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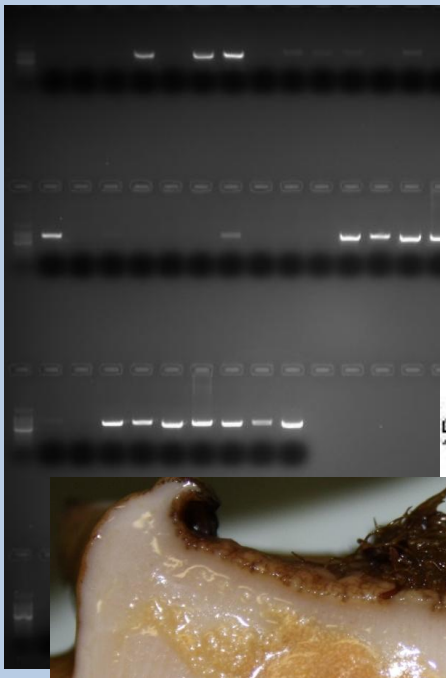
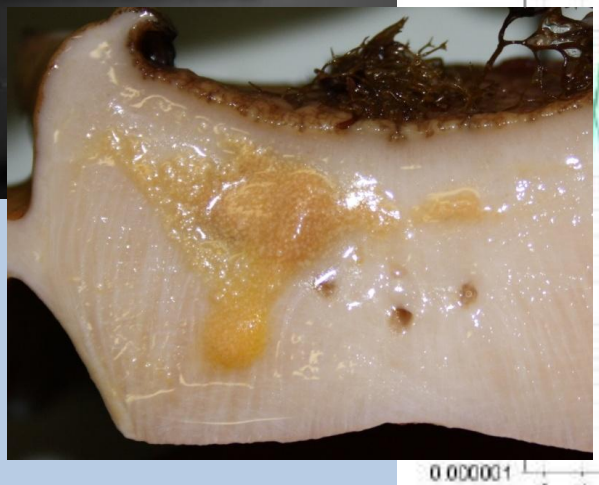
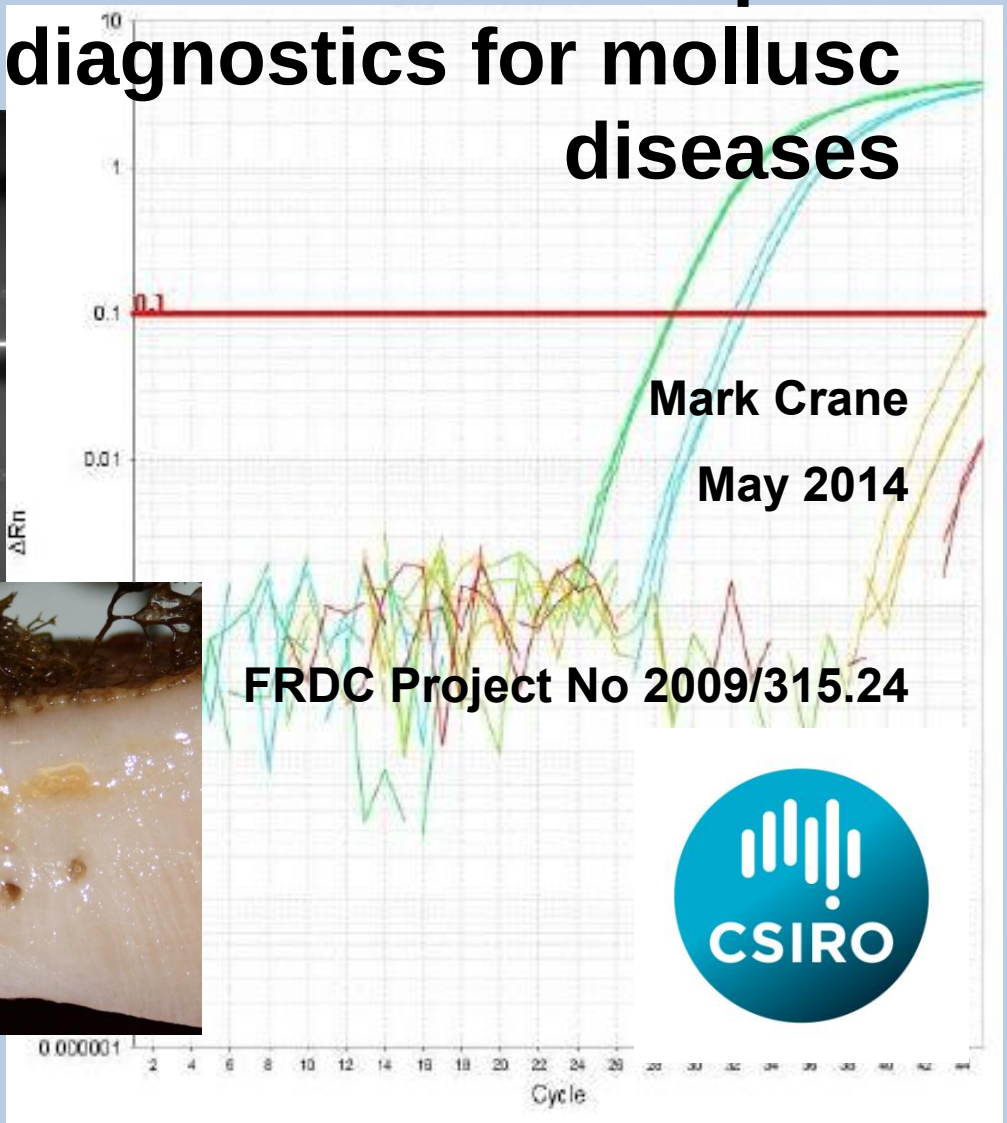
People development program: Aquatic animal health training scheme – KBBE workshop on diagnostics for mollusc diseases







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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Acknowledgments

The significant contributions by the workshop participants through preparation and presentation of comprehensive information concerning mollusc diseases within their respective countries are greatly appreciated as is the open and frank discussions that were held during the workshop. Without this level of collaboration the workshop outputs and value would have been severely limited.

The workshop organisers also thank FRDC for providing financial support that permitted the workshop to be hosted in Geelong.

Dr Ingo Ernst, as workshop facilitator, is also thanked for ensuring the workshop objectives were achieved and for his insightful contributions to the workshop.

The workshop organisers greatly appreciated Great Southern Waters abalone farm for opening their doors to the workshop participants, for the informative presentations and tour of the farm provided by GSW staff.

Abbreviations

AAHL	Australian Animal Health Laboratory
AU	Australia
CSIRO	Commonwealth Scientific and Industrial Research Organisation
EAFP	European Association of Fish Pathologists
EU	European Union
FRDC	Fisheries Research and Development Corporation
GSW	Great Southern Waters Pty Ltd, Indented Head, Victoria
KBBE	Knowledge-Based Bio-Economy
NZ	New Zealand
OIE	Office International des Epizooties - the World Organisation for Animal Health
OsHV	Ostreid herpesvirus
OsHV-1	Ostreid herpesvirus Type 1
SCAAH	Sub-committee of Aquatic Animal Health – a sub-committee of the Federal Government's Animal Health Committee

Executive Summary

What the report is about

Following on from the 2012 Knowledge-Based Bio-Economy (KBBE) workshop on “Disease mitigation and prevention in mollusc aquaculture” [Nantes, France, 11-15 June 2012] and addressing a recommendation from that workshop, the 2013 international KBBE workshop on “Mollusc Disease Diagnosis” was organised by CSIRO AAHL Fish Diseases Laboratory and took place in Geelong on 22-24 October 2013. The workshop provided a forum for international experts on mollusc diseases to agree on priorities and recommendations for future research on those diseases of common interest to the participating countries. This report details the outputs from the international workshop.

Background

The European Union, New Zealand, Canada and Australia are participants in the International Knowledge-Based Bio-Economy (KBBE) Forum, a cooperation initiative launched in September 2010. Four work streams have been identified:

- Bio-refineries & Bio-based materials (led by Canada in 2010-2011)
- Food & Health (led by Australia in 2010-2011)
- Fisheries & Aquaculture (led by EU in 2010-2011)
- Sustainable agriculture (led by New Zealand in 2010-2011)

For information on KBBE see http://ec.europa.eu/research/bioeconomy/international-cooperation/forum/index_en.htm.

Within the Fisheries and Aquaculture work stream, KBBE identified mollusc aquaculture as a high priority area and an international workshop was held in 2012 on "Disease Mitigation and Prevention in Mollusc Aquaculture", Nantes, France. At that workshop it was recommended that another international workshop on “Mollusc Disease Diagnosis” be held in 2013. Apart from promoting international collaboration, hosting this workshop in Australia was viewed as an excellent opportunity to promote Australia’s capacity/capability in mollusc disease diagnosis internationally and in particular to colleagues from our international trading partners. The workshop was well-attended with 10 participants from the European Union (EU), 5 from New Zealand (NZ) and 12 from Australia (AU) including several members of the Sub-committee for Aquatic Animal Health.

Aims/objectives

1. Plan and organise an international workshop "Diagnostics for Mollusc Diseases"

2. Host an international workshop "Diagnostics for Mollusc Diseases"

3. Submit a report to FRDC and KBBE Forum on the international workshop "Diagnostics for Mollusc Diseases"

Methodology

In a workshop format, invited mollusc disease experts from KBBE participant countries exchanged information through MS PowerPoint presentations and discussion on mollusc disease priorities, diagnostic capabilities and mollusc disease diagnostic problems specific to their respective countries

Results/key findings

During the course of the workshop four priority disease agents were identified (*Bonamia*, *Perkinsus, Mikrocytos* and OsHV-1) that were of common interest to the participating countries. In addition, to address knowledge gaps, short-, medium- and long-term research activities were identified, some of which were common to all disease agents while others were disease-specific. Many of the short-term activities do not require extra resources and can be initiated immediately. The medium- and long-term activities, however, do require further resources, and extra funding will need to be sourced.

Implications for relevant stakeholders

While research activities have been identified, specific research projects need to be developed, and applications for funding submitted to appropriate agencies. Development of these projects requires leaders/coordinators from each jurisdiction (EU/AU/NZ). Research on mollusc diseases has been included in the Work Programme 2014-2015 of the recently announced new EU framework programme for research and innovation (Horizon 2020; see: http://ec.europa.eu/programmes/horizon2020/en/), that will run from 2014 to 2020. While provision has been made to facilitate engagement between EU, AU and NZ KBBE bio-triangle participants, AU and NZ are expected to contribute resources to the research programme through collaborative projects.

Recommendations

Specific recommendations included ensuring that funds are available to permit on-going international KBBE workshop/meetings to facilitate collaborative research on the priority mollusc diseases. For example, travel to the 2015 European Association of Fish Pathologists (EAFP) Conference with the aim to (1) contribute (chair and present) to a special session on OsHV-1 and (2) participate in 2015 KBBE workshop to discuss progress on research undertaken on each of the priority agents (*Bonamia*, *Mikrocytos*, *Perkinsus* and OsHV-1).

Keywords

Mollusc disease; diagnosis; *Bonamia*; *Microcytos*; *Perkinsus*; OsHV-1

Introduction

In recent years several mollusc diseases (e.g. Perkinsosis, Bonamiasis, abalone viral ganglioneuritis, oyster oedema disease, ostreid herpes viral disease) have impacted, and continue to impact, Australian fisheries and aquaculture. Similar diseases also affect mollusc aquaculture overseas, e.g. Bonamiasis of oysters in Europe, ostreid herpes viral disease in Europe and New Zealand. The outcomes of past and present efforts to manage diseases in farmed molluscs have been poor, and few controls have been devised for disease outbreaks in wild populations. Thus these diseases have continued to spread partly due to poor international coordination of response to emerging diseases, lack of sensitive and standardised diagnostic tests, no understanding of whether these diseases arose separately in different countries or whether they have spread internationally, poor understanding of mollusc immunity, poor understanding of pathogen biology in the context of host and environmental change, little information about how the infectious agents are spread within populations, lack of coordinated research on improved measures to avoid disease, inability to predict where and when future threats will arise. These factors suggest a need for an internationally based and coordinated multidisciplinary research approach to ensure the sustainability of mollusc aquaculture and wild mollusc populations. This need was recognised internationally and led the 'International Knowledge-Based Bio-Economy (KBBE) Forum' (a new cooperation initiative between the European Commission, Australia, Canada and New Zealand launched in September 2010) to organise the 2012 KBBE Forum workshop on "Disease Mitigation and Prevention in Mollusc Aquaculture".

At the KBBE Forum workshop on "Disease Mitigation and Prevention in Mollusc Aquaculture", held in Nantes, France, June 2012, harmonisation of diagnostic methods and implementation of new diagnostic methodologies for mollusc diseases were identified as scientific priorities. One recommendation from the workshop was to hold a future KBBE workshop to address issues concerning mollusc disease diagnostics. Australia was identified as the potential workshop host pending funding approval. In consultation with FRDC and DAFF, the Aquatic Animal Health Training Scheme was identified as an appropriate source of funding to off-set the costs associated with running the workshop since it would align with several key research areas within the FRDC R&D Plan and be of interest to industry and regulators.

Objectives

1. Plan and organise an international workshop "Diagnostics for Mollusc Diseases"
2. Host an international workshop "Diagnostics for Mollusc Diseases"
3. Submit a report to FRDC and KBBE Forum on the international workshop "Diagnostics for Mollusc Diseases"

Method

Objective 1: Plan and organise an international workshop "Diagnostics for Mollusc Diseases"
Through the KBBE Forum, invitations to the workshop were forwarded to representatives of participating countries (Australia, New Zealand, Canada and countries of the EU). The invitations provided information on the dates, venues and activities of the workshop. In addition, a notice of the workshop was provided to the Sub-committee of Aquatic Animal Health (SCAAH) and SCAAH representatives were invited to nominate participants from their jurisdictions.

Objective 2: Host an international workshop "Diagnostics for Mollusc Diseases"
The workshop was hosted over a 3-day period in October 2013 by the AAHL Fish Diseases Laboratory, CSIRO Animal, Food and Health Sciences, Geelong, Victoria.

Days 1 and 2 involved presentations from each participating laboratory/country on:

- Infectious diseases of oysters, mussels, abalone, other molluscs and current diagnostic methods
- Laboratory networks
- Proficiency Tests
- Quality Assurance
- Test validation
- R&D towards improved disease diagnosis
- Diagnostic gaps: For established diseases and for emerging diseases – strategy for filling the gaps
- Potential lead agencies and collaborating laboratories
- Regulatory issues (EU, Canada, NZ, Australia)
- Discussion and conclusion

Day 3 involved completion of actions from Days 1 and 2 followed by study tours. There were two tour options (1) Great Southern Waters (GSW) abalone farm (2) AAHL bio-secure facility. It was not possible for all workshop participants to go on both tours (there are restrictions on visitors to the AAHL bio-secure facility, for example, visitors cannot go on to farms, including aquatic animal farms and hatcheries, for 7 days after entering the AAHL bio-secure facility).

Objective 3: Submit a report to FRDC and KBBE Forum on the international workshop "Diagnostics for Mollusc Diseases"
A draft report on the international workshop "Diagnostics for Mollusc Diseases" was prepared by the workshop leaders and forwarded to workshop participants for review and comment. The report detailed the workshop activities including any recommendations for further action. Following review and feedback from workshop participants the report was finalised and submitted to FRDC, the KBBE Forum and distributed to all workshop participants, all SCAAH members and the OIE Aquatic Animals Health Standards Commission.

Results Discussion & Conclusion

The project progressed as planned with the KBBE Workshop on “Mollusc Diseases Diagnosis” being held in Geelong on 21-24 October 2013. There were 10 participants from EU, 12 participants from Australia (including SCAAH representatives) and 5 from New Zealand. Unfortunately, Victoria State was unable to participate due to earlier commitments. Queensland and Northern Territory did not send representatives - mollusc diseases are not a high priority for these jurisdictions.

The workshop was facilitated and led by Dr Ingo Ernst, Director – Aquatic Animal Health, Australian Government Department of Agriculture, and Dr Mark Crane, Senior Principal Research Scientist, Research Team Leader, Project Leader - AAHL Fish Diseases Laboratory, CSIRO Animal, Food and Health Sciences.

Following the workshop a draft Workshop Report was prepared and distributed to participants for comment. On receipt of feedback from participants a final report was produced and distributed to workshop participants, KBBE, FRDC and CSIRO Marine and Atmospheric Research (see Appendix 1).

During the course of the workshop four priority disease agents were identified (*Bonamia*, *Perkinsus, Mikrocytos* and OsHV-1) that were of common interest to the bio-triangle participants. In addition, to address knowledge gaps, short-, medium- and long-term research activities were identified, some of which were common to the disease agents while others were specific. Many of the short-term activities do not require extra resources and can be initiated immediately. The medium- and long-term activities, however, do require further resources, and extra funding will need to be sourced.

Implications

This workshop has fulfilled a recommendation of the 2012 KBBE workshop on "Disease Mitigation and Prevention in Mollusc Aquaculture". The report from the 2013 KBBE workshop on “Mollusc Disease Diagnosis” provides the basis for planning of future collaborative research projects on pathogens of molluscs leading to harmonisation of diagnostic methods and implementation of new diagnostic methodologies for mollusc diseases, such as those that may be considered as part of the new EU framework programme for research and innovation (Horizon 2020; see: http://ec.europa.eu/programmes/horizon2020/en/), that will run from 2014 to 2020.

Moreover, the workshop provided a forum for leading international diagnostic and research laboratories to discuss issues of common interest facing the mollusc aquaculture sector globally. There was agreement on the priority diseases/agents and issues that require addressing by this international forum. A high level of trust between the workshop delegates developed over the duration of the workshop, and those recommendations (e.g. exchange of information on diagnostic methods) that do not require extra resources are already being implemented.

In addition, participants agreed that a letter should be forwarded to the OIE Aquatic Animals Health Standards Commission concerning the diagnostic methods for mollusc diseases that are presented in the OIE Manual of Diagnostic Tests for Aquatic Animals. Participants considered that there are some areas (test validation, rating of tests against purpose of use, chapters on Infection with *Perkinsus marinus* and Infection with *Perkinsus olseni*) in which the manual could be improved and agreed that this information should be provided (by Dr Ingo Ernst) to the OIE.

Thus, this workshop was very successful and provided excellent value for the relatively small investment. International collaborations have been established as a result, recommendations have been made to the OIE, and it is likely that further collaborations of benefit to Australia will develop.

Recommendations

Specific recommendations included ensuring that funds are available to permit on-going international KBBE workshop/meetings to facilitate collaborative research on the priority mollusc diseases. For example, travel to the 2015 EAFP Conference with the aim to (1) contribute (chair and present) to a special session on OsHV-1 and (2) participate in 2015 KBBE workshop to discuss progress on research undertaken on each of the priority agents (*Bonamia*, *Mikrocytos*, *Perkinsus* and OsHV-1).

Further development

While research activities have been identified, specific research projects need to be developed, and applications for funding submitted to appropriate agencies. Development of these projects requires leaders/coordinators from each jurisdiction (EU/AU/NZ). Research on mollusc diseases has been included in the WP2015 of the recently announced new EU framework programme for research and innovation (Horizon 2020; see: http://ec.europa.eu/programmes/horizon2020/en/), that will run from 2014 to 2020. While provision has been made to facilitate engagement between EU, AU and NZ KBBE bio-triangle participants, AU and NZ are expected to contribute resources to the research programme through collaborative projects.

There was not sufficient time for the workshop to undertake prioritisation of mid- and long-term research activities or to develop a roadmap for 2014 onwards, nevertheless it was recognised that this is an important next step and needs to occur in concert with, and complementing, Horizon 2020 programme.

Extension and Adoption

The workshop report (Appendix 2) has been made available to KBBE, OIE, CSIRO Marine and Atmospheric Research, SCAAH and all participating jurisdictions to be used for future planning.

Appendices

Appendix 1. List of researchers and project staff

Appendix 2. Workshop Report

Appendix 1. Participant list

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Appendix 2. Workshop Report

International Knowledge-Based Bio-Economy (KBBE) Forum

Report from the Workshop on "Mollusc Disease Diagnosis"

Geelong, Australia, 21-24 October 2013

A collaborative initiative between the EU, Australia and New Zealand

Acknowledgements

This workshop was conducted as part of the Fisheries Research Development Corporation (FRDC) project (2009/315.24), People Development Program: Aquatic animal health training scheme – KBBE workshop on diagnostics for mollusc diseases which was partially funded by the FRDC on behalf of the Australian government.

Summary

Following on from the 2012 KBBE workshop on "Disease mitigation and prevention in mollusc aquaculture" [Nantes, France, 11-15 June 2012] and addressing a recommendation from that workshop, a further international KBBE workshop on "Mollusc Disease Diagnosis" was organised and held in Geelong, Australia, 21-24 October 2013. The workshop was well-attended with 10 participants from the European Union (EU), 5 from New Zealand (NZ) and 12 from Australia (AU).

The workshop was successful in achieving its aims which were:

- To exchange information to determine mollusc disease priorities and diagnostic capabilities of participating countries.
- To identify mollusc disease diagnostic problems.
- To identify research strengths and opportunities for collaboration to address diagnostic problems.

During the course of the workshop four priority disease agents were identified (*Bonamia*, *Perkinsus, Mikrocytos* and OsHV-1) that were of common interest to the bio-triangle participants. In addition, to address knowledge gaps, short-, medium- and long-term research activities were identified, some of which were common to the disease agents while others were specific. Many of the short-term activities do not require extra resources and can be initiated immediately. The medium- and long-term activities, however, do require further resources, and extra funding will need to be sourced.

Specific recommendations included ensuring that funds are available to permit on-going international KBBE workshop/meetings to facilitate collaborative research on the priority mollusc diseases. For example, travel to the 2015 EAFP Conference with the aim to (1) contribute (chair and present) to a special session on OsHV-1 and (2) participate in 2015 KBBE workshop to discuss progress on research undertaken on each of the priority agents (*Bonamia*, *Mikrocytos*, *Perkinsus* and OsHV-1).

While research activities have been identified, specific research projects need to be developed, and applications for funding submitted to appropriate agencies. Development of these projects requires leaders/coordinators from each jurisdiction (EU/AU/NZ). Research on mollusc diseases has been included in the WP2015 of the recently announced new EU framework programme for research and innovation (Horizon 2020; see: http://ec.europa.eu/programmes/horizon2020/en/), that will run from 2014 to 2020. While provision has been made to facilitate engagement between EU, AU and NZ KBBE bio-triangle participants, AU and NZ are expected to contribute resources to the research programme through collaborative projects.

There was not sufficient time for the workshop to undertake prioritisation of mid- and long-term research activities or to develop a roadmap for 2014 onwards, nevertheless it was recognised that this is an important next step and needs to occur in concert with, and complementing, Horizon 2020 programme.

1. Background

In 2012, as part of the International Knowledge-Based Bio-Economy (KBBE) Forum priority on mollusc aquaculture (within KBBE Fisheries & Aquaculture work stream), an international workshop was organised on "Disease mitigation and prevention in mollusc aquaculture" [Nantes, France, 11-15 June 2012]. The workshop was attended by 31 participants from the European Union (21), New Zealand (5) and Australia (5) and provided a forum where mollusc production and diseases in EU, NZ and AU were discussed together with current research activities, knowledge gaps, research priorities, opportunities for international cooperation, and research funding. Two proposed actions were recommended from the 2012 Nantes workshop:

1) Elaboration and launching of a joint research initiative on mollusc diseases, with emphasis on Oyster Herpes Virus OsHV-1

1a) (short term) To draft a common text for a call for proposals to be endorsed by the KBBE Forum and funded by the respective governments of AU/NZ/CA and the EU. Based on the analysis of the priorities identified by Australian, New Zealand and European Nantes workshop participants, three main subjects were found suitable for such a call:

- Investigation of genetic diversity of OsHV-1 and related viruses in order to better understand virus spread, pathogenicity and key drivers of virus emergence (in different parts of the world) including effects of global change.
- Investigation of genetic basis of bivalve resistance/tolerance to OsHV-1 and related viruses in order to define effective anti-viral defence mechanisms, to develop programs on genetic selection of shellfish strains resistant/tolerant to these viruses and to study the resistance of selected animals to other pathogens including bacteria and parasites.
- Developing a network between Australia, New Zealand, Canada (CA) and EU in order to share information about oyster mortality events related to OsHV-1 and its different genotypes.

1b) (medium term): Launching of the call AU/NZ/EU (and maybe CA?) (Summer, 2013). Instruments aiming at ensuring efficient clustering between the projects that will be selected in AU, NZ, AU, (CA?) should also be foreseen in the call.

1c) (medium term) Submission – evaluation – selection of proposals and implementation of the parallel research initiatives (projects in AU/NZ/EU/CA) (2014).

2) Workshop on diagnostics of mollusc diseases (Australia, 2013)

The aim of the workshop will be to share experiences and best practices, benchmark existing diagnostic methods and tools and pave the way to the standardisation of diagnostic approaches.

Thus to address action 2, an international KBBE workshop on "Mollusc Disease Diagnosis" was organised and held in Geelong, Australia, 21-24 October 2013. The workshop was attended by 27 participants from EU (10), NZ (5) and AU (12), including a workshop facilitator.

2. Workshop objectives

- To exchange information to determine mollusc disease priorities and diagnostic capabilities of participating countries.
- To identify mollusc disease diagnostic problems.
- To identify research strengths and opportunities for collaboration to address diagnostic problems.

3. Report on sessions

For each session there were a number of presentations by representatives from EU, NZ and AU providing information on each of the workshop topics leading towards achievement of the workshop objectives. The presentations are provided in Annex 4.

3.1 Session 1: Significant diseases of molluscs and their diagnosis. Identify the high priority diseases, the status of diagnostic tests and the stage of validation.

3.1.1 Mollusc diseases of significance in EU and their diagnosis: Presented by Isabelle Arzul on behalf of EU (Presentation 01).

Summary:

- Mollusc production was detailed: In summary, while production varies in each country, mussels and oysters are the most important sectors, by value (others include clams (see Italy, Portugal), cockles (see Spain, Portugal, UK), scallops (see UK), abalone).
- Regulatory processes were summarised:
  - EU listed pathogens (Directive 2006/088/EC)
  - Regulatory process
  - Emerging diseases (Article 41 of 2006/088/EC – regarding emerging diseases)
  - Diagnostic approach:
  - OIE Manual:
    - SOPs (EURL Website)
    - Free status
  - Mortality investigations
  - Confirmatory testing
  - Histology used widely with ISH
- Significant disease agents included *Bonamia, Marteilia, Perkinsus, Microcytos* spp., OsHV-1 µvar, *Vibrio* spp.
- The main issues discussed were:
  - Geographical range of some pathogens is changing
  - Pathogen characterisation required
  - Role of OsHV-1 µvar/*Vibrio* co-infections in disease/mortality
  - Pathogen species identification
  - Diagnostic test performance characteristics are often lacking

Discussion post-presentation

- Mortality monitoring discussed with some disagreement about approach and so deferred for further discussions until later in the workshop. There was some discussion about the need for better industry-friendly interaction with the industries that we report to. It was suggested that there should be more emphasis on practical application of knowledge from diagnostics and research to provide producers with information on, for example, likelihood and timing of disease outbreaks.
- Other issues discussed included the fact that there are two species of mussels but considered together despite the likelihood that they have different disease susceptibilities.
- The geographical, temperature, water quality (environmental) influences on disease were also discussed as well as the differences within and problems with the whole *Bonamia* spp group.
- The importance of using a range of diagnostic tools for reaching causality diagnosis was mentioned.

3.1.2 Mollusc diseases of significance in New Zealand and their diagnosis: Presented by Andrea Alfaro on behalf of NZ (Presentation 02).

Summary:
The strengths of AUT (multi-disciplinary approach to R&D; metabolomics application to disease investigation) were covered followed by the importance of seafood (e.g. scallops, mussels, paua, clams, cockles, geoduck) as a major export industry for NZ.

- Cawthron Report No. 1334 (Jan. 2013) provided a list of significant pathogens. Top ten:
  - OsHV-1
  - *Bonamia*
  - Apicomplexan protozoan APX (mussel/flat oyster)
  - *Perkinsus*
  - Digestive epithelial virosis DEV (mussel/scallop/oyster)
  - *Haplosporidium* sp. (paua)
  - Flat worms (Pacific oyster/mussel)
  - Shell mycosis (paua)
  - Scallop mycoplasma
  - *Rickettsia* (oysters/scallops)
- Main issues included:
  - *Perkinsus olseni* (NZ geographical strain transmission/pathogenicity in paua)
  - DEV purification, characterisation and sequence analysis
  - APX
  - Identification of scallop inclusions (tentative vermiform gregarine)
  - Mussel aquabirnavirus characterisation

3.1.3 Mollusc diseases of significance in Australia and their diagnosis: Presented by Serge Corbeil on behalf of Australia (Presentation 03).

Summary:
The presentation was divided into four sections.

- Significant diseases:
  - Oyster oedema disease (OOD)
  - Abalone viral ganglioneuritis
  - OsHV-1
  - *Perkinsus*
  - *Bonamia* spp.
  - QX disease (*Marteilia sydneyi*)
- Diagnostic strategy:
  - State diagnostic laboratories will use histology as a front-line test. Suspect disease followed up with more specific tests.
  - Emerging or exotic disease cases submitted to AAHL:
    - Preliminary screening by qPCR (if available)
    - Confirmation by conventional PCR and sequencing
- Diagnostic tests:
  - Abalone viral ganglioneuritis: See OIE Manual for tests
  - OsHV-1: Martenot and EMAI qPCR and C2/C6 conventional PCR
  - QX: cPCR Leg1-Pro2 (Kleeman et al., 2000, 2002)
  - *Bonamia*: cPCR Bo-Boas RFLP; qPCR TaqMan Bon ITS-Taq-F/R
  - *Perkinsus*: cPCR (Perk-ITS 85F / 750R) (OIE); *P. olseni*-specific primers PolsITS-140F / 600R (OIE); qPCR (AAHL) (Taq 108 For / 171 Rev) (genus-specific); qPCR (AAHL) (species-specific in development)

- OOD: Research, funded by FRDC, on agent identification is on-going with the aim to develop diagnostic tests.

- Priority issues:
  - Characterisation of *Bonamia* spp. in Australia
  - Characterisation of *Perkinsus* spp. in Australia
  - Identification of OOD pathogen
  - Characterisation of OsHV-1 in Australia
  - Characterisation of AbHV genotypes in Australia

3.1.4 Discussion

Following the completion of the presentations in this session it was clear that there were some common themes coming through, in particular, diseases/agents in common which included:

- *Bonamia*
- *Perkinsus*
- OsHV-1

3.2 Session 2: Diseases of molluscs - Identification of diagnostic problems. For example, which diseases and/or what diagnostic tests are problematic. What are the specific problems, e.g., lack of sensitivity, lack of specificity, lack of validation data? Are there any new diseases for which diagnostics are not well-developed?

3.2.1 Diseases of molluscs: Identification of diagnostic problems in EU: Presented by Beatriz Novoa on behalf of EU (Presentation 04).

Summary:

- Firstly, introduced the NRL – Spain, and capabilities/research interests:
  - Fish immunology response Zebrafish genomic tools
  - Molluscs antimicrobial responses: peptides and myticins
  - NGS and genomics
  - Zebrafish Biomedicine
  - Research in biotechnology
  - Galicia zone for cultured mussels
- Within the EU the key diagnostic problems related to the following:
  1. Validation and comparison of diagnostic techniques
  2. Dealing with disease outbreaks with novel pathogens
  3. Occurrence of different results with different diagnostic approaches
  4. *Vibrio* species discrimination
  5. Adequate networking to deal with diagnostic problems

It was recognised that the European Reference Laboratory (EURL) has responsibilities specifically related to dealing with 1, 2, 3 & 5 above and continues to address these as part of their ongoing activities.

Validation and comparison of diagnostic techniques

The benefits of using histology were outlined, namely the technique's ability to discern several pathogen groups (including co-infections), pathogenicity and the presence of toxico-pathic or environmentally induced lesions. It is considered the most important tool used by pathologists (but requires trained/experienced histopathologists): The technique provides the overall aspect of tissue response to an infecting pathogen. The importance of relating histological changes to results from tissue imprints (cytology), *in situ* hybridisation and molecular tools (PCR and qPCR) was stressed

(histological changes are not necessarily specific and not sufficiently sensitive to detect sub-clinical infections reliably). It was noted that many molecular detection methods were developed for research rather than diagnostic purposes. For OsHV-1, there is a lack of validation for most techniques.

Dealing with disease outbreaks with novel pathogens
The example of the abalone *Haliotis tuberculata* was used to illustrate the situation where multiple infections may be present and there is the need to apply a suite of techniques. However, it was noted that no cell lines for molluscan virology are available and that Koch's postulates are rarely fulfilled.

Occurrence of different results with different diagnostic approaches
Not all standard techniques are available for all pathogens (e.g. no qPCR for *Marteilia refringens*). In addition, there remains the need for standardisation of:

- Tissue selection
- Conditions for tissue storage
- DNA extraction and the need for internal controls
- Choice of extraction methods and equipment (e.g. thermocyclers)

Recognition that many problems occur when DNA levels are low and that there is a need to decide on a 'cut-off point' for very low levels where reproducibility of results may be variable. With co-infections, for example OsHV-1 with *Vibrio aesturanius*, the role of the bacteria is unclear since its presence may not be associated with mortalities.

Networking
Improvements in interpretation of histological sections may be realised through use of scanned slides accessible to participating laboratories for agreement.

3.2.2 Diseases of molluscs: Identification of diagnostic problems in New Zealand: Presented by Steve Webb on behalf of NZ (Presentation 05).

Summary:
In New Zealand there is investigation of mortalities, routine monitoring and testing for translocation but to date there is less emphasis on management of disease issues. In addition, there is a stringent requirement for cost-effectiveness since the industry pays. A range of diagnostic techniques (gross examinations especially for paua; tissue smears including heart imprints and haemolymph sampling; histopathology; PCR and ISH; RFTM for *Perkinsus*) are employed, with histology playing a key role (with the use of several staining techniques for visualisation of pathogens and host response).

General issues/problems identified in disease diagnosis are that (1) tests must be affordable and appropriate (e.g. for translocation – needs to be quick and cheap) but (2) as to be expected with histopathology there may be issues with sensitivity and specificity. This is exemplified in distinguishing microcells. Although only *Bonamia exitiosa* is reported extant in NZ (and may occur at very low intensities), other microcells may present a similar appearance and escape specific identification or even detection. (3) A further deficiency is a lack of species-level tests for Vibrios. Finally (4) we need to know more about the basic biology and lifecycle of the host and response to environmental stress so that natural fluctuations in the histological picture can be accounted for when assessing the presence and effects of pathogenic conditions.

Benefits of histological techniques:

- Broad spectrum and inexpensive (cf. other tests, such as PCR and ISH, may require the investigator to know or at least suspect the identity of the target).
- Can give a general health indication as well as path ID, although sensitivity/specificity can vary greatly.
- Permanent record
- Several host animals can be examined on one slide – with resultant economies.
- Cowdrey Type A, aka Lipschutz bodies (same thing but not listed in the literature as such) are commonly associated with vertebrate herpesviral infections and have been reported infrequently in infected oysters.
- Other nuclear figures such as pyknosis and chromatin margination are associated with cell death and can suggest viral infection as a possibility.
- APX: another NZ infection - extremely small cell, hard to spot due to lack of host response. *Ostrea chilensis* more susceptible. PAS stains makes APX stand out in sections.

Current activities:

- Sampling for routine health testing of land-based oysters and mussels [n=150 animals providing detection of 2% prevalence or higher with 95% confidence assuming 100% Se/Sp]
- Sampling *Haliotis iris* for reseeding

Which diseases and what diagnostic tests are problematic:

- DEV: is it a real disease or an extreme manifestation of normal development cycle?
- Ganglioneuritis sensitivity and sentinel populations in NZ
- Unattributable deaths of oysters and mussel juveniles
- Detailed diagnostics for ciliates, Vibrios, OsHV-1 and possibly *Nocardia*

Discussion:
So how does routine sampling (surveillance) help control and manage disease? Is there any documented evidence that routine testing (n=150) has more utility than sampling in response to a disease outbreak? How much effort should be apportioned to detecting specific disease? Self-reporting (by producers) can be a challenge – accredited farmers for sampling selection.

3.2.3 Diseases of molluscs: Identification of diagnostic problems in Australia: Presented by Nick Moody on behalf of Australia (Presentation 06).

Summary:
In Australia the main issues related to the diagnosis of:

- Abalone herpesvirus (AbHV)
- *Bonamia* spp.
- *Perkinsus olseni*
- Oyster oedema disease (OOD) which has an unknown aetiology (research is on-going through a current FRDC project).

The need for standardisation of each stage of each protocol was stressed and choice of assay was recognised as being critical.

With respect to AbHV, a series of requirements were outlined with work on-going that included the use of nested PCR to obtain more genome sequence for various strains of AbHV, and increasing the number of replicates to determine genetic variation between and within strains.

Aspects still to be resolved related to whether samples should/could be pooled (particularly for surveillance of healthy populations to detect sub-clinical carriers re: translocation, appropriate sample size, limited known positive samples for assay validation).

3.2.4 Discussion

Following this session it was agreed that the diseases/agents in common were:

- *Bonamia*
- *Marteilia*
- *Perkinsus*
- OsHV-1 µvar

Further discussion included a desire to provide producers with practical information and recommendations to assist in on-farm disease control and management.

3.2.5 Sessions 1 and 2 Summary: Common themes

The issues which seemed to recur throughout the presentations from each of the three regions/countries were listed and prioritised. There was a range of diagnostic issues as well as a number of diseases/agents. These are listed below in priority order with an indication of the top three priorities for the diagnostic issues common to EU/NZ/AU, and for the diseases/agents in common, as discussed and agreed by the workshop participants:

COMMON DIAGNOSTIC ISSUES

1. Test validation:
   - Repeatability: Samples and sample processing/extraction methods
   - Pooling of samples? Re: Surveillance
   - Fitness for purpose
   - OIE Chapters – Tables (additional notes required)
2. qPCR Ct cut-offs, confirmatory testing of samples with high Ct values
3. Histology
   - Criteria for histological sections: Sharing of protocols can be done immediately as an initial activity
   - Proficiency testing
   - Accreditation
   - Other histological stains
   - *In situ* hybridisation

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4. Lack of molluscan cell lines for virology
5. Capability (e.g. EM)
6. Laser capture micro-dissection (including for use in co-infections)

DISEASES

1. OsHV-1/Vibrios:
   - Some research undergoing in Europe on the significance of *Vibrio* spp.
   - No evidence for *Vibrio* spp. involvement in Australia. Transmission experiments required.
   - NZ: Unknown. Needs to be investigated further.
   - Other OsHV-1 variants. Why is OsHV-1µVar so pathogenic?

2. *Bonamia* – all species. Identification down to species level – with attention to other microcells (*Mikrocytos*).
3. *Perkinsus* spp.

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4. *Marteilia* spp.
5. *Haplosporidium* spp.
6. Co-infections – laser capture micro-dissection (see above)
7. Mortalities with unknown aetiology
8. Definition of abnormal/significant mortality? – need information from operators [Major industries: Mussels, oysters, abalone]
9. *Chlamydia*/RLO

3.3 Session 3: Diagnostic laboratory harmonisation. Diagnostic networks, QA systems (ISO 17025 or equivalent), laboratory proficiency tests.

3.3.1 State of laboratory network in EU: Presented by Marc Engelsma, on behalf of EU (Presentation 07).

Summary:

- EURL and NRL network
- EU Reference Laboratory (IFREMER, France) for mollusc diseases
- National reference laboratories for molluscs (22) (there are no landlocked reference laboratories)
- EU legislation: 2006/88/EC
- EURL (in consultation with EU commission) coordinates methods, provides reference materials, develops and validates tools, molecular characterisation of parasites/pathogens, assists NRL in diagnosis, training (through annual meetings/workshops), production of control materials for inter-laboratory comparisons (proficiency testing), collaborates with laboratories in other countries (e.g., AU, NZ, etc), has tools such as website SOPs, Mscope (scanned slide viewer), development/validation of tools, for example, real-time PCR for *B. ostreae*.
- NRLs – notify Competent Authority (CA) of detection of EU listed diseases, use EURL methods at national level, assist with diagnosis of disease outbreaks, comparison tests, facilitate training
  - Regional laboratories in France, Spain, Italy and Germany (harmonised by NRLs)
- Other networks:
  - Bivalife: *C. gigas, M. edulis* and *M. galloprovincialis*. Participating laboratories complement (rather than duplicate) each other. Transfer and validate existing methods for detection and identification of oyster and mussel pathogens (OsHV-1 uvar, *Vibrio* spp., *Marteilia refringens*, *Norcardia*). Characterise culture sites in EU; life cycle; virulence; identify recommendations for control. 12 partners. IFREMER coordinator. Industry involvement (EU shellfish producers' associations). Ends January 2014.
  - Oysterecover: Flat oyster to tackle Bonamiosis. Identification of invertebrates involved in life cycle among other things. Finishing this year. 8 research organisations and subject matter experts.
- Expertise of EU laboratories
  - IFREMER (France) – NRL (and EURL) for mollusc diseases.
    - Involved in establishment of quality and uniformity of techniques
    - OsHV-1 and *Vibrio* diagnostics
    - Histology and validation of some diagnostic tools

- CEFAS – NRL (England and Wales). *Bonamia*, *M. refringens*, *Mikrocytos* spp., OsHV-1, haplosporidians. Tools: Epidemiology, histology, molecular biology (conventional and real-time PCR, laser capture micro-dissection (LCMD) and electron microscopy (EM).
- CSIC – NRL (Spain). *Bonamia*, *M. refringens*, *Mikrocytos* sp, OsHV-1, *Perkinsus*. Range of tools for exotic and non-exotic diseases
- IRTA (Catalonia) – research laboratory, *Vibrio*, OsHV-1, *Perkinsus*. Tools: histology, classical microbiology, RFTM, real-time PCR and conventional PCR.
- IZSV – NRL (Italy). *Perkinsus*, *M. refringens*, *Bonamia*, OsHV-1, Tools: Histology, PCR, bacteriology, histochemistry, TEM, MS Maldi-Tof.
- Marine Institute - NRL and CA (Ireland). *Bonamia*, *M. refringens*, *Mikrocytos* sp, OsHV-1, *Perkinsus*, *Vibrio tapetis*. Tools: Histology, real time PCR
- UCC (Ireland) – University: Areas of interest include epidemiology and host/parasite interactions.
- CCMAR (Portugal) – University research. *Perkinsus* in clams. Host-parasite interactions. Tools: NGS, microarrays, EM, histology, bioinformatics, database curation and support.
- CVI – NRL (Netherlands and Belgium). *Bonamia*, *M. refringens*, OsHV-1. *Nocardia*, Haplosporidians, *Vibrio* spp. Tools: Histology and real-time PCR.

Discussion: Need to better link the findings/implications of the laboratory diagnostics with the growers in a language that can be understood (e.g., production of grey literature). Another EU FP7 project, EUROSHELL, is intended to bridge the gap between science and producers to support the European marine mollusc production sector.

3.3.2 State of laboratory network in New Zealand: Presented by Brian Jones on behalf of NZ (Presentation 08).

Summary:

- NZ located across sub-Antarctic waters (3-5^{o}C during winter) in the south, and warm sub-tropical waters in the north - large range in water temperatures across the country. Warm water currents from Australia flow around the North Is.
- Laboratory activity in aquatic diseases: Cawthron (Nelson), NIWA (Top of North Island), Massey University (Palmerston North), and MPI (Wallaceville) involved in diagnosis. Otago University has expertise, but not funded.
- Oysters – Flat (*O. chilensis*) and Pacific (*C. gigas*)
- Mussels - Blue (*M. galloprovincialis*) and Greenshell (*Perna canaliculus*)
- MPI animal health laboratory: 35 staff (minority focused on aquatic diseases). Based at National Centre for Biosecurity and Infectious Diseases, Wallaceville. Identification of all suspected exotics and new and emerging diseases of production, companion and aquatic animals. Diagnostic testing for high impact diseases, developing new tests. Very little R&D except on new diagnostics (similar to CSIRO-AAHL).
- No functional laboratory network because (1) few personnel (small country), (2) no common forum and (3) competition for limited funding between research providers (e.g., Cawthron, NIWA).

3.3.3 State of laboratory network in Australia: Presented by Paul Hick on behalf of Australia (Presentation 09).

Summary:

- Diagnostic capacity – federal and state government veterinary laboratories, university and private laboratories.

- Interest: Aquaculture, wild fisheries and biodiversity (disease reporting and appropriateness of samples differ between aquaculture and fisheries/biodiversity – natural resources - interests).
- Limited specialists (aquatic animal health professionals) working between the production sector (field veterinarians/extension officers) and the diagnostic laboratories (virologist, microbiologists, parasitologists).
- End-users of results are resource managers: usually government (State/Federal) or animal health specialists. They will interpret results and implement policy based on those.
- Focus on notifiable/known diseases. Very little known outside of those (self-fulfilling prophesy in terms of finding what is being looked for).
- Other data e.g., environmental, is collected but not clear if this is integrated into diagnostics.
- State-funded veterinary laboratories – have histopathology, microbiology and molecular diagnostics except for South Australia (SA uses private laboratory for histopathology and uses AAHL for molecular diagnostics). Other methods vary between the states. AAHL has overall responsibility for exotic diseases and collaborates with State laboratories/agencies on new and emerging diseases.
- Nation-wide, 20 laboratories self-assessed as having some level of capability in aquatic animal health disease diagnostics. It was noted that expertise is retiring (succession planning is required). Laboratory services provided on cost-recovery basis.
- Expertise tends to be developed through research funding (a significant issue considering funding is declining).
- Animal Health Committee (AHC), which consists of state government chief veterinary officers and Australian chief veterinary officer, determines national policy, with sub-committees (e.g., Sub-committee on aquatic animal health; SCAAH) consisting of technical experts from government, universities and the private sector used for advice on policy and technical issues.
- AHC Sub-committee on animal health laboratory standards (SCAHLS; www.scahls.org.au): Manages diagnostic laboratory standards e.g. Australia and New Zealand Standard Diagnostic Procedures (ANZSDPs). Network of diagnostic specialists.
- Laboratories for emergency animal disease diagnosis and response (LEADDR) Network (covered later presentations): to ensure inter-state harmonisation of diagnostic testing.
- Case study presented: Abalone viral ganglioneuritis in NSW.
    - High value species (2012 commercial quota 110 t = $2.8 million)
    - Previous mass mortality events associated with *Perkinsus*
    - Other reasons for population decline include poaching and recreational fishing (total population decline estimated ~90%)
    - Disease in live abalone reported in a restaurant aquarium
    - Histology and qPCR identified pathogen (in parallel at AAHL and NSW State diagnostic laboratory)
    - Discovery of identical pathogen in Tasmanian processing plant – traced forward to live transfer to Sydney Fish market (wholesale outlet)
    - Protocol developed for seizing animals (n= 156 animals from 40 sites)
    - Emergency disease response identified at key risk sites
    - High throughput PCR used to test almost 400 samples (135 positive)
    - Following clean-up and disinfection, sentinel animal program established:
        - 10 sentinel animals per affected tank tested at days 10 and 15
        - No single PCR test detected all of the positives
    - Follow up programme to educate communities
        - No abalone guts to be used as bait

Questions/Discussion

- NZ and AU are small communities, so informal processes, telephoning/emailing people, occur when issues arise. It was noted that informal processes play an important role (often quicker than formal processes). It was suggested that it is important to get all laboratories (government, university, private) linked into the network to ensure effective co-operation between government and university laboratories. For example, Richard Whittington (as the university representative on SCAAH) surveys university research in aquatic animal diseases annually to get a complete picture on what is taking place and an idea of the expertise being developed.
- In AU (and NZ) there is an obligation to notify national authorities of any suspected notifiable/emergency/exotic disease outbreak. This is important because diagnostic/health-related activities in the private sector are increasing while the Government sector is decreasing.
- There were questions about whether or not researchers outside official diagnostic laboratories would report disease events due to loss of control of data and potential publication (national obligation to report vs. interest in publication). It was noted that environmental scientists in the UK are using histology to look at mussels as biomarkers. In theory could identify pathogens, including listed pathogens but lack of awareness of official obligations to notify competent authority could be a problem, but not clear if those people would notify.
- In EU: More difficult to have connection between research and the NRL/EURL. Issues around use of data, publication and sensitivities – especially if a laboratory is both a NRL and a research group.
- Expertise reaching retirement age: This is an issue that needs to be addressed. Expertise is based on available research funding – and this is limited/under threat – therefore there is a need to make the point that research is required to underpin capability and assist with succession planning. In AU, the major funding body (FRDC) for aquatic animal health has a People Development programme aimed at building future capability.

3.3.4 Validation status of current diagnostic tests in the OIE manual: Presented by Steve Feist (Presentation 10).

Summary:

- Validation = fitness of an assay, properly developed, optimised and standardised for an intended purpose (e.g., surveillance, diagnosis, confirmation, etc).
- Set of criteria for assay development and validation articulated in OIE guidelines
  - Definition of purpose, optimisation, standardisation, repeatability, etc
- Four steps for validation.
  1. Analytical performance characteristics (including analytical accuracy of adjunct tests, reagents etc).
  2. Diagnostic performance of the assay (reliant on known population health status – important but probably the least completed step).
  3. Reproducibility (ring tests, etc).
  4. Program implementation (including fitness for use, interpretation of results, international recognition, deployment of the assay).
- Monitoring performance after initial validation (modification, enhancing confidence, verifications of existing assays (in-house validation), QA/QC proficiency testing).
- The process is onerous and costly.
- Rating of tests A-D where:
  A) Recommended (availability, utility, good sensitivity and specificity);
  B) Standard (good sensitivity and specificity)

C) Limited application in some situations because of cost, accuracy, etc; and
D) Not recommended.

- For each disease chapter in OIE, there is a table for the assessment of specific methods and when those methods are suitable (e.g., for different life stages and purposes - surveillance, diagnosis etc.). It is unclear who actually developed these tables for each pathogen. Question whether or not these methods have actually been validated.
- OIE manual inclusion of validation protocols is new, as are the tables. Different authors for each chapter. Draft chapters go out to all OIE member countries for review and it can be quite difficult to resolve comments (particularly if they are not relevant to situation). Tables are a useful starting point. Each person who fills out the tables probably fills them out quite differently, e.g., an "A" maybe interpreted as relative to "no test" i.e. best available situation.
- Would be good to look at how these tables might be improved. Should probably be sent to experts before going to each member country. Table might benefit from an extra column that states how the validation was assessed, or an explanation of assigned values (e.g., according to OIE Validation Chapter 3 – what validation stages are completed/not completed). Noted that it is better to have a table than none in emergency situations. Also noted that the tables don't consider testing in series or follow the diagnostic sequence.
- How do the OIE and EU align? Which has precedence?
  - In absence of EU-generated information, refer to the OIE manual. However, in the main, EU procedures tend to be in line with OIE.

Discussion

It was recognised that few tests are fully validated to OIE standards. This is an aspiration that is rarely achieved for various reasons (priorities, lack of funding, lack of time, new tests emerging). Can this be addressed? If so, what is needed? At what stage of validation are the tests? – No information in OIE Manual. Workshop participants agreed that the stage of validation should be stated in the OIE Manual.

3.3.5 Quality assurance systems in diagnostic laboratories in EU: Presented by Deborah Cheslett on behalf of EU (Presentation 11).

Summary:

- Legislated EURL/CRL must have accreditation for ISO or EC
- EURL accredited for a range of tests ISO17025 and ISO9001
- NRLs have a mix of accreditation status:
  - Some have none (Bulgaria, Greece, Slovenia, Sweden, Croatia),
  - Some operate under quality system (Denmark, Montenegro, Portugal, Netherlands and Belgium), and
  - Some fully accredited (France, Spain, Italy, Germany, UK (England and Wales), UK (Scotland), Ireland, Turkey, Romania, etc.
- Breakdown of accreditation by pathogen and method (refer to presentation for detail – For histology, this includes competency of slide production, training/competency of staff, status of equipment, ring testing). For example:
  - Histology (range of pathogens, range of laboratories)
  - Cytology
  - Molecular diagnostics (few specific tests; only for Germany, UK and Ireland).
  - There is a vast difference in what each country has accreditation for and the accreditation systems within each country.
- National Accreditation Boards will certify the tests, so there can be variation between different countries in terms of what is required to get accreditation. The accreditation

boards look at validation, SOPS, what the laboratory does to operate a quality system. They will take a specific test and look at it. It is a big task to get a test ready for accreditation.
    - Accredited histopathology based on both the procedures and the person by the accreditation organisation of the specific country (also based on documentation of staff training, calibration status of equipment, etc)
- Need ring tests to retain accreditation.
- Quite a complex network of regional laboratories operating under different QA systems. Lots of variation in accreditation depending on the country.
- New methods for accreditation – range of laboratories looking to accredit PCR tests for range of pathogens, *Bonamia*, *Marteilia* and OsHV-1.
- Most laboratories have histology and cytology for *Bonamia* and *Marteilia* but accreditation in molecular methods is low.
- Bivalife consortium: Range of accreditation status (according to ISO 17025) across laboratories involved in this program (certain laboratories, specific tests only).
- Oysterecover: Very few accreditations
- Issues: Cost, lack of official samples, lack of resource
- Benefits: Traceability, common and standardised procedures, confidence in results, determination of specificity and sensitivity, continuous improvement
- Problems: Cost, proficiency tests unavailable, time-consuming, QA people lose touch with the work they are supposed to be doing.
- Separation of QA diagnostics and research activities. Research/university laboratories and reference laboratories operate under different environments. Research laboratories don't need to operate to the same standard re: QA. It would be very expensive and time-consuming for research laboratories to operate this way.

3.3.6 Quality assurance systems in diagnostic laboratories in NZ: Presented by Brian Jones on behalf of NZ (Presentation 12).

Summary:

- Government regulations require all laboratories that undertake testing for export must be approved by MPI under the laboratory approval scheme (LAS) or export laboratory program.
- Need to be under IANZ – NZ equivalent of NATA QA system.
- Includes export laboratory testing of all live animals and germplasm (unless certified as food) for the purpose of obtaining an export licence (under Animal Products Act 1999).
- Laboratory approval scheme – micro- and chemical or other laboratory testing for market access assurances.
- IANZ independent (under IS0 17025) – member of international group.
- Test competence and experience of staff, integrity and traceability of equipment and materials, validating of the suitability of methods and results, management systems and standards are in place.
- Recognised ongoing requirement for monitoring and associated cost.
- Maintained by internal and external audits, internal quality improvement program and internal and external proficiency testing.
- Accreditation of individual tests rather than class of tests (e.g., individual PCRs must each be accredited rather than the PCR process) – multiplies the work.

3.3.7 Quality assurance systems in diagnostic laboratories in AU: Presented by Nick Moody on behalf of Australia (Presentation 13).

Summary:

- A number of standards are used:

- ISO 9001
- ISO 17025
- ISO 17043
- National standards as well (AS/NZS 2243.3)
- ISO14001 – Environmental management system

- There are a number of national accreditation authorities:
  - NATA
  - Standards Australia
  - National Measurement Institute
  - JAS-ANZ
- Other standards for animal health laboratories in AU:
  - SCAHLS (administers ANZSDPs and "slide of the quarter")
  - OGTR/GMOs
  - Biosecurity Australia (for quarantine approved premises)
  - Security sensitive biological agents, audited by Biosecurity Australia
- Most time/effort is spent on methods and staff training and required documentation:
  - Use OIE standards if available, ANZSDPs if no OIE standard, then EU regulations, peer-reviewed published methods, in-house methods.
  - Validation, equivalency testing of new methods.
  - SOPs developed within laboratories. Looking for harmonisation between laboratories, not standardisation (as different laboratories have different requirements).
  - Lot of work on quality controls – monitor performance of enzymes, equipment etc.
  - Validation and equivalency testing of new methods.
  - Check-testing every 6 months
  - Use competency to document initial staff training. Increase in staff workload.
- QA is a process of continual improvement, monitored by internal and external audits, ring testing etc., that helps to identify improvement opportunities (=mistakes), but needs leadership/enthusiasm.
- AAHL has no operational differences between accredited and un-accredited tests.
- Documentation:
  - Maintain knowledge of changing requirements
  - Greater use of electronic records – issues around this that need to be sorted (e.g., version control, permanent record)
- <u>IMPORTANT</u>: Accreditation is a requirement for all official state/diagnostic laboratories (AHC policy).

3.3.8 Proficiency testing activities for mollusc disease diagnostics in EU: Presented by Isabelle Arzul on behalf of EU (Presentation 14).

<u>Summary</u>:

- Why proficiency testing (PT)?
  - Universal conclusions from tests
  - monitor capability
  - harmonise methods, etc
- Inter-laboratory comparisons – organised by EURL or NRC or within EC context
- EURL tries to organise one every 2 years, e.g., histology for *Bonamia*, *Marteilia* and cupped oyster pathogens: Up to 21 participants (increasing every year), between 30-60 slides.
- PCR PT since 2008. 13-15 participants, 24-30 samples, testing for *Bonamia*, *Marteilia*, and OsHV-1.
- Process: For example with histology: (1) slide preparation, (2) check quality and status by double-reading, (3) announce the test and request for laboratory participation, (4) send

slides to first participant (1 week, 60 slides), (5) participant sends slides onto the next laboratory, (6) results collated and (7) report results to NRLs (lengthy process).

- Similar process for PCR. Test sample prepared and processed (QA). Tissue sample sent. PT to examine entire process from extraction onwards.
- Constant mean between years, but some disparity between laboratories (fluctuates)
- Tend to get better results from heart imprints compared to histology slides
- PCR: look at variation. Develop a Kappa value.
- Inter-laboratory (NRLs): Compare competency but also can compare diagnostic methods (e.g., comparison of two diagnostic methods for OsHV-1).
- EURL provides samples (infected or non-infected) and give to participants to prepare samples, run tests and compare these.
- Saw that greater discrepancy with one PCR test over the other (i.e., one test seemed to be easier to replicate)
- Proficiency testing in EU projects (e.g., Bivalife, Oysterecover): When number of participants using same test in different situations. Useful to determine competency of laboratory network, compare techniques and identify potential optimisation work. Identify areas for training (i.e., future workshops).
- mScope (Http://mscope.ifremer.fr) - Electronic system for storing slides, can share with collaborators and discuss using skype, can use for self-testing and training. Similar to DAFF Project Neptune.

3.3.9 Proficiency testing activities for mollusc disease diagnostics in NZ: Presented by Brian Jones on behalf of NZ (Presentation 15).

Summary:

- Participates in AU national quality assurance program (OsHV-1, AVG)
- Participates in EU Inter-laboratory PT for finfish viruses (no mollusc diseases currently)
- Internal proficiency testing (e.g., *Perkinsus*)
- IDC – good reference slides of mollusc diseases. Not currently accredited for histology (will change that soon). Participates in the "slide of the quarter" with AU – state laboratories send a typical histology slide from its case load, to other state (and NZ) laboratories for review. This is a tool for training rather than for PT.

3.3.10 Proficiency testing activities for mollusc disease diagnostics in AU: Presented by Mark Crane on behalf of Australia (Presentation 16).

Summary:

Current PT activities:

- EU Reference Laboratory for fish diseases (Denmark) – annual ring test (only test where finfish viruses exotic to Australia are examined). AAHL participation is essential (participation in PT is a requirement for accreditation).
- DAFF SE Asia Inter-laboratory PT program:
  - 10 Asian countries (up to 40 laboratories), 10 disease agents (finfish and crustacean viruses), molecular testing only
  - AAHL provides the QC (homogeneity- and stability-tested) material (EtOH-fixed, homogenised tissues from infected animals) to ANQAP
  - ANQAP prepares the PT panels, distributes to participating diagnostic laboratories (de-identified, coded), collates results and prepares annual reports
  - No mollusc diseases in this program
  - 1 year into the 3-year program

- DAFF National aquatic animal health PT program:
  - 2 of the 7 agents are mollusc pathogens (oyster herpes virus and abalone herpes virus)
  - 3-year programme (2013-2015)
  - Annual testing
  - AAHL provides the QC (homogeneity- and stability-tested) material (EtOH-fixed, homogenised tissues from infected animals)
  - ANQAP prepares the PT panels, distributes to participating diagnostic laboratories (de-identified, coded), collates results and prepares annual reports
  - OIE manual recommended tests
  - Program just started, so no results yet
  - This is the second such national project funded by DAFF. The previous project (2010-12) was similar and on its completion a review process indicated benefits to the participation laboratories. The laboratories agreed to continued participation in the second project contingent on DAFF funding.
- The LEADDR network
  - Laboratories for emergency animal disease diagnosis and response (LEADDR)
  - Objective: To ensure all national network laboratories obtain consistent results when testing the same PT panels.
  - PT panels: OsHV-1 and white spot virus (crustacean pathogen)
  - Results are open among participants, no anonymity – results are discussed in an open forum (by teleconference) to trouble-shoot and improve performance (consistency between laboratories).
  - Network QC (NQC) material = non-infectious plasmid controls (for monthly reporting). Used to establish tests, test reagent quality, test equipment performance and test competency of laboratory staff running the test. No tissues, so influence of tissues on nucleic extraction is not tested.
  - PT material (for 6 monthly reporting): EtOH-fixed, homogenised tissue samples provided by AAHL (tests whole process from extraction onwards).
  - AAHL has undertaken equivalency testing using field samples for comparing different assays (e.g. Orf49, Orf66 and Orf77 qPCRs for AbHV).
  - Equivalency testing for two OsHV-1 molecular tests (Martenot and EMAI) has been undertaken:
    - The two tests target different parts of the OsHV-1 genome
    - Results demonstrate that the tests are equivalent when run under identical conditions
  - PT is expensive and time-consuming, but provides benefits: benchmarks, confidence in the laboratory capability, and regulatory confidence in results.
  - Requires commitment of resources (people, time, funds) from both the organising and participating laboratories.

3.3.11 The epidemiological picture *per se* related to a European inter-site causal analysis: Presented by Dolors Furones (Presentation 17).

Summary:
- Increasing mortalities in *C. gigas* since 2005.
- In 2008, 2009 large mortalities in France
- OsHV-1 µvar – evolving pathogen (strain differences since 2005)
- Characterisation of study areas
- Standard operating procedures for sampling
- Examination of potential risk factors associated with mortalities:
  - Vibrios, environmental conditions, culture conditions

- Sampling sites: France (3 sites), Ireland (3), Spain (2), Italy (1), Netherlands (1)
- In some areas of Spain, there are moves away from mussel to oyster farming because of high values associated with oysters.

- OsHV-1 mortalities have different patterns, e.g., some reach up to 100% mortality, others don't.
- In Spanish sites, virus appears to be present all year round, but prevalence higher in spring where greater mortalities are observed.
- In Spanish sites, high prevalence of *V. spendidus* before mortalities.
- In Spanish sites, only one detection of *V. aesturianus* in two bays in two years.
- When temperature reaches 16^{o}C – problems with OsHV-1 prevalence and mortalities begin (associated with both temperature increase (Spring) and decrease (Autumn)). Pattern of *V. spendidus* with temperature appears to be similar (though preliminary).
- When OsHV-1 not detected, low mortalities were observed.

Difficulties

- 2011/2012 – atypical climatology
- Difficult to track batches throughout production cycle

Management

- Which time of the year do you release spat into the field?
- Do you engage in over wintering to help to reduce mortalities?
- Appears to be a correlation with stress (temperature) – lower mortalities observed in offshore long-lines than inside bays. However for long-lines, there was higher OsHV-1 prevalence and mortalities on the top of the lines than seen at the bottom. Lines are typically 10-12 m (water depth 16 m). No tide. Top levels have high temperature fluctuations.

3.3.12 eDNA approach for pathogen diversity and disease risk: Presented by Stephen Feist.

Summary:

- eDNA: detection of macro- and micro-organisms/pathogens in water/sediment samples
- Applications for epidemiology/pathogen ecology
- Potential for determining pathogen range
- Potential for assessing emerging disease risks
- Detection of exotic macro-species
- Detection of novel microorganisms/pathogens

3.3.13 Discussion: Summary

Following the presentations in this session, the workshop participants revisited the list of priorities generated at the conclusion of the previous day to determine whether there were any other issues/diseases/agents that needed to be added or whether priorities needed to be changed. The participants agreed on the priority lists.

3.4 Session 4: Knowledge gaps: Collaborative opportunities. Sharing of resources, e.g. positive control material. Progress of test validation across multiple laboratories.

For this session, workshop participants worked in three groups to identify projects that would address priorities common to EU, NZ and AU, i.e. the top three diseases/agents (*Bonamia*, OsHV-1, *Perkinsus*) and the diagnostic issues that were identified through the course of the workshop. The three groups were assigned one of each of the three priority agents (*Bonamia*, OsHV-1, *Perkinsus*)

for which they developed the potential projects and then reported back to the full group for further discussion.

3.4.1 Group 1: *Bonamia*

1. Background

Bonamia is essentially a parasite of flat oyster species, although mechanical carriage could occur on other mollusc species. The disease had a history of severely impacting flat oyster industries, necessitating a move to alternative species such as *C. gigas*. With reduced flat oyster populations, *Bonamia* represented a threat to endangered flat oyster species in the wild at particular locations. Identification to a species level has been difficult historically.

2. Priority objectives

- To identify the geographical range of *Bonamia*
- To establish effective translocation protocols (including demonstration of freedom) in order to reduce risk of spread to free areas
- To be able to identify isolated *Bonamia* to a species level with confidence
- To be able identify prevalence levels as an aid to producers, e.g. ability to predict timing and location of disease outbreaks
- To assist in the development of disease-resistant mollusc lines
- To better understand other causal factors contributing to disease events – host/pathogen/ environment interaction. Susceptibility of different host populations in different environments. Identification of pathogen virulence factors.

3. Specific discussion topics relating to diagnostics

Validation

- It was noted that a combination of cytology/histology and PCR was currently used for diagnosis. Initial screening using histology then testing any negatives with PCR. Other laboratories used real-time PCR as the primary screening test then tested any positive results using RFLP and sequencing.
- It was also noted that PCR was currently used to distinguish *B. exitiosa* from *B. ostrea* (Robert et al., 2009; Ramilo et al 2013).
- Although some data on validation of individual tests has been undertaken (Corbeil et al., 2006), it was noted that none of the tests described had been validated to OIE standards and standardization and validation for cytology and PCR (both real-time and conventional) was required.
- Further data necessary for validation may also be available at other laboratories.

Histology

- There was a need for good quality reference material and histological slides by some laboratories (CVI, The Netherlands can assist). Better understanding of host/environmental influences on histological lesions is required.
- lt was noted that PT for histopathology of *Bonamia* cases was important and that international cooperation was necessary to achieve this.
- Validation of histology, in particular sensitivity, was also considered important, but it was noted that validation of the PCR test would assist this process by providing a comparative test.
- Confirmatory test for definitive diagnosis (to species level) is required.

Project wish-list

1. Validation of the PCR tests, in particular qPCR. It was noted that comparison of available data from various laboratories may facilitate this process.
2. Establishment of training schemes for histopathologists to enable experience in detecting and diagnosing *Bonamia* infection. Exchange of personnel between laboratories, especially younger pathologists was considered important in achieving this.
3. Development of an international proficiency testing scheme for histopathologists, for example.

[*Efficacité des tests diagnostiques sans frontières* (courtesy of Richard) ETDSF]

3.4.2 Group 2: OsHV-1

Desired Outcome

Harmonisation of methodology to increase the quality of diagnosis and to assist with research efforts in support of improved diagnostics.

