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DE SÉCURITÉ SANITAIRE
DES ALIMENTS

Maisons-Alfort, 4 December 2009

OPINION

of the French Food Safety Agency on the system for monitoring lipophilic phycotoxins in shellfish farming areas concerning the determination of at-risk periods and reference sampling points

THE DIRECTOR GENERAL

1. CONTEXT OF THE REQUEST

The French Food Safety Agency (AFSSA) was requested by the Directorate General for Food (DGAL) on 23 July 2009 to issue an opinion on the system for monitoring lipophilic phycotoxins in shellfish farming areas concerning the determination of at-risk periods and reference sampling points.

2. CONTEXT AND QUESTIONS RAISED

The requirements for monitoring phycotoxins have been defined in European Community Regulations (EC) nos. 853/2004, 854/2004 and 2074/2005. In France, production areas are monitored by Ifremer[1] through the REPHY[2] network, whose technical specifications for monitoring procedures are revised annually.

For offshore communities (Pectinidae and other shellfish, such as carpet-shell clams, queen scallops,[3] etc.), where the distance from the coast and the depth of the exploited communities (benthic or buried) does not allow for a representative sample of the phytoplankton in a water column, shellfish are tested systematically[4] for three families of toxins one month and two weeks before the fishing period. During the fishing period, as long as no toxins are detected, shellfish are sampled once every two weeks; during a toxic episode, this is done once a week.

For coastal communities and farms, shellfish are systematically monitored for lipophilic phycotoxins in **at-risk areas** and during **at-risk periods** (see definitions in section 4). Outside of these at-risk periods, the strategy is the same as that applied for paralysing and amnesic phycotoxins. This means that it is based on the detection in water of phytoplankton species that are known to produce phycotoxins. When the phytoplankton alert level is exceeded, shellfish are tested for the corresponding phycotoxins.

In practice, this monitoring relies on:

- seawater samples, which are collected from once a week to once a month, to test for the presence of toxinogenic phytoplankton species;
- testing for lipophilic phycotoxins in shellfish in various situations:
  - when toxinogenic phytoplankton species have been identified in seawater samples and are therefore likely to have contaminated shellfish (once a week);
  - systematically at 10 reference locations spread out along the French coast (once a month);
  - systematically in at-risk areas during at-risk periods (once a week).

Reference sampling points were created in 2006: there were initially 7 sampling points and these were extended to 10 in 2008. Reference sampling points and at-risk periods were specified chiefly in response to a request from the European Commission's Food and Veterinary Office (FVO) (further to an inspection in 2004) to reinforce shellfish testing including in situations where toxinogenic phytoplankton are not detected.

[1] Ifremer: French Research Institute for Exploitation of the Sea
[2] REPHY: Phytoplankton and phycotoxin monitoring network
[3] Common UK term: dog-cockle
[4] i.e. a direct analysis of toxins found in shellfish, not based on excess levels of toxinogenic phytoplankton, which is an indicator.

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In this context, AFSSA was requested to suggest improvements concerning:

- the determination of at-risk periods (procedures used, monitoring frequency, etc.);
- monitoring at reference sampling points (number, location, frequency, etc.).

AFSSA was also invited to make recommendations to adapt the monitoring system for lipophilic phycotoxins in shellfish farming areas.

The conclusions may be taken into account in the framework of the annual revision of REPHY's programming and procedure specifications in 2010.

3. EXPERT ASSESSMENT METHOD

The emergency collective expert assessment group (GECU) on 'Phycotoxins/At-risk periods' set up by the Deputy Director General of the French Food Safety Agency, together with the chairperson of the Scientific Panel on 'Chemical and physical contaminants and residues', was in charge of this assessment.

This 'Phycotoxins/At-risk periods' GECU includes:

- experts from the Scientific Panel on 'Chemical and physical contaminants and residues';
- experts from Ifremer, the EMP (Environment, Microbiology, Phycotoxins) Department and the DYNECO/VIGIES (Dynamics of the Coastal Environment/Development of Information for Integrated Management and Monitoring) Department;
- experts from the Toxin Characterisation Unit (CAT) of the Laboratory for study and research on food quality and processing (AFSSA/LERQAP), which is the National reference laboratory for marine biotoxins;
- experts from the Department for the Assessment of Nutritional and Health Risks (DERNS).

Following consultation of the 'Phycotoxins/At-risk periods' GECU and the Scientific Panel on 'Chemical and physical contaminants and residues', which met on 13 and 20 November 2009, AFSSA has reached the following conclusions.

4. QUESTION 1: AT-RISK PERIODS

The determination of **at-risk periods** was initiated in 2005 according to an empirical criterion used up to and including 2008: an at-risk period covers "all months during which lipophilic toxins are found, for each of the at-risk areas, according to the following rule: all months over the past six years that were affected at least twice by toxicity[5] should be included; however, one single observation during one of the past two years (2006-2007) is enough to include the concerned month in the at-risk period" (extract from the REPHY 2008 book).

For 2009, the at-risk period included, for all of the at-risk areas, all of the months for which there was a positive bioassay in at least two of the six previous years.

At-risk areas are areas for which there was a positive bioassay in at least two of the past six years (before 2009, one single positive bioassay was needed to characterise the area as at-risk).

To identify non-empirical criteria to be taken into account for determining at-risk periods, the 'Phycotoxins/At-risk periods' GECU performed a statistical analysis of data submitted by Ifremer in order to determine the number of years and events to be taken into consideration. This analysis, which is described in detail in annexes 1 and 2, was based on the results of:

- the mouse bioassay,
- chemical analyses exceeding the regulatory limits[6].

The preliminary analyses undertaken by this GECU suggest that the period that provides the most information covers the past 3 years (the last 2 full years and the available results for the

[5] Corresponding to a positive mouse bioassay: 2/3 or 3/3 mice dead in a 24-hour period.
[6] See point 6 for details on regulated phycotoxins and current limits.

current year). The information supplied is no longer significant after 3 years for the mouse bioassay and after 2 years for the chemical analysis.

However, it should be noted that this conclusion is based on monitoring data, which are unbalanced in terms of the number of analyses per site and per month. Moreover, chemical analysis was only introduced a few years ago and the number of analyses of a given site and a given month for a period of several years is limited (see annexes).

The graph below shows the number of events to be taken into account to determine the at-risk period. In light of the number of available analyses (complete series for a given site and month), only the mouse bioassay results were taken into account, for data acquired later than 2003[7] (annexe 1a).

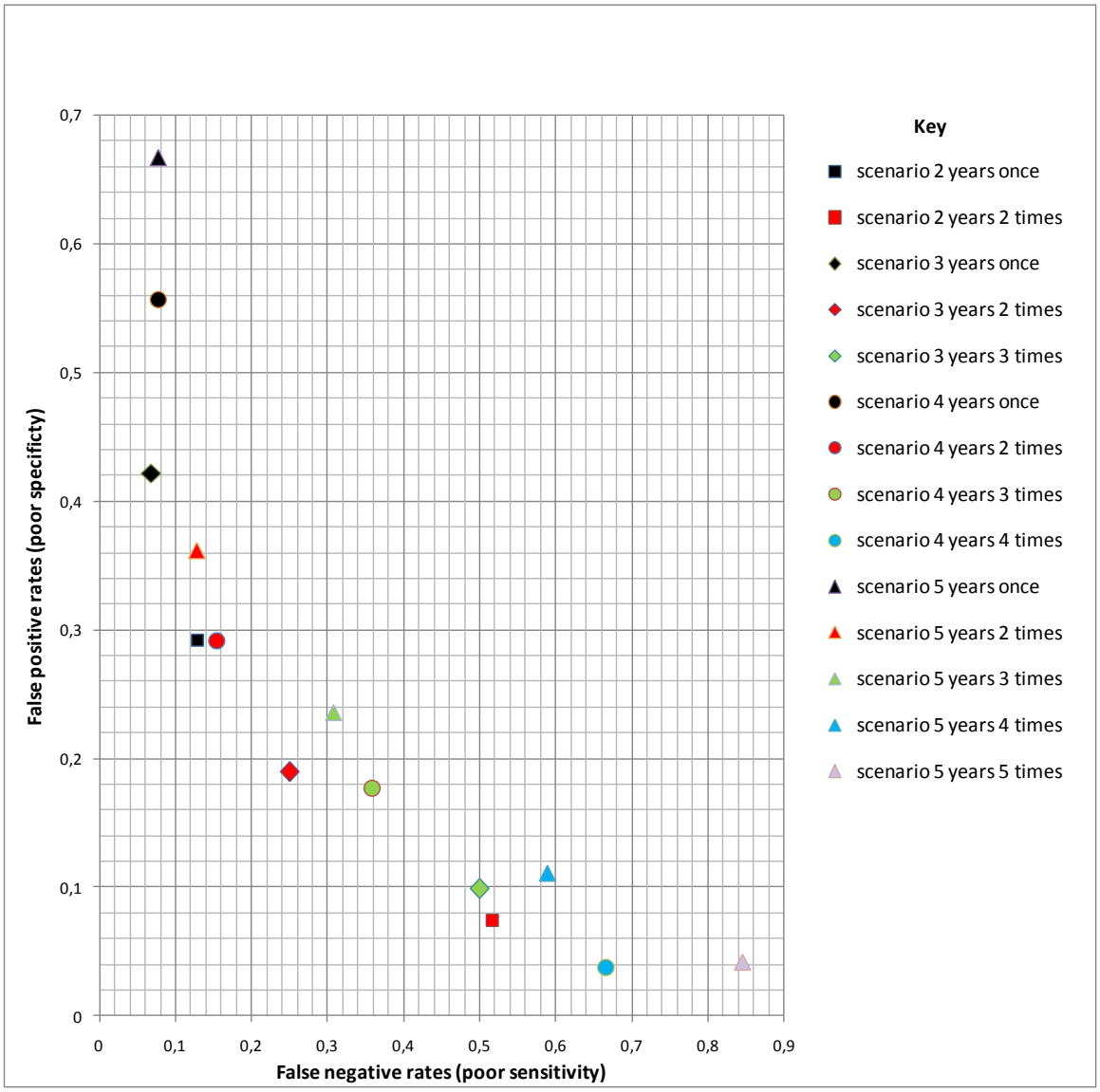
The ROC (Receiver Operating Characteristic) curve is a graph which is used to optimise the balance between sensitivity and specificity. Sensitivity and specificity are both independent of the prevalence of contaminated sites and illustrate the performance of the proposed monitoring system. Monitoring sensitivity and specificity are defined as follows:

- Sensitivity (Se) is the detection of a positive sample (M+) that was accurately predicted by the information corresponding to a given scenario (T+), corresponding to a rule for interpretation of historical data of a site. One of the possible scenarios studied, for example, considers that if a site, during a given month, was found to be positive twice in the past 5 years, it is predicted as positive. In mathematical terms, this can be written as follows, P being a conditional probability: Se=P(T+/M+).
- Specificity (Sp) for the same scenario is the detection of a negative sample (M-) that would have been predicted as negative according to the same scenario (T-). If we use the previous example, the same site, for the month in question, would have been found positive less than twice over the past 5 years: Sp=P(T-/M-).
- Sensitivity and specificity are not positively correlated. They are directly related to the false positive rate (FPR) and false negative rate (FNR) that the rule of interpretation will generate. In mathematical terms, this can be written as follows: FPR=1-Sp and FNR=1-Se.

