siriyong (1976), Medeiros *et al.* (2006), Métongo & Kouamenan (1991), Ordiano-Flores *et al.* (2011), Ortega-García *et al.* (2009), Sompongchaiyakul *et al.* (2006), Thomson & Lee (2009) and Yamashita *et al.* (2011).

A total of 33 lots (1888 tunas) of yellowfin tuna were analysed by the Whitaker method.

For albacore tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Food and Drug Administration (2010), Greig & Krzynowek (1979), Hisashimi *et al.* (2010), Kaneko & Ralston (2007), Kumar *et al.* (2003), Morrissey *et al.* (2004), Morrissey & Geise (2006), Storelli *et al.* (2002) and Storelli & Marcotrigiano (2004).

The native habitats of albacore tuna are the Pacific Ocean and the Indian Ocean. A total of 12 lots (468 tunas) of albacore tuna were analysed by theWhitaker method.

For bigeye tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Aldrin *et al.* (1973), Boush & Thieleke (1983), Chen *et al.* (2011), Food and Drug Administration (2006), Food and Drug Administration (2010), Kaneko & Ralston (2007), Kumar *et al.* (2003), Lowenstein *et al.* (2010), Menasveta & Siriyong (1976), Yamashita *et al.* (2005) and Yamashita *et al.* (2011).

The native habitat of big-eye tuna are the Atlantic Ocean, the Pacific Ocean and the Indian Ocean. A total of 13 lots (557 tunas) of bigeye tuna were analysed by theWhitaker method.

For Atlantic bluefin tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Burger & Gochfeld (2011), Food and Drug Administration (2006), Focardi (2012), Hardisson *et al.* (1999), Licata *et al.* (2005), Morales-Nin & Fortuno (1990), Ouedraogo & Amyot (2011), Relini *et al.* (2007), Storelli *et al.* (2002), Thibaud (1971) and Yamashita *et al.* (2005).

The native habitats of bluefin tuna are the Atlantic Ocean and the Mediterranean Sea. A total of 26 lots (646 tunas) of bluefin tuna were analysed by the Whitaker method.

For skipjack tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Al-Busaidi *et al.* (2011), Greig & Krzynowek (1979), Kaneko & Ralston (2007), Kojadinovic *et al.* (2006), Kumar *et al.* (2003), Sompongchaiyakul *et al.* (2006) and Yamashita *et al.* (2011).

The native habitats of skipjack tuna are the Atlantic Ocean, the Pacific Ocean and the Indian Ocean. A total of 10 lots (232 tunas) of skipjack tuna were analysed by the Whitaker method.

B. Canned tuna

For yellowfin tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Food and Drug Administration (2010), Food Department Agency (2003), Ruelas-Inzunza *et al.* (2011) and Zook *et al.* (1976). A total of 15 lots (158 cans) of yellowfin tuna from Mexico, USA and UK were analysed by the Whitaker method.

For light tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Burger & Gochfeld (2004), Forsyth *et al.* (2004), Food and Drug Administration (2010), Food Department Agency (2003), Gerstenberger *et al.* (2010), Holloman & Newman (2010), Kumar *et al.* (2003) and Thomson & Lee (2009).

A total of 14 lots (1449 cans) of light tuna from USA, Canada, UK, Fiji and New-Zealand were analysed by the Whitaker method..

For white tuna, the following publications were used for the regression curve (relationship between the total variance and mercury concentration): Burger & Gochfeld (2004), Burger & Gochfeld (2006), Forsyth *et al.* (2004), Food and Drug Administration (2010), Food Department Agency (2003), Gerstenberger *et al.* (2010), Holloman & Newman (2010), Kumar *et al.* (2003) and Rasmussen & Morrissey (2007).

A total of 20 lots (1820 cans) of white tuna from USA, Canada, UK and Fiji were analysed by the Whitaker method.

Sampling plans tested

Eleven sampling plans were tested by this mathematical method:

1, 2, 5 or 10 tunas or cans
1 pool of 1, 5 or 10 tunas or cans
2 pools of 2, 3 or 5 tunas or cans
3 pools of 2 tunas or cans

The rejection probability for the lot was defined as at 0.85 µg Hgt/g ww (producer risk) and the acceptance probability for the lot was defined as 1.15 µg Hgt/g ww (consumer risk).