Description of activity

1) Combined OsHV-1 ringtest EU-AU-NZ
   - include population testing

2) Evaluating the effect of pooling samples
   - Pooling after digestion
   - Pooling before digestion
   - Multilocations/sharing data

3) Guidelines for criteria on validation of molecular testing (aquatics) (including addressing fit for purpose)

4) Diversity of OsHV-1 (also needed for diagnosis)
   - Diversity in time and space
   - Whole genome sequencing

5) Histopathology: Testing with polyclonal antibodies and *in situ* hybridisation to determine if observed lesions are caused by OsHV-1 – to study pathogenicity.

Other links

- EC Directorate-General for Research and Innovation
- EC Directorate-General for Health and Consumers [DG Sanco]

3.4.3 Group 3 - *Perkinsus*

A range of issues were incorporated into a potential collaborative project:

OIE Chapter

The OIE Chapter needs to be revised.

- Requirement for up-dating, e.g. qPCR inclusion in OIE Chapter - the issue concerning high Ct values would need to addressed (validation)
- Ray's fluid thioglycollate culture method (RFTM) is cheap with high sensitivity but low specificity – requirement for fresh material was questioned. Positive control material should

be required as part of the diagnostic process to demonstrate test validity. PCR should be the gold standard rather than RFTM
- Cell culture method (positive controls?)
- Standardisation/harmonisation of histological procedures is required

Species identification
- With seven known species there is a need for speciation tests which will require whole genome sequencing leading to molecular test development and validation. The ITS region as the appropriate target for genetic variation between and within species was questioned.
- Co-infections – how can this be resolved (laser capture micro-dissection offers a means to achieve this).

Environmental triggers
While this is not a diagnostic issue *per se* a better understanding of environmental triggers for disease outbreaks would lead to better disease control.

Outcomes
- Better OIE Chapter
- Full genome sequence – Species identification and better understanding of variation within species

3.5 Session 5: Workshop review and future actions

In this session, the projects concerning the four priority disease agents (*Mikrocytos* has been included) were re-visited and refined with respect to:
- Desired outcomes
- Specific activities leading to the desired outcomes
- Required resources to support the activities
- Potential participating laboratories

See the following tables for summary.

3.5.1 Activities common to all priority agents

	OUTCOME	ACTIVITY	RESOURCES	PARTICIPANTS
Short-term <1 year	Improved characterization and understanding of the range of tissue lesions and disease processes through development of a reference collection	Sharing of material including histological sections, ISH, IHC, EM and smears. On-line collaboration using whole slide images.	In-kind; Slide-scanning capability	All consortium laboratories; lead coordinating laboratory: Cefas/CSIC/ Ifremer
Short-to-medium <2 years	Identification of the most appropriate diagnostic/surveillance PCR tests currently in use	Sharing of protocols & comparison of tests used by consortium laboratories; information on test usage, issues (advantages and limitations), sequencing strategies.	In-kind	All consortium laboratories; CVI willing to lead (not *Perkinsus* – not a priority for The Netherlands)
Medium 1-3 years	Improved OIE Chapter including definition of suspect case; confirmed case; fit-for-purpose tests: diagnosis and surveillance, including "gold standard" and status of validation	Revision of OIE Aquatic Manual Chapters to reflect new information on diagnostic tests	In-kind	OIE Ref Labs; suggestion for a southern hemisphere ref lab contribution to deal with regional differences
Medium-to-long 1-5 years	Improved/demonstrated molecular diagnostic competence among laboratories and validation of molecular tests	International PT – molecular methods [qPCR, cPCR, ISH sequencing]; Some work on-going in France; testing populations displaying different level of prevalence to determine Sp and Se values	Funding for preparation & distribution of quality controlled material	All consortium laboratories; lead coordinating laboratory to be identified; Ifremer could lead the testing of populations with different levels of prevalence

3.5.2 *Bonamia*, *Perkinsus* and *Mikrocytos* spp.

	OUTCOME	ACTIVITY	RESOURCES	PARTICIPANTS
Medium 1-5 years	Improved/demonstrated histopathology & cytology competence among laboratories and validation of histological and cytological tests	International PT – histology; training & staff exchanges; on-line collaboration using whole slide images. Material exchanged for validation activity.	EURL slide sets supplemented with more slides from AU and NZ; Cefas Registry of Aquatic Pathology (RAP)	All consortium laboratories; Ifremer to lead; CSIRO-AAHL to coordinate in AU and MPI in NZ.
Medium 1-5 years	Improved methods for species identification; improved understanding of genetic variation within a species and its significance; determination of host and geographical ranges, and identification of potential transfer/introduction routes; tools/diagnostic approach to detect co infections; improved understanding of co-infections	Parasite purification from culture, application of cPCR; NGS including full genome/transcriptome sequencing (multi gene taxonomy); LCMD; EM; ISH; IHC; eDNA and newer technologies as they become available (transcriptomics approach; study of population genetics). On-going work on *Perkinsus* at AAHL	Funding for collaborative effort for different species	All consortium laboratories; Ifremer willing to lead. CSIC/Cefas/CVI could help to lead. AAHL to coordinate in AU; coordinating laboratory in NZ to be identified.
Long >5 years	Better understanding of host/pathogen/ environment interaction & immune response. Determination of virulence factors; evaluation of the impact of parasites especially where several congeneric species occur; development of resistant lines; better understanding of the relationship of mortality with pathogen prevalence and intensity of infection	Infection model; application of diagnostic and research tools. Preliminary work on NSW organisms by USyd and NSW DPI.	Funding for collaborative study of *Bonamia/ Mikrocytos* in different geographical regions; sharing of information and material	Cawthron, MI, Ifremer, UCC, USyd, NSW DPI. CSIC/Cefas could help to lead
Long >5 years	Development of effective translocation protocols between zones	Use new information on diagnostic tests to develop generic translocation protocols		To be determined; CSIC willing to lead

3.5.3 OsHV-1

	OUTCOME	ACTIVITY	RESOURCES	PARTICIPANTS
	Overall outcome: Harmonisation of methodology to increase quality of OsHV-1 diagnosis, and research in support of improved diagnostics			
Short-term <1 year	Optimisation of sample preparation including extraction protocol, tissue type, determination of effect of pooling samples for diagnostic/surveillance testing and identification of factors impacting on test performance (Se and Sp)/reliability	Sharing of available data on the effect of pooling	In-kind	All consortium laboratories; Ifremer is interested to lead
Short-to-medium <2 years	Communication and collation of disease management tools demonstrated to be effective	Sharing of epidemiological info, husbandry measures, effective disinfection protocols and biosecurity procedures; current epidemiological database (Bivalife) to be maintained and data made available as a web application	Work is on-going; funding required for additional database development/ maintenance	Collaborating laboratories to include Cefas, IRTA, CSIC, MI, CVI, USyd, NSW DPI
In 2015	Exchange of information on OsHV-1 research among consortium members and the broader scientific community	2015 EAFP Conference special session on OsHV-1	Funding for invited presenters	All consortium laboratories; Lead laboratory: Ifremer
Medium 1-5 years	Establishment of improved tools for diagnosis of, and research on, OsHV-1	Polyclonal antibody production using engineered proteins as antigens; some polyclonal antibodies already produced; development of IHC and ISH for diagnostic and research purposes	Funding for research projects	Collaborating laboratories to include Ifremer, Cawthron, CSIC, USyd, NSW DPI

Medium-to-long 5 years	Improved molecular characterisation of OsHV-1 from different regions / collected at different periods and determination of OsHV-1 diversity (space and time); establishment of OsHV-1 isolate database. Development of genotyping techniques for virus identification (a genotyping approach is under development)	Whole genome sequencing of isolates from different regions and from archival material; sharing of partial sequence data where/when available if distinct variants are observed; current epidemiological database (Bivalife) adapted to include pathogen characterization; on-going research (ifremer) through an ERANET EMIDA funded project (Moltraq) and a SUDOE funded project (Aquagenet). Standard approach to sequencing would also be useful	Some work is underway at ifremer, USyd, AAHL; need for additional funding?	All consortium laboratories; Ifremer to lead and coordinate
Long >5 years	Better understanding of pathogenicity/ pathogenesis/virulence and the relationship of mortality with pathogen prevalence and intensity of infection	Viral gene expression studies; (transcriptomics approach; study of population genetics); identification of risk factors; on-going research (ifremer) through EU funded project (Bivalife): Development of a qPCR method to quantify virus RNAs	Funding to conduct research project	Lead laboratory to be identified; others: UNIPD (P. Venier); UNITS (A. Pallavicini) in collaboration with NRL Italy and CSIC Spain; (Cawthron, Cefas) to assist; IRTA; CSIC could help to lead; MI, UCC, USyd, NSW DPI
Long >5 years	Identification of host range and reservoirs of OsHV-1	Collaborative research project using eDNA approaches (including ISH and PMA PCR) or surveillance using fit-for-purpose tests	Some work is underway; further funding to conduct research project	Cefas; USyd; IRTA, CSIC, MI, UCC, CVI, NSW DPI

3.6 Specific recommendations

3.6.1 Short-term activities

To commence collaborative activities which do not require additional resources, e.g., exchange of information on diagnostic protocols and comparison of tests; exchange of information on test usage, issues and sequencing strategies; exchange of reference material (histological sections) for each of the priority disease agents (*Bonamia*, *Mikrocytos*, *Perkinsus*, OsHV-1).

3.6.2 KBBE Funding

3.6.2a 2014 WAS Conference/KBBE Workshop on "Genetic Resistance"

To seek funding from KBBE for travel to World Aquaculture Society Conference, Adelaide, 7-11 June 2014 (http://www.aquaculture.org.au/) with the aim to (1) contribute (chair and present) to health sessions on mollusc aquaculture and (2) participate in the KBBE workshop on "Breeding for resistance" (to be confirmed).

3.6.2b 2015 EAFP Conference Special Session

To seek funding from KBBE for travel to 2015 EAFP Conference with the aim to (1) contribute (chair and present) to a special session on OsHV-1 and (2) participate in 2015 KBBE workshop to discuss progress on research undertaken on each of the priority agents (*Bonamia*, *Mikrocytos*, *Perkinsus*, OsHV-1).

3.6.3 Collaborative R&D

To develop collaborative R&D projects addressing identified priorities for each of the four disease agents of common interest to EU/NZ/AU viz. *Bonamia*, *Mikrocytos*, *Perkinsus*, OsHV-1 (taking into account the recommendations from the 2012 international KBBE workshop organised on "Disease mitigation and prevention in mollusc aquaculture", Nantes, France, 11-15 June 2012).

3.6.4 R&D Funding Sources

To identify funding bodies with a mandate to support international collaborative research on infectious diseases of commercially significant mollusc species.

3.6.5 Research Projects

To submit research proposals to relevant funding bodies within EU/NZ/AU, including KBBE Forum.

3.6.6 Validation of Diagnostic Tests

To develop guidelines for criteria on validation of diagnostic, particularly molecular, tests (including fitness-for-purpose) specific for all aquatic animals.

3.6.7 Coordination

It is suggested that a coordinator from each jurisdiction be elected to be the contact point for activities involving "all consortium laboratories".

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Annex 1. Workshop summary

1. Workshop venues

The workshop will be held at two venues: The Mercure Hotel, Geelong, Victoria, Australia (http://www.mercure.com/gb/hotel-3033-mercure-geelong/index.shtml) for the first two days, and CSIRO Australian Animal Health Laboratory, Geelong (http://www.csiro.au/places/aahl) for the final day. The workshop will conclude with a tour of Great Southern Waters abalone farm, Indented Head, Victoria (http://www.gsw.com.au/home/welcome/) and the workshop dinner at Vue Grand Hotel, Queenscliff, Victoria (http://www.vuegrand.com.au/).

2. Summary of Workshop schedule

Day 1: Monday 21 October 2013

18.00-20.00 Welcome cocktails (Mercure Hotel, Geelong)

Day 2: Tuesday 22 October 2013

09.00-17.00 Workshop sessions 1 and 2 (Mercure Hotel, Geelong)
Evening – free time

Day 3: Wednesday 23 October 2013

09.00-17.00 Workshop sessions 3 and 4 (Mercure Hotel, Geelong)
19.00-22.00 Dinner @ Mercure Hotel

Day 4: Thursday 24 October 2013

08.30 Depart hotel (transportation will be arranged)
08.45 Arrive CSIRO Australian Animal Health Laboratory
09.00 Workshop session 5
11.00 Tour of CSIRO Australian Animal Health Laboratory
13.30 Depart CSIRO Australian Animal Health Laboratory (transportation will be arranged)
14.00 Arrive Great Southern Waters abalone farm, Indented Head: Tour
17.30 Depart Great Southern Waters abalone farm (transportation will be arranged)
18.00 Arrive Vue Grand Hotel, Queenscliff: Workshop dinner
22.00 Depart Vue Grand Hotel, Queenscliff (transportation will be arranged)
22.45 Arrive Mercure Hotel, Geelong

End of Programme

Annex 2. International (AU/EU/NZ) workshop on mollusc disease diagnosis, Geelong, Australia, 22-24 October 2013

FINAL WORKSHOP AGENDA

Tuesday 22 October 2013

Introduction

9.00 Welcome and introduction (Dr Mark Crane, CSIRO-AAHL Fish Diseases Laboratory)

9.10 Preliminary briefing by workshop facilitator (Dr Ingo Ernst, Director – Aquatic Animal Health, Australian Government Department of Agriculture Fisheries and Forestry)

SESSION 1: Significant diseases of molluscs and their diagnosis. Identify the high priority diseases, the status of diagnostic tests and the stage of validation

9.30 Mollusc diseases of significance in EU and their diagnosis (Dr Isabel Arzul, EU Reference Laboratory for Mollusc Diseases, Ifremer, France)
Discussion

10.30 Coffee Break

11.00 Mollusc diseases of significance in New Zealand and their diagnosis (Dr Andrea Alfaro, Auckland University of Technology)
Mollusc diseases of significance in Australia and their diagnosis (Dr Serge Corbeil, CSIRO-AAHL Fish Diseases Laboratory)
Discussion

12.30 Lunch break

SESSION 2: Diseases of molluscs: Identification of diagnostic problems. Which diseases and/or what diagnostic tests are problematic. What are the specific problems, e.g. lack of sensitivity, lack of specificity, lack of validation data? Are there any new diseases for which diagnostics are not well-developed?

13.30 Diseases of molluscs: Identification of diagnostic problems in EU (Dr Beatriz Novoa, CSIC, Spain)
Diseases of molluscs: Identification of diagnostic problems in New Zealand (Dr Steve Webb, Cawthron Institute)
Diseases of molluscs: Identification of diagnostic problems in Australia (Dr Nick Moody, CSIRO-AAHL Fish Diseases Laboratory)
Discussion

15.00 Tea break

15.30 Diseases of molluscs: Identification of common issues (summary of the day's discussion to be led by Drs Ingo Ernst and Mark Crane)
Discussion

17.00 End of Day 2

Wednesday 23 October 2013

SESSION 3: Diagnostic laboratory harmonisation. Diagnostic networks, QA systems (ISO 17025 or equivalent), laboratory proficiency tests.

9.00 State of laboratory network in EU (Dr Marc Engelsma, Wageningen UR, the Netherlands)
State of laboratory network in New Zealand (Dr Brian Jones, Ministry of Primary Industries)
State of laboratory network in Australia (Dr Paul Hick, University of Sydney)
Discussion

10.30 Coffee break

11.00 Validation status of current diagnostic tests in the OIE manual (Dr Steve Feist, cefas, UK)

Quality assurance systems in diagnostic laboratories in EU (Dr Deborah Cheslett, Marine Institute, Ireland)
Quality assurance systems in diagnostic laboratories in NZ (Dr Brian Jones, Ministry of Primary Industries)
Quality assurance systems in diagnostic laboratories in AUS (Dr Nick Moody, CSIRO-AAHL Fish Diseases Laboratory)
Proficiency testing activities for mollusc disease diagnostics in EU (Dr Isabelle Arzul, ifremer, France)
Proficiency testing activities for mollusc disease diagnostics in NZ (Dr Brian Jones, Ministry of Primary Industries)
Proficiency testing activities for mollusc disease diagnostics in AUS (Dr Mark Crane, CSIRO-AAHL Fish Diseases Laboratory)
The epidemiological picture *per se* related to a European inter-site causal analysis (Dr Dolors Furones, IRTS, Spain)
eDNA approach for pathogen diversity and disease risk (Dr Stephen Feist, CEFAS, UK)
Discussion

12.30 Lunch break

SESSION 4: Knowledge gaps: Collaborative opportunities. Sharing of resources, e.g. positive control material. Progress of test validation across multiple laboratories.

13.30 Synthesis of discussions with emphasis on identification of high priority issues of common interest leading to collaborative opportunities (Drs Ingo Ernst and Mark Crane)
Discussion
15.00 Tea break
15.30 Workshop review and future actions (Drs Ingo Ernst and Mark Crane)
Discussion
17.00 End of Day 3

Thursday 24 October 2013

SESSION 5: Workshop review and future actions (Venue: Australian Animal Health Laboratory)

9.00 Wrap up. Draft report preparation
10.30 Coffee break
11.00 TOUR: CSIRO Australian Animal Health Laboratory
12.30 Lunch break
14.00-17.30 TOUR: Great Southern Waters abalone farm (Indented Head)
18.00-22.00 Official Workshop Dinner (Vue Grand Hotel, Queenscliff)
22.00-22.45 Return to Mercure Hotel

End of Programme

Annex 3. Participant list

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23	Steve Feist	CEFAS Weymouth Laboratory, Weymouth, UK	stephen.feist@cefas.co.uk
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25	Dolors Furones	Research & Technology Inst for Agriculture & Food-IRTA, Spain	dolors.furones@irta.cat
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Annex 4. Presentations

01 Mollusc diseases of significance in EU and their diagnosis (Isabelle Arzul)

02 Molluscan diseases of significance in New Zealand (Andrea Alfaro)

03 Mollusc diseases of significance in Australia and their (molecular) diagnosis (Serge Corbeil)

04 Diseases of molluscs: Identification of diagnostic problems in EU (Beatriz Novoa and Antonio Figueras)

05 Identification of diagnostic problems in NZ (Steve Webb)

06 Mollusc disease diagnosis: Identification of diagnostic problems in Australia (Nick Moody)

07 State of laboratory network in EU (Marc Engelsma)

08 State of laboratory network in New Zealand (Brian Jones)

09 Australian diagnostic laboratory network (Paul Hick)

10 Validation status of current diagnostic tests in the OIE manual (Stephen Feist, David Stone, Mark Crane)

11 Quality assurance systems in diagnostic laboratories in EU (Deborah Cheslett)

12 Quality assurance systems in New Zealand (Brian Jones)

13 Quality assurance systems in diagnostic laboratories in Australia (Nick Moody)

14 Proficiency testing activities in EU (Isabelle Arzul)

15 Proficiency testing for mollusc disease diagnostics in New Zealand (Brian Jones)

16 Proficiency testing activities for mollusc disease diagnostics in Australia (Mark Crane)

17 The epidemiological picture *per se* related to European inter-site causal analysis (M. Dolors Furones, K.B. Andree, A. Roque, N. Carrasco, B. Lacuesta, M. Gonzalez, I. Gairin, E.J. Peeler, A. Reese, C. Rodgers)


Mollusc diseases of significance in EU and their diagnosis
fppt.com
Ifremer

1-Mollusc production in Europe

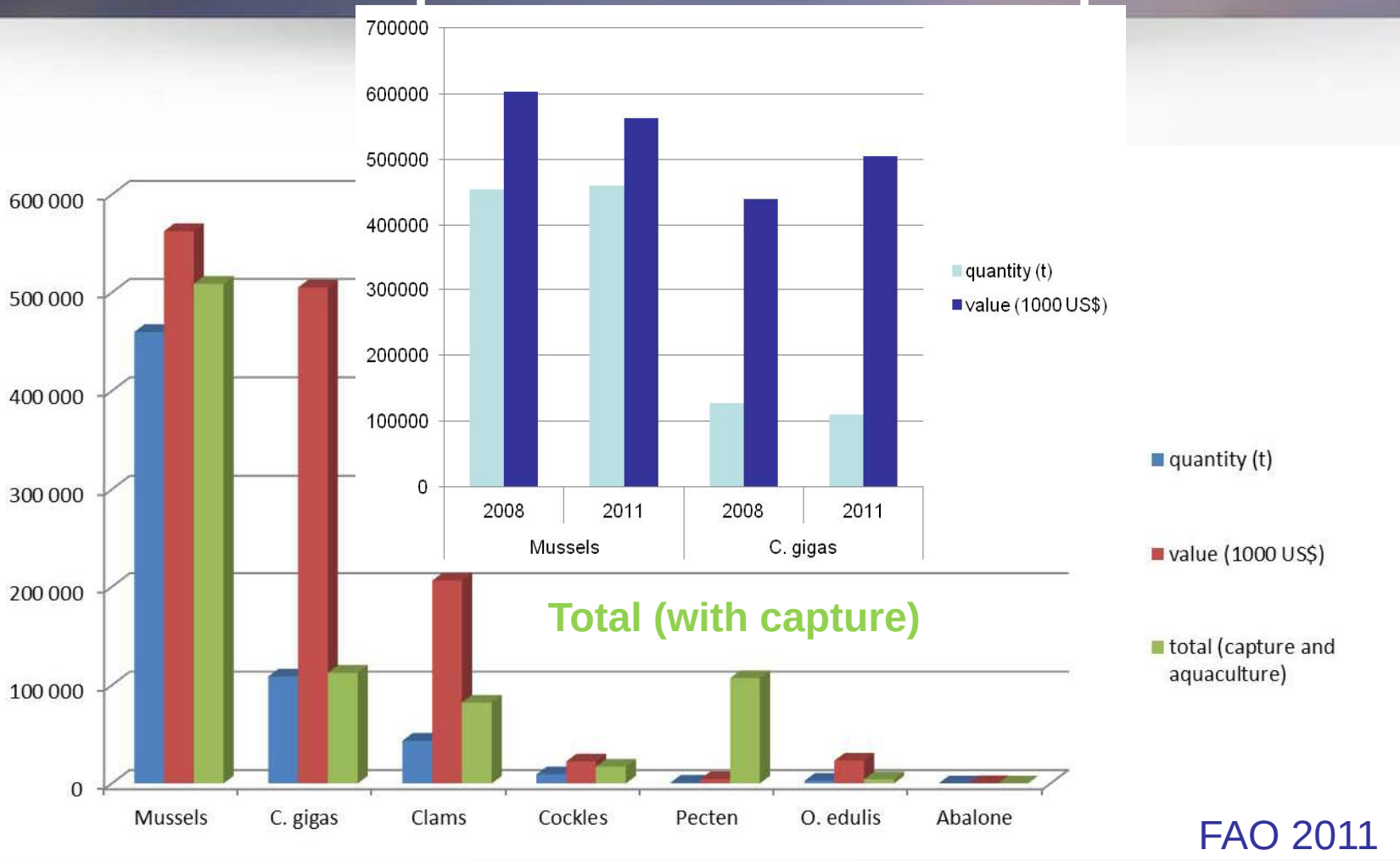
2-Regulatory framework

3-Diagnosis approach

4-Diseases of significance

Production context

Mollusc production in Europe



FAO 2011

FAO (total fishery) 2011

Iceland
Mussel 110 t
Clams 5 t

Norway
Mussels 1 742t
Scallops 752 t
Cockles 5 t
O. edulis 2 t

United Kingdom and Islands
Scallops 65 772t
Mussels 16 324t
Cockle 7 639t
C. gigas 1 331 t
O. Edulis 298 t

Sweden
Mussels 1 555 t
O. edulis 14 t
Cockles 5 t

The Netherlands
Mussels 32418 t
C. gigas 3 500t
Clams 3097 t
O. edulis 90 t

Denmark
Mussels 34 980 t
O. edulis 804 t
Cockles 794 t

Ireland
Mussels 22 671t
C. gigas 11 001 t
Scallop 2 342 t
Clam 715 t
Cockles 372 t
O. edulis 279 t
Abalone 0.5 t

Germany
Mussels 20 830 t
C. gigas 80 t

France
C. gigas 95 073t
Mussels 79 843 t
Clam 4 667t
Cockles 2 282 t
O. edulis 1 297t
Abalone 45 t

Adriatic
Mussels 2417 t
O. edulis 54 t
Clams 35 t

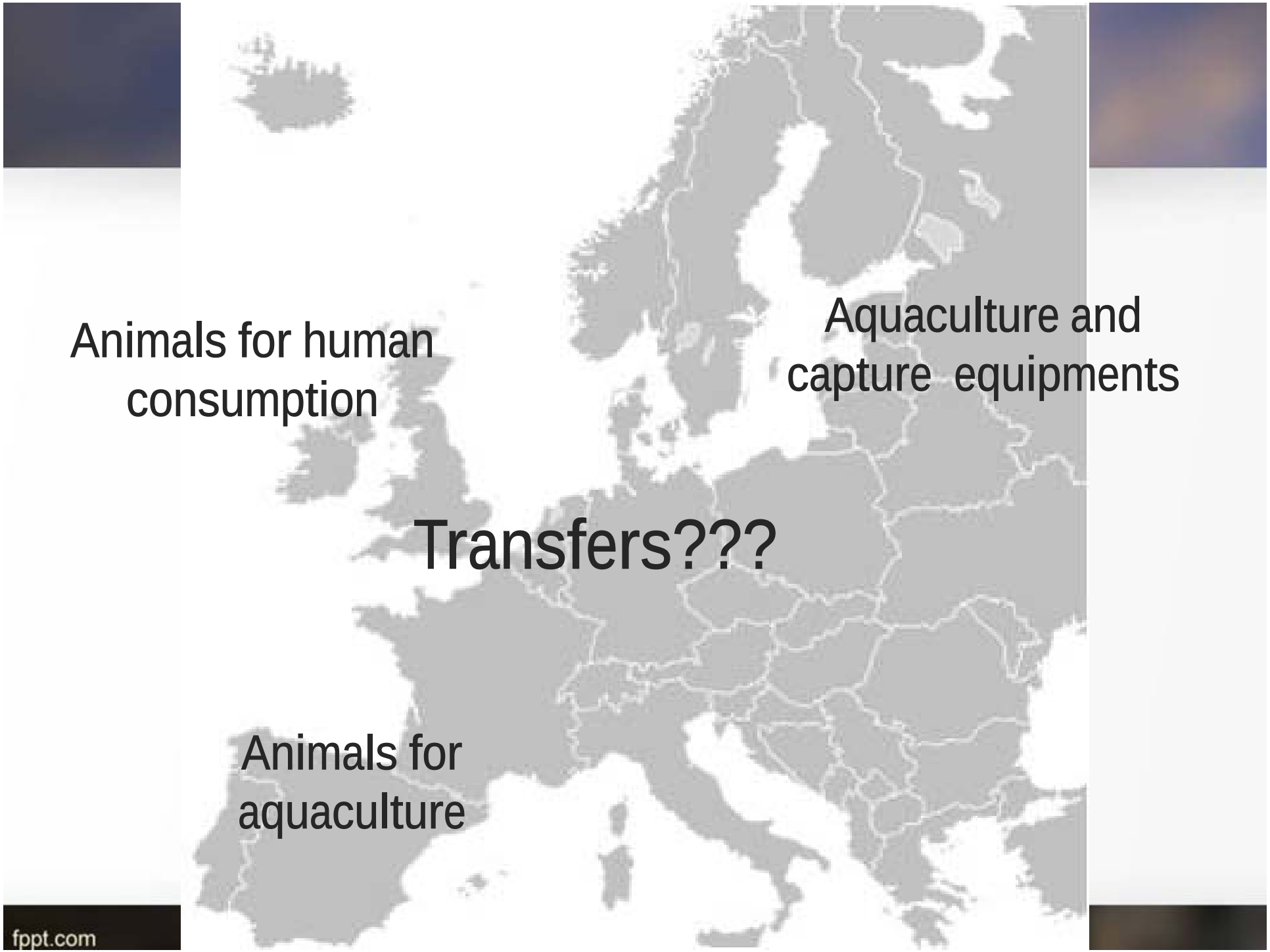
Black sea
Mussels 644 t

Portugal
Clam 4 646 t
Cockles 1695 t
Oysters 490 t
C. gigas 453 t
Mussels 384 t

Spain
Mussels 208 849t
Clams 8 300 t
Cockles 4302t
C. gigas 1 120t
O. edulis 748 t
Scallops 226 t

Italy
Mussels 64 300 t
Clams 58 611 t
Scallops 301 t
O. edulis 27 t
Cockles 11 t
C. gigas 10 t

Greece
Mussels 20 3130 t
O. edulis 100 t
Clams 241 t


Animals for human consumption
Aquaculture and capture equipments
Transfers???
Animals for aquaculture

Mollusc species of significance

Production importance (Mussels / *C. gigas*)

Capture importance (Scallops)

Wide geographic range (Mussels / *O.edulis*)

Diversity (Clams)

Local importance (Cockles)

High market value (Abalone)

Endangered species (*O. edulis*)

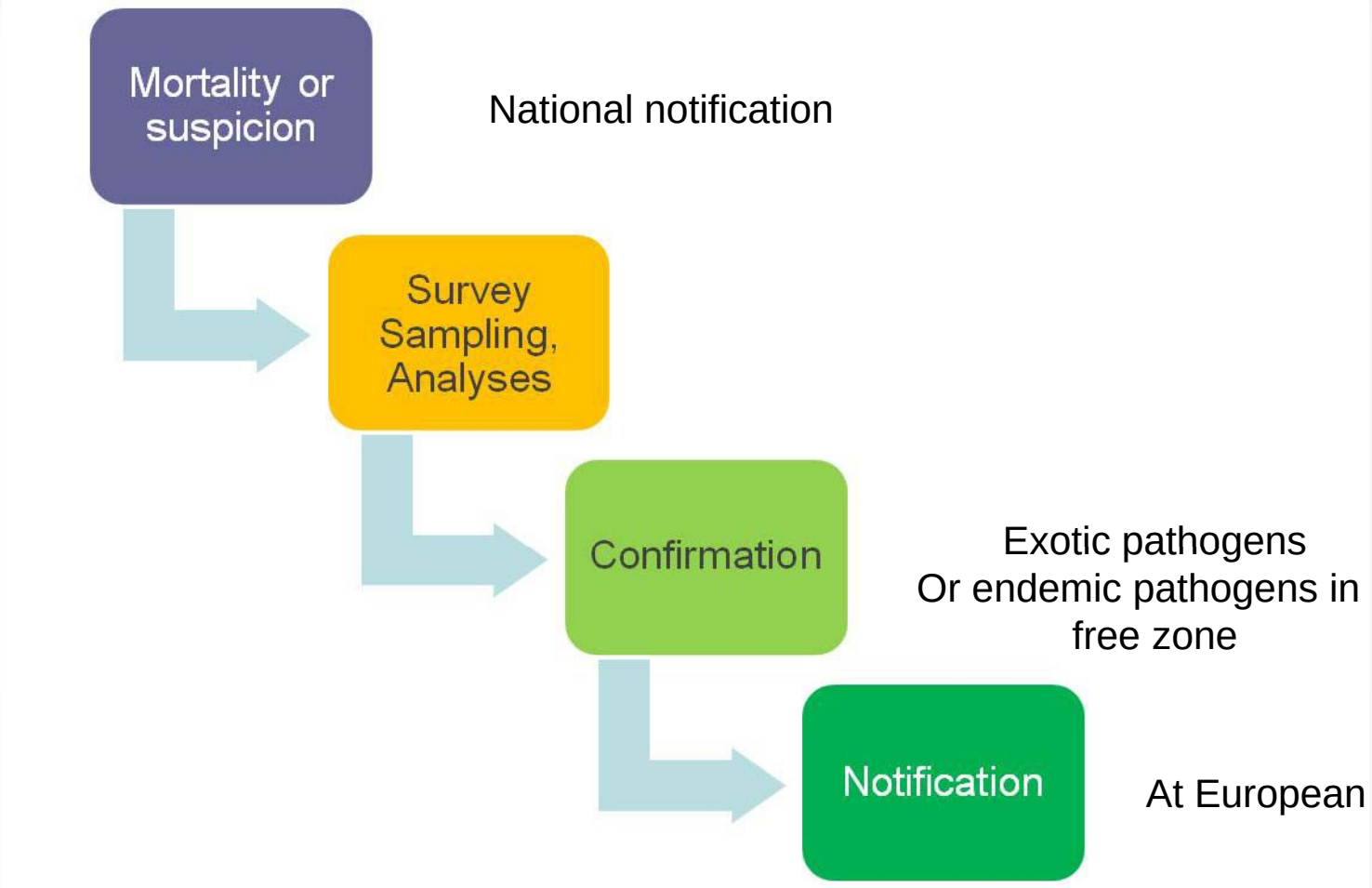
Regulatory framework:

Directive 2006/088/EC

Listed pathogens : Annex IV, Part II

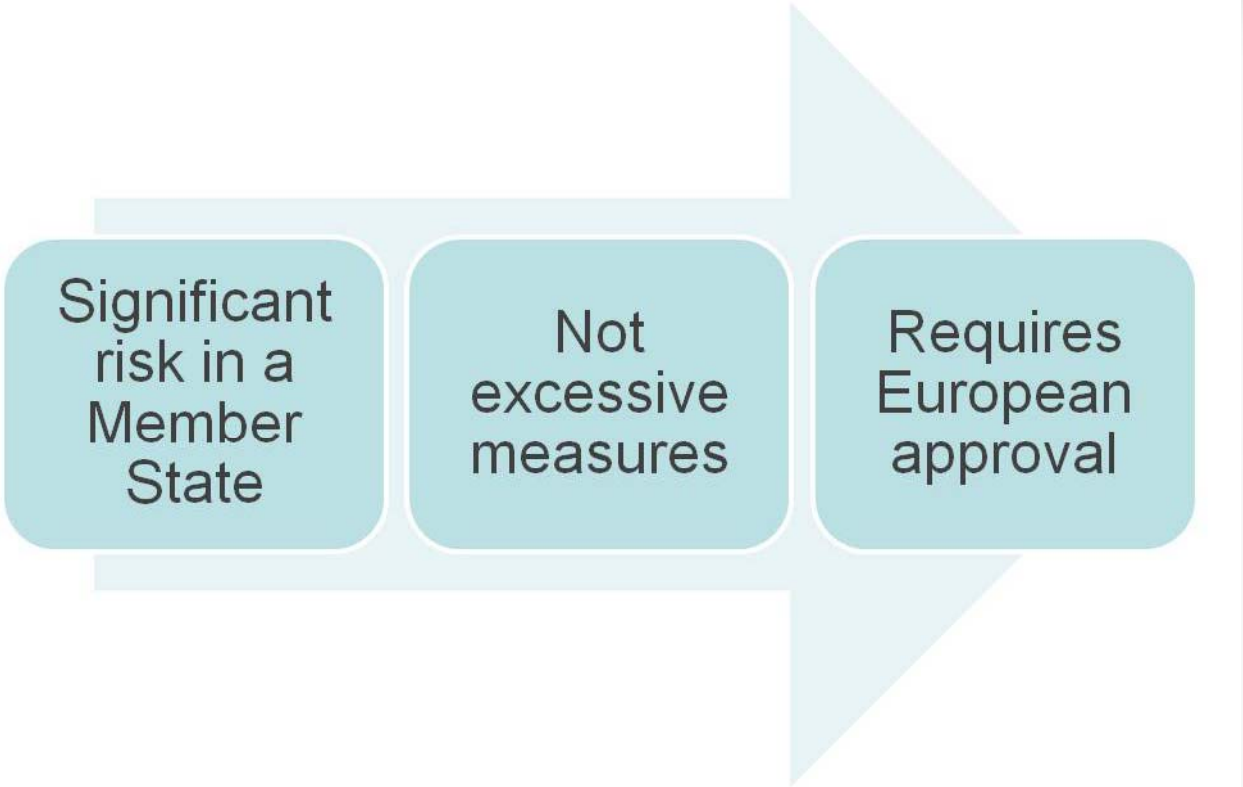
Exotic diseases	
Infection with *Bonamia exitiosa*	*Ostrea angasi*, *O. chilensis*
Infection with *Perkinsus marinus*	*Crassostrea gigas*, *C. virginica*
Infection with *Mikrocytos mackini*	*Crassostrea gigas*, *C. virginica*, *Ostrea conchaphila* and *O. edulis*
Non exotic diseases	
Infection with *Bonamia ostreae*	*Ostrea edulis*, *O. chilensis*, *O. conchaphila*, *O. denselammelosa*, *O. puelchana*
Infection with *Marteilia refringens*	*Ostrea edulis*, *O. chilensis*, *O. angasi*, *O. puelchana*, *Mytilus edulis* and *M. galloprovincialis*

Listed pathogens



National provisions for not listed diseases

Article 43

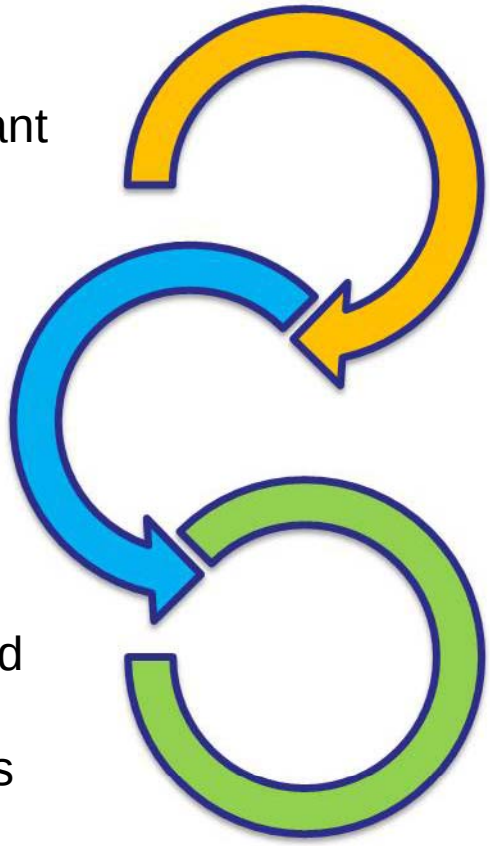


Diseases for which national measures are approved under Decision 2010/221/EU	
Infection with OsHV-1µvar	*Crassostrea gigas*

What about emerging diseases?

Article 41

Emerging significant situation



MS and European Commission information

Eventually included in the list of notifiable diseases

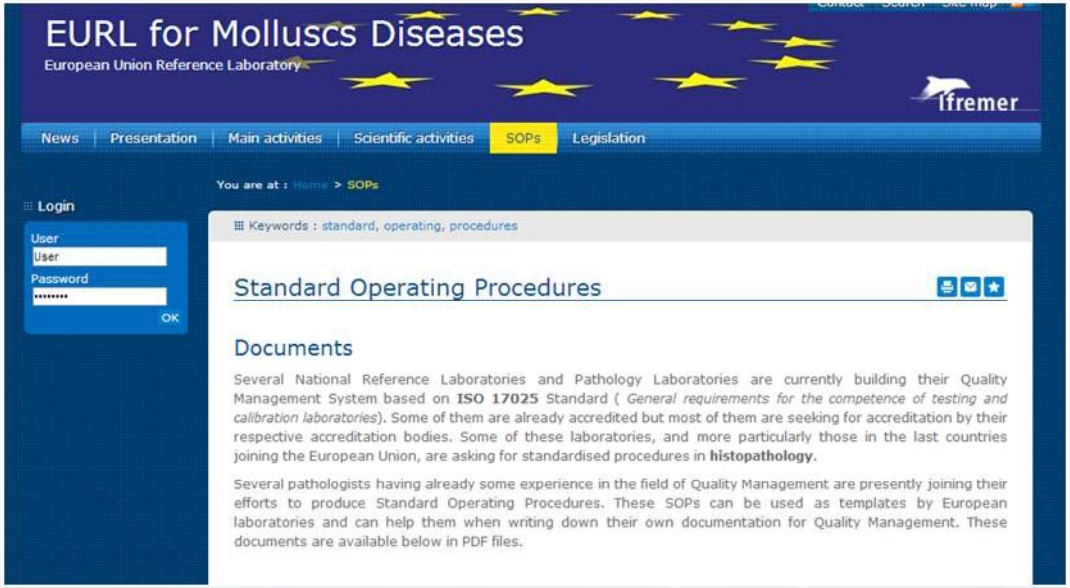
Diagnostic approach

Diagnostic approach

1. Soon : a desision on surveillance and diagnostic of endemic EU diseases

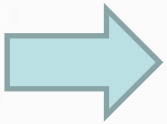
2. In accordance with methods recommended in the OIE aquatic Manual

3. Stantard Operating Procedures available on the EURL website



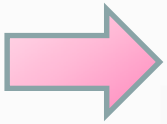
Diagnostic approach

1. To demonstrate and maintain free status
   - Regarding endemic listed diseases (at EU and national levels)



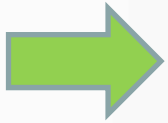
Tools specific to the diseases

2. To investigate mortality outbreaks
   - Regarding all the diseases (endemic, exotic, potentially emerging diseases)



General and presumptive tools

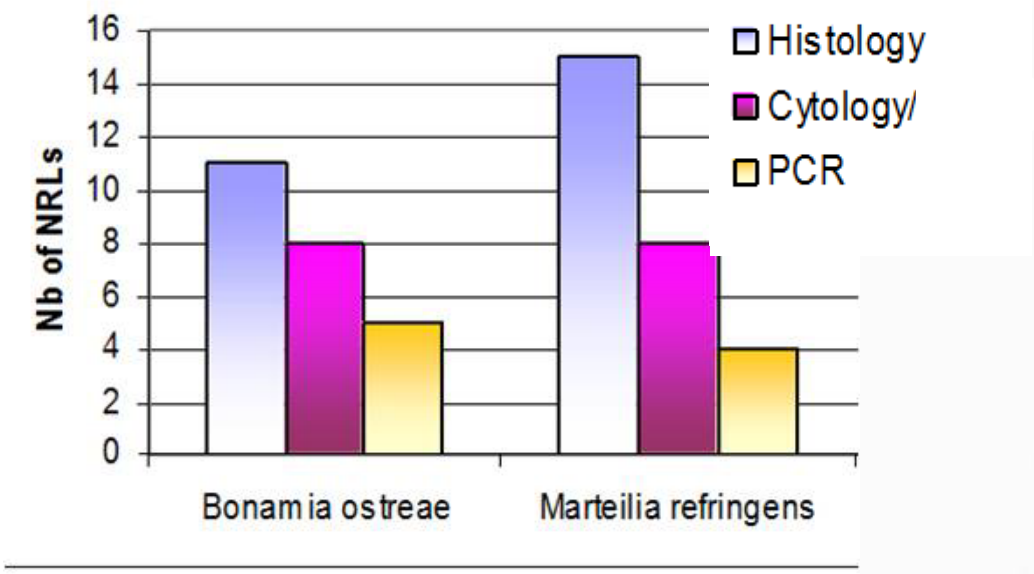
3. To confirm a suspicion
   - Molecular characterization, tissue distribution…



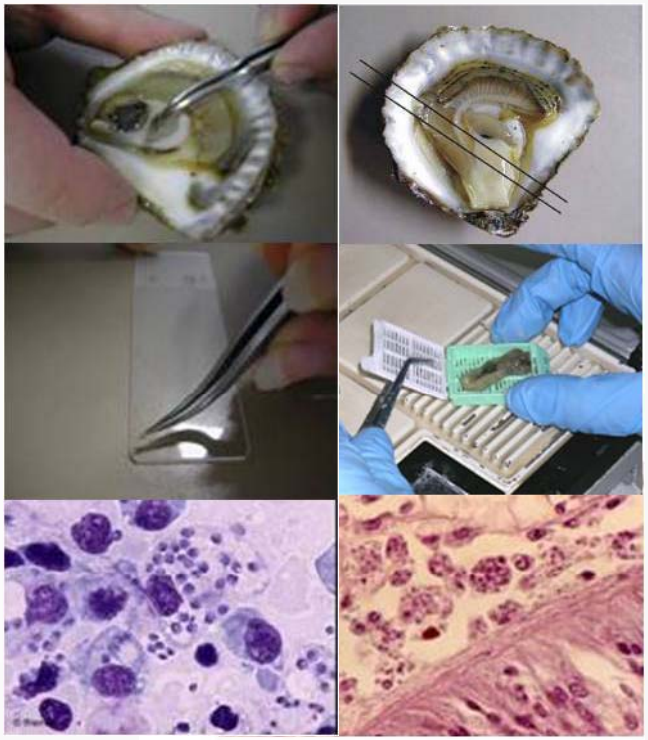
Complementary tools

To demonstrate and maintain free status

Endemic EU listed diseases



Data collected in 2012 based on 2011 national reports



Photos: Ifremer ©

Whereas PCR could be helpful, histology is still widely used

To investigate mortality outbreaks

All the diseases (endemic, exotic, potentially emerging diseases)

General tool= Histology and bacteriology

To observe any abnormal lesions/pathogens and to isolate main bacterial strains

+

Presumptive methods=Cytology, PCR, fresh smears, RFTM

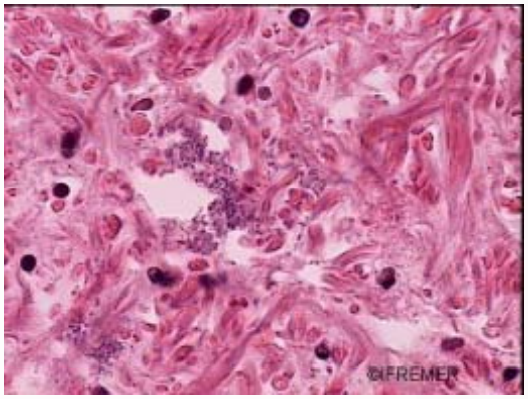
To be selected depending on the affected species

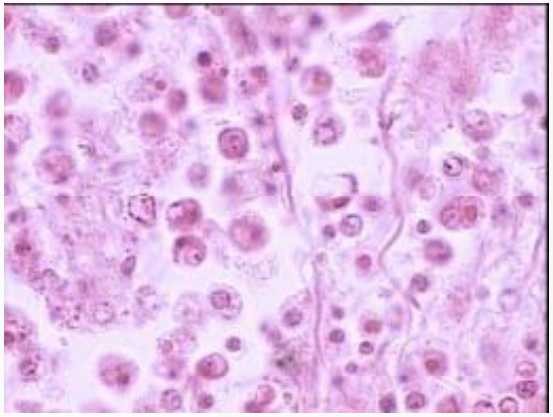
To investigate mortality outbreaks

All the diseases (endemic, exotic, potentially emerging diseases)

General tool= Histology

Histology allows detecting a wide range of pathogens but is not suitable for most of bacterial and viral diseases





To investigate mortality outbreaks

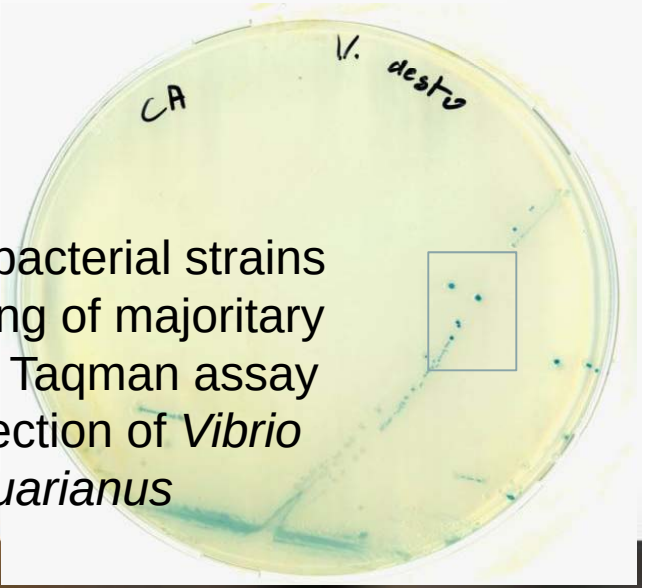
More probable pathogens based on available information

Presumptive methods=Cytology, PCR, RFTM, fresh smears

PCR assays for the detection of OsHV-1 (µvar)

Example: mortality outbreak reported in *Crassostrea gigas*

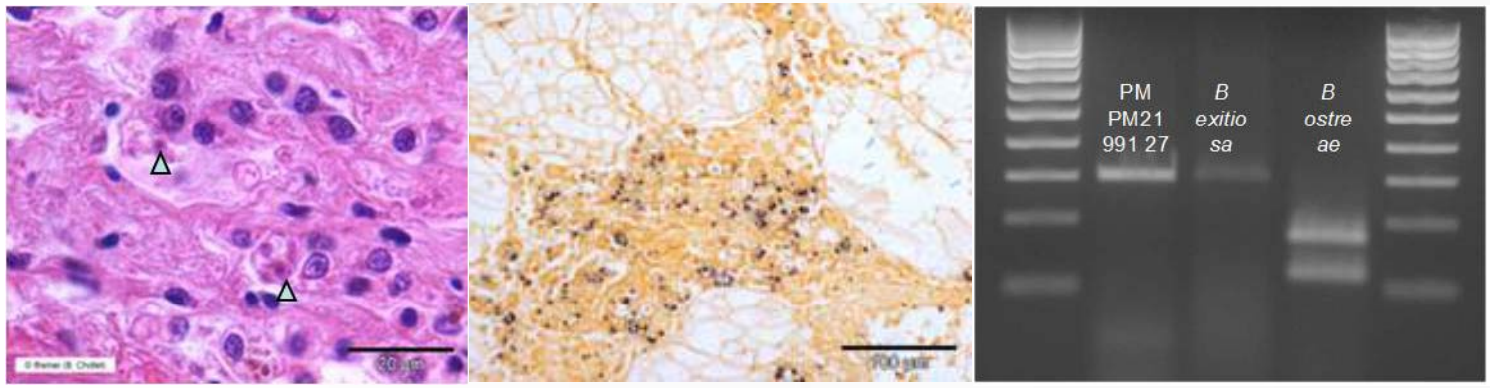
Isolation of bacterial strains and screening of majoritary strain using Taqman assay for the detection of *Vibrio aestuarianus*



To confirm a suspicion

Better characterization and demonstration of true infection

Example : confirmation of the presence of *Bonamia exitiosa* in flat oysters from EU



Combination of histology, *in situ* hybridization, PCR-RFP and sequencing

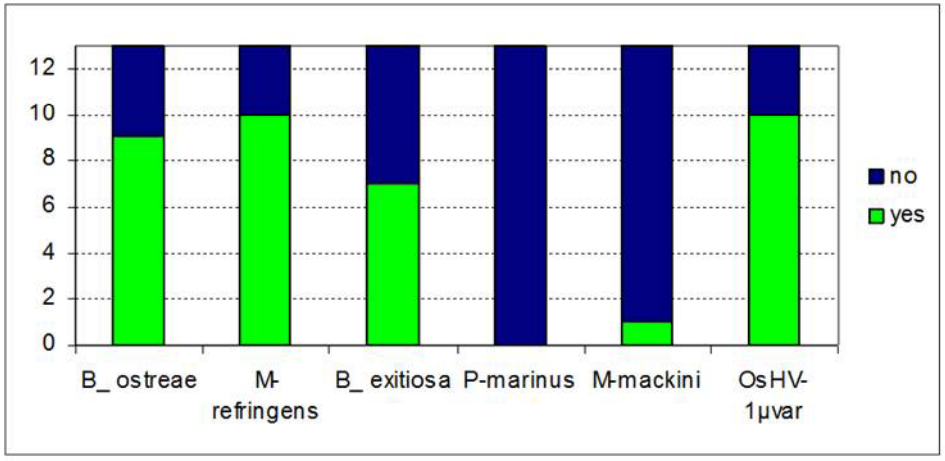
Diagnostic approach in EU

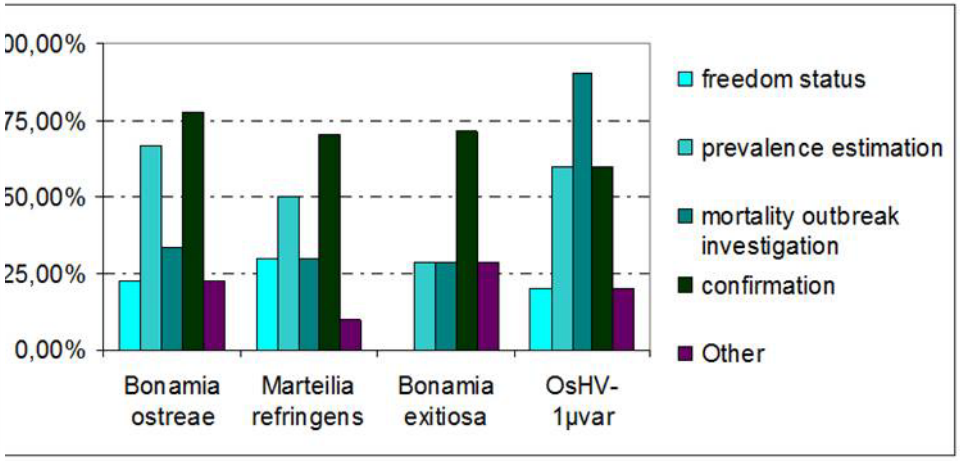
1. Histology remains an important tool for the detection of mollusc diseases

   → Workshops, training sessions
   Interlaboratory competency tests

2. When appropriate, more and more MS move from histology to PCR

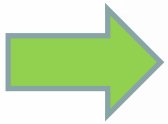
   → Validation, comparison efforts still required





Diagnostic approach in EU

3. Transfer of some confirmatory techniques to the NRLs network



Example: workshop on *in situ* hybridization for the detection of *Bonamia* spp. and *Marteilia refringens* organised in La Tremblade in 2013
Standard operating procedures available on the EURL website

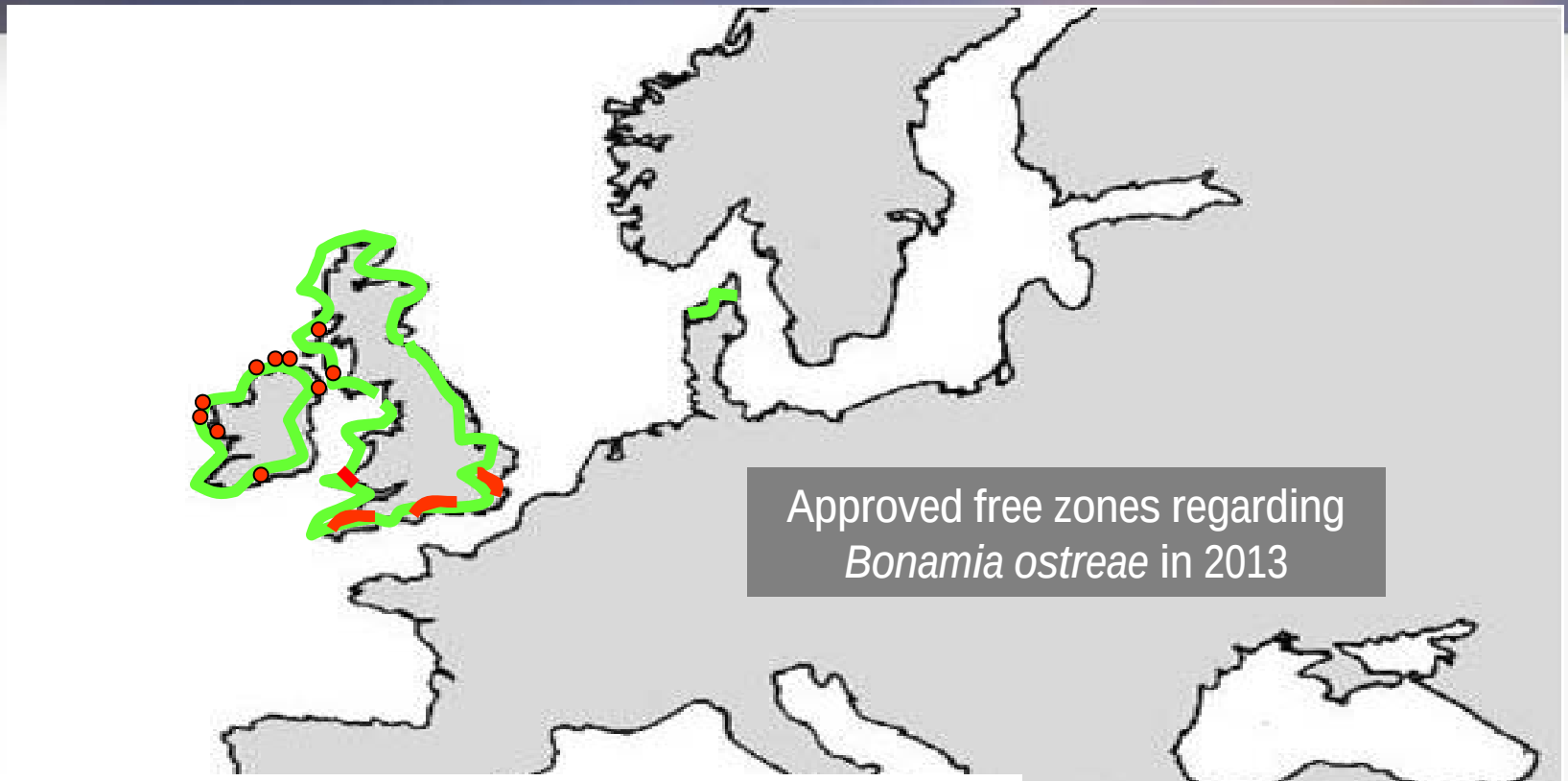
However, in case of detection of an exotic pathogen, confirmation by the EURL is requested by the European Commission

Main mollusc diseases in EU

Which are the mollusc diseases of significance in EU ?

	Pathogens	Susceptible species
Listed European pathogens	*Bonamia ostreae* *Marteilia refringens*	Flat oysters Mussels
Listed exotic pathogens	*Perkinsus marinus* *Mikrocytos mackini* *Bonamia exitiosa*	Cupped oysters Flat oysters
Listed at national level	OsHV-1 µvar	Cupped oysters
OIE listed but not EU listed pathogens	*Perkinsus olseni* *Xenohaliotis californiensis* Abalone Herpesvirus	Clams and abalone Abalone Abalone
Other pathogens	OsHV-1 *Vibrio aestuarianus* Other *Vibrio* species *Nocardia crassostreae* *Mikrocytos* sp. Trematods *Marteilia cochillia* *Haplosporidium spp.* …	Bivalves Cupped oysters + cockles? Cupped oysters Cupped and flat oysters Clams and donax Infaunal species Cockles Molluscs

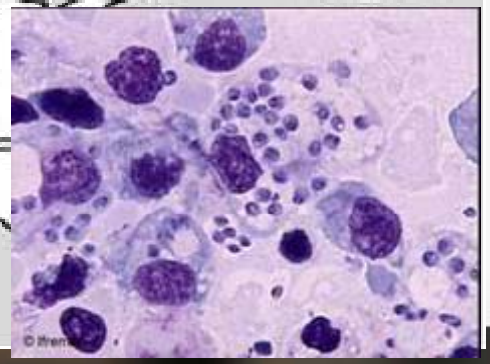
Infection with *Bonamia ostreae* in EU



Approved free zones regarding *Bonamia ostreae* in 2013

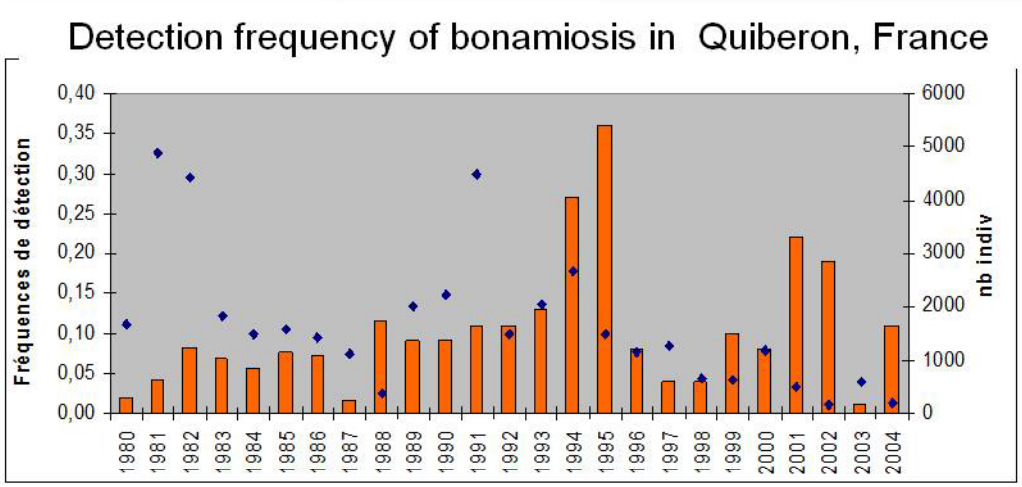
New sites detected infected during surveillance programmes carried out these last years in Ireland, in UK and in Norway

Enlargement of the distribution of the pathogen?

