Bivalife

Improving European mollusc aquaculture: disease detection and management

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General and specific objectives of the project

The two core objectives of the Bivalife project were (i) to provide innovative knowledge on pathogens infecting oysters and mussels and (ii) to develop practical approaches for the control of infectious diseases and resulting mortality outbreaks these pathogens induce.

The project addressed the major issue identified by the European Commission on the risk of pathogen transfer and infectious disease outbreak occurrence among marine bivalve molluscs, through the specific objectives:

- *(i) improve, validate and transfer existing methods for detection and identification of Pacific oyster and mussel pathogens;*
- *(ii) search and characterise Pacific oyster and mussel pathogens in relation to the presence or absence of mortality;*
- *(iii) assess the relationship between the presence of Pacific oyster and mussel pathogens and environmental risk factors in the development of mortality or disease;*
- *(iv) investigate mechanisms allowing Pacific oyster and mussel pathogens to survive outside the host;*
- *(v) identify pathogen intrinsic virulence factors and effects on host defence mechanisms;*
- *(vi) develop methods and recommendations for pathogen control and eradication in Europe.*

The project focused on three mollusc species: the Pacific oyster, *Crassostrea gigas* and two mussel species *Mytilus edulis* and *M. galloprovincialis*, the most important species in terms of European production.

Bivalife validated and updated efficient methods for identification and detection of relevant pathogens that affect oysters and/or mussels. The selected pathogens were the virus OsHV-1, *Vibrio* species including *V. splendidus* and *V. aestuarianus*, as well as the parasite *Marteilia refringens* and the bacterium *Nocardia crassostreae*. OsHV-1 and *Vibrio* species have been detected in association with mortality outbreaks affecting the Pacific oyster in Europe. *Marteilia refringens* is reported being associated with mortality of mussels in some cases while it does not seem to be able to develop in *C. gigas*. *Nocardia crassostreae* infects the Pacific oyster and was recently reported in The Netherlands in association with mortality of Pacific oysters.

The project studied the relationship between presence of a given pathogen and its actual implication in observed mortality through the development of a database related to site and pathogen characterization and the identification of any potential determinants related to disease manifestation. Pathogens are often necessary but not sufficient cause of disease, which is an outcome of interaction between host pathogen and environment. The final objective is the use of a common methodological approach shared by Member States to identify and quantify potential risk related to the presence of relevant pathogenic agents in Europe.

Considering that information about Pacific oyster and mussel pathogens outside their hosts is lacking, the project explored the mechanisms that allow pathogens to resist outside the host based on

(i) defining criteria and methods to measure pathogen survival,
(ii) searching for infectious (viable) pathogens in different compartments in the field,
(iii) and monitoring survival of targeted pathogens in laboratory assays.

Bivalife also delivered new information on the pathogenicity of relevant pathogens through comparing contrasted biological material (infected versus non-infected individuals, resistant versus susceptible groups, susceptible versus non-susceptible species) originating from experimental trials.

Bivalife also developed efficient methods and techniques aimed at eradicating pathogens in controlled culture conditions. Practical methods and general recommendations drawn from knowledge acquired during the course of the project.

State of the art

Aquaculture is a dynamic activity in Europe where the growth rate of the sector has been around 4% during the last decade. Europe was the third largest contributor with around 4.2% of world aquaculture production. European aquaculture is characterised by a focus on a limited number of species including oysters and mussels being raised at an industrial level. The marine bivalve industry has grown to be very important for many regions of the European Union contributing substantially to social and economic activity in the coastal zones. The yearly European oyster production is the 126 000 tonnes, France being the leading Member State (80 000 tonnes/year). The mussels *Mytilus edulis* and *M. galloprovincialis* are the bivalve species with the highest production output in Europe (491 000 tonnes/year). Spain is the second world producer and the top European producer with an output of 300 000 tonnes/year (*M. galloprovincialis*).

For some years, the European shellfish industry recognised a rather slow growth, compared to other aquaculture activities. European shellfish farming, relying directly upon natural environments and feeding resources, is facing various risks and limiting factors which include primarily, as well as in every farmed species, infectious diseases. Historically, infectious diseases have seriously affected the marine bivalve industry in Europe in various occasions. In the early 1970's, the French oyster industry in particular faced a severe blow when irido-like virus infections led to the almost total extermination of the Portuguese oyster, *Crassostrea angulata*, in French and European Atlantic waters. In the late 1960's and 1970's, devasting effects on the European flat oyster, *Ostrea edulis*, production were reported related to two protozoan diseases, bonamiosis and marteilisois. Since 2008 massive mortality outbreaks affecting *C. gigas* oysters are reported in different Member States. These are attributed to a combination of adverse environmental factors together with the presence of ostreid herpesvirus type 1 (OsHV-1) and the presence of bacteria belonging to the genus *Vibrio*.

The control of farmed shellfish health is one of the key elements to maintain the competitiveness and to increase the sustainability of the industry as a whole. The Council Directive 2006/88/EC "on animal health requirements for aquaculture animals and products thereof and on the prevention and control of certain diseases in aquatic animals", underlines the necessity for the development of the aquaculture sector in the European Union to increase the awareness and preparedness of the competent authorities and aquaculture production business operators with respect to the prevention, control and eradication of aquatic animal diseases.

The project focused on three mollusc species, namely the Pacific oyster *Crassostrea gigas* and two mussel species *Mytilus edulis* and *M. galloprovincialis*, the most important species in terms of European production. The pathogens the project selected are mainly pathogens associated with mortality outbreaks in Pacific oysters, *C. gigas*. The targeted pathogens were the virus OsHV-1, *Vibrio* species including *V. splendidus* and *V. aestuarianus*, as well as the parasite *Marteilia refringens* and the bacterium *Nocardia crassostreae.* Epizootic diseases, like those that have devastated the oyster culture industry in Europe have not been encountered by the mussel culture industry. Mussels effectively appear less sensitive to a large range of well known infectious diseases. Consequently, mortality outbreaks have more rarely been reported in Europe in the mussel species, *Mytilus galloprovincialis* and *M. edulis*.

(i) Key drivers to improve disease diagnosis: harmonisation of diagnosis techniques for oyster and mussel pathogens through method transfers and validation (inter-laboratory assays)

Effective health management measures are based on diagnosis procedures such as: (i) a systematic approach to disease monitoring including the early detection of early infected animals, (ii) the use of sensitive and rapid diagnosis techniques, (iii) the collection of diagnosis data, and (iv) the establishement of diagnositic laboratories using standardised procedures with samples collected according to defined rules. Mollusc health surveillance/control programmes require both adequate legislation and standardised procedures for pathogen diagnosis. Valid laboratory results are essential for diagnosis, surveillance and trade.Molecular techniques for diagnosing bivalve infectious agents have been developed during the last decade. Real-time Polymerase Chain Reaction (PCR) techniques are already used for routine detection of pathogens infecting oysters and mussels including OsHV-1, *V. splendidus*, *V. aestuarianus*, *N. crassostreae* and *M. refringens*. Although they are now moving from a developmental phase in specialized laboratories for research purposes to routine application, molecular tools need formal validation. These diagnostic tools are not standardized and differences exist in the quality of reagents quality and preparation, in controls, as well as in the interpretation of results. Obviously, the use of a "standardized" diagnostic tool for routine analysis should allow the implementation of a calibrated and controlled process in laboratories. It is recognised that for such purpose, studies conducted in parallel with the same isolates in several laboratories would be necessary.

The transfer and the validation of already existing molecular tools for notifiable and non-notifiable pathogens (*M. refringens*, OsHV-1, *V. splendidus*, *V. aestuarianus* and *N. crassostreae*) were performed during the course of the project based on interlaboratory comparison assays involving several laboratories.

(ii) Control of animal movements and transfers

Specific objectives of Bivalife were (i) determination of the geographical distribution of certain known oyster and mussel pathogens in Europe based on the use of validated techniques, (ii) identification of putative emerging pathogens in Europe related to mortality outbreaks , (iii) production of a data base related to site and pathogen detection, (iv) dentification of relevant potential determinants related to disease manifestation and (v) development of practical methods and general recommendations infectious disease control drawn from knowledge acquired during the course of the project

Considering that the trade of live molluscs is a major cause of epizootics, restriction of animal movements is a basic way to control infectious diseases. Transfer regulations at international and European levels have been developed in order to avoid the introduction of animals from an enzootic area to a pathogen-free area. Minimum measures for the control of certain diseases affecting bivalve molluscs were established by the Council Directive 2006/88/EC. This Directive lays down harmonised animal health provisions for the placing of aquaculture animals and products thereof on the market, including specific provisions applicable to species susceptible to certain exotic and non-exotic diseases (notifiable). However, in Pacific oysters infectious diseases are frequently reported in larvae and spat and are involved in mortality outbreaks. These diseases are not currently notifiable diseases subjected to specific control measures under EU or WOAH (World Organisation for Animal Health) legislation. Although, pathogens associated with these mortalities, mainly virus and bacteria, generate important economic losses, their impact may be misunderstood or seriously underestimated.

During the course of Bivalife, relevant - mainly non-notifiable - pathogens (OsHV-1, *V. splendidus*, *V. aestuarianus*, *N. crassostreae* and *M. refringens*) were searched in samples collected from France, Ireland, Italy, Spain and The Netherlands. Information was obtained about the presence/absence of these pathogens in the main oyster and mussel producing Members states using previously validated techniques.

(iii) Understanding complex interactions between animal, pathogen and environment: development of tools to measure pathogen survival in various environments and detection of viable/infective relevant pathogens in different compartments (animals, water, sediment)

Reducing the impact of pathogens is likely to rely on knowledge of their biology, their life cycle and the mechanisms that allow them to survive outside the host. Marine bivalves including oysters and mussels are typically reared in estuarine environments frequently subjected to fluctuations of environmental factors such as temperature, salinity and pollution. Outside the host, the environment of pathogens is thus composed of many different types of factors. The pressure of different stressors, including variations of salinity and temperature, may lead to potentially irreversible alterations of pathogens and host metabolism.

Viable/infective relevant pathogens were searched in different types of samples (water, sediment, zooplankton, phytoplankton and macrofauna) collected from France, Ireland, Italy, Spain and the Netherlands. In order to complete information about the detection and the viable/non viable status of pathogens in different compartments in the field, the effects of physico-chemical parameter (e.g.temperature, salinity, UV) variations on pathogen survival were monitored based on laboratory trials.

(iv) Understanding immunity of aquacultured mollusc bivalve species : identification of relevant host defense mechanisms in contrasted biological material and identification of virulence factors in oyster and mussel pathogens

The development of an infectious disease results from an imbalance between the host and the pathogen due to different factors including internal factors of both protagonists (virulence of the pathogen, susceptibility of the host). During the last decade, various molecular techniques including mRNA differential display, Suppressive Subtractive Hybridization (SSH) and Expressed Sequence Tag (EST) libraries have been applied in bivalves in order to identify immune genes and to assess the level of gene expression. About 50,000 ESTs from bivalves are now available in various databases. Such data provides the basis for understanding the role of the innate immune system against various pathogens and may be useful in defining selection markers for improved resistance to infections.

In order to better understand pathogenesis and pathogen resistance, the innate immune response was studied in oysters in oysters and mussels from experimental trials. Contrasted biological material (infected versus non-infected individuals, resistant versus susceptible individuals (selected oyster and mussel families), susceptible versus non-susceptible species) was compared targeting genes of interest.

(vi) Developing and adopting new pathogen inactivation methods

Although hatchery technology is constantly being improved, significant production problems including infectious diseases must be solved before hatcheries become a major supplier of juveniles for the industry. Mollusc hatcheries are unique in having closed facilities that enhance disease control capabilities. Hatcheries may produce checked progeny, namely specific pathogen free, and reduce circulation of infected stocks. Moreover, they may have a pivotal role in the development and implementation of health management strategies based on improved resistance to infectious diseases.

The involvement of hatcheries in the control of infectious diseases would offer producers the possibility to contribute proactively in the early detection of possible adverse conditions for bivalve growth and survival before a major problem occurs, contributing to the minimisation of the effects of disease outbreak. Adequate control of larvae and spat from hatcheries in controlled facilities may avoid costly epizooties. Moreover, the availability of efficient tests for relevant pathogen detection may facilitate screening of brood stock, spat and larvae before commercial transactions and therefore constitute a guarantee of product quality for oyster producers.

