New species for EU aquaculture

Deliverable Report

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Deliverable Title	Protocol for early diagnosis of epitheliocystis during early stages of greater amberjack culture		
WP No:	25	**WP Lead beneficiary:**	P1. HCMR
WP Title:	Fish health-greater amberjack		
Task No:	25.1	**Task Lead beneficiary:**	P1. HCMR
Task Title:	Study of Epitheliocystis during larval rearing		
Other beneficiaries:			
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Objective: The objective of the task is to provide early diagnosis tools for Epitheliocystis infections in greater amberjack (*Seriola dumerili*)**.**

Introduction

Epitheliocystis is a bacterial disease affecting gills and skin in fishes. It has been reported in more than 50 freshwater and marine species worldwide, both wild and cultured (Nowak and LaPatra, 2006). The pathological condition is mainly diagnosed by the characteristic spherical inclusions -or cysts - in the infected cells (mostly epithelial cells but also chloride cells, mucous cells and pillar cells), containing the replicating etiological agent (**Figure 25.4.1**). The infection results in inflammation, epithelial hyperplasia, proliferative cell response and fusion of lamellae with consecutive reduction of oxygen uptake rates. It is lethal in the early developmental stages of the fish and benign for the adults (Katharios et al., 2008).

Epitheliocystis can be caused by various bacterial agents, initially identified as Gram-negative bacteria of the Chlamydiae phylum, and lately associated also with other β- and γ- proteobacteria. All those pathogens can be either obligate or facultative intracellular bacteria, residing and replicating within the host cell.

Intracellular pathogens have developed strategies to avoid the host response mechanism. Their progressive adaption from a free-living state to the intracellular life-style often leads to the phenomenon of genome reduction: irreversible genome loss with progressive dependence from the host (niche specialization)

(Casadevall, 2008). Due to the inability of bringing these bacteria into culture, new genomic methodologies have been lately applied in order to understand their structure, metabolism, life strategies and evolution (Seth-smith et al., 2015).

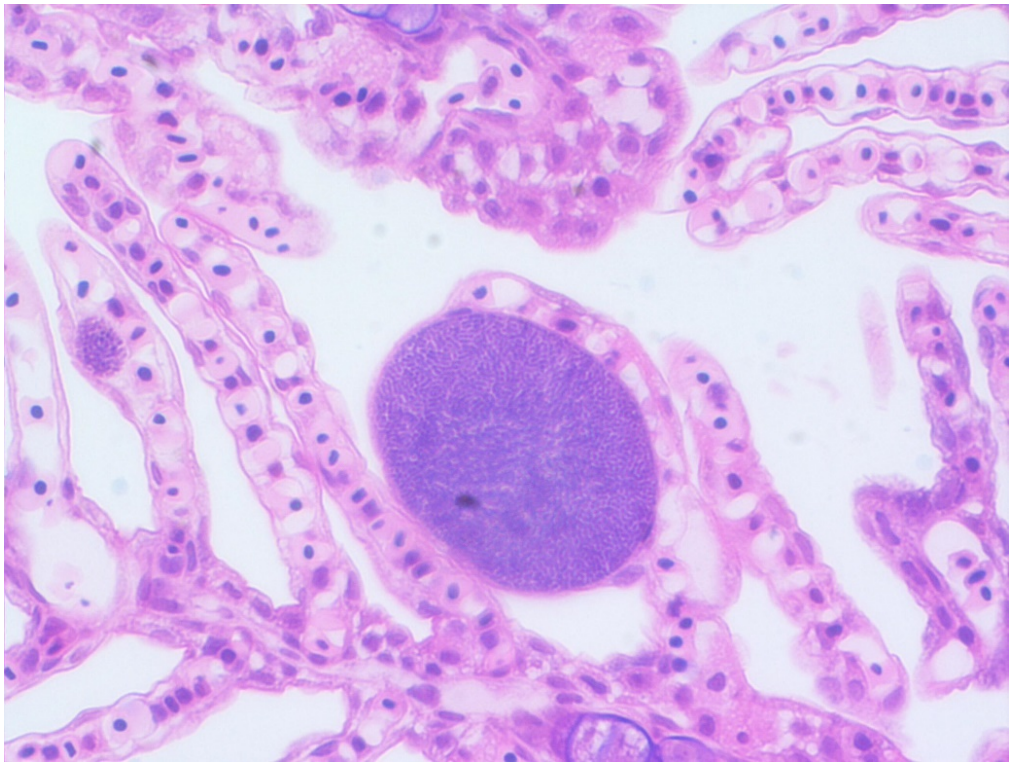

Figure 25.4.1. Histological section of juvenile common dentex (*Dentex dentex*) gill affected by epitheliocystis (Katharios personal collection).

In the past years, epitheliocystis in Greece has been investigated by Katharios *et. al* in the Hellenic Centre of Marine Research (Crete), in partnership with Vaughan *et al.* from the Veterinary Pathology department of the University of Zurich (Switzerland) and in collaboration with different commercial fish farms contributing samples (Katharios et al., 2015; Seth-smith et al., 2015). Moreover, within the frames of DIVERSIFY and more specifically in Task 25.1 a study was undertaken by Angelikì Antonakaki (MSc student of the University of Crete) which has provided a general epidemiological survey on different hosts: sea bream, amberjack, sea bass and sharpsnout sea bream. Those studies have shown the diversity of the bacterial agents causing the disease in Greece and provided thorough morphological characterization using advanced imaging techniques but also insights on their biology and life style through the direct sequencing of their genomes (Qi et al., 2016). In addition, the seasonality of disease occurrence has been mapped confirming that the susceptibility of the host depends on the developmental stage but also on a compromised immune system and high stress level. The most critical phase of the fish is during their transition from the hatchery environment to the open sea. This is the time window where most of the epitheliocystis outbreaks occur and can become responsible for losses as high as 20%. In addition, larval stages are also a critical phase in cases where natural sea water is used instead of borehole water (eg mesocosm hatcheries).

Greater amberjack is a sensitive species to epitheliocystis. Both wild and cultured species have been affected by this disease (Crespo et al., 1990; Grau and Crespo, 1990; Venizelos and Benetti, 1996). Therefore, the main objective of the current task was to develop tools for early diagnosis of the disease; this task gave the opportunity to study the disease in this species within the new context of molecular diagnostics that provide higher resolution in terms of identifying the aetiological agent.

Materials and methods

Experimental design

Since the main objective of the task was to develop and validate tools for the early detection of epitheliocystis, we exposed larval fish to untreated seawater using the mesocosm technology at the facilities of HCMR assuming that they will be infected naturally. This is because all microbial agents related to the disease are not cultivable *in vitro*. The experimental design followed the one proposed and applied earlier in sharpsnout seabream (Katharios et al 2015). According to this protocol, fish are monitored daily by visual inspection for lesions, and every other day using PCR for the presence of the pathogens. At the same time, rearing water is sampled at the inlet using fractionation following serial filtration and filtrates are also screened using molecular tools.

In addition to the larval cultures, a nation-wide sampling was also implemented in order to study the prevalence of the disease but also the diversity of the bacterial agents responsible for the lesions. We implemented 3 surveys with the participation of the biggest fish farms in Greece which provided samples for the analysis.

Larval rearing monitoring

2014 experiment

One 5-m^3 mesocosm tank receiving unfiltered natural sea water was stocked with 5,000 greater amberjack eggs on 25th of June 2014. The environmental parameters of the tank water were monitored daily. Following mouth opening the fish were provided live rotifers while fresh microalgae *Chlorella minutissima* were added in the rearing water daily.

Fish were visible at the beginning of the culture however their population density was decreasing daily which together with water turbidity due to the microalgae made fish sampling extremely difficult. At 20 dph (14th of July) the rearing was terminated due to the extremely low number of fish present in the tank.