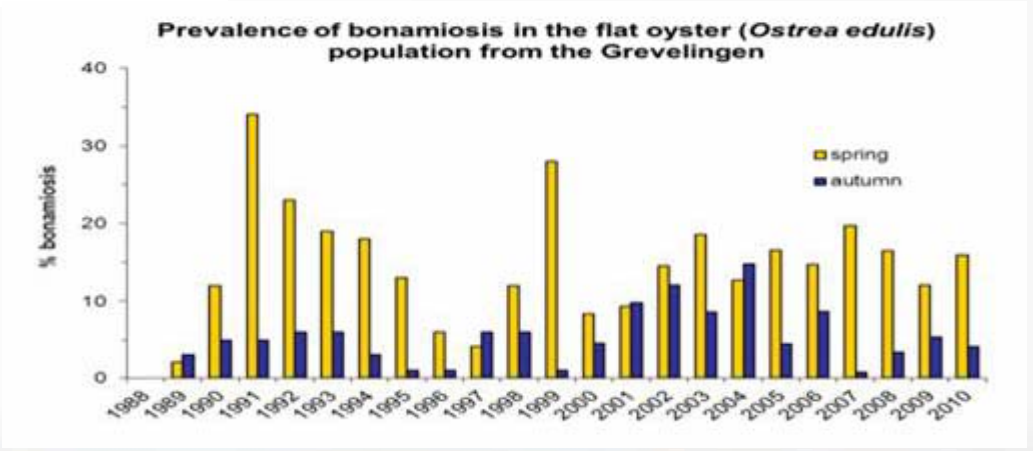


Infection with *Bonamia ostreae* in *Ostrea edulis*

No systematic monitoring in infected sites

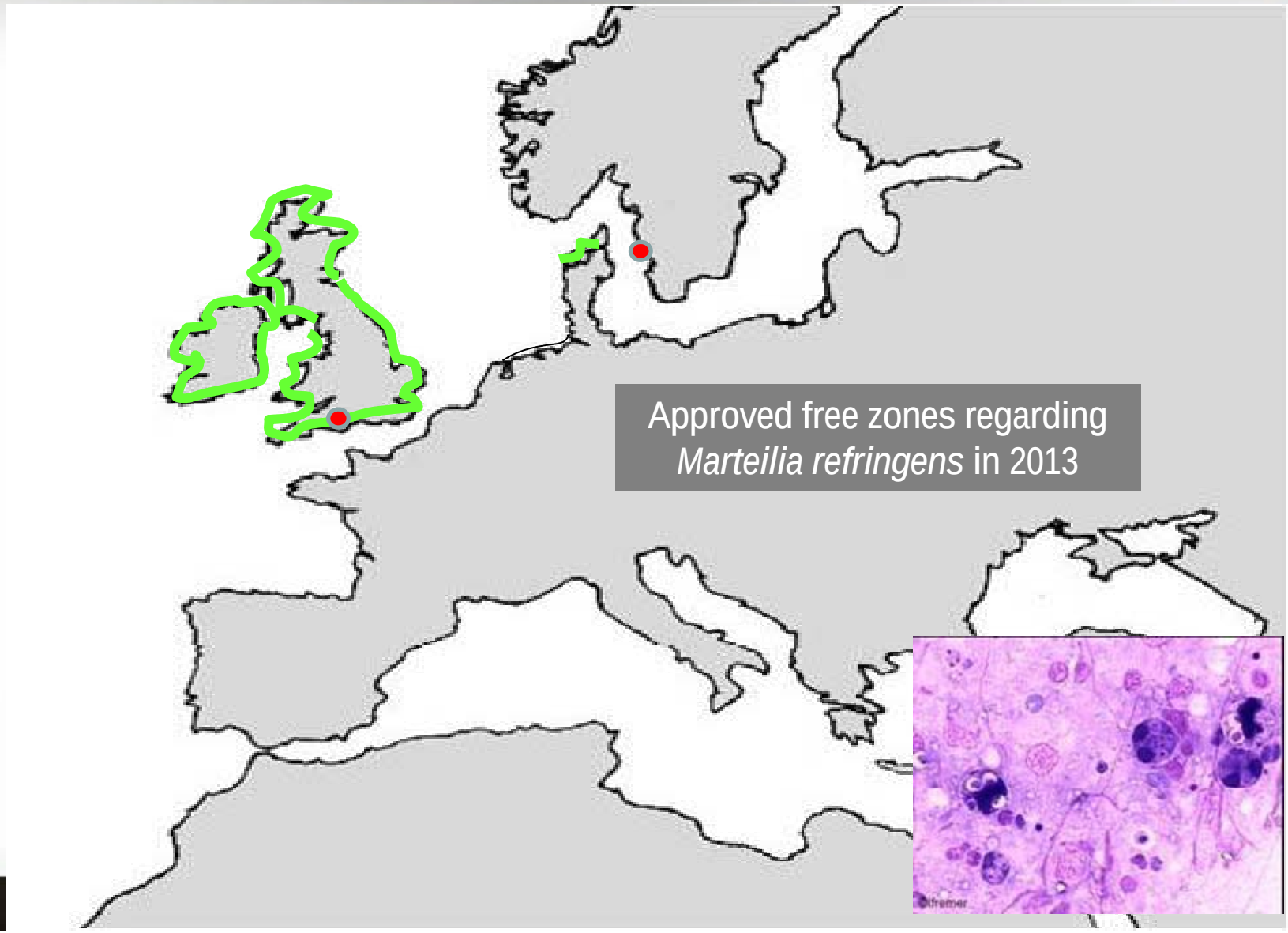


Data from I. Arzul



Data from Marc Engelsma

Infection with *Marteilia refringens* in EU



Bivalve host range of *Marteilia refringens* in EU

Host species	Suspected host species	Non susceptible species
Ostrea edulis	*Ruditapes decussatus, R. philippinarum*	*Crassostrea gigas :no mature stages*
Mytilus edulis *Mytilus galloprovincialis* *Xenostrobus securis*	*Tapes rhomboides, T. pullastra*	
Solen marginatus *Chamelea gallina*	*Ensis minor, E. siliqua*	

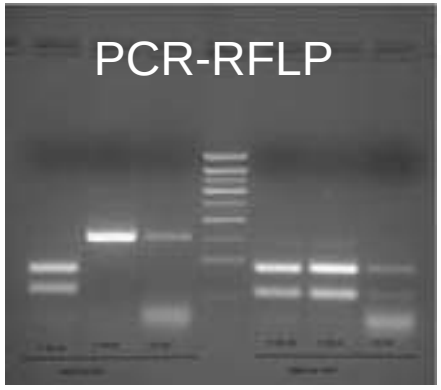
Infection with *Marteilia refringens* in EU

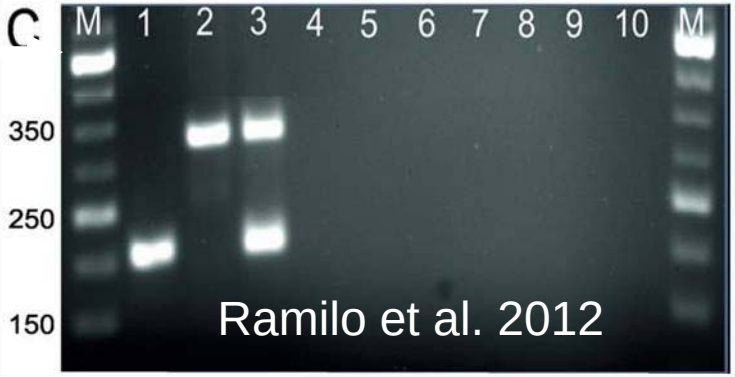
- These last years, new sites detected infected during surveillance programmes in mussels in Sweden and in UK or during research studies (Slovenia)
- The « enlargement » of the list of susceptible species has contributed to enlarge the official geographic range, however surveillance programmes regarding marteiliosis only target flat oysters and mussels
- No systematic monitoring in infected sites
- Systematic molecular characterization (typing) of the parasite when it is detected

Infection with *Bonamia exitiosa*

Since the notification of *Bonamia exitiosa* in Galicia, Spain in October 2007(Abollo et al. 2008), diagnostic of bonamiosis includes species characterization :

- PCR- RFLP + sequencing
- Specific PCR tools (Robert et al. 2009; Ramilo et al. 2012, …)

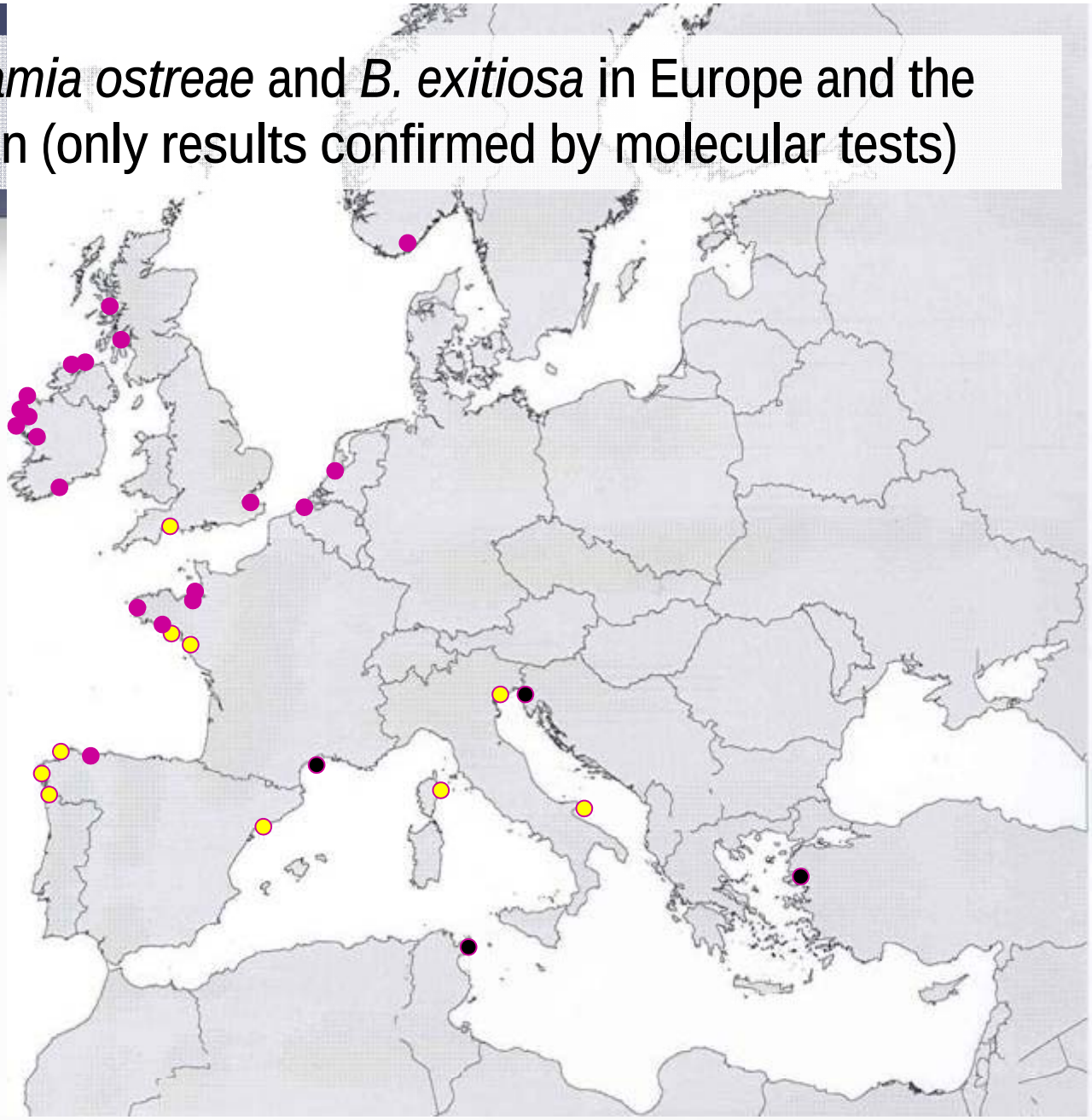




Cloning
PCR-RFLP and sequencing

Distribution of *Bonamia ostreae* and *B. exitiosa* in Europe and the Mediterranean basin (only results confirmed by molecular tests)

- *Bonamia ostreae*
- *Bonamia ostreae and B. exitiosa*
- *Bonamia exitiosa*

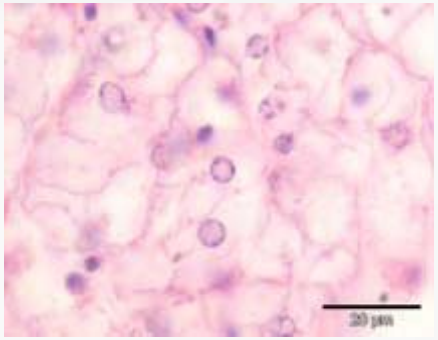


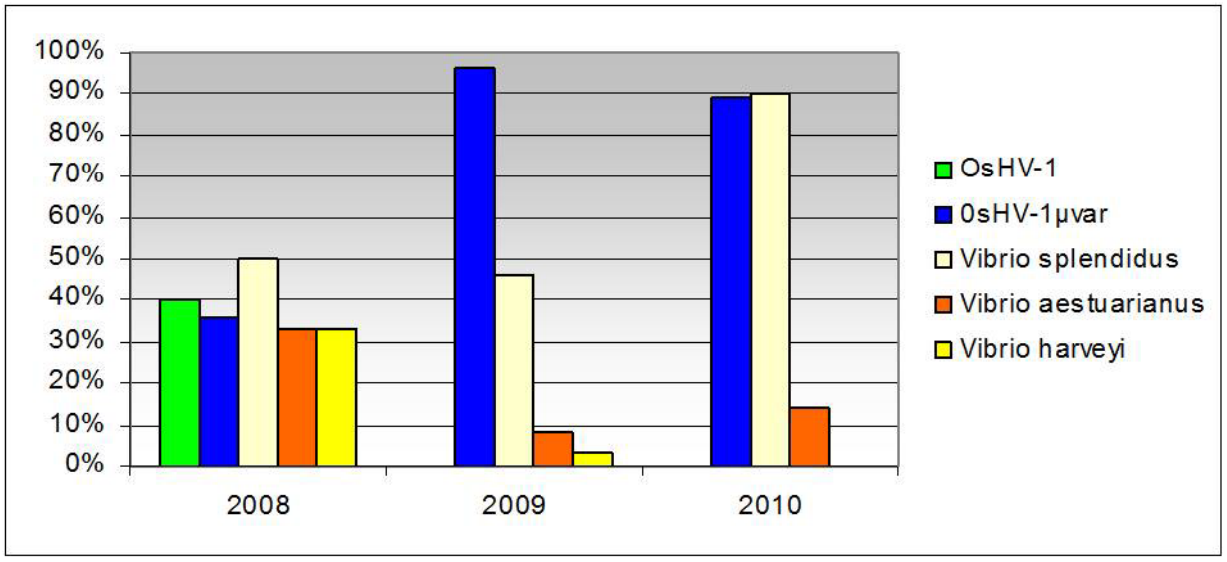
Infection with OsHV-1µvar

Pacific oyster mortality outbreaks in France

Histology : No detection of listed pathogens

Bacteriology and PCR for the detection of OsHV-1



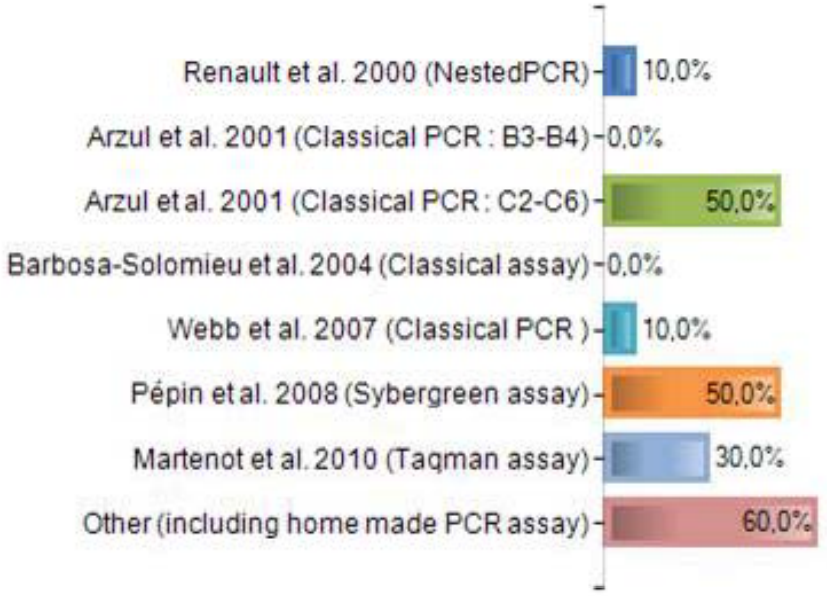


Several cases of co-infections (OsHV-1 and *Vibrio splendidus* and/or *V. aestuarianus)*

08 and 2009

Infection with OsHV-1µvar

Which PCR assays ?



Nested version of the C2C6 primers

EURL Locked Nucleic acid probe PCR (uVar specific)

1) Webb et al is used in SYBR Green setting;

2) Unpublished real time PCR Ifremer

Aranguren et al. , 2011

CF/CR primers - COMMISSION REGULATION (EU) No 175/2010

AFBI's in house designed Taqman assay

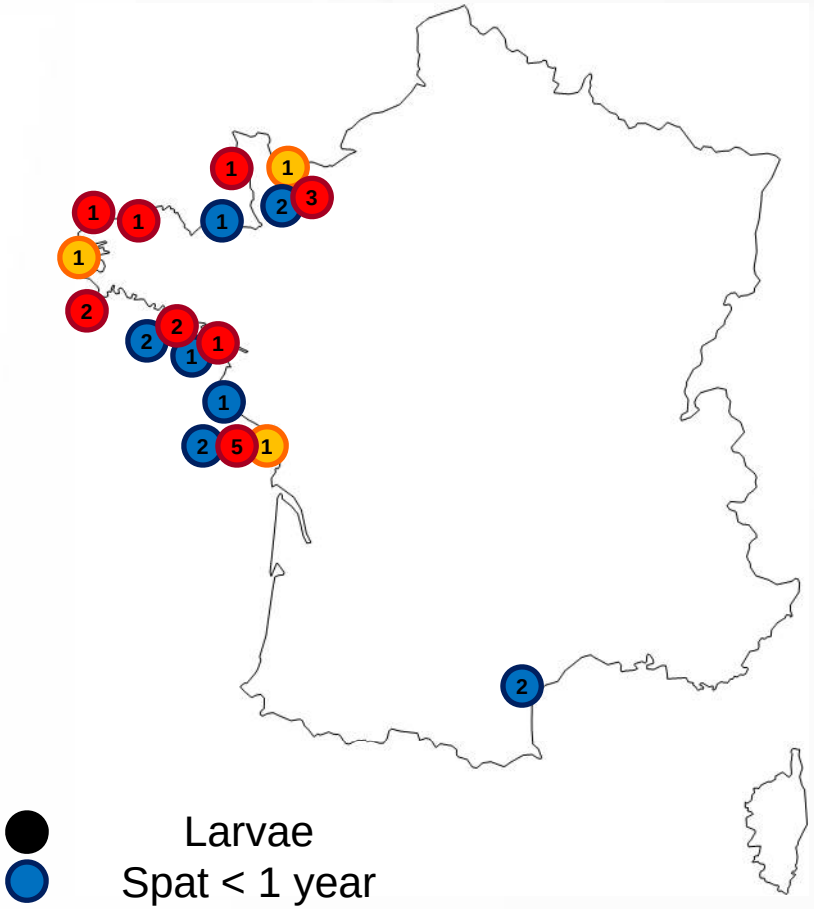
Distribution of OsHV-1 µvar in Europe

Detection of OsHV-1 µvar

(not always associated with mortalilty of *C. gigas*)

Vibrio aestuarianus in France

Vibrio aestuarianus in abnormal mortality analyzed – Repamo 2012



47 batches tested

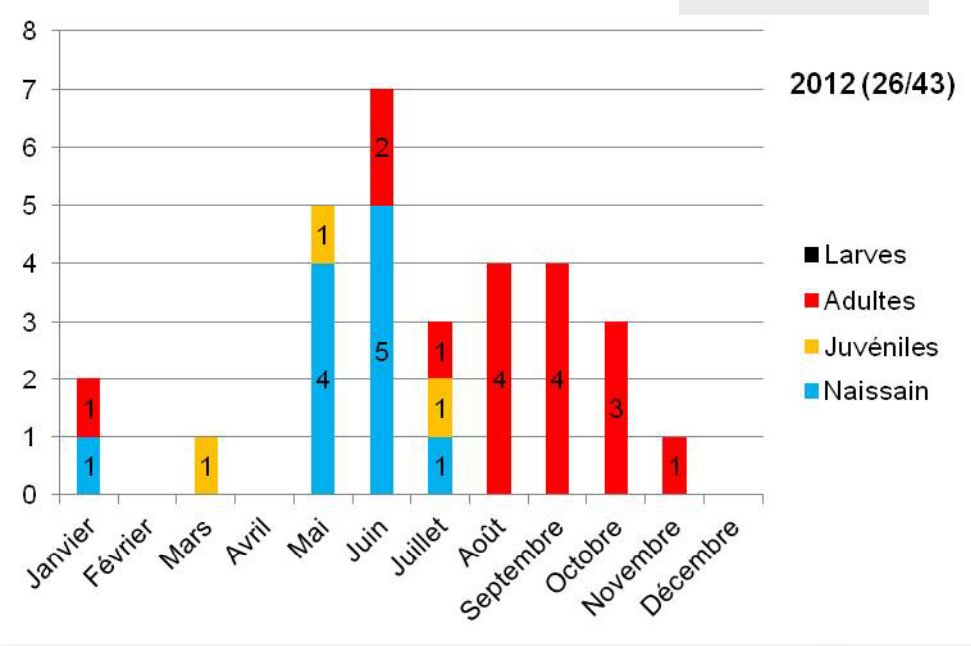
Detection of *V. aestuarianus* DNA using a specific Taqman assay in **30** batches :

11 spats

3 juveniles

16 adults

2012

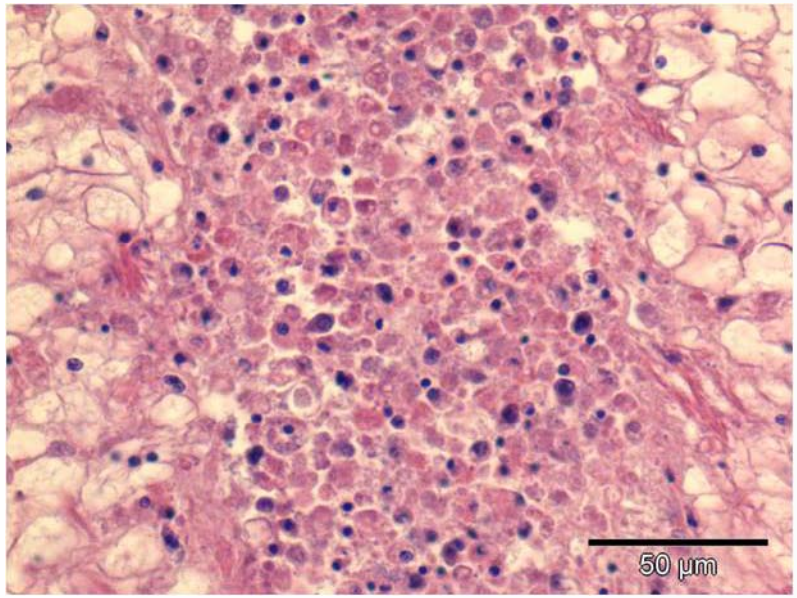


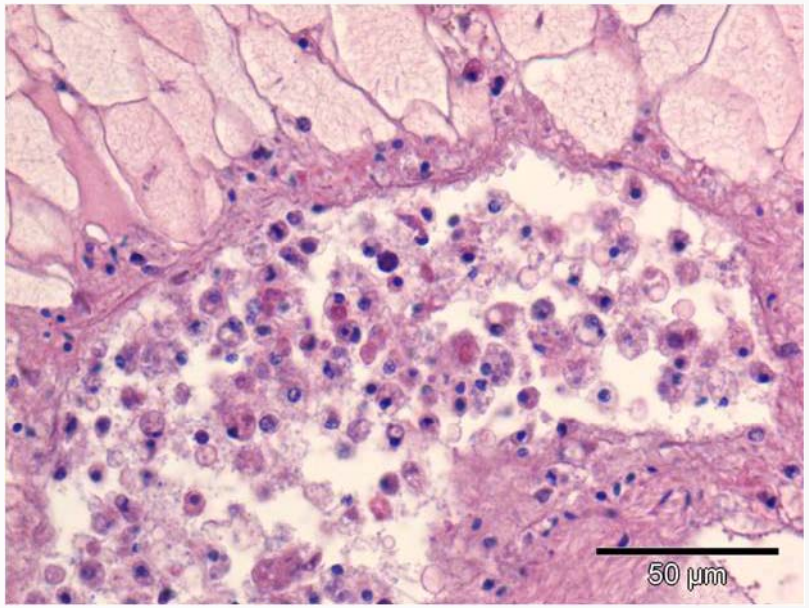
Data from Agnès Travers

Vibrio aestuarianus in France

In case of mortality:

- High concentration (up to 10^9 bacteria/ml) in oyster hemolymph
- No tissue lesions, except in hemolymphatic compartment

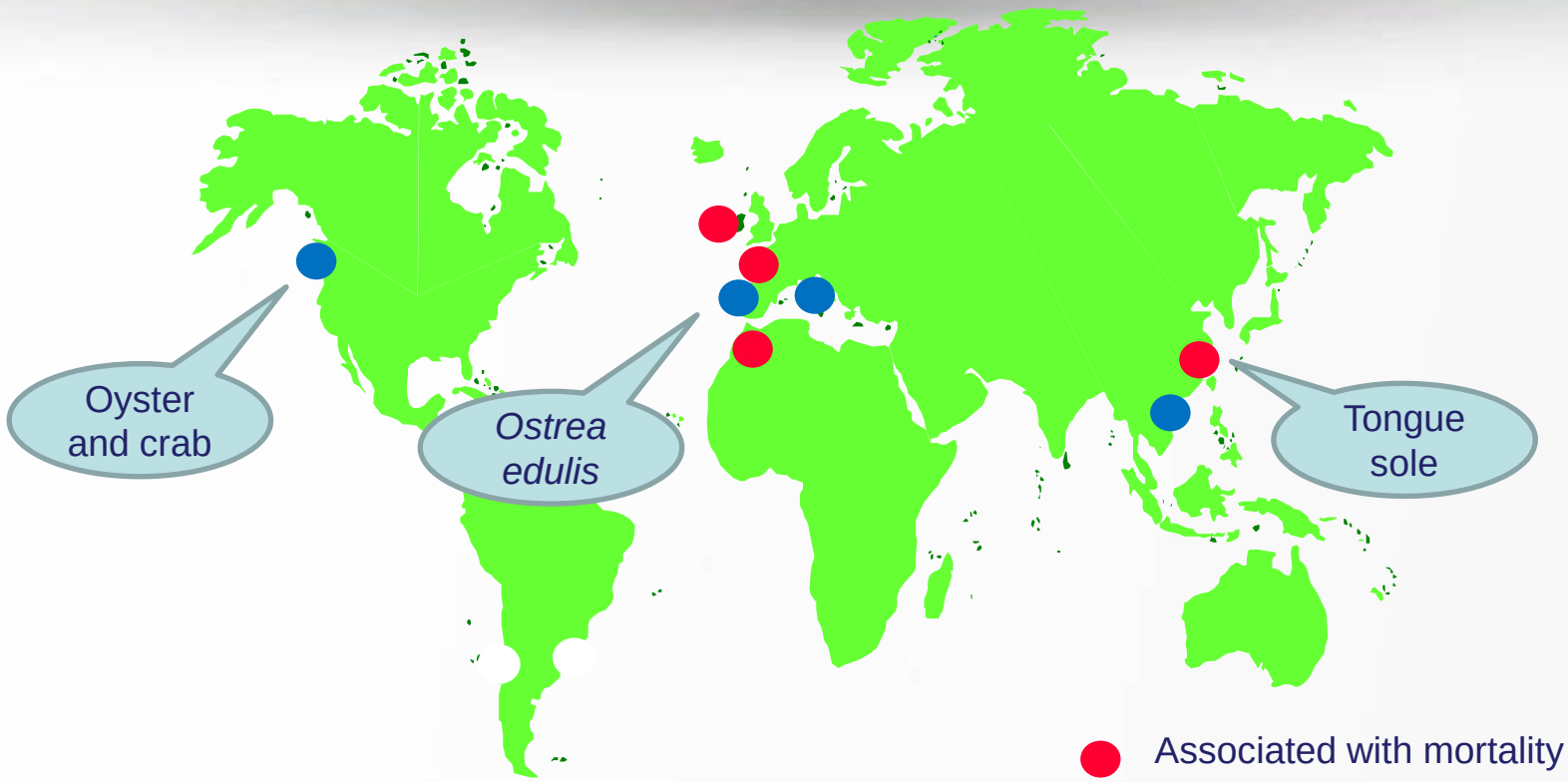




Photos JP Joly

Data from Agnès Travers

Vibrio aestuarianus in the world



Detection of *Vibrio aestuarianus* in *Crassostrea gigas* and other species

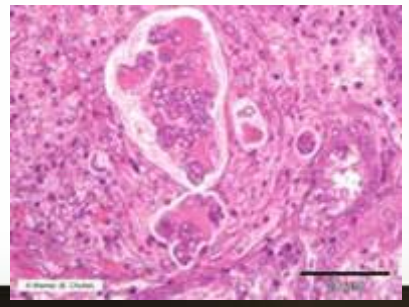
Infection with parasites of *Perkinsus olseni* and other related species

Perkinsus olseni= wide host and geographic ranges - Associated with mass mortalities in some parts of the world

-reported in southern European countries (France, Portugal, Spain, Italy)

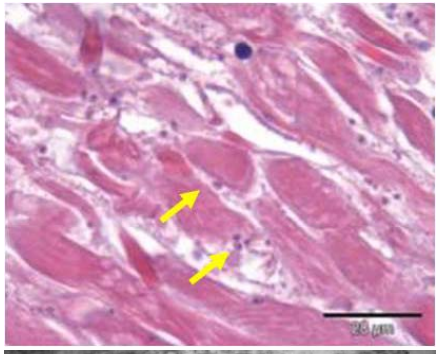
-diagnosis usually based on thioglycolate medium culture and histology

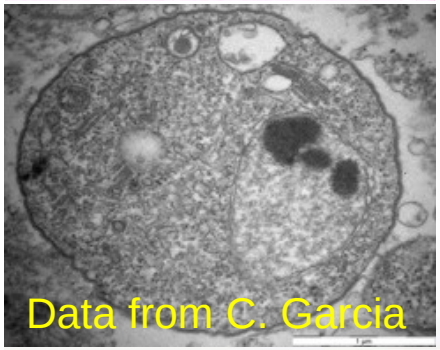
-recent characterization effort has been performed allowing the detection of two species : *Perkinsus olseni* and *P. chesapeaki* in France and Spain



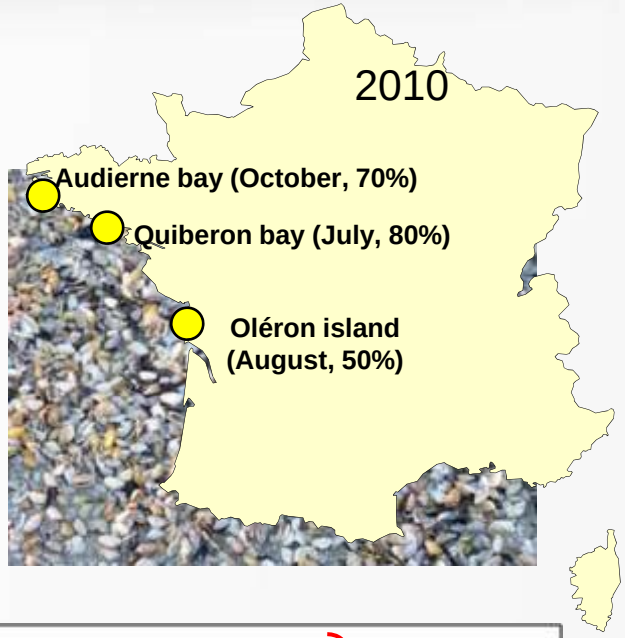

A newly detected pathogen associated with *Donax trunculus* mortality outbreaks

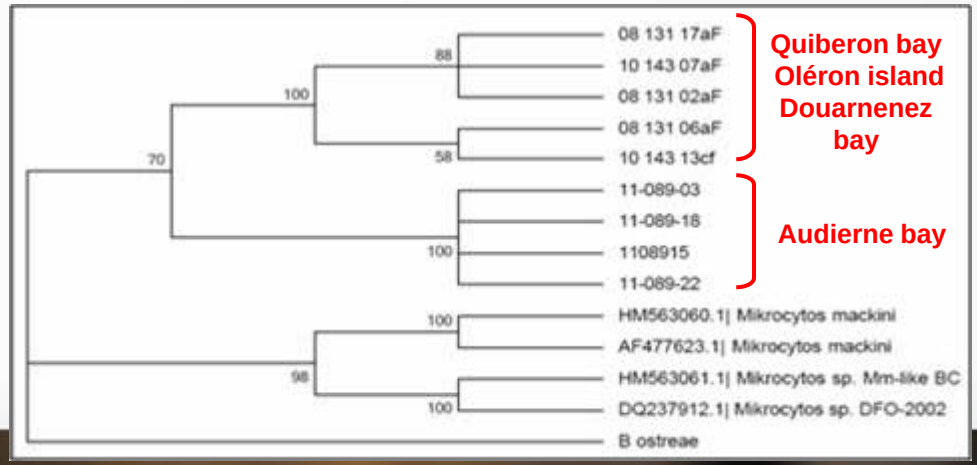
- Declaration of massive mortalities (up to 80%) of *Donax trunculus* juveniles and adults in 2008, 2010, 2011 and 2013
- Observation of microcell parasites (2-3µm) in different tissues, particularly in muscular tissue and mantle, intra or extracellular position, associated with haemocytic infiltration and necrosis





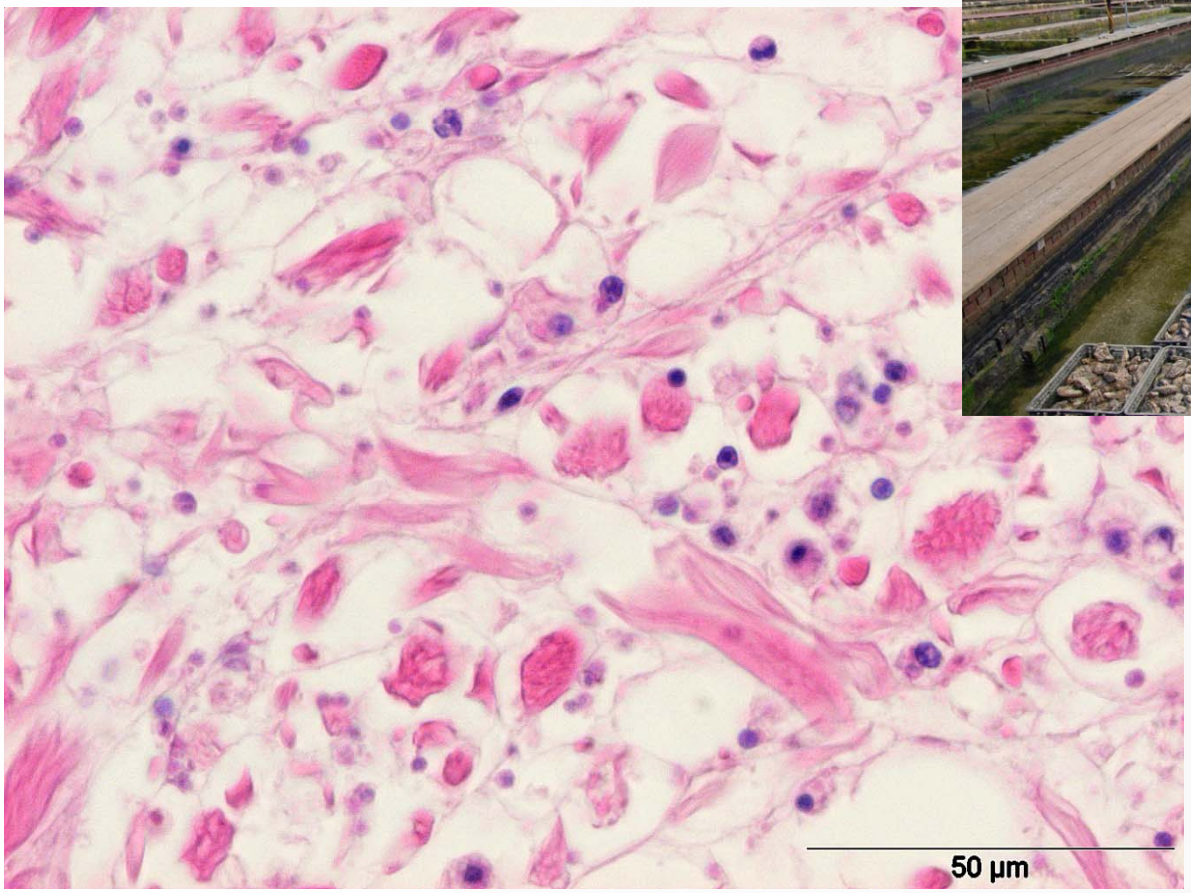
- 18S rRNA sequence : 79% of homology with other *Mikrocytos* sp.



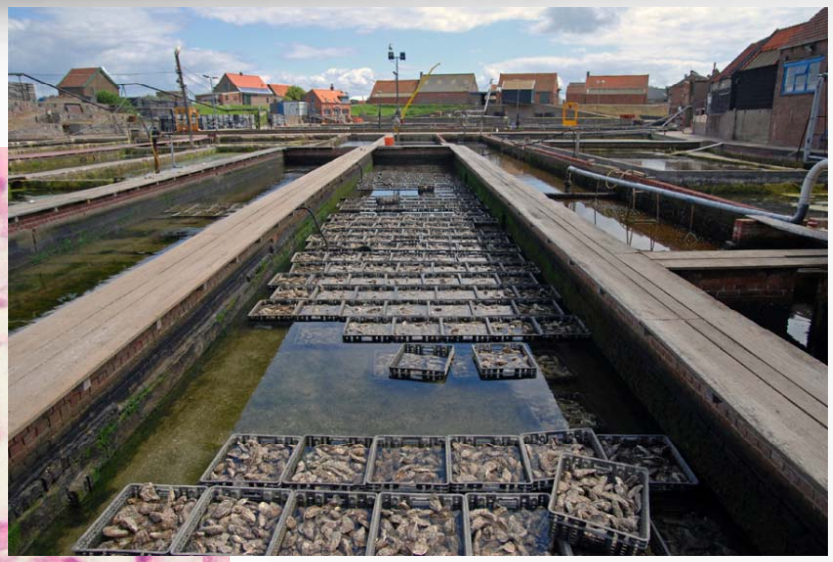


The same parasite was detected in other Member States in association with mortality

In Manila clam, *Ruditapes philippinarum* in The Netherlands



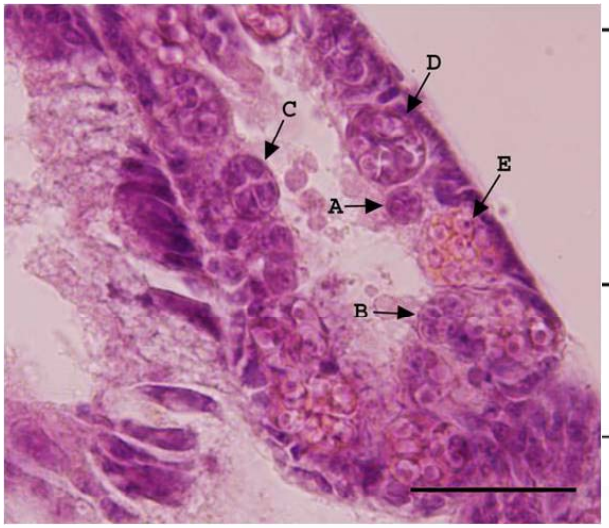
Data from M. Engelsma

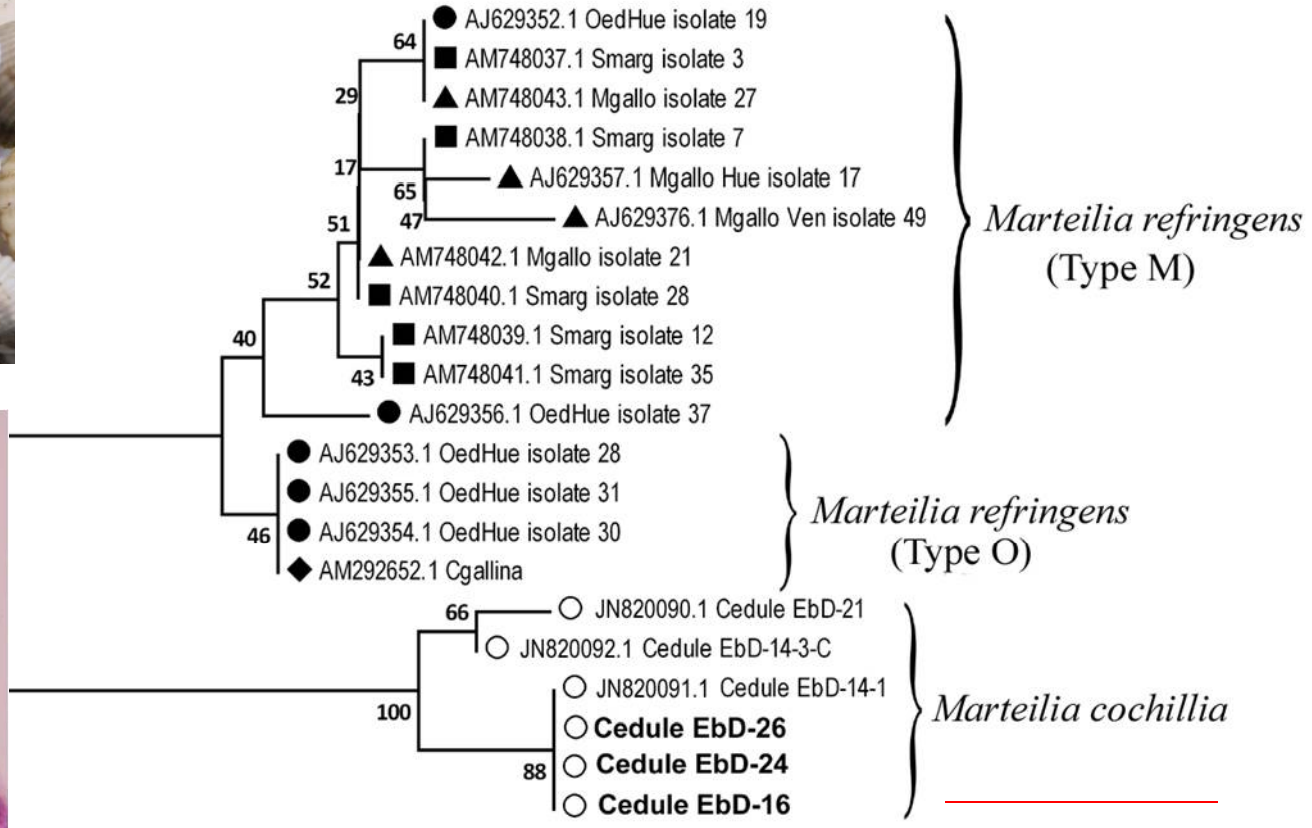


And in Manila clams in Galicia, Spain (Villalba et al.)

Marteilia cochillia affecting the edible cockle *Cerastoderma edule*

In cockles in delta del Ebro, Spain (Carrasco et al. 2013)





Data from N. Carrasco

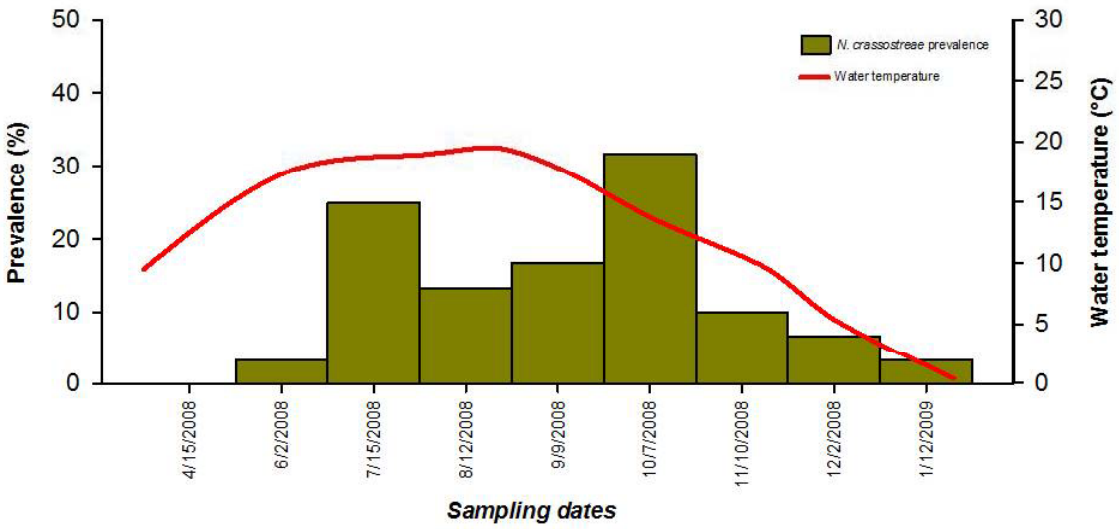
A similar pathogen has been characterized from cockles in Galicia (Villalba et al .2013 EAFP)

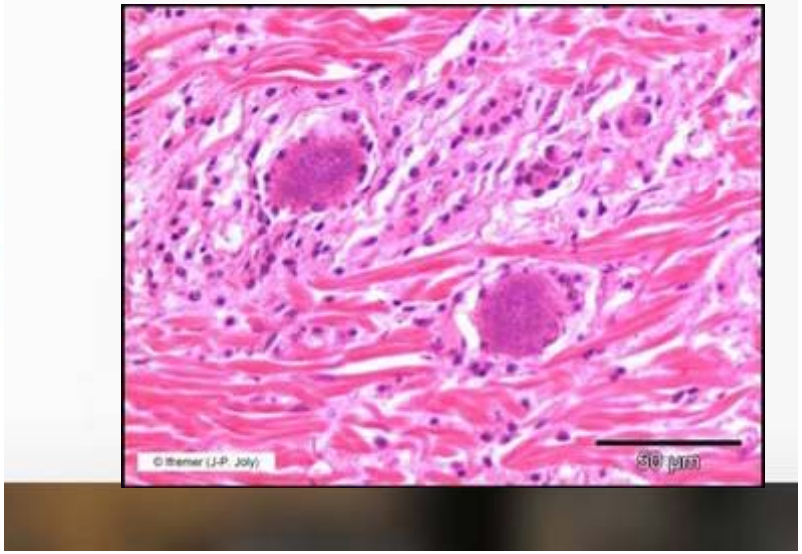
Infection with *Nocardia crassostreae*

Nocardia crassostreae reported in *Crassostrea gigas* from Japan, North America and sometimes associated with mortalities

Recently, *N. crassostreae* has been isolated in *C. gigas and Ostrea edulis* from **The Netherlands** (Engelsma *et al.* 2008) and *in O. edulis* and *Mytilus galloprovincialis* from **Italy** (Carella et al. 2013)

(data from M. Engelsma)





Conclusions perspectives

The geographic distribution of some pathogens has recently changed :

- apparent spread of some endemic pathogens

A better characterization of detected pathogens:

- *Bonamia exitiosa/B. ostreae*
- *Perkinsus olseni/P. chesapeaki*
- OsHV-1/OsV-1 µvar

Some emerging phenomenon :

- Mortality of *Crassostrea gigas* associated with OsHV-1 µvar and *Vibrio aestuarianus*
- Mortality of infaunal species associated with *Mikrocytos* sp. and *Marteilia cochillia* cause concern in some regions

Conclusions perspectives

Diagnostic approach evolves according to those changes

- Association of wide and specific tools
- Interest of multiplex tools

Some questions remain open

- pathogen species definition ?
- which target and for which purpose ?

Diagnostic is one part of the surveillance of diseases

- importance of sampling design
- interpretation of results depends on technique performances which are often lacking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Molluscan diseases of significance in New Zealand

Professor Andrea C. Alfaro

Aquaculture Biotechnology Group

Institute of Applied Ecology

Auckland University of Technology

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APPLIED ECOLOGY NEW ZEALAND
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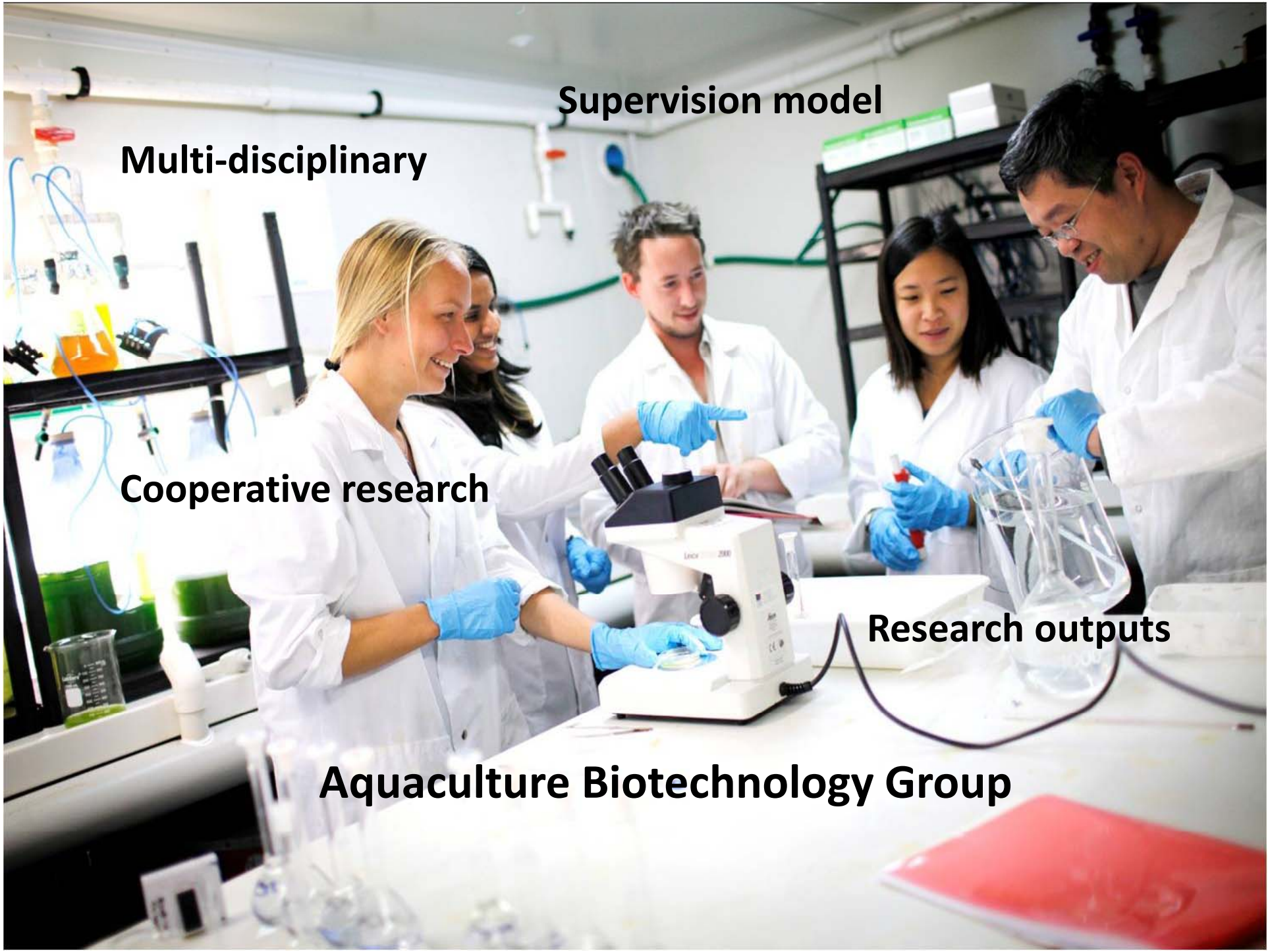
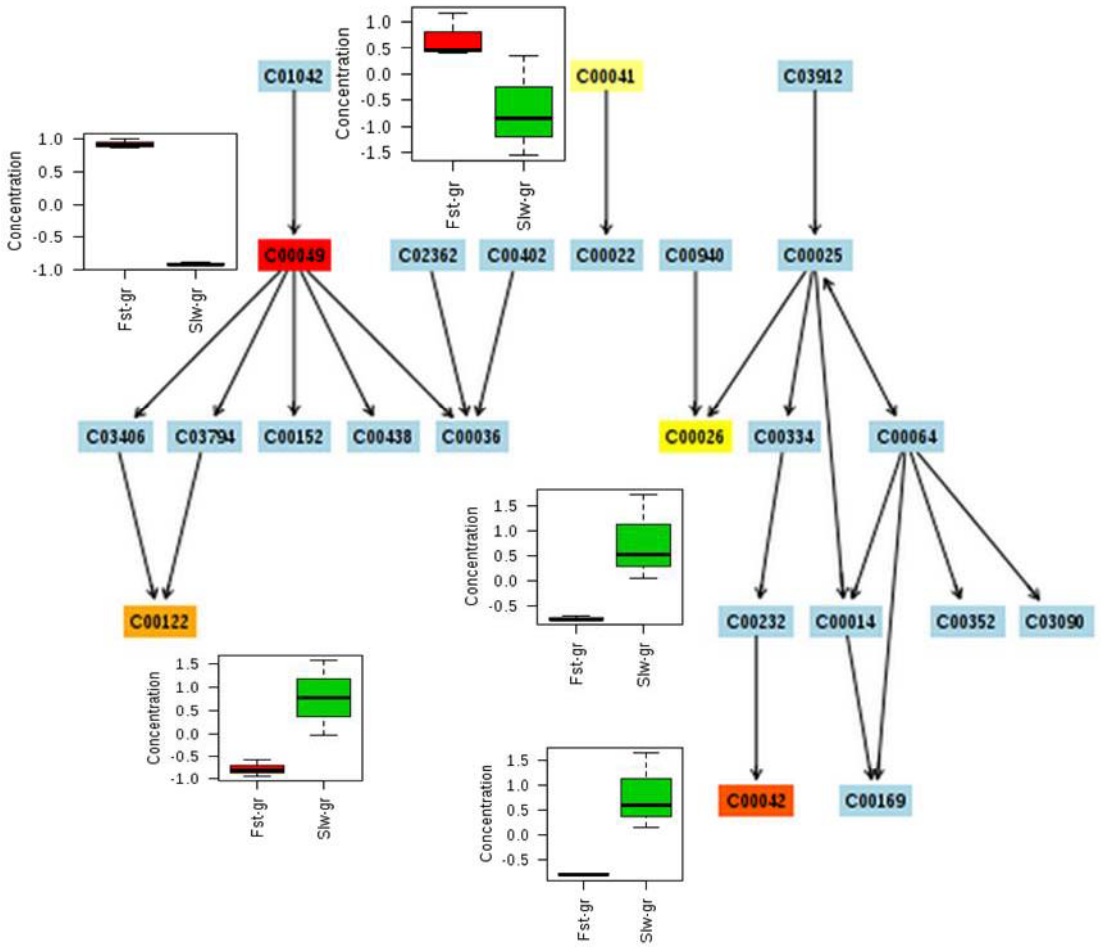
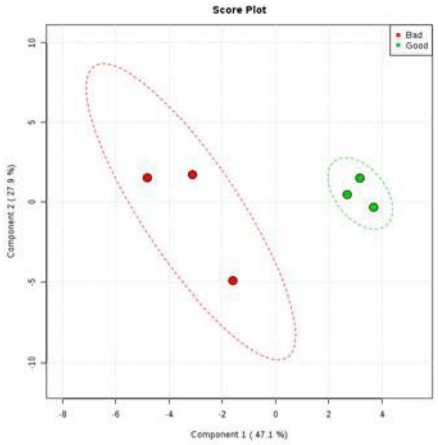
AUT
UNIVERSITY
TE WANANGA ARONUI
O TAMAKI MAKAU RAU


Supervision model
Multi-disciplinary
Cooperative research
Research outputs
Aquaculture Biotechnology Group

Mapping of relevant metabolites onto known biochemical pathways



Multivariate classification and feature reduction of individual metabolites



Energy production

Growth

Respiration

Waste removal

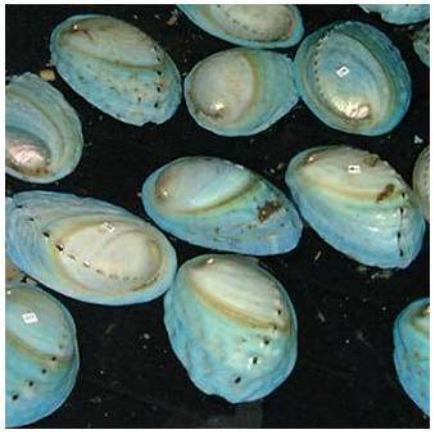
Cellular signalling

+ Neurotransmission

Immune system deficiencies


New Zealand Aquaculture
• Seafood is NZ's 4th or 5th largest export earner
• Target increase to $1 billion by 2025

Key Species



CAWTHRON INSTITUTE

REPORT NO. 1334

ASSESSMENT OF PATHOLOGY THREATS TO THE NEW ZEALAND SHELLFISH INDUSTRY

STEPHEN CHARLES WEBB

ISSUE DATE: 15 January 2013

Table i. Shortlists of significant pathogens. (note: this is not a systematic risk assessment) 1-10 (in blue) are resident/indigenous NZ pathogens - Table 6 in the text gives host/ pathology details. 1-26 in black are foreign agents posing a threat to NZ shellfish - Table 7 in the text gives host/pathology details.

	Agent/condition	Actual or potential NZ Hosts
1	Ostreid Herpes Virus	Flat oyster *(Ostrea chilensis)*, Pacific oyster *(Crassostrea gigas)*
2	*Bonamia exitiosa* (Protozoa)	Flat oyster *(Ostrea chilensis)*
3	Apicomplexan X (Protozoa)	Greenshell™ mussel *(Perna canaliculus)*, Flat oyster *(Ostrea chilensis)*
4	*Perkinsus olseni* (Protozoa)	NZ cockle *(Austrovenus stutchburyi)*, *Macomona liliana*, *Barbatia novaezelandia*, *Paphies* sp.
5	Digestive epithelial virosis	Greenshell™ mussel *(Perna canaliculus)*, Blue mussel *(Mytilus galloprovincialis)*, Scallop *(Pecten novaezelandiae)*, NZ cockle *(Austrovenus stutchburyi)*
6	Paua *Haplosporidium* sp. (Protozoa)	Paua *(Haliotis iris)*
7	*Enterogonia orbicularis* and other flatworms (Platyhelminthes)	Pacific oyster *(Crassostrea gigas)*, Greenshell™ mussel *(Perna canaliculus)*
8	Shell mycosis (Fungus)	Paua *(Haliotis iris)*
9	Scallop Mycoplasmas (bacteria)	Scallop *(Pecten novaezelandiae)*
10	Rickettsioses (Bacteria)	Pacific oyster *(Crassostrea gigas)*, Flat oyster *(Ostrea chilensis)*, Scallop *(Pecten novaezelandiae)*, NZ cockle *(Austrovenus stutchburyi)*, Rock oyster *(Saccostrea glomerata)*, Fan scallop *(Chlamys delicatula)*
1	*Marteilia maurini/refringens* (Protozoa)	Greenshell™ mussel *(Perna canaliculus)*, Flat oyster *(Ostrea chilensis)*
2	Disseminated hemic neoplasia (Virus?)	Greenshell™ mussel *(Perna canaliculus)*, Blue mussel *(Mytilus galloprovincialis)*
3	Oyster velar virus disease	Pacific oyster *(Crassostrea gigas)*
4	Viral ganglioneuritis	Paua *(Haliotis iris)*
5	*Ctyophaga* spp., hinge ligament disease. (Bacteria)	Pacific oyster *(Crassostrea gigas)*
6	*Bonamia ostreae* (Protozoa)	Flat oyster *(Ostrea chilensis)*
7	*Mikrocytos (Bonamia) roughleyi* (Protozoa)	Rock oyster *(Saccostrea glomerata)*
8	*Xenohaliotis californiensis* (Bacteria)	Paua *(Haliotis iris)*
9	*Labyrinthuloides haliotidis* (Fungus-like protozoan)	Paua *(Haliotis iris)*
10	Gill necrosis virus disease	Pacific oyster *(Crassostrea gigas)*
11	*Francisella* sp. (Bacteria)	Paua *(Haliotis iris)*
12	*Marteilioides chungmuensis* (Protozoa)	Pacific oyster *(Crassostrea gigas)*, Rock oyster *(Saccostrea glomerata)*
13	QX disease - *Marteilia sydneyi* (Protozoa)	Rock oyster *(Saccostrea glomerata)*
14	Brown ring disease (Bacteria)	Venerid clams
15	Chytrid-like disease (Fungus-like protozoan)	Clams
16	Scallop *Marteilia* sp. (Protozoa)	Scallop *(Pecten novaezelandiae)*
17	Mycoplasmosis (Bacteria)	Scallop *(Pecten novaezelandiae)*
18	*Terebrasabella heterouncinata* (Annelid worm)	Paua *(Haliotis iris)*
19	*Sirolpidium zoophthorum* (Fungus)	Venerid clams, oysters and scallops
20	Amyotrophia Wasting disease (Possibly viral)	Paua *(Haliotis iris)*
21	Gill Rickettsia (Bacteria)	Scallop *(Pecten novaezelandiae)*
22	*Perkinsus qugwadi* (Protozoa)	Scallop *(Pecten novaezelandiae)*
23	*Perkinsus marinus, P. andrewsi, P. chesapeaki* (Protozoa)	Clams
24	*Steinhausia mytilovum* (Protozoa)	Greenshell™ mussel *(Perna canaliculus)*
25	*Vibrio harveyi* (Bacteria)	Paua *(Haliotis iris)*
26	Infectious Pancreatic Necrosis virus (IPNV)	Venerid clams (possible moderate to severe threat to salmonids)

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2	*Bonamia exitiosa* (Protozoa)	Flat oyster *(Ostrea chilensis)*
3	Apicomplexan X (Protozoa)	Greenshell™ mussel *(Perna canaliculus)*, Flat oyster *(Ostrea chilensis)*
4	*Perkinsus olseni* (Protozoa)	NZ cockle *(Austrovenus stutchburyi)*, *Macomona liliana*, *Barbatia novaezelandia*, *Paphies* sp.
5	Digestive epithelial virosis	Greenshell™ mussel *(Perna canaliculus)*, Blue mussel *(Mytilus galloprovincialis)*, Scallop *(Pecten novaezelandiae)*, NZ cockle *(Austrovenus stutchburyi)*
6	Paua *Haplosporidium* sp. (Protozoa)	Paua *(Haliotis iris)*
7	*Enterogonia orbicularis* and other flatworms (Platyhelminthes)	Pacific oyster *(Crassostrea gigas)*, Greenshell™ mussel *(Perna canaliculus)*
8	Shell mycosis (Fungus)	Paua *(Haliotis iris)*
9	Scallop Mycoplasmas (bacteria)	Scallop *(Pecten novaezelandiae)*
10	Rickettsioses (Bacteria)	Pacific oyster *(Crassostrea gigas)*, Flat oyster *(Ostrea chilensis)*, Scallop *(Pecten novaezelandiae)*, NZ cockle (*Austrovenus stutchburyi)*, Rock oyster *(Saccostrea glomerata)*, Fan scallop (*Chlamys delicatula)*
		Greenshell™ mussel *(Perna canaliculus)*, Flat oyster *(Ostrea*

Table ii. Assessment of pathogen threat to important New Zealand mollusc culture species. Pathogen species names in blue signify that they are in NZ. Information levels R = Report derived, E = Estimate, S = Surmise.

	Known parasite/pathogens	Pathological threat	
		Actual	Potential
Greenshell™ mussel *(Perna canaliculus)*	*Marteilia maurini*	Not here R	Extreme E
	Marteilia refringens	Not here R	Extreme E
	Disseminated hemic neoplasia	Not here R	High E
	Digestive epithelial virosis (DEV)	Low R	Moderate R
	Vibrio splendidus and a *V. coralliilyticus/neptunius*-like	Low R	Moderate R
	Vibrio harveyi	Not here?	Moderate E
	Cercaria haswelli - Tergestia agnostomi	Low R	Moderate S
	Flatworms eg: *Enterogonia orbicularis*	Low R	Moderate R
	Orchitophryid ciliates	Low R	Moderate R
	Steinhausia mytilovum	Not here R	Moderate S
	Proctoeces spp.	Not here R	Low/moderate E
	Haplosporidium costale	Not here R	Low/ moderate S
	Marteilia sp.	Not here R	Low E
	Apicomplexan X	Low R	Low E
	Mytilicola orientalis/intestinalis	Not here R	Low S
	Haplosporidium sp.	Not here R	Low S
	Polydorids (native and alien)	Low/high R	Moderate/high S
Pacific oyster *(Crassostrea gigas)*	Ostreid Herpes Virus (OsHV-1)	Extreme R	Extreme R
	Hinge ligament disease *Ctyophaga* spp.	Not here R	Extreme E
	Oyster velar virus disease	Not here R	Extreme E
	Flatworms eg: *Enterogonia orbicularis*	Low/moderate R	Moderate /high S
	Rickettsiae	Low/moderate R	Moderate S
	Gill necrosis virus disease	Not here R	Low S
	Marteilioides chungmuensis	Not here	Moderate S
	Polydorids (native and alien)	Low/high R	Moderate/high S
	Echinocephalus crassostreai	Not here R	PR issue – impact unknown
	Juvenile oyster disease	Not here	Low S
	Nocardia crassostreae	Not here R	Moderate R
	Mikrocytos mackini	Not here	Low R
	Haplosporidium nelsoni MSX	Not here R	Low R
	Sirolpidium zoophthorum	Not here R	High E
	Stylochus spp.	NZ status uncertain	Moderate S
Paua *(Haliotis iris)*	Viral ganglioneuritis	Not here R	Extreme S
	Haplosporidium sp.	High R	Extreme S Threat to export?
	Labyrinthuloides haliotidis	Not here R	Extreme E
	Xenohaliotis californiensis	Not here R	Extreme E
	Francisella sp.	Not here R	Extreme E
	Polydorids (native and alien)	Low/high R	Moderate/high S
	Terebrasabella heterouncinata	Not here R	High E
	Amyotrophia	Not here R	High E

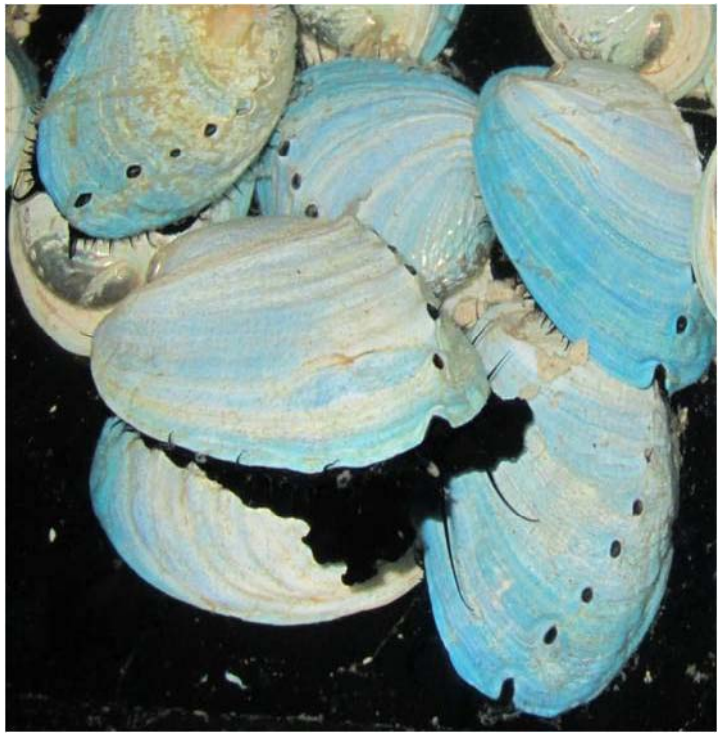
Greenshell™ mussel (*Perna canaliculus*)

- No serious pathological threats.
- Mortalities (50-100%) reported to be due to virus-like particles and digestive tubule damage.
- Sporadic mortalities due to an unenveloped RNA virus

Pacific oyster (*Crassostrea gigas*)

- First introduced in the 1940s.
- Ostreid herpes virus (OsHV-1).
- Reported: oyster velar virus, *Nocardia*, rickettsiae, vibrosis, *Enterogonia orbicularis* and polydorids.

Abalone (*Haliotis iris*)



- Reported: Haplosporidia, epithelial erosion, rickettsial inclusions in gut, protozoa in foot epithelium, bacterial infections, non-specific necrosis, granuloma-like lesions, hemocytic neoplasia-like inflammation and gregarines (apicomplexans).
- Postule disease by *Vibrio*, fungal shell mycosis, and shell-boring spionid mud worms.

Flat or Bluff oyster (*Ostrea chilensis*)

- Most significant pathogen: *Bonamia exitiosa*.
- Mortalities due to haplosporidian protozoan and apicomplexan X (APX) protozoan.
- Others: Mudworm infestations, the protozoan *Microsporidium rapuae*, the digenean trematode *Bucephalus longicornutus* , the copepod *Pseudomyicola spinosus*, etc.

Scallop (*Pecten novaezelandiae*)

- Numerous parasites, but not serious.
- Significant ones: digestive epithelial virosis (DEV), Rickettsia-like organisms (RLO) and a new unidentified inclusion.

Geoduck (*Panopea zelandica*)

- Little information, but reported: tubule changes suggestive of digestive epithelial virosis, nephridial granules, ciliates on the siphon, bryozoan on the siphon and copepods in the gut.

Cockle (*Austrovenus stutchburyi*)

- Potential threat by parasite *Perkinsus olseni*.
- Others: digestive tubules, etc.

Pathology Threats

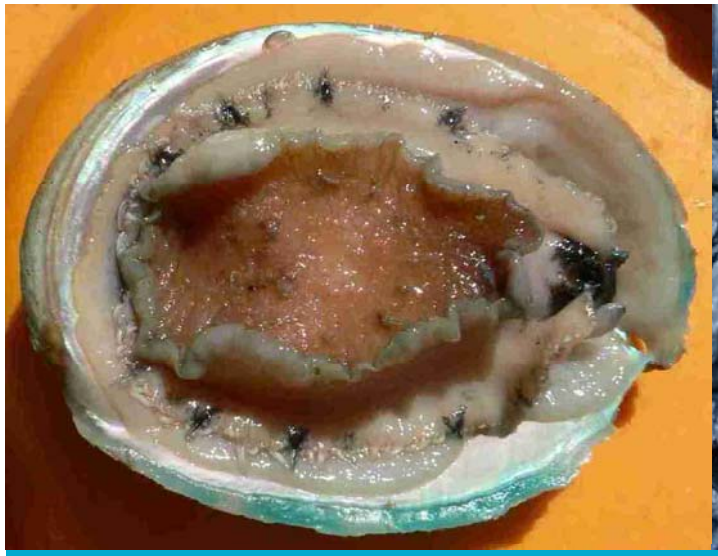
- Indigenous pathogens
- Alien pathogens
- Factors: transfer, establishment and dispersal pathways
- Need for basic biology
- Threats to exports

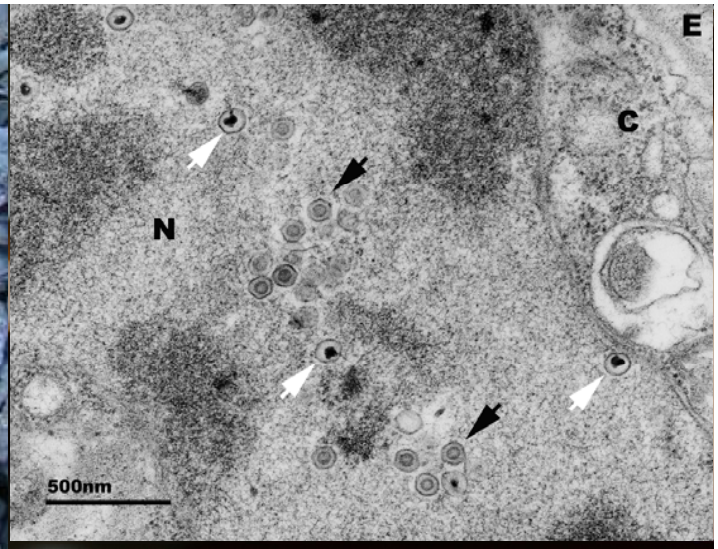
Recommendations

- Formulation of credible policies for managing the risks our pathogens pose to overseas customers
- Knowledge of current acceptable import standards
- Active surveying and collation of data on NZ pathologies
- Planning for support of these activities

Further Research

- Transmission experiments of NZ *Perkinsus olseni* on abalone.
- Parasite load or pathology on *Perna canaliculus* and *Mytilus galloprovincialis.*
- Assessment of *E. orbicularis* numbers in export mussels.
- Purification and sequencing of DEV virus.
- Examination of genetic affinities of the various DEV viruses of this type world-wide.
- Assess transmission and virulence in and between different hosts.
- Assess APX transmissibility and virulence to other bivalves.
- Purification and sequencing of APX for identification and detection.
- Surveying for IPNV and related aquabirnaviuses viruses of mussels cultured susceptible finfish.
- Identify inclusion in scallops with transmission electron microscopy.