Creating a controlled growth environment during the inland growth cycle of the shellfish by reducing the presence of pathogens through the use of a disinfection barrier is one of the main objectives for sustainable aquaculture.The disinfection barrier is a state of the art disinfection technology based on Ultra-Violet (UV) light. UV light has the ability to inactivate all microorganisms without creating collateral damage in the environment. The inactivation level is defined by the amount of germicidal UV light that each microbe absorbs during the exposure time in the UV system. The necessary dose level is "microbe dependent" - meaning that each microbe has its characteristic inactivation dose. Relying on advanced fiber optic principles, the Hydro Optic Disinfection^{TM} (HOD) technology "traps" light photons to provide uniform UV dose distribution within the reactor and eliminate low dose escape routes. The HOD technology has proven field capabilities in the aquaculture farming industry such as in the salmon egg production, hatcheries and smolts, as weel as in cod farms, sea bass and sea bream industries and in shrimp industries.

Bivalife developed and promoted (i) diagnosis efficacy (ii) integrated bio-security measures and (iii) efficient methods and techniques aimed at eradicating pathogens that meet the time constraints experienced under hatching conditions, and are of the utmost importance for efficient control of diseases in bivalve hatcheries. The project implemented the HOD technology to block each potential contamination source at the pre-defined inactivation level (UV dose). This activity focused on defining the UV germicidal dose to inactivate the predefined pathogens, selectively inactivate pathogens in the live feed without affecting the live feed itself.

Bivalife promoted a re-inforced collaboration among those stakeholders involved in mollusc health issues:

(i) Research institutes that developed relevant knowledge on oyster and mussel pathogens and interactions with their hosts;
(ii) European national laboratories involved in mollusc disease diagnosis and control, which aim is to support farmers in the implementation of control measures and bio-safety procedures;
(iii) a high tech SME that bring its extensive knowledge, methodology and technology in the field of new disinfection capabilities;
(iv) and the EU shellfish producers, who are in the front-line and face the negative impact of pathogens and diseases on their livestocks, will be beneficiary of the results through the participation of their representatives to Bivalife ’s exploration and development meetings.

WP1. Project management during the period

During the course of the project, different administrative and financial documents have been finalized, including the signature of the Grant Agreement.

The coordinator as the representative of the Consortium interacted with the European Commission Project Officer on different aspects including the amendment of the Consortium Agreement in order to integrate an additional participant. A new beneficiary to the consortium, the European Molluscs' Producers Association (EMPA) was integrated as "beneficiary no.12. The EMPA gathers since 1999 the 12 professional national or regional organizations representative of 9 European countries: France, Spain, Ireland, the United Kingdom, Greece, Italy, the Netherlands, Denmark and Germany. The involvement of producers' representatives (EMPA) in the consortium requested by the Commission services needed necessary quite long discussions in order to determine with the EMPA their potential involvement and with all beneficiaries possible allocation of a reasonable budget for EMPA tasks without changing the total EU requested contribution.

Acting as coordinator for the Grant Agreement Number 266157 (Bivalife) on the behalf of all beneficiaries, Ifremer ("beneficiary no.1") also submitted a request to add a third party, the Galway-Mayo Institute of Technology (GMIT) linked to the Marine Institute (Participant 6). This is a pre-existing relationship which involves research collaboration as well as staff transfer through adjunct lectureships i.e. the relationship involves academic, scientific and teaching collaboration. An official Memorandum of Understanding (MOU) is in place and has been signed by the Chief Executive of the Marine Institute and the President of GMIT. For the project, together, the MI and GMIT were collaborating on the collection of oyster samples, the collection of accurate mortality data and the collection of environmental data.

Four meetings took placed during the course of the meeting. To further organize the consortium and the schedule of the project, the kick-off meeting was held in Issy les Moulineaux (France) at the Ifremer headquarters building near Paris on the 7th and 8th of April 2011. In total, 27 participants from the 12 participants attended the meeting. A second meeting was organized in June 2012 at the same place (Issy les Moulineaux). A representative of DG RTD has been invited at both meetings and the Bivalife scientific officer joined the second meeting held in June 2012. Two additional meetings took place in 2013. The first one was held in Issy les Moulineaux (France) on the 3rd and 4th of April 2013. A second meeting has been organized in November 2013 (28th and 29Th November) in Padova (Italy).

WP2. Detection and identification of relevant pathogens and associated risk factors

WP2 - T1: Transfer and validation of existing diagnostic tools (including inter laboratory validation)

The transfer and the validation of techniques already existing for the detection of relevant bivalve pathogens including OsHV-1, *Vibrio* species including *Vibrio splendidus*, *V. aestuarianus*, *Nocardia crassostreae* and *Marteilia refringens* was carried out. Thus, participants used the same techniques to search relevant pathogens in samples collected in their own country. In order to transfer diagnostic tools previously developed for OsHV-1, *Vibrio splendidus, V. aestuarianus and Nocardia crassostreae*, interlaboratory comparison assays werer carried out. A collection of reference material (positive controls) was constituted that each participant could share in this project. The reference material for the highlighted pathogens was sent to the participants involved in the interlaboratory comparison assays.

WP2 - T2: Search and identification of relevant pathogens

Some characteristic sites (detection or absence of mortality events in previous years, presence of Pacific oysters and mussels) have been selected. Mussel and Pacific cupped oyster sites known to be impacted or not by mortality outbreaks according to pre-existing data from previous epidemiological surveys have been be identified. Field sampling included sites where disease outbreaks are known to occur (positive control) and where they are not known to occur.

The sampling guide compiled by Participant 9 and Participant 5 was completed in modified form in April 2012 and was used by all partners committed to field sampling in 2011, 2012 and 2013. The modified sampling guide was included in the previous project report as an annex.

France (Participant 1)

Three (3) sites have been identified along the French coast: (1) Thau lagoon, (2) Marennes Oléron (Atlantic coast) and (3) bay of Brest (Brittany). Oyster spat from a same batch and mussels (except for Brest) were monitored and sampled in 2011 and 2012. Other species were also collected for monitoring (pectinids, barnacles, …) as well as sediment and seawater samples. As planned, no sample collection was carried out on the 3 French sites in 2013.

In 2011, no sample was collected from the Thau lagoon as mortality events occurred quite early and main oysters died quickly after deployment in the field. On the Marennes Oléron site, Pacific oyster cumulative mortality reached 75% with a peak observed in May. On the Bay of Brest site, oyster cumulative mortality reached 70% with a peak observed between May and June. During those mortality events, OsHV-1 DNA was detected in oysters with viral DNA content reaching 10^6 - 10^7 copies/mg of tissue. Strains belonging to *V. splendidus* clade were isolated before, during and after mortalities without any correlation with the observed mortalities.

On the opposite, *V. aestuarianus* was only detected during a second oyster mortality event that occurred in the Bay of Brest in December 2011. No mortality was recorded on mussels, but OsHV-1 DNA was detected with a high frequency but a low copy number at the end of the summer, after the mortality events.

OsHV-1 detection trends seen during a given year allowed a reliable prediction of mortality onset as this coincided with water temperature increases. Mortalities peaked in May and June (depending on water temperature increase). Annually, mortalities peaked approximately during the peak of virus detection. *Vibrio splendidus* was also detected frequently and in high prevalences (before and during mortalities).

Other species were also collected for monitoring as well as sediment and seawater samples. Although it was not possible to detect OsHV-1 DNA in all sediment samples using real-time PCR, several isopod crustacean (*Sphaeroma sp.*) and seawater samples tested positive. Large amounts of OsHV-1 DNA were detected in some crustacean samples in Thau lagoon in May 2012. Moreover, viral DNA was also detected in a single sample of *Balanus sp.* in Thau lagoon in June 2012.

Spain (Participant 2 and Participant 5)

Spanish Mediterranean sites (Participant 5)

Two relevant Spanish Mediterranean sites were sampled, Alfacs and Fangar bays (IRTA, Participant 5) of the Ebro River delta (Tarragona, Spain).

OsHV-1 detection trends seen during a given year allowed a reliable prediction of mortality onset as this coincided with water temperatures rising above 16 °C. Mortalities peaked in April or early May. Long term trends (3 years) suggest there has been an overall reduction in the detection of OsHV-1. Annually, mortalities peaked approximately during the peak of virus detection. A second peak of mortality, although smaller, was seen in autumn during the transition from warmer to colder temperatures but in older *C. gigas*. When looking at bivalve compartments (i.e. bivalves other than *C. gigas*) for OsHV-1, real-time PCR positive individuals of *M. galloprovincialis* with low virus DNA amounts were detected coinciding with high virus in *C. gigas* (i.e. large number of positives detected in *C. gigas*) in Alfacs Bay, where there are more mussels and less Pacific oysters. Persitence of the virus in positive *M. galloprovincialis* remains to be determined. It was not possible to detect OsHV-1 in other compartments (sediment, water and zooplankton) in Alfacs Bay using the techniques employed.

Vibrio spp. were found at consistently high levels in most samples (10^{3-4} CFU) and *V. splendidus* was confirmed from most of them. Prevalence of *V. splendidus* decreased to 0% in the final samples tested in 2012 and the decrease generally coincided with rising water temperatures. The significance of this in relation to oyster mortalities requires further study.

Vibrio aestuarianus was detected only once during 3 years of sampling. Perhaps for the Spanish Mediterranean coast, *V. aestuarianus* does not play a role in *C. gigas* mortality outbreaks due to its temperature preferences.

Marteilia sp. was not observed in *M. galloprovincialis* in 2011 and 2012. When looking at bivalve compartments for *Marteilia* sp., a *Marteilia* sp. parasite was observed in cockle *C. edule* sampled in 2013 (up to 100%). The *Marteilia* sp. parasite observed in cockles has been described as *Marteilia cochillia* nov. sp. *Marteilia cochillia* targets the digestive gland of the *C. edule* cockle and has been observed associated with high mortality rates. No *Marteilia* sp. presence was detected in environmental compartments (sediment, column water and zooplankton) sampled in the same location where mussels were collected.

Sites in Galicia (Participant 2)

Two (2) different sampling points were selected in Galicia: Alcabre (Pontevedra) and Sor River (A Coruña) (Figure 9) (CSIC, Participant 2).

During the sampling period, no mortalities were found in the bivalves from the two selected localities. Only one sample was lightly positive for herpesvirus in mussel and Nocardia was never detected. However, the two bacterial species (*Vibrio aestuarianus* and *V. splendidus*) were detected by real-time PCR in the two locations associated to the different samples (both bivalves and environmental samples). Interestingly, most of the detections were done in mussels and not in oysters.

Experimental infections of mussels were conducted with the field *Vibrio* isolates. Intramuscular injections revealed that *V. splendidus* and *V. aestuarianus* were only moderately pathogenic in mussels compared with previous data on oysters. *Vibrio* populations were influenced by changes in water temperature and salinity and also that sediment was an important natural reservoir for bacteria. Athough the pathogenicity of the *Vibrio* strains isolated from the wild was low for mussels, their potential risk for other bivalves cannot be ignored.

The Netherlands (Participant 4)

Within the Bivalife project the epidemiology of *N. crassostreae* in Pacific oysters and its environment was studied. Lake Grevelingen, an artificially enclosed seawater lake in the Netherlands and culture area for Pacific oysters and flat oysters, has been selected as study site for *N. crassostreae*. During the period of sample collection the estimated mortalities in the Pacific oyster population at the sampled locations ranged from 0% to 5.9%. Although 5.9% mortality is above the average mortality of 1.1% for this area it is still considered within the range of back ground mortality for Pacific oysters in the area. Hence, no elevated mortalities were observed in the course of the Bivalife project.

Despite the fact that no elevated mortalities were observed *N. crassostreae* was present in the Pacific oyster population at low frequency. The results from the Bivalife study showed that, while no difference in susceptibility can be made among adult Pacific oysters from different size classes, adult oysters were more susceptible to infection with *N. crassostreae* compared to juvenile oysters. Although a seasonal pattern could be discerned in the prevalence of *N. crassostreae* in the Pacific oysters from Lake Grevelingen there were strong fluctuations in prevalence between the different years. Presumably some environmental factors (e.g. summer temperature) play a significant role in the susceptibility of the oysters for *N. crassostreae*.

As a range of environmental parameters was collected in the cause of the project a number of these parameters could be assessed for their involvement in the epidemiology of *N. crassostreae*. In general the observations from this study support the idea that *N. crassostreae* is an opportunistic bacterium which can infect oysters under suboptimal environmental conditions for the oyster.