Five samplings were made during the course of the experiment. At each sampling 10 L of rearing water was passed through serial filters of 250, 120, 53, 25 μm and 1 L of the last filtrate was passed through 0.22μm filters using sterile syringes. All filtrates were collected in 3 replicate 50 mL vials and stored in -80^{o}C, or RNA*later* and phosphate buffered formalin following centrifugation at 3,000 rpm for 5 min.

DNA from each filtrate and from the filters of 0.22μm was extracted at the same day of the sampling and subjected to PCR using universal chlamydia primers but also specific primers for bacteria which were associated with epitheliocystis previously. The primers used in the PCR analysis are shown in **Table 25.4.2**. DNA from positive controls was also included using previously studied epitheliocystis agents from Crete and other places in Greece.

2015 experiments

Two parallel larval rearing trials were made in HCMR using the mesocosm technology described earlier with two fish species; *Seriola dumerili* and *Argyrosomus regius*. **Table 25.4.1** contains the information concerning these trials and the samples obtained.

Ten L of water were taken from the water column of the tanks at each sampling in triplicates. Samplings were made using a specially designed sampler.

Samples were fractionated. In particular, they were consecutively filtered through decreasing pore diameter filters: **250 μm, 120 μm, 53 μm** and **25 μm.** The samples obtained (filtrates & larvae) were used for **1)** histology, **2)** molecular analysis and **3)** preserved and stored for future use. In addition, 1-2 L of flow-through water from the abovementioned fractionation were passed through a **0.22 μm** filter. This filter paper was divided in three equal and used for **1)** molecular analysis, **2)** isolation of bacteria in general nutrient media and **3)** preservation and storage for future need.

Table 25.4.1. Data of the rearing trials and the samples obtained

Date	Water source	*Argyrosomus regius*		*Seriola dumerili*		Samples
		sampling	age (dph)	sampling	age (dph)	
2/7/2015	Borehole	1	hatching			filtrate + eggs + larvae
3/7/2015	Borehole		1			
4/7/2015	Borehole		2			
5/7/2015	Borehole		3			
6/7/2015	Borehole		4			
7/7/2015	Borehole	2	5			filtrate + larvae
8/7/2015	Sea	3	6	1	hatching	filtrate + larvae
9/7/2015	Sea		7		1	
10/7/2015	Sea	4	8	2	2	filtrate + larvae
11/7/2015	Sea		9		3	
12/7/2015	Sea		10		4	
13/7/2015	Sea	5	11	3	5	filtrate + larvae
14/7/2015	Sea		12		6	larvae
15/7/2015	Sea	6	13	4	7	filtrate + larvae
16/7/2015	Sea		14		8	larvae
17/7/2015	Sea	7	15	5	9	filtrate + larvae
18/7/2015	Sea		16		10	
19/7/2015	Sea		17		11	
20/7/2015	Sea	8	18	6	12	filtrate + larvae
21/7/2015	Sea		19		13	larvae
22/7/2015	Sea	9	20	7	14	filtrate + larvae
23/7/2015	Sea		21		15	
24/7/2015	Sea	10	22	8	16	larvae
25/7/2015	Sea		23		17	
26/7/2015	Sea		24		18	
27/7/2015	Sea		25	9	19	larvae
28/7/2015	Sea		26		20	
29/7/2015	Sea		27	10	21	larvae
30/7/2015	Sea		28	11	22	larvae
31/7/2015	Sea		29	12	23	larvae

1/8/2015	Sea		30		24	
2/8/2015	Sea		31		25	
3/8/2015	Sea		32	13	26	larvae

In total, 10 samplings were made for *Argyrosomus regius* and 13 for *Seriola dumerili* in 2015.

The filtrates were placed in 50 mL falcons and centrifuged immediately at 5,000 rpm for 15 min. The supernatant was discarded and the pellet was transferred into a 1.5 mL Eppendorf, recentrifuged at 20,000 rpm and the resulting pellet was stored until further analysis. The filtrates for histology and molecular analysis were preserved in the appropriate volume of preservative, 10% PBF and RNA later respectively while the filtrates for future use were stored in -80ºC. Filtrates from the 0.22 μm filters were preserved in 25% glycerol at -80ºC.

Total DNA extraction was performed from the filtrates, the individual larvae samples and the bacteria isolated in bacterial cultures. Filtrate and larvae samples were digested using Proteinase K (1-3 h at 56ºC) and a DNeasy Blood & Tissue Kit was used for the DNA extraction. DNA of the microorganisms from the 0.22 μm diameter filter was similarly extracted whereas DNA from the cultivated bacteria was extracted via boiling.

Specific pairs of primers were used for each pathogen. The primers and PCR conditions are presented in detail in **Table 25.4.2.**

Table 25.4.2. Primers and the PCR conditions for detection of pathogens, the microorganism targets.

Pathogen	Primer	Primer's sequense (5'-3')	Annealing temperature	Extension duration	Product's length
Endozoicomonas spp.	Endo sp F	AGTAGGGAGGAAAGGTTGAAGG	60ºC	30 sec	400 bp
	Endo sp R	CCCAGAATACAAGACTCCGGAC			
Ichthyocystis spp.	Ichthyo sp F	AACTARGATGGTGGCGAGTG	60-62ºC	1 min	900 bp
	Ichthyo sp R→	CGCACATGTCAAGGGTAGG			
Chlamydiaceae	IGF	GACTAGGTTGGGCAAG	55ºC	30 sec	300 bp
	IGR	AGCTCTTA(T/G/A)(C/T)AACTTGGTCTGTA			

F=Forward primer; R=Reverse primer.

One third of the 0.22 μm filter was used for the isolation of microorganisms of each sampling day. In order to achieve the isolation of the etiological agents, pieces of the filter were cultivated in Marine agar and Marine broth.

Fish farm survey

Survey 1

Apart from the experimental larval rearing that was performed for pathogen isolation, the observation and the understanding of the biology and the life cycle of epitheliocystis agents at the HCMR facilities in Crete, gill samples of cultured gilthead seabream with confirmed epitheliocystis were collected from collaborating fish farms of Greece. The samples collected were from cage farms in various regions, including Arkadia, Argolida, Galaxidi, Astakos, and Euvoia. The samples were analyzed by PCR and histology in HCMR while analysis was completed at the Veterinary Department of Zurich University (Prof. Lloyd Vaughan) within the

framework of cooperation of other research programs (Aquaexcel, KRIPIS). qPCR, fluorescent in situ hybridization (FISH), TEM and genomic analysis were performed in Zurich.

Figure 25.4.2 illustrates the typical morphology of the cysts of *Ca.* Ichthyocystis sp. in gilthead seabream gills. *Ca.* Ichthyocystis spp. are the most prevalent bacterial agents causing epitheliocystis in Greece.

