TECHNICAL MANUAL FOR MEAGRE HEALTH

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DIVERSIFY
New species for EU aquaculture

Contents

Introduction

The meagre is found in the Mediterranean and Black Sea, and along the eastern Atlantic coast. It has attractive attributes for the market that include **large size, good processing yield, low fat content, excellent taste and firm texture**. The species also has the biological characteristics required for commercial aquaculture using well-established culture technologies. These characteristics include a **fast growth of ~1 Kg per year**, a **low feed conversion ratio of 0.9-1.2** –which is similar to the Atlantic salmon- relatively easy larval rearing and established induced spawning protocols for the production of viable eggs. Meagre was first produced in 1997 in a commercial hatchery in France and since then it has exhibited annual production increases as high as 7 fold (FAO, 2012). In 2010, European meagre aquaculture production was 2,387 t, mainly in Spain, with smaller quantities from France, Portugal, Italy, Greece and Croatia (FAO, 2012).

One of the major obstacles of integrating a new fish species into the commercial rearing procedures and production is the emerging and new pathologies that may arise. In this technical manual, all major diseases and health-related issues studied and recorded during the course of DIVERSIFY project are presented.

The manual which was prepared in the frame of the DIVERSIFY project is divided in two parts; **Part A**, containing the non-infectious diseases (Systemic Granulomatosis and Chronic Ulcerative Dermatopathy) and **Part B**, containing diseases caused by bacteria and parasites.

PART A

NON-INFECTIOUS DISEASES

Co-funded by the Seventh Framework Programme of the European Union

1. SYSTEMIC GRANULOMATOSIS

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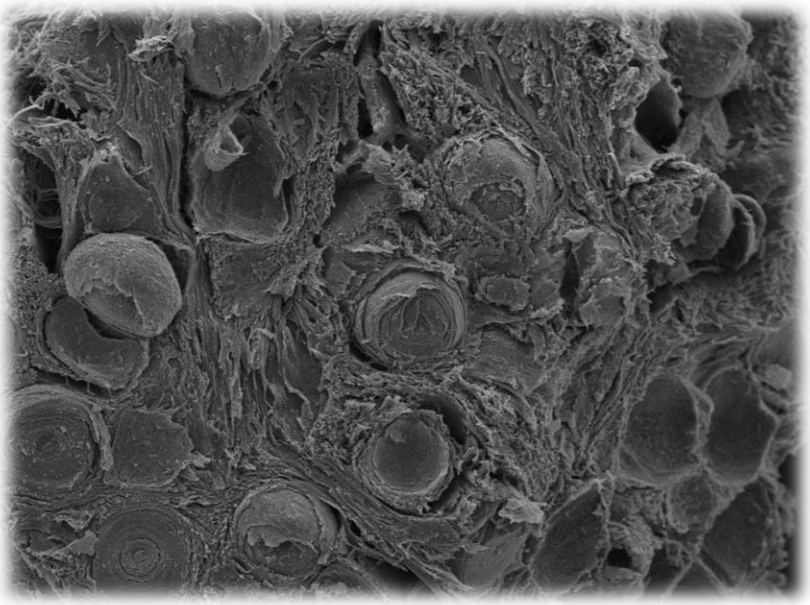

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Systemic Granulomatosis

Systemic Granulomatosis (SG) is a pathological condition affecting the majority of farmed populations of meagre. SG is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic (Ghittino et al., 2004; P Katharios et al., 2011). SG is not associated with high mortalities; however, it may lead to reduced growth and physiological performance during grow-out and, in addition, it affects the final product, making it unacceptable to the consumer. The aetiology of the disease is unknown; however, two hypotheses have been raised. The first is that it is caused by bacterial pathogens most likely *Nocardia* spp. (Elkesh et al., 2013) and the second that it may be a metabolic disorder (Ghittino et al., 2004; P Katharios et al., 2011) similar to systemic granulomas observed in other cultured fish species.