On the basis of the mouse bioassay results, the graph below shows the false negative rate (x-axis) and the false positive rate (y-axis) on a scale from 0 to 1, adhering to the principle of the ROC curve. As a result, according to this analysis:

- the scenario '**2 positive results over the last 3 years**' is the best balance between poor sensitivity (not enough positive sites detected, too many false negatives) and poor specificity (too many unusable samples, too many false positives);
- the scenario '**1 positive result during the last 3 years**' is the scenario that provides consumers with the highest safety level (lowest poor-sensitivity value).

[7] The mouse bioassay protocol was modified in 2002.



Graph 1: Poor surveillance sensitivity and specificity according to the criteria used to determine at-risk periods on the basis of mouse bioassay results.

Nevertheless, before this criterion may be applied by REPHY, its effectiveness should be verified in terms of prediction of toxicity episodes through simulations of a few previous years.

Time scale of at-risk periods and monitoring frequency

Given the kinetic variability of shellfish contamination and decontamination with lipophilic phycotoxins, AFSSA considers that:

- at-risk periods should still be determined on a monthly basis (and not on a weekly basis);
- samples should still be taken weekly during at-risk periods (and not every two weeks as it has been observed in some situations).

5. QUESTION 2: REFERENCE SAMPLING POINTS

Reference sampling points are points where two systematic analyses, i.e. mouse bioassay and chemical analysis are performed, once a month all year long (this sampling increases to once a week during at-risk periods or during toxic episodes, as for the other sampling points).
The choice of locations for these points takes into account the need to comply with the European Commission's requirements: monitoring of the entire coast to make up for France's inability, due to the high number of production areas, to precisely comply with the regulatory requirement of conducting systematic water/shellfish analyses at all points. The selection criteria are:

- the most homogeneous geographic distribution possible over the entire French coast;
- location in production areas (for mussels or oysters) that are active the whole year round (fishing areas have been excluded since 2009);
- suspicious or unexplained results obtained on several occasions for approximately half of these points;
- location in risk-free areas, for the potential detection of emerging phycotoxins, for the rest of the points.

For each of the reference sampling points, a table in annexe 3 gives information on their location, the year the monitoring started, the sampled shellfish species and selection criteria. This table is combined with a map illustrating the distribution of the 10 reference sampling points in 2009 (annexe 4).

The analysis of the results from the 10 reference sampling points revealed (details in annexe 5):

- no positive mouse bioassays in the at-no-risk areas;
- a few toxicity episodes outside of the at-risk periods.

The overall result is therefore favourable because these points not only corroborated the concept of at-no-risk areas but they also improved the monitoring system, as the positive bioassays led to management measures being taken (closures of production areas).

Nevertheless, care should be taken when interpreting these results. Out of the 10 sampling points monitored in 2009, only 4 have historical data since 2006 and 4 since 2008 (the latter 2 are new sampling points).

Moreover, it should be noted that none of the reference sampling points monitored in 2009 or before are representative of the open sea on the Mediterranean coast. The 'Parc Leucate' and 'Diana Centre' sampling points are representative only of their respective marshes (Salses-Leucate and Diana). This is due to the lack of shellfish availability for surveillance. In accordance with the AFSSA opinion of 11 July 2008[8], this system should incorporate risks related to the phycotoxins produced by various species belonging to the *Ostreopsis* genus found on the Mediterranean coast.

6. INITIAL RECOMMENDATIONS FOR A VIGILANCE SYSTEM REGARDING RISK RELATED TO LIPOPHILIC PHYCOTOXINS IN THE FRAMEWORK OF A MONITORING SYSTEM BASED ON THE CHEMICAL ANALYSIS OF PHYCOTOXINS THAT ARE SUBJECT TO REGULATIONS

The goal of a monitoring system for lipophilic phycotoxins in shellfish based on chemical analysis is to monitor those that are subject to regulations:

- okadaic acid and dinophysistoxins (DTX1, DTX2, DTX3);
- pectenotoxins: PTX1 and PTX2;
- yessotoxins: YTX, 45 OH YTX, Homo YTX, and 45 OH Homo YTX;
- azaspiracids: AZA1, AZA2 and AZA3.

Maximum limits in shellfish (whole body or any edible part separately) are:

- 160 micrograms of okadaic acid equivalents per kilogram for okadaic acid, dinophysistoxins and pectenotoxins;
- 1 milligram of yessotoxin equivalents per kilogram;
- 160 micrograms of azaspiracid1 equivalents per kilogram for azaspiracids.

[8] Opinion of the French Food Safety Agency of 11 July 2008 on the relevance of taking the *Ostreopsis* epibenthic microalgae into account as part of the general monitoring system for the marine environment and marketed foodstuffs.

A **vigilance system** is being introduced as part of an effort to improve the national monitoring system for lipophilic phycotoxins in shellfish, based on the chemical analysis of phycotoxins subject to regulations (LC/MS-MS) instead of the mouse bioassay, for the purpose of:

- detecting the occurrence of known lipophilic phycotoxins not subject to regulations, new analogues of known phycotoxins and emerging phycotoxins;
- conducting regular monitoring outside at-risk periods and/or in the absence of toxic phytoplankton.

The vigilance system will therefore ultimately improve consumer safety particularly at the beginning of the transition from the mouse bioassay to the chemical analysis.

Concerning the monitoring of known or emerging lipophilic phycotoxins not subject to regulations, the mouse bioassay appears to be suitable with implementation starting in 2010. Pending the development of rapid detection tests that cover the widest possible range of toxicity types corresponding to known toxins (gastro-intestinal effects, hepatotoxicity, neurotoxicity, etc.), AFSSA recommends maintaining the mouse bioassay as a global testing tool.
However, it is useful to review this tool's main limitations, due to problems with regard to:

- specificity and sensitivity that are partially related to the variability of the biological material (mouse);
- extrapolation to humans.

The relevance of maintaining the mouse bioassay in a vigilance system should be reconsidered once rapid detection tests are available, given the progress of work on *in vitro* cell lines.

Concerning vigilance outside of at-risk periods and/or when no toxic phytoplankton are found, the 10 reference sampling points defined for 2009 (see section 5) should be kept for 2010, to continue data acquisition and collect historical data for these sampling points.
As stated above, this system should be enhanced as quickly as possible to incorporate risks related to the phycotoxins produced by various species in the *Ostreopsis* genus found on the Mediterranean coast. For example, it might be possible to use underwater mussel bags as a bio-indicator and this should be studied.

For the purpose of consolidating the system, the number and geographic distribution of these vigilance points could be analysed further on the basis of the data already collected by Ifremer.

Sampling could initially be done monthly and then adjusted following a study at several sampling points to determine the optimal interval (for example, once or twice a month).

The monitoring procedures could include:

- a chemical analysis of known lipophilic phycotoxins not subject to regulations and new analogues of known phycotoxins;
- a bioassay (mouse bioassay pending new alternative tools);
- phytoplankton monitoring (whenever possible).

In the event of a positive mouse bioassay result not explained by chemical analysis and coinciding with an unusual situation (e.g. in terms of place, period or mouse symptomatology), such an episode should be examined by a **Vigilance Unit** that could comprise the Steering Committee's members (DGAL, DPMA, DGCCRF, DGS, Ifremer, AFFSA/Marine biotoxins NRL) and representatives of the InVS and AFSSA/DERNS to bring expertise in the risk assessment area, in order to propose, depending on the situation, management measures and/or additional investigations including the epidemiological aspect and/or alert measures.

Concerning paralytic (PSP) and amnesic (ASP) toxins, a vigilance system does not appear to be necessary. The current system, based on shellfish analyses when the alert level is exceeded for the phytoplankton that produce these toxins, provides consumers with an adequate level of protection.

The fact that, over the past few years, the monitoring systems implemented by the DGAL did not reveal any situations in which the regulatory limits of PSP or ASP were exceeded in marketed

shellfish batches indirectly proves that the system for monitoring the marine environment is effective. In comparison, concerning lipophilic phycotoxins between 2006 and 2009, 4 shellfish samples of French origin prescribed by monitoring systems showed levels that exceeded the regulatory limit.

6. CONCLUSIONS AND RECOMMENDATIONS

Concerning the determination of at-risk periods, preliminary analyses tend to show that the period that provides the most information is the past 3 years (the last 2 full years and the current year's available results).

Concerning the number of events to be taken into account, it could be 1 or 2 positive results over the past 3 years, depending on the objective (balance between sensitivity and specificity or the highest degree of consumer protection).

Nevertheless, before this criterion may be applied by REPHY, its effectiveness in predicting toxicity episodes through simulations of a few previous years should be verified.

Concerning the initial recommendations for a vigilance plan for lipophilic phycotoxins, the 10 reference sampling points defined for 2009 should be kept for 2010, to continue data acquisition and establish an historical data record of these sampling points.
This system should however be improved as quickly as possible however to incorporate risks related to the phycotoxins produced by various species in the *Ostreopsis* genus found on the Mediterranean coast.

Given that implementation will begin in 2010, AFSSA recommends maintaining the bioassay as a global testing tool for this vigilance plan.