Mollusc diseases of significance in Australia and their (molecular) diagnosis

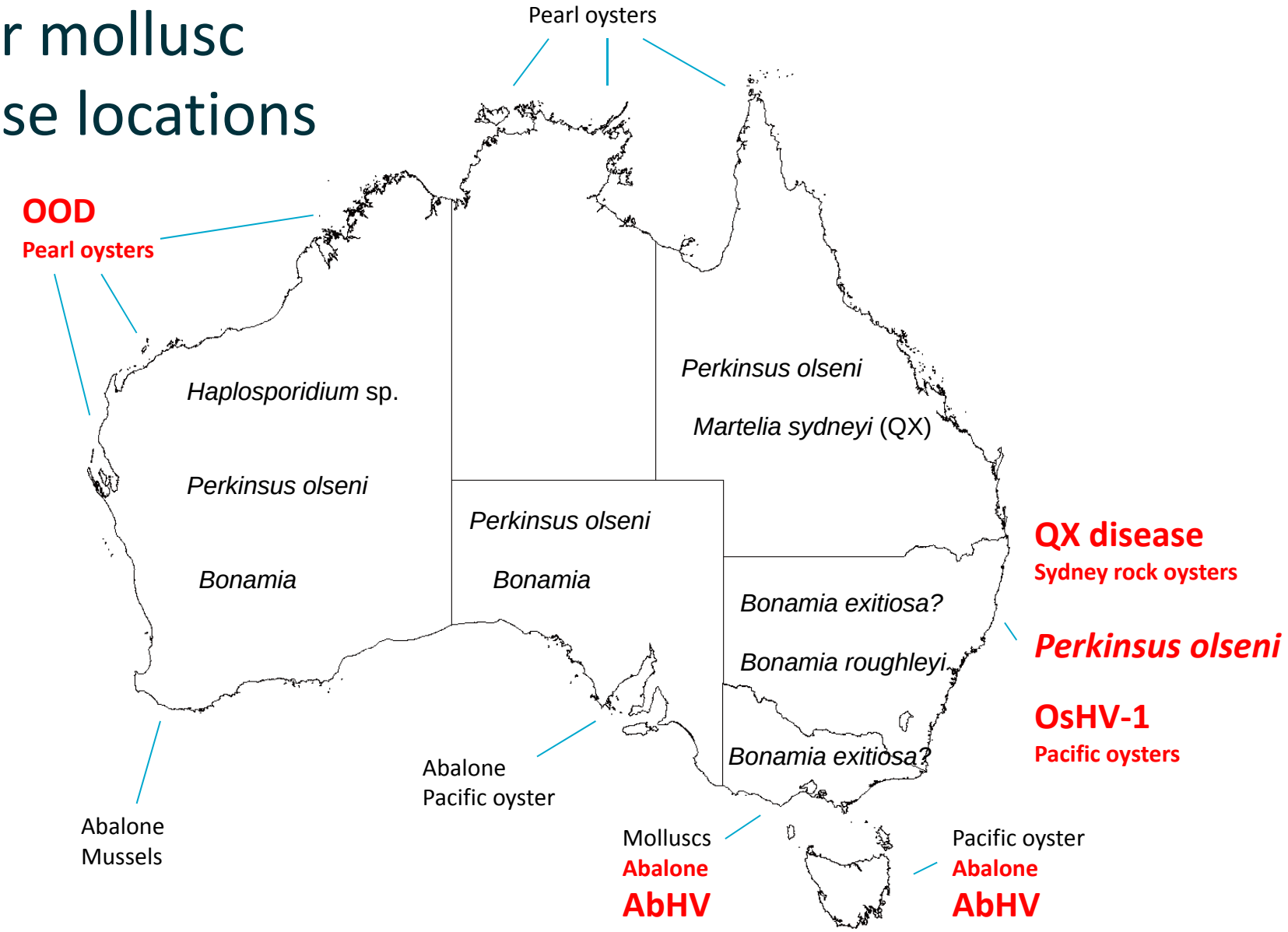
MOLLUSC DIAGNOSTIC WORKSHOP

Serge Corbeil| Aquatic Animal Virologist

October 21-24, 2013

LIVESTOCK INDUSTRIES
www.csiro.au

Major mollusc disease locations



Strategy used:
Screen by qPCR (if available) and confirm by cPCR and sequencing.

Abalone Herpesvirus (AbHV)

Abalone Viral Ganglioneuritis (AVG)

Blacklip abalone

Greenlip abalone

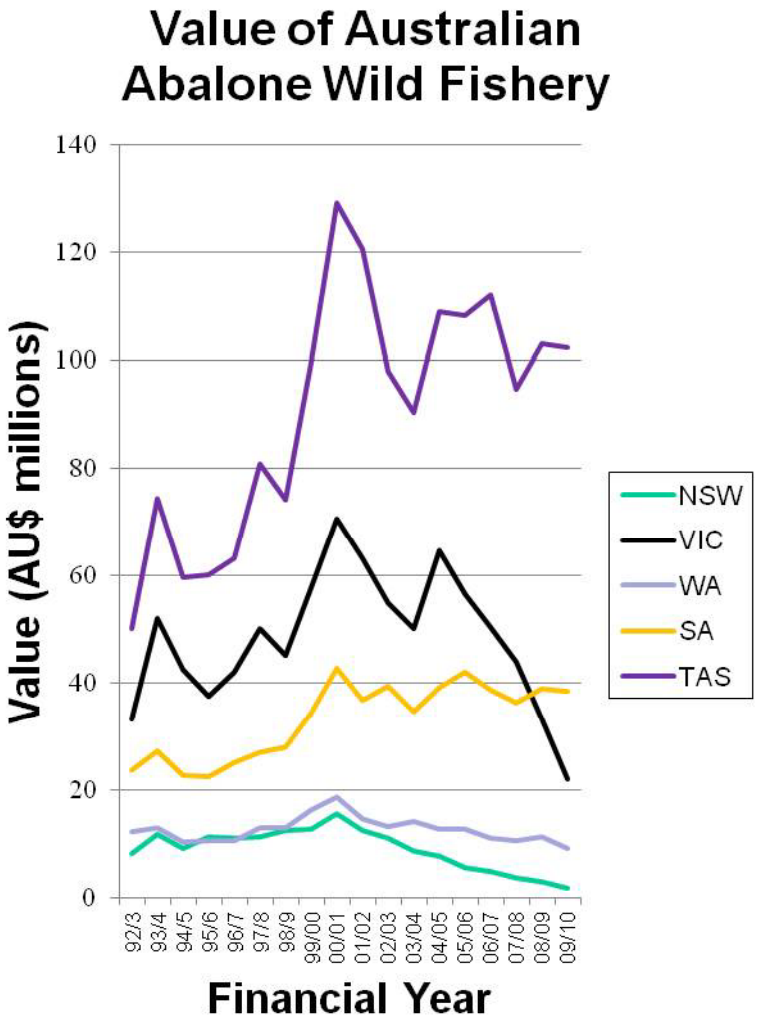
Hybrids

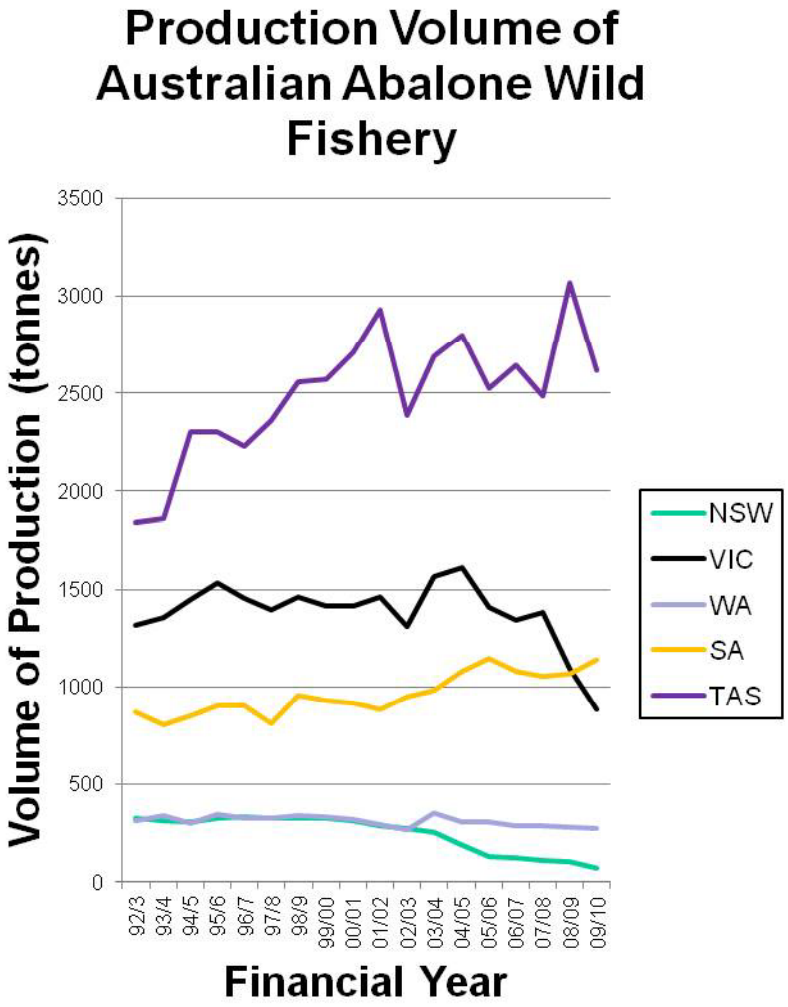
Abalone Herpesvirus (AbHV)

- December 2005: Mortalities farmed abalone in Victoria
- May 2006: Mortalities in wild abalone in Victoria
- Abalone herpesvirus (AbHV) causes Abalone Viral Ganglioneuritis (AVG)

From Corbeil et al 2010 Dis Aquat Org 92: 1-10

AbHV


Value of Australian Abalone Wild Fishery
Value (AU$ millions)
140
120
100
80
60
40
20
0
92/3
93/4
94/5
95/6
96/7
97/8
98/9
99/00
00/01
01/02
02/03
03/04
04/05
05/06
06/07
07/08
08/09
09/10
Financial Year
NSW
VIC
WA
SA
TAS


Production Volume of Australian Abalone Wild Fishery
Volume of Production (tonnes)
3500
3000
2500
2000
1500
1000
500
0
92/3
93/4
94/5
95/6
96/7
97/8
98/9
99/00
00/01
01/02
02/03
03/04
04/05
05/06
06/07
07/08
08/09
09/10
Financial Year
NSW
VIC
WA
SA
TAS

AbHV

Diagnostic tests developed and used:

-cPCR (3) (AAHL) (Vic and Tas)

-qPCR (TaqMan) (3) (AAHL and VicDPI)

-In situ hybridisation assay (VicDPI)

Ostreid Herpesvirus (OsHV-1)

Pacific Oyster Mortality Syndrome (POMS)

OsHV-1

-Identified in NSW in November 2010.

-Estimated loss for 2013 season $3M.

-Zero prospect of restocking juvenile oysters therefore 2014 production will be zero.

OsHV-1

Diagnostic tests used at AAHL:

-cPCR C2-C6 (Ifremer-OIE)

-qPCR (TaqMan) (2) (OIE and EMAI)

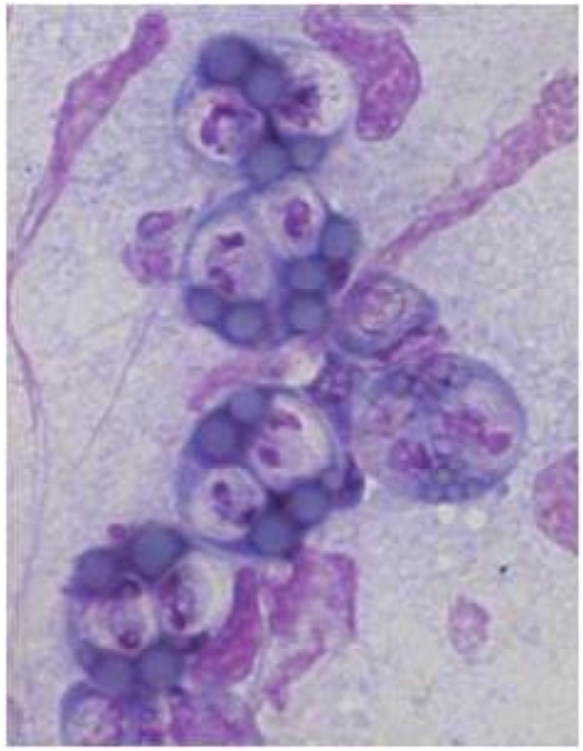
Martelia sydneyi

QX disease

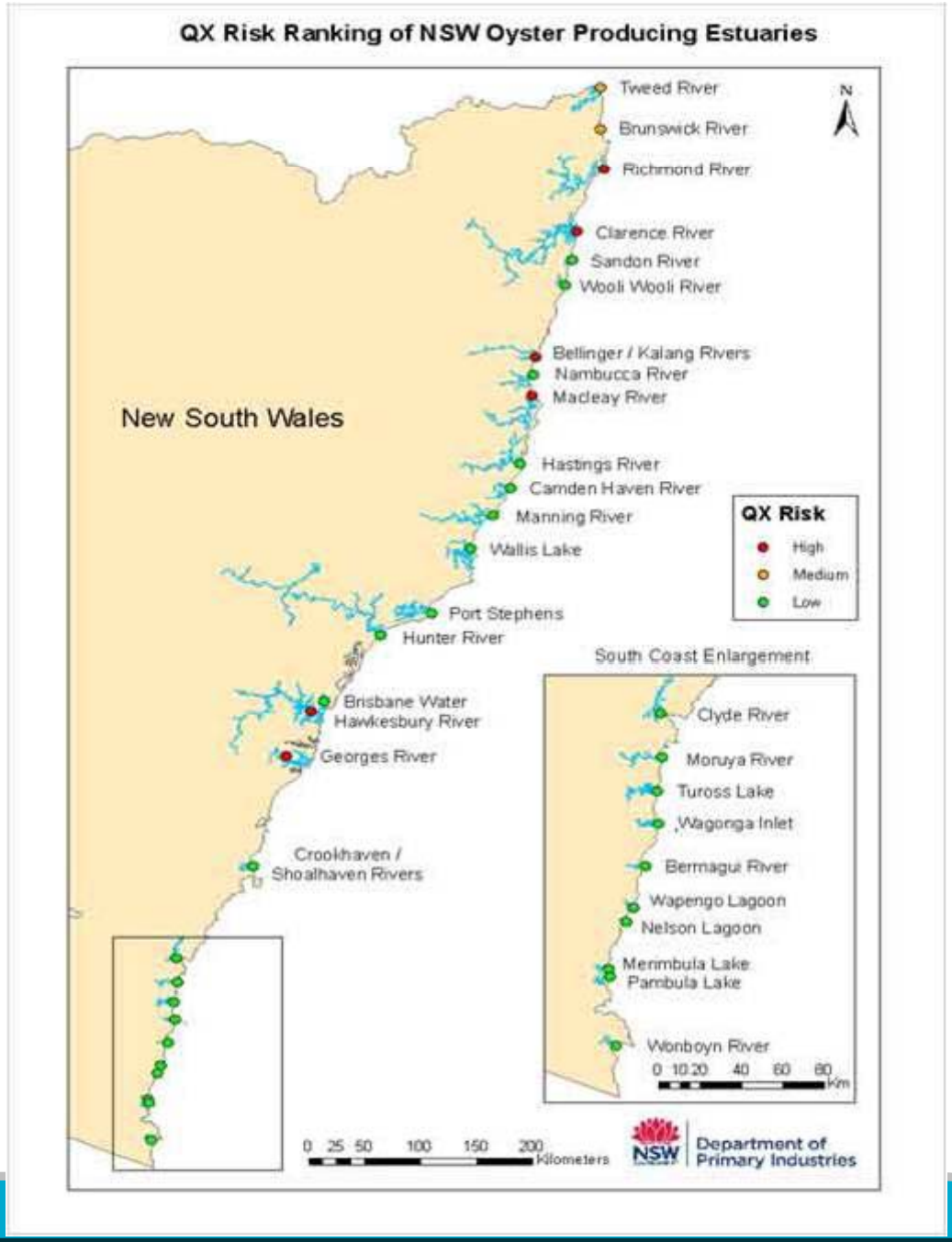
Sydney Rock Oyster

Martelia sydneyi

- Protozoan, *Martelia sydneyi*, identified as the cause of QX disease in 1967
- First diagnosed in Sydney Rock Oysters in the Georges River in 1994 (devastated production).



http://www.qm.qld.gov.au/Find+out+about/Animals+of+Queensland/Parasites/Animal+parasites/QX+disease+in+oysters


QX Risk Ranking of NSW Oyster Producing Estuaries
Tweed River
Brunswick River
Richmond River
Clarence River
Sandon River
Wooli Wooli River
Bellinger / Kalang Rivers
Nambucca River
Macleay River
New South Wales
Hastings River
Camden Haven River
Manning River
Wallis Lake
Port Stephens
Hunter River
Brisbane Water
Hawkesbury River
Georges River
Crookhaven /
Shoalhaven Rivers
QX Risk
High
Medium
Low
South Coast Enlargement
Clyde River
Moruya River
Tuross Lake
Wagonga Inlet
Bermagui River
Wapengo Lagoon
Nelson Lagoon
Merimbula Lake
Pambula Lake
Wonboyn River
0 10 20 40 60 80
Km
0 25 50 100 150 200
Kilometers
N
NSW
Department of
Primary Industries

Martelia sydneyi

"I have seen QX cause 98-100% mortality of market sized oysters in some farms in SE QLD during bad years (wet years)" (Dr Ben Diggles).

Martelia sydneyi

Diagnostic tests used:

-cPCR Leg1-Pro2 (Kleeman et al., 2000, 2002)

Bonamia spp.

Bonamiasis

Ostrea angasi

Winter mortality

Sydney Rock Oyster

-Winter mortality has affected SRO in NSW (up to 60 to 80%, just before oysters reach market size).

-*Bonamia s*topped the budding flat oyster development in the late 1980's in Victoria.

-Currently flat oysters appear to be doing well in NSW, Victoria and South Australia.

Bonamia

Diagnostic tests used:

-cPCR Bo-Boas RFLP (Cochennec *et al*., Ifremer)

-qPCR TaqMan Bon ITS-Taq-F/R (AAHL-Ifremer)

Perkinsus olseni

Abalone – South Australia and NSW

Oyster – Western Australia

Impact of Perkinsosis on South Australian fisheries

"A loss of at least $500 000/year since the early 1980s if you take into account the loss of greenlip abalone catch from Western Gulf St Vincent (there are no longer fishable populations of abalone there). "

"Loss of $10 000 per year in the currently fished areas."

(Dr Marty Deveney, SARDI)

Impact of Perkinsosis on NSW fisheries

Large part of abalone fisheries closed in 2005.

94 tons harvested in 2011 (300 tons in 2001).

Perkinsus olseni

Diagnostic tests developed/used at AAHL:

-cPCR (Perk-ITS 85F / 750R) (OIE)

-qPCR (AAHL) (Taq 108 For / 171 Rev) (genus specific)

-qPCR (AAHL) (Prototype) (Nick Gudkov) (species specific)

Perkinsus in WA

Department of Fisheries WA

cPCR Genus-specific primers PerkITS-85F / 750R (OIE)

cPCR *P. olseni*-specific primers PolsITS-140F / 600R (OIE)

Oyster Oedema Disease

Pearl oysters

Western Australia

Oyster Oedema Disease (OOD)

- From 1996 to 2010, 16 vessels in the industry was reduced to only 2
- Today, 25% of farms are “mothballed” and seeding has been reduced to 40%.
- Two of the three biggest companies in 2006 have now left the pearl aquaculture industry.
- Five hatcheries now reduced to one.
- Mortalities first notified from Gales Bay on 27th Oct. 2006, followed by Whalebone Island on 30th Oct.

Oyster Oedema Disease (OOD)

A new FRDC / WA Fisheries project is starting to determine the cause of mortalities.

Acknowledgements

Fisheries Research and Development Corporation
Bio-Triangle
Department of Fisheries – Western Australia
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University of Sydney
Nick Moody
Ben Diggles
Ana Rubio
David Maitment
Melanie Crockford
Marty Deveney
Jeffrey Go

AAHL Fish Diseases Laboratory

Diseases of molluscs: Identification of diagnostic problems in EU

Beatriz Novoa

Group of Immunology and Genomics, Institute of Marine Research, CSIC

Antonio Figueras

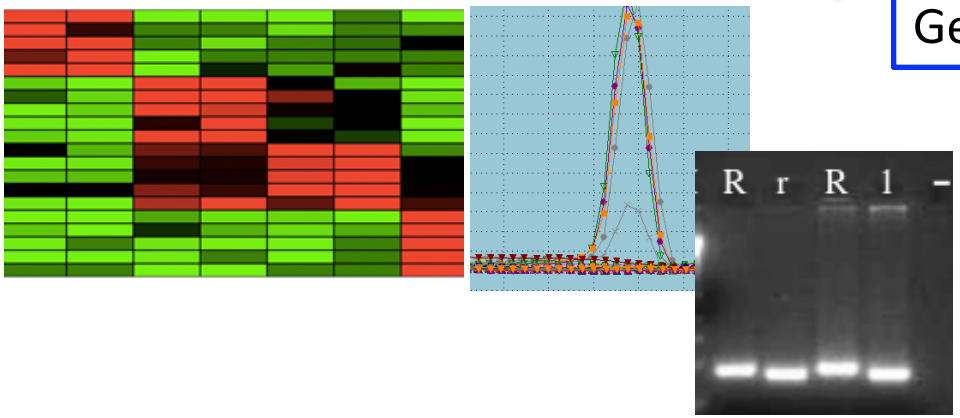
Spanish National Reference Laboratory
Vicepresident of the Spanish National Research Council , CSIC

Geelong, AUSTRALIA
21-24 October 2013

-Spanish National Reference Laboratory for molluscs diseases

-Fish Immune responses (also zebrafish). Genomic tools

-Applied Research: Aquaculture

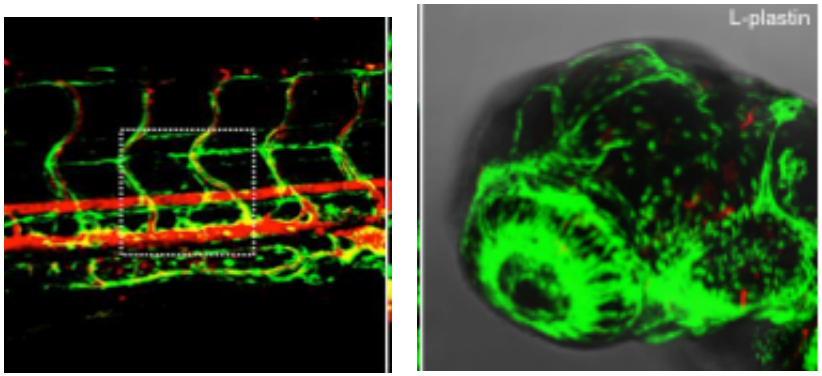


- Basic Research

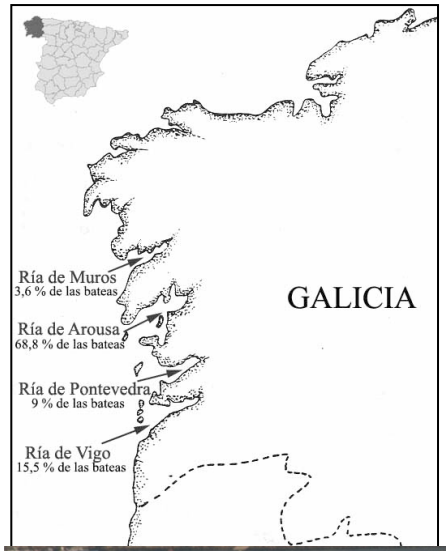
- Molluscs: Antimicrobial peptides. Myticins

- NGS and genomes

- Zebrafish: Biomedicine



Spain is the leading European producer of the Mediterranean mussel (*Mytilus galloprovincialis*)



Galicia (NW Spain) is the most important productive region of Spain
Mussel (*Mytilus galloprovincialis*) represents >95% of production

Identification of diagnostic problems in EU

Do we have specific diagnostic problems in EU?

1) **Validation and comparison of diagnostic techniques**

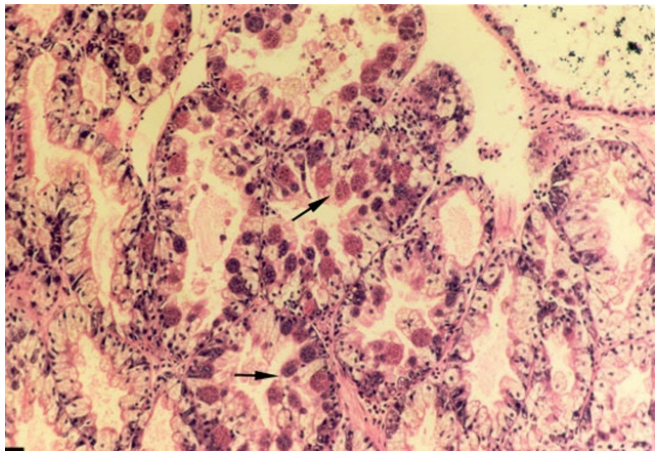
2) **Unknown pathogens and coinfections**

3) ***Different results with different approaches: qPCR***

4) ***Vibrios: bacterial infections***

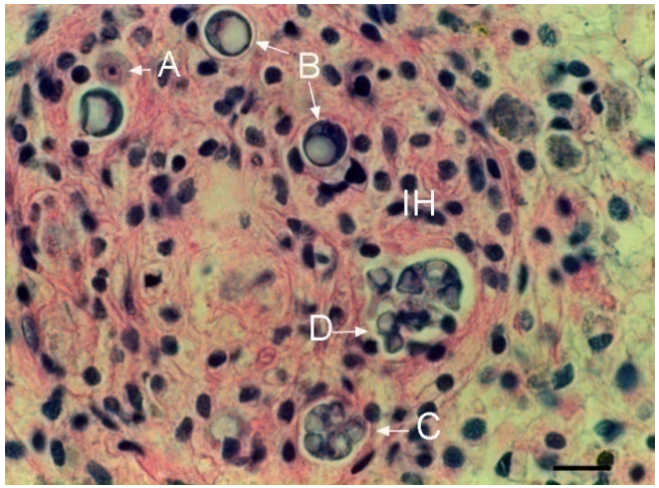
5) ***Networking to solve diagnostic problems***

HISTOLOGY: First and probably the most important technique for the diagnosis of molluscs diseases



Pathogens:

-Marteilia
-Bonamia
-Perkinsus
-All the unknown problems



Advantages:

-General aspect of tissues: Real impact of the disease
-Posibility to detect "other" pathogens
-Reference for other techniques such as PCR.

Problems:

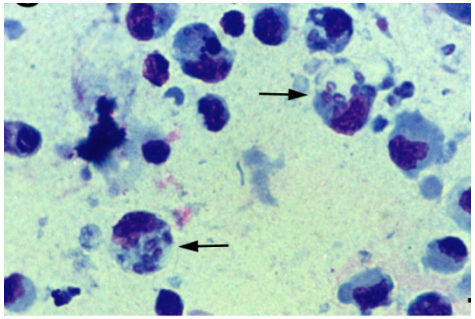
- Need trained people
-Some problems with people of the different

to recognize "difficult pathogens" (very small, low prevalences, exotic diseases, Virus diseases?? Better detection of "big pathogens"??

-Expensive
- Difficulties to share information and even to store blocks and slides

Imprints:
Martelia
Bonamia

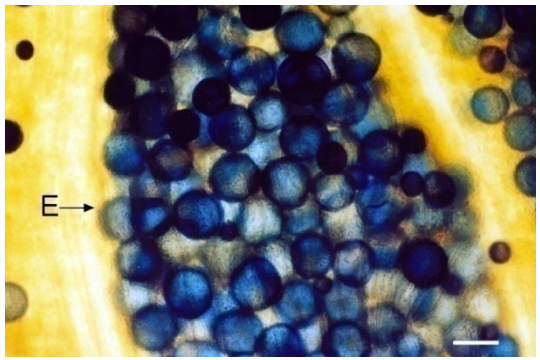
Easy



RFTM

Perkinsus

Easy

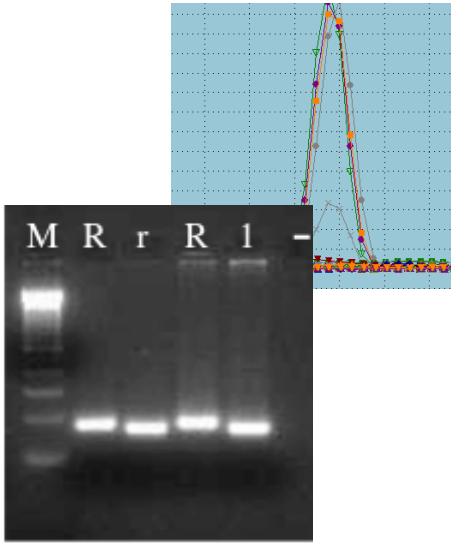


PCR and qPCR

All pathogens

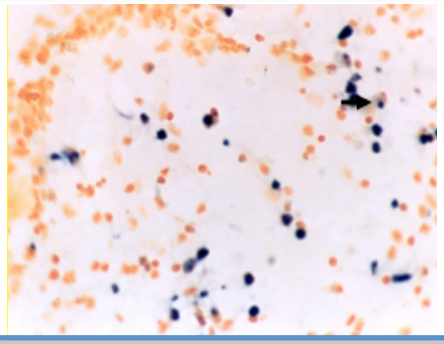
Need to standarize all the conditions among the labs

Able to distinguish species, strains. Detect viruses…



Bacterial growth in specific media

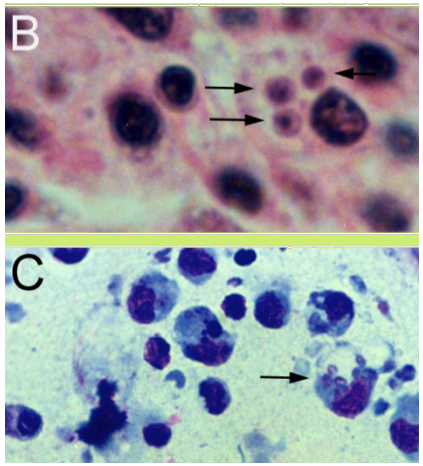
ISH: Combination of histology and molecular techniques

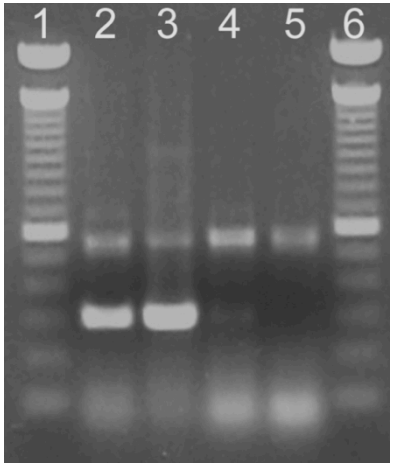


1) Validation and comparison of diagnostic techniques

Problem: The lack of validation approach for most of the techniques we use !

Bonamiosis





Two Laboratories

Three Tecniques:

- Histology
- Cytology
- Histology+ Citology
- PCR

Epidemiological parameters

- **Sensitivity**
- **Specifity**
- **Positive predictive value (PPV):** Measure of the probability that a positive result actually corresponds to a carrier animal.
- **Negative predictive value (NPV):** Measure of the probability that a negative result actually corresponds to a healthy animal.

(Balseiro et al., 2006)

Table 2
Comparison among the different techniques employed in this study, PCR, histology, cytology and the combination of histology and cytology data integrated

		PCR			Cytology		
		Positive	Negative	Total	Positive	Negative	Total
Histology	Positive	51–50	4–3	55–53	25–33	30–20	55–53
		(21.3–20.8)	(1.7–1.3)	(22.9–22.1)	(10.4–13.8)	(12.5–8.3)	(22.9–22.1)
	Negative	23–28	162–159	185–187	0–1	185–186	185–187
		(9.6–11.7)	(67.5–66.3)	(77.1–77.9)	(0–0.4)	(77.1–77.5)	(77.1–77.9)
	Total	74–78	166–162	240–240	25–34	215–206	240–240
		(30.8–32.5)	(69.2–67.5)	(100–100)	(10.4–14.2)	(89.6–85.8)	(100–100)
Cytology	Positive	24–33	1–1	25–34	NA		
		(10–13.8)	(0.4–0.4)	(10.4–14.2)			
	Negative	50–45	165–161	215–206			
		(20.8–18.8)	(68.8–67.1)	(89.6–85.8)			
	Total	74–78	166–162	240–240			
		30.8–32.5)	(69.2–67.5)	(100–100)			
Combination of histology and cytology	Positive	51–50	4–4	55–54	NA		
		(21.3–20.8)	(1.7–1.7)	(22.9–22.5)			
	Negative	23–28	162–158	185–186			
		(9.6–11.7)	(67.5–65.8)	(77.1–77.5)			
	Total	74–78	166–162	240–240			
		(30.8–32.5)	(69.2–67.5)	(100–100)			

In each cell, the number on the left corresponds to laboratory A and the number of the right to laboratory B. Above: number of samples; Below: percentage.

PCR assay:

high sensitivity

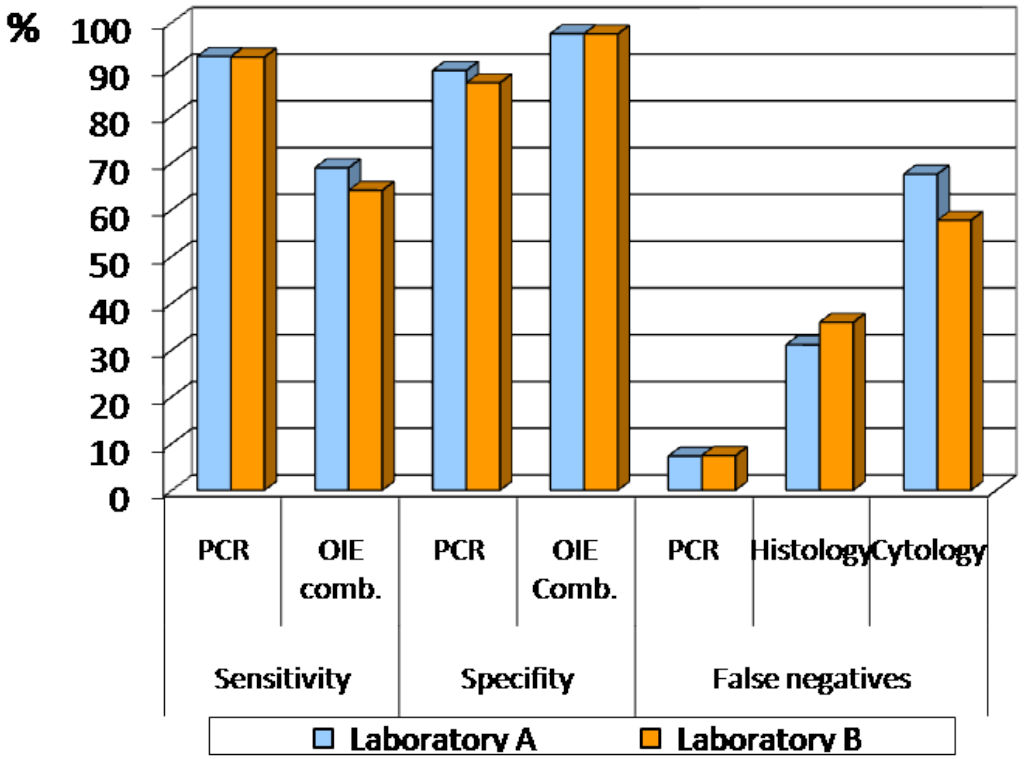
lower number of false negative results

Suitable for screening?

Classical methods (histology and cytology):

higher specifity

Suitable for confirmatory analysis?

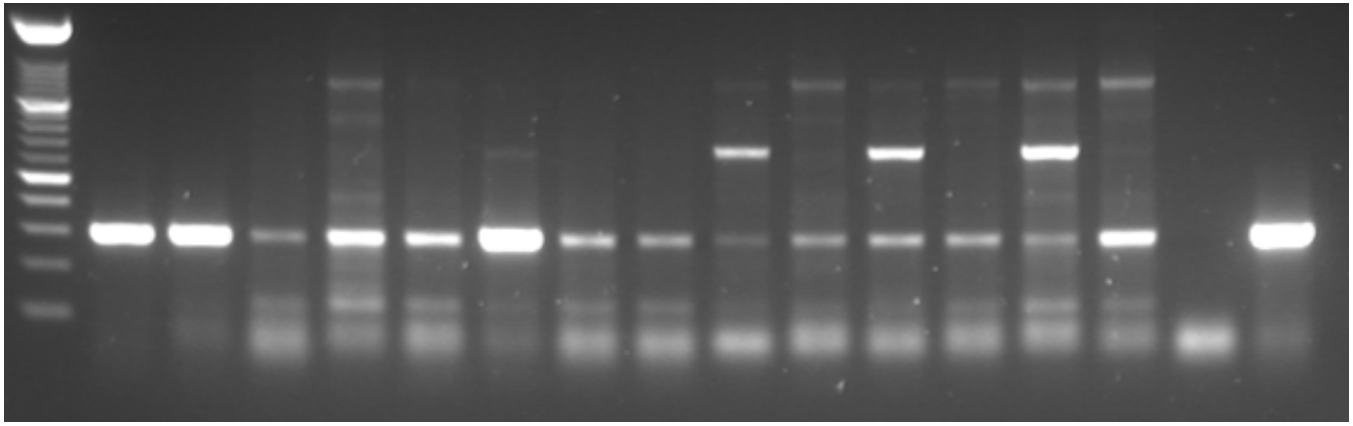


- Most molecular detection methods described in OIE manual were not developed specifically for diagnostics but intended for research purposes!

- Most PCR's and real time PCR's for detection of *Bonamia* not species specific (Few exceptions e.g. Engelsma et al 2010, Robert et al 2009 and Ramilo et al 2013 but these need further validation)

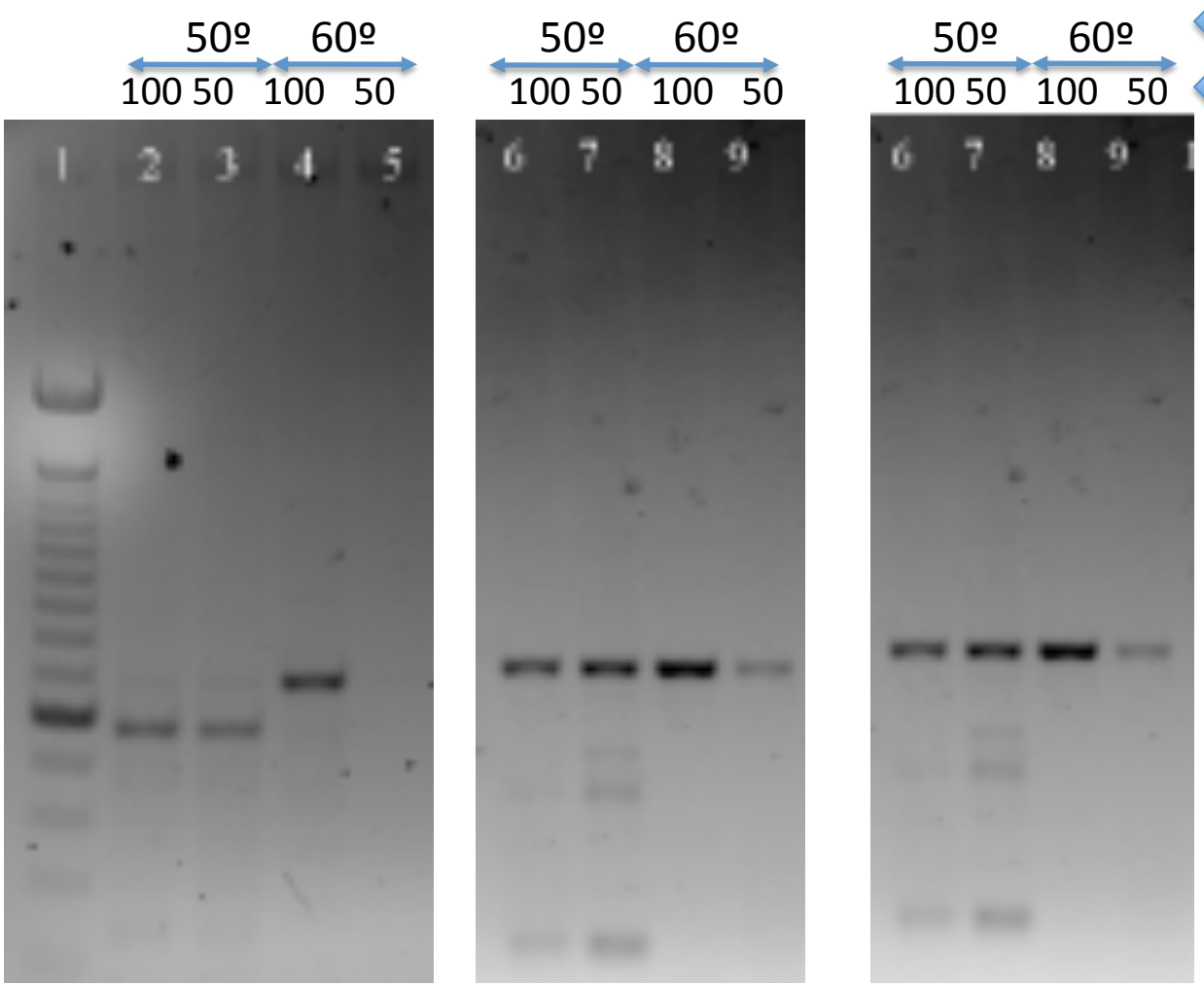
- BO-BOAS primer-set Cochennec et al. 2000 . At lower annealing temperatures an a-specific product is generated of 295 bp (expected product size 300 bp). Might depend on brand of Taq used! Tested with *O. edulis* from *Bonamia*-free zones in France and Ireland

BO-BOAS



Oyster herpesvirus

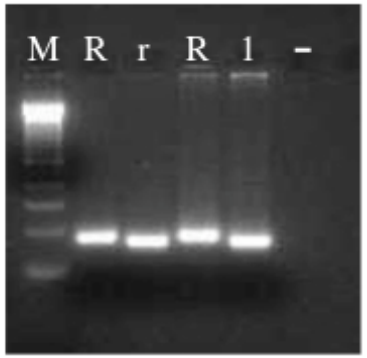
Optimization of the diagnosis protocol. Classical PCR for Oyster herpesvirus (C2-C6)



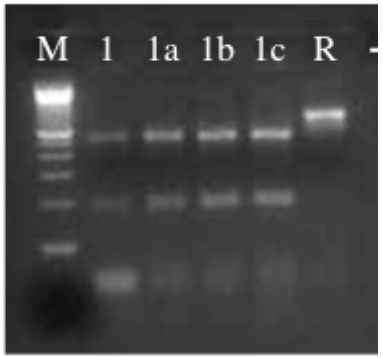
Tª

DNA (ng/ml)

CF-CR primers



C2-C6 + restrictio n



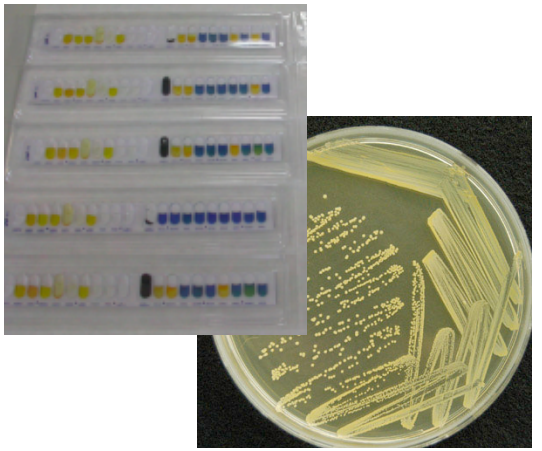
(Aranguren et al, 2013)

Lack of validation approach for most of the techniques… but it is time consuming: we spend time for one PCR technique and 1 year later when it is finished you have new PCR assays published sometimes more promising!!!!

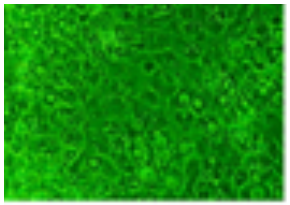
2. Unknown pathogens and coinfections

European abalone
Haliotis tuberculata

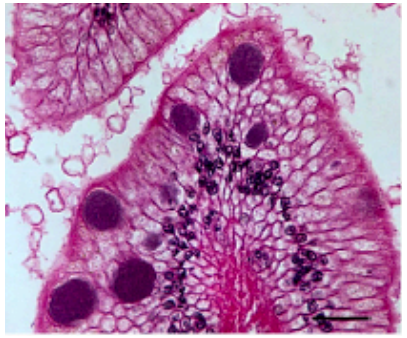
Bacteriological analysis.
Koch postulates



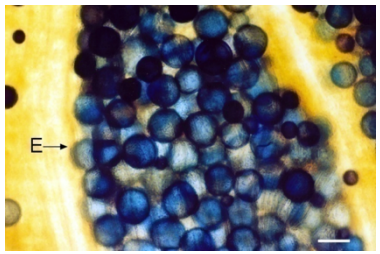
Virological analysis?? Cell line??



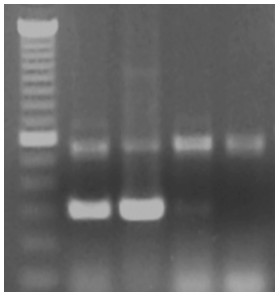
Histology

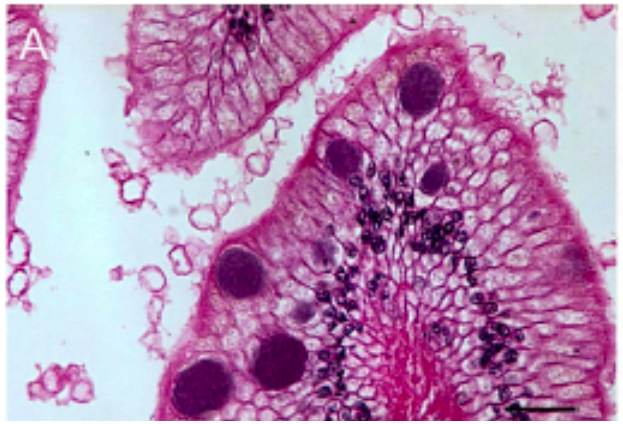


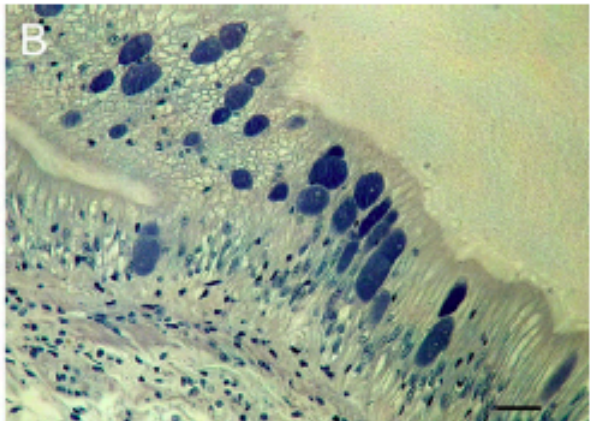
RFTM



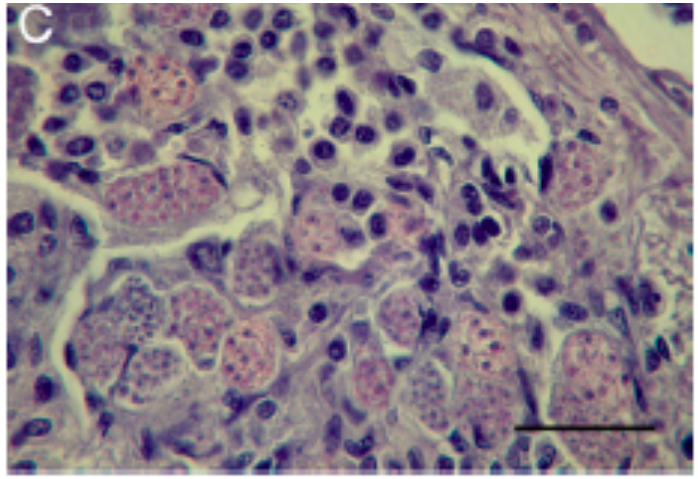
Molecular analysis??



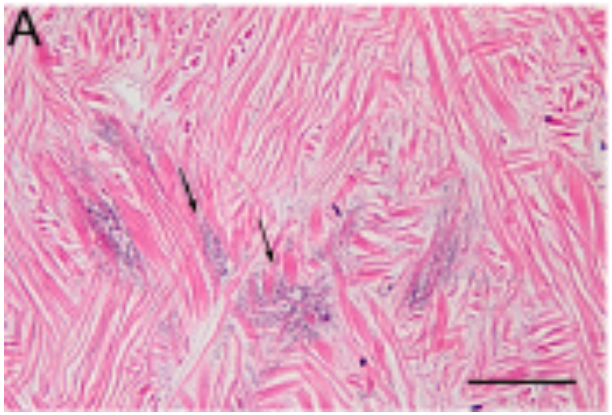


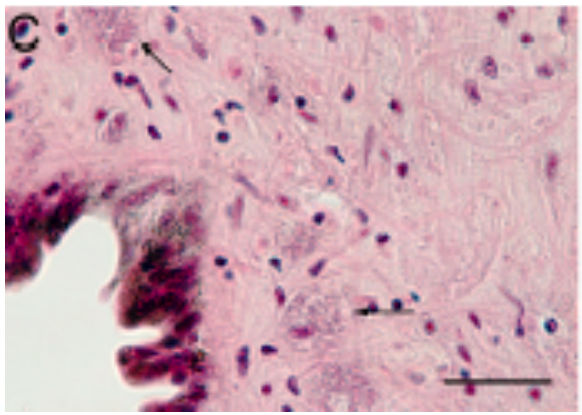


Candidatus Xenohaliotis californiensis



Haplosporidian parasite

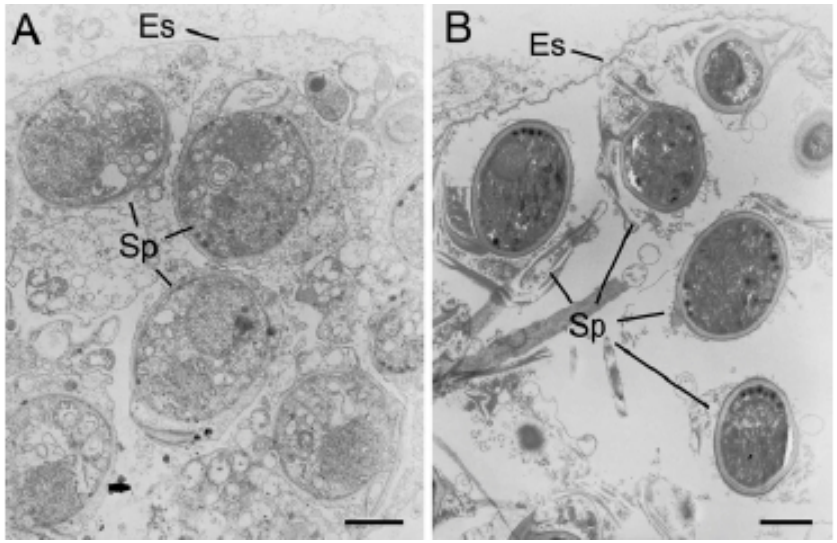




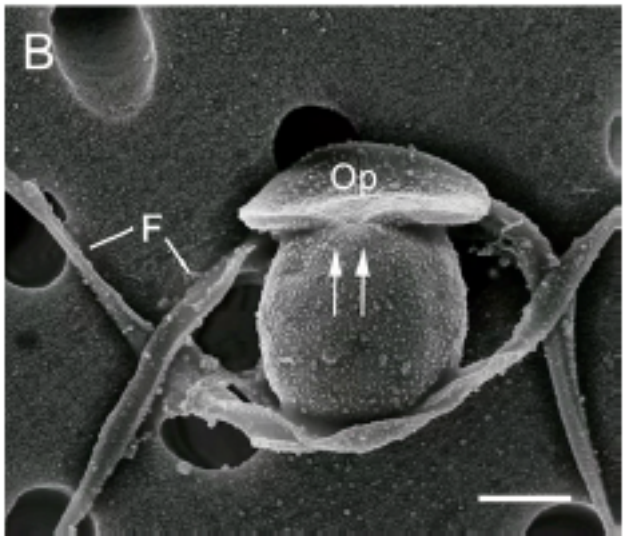
Bacterial infection (*Vibrio campbellii*)

(Balseiro et al., 2006a, 2008)

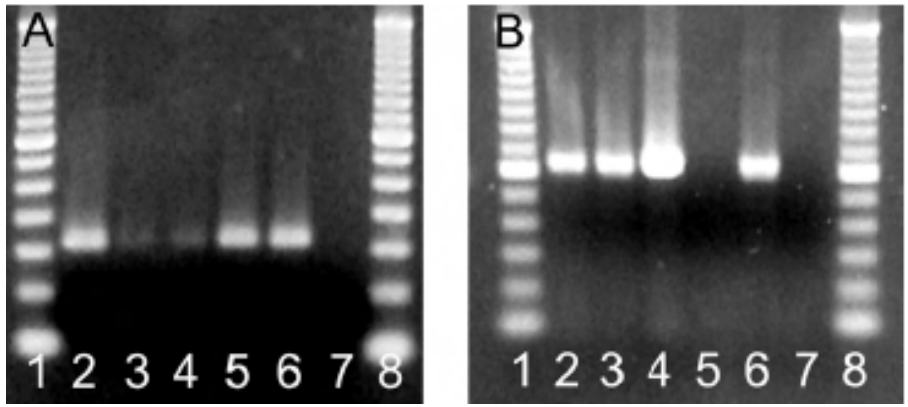
TEM, SEM



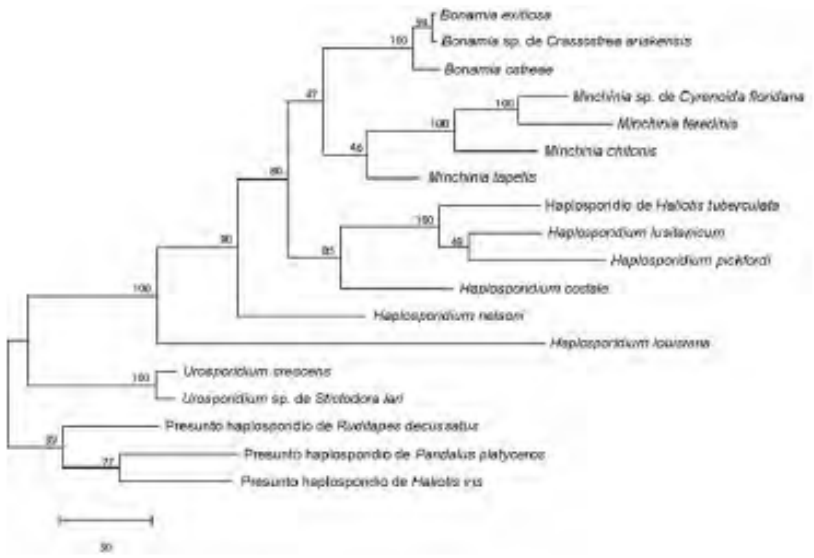
Haplosporidium montforti n. sp.



(*Azevedo et al., 2006*)



The sequences of the amplified fragments of the 16s rRNA revealed that it was Candidatus Xenohaliotis californiensis



Different results with different approaches: qPCR

Not developed for all pathogens: (ex: no qPCR for *Marteilia refringens. In house* real time PCR was developed by CVI together with IRTA)

There are some aspects that can affect DNA quality and quantity and RESULTS:

1. Tissues and storage

- Tissue selection for DNA extraction (gill, heart, haemolymph)
- Tissue storage. Fresh frozen (-20°C/-80°C) seems to give optimal results as ethanol can act as a PCR inhibitor and paraffin embedded tissue results in fragmented DNA with products < 500 bp only being amplified
- Tissue storage time

2. DNA Extraction. Need of internal control

- Some systems are cheaper and more rapid
- Fewer steps (Chelex-100, Sigma) prevents the risk of cross contamination (important when handling viral samples),
- Loss of DNA can occur during the multiple spin steps using the extraction kits
- PCR inhibition in some tissue especially in clam

3. Reactives and qPCRs machines

- When a company changes their reactives (ex: Agilent Real Time PCR mix, we sometimes do not obtain similar results with the new reactives)

- Whay about different qPCR machines or chemical reactives?

- Light positive results in PCR or High Ct values: what is the meaning of this light DNA detection?

HOW SHOULD WE USE REAL TIME PCR?

Nocardia Ring Test (Marc Engelsma)

+: Ct < 40 and Tm: 76,5
-: No Ct or Ct OK but out of the Tm range of 76,5 ± 0,8ºC

Individual Ct and Tm values

Sample number	A			
	Ct	Tm	Ct	Tm
1	24.59	75.6	24.43	75.6
2	-		-	
3	-		-	
4	28.74	76.2	28.69	76.2
5	21.54	76.5	21.40	76.5
6	24.86	76.2	24.71	76.2
7	18.18	76.2	18.14	76.2
water	-		-	
control	16.04	75.9	16.51	76.2

OUT OF RANGE

AB 7300 system

Agilent Technologies Mx3005P machine

Now, the Ct values obtained with Agillent machine were even lower than the values obtained with the AB machine.Regarding to the Tm values, our results are again clearly our of the range described in the protocol (76.5, SD=0.8), and it seems to be clearly dependent on the machine used.

	Ct	*Ct Mean*	Tm	Result
n°1	24,59 24,43	**24,51**	75,6	Positive??
n°2	- -	**0,00**	70,05	Negative
n°3	- -	**0,00**	71,4 70,05	Negative
n°4	28,74 28,69	**28,72**	76,2	Positive
n°5	21,54 21,40	**21,47**	76,5	Positive
n°6	24,86 24,71	**24,79**	76,2	Positive
n°7	18,18 18,14	**18,16**	76,2	Positive
water	- -	**0,00**	88,8	Negative
Nocardia	16,04 16,51	**16,28**	75,9 76,2	Positive

Applied Biosystem 7300

Ct	*Ct Mean*		Tm	Result
23,58 23,36	**23,47**		79,5 79,5	Positive
- -				Negative
- -				Negative
27,25 27,75	**27,50**		79,03 79,03	Positive
20,30 20,45	**20,38**		79,53 79,53	Positive
23,84 23,82	**23,83**		79,05 79,05	Positive
17,13 17,21	**17,17**		79,6 79,6	Positive
- -				Negative
14,93 14,93	**14,93**		79,59 79,59	Positive

Agilent Tech Mx 3005P

OUT OF RANGE!!!!!

Tm: 75,8- 77,3

Vibrios: bacterial infections

Irland: ***V. aesturianus***.

Mortality *in C. gigas* that affects not only spat but half grown or adults as well.

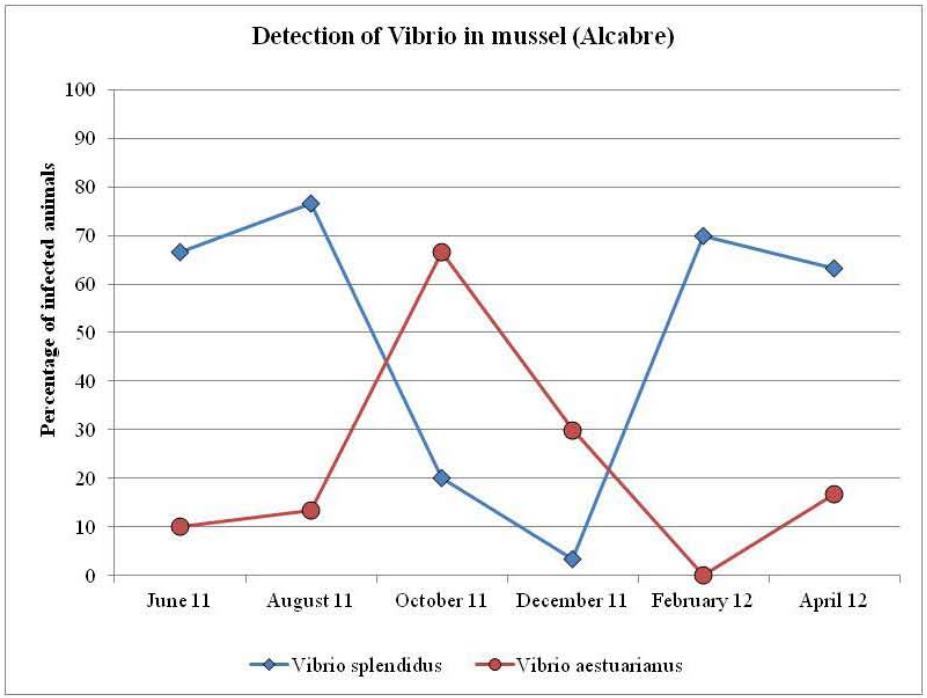
Other pathogens on top of OsHV-1?

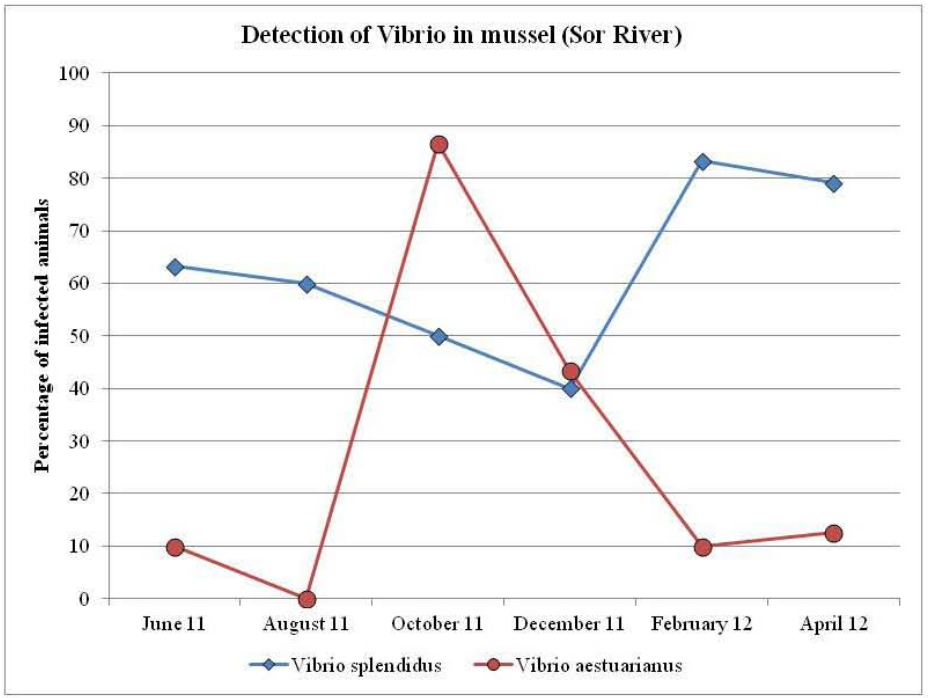
- *V. aestuarianus* can be detected by qPCR in most samples (low CT values with all animals tested being positive)

-However, only rarely it was possible to culture the bacteria from gills

- No pathology could be directly tied to the presence of *V. aesturianus*.

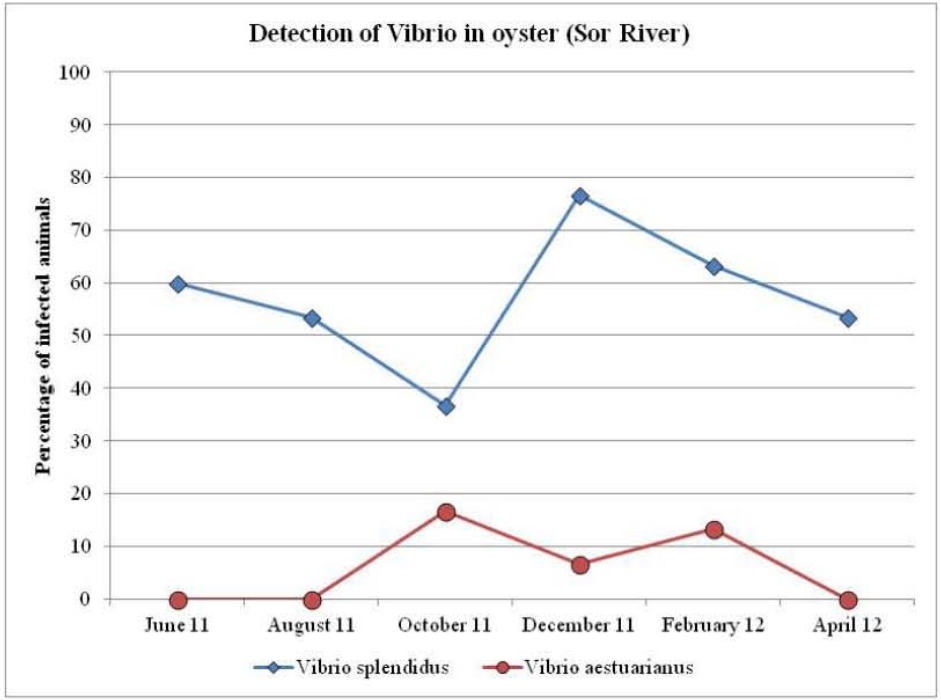
We know V. aes is present but we do not know what role it plays in the mortality if any. Many of the sites where we find V. aes we also find OsHV-1uVar.





qPCR:

(Romero et al, submitted)



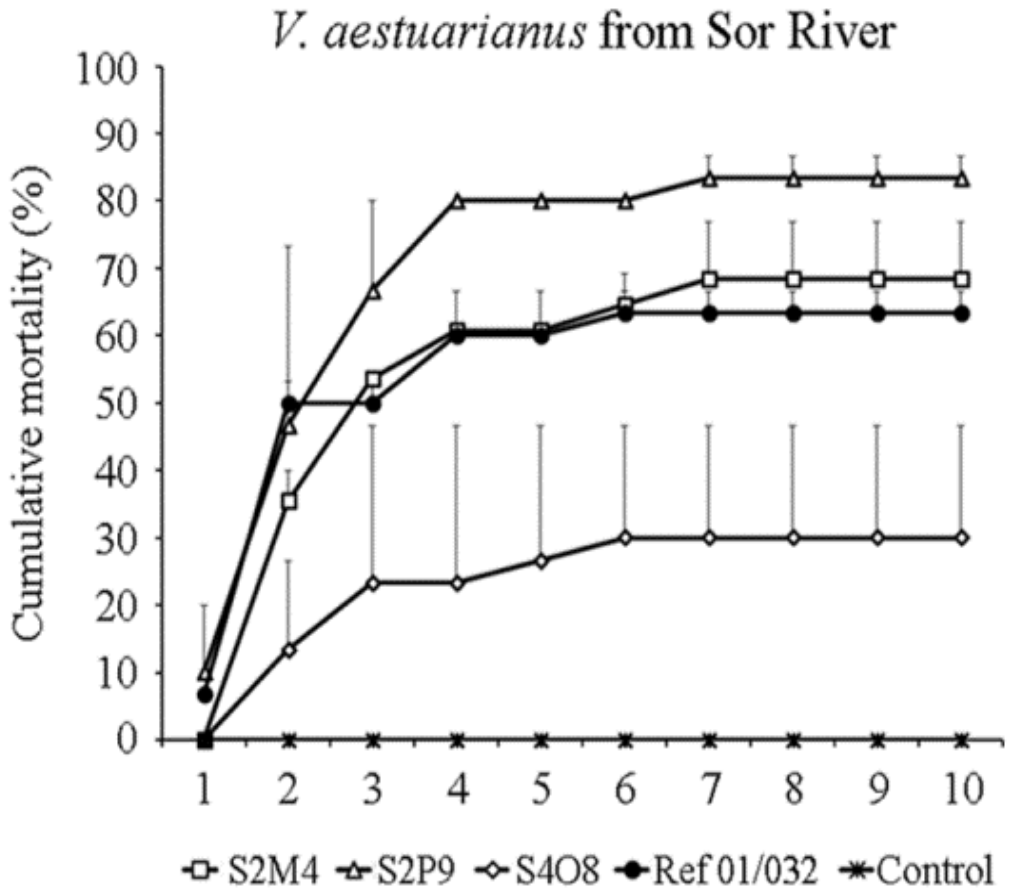
Percentage of positive colonies, previously grown in TCBS, identified by specific qPCR as *V. splendidus* (*V. spl*) or *V. aestuarianus* (*V. aest*). Dash (-) indicates the absence of colony growth in TCBS plates.