Gross pathology and histology

Externally, heavily affected fish can be emaciated, with fin erosion, exophthalmia and in several cases unilateral blindness. Visible granulomas of various sizes are usually scattered in the internal organs. Liver, kidney and spleen are the organs affected more by SG. In heavily affected fish with many visible granulomas, large part of the liver and the kidney can be necrotic and calcified, while the heart can be completely covered by white to cream colored nodules (**Figure 1.1, 1.2**). At stereoscopic level, fresh squash preparations of affected tissues reveal the presence of granulomas encapsulated by several layers of fibrous tissue with an "onion-like" appearance (**Figure 1.3**). This characteristic "onion-like" appearance was also evident in photos from scanning electron microscopy (**Figure 1.4).**

Figure 1.1. A,B. External lessions on the eye and the tail fin of meagre realated to SG. **C,D.** Dystrophic calcification of the liver and the kidney.

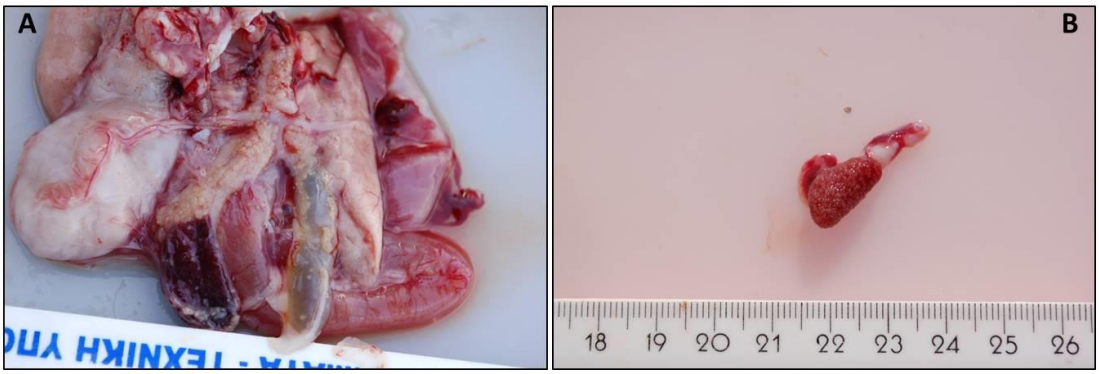

Figure 1.2. **A**. Multiple granulomas in the soft tissues of meagre. **B**. Heart of meagre fully covered by granulomas.

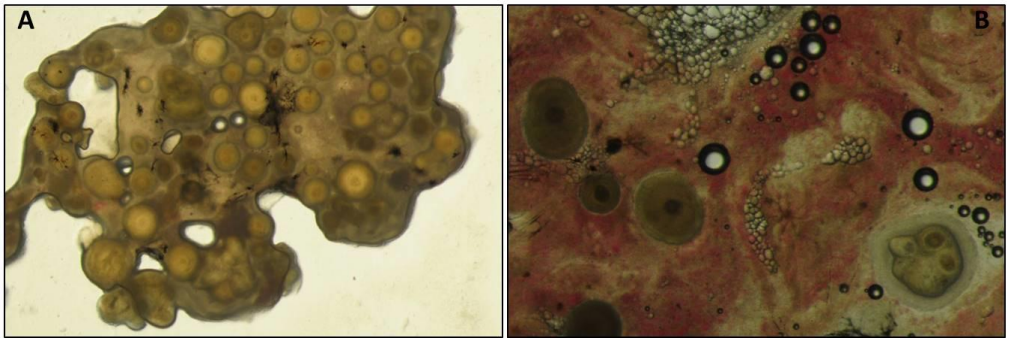

Figure 1.3. Fresh squash preparation from liver (**A**) and kidney (**B**) with granulomas (A:stereoscope x1, B:stereoscope x2.5).