In the event of a positive mouse bioassay result that is not explained by chemical analysis and which coincides with an unusual situation (for example, in terms of area, period or mouse symptomatology), such an episode should be examined by a Vigilance Unit that would propose, according to the situation, management measures and/or additional investigations including the epidemiological aspect and/or alert measures.

7. KEYWORDS.

Lipophilic phycotoxins, marine biotoxins, at-risk periods, reference sampling points, vigilance.

The Director General of the French Food Safety Agency

Marc MORTUREUX

Annexe 1a:

Analysis no. 1 of the predictive potential of a site's historical data based on mouse bioassay results

The work was undertaken based on a file submitted by Ifremer with compiled data from 9,677 analyses (mouse bioassay) conducted at 245 sites between January 1995 and December 2008.

The purpose was primarily to assess the relevance of the current definition of an 'at-risk month' for a given site. Currently, a month is considered to be an at-risk period if it has been found to be positive twice over the past 6 years.

For a given site, a month is declared to be *positive* if at least one mouse bioassay concluded '+MT' (positive mouse test). It is considered to be *negative* if all the mouse bioassays performed in the month concluded '-MT' (negative mouse test) or 'NA' (not analysed), with at least one test concluding '-MT'. Furthermore, a month is declared to be *undefined* if no tests were undertaken in this month or if the tests all concluded 'NA'.

The classification of a month as an at-risk period is not included in the data in this file. This therefore had to be recalculated using the previous rule. In view of the period covered by the data, each month was evaluated as either an at-risk period or a at-no-risk period only for the period ranging from January 2001 to December 2008, i.e. 96 months.

In order to limit the proportion of undefined months (months for which no analyses were undertaken at the area in question), the study took into account only the 25 sites for which more than 100 analyses had been conducted during the period from January 1995 to December 2008 (minus two sites used as reference sampling points[9]). These sites correspond to 5,409 analyses out of the 9,677 recorded analyses and cover a wide variety of situations. For example, for site 36083010 (Salses-Leucate), 307 analyses were conducted. Only 15 of the 96 months were undefined and 53 months (more than half) were positive. Phycotoxins are therefore found very frequently at this site. Conversely, site 32071013 (Pertuis de Maumusson/Ronce) was regularly studied (39 undefined months and 57 analysed months) whereas phycotoxins were found during only one of the 96 months. Other sites appear to be studied less regularly. For example, for site 07015025 (Barfleur community), 76 months were undefined, and 101 analyses were conducted from 1995 to 2008, but only 20 of the 96 months taken into account here are concerned and all of the analyses were negative.

RESULTS

In total, 2,400 observations (25 sites x 96 months) were examined using the following procedures:

- classification as an *at-risk month* or *at-no-risk month*;
- results of the month's analyses: *positive, negative* or *undefined*.

Current situation: 2 positive results over the past 6 years

The results are as follows:

		Classification	
		At-risk month	Risk-free month
Analysis result	Undefined	0	1231
	Positive (+MT)	267	178
	Negative (-MT)	171	553

This table shows that the current classification of at-risk periods enabled prediction of positive results in 267/(267+178) = **60% of cases**. For the remaining 40%, other factors (plankton bloom, etc.) were taken into account when deciding to conduct an analysis.

Scenario no. 1: 1 positive result over the past 6 years

[9] The two sites 19036004 ('Basse Jaune') and 21041001 ('Les Glénan') were not used because they correspond to shellfish fishing areas. The sites 43114102 ('Etang d'Urbino – Centre') and 37089003 ('Ingril Sud'), for which 98 and 96 analyses were conducted, were used because they almost satisfied the inclusion criterion requiring 100 analyses.

A month is defined as an at-risk period if this month was found to be positive at least once over the past 6 years. In this case, the examined data give the following result:

		Classification	
		At-risk month	Risk-free month
Analysis result	Undefined	*220*	1011
	Positive (+MT)	358	87
	Negative (-MT)	326	398

Some months, classified as at-risk by this new definition, were not subject to an analysis that might have confirmed the positive result. The information obtained should therefore be cautiously considered. However, this definition of at-risk periods predicted positive results in 358/(358+87) = **80.4% of cases**. Only 19.6% of the positive results were obtained during at-no-risk periods (false negatives). The forecast quality would therefore be doubled with respect to the current situation.

With the available data, it is not possible to precisely evaluate the number of additional analyses that would be needed by this new rule. However, the number would at least equal the number of months classified as at-risk by this rule and for which no analyses were conducted, i.e. 220.

Scenario no. 2: 1 positive result over the past 4 years

A month is defined as an at-risk period if this month was found to be positive at least once over the past 4 years. In this case, the examined data give the following result:

		Classification	
		At-risk month	Risk-free month
Analysis result	Undefined	*188*	1043
	Positive (+MT)	339	106
	Negative (-MT)	294	430

This definition of at-risk periods predicted positive results in 339/(339+106) = **76.2% of cases** while 23.8% of the positive results were obtained during at-no-risk periods (false negatives). The forecast quality is between that observed in the current situation and that of scenario no. 1, but remains close to that of scenario no. 1. Moreover, the number of additional analyses, evaluated as described above, would be at least 188.

This scenario gives results similar to those obtained in scenario no. 1.

Scenario no. 3: 1 positive result over the past 2 years

A month is defined as an at-risk period if this month was found to be positive at least once over the past 2 years. In this case, the examined data give the following result:

		Classification	
		At-risk month	Risk-free month
Analysis result	Undefined	*122*	1109
	Positive (+MT)	286	159
	Negative (-MT)	218	506

This definition of at-risk periods predicted positive results in 286/(286+159) = **64.3% of cases** while 35.7% of the positive results were obtained during at-no-risk periods (false negatives). The number of additional analyses, evaluated as described above, would be at least 122.

Scenario no. 4: 1 positive result over the past 3 years

A month is defined as an at-risk period if this month was found to be positive at least once over the past 3 years. In this case, the examined data give the following result:

	Classification

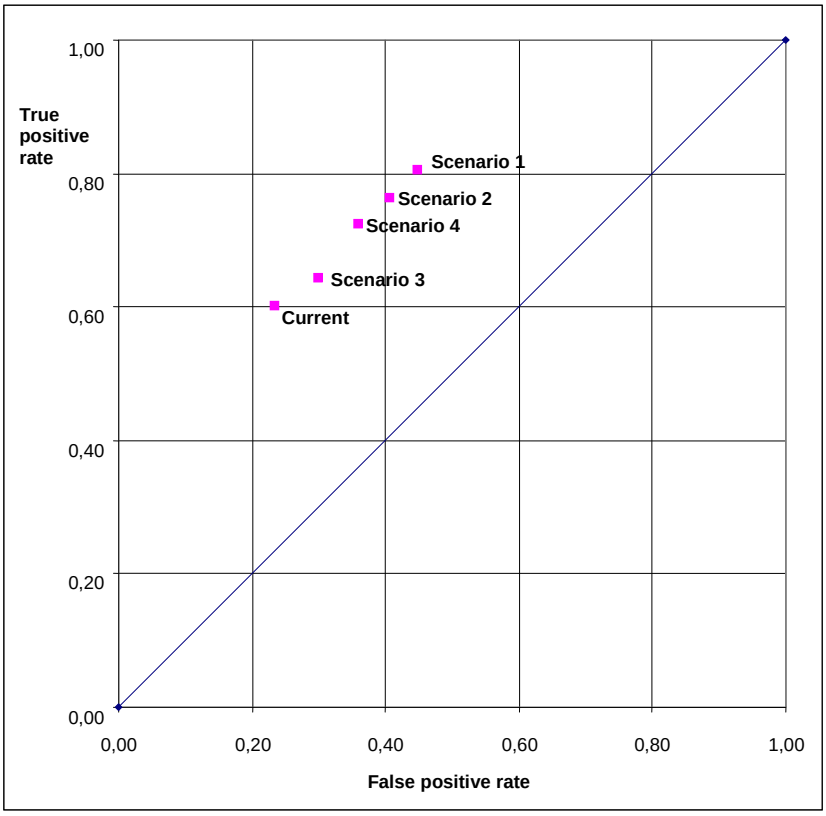
		At-risk month	Risk-free month
Analysis result	Undefined	*162*	1069
	Positive (+MT)	322	123
	Negative (-MT)	261	463

This definition of at-risk periods predicted positive results in 322/(322+123) = **72.4% of cases** while 27.6% of the positive results were obtained during risk-free periods (false negatives). The number of additional analyses, evaluated as described above, would be at least 162.

CONCLUSION

The comparison of the current definition and the four proposed scenarios gives the following ROC-type diagram. It should be noted that the false positive rate systematically deteriorates as the false positive rate improves. When choosing the ideal scenario, these two types of information need to be balanced. It is however difficult to conclude with certainty because the numerous undefined months in the various hypothetical scenarios could significantly modify the results.

After a preliminary analysis we may conclude that **the current method favours a low false positive rate** (analyses in at-risk periods account for only 23% of negative analyses) whereas scenario 1 favours the true positive rate (80% of the positive tests were detected in at-risk months).



Preliminary ROC-type diagram (several months remain undefined).

List of the 25 sites taken into account and the corresponding number of analyses.

Marine area ID	Marine area name	Number of analyses	Site ID	Site name
097	Etang de Salses-Leucate	642	36083002	Parc Leucate 2
087	Arcachon aval	432	34077060	Banc Arguin sud
068	Traicts du Croisic	343	27059002	Le Grand traict
049	Rade de Lorient - Groix	319	23045001	Groix nord
097	Etang de Salses-Leucate	307	36083010	Salses-Leucate
040	Baie de Douarnenez	245	19039001	Kervel
038	Iroise - Camaret	233	19036003	Dinan Kerloc'h
088	Bassin d'Arcachon	231	34077037	Grand Banc
048	Aven - Belon - Laïta	216	22044004	Poulguin
010	Baie de Seine et Orne	199	05010002	Antifer ponton pêche
066	Pen Bé	185	27057018	Pont-Mahé
055	Baie de Quiberon	183	25049001	Men er Roue
065	Estuaire de la Vilaine	178	27057002	Le Halguen
097	Etang de Salses-Leucate	172	36083013	Coudalère
065	Estuaire de la Vilaine	165	27057001	Kervoyal
067	Traict de Pen Bé	144	27057007	Pointe Pen Bé
042	Baie d'Audierne	142	20040001	Tronoen
063	Baie de Vilaine - côte	128	27057004	Le Marescle
047	Baie de Concarneau	124	21043001	Penfoulic
015	Ravenoville - Saint Vaast - Barfleur	114	07015025	Barfleur gisement
018	Cotentin Ouest	104	09021025	Les Minquiers
053	Rivière d'Etel	104	24047006	Beg er Vil
082	Pertuis de Maumusson	101	32071013	Ronce
117	Plaine Orientale	98	43114102	Etang d'Urbino - Centre
105	Etangs Palavasiens	96	37089003	Ingril sud

Annexe 1b:

Analysis no. 2 of the predictive potential of a site's historical data (since 2003) based on mouse bioassay results

Due to the methodological changes in the implementation of mouse tests in 2002, only data obtained after 2002 (2003-2008) were used for the analysis.