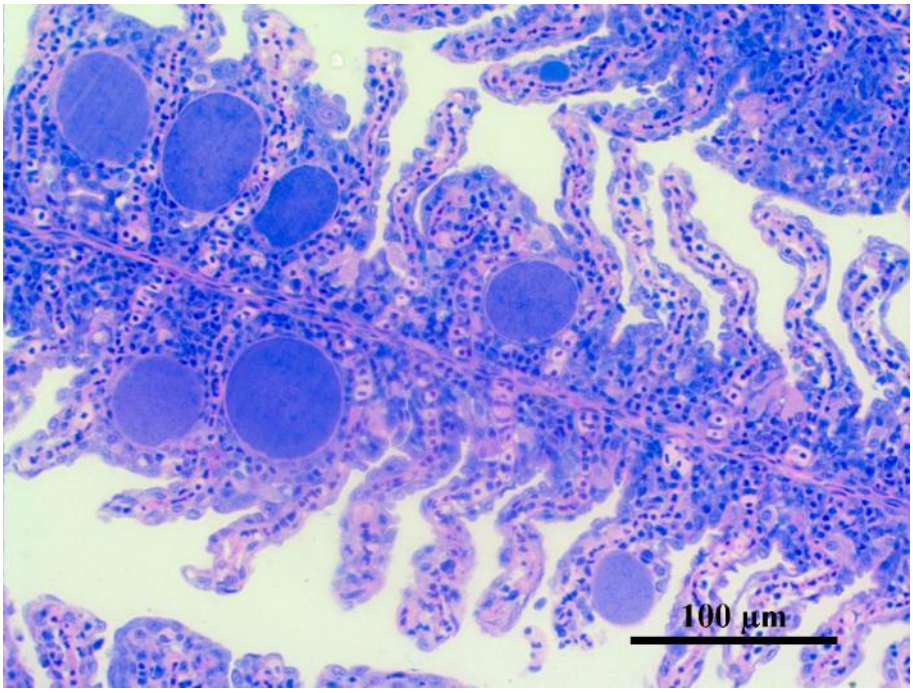

Figure 25.4.2. Typical appearance of *Ca.* Ichthiocystis sp. Intracellular inclusions in the gill epithelium of the secondary lamellae can easily be observed. The sample is from a sea bream farmed in Argolida.

Survey 2

Since the study of epitheliocystis is an active and ongoing interest for our group, trying to acquire comprehensive knowledge of the disease, the life cycle of the pathogens and its effect on the host, more samples of sea bream gills are being collected from the collaborating fish farms. In combination with the molecular, histology and electron microscopy analysis that are being conducted, we are trying to observe if there is a correlation between:

- Season (collecting samples whenever an incident of epitheliocystis emerges in a fish farm)
- the water temperature of the farm cages in the regions sampled
- the fish age
- the time period from when fish were put out in the sea cages to when they got infected
- the prevalence (how many fish were infected out of the total number screened during the sampling from the cages)
- and the intensity (an estimation of how severe the infection is related to the number and size of the cysts observed on the gills).

The new samples covered the period March 2015 – September 2016. They were derived from 3 different hosts, gilthead seabream, European seabass and greater amberjack cultured in 8 different geographic localities in Greece (**Figure 25.4.3**).

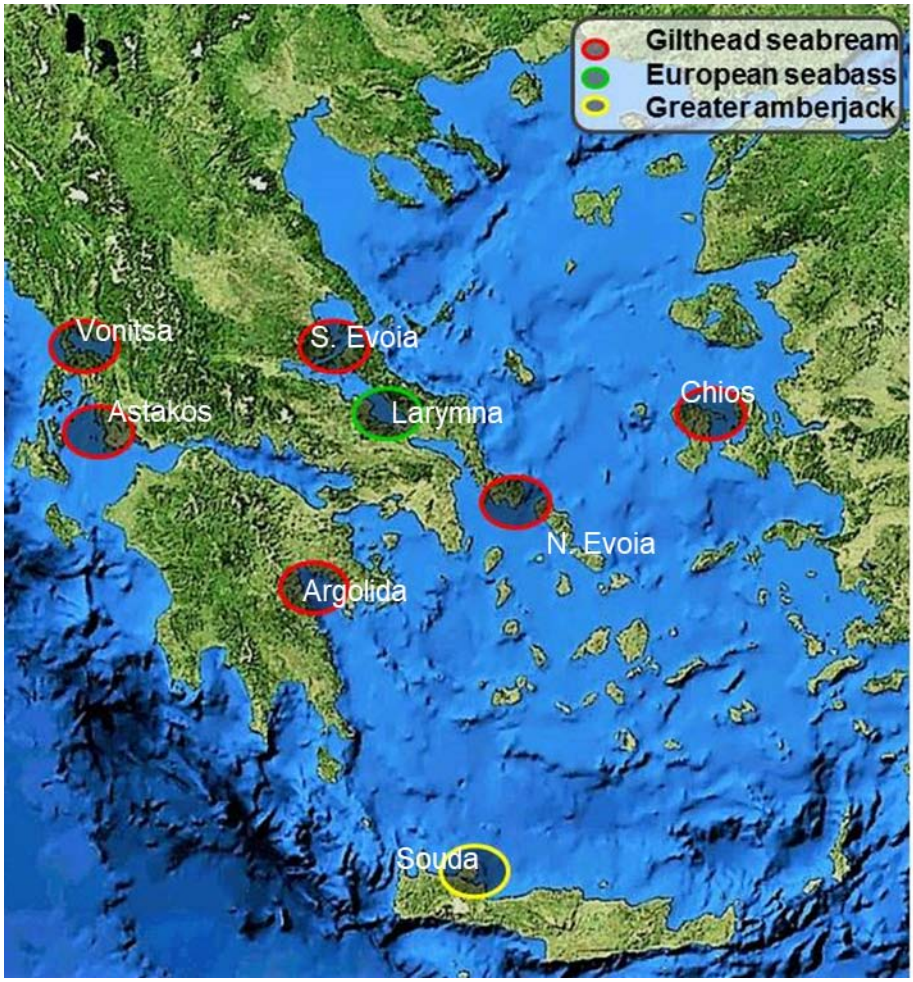

Figure 25.4.3. Sampling stations. Different color code indicates the fish species sampled.

The number of fish sampled per species together with all information regarding culture conditions are shown in **Table 25.4.3.**

Table 25.4.3. Data on the samples processed for the detection of Epitheliocystis

Fish species	n	Age (months)	Time in the sea (months)	Weight (g)	Temperature (°C)
Gilthead seabream	21	4 - 9	1.5 - 6	10-70	13.8-23
European seabass	3	10.5	6	15	12
Greater amberjack	9	4	2	60 - 139	26

Survey 3. Epitheliocystis outbreak in farmed greater amberjack at the pilot farm of HCMR in Souda, Chania

An incidence occurred in a population of on-growing greater amberjack farmed at the cage farm of HCMR in Souda (Crete, Greece). Greater amberjack adults of average weight 225g suffered cumulative mortalities of approximately 0.5% from November until December 2017. Initially, fish samples (n=5) were sent to the Fish Diseases Lab of HCMR for analysis where visible lesions consistent with epitheliocystis were found on the gills of the fish. Gill samples were taken immediately for microbiology, histology, TEM, PCR and genomic analysis. Sampling was also made after 2 days on site where another 20 fish were sampled. The population of greater amberjack is being followed on a monthly basis together with juvenile fish of other species farmed in the site including gilthead seabream, seabass and meagre.

