FINAL REPORT (DEVELOPMENT AWARD)

AWARD CODE and TITLE

2009/315.11 Aquatic Animal Health Training Scheme: Nicholas Moody

AWARD RECIPIENT: Dr Nicholas Moody

ADDRESS: CSIRO AAHL, 5 Portarlington Rd, Geelong 3220 AUSTRALIA

HOST ORGANISATION: CSIRO Livestock Industries

DATE: 06 February, 2012

ACTIVITY UNDERTAKEN

Visited the CEFAS Laboratory (Weymouth, United Kingdom) 6 to 8 September, 2011, the EU Central Reference Laboratory for Fish Diseases (Århus, Denmark) from 19 to 22 September, 2011 and the EU Central Reference Laboratory for Mollusc Diseases (La Tremblade, France) from 26 to 27 September, 2011.

OUTCOMES ACHIEVED TO DATE

I learnt that Australia's expertise regarding exotic and emergency enzootic pathogen diagnostic test selection, evaluation, optimisation and implementation are at the leading edge of international standards. An example is the rapid confirmation of the identity of the causative agent and implementation of diagnostic tests during the response to the detection of OsHV-1 in NSW. Through discussions with CEFAS staff regarding test selection for OsHV-1, comments were provided to DAFF to ensure greater emphasis is placed on the probe-based qPCR assay used in Australia as a viable alternative to the Sybr Green assay favoured by the French authors in the draft OIE Chapter of OsHV-1. This will ensure that the assays we are using are documented as equivalent alternatives in the international standards. AFDL is increasing the use of multiple assays targeting different regions of a pathogen for greater confidence in the advice provided to authorities in the event of an exotic or emergency enzootic disease outbreak and this was seen as an excellent and highly desirable approach.

It was valuable to learn that laboratories overseas have the same issue as we do regarding the "suspect" or "grey" range for real-time molecular tests where the target organism is at the limits of detection of the test and there was a general consensus that this issue should be discussed at an international level.

Increased confidence in exotic agent test selection, development and/or implementation will be accomplished through greater access to viral isolates from overseas laboratories for test validation, (e.g. different VHSV and ISA genotypes from the EU Reference Laboratory for Fish Diseases) and access to the pre-publication protocols (e.g. VHSV qPCR). The quality systems in place concerning our tests are also of a very high standard and are amongst the best in the world. After presentations at each of the laboratories all staff were very impressed with the AAHL and AFDL facilities and the range of pathogens we have the capability to test for and the range of species our samples are sourced from. Our international reputation was further

enhanced and this, combined with ongoing maintenance of the networks developed will increase our ability to establish collaborative projects with overseas laboratories and increase our leverage for international research funds.

In addition, these visits served to increase rapport and trust between AFDL and other national fish disease laboratories in UK, Denmark and France which will assist in open communication in the future. The overseas laboratories have a greater understanding of aquatic animal health systems in Australia and, in turn, we have an insight to operations in these overseas countries.

Acknowledgments

Funding for return airfares from Melbourne to London, from London to Split, Croatia and attendance at the EAFP 15th International Conference on Disease of Fish and Shellfish from 11 to 17 September, 2011) was provided by CSIRO through the AAHL Fish Diseases Laboratory. Personal contributions for meals were made by the recipient. The Aquatic Animal Health Training Scheme is jointly funded by the Department of Agriculture Fisheries and Forestry (DAFF) and the FRDC.

Background

The aim of the Aquatic Animal Health Training Scheme (AAHS TS) is to improve knowledge and skills in aquatic animal health management to support Australia's fishing and aquaculture industry, including the aquarium sector, with the focus of the scheme is on up-skilling rather than basic training. During discussions with Dr Mark Crane, AFDL Project Leader, regarding travel to Croatia for the 15th EAFP International Conference, the opportunity for Dr Nick Moody to visit CEFAS (Weymouth, UK) and the EU Reference Laboratory for Fish Diseases (Århus, Denmark) to value add to the trip was raised. Discussions with Jo-Anne Ruscoe at the FRDC Annual Planning Workshop (7 April 2011, Canberra) identified that the AAH TS was an appropriate source of funding for the additional travel and an application should be submitted. Dr Keith Way confirmed with the Head of Lab/Division of CEFAS Laboratory in Weymouth, UK that Nick is very welcome to visit prior to the EAFP Conference. Dr Niels Jorgen Olesen, Head of the EU Central Reference Laboratory for Fish Diseases in Denmark was happy for Nick to visit after the EAFP Conference so an application was submitted. The application was reviewed and supported by the FRDC AAHS and approved by the FRDC. During the First FRDC Australasian Aquatic Animal Health Scientific Conference (5 to 8 July, 2011, Cairns) and subsequent OsHV-1 µVar International Workshop (9 to 10 July, 2011, Cairns) Dr Isabelle Arzul was very supportive of an additional visit to the EU Central Reference Laboratory for Mollusc Diseases (La Tremblade, France). This additional visit would value add to the trip and enable compliance with a condition of the FRDC approval to consider skills/training development and information exchange as it relates to current oyster health issues (e.g. OsHV-1). A variation to the award to add this travel (without increasing the funds requested from FRDC) was approved.

Need

A Current Priority in the 2011 FRDC AAHS R&D plan (6.2.3 Endemic and exotic aquatic animal disease diagnostics) is "Diagnostics for agents of national/international significance".
The AAHL Fish Diseases Laboratory (AFDL) role involves detection/confirmation of exotic and newly emerging diseases (particularly those in Australia's National List of Reportable Diseases of Aquatic Animals), index case confirmation and supporting national response capability. Ideally, AFDL diagnostic assays are adopted from the OIE Manual of Diagnostic Tests for Aquatic Animals 2010 or ANZSDPs. Unfortunately, for some agents there are either multiple protocols in the OIE Chapter (e.g. ISAV, IHNV, and VHSV), real-time PCR protocols for high-throughput screening are not in the OIE Chapter (e.g. VHSV, IHNV) or there is no OIE Chapter (e.g. NNV, IPNV). Where no assays are described, published literature is the only source of information. Due to a time lag between updating the OIE Chapters or publication of test protocols, it is not always possible to be confident that the most current protocols have been implemented. Published methods may not adequately describe the specificity of the tests (e.g. detection of different genotypes by ISAV and VHSV RT-qPCRs).

One way to ensure awareness of the latest developments in diagnostic protocols and to compare AFDL's capability is through networks with overseas reference laboratories who routinely deal with agents that are exotic to Australia. Nick Moody's attendance at the EAFP Conference in September will provide an opportunity to also visit the CEFAS Laboratory in the UK, the EU Reference Laboratory for Fish Diseases in Denmark and the EU Central Reference Laboratory for Mollusc Diseases in France. Discussions with scientists in each of these laboratories will cover current protocols (published and unpublished) in use for detection of agents on Australia's National List of Reportable Diseases of Aquatic Animals, general aspects of laboratory testing, including quality assurance procedures and high through-put sample processing and testing.

