Technical Report from the Community Reference Laboratory for Mollusc Diseases

2009

Content:

Introduction p. 2

Functions and duties for the Community Reference Laboratory for mollusc diseases p. 3

Technical report p. 4

- Organise the 2009 Annual Meeting and the 7th Technical workshop for the National Reference Laboratories p. 4
- Produce a report from this meeting p. 4
- Survey and diagnosis of mollusc diseases p. 6
- Establish and maintain the library of mollusc pathogens p. 7
- Update the www site of the Community Reference Laboratory p. 8
- Supply available reference reagents and material to the National Reference Laboratories in Member States p. 9
- Identify and characterize mollusc pathogen isolates p. 12
- Organise an Inter-Laboratory Proficiency test for pathogens included in Directive 2006/88 p. 18
- Assess alternative methods for the identification of listed pathogens p. 19
- Provide opportunities of training in laboratory diagnosis of mollusc diseases p. 20
- Welcome and host visitors to the Community Reference Laboratory p. 21
- Attend international meetings and conferences p. 21
- Prepare a handbook for diagnostic procedures in a CD-ROM format p. 22
- Promote Quality Assurance in diagnostic laboratories for mollusc diseases p. 23

Annex 1: Report from the 2009 Annual Meeting of the National Reference Laboratories for Mollusc Diseases and Technical Workshop p. 25

Annex 2: Reference Material sent by the CRL during 2009 p. 65

Annex 3: Characterization and analysis performed by CRL in support of other laboratories during 2009 p. 69

Annex 4: Training and scientific collaboration in 2009 p. 70

Annex 5: Publications relevant to the work of the CRL p. 71

Annex 6: Presentations at international conferences and meetings p. 73

Cover picture: Pacific oyster (Crassostrea gigas) farming in Marennes-Oléron, France (© L. Miossec, Ifremer)
A. Introduction:

The Ifremer laboratory for Genetics and Pathology in La Tremblade (Laboratoire de Génétique et Pathologie) has been appointed as the Community Reference Laboratory for mollusc diseases (CRL) since December 1995. Functions and duties of the Reference Laboratory are established by the Council Directive 2006/88/EC, Annex VI, Part I, and follow this introduction for your information. These duties of the CRL cover pathogens of molluscs as listed in the Council Directive 2006/88/EC Annex IV Part II namely *Bonamia ostreae*, *Bonamia exitiosa*, *Marteilia refringens*, *Perkinsus marinus* and *Mikrocytos mackini*.

In 2009, the CRL included eight staff members consisting in three technical assistants and five scientists. The CRL was accredited in histopathology on the 1st October 2009 and is presently building another quality management system for the organisation of interlaboratory comparison tests.

In March 2009, the Annual Meeting for National Reference Laboratories for Mollusc Diseases was held in La Tremblade in combination with a technical workshop on the detection of *Mikrocytos mackini* by histology and detection and typing of *Marteilia refringens* by PCR-RFLP. Beside the traditional presentation of the current mollusc disease situation of individual countries, a session focused on the situation of *Bonamia exitiosa* in Europe and another one was devoted to abnormal mortality events especially those which affected *Crassostrea gigas* in 2008.

This report is based on the tentative working programme previously submitted to the Commission for the year 2009 and describing activities to be undertaken on each item as well as current status of projects.

La Tremblade, March 2010

Isabelle Arzul, Céline Garcia and Jean-Pierre Joly

The Community reference laboratories shall:

(a) coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing the disease concerned, specifically by:

(i) typing, storing and, where appropriate, supplying strains of the pathogen of the relevant disease to facilitate the diagnostic service in the Community,

(ii) supplying reference reagents to the national reference laboratories in order to standardise the tests and reagents used in each Member State,

(iii) organising periodic comparative tests (ring tests) of diagnostic procedures at Community level with the national reference laboratories designated by the Member States, in order to provide information on the methods of diagnosis used and the results of tests carried out in the Community;

(iv) retaining expertise on the relevant disease pathogen and other pertinent pathogens to enable rapid differential diagnosis;

(b) assist actively in the diagnosis of outbreaks of the relevant disease in Member States by receiving pathogen isolates for confirmatory diagnosis, characterisation and epizootic studies;

(c) facilitate the training or retraining of experts in laboratory diagnosis with a view to harmonising diagnostic techniques throughout the Community;

(d) collaborate, as regards methods of diagnosing animal diseases falling within their areas of competence, with the competent laboratories in third countries where those diseases are prevalent;

(e) collaborate with the relevant OIE reference laboratories with regard to exotic diseases listed in Part II of Annex IV under their responsibility;

(f) collate and forward information on exotic and endemic diseases, that are potentially emerging in Community aquaculture.
C. Technical report:

Objectives for the period January December 2009

Organise and prepare the 2009 Annual Meeting and the 7th technical workshop of the National Reference Laboratories of the National Reference Laboratories.

TECHNICAL REPORT

The 2009 Annual Meeting of the National Reference Laboratories for mollusc diseases (NRLs) was held from the 16th to the 19th of March 2009 in La Tremblade in combination with a Technical workshop on the detection of Mikrocytos mackini by histology and the detection and typing of Marteilia refringens by PCR-RFLP. In total, 42 participants attended this meeting. The Commission (DG Sanco unit E2) and 17 countries (Belgium, Bulgaria, Canada, Croatia, Denmark, France, Germany, Greece, Ireland, Italy, The Netherlands, Norway, Poland, Romania, Spain, Sweden and United Kingdom) were represented by their delegates or by invited private experts.

The agenda of the Annual Meeting included seven sessions, namely:

1) Diagnosis and survey of mollusc diseases
2) Situation regarding Bonamia exitiosa
3) The new Directive and related issue
4) Results of recent field studies
5) Abnormal mortality events
6) News from the bench
7) Community Reference Laboratory day life activities

The agenda of the Technical Workshop included two sessions, as follows:

8) Detection of Mikrocytos mackini by histology
9) Detection and typing of Marteilia refringens by PCR-RFLP

1. The first session concerned the epidemiological situation of mollusc diseases in Europe. Participating countries reported on their own situation with regards to diseases of molluscs and abnormal mortality outbreaks, case studies and investigations. Major epidemiological changes in EU in 2008 were (1) the detection of Bonamia ostreae in
Belgium and in two previously free areas, Lough Strangford (Northern Ireland) and Kent (England) (2) the suspension of bonamiosis in flat oysters from Norway for the second year which needed to be confirmed (3) the detection of Bonamia exitiosa in France in flat oysters from a Corsican lagoon and Mediterranean Sea (4) the mass mortalities associated with the presence of herpesviruses OsHV-1 and Vibrio species which affected Crassostrea gigas in France and also in Ireland.

2. The second session focused on the situation of Europe regarding Bonamia exitiosa. So far the parasite has been detected and confirmed in 3 Member States: France, Italy and Spain. However, where Bonamia exitiosa was not detected, it is not possible to conclude that these locations are free of Bonamia exitiosa. Mixed-infection with B. exitiosa and B. ostreae in same location and even in same oyster was observed explaining the difficulty to appreciate the impact of B. exitiosa on European flat oyster populations. The notion of pathogen species has been discussed. For example, what is the relative taxonomic position of Bonamia ostreae and B. exitiosa: are these parasites two different species or two strains of a same species? The lack of knowledge in the actual epidemiology of different aquatic pathogen strains, species, their temporal and spatial distribution, their involvement in natural outbreaks, and their phylogenetic interrelations, is hampering the appropriate and adequate approach to strain or species differentiation.

3. The third session included presentations about the fish and shellfish health Directive 2006/88/EC and more specifically about the surveillance of mollusc diseases and the risk categorisation of farms and mollusc farming areas. Results from a review conducted by EFSA to establish the list of susceptible species to notifiable pathogens were presented underlining the need of combined and multidisciplinary studies to assess susceptibility to a disease.

4. During the fourth session, results from field studies were presented (1) the dynamics of infection with Nocardiocrassostreeae in Crassostrea gigas from a Dutch estuary, (2) the detection by PCR of Candidatus Xenohaliotis californiensis in abalone from some French hatcheries without possible confirmation by histology or in situ hybridization, (3) the eradication programme put in place in Loch Sunart, Scotland against Bonamia ostreae.

5. The fifth session was entirely dedicated to mortality events which occurred in Europe in 2008 more particularly in
France and Ireland with *Crassostrea gigas*. The role of some pathogens including OsHV-1 and bacteria of the genus *Vibrio* was questioned.

6. The sixth session included some presentations on recent research studies. These works highlighted the interest of transcriptomic approaches for studying non-specific defence mechanisms for example in response to a pathogen like OsHV-1, *Vibrio splendidus* or *Bonamia ostreae*. Development of new diagnostic tools for the specific detection of parasites of the genus *Bonamia* was also presented and discussed.

7. In the last session some information related to CRL activities were presented like the preparation of a quality management system for the organisation of inter laboratory comparison tests and results of the inter laboratory proficiency test based on the detection of *Bonamia* by PCR organised in 2009.

8. The workshop included a session on the detection of *Mikrocytos mackini* by histology with a general lecture on the disease and some time for histological slide examination. The workshop also included a training session on the detection and the typing of *Marteilia refringens* by PCR-RFLP.

A report of the meeting is appended at the end of this document (Annex 1).

**Collect material for and produce report covering the 2009 Annual Meeting of National Reference Laboratories**

As stated in the above section, the 2009 Annual Meeting and 7th Technical Workshop of NRLs were held in March 2009 in La Tremblade, France. A report from this meeting and workshop was produced providing information on the content of communications and discussions held during the different sessions.

A report of the meeting is included in annex 1.

**Collate material concerning the results of diagnosis and surveys regarding mollusc diseases**

Countries attending the Annual Meeting were asked to fill the questionnaire sent annually by the CRL comprising three sessions: 1) General data on mollusc production 2) Diagnostic methods used by laboratories for the detection of mollusc diseases 3) Epidemiological results for the surveillance of mollusc diseases in 2008.

This task is of central importance in the duties of the CRL. Given the pivotal role of histology in diagnostic procedures for diseases of molluscs, a particular emphasis is given to histological blocks,
sections and slides.

However more and more requests concern samples for molecular tests. In that case, tissues collected from infected or non-infected specimens or DNA extracted from such tissues are provided as positive and negative controls. Plasmid DNA suspensions (containing PCR fragments) can also be provided for example as controls for PCR-RFLP for *Bonamia* species determination or *Marteilia refringens* typing.

Establishing and maintaining the library of mollusc pathogens covered the following items in 2009:

**Maintain the existing library:**

A library of histological sections was created in 1997 which, now, includes materials covering the main pathogens of molluscs found in Europe and more broadly in the World (*Bonamia ostreae, B. exitiosa, B. roughleyi, B. perspora, Bonamia* isolates from Chile and from Argentina, *Marteilia refringens, M. sydneyi, Perkinsus olsenii, P. marinus, P. chesapeakei, P. qugwadi, Iridovirus, Herpesvirus, Haplosporidium nelsoni, H. costale, Mikrocystos mackini, Vibrio tapetis, Vibrio harveyi, Vibrio spp., Nocardia crassostreae, Marteilioides chungmuensis, Candidatus Xenohaliotis californiensis, Minchinia tapetis, Marteilia isolates from Ruditapes philippinarum and Ensis ensis etc...*). Bacterial strains are also conserved in the CRL collections. Most of them are type strains of *Vibrio* species or *Vibrio* strains isolated from mortality outbreaks.

**Acquire new reference material (strains, slides, blocks):**

The collection is continuously enriched with new reference material, requested on a regular basis from scientists publishing papers in the field of mollusc pathology.

**Give an easy access to the library:**

This has been done in the previous years by editing and updating a CRL catalogue, under a Microsoft Excel format, of the library giving access to the reference material by key words such as disease, pathogen, host species, lesion type, geographic origin, etc…

The collection of photos, created in 1999, has been included in a practical guide for diagnostic in histology established under a html format. This guide was burnt on CDs and distributed to participants of workshops and visitors.

A new tool for training in histology and relying on some virtual slides accessible by NRLs from the mollusc CRL website was put

As stated above, the library of mollusc pathogens of the CRL includes histological paraffin blocks as well as ethanol fixed infected tissues of pathogens listed in the Directive 2006/88/EC which are used to prepare and distribute, on request, hemalun-eosin (H&E) stained histological slides and Hemacolor® stained imprints or DNA suspensions for molecular test controls.

Update the www site of the Community Reference Laboratory.

The CRL website is particularly dedicated to all European National Reference Laboratories looking for information regarding mollusc diseases (www.ifremer.fr/crlmollusc). The website is organised in six sessions:
- Main activities,
- Scientific activities,
- Legislation,
- Network of NRLs,
- SOPs and Quality,
- Tutorials.

Pages make available general information, events, legislation and leaflets on some pathogens of concern. Links to other resources of interest for people working in the field of mollusc health certification and disease survey programmes are also provided like:
- OIE Aquatic Animal Diseases Commission,
- DFO synopsis of infectious diseases of molluscs,
- Aquatic animal pathology database AAPQIS,
- FAO database reporting the movements and transfers of aquatic species.

The website is also a place of advertisements for events in the field of mollusc health management (workshop, annual meeting, histopathology and molecular biology proficiency tests).

The website of the CRL has been regularly updated during 2009 but the main change although not visible from outside was the migration from the Frontpage software to the now widely known and free eZ Publish software.
Supply available reference reagents and material to the National Reference Laboratories in Member States.

Some documents have been updated like SOPs on diagnosis of Bonamia sp. and Marteilia sp. by histopathology, diagnosis of Marteilia refringens by PCR-RFLP and some new SOPs have been added like SOPs on Perkinsus sp., Haplosporidium sp. and Mikrocytos sp. diagnosis by histopathology, particularly to help the European pathologists taking the 2009 interlaboratory comparison test. A new SOP for Marteilia refringens detection and characterization by in situ hybridization (ISH) has also been added.

The “News” page is now directly accessible from the website home page (see picture) and has been regularly updated, particularly with information on the mortality events of oysters Crassostrea gigas during summer 2009 in France.

Reference material provided to laboratories working on mollusc diseases usually consists of H&E stained histological sections and paraffin blocks when available, bacterial strains as well as DNA suspensions.

In 2009, reference materials were provided to the National Reference Laboratories and other laboratories within the Community and from third countries. A table summarizing
material providing to the different laboratories in 2009 is included in Annex 2.

1. Two plasmidic DNA suspensions including M2A-M3AS fragment from *Marteilia refringens* type M, M2A-M3AS fragment from *M. refringens* type O were sent to the Marine Institute Rinville, Co Galway, Ireland.

2. Three histological slides corresponding to *Ostrea edulis* infected with *Marteilia refringens*, *O. edulis* infected with *Bonamia ostreae* and a healthy *O. edulis* were sent to INTECMAR, Pontevedra, Spain.

3. Three paraffin blocks corresponding to *Ostrea edulis* infected with *Marteilia refringens*, a healthy *O. edulis* and a healthy *Mytilus edulis* were sent to ECOLAG, University of Montpellier, France.

4. Four plasmidic DNA suspensions including Bo Boas fragment amplified from *Bonamia exitiosa*, Bo Boas fragment amplified from *Bonamia ostreae*, M2A-M3AS fragment from *Marteilia refringens* type M, M2A-M3AS fragment from *M. refringens* type O were sent to the Istituto Zooprofilattico Sperimentale delle Venezie, Via Leonardo Da Vinci, 39 45011 Adria, Italy.

5. Ten histological slides including *Ostrea edulis* infected with *Marteilia refringens*, *O. edulis* infected with *Bonamia ostreae*, *O. chilensis* infected with *B. exitiosa*, *Crassostrea gigas* infected with *Marteilioides chungmuensis*, *C. virginica* infected with *Haplosporidium costale*, *C. virginica* infected with *H. nelsoni*, *C. gigas* infected with *Mikrocytos mackini*, *C. virginica* infected with *Perkinsus marinus*, *Ruditapes decussatus* infected with *P. olseni*, *Haliotis discus hannaï* infected with *Xenohaliotis californiensis* and one Cdrom of the CRL histological collections were sent to the Service de la perliculture, Polynésie française.

6. Twenty one histological slides corresponding to *Crassostrea gigas* infected with *Mikrocytos mackini*, twenty two plasmidic DNA suspensions including M2A-M3AS fragment from *Marteilia refringens* type M, and twenty two plasmidic DNA suspensions including M2A-M3AS fragment from *M. refringens* type O were sent to the participants of the 2009 annual meeting and technical workshop of NRLs for mollusc diseases.

7. DNA corresponding to an internal standard for the detection of OsHV-1 was sent to IRTA, Spain.
8. Two paraffin blocks corresponding to *Ostrea edulis* infected with *Marteilia refringens* were sent to the Pacific Biological Station Nanaimo, Canada.

9. One Cdrom of the CRL histological collections was sent to INTECMAR, Pontevedra, Spain.

10. Six histological slides and five cytological slides corresponding to *Ostrea edulis* infected with *Bonamia ostreae* and *O. edulis* infected with *Marteilia refringens* and one Cdrom of the CRL histological collections were sent to el Instituto de Biologia marina y pesqueria “Alte Storni” Argentina.

11. One bacteriological strain (of the genus *Photobacterium*) was sent to the LD40, Mont de Marsan, France.

12. One bacteriological strain corresponding to LGP 32 (*Vibrio splendidus*) was sent to the University of Cork, Ireland.

13. Two sets of the following material were sent to NFIS, South Korea: nine histological slides corresponding to *Crassostrea gigas* infected with *Mikrocytos mackini*, *C. gigas* infected with *Marteilioides chungmuensis*, *C. virginica* infected with *Perkinsus marinus*, *C. virginica* infected with *H. nelsoni*, *Ruditapes decussatus* infected with *P. olseni*, *Mytilus edulis* infected with *Marteilia refringens*, a healthy *O. edulis*, three cytological slides corresponding to *O. edulis* infected with *B. ostreae*, *M. edulis* infected with *M. refringens* and *O. edulis* infected with *M. refringens*, one Cdrom of the CRL histological collections and four plasmidic DNA suspensions including Bo Boas fragment amplified from *Bonamia exitiosa*, Bo Boas fragment amplified from *Bonamia ostreae*, M2A-M3AS fragment from *Marteilia refringens* type M, M2A-M3AS fragment from *M. refringens* type O.

14. Two plasmidic DNA suspensions including M2A-M3AS fragment from *Marteilia refringens* type M, M2A-M3AS fragment from *M. refringens* type O were sent to the Veterinary faculty of Ljubljana, Slovenia.

15. Seven DNA suspensions extracted from gills of *Ostrea edulis* were sent to the National Veterinary Institute Oslo, Norway.

16. Four histological slides corresponding to *Crassostrea gigas* infected with *M. mackini*, *C. virginica* infected with *Haplosporidium nelsoni*, *C. virginica* infected with *Perkinsus marinus*, *Ruditapes decussatus* infected with *P. olseni* were sent to the Marine Laboratory, Aberdeen,
The CRL has been involved in investigations and diagnostic of different cases on request of NRLs or diagnostic laboratories from third countries (See table in Annex 3). Technical assistance and advice were provided by the CRL to colleagues from:

1. INRH, Casablanca, Morocco for histological examination of Crassostrea gigas (77 slides),

2. National Veterinary Institute of Bergen, Norway for histological examination of Ostrea edulis and parasite identification by in situ hybridization PCR, Real Time PCR and sequencing (material corresponding to 9 oysters),

3. Universidade Federal de Sergipe, Centro de Ciencias Biologicas e da Saude, Aracaju, Brasil for histological examination of Crassostrea gigas and parasite identification by in situ hybridization and PCR, (material corresponding to 1 oyster),

4. Veterinary Control and Research Institute, Izmir, Turkey for histological examination of Ostrea edulis (9 slides) and parasite identification by in situ hybridization, PCR-RFLP and sequencing,

5. VNIRO, Moscow, Russian Federation for histological examination of Ostrea edulis (18 slides and paraffine blocks), in situ hybridization and PCR tests,

6. Institut National Agronomique de Tunisie, Tunis, Tunisie, to test the presence and characterize Bonamia sp. and Marteilia sp. parasites by PCR-RFLP in 90 DNA suspensions extracted from Ostrea stentina,

7. INSTM, Unité de Pathologie des animaux aquatiques 20025 Salammbô, Tunisia for the double reading of 6 slides.

Formal studies had been scheduled for the year 2009 in the CRL working plan. The background of these studies, overall goals, methodology used and main outputs are given in the following sections.