Alcabre	June 2011		August 2011		October 2011		December 2011		February 2012		April 2012	
	V. spl	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*
Mussel	-	-	-	-	12,5	0	0	0	0	0	25	0
Water	-	-	0	0	75	0	10	0	40	0	100	0
Sediment	0	0	0	0	28,5	0	50	0	60	0	20	0
Plankton	0	0	0	0	88,8	0	0	0	20	0	80	0

Sor River	June 2011		August 2011		October 2011		December 2011		February 2012		April 2012	
	V. spl	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*	*V. spl*	*V. aest*
Mussel	25	25	-	-	-	-	100	0	-	-	25	0
Oyster	0	0	0	12,5	-	-	0	0	-	-	100	0
Water	0	0	0	0	40	0	25	0	100	0	60	0
Sediment	0	0	0	0	60	0	25	0	80	0	60	0
Plankton	0	10	0	0	80	0	0	0	0	0	100	0

**Environmental samples were enriched in alkaline peptone water

V. aestuarianus isolates from the field. Effect on mussel



Plankton isolate (89%)

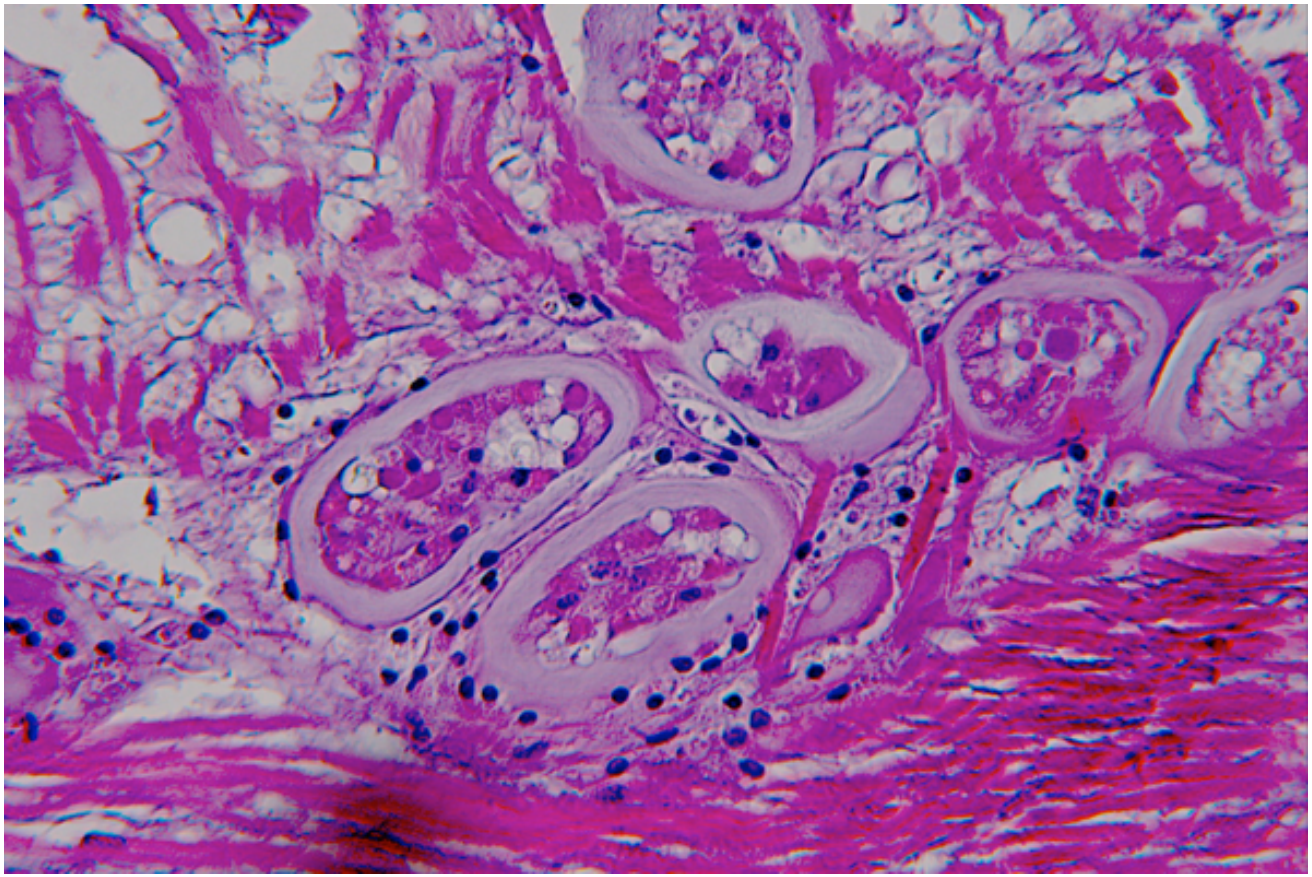
Mussel isolate (64%)

Reference strain (63 %)

Oyster isolate (30 %)

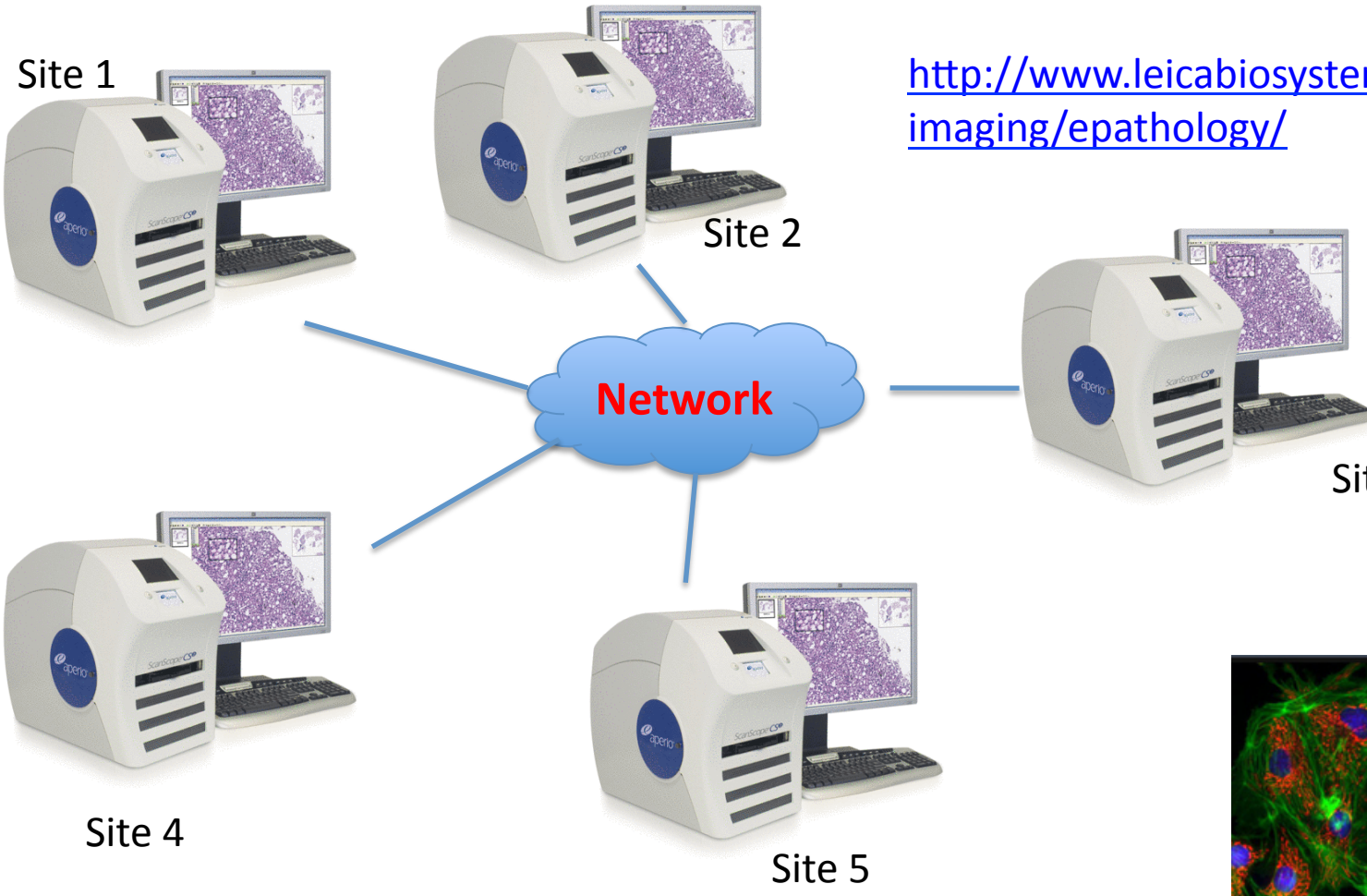
But… intramuscular injection only and…. 10^{10} CFUs!!!

Networking to solve diagnostic problems

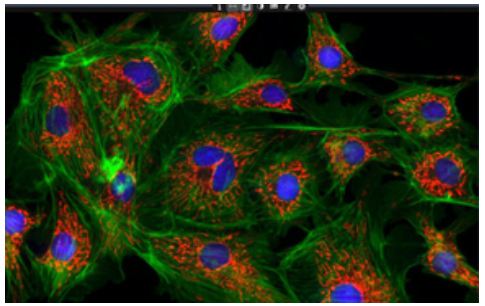


Parasites found in Challista chione , wild beds: coccidia?
Often they are present, numerous, in mass mortality events.

Giuseppe Arcangeli



http://www.leicabiosystems.com/pathology-imaging/epathology/



http://apac.slidepath.com/dih/webViewer.php?snapshotId=13813966550219

http://apac.slidepath.com/dih/webViewer.php?snapshotId=13813966864147

Thanks for your attention

Bivalife Project

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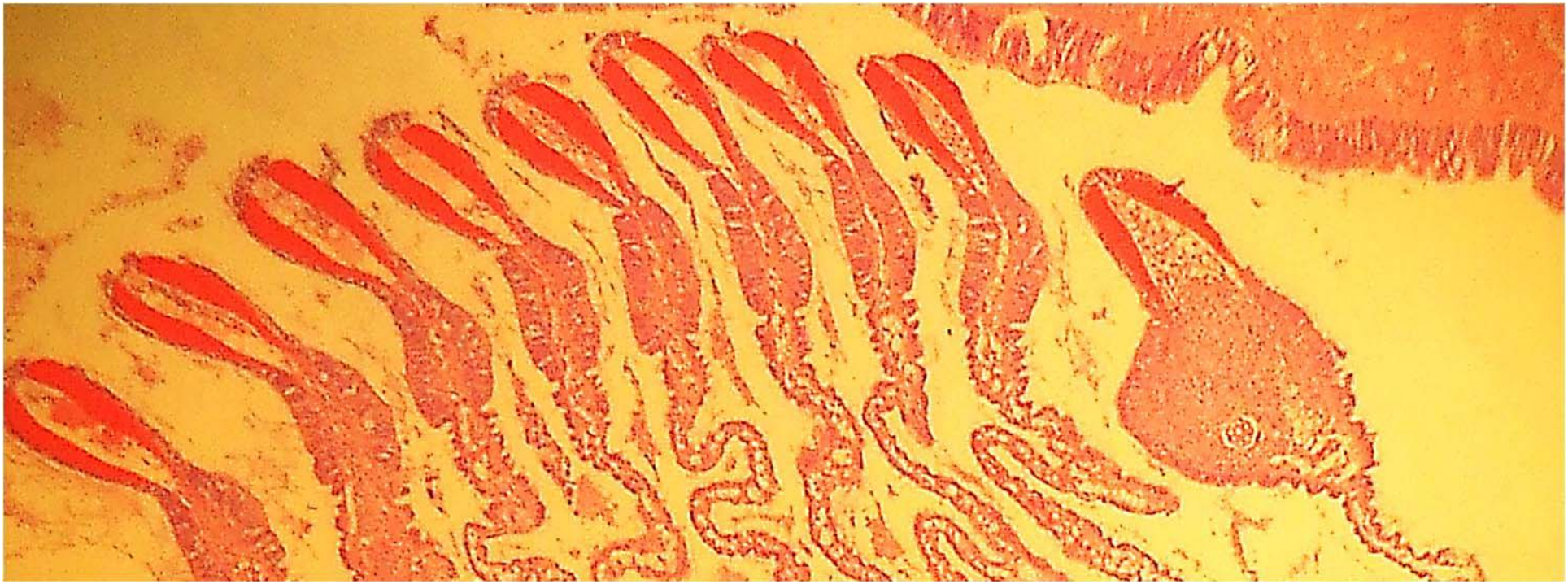
KBBE workshop: Mollusc Disease Diagnosis

A collaborative initiative between the EU, Australia and New Zealand.
Geelong, Australia 21-24 October 2013

Identification of diagnostic problems in NZ | 22 October, Session 2



Purposes of disease testing

- Management of disease issues
- **Investigation of mortalities/morbidities**
- **Routine monitoring of healthy populations**
- **Testing for translocation**

Must be cost-effective for aquafarmers

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Testing Methods

- Gross examination
- Tissue smears including heart prints and hemolymph sampling
- Histopathology
- Molecular: PCR and ISH
- Other: Ray’s Fluid for *Perkinsus*

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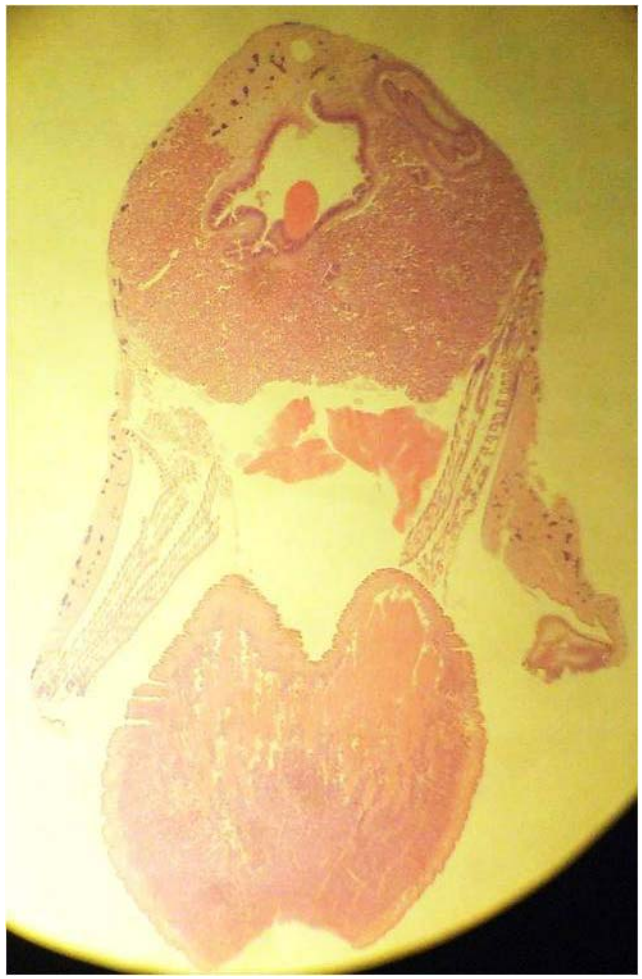
Histopathology

- Broad spectrum - inexpensive
- Gives an indication of general health: feeding, reproduction etc
- Varying sensitivity
- Specificity can be good if no pathogen congenerics are reported/expected
- Permanent record

Typical result in bivalves



Problems:

- Ensuring inclusion of all tissues of interest in one section - more sections = higher cost
- Multiple larvae, spat and juveniles in one section are difficult to orientate
- Criteria for acceptability?

Histopathology:
general indicators

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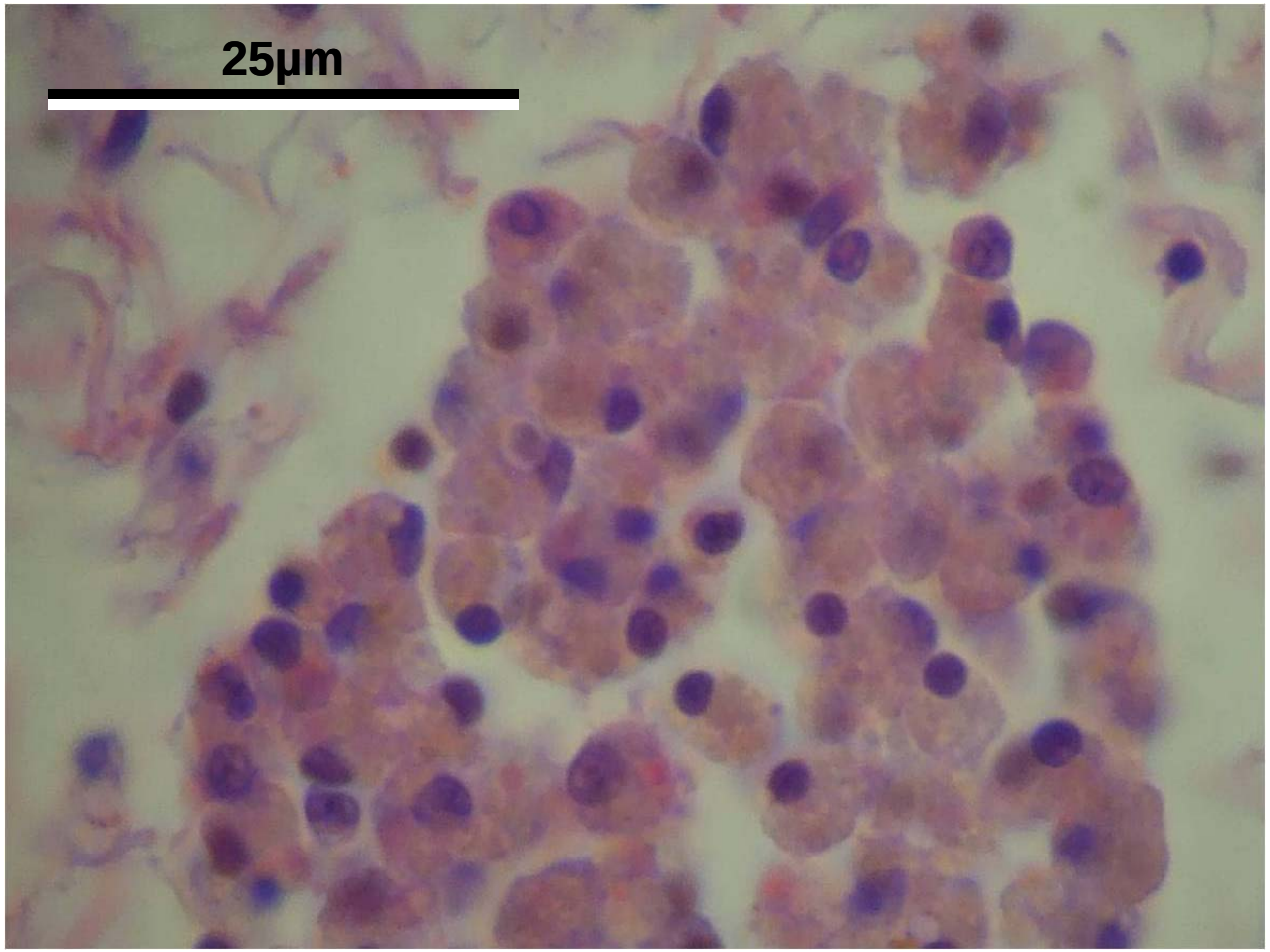
Brown cells

Spisula aequilatera



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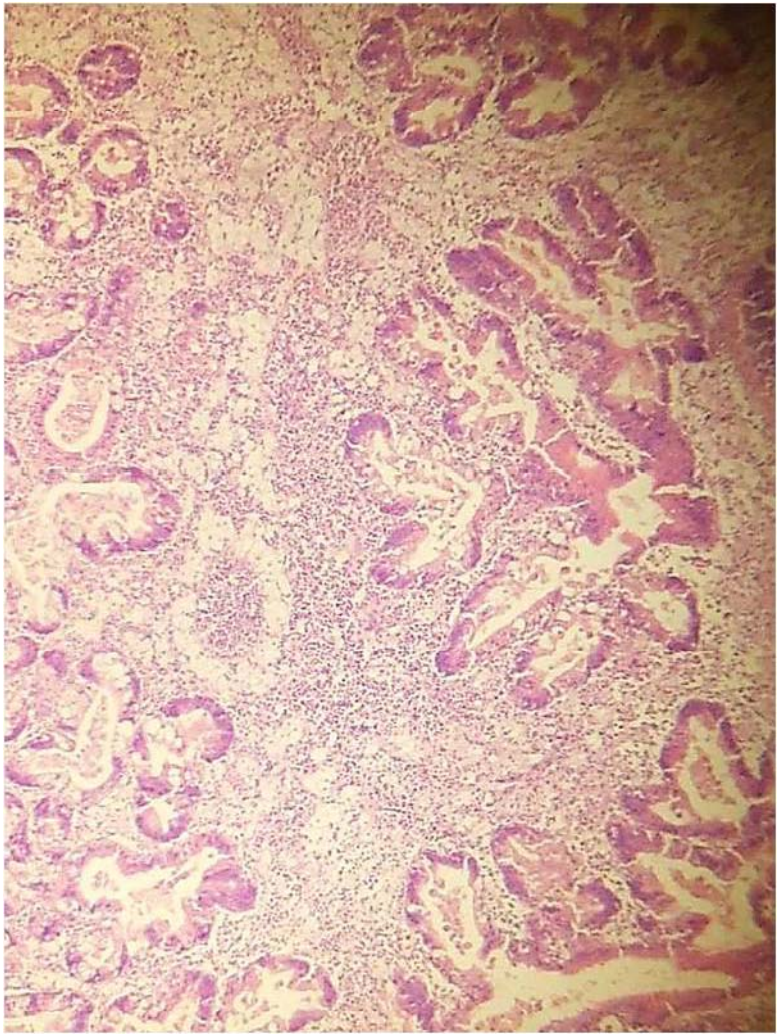
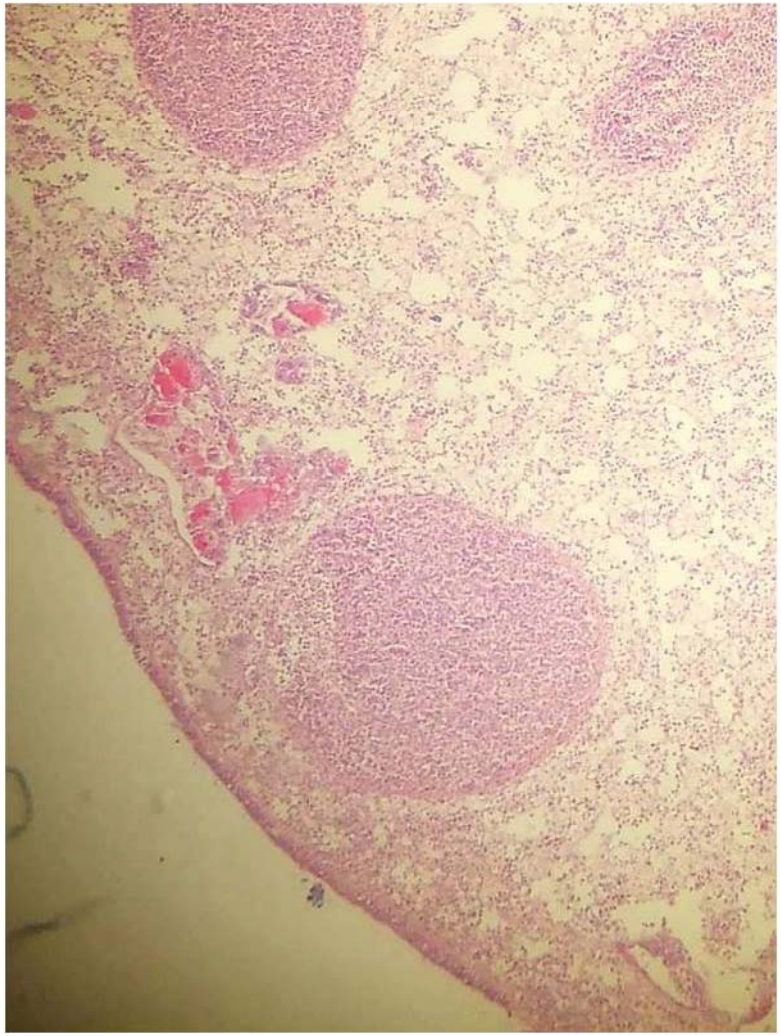
Hemocytosis



Diffuse



Focal

Histopathology:

hemocytosis alerting to other observable agents

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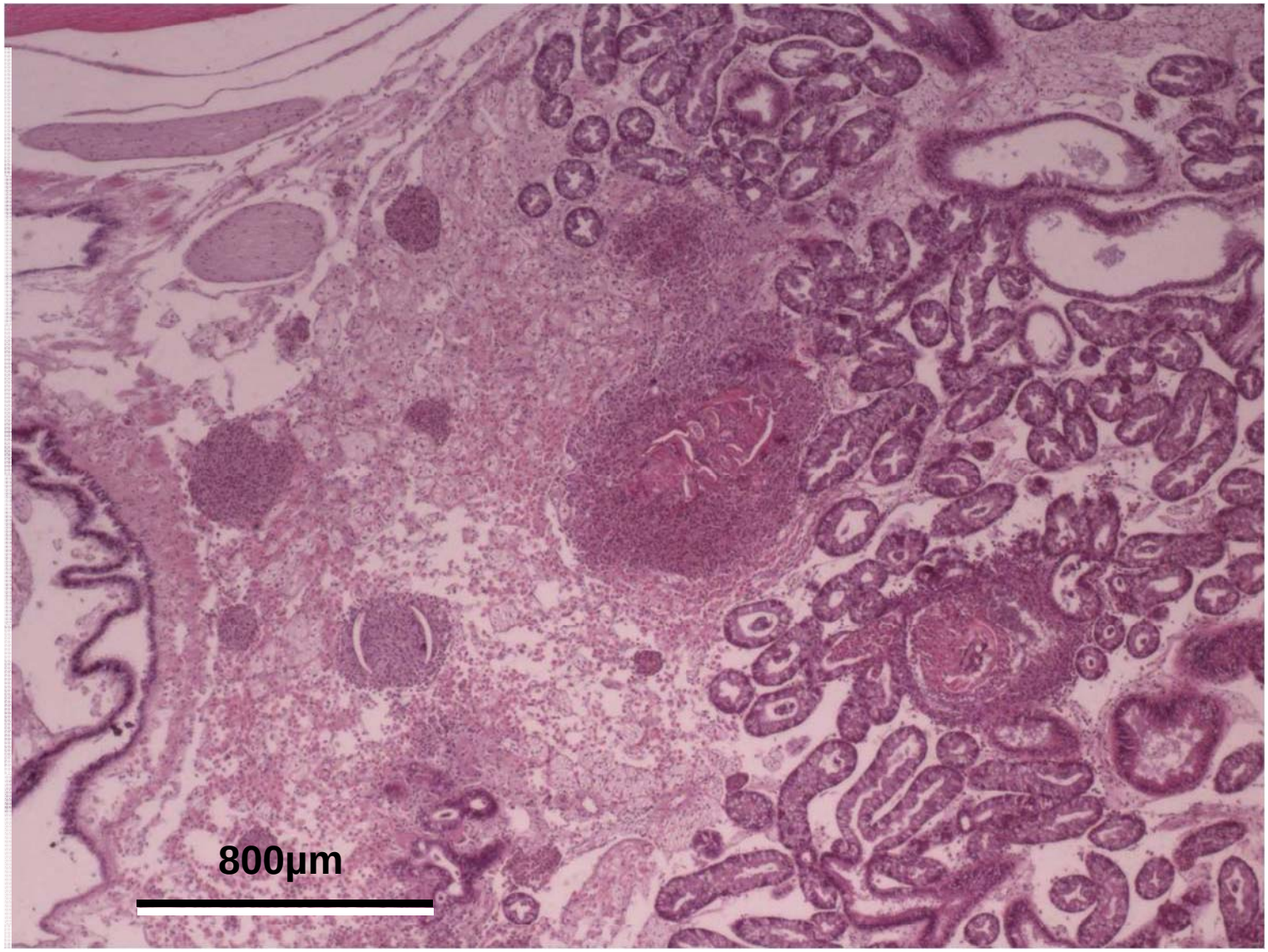
Copepod

Ostrea chilensis



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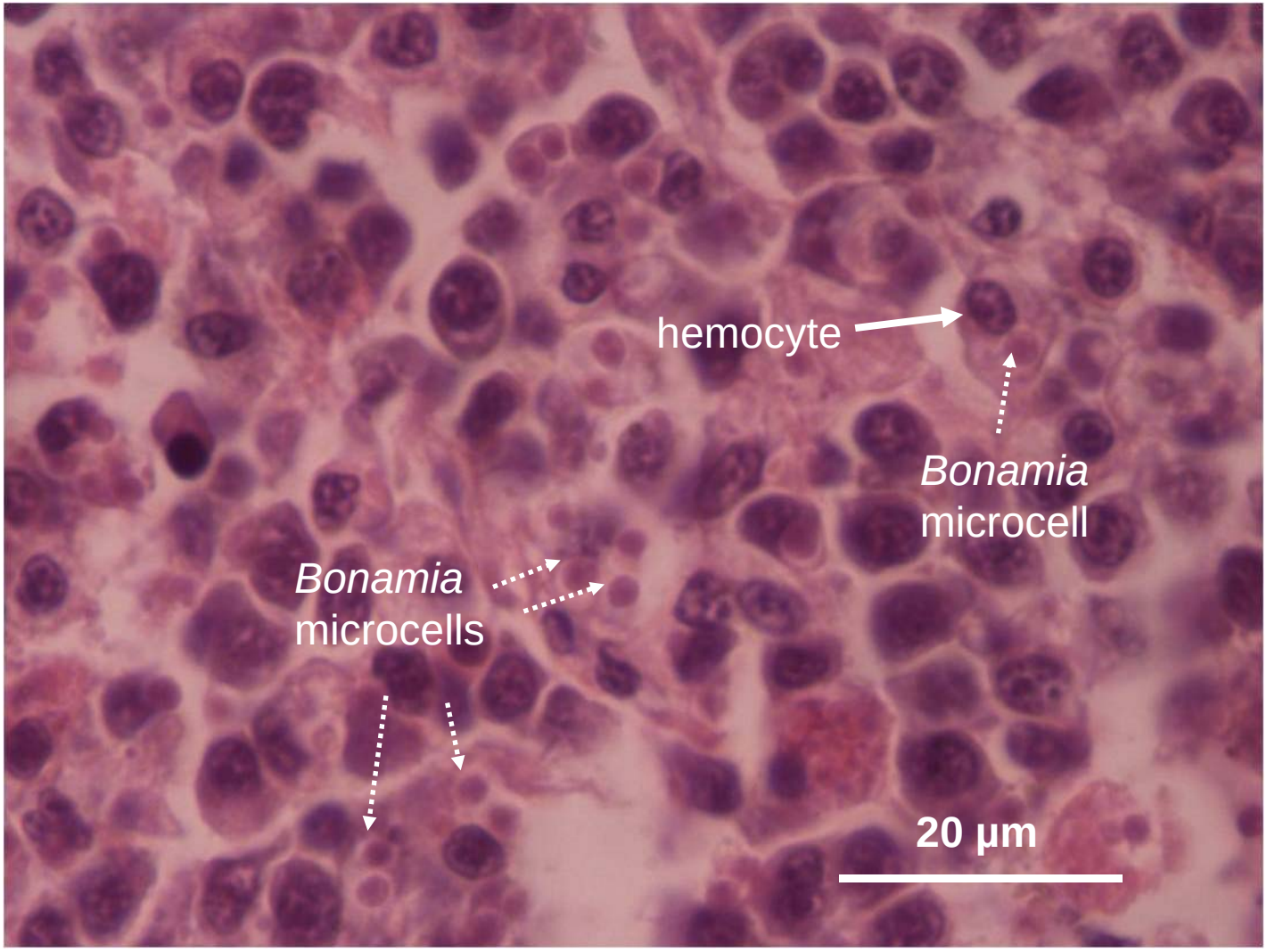
Moderate infection with *Bonamia exitiosa*

digestive tract connective tissue

Ostrea chilensis



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Histopathology:
signs of viral infection

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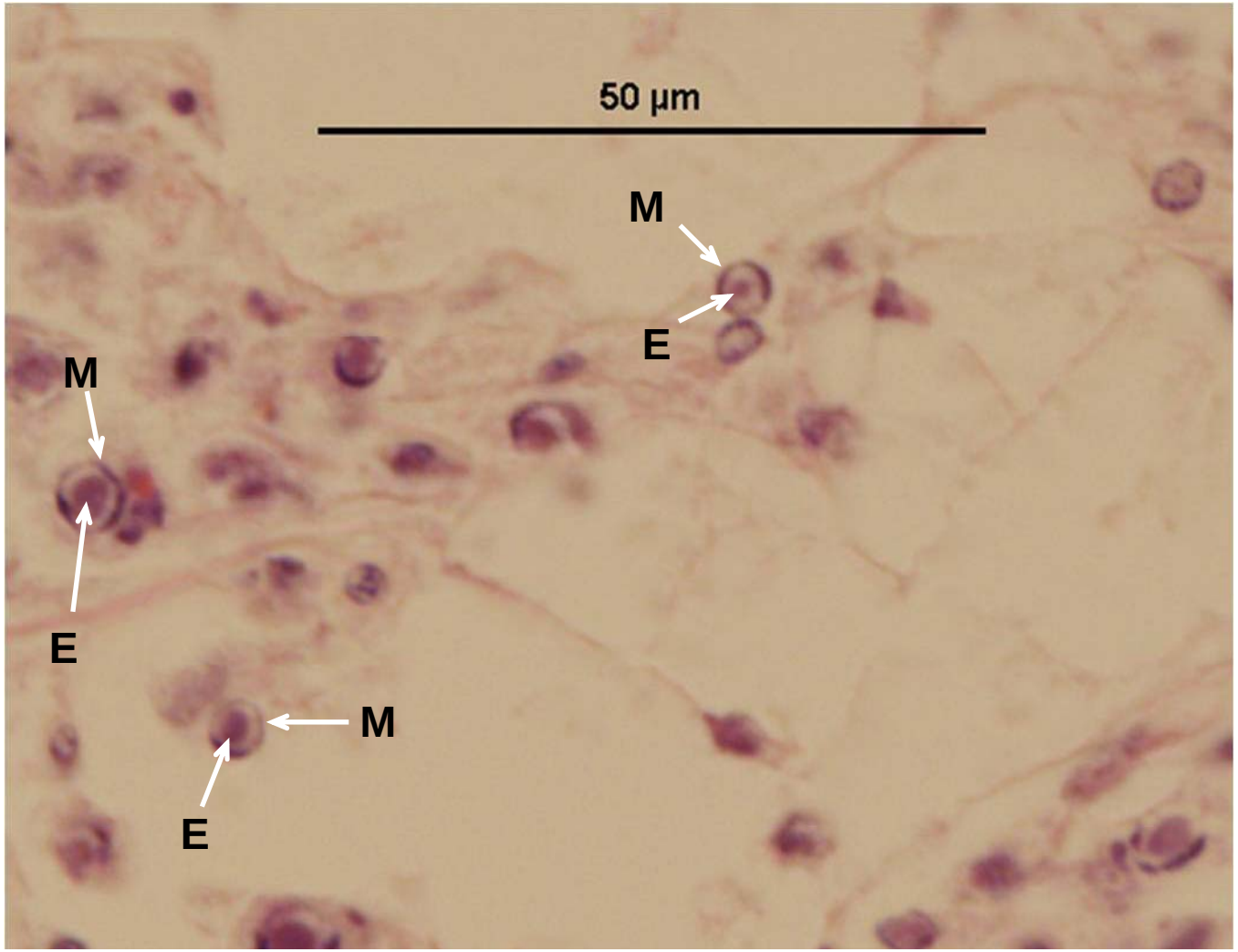
Cowdrey type A bodies - aka Lipschütz bodies



Ostreid herpesvirus - 1

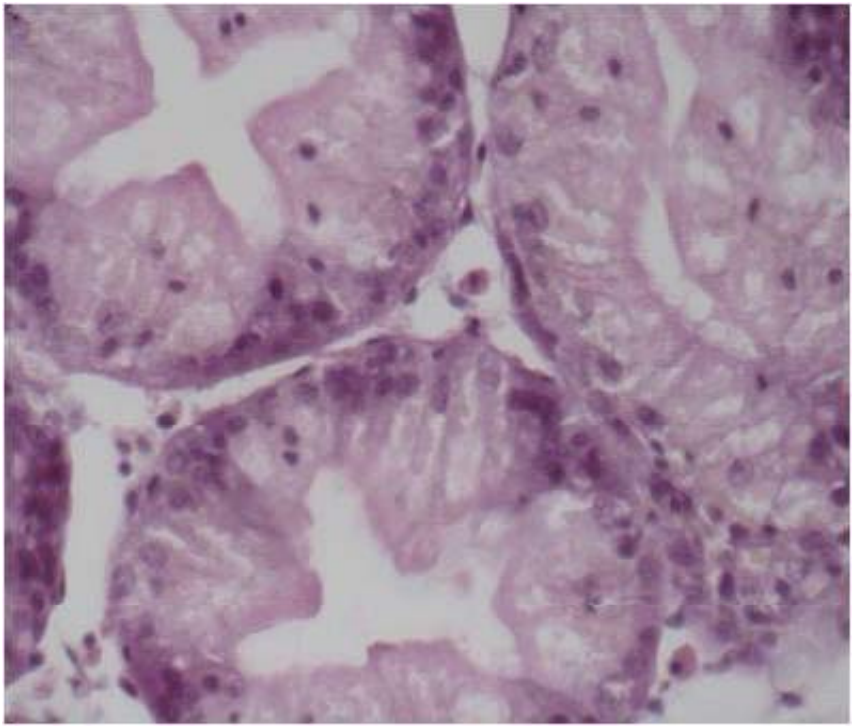
Classic indicator of herpes infection

For OsHV-1 particularly in connective tissue

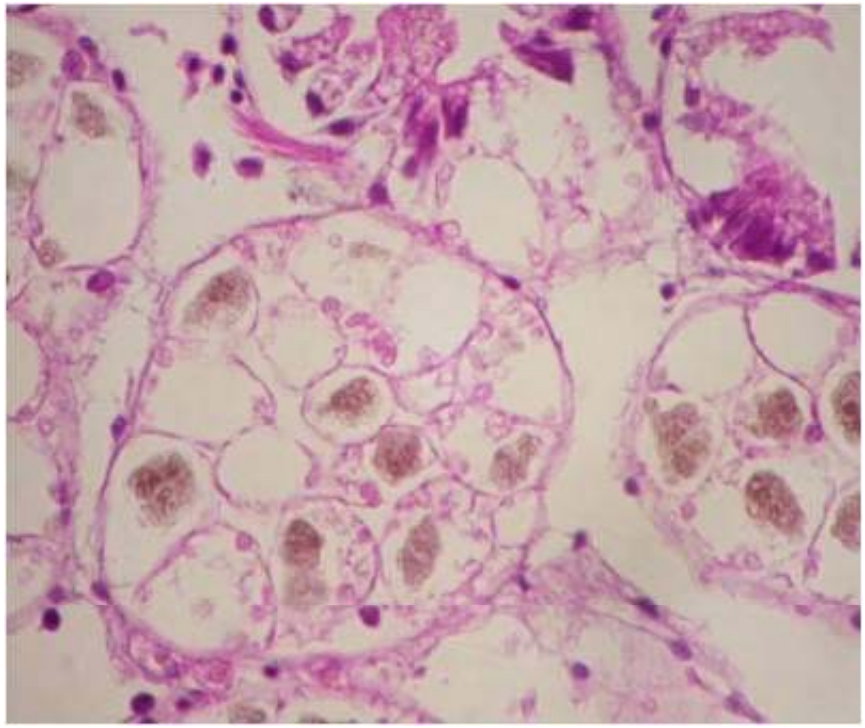
M = chromatin margination
E = Eosinophilic centre

Digestive epithelial virosis

Associated with mortalities in scallops, clams & mussels



Normal



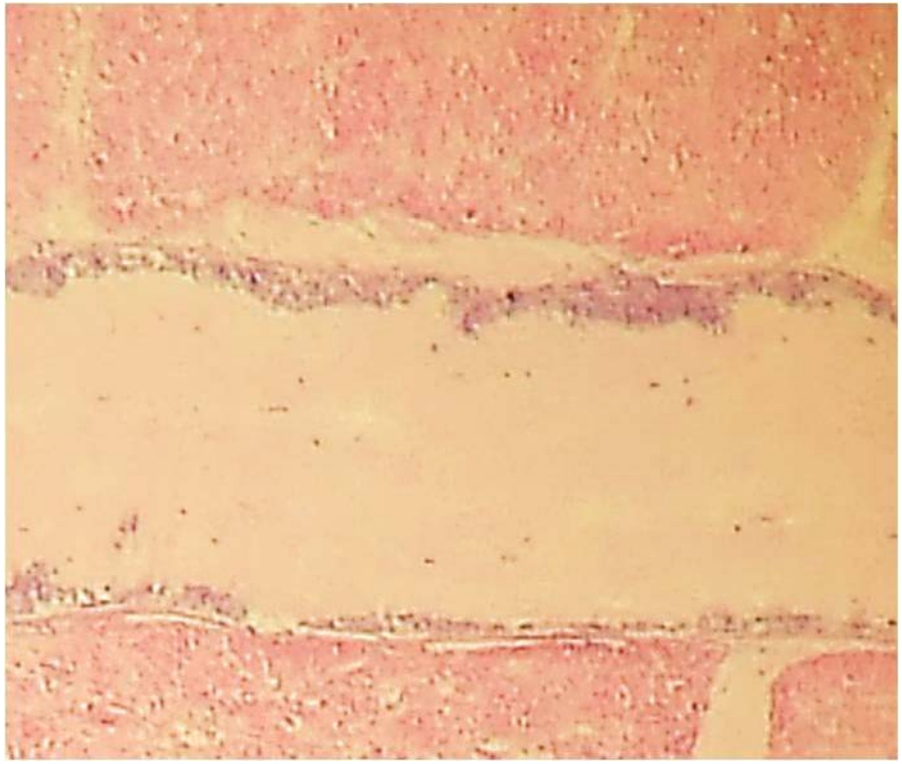
Infected

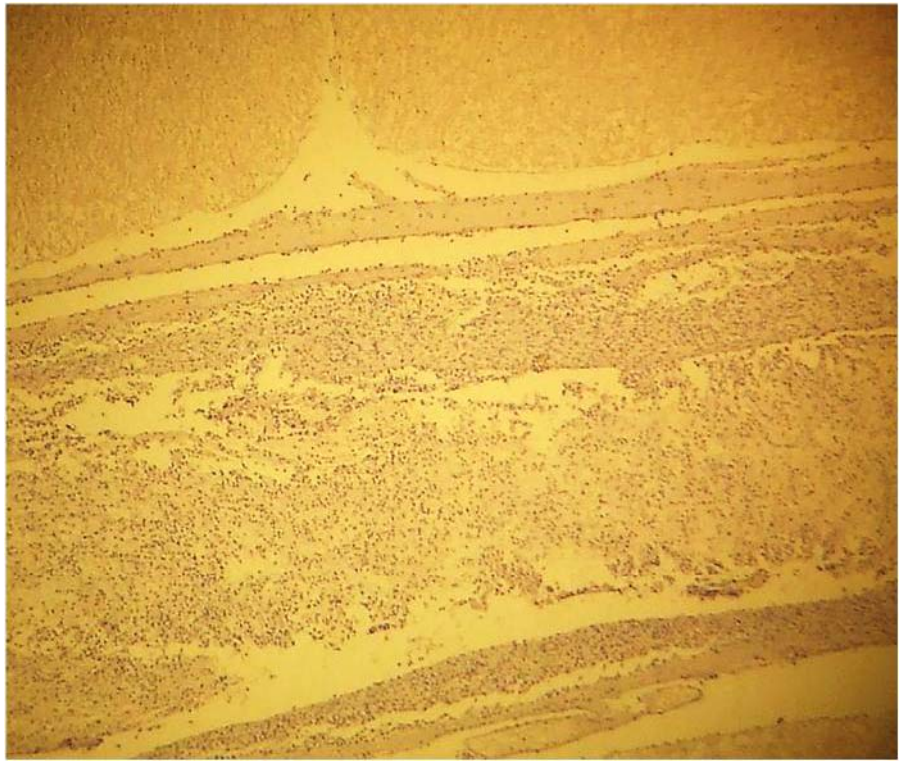
Necrotic cells filling the digestive lumen
Voids in the epithelium
Nuclear pycnosis and chromatin margination

Problem: is it a viral infection or an extreme form of normal development cycle?

Ganglioneuritis in *Haliotis*

Current testing combines gross examination with histo





Not in NZ - yet

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Histopathology:
direct visualisation of pathology agents

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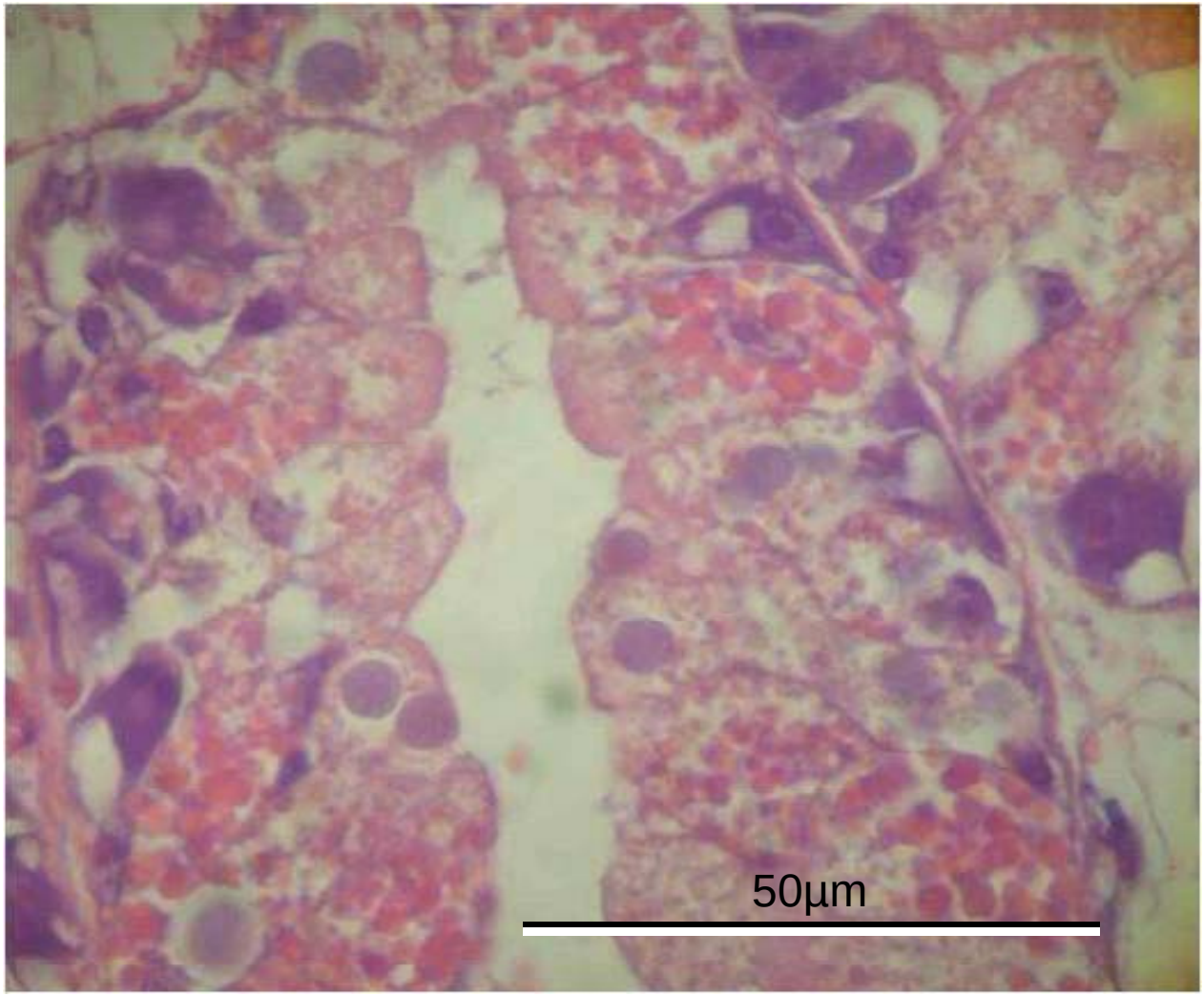
Intestinal tubule rickettsiae

Perna canaliculus



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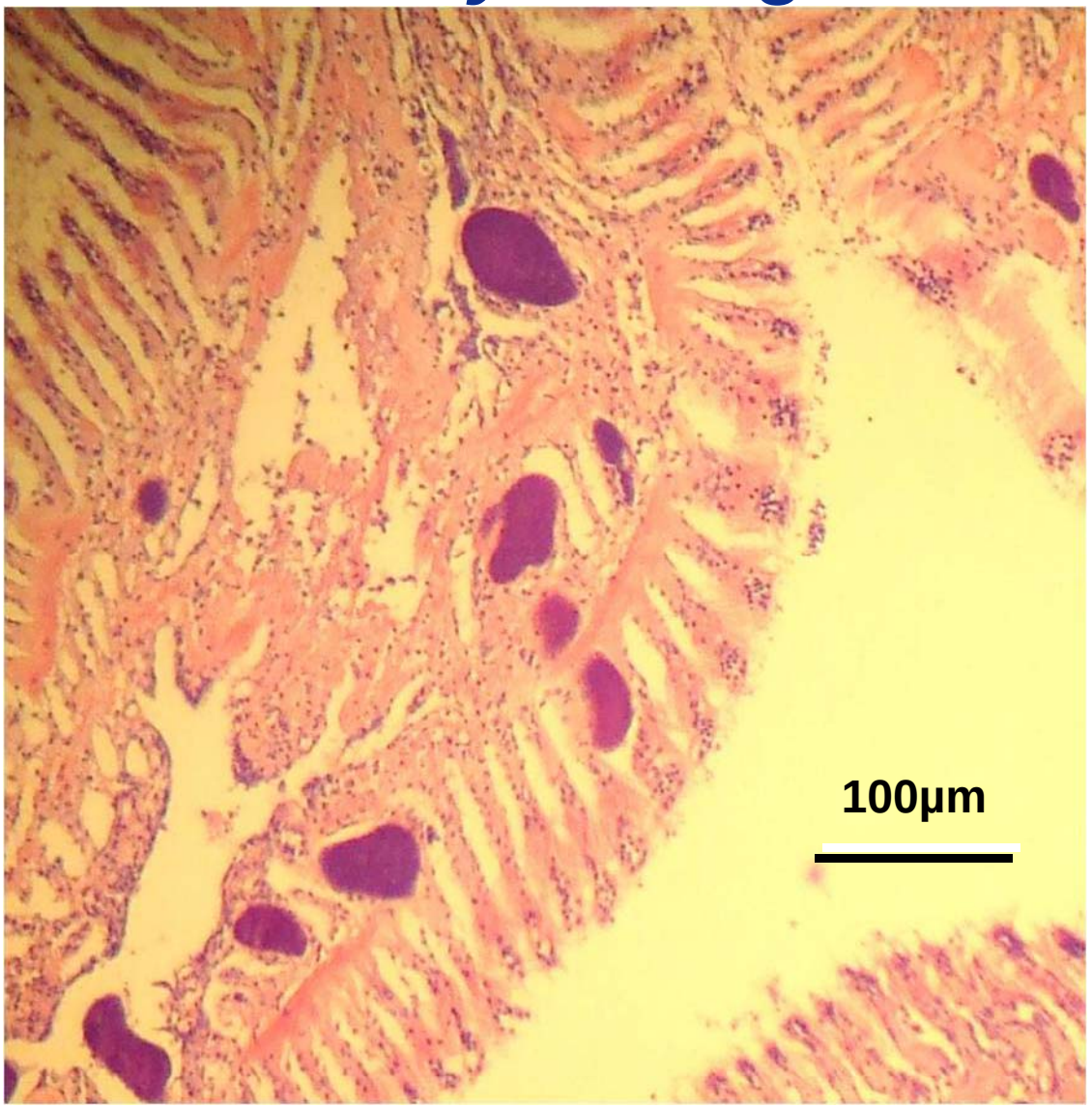
Chlamydia in gill

Spisula aequilatera



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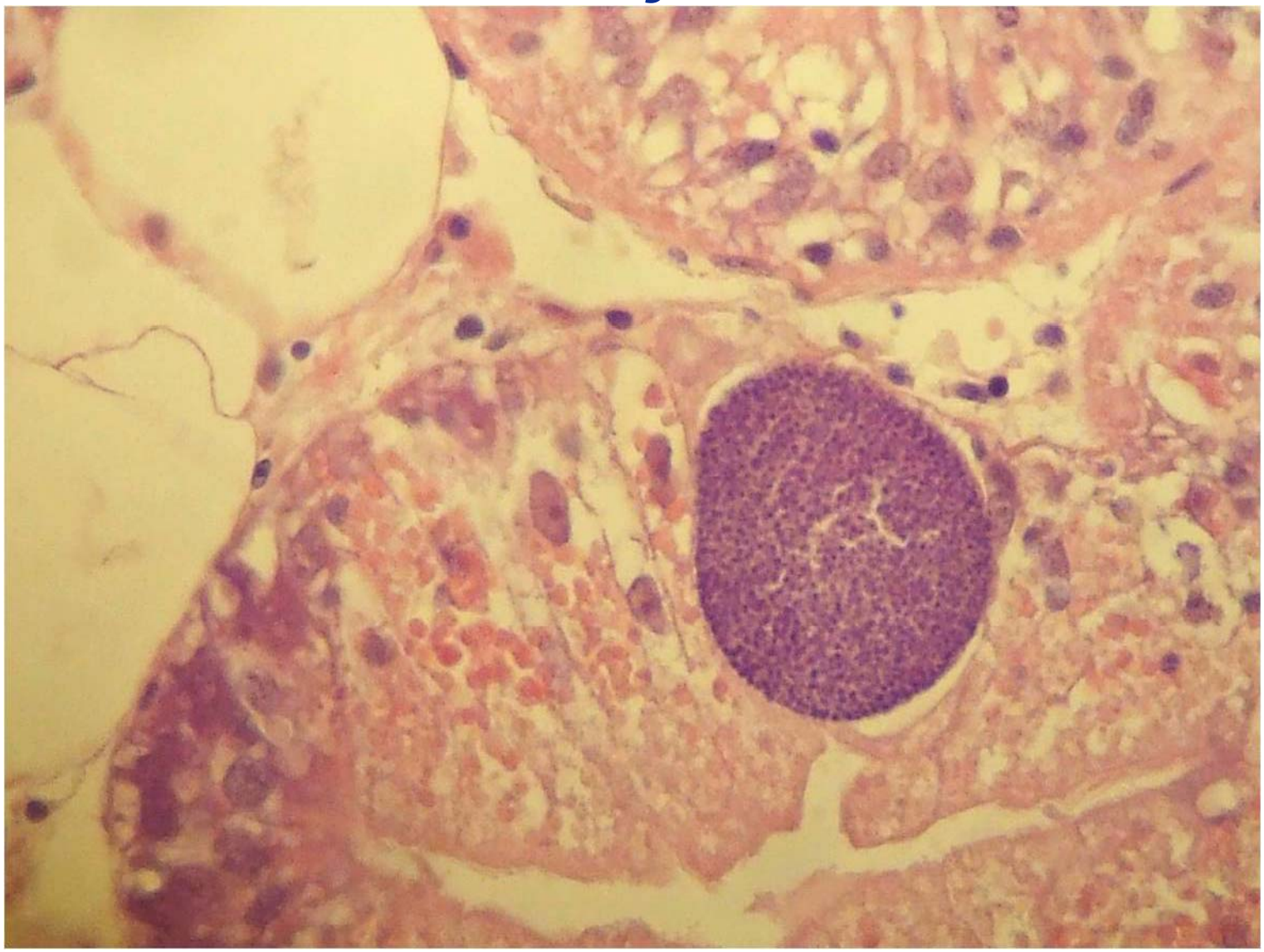
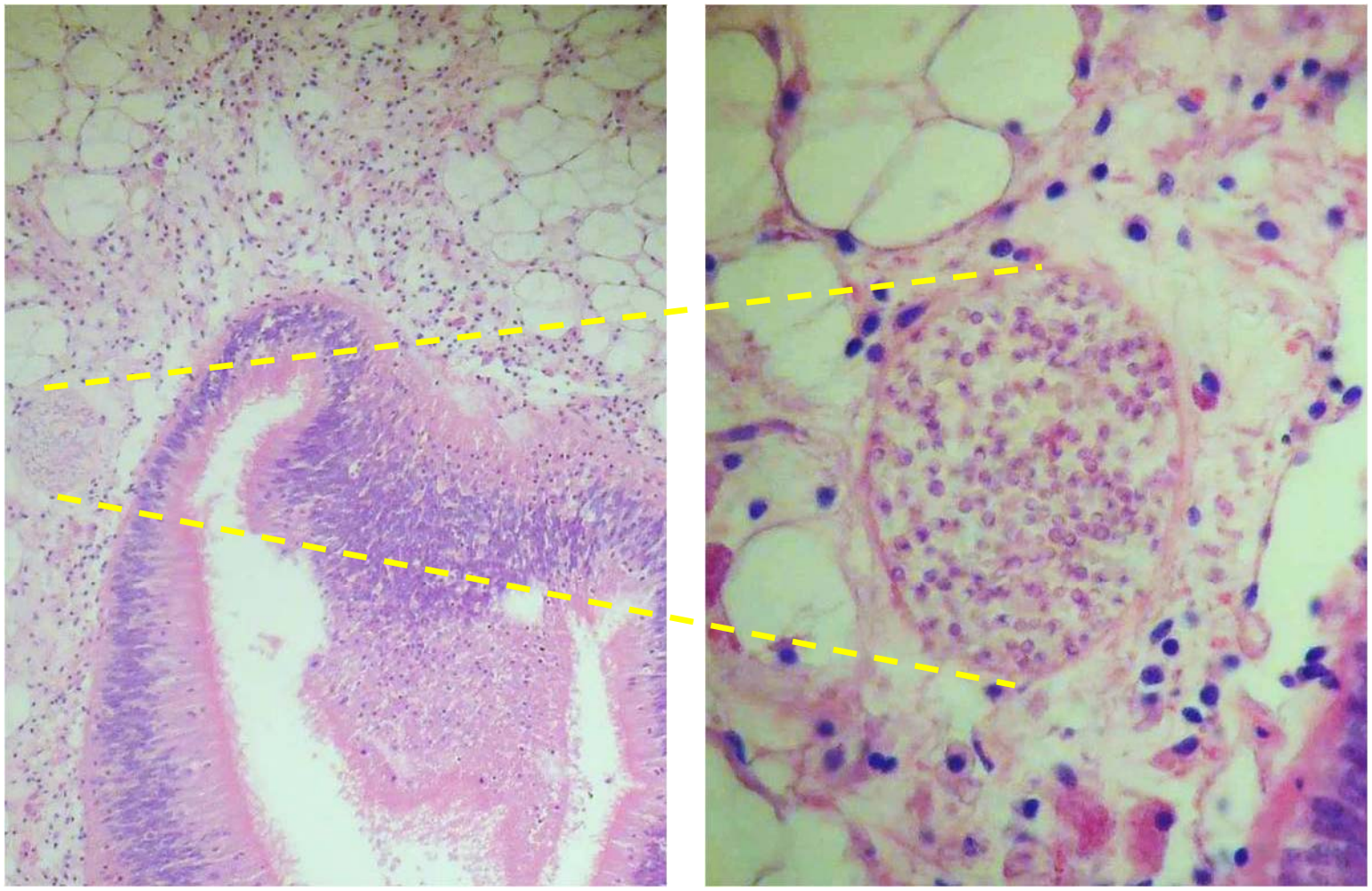
Chlamydia?



Microsporidium rapuae

Ostrea chilensis, *Perna canaliculus*



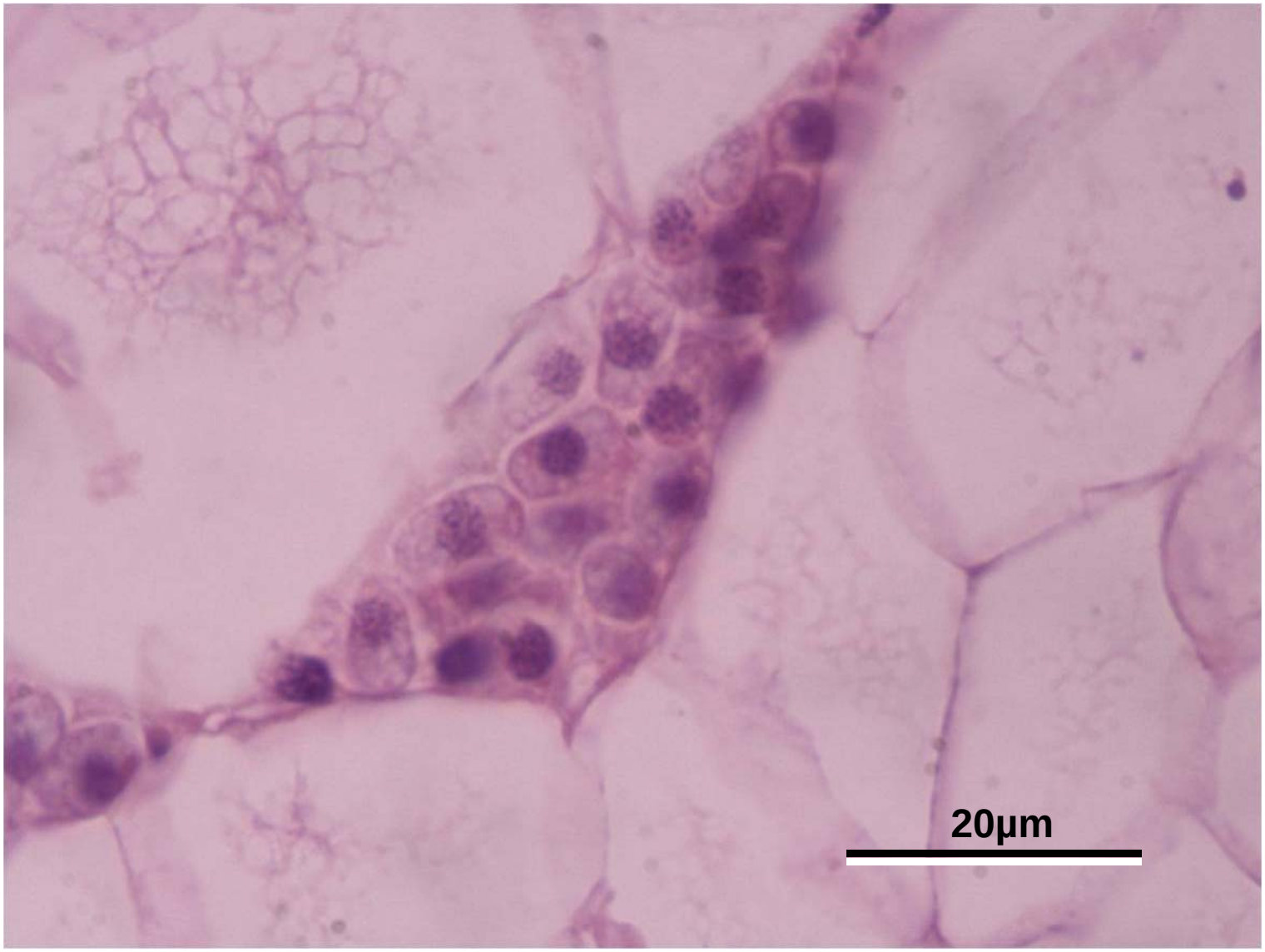
Problems

Same species in both hosts?

Susceptibility of other hosts?

APX

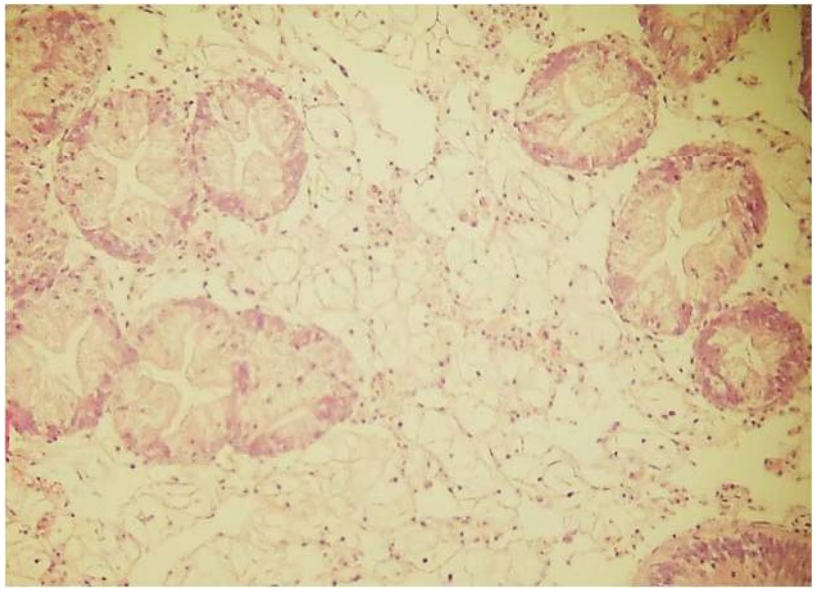
Ostrea chilensis, Perna canaliculus

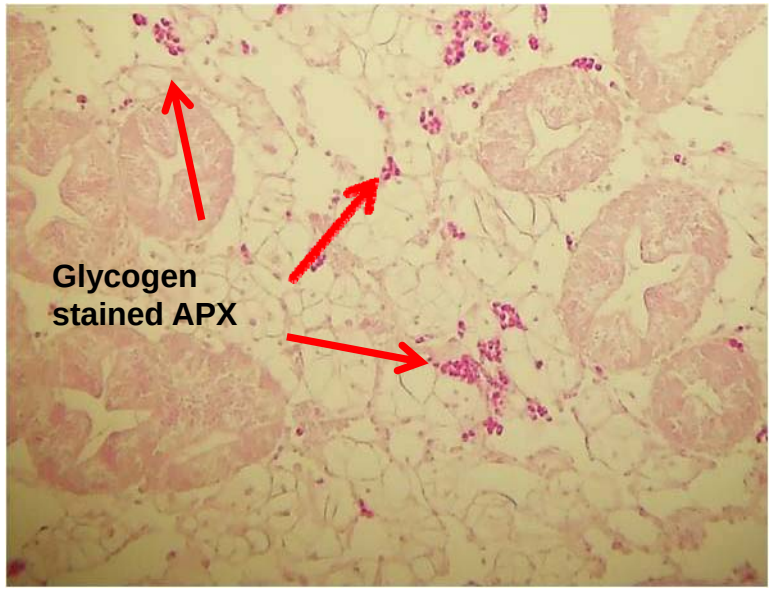


CAWTHRON

APX

Ostrea chilensis, Perna canaliculus





H&E

PAS

Problems

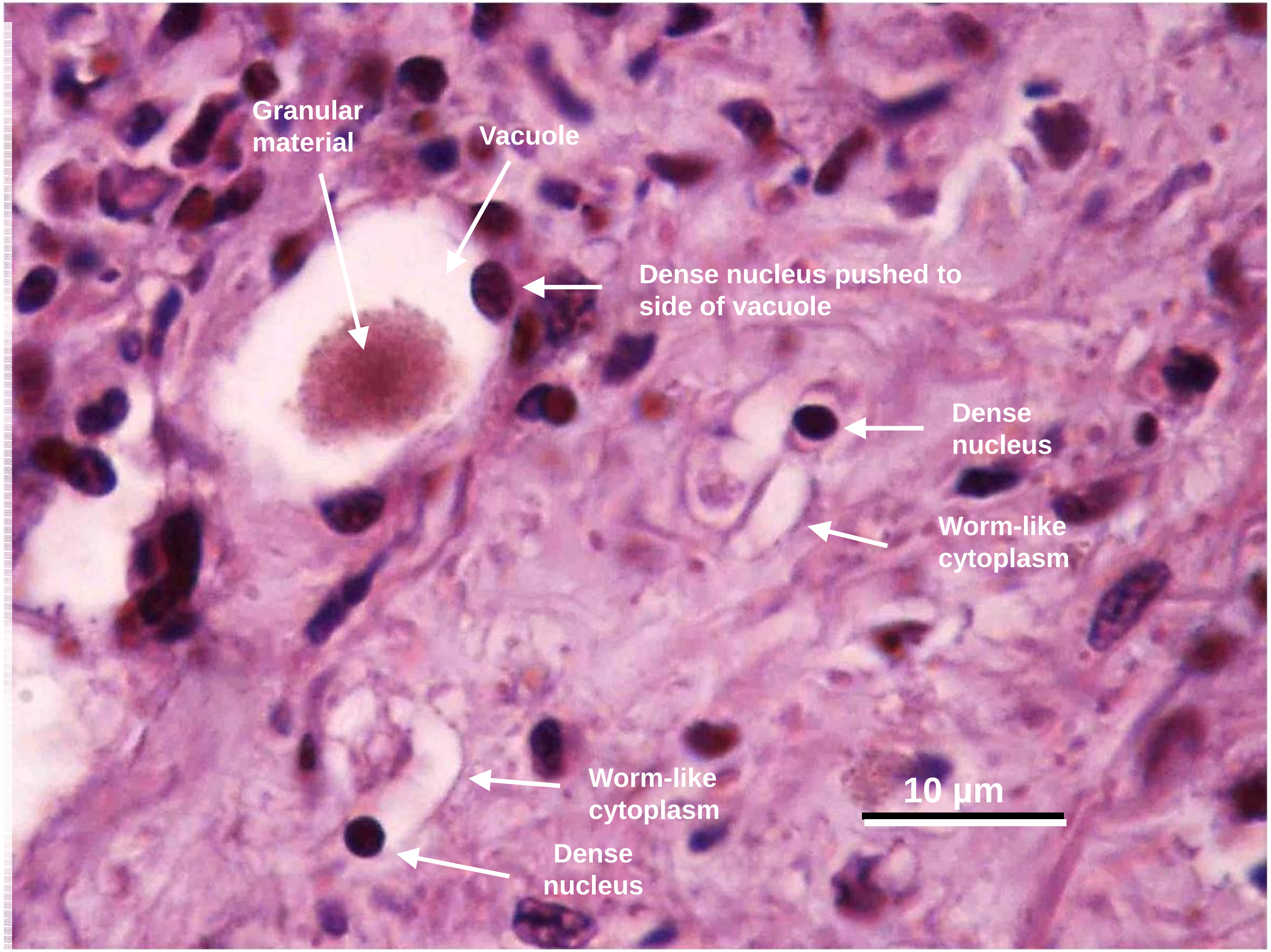
Same species in both hosts?