Sample analysis

The pipeline for the analysis of the samples is shown in **Figure 25.4.4.**

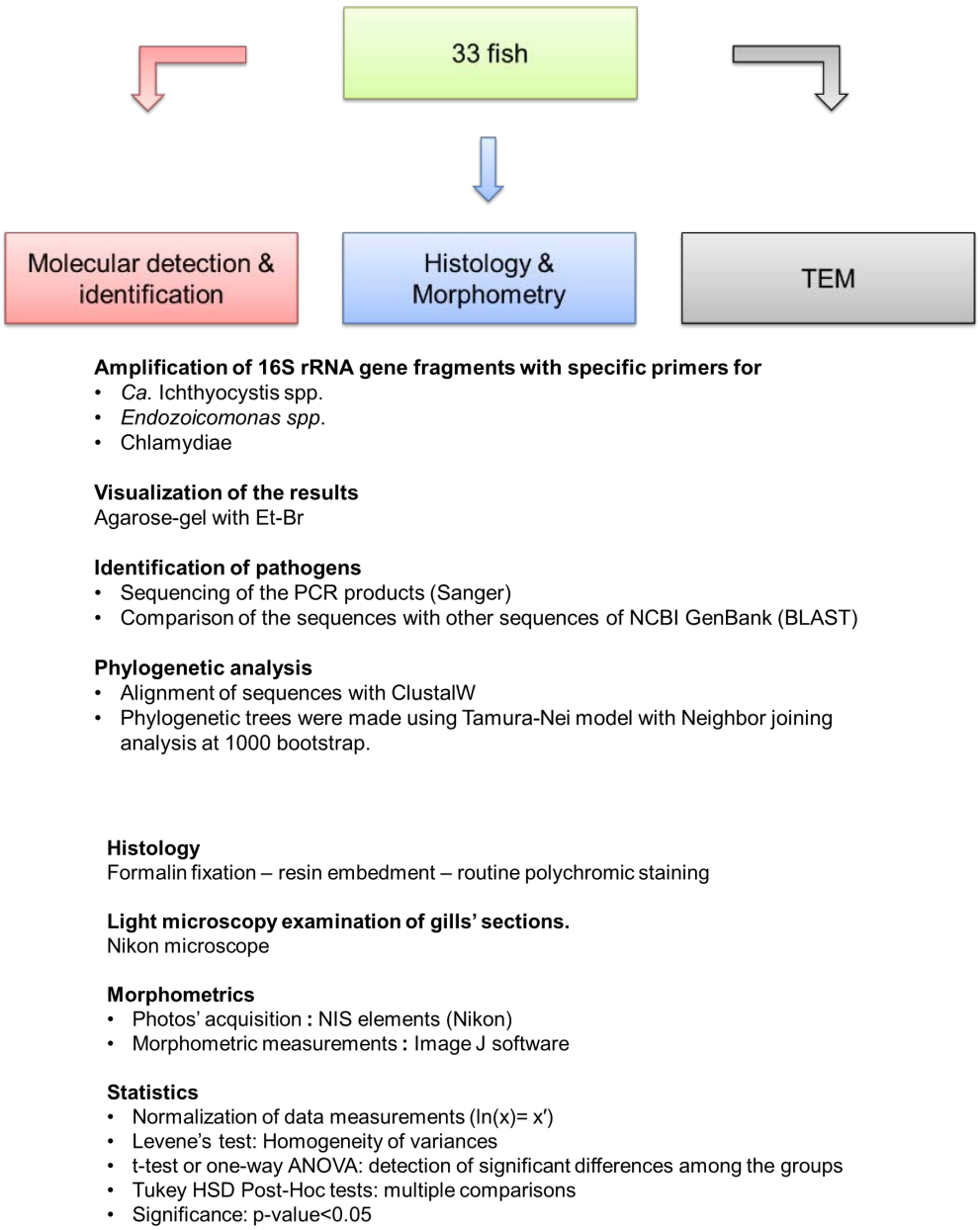

Figure 25.4.4. The identification pipeline for the processed samples.

Electron Microscopy

Selected samples were also processed for Electron Microscopy at the EM Lab of the University of Crete. Gill samples were fixed in 2% glutaraldehyde in sodium cacodylate buffer and were embedded into epoxy resin and prepared for TEM according to standard procedures. Gill sections containing epitheliocystis lesions were selected from epoxy resin blocks using semithin sections (1 μm) stained with toluidine blue (Sigma–Aldrich). Ultrathin sections (80 nm) were mounted on copper grids (M, contrasted with uranyl acetate dihydrate (Sigma–Aldrich) and lead citrate (Merck Eurolab AG) and investigated using a JEOL JEM2100 transmission electron microscope.

Results

Larval rearing experiments

There was no evidence of the disease in any of the greater amberjack or the meagre (*Argyrosomus regius*) larvicultures conducted. The survival of fish and the duration (12 days) of the rearing process was remarkably low in the experimental larval rearing in 2014. Possibly, this problem was related to bad quality of the eggs and some technical issues (*e.g.* regulation of temperature of the tanks, shading of the tanks etc.), since there were no pathogens isolated from the fish. For the experiment in 2015 the technical issues were solved resulting in a 33-day rearing duration with the survival and development of the larvae being similar to that referred to in the bibliography.

Briefly, in the two parallel cultures none of the microorganism-targets were detected on the larvae. This was in accordance with the fact that epitheliocystis disease clinical signs (eg inclusions in gills and skin) were not found in either fresh preparation or by histology. A positive signal for *Chlamydiaceae* and *Endozoicomonas* spp. appeared for some of the filtrates. A positive signal for *Chlamydiaceae* was observed in different filtrates of both experiment and algal samples from the microalgae cultures used for the rearing process during the early developmental stages of the larvae. A positive signal for *Endozoicomonas* spp. was observed in different filtrates of the same sampling days during the first days of the experiment (2015) but it was not detected in any of the following samplings. Finally, there was no positive signal for *Ca* Ichthyocystis spp in the samples analyzed.

Fish farm surveys

Survey 1

Two different morphological types of cysts were observed in gill histology. The majority of the cysts were big with a diameter of 80-100 μm circumscribed in a fine, thin membrane whereas there were a few others of smaller size with the outer membrane slightly thicker and the content of the cyst being intensely granular.

Gill samples with Epitheliocystis from the different fish farms of Greece were screened using PCR with specific primers for the particular species in order to identify the presence of the pathogen in other regions of Greece as well.

More than 60 samples were screened in total, covering the time period 2012-2015 and the regions of: Argolida, Arkadia, Astakos, Saronikos, Galaxidi and Euvoia. All samples were positive for Ichthyocystis and Chlamydia of the Piscichlamydia genus. qPCR analysis showed that the majority of the load belonged to Ichthyocystis identifying it as the main and dominant pathogen in Greece with wide geographical distribution.

The results of the experiments, the samplings and the analysis process revealed that, in contrast to the prevalent belief that epitheliocystis is attributed to Chlamydia, at least in Greece the main pathogens causing epitheliocystis disease are bacteria that belong to the β- or γ-proteobacteria. These bacteria have a mainly intracellular life cycle and their cultivation in vitro has not been accomplished yet.

Survey 2

Gill samples with confirmed epitheliocystis from the different fish farms of Greece

Of the 33 samples analyzed,

- All (33) samples were positive for *Ca.* Ichthyocystis spp.
- 15 samples were positive for Chlamydiae
- 2 samples were positive for *Endozoicomonas spp.*

Chlamydiae were found in gilthead seabream from Astakos, S. Evoia, N. Evoia, Argolida, Vonitsa and in European seabass from Larymna. *Endozoicomonas* was found in gilthead seabream from Chios.

The phylogeny of the 3 different aetiological agents of Epitheliocystis was analyzed separately for each bacterial species (**Figures 25.4.5-7**).