**Molecular and epidemiological investigations on Bonamia ostreae and other related parasites**

*Bonamia ostreae* is a small-size (2-3 µm) uninucleate protozoan parasite affecting populations of European flat oyster *Ostrea edulis*. Other known and characterized species of the genus are *B. exitiosa*, infecting *O. chilensis* from New Zealand.
and *O. angasi* from Australia; *B. roughleyi* infecting *Saccostrea commercialis* from Australia and *B. perspora* described in *Ostreaola aequitatis* from North Carolina, USA. Besides these four species, no fully characterized *Bonamia* spp. were reported in *O. chilensis* in Chile and in *O. puelchana* in Argentina.

The notification of *Bonamia exitiosa* in Spain in October 2007 and its detection in Adriatic Sea in Italy in *Ostrea edulis* outside mortality occurrence supports the apparent wide distribution of the parasite all around the world and questioned its impact on European flat oyster populations. The presence of both parasites *B. exitiosa* and *B. ostreae* in same areas and even in same individuals reinforces the need of specific diagnostic tools.

Diagnostic techniques routinely used in the past for the detection of *Bonamia* species were not species specific (e.g. histology, cytology, PCR) and presently need to be combined with additional techniques like RFLP and sequencing to specify the species.

More recently a Real Time PCR assay targeting one actine gene from *B. ostreae* was developed and validated against heart imprints for the detection and quantification of *Bonamia ostreae* (Robert et al. 2009). The analytical specificity of this assay was demonstrated by the lack of amplification of oyster samples infected by other close related pathogens. This technique was also shown 10 to 1000 times more sensitive than conventional PCR. This assay should be very useful for epidemiological study and to appreciate the distribution of the parasite within the different organs of the oysters or in the environment.

Most of epidemiological studies carried out on infection with *Bonamia* species concern the parasite within its host. Despite some recent works aiming at investigating the presence of *B. ostreae* in zooplankton no data is available about the behaviour of the parasite in the water column. In this context we undertook a study on the survival of the parasites in sea water and the effect of temperature and salinity on parasite viability. Flow cytometry measures allowed us to show an impact of both environmental parameters on parasite survival. *B. ostreae* seems to prefer hypersaline and cold waters (Arzul et al. 2009). These results provide further information to aid management decisions and control of the disease.

The susceptibility of flat oyster larvae to bonamiosis has never been investigated. It was commonly admitted that the disease mainly affect older stages. However recent works have shown that spat less than 6 months can be detected infected and can even die from the disease (Lallias et al. 2008). Considering that flat oysters are larviparous (they incubate larvae for 10-15 days within the palleal cavity before releasing them in the water column), we took benefit from a survey on the reproduction of flat oyster carried out in Quiberon Bay, Brittany in France to obtain
some oysters incubating larvae between 2007 and 2009. We tested both genitors and larvae by PCR and in situ hybridization and were able to detect *Bonamia ostreae* in both stages suggesting that the parasite can be transmitted from genitor to larvae during this incubation period. Larvae might thus contribute to spread the disease during their planctonic life.

Some of these results have been published:


**Workplan to characterise *Bonamia* parasites in Europe**

In October 2007, Spain notified the presence of *Bonamia exitiosa* in some flat oysters collected from Ria de Arousa, in Galicia. The CRL was asked to perform some confirmatory analysis. Histological features were very similar to infection with *Bonamia* parasites but the central location of nuclei and the size of parasites could suggest that it was *B. exitiosa*.

*Picture: Bonamia exitiosa* cells inside the connective tissue of a flat oyster *Ostrea edulis* from Galicia, Spain.
Restriction profiles as well as 18S gene and ITS1 sequences were similar to *Bonamia exitiosa* ones. These results confirmed the presence of *B. exitiosa* in flat oysters *Ostrea edulis* from Spain.

Subsequently, *Bonamia exitiosa* was also detected in flat oysters *Ostrea edulis* from Manfredonia Gulf (Adriatic sea, Italy).

Following these two reports and considering that *Bonamia* is routinely detected by histology and/or imprints within the European Union, which does not allow determining the species, it appeared necessary to investigate the possible presence of *B. exitiosa* in areas usually considered as infected with *Bonamia ostreae*.

In this context, in 2008, a workplan to characterise *Bonamia* parasites in Europe has been proposed by the CRL to NRLs with the support of the European Commission. The outcome of this study was to determine the spread of *B. exitiosa* in the EU.

All NRLs were invited to participate in this working programme, especially those from countries where *Bonamia* had already been reported. Three Member States (Spain, Ireland, France) closely followed the working programme proposed by the CRL/European Commission which was a screening of populations in known infected zones by histology or heart imprints, PCR-RFLP on infected oysters, cloning of some PCR products and PCR-RFLP on about 10 clones per PCR products, sequencing of some clones. Three Member States (The Netherlands, Belgium and Italy) partly applied the proposed protocol by the use of other detection tool and/or by not including a cloning step in their approach. Two Member States did not detect any parasite of the genus *Bonamia* in the investigated zones (UK-Scotland and Portugal).

In the context of this survey, Spain detected again the presence of *Bonamia exitiosa* in cultivated but also wild flat oysters from Galicia. In addition to this working programme, some parasites identified as *Bonamia exitiosa* were detected in France: during a study carried out on a natural bed of flat oysters in Corsica, and in association with a mortality event which affected flat oysters in the Mediterranean Sea near Thau lagoon.

As conclusions, so far *Bonamia exitiosa* has been detected and confirmed in 3 Member States: France, Italy and Spain. However, where *Bonamia exitiosa* was not detected, it is not possible to conclude that these locations are free of *Bonamia exitiosa*. Mixed-infection in same location and even in same oyster was observed. The map needs to be completed and more studies are required to understand why this parasite is there and to evaluate its impact on flat oyster populations.

**Studies on the life cycle and host range of Marteilia refringens**
The CRL investigated questions related to marteiliosis, caused by the paramyxean parasite *Marteilia refringens*. The parasite occurs in Europe where it infects flat oysters, *Ostrea edulis*, and mussels, *Mytilus edulis* and *M. galloprovincialis*. The CRL has developed confirmatory methods to be used by Member States in order to clarify the host and geographic distributions of these species in Europe.

Co-infection exists in mussels and oysters that are possibly infected by both *Marteilia refringens* type M and type O. The event seems to be restricted to areas where the prevalence of the disease is high.

The CRL was also involved in studies on the life cycle of the parasite. Part of this cycle was apparently solved and the copepod *Paracartia grani* is more than suspected to act as an intermediate host since the parasite could be transmitted from infected oysters and infected mussels to healthy copepods. A study performed in Delta del Ebro (Spain) showed the possible involvement of some other zooplankton species in the parasite cycle. A similar study was carried out in the Diana lagoon in Corsica, France where flat oysters and mussels cohabit as well as *Marteilia refringens*. The dynamics of the infection with the parasite in both host species has been followed as well as the distribution of parasite stages within the different organs of infected specimens. Zooplankton was also collected every two weeks. First analyses performed by PCR suggest that different species (not only copepod species) might be infected or carriers. Further studies relying on in situ hybridization will allow confirming or not these PCR results.

Some of these results have been presented:


**Characterization of bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity**

A four-year bacteriological survey (2003-2007) of four molluscs cultivated in France and faced with mortality episodes was performed by the French shellfish pathology network. The more abundant bacteria isolated during 92 mortality episodes, occurring mainly in Pacific oyster *Crassostrea gigas*, were identified by genotyping methods.

It allowed us both to confirm the representativeness of *V.*
splendidus and V. aestivalis bacterial strains and to identify both a large number of V. harveyi-related strains mainly detected during 2007 oyster mortality outbreaks and to a lesser extent bacterial strains identified as Shewanella colwelliana. Because metalloprotease has been reported to constitute a virulence factor in a few Vibrio strains pathogenic for C. gigas, several bacterial strains isolated in this study were screened to evaluate their pathogenicity in C. gigas spat by experimental infection and their ability to produce metalloprotease-like activity in the culture supernatant fluids. A high level (84%) of concordant results between azocaseinase activities and virulence of strains was obtained in this study. Because bacterial metalloprotease activities appeared as a common feature of pathogenic bacteria strains associated with mortality events of C. gigas reared in France, this phenotypic test could be useful for the evaluation of virulence in bacterial strains associated with such mortality episodes.

Some of these results have been presented:


Contribution to the characterization of protozoans of the genus Perkinsus present in clam production areas in Europe

The genus Perkinsus includes protozoan parasites of marine molluscs in many different locations around the world. Infection may be associated with high mortality rates. Two species, Perkinsus marinus and P. olseni, are given particular attention because of their impact on aquaculture.

Considering the geographical distribution of Perkinsus olseni, occurring from Pacific Islands through Australia, Southeast Asia, to Europe and to Uruguay and its large host range (clams, oysters, cockles, abalones), one could wonder if this pathogen fulfills OIE listing criteria. However, within the geographic range of P. marinus, differences in virulence between isolates were demonstrated, suggesting existence of several strains of this parasite might exist with differences in genetic composition, geographic distribution and virulence. Similarly, variability in the pathogenicity of Perkinsus olseni raises questions on the existence of types or strains of the parasite or differences in host responses under different environmental conditions. In 2008 and 2009, the CRL participated in the characterization of parasites of the genus Perkinsus detected in different clam production areas in France and in some other
locations from the Mediterranean Basin. The species *P. olseni* could be detected in some places but some samples are still tested to have a more exhaustive picture of the distribution of this species in Europe.

Since 1998, six inter-laboratory proficiency tests have been organised by the CRL for the detection of some mollusc pathogens by histology and cytology (imprints). The goal of these proficiency tests is to establish that the examination of a given sample lead to the same conclusions in any laboratory within the National Reference Laboratory network.

All these tests included some slides of flat oysters infected or not by *Bonamia ostreae* and *Marteilia refringens* (listed pathogens present in Europe). In addition, some slides of other bivalve species infected or not by listed pathogens exotic to Europe were also included and the focus of each test was adapted according to results previously obtained by NRLs and according to the evolution of the regulation regarding listed pathogens.

The four first tests included 30 slides while the two last tests included 60 slides corresponding to two sets of tissue sections: 30 sections for European pathogens and 30 other sections for other selected pathogens.

The number of participants increased from 10 laboratories in 1998 to 20 in 2007. This increase is partly linked to the enlargement of EU.

Percentages of good answers generally increased suggesting that participating laboratories improved their ability to detect mollusc pathogens. However, results generally highlight the difficulty to detect low levels of infection and also depend on targeted pathogens.

In 2008, for the first time the CRL organised an inter laboratory proficiency test for the detection of *Bonamia* spp. by PCR. The objective of this ring test was to test the ability of NRLs to detect *Bonamia* spp. in flat oyster *Ostrea edulis* by PCR, from the DNA extraction up to the PCR test using reagents and the protocol sent with the samples. Participation in this ring test was considered optional since PCR for the detection of *Bonamia* spp. was not yet used routinely by all the NRLs. Percentages of good responses were good, above 60% for all participating laboratories. General Kappa coefficients (statistical measure of inter-rate reliability) calculated according to Fleiss (1981) were estimated between 0.49 (moderate agreement) and 0.79 (substantial agreement).

In 2009, a seventh inter laboratory proficiency test based on histology/cytology was organised by the CRL. The aim of this test is to evaluate the competency of participants regarding (1) the
detection of EU listed diseases in Directive 2006/88/EC present in Europe, (2) the detection of exotic pathogens presently described in oysters from North America. This test includes 60 slides among which 30 correspond to flat oyster *Ostrea edulis* sections or imprint slides and 30 corresponded to cupped oysters *Crassostrea gigas* and *C. virginica* sections. 19 laboratories positively answered to the announcement and last participants are presently still performing the test.

Assess alternative methods for the identification of listed pathogens.

Surveillance of mollusc diseases is routinely performed by histology or cytology and by PCR. When outbreaks of mortality occur, histology is indicated if no pathogen is previously suspected but various presumptive diagnostic methods can be used in addition to histology. When a pathogen is encountered, electron microscopy and/or molecular probes when available should be used for specific identification.

Recent efforts have led to the development of nucleic acid based diagnostic methods. These techniques offer the advantages of high sensitivity and high specificity, and possible rapid screening of aquatic organisms for the presence of a pathogen.

They are moving from development in specialised laboratories to routine application and are expected to find an increasing use in routine mollusc disease monitoring programmes.

The further development and use of DNA based diagnostic techniques like real time PCR also holds promise for international efforts to control the introduction of exotic diseases into new geographic areas. On the other hand, the routine use of DNA based diagnostic techniques is hampered by a number of problems, which may result in false positive or false negative results. However, efforts should be put forwards to develop, validate and standardise rapid diagnostic techniques for major mollusc diseases and pathogens.

This is undertaken by the CRL in collaboration with several European and non European laboratories especially for *Bonamia ostreae* and *Marteilia refringens*.

During the last couple of years a study has been carried out to compare gill and heart imprints and PCR. Specificity and sensitivity values have been determined using classical (based on a gold standard) and latent tests showing the interest of latent tests in the case of diagnostic of mollusc diseases.

In 2008, the CRL was involved in the development of a real-time PCR assay for the detection and quantification of *Bonamia ostreae* (Robert et al. 2009). Analytical specificity was confirmed by the lack of amplification observed in oyster samples infected by close related parasites including *B. exitiosa*. The assay had a minimum detection limit of 50 gene copies per reaction when plasmid DNA was used as template. Using infected oyster
samples as template, the assay was at least 10-fold more sensitive than conventional PCR. 132 oysters were tested by this quantitative real-time PCR assay and by semi quantitative approach based on heart imprint examination. A strong correlation was observed between both techniques which validate the real-time PCR assay.

In 2009, the CRL initiated a comparison study between digestive gland imprints and PCR for the detection of *Marteilia refringens* in flat oysters *Ostrea edulis*.

Some of these results have been published:


Several periods were scheduled and proposed in order to give an opportunity to train technical staff of the National Reference Laboratories and other laboratories involved in the diagnosis of mollusc pathogens (see below). During these periods, the staff of the CRL helps trainees to improve their practice in mollusc disease diagnosis procedures.

Besides the workshop organised jointly with the 2009 annual meeting, one training period in the laboratory was specifically organised for the following colleagues:

- Gwang-Jin Choi, from the National Fisheries Products Quality Inspection Service, from Gangneung City, and Myoung Hee, from the National Fisheries Products Quality Inspection Service, from Busan City, South Korea. This training aimed at (1) detecting pathogens of mollusc by histology especially those listed in the OIE aquatic Code (2) detecting and characterizing *Bonamia* sp. by PCR-RFLP (3) detecting and typing *Marteilia refringens* by PCR-RFLP (4) detecting *Bonamia* sp. by *in situ* hybridization (5) detecting *Perkinsus* sp. by thioglycolate medium culture.

In the context of the evaluation of Community Reference Laboratories in the field of animal health and live animals requested by the European Commission, DG Sanco, the CRL welcomed two auditors, Jan Goudswaard and Lucia Russo.
In addition, the CRL welcomed the following visitors in 2009:

- David Hurwood, School of Natural Resources Sciences
  Queensland University of Technology, Australia
- Lasse Jensen, Fisheries and Maritime Museum of
  Esbjerg, Denmark
- Jose A. Aranda Burgos, CIMA, Spain

Meetings and conferences provide opportunities for contacts and collaboration with colleagues as well as a way to keep abreast of new development in the field of mollusc pathology. Members of the CRL attended the following meetings in 2009:

- 101th Annual Meeting National Shellfisheries
  Association, Savannah, Georgia, USA, 22-26 March 2009.
- 14th EAFP conference, held in Prague, Czech Republic,
  14-19 September 2009. A workshop “Diagnostic approach
  in mollusc disease surveillance” was organised in the
  context of this conference
- Workshop “Epidemiology of different agents causing
  disease in aquatic animals: scientific review and database
  development (EFSA/AHAW/2008/01)” 21-22 October
  2009, CIFIV, Colleatterrato, Teramo, Italy
- Workshop on implementation of aquatic animal health
  surveillance based on Council directive 2006/88/EC » 6-7
  April 2009, Zadar, Croatia
- European Society of Marine Biotechnology (ESMB).
  Conference, Concarneau, France 1-3 septembre 2009
- International Oyster Symposium, Taipe, Taïwan, 2-4
  Novembre 2009.
- Colloque AEEMA (Association pour l'Étude de
  l'Épidémiologie des Maladies Animales), Maison-Alfort,
  05 juin 2009
- 12th Symposium of the International Society for
  Veterinary Epidemiology and Economics, Durban, South
  Africa, 10-14/08/2009
- Immuninv, Poitiers, France, 21-23 octobre 2009

The Community Reference Laboratory hands a high number of materials and documents that constitute a significant interest for diagnostic laboratories in the Member Countries.

It was proposed to organize these elements in order to set a handbook for diagnostic procedures. A first version of a CD-
ROM presenting CRL’s collections has also been developed since 2002. An updated copy was given to participants of the last Workshops held in La Tremblade.

The objective of this material is to provide diagnostic laboratories with a practical guide in histology of main mollusc species of commercial interest. The material is devoted to diagnostic laboratories for mollusc diseases, National Reference Laboratories for mollusc diseases, and scientists, veterinarians, or technicians, involved in diagnosis of molluscs diseases. It is available for potential users, free from charge, on request to the Community Reference Laboratory.

Species by species, tissue by tissue, healthy and abnormal features, the CD-ROM proposes illustrations believed to be valuable for mollusc diseases diagnostic, especially diseases notifiable to the EU and OIE. This first edition was focused on the two oyster species *Ostrea edulis* and *Crassostrea gigas* and is subject to permanent reviewing and updating. The second edition (version 1.1), available in 2003, included oyster species, mussels, pearl oysters and abalone. The last edition (version 1.3) edited in 2007 includes clam species in addition.

**Quality Management**

In 2006 French and European regulations (directive 2006/88/EC) were issued stating that National Reference Laboratories should be accredited for their activities. The CRL, like many other European National Laboratories, had already anticipated these regulations and started to operate a quality management system since 2003. The CRL was ready to be accredited against the
International Standard ISO 17025 in summer 2006 but the French accreditation body (Cofrac: Comité Français d’Accréditation) had no technical auditor in the field of mollusc diseases and specially in diagnosis techniques using histopathology. After waiting more than 2 years, the first audit was organised by the Cofrac in April 2009. It lasted 2 days and a half and was performed by a French quality auditor and an Italian technical auditor. A few non conformities were found with only one critical non conformity (validation of the techniques used) that was solved within a month. The CRL was finally fully accredited in histopathology on the 1st October 2009.

The laboratory is accredited against the International Standard ISO 17025 for the following diagnosis techniques:

- Diagnosis of *Bonamia* sp. by histopathology
- Diagnosis of *Marteilia* sp. by histopathology
- Diagnosis of *Perkinsus* sp. by histopathology
- Diagnosis of *Mikrocytos* sp. by histopathology

The CRL is presently building another quality management system for one of its main task - i.e. the organisation of interlaboratory comparison (ILC) tests. Some documents have already been written and used for the organisation of the 2009 European ILC in histopathology. The CRL is seeking accreditation for the organisation of ILC by 2011.