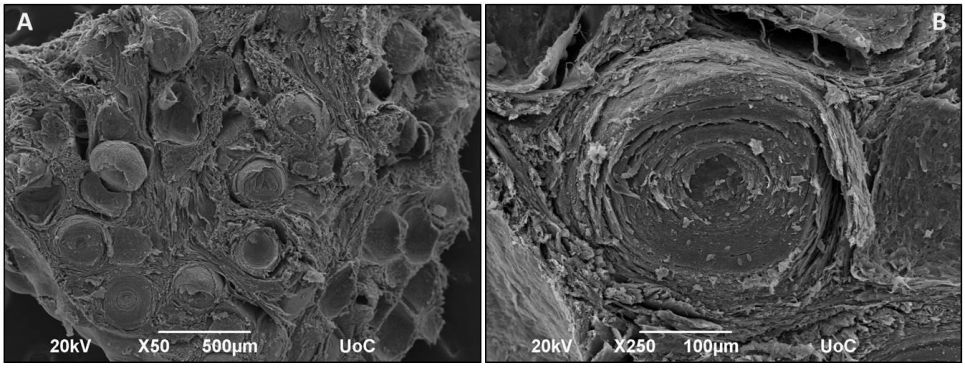

Figure 1.4. A. SEM microphotograph of the heart covered by granulomas. **B.** SEM microphotograph of a heart granuloma with "onion-like" appearance.

Histologically, the morphology of the granulomas consists of a central necrotic amorphous area surrounded by a multilamellar layer of epithelioid cells and fibrous tissue. Several stages of the granuloma formation with the characteristic epithelioid cells can be identified in the examined tissues ranging from immature granulomas, multilayer mature granulomas to big nodules, possibly a result of the merging of several adjacent granulomas that had big areas of central necrosis with dystrophic calcification (**Figure 1.5, 1.6**).