Compared to the previous approach, a scenario that analyses results over several years should not lack any analyses for any of the years in question. For example, if a site was studied in the month of March and if the criterion includes 4 years of back data, the value for this site will be analysed only if at least one analysis was conducted for 5 consecutive years. The most recent observed result is considered to be the variable of interest that should be explained by the previous years' results.

In total, the data file contains 515 lines, each corresponding to one month, one site and several years of mouse bioassay results. The number of results that can be used varies according to the period in question. For example:

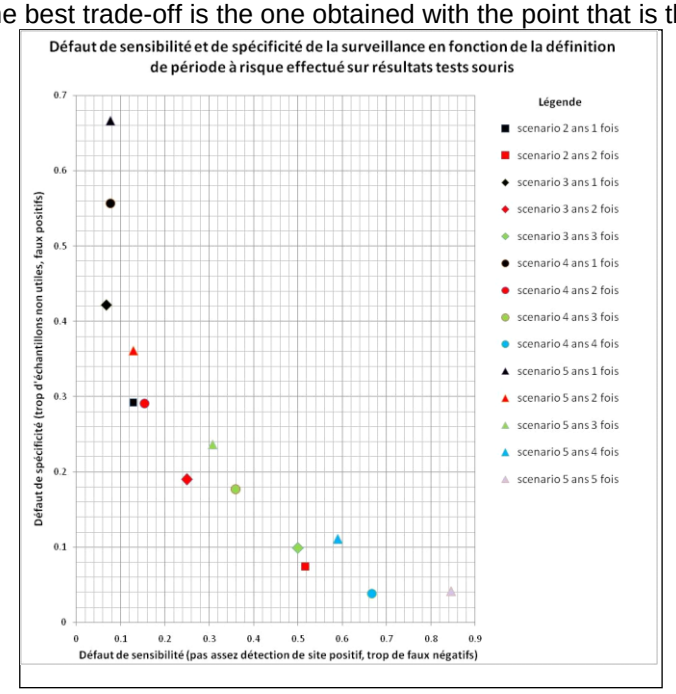
- for the 2-year history, 264 results are available;
- for the 3-year history, 165 results are available;
- for the 4-year history, 118 results are available;
- for the 5-year history, 111 results are available.

A calculation example is given below. If the interpretation rule covers 3 years with at least 2 years in which positive results were observed for a given month, at a given site, for a period classified as at-risk, the 165 available results can be classified according to the following table:

	At-risk period	Risk-free period	TOTAL
Negative observation, in the most recent period	23	98	121
Positive observation, in the most recent period	33	11	44
TOTAL	56	109	165

- Sensitivity is 33/44;
- Specificity is 98/121;
- The false positive rate is 23/121 (or 1-Sp) (numerical value 0.19);
- The false negative rate is 11/44 (or 1-Se) (numerical value 0.25).

In the graph below (same as on page 4), the analysed point corresponds to the scenario for 3 years and 2 times, x-axis 0.25 and y-axis 0.19. The closer the point is to the origin of the axes (0,0), the lower the false positive and false negative rates. If the same significance is assigned to false positives and false negatives, the best trade-off is the one obtained with the point that is the closest to (0,0).



Annexe 2:

Analysis of the predictive potential of a site's historical data based on mouse bioassay results

ON THE BASIS OF THE CHEMICAL ANALYSIS

The analysis examined the 9,678 records in the REPHY database submitted by Ifremer for this expert assessment. Only 1,348 of these records have a chemical analysis result expressed as a binary value, i.e. a value that does or does not exceed the regulatory limit.
The number of data per site or per month varies. The data taken from a site, for a given month and a given year, are considered to be equiprobable and follow a binomial distribution with a probability related to the site, the month and the year, which can be estimated from the observations.
The question can be expressed as follows: Does the frequency of positive samples measured in years n-1, n-2, n-3, n-4 and n-5 for a given month and a given site provide information enabling us to predict the frequency of positive samples measured in year n? In order to avoid statistical estimation problems of data independence and a too high colinearity of explanatory values, if the measurement was taken several years in a row at a given site, in the same month, only the most recent frequency estimate is kept in the data set. The values obtained in previous years are kept as explanatory variables of this frequency and are expressed as mean frequency (same site, same month).

The final file obtained for the chemical analyses, including at least one value obtained during the previous years, for a given month and a given site, corresponds to 303 analyses or 102 frequency measurements. **Of these 102 measurements, 76 are linked to a measurement from the previous year (n-1), 72 to a measurement from year (n-2), 43 to a measurement from year (n-3), 17 to year (n-4) and 5 to year (n-5).** The estimated measurement of effect for years n-4 and n-5 is therefore considered to be unreliable, and is abandoned for year n-5.

A common linear model, such as a binomial model with a logistic function, is used (model commonly used in epidemiology).

The relationship is as follows: $logit(P) = log\ (P/(1-P)) = \alpha + \beta_{n-1}P_{n-1}$

The factor analysis shows that the measurements taken in previous years had a good predictive potential. It should be noted that this predictive potential decreases over time, even though no direct comparison can be made due to the different number of available results.

The prediction P (for year n) can be calculated with the following equation:

$$P = \frac{\exp\ (\alpha + \beta_{n-1}P_{n-1} + \beta_{n-2}P_{n-2} \cdots)}{1 + \exp\ ((\alpha + \beta_{n-1}P_{n-1} + \beta_{n-2}P_{n-2} \cdots)}$$

RESULTS:

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	-2.41	0.26	-9.112	< 2.10-16
12 months	2.66	0.37	7.01	**$1.53.10^{-12}$**
(Intercept)	-2.54	0.33	-7.69	$1.4.10^{-14}$
24 months	2.27	0.44	5.17	**$2.34.10^{-07}$**
(Intercept)	-2.31	0.377	-6.13	$8.52.10^{-10}$
36 months	2.03	0.57	3.58	0.000345
(Intercept)	-2.80	0.67	-4.18	$2.9.10^{-05}$
48 months	4.98	1.52	3.26	0.0011

To assess the usefulness of adding information from the previous year, a multivariate approach was used. The models with 12-month information were compared with several other models containing several covariables corresponding to the information observed in previous years.

The only model that significantly (5% level) improved the prediction relied on information from the past 2 years (detailed results not given).

There are too few available results to analyse the effect of years >3 (50 values) on the basis of chemical results, so while the contribution is not significant it does not necessarily mean that the information is not relevant for some sites.

Moreover, the number of analyses conducted in the month at one site or by site is highly variable. The accuracy of an estimate is therefore not the same from one site to another or from one year to another. For example, if only one analysis was conducted, the information provided is weaker than if there had been 10 analyses. The model described here does not take this into account.

ON THE BASIS OF THE MOUSE BIOASSAY

The same analysis was conducted using mouse bioassays on the 515 records obtained after 2002 (see annexe 1a). But in this case, the analysis method relied on the frequency of positive responses. It is therefore independent from the interpretation rule.

Out of these 515 measurements, 322 are linked to a measurement from the previous year (n-1), 322 to a measurement from year (n-2), 238 to a measurement from year (n-3), 194 to year (n-4) and 122 to year (n-5).

The association between the positive frequency observed in the most recent year and the frequency observed in one or more previous years is still found to be significant, factor by factor at the 5% level. In a multivariate analysis, the analysis shows that it is useful to keep the information from the 3 previous years at the level of 5%, but that past this time, the information is no longer useful for predicting contamination.

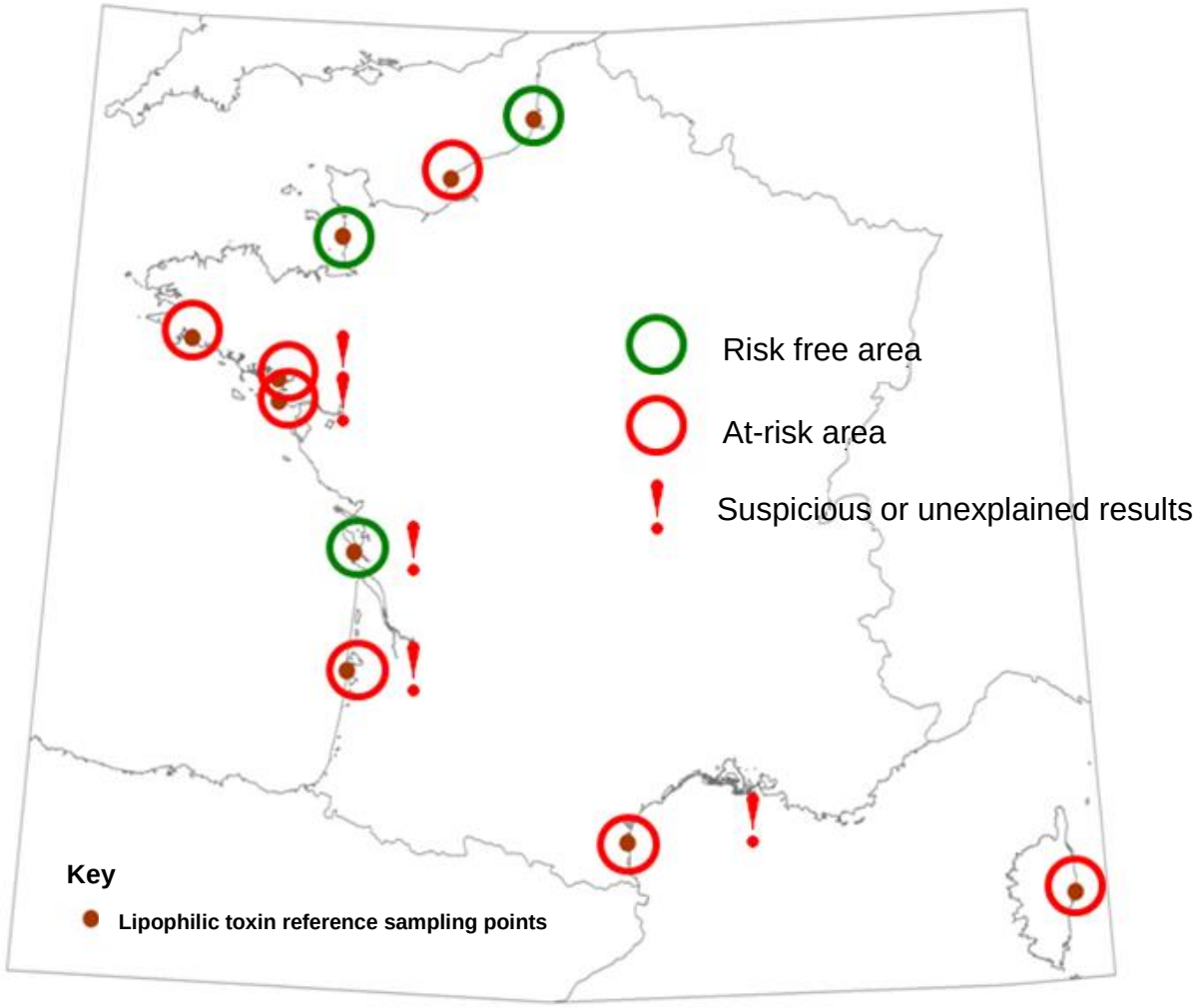
The number of analyses conducted in the month at one site or by site is highly variable. The estimate's accuracy is therefore not the same from one site to another or from one year to another. For example, if only one analysis was conducted, the information provided is weaker than if there had been 10 analyses. The model described here does not take this into account.