Objectives

1. Obtain knowledge of current test protocols and positive control strategies for detection of aquatic animal diseases in the European Union, both exotic and endemic to Australia.

2. Obtain knowledge of current methods for high-throughput molecular testing for aquatic animal diseases in the European Union.

Methods

At each laboratory I had a general introduction to the roles and responsibilities by the Laboratory Manager followed by specific discussions with senior scientists and tours of the laboratories they were responsible for. Discussions included general laboratory set-up and work practices, testing regimes and quality assurance practices and more discipline-specific issues that were identified as we were talking. Scientists/staff I had discussions with included:

CEFAS Laboratory – Weymouth, United Kingdom

Kevin Denham – CEFAS Fish Health Inspectorate (FHI)
David Stone – Molecular Testing
Grant Stentiford – CRL for Crustacean Diseases
Keith Way – Virology
Ed Peeler – draft OIE Manual chapters

EU CRL for Fish Diseases – Århus, Denmark

Jette Mølgard - Tissue Culture
Søren Kahns and Søren Peter Jonstrup - Molecular Testing and Virology
Nicole Nicolajsen - EU Ring Testing
Niels Jørgen Olesen – all aspects

EU CRL for Mollusc Diseases – La Tremblade, France

Isabelle Arzul - Molecular Testing, OsHV-1 and Parasites
Jean-François Pépin – OsHV-1 and Research Projects
Tristan Renault – OsHV-1 and Research Projects

At each facility I also gave a 60 minute presentation about the role of the AAHL Fish Diseases Laboratory and highlighted some of the current FRDC research projects and emergency animal disease response work that had been undertaken.

Results/Discussion

Overall, all staff I talked to were very impressed with the AFDL facility and work undertaken, in particular the range of species and pathogens we have responsibility for and the response work we have done. Any differences between AFDL and the laboratories visited were very minor and inconsequential. Differences were based on operator preference due to experience and have no impact on the standard or quality of the work done. It was however, very beneficial to see the approaches taken in other laboratories as there are always individual facility variations to adoption of standard procedures. Detailed notes taken during the laboratory visits (and during the EAFP Conference in Split, Croatia) are included as Appendix A. The test strategies and work-flows we have in place are at least to the standard of laboratories in the UK, Denmark and France. Discussions regarding variable results at higher Ct values for real-time testing were very valuable – all laboratories agreed there is a variable suspect range where there are low levels of target in the sample.

Molecular Virologists at Weymouth and Århus considered the FRDC application to produce synthetic DNA and RNA positive controls to be very good idea and were particularly interested in the approaches to be used to produce stable synthetic ssRNA.

Benefits and Adoption

Wild, cultured, recreational and environmental aquatic animal sectors will benefit from the funding used through improved exotic pathogen test capability (increased access to exotic viral isolates will enable enhanced cell culture, immunodiagnostic and molecular test development, evaluation, optimisation and implementation as will access to pre-publication information and protocols). Australian and State CVOs can be confident that the knowledge and service provided by AFDL is world-class based on open and honest discussion with colleagues from world-renowned laboratories overseas who perform the same tasks as AFDL. Colleagues overseas are interested in exploiting collaborative opportunities so there may be increased access to international funding opportunities.

Knowledge gained will be directly applied to the FRDC through suggested methodology improvements to funding proposals through the FRDC AAHS SAC. Information learned regarding the UK and French approaches to OsHV-1 has already been provided to the SCAAH Pacific Oyster Health Management Working Group. Additionally, knowledge will be shared with interstate colleagues through already established national networks.

Further Development

Information and/or reagent exchanges were discussed and requests to and from Australia are detailed below:

<u>EAFP Conference</u>

AFDL to collaborate with Marty Deveney (PIRSA) to obtain RNA 1 and RNA2 genome sequences for Australian nodavirus isolates through pyrosequencing.
AFDL to source *Renibacterium salmoninarum* DNA for Spain
AFDL to provide NNV PAb to Vikram Vakharia
AFDL to prepare 25 NNV Positive and 25 NNV Negative sets of serial section for comparative NNV IHCT and in situ hybridizations studies with Dave Groman in Canada

<u>CEFAS LABORATORY – WEYMOUTH, UNITED KINGDOM</u>

Request from AFDL:
AFDL to identify strains of EHNV pathogenic to rainbow trout and redfin perch
AFDL investigate possibility of flexible accreditation for molecular tests
AFDL to provide NNV PAb to CEFAS

<u>EU CRL FOR FISH DISEASES – ÅRHUS, DENMARK</u>

Request from AFDL:
AFDL to identify strains of EHNV pathogenic to rainbow trout and redfin perch
AFDL to source isolates of *A. invadans* (EUS)
AFDL to provide the Cox I Reference and website for identification of fish cell lines
AFDL to provide Nodavirus polyclonal antibody (send VHSV and SVCV PABs as well)
AFDL to insert sequences of AUS NODA viruses (RNA1 and RNA 2) in new fishpathogens database

AFDL to provide the protocol for the NNV real time RT-PCR
AFDL to consider possibilities for subcontracting EM for EU CTL for Fish Diseases
AFDL investigate possibility of flexible accreditation for molecular tests

Deliveries to AFDL:
EU CRL to send VHS and ISA isolates: VHSV Genotype II and III primarily (NM will email detailed request when AFDL database has been interrogated)
EU CRL to send Ranavirus isolates including the 2 frog virus isolates and the turbot and cod virus isolates.
EU CRL to send training course material (SOP´s etc- HFSK will prepare a pdf file).
EU CRL to provide the VHSV TaqMan assay procedure.

EU CRL for Mollusc Diseases – La Tremblade, France

Request from AFDL:
AFDL to investigate whether we have an appropriate TEM fixation protocol for copepods (primarily for *Marteilia* investigations.)
AFDL to investigate MTA for transfer of LNA OsHV-1µVar-specific qPCR protocol transfer.

Deliveries to AFDL:
AFDL to investigate availability of material infected with Bonamia sp. in Tasmania for laser microdissection, PCR and sequence analysis.
AFDL to investigate availability of material suffering from Winter Mortality to determine if *B. roughleyi* or *B. exitiosa* is present.