One of the main objectives of the International Standard 17025 «General requirements for the competence of testing and calibration laboratories» is to facilitate cooperation between laboratories particularly for the harmonisation of standard operating procedures (SOPs). Being aware that some European laboratories need some help in writing the documentation of their Quality Management system, the CRL proposes some standard operating procedures that can also help them to diagnose some of the most important diseases to report in Europe and the world. These documents are available on the CRL website on the “SOPs and Quality” page and can be downloaded as PDF files:

http://wwz.ifremer.fr/crlmollusc/presentation_1/sops_quality

- In the field of **histopathology** diagnosis are available:
  - Diagnosis by histo-cytopathology of *Bonamia* spp. in the flat oyster *Ostrea edulis* (4th edition, 2009)
  - Diagnosis by histo-cytopathology of *Marteilia* spp. in the flat oyster *Ostrea edulis* and the mussels *Mytilus edulis* and *M. galloprovincialis* (2nd edition, 2009)
  - Diagnosis by histopathology of *Perkinsus* sp. in molluscs (1st edition, 2009)
• Diagnosis by histo-cytopathology of *Mikrocytos* sp. in oysters (1st edition, 2009)
• Diagnosis by histopathology of *Haplosporidium* sp. in oysters (1st edition, 2009)
• Molluscs processing for diagnosis by histology (2nd edition, 2006)
• Koehler illumination system for the microscope (1st edition, 2009)

- In the field of **molecular biology** diagnosis you can find:
  • *Bonamia* spp detection by Polymerase Chain Reaction and species characterisation by Restriction Fragment Length Polymorphism (1st edition, 2008)
  • *Marteilia refringens* detection and characterisation by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (2nd edition, 2009)
  • *Marteilia refringens* detection and characterization by *in situ* hybridization (ISH) (1st edition, 2009)

These SOPs are regularly updated following technical improvement or regulation evolution. Work on other standard operating procedures will proceed on similar topics.
Annex 1: Report from the 2009 Annual Meeting of the National Reference Laboratories for Mollusc Diseases

Executive summary

Follow highlights of the discussions, expert opinion and recommendations formulated during the 2009 Annual Meeting and the 7th Technical workshop of National Reference Laboratories (NRLs) for Diseases of Molluscs.

- In 2008, major epidemiological changes in EU were (1) the detection of *Bonamia ostreae* in Belgium and in two previously free areas, Lough Strangford (Northern Ireland) and Kent (England) (2) the suspicion of bonamiosis in flat oysters from Norway for the second year which needs to be confirmed (3) the detection of *Bonamia exitiosa* in France in flat oysters from Corsica, Mediterranean Sea and Vendée (4) the mass mortalities associated with the presence of herpesviruses OsHV-1 and *Vibrio* species which affected *Crassostrea gigas* in France and also in Ireland.

- So far *Bonamia exitiosa* has been detected and confirmed in 3 Member States: France, Italy and Spain. However, where *Bonamia exitiosa* was not detected, it is not possible to conclude that these locations are free of *Bonamia exitiosa*. Mixed-infection with *B. exitiosa* and *B. ostreae* in same location and even in same oyster was observed explaining the difficulty to appreciate the impact of *B. exitiosa* on European flat oyster populations. The origin of this parasite remains unknown.

- The notion of pathogen species has been discussed. For example, what is the relative taxonomic position of *Bonamia ostreae* and *B. exitiosa*: are these parasites two different species or two strains of a same species. The lack of knowledge in the actual epidemiology of different aquatic pathogen strains, species, their temporal and spatial distribution, their involvement in natural outbreaks, and their phylogenetic interrelations, is hampering the appropriate and adequate approach to strain or species differentiation. In this context a project supported by EFSA started in January 2009. This project aims at (1) identifying which type of information is needed to address the issue of strain/species differentiation and (2) developing a database to collect and analyse the appropriate and relevant information.

- It was reminded that the detection of listed exotic and endemic pathogens must be notified to the European Commission and should be followed by containment and eradication programmes. Eradication is possible in closed or semi closed structures like hatcheries, nurseries or purification centres, but much more difficult to implement in natural beds. The eradication programme put in place in Loch Sunart, Scotland against *Bonamia ostreae* seems to be successful but is very expensive and is not applicable in all new infected areas. Considering the difficulty to eradicate mollusc diseases, once again the importance of transfer restriction from infected to free zones was highlighted.

- The essence of the New Directive 2006/88/EC is to prevent aquatic disease introduction/emergence and spread. One key point to achieve this objective is disease surveillance. The Directive provides elements for the implementation of a risk based surveillance system through Article 10 and through the related draft Decision 2008/896/EC. Scottish and French exercises for risk categorisation of farms and
mollusc farming areas according to these guidelines were presented as examples. A decision on sampling and diagnostic procedures for listed diseases is under preparation and was presented during the third session of this meeting.

- The situation of zones or countries free of *Marteilia refringens* raised some questions especially when this status is based on flat oyster situation and does not consider mussels. The inclusion of mussels *Mytilus edulis* and *M. galloprovincialis* in the list of susceptible species to *M. refringens* does not question the situation of these zones or countries but a targeted surveillance including mussels would be recommended particularly when the country is not totally free.

- The approach followed by EFSA and results of the review conducted to establish the list of susceptible species to notifiable pathogens were presented underlining the need of combined and multidisciplinary studies to assess susceptibility to a disease. Pathogens not being properly identified or unsettled taxonomy have been a problem in many instances. A report is accessible under the following link: [http://www.efsa.europa.eu/EFSA/efsalem-page-1178620753812_1211902178477.htm](http://www.efsa.europa.eu/EFSA/efsalem-page-1178620753812_1211902178477.htm)

- The detection of DNA from *Candidatus Xenohaliotis californiensis* in abalone from some French hatcheries without possible confirmation by histology or in situ hybridization illustrates the difficulty to diagnose pathogens present at low level of infection without inducing any clinical sign or lesions. Placing animals in conditions conducive to disease development could help to increase chance to observe the pathogen in tissue by histology.

- Having in mind that *Nocardia crassostreae* was associated with mortalities of *Crassostrea gigas* in North America and that it has been detected in Europe since 2003, this bacteria should be regarded as a potential risk for European oyster production. The development of a real time PCR assay allowed studying the dynamics of infection in *Crassostrea gigas* from a Dutch estuary. Results showed a seasonal pattern of the infection with peak of prevalence observed in autumn.

- In the context of mollusc disease surveillance, mortality reports constitute one of the only warning signals of a pathogen emergence/introduction. However, the definition of abnormal mortality is still under debate and one difficulty is to differentiate normal or routine from abnormal mortalities. Studies investigating normal mortality rates of some mollusc species in some specific environmental conditions should be encouraged.

- In 2008, French oyster production suffered from massive mortalities. The context of this abnormal mortality event as well as studies investigating the role of herpesviruses OsHV-1 and bacteria of the genus *Vibrio* were presented during the fifth session. An epidemiological study aiming at identifying factors associated with, and the cause (or causes) of these mortalities is under progress. Considering the importance of transfer of *C. gigas* in France but also from France to European countries, one can wonder how to prevent a such outbreak. Despite transfer restriction after mortality reports, some mortality cases have been reported in Ireland on some oysters imported from France prior to mortality outbreaks.
A multidisciplinary work programme aiming at understanding a mortality event affecting cockles in Wales was presented. The specificity of this mortality to cockles as well as the spread pattern support an infectious aetiology. However, regarding the presence of numerous pathogens -some of them previously implicated in mortalities of cockles- studies investigating the dynamics of these different pathogens and their potential correlation with mortality outbreak are needed showing that understanding mortality cases generally requires more than one sample at one date.

Histopathology is the golden standard method for the OIE with regard to detection of numerous mollusc pathogens including *Bonamia ostreae* and *B. exitiosa*. However in some contexts, more sensitive and specific tools might be required. Some PCR assays have been developed, few of them validated. These assays usually do not allow alone to discriminate *Bonamia* species and need to be associated with RFLP and/or sequencing. More recently some **Real time PCR assays** targeting the 18S or the ITS1 regions have been developed or adapted from conventional PCR assays. Their specificity to discriminate *B. ostreae* and *B. exitiosa* seems to be promising. In parallel a Real Time PCR assay has been developed and validated against heart imprint and conventional PCR assay for detection and quantification of *Bonamia ostreae*. This assay, targeting the actin gene was shown to be sensitive, specific (only *B. ostreae* detected) and reproducible.

Given that in an infected area, eradication is most of the time not possible, knowledge of **host-pathogen interactions** is of central interest in order to propose some alternative measures to producers like advices for stock management or development of resistant host strains. Some recent results concerning host-pathogen interactions were presented included *Ostrea edulis - Bonamia ostreae*, *Vibrio* species – *Crassostrea gigas* and OsHV-1- *C. gigas* models.

The CRL is currently building a quality management system for the organisation of **interlaboratory comparison tests**. Organisation and results of the comparison test organised in 2008 to test the ability of NRLs to detect *Bonamia ostreae* by PCR were presented. Material sent to participants and the analyses of results were widely discussed. Moreover, some NRLs expressed their wish to have more time during the workshop to observe slides from previous histology comparison test. In addition NRLs will have access to the scanned slides through the CRL website.

The **workshop** included a session on the detection of *Mikrocytos mackini* by histology with a general lecture on the disease and some time for histological slide examination. The workshop also included a training session on the detection and the typing of *Martellia refringens* by PCR-RFLP introduced with presentation explaining the situation regarding *M. refringens* type O and type M. One histological slide with *M. mackini* and plasmidic DNA suspensions from both *M. refringens* types will be provided to each participant after the workshop.

The CRL proposes to plan the **next annual meeting** for the beginning of 2010 (March) in Nantes. NRLs are invited to propose topics on which they are working or for which they need information and/or training.
Introduction

The 2009 Annual Meeting and the 7th Technical Workshop of National Reference Laboratories for Mollusc Diseases was held in La Tremblade on the 16th to the 19th of March 2009. In total, 42 participants from 17 countries (Belgium, Bulgaria, Canada, Croatia, Denmark, France, Germany, Greece, Ireland, Italy, Norway, Poland, Romania, Spain, Sweden, The Netherlands and United-Kingdom) attended the meeting.

The Annual Meeting included seven sessions: 1/ Current epidemiological situation in the Member States 2/ Situation regarding *Bonamia exitiosa* 3/ The new directive and related issues 4/ Results of recent field studies 5/ Abnormal mortality events 6/ News from the bench 7/ CRL day life activities. The technical workshop concerned the detection and typing of *Martelilia refsingens* by PCR-RFLP and the detection of *Mikrocytos mackini* by histology.

This report provides summaries of the questions discussed during the meeting and the workshop and outstanding facts for follow-up activities. It contains collective expert opinion and recommendations made during the meeting.

**ANNUAL MEETING SESSION I: DIAGNOSIS AND SURVEY OF MOLLUSC DISEASES**  
*(Secretary: I. Arzul)*

Current epidemiological situation in the Member States

National delegates participating in the Annual Meeting briefly presented outstanding issues and major changes with regards to the mollusc health status in their country. The detailed reports received from NRLs are given in annex of this report (Annex D). A summary of the reports is given here country by country followed by questions and discussion which took place at the end of each presentation.

**Belgium:** The mollusc health status in Belgium was not presented during this session.

*Brigitte Pochet from the Belgium Competent Authority explained that there was presently no National Reference Laboratory for Mollusc Diseases in Belgium and that she was not aware about the situation of Belgium regarding mollusc health status. However, Isabelle Arzul reported that some PCR tests were conducted on flat oysters collected from Oostende area and some positive results were obtained. However no complementary analysis was performed by histology because the animals were not treated for such analysis.*

**Bulgaria:** The Bulgarian mollusc production is represented by mussels, *Mytilus galloprovincialis*, (190 tons) from 8 registered farms and 750 tons harvested from natural beds. At the present moment it accounts for less than 1% of the total aquaculture production. There is no hatchery and nursery.
It has good perspective for future development and during the last 3 years there was a steady tendency for development of marine aquaculture. There are two zones for surveillance – in North (6 farms) and South (2 farms) Black Sea coast. In 2008, 138 mussels were collected in August and 32 specimens in September-October from 2 mussel producing areas in order to determine the distribution of marteliosis. These individuals were tested regarding the presence of Marteilia refringens. Screening methods used for Marteilia refringens are histology and tissue imprints. No parasite was recorded in these samples. There is no official data for abnormal mortality event in bivalve population and no disease was reported in 2008.

Croatia: In 2008, in Croatia 3716t of bivalve molluscs were put on the market out of which 3621t were produced and 95 t were collected from natural beds. There are 124 farms cultivating flat oysters (Ostrea edulis) and mussels (Mytilus galloprovincialis) and one experimental hatchery for oysters in Zone III. During the last year several new sampling points were included in the Zone I and Zone II. Surveillance is performed according the National Laws and mainly oysters and mussels were monitored by means of cytology and histology to test the presence of Bonamia ostreae and Marteilia refringens. Sampling was carried out during April and from August to October and the results of the analysis revealed presence of Marteilia refringens in mussels from all zones with prevalence of 12.16%. In wart venus (Venus verrucosa) from natural beds presence of Perkinsus sp. was noticed.

Denmark: The Mytilus edulis production in Denmark dropped from 57,335 tons in 2007 to 34,959 tons in 2008, whereas the production of Ostrea edulis, located in Limfjorden, rose from 1,212 tons to 1,489 tons. Around 30 individuals were collected in spring and autumn 2008 in three sites from Limfjorden. Investigations for 2008 were not finished at meeting dates. Denmark is officially recognized Bonamia ostreae and Marteilia refringens free in the Limfjorden area since December 2004. The Danish mussel industry has shown interest in having areas outside Limfjorden given the same status, wherefore a screening programme has been proposed by the Danish competent authorities. Sampling of blue mussels has been done from four different locations (three sites in each location) both in 2008 and 2007, but these samples (1,800 individuals per year) have not gone further than the formaldehyde stage, as the mussel industry and the competent authorities have not come to a decision on the payment of expenditures in connection with a screening programme. An investigation of blue mussels with “red worms” from a certain area in Limfjorden confirmed that these “worms” were Mytilicola intestinalis.

France: The mollusc production remains constant with an annual production of 195,800 tons. The main productions are Pacific oyster, Crassostrea gigas and mussels, Mytilus edulis and M. galloprovincialis. In 2008, France participated in the programme settled by the CRL in order to characterize the parasite Bonamia present in Europe. For 2008 only the main production areas of flat oysters, Ostrea edulis (zone III = Brittany) were sampled. Eight samples accounting 454 individuals were analysed in histology; 80 individuals were infected by Bonamia ostreae (confirmation by PCR-RFLP, cloning and sequencing). B. exitiosa was not detected in these samples. Meanwhile, a survey on marteliosis in flat oysters and mussels was carried out in Corsica in 2007 and 2008. Analyses are in progress but Bonamia exitiosa was detected in one Ostrea edulis sample (3/30 individuals).
In 2008, important abnormal mortality events occurred in most oyster producing areas in France during the summer. The mortalities were sudden and severe (up to 100%) and mainly affected 6 to 18 month old juveniles. Only *Crassostrea gigas* species was affected. The cause of the mortality was unclear but OsHV-1 virus and bacteria belonging to Vibrionaceae family were frequently detected in affected populations.

In addition, *Bonamia exitiosa* was detected in flat oyster *Ostrea edulis* during a mortality event in Mediterranean Sea in August 2008. 32 on 76 tested individuals were infected by this parasite. An investigation occurred in order to know the sample origin. The samples came from 2 different French areas (Brest bay end Bourgneuf bay). A sampling was done in these areas and *Bonamia exitiosa* was detected in Bourgneuf bay (no detection in Brest bay).

Few other mortality events occurred in 2008 and concerned cockles (1 case), *Donax trunculus* (1 case), clams (1 case) and mussels (3 cases). In one case of mussel mortality, there is an atypical detection of *Marteilia refringens*; this parasite was mainly observed in histology in connective tissues of mantle, labial palps and gills whereas *M. refringens* is classically a parasite of the mollusc digestive gland.

For 2009, France will maintain its efforts on the study of mortality and on the characterisation of the different zones regarding the presence of *Bonamia exitiosa*.

Antonio Villalba said that several cases of *Marteilia* had already been reported in the connective tissue of gills and mantle in mussels from Galicia associated with high haemocytic infiltration.

Ian Laing questioned the French representatives about the impact of *Crassostrea gigas* mortality on the production of this species. The impact has been evaluated through the mortality cases notified by the farmers to the Competent Authority but might have been biased because these notifications notably aimed at obtaining financial compensation.

Pedro Rosado Martin asked if there is a hypothesis regarding the origin of *Bonamia exitiosa* along the French Mediterranean coast and in Corsica. At the moment there is no explanation. Pedro Rosado Martin asked if French authorities put in place containment or eradication programmes to control the presence of *Bonamia exitiosa*. In natural beds, it is not possible to envisage eradication while in structures like hatcheries-nurseries and in rafts in Mediterranean Sea, infected stocks are being destroyed. Moreover, transfers of flat oysters from sites found infected to sites considered free from this pathogen are not permitted;

**Germany**: There is no information about disease mollusc situation in Germany for 2008.

**Greece**: Mollusc production in Greece mainly relies on mussel *Mytilus galloprovincialis* with an annual production of 38 300 tons. This production essentially takes place in Thessalonica where about 300 farms are recorded. In 2008, 6 samples of flat oysters *Ostrea edulis* and 28 samples of mussels *M. galloprovincialis* were collected and tested by histology. Both species were found infected with parasites *Marteilia* spp. in two sites (Fthiotida and Lesvos).

**Ireland**: The disease status for the non-exotic and exotic diseases listed in 2006/88/EC remains the same. The entire country is deemed free from Marteiliosis whilst bonamiosis has been confirmed to be present in 8 bays around the coast. The rest of the country is considered to be free of the disease. In the spring of 2008 Ireland carried out a comprehensive screening program to establish whether the *Bonamia* parasite present in the country was *B. ostreae* as believed. 93 positive oysters were tested by PCR, PCR-RFLP and sequencing and all were
shown to be infected with *B. ostreae* only. Three reports of abnormal mortality were investigated in pacific oysters in 2008. OsHV1 was found in association with mortalities in all three areas and *Vibrio splendidus* was also detected in two areas. A link to the mortalities that occurred in France is suspected.

**Italy:** During 2008, in Italy mollusc health situation was similar to the previous years. In Manila clam farms, *Perkinsus* was present with high prevalence. *Marteilia* infection was moderate and mainly present in farms located in Tirreno Sea and Ligure Sea. *Bonamia ostreae* was reported in flat oysters in Venice lagoon. Oysters came from France. Subsequently to this detection, flat oysters were destroyed.


Pedro Rosado Martin asked if the detection of pathogens like *Bonamia ostreae* in Italy is systematically followed by an eradication of the pathogen. The Italian representative replied that eradication was envisaged case by case.

It seems that some clam seed are imported from U.S.A. and U.K.. Such imports from U.S.A. should not presently be authorised.

**Norway:** According to official data, 4 t of *Ostrea edulis*, 3500 t of *Mytilus edulis* and 6 t of *Pecten maximus* were produced in 2008. In addition, 746 t of *P. maximus* were harvested from natural beds. Two hatcheries were active, one producing *O. edulis* (1 million spat) and one *P. maximus* (1 million spat). One nursery produced *O. edulis* (500 000 2 g). There were 807 shellfish farm licences in 2008 (417 *M. edulis*, 98 *P. maximus*, 117 *O. edulis*) and 421 active farms in 2007. Mollusc movements-transfers are only for consumption.

For diagnostic methods, histology is used for screening for endemic and exotic diseases. PCR for *Bonamia* has been established and is used for presumptive and confirmatory diagnosis. All other diseases will be referred to CRL. Histology for *Bonamia* and *Marteilia* detection is accredited. Six sampling points were included in the sampling plan for 2008. However, due to low or no production of oysters, four points were sampled in spring and three in autumn. In spring 12-40 oysters were sampled from each point, in autumn 30-50 oysters. In total, 212 oysters were collected.