Susceptibility of other hosts?

CAWTHRON

Unidentified Inclusion: vermiform gregarine?

Pectin novaezelandiae



Problem: time/resources to pursue non-critical issues

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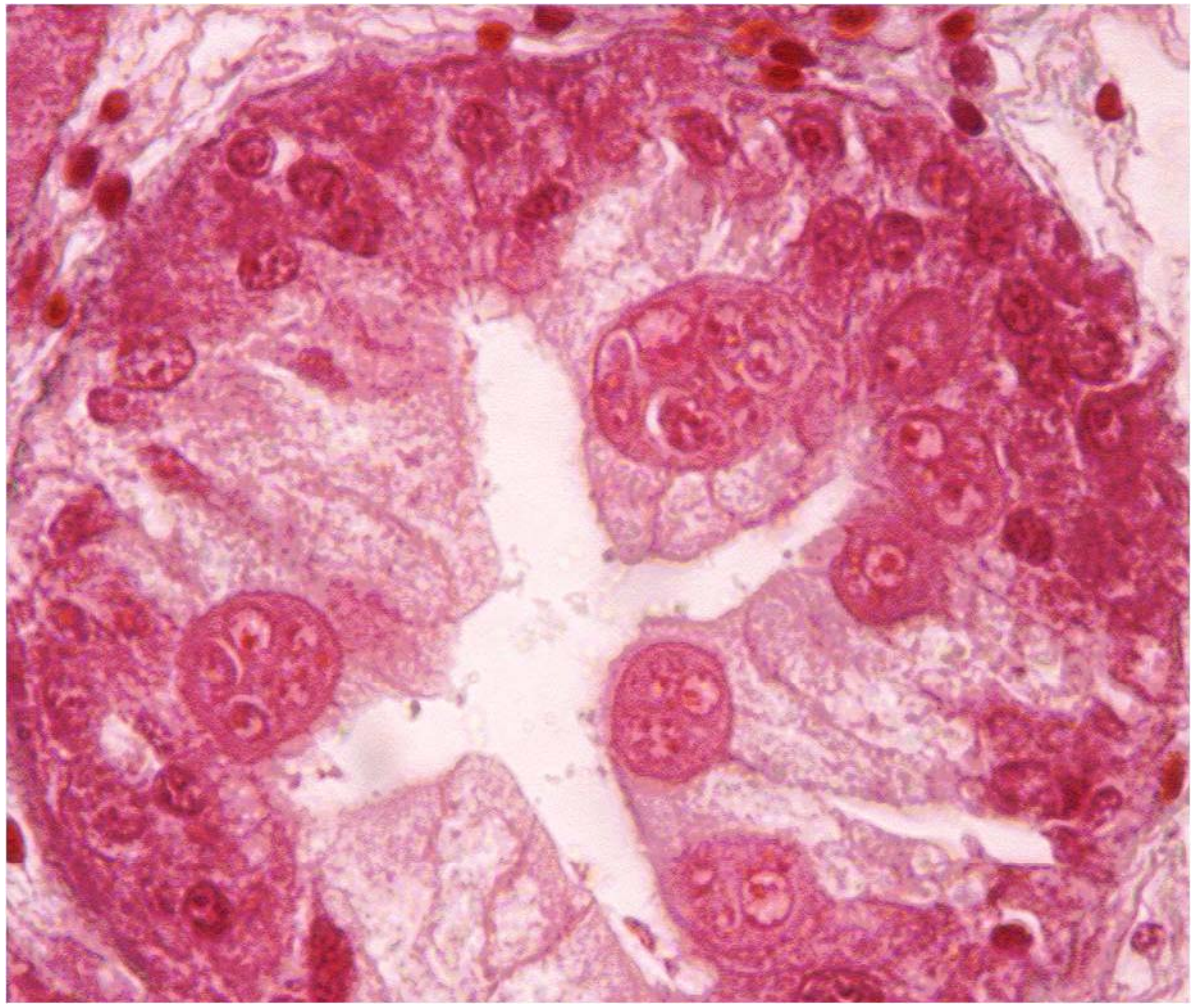
Marteilia in mussels and oysters

Not in NZ – yet



***M. Sydneyi* – threat to NZ Rock oyster (*Saccostrea*)**
M. refringens/maurini* – potential threat to *Perna canaliculus
Intermediate host required – suitable host in NZ?

CAWTHRON

Current activities

Sampling

- Testing at Glen n = 150 per species per 6 months (*2% prevalence at 95% confidence)
- *Haliotis iris* for reseeding n=60 (*5% prevalence at 95% confidence)
- Investigation of mortalities/morbidities - up to 30 affected animals.

*Assumes 100% specificity and sensitivity

Research activities at Cawthron on diagnostic methods

- OsHV-1 PCR
- *Bonamia* PCR
- APX (PAS) and Microsporidian (NR/NB) histopathology

Which diseases and/or what diagnostic tests are problematic?

DEV - is it a real disease or an extreme manifestation of a normal cycle of epithelial development?

Ganglioneuritis - what level of prevalence detection is useful? Histopathology is good for moderate/severe infections. When will more sensitive methods be needed in NZ? Selection of sentinel populations for testing?

Unattributable deaths of oyster and mussel juveniles – detailed diagnostics for ciliates, *Vibrios* (gram –ve), OsHV-1, and possibly *Nocardia* (gram +ve).

CAWTHRON INSTITUTE

What are the specific problems?

Provision of appropriate diagnostic capacity without making testing unaffordable.

Lack of sensitivity – OsHV-1, *Bonamia*, *Perkinsus*, Ganglioneuritis, abalone haplosporidian.

Lack of specificity - Vibrio spp. *Mikrocytos* vs *Bonamia*, *Marteilia* spp, *Perkinsus* spp.

Resources required to pursue interesting but non-critical occurrences such as the putative vermiform gregarine in scallops and systemic encysted ciliates in a range of bivalves.

Sampling issues

The first sign of a pathogen as a problem is often during a mortality/morbidity event – not through routine testing. NZ examples: abalone haplosporidian, OsHV-1, ciliates in larval mortalities, *Perkinsus*.

So how does routine sampling help control/manage disease?

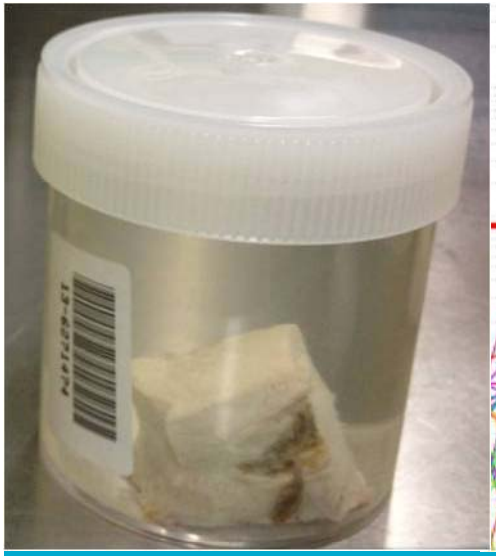
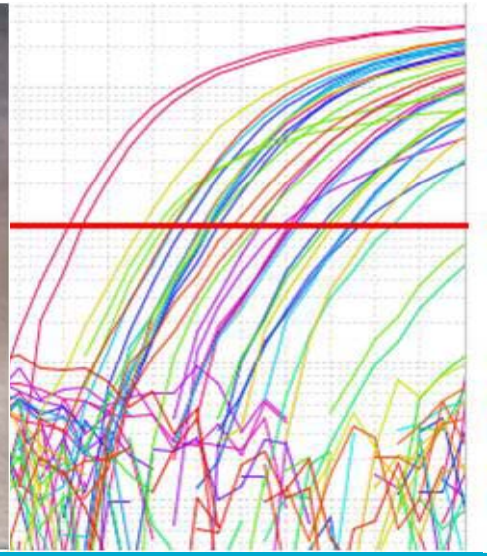
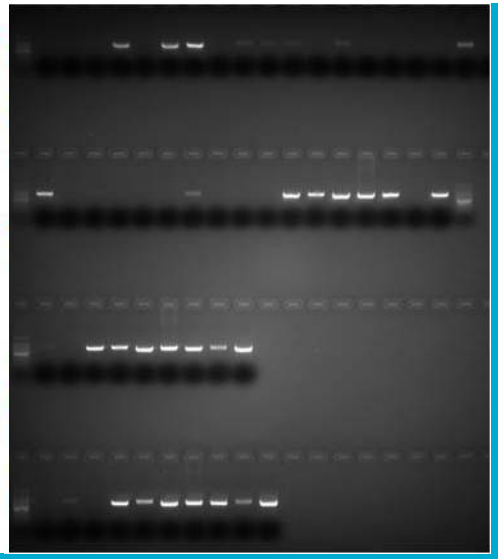
Is there any documented evidence that routine testing at n=150 has more utility than sampling at n= 60?

How much effort should be apportioned to detecting specific diseases?
To answer this more work on basic biology of pathogen/host interactions is required. Critical factors such as prevalence, intensity and conditions favourable to transmission and virulence in any host population require clarification.

Self-reporting of unusual mortalities/morbidities by farmers?

If farmers could be accredited to report/submit mortality samples, would this result in a lesser need for routine sampling? Create incentive to get accredited.

Criteria for accreditation?

?

Mollusc disease diagnosis: Identification of diagnostic problems in Australia

Nick Moody | Aquatic Animal Health Specialist

Workshop on Mollusc Disease Diagnosis, 21-24 October, 2013, Geelong, Australia

CSIRO ANIMAL, FOOD AND HEALTH SCIENCES
www.csiro.au

Diseases of concern

- Abalone herpesvirus (AbHV)
  - Abalone (*Haliotis laevigata and H. rubra)*
- Ostreid herpesvirus (OsHV-1 µVar)
  - Pacific oysters (*Crassostrea gigas)*
- *Perkinsus olseni*
  - Abalone *(H. rubra)* and Pearl oysters (*Pinctada maxima)*
- *Bonamia exitiosa*
  - Flat oysters (*Ostrea angasi*)
- Oyster oedema disease (OOD)
  - Pearl oysters (*Pinctada maxima)*

Unknown aetiology

Oyster Oedema Disease

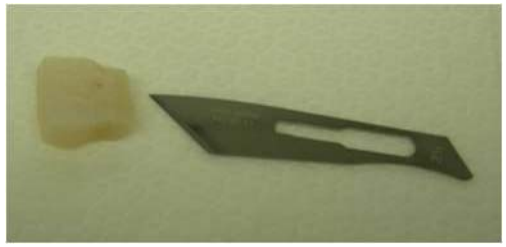
- Unknown aetiology
- FRDC 2013/002: Identifying the cause of Oyster Oedema Disease (OOD) in pearl oysters (*Pinctada maxima*), and developing diagnostic tests for OOD (June 2013 – Jun 2016)
- Objectives:
  1. Identify the presence of any pathogens associated with OOD-affected oysters using a next generation sequencing approach.
  2. Investigate the association of the pathogens detected by next generation sequencing with the severity of disease in P. maxima using a combination of quantitative (q) PCR, pathology and histopathology (including in situ hybridisation).
  3. Develop diagnostic tests that can detect and identify the causative infectious agent(s) of OOD

Samples

Submitted samples

- EtOH-fixed abalone tissue
- Methanol is not a good fixative

- More an issue with new/emerging diseases
- Solved by knowledge transfer to submitters



Sample processing

- Proteinase K digestion vs Bead beating
  - AbHV
  - OsHV-1

- OsHV-1 example:

Sample No	Digested (A)	Bead beater (B)	Difference (A – B)
1	30.88	Undetermined	**Significant**
3	17.82	25.98	**-8.16**
5	14.34	21.39	**-7.05**
6	26.47	34.91	**-8.44**
9	28.66	35.93	**-7.27**
2	35.30	Undetermined	**Significant**

- Proteinase K digestion more time consuming but results in a more sensitive procedure

Extraction

Columns vs Magnetic beads

- High throughput vs low throughput

Sample No	Spin columns (A)	Magnetic beads (B)	Difference (A – B)
1	28.40	31.59	**-3.19**
2	24.50	28.63	**-4.13**
3	17.90	21.04	**-3.14**
4	24.70	28.71	**-4.01**

- Issue for surveillance or translocation samples (low prevalence, low levels of target)
- Evaluate different Magnetic bead extraction chemistries

Choice of assay

Real-time assays: AbHV

- Late 2005: Mortalities in Victorian abalone: VIC-1
- Mid 2008: mortalities in Tasmania in a processing plant: TAS-1
- 2009 – 2010: mortalities in Tasmania in a processing plant: TAS-2
  - Not all lesion positive samples were positive by current qPCR
  - Multiple assays and confirmation by sequencing
- 2010 – 2011: mortalities in Tasmania in a processing plant: TAS-3 & TAS-4
- Identified 4 Tasmanian strains of AbHV (possibly TAS-5)

AbHV real-time assays

- Current AbHV real-time assays: ORF66 and ORF77 (and ORF49)

STRAIN→	VIC-1	TAS-1	TAS-2	TAS-3	TAS-4	TAS-5?
	2005/6	Aug 2008	Aug 2009 Nov 2011	Dec 2010 Jan 2011	Feb 2011	Nov 2011
ORF49	✓	✓	✘	✘	✓	✓
ORF66	✓	✓	✓	✓	✓	✓
ORF77	✓	✓	✓	✓	✓	✓

- Multiple assays targeting different regions of the viral genome
  - e.g. OsHV-1: Martenot and EMAI qPCRs

Limits of detection
Low levels of target
Grey zone

Very low levels of target

- Issue with AbHV and OsHV-1

- Surveillance, confirmation of infection and translocation
  - Have to confirm a “suspect”, “indeterminate”, “ambiguous” etc., etc., result

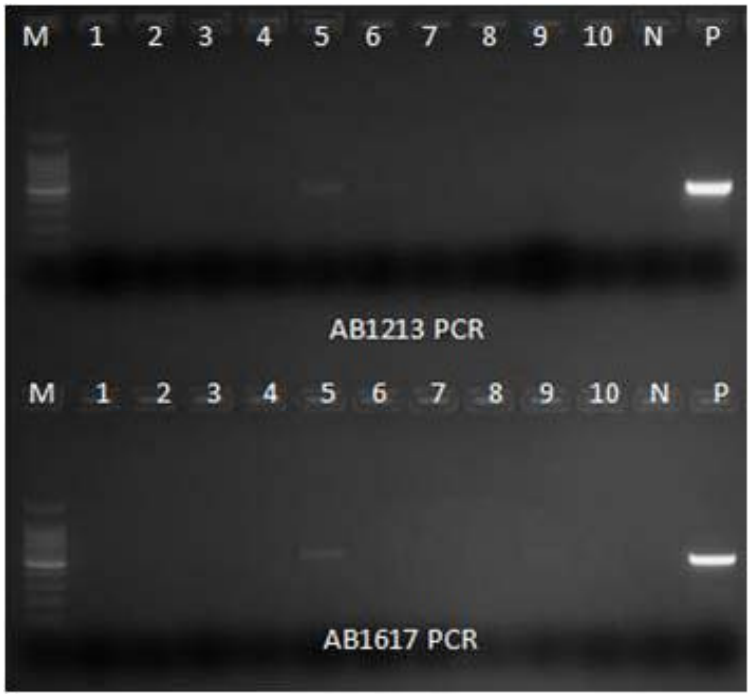
Sample	AbHV66 qPCR	AbHV49 qPCR	AbHV77 qPCR
14	25.89	26.53	28.57
03	30.71	30.15	32.01
16	36.31	Und/36.35	39.17
19	38.71	Und/38.85	Undet
29	39.06	Undet	Undet
05	**Undet/36.49**	**Undet**	**Undet/40.38**
28	**Undet/36.56**	**Undet**	**Undet/41.78**
15	**Undet/37.37**	**Undet**	**Undet**
02	**Undet/38.50**	**Undet**	**Undet/38.9**

Things that didn't work

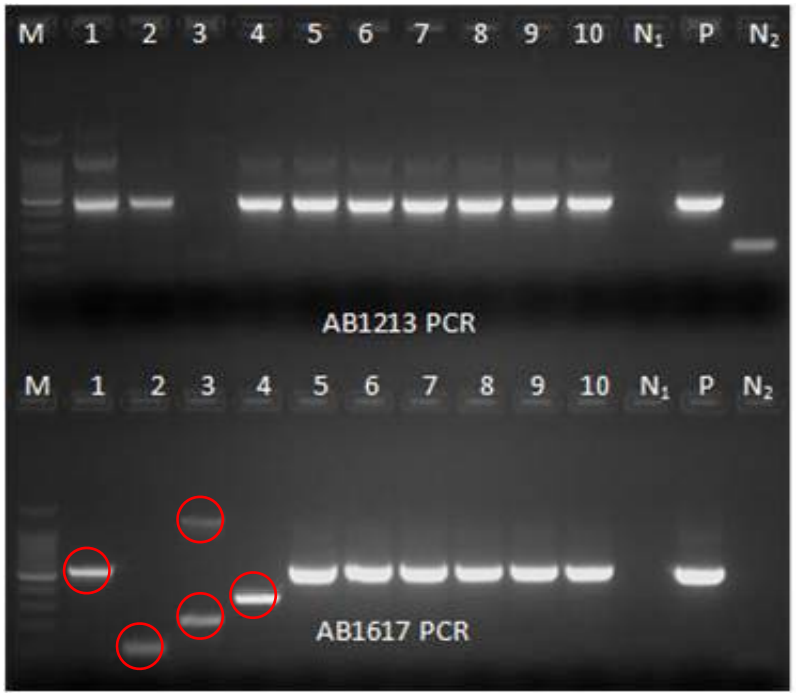
- Increasing template volume
  - Inhibition of PCR due to excessive levels of host template
  - Dilution possible solution but dilute out target
- cqPCR
  - Conventional PCR followed by real-time PCR
- nnPCR
  - Nested nested PCR
- Multiplexing with 18S
  - Two single assays slightly (~1C_T more sensitive)

Possible solutions

- AbHV: important to get sequence for new isolates



2µL DNA, 23µL reaction mix, 40 cycles



5µL DNA, 50µL reaction mix, 70 cycles

- Nested PCR as more whole genome sequence is generated

Possible solutions

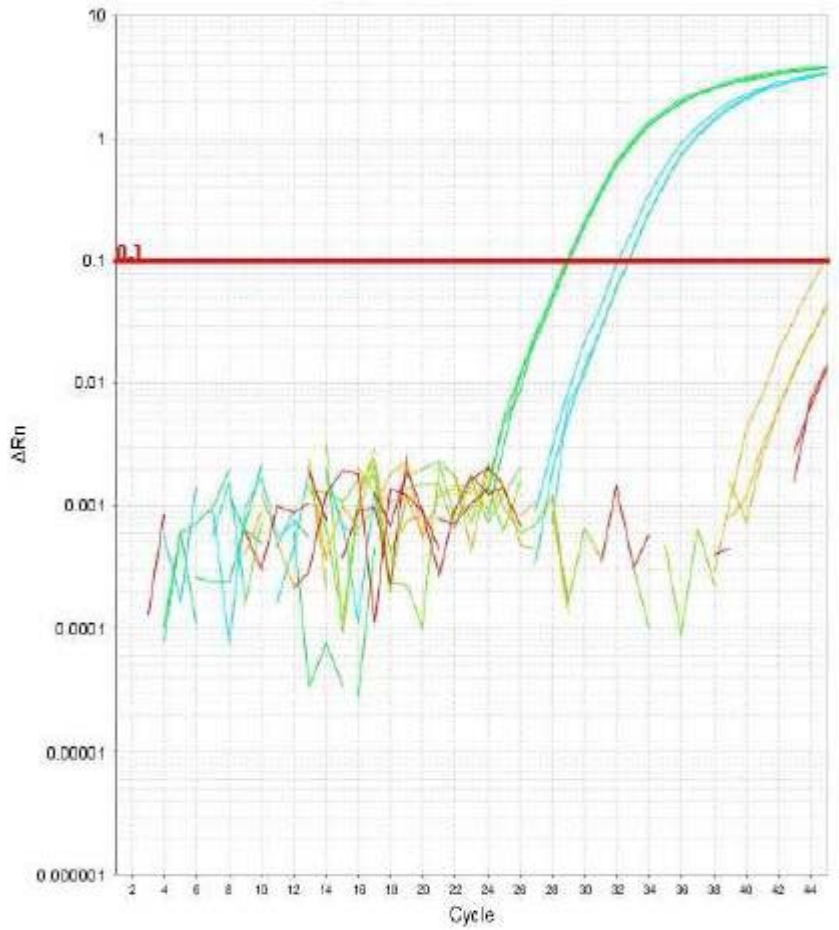
- AbHV: important to clarify true status (POS/NEG)
- 50µL template + 575µL Master Mix → 25 replicates

Cт Mean	Cт SD	Positive	Negative
44.20	0.94	41.90	Undetermined
		43.34	Undetermined
		44.34	Undetermined
		45.13	Undetermined
		44.40	Undetermined
		44.77	Undetermined
		44.76	Undetermined
		44.67	Undetermined
		44.36	Undetermined
		44.36	Undetermined
			Undetermined
			Undetermined
			Undetermined
			Undetermined
			Undetermined
	n	10	15

Cт Mean	Cт SD	Positive	Negative
43.03	1.34	43.68	Undetermined
		42.68	Undetermined
		41.77	Undetermined
		41.36	Undetermined
		45.70	Undetermined
		41.50	Undetermined
		41.98	Undetermined
		43.20	Undetermined
		43.59	Undetermined
		43.33	Undetermined
		44.51	Undetermined
			Undetermined
			Undetermined
			Undetermined
	n	11	14

Possible solutions

- Don't report C_T values
- Arbitrary threshold of 0.1 for positive control monitoring
- Assess the result based on a typical amplification curve
- Increased number of cycles to 50 (or 55)
  - Sample with C_T of 48 confirmed as positive by 70-cycle PCR and sequencing
- Educating people that this is real result



To resolve

Things to sort out

- Pooling
- Number of samples per specimen for non-homogenously distributed pathogens
- Limited known-positive samples for assay validation

Acknowledgements

- AAHL Fish Diseases Laboratory
- State laboratory colleagues
- Fisheries Research and Development Corporation
- Department of Agriculture, Fisheries and Forestry

Thank you

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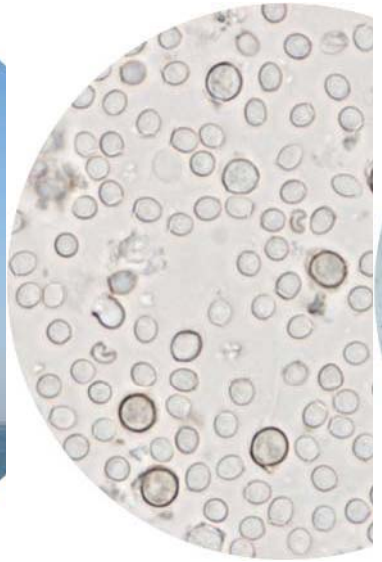
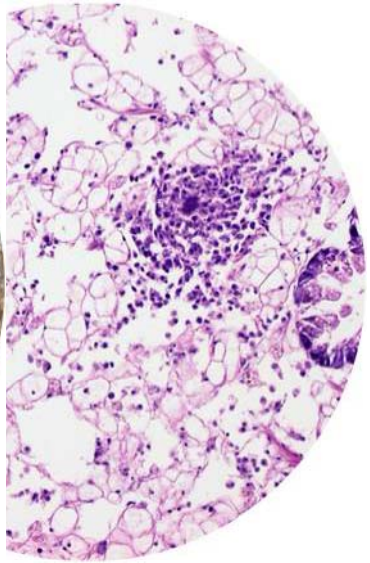
State of laboratory network in EU

Workshop on "Mollusc Disease Diagnosis"

Geelong, Australia, 23 October 2013

Marc Engelsma



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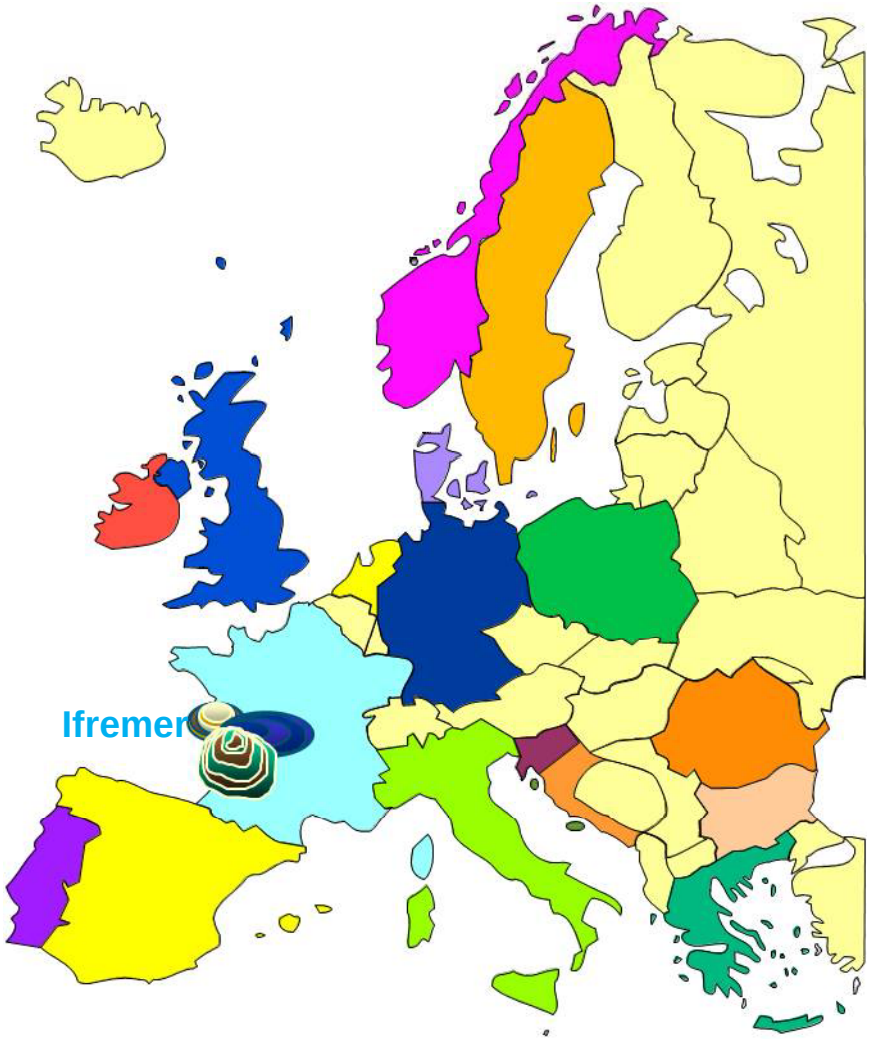
EURL-NRL network for mollusc diseases

- EU Reference Laboratory for mollusc diseases
  - IFREMER, La Tremblade
- National Reference Laboratory for mollusc diseases
  - 22 NRLs
- Function and duties of EURL and NRL are defined in the EU legislation 2006/88/EC

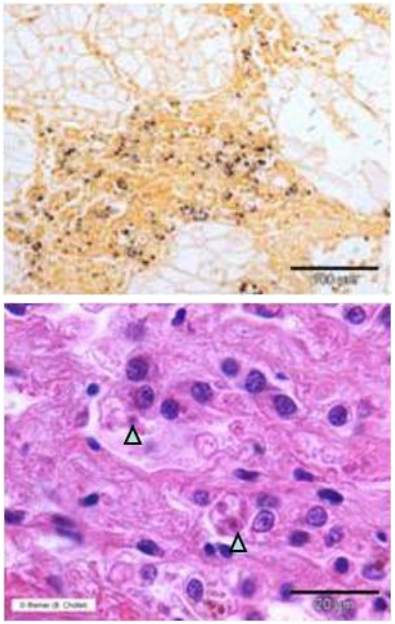


EURL function and duties

- Coordinate in consultation with EU Commission the methods employed by MS for diagnosing mollusc diseases
  - Supplying control materials
  - Organizing inter-laboratory comparison tests
- Assist in diagnosis of disease outbreaks in MS: confirmatory diagnosis, characterisation and epizootic studies
- Facilitate training in diagnosis; harmonise diagnostic techniques throughout Europe
- Collaborate with competent laboratories in third countries on methods of diagnosing exotic diseases

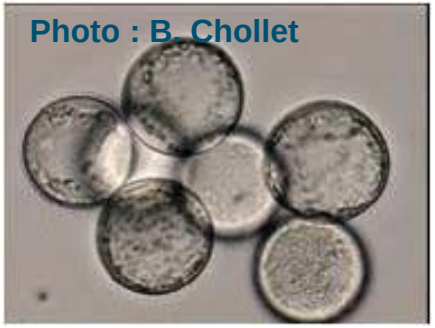
Examples of tasks carried out by the EURL

- Collection of Reference material
  - Paraffin blocks, slides, tissue, DNA, bacteria, cultures…
- Assistance to NRLs
  - Reception and characterization of material
- Organisation of Interlaboratory Comparison Tests
  - Histocytopathology / PCR
- Training:
  - annual meetings, workshops, trainees …
- Collaborations :
  - Australia, Mexico, South Korea, Tunisia…
- Some tools: website, mscope



Examples of tasks carried out by the EURL

- Development and validation of diagnostic tools
  - Real time PCR for detection and quantification of *B. ostreae* (Robert et al. 2009)
  - Development of real time PCR for specific detection of OsHV-1 µvar (Pépin et al. *in prep*)
- Characterization of genes of diagnostic and taxonomic interest
  - actin gene (*Bonamia exitiosa*) ; HSP90 (*Bonamia ostreae*) (Prado et al. 2012)
- Molecular characterization of parasites of the genera *Bonamia*, *Marteilia* and *Perkinsus*
  - Distribution of *B. exitiosa* in Europe
  - *M. refringens* typing
  - Detection of *P. chesapeaki*-like in France (Arzul et al. 2012)



NRL function and duties

- Notify the competent authority (CA) on suspicion of EU listed disease
- Coordinate in consultation with EURL methods employed at national level for diagnosing mollusc diseases
- Assist in diagnosis of disease outbreaks at MS level by confirmatory diagnosis and characterisation and ensure confirmation of positive results of EU listed diseases
- Participate in comparison tests organized by EURL
- Operate under QA ISO9001/ISO17025
- At national level:
    - Facilitate the training with a view to harmonise diagnostic techniques
    - Organise ring tests

Other networks

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BIVALIFE

- The project focuses on *Crassostrea gigas*, *Mytilus edulis* and *M. galloprovincialis* and targets the pathogens: OsHV-1, *Vibrio* species, *Marteilia refringens* and *Nocardia crassostreae*.
- The objectives of BIVALIFE are:
  - Transfer and validate existing methods for detection and identification of oyster and mussel pathogens
  - Characterise culture sites in Europe regarding presence of oyster and mussel pathogens and assess their role in observed mortality
  - Investigate the life cycle, mechanisms allowing pathogens to survive outside the host and their original source
  - Identify pathogen intrinsic virulence factors and effects on host defence mechanisms
  - Develop methods and recommendations for pathogen control and eradication in Europe

BIVALIFE

- Partners
  - IFREMER, FR (Coordinator)
  - CSIC, SP
  - UCC, IE
  - CVI-IMARES, NL
  - IRTA, SP
  - Marine Institute, IE
  - UNIGE, IT
  - UNIPD, IT
  - CEFAS, UK
  - CNRS, FR
  - ATLANTIUM, I
  - EMPA

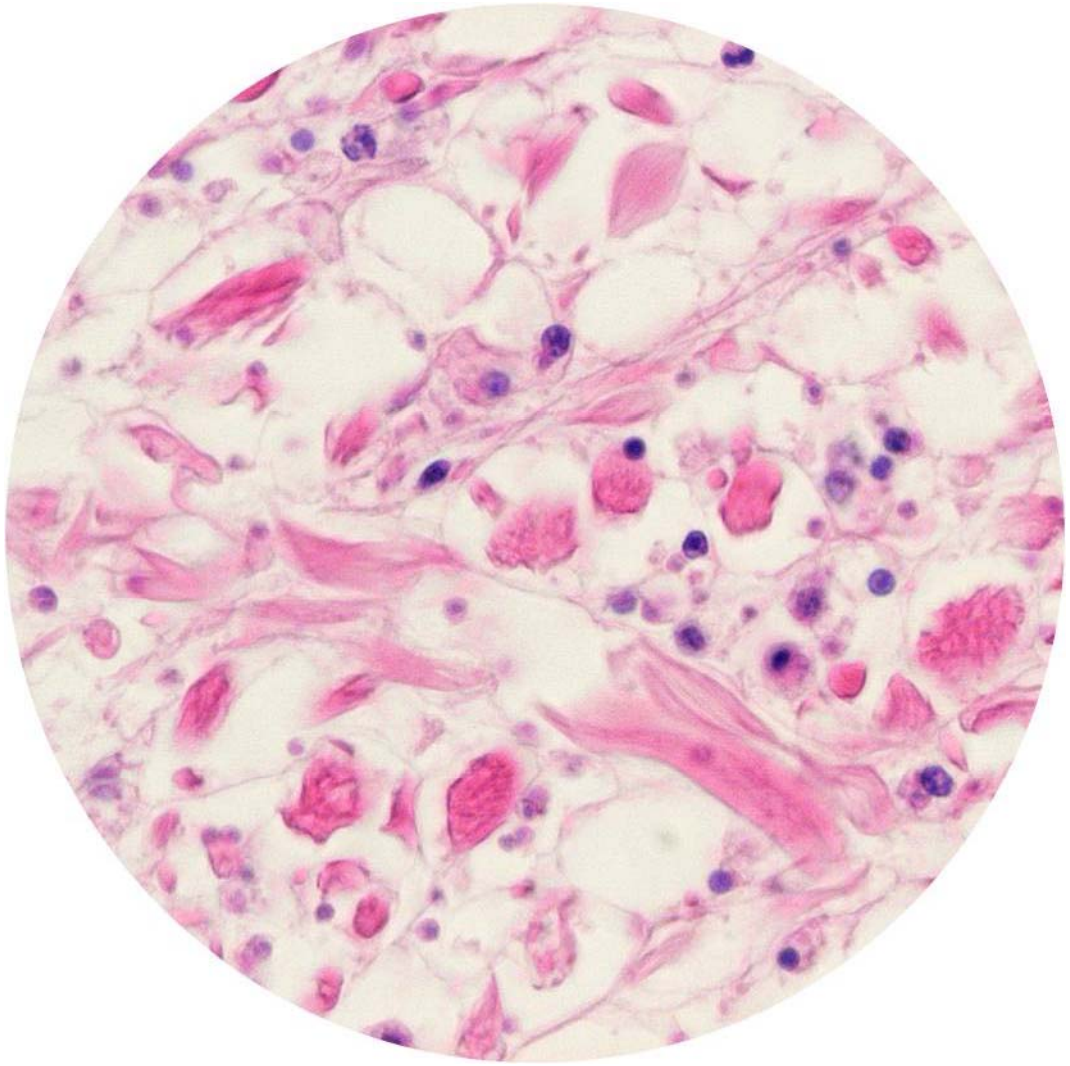
OYSTERECOVER

- Aim: Recovery of European flat oyster production, through development of strategies to tackle bonamiosis
- Objectives:
  - Validate diagnostic methods
  - Determine possible involvement of invertebrates in the life cycle of *Bonamia ostreae* (vectors or intermediate hosts)
  - Study the ability and the mechanisms of haemocytes to destroy *B. ostreae* between oyster stocks with different susceptibility to bonamiosis
  - Study gene expression profiles in response to bonamiosis challenges, to identify candidate genes for tolerance
  - Evaluation of the gain of selective breeding programmes for oyster tolerance to bonamiosis
  - Genetic characterization of flat oyster populations with genetic markers and markers linked to tolerance to bonamiosis

OYSTERECOVER

- European Research Centres and Universities:
  - CETMAR, SP (coordinator)
  - CIMA, SP (scientific coordinator)
  - USC, SP
  - UCC – IE
  - CEFAS, UK
  - IMARES, NL
  - Danish Shellfish Centre, DK
  - AGROCAMPUS, FR
- SME Associations:
  - Confraría de Pescadores S. Bartolomé de Noia, SP
  - Clew Bay Marine Forum Ltd, IE
  - Clew Bay Oyster Co-operative Society Ltd, IE
  - Nederlandse Oestervereniging, NL
  - Comité Régionale de la Conchyliculture Bretagne-Nord, FR
- SMEs:
  - José María Daporta Leiro e Hijos S.L., SP
  - Atlantic Shellfish Ltd, IE

Specific expertise of EU laboratories



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IFREMER (FR)

- EURL and French NRL for mollusc diseases: Functions defined in 2006/88/EC directive and in French legislation (décret n°2006-7 du 4 janvier 2006)
- Main goals: 1) to contribute to the study of the health of French marine molluscs and 2) to ensure a high level of quality and uniformity of analytical results in official laboratories.
- In France, network of 9 official laboratories and 5 recognised laboratories
  - OsHV-1 and *Vibrio* diagnostic
- In 2013:
  - expertise on *C. gigas* adult mortalities
  - development of a new laboratory network for histological analyses
  - validation of some diagnostic tools

CEFAS (UK)

- CEFAS is the National Reference Laboratory for England and Wales
- Full set of techniques applied (histology, molecular, EM) for:
  - *Bonamia ostreae / Bonamia exitiosa*
  - *Marteilia refringens*; including research project looking at genetics of mussel and oyster forms
  - *Mikrocytos* spp.; recently diagnosed new species
  - OsHV-1
  - Haplosporidians
- Expertise includes epidemiology
- Availability of experimental lab facilities and possibilities for field trials

CSIC (SP)

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

- National Reference Laboratory for Spain
  - Diagnostic tools carried out in the Spanish NRL

Diagnostic tools	**Non exotic pathogens**		**Exotic pathogens**		
	Bonamia ostreae	*Marteilia refringens*	*Bonamia exitiosa*	*Perkinsus marinus*	*Microcytos mackini*
Targeted surveillance	Histology Tissue imprints PCR	Histology PCR	Histology Tissue imprints PCR	Histology RFTM	Histology
Presumptive diagnosis	Tissue imprints, PCR	Tissue imprints, PCR	Histology Tissue imprints, PCR	PCR	Histology PCR
Confirmatory diagnosis	Sequencing/ PCR-RFLP	PCR/ Sequencing	Sequencing/ PCR-RFLP	sequencing	sequencing

  - Oyster herpes virus µvar: qPCR/PCR-RFLP/sequencing

IRTA (SP)

- Methods used for diagnosis of mollusc diseases:
  - Histology
  - Other: classical microbiology, RFTM, PCR, real time PCR, PCR-RFLP, DIG-ISH, MLST analysis
- Diseases of importance:
  - OsHV-1: Main screening PCR (Webb et al and C2/C6, C9/C10) and in addition histology and ISH
  - *Vibrio* spp.: Classical bacteriology and Taqman real time PCR (SOP Bivalife)
  - Parasites: emphasis on *Perkinsus* detection

IZSV (IT)

Istituto Zooprofilattico Sperimentale delle Venezie

- Diagnostic methods applied at NRL Italy:
  - *Perkinsus olseni*: RFTM culture, cytology, histology, PCR
  - *Marteilia refringens*: Cytology, histology, PCR
  - *Bonamia ostrea* and *B. exitiosa*: Cytology, histology, PCR
  - OsHV-1 and variants: PCR
  - Other pathogens: histology
- Other available assays as support and to investigate mass mortality events, emerging pathogens or to study the interaction host-pathogen:
  - Bacteriology (culture methods, PCR: one step, real time, multiplex, RFLP, multi-locus-seq.): i.e. *Vibrio*'s
  - Histochemistry; Transmission electron microscope; DNA analyzer; MS Maldi-tof
- In close collaboration with university of Padova and Teramo: flow cytometry, transcriptomics techniques

Marine Institute (IE)

- Fish Health Unit, Marine Institute designated NRL & CA Ireland
- Diagnostic methods for molluscs:
  - *Bonamia ostrea* / *Bonamia exitiosa*: Histology, cytology, real time PCR (Ramilo et al 2013)
  - *Marteilia Refringens*: Histology, real time PCR (Ifremer, EURL)
  - *Perkinsus marinus*: Histology (undergoing validation)
  - *Mikrocytos mackini*: Histology (undergoing validation)
- Diseases of national importance
  - OsHV-1: Main screening Taqman PCR (Martenot et al 2010) and species confirmation LNA real time PCR (Ifremer, EURL)
  - *Vibrio* sp.: Classical bacteriology, 16S sequencing and Taqman Real time PCR for *V. aestuarianus* (*in house*) and *V. splendidus* (Ifremer, EURL)
  - BRD / *Vibrios tapetis*: Classical bacteriological methods and conventional PCR (Romalde et al, 2007)

UCC (IE)

PI (Sarah Culloty plus currently team of 13)

Working on Oysters (*O. edulis* and *C. gigas*), mussels (*M. edulis* and *M. galloprovincialis*), clams, urchins

- Main interests: Epidemiology, Host parasite interactions, Life cycle studies (mechanisms for pathogens to maintain themselves outside the host), Diagnostics (validation, development of primers, etc), Development of resistance to disease

Some current projects:

Herpes virus:

- EU FP7 Bivalife – herpes virus outside of the host and factors impacting on development of infection)
- EU FP7 Herpish – development of resistance to herpes virus

Bonamia ostreae

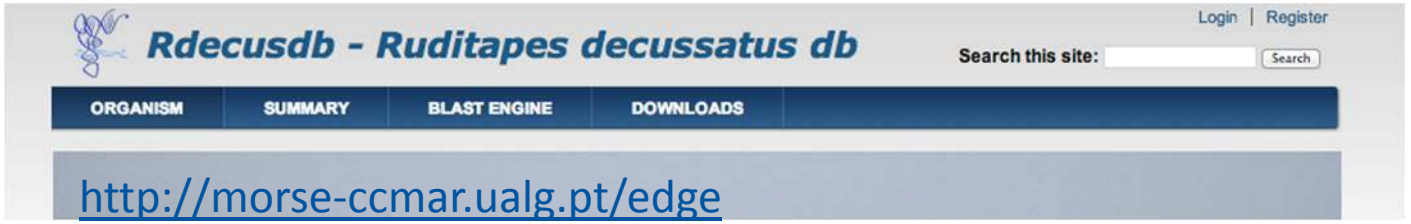
- EU FP7 Oysterecover – conservation of native oyster through control of *Bonamia* (validation of diagnostics, life cycle, genetic basis for resistance)

CCMAR (PT)

CCMAR
CENTRO DE CIÊNCIAS DO MAR

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

- Evolutionary cell biology/ bioinformatic group /translational to human, animal health; Host – parasite interaction (cell inside cell)
- Tools developed for host: Transcriptome sequencing and custom microarrays; for parasites (Digital Differential gene expression – genome and transcriptomic sequencing)



Available resources:
- NGS – Illumina
- Microarrays – Affymetrix ; Agilent
- Electron Microscopy; Histology facility; qPCR; flow cytometery
- Bioinformatics cluster (EU elixir network)
- Database curation and support

CVI (CVI)

- The Central Veterinary Institute is the NRL for the Netherlands and Belgium
- Diagnostic methods applied for EU listed pathogens:
  - *Bonamia ostreae* / *Bonamia exitiosa*: histology and real time PCR (Marty et al 2006)
  - *Marteilia refringens*: histology and real time PCR (*in house*)
  - Other listed pathogens: histology
- Diagnostic methods for other mollusc diseases:
  - OsHV-1: SYBR Green (Webb) and LNA real time PCR (Ifremer)
  - *Nocardia crassostreae*: real time PCR (Carrasco et al. 2013)
  - Haplosporidia: molecular detection methods (incl. ISH)
  - Classical bacteriology, 16S sequencing, MALDI-TOF and Taqman real time PCR for *V. aestuarianus* and *V. splendidus* (Ifremer)

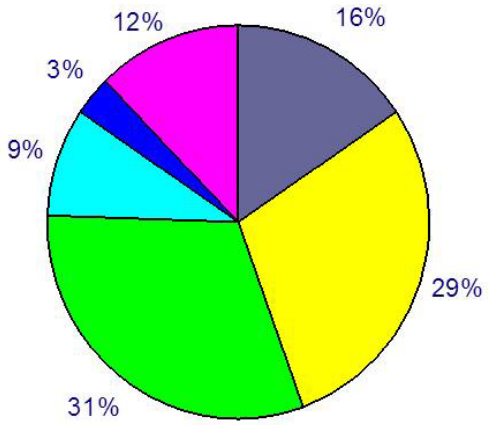
Jan Steen

CENTRAL VETERINARY INSTITUTE
WAGENINGEN UR

EURL function and duties

- Time spent on each activity by the EURL Ifremer

- Harmonisation of the diagnosis approach
- Assistance to NRLs
- Training, synthesis and transfer of information
- To acquire and maintain expertise
- International collaboration
- Epidemiological and experimental investigations



Ministry for Primary Industries
Manatū Ahu Matua

Mollusc Disease Diagnosis

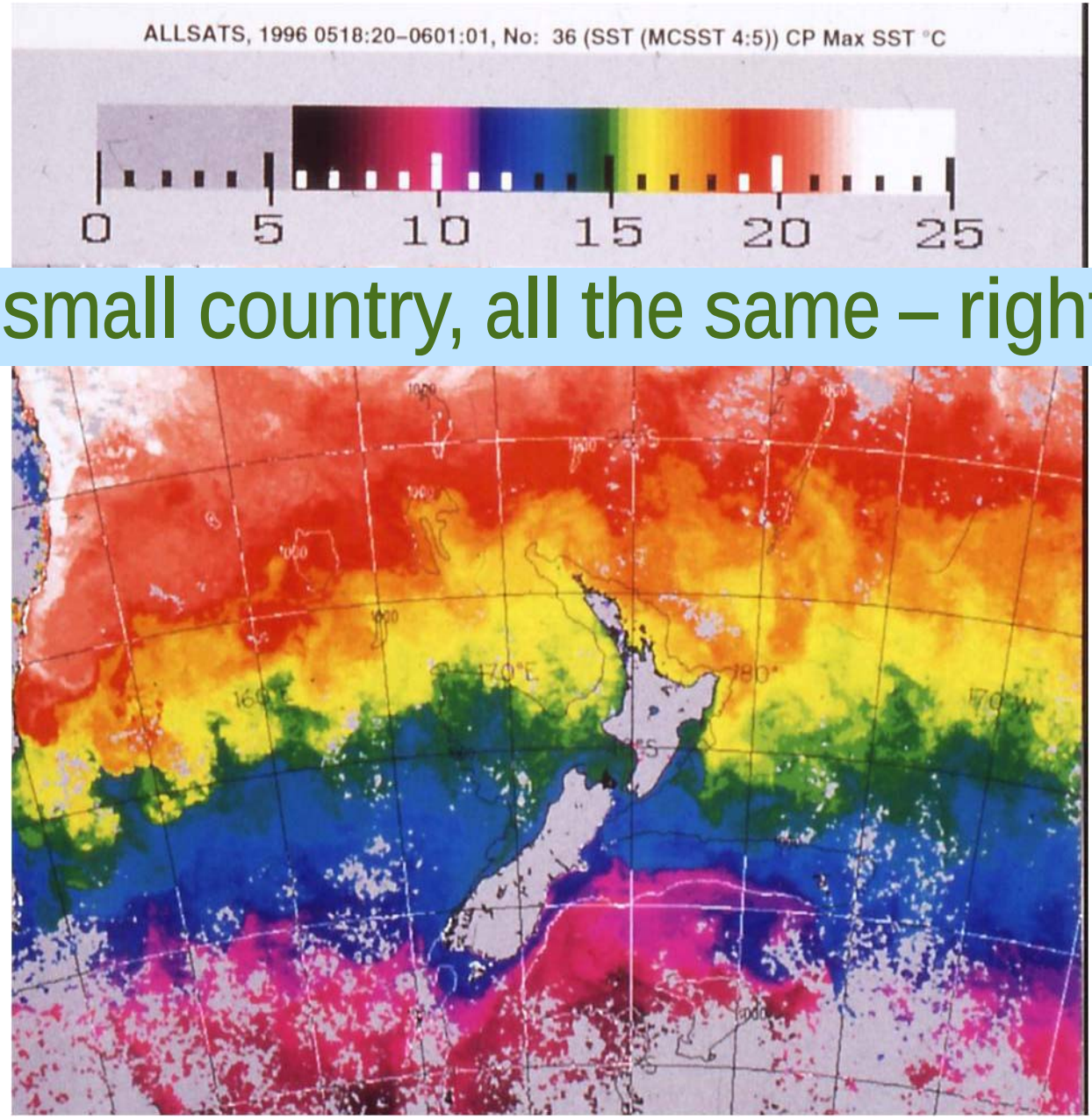
State of laboratory network in New Zealand

Brian Jones

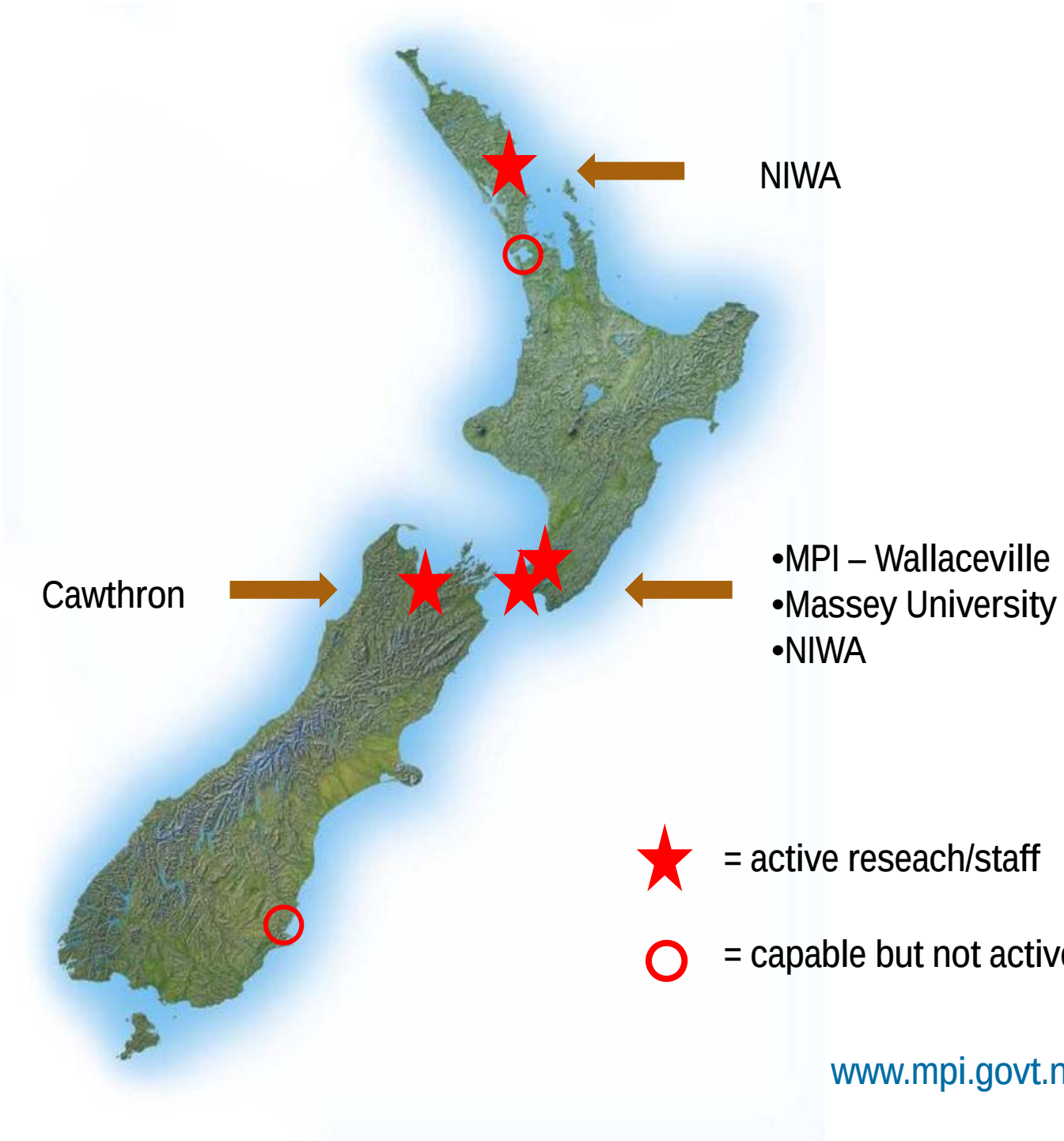
23 October 2013

www.mpi.govt.nz

Where is New Zealand?



A small country, all the same – right?


NIWA
Cawthron
•MPI – Wallaceville
•Massey University
•NIWA
= active reseach/staff
= capable but not active

MPI Animal Health Laboratory (PC3+)

- Based at the National Centre for Biosecurity and Infectious Disease campus at Wallaceville
- Responsible for the identification of all suspected exotic, new and emerging diseases of production, companion and aquatic animals, wildlife and introduced fauna.
- Has capability for diagnostic testing for high impact diseases and developing diagnostic tests for new and emerging diseases, including zoonotic diseases that impact on human health.

So - why no functional laboratory network?

- Few personnel
- No common forum
- Competition for limited funding

Australian diagnostic laboratory network

Capacity for diagnosis of disease in marine molluscs

FACULTY OF
VETERINARY SCIENCE

THE UNIVERSITY OF SYDNEY

Paul Hick

International Bio-based Economy Forum

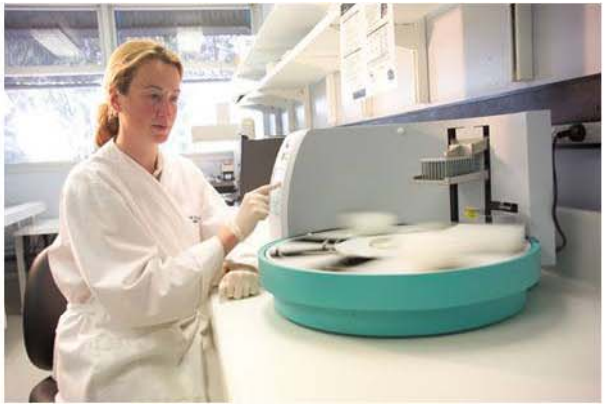
Workshop on Marine Mollusc Health | Geelong | October 2013

Diagnostic capacity

Government | Private | Research Laboratories

Equipment

Human resources and networks



Diagnostic capacity

Government | Private | Research Laboratories
Equipment
Human resources and networks

Limited specialists

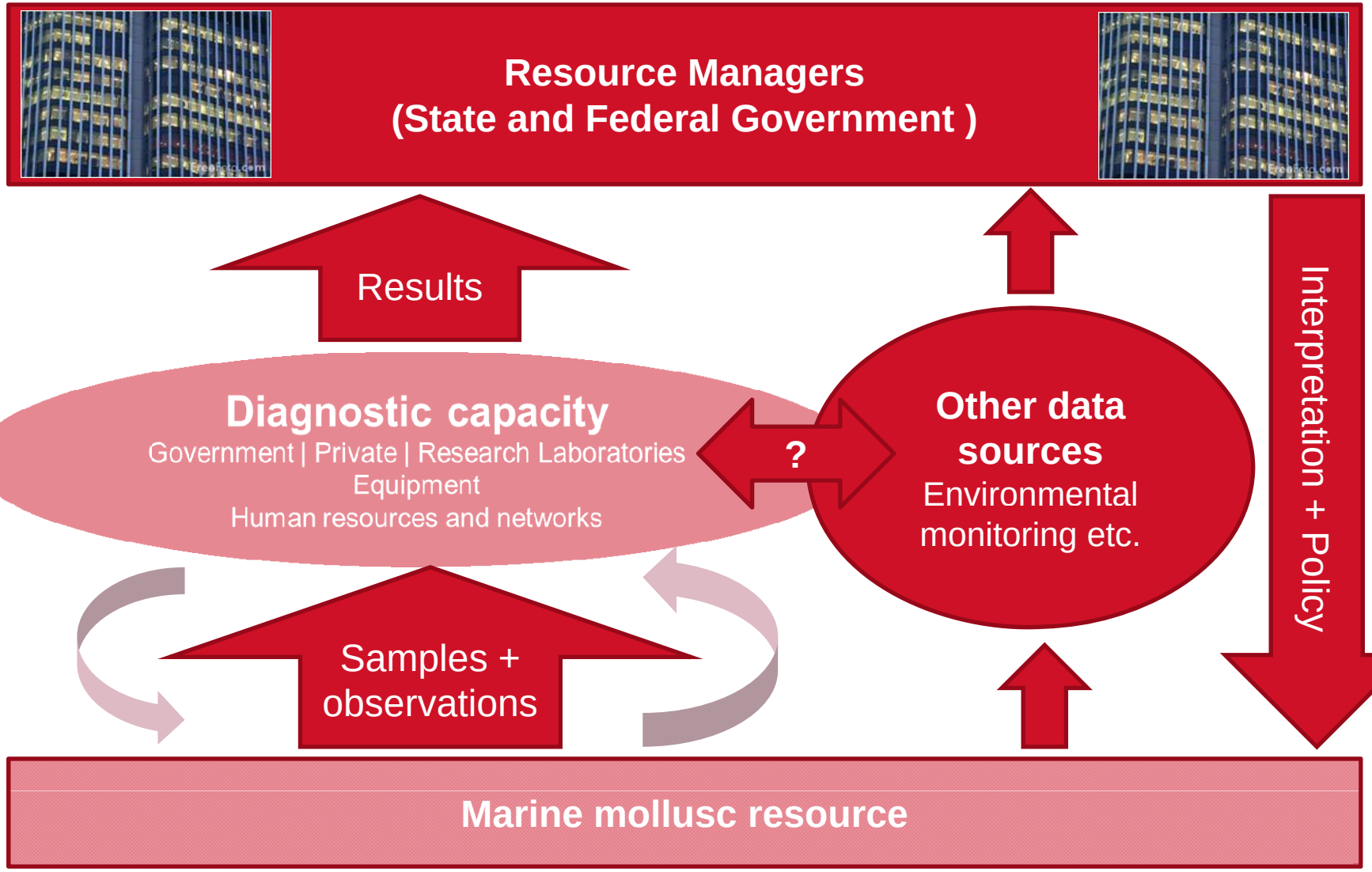
Disease reporting
Appropriate samples

Ma ne mollusc resou ce

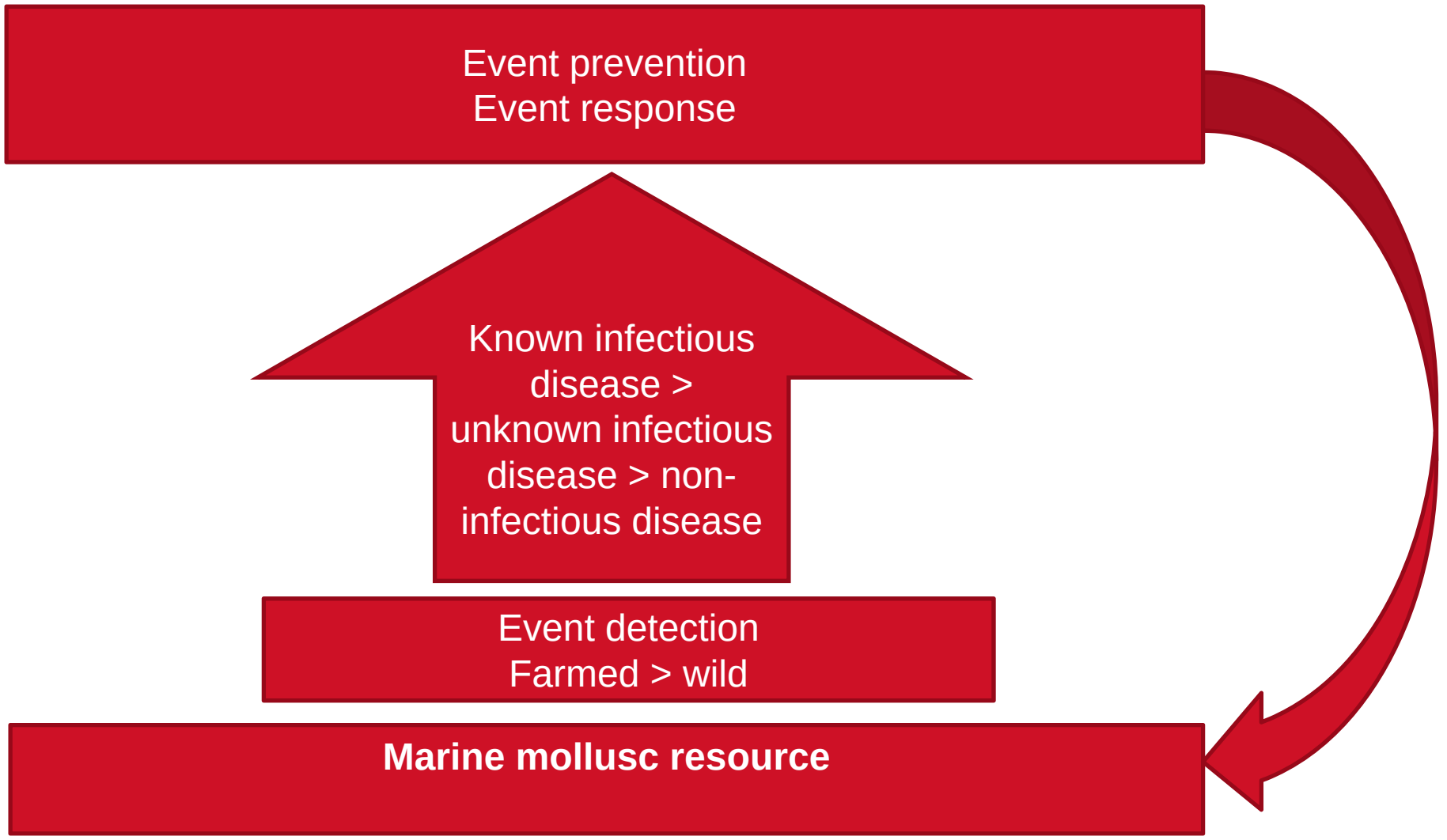
Farmed stock

Wild stock
Commercial fishery
Recreational fishery
Ecosystem / Biodiversity

Introduction



Introduction



Laboratory Infrastructure

	State	Histopathology	Molecular	Other
1.	New South Wales	✓	✓	Variable
2.	Victoria	✓	✓	
3.	Tasmania	✓	✓	
4.	South Australia	(private lab)	AAHL	
5.	Western Australia	✓	✓	
6.	Northern Territory	✓	✓	
7.	Queensland	✓	✓	

Australian Animal Health Laboratory (AAHL): responsibility for index and primary emergency (exotic or emerging) disease cases;

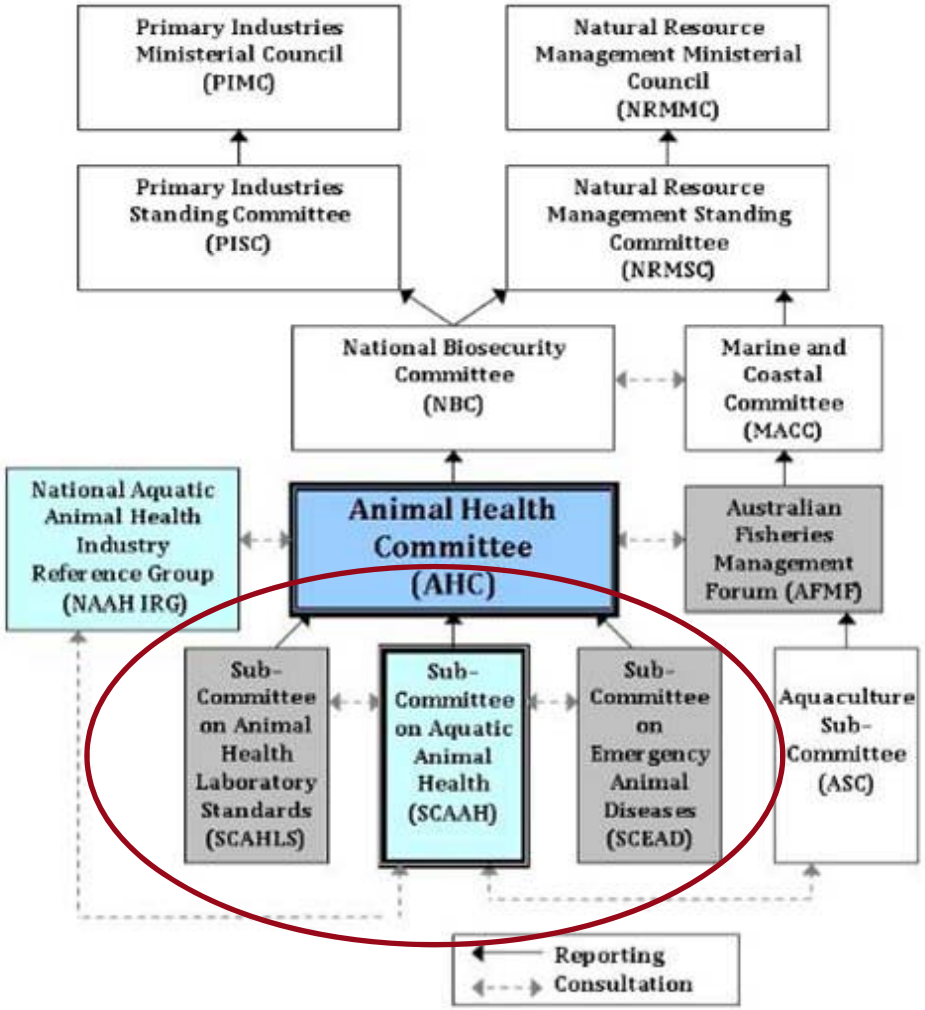
Mulluscan disease diagnosticians

- Capacity within the aquatic diagnostic network was last audited in 2005 [1.]
  - 20 laboratories self-assessed as having expertise in Aquatic animal health
  - Expertise reaching retirement age
  - Laboratory services provided on a cost recovery funding model = low case load

- Test methods and expertise and in diagnostic laboratories is generated by research funding.