These two mercury levels are defined by calculating the maximum measurement uncertainty for mercury analysis with this formula (European Commission, 2011):

$$Uf = \frac{\sqrt{(\frac{LOD}{2})^2 + (aC)^2)}}{1000} = 0.15\ \mu g/g$$

With:

Uf : maximum standard measurement uncertainty (µg/kg)

LOD : limit of detection of the method (maximal LOD in studied publications = 50µg/kg)

a : numeric factor defined in the Regulation n°836/2011 (a = 0.15)

C : concentration of interest (µg/kg) (C = 1000µg/kg)

The probabilities (consumer and producer risks) must strive for 0% to define the best sampling plan. However, in reality, it is not possible. So, these risks must be minimal and equivalent as much as possible.

Task achievements

The results for three steps of the Whitaker's method (goodness-of-fit test of observed contamination data, equation of regression curve and calculation of producer and consumer risks) are detailed below.

Goodness-of-fit test.

The observed data have been tested using the Kolmogorov-Smirnov goodness-of-fit test for three distributions: Normal, Log-normal and Gamma. The best theoretical distribution, for each species, is detailed in the table 11.

Table 11. Results of goodness-of-fit test for each species of fresh and canned tuna

	Species	Best theoretical distribution
Fresh tuna	Skipjack tuna	Gamma
	Yellowfin tuna	Gamma
	Bigeye tuna	Log-Normal
	Albacore tuna	Log-Normal
	Bluefin tuna	Gamma
Canned tuna	Light tuna	Log-Normal
	Yellowfin tuna	Log-Normal
	White tuna	Log-Normal

Relationship between total variance and total mercury level

The total variance equations of the regression curve (total variance in function of Hgt levels) for each species are detailed in the table 12 for fresh tuna and the table 13 for canned tuna.

Table 12. Regression curves and total variance equations for each species of fresh tuna

Species	Number of lots	Type of regression curve	Coefficient of determination (R^2)	Total variance equation

Albacore tuna	15	Exponential	0.83	Variance = $0.0019.\exp^{(3.0384 \times [Hgt])}$
Yellowfin tuna	33	Polynomial	0.71	Variance = $0.2631\ [Hgt]^2 + 0.0297\ [Hgt] + 0.0003$
Altantic Bluefin tuna	26	Puissance	0.64	Variance = $0.2091\ [Hgt]^{1.738}$
Bigeye tuna	13	Polynomial	0.74	Variance = $0.2757[Hgt]^2 - 0.0592\ [Hgt] – 0.0043$
Skipjack tuna	10	Linear	0.002	Variance = $-0.0026\ [Hgt] + 0.0078$

For skipjack tuna, the total variance is the same regardless the total mercury concentration. So, the used variance is 0.0078 $(\mu g/g)^2$ for the calculation of producer and consumer risks.

Table 13. Regression curves and total variance equations for each species of canned tuna

Species	Number of lots	Type of regression curve	Coefficient of determination(R^2)	Total variance equation
White tuna	20	Linear	0.61	Variance = $0.0823\ [Hgt] – 0.0134$
Yellowfin tuna	15	Linear	0.46	Variance = $0.0733\ [Hgt] – 0.0008$
Light tuna	14	Logarithm	0.67	Variance = $0.0165 \times Ln([Hgt]) + 0.0046$

Producer and consumer risks

Accept and reject probabilities are calculated thanks to the parameters of the best theoretical fit (α and β for gamma distribution and μ and σ for log-normal distribution) (§3.A) and the total variance equation of the regression curve (§3.B).

Results for fresh tuna are presented in the table 14 and for canned tuna presented in the table 15.