*Marteilia refringens* was not detected in the 212 oysters. *Bonamia ostreae* was not detected in 162 oysters. One sample is not concluded as *Bonamia*-like structures were observed in haemocytes of several oysters from a wild population. This was also observed in 2007 and samples sent to CRL. *Bonamia* was not detected by ISH or PCR of gill tissue. In 2008, sample size from this population was increased to 40 oysters spring and 50 autumn. *Bonamia*-like structures were detected in autumn sample. PCR for *Bonamia* on gill samples were not conclusive. PCR-product will be sequenced and samples referred to CRL for further examination.

In addition, mortality in brood scallops in hatchery and neoplasia in brood oysters has been reported from another laboratory.
Poland: In spite of an access to the sea, Poland does not produce any molluscs at all for the commercial scale. The reasons are environmental conditions like low level of salinity of the sea (average value for Polish coast of Baltic Sea is 7‰, in Pucka Bay 6 - 1‰) and temperature of the water (in the inshore zones from -0.5°C during winter time to 17°C in the summer) and also the water pollution. For these reasons the size of wild Mytilus edulis and Macoma baltica living there only reaches 1-3 cm. Some of the species of molluscs, like Crassostrea gigas and M. edulis, are imported to Poland for human consumption.

Examinations of wild population of M. edulis were carried out in 2008. The methods we used are cytology and histology. None of these methods are accredited yet but the accreditation process should be finished till a half of the year. Samples of wild population of M. edulis from Pucka Bay were collected in August. Two sampling points were chosen and 30 individuals were collected from depth of 4 m; the water temperature was about 15°C. No Marteilia refringens was detected. Abnormal mortality wasn’t observed either.

The plan for the next year is to continue sampling from this area and if it will be possible to increase the amount of the sampling points on another point of the coast of Baltic Sea.

Portugal: Portuguese representative could not attend the Annual Meeting but sent an abstract and a presentation about the situation of Portugal regarding mollusc diseases.

During 2008 the Laboratory of aquatic animal diseases of IPIMAR designated by the Portuguese CVO as NRL for Molluscs Disease, proceed with a national survey along the Portuguese coast in order to identify the most important pathogens in bivalve populations commercially exploited and quantifying their role in commercial operations.

Following the alert launched by of the CRL in last summer, we also proceed with an additional survey at the tow most important production areas of Japanese oyster (Algarve and Aveiro) in order to detect massive mortalities, presumably associated with the Summer Mortality Syndrome of this species.

During those campaigns the laboratory collect from 20 distinct sampling sites, along the coast, more than 500 samples of 7 different bivalves species. Approximately 500 histology and 190 cytology analysis were done as well as 120 RFTM cultures.

As result of those surveys we remark that:

- Marteiliosis is endemic in wild sea beds of flat oyster along the coast with low levels of prevalence. Bonamia presence was not observed both in cytology and histology analysis. No evidences of massive mortality were reported by fishermen or observed in sea beds during sampling.
- Perkinsiosiis is present in culture settlements of clams, «viveiros», with high prevalence of the disease. Less massive mortalities were reported.
- The cultivation of Japanese oyster, using imported seed, reports a peak of mortality during last summer. In the tow farms surveyed between May and June at Algarve the mortality rate reaches 90% during that period. Besides the weak appearance and gaping observed in the animals, no evidence of pathogen had been observed.
- Cockles cultured together with clams in «viveiros» shows also high prevalence of Perkinsiosis and up to 42% of prevalence of digenetic trematode, Bucephalus sp., severe mortalities of this specie continued to be reported by farmers.
- Natural and cultivated populations of Portuguese oysters in Sado are quite healthy, showing low (10%) prevalence of gill lesions. New settlements continue to expand along the old sea beds of the estuary.
• Cultivated mussel populations have high prevalence of Marteliosis but no massive mortalities were reported.

**Romania:** In the year 2008, Romania carried out sanitary veterinary surveillance of farmed population of molluscs. Molluscs samples were represented by *M. galloprovincialis*. The main aim of surveillance was *Marteilia refringens*. No abnormal mortality was reported during the year. The position of sampling was located in zone 4 (between Agigea and Mangalia places) in the area where the molluscs farm is and, the sampling time was in summer (July). Negative results for *Marteilia refringens* were found by means of histology.

**Slovenia:** Slovenian representative could not attend the Annual Meeting but sent an abstract and a presentation about the situation of Slovenia regarding mollusc diseases. The Slovene sea, situated in the Gulf of Trieste, lies at the very north end of The Adriatic Sea. The entire Slovene coast is 46 km long. The sea temperature varies considerably: from up to 30° C during the summer to 0° C during very cold winters. Many rivers, groundwater and underwater springs have a strong influence on salinity, which fluctuates from 20 ppt after abundant rainfall to 38 ppt during the late summer and winter. Thirty eight taxons of Bivalvia are living freely in the Slovene Sea, but only Mediterranean mussels (*Mytilus galloprovincialis*) are breed on three locations, i.e., Seča, Strunjan and Debeli rtič. Mussel seeds are bought or collected from natural beds, put in nets and cultured on ropes, which are hanging from rafts. They reach commercial size after 14 to 18 months and are up to 7 cm long at the time. 250 tons of Mediterranean mussels were produced in 2009. Two sampling sites, i.e. Seča and Strunjan were established in Slovene sea. Although no mortality occurred in any of farms in 2009 one sampling was performed in June, during which 150 cultured Mediterranean mussels were collected from a farm in Strunjan. There were no abnormalities nor lesions detected during the macroscopic inspection of mussels. The histological examination was negative for the presence of *Marteilia sp.*

**Spain:** Most of the Spanish production of shellfish is located in Galicia based mainly in mussel aquaculture although no official data of the total production could be recorded. 870 flat oysters harvested from different production areas were tested in Spring and Autumn regarding the presence of *Bonamia exitiosa*, *Bonamia ostreae* and *Marteilia refringens* in the Atlantic coast (Galicia, Asturias and Cantabria) and in the Mediterranean coast (Cataluña, Comunidad Valenciana and Islas Baleares). *Bonamia ostreae* was detected in the Atlantic coast (Galicia and Asturias). *Marteilia refringens* showed a wider distribution all over the Mediterranean coast and in one point in the Atlantic coast (Cantabria). *Bonamia exitiosa* was exclusively detected in Galicia both in cultured and wild flat oysters. Co infection with *Bonamia ostreae* was also detected. 600 mussels were collected from 20 sites in Galicia and 150 mussels from 1 site in Ceuta in order to study the distribution of *Marteilia refringens* in *Mytilus galloprovincialis*. Both regions were found infected by the parasite. *Perkinsus olseni* was detected in *Ruditapes decussatus*, *R. philippinarum* and *Venerupis pullastra* collected in Galicia, in *R. decussatus* from Asturias and in *R. philippinarum* from Cataluña. Two mortality events of *Crassostrea gigas* were recorded in May and October in Cataluña and in June in Cantabria. A mortality event of *Cerastoderma edule* was also reported in
Cataluña in August.

*Antonio Villalba questioned the use of gill tissue as a target for the PCR for the detection of Bonamia exitiosa and eventually recommended to include gonad tissue.*

**Sweden:** The Swedish mollusc production (farms) is dominated by blue mussels, with approximately 2,000 tons produced in 2008. Only small amounts of oysters (*Ostrea edulis*) are produced in farming activities. One hatchery/nursery has started up and will contribute to increase the production in the coming years. During 2008 there was no active screening ongoing regarding mollusc diseases. Sweden has applied for freedom for Bonamiosis and Marteiliosis on historical grounds and has during 2009 been declared free from *Bonamia ostreae*. Further sampling will be performed targeting blue mussels to gain the same status for the mollusc pathogen *Marteilia refringens*.

**The Netherlands:** *Mytilus edulis* is the most important mollusc production in the Netherlands with 37,278 tons produced per year. Since 1988 *Bonamia ostreae* has been present in flat oysters, *Ostrea edulis*, from Lake Grevelingen. In the spring of 2008 *B. ostreae* was observed in 17% of the investigated flat oysters. The species was confirmed to be *B. ostreae* by PCR and sequencing of the positive specimens. No *Marteilia refringens* was observed in the investigated oysters and mussels. In the spring samples of *Mytilus edulis* a *Haplosporidium* species was detected by histopathology from a single specimen. No abnormal mortality event was reported in 2008.

*Gary Meyer reported that Haplosporidium parasites have already been observed in mussels in Canada. David Fraser asked why Crassostrea gigas were monitored in The Netherlands. The surveillance of this species is included in the routine surveillance programme.*

**United Kingdom:** Scotland presented the UK epidemiological report for 2008, including shellfish technical and production data. A total of 457 farm sites produced 28,301 tons of cultured molluscs, including *Mytilus edulis, Crassostrea gigas* and *Ostrea edulis*; plus 16,365 tons of molluscs from natural beds, including *Cerastoderma edulis, Ostrea edulis* and *Ruditapes philippinarum*. There were four hatcheries in the UK registered for the production of *Crassostrea gigas, Ostrea edulis, Ruditapes philippinarum* and *Pecten maximus*. Mollusc movements or transfers to Member States included imports of *C. gigas* to the UK from the Channel Isles and from France via Jersey. Scotland made two exports: *O. edulis* to N Ireland; and *P. maximus* to the Isle of Man. Scotland did not trade with Third Countries during 2008.

Methods in Manual of Diagnostic Tests for Aquatic Animals include: screening methods routinely performed for surveillance purpose; presumptive diagnostic methods, and confirmatory methods for specific identification, e.g. PCR-RFLP & sequencing to differentiate between *Bonamia ostreae* and *Bonamia exitiosa*. The UK is accredited to ISO 17025 standard for histology.

Surveillance and monitoring of *Bonamia ostreae* and *Marteilia refringens* in *Ostrea edulis* resulted in confirmation of *Bonamia* in a new area off the North Kent coast of England. The original positive sample was taken in late 2007 from a private bed of native flat oysters in the River Thames estuary. These oysters were originally moved from wild beds in a public
fishery in an adjacent area. Results from further sampling of the public beds have not shown any evidence of the presence of the disease organism. It is not known how the pathogen may have been spread into this previously uninfected area; however boats which fish in infected areas also visit this fishery. As a result of the outbreak, the Zone C controlled area for Bonamia was extended.

Continued surveillance of wild and aquaculture sites resulted in no change to the Bonamia status of Scotland in 2008; although two sites remain confirmed, 1 in 2006 cleared and considered as fallow, second in 2007 which contains wild stock. Northern Ireland reported a Bonamia positive site at Strangford Lough, situated at north east Ireland; no further details were available prior to the meeting. All UK Bonamia infected areas remain under control. Apart from annexed areas for Bonamia, the UK maintains disease free status for Marteilia and other listed pathogens.

No abnormal mortalities in shellfish sites were reported in Scotland where 90 sites were routinely inspected during the year. In England, mortality in harvested beds of Ruditapes philippinarum in Poole Harbour and cultivated lays off the north Kent coast were reported. Testing revealed the presence of picorna-like virus particles at high prevalence at all sites. The hatchery at Whitstable supplies clams for the beds in North Kent but samples of juveniles from here were negative for virus. Since July 2003 mass mortalities of cockles have been observed at two commercially exploited sites, Three Rivers and Burry Inlet, in South Wales. Other mortality has also been reported more recently from sites in Cornwall. Investigations are continuing and a range of potential causative factors are being studied by various agencies. This work is being co-coordinated by the Environment Agency. CEFAS is dealing with disease aspects and taking monthly samples. Several parasites, including pathogenic species, have been detected. The pattern of occurrence and prevalence of some of these in the cockles suggest that they may be a contributory factor in the mortality events. Matt Longshaw presented a full report at the annual meeting.

In response to the 2008 summer mortality of oysters in France the two growers who import seed or half-grown Crassostrea gigas from France were contacted. Their oysters, from Saint Germain Suray, Blainville Sur Mer and Marennes-Oleron, showed good growth and survival and there was no mortality. There were no reports of unusual mortality from any oyster farms in England and Wales. The main grower on Jersey reported low-level mortality in his stocks that was accounted for by environmental factors and husbandry practice.

In summary, there was no significant change to the disease status of UK during 2008: atypical mortality incidents in England are under investigation; in 2009, risk based, intelligence led and training awareness surveillance is to be introduced under the EC Directive 2006/88/EC.

Giuseppe Arcangeli asked if picornaviruses were significant in Manila clam mortality. English representatives replied that these viruses were probably one cause of the reported mortality because of the high detection frequency and the associated histopathological lesions.

Pedro Rosado Martin pointed out that the presence of listed pathogens (European and exotic ones) must be notified.

During these presentations several points were discussed and are listed after each National situation abstracts. In addition, Stein Mortensen questioned the confidence we could have regarding the mollusc species we sample for surveillance purpose. Indeed, sometimes it is not easy to differentiate some oyster or mussel species like Mytilus edulis and M. trossulus.
**ANNUAL MEETING SESSION II: SITUATION REGARDING BONAMIA EXITIOSA**
*(Secretary: C. François)*

*Bonamia exitiosa* parasitizing the flat oyster *Ostrea edulis* in Galicia (NW Spain)

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The haplosporidian *Bonamia exitiosa* was found infecting the European flat oyster *Ostrea edulis* in the Galician coast (NW Spain), which was officially noticed on October 2007. It involved the first report of this parasite along European waters and its first detection in that host species. Histopathology and analysis of the nucleotide sequence of the small subunit ribosomal DNA gene allowed to identify this species in oysters taken from a raft located in an oyster culture area in the Ría de Arousa close to Cambados. Subsequent analyses of samples from 5 oyster batches with different origins that had been introduced in the same raft for on growing were performed by PCR, using the Bo-Boas primer set, followed by RFLP analysis, combined with histopathology. The analyses showed infections by *Bonamia ostreae*, *B. exitiosa* and mixed infections by both *Bonamia* species in 4 oyster batches and the prevalence reached up to 60% in one sample, thus demonstrating the success of *B. exitiosa* to infect the European flat oyster in the Galician marine ecosystem even in concurrent infections with *B. ostreae*.

Because the primer set Bo-Boas amplifies DNA of both *B. exitiosa* and *B. ostreae*, further analysis is required for species identification, being RFLP analysis a choice. Sometimes the PCR amplification does not yield enough product quantity to perform RFLP analysis. Furthermore, in cases of mixed infections in which one of the parasite species is overrepresented, the other species could be undetected because of the competition for the primers. To overcome these limitations new species specific primer sets have been designed, which amplified regions of the SSU rDNA genes of *B. exitiosa* and *B. ostreae* respectively. The primer set specific for *B. exitosa* in combination with histopathology has been used in a survey to assess the geographic range of *B. exitiosa* along the Galician coast, involving 9 sampling stations, 5 of them corresponded to natural beds and 4 to culture areas. The parasite has been detected in 5 stations located in 3 Galician Rías (Arousa, Vigo and Ortigueira); 3 stations corresponded to natural beds.

Additionally, an analysis of the ultrastructure of *B. exitiosa* parasitizing *Ostrea edulis* is being performed with TEM. Early results have shown that the Galician parasite shows similar ultratstructural morphology to that of *B. exitiosa* infecting *Ostrea chilensis* in New Zealand.

Raquel Aranguren asked Antonio Villalba what kind of primers he used for the first detection of *Bonamia exitiosa* in Galicia. The parasite was firstly identified using Bo-Boas PCR followed by a digestion with BglI. Then some primers specific to *B. ostreae* and others specific to *B. exitiosa* have been designed and allowed detection of mixed infection (46 oysters/166), *B. ostreae* only (45 oysters/166), and then infection with *Bonamia exitiosa* only (18 oysters/166).
Stein Mortensen asked if the animals of the different batches came from foreign countries. The oysters had different origins (France mainly, Italy, Denmark, Turkey).

Isabelle Arzul asked if there are some Ostrea stentina in Spain. Antonio Villaba said Yes and this raises an interesting question: which is the role of Ostrea stentina in the detection of Bonamia exitiosa?

Pedro Rosado-Martin asked if it is possible to estimate the impact of Bonamia exitiosa on flat oysters production. Regarding the few data presently available, Antonio Villalba said that the industry was so affected by Bonamia ostreae, that it should be first restored, keeping in mind that Bonamia exitiosa is present.

Pedro Rosado-Martin questioned Antonio Villalba about the measures which could be proposed following the detection of this exotic pathogen. Antonio Villalba said that the priority is transfer restriction. However further studies are needed including studies on the susceptibility of the hosts to Bonamia exitiosa and the development of more sensitive tools.

Isabelle Arzul asked Antonio Villalba if some lesions due to Bonamia exitiosa could be observed considering that in France infected flat oysters did not present any gross lesion like gill indentation. Antonio Villalba replied that haemocytic cell infiltration could be observed in the gills by histopathology but no gross sign was reported. However, Antonio Villalba added that no proper epidemiological study has been carried out.

Finally Isabelle Arzul wanted to know the age of the oysters found infected by Bonamia exitiosa. These oysters were older than one year.

Distribution of Bonamia exitiosa in Europe.

Isabelle Arzul

Community Reference Laboratory for Molluscs Diseases, Ifremer, La Tremblade, France

Parasites of the genus Bonamia and considered as being “Bonamia ostreae” have been detected in flat oysters in several European countries including Ireland, UK, the Netherlands, France, Spain, Portugal and Italy. Some areas are free or under an approved programme (Decision 2009/177/EC).

Routine diagnosis is generally based on histology and cytology which do not allow differentiation between parasites of the genus Bonamia.

In October 2007, Spanish authorities notified the finding of Bonamia exitiosa in flat oysters Ostrea edulis to the Commission on 16th October 2007. This report changed the picture we had regarding the distribution of Bonamia species in the world. Indeed, previously B. exitiosa was reported in Australia and New Zealand while B. ostreae was reported in Europe and North America.

The detection of Bonamia exitiosa in Ria de Arousa in Galicia, Spain was the first report of this pathogen in the EU and in the European flat oyster, Ostrea edulis. Subsequently, Bonamia exitiosa was detected in flat oysters Ostrea edulis from Manfredonia Gulf (Adriatic Sea, Italy).

Following these two reports, a working programme was proposed by the CRL to the NRLs for mollusc diseases with the support of the European Commission. This working programme aimed at characterizing parasites looking like Bonamia usually detected in Europe and at collecting epidemiological information on Bonamia exitiosa to find out the actual spread of the parasite in the EU.

It was proposed to

- identify zones known to be infected by Bonamia parasites
• collect oysters preferably in winter-spring 2008
• collect information about oyster origins and culture conditions for each sample
• process material to ensure access to DNA, TEM, ISH and histological technology

All NRLs were invited to participate in this working programme, especially those from countries where *Bonamia* has already been reported.

• Scottish NRL investigated the presence of parasites of the genus *Bonamia* in 6 areas by histology and did not detect any parasite.
• Northern Ireland England and Wales did not perform additional sampling.
• Portuguese NRL tested twice a year the presence of *Bonamia ostreae* in wild bed but have never detected the parasite yet;
• The Italian NRL tested between 30 and 347 oysters in three areas from the Adriatic Sea and noticed the presence of *Bonamia ostreae* in Venise in flat oysters originating from France. *B. exitiosa* could not be found again in Manfredonia Gulf.
• In Ireland, nine zones were investigated and *Bonamia* parasites were observed by histology in 6 zones with prevalence ranging from 0 to 33% according to the sampling sites. Infected specimens were selected for further analysis by PCR-RFLP and cloning. 930 clones were finally sequenced. Results showed that only *Bonamia ostreae* was detected in the Irish samples.
• In The Netherlands, 39% of tested flat oysters appeared infected by *Bonamia* parasites using Real Time PCR. This technique suggested that all the detected parasites were *Bonamia ostreae*.
• In France, 3 zones were investigated and samples were collected from eight sites. *Bonamia* parasites were detected by histology in all the samples with prevalence ranging between 6.6% and 26.4% according to the site. Infected specimens were tested by PCR-RFLP and 80 PCR products were cloned. 246 clones were tested in PCR-RFLP and some of them were sequenced (69). Results showed that only *Bonamia ostreae* were detected in these samples.
• In Belgium, 242 flat oysters collected from one zone were tested by PCR. 6% of the oysters appeared positive and 14 PCR products were digested and showed *Bonamia ostreae* restriction profiles.
• In Spain, six zones were subject to sampling. A total of 639 oysters were tested by PCR-RFLP and prevalence of 0 to 20% were found in Galicia. 26 PCR products were cloned and 10 clones per PCR-products were tested again by PCR-RFLP. 7 oysters were found infected by *Bonamia exitiosa* and one case of mixed infection by *B. ostreae* and *B. exitiosa* was detected.