Annexe 3:
History of lipophilic phycotoxin reference sampling points (source: Ifremer)

marine area		LER	2009 reference points			monitored since	shellfish		comments	sampling
code	name		id	mnemo	name					
006	Baie de Somme - large	LER/BL	3006102	006-P-009	Pointe de St Quentin	2009	mussels	bouchot	area not at risk, with no history of toxic episodes	no results available
010	Baie de Seine et Orne	LER/N	5010002	010-P-002	Antifer ponton pêche	2008	mussels	specific struct.	at-risk area + national maximum levels of *Dinophysis*	mussels regularly sampled since March 2008
018	Cotentin Ouest		9021013	018-P-056	Pointe Agon nord	2009	mussels	bouchot	area not at risk for coastal shellfish, with no history of toxic episodes	mussels regularly sampled since March 2009
							cupped oysters	rack culture		
047	Baie de Concarneau	LER/FBN/CC	21043003	047-P-003	Le Scoré	2008	mussels	longline	at-risk area	mussels regularly sampled since May
							cupped oysters	rack culture		
065	Estuaire de la Vilaine	LER/MPL/TM	27057001	065-P-001	Kervoyal	2008	mussels	bouchot	at-risk area + short survival times with neurological symptoms in 2006 and 2007	mussels regularly sampled since May 2008
068	Traicts du Croisic	LER/MPL/NT	27059002	068-P-002	Le Grand traict	2006	mussels	bottom culture	at-risk area + observation of questionable results on several occasions, some with neurological symptoms in 2006, 2007 and 2008	mussels (mainly) or oysters or cockles or clams regularly sampled since May 2006
							cupped oysters	rack culture		
							cockles	natural bed		
082	Pertuis de Maumusson	LER/PC/LR	32071013	082-P-009	Ronce	2006	cupped oysters	rack culture	area not at risk, but questionable results observed on several occasions before 2007	oysters regularly sampled since April 2006
							cockles	natural bed		
087	Arcachon aval	LER/AR	34077060	087-P-009	Banc Arguin sud	2006	mussels	natural bed	several atypical toxic episodes since 2005	mussels AND oysters regularly sampled since January 2006
							cupped oysters	rack culture		
							cockles	natural bed		
097	Etang de Salses-Leucate	LER/LR	36083002	097-P-002	Parc Leucate 2	2006	mussels	longline or rope	at-risk area over a long period + questionable results observed on several occasions	mussels AND oystesr regularly sampled since January 2006
							cupped oysters	longline or rope		
118	Etang de Diana	LER/PAC/CO	43114001	118-P-001	Diana centre	2008	mussels	longline or rope	at-risk area	mussels (mainly) or oysters regularly sampled since January 2008
							cupped oysters	raft		

Annexe 4:

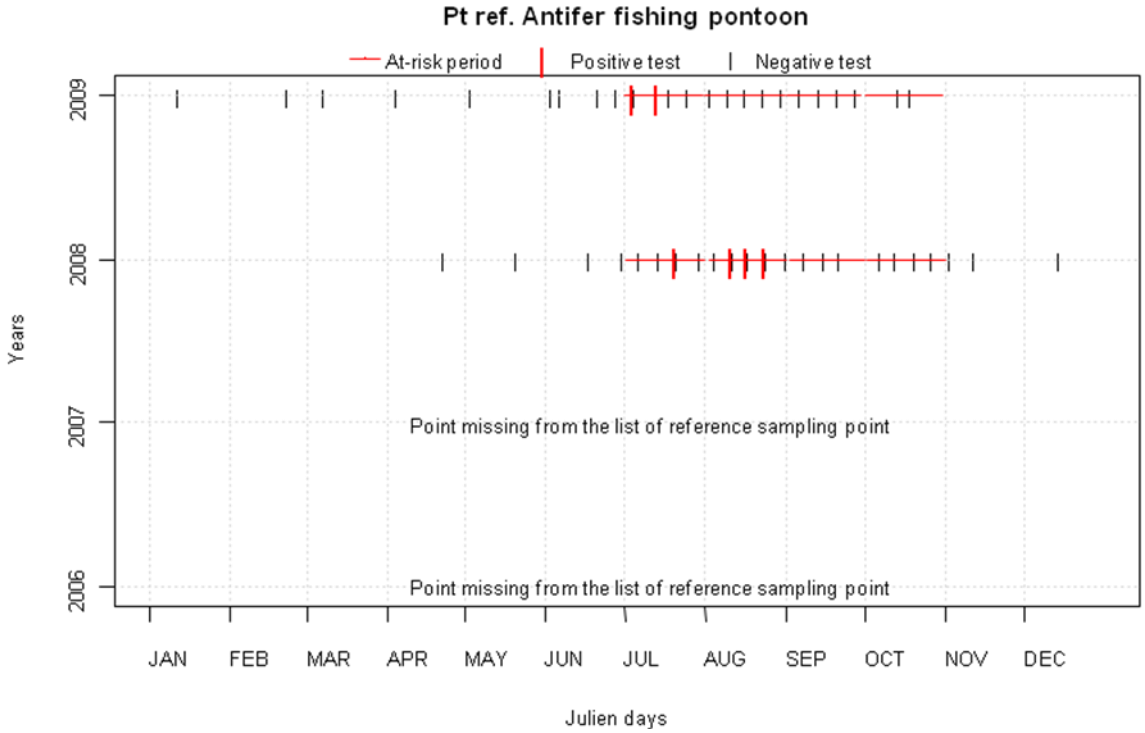
Map of lipophilic phycotoxin reference points (source: Ifremer)



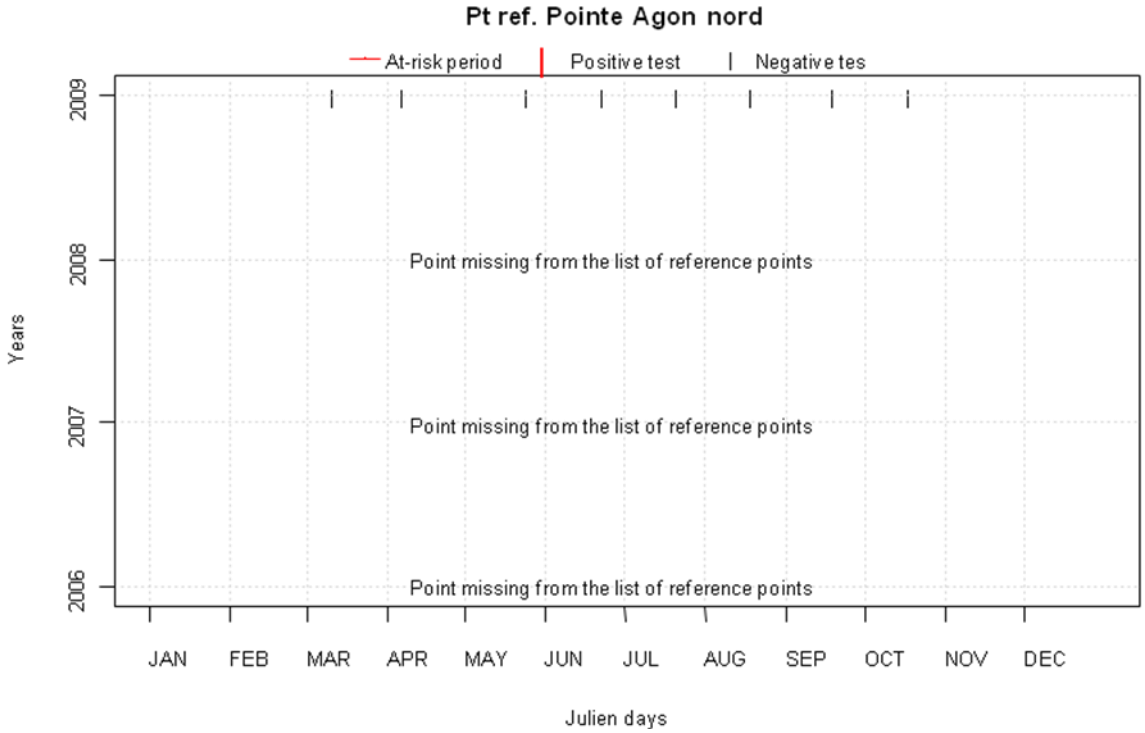
Annexe 5

Results from the 10 reference sampling points monitored in 2009 (+3 other points abandoned in 2009; source: Ifremer)