Table 14. Rejection probabilities for a compliant lot (producer risk) and acceptance probabilities for a non-conforming lot (consumer risk) for fresh tuna

		1 tuna	2 tunas	3 tunas	5 tunas	10 tunas	1 pool of 2 tunas	1 pool of 3 tunas	1 pool of 5 tunas	1 pool of 10 tunas	2 pools of 2 tunas	2 pools of 3 tunas	3 pools of 2 tunas	2 pools of 5 tunas
Albacore tuna	Rejection probability at 0.85 µg/g	17 %	30%	42%	60%	84%	10%	6%	2%	0%	18%	11%	57%	5%
	Acceptance probability at 1.15 µg/g	29 %	9%	3%	0%	0%	20%	15%	8%	2%	15%	2%	3%	1%
Yellowfin tuna	Rejection probability at 0.85 µg/g	31 %	53%	68%	85%	98%	29%	26%	22%	15%	49%	46%	59%	40%
	Acceptance probability at 1.15 µg/g	47 %	22%	10%	2%	0%	41%	37%	31%	23%	17%	14%	3%	10%
Bluefin tuna	Rejection probability at 0.85 µg/g	30 %	52%	66%	84%	97%	27%	24%	19%	12%	47%	42%	54%	35%
	Acceptance probability at 1.15 µg/g	44 %	19%	8%	2%	0%	37%	33%	27%	18%	14%	11%	2%	7%
Bigeye tuna	Rejection probability at 0.85 µg/g	28 %	41%	62%	73%	93%	25%	22%	18%	11%	43%	39%	62%	33%
	Acceptance	46 %	18%	10%	1%	0%	39%	35%	28%	19%	15%	12%	7%	8%

	probability at 1.15 µg/g													
Skipjack tuna	Rejection probability at 0.85 µg/g	3%	5%	8%	13%	24%	0%	0%	0%	0%	1%	0%	0%	0%
	Acceptance probability at 1.15 µg/g	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

For yellowfin tuna, bluefin tuna and bigeye tuna, the best sampling plan is one pool of ten tunas. More precisely, the producer risk for tuna at a concentration level of 0.85 µg/g is at 15%, 12% and 11% for yellowfin tuna, bluefin tuna and bigeye tuna, respectively. For consumer risks, the levels these species, for a concentration at 1.15 µg/g are at 23%, 18%, and 19%, respectively. For albacore tuna, even if the best plan is the same as above one pool of five tuna gives a producer risk at 2% for a contamination at 0.85 µg/kg and a consumer risk at 8% for a contamination at 1.15 µg/g and this sampling plan is cheaper. For skipjack tuna, one pool of two tunas is sufficient with a producer and consumer risks that are null due to homogenous distribution of mercury compounds in this specie.

Table 15. Rejection probabilities for a conforming lot (producer risk) and acceptance probabilities for a non-compliant lot (consumer risk) for canned tuna

		1 can	2 cans	3 cans	5 cans	10 cans	1 pool of 2 cans	1 pool of 3 cans	1 pool of 5 cans	1 pool of 10 cans	2 pools of 2 cans	2 pools of 3 cans	3 pools of 2 cans	2 pools of 5 cans
White tuna	Rejection probability at 0.85 µg/g	23 %	41%	55%	73%	93%	18%	14%	9%	3%	32%	26%	60%	16%
	Acceptance probability at 1.15 µg/g	33 %	11%	3%	0%	0%	24%	18%	11%	4%	6%	3%	4%	1%
Yellowfin tuna	Rejection probability at 0.85	24 %	42%	56%	74%	93%	18%	15%	9%	4%	34%	27%	60%	18%

	µg/g													
	Acceptance probability at 1.15 µg/g	33 %	11%	4%	0%	0%	24%	19%	12%	4%	6%	3%	4%	1%
Light tuna	Rejection probability at 0.85 µg/g	21 %	38%	51%	70%	91%	15%	11%	6%	2%	28%	21%	59%	12%
	Acceptance probability at 1.15 µg/g	26 %	7%	2%	0%	0%	17%	11%	6%	1%	3%	1%	3%	0%

For all type of canned tuna, the best sampling plan is one pool of ten cans with producer and consumer risks very low (less than 5%). Moreover, this plan would not be expensive for the producer on account of the price of cans.

In conclusion pooled samples with 10 tuna isthe best trade-off between reliability and costs for yellowfin, bluefin and bigeye tuna. For albacore tuna, one pool of 5 tunas is sufficient and for skipjack tuna ~~which~~, one pool of 2 tunas is the best sampling plan. For canned tuna, pooled samples with 10 cans seem to be the best trade-off between reliability and costs.

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