Ireland (Participant 3 and Participant 6)

The sites were selected on the basis that they had been impacted by the OsHV-1 (variant µVar) in 2009 and 2010 and gave a fair representation of the affected sites within Ireland. The sites selected are Donegal Bay, Carlingford Lough, Dungarvan Bay

Mortalities were minimal to absent on all three farms from 2011 to 2012. Dungarvan Shellfish Ltd. reported no production losses of *C. gigas* to mortality events during 2011 to 2012, while Carlingford Oyster Company had experienced approximately 20% mortality during the mid/late summer of 2012. Mortality at Donegal Oceandeep Oysters was more complicated. Heavy mortalities in excess of 60% were reported in July of 2012 and attributed to a bloom of the dinoflagellate *Karenia mikimotoi*. These heavy mortalities also extended to some of the associated invertebrate community. Several species of polychaetes typically found at this site were absent from the area following the bloom event. In 2013, mortalities were minimal (<0.5%) to absent in Carlingford during the field trial period.

At Carlingford site, in 2011 and 2012 mortality did not materialise until late July/early August. Profile showed peak of OsHV-1 positives at the time of active mortality and levels dropping as we move into winter. At Dungarvan site, slight mortality (15%) was observed in *C. gigas* seed batches in August 2012, however, no mortality was reported in the Bivalife batch. 5-10% mortality was reported in seed stocks overall for the 2012 season. A higher detection of OsHV-1 was reported shortly after the seed arrived in the bay. This differs from the previous years profile, where stock was negative for OsHV-1 detection when received and appeared to stay negative over the sampling period with only a few real-time PCR positives detected with high CT values.

At Donegal Bay site, mortality in all stocks was reported from early July 2012 due to the presence of an algal bloom (*Karenia mikimoti*) - estimated counts suggested there was 30-40% mortality in Bivalife batch, this was later confirmed to be 70-80% mortality following grading of this stock in spring 2013. Results would suggest the seed stock was OsHV-1 positive when it arrived as 131/150 individuals were found OsHV-1 positive. The picture in Donegal Bay was further complicated by the presence of a *Karenia mikimotoi* bloom which resulted in significant losses from North County Donegal to county Mayo in both OsHV-1 infected and non-infected bays.

The results show frequent detection of *V. aestuarianus* both in sites with and without mortality although in the sites where there was no mortality at the time of sampling the lowest CT value observed was 29.

In 2013, Participant 6 (MI) implemented a new TaqMan real time PCR assay for *V. aestuarianus*. The method has undergone validation in house and was used during the summer of 2013 to test samples of *C. gigas* collected during mortality outbreaks for the presence of *V. aesturianus*. Samples in which the pattern of mortality was not typical of that expected for OsHV-1 were targeted. Prior to 2013, the methodology used for screening for *V. aesturianus* had involved culture followed by PCR on suspect isolates. In 2013, Participant 6 began screening DNA extracts as it seems that culturing the bacteria is not always successful and hence the presence of *V. aesturianus* may have been underestimated prior to the date. In samples collected in 2013, *V. aestuarianus* was frequently detected at varying levels but no pathology was associated with the findings. As a result a second study was undertaken to test archive material collected between 2008 and 2012 for the presence of *V. aesturianus*. The archive material composed samples taken from both areas experiencing mortality and those where no mortality occurred as well as from areas both positive and negative for OsHV-1. The aim of the study was to gain a better understanding of the prevalence of *V. aestuarianus* in Ireland and its role in the mortalities observed.

Detection of target pathogens in invertebrate, plankton, biofilm and sediment samples (Participant 3)

Sampling of the invertebrate community associated with commercial culture of *Crassostrea gigas* began in the autumn of 2011 to 2012, with subsequent sampling over the summer of 2013. Invertebrates from two control non-culture sites, Ardmore, Co. Waterford and Bullen's Bay, Co. Cork, were also screened for OsHV-1 detection in 2013. Ardmore is 25km from Dungarvan, Co. Waterford, an OsHV-1 (variant µVar) endemic site and Bullen's Bay is 10 Km from Oysterhaven, Co. Cork, an OsHV-1 (variant µVar) endemic site.

A total of 45 biofilm samples, 34 plankton samples and 45 sediment samples were collected from all three sites during 2011 to 2012. A total of 30 different species of invertebrates were collected from the three sites in 2011 to 2012 with an overall total of 1062 individual samples being sampled, 538 from Carlingford Lough, 251 from Dungarvan and 273 from Donegal. The highest species diversity was found in Carlingford (n=27) followed by Dungarvan (n=21), while considerably fewer macroinvertebrate species were observed at the Donegal (n=16) location. In 2013, a total of 60 biofilm samples, 45 plankton samples and 45 sediment samples were collected from Carlingford Lough. A total of 22 different species comprising of 605 invertebrate samples including 300 *C. gigas* were sampled. The largest number of invertebrates collected was in July 2013 (n=97), which also had the highest species diversity (n=26), while September had the least number of specimens being collected (n=102) and had the lowest species diversity (n=54). A total of 36 invertebrates consisting of 4 groups and 8 species were collected from the control site at Ardmore and 68 invertebrates consisting of 2 groups and 8 species from Bullen's Bay were screened.

Positive samples for OsHV-1 were relatively limited. A total of 2% (23/1062) of samples tested were observed at the Carlingford Oyster Company site (CF, n=20). The three additional samples originated from Donegal Ocean Deep Oysters (DL), while no positive samples were observed from Dungarvan Shellfish Ltd. (DG). Nearly half the samples testing positive (n=10) originated from water column samples. The remaining samples types testing positive were *C. gigas* (n=5), an unidentified Porifera (n=3) and biofilm samples (n=2). One positive sample was observed from *Spirobis borealis*, *Asterias rubens* and a sediment sample.

Subsequently in 2013, screening of the 2011-2012 samples (n=1062) was repeated using the newly developed UCC primers. A higher detection was observed in the *C. gigas* and macroinvertebrates at Carlingford Lough, Dungarvan and Donegal using the UCC primers compared to the C9/C10 real time PCR screening. A significant difference ($P<0.0001$) in the screening results was observed with an overall infection of 39% being detected from all three sites combined using the UCC primers while only 2% infection was detected using real time PCR and C9/C10 primers, with the majority of those infections being detected in Carlingford samples. Samples collected in 2013 were screened with the UCC primers and detected OsHV-1 with a range of 45-100% in *C. gigas* and 3-69% in the grouped macroinvertebrates at Carlingford Lough over the study period. All invertebrate samples screened at the two control sites, Ardmore and Bullen's Bay, were negative for OsHV-1. No significant differenres in terms of OsHV-1 detection were observed at Carlingford Lough in 2013 (39%) compared to 2011-2012 (32%).

In the 2013 invertebrate groups screened by PCR, OsHV-1 was most frequently observed in bivalves followed by gastropods, tunicates, crustaceans, polychaetes and the chordate. Bivalve species, other than *C. gigas*, consisted of the edible cockle *Cerastoderma edule* and the blue mussel *Mytilus edulis*. All four monthly samples of *C. gigas* were PCR positive for OsHV-1 while three out of the four *C. edule* samples and two out of the four *M. edulis* samples were PCR positive. OsHV-1 DNA was deected in three gastropod species, the periwinkle *Littorina littorea*, the limpet *Patella vulgata* and the dog whelk *Nucella lapillus*. Two monthly samples of *L. littorea* and *P. vulgata* were PCR positive while a single sample of *N. lapillus* collected in September 2013 was positive. Several tunicate *Ascidiella aspersa* samples were PCR positive with OsHV-1 while a single sample of the polychaete *Aurelia aurita* and the fish species *Lipophyrys pholis* were positive for OsHV-1 in the PCR screening.

In the rescreening of the 2011-12 sediment, plankton and biofilm samples in 2013, a higher level of OsHV-1 detection was reported compared to the real-time PCR screening. In the seasonal samples rescreened at Carlingford Lough, a detection range of 0-100% was observed in the plankton, 0-40% in the sediment and 0-80% in the biofilm. In the Dungarvan samples screened, a detection range of 0-100% was reported in the plankton, 100% in the two sediment samples screened and 0-20% in the biofilm. In the Donegal samples screened, a detection range of 0-100% in the plankton, 0-100% in the sediment and 0-100% in the biofilm was observed.

Of significance, the results from the invertebrate screening from the two control non-culture sites, which were all negative for OsHV-1, would indicate that a relationship between the farming of *C. gigas* and OsHV-1 detection exists and that once the virus becomes endemic in an area it can be maintained for an extended period of time at that site. Positive results were obtained year round for the detection of OsHV-1 in *C. gigas*, macroinvertebrates and water column samples at the three Irish oyster culture sites. OsHV-1 DNA was detected in the biofilm coating the oyster bags year round and in all seasons (spring, summer and autumn) screened for the sediment samples. An inverse relationship was observed between the presence of the virus in *C. gigas* and other invertebrates, when detection of OsHV-1 was high in *C. gigas* it was low in the other invertebrates and vice versa.

Certain invertebrate groups and species were consistently positive for OsHV-1 DNA detection at all sites and years. These include bivalves (*Mytilus edulis*, *Cerastoderma edule*), gastropods (*Patella vulgate*, *Nucella lapillus*, *Littorina littorea*), ascidians *(Ascidiella aspersa*), crustaceans (*Carcinus maenas*) and a Porifera sp. The high level of OsHV-1 detectio in other filter feeding bivalves, *M. edulis* and *C. edule*, may be due to the intake of the virus during suspension feeding, while the high prevalence in *C. maenas* may be due to the ingestion of the virus by feeding on dead or dying infected *C. gigas*. Grazing gastropods may have ingested the virus by feeding on the biofilm. Further studies are required to indicate if the virus is replicating in these other macroinvertebrate species, where the virus was observed. *In situ* hybridisation (ISH) is required to localise the virus (DNA/RNA) within the tissues of such organisms. Positive results for the detection of *V. splendidus* in macroinvertebrates at all three Irish sites were observed all year round.

Vibrio splendidus was detected at all three sites at different times of the year. The presence of *V. splendidus* was detected in a wide variety of the invertebrate community in 2012 originating from all three sites. In contrast, *V. aestuarianus* was not detected in any of the samples screened. It was not known based on the primers available for the screening if the positives for *V. splendidus* were pathogenic strains or not. Due to this uncertainty further screening of samples was not carried out in 2013.

Italy (Participant 7 and Participant 8)

Participant 8 and Participant 7 have extended the study of bivalve pathogen occurrence in the Goro lagoon, Italy, over 3 three seasonal periods (2011-2013) according to cohabitation experiments performed with *C. gigas* and *M. galloprovincialis*, both juvenile and spat.

Mortality rates observed in juvenile-adult bivalves in 2011 could be classified normal in consideration of the Mediterranean climate and the conditions of cohabitation in the shallow lagoon waters. In 2012 the detection of OsHV-1 DNA preceded the massive oyster spat mortality, facts supporting the virus infectivity and its causal role in spat killing. The events occurred in 2013 remain to be understood, either the viral burst or other factors concurred to the significant observed mortality were missed. These results suggest that OsHV-1 (variant µVar) can cause big problems also in the Italian coastal waters.

The presence and concentration of *Vibrio* spp., *Vibrio splendidus* and *V. aestuarianus* were analyzed in water, sediment, zooplankton and bivalve samples (*Mytilus galloprovincialis* and *Crassostrea gigas*) by both culture dependent and molecular assays. Vibrios were found all year round in all matrices and did not show a clear seasonal trend. *Vibrio* spp. concentrations by real-time PCR were at least one order of magnitude greater than MPN counts. Bacteria belonging to the *V. splendidus* group were generally found in higher concentration than *V. aestuarianus*. Both species were mostly found in association with zooplankton and were also frequently detected in oysters and mussels. The absence of a clear temporal trend in *Vibrio* spp. abundance as well as the presence of culturable vibrios in seawater during cold months in the Goro Lagoon could be related to the high nutrients content of these waters. This condition is known to favor bacterial persistence and maintenance of culturability in *Vibrio* species. Bacteria belonging to the *V splendidus* clade were generally found in higher concentration than *V. aestuarianus*. *Vibrio splendidus* clade bacteria were always found in sea water and sediment. *Vibrio aestuarianus* bacteria were never found in sea water but they were found in the sediment. Interestingly, most of the plankton samples were positive for *V. aestuarianus*.