Appendices

Appendix A

Notes from European Study Tour – Sept 2011

15TH INTERNATIONAL CONFERENCE ON DISEASES OF FISH AND SHELLFISH, EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS - SPLIT, CROATIA

The conference provided an opportunity to listen to a wide range of presentations covering topics including aquatic parasitology, immunology, bacteriology, virology and epidemiology that I would not normally investigate during normal work duties, due to time pressures. The standard of the presentations were comparable to those of the biannual FRDC AAHS Scientific Conferences that I have regularly attended in Australia since 2003. The range of topics was broader than that of the Cairns conference. The opportunity to have face-to-face discussions with the authors/presenters and others after the presentations was very beneficial. It was also excellent to meet authors whose publications I rely on as part of my daily duties to discuss more detailed elements of the work. Similarly, it was encouraging to have discussions with people after both my presentations, who were impressed with the specific work I presented and the overall role and capabilities of AFDL. Without attendance at the conference this would not have happened. Similarly, the conference enabled me to have more detailed discussions and network with colleagues I had visited in the United Kingdom beforehand and was going to visit in Denmark and France after the conference.

Attendance at the conference also highlighted and reinforced the need to find time to publish work that AFDL has done in the past, as while the science we do is of an international standard (and the results get to the stakeholders who require it within Australia) there are collaborative opportunities we cannot exploit as our international colleagues are not aware of what we do. An excellent example was the Nodavirus Workshop at

the conference. Four presentations were given followed by a general discussion of current issues. Overall, the work being done by the Europeans was completed within Australia 10 years ago and current research efforts appear to be involved with re-developing molecular detection tests and re-establishing phylogeny. The two major issues in Australia are emergence of clinical disease in older fish and detection of virus in subclinical carrier fish. These are also issues for Europe but research efforts are not being directed towards addressing them. There is a potential opportunity for international collaborative research in this area.

Gave two oral presentations:

- NJG Moody, LM Williams, KR Davies, GA Carlile, J Young, JR White, T Pye, E Hansson, T McDonald, AJ Foord and MStJ Crane (2011) Molecular detection of Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) in farmed *Penaeus monodon* in Queensland.
- NJG Moody, IG Anderson, RJ Whittington and MStJ Crane (2011) Implementation of Betanodavirus diagnostic tests in Australia.

ACTION: AFDL to collaborate with Marty Deveney (PIRSA) to obtain RNA 1 and RNA2 genome sequences for Australian nodavirus isolates through pyrosequencing.
ACTION: AFDL to source *Renibacterium salmoninarum* DNA
ACTION: AFDL to provide NNV PAb to Vikram Vakharia
ACTION: AFDL to prepare 25 NNV Positive and 25 NNV Negative sets of serial section for comparative NNV IHCT and in situ hybridizations studies with Dave Groman in Canada

CEFAS LABORATORY – WEYMOUTH, UNITED KINGDOM

Kevin Denham – CEFAS Fish Health Inspectorate (FHI)
David Stone – Molecular Testing
Grant Stentiford – CRL for Crustacean Diseases
Keith Way – Virology
Grant Stentiford – CRL for Crustacean Diseases

The Fish Health Inspectorate is responsible for the enforcement of the EU aquatic animal health regime in England and Wales which includes statutory inspection, sampling and testing programs for fish, shellfish and crustacean stocks, enforcement of statutory disease controls and implementation of controls on the import and export of live fish, shellfish and crustaceans. CEFAS is an executive agency of DEFRA whose role is to provide scientific and research advice to DEFRA. FHI farm database contains details of 8,500 aquaculture facilities and 40,000 catch-and-release facilities. There are a lot of multi-species facilities. Farms are organised by location, owner, national grid reference with a Google Earth image. Records include fish in and out details, water in and out details, disease events, farm visit schedules, treatments reported by inspectors etc.

There are two main laboratories, in Lowestoft and Weymouth. The Weymouth Laboratory provides specialist monitoring, diagnostic and research work on behalf of DEFRA and the Food Standards Agency on fish and shellfish health and shellfish hygiene, respectively. Each financial year, a service level agreement is produced between the FHI and diagnostic functions to define delivery of testing services for screening and confirmation of listed and non-listed diseases of fish, bivalve mollusks and crustaceans. In particular the SLA prescribes the diagnostic test capabilities required, including test protocols to be followed and the timescales for completion of tests and reporting of test results. There are four primary laboratory functions within the Aquatic Animal Disease Group; Virology, Bacteriology, Histopathology, Molecular. Each discipline has a function manager and a technical manager. The laboratories are accredited to EN ISO/IEC ISO 17025:2005 with flexible scope accreditation[1] through the United Kingdom Accreditation Service (UKAS) with accreditation covering the type of test (i.e. PCR, RT-PCR, qPCR, RT-qPCR) and tests are added to the list as they are implemented. Use Q-Pulse

[1] Flexible scopes of accreditation can allow a laboratory to undertake certain tests/calibrations, and to report the results as accredited, even though they may not be explicitly stated on their accreditation schedule. This may involve:
(a) the inclusion of new or amended tests in accordance with a generic method;
(b) the modification of existing methods to broaden their applicability (e.g. to deal with new materials tested or properties measured, etc);
(c) the inclusion of newly revised or technically equivalent standard methods that are already covered by accreditation.
[http://www.ukas.com/library/Technical-Information/Pubs-Technical-Articles/Pubs-List/LAB39.pdf]

(http://www.q-pulse.com) for managing SOPS. Seems a good system to ensure amendments are authorized in a timely manner. Sample and test data are held in an electronic database which acts as an interface between the laboratories and FHI.

- Scotland dominate cultured species through value and volume with Atlantic salmon (more Atlantic salmon in Scotland than cattle) although shellfish are also very valuable – export £10 million in mussel seed. High health status so it is difficult to import fish. Regions are based on major river catchments.
- FHI subcontracts the diagnostic testing to the CEFAS laboratory.
- Fish smuggling is a major problem which requires intelligence gathering. An ex-policeman does this.
- *Garra rufa* (Doctor fish – an imported ornamental species) is a big issue as 10,000 to 30,000 are imported per week. Targeted sampling of imported ornamental fish.
- Inspectors collect and process samples and are accredited for sampling fish, crustaceans and mollusks. SOP for each species.

Aquatic animal facility

The aquatic animal facility features control of salinity and temperature of water into the tanks with dedicated staff to manage to facility. Keep individual animals, or smaller buckets with holes in them, in the one common recirculated water source (less variation between animals due to different environments in individual mutually exclusive buckets. Possibly something to consider for the individual abalone (same animal treatments in the same common water system). Currently investigating the use of tench and chub as sentinels for VHSV.
Molecular Testing

There is the standard separation of steps. They use QIAGEN robotic extraction systems of 6, 48 and 96 sample capacities for all extractions with the justification being to reduce contamination. Most samples tested are infected cell cultures although they use an MP Biomedicals bead beater for tissue samples. Generally extract 50µL cell culture supernatant or 5mg tissue and elute in 50µL but for OsHV-1 10mg tissue of tissue is extracted and eluted in 100 µL. The extraction control is bovine thymus calf DNA where 1 sample per rack of extractions is seeded. For increased sample numbers (96 well extraction robot) there is an extraction control every 10 samples. The extraction control is only tested if sample is test negative. They also use a no template control for the extraction although this in not routinely tested. Use conventional assays for screening, most of which are nested. Confident using nested PCRs and do not have many real-time assays. Similar issues to AFDL regarding high Ct values in the "grey zone" for real-time PCRs.