[1.] Landos, Dhand and Whittington 2006. FRDC final report: Aquatic Animal Health Subprogram: Establishment of a national aquatic animal health diagnostic network.

Governance / Framework



The 2 committees to which the Aquatic Animal Health Program has direct input are the Sub–Committee on Aquatic Animal Health (SCAAH) and Aquatic Consultative Committee on Emergency Animal Disease (Aquatic CCEAD).

Sub committee on Animal Health Laboratory Standards (SCAHLS)

- SCAHLS represents the activities of the veterinary laboratory network of Australia and New Zealand.
- Aquatic ANZSDP e.g.
  - Bonamiosis. Unique Australian situation necessitates test approaches specific to Australia (Jan 2009).
  - *Perkinsus olseni* standard tests are under development

Governance / Framework

SCAHLS
Sub-Committee on Animal Health Laboratory Standards

Network of specialists for tests in routine use e.g.

Agent	Species	Test	Status	Principal/Contact	SCAHLS Rep	Laboratory
Abalone herpes virus	Aquatic - mollusc	qPCR	RT, PV	M Crane	AAHL	AAHL
Abalone herpes virus	Aquatic - mollusc	PCR	ACC	M Crane	AAHL	AAHL
Abalone herpes virus	Aquatic - mollusc	qPCR	RT, PV	C Brosnahan	NZ	AHL, NZ
Abalone herpes virus	Aquatic - mollusc	PCR		M Fegan	VIC	DPI Vic, Bundoora
Abalone herpes virus	Aquatic - mollusc	qPCR	ACC	M Fegan	VIC	DPI Vic, Bundoora
Abalone herpes virus	Aquatic - mollusc	ISH		M Fegan	VIC	DPI Vic, Bundoora
Abalone herpes virus	Aquatic - mollusc	qPCR	PV	M Crockford	WA	AHL, DAFWA
Abalone herpes virus	Aquatic - mollusc	ISH	PV	M Crockford	WA	AHL, DAFWA

http://www.scahls.org.au/new_tests/register/tests-in-routine-use 22-10-2013

Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR)

- Laboratory network for harmonisation of tests for important emergency animal diseases
  - Validation at the laboratory rather than assay level
  - E.g. Control samples and proficiency panels for detection of testing OsHV-1 by PCR.
- **AQUAVET Plan**
  - Procedures for dealing with exotic disease incursions e.g. withering disease of abalone
  - Operational procedures e.g. disinfection and disposal
  - Diagnostic resources e.g. field guide

Case study: Abalone viral ganglioneuritis in New South Wales 2011

THE UNIVERSITY OF SYDNEY

Case Study: Abalone Viral Ganglioneuritis in NSW

Background

- New South Wales abalone – a resource under pressure
  - Commercial quota - TAC 2012 = 110 tonnes, $2.8 million [1.]
  - Recreational fishing - bag limit 2 (or 0 on weekdays)
  - Disease - Mass mortality events + *Perkinsus sp*.
  - Illegal poaching

- Total decline in population by 90% of virgin abundance in some areas [2.]

1. Total Allowable Catch Committee – Abalone Determination and Report for 20012/13
2. Liggins and Upston 2010. FRDC Project 2004/84 Final Report

Case Study: Abalone Viral Ganglioneuritis in NSW

Event detection

- Disease in live abalone reported in a restaurant
  - Timely reporting network: confirmed AbHV infection at a processing plant in Tasmania (16/11/2011).
- NSW Aquatic Biosecurity and fisheries compliance officers seized animals on 24/11/2011

Case Study: Abalone Viral Ganglioneuritis in NSW

Initial diagnosis

› Parallel submission – AAHL and NSW laboratory.

- Histopathology – characteristic ganglioneuritis

(diffuse infiltration of haemocytes predominantly at the periphery of the pleuropedal ganglion surrounding neuronal cells and extending along the pedal nerve cords)

- qPCR positive
- conventional PCR positive (+ sequence)

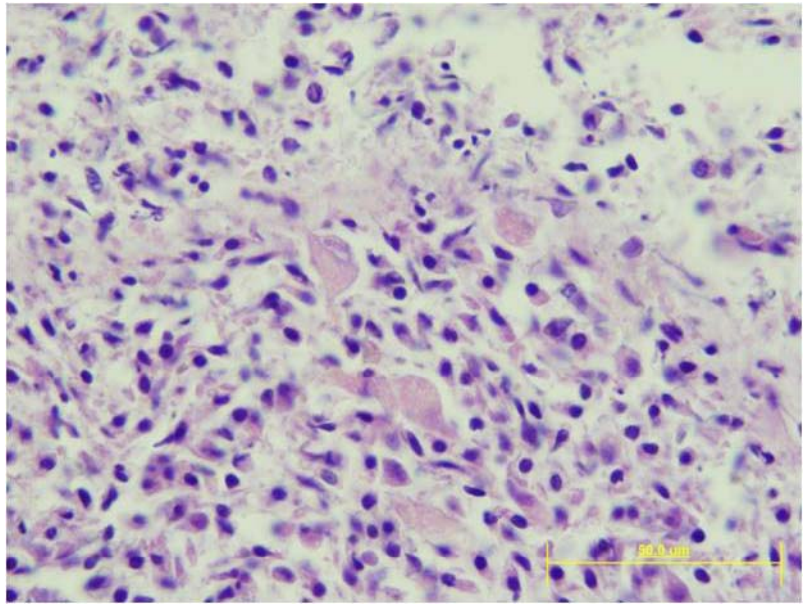


Image R. Reece: abalone ganglia

Case Study: Abalone Viral Ganglioneuritis in NSW

Trace forward

- Emergency disease response identified at risk premises
- NSW I&I Emergencies & Animal Welfare Unit produced a protocol:
  - Collection of suitable diagnostic specimens,
  - Prevention of cross contamination between locations

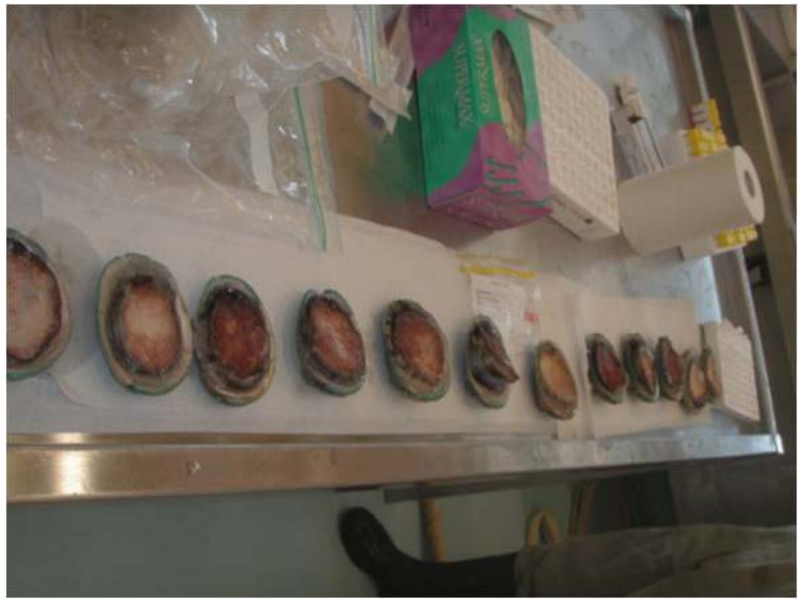
- A total of 156 abalone were seized for testing from ~ 40 premises

Case Study: Abalone Viral Ganglioneuritis in NSW

Laboratory Testing

- Responsibilities return to state veterinary diagnostic laboratory.
- Focus on PCR for detection AbHV (rather than diagnosis of AVG)
  - The laboratory network – PCR protocols and positive control from state vet lab in Tasmania, dissection guide from AAHL. All underpinned by previous research.

- High throughput testing by real-time PCR:
  - 399 abalone tested in 2 months
  - 135 PCR positive abalone
  - 30 infected premises.



Case Study: Abalone Viral Ganglioneuritis in NSW

Follow-up

- Sentinel program
  - To demonstrate effective decontamination of the premises:

    place 10 negative abalone per tank and sample on day 10 and 15.
- Research
  - PCR positive samples referred to AAHL for molecular epidemiology
  - Highlight need for ongoing test validation, development and diversification
  - More than 1 assay was required to detect all the positive samples in the NSW outbreak
- Reporting
  - OIE notified December 2011. “New South Wales eradicated abalone herpesvirus which was contained in aquaria, and is now free from this disease.
- Policy adaptation
  - Live abalone import restrictions; culturally targeted extension material e.g. prohibition of using abalone gut as bait.

Discussion

KBBE Workshop on Mollusc disease diagnosis

Validation status of current diagnostic tests in the OIE manual

Stephen W Feist*, David Stone*, Mark Crane**

*OIE Collaborating Centre for Information on Aquatic Animal Diseases Cefas Weymouth Laboratory, Weymouth, UK.

**AAHL Fish Diseases Laboratory, CSIRO Animal, Food and Health Sciences, Geelong, Australia.

What and Why

- Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose.

  - demonstration of freedom (in a population; for trade purposes; re-establishment of freedom after outbreaks)

  - contribution to eradication

  - confirmatory diagnosis, suspect or clinical

  - estimations of prevalence (for RA)

  - determine immune status (serological test)

OIE listed mollusc diseases

Infection with:

- Abalone herpes-like virus
- *Bonamia exitiosa*
- *Bonamia ostreae*
- *Marteilia refringens*
- *Perkinsus marinus*
- *Perkinsus olseni*
- *Xenohaliotis californiensis*
- OsHV – 1 microvariant (from May 2013)

OIE Validation criteria

Criteria for Assay Development and Validation

1. Definition of the intended purpose(s)
2. Optimisation
3. Standardisation
4. Repeatability
5. Analytical sensitivity
6. Analytical specificity
7. Thresholds (cut-offs)
8. Diagnostic sensitivity (DSe)
9. Diagnostic specificity (DSp)
10. Reproducibility
11. Fitness for intended purpose(s)

Validation

Four steps - achieving the ideal:

1. Step 1 – Analytical performance characteristics
   - repeatability; analytical specificity; analytical sensitivity; analytical accuracy of adjunct tests
2. Step 2 – Diagnostic performance of the assay
   - reference animal populations; experimentally infected/vaccinated; threshold determination; calculation on DSe and DSp ; provisional assay recognition. (RELIANT ON KNOWN POPULATION HEALTH STATUS)
3. Step 3 – Reproducibility
   - reproducibility and designation of a validated assay (ring tests etc)
4. Step 4 – Programme implementation
   - fitness for use; interpretation of results; international recognition; deployment of the assay (have been implemented to demonstrate freedom in some cases)

Monitoring assay performance after initial validation

- Modifications
- Enhancing confidence
- Verification of existing assays (in-house validation)

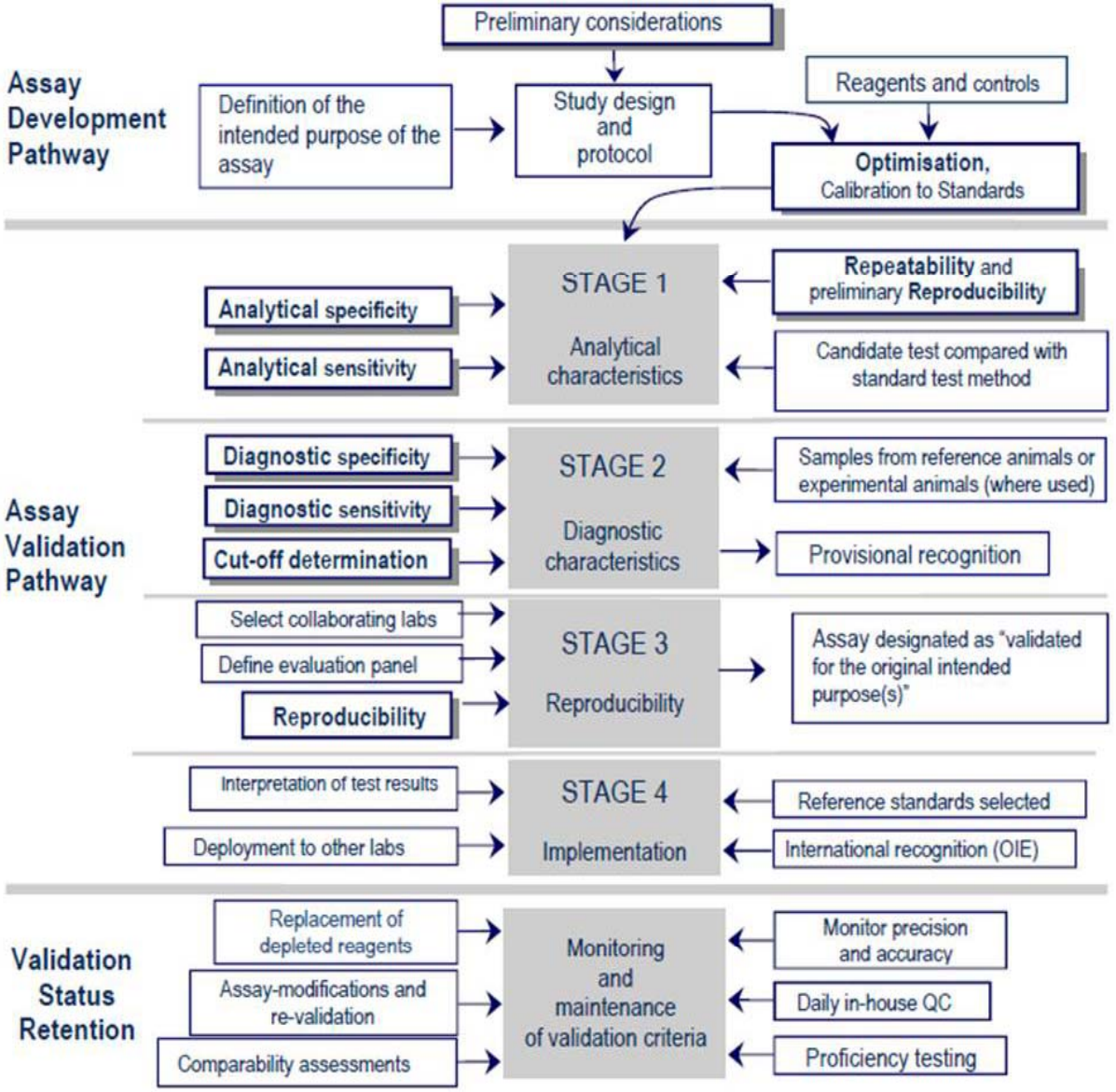


Figure 1. The assay development and validation pathways with assay validation criteria highlighted in bold typescript within shadowed boxes. (***From OIE Manual of Diagnostic Tests for Aquatic Animals***)

Rating of tests

a. recommended for reasons of availability, utility, DSe, DSp
b. standard method with good DSe and DSp
c. has application in some situations but limited because of cost, accuracy etc
d. not recommended

Abalone herpesvirus

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis	
	Larvae	PLs	Juveniles	Adults			
Gross signs	d	d	c	c	c	d	N
Bioassay	d	d	d	d	d	c	N
Direct LM	d	d	d	d	d	d	N
Histopathology	d	d	b	b	a	a*	N
Transmission EM	d	d	d	d	d	c	N
Antibody-based assays	d	d	d	d	d	d	N
DNA probes – *in situ*	d	d	c	c	d	a*	Y?
PCR	d	d	a	a	a	a	Y
PCR and Sequence	d	d	d	d	d	a	Y

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction; *Histopathology can be confirmed using *in-situ* hybridisation (ISH).

B. exitiosa

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis	
	Larvae	PLs	Juveniles	Adults			
Gross signs	d	d	c	c	c	d	N
Tissue imprints	d	d	a	a	a	c	Y?
Histopathology	d	d	a	a	a	c	Y?
Transmission EM	d	d	d	d	d	b	N
DNA probes – *in situ*	d	d	d	d	d	b	N
PCR and TaqMan PCR	a	a	a	a	a	c	Y?
PCR-RFLP	d	d	d	d	d	b	N
Sequence	d	d	d	d	d	a	Y

PLs = postlarvae; EM = electron microscopy; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

B. ostreae

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis	
	Larvae	PLs	Juveniles	Adults			
Gross signs	d	d	c	c	c	d	N
Tissue imprints	d	d	a	a	a	c	Y?
Histopathology	d	d	a	a	b	c	Y?
Transmission EM	d	d	d	d	d	a	Y?
DNA probes – *in situ*	d	d	d	d	d	b	N
PCR and TaqMan PCR	a	a	a	a	a	c	Y?
PCR-RFLP	d	d	d	d	d	b	N
SYBR® Green real-time PCR	a	a	a	a	a	c	Y?
Sequence	d	d	d	d	d	a	Y

PLs = postlarvae; EM = electron microscopy; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

Not an ‘a’ for B. exitiosa.
Is this specific?

Marteilia refringens

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis	
	Larvae	PLs	Juveniles	Adults			
Gross signs	d	d	c	c	c	d	N
Wet mounts	d	d	c	c	c	d	N
Imprints	d	d	b	b	a	c	Y
Histopathology	d	d	a	a	b	c	Y?
DNA probes – *in situ*	d	d	d	d	d	b	N
PCR	a	a	a	a	a	a	Y
Sequence	d	d	d	d	d	a	Y
Transmission electron microscopy	d	d	d	d	d	b	N

PLs = postlarvae; PCR = polymerase chain reaction.

Perkinsus marinus

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis	
	Seed	Juveniles	Adults			
Gross signs	d	d	d	d	d	N
Haemolymph smears	d	c	c	c	d	N
Histopathology	b	b	b	b	d	N
RFTM, tissue assay*	d	a	a	b	d	N
RFTM, body burden assay*	d	c	c	c	d	N
PCR	a	b	b	a[1]	b[1]	Y?
DNA probes – *in situ*	d	b	b	b	a	Y?
Sequence	d	d	d	d	b[1]	Y?

RFTM = Ray's fluid thioglycollate culture method; *the technique is not species specific, but can be used reliably in hosts/areas where only one species of *Perkinsus* is present or predominant; [1]should be used only if infections visualised by smear, RFTM or histology; PCR = polymerase chain reaction.

Perkinsus olseni

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis	
	Seed	Juveniles	Adults			
Gross signs	d	d	d	d	d	N
Haemolymph smears	d	c	c	c	d	N
Histopathology	b	b	b	b	d	N
RFTM, tissue assay*	d	a	a	b	d	N
RFTM, body burden assay*	d	c	c	c	d	N
PCR	a	b	b	a[1]	b[1]	Y?
DNA probes – *in situ*	d	b	b	b	a	Y?
Sequence	d	d	d	d	b[1]	Y?

RFTM = Ray's fluid thioglycollate culture method; *the technique is not species specific, but can be used reliably in hosts/areas where only one species of *Perkinsus* is present or predominant; [1]should be used only if infection is visualised by smears, RFTM or histology; PCR = polymerase chain reaction.

Not sure why the PCR and sequencing is not considered an 'a' for confirmation of RFTM and histopathology results

Xenohaliotis californiensis

Table 5.1. *Methods for targeted surveillance, detection and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis	
	Larvae	Juveniles	Adults			
Gross signs	d	c	c	c	d	N
Bioassay	d	d	c	c	c(a)[1]	N?
Tissue imprint – Giemsa stain	d	c	c	b	b(c)[2]	N
Histopathology	d	b	b	a	a[3]	Y?
Transmission EM	d	d	d	b	c	N
DNA probes – *in situ*	d	c	c	a	a	N
PCR	d	a	a	a	c(a)[4]	Y
SSU rDNA sequence	d	d	d	a	a	Y

[1]For valuable broodstock, it is possible to use polymerase chain reaction (PCR) of faeces as a first screen and, if negative, subsequently to use the bioassay method in combination with histology (See Section 6). [2]Tissue imprints should be used in combination with PCR and possibly sequencing to confirm the agent. [3]In new cases, such as a new geographical location, PCR and sequencing are recommended to confirm identity of the bacterium. [4]PCR alone is not confirmatory but when used in combination with histology, it may be considered confirmatory. EM = electron microscopy; SSU rDNA = small subunit ribosomal DNA.

OsHV-1 µVar

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance			
	Larvae	Juveniles	Adults	
Gross signs	d	d	d	N
Bioassay	d	d	d	N
Histopathology	d	d	d	N
Transmission EM	d	d	d	N
Antibody-based assays	d	d	d	N
DNA probes – *in situ*	c	c	c	N
PCR	a	a	a	N?
qPCR	a	a	a	N?
Sequence	d	d	d	Y?

EM = electron microscopy; PCR = polymerase chain reaction; qPCR = real-time PCR.

For confirmation of 12bp deletion

Quality assurance systems in diagnostic laboratories in EU

KBBE Workshop, Geelong, Australia

21-24 October 2013

Deborah Cheslett

Marine Institute

Marine Institute
Foras na Mara

Content

- Why Quality Assurance?
- QA in the CRL/NRL network
- QA in the BIVALIFE consortium
- QA in the OYSTERECOVER consortium
- Feedback on QA

Marine Institute
Foras na Mara

Why Quality Assurance?

- Consistent, reliable, repeatable results
- Higher degree of confidence in results
- Better traceability
- Demonstrate due diligence in the event of legal action
- Marketability of accreditation

Why Quality Assurance?

Legislation: EURL/CRL

"the Commission may designate only laboratories that operate and are assessed and accredited in accordance with the following European Standards, account being taken of the criteria for different testing methods laid down in this Directive:

(a) EN ISO/IEC 17025 on 'General requirements for the competence of testing and calibration laboratories';

(b) EN 45002 on 'General criteria for the assessment of testing laboratories';

(c) EN 45003 on 'Calibration and testing laboratory accreditation system — General requirements for operation and recognition'"

Marine Institute
Foras na Mara

Why Quality Assurance?

Legislation: NRLs

"These national reference laboratories shall:
...operate and be assessed and accredited in accordance with the following European Standards account being taken of the criteria for different testing methods laid down in this Directive:
(i) EN ISO/IEC 17025 ...." OR

"The Member States may designate national reference laboratories which do not comply with the requirements referred to in point 1(i)(i) of this Part, where operation under EN ISO/IEC 17025 is practically difficult, provided the laboratory operates under quality assurance in line with the guidelines in ISO 9001."

QA in the CRL/NRL Network: The EURL - IFREMER

- Operating to ISO 17025
- Accreditation held for:
  - Histopathology for all mollusc species
  - Cytology & Histology: *Bonamia* sp., *Marteilia* sp, *Mikrocytos* sp.
  - Histology: *Perkinsus* sp.
- Operating under a laboratory Management system (ISO 9001) for all activities related to the EURL duties

Marine Institute
Foras na Mara

QA in the CRL/NRL Network: The NRLs

- Laboratories with no accredited methods & not operating under a quality system:
  - Bulgaria, Croatia, Greece, Slovenia, Sweden
- Laboratories with no accredited methods but operating under a quality system:
  - Denmark, Montenegro, Portugal, Netherlands & Belgium
- Laboratories with accredited methods:
  - France, Spain, Italy, Germany, UK (Eng & Wales), UK (Scotland), Ireland, Turkey, Romania, Poland, Norway

QA in the CRL/NRL Network: Accredited tests

Histology

All mollusc species/general diagnostics	France, Germany, UK (England & Wales)
Bonamia sp.	France, Italy, Spain, Turkey, UK (Scotland)
Marteilia sp.	France, Ireland, Italy, Spain, Turkey, UK (Scotland)
Perkinsus sp.	France, Spain, Turkey
Mikrocytos sp.	France, Turkey

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Foras na Mara

QA in the CRL/NRL Network: Accredited tests

Cytology

Bonamia sp.	France, Ireland, Italy, Poland, Spain
Marteilia sp.	France, Italy, Poland, Romania (mussels only), Spain
Perkinsus sp.	Turkey
Mikrocytos sp.	France

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QA in the CRL/NRL Network: Accredited tests

Molecular Diagnostics

All mollusc species/general diagnostics	Germany
B. Ost/B. exit by PCR	UK (Scotland)
OsHV-1	Ireland

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Foras na Mara

QA in the CRL/NRL Network: Accredited tests

Other

Classical diagnostic tests	Germany
H&E for mollusc pathogens	Romania
Trichromic staining for mollusc pathogens	Romania

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QA in the CRL/NRL Network: Regional/Designated labs

France: All working under Quality Management system and hold accreditation for several techniques including PCR. One has accreditation for OsHV-1

Italy: 9 regional labs, 6 hold accreditation for various techniques including histology / cytology for *Bonamia sp*. in oysters and *Marteilia sp*. in mussels

Spain: 5 regional labs, only INTECMAR has accreditation for histology

Germany: 1 (Lower Saxony), accreditation for Histology

UK: AFBI in NI, no accreditation for mollusc diseases

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QA in the CRL/NRL Network: New methods for accreditation

- Real time PCR for *Bonamia*/*Marteilia*
- Real time PCR for OsHV-1
- PCR-RFLP & ISH for mollusc pathogens
- PCR for *Marteilia* (le Roux et al, 2001)
- PCR for *Bonamia* (Cochennec et al, 2001)
- Histo/cytology for *Bonamia*/*Marteilia*
- PCR-RFLP for *B. ost*/*B. exit*
- Taqman PCR for *Marteilia* sp.

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QA in the CRL/NRL Network: Accredited tests

Summary

- High diversity in tests accredited
- Histology & cytology for *Bonamia sp./Marteilia sp.* highest representation
- Molecular Accreditation low for mollusc diseases
- Differences within this group in approach to accreditation

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Bivalife Consortium

Accredited for certain tests under ISO 17025

- IFREMER MI, CSIC, CEFAS

Operating under quality system under ISO 9001 /ISO 9002 / 17025

- CVI-IMARES, IRTA

Involved in mollusc diagnostics but no accreditation/not operating under a quality system

- UCC, UNIGE, UNIPD, CNRS(?)

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OYSTERECOVER Diagnostic Labs

- CVI-IMARES: operate under ISO 9001
- CEFAS: Accreditation for histology
- UCC: No accreditation
- CIMA: No accreditation
- USC: ?

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Feedback on Accreditation

- Issues in gaining accreditation:
  - Cost, lack of official samples, lack of resources
- Benefits of accreditation:
  - Traceability, common and standardised procedures, better confidence results, continuous improvement of qualifications and competence of staff, determination of specificity and sensitivity
- Problems associated with accreditation:
  - Cost, Proficiency tests unavailable, time consuming, QA people losing touch with the work they are supposed to be doing

Marine Institute
Foras na Mara

Thank you for your attention

Marine Institute
Foras na Mara

Ministry for Primary Industries
Manatū Ahu Matua

Mollusc Disease Diagnosis

Quality Assurance systems in New Zealand

Brian Jones

23 October 2013

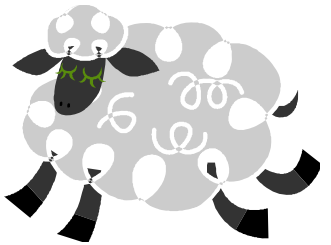
www.mpi.govt.nz

Primary Industries Laboratories

- Government regulations require all primary industries lab's testing New Zealand's export produce to be approved under the MPI **Laboratory Approval Scheme (LAS)** and/or **Export Laboratory programme (ELP).**
- This supports MPI's official assurances about exported produce and overseas markets access requirements are met.
- IANZ conducts assessments of such lab's, which must be accredited as a pre-requisite for approval by the MPI.

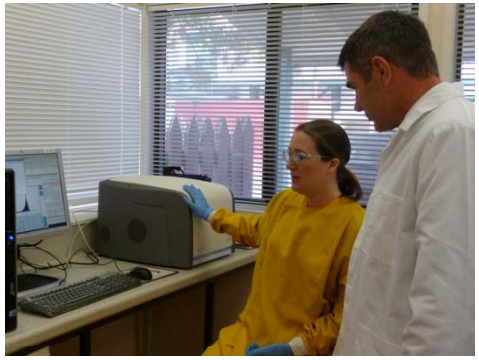
What is Export Laboratory Programme?

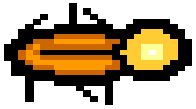
- Consolidates the requirements of the *Animal Product Act 1999*
- Applies to export laboratory testing in respect of **live animals** and germplasm (*other than live animals and germplasm certified as food*) for the purpose of obtaining official assurance for export



What is the Laboratory Approval Scheme?

The Laboratory Approval Scheme specifies requirements for laboratories that carry out microbiological and chemical, or any other specified laboratory testing for market access assurances.





Laboratory quality Assurance systems

- IANZ (International Accreditation New Zealand)
  - ISO 17025
  - IANZ Accreditation is a requirement for:
- MPI (Ministry for Primary Industries)
  - Laboratory Approval Scheme, Food Safety (LAS) and/or
  - Export Laboratory Programme, Animal Exports (ELP)

Who are IANZ?

- IANZ = International Accreditation New Zealand. They are an independent “not for profit” accreditation body
- Full signatory member of the ***International Laboratory Accreditation Cooperation*** (ILAC) and the regional body, ***Asia Pacific Laboratory Accreditation Cooperation*** (APAC)

IANZ Accreditation

Means formal recognition that a laboratory has been independently assessed by IANZ in five key areas:

1. Competence and experience of staff
2. Integrity and traceability of equipment and materials
3. Technical validity of methods
4. Validity and suitability of results
5. Compliance with appropriate management systems & standards and found to be competent to carry out its services in a professional, reliable and efficient manner.

IANZ Accreditation

- Maintained through:
- A system of internal and external audits
- An internal quality improvement program
- Both internal and external proficiency testing

Mollusc disease diagnosis: Quality assurance systems in diagnostic laboratories in Australia

Nick Moody | Aquatic Animal Health Specialist

Workshop on Mollusc Disease Diagnosis, 21-24 October, 2013, Geelong, Australia

CSIRO ANIMAL, FOOD AND HEALTH SCIENCES
www.csiro.au

Regulations

International Standards

- ISO/IEC 17025:2005
  - General requirements for the competence of testing and calibration laboratories
- ISO 9001:2008
  - Quality Management Systems
- ISO/IEC 17043:2010
  - Conformity assessment - General requirements for proficiency testing

National Standards

- AS/NZS 2243.3:2010
  - Safety in laboratories - Microbiological safety and containment

- National Association of Testing Authorities (NATA)
  - One of four organisations recognised by the Australian Government that form Australia's standards and conformance infrastructure
    - NATA
    - Standards Australia
    - National Measurement Institute (NMI)
    - Joint Accreditation System of Australia and New Zealand (JAS-ANZ)

  - Veterinary Testing
  - Proficiency Testing Scheme Providers

SCAHLS

- Sub-Committee on Animal Health Laboratory Standards
  - SCAHLS represents the activities of the veterinary laboratory network of Australia and New Zealand and is a sub-committee of the Animal Health Committee, reporting to Primary Industries Standing Committee
  - The primary strategy is effective communication and commitment to the mission by laboratory directors

- National, State & Private Laboratories, Universities, Commonwealth, Aquatic representatives

- Administer Australian and New Zealand Standard Diagnostics Protocols (ANZSDPs)

- Facilitate development, evaluation and communication of new tests developed in Australia and New Zealand

Other Standards/Regulations

- Office of the Gene Technology Regulator (OGTR)
  - GMOs
- Biosecurity Australia (BA)
  - Imported biologicals
  - Destruction
  - Movement
- Security Sensitive Biological Agents (SSBA)
  - To limit the opportunities for acts of bioterrorism or biocrime to occur using harmful biological agents and to provide a legislative framework for managing the security of SSBAs
- ISO 14001: Environmental Management System
- Organisational requirements

Laboratory implementation

Laboratory QA System components

- Organisation and Management
- Document control
- Management review
- Internal audits
- Quality control (internal/external)
- Contract review
- Control of Nonconformance and Corrective/Preventative Action
- Staff and Training
- Accommodation and Environment
- Equipment
- Calibration
- Test Methods
- Specimen management
- Records
- Reports
- External Resources
- Complaints
- Microbiological Security
- AAHL External Proficiency Testing Scheme

Methods

1. OIE Standards

2. Australian and New Zealand Standard Diagnostics Protocols (ANZSDPs)

3. EU Commission Regulations

4. Peer-reviewed published methods

5. In-house methods

- Compare a number of protocols (if available)
  - Real-time for screening, conventional for confirmation

Methods

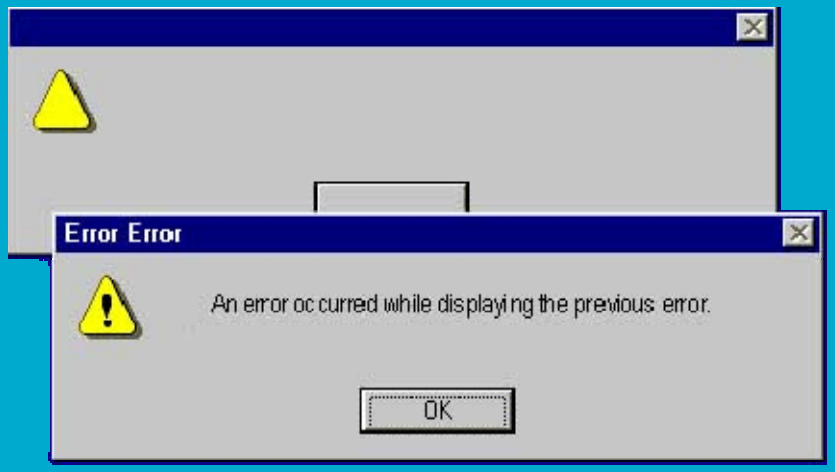
- Standard protocols within laboratories
- Variability between approaches in different laboratories
  - Harmonisation not standardisation
- Document performance of controls
  - Plasmids/synthetic RNA (more stable, monitor contamination)
  - Network Quality controls (NQCs)
- Monitor performance of enzymes, equipment etc.
- Validation, equivalency testing of new methods
- Check testing (6-montly)

Staff

- Increasing workload
- Training (internal/external auditors)
- Competency
  - Document initial gaining of competency
  - Annual review of competency
  - Can be a sensitive issue
- Explain that QA is an evolving process (e.g. sequencing and analysis)
- Networking helps identify ~~mistakes~~ improvement opportunities
  - Working with biological systems
- If you can't make it exciting at least appear enthusiastic yourself

Documentation

- Requirement to maintain knowledge of changing QA requirements
  - Technical updates
  - Field Application Document updates
- Lot of paperwork
- Greater reliance on electronic records
  - Permanent record
  - Version control
  - Operator identification
  - Organisational record-keeping requirements
- Trying to remember to do it all



Thank you

AAHL Fish Diseases Laboratory
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Aquatic Animal Health Specialist

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w www.csiro.au

Proficiency testing activities in EU

Isabelle Arzul, Ifremer, LGPMM, La Tremblade, France

Inter laboratories comparison (ILC) :

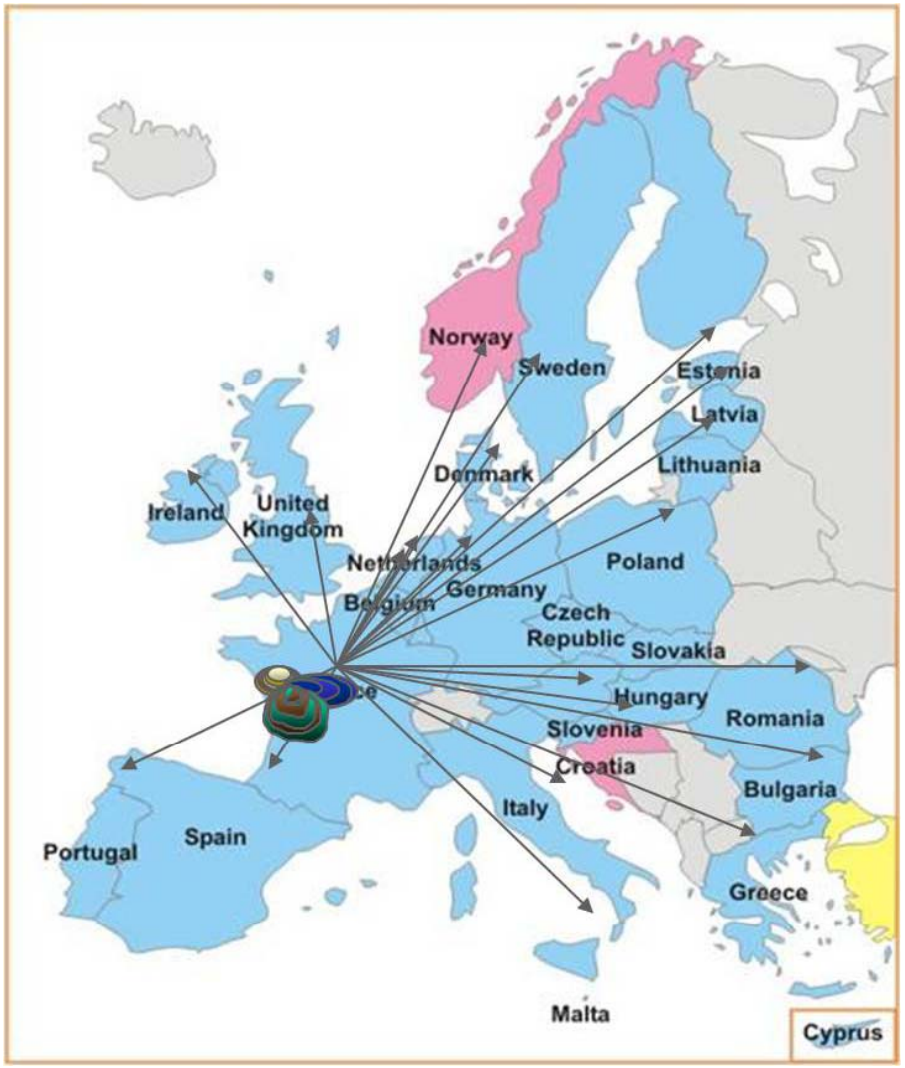
- To establish that the examination of a given sample leads to the same conclusions in any laboratory
- To determine a laboratory's capability to conduct specific diagnostic tests
- To harmonise existing test methods (reading of histological and cytological preparations; performing a PCR test)
- To evaluate new test methods

1- ILCs organised by the EURL for the NRLs

2- ILCs organised at the national level by NRLs

3- ILCs organised in the context of European projects

1 EURL


Norway
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Denmark
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United Kingdom
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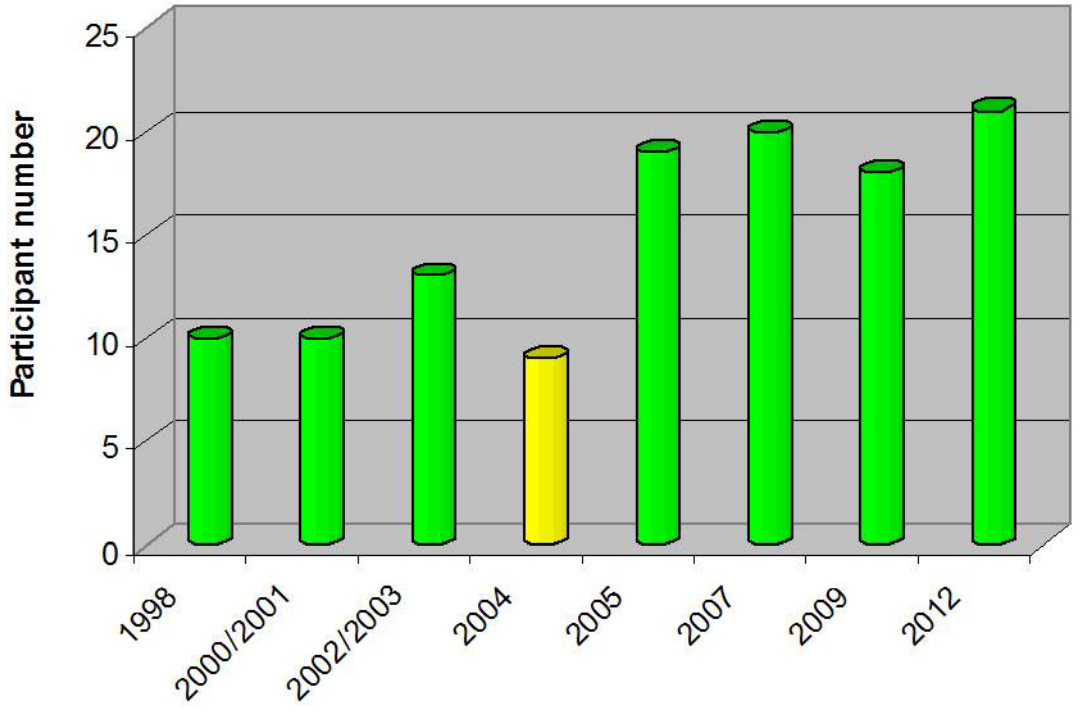
Hungary
Romania
Slovenia
Croatia
Bulgaria
Italy
Portugal
Spain
Greece
Malta
Cyprus

1 EURL

ILC based on histology every two years

Year	Pathogens	Slides	Participants
1998	Bo & Ma	30	10
2000	Bo & Ma, exotic, clam and mussel pathogens	40	10
2002	Bo & Ma, C. gigas, clam and mussel pathogens	30	13
2004	Bo & Ma	30	9 CCs
2005	Bo & Ma, C. gigas, pathogens	60	19/14
2007	Bo & Ma, cupped oyster pathogens	60	20/18
2009	Bo & Ma, cupped oyster pathogens	60	18/17
2012	Bo & Ma, cupped oyster pathogens	60	21

ILC based on histology every two years



1 EURL

ILC based on PCR every two years

Year	Pathogens	Samples	Participants
2008	*Bonamia* sp.	30	13
2010	*Marteilia refringens*	24	13
2011	OsHV-1 µvar	24	15

1 EURL

ILC based on histology organisation

Slide preparation

Selection, preparation and checking of the quality and status of each slide (double reading of slides)

Proficiency test announcement and inscription

Test presentation
Participants inscription

Slide sending to the first participant

1 week for reading 60 slides
Sending to the next participants

Reception and analysis of all NRL results by the EURL

Sending a personal response to each NRL

Sending a synthesis of all results to European Commission

Quality management

1 EURL

ILC based on PCR organisation

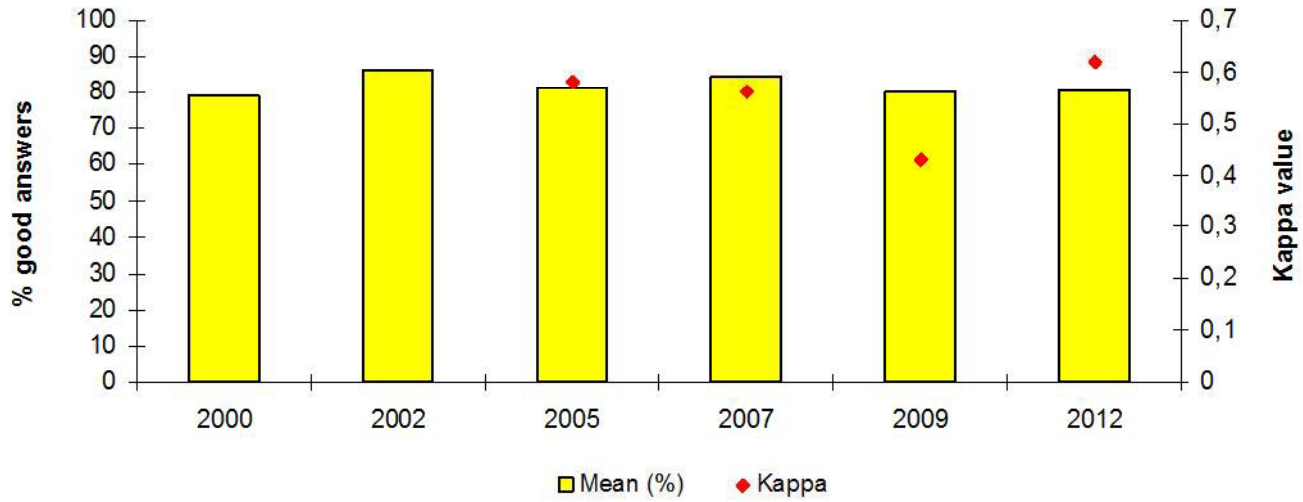
Design and sample preparation	Selection, preparation and checking of the quality and status of each sample
Announcement and inscription	Through reflabnet explaining the aim of the test, contents and asking who will participate in
Sample sending	Samples packaged to ensure their protection and sent by express mail. Sending and reception dates, package tracing codes recorded for each participant.
Results sending	Participants had to return their results (+ or -) to EURL
Result analysis	Report sent to DGSanco and coded version of the report sent to each participant (with their own code)

1 EURL

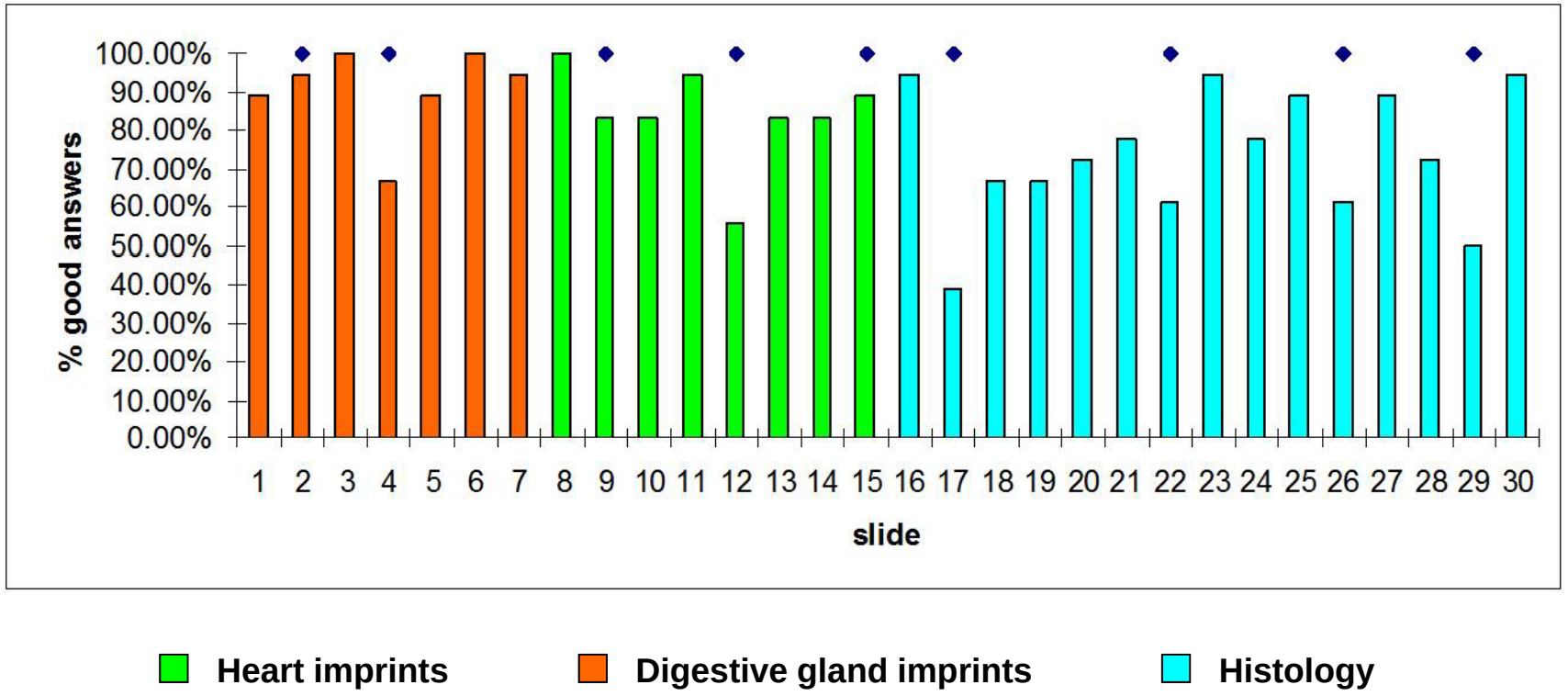
ILC based on PCR : evaluation of the complete process

- DNA extraction
- DNA concentration estimation and adjustment
- PCR test
- Interpretation of results
- Standard Operating Procedure

ILC based on histology results (*O. edulis*)



ILC based on histology results (*O. edulis*)



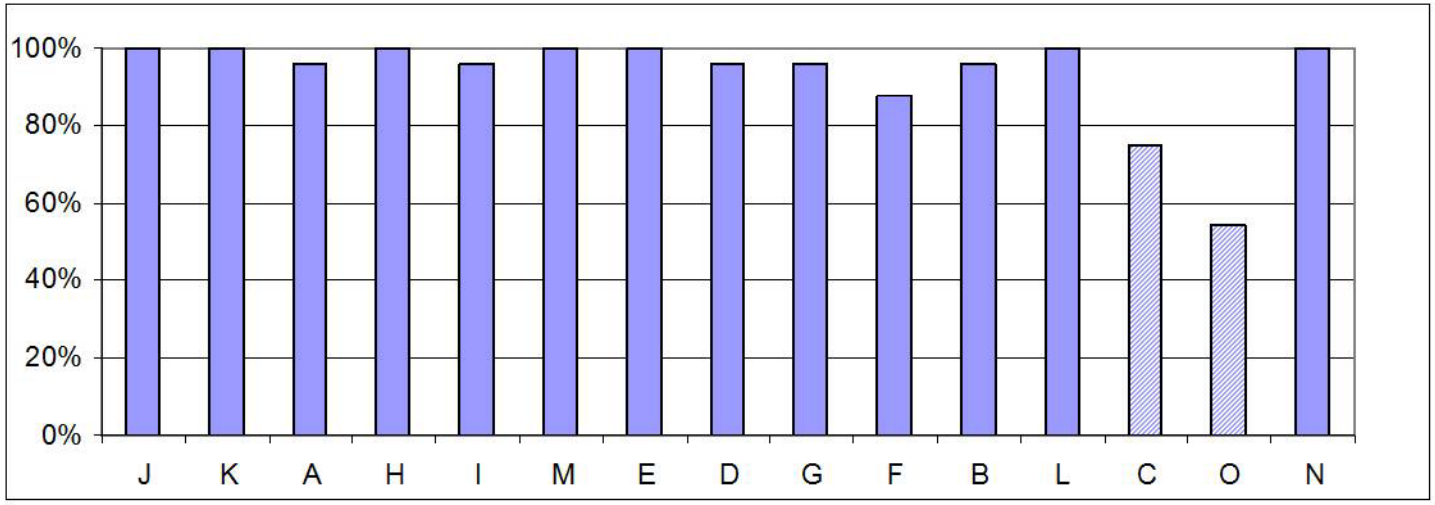
Better results obtained for imprints slides compared to histological slides

1 EURL

ILC based on PCR results

For each participant, we establish

Percentages of good answers (compared to reference results)



Example of results obtained during last ILC based on PCR

1 EURL

ILC based on PCR results

From percentages of good answers per participant, we establish:

Average (%)	Minimum value (%)	First quartile (%)	Median (%)	Third quartile (%)	Maximum value (%)	Kappa value
93,06%	54,17%	95,83%	95,83%	100%	100%	0,76

2 NRLs

Some EU NRLs organised ILC

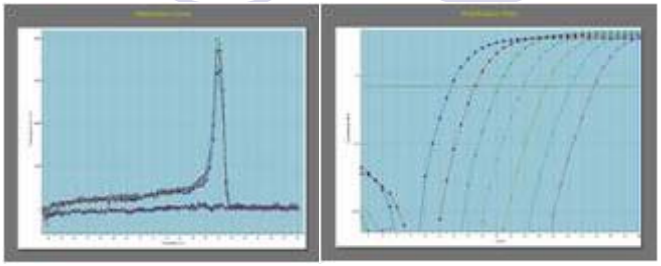
When there are designated laboratories for the diagnosis of mollusc diseases in Member states:

- Italy and Spain: ILC based on histology and cytology
- France : ILC based on PCR

Example of an ILC organised in France in 2011

2 NRLs

Objectives



- Evaluate competency of participants regarding the detection of :
  - OsHV-1
  - *Vibrio aestuarianus*

- Compare two diagnostic methods for the detection of OsHV-1:
  - PCRQ SYBR®Green de Pépin *et al.* (2008) (official method)
  - PCRQ TaqMan® de Martenot *et al.* (2010)

⇩

In order to have two official methods for the detection of *OsHV*-1

2 NRLs

Participants:

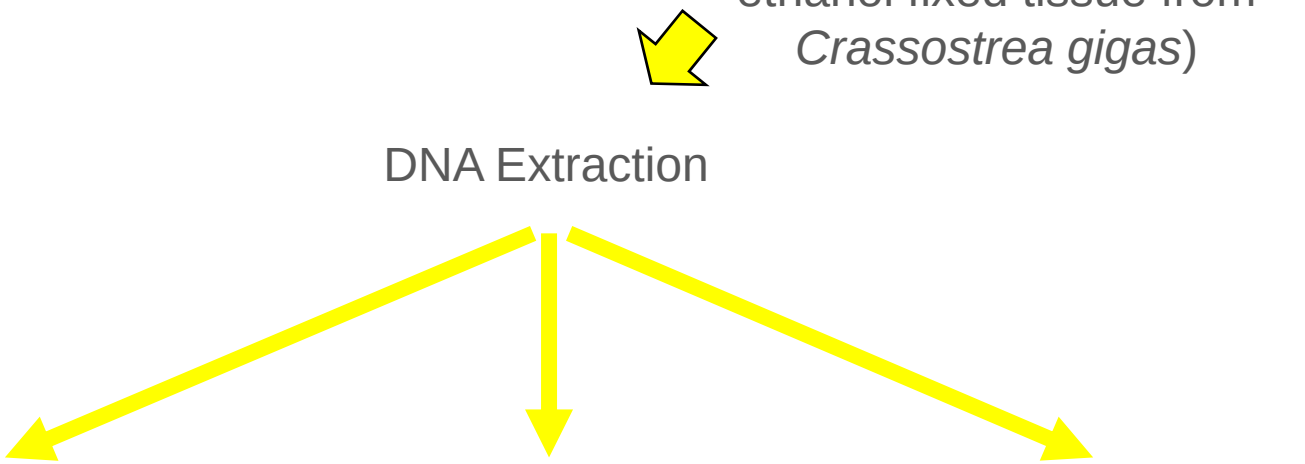
14 laboratories:

- 8 official laboratories
- 4 recognised laboratories
- 1 laboratory under agreement process
- the NRL

2 NRLs

Sample composition:

30 samples
(infected or non infected ethanol fixed tissue from *Crassostrea gigas*)



Only qualitative results were evaluated: presence or absence

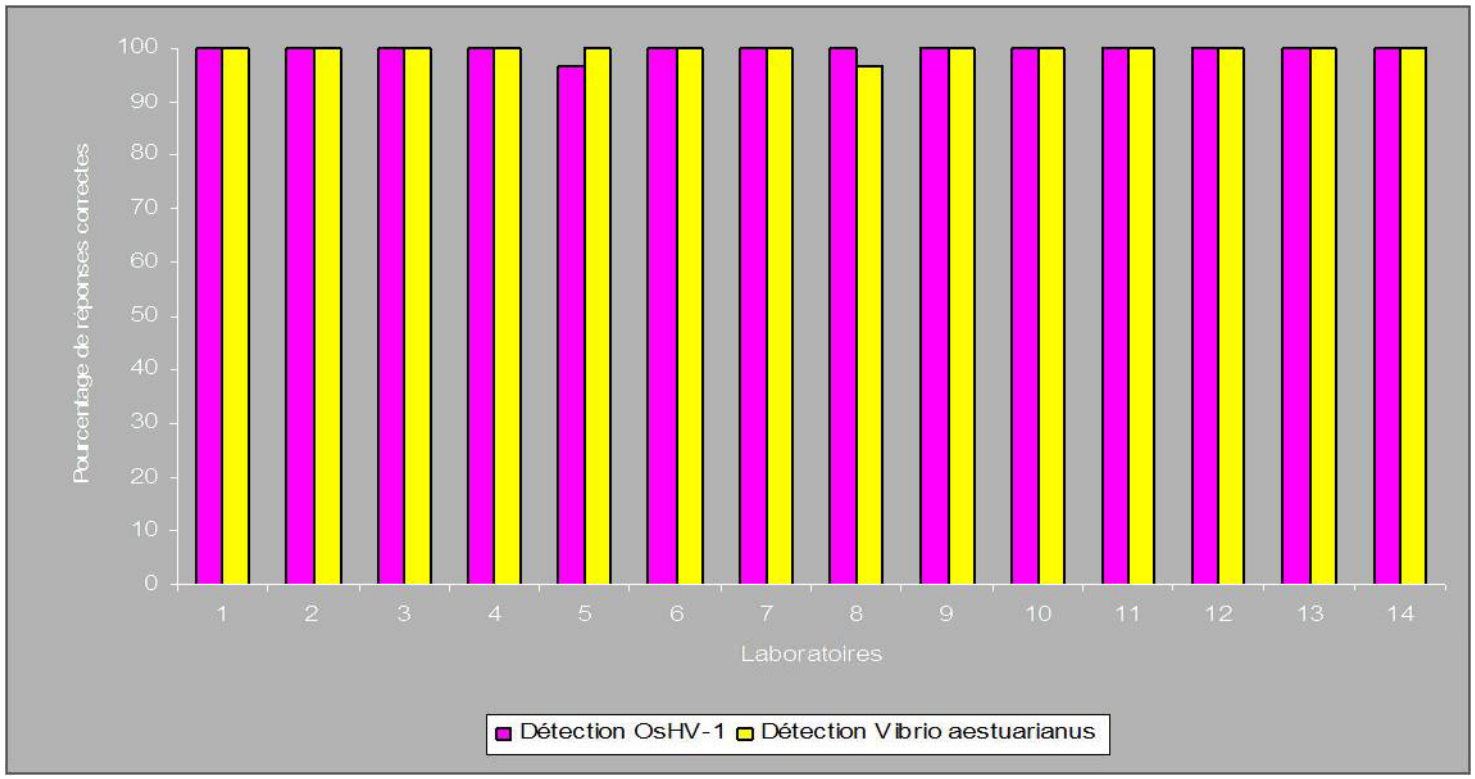
2 NRLs

Sample composition:

Samples	Nature	
	OsHV-1	*Vibrio aestuarianus*
1=2=3	Negative	Negative
4=5=6	Negative	Negative
7=8=9	Highly positive	Lightly positive
10=11=12	Highly positive	Lightly positive
13=14=15	*Detection limit*	Highly positive
16=17=18	*Detection limit*	Highly positive
19=20=21	Highly positive	*Detection limit*
22=23=24	Highly positive	*Detection limit*
25=26=27	Lightly positive	Highly positive
28=29=30	Lightly positive	Highly positive

2 NRLs

Results of participants:



OsHV-1 *Vibrio aestuarianus*

2 NRLs

Results of the comparison study:

	SYBR®Green OsHV-1	Taqman® OsHV-1
Specificity (%)	**100**	**96,4**
Sensitivity (%) Detection limit	5,9	40,5
Sensitivity (%) Light infection	98,8	100
Sensitivity (%) High infection	100	100
Global sensitivity (%)	**76,2**	**85,1**

2 NRLs

Results of the comparison study:

Infection level	Agreement level (%) SYBR®Green OsHV-1	Agreement level (%) PCRQ Taqman® OsHV-1
Non infected	100	95,2
Detection limit	93,6	74,6
Lightly infection	98,4	100
Highly infected	100	100
Global	98,4	94

2 NRLs

Conclusions of the comparison study:

- Good sensitivity and specificity of both techniques
  - Sensitivity of the Taqman assay slightly higher
  - Specificity of the Taqman assay slightly lower

- Discrepancies between laboratories were slightly higher when using the PCRQ Taqman® OsHV-1.

- The PCRQ Taqman® OsHV-1 assay was subsequently recognised as an official method for the detection of OsHV-1 in the context of mortality of *Crassostrea gigas* in France

ILC organised in EU projects

For example, when several participants use same diagnostic tools to evaluate the situation in different sites:

- Oysterrecover: tools to detect *Bonamia* sp.
- Bivalife : tools to detect OsHV-1, *Vibrio* spp. and *Nocardia crassostreae*

Example of the ILC organised by CVI-IMARES

3 EU projects

ILC for the detection of *Nocardia crassostreae* by Real Time PCR in *Crassostrea gigas*

7 samples of mechanically lysed *C. gigas* tissue. Five samples were spiked with a dilution series of cultured *Nocardia crassostreae*.

Sample	Contents	Reference result	Ct value	Tm
1	Lysed *C.gigas* tissue + Nocardia 10^{-3} dilution		27.06	75.8
2	Lysed *C.gigas* tissue	-	-	
3	Lysed *C.gigas* tissue	-	-	
4	Lysed *C.gigas* tissue + Nocardia 10^{-4} dilution		29.78	76.1
5	Lysed *C.gigas* tissue + Nocardia 10^{-2} dilution		23.90	76.1
6	Lysed *C.gigas* tissue + Nocardia 10^{-3} dilution		25.56	76.5
7	Lysed *C.gigas* tissue + Nocardia 10^{-1} dilution		19.17	76.5

3 EU projects

Average Ct value obtained by the laboratories compared with the reference results.

	Ref	A	B	C	D	E
1	27.06	24.51	25.50	28.55	26.67	26.51
2	-	-*	-	-	35.91**	-
3	-	-*	-	-	-	-
4	29.78	28.72	29.46	21.35	29.40	30.61
5	23.90	21.47	22.19	24.80	22.90	23.52
6	25.56	24.79	25.58	28.40	26.01	26.48
7	19.17	18.16	18.62	20.05	20.63	19.50

3 EU projects

Average Tm of all samples.