Overall, the results suggest that *V. splendidus* clade bacteria are in general more capable to persist in the aquatic environment than *V. aestuarianus*. This observation is confirmed by data obtained in microcosm experiments (see WP3). Zooplankton and the sediment compartment seem to represent important environmental reservoirs for these species where the bacteria can persist in high abundance and/or during cold and unfavorable environmental conditions. For a proper evaluation of the presence of *V aestuarianus* and *V. splendidus*-clade bacteria in the environment, microbiological analysis should be performed not only in sea water and bivalve samples but also in sediment and plankton samples from the same area. These matrices could carry potentially pathogenic bacteria, which, under appropriate conditions, might prosper and, when inside bivalves, participate to tissue damage.

WP2 - T3: Pathogen characterisation including genotyping *(Participants 1, 8 and 10)*

The general objective of this task corresponds to characterisation of pathogen isolates detected in the previous task (WP2 - Task 2) for typing and differentiating pathogen strains (related to geographical origin or virulence) collected during the course of th e project.

- *Setting up a VNTR based tool for the V. splendidus clade (Participant 10 and Participant 1)*

A set of 27 loci were deduced from the genome of the type strain *V. splendidus* LGP 32 leading to the synthesis of 18 primer pairs that were assessed on a set of type and non type *V. splendidus* strains. Among those, specific primer pairs were selected for their discrimination capability among *V. splendidus* and *V. splendidus* like species but also *Vibrio* from the *V. splendidus* (*Vibrio* website, www.vibriology.net). Other primer pairs were selected for their ability to differentiate isolates from *V. splendidus sensus stricto* species.

Primer sets were validated on type and not type strains of *V. splendidus* and on the type strain collection of Participant 1 (Ifremer). This included type strains of the *V. splendidus* clade (*V. gigantis*, *V. crassostreae*, *V. cyclotrophicus*, *V. tasmaniensis*, *V. pomeroyi*, *V. lentus*, *V. kanaloae*) which all showed a signal with at least one of the designed primer sets. More distant strains of *Vibrio* species (*V. harveyi*, *V. tapetis*, *V. aesturianus*, *V. pelagius*, *V. fortis*, *V. chagasii*) were also tested. Except for one primer pair, no amplification was detected with those more distant strains, demonstrating the specificity of the tools.

Application of the developed tool set to the different *Vibrio* strains isolated within the framework of Bivalife WP2 field survey already showed positive answers and was assessed in the frame of WP4 task 2. The VNTR approach should thus constitutes a powerful, rapid and inexpensive tool for direct, one-step screening of infected oysters and simultaneous typing of *V. splendidus* and related species during current epidemic outbreaks.

- *Setting up VNTR based tools for V. tapetis, V. harveyi* and *V. aestuarianus (*Participant 10)

For *V. tapetis* species, 118 minisatellites were found. Eighteen VNTR that matched good criteria in terms of length (9-2000 pb and total over 50pb), species specificity, conservation (80-100%) and polymorphism between the two reference strains, and then designed the corresponding primer pairs were selected. In all, 8 VNTRs were retained and used to characterize 11 pathogen isolates.

The same strategy was used to design VNTRs for *V. aestuarianus*. However, among the 10 VNTRs selected, only 2 displayed a significant polymorphism between strains. Most probably the availability of only one genomic sequence was too limiting for this kind of strategy. For typing *V. aestuarianus* isolates, other methods, based notably on virulence factors have thus been developed (collaboration with Particpant 1, Ifremer Brest).

- *OsHV-1 specimen partial sequencing (Partipant 1)*

Partcipant 1 developed a previous study targeting 3 different areas of the viral genome. Seventy-two OsHV-1 “specimens” collected mainly in France over an 18 year period, from 1993 to 2010 were analysed. Additional samples were also collected in Ireland, USA, China, Japan and New Zealand. Three virus genome regions (ORF4, ORFs 35/36/37/38 and ORFs 42/43) were selected for PCR analysis and sequencing. Although ORF4 appeared as the most polymorphic genome area, distinguishing several genogroups, ORFs 35/36/37/38 and ORFs 42/43 also showed variations useful in grouping subpopulations of this virus.

During the course of Bivalife project, virus positive DNA samples collected from oysters or mussels in 2011 and 2012 (France, Ireland, Spain, the Netherlands) have been selected in order to be determined regarding genotype status, namely the variant µVar or different variants, using sequencing with PCR products obtained from the 3 virus genome areas previously selected. Morover, OsHV-1 positive samples collected in other countries Australia, Brasil, China, Ireland, Japan, Korea, Mexico, Morocco, Netherlands, New Zealand, Spain, Sweden (*O. edulis*), Tunisia, UK, and USA) were also analyzed.

French specimens collected from 2008 to 2013 and all samples collected in other Members States (Ireland, Portugal, The Netherlands, Spain and UK) including samples collected during the course of the Bivalife project grouped together. The sequence of the variant µVar deposited in GenBank (accession n° HQ842610) was included in this group. It also integrated samples from New Zealand, Brazil and Korea. Although the C2/C6 fragment sequence for these specimens (New Zealand, Brazil and Korea) was quite similar to the sequence deposited in GenBank under the accession number HQ842610 (µVar), some differences were reported. A large deletion (605 bp) was reported for some PCR products obtained with the primer pair Del 36-37F2/Del 36-37R. The large deletion was observed for some French samples collected in 2008, all French samples collected after 2008, all samples collected in other Members States (Ireland, Portugal, The Netherlands, Spain and UK) including samples collected during the course of the Bivalife project. This deletion was also noticed for samples collected in the USA, New Zealand and Tunisia. Although the PCR products obtained from samples collected in France from 1993 to 2007 and some of the samples collected in 2008 did not demonstrate the 605 bp deletion, a few point mutations were reported differentiating 4 groups, one of them presenting 100% of identities with the reference type (GenBank accession n° AY509253).

-. OsHV-1 genotyping (Participant 1)

A genotyping approach was developed for OsHV-1 specimens based on microstellites. Deteremination of nucleotide sequences of PCR-amplified virus DNA fragments is the most accurate method for virus genotyping. However, virus DNA sequencing is time-consuming in the high-scale format. Microsatellites have been reported from different herpesviruses including human cytomegalovirus and used as molecular markers to define virus polymorphism. The variant µVar demonstrated a deletion of 12 bp in a microsatellite zone located up-stream of the ORF4.

In this context, during the course of the Aquagenet project (SOE2/P1/E287), 47 clinical OsHV-1 specimens were characterized targeting ORF 4 and its related upstream zone. DNA sequencing and genotyping of a microsatellite locus were performed. The results obtained with both techniques were compared: they appeared to be equally useful to differentiate clinical OsHV-1 specimens and were thus analysed in order to describe OsHV-1 diversity. Genotyping based on a microsatellite locus appeared as a powerful tool to study OsHV-1 polymorphism and can offer a first level of discrimination between specimens in order to select best candidates for complete genome sequencing. These results have been published in 2013.

Duing the course of the Bivalife project, the work was pursued developing the genotyping approach targeting 6 microsatellite loci and a collection of 263 samples including samples collected for Task 1 of the WP2. Genotyping French samples using the 6 microsatellites identified 2 groups of samples: i) the first one containing samples collected in 1994/1995/1996 and in 2008/2009/2010 and ii) a second one containing samples collected in 2005/2006 and 2007. Genotyping all samples using the 6 microsatellites identified 3 groups: i) one containing samples from Korea, New Zealand and Australia, ii) samples collected in Europe and iii) USA and Sweden. Genotyping samples considered as µVar or close related variants (microvariants) using the 6 microsatellites identified 3 groups: i) samples from New Zealand and Australia, ii) samples collected in Europe and iii) samples from Korea.

The genotyping approach using the 6 microsatellites showed that all the samples from the collection collected since 2009 in Europe (including samples collected during the course of Bivaliofe project) grouped together. Although based on partial sequencing the samples collected in New Zealand and in Australia have been previously identified as variants close from the variant µVar, genotying using the 6 markers demonstrated a distinct group containing these samples.

WP2- T4: Production of a database and data collection (Participants 5 and 9)

A database was designed in MS Access for the duration of the project and the data was exported to MS Excel for analysis using standard software packages. The database design was based on the template related to the field sampling sheets previously produced. The database itself was comprised of a series of interlinked tables for storing the data on sampled bivalves and their associated parameters, which are accessed via a data entry "switchboard". Data were collected at different levels, e.g. batch, farm, bay, … Consequently, the design was closely related to the experimental design of WP2 and WP3 to enable the most adequate data to be collected for subsequent data analysis. The basic input data was derived from the characterization of bivalve mollusc sites in five European countries (France, Ireland, Italy, Netherlands and Spain) with the recorded presence of five specific pathogens (OsHV-1, 2 *Vibrio* spp., *Nocardia crassostreae* and *Marteilia refringens*).

The production of a database related to site and pathogen characterization was used for evaluating epidemiological criteria affecting disease manifestation (pathogen presence and mortality). From a disease and epidemiological point of view, 'pattern and frequency' can be interpreted through the level of mortalities. In addition, the mortalities may be seasonal and could only occur, for instance, in the spring, although this may vary with the pathogen and the water temperature or salinity.

The main data registry entries finally included in the database were:

(i) study areas - 12 (including associated data on shellfish species present, their culture type and annual production quantity, as well as non-aquaculture local activities in the area and types of contamination),
(ii) production sites - 54 (sampled sites broken down into shellfish species farmed, their origin and production details, size and density, production method and handling data, estimated and expected mortality, as well as the nearest site to the one sampled),
(iii) shellfish batches - 148 (characterisation data for each production site related to the first sampling visit, including origin detail, age, size, as well as basic water parameters, such as depth and tidal exposure)
(iv) sampling visits - 655[1] (including data for stock movements, grading and cumulative mortalities),
(v) diagnostic tests requested - 1,829 (including data on type of test, pathogen expected, pathogen found, viral DNA amount ("viral load" = viral DNA copies ng DNA^{-1}) [in the case of OsHV-1], estimated infection, calculated prevalence),

(vi) environmental recordings - 164 (temperature, oxygen and salinity for each sample visit),
(vii) abnormal mortalities - 40 (including shellfish age, water depth, estimated mortality, detected pathogen).

WP2-T5: Descriptive epidemiology and definition of causal links in the context of mollusc mortalities (Particpant 5 using data from Particpants 1, 2, 3, 6, 7 and 8)

In this project field data were collected from 12 study areas in 5 European countries, firstly, to characterize the study areas and assess levels of mortality. Secondly, at selected sites, environmental parameters were monitored over time and shellfish were periodically sampled for the presence of potential pathogenic agents, with the aim of identifying factors associated with mortality and the presence of pathogenic agents. The study focused almost exclusively mortality and pathogen detection in *Crassostrea gigas* at selected study areas in 5 countries from the Republic of Ireland in the northern Europe to eastern Spain and Italy in the south. Thus a wide range of geographic, climatic and environmental factors, as well as the methods and scale of production were captured.

There was considerable variation in mortality from between sites and years. It can be concluded for many production areas cumulative mortality in a 12 month period of up to 40% may be considered ‘normal’ or ‘expected’. However, variation between sites indicates that different thresholds are ideally geographically specific.

The results confirm previous observations that the presence of the virus is associated with mortality in spat. The frequency and accuracy of mortality recording was not sufficient to draw firm conclusions about the level of impact, however, there was evidence of considerable variation between production areas and sites. The number of study areas and sites were insufficient for the role of other environmental factors to be investigated.

From the investigations of pathogens in environmental compartments vibrios can be detected and cultured from seawater and sediment. The results from Ireland show that OsHV-1 can be detected (by PCR) from a range of aquatic animals and biofilm. These animals could potentially act as vectors and spread the pathogens to uninfected areas, and this would be worth exploring further.

Detailed recommendations for future epidemiological studies are made. Arguably this study was too ambitious. Careful planning, based on sample size calculations, is needed to ensure that resources are matched to the objectives. Studies can be hypothesis generating or hypothesis testing; study designs appropriate to each are discussed. Since signs of disease in shellfish are generally impossible to detect, we rely on mortality as a key outcome. Mortality data in this report was estimated by a number of different methods. Future studies should establish and consistently apply a well described method for mortality estimation.

WP3. Mechanisms allowing pathogens of interest to survive outside the host

WP3 - T1: Definition of criteria and development of methods for pathogen survival evaluation (Participants 1, 4, 5 and 7)

For bacteria belonging to *Vibrio* genus, criteria and methods included viability assays, assessment of viable but non-culturable state (VBNC), analysis of adhesive properties and capability to form biofilms on biotic and abiotic substrates, persistence in sediments and bulk water, evaluation of virulence-related traits (link with WP4), and transmission/exposure trials. Methods to measure pathogen survival were developed in order to establish the viability (i.e. live/dead) status and/or pathogenicity potential and/or infective/non-infective status of pathogens including OsHV-1 and *Marteilia refringens*.