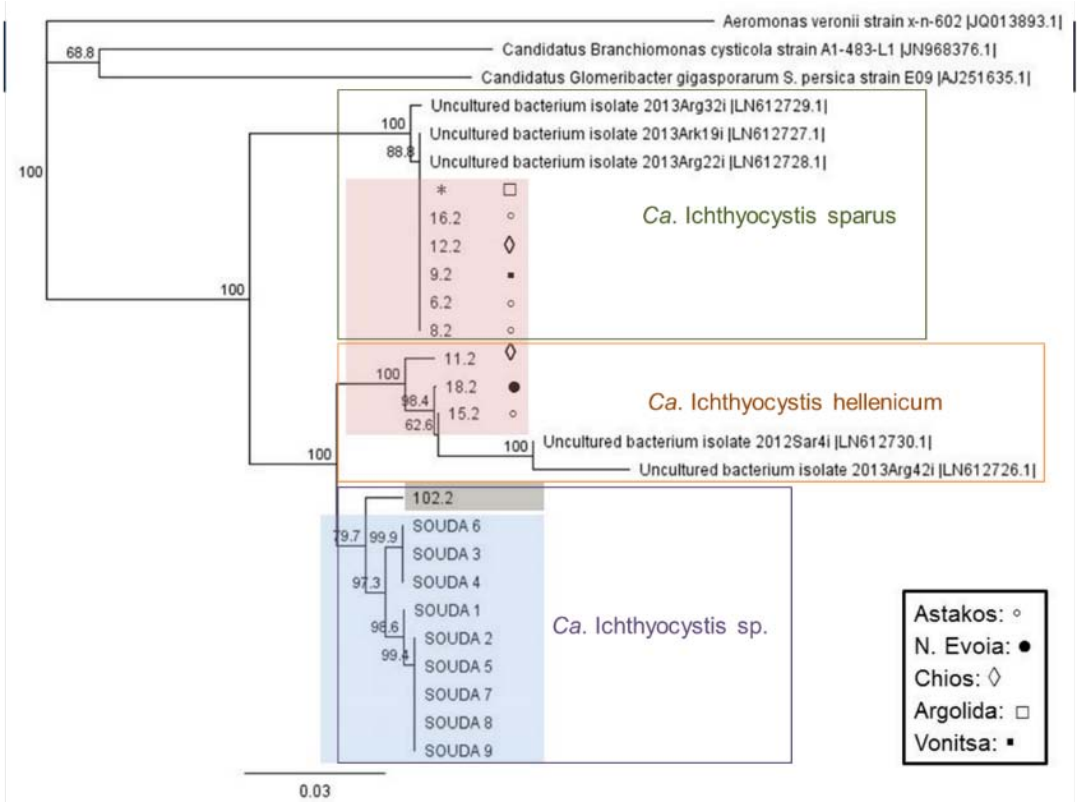

Figure 25.4.5 Phylogenetic analysis of the *Ca.* Ichthyocystis spp.-positive samples. The majority of the samples from mainland Greece are clustered together with the two species of the genus already described previously; *Ca. Ichthyocystis sparus* and *Ca. Ichthyocystis hellenicum*. However, there is a third cluster (blue color) containing the samples from on-growing adult greater amberjack from Crete (Souda) and a sample from seabass from Larymna (102.2). This cluster may represent a novel species of the genus.

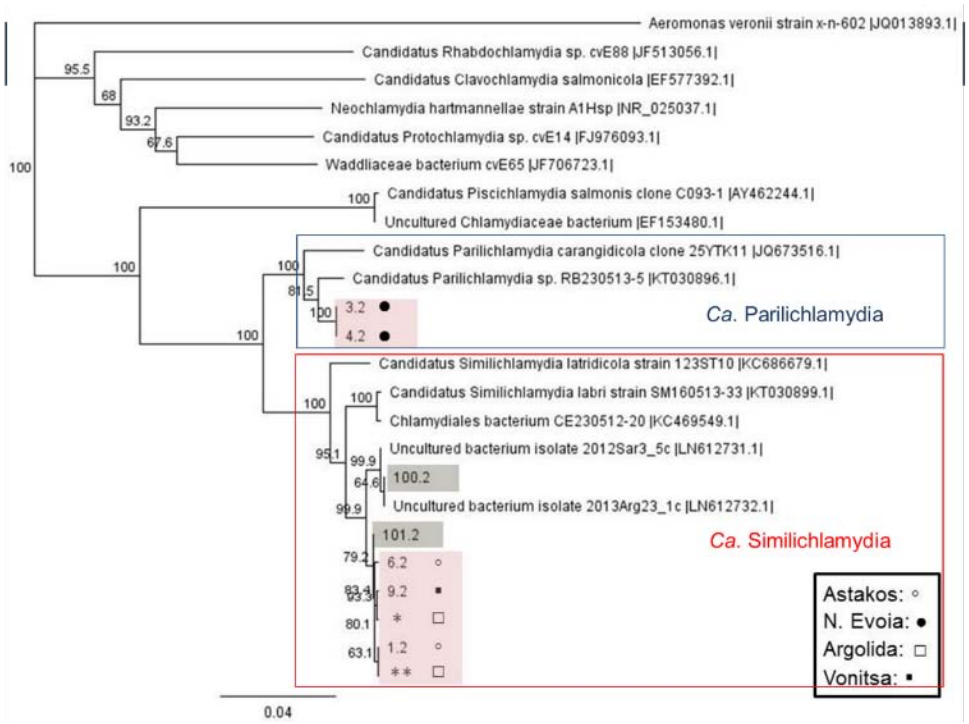

Figure 25.4.6. Phylogenetic analysis of the *Chlamydiae*-positive samples. The samples obtained from this survey cluster with *Ca.* Similichlamydia and *Ca.* Parilichlamydia.

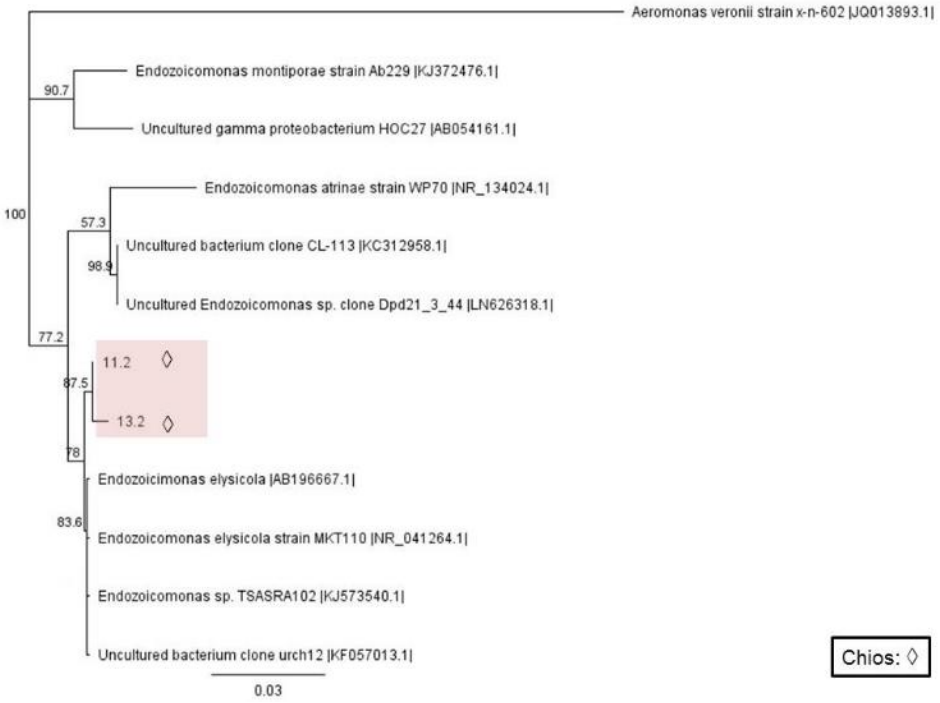

Figure 25.4.7. Phylogenetic analysis of the *Endozoicomonas*-positive sample. This sample was obtained from seabream cultured in Chios.

Histologically, the cysts of epitheliocystis can be divided in 4 morphologically distinct groups. Type a: big cysts containing fine granular material, type b: big cysts with coarse granular material, type c: cysts with coarse granular material surrounded by a "ring" formation and type d: small cysts with a ring surrounded by a capsule **(Figure 25.4.8)**.