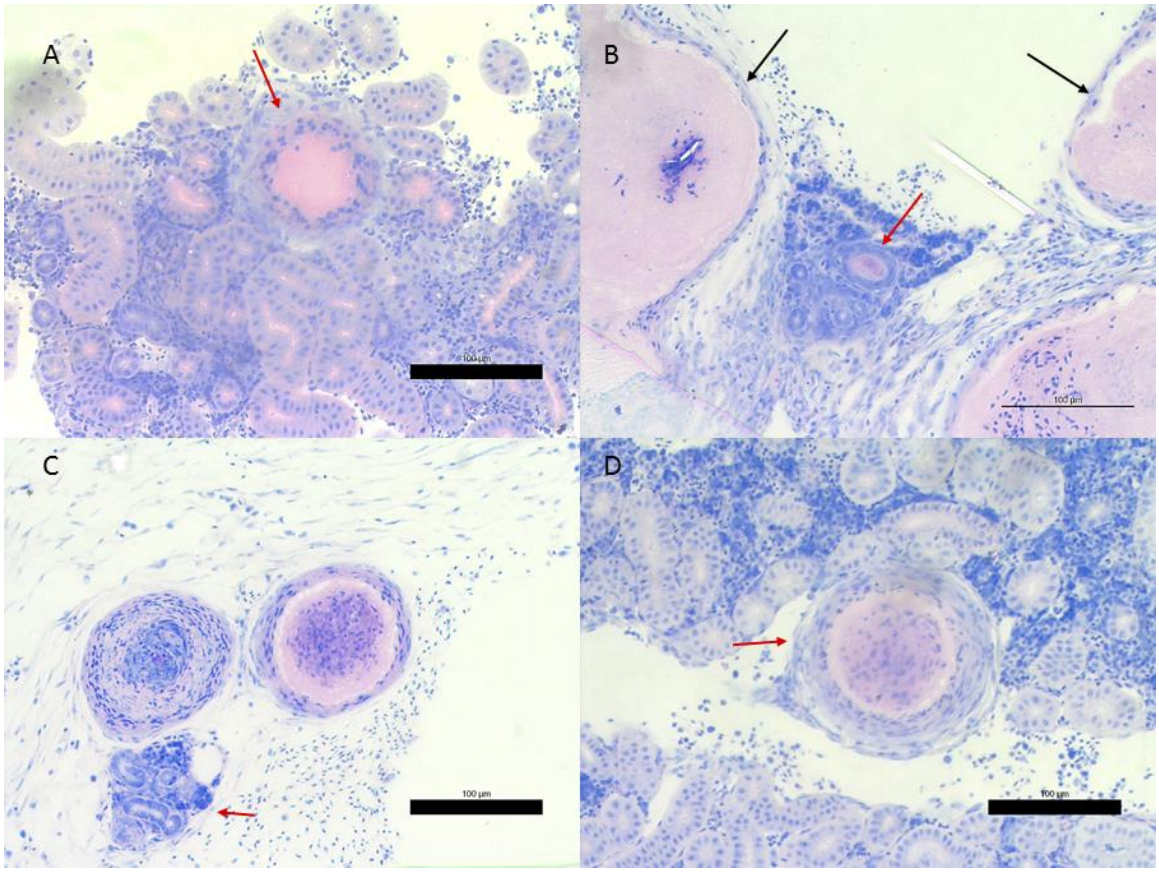

Figure 1.5. A. Immature granuloma in the kidney of meagre. There is an amorphous, acellular area, which is surrounded by inflammatory cells. **B.** An immature granuloma in the kidney (red arrow) in a small area of a kidney with normal appearance. In this particular fish there was extensive caseous necrosis in this organ, a small part of which is indicated with black arrows. **C**. Two adjacent granulomas sectioned at different levels over a small part of renal tissue (red arrow). **D.** Typical appearance of a "young" granuloma in kidney.