- Assay details:
    - Conventional reaction volume is 50µL and real-time reaction volume is 20µL.
    - Use 2.5 µL template.
    - No spectrophotometer quantification of template.
    - Positive controls:
        - Weak product in 1° PCR.
        - Additional control in 2° PCR.
        - Plasmids diluted for conventional PCR.
        - Real-time plasmids diluted in matrix of sample (negative host DNA).
        - Consider FRDC Positive control project to be a good idea. Interested in synthetic RNA particularly in a robust, stabile synthetic ssRNA control. Consider use of insertions or deletions to be an excellent idea.

Tissue Culture

Follow OIE Guidelines with any tweaks required by the EU Commission as standard operating procedures and provide cells for Diagnostic and Research groups. Records kept for traceability for all reagents, passage levels etc. and use pre-made media and add additives, if required and undertake performance checks of new batches of media, NCS, FBS, trypsin and PBS. Undertake *Mycoplasma* sp. testing every 3 months by cell staining but they maintain continuously growing stocks of cells. Control tray of cells for each lot provided which is checked and signed by the producer and user. Always have cover of 3 or 4 people that can do cell culture although there is one core staff member who usually does it.

Virology

Virology group undertakes cell susceptibility testing monthly. Samples processed into VTM, supplemented with antibiotics and antimycotics, and left at 4°C overnight. Virus isolation is undertaken in 12 and 24 well plates prepared in the clean cell culture area. No CO_2 incubators – use gas bags with a predetermined air/CO_2 mix and use different media (e.g. TRIS-buffered). Samples are given a consecutive Virology accession number (Inspectorate numbers are assigned to blocks for pre-planned surveillance and diagnostic testing so are not consecutive). Prescribes tests for routine diagnostics are assigned by the Inspector but they will have discussions for any unusual cases.

- Cell lines used:
  - Use CCB for KHV, SHK for ISA and SSN for NNV
  - Goldfish herpesvirus isolated on KF cells – can isolate it but won't continue to grow
  - KF cells are good for carp pox.
  - Problems with BF2 cells occasionally.

General comments

- Gave a presentation to staff titled "AAHL Fish Diseases Laboratory (AFDL)".
- Spoke to Ed Peeler about the draft OIE OsHV-1 protocol. Suggested some changes which I agreed with so I incorporated these into the reviewed method I emailed to Mark. EU only gets to submit one set of comments for each OIE method so the comments Australia makes are very important.
- Jason Weeks (Aquatic Health and Hygiene Business Development Officer) – very interested in collaborative opportunities including, staff transfer (work experience).
- CRL for Crustacean Diseases is separate to CEFAS diagnostic laboratories with regard to crustacean diseases and is primarily concerned with TSV, YHV and WSSV (due to EU Commission interest). Need to demonstrate freedom from WSSV by testing. Can buy WSSV positive prawns in the supermarket. Wide range of crustacean pathogens – use a combination of histology, TEM and molecular testing.

ACTION: AFDL to identify strains of EHNV pathogenic to rainbow trout and redfin perch
ACTION: AFDL investigate possibility of flexible accreditation for molecular tests

<u>EU CRL FOR FISH DISEASES – ÅRHUS, DENMARK</u>

Jette Mølgard - Tissue Culture
Søren Kahns and Søren Peter Jonstrup- Molecular Testing and Virology
Nicole Nicolajsen - EU Ring Testing
Niels Jørgen Olesen – all aspects

The main purpose of the CRL for Fish Diseases is to ensure the quality of diagnostics of fish diseases in EU Member States and to harmonise the procedures and methodologies applied. The work is mainly concerned with the exotic (Epizootic Haematopoietic Necrosis Virus and Epizootic Ulcerative Syndrome) and non-exotic (Viral Hemorrhagic Septicemia, Infectious Hematopoietic Necrosis Virus, Koi Herpes Virus, and Infectious Salmon Anemia Virus) diseases mentioned in Council Directive 2006/88/EC. The CRL co-ordinates those activities of the National Reference Laboratories (NRLs) for Fish Diseases in EU that aim to harmonise diagnostic techniques and disseminate information of mutual interest. The work of the CRL also includes development of a database (Fishpathogens.eu) that aims to collect information on fish disease isolates and their sequences. Details of the work program are decided at the Annual Meeting of the NRLs for Fish Diseases.

Role

- Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2
- Identify and characterise selected isolates of listed viruses (serological and genetic characterisation) and produce antisera against selected isolates when necessary.

- Assess and standardise of real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases. Evaluate available kits and reagents for diagnosis of the listed non-exotic diseases VHS, IHN, ISA and KHV including consideration of their sensitivity and specificity.
- Update and maintain a library of isolates of Infectious Salmon Anaemia Virus (ISAV), Viral Haemorrhagic Septicaemia Virus (VHSV) and Infectious Haematopoietic Necrosis Virus (IHNV), Koi Herpes Virus (KHV) and Enzootic Haematopoietic Necrosis Virus (EHNV).
- Update and include standard operating procedures on the CRL web page for the listed diseases.
- Prepare the Annual Inter-laboratory Proficiency Test for the National Reference Laboratories (AFDL is a participant).
- Facilitate and provide training in laboratory diagnosis.

Tissue Culture

Accredited to EN ISO/IEC ISO 17025:2005 with flexible scope accreditation[2]. Maintain continuous stock cell cultures for up to 1 year in cell banks at 15°C and 21°C. Back-up cell culture bank. Make their own media and reagents and batch test with details of all media, trypsin, etc. etc and passage of cell cultures recorded. *Mycoplasma* sp. testing is done twice a year by cell staining. Purchased FBS and NCS are batch tested with the final choice made based on cell appearance and sensitivity test results. Five technical staff in cell culture and virology sections. No CO_2 incubators - use different media (e.g. TRIS-buffered). Use penicillin and streptomycin in flasks and add Nystatin to plates and Gentamycin when samples are added.