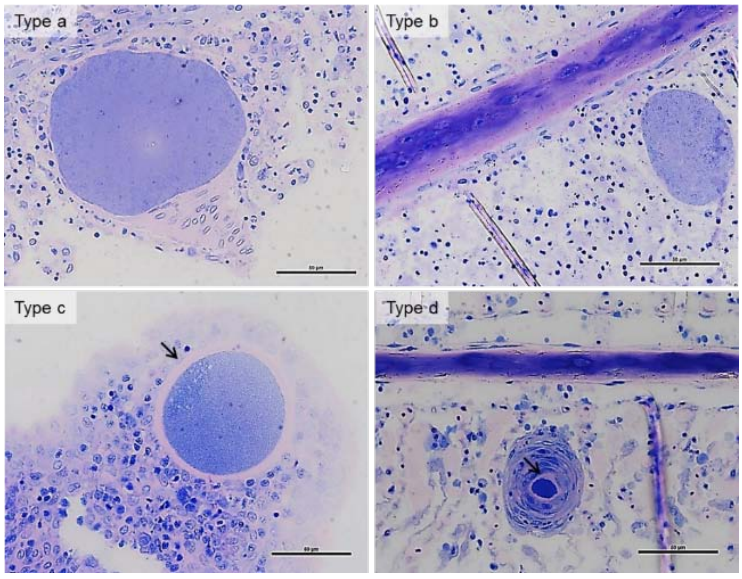

Figure 25.4.8. The four different types of cysts identified in the survey.

The morphometric characteristics of the cysts were compared based on the cyst type (**Figure 25.4.9**) and the fish species (**Figure 25.4.10**). Morphometry of the cysts obtained from greater amberjack are shown in **Figure 25.4.11**.

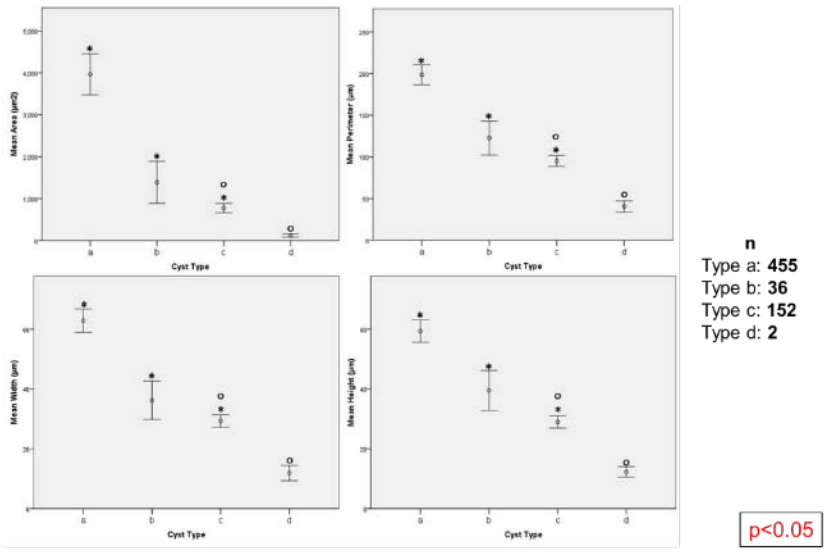

Figure 25.4.9. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the 4 cyst types.

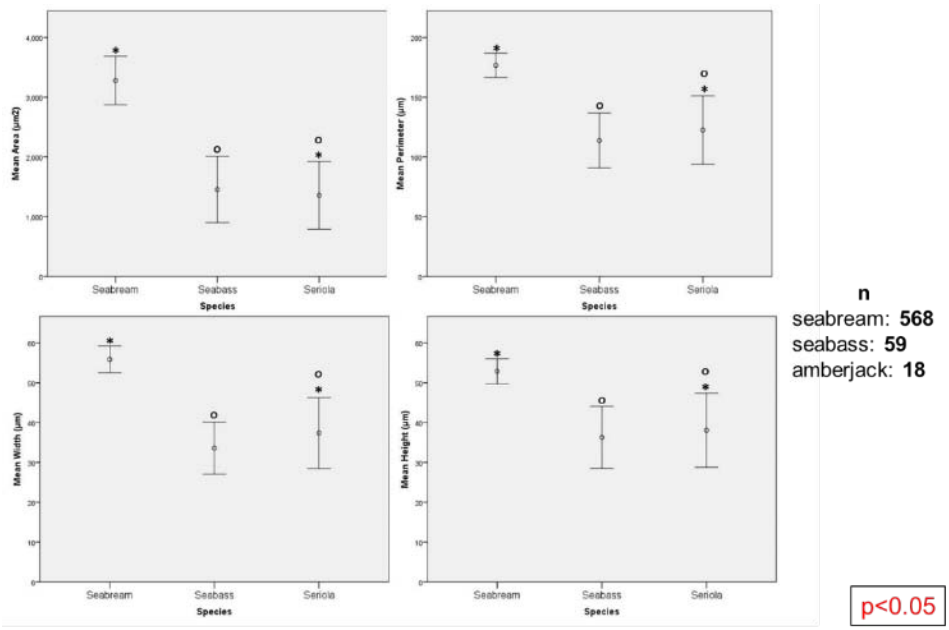

Figure 25.4.10. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the cysts in the three species studied.

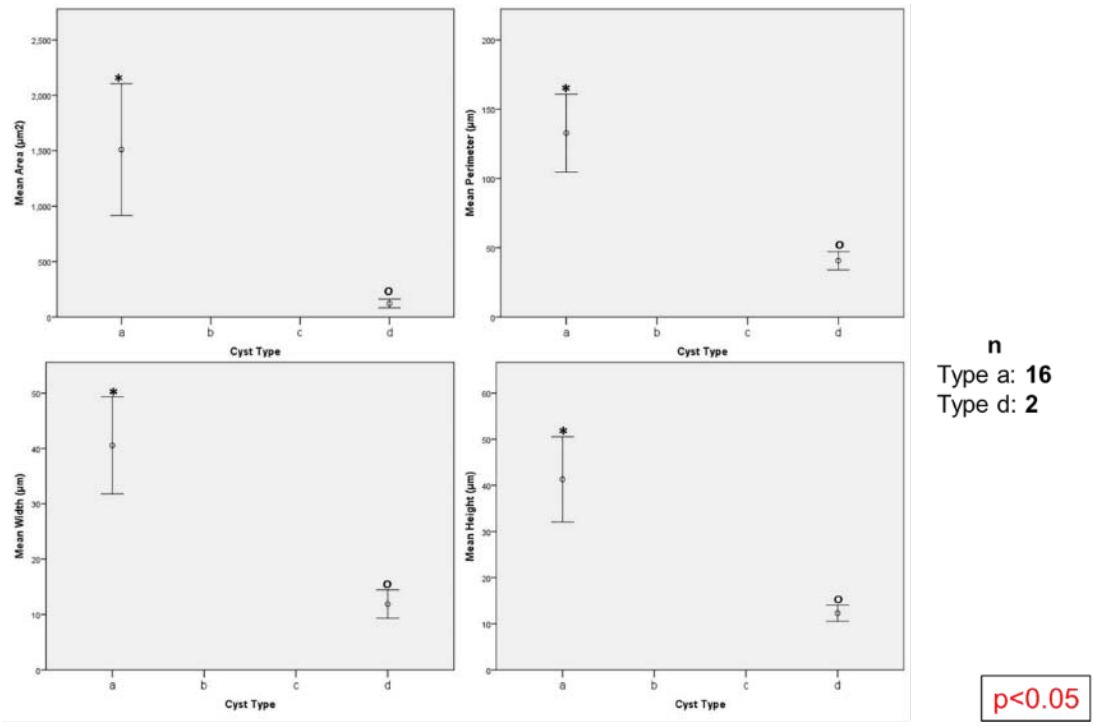

Figure 25.4.11. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the cysts in the samples of greater amberjack.