Pointe de St Quentin (Baie de Somme large): new point in 2009, no data in Quadrige²

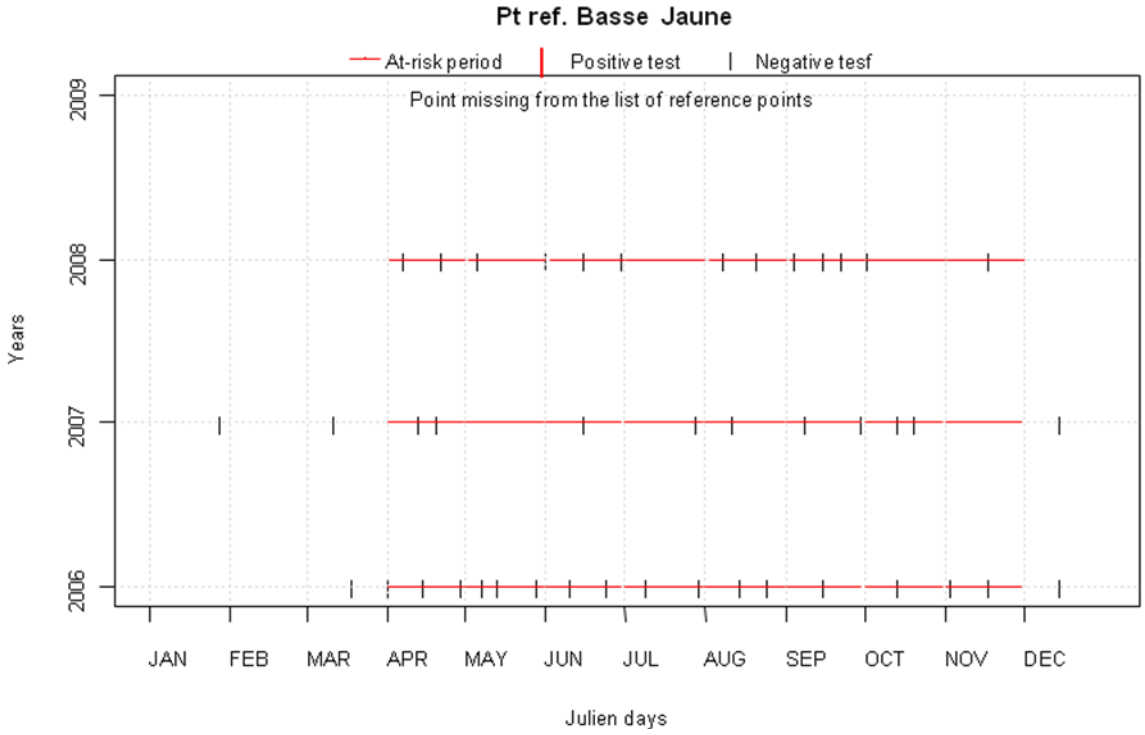


Area with the highest concentrations of *Dinophysis*
➔ **No positive bioassays outside of the at-risk period**



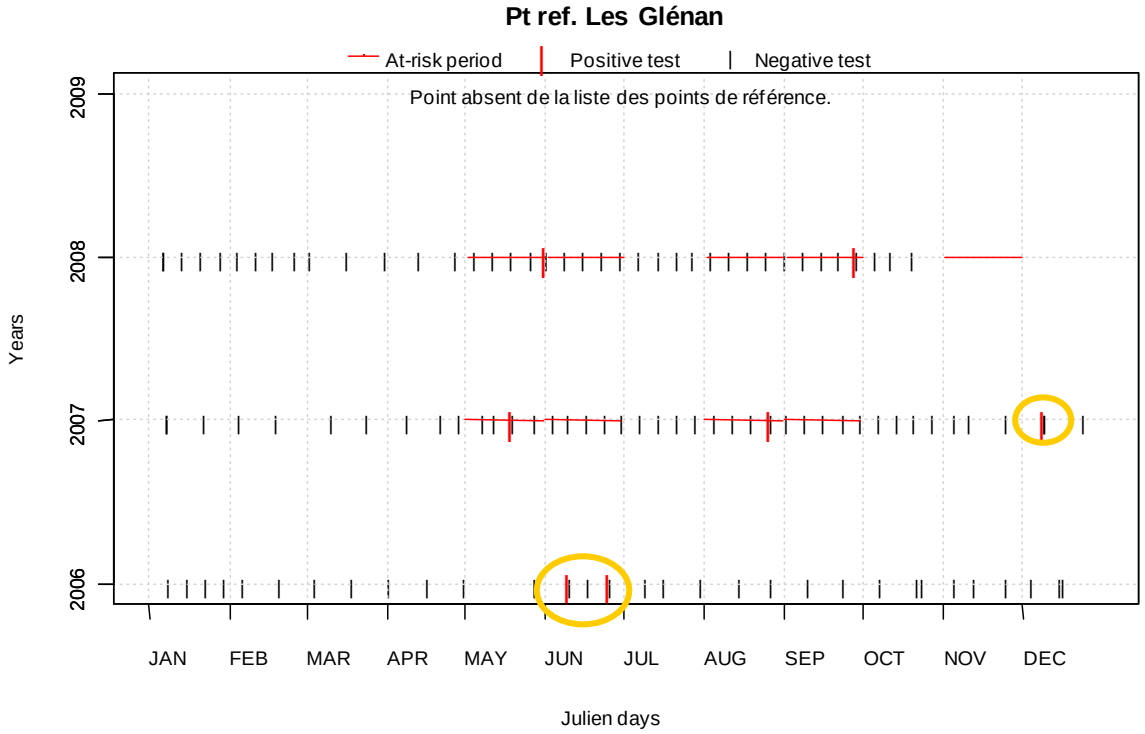
Area with no at-risk periods
➔ **No positive bioassays**

Point abandoned in 2009 since these are shellfish intended for fishing (the reference points now concern only coastal communities)

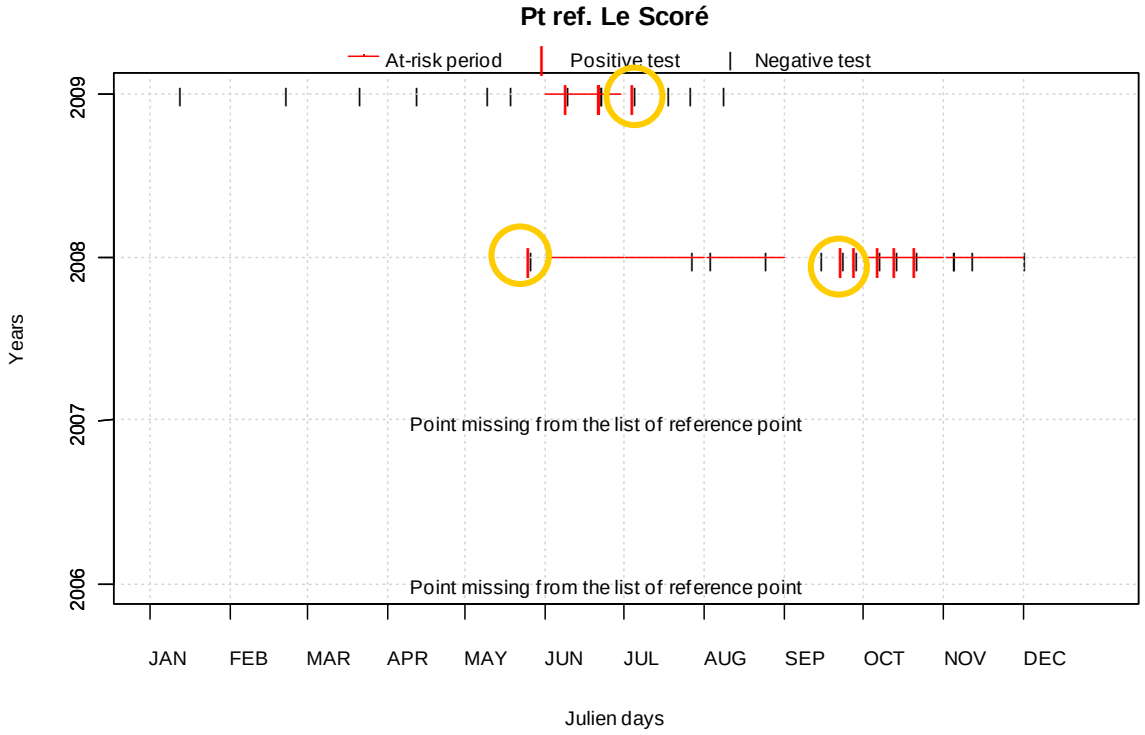


➔ **No positive bioassays outside of the at-risk period**

Point abandoned in 2009 since these are shellfish intended for fishing (the reference points now concern only coastal communities)