- SSN-1 and ASK cells are more sensitive when 2-β-mercaptoethanol is added.
- Susceptibility testing:
    - Same virus is titrated on the cells.
    - Reduced the range of cell types used to target the specific virus they would be used to isolate.
    - Two strains of IPNV on BF2 and CHSE cells.
    - Two strains of VHSV on BF2 and FHM cells.
    - One strain of IHNV on EPC and FHM cells.
    - One strain of SVCV on EPC and FHM cells.
    - One strain of EHNV on BF2 and EPC cells.

Virology

Use standard OIE protocols with general testing using pools of spleen, some kidney and heart. Dilute 1/10, homogenize with a mortar and pestle then centrifuge the samples.

Seminar by Anna Amanda Schönherz "Rainbow trout susceptibility to oral transmission of VHSV following feeding on infected material: MSc studies.

- EU freshwater VHSV genotypes (Ia, Ic & Id) are adapted to rainbow trout.
- EU marine VHSV genotypes (Ib, Ic, II and III) are not adapted to rainbow trout.
- Identified stomach as a viable route of VHSV entry after infection by intubation
    - RT-qPCR: VHSV detected in the kidney in the absence of feed in the stomach indicated movement of virus from the stomach (strong indicator of replication).
    - Could use IHCT to localize the virus.

Aquatic animal facility

Flow through tanks next to each other which are an efficient use of space with ability to adjust water hardness, salinity and temperature, with dedicated staff to look after the facility. Currently holding trout, albino trout, turbot and herring. Interesting that they source eggs so they can be decontaminated (otherwise as the fish are

[2] Flexible scopes of accreditation can allow a laboratory to undertake certain tests/calibrations, and to report the results as accredited, even though they may not be explicitly stated on their accreditation schedule. This may involve:
(a) the inclusion of new or amended tests in accordance with a generic method;
(b) the modification of existing methods to broaden their applicability (e.g. to deal with new materials tested or properties measured, etc);
(c) the inclusion of newly revised or technically equivalent standard methods that are already covered by accreditation.
[http://www.ukas.com/library/Technical-Information/Pubs-Technical-Articles/Pubs-List/LAB39.pdf]

from all over the place they will be infected with something) and grow the fish themselves. UV-irradiate the water before use and heat-treat (pasteurize) water before release.

Molecular Testing

Use MagNA Pure robotic extraction system and QIAGEN spin columns and a bead beater (also for EUS) for sample homogenization. Extraction control is spiked with a plasmid that has a deletion of insertion for real-time and conventional assays. Similar issues to AFDL regarding high Ct values in the "grey zone". Very long discussion and decided it should be addressed internationally. Also discussed whether it would be more sound to undertake 2 extractions from the one sample and test in singlicate as opposed to the current practice of undertaking one extraction and testing in duplicate. Endogenous controls are very important and consider FRDC Positive control project to be a good idea. Interested in *Cox* 1 PCR protocol. Discussed the new VHSV RT-qPCR. Primer and probe sequences will be emailed to me for comparison with the Garver RT-qPCR. The Danish assay is more sensitive for genotypes II and III and having two assays, targeting different regions is desirable.

- Assay details:
    - Conventional and real-time reaction volume is 25µL.
    - Use 5 µL template added.

EU Ring Testing

Discussed all aspects of the EU Ring Test. In 2010, 35 laboratories participated so very detailed records of sample preparation, homogeneity testing and stability testing are required. In 2011 there will be two panels; PT1 will be normal cell culture and PT2 will be molecular testing. Check representative vials for each sample for everything by serology and molecular test to ensure quality. Devised a better protocol for storing material in ampoules. Test samples with IFAT in a 96-well plate format using a protocol that takes 60 minutes. Suggestion is to test each vial by titration in 96-well plates and culture on 24-well plates at the same time and using one ampoule at a time, decontaminating and then doing the next vial. Staff visit countries with lower scores while they are doing the assays and watch. Then provide tips on how to do things better. Discussed end-point diluting the samples for molecular testing to get a better estimate of sensitivity for conventional assays.

- Quality control
    - Test 5 replicates for each sample with no greater than 1 log difference acceptable.
    - Keep one ampoule at 30°C for 3 months prior to testing

General comments

- Gave a presentation to staff titled "AAHL Fish Diseases Laboratory (AFDL)".
- Suggested that Mark Crane nominate to become the President of the OIE Aquatic Animal Health Standards Commission (OIE AAHSC) (formally the Fish Diseases Commission) and that the nomination would be supported by the EU. Niels also suggested I go straight the Australian CVO and suggest he promote and nominate Mark. This is quite flattering as the Members are required to be internationally recognised specialists in the fields of methods for surveillance, diagnosis and prevention of infectious aquatic animal diseases and have extensive international experience, at the regional or global level, of aquatic animal infectious disease surveillance, diagnosis, control and disease prevention methods.
- Very interested in collaborative opportunities, including test development (inter-laboratory robustness testing). Share the same view as AFDL that if another appropriate assay has been developed, there is no point re-inventing the wheel.
- *http://www.fishpathogens.eu/*
    - Electronic depository of viral isolate and sequence data. Currently incorporates VHSV and IHNV sequences with implementation of VNN sequence submission underway. They prefer to have sequences published to Genbank as well limited private/public access (private access restricted to a limited period of time as it is expected you would publish.
    - Requested to add Australian NNV sequences when the database is established.

Request from AFDL:
ACTION: AFDL to identify strains of EHNV pathogenic to rainbow trout and redfin perch
ACTION: AFDL to source isolates of *A. invadans* (EUS)

ACTION: AFDL to provide the Cox I Reference and website for identification of fish cell lines
ACTION: AFDL to provide Nodavirus polyclonal antibody (send VHSV and SVCV PABs as well)
ACTION: AFDL to insert sequences of AUS NODA viruses (RNA1 and RNA 2) in new fishpathogens database
ACTION: AFDL to provide the protocol for the NNV real time RT-PCR
ACTION: AFDL to consider possibilities for subcontracting EM for EU CTL for Fish Diseases
ACTION: AFDL investigate possibility of flexible accreditation for molecular tests

Deliveries to AFDL:
ACTION: EU CRL to send VHS and ISA isolates: VHSV Genotype II and III primarily (NM will email detailed request when AFDL database has been interrogated)
ACTION: EU CRL to send Ranavirus isolates including the 2 frog virus isolates and the turbot and cod virus isolates.
ACTION: EU CRL to send training course material (SOP´s etc- HFSK will prepare a pdf file).
ACTION: EU CRL to provide the VHSV TaqMan assay procedure.