- *Development of a PMA real- time PCR for Vibrio splendidus and V. aestuarianus detection (Participant 7)*

A PMA-real Time PCR protocol for the detection and enumeration of viable *V. splendidus* and *V. aestuarianus* strains in environmental samples. Propidium monoazide (PMA) cross-linking for Live/Dead cell quantification by PCR PMA has been shown to be useful for live/dead distinction in combination with real-time PCR. It selectively enters cells with compromised membranes and, once inside the cell, intercalates into the DNA with high affinity. PMA has a light-activated azide group which allows covalent cross-linkage to organic moieties in its proximity upon light exposure. The covalent binding of the dye to DNA results in strong inhibition of PCR amplification.

The traditional real-time PCR protocol was detecting and measuring all dead cells in the samples. In contrast, the PMA-based protocol did not detect any dead cells (when the number of total bacterial cells were equal or lower than 10^5 cell ml^{-1}) or it detected only a very small fraction (less than 1% when the number of total bacterial cells are greater than 10^5 cell ml^{-1}). The protocol was able to correctly quantify the number of viable *V. aestuarianus* cells in mixed samples as it was observed that, in the presence of the PMA treatment, the real-time quantification of suspension containing 0% and 10% of live cells were 2 log and 1 log lower respectively than quantification by the standard real-time PCR protocol. Comparable results were obtained using *V. splendidus* LPG32.

- *Development of a PMA real- time PCR for OsHV-1 detection(Participant 1)*

PMA real-time PCR was developed and optimized for the detection and enumeration of non-broken viral capsids samples by conducting a number of experimental trials using different concentrations of virus (without treatment or heat treated for 60 min at 99°C) in ASW and testing different PMA concentrations (50, 100, 200, 300, 400 and 500 µM) using the PhAST blue system.

The PMA-based protocol only detected a fraction of the DNA detected using the conventional real-time PCR (around 10 times less). Viral DNA copies detected using the PMA-based protocol were interpreted as non-broken capsids avoiding PMA penetration and fixation. Results of trial experiments conducted with 4 different viral suspensions showed thet percentages of non-broken capsids ranged from 2 to 13%. This percentage was around 0% after treatment of the viral suspension 1 hour at 99°C.

- *Detection of OsHV-1 gene expression by real-time PCR (Participant 1)*

Recently, techniques including reverse transcription real time PCR, microarray or RNAseq have been used to study virus gene expression. In view of the low number of genes encoded by the virus, the reverse transcriptase real-time PCR technique was selected to confirm OsHV-1 mRNA presence. Thirty-nine OsHV-1 mRNAs were studied in Pacific oyster spat during an experimental infection (0, 2, 4, 18, 26, and 42 hours post injection). Relative expression was also performed on viral genes detected at all times. Eight viral genes were analysed using a host housekeeping gene (oyster Elongation factor gene). Thus, 8 virus genes were selected. They belong to 4 groups/families: (i) unknown proteins with ORF 43 and ORF 57, (ii) enzyme group with ORF 20 and ORF 24, (iii) menbrane protein group with ORF 25 and ORF 72 and, (iv) apoptosis inhibitors with ORF 87 and ORF 106. No ORFs belonging to ring finger protein groups were detected at all times.

This study is the first to demonstrate OsHV-1 RNA detection early after exposure. Main differences in terms of RNA detection between virus genes were observed at 2 and 4 h pi. Moreover, virus transcripts were detected from 18 h pi for the 39 genes monitored in this study. This result was in accordance correlated with virus DNA quantity detected detection by real time PCR with high amounts detected from 18 h pi. Viral RNA detection by real-time PCR is an additional tool in order to acquire a better understanding on the viral cycle, viral gene expression and spread of the virus.

- *Detection of OsHV-1 gene expression by in situ hybridization (Participant 1)*

An *in situ* hybridization protocol for detecting mRNAs of ostreid herpesvirus 1 (OsHV-1) which infects Pacific oysters, *Crassostrea gigas*, was developed. The three targeted viral mRNAs (ORF7, ORF 25 and ORF 87) were detected mainly in connective tissues in mantle, gills and gonads, this being consistent with previous histological descriptions of infection by OsHV-1 and results obtained using DNA ISH. In ISH, RNA probes were able to localise individual cells within paraffin-embedded oyster tissues. Absence of discrete precipitates in negative control oyster tissue sections confirmed the specificity of the probes. The low level of signal detection of virus RNA contrasted with virus DNA detection by ISH and by real time PCR. RNA transcripts could be less stable than viral DNA in oysters. However, the PCR platform provides no precise information concerning viral replication sites at a cellular level. The ISH assay developed in this study provides a platform to establish accurately physical and temporal distribution of OsHV-1 mRNAs during experimental infections in oysters.

- *Development of a Marteilia refringens reverse transcription real-time PCR assay to study parasite viability outside the host (Participant 5)*

One of the specific objectives of WP3-T1 was to develop a *M. refringens* reverse transcription real-time PCR assay in order to study parasite viability outside the host. An RNA extraction protocol was needed to give high-quality RNA for reverse transcribing into cDNA and the analysis of gene expression via real-time PCR. It was necessary to obtain at least one partial open reading frame of a gene that would provide evidence of "proof of life" and not merely the presence of RNA or DNA from non-viable cells. Therefore, β-actin from *Marteilia* sp. was the chosen target gene for this work.
Attempts, without success, have been made to isolate the β-actin gene from infected material in order to sequence and develop it as a marker of cellular metabolism for real-time PCR detection. The key to solving this bottle-neck could be a source of pure *Marteilia* DNA and massive whole genome sequencing. In addition, other detection methods with the same aim could include the amplification of ribosomal DNA with a RNA-based approach. However, RNA from sediment samples may not be useful for this type of study given the level of degradation observed in the samples that were obtained in the present study. Moreover, more work is needed for validation. Nevertheless, if an appropriate target gene is identified and there are viable *Marteilia* parasite stages present this approach should be potentially productive.

WP3 - T2: Definition of criteria and development of methods for pathogen survival evaluation (Participants 1, 4, 5 and 7)

- *Detection/isolation of Nocardia crassostreae from sediment (Participant 4)*

Nocardia crassostreae is assumed to be an environmental bacterium from the sediment which can infect oysters under suboptimal environmental conditions. The source of the infection would be *N. crassostreae* from the sediment while there is no or only marginal direct transmission from oyster to oyster. Within WP3-T2 three lines of experiments were carried out in order to evaluate the possibility of sediment to act as source of infection for *N. crassostreae* in Pacific oysters: (i) test the survival of *N. crassostreae* in seawater and sediment *in vitro*, (ii) isolation/detection of *N. crassostreae in vivo* and (iii) comparison of on bottom and off bottom culture of Pacific oyster in a *N. crassostreae* endemic area. Furthermore, the possibility of the existence of different morphotypes of *N. crassostreae* was evaluated by prolonged culture of the bacterium on Bennets agar (iv).

Initially strong inhibition of the recovery of *N. crassostreae* from the sediment was observed. After subsequent testing different extraction methods after spiking sediment with *N. crassostreae*, successful results could be obtained with the MoBio Power Soil kit. Furthermore, in addition to the standard Brain Heart Infusion (BHI) agar plates *N. crassostreae* was shown to grow at the specific spore stimulating media Bennets agar and Arret-Kirshbaum agar. A specific DNA extraction kit was shown to be successfull in countering the initially observed inhibition. This method was subsequently adapted for use in *in vivo* and *in vitro* trials to detect *N. crassostreae* in sediment samples (WP3-T2).

In the UV dose response tests carried out by Participant 11 in WP3-T2, two phases in the dose response curve of *N. crassostreae* were observed. In the initial phase a log inactivation of *N. crassostreae* was observed with increase of the UV dose. In the second phase there was only a limited effect with the increase of the UV dose. This suggests the possible existence of other morphotypes of *N. crassostreae* in addition to its vegetative state. Growth of *N. crassostreae* reference strains was tested on the spore stimulating media, Bennets agar and Arret-Kirshbaum agar in order to be able to evaluate the possibilities of *N. crassostreae* to enter a state of dormancy. Bennets agar was selected as culture agar to initiate a potential state of dormancy in *N. crassostreae* (WP3-T2). The ultrastructure of *N. crassostreae* bacteria cultured for 3 months on Bennets agar were compared to fresh cultures under an electronmicroscope. Although differences were observed between fresh and dormant cultures, non of these indicated alterations of the cell membrane leading to formation of spores.

Several experiments have been carried out to investigate in what respect sediment could be a source of *N. crassostreae*. Although *N. crassostreae* could not be isolated by culture from Lake Grevelingen sediment, using real-time PCR genetic material of *N. crassostreae* could be detected in the sediment from a *N. crassostreae* endemic site. Within the laboratory *N. crassostreae* survived in sediment for at least 12 days at 4°C, as well as at 22°C. However, so far prolonged culture on spore stimulating media showed no evidence for the existence of spore-like morphotypes of *N. crassostreae*.

The comparison of on bottom with off bottom culture of Pacific oyster was carried out on a small scale, and the results would need confirmation. However, the detection of 2 positive specimens in the basket at the end of the experiment at least shows that raising oysters from the sediment by cultivation in baskets does not protect the oysters from infection in *N. crassostreae* endemic areas.

- *Detection/isolation of Vibrio splendidus and V. aestuarianus (Participant 7)*

Persistence, viability and culturability of 2 strains (*V. splendidus* LGP32 strain and *V. aestuarianus* 01/32 strain) were studied in seawater and sediment laboratory microcosms, their capability to interact with chitin containing surfaces and to form biofilm. In addition, their capability to activate a viable but nonculturable (VBNC) state after extended incubation at 5°C was analyzed.

By conducting laboratory experiments under natural setting conditions *V. splendidus* LGP32 strain generally exhibited longer persistence in seawater and sediment than *V. aestuarianus* 01/32 strain, suggesting that the latter bacteria are possibly more demanding in term of living requirements and conditions than the former.

Both strains maintained viability and culturability for longer times in the sediment suggesting that the sediment may represent a more suitable environment for their persistence in the aquatic environment. This is probably linked to the fact that sediment provides biotic and abiotic surfaces for bacterial development, and concentration of organic matter in this compartment in natural conditions is 10,000 to 100,000 fold higher than in the overlying water column.

The two strains are capable to attach to chitin surfaces and to form biofilm, although with different efficiency, suggesting that the adhering phase may represent a survival strategy for both strains in the aquatic environment. Results also showed that another survival strategy of these bacteria is the induction of the VBNC physiological state after extended incubation at 5°C, a condition that could be met during the winter season in shallow brackish environment where bivalve farming generally takes place.

Overall, the above results suggest that persistence in the sediment, adhesion to chitin surfaces, biofilm formation and induction of the VBNC state may represent important survival strategies of *V. splendidus* and *V. aestuarianus* in the environment. These *in vitro* data together with those obtained in the field (see WP2) suggest that, for a proper evaluation of the presence of *V. splendidus* and *V. aestuarianus* in the aquatic environment, plankton and sediment samples should be analyzed in addition to water samples. Due to the capability of these bacteria to activate the VBNC state, it appears that culture-dependent methods currently used for monitoring the presence of these bacteria in the environment may be not sufficient since they do not detect bacteria in the VBNC state. Results emphasize a need for DNA-based methods for the detection of *V. splendidus* and *V. aestuarianus* in environmental matrices and bivalves.

- *Laboratrory trials with OsHV-1 (Participant 3)*

Several laboratory trials were carried out to determine the influence of varying environmental conditions (temperature, salinity and nutrient concentration) on the variant µVar and its impact on *C. gigas*.

Overall the detection of OsHV-1 DNA was quite low in all laboratory trials. Higher salinities (38‰) appeared to act as a stressor for *C. gigas* with the oysters performing better when kept at a reduced salinity. Mortality in oysters held at a reduced salinity did not increase, even when seawater temperatures were elevated above normal environmental temperatures. Although some oysters held at a reduced salinity were positive for viral DNA they did not showed consistant mortality. Mortalites (0-3%) were minimal in all of the nutrient treatments.

- *Define the specific UV dose for the selected pathogens (Participants 11, 8 and 1)*

The UV resistivity tests of *Vibrio* cultures were performed with the LP-CBA device and the UV resistivity of *N. crassostreae* was tested with the MP-CBA device, which composed of an Atlantium MP lamp. The UV resistivity of *N. crassostreae* under LP lamp was also tested.