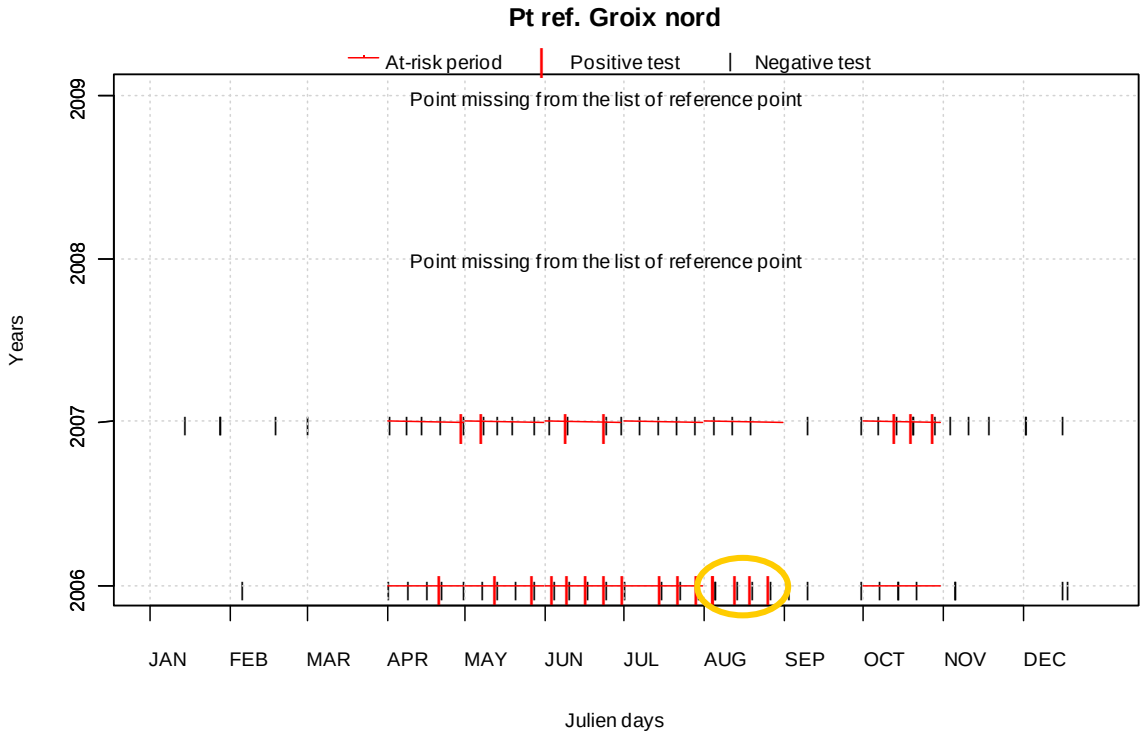


➔ **3 positive bioassays outside of the at-risk period: 2 in 2006 and 1 in 2007 (but the at-risk periods used are for coastal communities whereas these are shellfish intended for fishing that are systematically monitored during harvesting periods)**

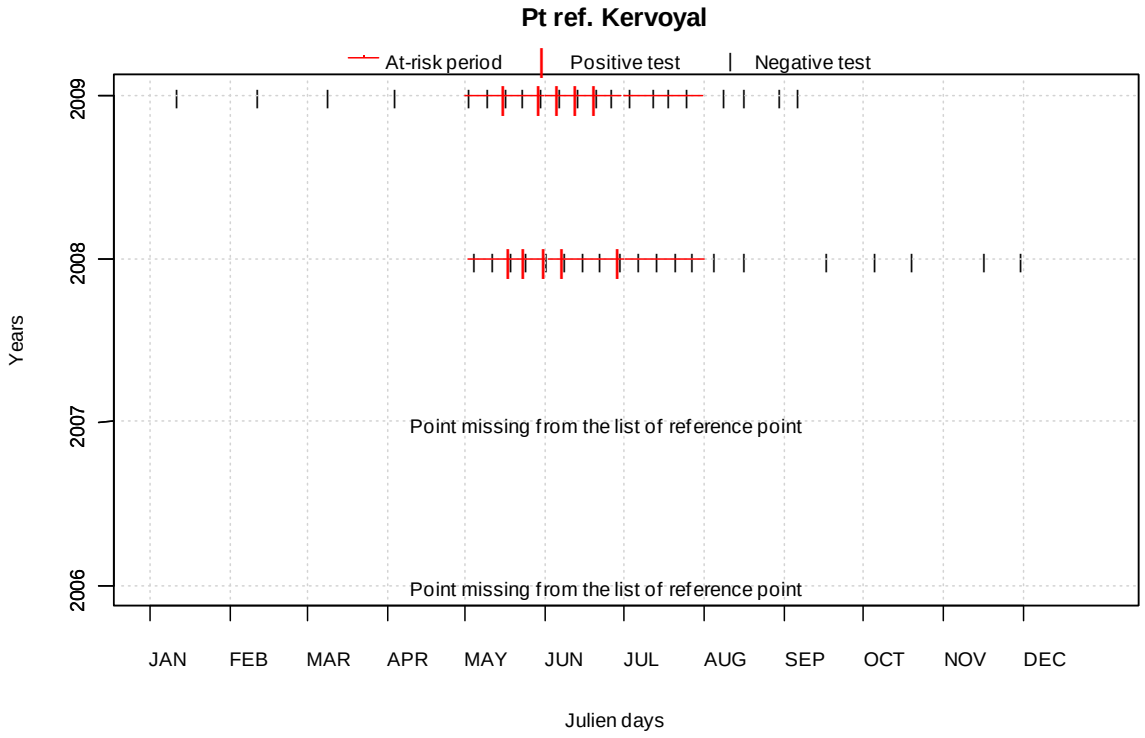


➔ **4 positive bioassays outside of the at-risk period: 3 in 2008 and 1 in 2009**

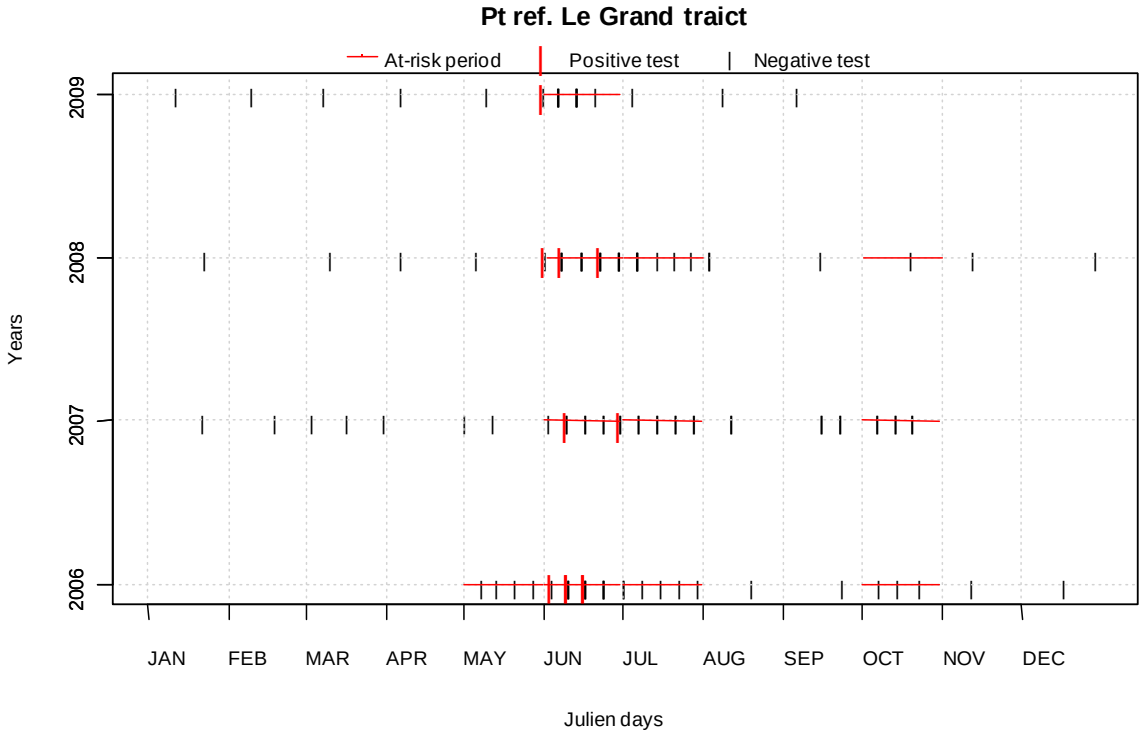
Point abandoned in 2009 since there are no more longline mussels available (production stoppage)



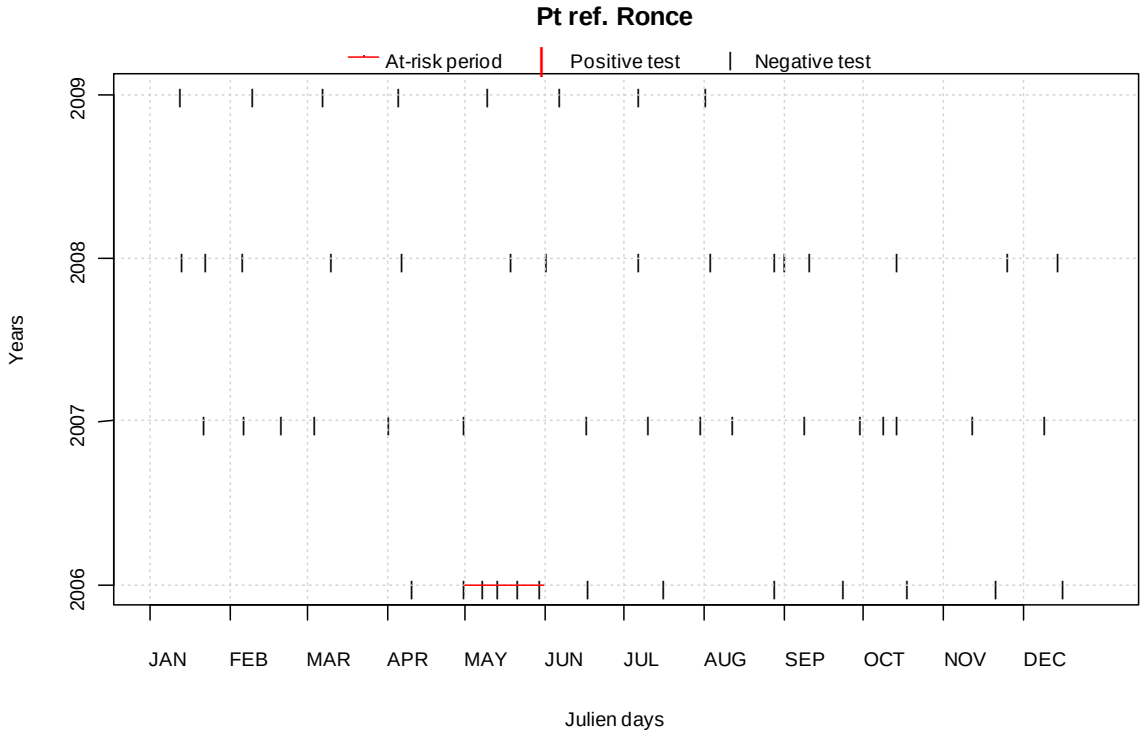
➔ **4 positive bioassays outside of the at-risk period in 2006**