In some cases, mainly in livers, the initial stages of the granulomas are located at the blood vesicles resembling vasculitis (**Figure 1.7**). In these cases, there is also an involvement of rodlet cells. Rodlet cells are present in large numbers in all tissues. Rodlets are aligned like epithelial cells in the peritoneal membranes but they are also found in livers, pancreas and intestine. (**Figure 1.8**). The distinctive characteristics of these pear-shaped cells are the collection of the rodlets (linear crystal structures) within their cytoplasm and the thick surrounding membrane.

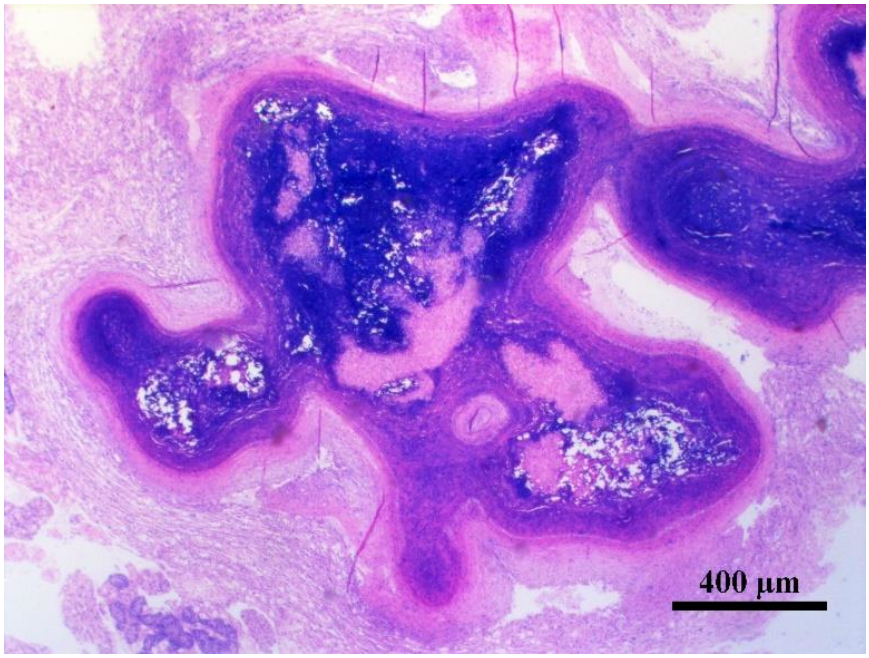

Figure 1.6. Dystrophic calcification in the kidney of meagre.