For *V. aesturianus* culture, 3 illumination tests were run. Each illumination test included 7 illumination procedures (seven different doses uniformly spread along the scale) with 5 different dilutions. More than 6 logs inactivation were achieved with UV dose of 4 mJ/cm^2 .For *V. splendidus* culture, 4 illumination tests were run. Each illumination test included 8 illumination procedures (eight different doses uniformly spread along the scale) with 5 different dilutions. More than 6 logs inactivation were achieved with UV dose of 5 mJ/cm^2. The UV sensitivity of *Vibrio* species is similar to the UV sensitivity of *V. cholerae* - 4 logs at 3-9 mJ/cm^2.

Six illumination tests were run on *V. harveyi* cultures. Each illumination test included 9 illumination procedures (nine doses uniformly spread along the scale) with 5 dilutions. More than 6 log inactivation was achieved with a UV dose of 5 mJ/cm^2. The UV sensitivity of *V. harveyi* is similar to the UV sensitivity of *V. cholerae* - 4 log at 3-9 mJ/cm^2. Five illumination tests were run on the *V. tapetis* culture. Each illumination test included 11 illumination procedures (eleven doses uniformly spread along the scale) with 5 dilutions. More than 6 log inactivation of *V. tapetis* was achieved by a UV dose of 25 mJ/cm^2. The UV resistivity of *V. tapetis* is the highest between the *Vibrio* species tested during the Bivalife project.

Four illumination tests were run on the *N. crassostreae* culture. Each illumination test included 9 illumination procedures (nine doses uniformly spread along the scale) with 5 dilutions. Achieving 4 log inactivation of *N. crassostreae* required a UV dose of 30mJ/cm^2 under the MP. On the contrary, less than 4 log inactivation was achieved under the LP with 150 mJ/cm^2. Although of the tested UV doses caused total inactivation of *N. crassostreae* by the LP and MP lamps, the MP (HOD lamp) seems to inactivate the *N. crassostreae* suspension more efficiently than the LP lamp. Moreover, 2 phases of UV response are observed. During the first phase, the log inactivation of the bacterium *N. crassostrea* increased dramatically with increased UV dose until ~20 mJ/cm^2. In the second phase, at a UV dose greater than 20mJ/cm^2, a moderate affectivity of the UV light is observed with an increasing UV dose. This observation may indicate two living forms of the bacterium (vegetative and spores).

WP4. Pathogens of interest: identification of intrinsic virulence factors and effects on host defence mechanisms

WP4 - T1: Development of experimental disease models (Participants 1, 2 and 11)

Experimental disease (bacterial and viral) models were used in order to explore the susceptibility of Pacific oysters to selected pathogens including OsHV-1 and *Vibrio* species and to study pathogen/host interactions in controlled conditions. Experimental disease models provided contrasted biological material in terms of disease susceptibility such as infected versus non-infected animals and susceptible versus non-susceptible animals of the same species (families displaying different susceptibilities to mortality/infection with OsHV-1 and *Vibrio* species) for the following tasks (WP4, Tasks 2 and 3).

- *Producing Pacific families (Participant 1)*

During the first periof of the project, 45 Pacific oyster families have been produced by bi-parental crosses in 2011 and grown at Ifremer facilities in La Tremblade and Bouin (France). The susceptibility of the 45 families was tested once by experimental infection with OsHV-1 (variant µVar). Reported mortality rates varied from less than 10 % to 100 % depending of the considered family demonstrating different levels of susceptibility to OsHV-1 infection in laboratory conditions. Sixteen families were selected and also tested for their susceptibility to 3 *Vibrio* species: *V. splendidus*, *V. aestuarianus* and *V. harveyi*.

Although some families demonstrated low mortality rates after bacterial or viral challenges, other families showed a contrasted susceptibility to different pathogens under the conditions tested: as an example, two families (F25 and F34) showed high mortality rates (around 80-90%) after an OsHV-1 challenge and mortality levels reported for these families after a *V. splendidus* challenge were less than 20%.

During the course of the bivalife project, a second generation and a third generation have been produced for 10 families presenting contrasted susceptibility to different pathogens.

Production of Pacific oyster families demonstrating variable susceptibility to OsHV-1, *Vibrio splendidus*, *V. aestuarianus* and *V. harveyi*, in laboratory conditions was effective and these families were used and shared with some Participants as biological material of interest: oyster families were used for WP3 (searching OsHV-1 RNAs) and for WP4 *(*studying the oyster immune response to viral and bacterial infections).

- *Experimental OsHV-1 infections in oysters and mussels (Participant 1 and Participant 2)*

A comparison for OsHV-1 susceptibility of mussels and oysters was also conducted through experimental assays by injection and cohabitation. Assays were conducted in mussels and oysters. Cumulative mortalities were registered for 15 days. No mortality was reported neither in control animals (mussel and oyster) nor in infected mussels. Mortalities were only registered in oysters infected with the viral homogenate. In this group mortalities reached values up to 90% in some replicates .

In one assay mussels were intramuscularly injected with OsHV-1 suspension, put then in contact with healthy appearing juvenile oysters. After 48h seawater from tanks was replaced: After 12 days, mussels have had only 2% of mortality while oysters had 25% of mortality associated with high amount of virus DNA (> 10^7 DNA copies/mg). These results suggested that a “transmission of viral infection” from mussels toward sensitive oysters might occur during the assay.

As a preliminary conclusion, in tested conditions mussels have seemed to be not sensitive to viral infection OsHV-1, but may be able to transmit the disease to healthy appearing oysters. Nevertheless, due to problems in control groups during this study, other trials need to be be carried out.

Replication of OsHV-1 was also conducted *in vitro* in mussels and oysters hemocytes. The viral DNA was measured by real-time PCR. *In vitro*, an increase of the viral DNA amount was only detected in infected oyster hemocytes. No incresase of the viral DNA amount was detected in mussel hemocytes during the course of the experiment.

- *Experimental trials in mussels with Vibrio splendidus and V. aestuarianus (Participant 2)*

Experimental trials were conducted with *V. splendidus* and *V. aestuarianus* in mussels (*M. galloprovincialis*).

Infections by intramuscular injection were conducted in mussels (2.8 ± 0.28 cm length). Ten µl of three different doses (10^9, 10^8 and 10^7 CFUs/mL) of each *Vibrio* (*V. splendidus* and *V. aesturianus*) were intramuscularly injected at 15 ºC. The cumulative mortality registered in mussel infected with *V. splendidus* was higher than those registered with *V. aestuarianus*. The mortalities reached 70 % and 40% for *V. splendidus* and *V. aestuarianus*, respectively as soon as 4 days post-infection. Lower concentration of bacteria induced less than 10 % of mortality.

Experimental infections were also conducted in mussels with 2 cm length by bath infection. When the experimental infections were conducted at 15 ºC, no mortalities were registered in mussel infected with *V. splendidus* or *V. aestuarianus*. High cumulative mortalities were obtained when animals were infected at 25 ºC and hypoxia stressed. In those conditions *V. splendidus* induced up to 100 % of mortalities at day 15 in some tanks. The infection with *V. aestuarianus* also induced high mortality levels (up to 60%) although always lover than those induced by *V. splendidus*.

- *UV inactivation of pathogens*

The effects of the UV inactivated pathogens on bivalves have thus been assessed through experimental trials. Oysters were subjected to OsHV-1 that was exposed to the HOD germicidal power by using a static UV setup -Collimated Beam Apparatus (CBA) -.based on monochromatic (Low Pressure UV lamps) UV light and polychromatic (Medium Pressure UV Lamps). Participant 11 manufactured and supplied the UV devices (CBA devices) and prepared the protocols for UV illumination tests on pathogens for evaluating. Thus Participant 11 manufactured and supplied LP-CBA and MP-CBA to Participant 1. Participant 11 trained Participant 1 researchers to operate the CBA devices.

The immune responses of oysters and mussels against OsHV-1 were investigated by Participant 2. Non-inactivated OsHV-1 and UV inactivated virus provided by Participant 1 (CBA device) and Participant 11 were used in the *in vitro* assays. *In vitro* challenges of oyster and mussel hemocytes with live and inactived OsHV-1 were conducted but no significant differences were found in parameters such as nitric oxide production. Phagocytosis of fluorescent latex beads was inhibited after *in vitro* infection of mussel and oyster hemocytes with both non-inactivated OsHV-1 and inactivated virus. Apoptosis only was induced in oyster hemocytes.

WP4 - T2: Effects of pathogens on host defence mechanisms (Participants 1, 2, 7, 8, and 10)

Sub task 2.1 - Available molecular tools and development of new ones

- *Phage Display technology: identification of PRRs of immunocompetent cells in M. galloprovincialis (Participant 8)*

UniTS undertook the development of a new tool for the molecular characterization of host-pathogen recognition which possibly supports the objectives of WP2 Subtask 2.2 (Functional immunology) by allowing the identification of the PRRs of immunocompetent cells involved in pathogen recognition in *M. galloprovincialis*. The Phage Display technology is based on the ability to express foreign peptides, as fusions to capsid proteins, on the surface of bacteriophages that infects *E. coli.* A cDNA pool is cloned into a phagemidic vector upstream to a sequence encoding the pIII superficial protein. Therefore, a library of peptide or protein variants, expressed on the outside of a phage virion, is obtained. This creates a physical linkage between each variant protein sequence and the DNA encoding it, which allows rapid partitioning based on binding affinity to a given target molecule by an *in vitro* selection process called 'panning'.

Due to the difficulties experienced in the final steps of the library preparation for the sequencing, the samples were sent to the sequencing centre with considerable delay in respect to the expected schedule, thus preventing the final bioinformatics analysis to be carried out. This step not completed at present time.

Sub Task 2.2 - Functional Immunology

- *Immune parameter modulation after bacterial challenge in mussels (Participant 2)*

Several immune parameters were measured in hemocytes after *Vibrio* sp. infection. Mussels were im injected with 100 µl of solutions containing *V. splendidus* or *V. aestuarianus* at a final concentration of 10^6 CFUs/m. After 3h and 24h hemocytes were extracted and the total hemocyte count, the phagocytosis (Latex beads-FITC, *E. coli*-FITC, Zymosan-FITC) and the ROS and NO production were evaluated by flow cytometry.

According to the flow cytometry results animals infected with *V. aestuarianus* showed higher number of hemocytes at the injection site than those obtained in control and also in *V. splendidus* infected animals after 3h post stimulation. Differences in phagocytosis were only registered 3h after infection. At this point hemocytes from animals infected with *V. aestuarianus* showed lower levels of ingested latex beads than the levels registered in control cells and also in animals infected with *V. splendidus*. The production of ROS and NO after *Vibrio* infection was also evaluated by flow cytometry, but no significant differences were observed between control and infected animals.

- *Exploring intrinsic or mitochondrial apoptotic pathways in mussels (Participant 2)*

Specific sets of real-time PCR primers were designed and validated to measure the relative expression of each gene after apoptosis induction by UV-light treatment. Moreover, several chemical components were used to modulate, to block or to inhibit different stages of the apoptotic cell death induced after UV-light treatment:

- two chemical antioxidants N-acetyl-L-cystein (NAC) and the ammonium pyrrolidine dithiocarbamate (PDTC) due to their ability to inhibit the ROS production
- the pifithrin-α hydrobromide (PFT-α) since it is a reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription
- the cell membrane stabilizer cyclosporin A (CsA) who blocks mitochondrial permeability transition pore formation and thus, inhibits apoptosis,
- and finally the pan caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone (Z-VAD-FMK).

The apoptotic levels were recorded by flow cytometry, in a FACS Calibur flow cytometer (BD) using Annexin V-FITC and 7-Amino-Actinomycin (7-AAD) staining.

The incubation of cells with the antioxidants NAC or PDTC prior to the UV-exposition did not reduce the apoptotic levels at any time. Moreover, NAC treatment induced a significant increase in the apoptotic levels. The treatment with PFT-α induced a significant decrease of the apoptotic levels induced by UV-light in haemocytes. In order to understand the molecular mechanisms by which the treatment with PFT-α induced a decrease of the apoptotic levels after UV-treatment the expression of the selected key genes was analyzed by real-time PCR. The PFT-α treatment was able to modulate, to decrease and to delay the expression of the different genes. The treatment of hemocytes with CsA before the UV-light exposure did not induce significant differences in the percentage of apoptotic cells, in comparison with the value registered in untreated hemocytes at 3 h and 24 h pt. The percentage of apoptotic cells in hemocytes treated with the caspase inhibitor Z-VAD-FMK was not different from the percentage registered in the control group in all time points after the UV-light treatment.