EU CRL FOR MOLLUSC DISEASES – LA TREMBLADE, FRANCE

Isabelle Arzul - Molecular Testing, OsHV-1 and Parasites
Jean-François Pépin – OsHV-1 and Research Projects
Tristan Renault – OsHV-1 and Research Projects

Role

- Coordinate in consultation with the Commission the methods for diagnosing diseases of bivalve molluscs.
- Assist actively in the diagnosis of disease outbreaks by receiving pathogen isolates for confirmatory diagnosis, characterisation and epizootic studies.
- Facilitate the training or retraining of experts in laboratory diagnosis with a view to the harmonisation of diagnostic techniques throughout Europe.
- Collaborate as regards methods of diagnosing exotic diseases, with competent laboratories in third countries where those diseases are prevalent.
- Research focus:
    - The cellular defense of oysters with *Bonamia*.
    - Oyster herpesvirus.
    - *Marteiliosis* of flat oysters.
    - Bacteriological studies in support of the mollusc hatchery/nursery operations.
- http://www.eurl-mollusc.eu/
    - Standard Operating Procedures (Histology, Molecular Testing),
    - Proficiency Testing
        - *Bonamia ostreae* by PCR
        - *Marteilia refringens* by PCR-RFLP

Molecular Testing

New diagnostic facility with standard separation of steps. Similar issues to AFDL regarding high Ct values in the "grey zone". For sequencing of conventional PCR amplicons they clone so miss co-infections are not missed. Test 3 animals per submission and 3 clones per animal.

- OsHV-1 Diagnosis
    - Need to consider both OsHV-1 and OsHV-1µVar in any work done and not just focus on OsHV-1µVar. Need to also consider other pathogens (e.g. *Vibrio* sp.)
    - Important to include ORF4, ORF42 and ORF43 for phylogeny.
    - New qPCR specific for OsHV-1µVar based on ORF4 which contains more relevant deletions and mutations
        - Difficult to multiplex both qPCR assays so use both as singleplex assays for testing.
        - New assay is based on Linked Nucleic Acid (LNA) technology using 2 mutations specific for the µVar. This assay has been validated and compared with the Martenot

probe-based assay and the new assay is slightly more sensitive. Tested with different populations and is being distributed to the network of French laboratories for evaluation and implementation.

- Can provide primer and probe sequences to AAHL but need a confidentiality agreement as it is unpublished.

OsHV-1

OsHV-1 is a real problem and the French are facing a crisis as OsHV-1µVar has been detected everywhere. The Ministry of Agriculture want to identify different strains of the host (*C. gigas)* to identify potentially resistant populations but the scientists are not convinced resistant oysters are the solution as they may be more susceptible to something else. There is also concern that importing OsHV-1-resistant *C. gigas* from overseas will introduce new pathogens. There is also the issue of what to select for (higher resistance when put in the ocean). Due to collection of wild-spat, farmers are also reluctant to use selected animals as they don't want to be reliant on hatcheries. Possibly too late to do anything about OsHV-1 in France as there has been; (1) too much collection of spat and movement of animals, (2) oyster banks are extensive and it is impossible to get rid of sick animals and (3) the use of bags on tables can cause issues as there are large tidal variations so in some areas you have to wait 2 weeks between tides before you can inspect the animals (suspension culture is not very common but it is being established using Australian bags).
Specific points:

- IFREMER has had sentinel animals at 13 locations since 2009. Environmental conditions are measured by probe at the same time. The aim is to try and get a correlation between the sentinel animals and what farmers report.
- Can be difficult to detect OsHV-1 7 days after the peak of mortality. If the animals are collected 8 days after the peak of mortality they do not test them.
- Important issue for hatchery production (6 main hatcheries that send spat everywhere). Specific pathogen free hatcheries could be established but need to define status of areas the spat are going to (i.e. Ireland).
- Management:
    - Oysters placed higher up the beach seem to be less susceptible.
    - Change dates when spat are seeded, but could facilitate the emergence of the disease.
    - Try placing spat after May (reduce mortality at the end of summer).
    - Look at reducing (or increasing) stocking densities.
- Infectivity trials – better to inoculate OsHV-1µVar into the adductor muscle than cardiac muscle then co-habitate.
- Trials to look at temperature affect and identified a 16°C threshold:
    - >16°C = disease and mortalities
    - <16°C = no disease and no mortalities
    - Need to look at how the temperature increases. Does a slow increase have the same effect as a rapid temperature change?
- 2009/10 Epidemiological Study targeting pathogen detection: OsHV-1 and *Vibrio*.
    - Monitored temperature, salinity, turbidity and chlorophyll A with probes.
    - Ran from March to September during 2009 and 2010.
    - When temperature went over 16°C, sampling was increased from monthly to more frequently. Also sampled benthic animals, sediment and old and young oysters, before during and after the outbreak – still to be published.
        - OsHV-1 negative before the start of mortality
        - Temperature got to 18°C then 3-7 days later at 19°C the viral load increased. First mortalities observed 3 days later with viral load increasing over the next 10 days corresponding to the peak of mortalities. Viral load then decreased and the mortalities stopped
        - Consistent results over the 2 years of the project.
    - Still to be published but possibly healthy carriers (in natural spat and not from hatcheries).
- Research Projects
    - EU Commission funding investigating *C. gigas* and mussels – 3 yr project which began in 2011.
        - Presence of pathogens in different states (e.g. OsHV-1 throughout Europe).

- Also investigating *Vibrio*, *Marteilia* and *Nocardia crassostrea*
- Same techniques will be used in the different countries.
- Monitor environmental conditions as well.

- Try to detect pathogens in the environment and survival outside the host by detection of viral RNA as an indication of replication.
- Investigating the relationship between the pathogen and the host, the Immune response (identified a new immune gene) and virulence (looking at virulence factors in bacteria).
- Define effective treatments (e.g. UV irradiation could be a good thing but what about turbidity?).

- EU Commission funding to gain a better understanding if OsHV-1 at the molecular level including a retrospective study of archival material
- IFREMER Post-Doctoral Fellowship
  - Selection project to test progeny through infection trials (OsHV-1 and *Vibrio*) to produce material to identify what the differences between lines are.
- PhD students investigating expression of viral genes as 70% of OsHV-1 genes correspond to nothing on Genbank. Investigate susceptibility to OsHV-1 and contact with pesticides.