Transmission Electron Microscopy (TEM)

Two samples were processed for TEM; both were gills from seabream, one from Astakos (8.3) and one from Chios (14.3). The morphology of the bacteria in both cases are consistent with *Ca.* Ichthyocystis spp. (**Figure 25.4.12**).

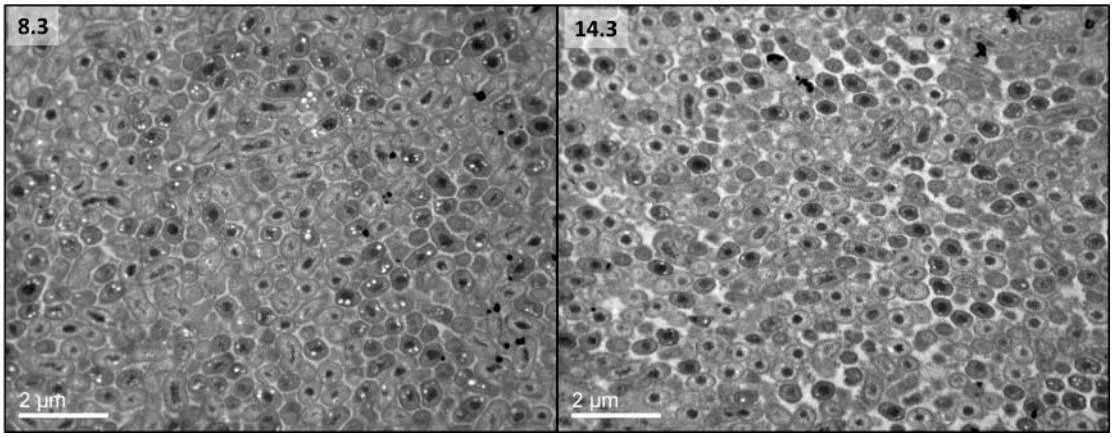

Figure 25.4.12. TEM micrograph of the bacteria inside the cysts. In both cases the bacteria are densely packed and their morphology is consistent with that of *Ca.* Ichthyocystis spp.

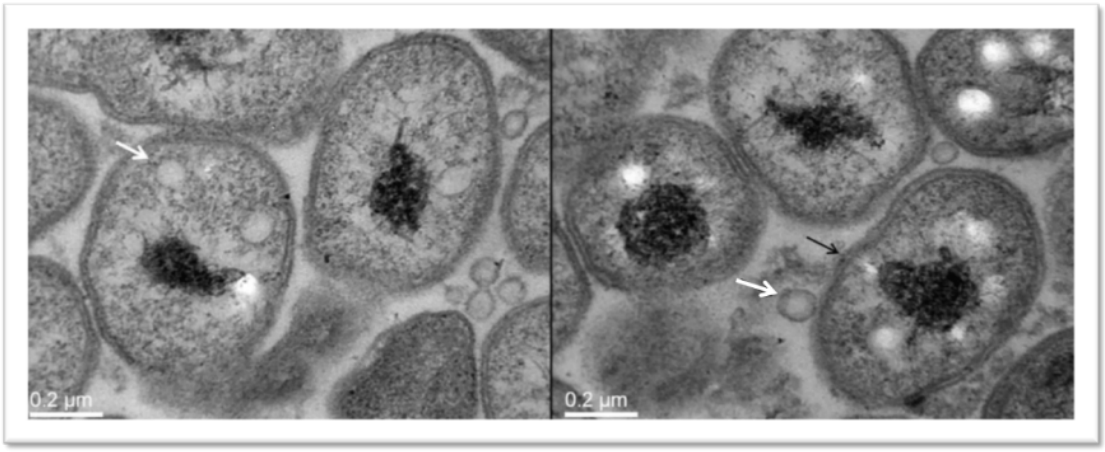

Figure 25.4.13. TEM micrograph of the bacteria inside the cysts. The bacteria have a visible double membrane (black arrow) and contain spherical bodies (white arrows). Compact nucleoids appear dark in the center of the bacteria.

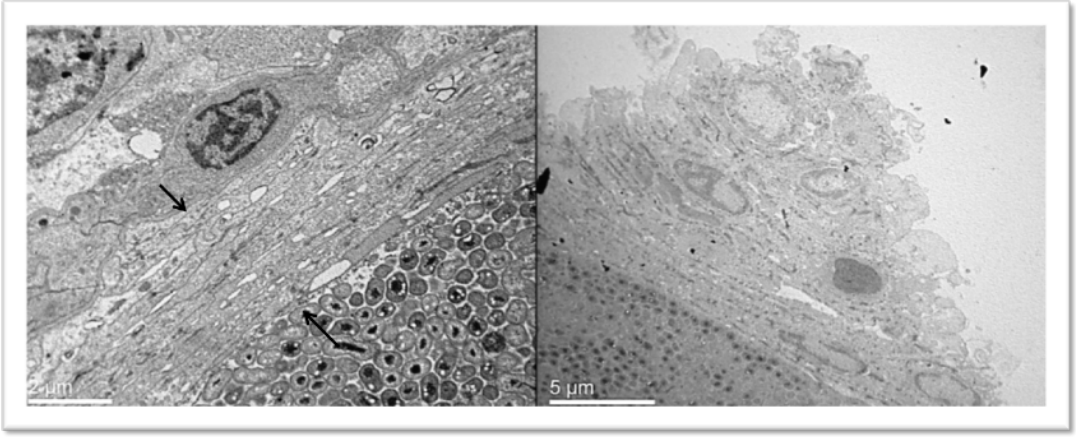

Figure 25.4.14. Bacteria are clearly separated from the fish tissue by interconnected epithelial cells which form a thick layer.

Survey 3. Epitheliocystis outbreak in farmed greater amberjack at the pilot farm of HCMR in Souda, Chania

In total 25 greater amberjack (MW: 225g) were sampled during the outbreak of epitheliocystis in December 2017. Affected fish had pale gills with excess mucous (**Figure 25.4.15A**). Fresh squash preparations of the gills were examined using a light microscope. Numerous cysts were readily visible (**Figure 25.4.15B**) indicating an intense infection of epitheliocystis.

Samples were processed for histology and PCR analysis using the primers developed in this task. All samples examined were positive for *Ca.* Ichthyocystis spp. The positive PCR bands were extracted and sequenced. The sequences obtained were identical to those obtained the previous year in the same location again in greater amberjack. It should be emphasised that for the previous case, detection was made only with PCR since no cysts were visible either in histology or by examination with light microscopy. This was probably due to either a very low bacterial load or possibly because of the stage of the infection (too early or too late). Using BLAST analysis, we have concluded that the Souda causative agents of epitheliocystis indeed belongs to a new species of the genus *Ca.* Ichthyocystis since the % similarity at the nucleotide level for the 16s gene is around 95% to the closest relative which is *Ca. Ichthyocystis hellenicum*. Further genomic analysis is currently underway in order to characterize this novel species.

The pathology of epitheliocystis in greater amberjack was distinct when compared to the pathology observed in other fish species caused by members of the Ichthyocystis genus. There was a massive inflammatory reaction with fusion of the secondary lamellae and multiple granulomas spread throughout the gill tissue (**Figure 25.4.15C-F)**. Granulomas consisted of epithelioid cells, a few lymphocytes and multinucleated giant cells of the Langhans type. The histological state of the gills indicated a late stage of the infection. The overall cumulative mortality that could be attributed exclusively to epitheliocystis was approximately 0.5% within a month. However, the population experienced higher mortalities earlier due to infection by the monogenean *Zeuxapta seriolae.* It is not possible to assess whether the mortalities observed during the first 5 months of rearing in the sea were due to the parasite, the epitheliocystis disease or a combination of the two diseases.