- *Bacterial sensitivity to killing by mussel serum and hemocytes (Participant 7)*

To study bacterium-hemocyte interactions, the number of hemocytes per milliliter of haemolymph was determined before each trial to obtain the desired experimental ratio of hemocytes to bacteria. 500µl of haemolymph were seeded onto 24 well plastic culture dishes and incubated at 18°C for 30 min. Non-adherent hemocytes were then removed. Bacterial suspensions (about 3×10^{7}-10^{8}CFU per ml each) were added either to whole serum or to hemocyte monolayers at 18°C either in the presence or absence of haemolymph serum.

In vitro experiments showed that *V. splendidus* LPG32 strain was not sensitive to killing by mussel serum. The same strain was also not sensitive to killing by hemocyte monolayers either in the presence or in the absence of serum. Similar to what observed for *V. splendidus*, *V. aestuarianus* 01/032 strain was not sensitive to killing by mussel serum after 60 and 90 min of incubation.

In contrast, *V. aestuarinaus* strain was partially cleared by hemocyte monolayers in the presence of haemolymph serum. No killing by hemocyte monolayers of *V. aestuarinaus* was observed in the absence of serum.

- *Phenoloxidase activity in Pacific oyters (Participant 10)*

Several lines of evidence showed that the activity of phenoloxidase (PO) in molluscs is correlated to infection by pathogens. Five families of Pacific oysters (Participant 1) were selected because of their contrasted differences reactions (survival vs death) after different challenge of vibrios, ie, F48: *Vibrio*-high survival oysters; F17: *Vibrio* low survival oysters ; F7: *V. aestuarianus* high survival oysters ; F39: *V. splendidus* high survival oysters ; F25: *V. harveyi* survival survival oysters. Phenoloxidase specific activities were determined in both control and infected (by injection) whole animals, at day 0 and at day 5.

In the absence of vibrio injection, animals from the family 48, surviving to all the tested vibrios, displayed a PO specific activity significantly lower than that observed for the other families. By contrast, for the animals which received injections, any clear trend in the PO activity levels was reported between families. No significant relationships have been found between individual bacterial pathogen concentrations and PO activity in oysters. PO activity was also determined in the extrapallial fluids of control and injected oysters. As for the tissues no clear trend was observed in the PO activity levels between the different families. Nevertheless, as for results obtained with whole animals, in the absence of vibrio injection, the PO specific activity level was significantly lower for family 48 oysters than that observed for the other families.

Sub task 2.3 - Molecular immunology

- *Identification of immune signal transduction pathways in M. edulis, M. galloprovincialis and C. gigas (Participant 8 and Particpant 10)*

Identification and functional characterization of relevant innate immunity- and defence-related molecules in *M. galloprovincialis* were carried out. To achieve this goal, Participant 8 took advantage of next generation sequencing technologies (RNAseq), which guaranteed the *de novo* assembly of a high quality reference transcriptome by the combination of new round of sequencing (UniTS-UniPD resources) and pre-existing sequence data. The availability of this resource permitted to identify with unprecedented depth a high number of full-length transcripts of interest. A particular attention has been focused on three main points:

i) the discovery of novel potential or bioactive peptides in mussel, by combining the research on already known AMP families to the *de novo* prediction of novel bioactive peptides;
ii) the identification of molecular characterization of components of the TLR pathway, which plays a critical role in the innate immune response, in collaboration with Participant 10;
iii) the detection of gene families under positive selection, taking advantage of the multi-individual and different geographical origin of the sequence data generated by RNA-seq, with a particular focus on pathogen-recognition receptors.

New immune-related genes related to several signal transduction pathways were discovered. Concerning the identification of Toll-like receptors and the downstream members of the TLR signalling cascade, significant results were achieved.

Given the promising results more functional studies need to be carried out in the future to clarify the role of MgCRP-I and other interesting AMP candidates in mussels. Transcriptomic analyses still running also represent a valuable resource for improving knowledge on the functional responses of the bivalves of interest.

- *Genes of mitochondrial apoptotic pathway in Mytilus galloprovincialis (Participant 2)*

An extensive data mining to identify genes potentially involved in the response against infectious agents was conducted. The MytiBase database base was used to identify genes of the mitochondrial apoptotic pathway in *Mytilus galloprovincialis* since apoptosis is one of the most important mechanisms involved in adaptive responses to environmental or biological stresses.

The sequences of six newly described key genes associated with the mitochondrial apoptotic pathway: p53, PDRP, Bcl_2, Bax and BI-1 and the DNA fragmentation factor A (Dff-A) were identified in the available databases. The complete sequences were obtained using RACE and their phylogenetic relations were studied. Moreover, the apoptotic process was induced in mussel hemocytes through UV-C light treatment and the modulation of the expression of the apoptotic genes was determined after the incubation of hemocytes with chemical reagents able to modulate, block or inhibit different stages of apoptotic cell death after UV irradiation. The analysis of apoptotic gene expression using real-time PCR revealed that the genes characterized here were modulated after UV radiation. With this study a clearer representation of the apoptotic Framework in bivalves was obtained that suggests that apoptosis in this group has a complexity similar to vertebrates.

- *Comparing Mytilus galloprovincialis and M. edulis (Participant 2)*

Moreover, with the help of the Reproseed project, the hemocytes transcriptomes *of Mytilus edulis* and *M. galloprovincialis* after an immune stimulation were compared. Many antimicrobial peptides (such as many variants of mytilin, myticin, defensin, etc) were found in both *Mytilus* species. These molecules were not present in transcriptomes of oysters or even clam species. If the GO terms differentially expressed in both mussel species were compared, there were not many differences in the distribution of modulated transcripts. The comparison using RNAseq of “immune transcripts” (from stimulated hemocytes, compared with that from other tissues (mantle, muscle and gill) identified that genes such as mytilin B, MGD2b, apextrin like proteína (apelP) or serine proteases were mainly expressed in hemocytes suggesting an important role on the immune defense of these animals.

At proteomic level, fibrinogen-related proteins, Myticins, c1q domain containing proteins or Heat shock protein 70 could be detected in hemocytes and also in hemolymph. The role of the different isoforms detected in the transcriptomic and proteomic studies needs to be further characterized.

- *Functional responses of bivalve hemocytes to in vitro and in vivo challenge with live V. splendidus and V. aestuarianus (Participant 7)*

Functional responses of bivalve hemocytes to *in vitro* and *in vivo* challesnge with live *V. splendidus* and *V. aestuarianus* were studied. Functional endpoints measured were lysosomal membrane stability (LMS) and lysozyme activity. In *in vitro* studies, responses of *Mytilus galloprovincialis* and *Crassostrea gigas* to bacterial challenge were compared. Moreover, the role of signaling components in mediating the activation of responses was investigated in mussel hemocytes by use of specific kinase inhibitors.

The in *vitro* studies clearly show that the hemocytes of *M. galloprovincialis* are less sensitive than those of *C. gigas* to these two pathogens. Lysozyme release does not appear as an efficient response of oyster hemocytes to vibrio challenge; the results underline that evaluation of LMS represents a rapid and sensitive tool for comparing the effects of bacteria on the hemocytes of different bivalves. Moreover, in mussel hemocytes activation PI-3K was identified as a crucial step in mediating the lysosomal damage induced by *V. splendidus*., with a minor role played p38 MAPK. The effects of *V. splendidus* may be due to the capacity of this strain to interfere with the signaling pathways involved in hemocyte function, thus partly escaping the bactericidal activity of the host cell.

In mussels, distinct functional responses to the two vibrio strains were observed both *in vitro* and *in vivo*. However, the capacity of recovery observed *in vivo* can be explained by distinct interactions between mussel hemocytes and different vibrio strains mediated by soluble serum components. This would account for the resistance of *Mytilus* spp. to infection not only with LGP32 and 01/032, but also to other vibrios.

- *Transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting different levels of susceptibility to OsHV-1 (Participant 1)*

The genetic basis that may underlie differences in susceptibility to virus infection among Pacific oysters was explored by challenging 2 Pacific oyster families presenting contrasted results in terms of mortality after an OsHV-1 challenge. Transcript profiling was carried out targeting virus and oyster genes. This work constitutes the first step towards elucidating the interaction between OsHV-1 and its host, the Pacific oyster and the first study of viral gene expression in families presenting contrasted susceptibility to the viral infection.

First, significant differences in terms of virus DNA and RNA detection were reported between both Pacific oyster families supporting a genetic variation in susceptibility to OsHV-1 that naturally infect Pacific oyster, *C. gigas*. Previous studies suggested that a genetic basis may underlie resistance to OsHV-1 infection in the Pacific oyster. Moreover, difference in susceptibility to infection with OsHV-1 was associated with differences in host gene expression profiles. A correlation between viral DNA amounts and oyster gene expression was also reported. These results suggested that up regulation of immune related genes may prevent virus replication. This opens new questions regarding the persistence and/or latency of the virus within the host. Genes of interest can be further evaluated in oyster breeding programs to determine if they confer genetic resistance to OsHV-1. This integrative strategy can be applied to other infectious diseases.

- *Host response during Vibrio infection in mussels (Participant 2)*

Experimental infections were conducted in order to analyze the expression of selected immune genes in infected and non infected animals. MPussles were im infected with 10 µl of a solution containing *V. splendidus* or *V. aestuarianus* (at a final concentration of 6.6 $x10^7$ and 9.33 $x10^7$ CFUs/mL). One, 3, 6 and 24 h post infection hemolymph, gills and mantle from 20 animals were collected to assess the expression of myticin C, mytimycin precursor, myticin B, mytilin B, lysozyme, MIF, C1q and Macp genes.

Vibrio splendidus induced high expression levels of myticin B, C, mytimicin and C1q at 3h pi. At this point other genes such as lysozyme and MACP showed low expression. No modulation of mytilin B and MIF was observed. *Vibrio aestuarianus* induced similar changes although myticin C and mytilin B were highly expressed at 1h pi. Moreover, lysozyme and MACP were also highly expressed at 24h pi.

- *Gene expression in challenged adult Mytilus galloprovincialis(Particpant 10)*

Adult *M. galloprovincialis* (Palavas-Prévost lagoon, France) were challenged by one injection of sterile seawater containing Gram-negative bacteria, *Vibrio splendidus* LGP32, *V. anguillarum*, Gram-positive bacteria, *Micrococcus luteus* spores of filamentous fungus, *Fusarium oxysporum*, LPS from *E. coli*, peptidoglycans from *M. luteus*, or beta-glucans from *Saccharomyces cerevisiae*.

Gene expression of intermediate molecules has been studied in several bivalves in response to various stimulations. None of these reports addressed the complete Toll/NF-κB pathway in the same animal. Moreover, nature of stimulant, duration of challenge, route of challenge (immersion, *in vivo* injection or *in vitro* incubation), and targeted tissues, were so diverse that comprehensive scheme and comparison between species cannot be outlined.

- *Molecular characterization and expression of TEP containing proteins in C. gigas oyster (Participant 10)*

Thioester containing proteins are molecules associated to the complement system and include complement proteins C3, C4, C5, the universal protease inhibitor α2 macroglobulin as well as a set of TEP initially identified in insects and only present in some invertebrates. In vertebrates, the complement system is a major biological reaction system in immune responses as part of both the innate and adaptive immune system. Several complement components were previously identified in invertebrates and associated to host defenses in arthropods and mollusks. The characterization in *Crassostrea gigas* of four thioester containing protein (TEP) was reported. Previous works on *Chlamys farreri* and on insects revealed the presence of alternative splicing at the highly variable region of insect TEP leading to structural change of TEP. These variations could increase the number of putative recognition motifs for microorganisms and could compensate the absence of the large repertoire of receptors of the adaptive immune response of vertebrates.

Results showed a large diversity of TEP containing proteins in *C. gigas*. Oyster TEP exhibited original characteristics in term of sequence structure as well as expression among tissues. Comparison of TEP expression in different oyster families revealed variations, which could be linked to the resistance of individual to specific *Vibrio* strains. However, additional researches need to be performed to identify the complete diversity of TEP containing proteins in *C. gigas*.