In addition to this work programme, some parasites identified as *Bonamia exitiosa* were detected in three zones in France: Corsica, outside Thau lagoon and in Bourgneuf bay.

As conclusions, so far *Bonamia exitiosa* has been detected and confirmed in 3 Member States: France, Italy and Spain. However, where *Bonamia exitiosa* was not detected, it is not possible to conclude that these locations are free of *Bonamia exitiosa*. Mixed-infection in same location and even in same oyster was observed. The map needs to be completed and more studies are required to understand why this parasite is there.

*During the presentation the representative from England, Ian Laing, explained that they did not plan additional sampling in spring 2008. Marc Engelsma added that in The Netherlands, in addition to the Real Time PCR test they performed sequencing and confirmed that only *Bonamia ostreae* was detected.*
After the presentation, Pedro Rosado-Martin wondered how sure we were that Bonamia ostreae and Bonamia exitiosa are different species. Isabelle Arzul recognised the relevance of the question and the difficulty to give an answer regarding the data available: ultrastructural studies tend to give a list of features allowing differentiation between both species. However these features might be specific to the host or environmental conditions more than to the parasite itself. Molecular data are presently very scarce and sequences available for both species do not allow designing specific and robust diagnostic tools. This question also highlights the need of more from other genome regions.

Cyrille François asked if Bonamia exitiosa was reported in third countries near Europe. Isabelle Arzul replied that Bonamia has been reported in Tunisia in Ostrea stentina.

Stein Mortensen underlined the lack of information about the trades of flat oysters. Antonio Villalba added that no abnormal mortality was reported in flat oysters in Galicia but the impact of this parasite is difficult to estimate. Bonamia exitiosa is probably present in Spain at least for 5 years (need to be confirmed).

Epidemiology of different agents causing disease in aquatic animals: scientific review and database development

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Research efforts to support the development of the European aquaculture sector have focused, among others, on infectious diseases of aquatic animals as a major production limiting factor, and particularly diseases listed in the Council Directives 91/67/EEC, 93/53/EEC, 95/70/EC and 2006/88/EC. As a result of the efforts to improve our understanding of aquatic pathogens, and in conjunction with the application of new biotechnology tools, the large biodiversity of these organisms has gradually been recognized with numerous strains and types. The challenge for regulators and risk managers is to determine which of these strains of pathogens should be covered by control measures and which could be regarded of lower significance. However, in the absence of guidance on the matter, approaches to address this issue across the different groups of pathogens, host species and regions of the world have varied.

The Scientific Panel on Animal Health and Welfare (AHAW) has recently adopted several Scientific Opinions dealing with the risk of transmission of diseases by means of transfer of vector and susceptible species. During the development of these opinions, the lack of uniformity in case definition of listed aquatic diseases, and their causative agents, was pointed as a source for potential difficulties in relation to pathogen identification and diagnosis. In some instances, the very wide variation in strain pathogenicity, and the wide distribution of non pathogenic strains were identified as issues to be further addressed.

The European Food Safety Authority is funding an initiative with the objective to establish and provide a scientific basis for pathogen definition and strain differentiation. The specific objectives of this project are to:

i) Provide updated critical scientific reviews with special focus on their causative agents, methods for testing and typing of the resulting isolates;

ii) Describe the current host and geographic strains distribution and their association with outbreaks;
iii) Establish a GIS multilayered database for mapping of the relevant polyphasic information;

iv) Identify data gaps and needs for further research to address the issues of pathogen definition and strain differentiation.

Franck Berthe added that the first outcomes of the project will be a report containing the scientific reviews and electronic form for the epidemiological database. Pedro Rosado-Martin questioned Franck Berthe about data management in the context of this project and more specifically how he expects to convince Member States to share their data. Franck Berthe explained that this database should be a skeleton that will be there to receive epidemiological data. If Member States and the European Commission want to collect information, this is the form which can suit to this purpose. Pedro Rosado-Martin asked Franck Berthe when he planned to obtain the first outcomes. Franck Berthe indicated that EFSA is trying to negotiate an extension of delay, from August to October 2009.

**ANNUAL MEETING SESSION III: THE NEW DIRECTIVE AND RELATED ISSUES**

(*Secretary: C. Garcia*)

**Surveillance of mollusc diseases**

*Pedro Rosado-Martin*

DG Sanco, European Commission, Brussels, Belgium

Council Directive 2006/88/EC lays down animal health requirements for aquaculture animals and products thereof and contains provisions on the prevention and control of certain diseases in aquatic animals. The Directive is focused in disease prevention. Being a key element of disease prevention, surveillance is addressed in several provisions of the Directive. Article 10 provides for the implementation of a risk based surveillance system. This general surveillance scheme aims at the detection of:

- any increased mortality (relevant for all farms and mollusc farming areas);
- listed diseases (relevant for farms and mollusc farming areas keeping species susceptible to the listed diseases).

To harmonise this risk based animal health surveillance system throughout the EU, the Commission has drafted Decision 2008/896/EC on guidelines for the purpose of the risk-based animal health surveillance schemes provided for in Council Directive 2006/88/EC. This system, once implemented, will contribute to increase the knowledge of the health status of all the aquaculture production businesses farming mollusc in the EU. Another type of surveillance is the disease specific surveillance aiming at achieving disease freedom status with regard to Infection with *Bonamia ostreae* and/or *Marteilia refringens*. Chapter VII of the Directive specifies the pathways to achieve disease freedom. Those pathways are:

- absence of the species susceptible to the diseases;
- pathogen is known not to be able to survive;
- based on historical grounds; and
• based on targeted surveillance.

The pathway based on targeted surveillance implies:
• routine inspection by the competent authority or by other qualified health services on behalf of the competent authorities;
• prescribed samples of aquaculture animals to be taken and tested for specific pathogen(s) by specified methods; and
• mandatory immediate notification of occurrence or suspicion of specified diseases or of any increased mortalities.

Stein Mortensen wondered if for example Norway could be considered as free of Marteilia refringens based on analyses performed on flat oysters. Should a surveillance including mussels be necessary? In Norway, mussels and flat oysters have two different distribution areas, mussels in the North and flat oysters in the South but, there are some transfers between these different areas.

Pedro Rosado-Martin replied that the status of Norway is not presently questioned because Norway has numerous historical data. However, he added that it would be interesting that Norway implements a targeted surveillance on mussels as the European Commission asked for Sweden.

David Fraser asked the same question for the United Kingdom: must surveillance on mussels be implemented? Pedro Rosado-Martin answered in the same way, a targeted surveillance would be necessary particularly when the country is not totally free, while those cases from countries with approved zone status would be considered on their merit.

Concerning the exotic species, David Fraser sought clarification if the same principle could be applied: Are historical data sufficient to conclude that the country is free? Can we trust these data?

Pedro Rosado-Martin answered that this fact is assumed by the European Commission.

Antonio Villalba wondered if two susceptible species are present in a same area, must these two species be sampled or just one of them. Pedro Rosado-Martin said that all susceptible species present in one area must be sampled if the country wants the free status for this area.

Hege Hellberg understood the principle to apply a discontinued surveillance once the free status is obtained but underlined the difficulty to know if and when abnormal mortalities occur in the field. So, is a discontinued surveillance acceptable for maintaining a freedom status?

Pedro Rosado-Martin specified that the new directive does not impose the discontinued surveillance, it is just a possibility; it is to each Member States to choose their own surveillance.

Risk based surveillance options for shellfish, Example of Scotland

David Fraser

Marine Scotland Marine Laboratory, Aberdeen, Scotland

In 2007-2008, an expert group, consisting of Scientists and policy makers from England, Scotland & Ireland, met to look at the statutory requirements of risk based surveillance under EC Directive 2006/88/EC, considering the risks of introduction and transmission of listed
diseases at farms and mollusc farming areas. Consultation with industry was integral to the development of a UK surveillance scheme consisting of regular inspections, visits, audits and where appropriate, sampling and including an element of training and intelligence led surveillance.

The Commission currently considers all member states free of exotic diseases, while the UK assumes freedom of certain non exotic diseases, applying risk based surveillance inspection at a frequency recommended under The Directive by fish health Inspector, veterinarian, biologist or health expert. Sampling is to be undertaken only on suspicion and considering abnormal mortality with regard to listed and emerging diseases. Targeted surveillance to be applied only on sites holding susceptible species where there is no historic evidence, by sample screening, of freedom from those diseases e.g. currently white spot disease of crustaceans.

Risk factors were identified as high medium and low and an initial attempt made to quantify those risks at each shellfish farm site. High risk sites included hatcheries, farms importing live shellfish e.g. spat importer and those farms relaying shellfish and growing shellfish susceptible to serious disease.

Medium risk sites are those in proximity to purification, dispatch and processing centres, while low risk are those where site conditions are conducive to disease expression, considering biosecurity and on site husbandry.

Of the 385 registered shellfish sites in 2009, 10% (39) were designated high risk, 27% (104) medium and 63% (242) low risk. Applying a frequency of inspection of one visit per year at high risk sites, every second year at medium risk sites and every four years at low risk sites (1/0.5/0.25) meant 39, 52 and 61 active surveillance inspections annually at high, medium and low risk sites respectively.

The proposed model focuses at high risk farms, employing diagnostic sampling only on suspicion of listed disease. The model requires periodic review, depending on zone health status, considering on site operational changes and, by necessity, be cost effective. Identification and quantification of risk factors is ongoing to ensure effective disease management.

Isabelle Arzul asked if Scottish competent authorities have planed to work at farm level or mollusc farming area level. David Fraser said that they had worked at site level; they categorized the risks for each site (transfers...) but they did not categorize the risks for diseases.

Pedro Rosado-Martin asked when this scheme would be apply. David Fraser answered that normally, this model would be applied very soon.

Stein Mortensen asked if they have a sufficient number of persons and how they train them. David Fraser said that they apply the same process as for fish, currently all inspectors are graduates with fish health or aquaculture experience, shadow an experienced inspector for up to a year training in Scotland depending on progress, then are audited to a high standard prior to working with autonomy. In addition each inspector should participate and pass a bespoke post graduate diploma through Stirling University.
Risk categorisation of mollusc farming areas, example of France

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Laboratoire de Génétique et Pathologie-Ifremer-La Tremblade -France

The EU directive 2006/88/EC stipulates in the Article 10 that member states shall ensure that a risk-based animal health surveillance scheme is applied in all farms and mollusc farming areas, as appropriate for the type of production. For this purpose a guideline was provided by the commission to help member states to establish this surveillance scheme (Commission decision, 2009/177/EC) and to determine the risk level of farms and mollusc farming areas. The risk factors relevant for this categorisation are the direct spread of disease via water and the movements of aquaculture animals.

We present a tentative categorisation of the French mollusc farming areas using an excel file provided by Ignacio de Blas from the University of Zaragoza. For each area, questions regarding presence of susceptible species, conditions about animal supply and delivery, water supply and delivery, geographical situation, and production management, are completed. Consequently, each French mollusc farming area is categorised as low, medium or high risk level.

Cyrille François specified that for the moment the French competent authority made no request on the risk categorization; he also indicated that for France, the exercise is difficult due to an important number of mollusc farms (> 3000) and for this reason, it is difficult to know which type of surveillance must be applied.
Aquatic species susceptible to diseases listed in Directive 2006/88/EC

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Following a request from the European Commission, the Panel on Animal Health and Welfare was asked to establish a list of aquatic animal species susceptible to the diseases listed in the Council Directive 2006/88/EC. More specifically, the question was to establish a) which species other than those listed in Part II of Annex IV to Directive 2006/88/EC could be considered as susceptible; and b) which of the species currently listed as susceptible in Part II of Annex IV to Directive 2006/88/EC cannot be considered as susceptible.

This was achieved through comprehensive literature review with considerations for: i) reflection of natural pathways provided by the experimental design of reported studies, ii) compliance with four objective criteria pertaining to susceptibility to infection, and iii) thorough identification of the causative agent. The four criteria used to assess susceptibility of host species were: evidence of replication or growth of the organism (A), presence of a viable organism (B), presence of specific clinico-pathological changes (C), and specific location of the pathogen (D).

This led to identification of two main groups: Group I, host species for which the quality of the data provided clear support for susceptibility, and Group II, host species for which incomplete or unclear data prevented a clear conclusion or the only available data was obtained from invasive experiments. Group I (susceptible species) contains i) traded and non-traded species, ii) species belonging to several genera, and iii) many were susceptible to several of the specified pathogens, so may represent different levels of risk. Within Group I, species were identified that currently are not listed in Directive 2006/88/EC and those species are recommended to be considered for possible inclusion. Partial evidence suggesting susceptibility was obtained for a large number of host species (Group II). Several host species, including some currently listed in Directive 2006/88/EC, were identified as potentially non-susceptible but it was not possible to confirm this status firmly due to the quality of the data.

Further scientific studies are required to resolve the uncertainty concerning the susceptibility of the host species identified in this group. Such studies should apply clear criteria, such as those used in this opinion, to assess susceptibility of host species and clear identification of the pathogen and affected host(s). In addition, the opinion noted that the lack of clear case definition for some of the specified pathogens compromised assessment of the susceptibility of some host species.

Franck Berthe specified that the EFSA report is available on the EFSA website.

Antonio Villalba asked why histology was not included in the list of techniques enabling demonstration of Bonamia ostreae replication considering that this technique allows the observation of binucleated parasite cells. Franck Berthe indicated that the list of techniques was not exhaustive.

Antonio Villalba noted that the definition of criteria was too strict particularly for the criterion “replication of Bonamia ostreae” which is defined as the observation of binucleated plasmodia. Sometimes, it is possible to observe an important proliferation of the parasite in
the whole host without the observation of binucleated plasmodia; but nevertheless, there is proliferation and replication of the parasite.

Franck Berthe approved this remark and added that in some publications, information related to each criterion were easy to collect but often, it was not the case highlighting the interest of requesting interpretation from a group of experts.

Isabelle Arzul underlined that the different criteria exposed in the EFSA report were not exhaustive; it was just a possible interpretation.

Antonio Villalba noted that pathogen species are questioned but host species should also be questioned. For instance, are Crassostrea gigas and C. angulata a same species or not? Franck Berthe agreed and evocated the detection of sporulation stages of Marteilia refringens in Crassostrea gigas which was not C. gigas but in fact Ostrea edulis.

ANNUAL MEETING SESSION IV: RESULTS OF RECENT FIELD STUDIES
(Secretary: I. Arzul)

Phylogeny, diagnosis and dynamics of the oyster pathogen *Nocardia crassostreae*.

Noèlia Carrasco1*, Ineke Roozenburg1, Inke Wijmenga1, Michal Voorbergen-Laarman1, Naoki Itoh2 and Marc Engelsma1

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*Nocardia crassostreae* is a gram-positive actynomicete causi ng the oyster disease, Pacific Oyster Nocardiosis (PON). This pathogen had been reported in the past in North America and Japan and related in some cases to *C. gigas* mortalities. Recently, *N. crassostreae* has been reported for first time in Europe, concretely in The Netherlands. Aim of this study is to increase the knowledge on this potential emerging pathogen in EU. Research has been focused on the study of the phylogenetical relationship between the European, Japanese and American isolates, the improvement of detection methods and the seasonal dynamics of the pathogen. First results regarding phylogenetical analyses show high homogeneity between different geographical isolates, possibly due to the history of *C. gigas* transfers. A species-specific SyBr Green Real-Time PCR based on *N. crassostreae* 16S-23S sequence with high sensitivity has been developed to improve the detection of this pathogen. This new detection tool for *N. crassostreae* has been compared with other detection methods (histology, conventional PCR and bacterial isolation) for field samples. Regarding the seasonal dynamics of *N. crassostreae* in a Dutch estuary, low or null levels of infection are observed during winter and spring, however prevalence of infection increases dramatically during summer and have a peak in autumn before decreasing at the end of this season. Furthermore, the potential role of the sediment as a pathogen reservoir is currently under study.

Isabelle Arzul reminded that Nocardia spp. (Pacific oyster nocardiosis) was included in the list of most serious aquaculture diseases and hazards, which threaten European aquaculture as identified through the second work package of the European project PANDA.
Bonamia ostreae in loch Sunart; is eradication possible?

David Fraser

Marine Scotland Marine Laboratory, Aberdeen, Scotland

Following confirmation of Bonamia at a registered aquaculture site in Loch Sunart, Scotland on 14 June 2006, a control zone to prohibit the relaying of shellfish within the loch was made under FH Regulations 1997. The subsequent introduction of Commission Decision 2007/104/EC annexed Loch Sunart from approved zone status for Bonamia ostreae.

The infected farm site, which is operated by a shellfish buying company, consists of two small bays into which animals too small for sale were seeded for growing. Low prevalence of infection was found on site, 3/159 animals tested positive by histology, only apparently healthy animals were found by survey and no infection found associated with cohabiting molluscs tested.

A sampling strategy involving shore and diver based surveys was undertaken: to clear the site of the susceptible species, the Flat Oyster (Ostrea edulis); and to provide evidence of the absence of natural recruitment or established native oyster populations within the loch system. The site is currently considered fallow of the Flat Oyster, Ostrea edulis and associated risk of direct transmission between oysters and potential carrier species considered low.

Results from survey provided evidence: that less than 10% of areas contain Flat oysters (at the 95% confidence level), which was backed by intelligence and good anecdotal evidence of their absence within the loch system; and that the site is fallow.

After at least a two year fallow period it is proposed to place, and test sentinel animals over a two year period for the presence of Bonamia infection. If tests prove negative, a case to the EC Commission will be prepared with proof of eradication, to have controls removed from the loch system to re-establish approved Zone status there. The Loch Sunart situation is atypical of most outbreaks which tend to occur on established and active fisheries where eradication is considered more problematic than the Loch Sunart situation.

At the end of the presentation, David Fraser asked the NRLs whether they considered the Scottish eradication programme feasible and requested suggested ways forward with regard to fallow period and restocking.

Stein Mortensen reminded that some years ago, an eradication programme took place in Limfjorden in Denmark and succeeded in eliminating Bonamia ostreae from the area.

Marc Engelsma asked if some oysters were used as sentinels. David Fraser informed us that some oysters were to be caught from a disease free area, be maintained in tanks for further analysis, prior to reseeding.
Survey of Candidatus Xenohaliotis californiensis in France.

Cyrille François*, Céline Garcia, Isabelle Arzul, Maeva Robert, Justine Michel, Géraldine Oréal, David Schikorski, Tristan Renault

Laboratoire de Génétique et Pathologie-Ifremer-La Tremblade -France

The infection with Candidatus Xenohaliotis californiensis, pathogen responsible for Withering Syndrome in several abalone species, is no more listed in UE since 2006 but has been maintained in OIE list of notifiable infections. Haliotis tuberculata is the only species of abalone present along the French coasts. Commercial fisheries of natural beds have been developed in Manche and Brittany and aquaculture of this species also exists in Vendée, North Brittany and Normandie.