Under specific conditions rodlet cells expel their rodlets into the extracellular environment. The composition of the rodlets is not known, however it has been shown that they contain the antimicrobial peptide piscidin. Thus, their secretory nature might be connected to the defense mechanism of meagre against infection. However, this cannot be fully supported since there is no data on the presence of these cells in normal or wild specimens.

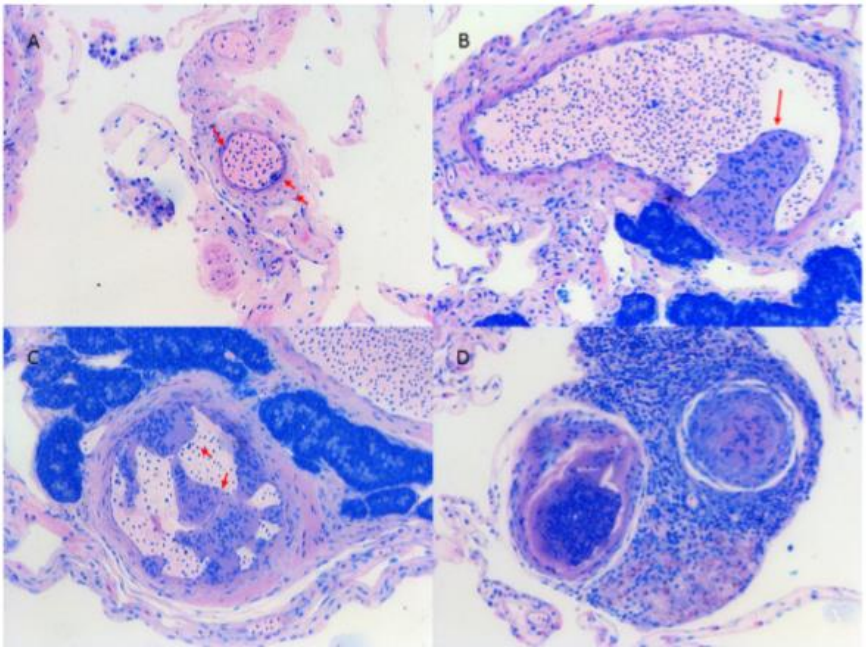

Figure 1.7. Blood vessel implication is evident in the manifestation of the disease. Various sections of blood vessels from the peritoneal membranes and the liver of affected fish are shown. There are specific growths composed of inflammatory cells at the endothelium of the vessels, which are indicated with red arrows. In more progressed stages (**C, D**) these growths seem to block the lumen of the vessel.

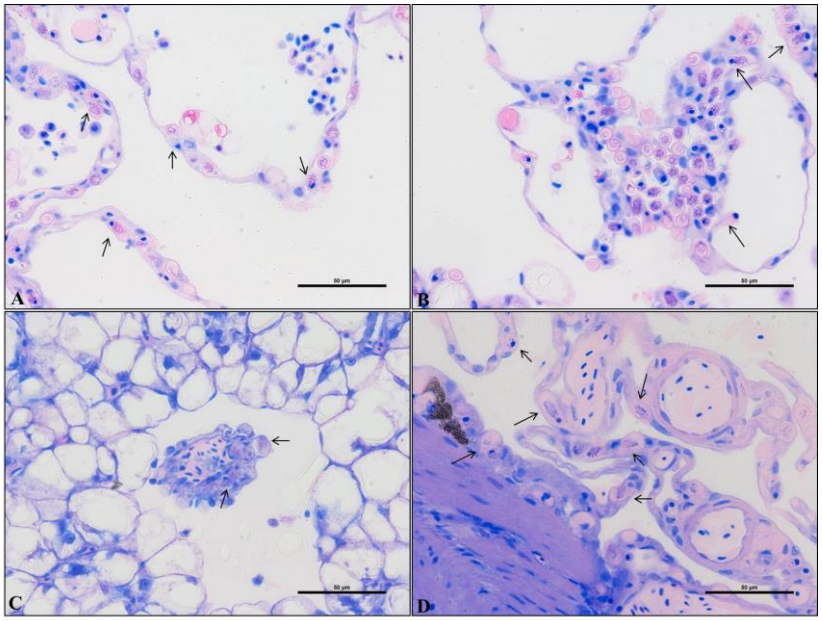

Figure 1.8. Rodlet cells (black arrows) aligned in the peritoneal membranes (**A, B**) and surrounding blood vessel walls (**C,D**).