Since the bacteria of the *Ca.* Ichthyocystis genus are obligately intracellular, we assumed that an intermediate host could be implicated serving as a vector for the transmission of the pathogens. To investigate this possibility, we screened commensal organisms from the fouled cage containing the infected greater amberjack.

Selected potential hosts growing on the cages were ascidians, bivalves, sponges and anemones. The following organisms were sampled: ascidian (pharynx and endostyle) (n=1), *Mytilus* sp. bivalve (gills) (n=2), pinctada type bivalve (gills and digestive gland) (n=3), anemone (whole body and tentacles) (n=2), sponges (n=2). Following DNA extraction, the samples were screened for Ichthyocystis and all were negative.

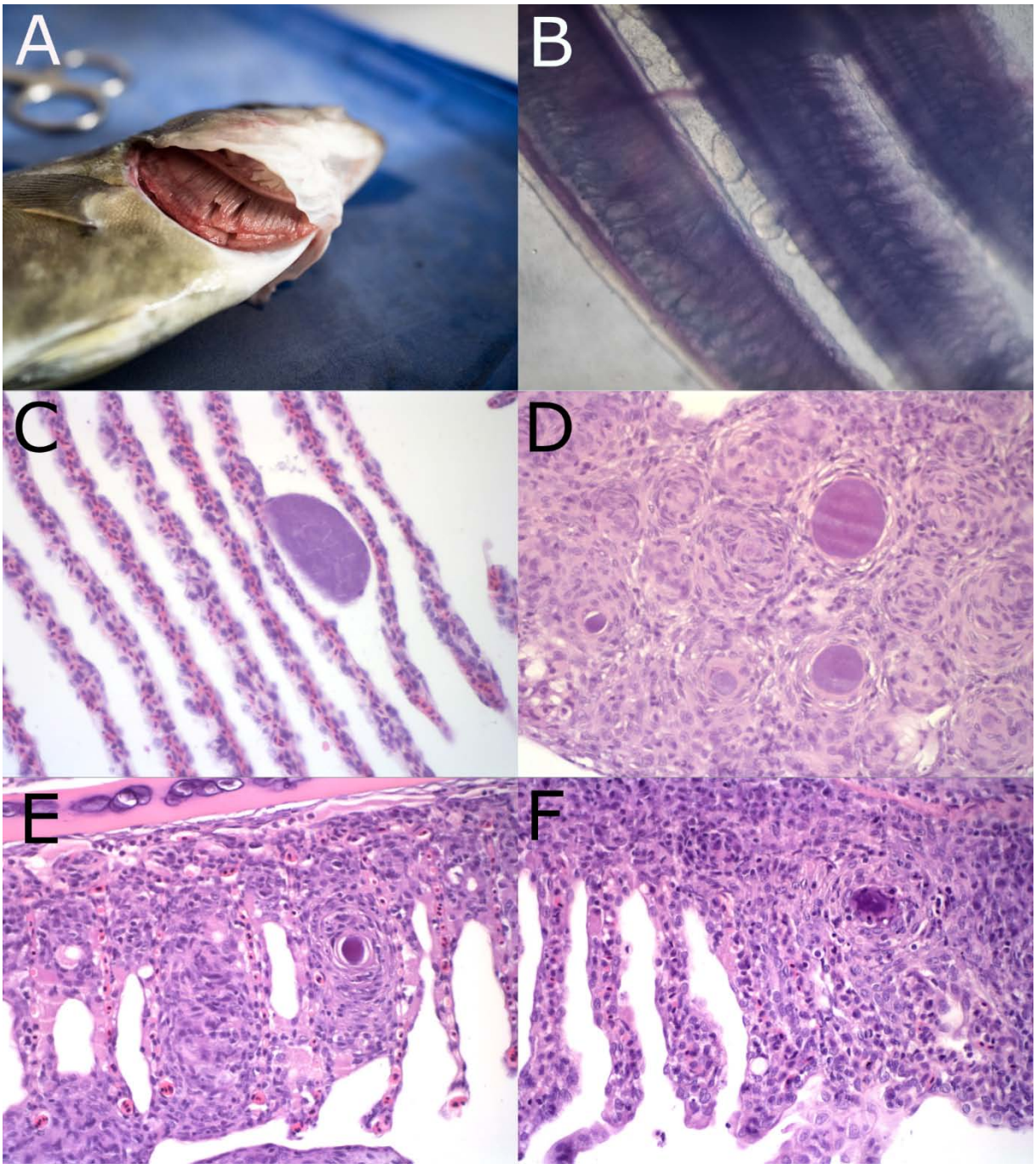

Figure 25.4.15. A. Pale gills with increased mucous in epitheliocystis-affected greater amberjack from Souda. **B.** Epitheliocysts were visible in fresh squash preparations of gill biopsies when examined under a light microscope. **C.** Typical inclusion containing granular material consistent with the description of epitheliocystis. **D.** Two large inclusions containing the bacterial agents and numerous granulomas that are remnants of the host response against the infection. **E** and **F**. Secondary lamellae are fused and massively inflamed following epitheliocystis infection.

Discussion

In HCMR facilities, Epitheliocystis has affected larviculture using 'Mesocosm' technique many times. The disease has emerged twice in sharpsnout seabream (*Diplodus puntazzo*) larviculture (Katharios et al., 2015, 2008) and in greater amberjack and common dentex (*Dentex dentex*) culture. From the incidents referred to above, molecular identification succeeded for the second incident of an Epitheliocystis outbreak in *Diplodus puntazzo* (Katharios et al., 2015, 2008) when the main pathogen related to the disease was named *Candidatus* Endozoicomonas cretensis n. sp. One of the main problems is that most of the time fish are simultaneously co-infected by more than one epitheliocystis-causing bacterial agent. As a result, the identification of the pathogen responsible for Epitheliocystis demands the use of techniques such as qPCR or FISH.

In this regard we have developed and tested molecular probes that can successfully detect the pathogens which are responsible for the disease.

From the survey we conducted in the Greek fish farms we confirmed our hypothesis that epitheliocystis can be caused by various unrelated pathogens which in many cases infect simultaneously the same host. We also confirmed that the most significant pathogen is *Ca.* Ichthyocystis spp. and we have possibly found a novel species of the genus, which infects adult greater amberjack. We will proceed with the analysis of the novel pathogen and it would be very interesting to assess whether this is species-specific for greater amberjack or is specific to the geographic area of detection. We have collected the necessary samples to proceed with whole genome sequencing of the pathogen and have also collected host tissue that will help us study the immune response of the host. One of the main findings of this task is that greater amberjack has a massive inflammatory reaction against this pathogen which is unique among the fish species which have been shown to be susceptible to this pathogen.

Epitheliocystis is a disease that affects greater amberjack. It can cause serious mortality at the early developmental stages or when it co-infects fish infested by monogenean parasites. The main pathogen of greater amberjack belongs to a novel species of the newly described genus *Candidatus* Ichthyocystis. In this work we have developed the necessary tools for the early and valid diagnosis of the disease.

Part of these results were presented in the European Association of Fish Pathologists Conference held in Belfast 4-8 September 2017 as "Antonakaki Angeliki, Vaughan Lloyd, Maja Ruetten, Seth-Smith Helena, Petropoulos Ioannis, Dourala Nancy, Kantham K. Papanna, Katharios Pantelis. *Epitheliocystis disease in Greece is caused by a wide variety of unrelated bacteria".*

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Deviations: A extension was requested for the delivery of the work, in order to include the analysis of an epitheliocystis outbreak in farmed greater amberjack.

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