Parasitology

Use standard operating procedures available on through the internet (http://www.eurl-mollusc.eu/). Had a discussion about Standard Operating Procedures:

- *Bonamia ostreae* and *B. exitiosa*
  - Project to sequence a lot of the *B. ostreae* genome.
  - More difficult for *B. exitiosa* as a lot of parasites are required and they are difficult to culture to large numbers.
  - Good 18S data and investigating ITS region sequences.
  - In flat oysters, larvae can be infected. And there are resistant oysters.
  - David Stone (CEFAS) has developed a TaqMan assay to discriminate between both.
  - Sybr Green assay to quantify *B. ostreae*, but it is better for research than diagnostic testing.
- *Marteilia* sp.
  - Steve Feist has funding for sequencing as it was recently found in English mussels.
  - 2 species indistinguishable by 18S and ITS only slightly better.
    - Mussel strain and flat oyster strain.
  - *M. refringens* infects both flat oysters and mussels.
    - Difficult to grow so are considering laser microdissection.
  - Conventional PCR but need to clone and screen up to 20 clones to detect co-infections and there is a multiplex qPCR to detect both.
  - Histology is a good tool as the infection is quite focal (can be missed by PCR due to the focal nature of the infection).
- *Perkinsus* sp.
  - Screen using thioglycolate medium
  - Detect 2 species with co-infections in some areas.

General

- Gave a presentation to staff titled “AAHL Fish Diseases Laboratory (AFDL)”.
- Went on a tour of the Aquatic Animal Facility and a tour of oyster farms.
- Interested in material from Australia (comment made that it is very difficult to get material from Australia):
  - Material infected with *Bonamia* sp.. Some work was done in Tasmania and it is important to incorporate into phylogenic analyses. If there is material in Australia, laser microdissection could be used followed by PCR and sequence analysis
  - Material from animals suffering from Winter Mortality. If *B. roughleyi* is considered the cause, it could actually be *B. exitiosa*.

Request from AFDL:

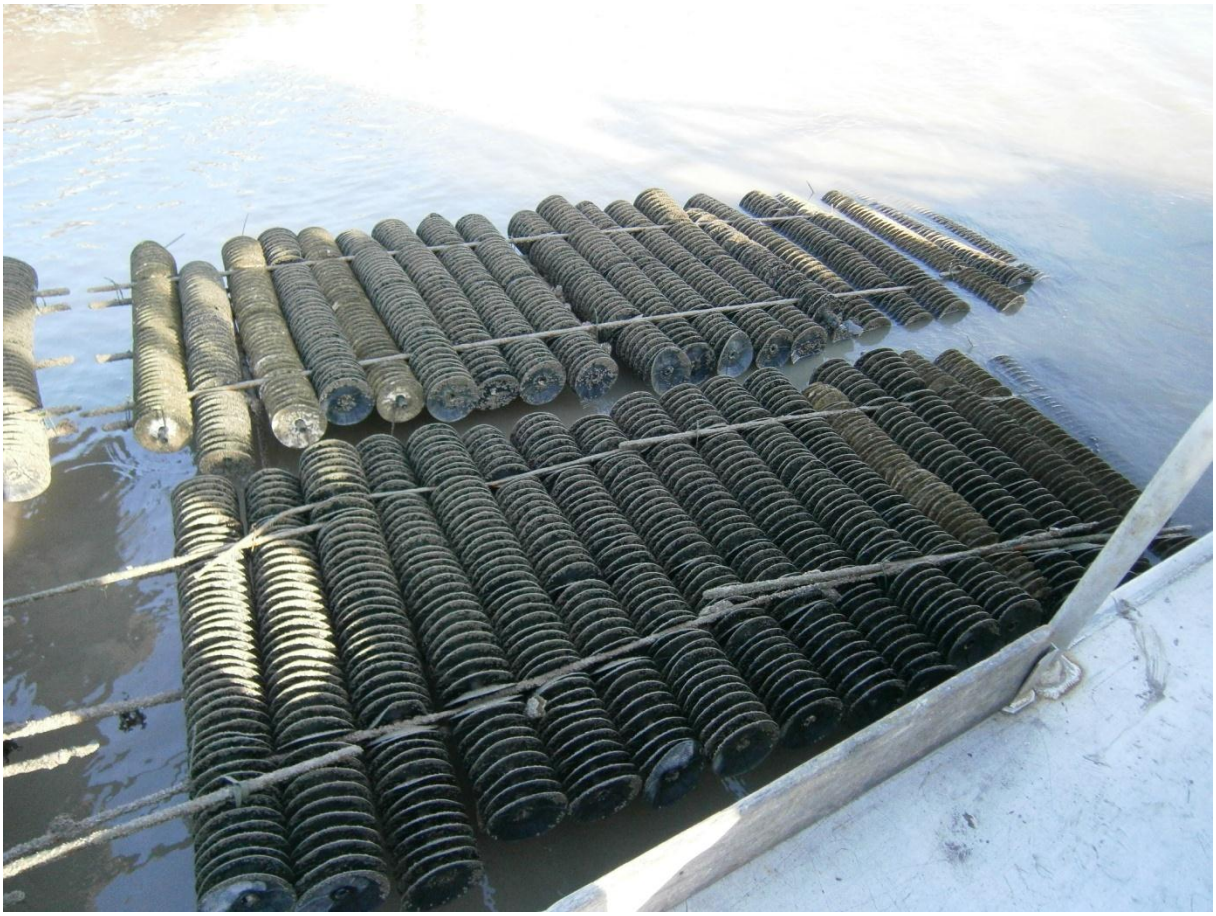
ACTION: AFDL to investigate whether we have an appropriate TEM fixation protocol for copepods (primarily for *Marteilia* investigations.)
ACTION: AFDL to develop MTA for transfer of LNA OsHV-1µVar-specific qPCR protocol transfer.

Deliveries to AFDL:
ACTION: AFDL to investigate availability of material infected with Bonamia sp. in Tasmania for laser microdissection, PCR and sequence analysis.
ACTION: AFDL to investigate availability of material suffering from Winter Mortality to determine if *B. roughleyi* or *B. exitiosa* is present.