Considering the increasing production in France and the recent description of C. Xenohaliotis californiensis in Europe, a survey has been carried out to verify if this pathogen is present in Haliotis tuberculata in France. The check-out of samples of abalones collected by Ifremer Pathology and Genetic Laboratory since 1998 does not show the presence of this pathogen. Samples have also been collected in five commercial hatcheries –nurseries in 2007 and 2008. The diagnostic tests performed were PCR (primers RA 3-6 and RA 5-1, OIE Manual of Diagnostic Tests for Aquatic Animals 2006) on every individual as screening technique, then photonic microscopy observation of histology slides, in situ hybridisation and sequencing depending on the result of the PCR. As results, C. Xenohaliotis californiensis has been detected in four hatcheries-nurseries and is suspected in the last one. These detections raise the question about the risk for the abalone production, even if no abnormality case was observed on French abalones in 2007-2008 associated with the detection of this pathogen.

Pedro Rosado Martin indicated that such a pathogen – Candidatus Xenohaliotis californiensis- could be listed at a national level if in some Member States it appears relevant to restrict transfers of molluscs regarding infection with this pathogen. Isabelle Arzul evocated the difficulty to deal with pathogens present in some conditions at very low level of infection without inducing clinical sign. Indeed, these pathogens are usually not detectable by histopathology and PCR is eventually able to detect DNA but must be completed and confirmed by other techniques like histology or in situ hybridization. When confirmation is not possible, one solution could be to take some animals and to maintain them in conditions considered more conducive for disease expression and/or development.

ANNUAL MEETING SESSION V: ABNORMAL MORTALITY EVENTS

(Secretary: J.P. Joly)

Abnormal mortality: theory and practise.

Raquel Aranguren

Instituto de Investigaciones marinas-Vigo-Spain

Marine bivalves are of considerable economic importance. Not least because of this, the amount of information available on bivalve diseases is extraordinarily large and comprehensive. One of the factors limiting the production is the presence of pathogens and
diseases which have particularly affected oyster and clams production. The role of environmental factors (including climate change) in disease development and their interaction with pathogen and host should also have to be taken into account. Different environmental factors will influence host-parasite interactions and pathogen persistence particularly for protozoan parasites and disease development. There is increasing evidence, however, that environmental stress and man-made water pollution in particular, may lead to debilitation and disease in numerous marine animals including bivalves. When considering the effects of pollution stress, one must take into account that bivalves are also subjected to biological (reproductive, competitive) stress and natural environmental stress (changes or marginal values of salinity, temperature, oxygen tension...). However, the effect of stress on the parasite or disease agent is another factor to be considered. Over the past decades, world molluscs production has been adversely affected by a number of diseases and, given their severe impact on economic and socio-economic development in many countries, some of these diseases have become a primary constraint to the development and sustainability of molluscs aquaculture. Disease agent transfer via transfers of live molluscs has been a major cause of disease outbreaks and epizootics.

But, what is an abnormal mortality in a population? If you look through different definitions we find that an abnormal mortality is a “sizeable mortality that occurs in a short time” (O.I.E Aquatic Manual 2003), or “unexplained mortalities above the level of what is considered to be normal” (Directive 2006/88/CE). Then, we can think of what is considered to be a normal mortality? When talking of close aquaculture facilities, it is relatively easy to estimate the real mortality. For example, a 100 % of mortality was recorded in an experimental abalone aquaculture raft in Galicia (NW Spain) caused by *Haplosporidium montforti* n.sp and *Candidatus Xenohaliotis californiensis*. Both pathogens were originally detected from abalone seeds transferred from other country where no mortalities had been recorded. The higher water temperature in Galicia was considered as a determining factor in the development of both pathogens.

In natural bed populations, it is important to know the real situation to manage in an appropriate way the natural resources. *In situ* controlled systems based on PVC cages buried into sand were designed to estimate the real mortality rate and to register the pathogenic burden on clam populations cultured in two different production areas of Galicia all over several years. Different situations were found; peaks of mortalities (57 %) with low pathogenic burden but adverse environmental conditions (7 ppt of salinity), high pathogenic burden of analyzed clams with no peaks of mortalities (no higher that 10 %) and no adverse environmental conditions and peak of mortality (45 %) with high pathogenic burden of the analyzed clams and no adverse environmental conditions.

As it has been demonstrated it is important to highlight the role of the environmental conditions in the development of the disease and the outbreaks of mortalities. *In situ* controlled systems should be designed according to each molluscs production system to be have a real vision of the different situations.

*Isabelle Arzul* reminded that mortality is the first sign to start sampling but studying the pathogens without mortality is important too.

*Antonio Villalba* suggested to collect information on mortality on important economic areas because a reference picture is needed to determine which mortality rate is “normal” or not. However such study is difficult to carry out, especially on wild beds of clams or cockles.

*Cyrille François* explained that in France the epidemiological surveillance network *Repamo* notably aims at surveying mollusc mortalities along the French costs. This network relies on people which have a good knowledge of the field and husbandry practices, which helps to determine if mortality is normal or not.
Hege Hellberg noted that Norway has low compliance of shellfish farmers to report abnormal mortalities. Cyrille François suggested to combine active and passive surveillance to estimate the normal and abnormal mortalities. Isabelle Arzul concluded that a same protocol should be used in all countries to estimate and compare mortality rates.

MORTALITY EVENT IN FRANCE IN 2008

Mortality event in France in 2008 – context
Cyrille François*, Laurence Miossec, Denis Saulnier, Jean-François Pépin, Céline Garcia, Isabelle Arzul, Jean-Pierre Joly, Maeva Robert, Emmanuelle Omnes, Bruno Chollet, Philippe Haffner, Nicole Faury, Laetitia Cobret, Tristan Renault

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In spring and summer 2008 massive mortalities occurred in almost all production areas of Pacific oyster in France. The phenomenon affected only *Crassostrea gigas* species, mainly spat and juveniles (< 2 year old), and was described as rapid with high mortality rates up to 100%. In view of this situation, a crisis unit was created and Ifremer agents were involved in sampling on the field and testing in the laboratories. Three hypothesis have been investigated by Ifremer : (1) environmental factors, (2) toxic algae, (3) pathogens.

The REPAMO network (réseau de pathologie des mollusques - Ifremer) carries on the survey of shellfish health status along French coasts and one of its protocol deals with the study of abnormal mortality. For this purpose, a procedure has been established between producers, local and national competent authorities and Ifremer to explain the roles of each partner. During this event, samples of different ages and origins of *Crassostrea gigas* have been carried out in almost all impacted and in some non-impacted areas for further tests in pathology by Ifremer Pathology and Genetic Laboratory: the detection and identification of pathogen(s), the study of transmission and virulence of pathogen(s) detected and finally a epizootic study.

Photonic microscopy on histology slides showed no infection by a listed pathogen (UE, OIE) in the oysters tested. Bacteriology and virology assays (bacterial isolation, PCR, PCRQ, sequencing) gave rise to the detection of *Vibrio splendidus*, *Vibrio aestuarianus* and herpes virus OsHV-1 in many samples, alone or in association. Bacteria of *Vibrio harveyi* group have been also described for the first time in Pacific oysters. The detection of OsHV-1 was confirmed by transmission electronic microscopy observations.

Summer mortality in 2008 and Ostreid Herpesvirus investigations
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In France, the year 2008 displayed high mortality rate ranging from 80% to 100% affecting only cupped oyster, *Crassostrea* gigas and mainly juvenile oysters from May to September. According to devastating of losses, it appeared important to investigate if this high mortality
rate could be associated to emerging or new pathogens or because of preliminary tests for herpesvirus detection displaying around 75% batches positive; if it could be related to the emergence of a new biotype of Ostreid Herpesvirus type 1 (OsHV-1).

We conducted at the Genetic and Pathology Laboratory of IFREMER La Tremblade (17) different studies in order to characterize aetiology and infectious pathogens associated to the outbreak:

i) experimental infections for inducing mortality;
ii) characterization of 2008 ostreid herpes virus isolates related to viral genomic polymorphism;
iii) detection of OsHV1 in plankton, larvae and seawater during an oyster larval abundance survey in Marennes Oléron basin.

i) Experimental infections for inducing mortality. Using biological material extracts prepared from field affected oyster batches we induce mortalities (> 80% IM, 40% cohabitation) in spat and juvenile oysters, for both route of infection, intramuscular injection or cohabition challenge. Using quantitative PCR, the viral loads associated with mortality in dead challenged animals were over \(10^7\) viral DNA copies/mg. Filtered extract lower than 0.1µm or UV inactivated did not induce any mortality. TEM observations in dead or moribund oysters tissues revealed numerous herpesvirus type particles and no other parasite or pathogen. Both biotypes identified in isolates, OsHV1 and OsHV1 µVar*, were virulent and generated mortality with the oyster stages used; it is the first time that such results trial were obtained.

ii) Characterization of 2008 ostreid herpes virus isolates related to viral genomic polymorphism. In order to characterize potential polymorphism in viral isolates, several biomolecular tools were carried out notably sequencing method. Analysis of various target sequences within viral genome present in infected batches demonstrated the presence of polymorphism. Polymorphism in some of the 2008 batches has been characterized and as a result, a new biotype* based on DNA sequence was described exhibiting: 96% to 99% sequence homology in two targeted regions C and IA, with reference OsHV-1 DNA; deletions in a micro-satellite zone and substitutions in coding zones. Consequently, at least two amino acids appeared modified in these isolates. Moreover, isolates that presented this new biotype were virulent and able for the first time to generate heavy mortality during challenge tests on spat. The work was completed by checking old batches from different geographical (France, USA, China, Japan) and chronological origins without any detection of the new biotype. The new biotype identified in 40% of studied spat batches in 2008 has been called «OsHV-1 µVar » and could be considered as an emerging viral biotype involved in oyster mortalities.

iii) Detection of OsHV1 in plankton, larvae and seawater during an oyster larval abundance survey in Marennes Oléron basin. A collaborative work carried out with the Regional Center for Aquacultural Experimentation and Application (CREAA) allowed to detect for three dates of the fourteen studied, viral DNA signal in zooplankton and in seawater. These results confirm that we are now able to detect OsHV1 DNA in seawater and we assume that such protocol could be helpful in epidemiological study.

*Vibrio* investigations in 2008: Is there an emergence of a new *Vibrio* species pathogenic for *C. gigas* oyster in France?
Preliminary to bacterial analysis performed in 2008, 115 bacterial strains were isolated by the French REPAMO surveillance network during 73 mortality events occurring in reared *C. gigas* oysters between 2003 and 2007. 16S rRNA gene sequencing analysis revealed that 50% of these strains belong to either *V. splendidus* or *V. aestuarianus* species. Testing 20 of these strains in experimental infection challenges revealed that most of them were virulent. These data suggest the involvement of both *Vibrio* species in *C. gigas* mortality events observed in the Atlantic and Mediterranean coasts of France, mainly during spring and summer. To facilitate further taxonomic studies we developed a multiplex qPCR test targeting specifically *V. splendidus* and *V. aestuarianus* strains, allowing us to rapidly identify dominant bacterial strains isolated by conventional culture methods in approximately 2h.

In the context of mortality events reported in 2008, exceptional by their extent, 241 dominant bacterial isolates were isolated from 45 batches of *C. gigas* oysters suffering abnormal mortality outbreaks between February and August. Fifty one percents of these strains (n=124) were identified as *V. splendidus* or *V. aestuarianus* based on our PCR diagnostic test. Interestingly, sequencing of other non identified isolates revealed that another *Vibrio* species, *V. harveyi* was found in abundance (22 isolates) and detected in 15 batches (33%). The virulence of nine *V. harveyi* bacterial strains was evaluated by experimental infection of *C. gigas* oysters. All strains induced precocious mortalities (in less than 24h of survey) and higher than 60%, when bacteria were injected in the adductor muscle, which was unusual by comparison to the delay of response (intensity and day of occurrence of mortality peak) obtained in comparable conditions with *V. splendidus* and *V. aestuarianus* pathogenic strains. Some *V. harveyi* strains associated to oyster mortality outbreaks have been isolated before 2008 but in low number before 2007 (only one strain during 2003-2006 survey) or in some restricted area in 2007 (in two lagoons on the Mediterranean coast, two hatcheries/nurseries in Atlantic coast and one experimental facility). Because *V. harveyi* is a polyphyletic group, our strains were more fully identified by a multilocus sequencing typing approach. Analysis of the obtained phylogenetical tree revealed that our *V. harveyi* related strains were grouped in three main clusters including type reference strains of *V. harveyi*, *V. alginolyticus* and *V. tubiashii*.

A strong correlation between 1) the ability of either *V. splendidus*, *V. aestuarianus* or *V. harveyi* strains to produce a toxin, metalloprotease in culture supernatants, and 2) virulence potential, as evaluated by experimental infection, was reported suggesting that measurement of metalloprotease activity could constitute a phenotypic marker of virulence of *C. gigas* bacterial strains. Furthermore haemolysins could be evidenced in some of our *V. harveyi* related strains.

These data may suggest the involvement of *V. harveyi* strains in the exceptional mortality outbreaks happened in France in 2008. Nevertheless, further epidemiological studies are needed to confirm it

**Epidemiological study on *Crassostrea gigas* mass mortality observed in 2008 in France: descriptive study**
Abnormal mortalities occurred in most oyster producing areas in France during the summer of 2008. The mortalities were sudden and severe (up to 100%) and mainly affected 6 to 18 month old juveniles. Only *Crassostrea gigas* species was affected. The cause of the mortality was unclear but OsHV-1 virus and bacteria belonging to Vibrionaceae family were frequently detected in affected populations.

An epidemiological study was commenced in autumn 2008 in order to identify factors associated with, and the cause (or causes) of these mortalities. We present here the first results of the descriptive part of this investigation. The objectives were to identify the affected population and to describe the pattern of mortalities in time and space. Mortality data, based on producers’ declarations, were collected from the departmental Offices of Maritime Affairs (local competent authority) and the regional mollusc producer bodies. They were supplemented by data from local and national monitoring networks (REPAMO and REMORA). More than 7000 records, consisting of location, date and level of mortality, age and origin of individuals and management (transfers and handling) were collected.

The initial descriptive analysis of this data has shown that the epidemic varied between regions. Map-based analysis using GIS allows a more precise spatio-temporal description of the outbreak within each production area. All age classes were affected but spats (< 12 months old) had a higher level of mortality than juveniles and adults. Initial observations do not show any difference between wild-caught and hatchery-bred spat mortality.

We discuss the limits and bias of the study, especially regarding underreporting of mortality events, analysis of the index cases in each production area. Additional data on cultivated oyster’s population and associated cultural practices are necessary to finalise the first part of this study and then to develop the analytical study including environmental parameters.

Antonio Villalba asked how we can be sure about farmer estimations of mortality rates. Laurence Miossec replied that it's difficult to rely on these estimations. She added that no mortality has been reported in Prevost lagoon nor in Corsica lagoon, so which environmental factor is implicated in mortality events?

Ian Laing asked if information on husbandry were available. Laurence Miossec explained that information on spat production are scarce but it seems that there is an evolution in husbandry, specially in nursing spat.

Pedro Rosado Martin questioned the possible actions to be put in place to avoid such abnormal event in 2009. Laurence Miossec reminded transfer restriction of spat or oysters in the event of a mortality outbreak is a priority.
EXPERIENCE FROM OTHER EUROPEAN MEMBER STATES

Mortalities in *Crassostrea gigas* in Ireland in 2008.

*Deborah Cheslett,*

Marine Institute-Rinville-C.o. Galway - Ireland

In the summer of 2008, widespread mortalities occurred in *Crassostrea gigas* along the French coast. The suspected cause of the mortalities was the presence of Ostreid herpes virus 1, frequently in association with *Vibrio splendidus*. The situation in France was a cause for grave concern amongst the Irish industry which relies to a large extent on imports of seed and half grown oysters from France.

In August, reports of increased mortality in *C. gigas* were received from 3 oyster growing areas in Ireland. All three areas had been supplied with seed from France in the immediate run up to the mortalities and so a link between the two events was suspected. An investigation into the mortalities showed the presence of OsHV1 in all three bays. In addition *V. splendidus* was detected in 2 of the bays. The investigation revealed other factors were at play but it seems likely that the presence of these two pathogens was at least in part responsible for some of the losses observed.

*Deborah Chesslet added that spat is usually imported from March to June but most spat is imported in May-June at a size of 6 to 10 mm.*

*David Fraser asked whether it was possible to use seed from hatcheries free from OHV/Vibrio to ensure that only healthy animals were moved and not carriers with resistance which could pass the disease onto naïve populations at country of destination. Isabelle Arzul explained that all French hatcheries were infected and that mortalities seem to occur under particular environmental and zootechnical conditions. David Fraser suggested that this could introduce problems as seen recently in Ireland.*

*Isabelle Arzul replied that it's presently impossible to certify that seed produced in hatcheries or in the wild are free of pathogens. Deborah Cheslett added that many growers buy their seed from France and sell back their oysters at the end of the production cycle restrictions on transfer of spat regarding the presence of pathogens like OsHV-1 and Vibrio spp. would have a dramatic effect on the industry. Sven Bergman wondered if there is export from Ireland to Germany. Deborah Chesslet replied that export occurred from a segregated island with no mortality reported.*
Investigations into commercially exploited cockles undergoing regular and persistent mortalities in south Wales

Matt Longshaw

CEFAS-Weymouth-England-UK

Cockles (Cerastoderma edule) are commercially exploited at a number of sites around the UK. Cockle mortalities in various beds around the coastline have been reported in the past few years. These mortalities are generally sporadic and acute and thus make investigations difficult to determine a cause of mortality. However, mortalities have been reported annually from the Burry Inlet in south Wales since summer 2004.

Whilst there have been reports of mortalities from this area previously, the more recent mortalities are considered unprecedented in their scale and severity. Typically, mortalities begin around April / May at several discrete cockle beds in the Inlet which then spreads to almost all other beds in the Inlet within a month. Mortalities continue to occur throughout the summer months, declining around September. Loss of up to 90% of single year classes has been reported. Given that cockles from the Burry Inlet grow rapidly, reaching sexual maturity within a year, this has proved problematic since some animals have died prior to reproducing.

As part of a multidisciplinary programme of work to determine possible causes of mortalities, samples of cockles have been collected from sites within the estuary, from the nearby Three Rivers estuary which has also experienced mortalities, from Crymlyn Burrows in Swansea Bay and from ad hoc sites around the UK for comparison. Contemporaneous biological and environmental data has also been collected from each site. Whilst studies are still on-going, it appears that the mortalities are cockle-specific, suggesting that an infectious agent may be involved. The spread of mortalities across the well-mixed Inlet adds weight to this theory. Histological studies of cockles have demonstrated the presence of a number of pathogens previously implicated in mortalities of cockles throughout its range. In particular prevalence and intensity of the digeneans Meiogymnophallus minutus, Bucephalus (=Labratrema) minimus and Himasthla spp. increases prior to the mortality event and decline during and after the mortality event suggesting loss of heavily infected animals from the population. Additionally, cockles from the Burry Inlet are affected by a number of other conditions including granulocytomas and disseminated neoplasias.

Recent efforts by the multidisciplinary team from various universities, government institutions and the industry have sought to carry out intensive, integrated sampling to further understand the mortality drivers in the cockles of the Burry Inlet.

Isabelle Arzul said that this multidisciplinary study of mortality events in cockle beds is very interesting. Such event does not affect the production of mollusc at a National level but has important economic consequences at a local scale.

Matt Longshaw added that indeed, 50 fishermen are living of this fishing so it's locally important.
Health Management of Bivalve Mollusc Hatcherries and Nurseries

Ralph Elston

AquaTechnics, Sequim, WA 98382-USA

During 2006 and 2007, we documented the re-emergence of severe episodes of vibriosis caused by *Vibrio tubiashii* in shellfish hatcheries on the west coast of North America. Lost larval and juvenile production from Pacific and Kumamoto oysters (*Crassostrea gigas* and *C. sikamea*) and geoduck clams (*Panope abrupta*), was documented. Losses were associated with the apparent mixing of an intrusion of unusually warm surface seawater and intermittently upwelled cooler, nutrient and *Vibrio* spp.-enriched seawater. The ocean temperature elevation anomaly in 2007 was not linked to an El Niño event, as was a similar episode in 1998. Concentrations of the dominant shellfish pathogenic vibrios were as high as 1.6 x 10^5 cfu mL^-1 in the cold, upwelled water. The bacteria possessed the genes coding for a protease and hemolysin described for *V. tubiashii*, and pathogenic isolates secreted these peptides. Lesions resulting from a classic invasive disease and a toxigenic noninvasive disease occurred in the oyster and geoduck clam larvae.