Since the infectious agent hypothesis was also investigated in the project, we screened a large number of fish for the isolation and characterization of *Nocardia* spp. which has been proposed as the aetiological factor of SG. In order to demonstrate the presence of infectious agents, we have applied special stains in many different meagre samples with granulomas. The results of these specific stains (Ziel-Neelsen, FiteFaraco and Gram stain) were negative. Following extensive sampling we have identified only one case of nocardiosis in meagre, originating from the same geographical are where it was first reported. Histological analysis of the Nocardia-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* spp. in meagre (Elkesh et al., 2013). Ziehl-Neelsen stain was weakly positive in the colonies located in the skin lesions. The bacterial colonies were not demarcated by a granulomatous formation (**Figure 1.9 A, B, C**). Typical granulomas were also present in all tissues examined. In these granulomas, no bacteria could be seen (**Figure 1.9D**).

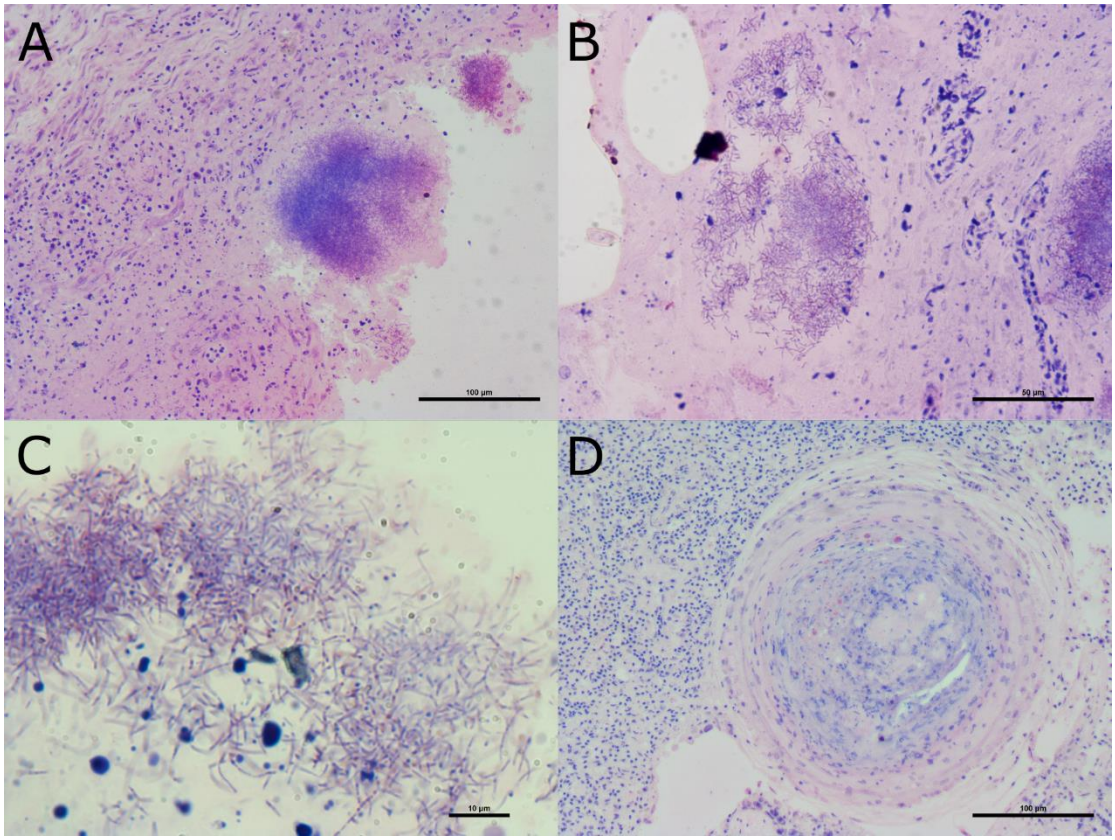

Figure 1.9. A, B. Histological section of a dermal lesion of a Nocardia-positive meagre (methylene blue/azure II/basic fuchsin stain). There are several bacterial colonies, which have elicited a moderate host response. **C**. Higher magnification of the bacterial colonies from dermal lesions. Note the filamentous branching morphology of the bacteria which are consistent with the descriptions of Nocardia spp. in other fish species. Ziehl Neelsen stain. **D**. A non-bacterial granuloma in the spleen with the typical morphology observed in SG (methylene blue/azure II/basic fuchsin stain).

Biochemical analysis

The analyses of serum liver enzymes such as ALT, AST and ALP have been proposed to be the main biomarkers for liver diseases. In general, the elevation of ALT and AST concentrations may indicate hepato-cellular diseases, while the elevation in ALP may indicate cholestatic diseases of the liver. Analysis of these serum enzymes showed that, regardless of the diets, ALP, ALT and AST activity increased in fish with granulomas or tissue calcification compared with fish without (**Figure 1.10**). Increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation, while elevated plasma ALP activity corresponds to an inflammatory reaction of the bile ducts. In damaged tissues, cell membranes become more permeable, releasing some

enzymes into the blood and thus modifying normal plasma values. In fish, elevated plasma ALP and AST have been associated with liver or bone disorders, so those results may be associated with SG, but further investigation needs to be done.