	Ref	A	B	C	D	E
Average Tm	76.2	76.1	76.5	78.9	76.1	76.5
St Dev	0.31	0.31	0.03	0.21	0.31	0.22
Min Value	75.8	75.6	76.4	78.5	75.6	76.4
Max Value	76.5	76.5	76.5	79	76.6	76.9

Better confidence in results obtained during field investigations

Possible comparison between different field situations

Identification of complementary works to better optimize the PCR assay

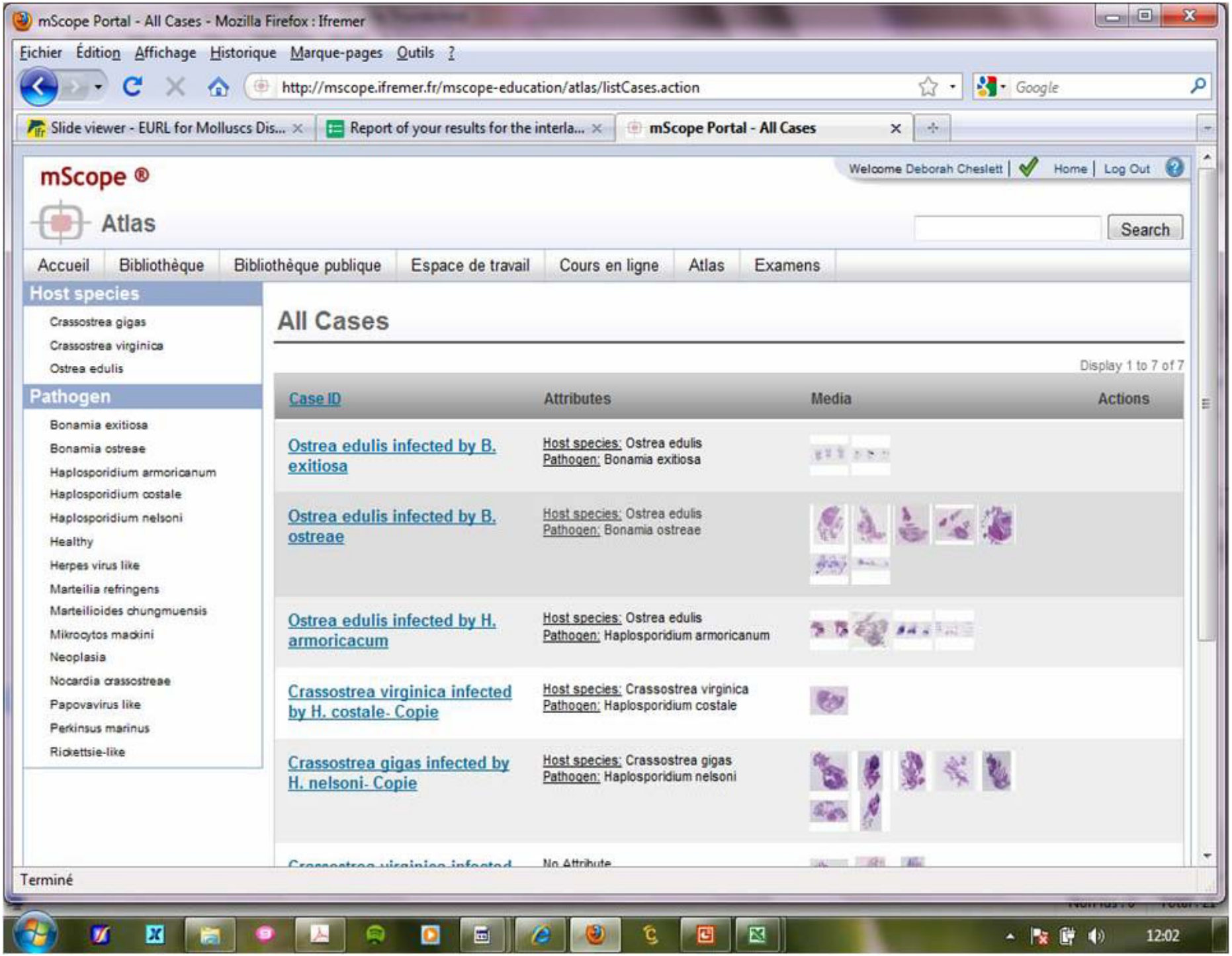
Conclusions

Results of ILC help to

Establish the competency of a network of laboratories
Identify potential optimization works
Compare techniques

Determine the composition of next ILC
Identify needs for training
- Workshop
- mScope®

…


mScope Portal - All Cases - Mozilla Firefox : Ifremer
Fichier Édition Affichage Historique Marque-pages Outils ?
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Google
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Welcome Deborah Cheslett | Home | Log Out
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Cours en ligne
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Examens
Host species
Crassostrea gigas
Crassostrea virginica
Ostrea edulis
Pathogen
Bonamia exitiosa
Bonamia ostreae
Haplosporidium armoricanum
Haplosporidium costale
Haplosporidium nelsoni
Healthy
Herpes virus like
Marteilia refringens
Marteilioides chungmuensis
Mikrocytos mackini
Neoplasia
Nocardia crassostreae
Papovavirus like
Perkinsus marinus
Rickettsie-like
All Cases
Display 1 to 7 of 7
Case ID
Attributes
Media
Actions
Ostrea edulis infected by B. exitiosa
Host species: Ostrea edulis
Pathogen: Bonamia exitiosa
Ostrea edulis infected by B. ostreae
Host species: Ostrea edulis
Pathogen: Bonamia ostreae
Ostrea edulis infected by H. armoricacum
Host species: Ostrea edulis
Pathogen: Haplosporidium armoricanum
Crassostrea virginica infected by H. costale- Copie
Host species: Crassostrea virginica
Pathogen: Haplosporidium costale
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Host species: Crassostrea gigas
Pathogen: Haplosporidium nelsoni
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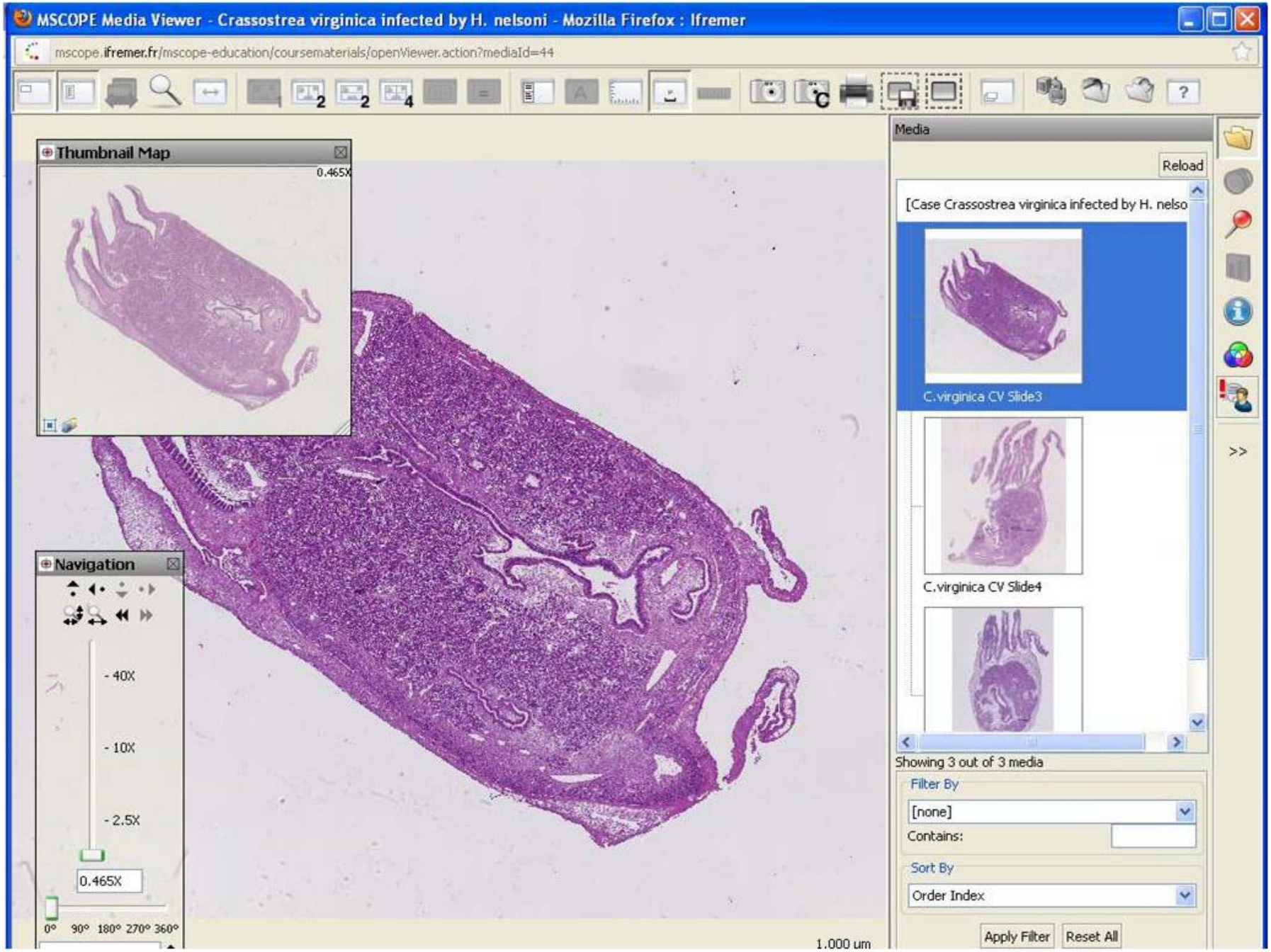
MSCOPE Media Viewer - Crassostrea virginica infected by H. nelsoni - Mozilla Firefox : Ifremer
mscope.ifremer.fr/mscope-education/coursematerials/openViewer.action?mediaId=44
Thumbnail Map
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Navigation
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- 10X
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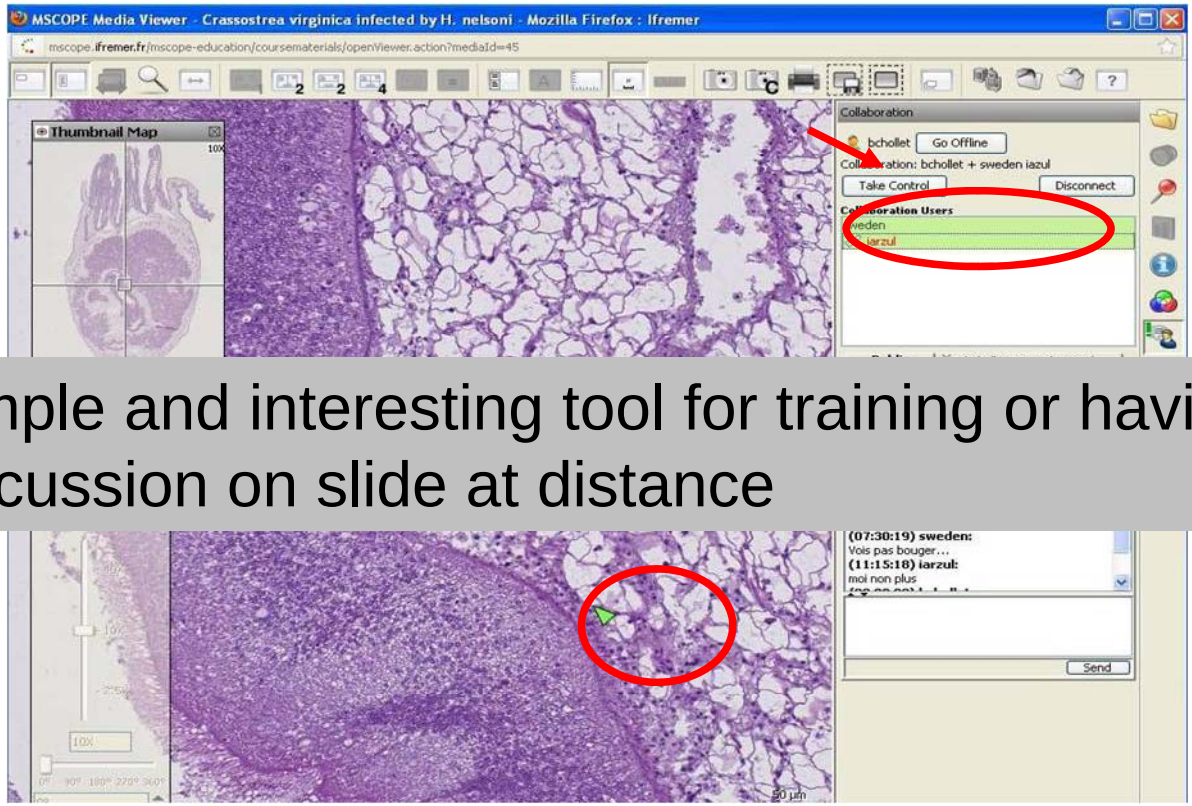
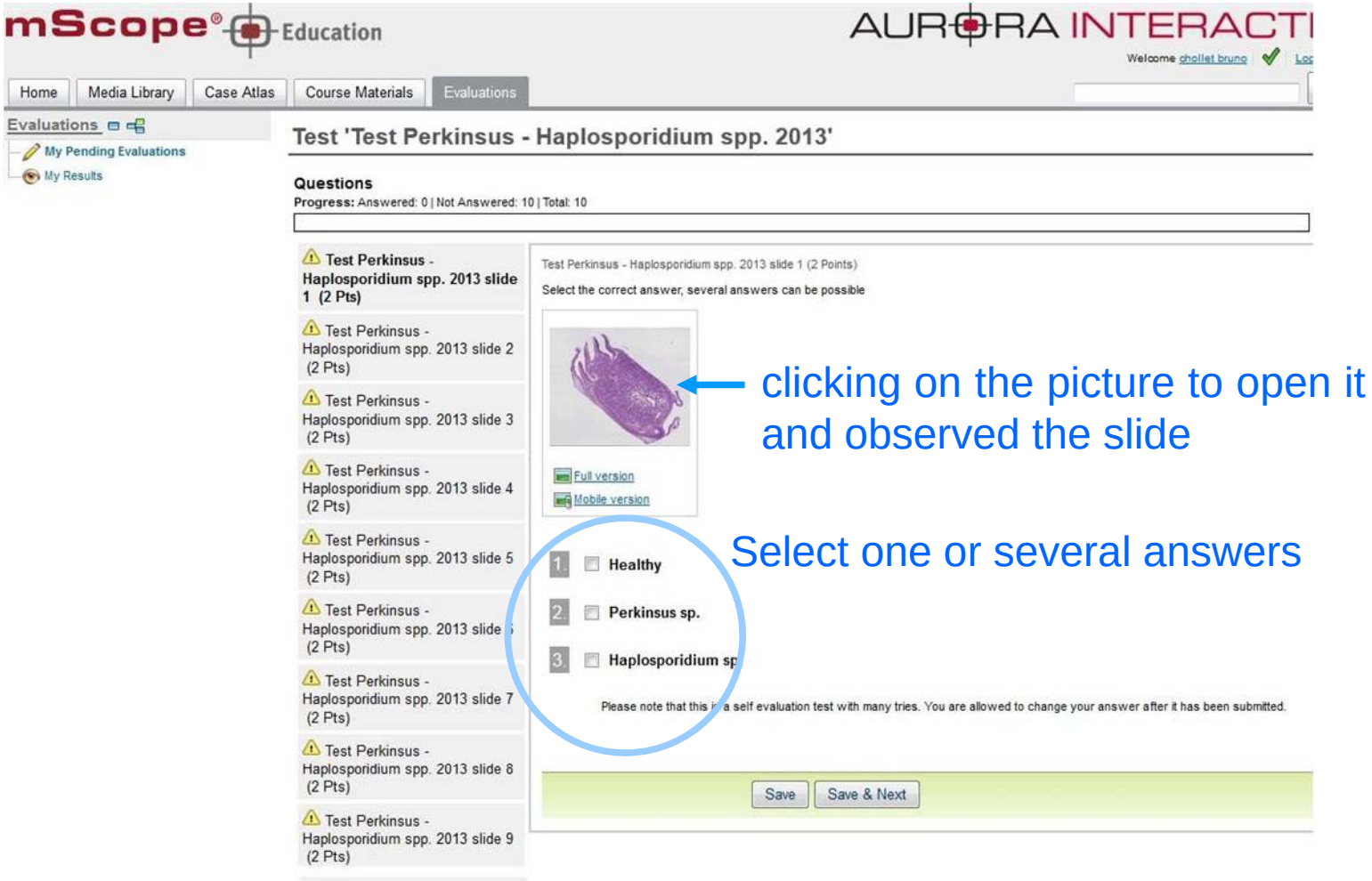
Media
Reload
[Case Crassostrea virginica infected by H. nelso
C.virginica CV Slide3
C.virginica CV Slide4
Showing 3 out of 3 media
Filter By
[none]
Contains:
Sort By
Order Index
Apply Filter
Reset All

Collaboration sessions allow several users to view the same slide simultaneously



Simple and interesting tool for training or having discussion on slide at distance

Self Testing



clicking on the picture to open it and observed the slide

Select one or several answers

Aknowledgements

All the NRLs network
Céline Garcia
Marc Engelsma

Ministry for Primary Industries
Manatū Ahu Matua

Mollusc Disease Diagnosis

Proficiency testing for mollusc disease diagnostics in New Zealand

Brian Jones

23 October 2013

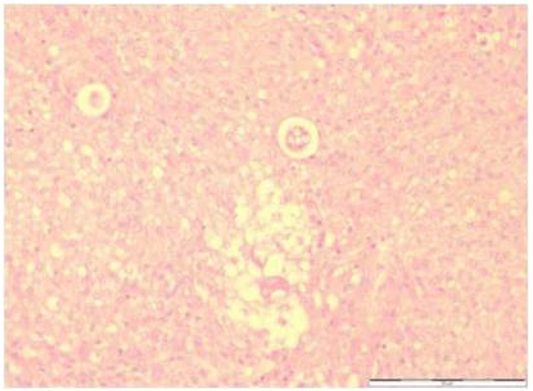
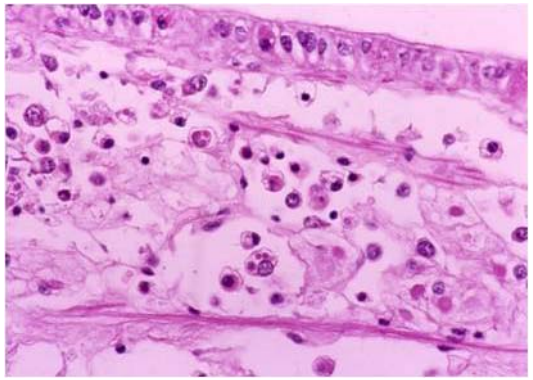
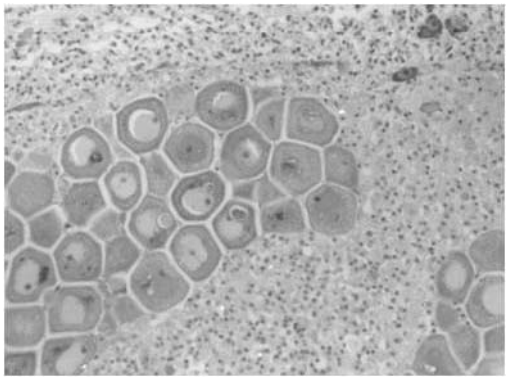
www.mpi.govt.nz

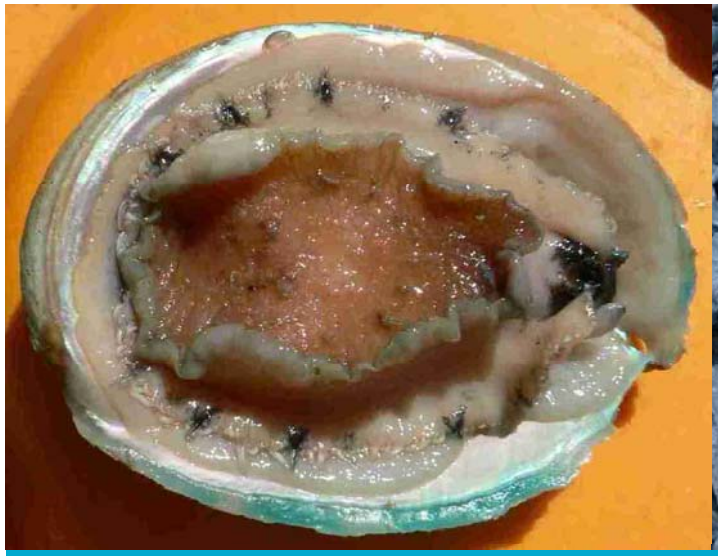
Proficiency testing for mollusc diseases at MPI

1. **Australian National Quality Assurance Program**
   - Ostreid Herpesvirus-1 Microvariant (OsHV-1 µvar)
   - Abalone viral ganglioneuritis (AVG)
2. **EU Inter-laboratory proficiency testing**
   - No mollusc diseases currently
3. **Internal proficiency testing**
   Perkinsus

Histology slides - IDCA

- We have a good collection of reference slides of mollusc diseases
- Participate in the “slide of the quarter” (which regularly has mollusc material).



Proficiency testing activities for mollusc disease diagnostics in Australia

WORKSHOP ON MOLLUSC DISEASE DIADNOSIS, GEELONG, VICTORIA, AUSTRALIA

Mark Crane | CSIRO AAHL Fish Diseases Laboratory, Geelong

21-24 October, 2013

DAFF/ANQAP/AFDL

- EU Ref Lab for Fish Diseases (Denmark) – Inter-laboratory Proficiency Test (annual)
- DAFF SE Asia Inter-laboratory Proficiency Test Program
- **DAFF National Aquatic Animal Health Proficiency Testing Program**
- **The Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR) network**

DAFF SE Asia Inter-laboratory Proficiency Test Program

DAFF SE Asia Inter-laboratory Proficiency Test Program

Viral disease	Test type
White spot syndrome virus of prawns (WSSV)	PCR
Yellowhead virus (YHV)	PCR
Taura syndrome virus (TSV)	PCR
Infectious myonecrosis virus (IMNV)	PCR
Infectious hypodermal and haematopoietic necrosis virus (IHHNV)	PCR
Macrobrachium rosenbergii nodavirus (*Mr*NV and XSV)	PCR
Megalocytivirus (RSIV)	PCR
Viral encephalopathy and retinopathy (VER/NNV)	PCR
Koi herpesvirus (KHV)	PCR
Spring viraemia of carp virus (SVCV)	PCR

DAFF: Funding and project management
NACA: SE Asia coordination
AFDL: PT material production and QA
ANQAP: PT panel production and distribution; collation of results/reporting

DAFF National Aquatic Animal Health Proficiency Testing Program

DAFF National Aquatic Animal Health Proficiency Testing Program

Viral disease	Test type
White spot syndrome virus of prawns (WSSV)	PCR
Yellowhead virus (YHV)	PCR
Gill associated virus of prawns (GAV)	PCR
Viral encephalopathy and retinopathy (VER/NNV)	PCR
Ostreid Herpesvirus-1 Microvariant (OsHV-1 µvar)	PCR
Abalone viral ganglioneuritis (AVG/AbHV)	PCR
Megalocytivirus (RSIV)	PCR

DAFF National Aquatic Animal Health Proficiency Testing Program

Australian Government Dept Agriculture, Fisheries and Forestry (DAFF):

- Provided funding for 3 years (2013-15) of annual PT

AAHL Fish Diseases Laboratory (AFDL):

- Provides material (EtOH-fixed, homogenised tissues) to ANQAP for PT panels (EtOH-fixed cell culture supernatant for RSIV)
- Homogeneity and stability tests PT panels

Australian National Quality Assurance Program (ANQAP):

- Aliquots material
- Distributes PT panels to diagnostic laboratories
- Collates results, prepares and distributes annual reports

DAFF National Aquatic Animal Health Proficiency Testing Program

Mollusc disease agents:

- OsHV-1 µVar
- AbHV

Material:

Ethanol-fixed, homogenised tissues from infected animals

- OsHV-1 µVar: Donated by Richard Whittington (U. Sydney)
- AbHV: Obtained from infected abalone ex Tasmania, donated by DPIPWE, Tasmania

Recommended tests:

- OIE Manual

PROGRAM HAS JUST COMMENCED – NO RESULTS YET

LEADDR
Aquatic Animal Health Proficiency Testing Program

LEADDR
Aquatic Animal Health Proficiency Testing Program (active since mid-2012)

Disease agents:
OsHV-1 µVar
WSSV

1. Network QC Material:
Non-infectious plasmid controls (for reporting monthly)

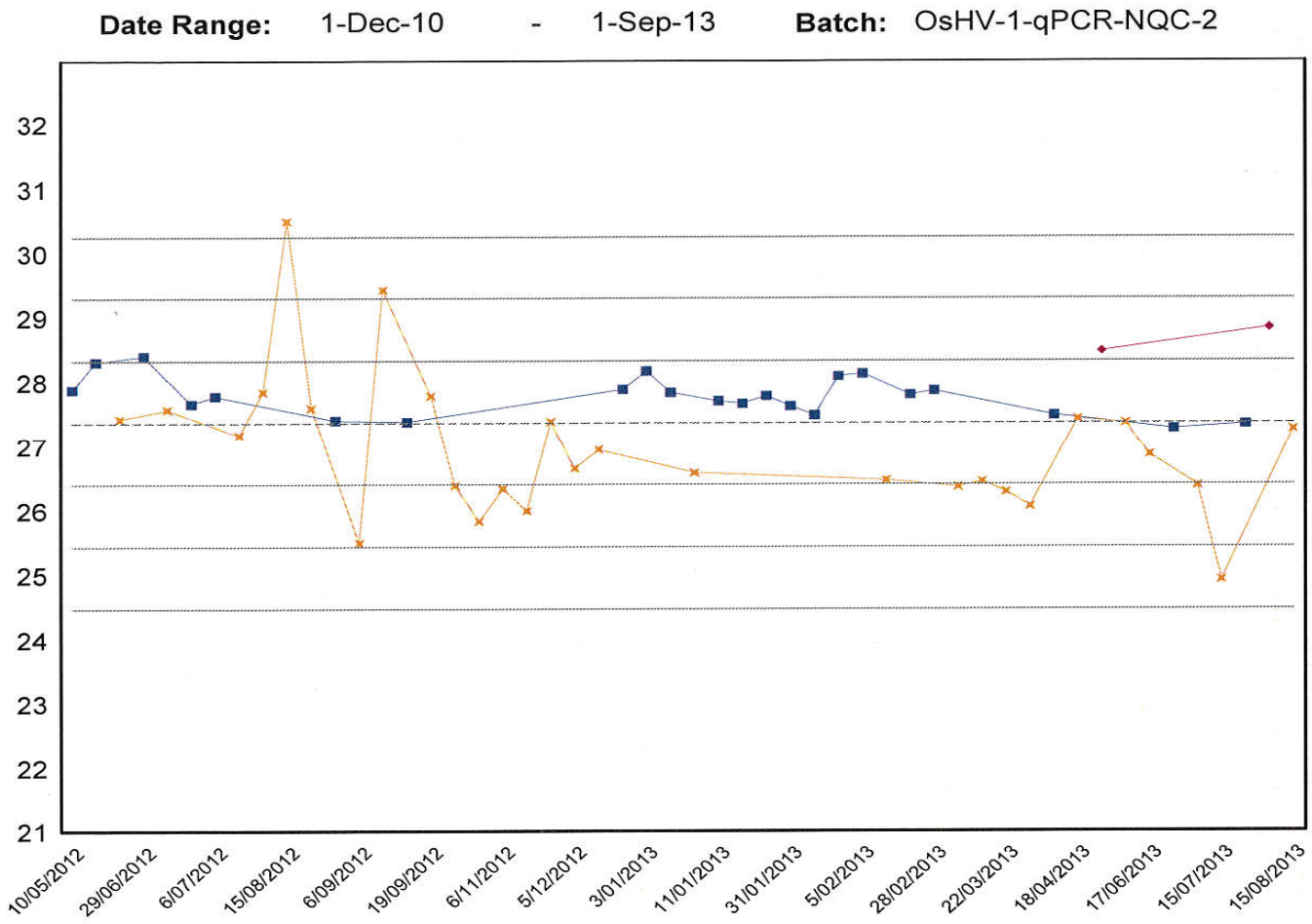
2. PT Material (6-monthly):

- OsHV-1 µVar: Ethanol-fixed homogenised tissue from infected oysters, donated by Richard Whittington (U. Sydney)
- WSSV: Ethanol-fixed homogenised tissue from experimentally infected shrimp

LEADDR
Aquatic Animal Health Proficiency Testing Program

NQC (OsHV-1)

Plasmid



LEADDR
Aquatic Animal Health Proficiency Testing Program

PT (OsHV-1)

Equivalency testing

Threshold 0.1	Martenot OsHV-1 FAST qPCR		EMAI OsHV-1 FAST qPCR	
SAN	2 µl template	5 µl template	2 µl template	5 µl template
13-00620-0001	Undet	Undet	Undet	Undet
13-00620-0002	30.6	29.3	30.1	28.8
13-00620-0003	26.7	25.5	26.5	25.3
13-00620-0004	20.2	18.8	19.9	18.8
13-00620-0005	26.9	25.6	26.7	25.4
No Template	Undet	Undet	Undet	Undet
Threshold 0.05	**Martenot OsHV-1 FAST qPCR**		**EMAI OsHV-1 FAST qPCR**	
SAN	2 µl template	5 µl template	2 µl template	5 µl template
13-00620-0001	Undet	Undet	Undet	Undet
13-00620-0002	29.6	28.5	29.3	27.8
13-00620-0003	25.8	24.5	25.7	24.5
13-00620-0004	19.5	17.9	19.1	18.0
13-00620-0005	26.0	24.7	25.8	24.6
NTC	Undet	Undet	Undet	Undet
Threshold 0.1	**2 µl template**		**5 µl template**	
SAN	Martenot	EMAI	Martenot	EMAI
13-00620-0001	Undet	Undet	Undet	Undet
13-00620-0002	30.6	30.1	29.3	28.8
13-00620-0003	26.7	26.5	25.5	25.3
13-00620-0004	20.2	19.9	18.8	18.8
13-00620-0005	26.9	26.7	25.6	25.4
NTC	Undet	Undet	Undet	Undet

LEADDR
Aquatic Animal Health Proficiency Testing Program

PT (OsHV-1): Ethanol fixed, homogenised tissues

Sample	Dilution	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Median
1	Negative	45.00	45.00	45.00	45.00	45.00	45.00
2	10^{-5}	30.57	31.34	32.96	31.59	31.10	31.34
3	10^{-4}	26.71	29.41	27.88	28.63	28.39	28.39
4	10^{-2}	20.20	21.81	21.70	21.04	22.14	21.70
5	10^{-4}	26.93	29.32	29.23	28.71	30.63	29.23

Summary

Summary

- Examples of proficiency testing programs have been presented, including equivalency testing of two molecular tests (OsHV-1)
- It is recognised that providing proficiency testing is a resource (time, material=$$)-intensive activity
- Requires commitment (resources=$$) from the PT organisers and the PT participating laboratories
- Provides participants with benchmarks
- Provides participants with confidence in their capability
- Provides regulators with confidence in their diagnostic laboratories
- Provides clients with confidence in diagnostic results
- Provides o/s trade partners with confidence in the national capability
- National requirement that diagnostic laboratories are NATA-accredited

Acknowledgements

Fisheries Research and Development Corporation
Australian Government Department of Agriculture, Fisheries and Forestry
University of Sydney
Department of Primary Industries, Parks, Water and Environment – Tasmania
Department of Primary Industries – New South Wales
Department of Environment and Primary Industries – Victoria (ANQAP)

AAHL Diagnosis, Surveillance and Response Group
AAHL Fish Diseases Laboratory

Thank you

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International Bio-based Economy Forum,
Workshop on Mollusc Disease Diagnosis,
Geelong, Australia, 21-24 October 2013

IRTA
INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA

The epidemiological picture *per se* related to European inter-site causal analysis

M.D. Furones[1,2], K.B. Andree[1,2], A. Roque[1,2], N. Carrasco[1,2], B. Lacuesta[1,2], M. Gonzalez[1,2], I. Gairin[1,2], E.J. Peeler[3], A. Reese[3], C. Rodgers[1,2]

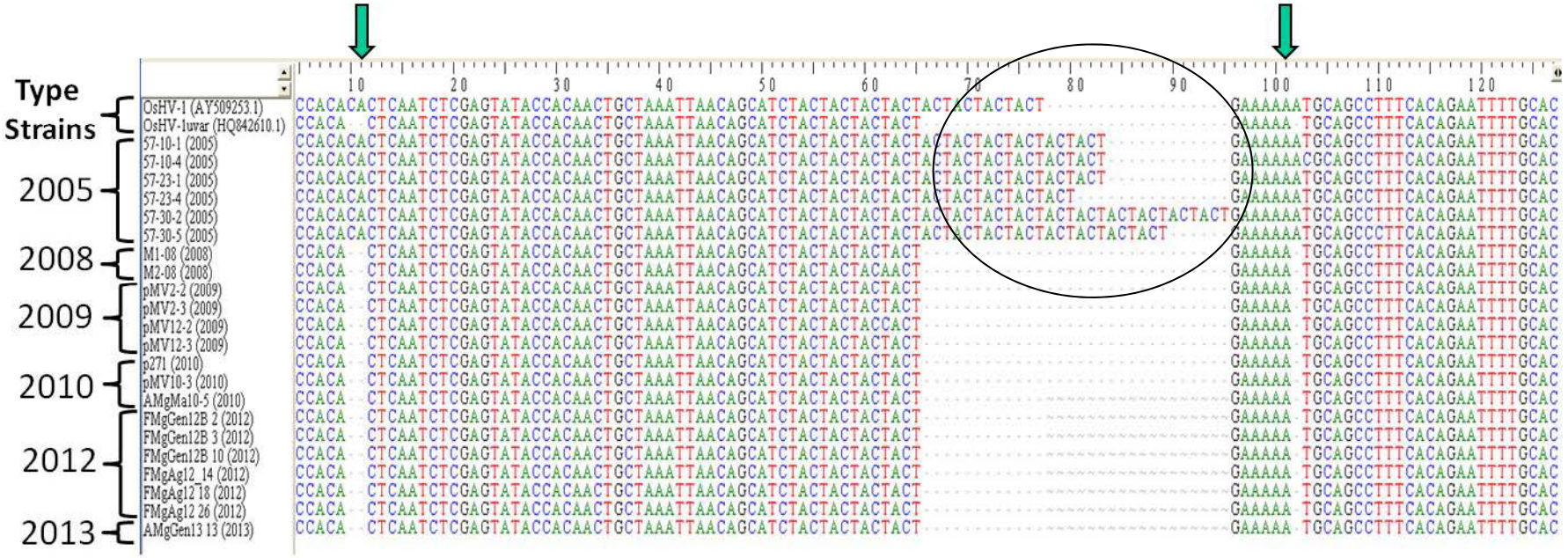
[1] *IRTA, St. Carles de la Ràpita, Spain*
[2] *Red de Referencia de Investigación, Desarrollo e Innovación en Acuicultura (XRAq)*
[3] *CEFAS, Weymouth, UK*

Generalitat de Catalunya
Gobierno de Cataluña

Scenario:

- More than a decade of recurring and increasing mortalities in Pacific oyster (*Crassostrea gigas*) production in Europe.
- Since approximately 2005, increasing mortalities linked to first detections of OsHV- Spain.
- In 2008 and 2009, different zones of *C. gigas* cultivation (e.g. the whole French coast, Ireland) experienced a significant increase in mortality, especially seed stock.
- Subsequent large impact in main production countries.
- Research projects at regional, national and European level.

Sequences obtained:



…an evolving picture

Components of the KBBE-Bivalife study

- Characterization of European study areas
- Sampling plan and guide
- Sample processing and results of OsHV-1 detection
- Potential risk factors associated with mortalities:
  - Vibrios
  - Environmental factors (temperature, salinity, compartments)
  - Bivalves (size, origin, time of immersion)

SEVENTH FRAMEWORK PROGRAMME

KBBE – Bivalife sampling

IRTA
INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA

SAMPLING AREAS
France (three)
Ireland (three)
Italy (one)
Netherlands (one)
Spain (four)

Generalitat de Catalunya
Gobierno de Cataluña

IRTA
INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA

KBBE – Bivalife sampling

Area characterization

Country	Study area	Salinity profile range	Size of area (Ha)	Tidal excursion	Annual TºC max.	Annual TºC min.	Annual rainfall (mm)	Water depth average (m)
France	Brest Bay	31-35	18,000	Yes	18.2	7.0	1,006	Variable
	Marennes Oléron	30-34	15,000	Yes	22.0	6.0	574	8.00
	Thau Lagoon	12-42	7,000	No	29.0	-1.0	500	4.00
Ireland	Carlingford Lough	30-35	1,220	Yes	20.0	1.0	1,200	3.00
	Donegal Bay	29-35	1,260	Yes	20.0	-1.0	1,000	4.50
	Dungarvan Bay	30-35	6,900	Yes	22.0	-1.0	1,100	3.00
Italy	Goro Lagoon	12-34	2,700	Slight	27.6	6.9	700	1.50
Netherlands	Lake Grevelingen	27.3-31.6	10,800	No	21.8	3.1	742	5.40
Spain	Alfacs Bay	25-35	4,900	No	31.0	7.0	532	3.13
	Fangar Bay	21-38	920	No	30.0	6.0	532	1.00
	Rio Sor	9-20	1,457	Yes	20.0	9.0	1,500	Variable
	Ría de Vigo	33-42	14,418	Yes	18.0	11.0	1,500	Variable

Grouped results 2011 - 2012

Detection of OsHV in *C. gigas*

2011 Prevalence OsHV — FANGAR (n = 357)

Pre-Mort (Mar 15) = 30%

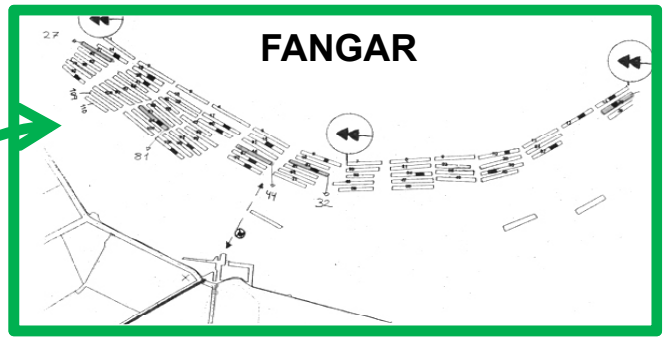
Mort (Apr 14) = 66.7%

Post-Mort I (Apr 27) = 46.43%

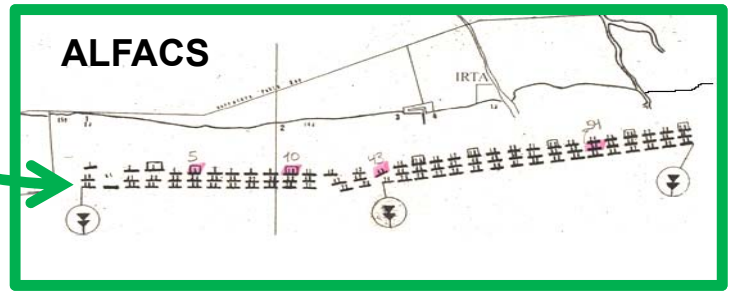
Post-Mort II (June 10) = 82.02% (*suspected new stock?)

2012 PrevalenceOsHV	FANGAR (n = 666)	ALFACS (n = 674)
	Pre-Mort (Mar 13) = 14.1%	Pre-Mort I (Mar 22) = 8.43% Pre-Mort II (Mar 27) = 0.0%
	Mort I (Apr 12) = 62.82%	Mort I (May 08) = 37.65%
	Mort II (May 02) = 48.28%	Mort II (May 29) = 12.07%
	Post-Mort I (June 19) = 29.2%	Post-Mort I (July 3) = 12.35%
	Post-Mort II (Aug 28) = 1.33%	Post-Mort II (Sept 12) = 20.0%

Example production and study areas (Spanish Mediterranean)

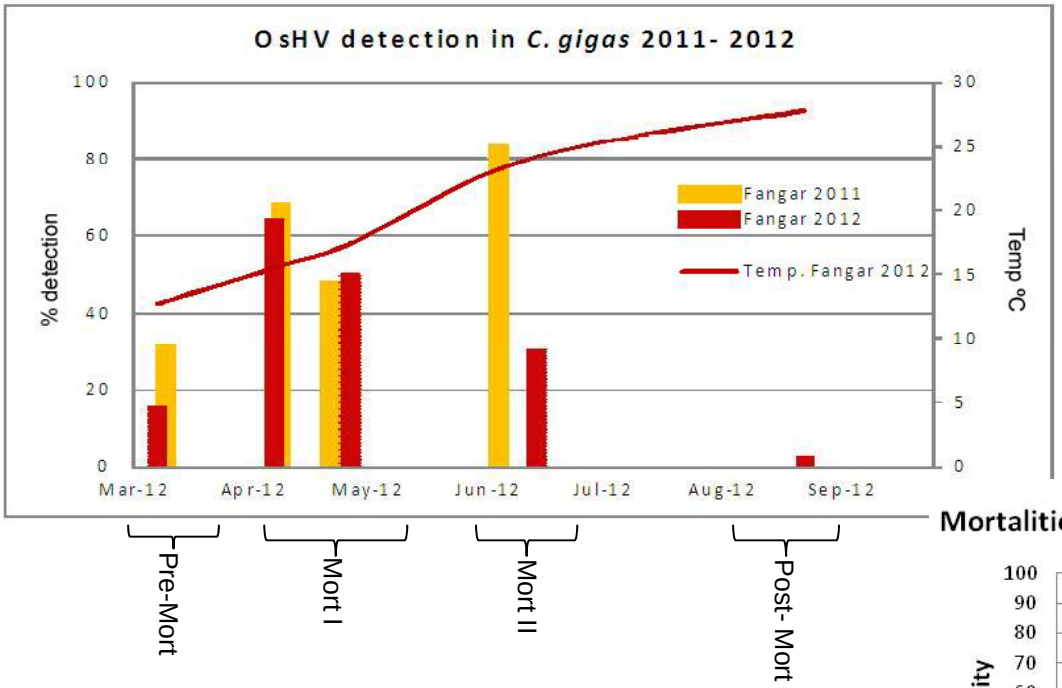


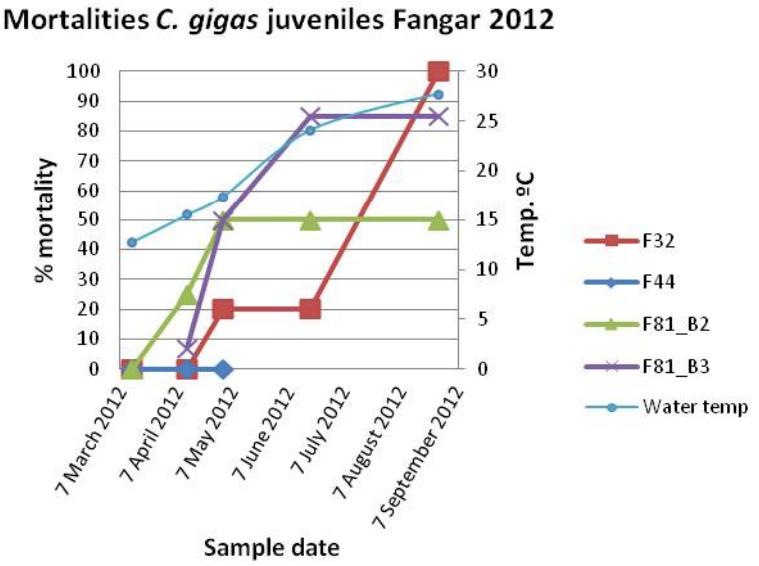
Fangar Bay
Temperature: 6 to 30 ºC
Salinity: 21 to 38 ‰
High renovation rate
Non-tidal



Alfacs Bay
Temperature: 7 to 31 ºC
Salinity: 25 to 35 ‰
Low renovation rate
Non-tidal

OsHV results - Fangar (2011 and 2012)





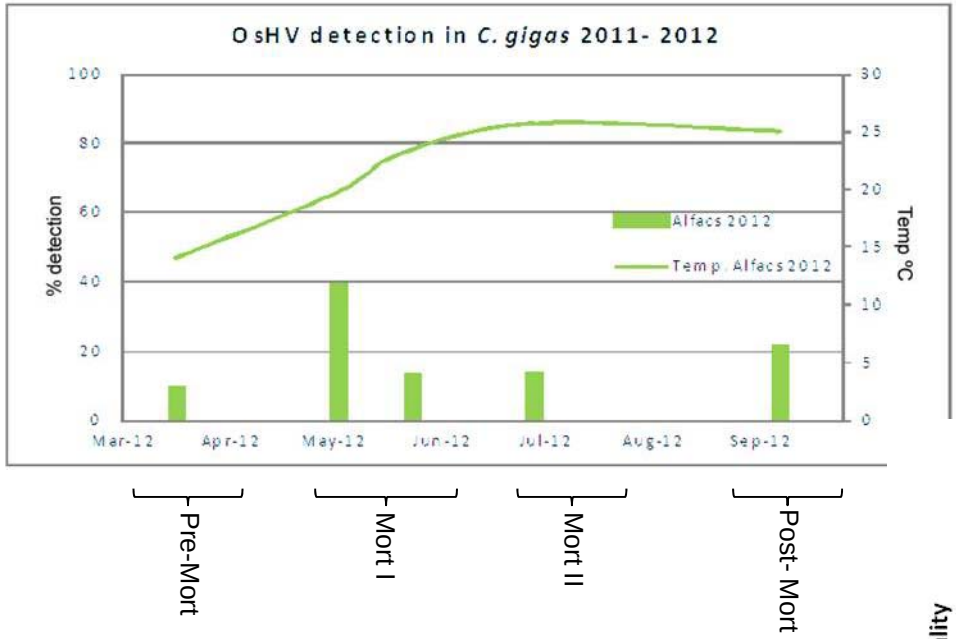
Batch	Origin		$t_{0\,(mm)}$	Date immersion
F32	Spain	Wild seed stock	<15.0	2011?
F44	-	Unknown	31.2	Unknown
F81_B2	France	Wild seed stock	<10.0	Unknown
F81_B3	IRTA	Hatchery	21.8	March 2012 June 2012*

*Removed and reimmersed

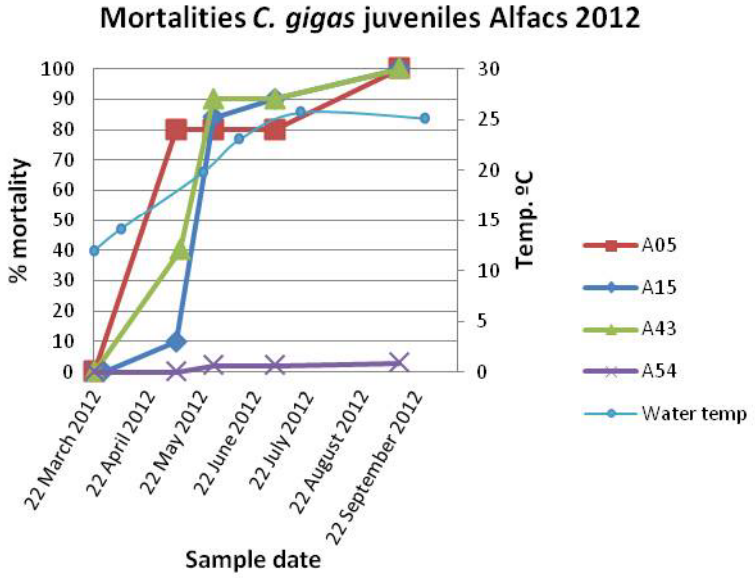
Seasonal detection of OsHV-1

Fangar Bay	Episodes	C2-C6
Autumn (December 2011)	No mortalities	4/30 2/30
Winter (March 2012)	Pre mortalities	2/30
Spring (April 2012)	Mortalities	22/30
Summer (August 2012)	Post mortalities	4/30

OsHV results - Alfacs (2012)



Batch	Origin		$t_{0\,(mm)}$	Date immersion
A05	France	Wild seed stock	<10.0	February 2012
A15	IRTA	Hatchery	27.0	January 2012
A43	France	Wild seed stock	<10.0	March 2012
A54	-	Unknown	39.6	Late 2011?



Grouped results 2011 - 2012

Identification of *Vibrio* spp. en *C. gigas*

2011 Prevalence *Vibrio* — FANGAR (n = 145)

Pre-Mort (Mar 15) = 97.3% (one detection of *V. aestuarianus*)

Mort (Apr 09) = 100%

Post-Mort I (Apr 27) = 100%

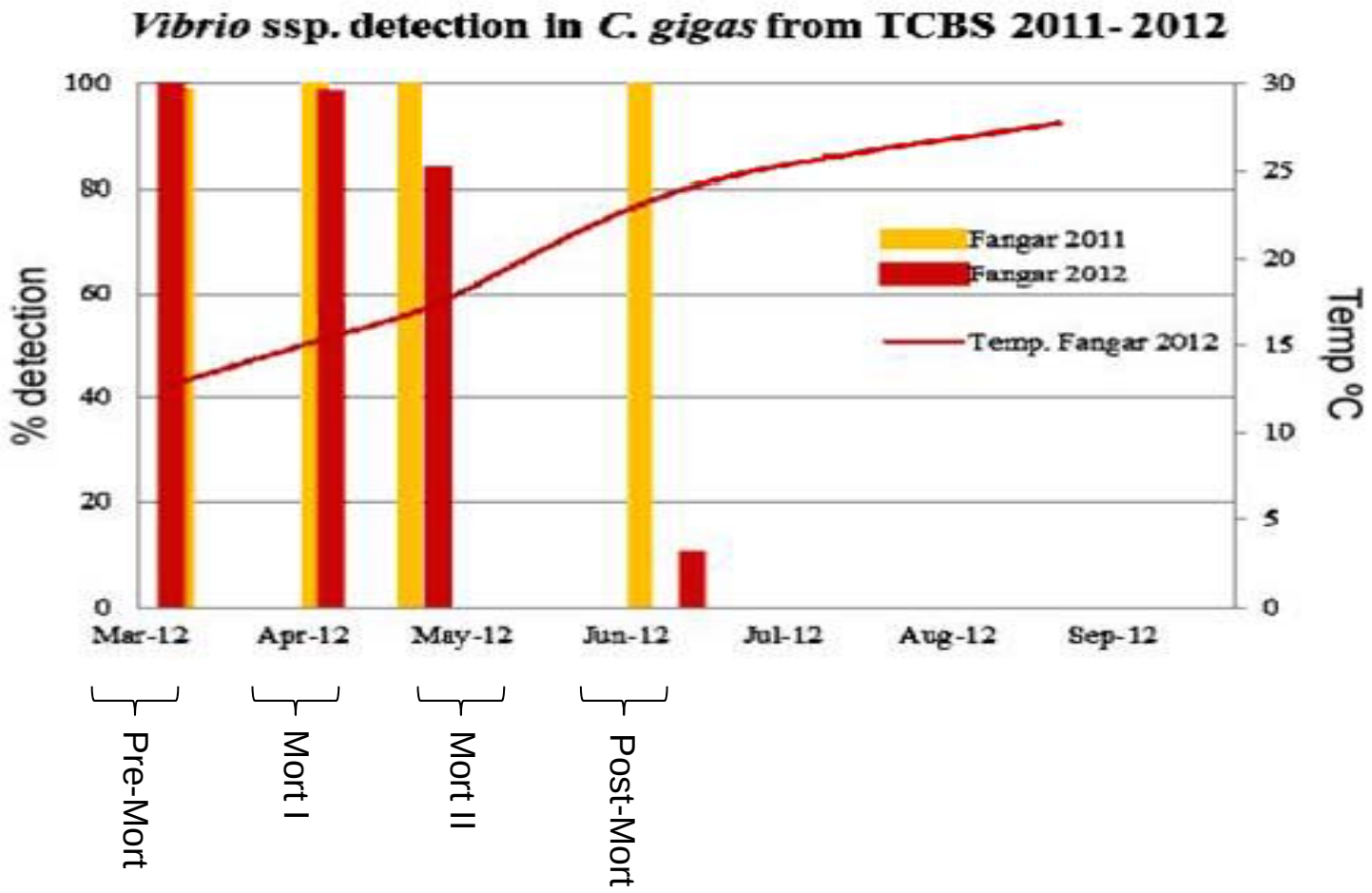
Post Mort II (June 10) = 100% (*suspected new stock?)

2012 Prevalence *Vibrio*

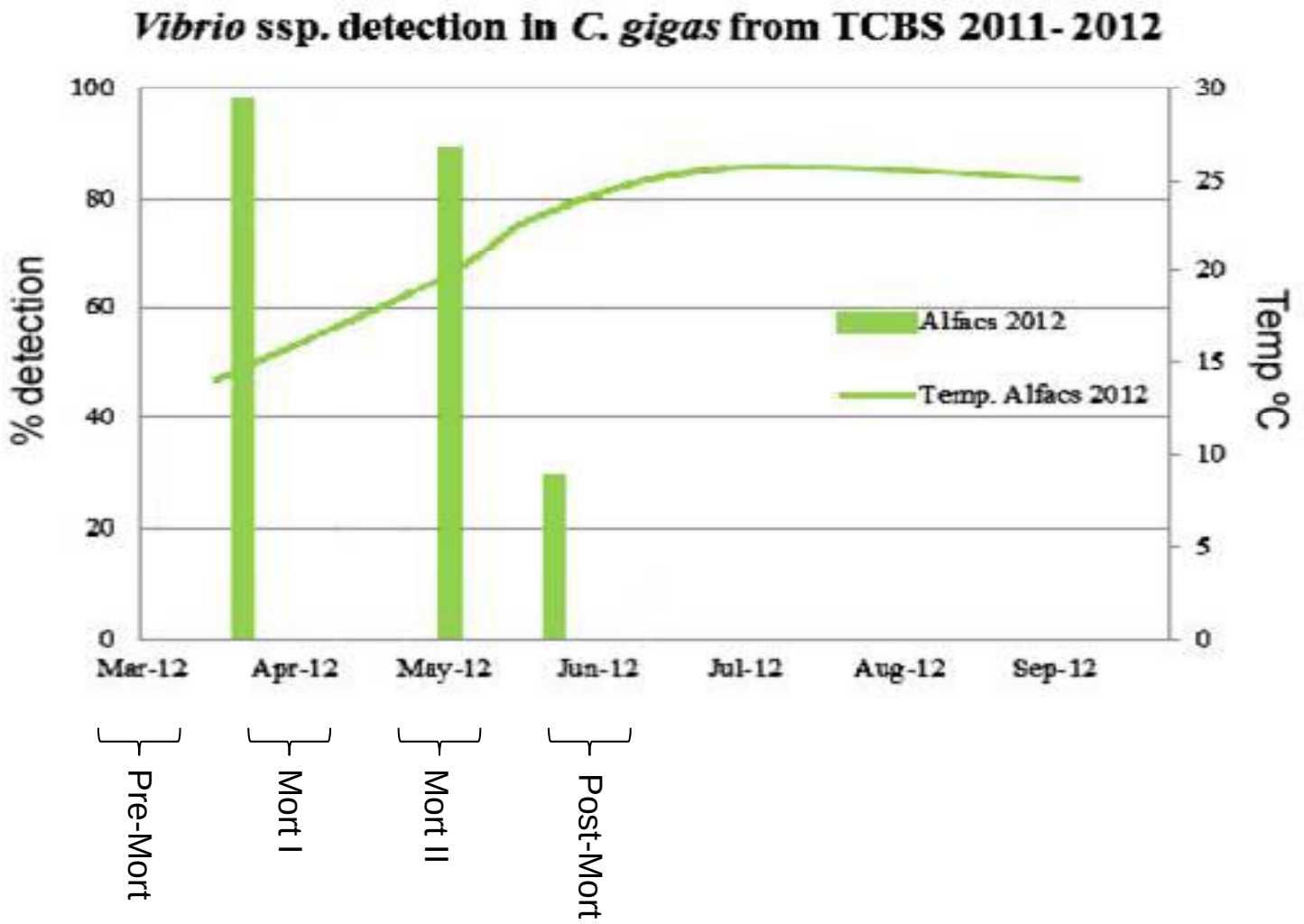
FANGAR (n = 288)	ALFACS (n = 279)
Pre-Mort (Mar 13) = 100.0%	Pre-Mort I (Mar 27) = 96.91%
Mort I (Apr 12) = 96.83%	Mort I (May 08) = 87.5%
Mort II (May 02) = 82.22%	Mort II (May 31) = 27.78%
Post-Mort I (June 19) = 8.43%	Post-Mort I (July 05) = 0.0%
Post-Mort II (Aug 28) = 0.0%	Post-Mort II (Sept 18) = 0.0%

One detection of *V. aestuarianus* in two Bays, in two years

Risk factors → *Vibrio* spp. – Fangar (2011/12)



Risk factors → *Vibrio* spp. – Alfacs (2012)



Crassostrea gigas OsHV prevalence (2011-2012) - France

Brest Bay

Visit Reference	Date	OsHV prevalence (%)	TºC
13	16/05/2011	100	16.5
14	15/06/2011	40	17.4
15	19/07/2011	36.67	16.8
16	11/10/2011	46.67	16.6
231	09/05/2012	0	13.5
232	06/06/2012	100	16.2
233	21/06/2012	53.57	16.2
234	01/08/2012	6.67	18.5
235	13/01/2013	16.67	--

Thau Lagoon

Visit Reference	Date	OsHV prevalence (%)	TºC
246	02/04/2012	13.3	16.3
247	29/05/2012	73.91	18.3
248	11/06/2012	50	(23.9)
249	09/08/2012	6.67	25.2
250	02/10/2012	3.33	19.2

Marennes Oléron

Visit Reference	Date	OsHV prevalence (%)	TºC
1	03/05/2011	30	16.1
2	16/05/2011	76.67	17.2
3	19/05/2011	56.67	18.2
4	06/06/2011	36.67	17.0
5	29/06/2011	30	19.7
6	11/10/2011	73.33	20.2
236	05/04/2012	3.33	12.0
237	22/05/2012	70	14.1
238	05/06/2012	70	17.2
239	19/07/2012	23.33	20.6
240	20/09/2012	26.67	17.8

Data source: Ifremer, France

Crassostrea gigas OsHV prevalence (2011-2012) - Ireland

Carlingford Lough

Visit Reference	Date	OsHV prevalence (%)	T°C
27	18/05/2011	3.02	--
28	03/08/2011	85.33	16.3
29	31/08/2011	53.33	15.3
30	12/10/2011	34.67	13.8
31	11/01/2012	28.67	7.6
187	08/06/2012	0	--
188	01/08/2012	83.33	15.0
189	14/08/2012	58.33	17.2
190	04/10/2012	18.33	11.8
191	13/12/2012	6.67	--

Donegal Bay

Visit Reference	Date	OsHV prevalence (%)	T°C
22	31/05/2011	12.67	--
23	29/08/2011	0	15.8
24	26/09/2011	3.33	13.3
25	26/10/2011	0.67	--
26	25/01/2012	0	8.1
192	06/06/2012	87.3	13.5
193	06/07/2012	8.3	15.0
194	26/07/2012	18.3	16.1
195	04/09/2012	65	15.8
196	02/11/2012	3.3	9.9

Dungarvan Bay

Visit Reference	Date	OsHV prevalence (%)	T°C
17	08/06/2011	0	12.3
18	24/08/2011	1.33	15.1
19	13/09/2011	0	14.0
20	24/10/2011	14.67	--
21	26/01/2012	0	7.2
197	21/05/2012	18	12.3
198	23/08/2012	15	16.3
199	18/09/2012	6.67	13.7
200	19/11/2012	0	10.9
201	18/12/2012	3.33	--

Data source: Marine Institute, Ireland

Crassostrea gigas Vibrio spp. prevalence (2011-2012) - France

Brest Bay

Visit Reference	Date	Vibrio prevalence (%)		TºC
		V. splendidus	*V. aesturianus*	
13	16/05/2011	6.67	0	16.5
14	15/06/2011	63.33	0	17.4
15	19/07/2011	56.67	0	16.8
16	11/10/2011	3.33	0	16.6
231	09/05/2012	32.99	0	13.5
232	06/06/2012	81.42	0	16.2
233	21/06/2012	74.59	0	16.2
234	01/08/2012	ND	ND	18.5
235	13/01/2013	26.22	0	--

Thau Lagoon

Visit Reference	Date	Vibrio prevalence (%)		TºC
		V. splendidus	*V. aesturianus*	
246	02/04/2012	63.60	0	16.3
247	29/05/2012	78.84	0	18.3
248	11/06/2012	37.25	0	(23.9)
249	09/08/2012	ND	ND	25.2
250	02/10/2012	0	0	19.2

Marennes Oléron

Visit Reference	Date	Vibrio prevalence (%)		TºC
		V. splendidus	*V. aesturianus*	
1	03/05/2011	20	0	16.1
2	16/05/2011	16.67	0	17.2
3	19/05/2011	6.67	0	18.2
4	06/06/2011	0	0	17.0
5	29/06/2011	63.33	0	19.7
6	11/10/2011	33.33	0	20.2
236	05/04/2012	39.80	2.38	12.0
237	22/05/2012	19.39	0	14.1
238	05/06/2012	19.39	2.38	17.2
239	19/07/2012	19.39	0	20.6
240	20/09/2012	63.60	2.38	17.8

Data source: Ifremer, France

Crassostrea gigas Vibrio spp. prevalence (2011-2012) - Ireland

Carlingford Lough

Visit Reference	Date	Vibrio prevalence (%)		T°C
		V. splendidus	*V. aestuarianus*	
27	18/05/2011	21.67	0	--
28	03/08/2011	96.67	0	16.3
29	31/08/2011	43.33	0	15.3
30	12/10/2011	28.33	0	13.8
31	11/01/2012	0†	0†	7.6
187	08/06/2012	1.00*	0*	--
188	01/08/2012	0†	0†	15.0
189	14/08/2012	0†	0†	17.2
190	04/10/2012	24.49	0	11.8
191	13/12/2012	0	0	--

*Single pooled sample
†*Vibrio* sp.

Donegal Bay

Visit Reference	Date	Vibrio prevalence (%)		T°C
		V. splendidus	*V. aestuarianus*	
22	31/05/2011	30.0	0	--
23	29/08/2011	35.0	0	15.8
24	26/09/2011	11.67	0	13.3
25	26/10/2011	8.33	0	--
26	25/01/2012	20.0	0	8.1
192	06/06/2012	53.37	0	13.5
193	06/07/2012	0†	0†	15.0
194	26/07/2012	0	12.56	16.1
195	04/09/2012	50.0	0	15.8
196	02/11/2012	29.59	0	9.9

†*Vibrio* sp.

Dungarvan Bay

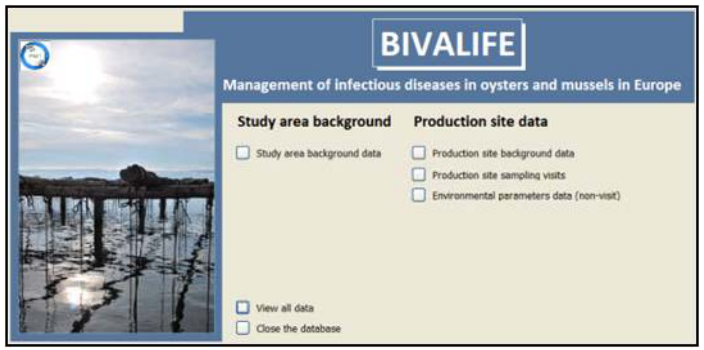
Visit Reference	Date	Vibrio prevalence (%)		T°C
		V. splendidus	*V. aestuarianus*	
17	08/06/2011	41.67	0	12.3
18	24/08/2011	13.33	0	15.1
19	13/09/2011	28.33	0	14.0
20	24/10/2011	0†	0†	--
21	26/01/2012	0†	0†	7.2
197	21/05/2012	58.47	0	12.3
198	23/08/2012	53.37	0	16.3
199	18/09/2012	58.47	0	13.7
200	19/11/2012	10.89	0	10.9
201	18/12/2012	0†	0†	--

†*Vibrio* sp.

Data source: Marine Institute, Ireland

Viral load analysis

Risk factors: Database and causal analysis



1. Information integration platform at the regional and EU level for all sampling:
   - Some historical analysis
   - Sampling guide for homogeneity
   - Field sampling (12 areas)
   - Study area data
   - Production site data
   - Environmental data
   - Diagnostic test results
2. Causal analysis and risk factors

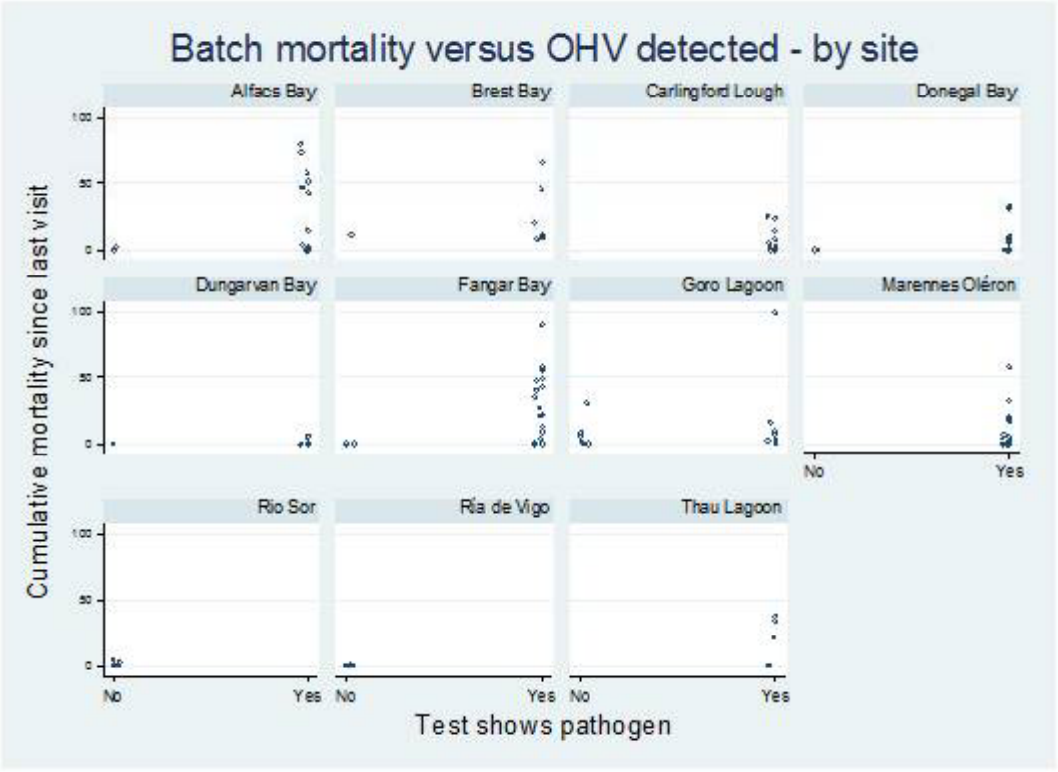
Observations on causal analysis

1. **Causal analysis:**
   - **Sequential monitoring for OsHV with a prospective outlook**
   - **Study areas selected by historical observations that condition the sampling strategy**
   - **Geographically spread, with wide range of climatic and hydrological covariates**
   - **Variety of species and culture methods (data on mussels and clams supports compartmental information)**
   - **Sampling over two years of atypical climatology**
   - **Difficult to track batches through the production cycles (except for own stock)**
   - **Analysis of data on-going**

Observations on causal analysis

2. **OsHV detection in batches**

   - **OsHV is detected at most of the sites and may be associated with no mortality up to large mortality**
   - **When OsHV was not detected, mortality was generally low but not always**



Discussion – OsHV

1. No detection of the OsHV-1 strain in the last eight years → Is it a natural genetic shift or displacement after introduction of the µvar strain?
2. Since 2005, there has been a decrease in *C. gigas* production coinciding with the appearance of the µvar strain.
3. Nearby production areas (climatologically similar) with distinct mesoscale phenomena (hydrodynamics, temperature, salinity, oxygen, etc.) have distinct prevalences of virus, different dates for onset of mortalities but similar mortalities levels.
4. The mortality starts later in Alfacs than in Fangar but the temperature profile is similar → Is it lower host or virus critical mass?
5. Unequal behaviour of batches that become infected but bivalve size and mortality have an inverse relationship.
6. The virus can be present all year round.

Discussion – Risk factors

1. Only one detection of *Vibrio aestuarianus* in two nearby bays in two years matches very low detection in other areas → Are the environmental conditions optimal in Europe?
2. The *V. splendidus* clade is detected in all positive samples.
3. The mortalities start one week after the water temperature reaches 16ºC, and there are indications that the same occurs in reverse in autumn.
4. An analysis of the risk factors, their variability and causality is in progress.
5. OsHV occurs in water, sediment, zooplankton, crustaceans, gastropods, and other bivalve species but not in all areas or at the same level → Which is the true reservoir?

Field observations (IRTA *C. gigas* spat 2011-2013)

Temperature

- **Temperature plays a "key" role in OsHV-1 µvar dynamics, and in spring/summer temperatures between 16-25ºC are critical.**
- **A second annual peak of virus/mortalities is suspected when temperatures decrease: important mortalities of *C. gigas* spat have been observed associated with high virus prevalences at the end of autumn/beginning of winter (decreasing temperatures to 11ºC).**
- **Under experimental thermal stress (25ºC), uninfected spat do not die while virus-infected spat show mortalities of 20% after 6 days.**

Field observations (IRTA *C. gigas* spat 2011-2013)

Stock manipulation

- **The spat immersion schedule (Tº>25ºC) is important to reduce risk of mortality.**
- **"Over-wintering" following autumn immersion helps to reduce mortalities.**
- **Correlation stress-mortality in equivalent conditions of virus infection: less mortality is observed in off-shore long-line cultures than inside the bays.**
- **In long-lines, higher herpesvirus prevalence and *C. gigas* mortality at the top of the rope than at the bottom.**
- **Non-infected hatchery spat can reach high prevalences and mortalities only 10 days after immersion in an affected area.**

Thank you for your attention

Acknowledgements:

- **KBBE - Bivalife** - Management of infectious diseases in oysters and mussels in Europe**.**
- **JACUMAR - Herpemol** - Caracterización de la situación sanitaria del litoral español relativa a la infección por virus herpes en moluscos bivalvos y evaluación del impacto de la enfermedad.
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- **PSQAM -** Plan de muestreo de aguas y de organismos de las zonas de marisqueo y cultivo de moluscos for data.

Generalitat de Catalunya
Gobierno de Cataluña

IRTA
INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA

Adentro estos trabajos hay tres metodologías usadas:

En estos trabajos se han usado tres metodologías:

- Herpes virus DNA polymerase (HVDP) qPCR by Webb et al. – detección de todas las cepas de herpes. Detecta genes de una copia.

- C2-C6 PCR seguido por RFLP utilizando digestión MfeI para diferenciar OsHV-1 de OsHV UVAR. Detecta genes de copia doble.

- C9-C10 PCR para la detección de OsHV y sus variantes (esto no era parte de la SOP oficial de cualquier proyecto). Lo usábamos como un ensayo SYBER Verde qPCR.

Pros	Cons
A) Con el uso de qPCR hay muy poco riesgo de contaminación y falsos positivos ya que los tubos de PCR nunca se abren.	A) Es cuantitativa pero detecta un gen de copia única por lo que hay una cierta pérdida de sensibilidad (aunque no extrema) en comparación con los cebadores de C2-C6.
B) Ya que detecta un gen que se produce dos veces en el genoma que tiene cierta sensibilidad añadida.	B) Debido a que es necesario correr un gel (el producto de la PCR para estos cebadores es demasiado grande para su uso en la qPCR) se requiere la abertura de los tubos de ADN amplificado, por lo que puede haber contaminación con productos de PCR en el laboratorio cuando se utiliza repetidamente y a largo plazo.
B) Un método de RFLP bien descrito y útil para diferenciar OsHV-1 y OsHVuvar	
C) Es más o menos equivalente al protocolo Webb (# A anterior), pero proporciona un segundo conjunto de cebadores para validar otros resultados.	
C) Aunque no se ha descrito originalmente como tal, puede ser utilizado como un qPCR ya que el producto de los cebadores es suficientemente pequeño, así se minimiza la contaminación de PCR.	C) Es cuantitativa pero detecta un gen de copia única por lo que hay una cierta pérdida de sensibilidad (aunque no extrema) en comparación con los cebadores de C2-C6.