Management and prevention require reduction of incoming concentrations of the bacteria, reduction of contamination in water and air supplies and in stock chemical solutions, removal of bacterial toxins, and interruption of the cycle of bacterial amplification in the hatchery and micro-algal food supplies.

Bivalve shellfish hatchery and nursery management goals include predictable production, high survival before and after sale, high growth rate, high health and condition and minimization of waste. Achieving these goals leads to efficient and profitable operation. Application of health management principles has been necessary to achieve consistent and efficient production in all forms of animal husbandry. Health management topics for bivalve shellfish intensive production include prevention and management of bacterial contamination, animal condition assessment and water quality monitoring and management. Contamination of hatcheries and nurseries is prevented by managing each component of the culture system that can serve as a source of contamination. These components include brood stock, algal feed stocks and the seawater source, in addition to the larval and juvenile cultures. High humidity in hatchery environments must be managed at critical steps to prevent maintenance of contamination. Water source suitability is site specific and water treatment and monitoring for both bacteriological contamination and water quality parameters need to be adapted to site specific needs. Microalgal food contamination by shellfish pathogenic bacteria can be a persistent and important source for introducing toxigenic and invasive bacteria into animal cultures. Approaches and methods for the management of larval and juvenile bivalve health, with emphasis on bacterial management, evaluation of animal condition and water quality evaluation will be presented.

Isabelle Arzul mentioned that Ralph Elston was not able to present his presentation but this one will be included in the CD rom. Moreover she expected that Ralph Elston would be present at the next meeting.
Diagnostic tools to detect and distinguish *Bonamia ostreae* and *B. exitiosa*.

Marc Engelsma*, Michal Voorbergen-Laarman

Laboratory for Fish and Shellfish Diseases, Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands

Histopathology is the golden standard method for the OIE with regard to detection of the oyster pathogens *Bonamia ostreae* and *Bonamia exitiosa*. The technique is very suitable for surveillance work. However it has some drawbacks: the method is not species specific and the sensitivity compared with some of the other methods is low.

In this presentation an overview is given on the diagnostic methods described in the OIE manual to detect *Bonamia* species. In addition a number of other methods potentially useful for detection and discrimination of *Bonamia* species is presented with focus on the molecular methods.

For diagnostic purposes detection with real time PCR assays has a number of advantages over conventional PCR methods. In particular the possibility of cross-contamination is reduced, as the system does not require opening of the reaction tubes after amplification. A number of published and unpublished PCR assays were tested for their suitability in a real time SYBR Green setting and their specificity to discriminate between *B. ostreae* and *B. exitiosa*. The results are promising but show also need for further optimization and validation of (real time) PCR assays.

Isabelle Arzul asked how many laboratories used these techniques, specially the real-time PCR. Hege Hellberg answered that the real-time PCR was used for the detection of fish pathogens. Anders Alfjorden specified that ISH was used in Sweden.

Isabelle Arzul concluded that there is a need to harmonize the techniques developed in European national reference laboratories and proposed Real Time PCR as a topic for the next workshop.

Real Time PCR for detection and quantification of *Bonamia ostreae*.

Maeva Robert, Céline Garcia, Bruno Chollet, Inmaculada Lopez-Flores, Sylvie Ferrand, Cyrille François, Jean-Pierre Joly and Isabelle Arzul*

Community Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade, France

*Bonamia ostreae* is an intracellular protozoan recognized as a cause of mortality among European populations of flat oysters *Ostrea edulis*. Based on the recent characterization of actin genes of *B. ostreae*, specific primers were designed for real-time PCR using SYBR® Green chemistry. Analytical specificity was demonstrated by the unique melting temperature peak observed in positive samples and by the lack of amplification in samples of oysters infected by closely related parasites including *B. exitiosa*. An artificial template consisting in
a plasmid clone was used to establish a calibration curve to evaluate the number of amplified targets per sample. The assay had a 6 log- dynamic range, mean inter- and intra-assay variation coefficients < 1% and a minimum detection limit of 50 gene copies per reaction when plasmid DNA was used as template. Using infected oyster samples as template the assay was at least 10-fold more sensitive than conventional PCR. Our quantitative assay was applied to test 132 oysters and results were compared with heart imprint ones. A strong correlation was observed between both techniques which validate the real-time PCR assay we developed and supports its use for improving our knowledge regarding B. ostreae life cycle within and outside its host.

Gary Meyer asked from which tissue DNA was extracted for these analyses. Isabelle Arzul answered that DNA was extracted from gills.

Gary Meyer asked from which tissue DNA was extracted for these analyses. Isabelle Arzul answered that DNA was extracted from gills. Basil Raggias wondered if the Real Time PCR assay using SYBR® Green chemistry could be adapted with a Taqman probe. Isabelle Arzul answered that the team started by developing a PCR using SYBR® Green chemistry. She underlined that Taqman probes can be interesting to detect simultaneously different species of Bonamia.

Marc Engelsma specified that he was quite happy with the SYBR® Green chemistry.

Modulation of gene expression in haemocytes from Ostrea edulis in response to an infection by the parasite Bonamia ostreae.

Benjamin Morga*, Isabelle Arzul, Nicole Faury, Amélie Segarra, Bruno Chollet, Tristan Renault.

Ifremer, Laboratoire Génétique et Pathologie, La Tremblade, France

Bonamiosis due to the parasite Bonamia ostreae is a disease affecting the flat oyster Ostrea edulis. Bonamia ostreae is a protozoan, affiliated to the order of haplosporidia and to the phylum of cercozoan. This parasite is mainly intracellular, infecting haemocytes, cells notably involved in the defence mechanisms of the oyster.

Suppression subtractive hybridisation cDNA library was performed to identify genes differently expressed (up or down-regulated) during an in vitro infection of haemocytes by Bonamia ostreae. Several genes of interest have been identified including genes involved in cytoskeleton, respiratory chain, membrane receptors, detoxification, regulation proteins and immune system. Real time PCR test were performed to study the relative expression of these candidate genes during an in vitro infection of haemocytes by purified parasites. The elongation factor alpha was selected as housekeeping gene.

Infection seems to particularly favour expression of genes likes actin related protein, filamin, liporeceptor, mitogen-activated protein kinase MAPK organizer 1 calmodulin and omega glutathione-s-transferase OGST. Interestingly, genes involved in defence mechanism like SOD, tetraspanin or TIMP appeared down-regulated suggesting that parasite escape degradation by inhibiting expression of such genes.

These results contribute to better understand how the parasite installs and survives within haemocytes.

Marc Engelsma asked which ratio between haemocytes and parasites was necessary for these experiments. Benjamin Morga answered that one haemocyte was necessary for five parasites.
What’s new about relationships between *Vibrio* and *Crassostrea gigas* summer mortality?

Sophie De Decker*, Julien Normand, Sophie Castagnet, Philippe Haffner, Pierre Boudry, Denis Saulnier

Ifremer, Laboratoire Génétique et Pathologie, La Tremblade, France

Two bacterial species, *Vibrio splendidus* and *Vibrio aestuarianus*, are frequently found associated to the Pacific oyster *Crassostrea gigas* during summer mortality events that occurred in France during the recent years. The pathogenicity for oysters of several *Vibrio* strains isolated during these events was demonstrated by experimental infection. Because of the seasonality of mortality firstly, the zoosanitary risk induced by *Vibrio* infection secondly, and finally the emergency to find out a way to improve disease management in oysters, the impact of three potentially correlating host factors, 1. sexual maturity, 2. ploidy level and 3. genetic basis on *C. gigas* susceptibility to vibriosis needed to be clarified. To explore vibriosis, an experimental infection model by co-injection of oysters with both *Vibrio* species has been developed and tested in diploid and triploid oysters, the mixture of the two *Vibrio* strains allowing to decrease the infective dose of the *Vibrio* mixture by a factor 10 to 100 when compared with each *Vibrio* species used separately, underlining a synergistic virulence effect of *V. splendidus* and *V. aestuarianus* when used in co-infection.

Firstly, diploid and triploid one-year-old *C. gigas* oysters, resulting from crossing 16 males and 1 female and reared under common environmental conditions, were tested *in vivo* by a standardized experimental co-injection model with *V. splendidus* and *V. aestuarianus* pathogenic strains. Four successive experimental infections were performed on these oysters over a reproductive cycle (i.e. from May 2007 to January 2008). As a result, the effect of several factors on survival after experimental infection was studied: family (through microsatellite-based parentage analysis), ploidy (2n versus 3n) and reproductive stage (determined by histological analyses over time). Results from a two-way ANOVA analysis (reproductive stage x ploidy) showed a significant correlation between the vibriosis susceptibility and the reproductive stage, suggesting a physiological weakness associated with maturity. This result allowed us to establish for the first time in *C. gigas*, a clear concordance between reproductive effort/spawning processes and *C. gigas* susceptibility to *V. splendidus* and *V. aestuarianus* infections. A limited ploidy effect was observed but probably shaded by an unwished but clearly observed reproductive effort on these triploids. The variation between families will also be discussed.

Secondly, we are also now developing a non invasive experimental infection model of transmission of *V. aestuarianus* and *V. splendidus* disease to healthy oysters by cohabitation with experimentally injected ones. Two novel quantitative Taqman PCR assays targeting the *dnaJ* gene of *V. aestuarianus* and the Green Fluorescent Protein gene of one *V. splendidus* GFP-tagged strain were developed to trace both pathogens. The specificity, sensibility and reproducibility of these Taqman PCR assays were assessed. These quantitative methods will allow us to quantify these *Vibrio* both spatially (different oyster tissues and seawater) and temporally during an experimental cohabitation challenge.

Overall, our results contribute to better understand interactions between *Vibrio* and their host and the relative role of different physiological and genetic factors modulating their susceptibility.
Stein Mortensen wondered if the correlation between the susceptibility to vibriosis and the reproductive stage was higher in spring. The answer was affirmative.

Stein Mortensen asked where the injection of Vibrios was performed in the animal and if it was done directly in the water. Sophie De Decker answered that it was in the adductor muscle and that injection through the water did not allow to transmit the disease.

Stein Mortensen asked if the filtering system was stopped during the experiment which was not the case according to Sophie De Decker.

Ostreid herpes virus infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: differences in viral DNA detection and quantification using real-time-PCR

Christopher Sauvage¹, Jean François Pépin¹, Sylvie Lapègue¹, Pierre Boudry² and Tristan Renault¹∗

¹ - Ifremer, Laboratoire Génétique et Pathologie, La Tremblade, France
² - Ifremer, UMR M100 Physiologie et Ecophysiologie des Mollusques Marins, Plouzané - France

Pacific oysters (*Crassostrea gigas*) on the French coasts experienced periodic mass mortalities during the summer months for at least 20 years. A herpes virus, Ostreid herpes virus 1 (OsHV-1), was frequently detected during mass mortality events in France. Because of the economical importance of oyster aquaculture, molecular diagnostic tools were developed to detect OsHV-1 as its complete genome was sequenced. Recently, the development of a real-time PCR assay allowed quantifying OsHV-1 DNA. This new diagnostic tool opens perspectives in terms of understanding mechanisms of viral replication, management of the disease or selective breeding.

In this context, an experiment was carried out at the Genetics and Pathology Laboratory (Ifremer, La Tremblade, Charente Maritime, France) during the summer 2006 in order to investigate the daily kinetics of summer mortality in three full-sib families of oysters reared under common conditions. The experiment was thus designed in order to monitor oyster mortality and sampling of individuals on a daily basis. Oysters were reared under identical environmental conditions throughout their life. OsHV-1 DNA was quantified using real-time-PCR. Dead and live oysters were collected during the mortality event. Surviving oysters were also sampled at the end of the experiment after the mortality event. This allowed recording daily changes in the individual viral DNA quantification, as a measure of the viral infection between dead, living and surviving oysters, and also within and between families.

A mortality outbreak was observed at the beginning of July 2006. The mortality event was severe and brief but significantly different between tested families. Cumulative mortality ranged from 1.2 to 49% and was significantly different between families. Real time-PCR assays revealed different viral DNA amounts among dead individuals between families. Live oysters showed a significantly lower amount of viral DNA compared to dead ones. The correlation between the level of mortality that occurred in families and the average level of infection by OsHV-1 was highly significant. Data showed a high variance in the viral prevalence between the 3 full-sib oyster families. This suggests that each group of oysters may be composed individuals with different degrees of tolerance to the viral infection.

This experiment showed for the first time daily changes of individual OsHV-1 DNA amounts during a mortality outbreak. Results also support a high genetic basis underlying the variance of survival of Pacific oyster to summer mortality, opening the possibility to improve
resistance to OsHV-1 by selective breeding. Previous studies suggest that a genetic basis may underlie the resistance to OsHV-1 infection of in the Pacific oyster.

*Isabelle Arzul concluded the session by encouraging people to present their own results.*

**ANNUAL MEETING SESSION VII: CRL DAY LIFE ACTIVITIES**  
*(Secretary: C. Francois)*

**Quality management and proficiency testing by interlaboratory comparison**

*Jean-Pierre Joly*

Community Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade, France

The CRL is currently building a quality management system for the organisation of interlaboratory comparison tests. For the accreditation purpose of the laboratory by the accreditation board of France (Cofrac: Comité Français d’Accréditation), most of the documents are written in French. However all the documents to participants are written in English. With the agreement of the European Commission, the CRL hopes that all the documents needed by the National Reference Laboratories of Europe (information about the test, registration form, instructions and recording sheets) could be available on a special page of the CRL website.

*When Jean-Pierre Joly asked NRLs if they agreed to have all the information concerning the Interlaboratory Comparison tests on the CRL website, they approved.*

**Proficiency tests for the detection of mollusc diseases: histology and PCR.**

*Isabelle Arzul*

Community Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade, France

Inter laboratory proficiency tests aim at establishing that the examination or the testing of a given sample lead to the same conclusions in any laboratory within the National Reference Laboratory network. Since 1998, the Community Reference Laboratory (CRL) for Mollusc diseases has organised 6 inter laboratory comparison tests based on the detection of some listed and important pathogens by histology and cytology.

In 2008, the CRL has invited NRLs to participate for the first time in a test based on the use of PCR. The objective of this ring test was to test the ability of NRLs to detect *Bonamia* spp. in flat oyster *Ostrea edulis* by PCR, from the DNA extraction up to the PCR test using reactives and the protocol sent with the samples.

Samples were selected following examination of heart imprints. Samples were sent mid January 2008 and participating laboratories had to return their results before February the 1st. All except one laboratory sent their results in time.

Thirteen laboratories participated in this test and two different sets of samples (T1 and T2)
were used. Each set of samples comprised 30 samples of ethanol fixed pieces of gill from flat oysters *Ostrea edulis* including a positive and a negative controls. Results obtained by each laboratory were compared to the results of the other laboratories which tested the same set of samples. In order to evaluate ability of laboratories to detect the parasite by PCR we determined the most probable status of the samples regarding presence or absence of parasite DNA. Indeed, each oyster was previously checked by tissue imprint to determine its *a priori* status. However, we know that imprints and PCR do not have same sensitivity and same specificity. We thus do not have the true status of each oyster by PCR so we determined its status *a posteriori* by taking the result (+ or -) obtained by the majority of the participating laboratories. Percentages of good responses were good, above 60% for all participating laboratories. General Kappa coefficients (statistical measure of inter-rate reliability) were estimated at 0.49 (moderate agreement) and 0.79 (substantial agreement) for test 1 and test 2 respectively. These values show that both sets of samples were not equivalent and results are not comparable between T1 and T2.

Stein Mortensen asked if it would be possible to have more time to discuss together about the histological slides which are the most difficult to read. Isabelle Arzul agreed about the need to spend time on histological examination but deplored the long time period between test performance by NRLs and the following workshop or annual meeting. Anna Turnbull emphasised the need to spend more time to examine histological slides because of the accreditation requirement and also to improve diagnostic. Bruno Chollet reminded that two years ago “difficult” ring test slides were presented and collectively discussed however it was time consuming. One solution would be to use the system of scanned slides to come back of slides just after the end of the test. Concerning the material that could be sent for the interlaboratory comparison test by PCR: Gary Meyer suggested to send frozen sample, instead of tissues fixed in ethanol. Isabelle Arzul explained that frozen samples need specific sending conditions and do not allow good DNA preservation. Ethanol is thus preferred. David Stone suggested to cut the tissues in several small pieces fixed in ethanol, to mix them and aliquot them before sending them to participants. Concerning the approach used to analyse the results of the comparison test, David Stone was not convinced by the use of consensus results as the good answers because the consensus could be the worst. Isabelle Arzul agreed but also mentioned that the OIE proposes this approach to evaluate results in such situation. David Stone suggested that the CRL repeats PCR tests several times on the same oysters in order to establish the reference results. Anna Turnbull made a parallel with their way of interpreting the reading of slides during a comparison test: slides are observed by three readers and results are a consensus of these three readings. Isabelle Arzul noted that usually it was easier to have a consensus for histology compared to PCR. Isabelle Arzul was surprised that nobody complained about the conclusions given at the end of the letter sent to each NRLs at the end of the interlaboratory comparison test by histology and cytology.

(We consider that:

1) an average of 80% of good responses or plus per collection of slides is good
2) an average of good responses between 60 and 80 % for one collection of slides is good but results obtained during surveillance programme should be taken with caution
3) an average of good responses of 60% or less is not enough and requires additional training.)

Anna Turnbull said that it should depend on the objectives fixed in each laboratory, in some cases it is a percentages of good responses.

Matt Longshaw explained that they adapt the results given by the CRL to their own objectives. Isabelle Arzul added that now the CRL includes more histological slides in the comparison test which allows to be more confident regarding results analysis and which also better reflects the context of routine diagnostic for mollusc disease surveillance.

Matt Longshaw asked if Bonamia spp could be missed in some countries considered free of the parasite. Isabelle Arzul replied that Marteilia spp. is easily detectable and is generally well diagnosed. However, regarding the small size of Bonamia spp., the detection of this parasite is not easy, especially for low level of infection.

Lastly, Hege Hellberg suggested the use of some ranges instead of unique values for some parameters included in the PCR protocol (e.g temperature). Indeed, usually it is easier to use a range of values in accreditation system than precise value.

Isabelle Arzul concluding this discussion by indicating that a new ring test based on histology and cytology should start by mid April 2009 and that in 2010 a ring test based on PCR should be organised for the detection of Marteilia refringens.

CRL activities in 2008 and perspectives for 2009
Isabelle Arzul

In 2008, the NRLs network for mollusc diseases included 22 NRLs from Member States and 3 NRLs from other countries (Norway, Croatia and Turkey). Functions and duties of the CRL for mollusc diseases are given in the Annex VI of the Directive 2006/088/EC.

One of the main aims of the CRL is to harmonize diagnosis within the EU. For this purpose the CRL has created and maintains a collection of pathogens available for laboratories in Member States. A CD-ROM on histology and anatomo-pathology has also been developed since 2002. The CD-ROM proposes illustrations and comments believed to be valuable for mollusc diseases diagnostic, especially diseases notifiable to the EU and OIE. It is subject to permanent reviewing and updating. Last update was done in 2007.

Reference material is sent on request to NRLs and mainly consists of histological slides or paraffin blocks. However, in 2008, some plasmidic DNA suspensions were also distributed as PCR positive controls on demand.

Inter-laboratory proficiency tests are regularly organised to test the ability of laboratories to identify listed and important pathogens by histology. In 2008, the CRL organised for the first time a ring test based on the use of PCR. The aim of this test was to evaluate the ability of NRLs to detect parasites of the genus Bonamia in flat oyster by PCR.