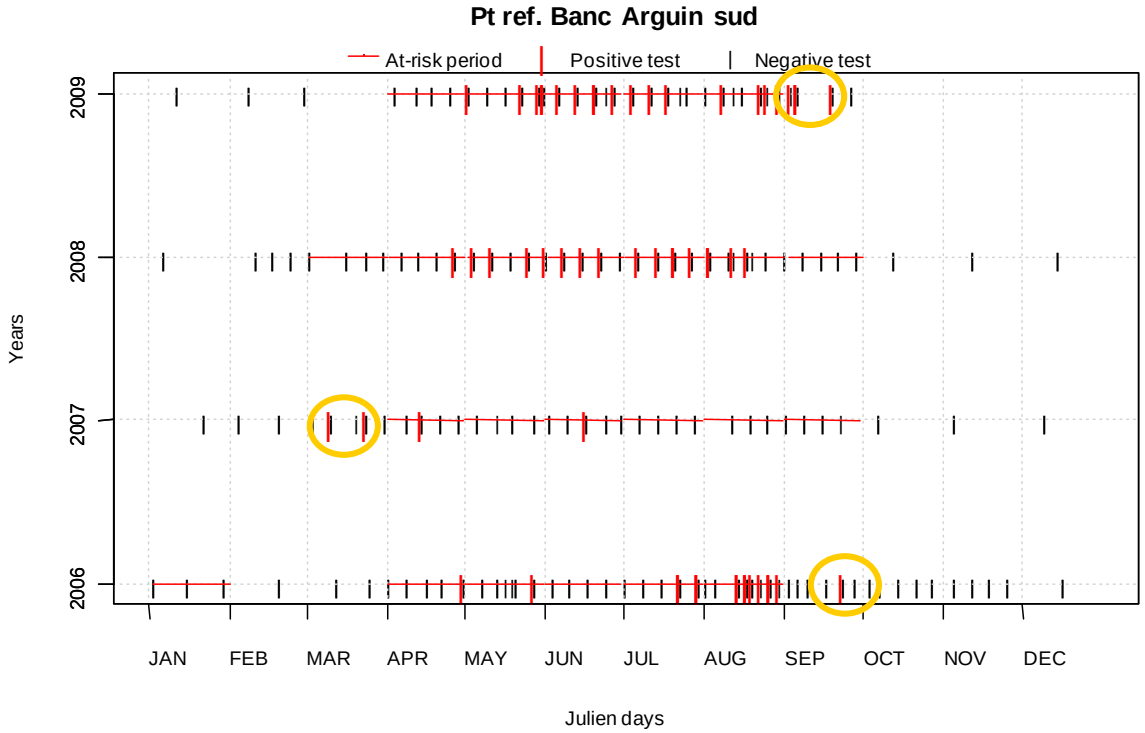
➔ **No positive bioassays outside of the at-risk period**



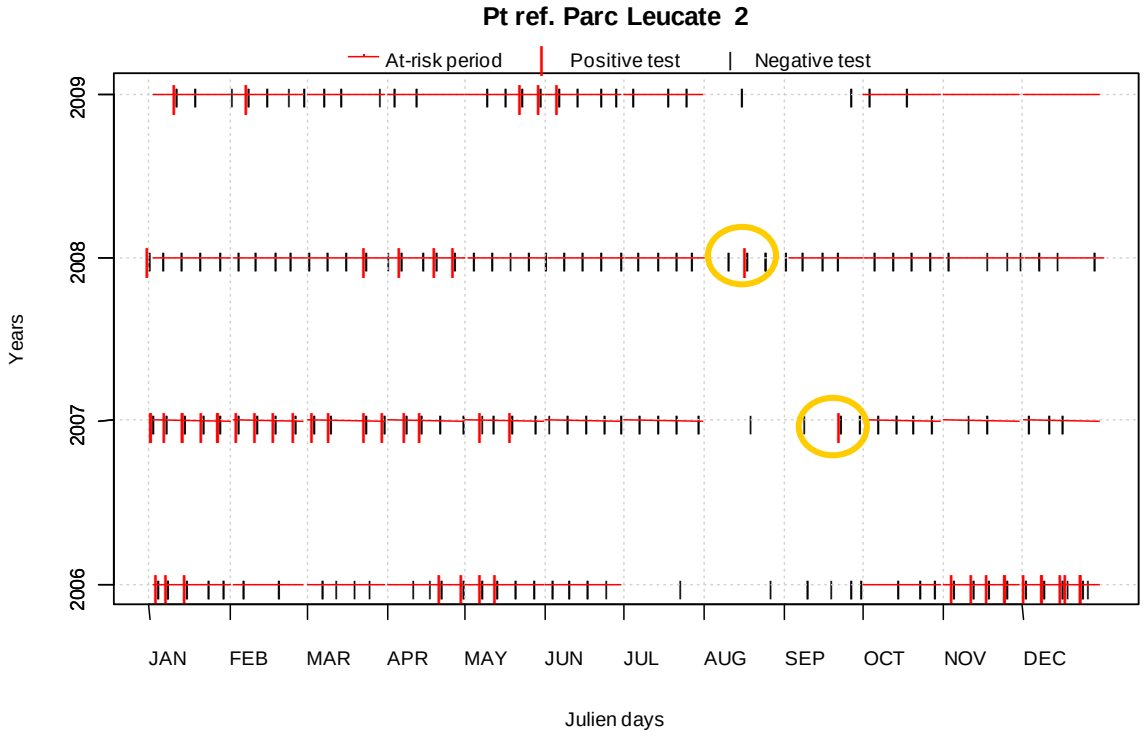
➔ **No positive bioassays outside of the at-risk period**



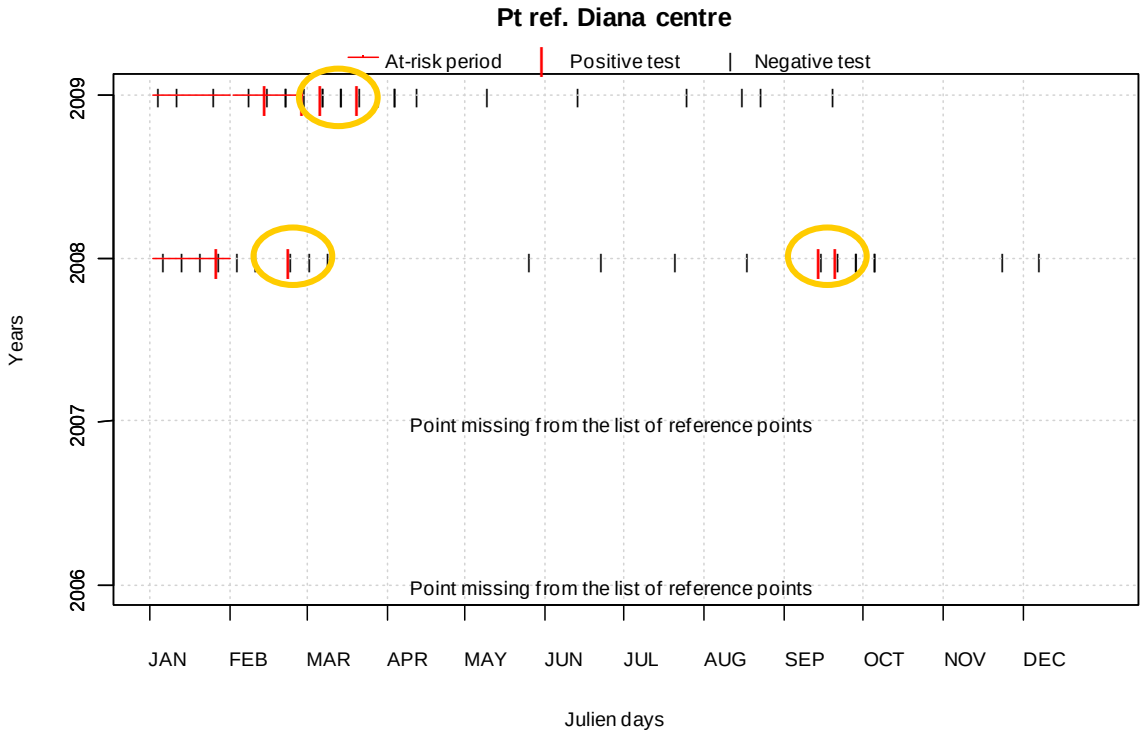
➔ **No positive bioassays outside of the at-risk period in 2006. Area risk-free since 2007 with no positive bioassays.**



➔ **6 positive bioassays outside of the at-risk period: 1 in 2006, 2 in 2007, 3 in 2009**



➔ **2 positive bioassays outside of the at-risk period: 1 in 2007 and 1 in 2008**



➔ **5 positive bioassays outside of the at-risk period: 3 in 2008 and 2 in 2009**

Annexe 6

Example of the Management Cell set up in Ireland, described in the Code of Practice No. 6 'Monitoring of marine biotoxins in bivalve molluscs', pre-publication draft, 2005[10],

In Ireland, the FSAI (Food Safety Authority of Ireland), the SFPA (Sea-Fisheries Protection Authority), the Marine Institute (Irish NRL) and the ISA (Irish Shellfish Association) is convened, at the request of any of its members, in the following situations:

- The results of test analyses are inconsistent with local or national trends
- A single, unexpected negative or positive result occurs
- Borderline results need consideration
- Sampling continuity has been interrupted
- A production area has been assigned an incorrect status
- Results from sampling indicate that the sampling frequency for a production area or shellfish species must be modified.

The Management Cell considers the following factors to reach a decision:

- The species of bivalve mollusc
- The details of the mouse bioassay result (mortality rate, survival time, etc.)
- Chemical analysis result
- Phytoplankton result
- Time of year/Toxicity Profile
- Status of adjacent production area
- Relevant historical data and sample analysis reports as provided by the Marine Institute
- Any other relevant data.

The results of self-controls professionals have carried out may be taken into account if they were performed in laboratories designated by the SFPA or FSAI and if the sampling/analysis protocols complied with the requirements of the SFPA, FSAI and Marine Institute.

Various types of management decisions may be made:

- Changing a production area's status
- Recommending a voluntary closure to producers
- Closing adjacent areas within the same bay
- Increasing sampling frequency and testing through an intensive series of chemical tests
- Decreasing sampling frequency according to profile and season
- Re-opening of an area
- Other actions as appropriate.

This Management Cell met 89 times in 2005, 103 times in 2006 and 35 times in 2007.

[10] http://www.fsai.ie/uploadedFiles/Monitoring_and_Enforcement/Monitoring/Shellfish_Monitoring/biotoxin_cop.pdf