Appendix B

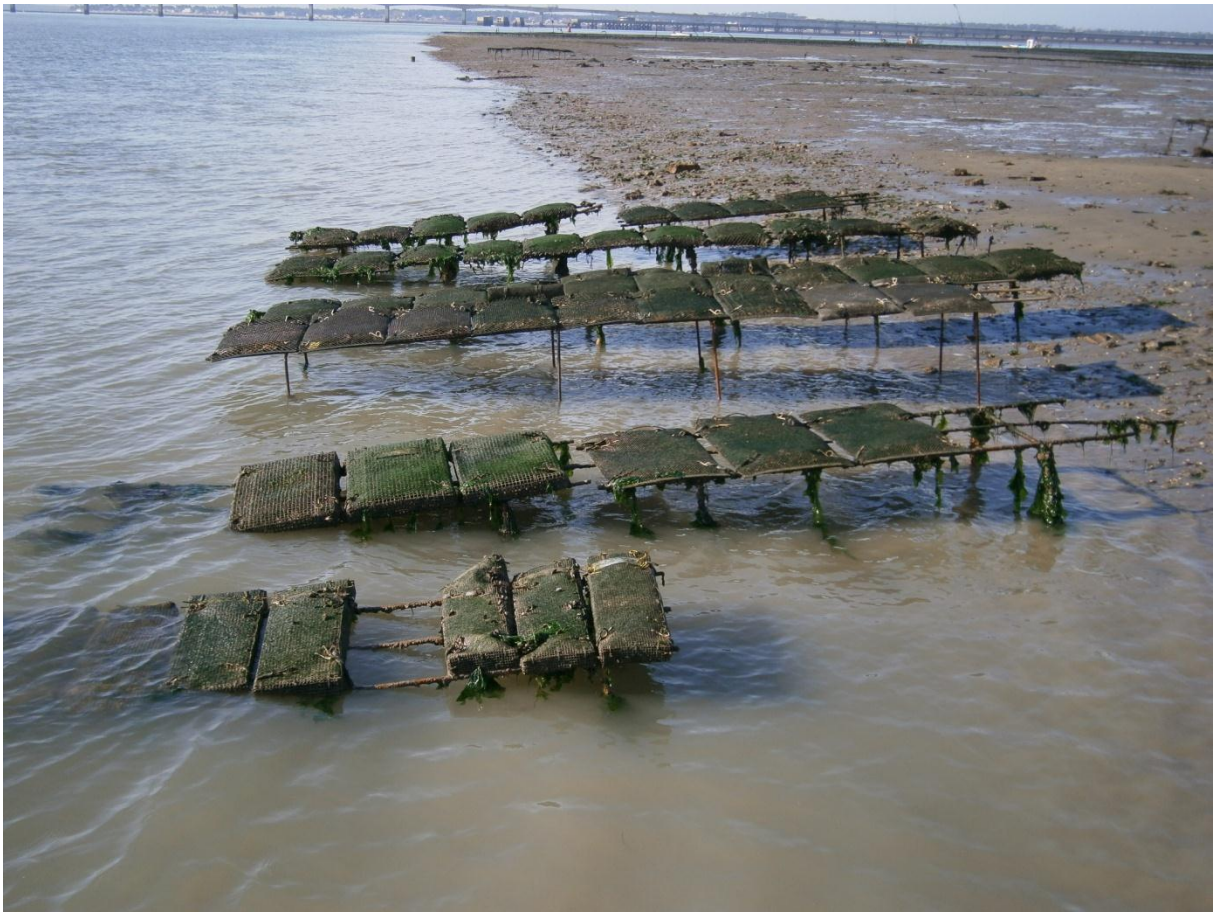
Attached are some photos of the sentinel oysters used by IREMER to monitor oyster health and the extensive oyster beds around La Tremblade, France.

Wild spat collectors, La Tremblade, France.

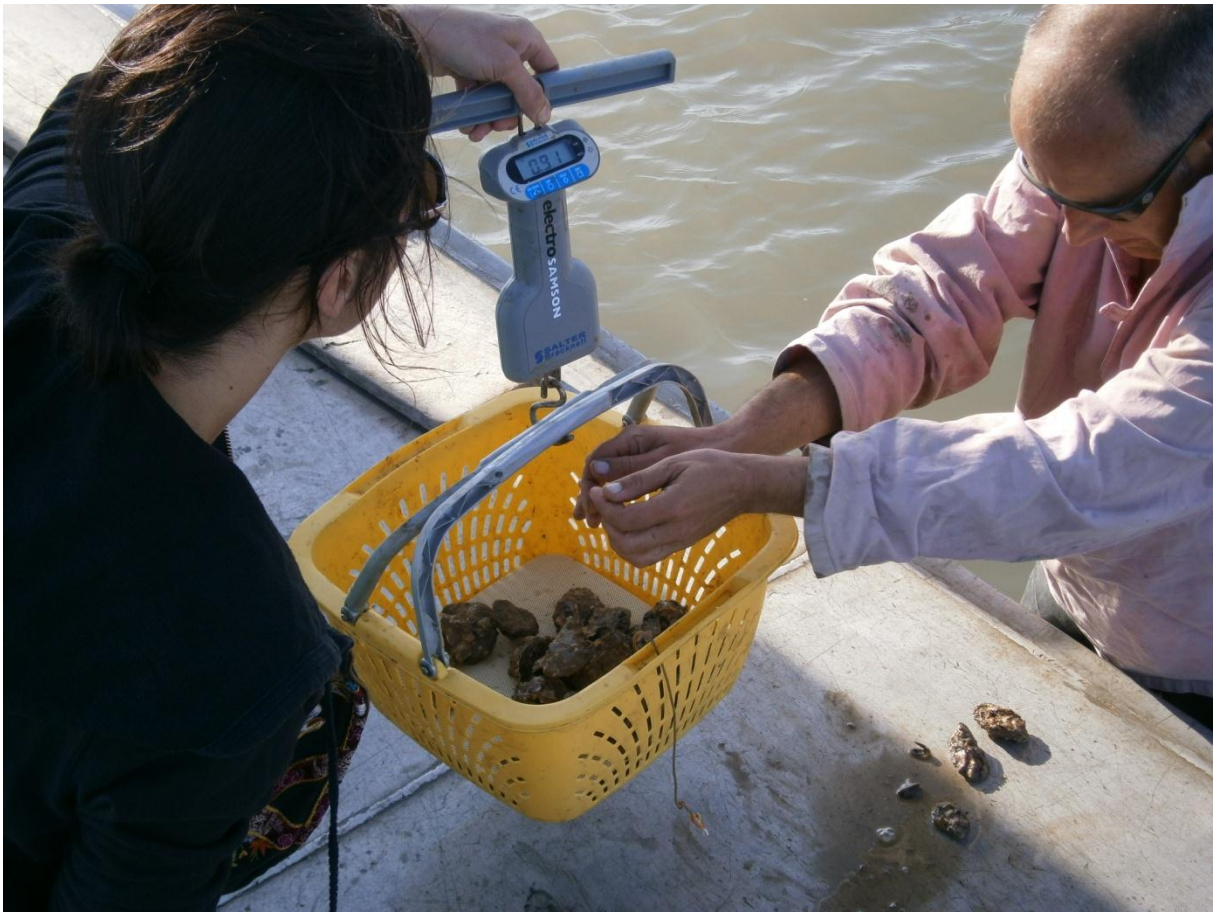


Wild spat collectors, La Tremblade, France.

Wild spat collectors, La Tremblade, France.



Sentinel oyster bags (foreground) amongst farmed oyster bags (background), La Tremblade, France.



Weighing sentinel oysters, La Tremblade, France.

IFREMER staff collecting oysters for health and genetic analysis, La Tremblade, France.

IFREMER Environmental monitoring equipment, La Tremblade, France.

Australian Government

Fisheries Research and Development Corporation