An advisory group in Quality Assurance was created in 2004. Exchange of information between NRLs during annual meetings or by e-mails allowed the CRL to write quality assurance documents that could be used by NRLs wishing to build their Quality System. These Standard Operating Procedures are available on the CRL website (http://www.ifremer.fr/crlmollusc/). Several SOPs were prepared in 2008 including the detection and characterization of parasites of the genera Bonamia and Marteilia by PCR-RFLP.

The CRL assists the NRLs in the diagnosis of disease outbreaks in Member States and provides opportunities of training and retraining through trainees, technical workshop and annual meetings. In 2008, the CRL organised the 4th Microcell working group meeting which
was held in La Tremblade on March 20-21. This meeting included different presentations and discussions about taxonomy, diagnosis, epidemiology and life cycle as well as host-pathogen interactions.

In addition the CRL carries out several studies on listed pathogens. In 2008, works have been done to characterize new genes in the genome of *Bonamia ostreae* and also to characterize *Bonamia* parasites present in Europe. In 2008, the CRL was also involved in studies on the life cycle and host range of *Marteilia refringens* as well as in the characterization of parasites belonging to the genus *Perkinsus* present in France.

A tool to access virtual slides through internet was presented. More particularly, NRLs have access to slides from the interlaboratory comparison test organised by the CRL in 2007 using the following link [http://www.ndpserve.com](http://www.ndpserve.com) (Login: IFREMER1, password: chollet).

*Isabelle Arzul* suggested for the next workshop time for histological slide observation, especially slides which had lead to misdiagnosis and training on real-time PCR. *Basil Raggias* asked if it would be possible to have something else than technique based on the SYBR® Green chemistry (Taqman for example).

**TECHNICAL WORKSHOP : PCR-RFLP FOR THE DETECTION AND TYPING OF MArTIIa Refringens**

*Marteilia refringens* typing.

*Isabelle Arzul*

Community Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade, France

In Europe, marteiliosis due to *Marteilia refringens* is a listed disease affecting flat oysters *Ostrea edulis* and in a lesser concern mussels *Mytilus edulis* and *M. galloprovincialis*. In the past, based on host specificity and ultrastructural criteria, flat oyster parasites were considered as *M. refringens* and mussel parasites were considered as a different species: *M. maurini*.

But, subsequent studies showed that host specificity was not strict and molecular investigations suggested that these two species were co specific and nowadays, flat oysters and mussels are in the list of susceptible species to *Marteilia refringens*.

By histopathology, primary parasites stages are observed in the epithelium of labial palps, stomach and sometimes in gills in oysters and mussels and in case of high infection levels, the parasite induces the destruction of the digestive gland. It is thus not possible to differentiate the parasite in both bivalves by this technique.

Moreover, the subtle differences in haplosporosome size and shape and existence of a multimembranous envelope next to the spore wall in *Marteilia maurini* used to differentiate *M. refringens* and *M. maurini* by TEM later appeared as probable artefacts related to sample treatment (Longshaw et al. 2001).

The 18S sequences are similar in parasites infecting *Ostrea edulis, Mytilus edulis* and *M. galloprovincialis* (Berthe et al. 2000) while ITS-1 sequences are different enough to identify a genetic dimorphism detectable by PCR-RFLP which allowed the characterization of types O (preferably found in oysters) and M (preferably found in mussels (Le Roux et al. 2001)).
The frequent detection of both types in flat oysters and mussels in addition to the characterization of the IGS sequences of both types which were not different enough to conclude that there corresponded to different species led to the present situation which is that both parasites are co-specific and that mussels are susceptible species to *M. refringens*. However, considering the few data available for the genome of this parasite and our poor knowledge of parasite dynamics in both bivalve species, *Marteilia refringens* typing appears relevant when this listed parasite is detected in Europe in flat oysters but also in mussels.

**TECHNICAL WORKSHOP: DETECTION OF MIKROCYTOS MACKINI BY HISTOLOGY**

**Everything you always wanted to know about Mikrocytos mackini.**

*Gary R. Meyer*

Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, BC. Canada

*Mikrocytos mackini*, the causative agent of Mikrocytosis (Denman Island Disease) is a small (2-4 µm diameter) unicellular protistan parasite of unknown taxonomic affiliation. The disease and associated mortality was first reported in Pacific oysters (*Crassostrea gigas*) from Henry Bay on Denman Island in British Columbia, Canada in the early 1960s. Gross signs of the disease occur only during the spring (March through May) and consist of the formation of yellow to green coloured pustules and abscesses (up to 5 mm in diameter) on the surface of the body, mantle, labial palps or within the adductor muscle. This disease significantly reduces the marketability of infected oysters. Four species of oysters (*C. gigas*, *C. virginica*, *Ostrea edulis* and *O. conchaphila* (=*lurida*)) are known to be susceptible to infection with *M. mackini*; however the geographic range of this pathogen remains limited to southern British Columbia and the northern part of Washington, USA. Infections are rarely detected in oysters less than 3 years old and in recent years the overall prevalence of infection in BC has been very low (<5%). Although *M. mackini* is no longer a disease listed by the Office International des Epizooties (OIE, 2006), international concern remains with respect to host specificity and risks associated with the movement of bivalves.

This presentation will encompass the disease signs, geographic distribution, epizootiology, host susceptibility and diagnostic procedures including: tissue imprints, histopathology, electron microscopy, polymerase chain reaction (PCR) and *in situ* hybridisation (ISH). In addition a brief overview of current research activities on *M. mackini* will be provided.
## Annex 2: Reference Material sent by the CRL during 2009

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<th>Date</th>
<th>Type of material</th>
<th>Number of samples</th>
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<th>Pathogens</th>
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<td>Plasmidic DNA</td>
<td>2 DNA suspensions</td>
<td>pCR®II-TOPO vector</td>
<td>M2A-M3AS from <em>M. refringens</em> type M</td>
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<td></td>
<td></td>
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<td><em>Bonamia ostreae</em></td>
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<td>02/02/2009</td>
<td>Paraffin block</td>
<td>One paraffin</td>
<td><em>Ostrea edulis</em></td>
<td><em>Marteilia refringens</em></td>
<td>France</td>
<td>ECOLAG, University of Montpellier, Place Eugene Bataillon, 34095 Montpellier, France</td>
</tr>
<tr>
<td></td>
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<td>block</td>
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<td>05/02/2009</td>
<td>Plasmidic DNA</td>
<td>4 DNA suspensions</td>
<td>pCR®II-TOPO vector</td>
<td>M2A-M3AS from <em>M. refringens</em> type M</td>
<td>Italy</td>
<td>Istituto Zooprofilattico Sperimentale delle Venezie, Via Leonardo Da Vinci, 39 45011 Adria, Italy</td>
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<td>M2A-M3AS from <em>M. refringens</em> type O</td>
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<td>Bo-Boas from <em>B. ostreae</em></td>
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<td>Bo-Boas from <em>B. exitiosa</em></td>
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<td>17/02/2009</td>
<td>Bacterial strain</td>
<td>1 bacterial strain</td>
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<td>of the genus <em>Photobacterium</em></td>
<td>France</td>
<td>LD40, Mont de Marsan, France</td>
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<tr>
<td>27/02/2009</td>
<td>Paraffin block</td>
<td>Two paraffin</td>
<td><em>Ostrea edulis</em></td>
<td><em>Mytilus galloprovincialis</em></td>
<td>France</td>
<td>ECOLAG, University of Montpellier, Place Eugene Bataillon, 34095 Montpellier, France</td>
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<tr>
<td>Date</td>
<td>Type of material</td>
<td>Number of samples</td>
<td>Host species</td>
<td>Pathogens</td>
<td>Country</td>
<td>Recipient</td>
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<tr>
<td>3/05/2009</td>
<td>Histological slides and CDrom</td>
<td>10 histological slides and 1 CDrom</td>
<td><em>Ostrea edulis</em>&lt;br&gt;<em>O. chilensis</em>&lt;br&gt;<em>Crassostrea gigas</em>&lt;br&gt;<em>C. virginica</em>&lt;br&gt;<em>Ruditapes decussatus</em>&lt;br&gt;<em>Haliotis discus hannai</em></td>
<td><em>Marteilia refringens</em>,&lt;br&gt;<em>Bonamia ostreae</em>,&lt;br&gt;<em>B. exitiosa</em>&lt;br&gt;<em>Marteilioides chungmuensis</em>&lt;br&gt;<em>Haplosporidium costale</em>&lt;br&gt;<em>H. nelsoni</em>&lt;br&gt;<em>Mikrocytos mackini</em>,&lt;br&gt;<em>Perkinsus marinus</em>&lt;br&gt;<em>P. olseni</em>,&lt;br&gt;<em>Xenohaliotis californiensis</em></td>
<td>Polynésie française</td>
<td>Service de la perliculture, BP 9047, Motu Uta, 98715 Papeete, Tahiti</td>
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<tr>
<td>23/06/2009</td>
<td>Plasmidic DNA</td>
<td>20 sets of the following material:&lt;br&gt;1 histological slide and 2 DNA suspensions</td>
<td><em>Crassostrea gigas</em>&lt;br&gt;pCR®II-TOPO vector</td>
<td><em>Mikrocytos mackini</em>&lt;br&gt;M2A-M3AS from <em>M. refringens</em> type M&lt;br&gt;M2A-M3AS from <em>M. refringens</em> type O</td>
<td>Participants of the technical workshop</td>
<td>20 European Laboratories</td>
</tr>
<tr>
<td>23/06/2009</td>
<td>Plasmidic DNA</td>
<td>1 DNA suspension</td>
<td>PCR®II-TOPO vector</td>
<td>Internal standard for a PCR for the detection of OsHV-1</td>
<td>Spain</td>
<td>IRTA, Crta. de Poble Nou Km 5,5, 43540-Sant Carles de la Ràpita, Tarragona, Spain</td>
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<tr>
<td>23/06/2009</td>
<td>Paraffin blocks and Plasmidic DNA</td>
<td>2 paraffin blocks and 2 DNA suspensions</td>
<td><em>Ostrea edulis</em>&lt;br&gt;pCR®II-TOPO vector</td>
<td><em>Marteilia refringens</em>&lt;br&gt;M2A-M3AS from <em>M. refringens</em> type M&lt;br&gt;M2A-M3AS from <em>M. refringens</em> type O</td>
<td>Canada</td>
<td>Pacific Biological Station, Aquatic Animal Health Program, 3190 Hammond Bay Road, Nanaimo, BC V9T 6N7, Canada</td>
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<td>26/06/2009</td>
<td>CD Rom</td>
<td>1 CD Rom</td>
<td></td>
<td></td>
<td>Spain</td>
<td>INTECMAR, Conseilleria de Pesca e Asuntos Maritimos, Peirao de Vilaxoàn, Pontevedra, Spain</td>
</tr>
<tr>
<td>Date</td>
<td>Type of material</td>
<td>Number of samples</td>
<td>Host species</td>
<td>Pathogens</td>
<td>Country</td>
<td>Recipient</td>
</tr>
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<tr>
<td>25/09/2009</td>
<td>CDRom, Imprint slides and histological slides</td>
<td>1 CDRom, 5 imprint slides and 6 histological slides</td>
<td><em>Ostrea edulis</em></td>
<td><em>Bonamia ostreae</em> and <em>Martelia refringens</em></td>
<td>Argentina</td>
<td>Laboratory of Parasitology and Histopathology of Bivalve Molluscs LABPAT-IBMP (SENASA LA 0116) Instituto de Biologia marina y pesqueria “Alte Storni” M. Guemes 1030-8520 San Antonio Oeste, Argentina</td>
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<td>01/10/2008</td>
<td>Bacterial strain</td>
<td>1 bacterial strain</td>
<td><em>Vibrio splendidus</em></td>
<td>Strain LGP 32</td>
<td>Ireland</td>
<td>Department of Zoology, Ecology and Plant Science, University College Cork, Enterprise Centre Distillery Fields, North Mall, Cork, Ireland</td>
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<tr>
<td>15/10/2009</td>
<td>CDRom, Histological slides, Imprints slides and Plasmidic DNA</td>
<td>1 CDRom, 9 histological slides, 3 imprint slides and 4 DNA suspensions</td>
<td><em>Crassostrea gigas</em> <em>C. virginica</em> <em>Ostrea edulis</em> <em>Ruditapes decussatus</em> <em>Mytilus edulis</em> <em>Mikrocytos mackini</em> <em>Martelia refringens</em> <em>Bonamia ostreae</em> <em>Marteilioides chungmuensis</em> <em>Perkinsus olseni</em> <em>Haplosporidium nelsoni</em> <em>Perkinsus marinus</em> <em>Healthy oyster</em> <em>pCR®II-TOPO vector</em></td>
<td>M2A-M3AS from <em>M. refringens</em> type M M2A-M3AS from <em>M. refringens</em> type O Bo-Boas from <em>B. ostreae</em> Bo-Boas from <em>B. exitiosa</em></td>
<td>South Korea</td>
<td>National Fisheries Products Quality Inspection Service, Gangneung Branch 318-2, Jumunjin Town, Gangneung City, Gangwon Province, South Korea</td>
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<tr>
<td>Date</td>
<td>Type of Material</td>
<td>Samples Provided</td>
<td>Reference Institution</td>
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<tr>
<td>26/10/2009</td>
<td>Histological slides, Imprints slides and Plasmidic DNA</td>
<td>9 histological slides, 3 imprint slides and 4 DNA suspensions</td>
<td>South Korea National Fisheries Products Quality Inspection Service, 10-4 6 Ga Jungang-Dong Busan City, South Korea.</td>
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<td>26/10/2009</td>
<td>Plasmidic DNA</td>
<td>2 DNA suspensions pCR®II-TOPO vector</td>
<td>Slovenia Institute of Pathology-Forensic and Administrative Veterinary Medicine, Veterinary faculty of Ljubljana, Gerbiceva 60. SI 1000 Ljubljana Slovenia</td>
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<td>27/10/2009</td>
<td>DNA extracted from tissues</td>
<td>7 DNA suspensions Ostrea edulis</td>
<td>Norway National Veterinary Institute Oslo, PO Box 750, Sentrum, 0106 Oslo, Norway</td>
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<td>11/12/2009</td>
<td>Histological slides</td>
<td>4 histological slides Crassostrea gigas C. virginica Ruditapes decussatus</td>
<td>United Kingdom Scotland Marine Laboratory, PO Box 101 Victoria Road, Aberdeen AB11 9DB, Scotland, United Kingdom</td>
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Annex 3: Characterization and analysis performed by CRL in support of other laboratories during 2009

<table>
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<tr>
<th>Date</th>
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<th>Number of samples</th>
<th>Host species</th>
<th>Pathogens</th>
<th>Country</th>
<th>Sender</th>
<th>Performed tests</th>
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<tr>
<td>26/02/2009</td>
<td>Histological slides</td>
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<td><em>Crassostrea gigas</em></td>
<td>Mortality</td>
<td>Morocco</td>
<td>INRH, 2 rue de Tiznit, Casablanca, Morocco</td>
<td>Histology</td>
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<td>18/03/2009</td>
<td>Paraffin blocks, ethanol fixed tissue</td>
<td>9 Paraffin blocks, 7 ethanol fixed tissues</td>
<td><em>Ostrea edulis</em></td>
<td>Bonamia ostreae</td>
<td>Norway</td>
<td>National Veterinary Institute of Bergen, PO Box 1263, Sentrumi, N5811 Bergen, Norway</td>
<td><em>In situ</em> hybridization, PCR, Real Time PCR and sequencing</td>
</tr>
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<td>23/03/2009</td>
<td>Paraffin block</td>
<td>1</td>
<td><em>Crassostrea gigas</em></td>
<td>Marteilia sp.</td>
<td>Brasil</td>
<td>Universidade Federal de Sergipe, Centro de Ciencias Biologicas e da Saude, Aracaju, Brasil</td>
<td>Histology, <em>in situ</em> hybridization and PCR tests</td>
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<tr>
<td>30/04/2009</td>
<td>Paraffin blocks, histological slides and ethanol fixed tissue</td>
<td>5 blocks, 9 slides and no countable ethanol fixed pieces of tissue</td>
<td><em>Ostrea edulis</em></td>
<td>Bonamia exitiosa</td>
<td>Turkey</td>
<td>Bornova Veterinary control and research institute Erzene Mah Ankara Cad N° 172/155, 35010 Bornova-Izmir-Turkey</td>
<td>Histology, <em>in situ</em> hybridization, PCR-RFLP and sequencing</td>
</tr>
<tr>
<td>02/06/2009</td>
<td>Paraffin blocks and histological slides</td>
<td>18 blocks and 18 slides</td>
<td><em>Ostrea edulis</em></td>
<td>Bonamia sp?</td>
<td>Russian Federation</td>
<td>107140 VNIRO, 17, V. Kranoselskaya, Moscow, Russian Federation</td>
<td>Histology, <em>in situ</em> hybridization and PCR tests</td>
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<td>03/09/2009</td>
<td>DNA suspensions</td>
<td>103</td>
<td><em>Ostrea stentina</em></td>
<td><em>Bonamia exitiosa</em></td>
<td>Tunisia</td>
<td>Institut National Agronomique de Tunisie, Departement des ressources animales, halieutiques et des technologies alimentaires, Tunis, Tunisie</td>
<td>PCR-RFLPモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキモンキMonteatsu*</td>
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<td>27/11/2009</td>
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<td>6</td>
<td><em>Ruditapes decussatus</em></td>
<td>Double reading</td>
<td>Tunisia</td>
<td>INSTM 20025 Salammbò Tunisia</td>
<td>Histology</td>
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### Annex 4: Training and scientific collaboration in 2009

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<th>Dates</th>
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<th>Name</th>
<th>Institute</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-16/10/ 2009</td>
<td>South Korea</td>
<td>Gwang-Jin Choi,</td>
<td>National Fisheries Products Quality Inspection Service, Gangneung City</td>
</tr>
<tr>
<td></td>
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<td>(1) detecting pathogens of mollusc by histology especially those listed in the OIE aquatic Code (2) detecting and characterizing <em>Bonamia</em> sp. by PCR-RFLP (3) detecting and typing <em>Marteilia refringens</em> by PCR-RFLP (4) detecting <em>Bonamia</em> sp. by <em>in situ</em> hybridization (5) detecting <em>Perkinsus</em> sp. by thioglycolate medium culture</td>
</tr>
<tr>
<td>5-16/10/ 2009</td>
<td>South Korea</td>
<td>Sung Myoung Hee</td>
<td>National Fisheries Products Quality Inspection Service, Busan City</td>
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<td>(1) detecting pathogens of mollusc by histology especially those listed in the OIE aquatic Code (2) detecting and characterizing <em>Bonamia</em> sp. by PCR-RFLP (3) detecting and typing <em>Marteilia refringens</em> by PCR-RFLP (4) detecting <em>Bonamia</em> sp. by <em>in situ</em> hybridization (5) detecting <em>Perkinsus</em> sp. by thioglycolate medium culture</td>
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Annex 5: Publications relevant to the work of the CRL.


Morga B., Arzul I., Chollet B. & T. Renault, 2009. Infection with the protozoan parasite Bonamia ostreae modifies in vitro haemocyte activities of flat oyster Ostrea edulis. Fish & Shellfish Immunology 26: 836-842


Annex 6: Presentations at international conferences and meetings


Arzul I., Robert M., Chollet B., Haffner P., Garcia C. Validation of diagnostic techniques: example of the pcr for *bonamia ostreae* detection. 14th EAFP International Conference Prague, 14-19 September 2009

Arzul I., Robert M., Garcia C., Chollet B., Langlade A. Can *Bonamia ostreae* infect larvae of flat oysters *Ostrea edulis*? 14th EAFP International Conference Prague, 14-19 September 2009


Boyer S., Arzul I., Bonnet D. A new comer in the Thau lagoon (South of France): *Paracartia grani* (Copepoda, calanoida) population dynamics and role in the ecosystem. 4ème Congrès européen sur les Lagunes Côtières, Montpellier, France, 14-18 December 2009