WP4 - T3: Characterization of pathogen virulence factors

- *Interactions between bivalve cells and V. splendidus and V. aestuarianus (Participant 7)*

Interactions between bivalve cells and *V. splendidus* and *V. aestuarianus* bacteria were studied. For both strains (*V. splendidus* LGP32 strain and *V. aestuarianus* 01/32 strain), an increase in bacterial adhesion to mussel hemocytes was observed in the presence of hemolymph serum compared with artificial sea water (ASW). Pretreatment of *V. aestuarianus* 01/032 with D-mannose significantly reduced the interactions of these bacteria with mussel hemocytes compared with the controls. The interactions of the same vibrios with *Crassostrea gigas* hemocytes were also analyzed to highlight possible differences in interactions with the two bivalve species, and performed experiments to identify serum component(s) involved in D-mannose sensitive association of *V. aestuarianus* 01/032 with mussel hemocytes.

- *A secretomic approach to identify new virulence factors in two mollusk-pathogenic vibrios (Participant 10)*
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At this time, the only virulence factor characterized in *V. aestuarianus* is the secreted zinc metalloprotease Vam, a member of the thermolysin family. No similar virulence factor was described up to date in *V. tapetis*, but a metalloprotease (Vsm), a homolog of Vam, was also identified as a major determinant of the toxicity of *V. splendidus* ECPs. As the *V. tapetis* genome does not contain any secreted zinc metalloprotease gene, the virulence mechanisms in this species are necessarily different from those in *V. aestuarianus*. In addition, several chromatographic fractions of ECPs displayed biological activity towards oyster- and clam-hemocytes, for *V. aestuarianus* and *V. tapetis*, respectively, indicating that other factors are also responsible for the biological effects on hemocytes.

Overall, 45 and 87 different proteins were identified in the active fractions of the *V. aestuarianus* and *V. tapetis* secretomes. However, it should be noted that whereas both genomes encode several candidate genes for toxins and hemolysins (unpublished results), except for the secreted RTX toxin from *V. tapetis*, none of these proteins were identified in the present study, raising the possibility that these genes and/or the genes encoding their secretion systems were not expressed in the tested conditions. The high proportion in the extracellular proteomes of normal periplasmic and outer membrane proteins, with no or very little inner membrane proteins suggest that the extracellular fractions contain outer membrane vesicles (OMVs).

Complete confirmation of the vesicular nature of this material will need more adapted method of fractionation and analysis (i.e. ultracentrifugation and electron microscopy). Release of OMVs appears to be a way to relieve envelope stress in Gram-negative bacteria. In *V. aestuarianus*, the Vpp protease and the triacylglycerol lipase were both associated with potential vesicle material whereas Vam was secreted in a free form. In *V. tapetis*, the lipase and a protease were also found in the void volume fraction. whereas the RTX toxin seems to be secreted in its free form. Future studies will be required to determine the role of OMVs from theses two species in virulence if any.

WP5. Pathogen control and eradication: development of methods, field tests and recommendations

The general objective of this WP is to define and propose practical methods and general recommendations drawn from knowledge acquired from previous WPs for infectious disease control. This WP includes an experimental work in order to evaluate pathogen inactivation and decontamination of system and animals.

During the course of the project, methods have been defined to estimate success of UV based inactivation of some pathogens in experimental and hatchery/nursery conditions. Prior works consisted in establishing protocols to estimate pathogen inactivation. Treated and no treated bacterial suspensions were tested on solid growth media (Zobell). Treated and no treated viral suspensions were tested by injecting healthy *C. gigas* spat or larvae. Different 'UV doses' (intensity) were tested in order to determine a UV range at which pathogen inactivation was successful. Finally, effectiveness of UV in hatchery conditions was assessed by comparison of survival and OsHV-1 detection in spat in connexion with diseased OsHV-1 spat with and without UV treatment. Impact of turbidity and flow on UV effectiveness was also investigated in nursery conditions.

WP5 - T1: Develop and evaluate methods for pathogen inactivation and decontamination of systems and animals (Participants 1 and 11)

Sub Task 5.1 - Inactivation of pathogens in sea water and on materials and equipments

During the course of the project, tests involving UV inactivation were conducted. However, other inactivation methods were not explored due to a lack of time.

- *Estimation of OsHV-1 inactivation using UV radiation*

Filtered OsHV-1 infected tissue homogenates (i.e. viral suspension in artificial seawater, <0.2 NTU turbidity) were subjected to UV treatment using device. UV treatment was carried out with low-pressure lamp at 254 nm, at different exposure times or energy conditions and with several pathogen concentrations (from $5x10^{+5}$ to $1.0x10^{+8}$ viral DNA copies/µl). One hundred µl of UV treated and no treated infected tissue homogenate were injected into the adductor muscle of anaesthetized healthy oyster spat. Negative controls consisted of oysters injected with sterile seawater. Inoculated oysters were then placed at 22°C in 2L tanks supplied with filtered (1µm) seawater. Mortality was daily monitored during a 7-day period.

In addition, three independent trials were conducted with 4 and 10 days old oyster larvae. Final infectious viral amounts ranged from $10+^{6}$ DNA copies/L to $10+^{8}$ DNA copies/L in 3L tanks. The mortality was estimated by binocular microscope observation and counting of three seawater/larvae samples from each aquarium at day 3, day 5 and day 7 post infection.
Injection of tissue homogenates without UV treatment in spat induced cumulative mortality rates of 60% to 100% at the end of the experiment. No significant mortality was recorded among oysters injected with UV treated tissue homogenates. Similarly, no significant mortality was recorded for the condition "UV inactivated" in the larvae trials while cumulative mortality ranged from 84% to 100% for 'untreated UV condition'.
OsHV-1 DNA was quantified in dead animals collected during the time course of the experiment and in surviving individuals collected at the end of the experiment. Over 10^{+6} DNA copies ng^{-1} of total DNA was measured in animals inoculated with untreated tissue whereas less than 10^{+2} DNA copies ng^{-1} of total DNA was measured in negative control groups or UV treated conditions.

- *Estimation of Vibrio spp. inactivation using UV radiation*

Effective inactivation (no growth on plate) of a fresh (24h broth culture) *V. splendidus* suspension (with a turbidity of 8NTU), was obtained using the 2 UV intensity levels: 15 mJ/cm2 30 mJ/cm2; with a suspension of untreated control bacteria corresponding to 8.0x10+7 CFU /mL.

Inactivation of pathogens in seawater was effective in the experimental conditions for all the tested UV intensity levels: 3 to 30 mJ for OsHV-1 and 15 to 30 mJ for *Vibrio splendidus*.

Sub Task 5. 2 - Inactivation of pathogens in hatchery-nursery conditions

- *Impact of turbidity and flow on UV effectiveness*

Seawater showing different turbidity (1.6 and 32 NTU) was used to test the inactivation of UV radiation on microbial flora (device for aquaculture, Bio-UV PE 870HO, UV-C 28W). Seawater flow ranged between 0.9 and 6.5 m^3/h. Microbial flora was measured by inoculating two bacterial growth media TCBS and Zobell. *Vibrio* spp. and bacteria appeared inactivated using UV radiation intensity (~30 mJ) however the treatment was less effective at 32 NTU compared to 1.6 NTU.

- *Use of UV radiation inside hatchery*

Survival and OsHV-1 detection were monitored in spat supplied with water in connexion with diseased OsHV-1 spat. Open flow seawater with or without UV treatment (UV-C) from two tanks containing 250 infected spat (OsHV1) fed two tanks of 200 healthy spat. Spat supplied with UV treated water did not show mortality while spat supplied with untreated UV seawater showed 54% of cumulative mortality after 37 days. PCR tests conducted to detect and quantify OsHV1 DNA in diseased group and in moribund animals confirmed the effective transmission of the viral infection by seawater flow in system without UV radiations.

Results showed experimentally the effectiveness of UV to inactivate targeted pathogens and their sensitivity to UV-C. These results answer questions raised in Task 1 and should be completed by testing HOD system in hatchery conditions and by further experimental works including ozone and chlorine inactivation approaches.

Participant 11 (Atlantium) conducted a survey in three Ifremer facilities to evaluate the required protection necessary for oyster and mussel pathogen control. Participant 11 identified a location for installation of the HOD system at Centre de Brest (Participant 1, Ifremer, Plouzané). Atlantium's Hydro Optic Water Disinfection System (HOD system) was planed to treat the wastewater from the mollusk hatchery to prevent cross contamination from the hatchery to the environment. The main targets of UV inactivation are: OsHV-1, vibrios and other bacteria, pathogenic algae and some larvae (up to 250µm). Atlantium sized the HOD system (which was sent to Participant 1) based on the known required UV doses for pathogen inactivation (as determined during WP3) and according to the field conditions (water system, water flow, water UV transmission and etc.) The functional parameters related to operational conditions are: maximum water flow of $10m^3$/hr and water UV transmission (UVT) of ~90%/cm at 254 nm. However, this part of the work is not done at present time due to lack of time.

WP5 - T2: Recommendations for surveillance and diagnosis (all Participants)

These recommendations follow the aims expressed in the DoW and therefore "focus on pathogens targeted in WP2, Task 2". As such, the recommendations are established from data obtained in other work packages, especially WP2 Task 2 and focuse on OsHV-1. They aim to contribute to the production of guidelines for sampling and surveillance of pathogens of interest for oyster and mussel culture by identifying: (1) the most suitable diagnostic tools, (2) the nature and size of samples (susceptible species and also other compartments of the field known to carry the pathogens) that should be tested, (3) the most suitable period and sites for sampling.

WP5 - T3: Recommendations for pathogen control in controlled culture conditions (hatcheries, nurseries) and in open water on-growing sites

These recommendations follow the aims expressed in the DoW and therefore "focus on pathogens targeted in WP2 - Task 2, as well as from data recorded in the database (WP2 - Task 4)". As such, they aim to support biosecurity measures for: (1) maintaining a free status regarding pathogens of interest in a controlled area, especially by suggesting water treatments and decontamination of imported animals, as well as materials and equipment used in controlled area containment and eradication in case of an outbreak. The recommendations are linked to WP2 that concerns potential management practices and environmental factors that may influence the impact of diseases (in terms of morbidity and mortality), since eliminating pathogens from open systems is generally not practical. Thus, WP5 - Task 3 uses the outputs from WP2 (i.e. risk factors for diseases and mortality) to develop recommendations and advice for shellfish farmers in order to minimise the impact of diseases and for their control in open water sites.

The proposed recommendations for biosecurity are divided in four main areas:

- Identification and use of reliable sources of stock
- Application of good management practices
- Effective disease recognition
- Identification of effective measures to take in the event of a disease outbreak or unknown mortality

WP6. Dissemination and exploitation

WP 6 - T1: Dissemination activities beyond the consortium (publications, conferences, workshops and web-based activities aimed at disseminating the knowledge and technology produced)

Bivalife participants joined meetings organised by local shellfish farmer associations in order to present the Bivalife project and its main objectives. Thus, some Participants provided information for the local bivalve aquaculture sector (local sector meetings). To support overall Bivalife objectives, EMPA contributed to disseminate information and results towards European shellfish farmers mainly through their representatives. The coordinator of the project participated to regular EMPA meetings and presented the results obtained during the course of the project. Shellfish farmer representatives have shown a positive perception of the BIVALIFE project. Communication to both the general public and end-users is done via our website (http://www.bivalife.eu), which presents the partners and main objectives of the project.

Moreover, information on the Bivalife project was also reported in journals dedicated to shellfish farmers. Some Participants also disseminated the aims of the work as a “highlighted project” through web sites (Spanish web portal MisPeces and the Fundación OESA (http://www.fundacionoesa.es/proyectos-i+d-destacados/kbbe-bivalife). In addition, information on the Bivalife project was also reported in specialised journals and presented on TV networks.

Several Bivalife participants joined the BioTriangle initiative launched at Geelong (Australia) in October 2013 in the context of the International Bio-based Economy Forum. The workshop entitled "Diseases mitigation and prevention in mollusc aquaculture" was a collaborative initiative between the EU, Australia, Canada and New Zealand aiming cooperation on diagnosis and epidemiology of main pathogens and mitigation of related diseases affecting aquatic farmed mollusc species.

WP6 - T2: IP Protection

There is no project of patents.

The protection of the structure and data content of the project database (WP2, Tasks 2 and 4) is envisaged.

Confidentiality issues: all consortium participants contributing sampling data to the Bivalife database decide that the information should be kept confidential within the partnership, and their interests should be preserved until permission is granted for its release. Each Particpant is responsible for dissemination and publication of their own data and any one partner cannot use the data from another partner without express permission.