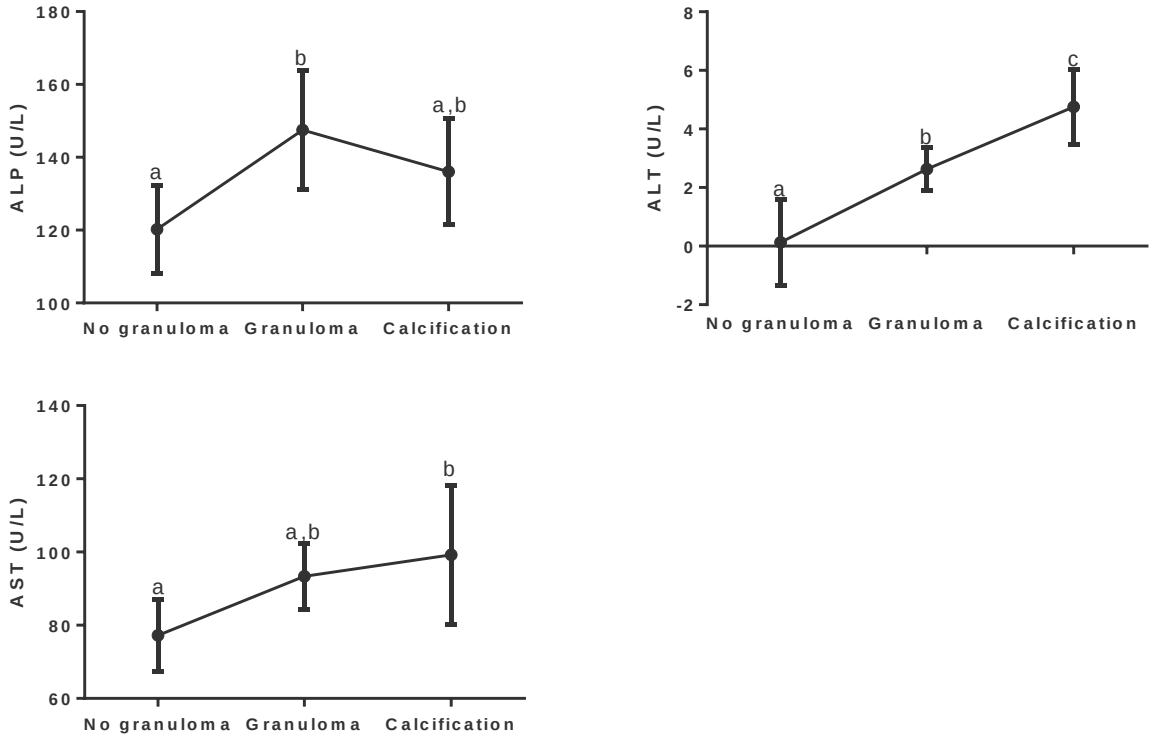

Figure 1.10. Mean concentrations (±SD) of ALP, ALT and AST in meagre with no granulomas, granulomas and calcification of even one tissue at the end of Vitamin D3 experiment (Task 24.1). Different letters (a, b) show statistically significant differences between the three conditions ($p<0.005$).

Feeding trials

Several nutritional trials were performed in the DIVERSIFY project in order to investigate the metabolic disorder hypothesis.

The metabolic disorder hypothesis (Ghittino et al., 2004; P Katharios et al., 2011) have been raised due to the similar systemic granulomas observed in other cultured fish species such as the gilthead sea bream (*Sparus aurata*), the rainbow trout (*Salmo gairdneri*) and the turbot (*Scophthalmus maximus*). In all cases, the development of the disease has been associated with nutritional imbalance in minerals and vitamins or inadequacy due to the use of plant protein sources or long-term stored formulated feeds or frozen fish.

We ran several feeding trials to assess the effect of vitamin D and Ca/P levels in feeds (HCMR), the effect of plant ingredients in the feeds (HCMR), as well as the effect of minerals and vitamins levels (FCPCT)

Trial 1. The effect of vitamin D_3 inclusions in diets in the development of SG in meagre (HCMR)

For vitamin D_3 trial four experimental diets with increasing levels of vitamin D_3 were prepared at HCMR (Athens, Greece). Meagre juveniles of 4 g average weight (n=600) were used for the feeding trial. Three replicates were used for each diet. The feeding trial lasted 93 days. At the end of the feeding trial samples were taken for granuloma evaluation and histology, estimation of specific biomarkers (CYP27, CYP24 enzymes) and antioxidant enzymes activity and plasma analysis.

To assess fish status regarding the presence of granulomas, a semi-quantitative method was developed based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. Each fish was dissected and internal organs were examined macroscopically. Fresh squash preparations of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney were assessed under a stereoscope. For the general state of each individual, the sum of the scores from the various tissues was calculated. The assessment scale used was according to the following scoring system shown in **Table 1.1**.

Table 1.1. The assessment scale used for the evaluation of granulomas.

Score 0	No granulomas
Score 1	Granulomas visible only with microscopy
Score 2	Granulomas visible macroscopically
Score 5	Tissue calcification

Main result: Supplementation with vitamin D_3 has no effect on the development of SG.

Trial 2. The effect of Ca:P ratio in the diet on the development of SG (HCMR)

For these trial nine experimental diets with different levels of Ca and P were formulated at the SKRETTING Aquaculture Research Centre (SARC), Norway. The basal diet was formulated to contain about 53% crude protein and 15% crude lipid. P was supplemented separately to the basal diet of the mixture to obtain various concentrations of P, while the amount of Ca that was supplemented in the basal diet was calculated to be either equal or double the amount of P. Meagre juveniles of 1 g average weight (n=1350 were used for the feeding trial. Three replicates were allocated to each diet. The feeding trial lasted 4 months.

At the end of the feeding trial samples were taken for granuloma evaluation and histology, body and mineral composition, estimation of specific biomarkers (CYP27, CYP24 enzymes) and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in Table 1.

Main result: The high P content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis.

Trial 3. The effect of high plant protein diets in the development of SG (HCMR)

The purpose of this third trial was to examine whether FM replacement by PP sources affects the development of SG. Furthermore, due to the results obtained in previous trial we also investigated whether P supplementation in PP diets has any effect on SG. Four experimental diets were formulated at the SKRETTING Aquaculture Research Centre (SARC) with 60% (FM) and 14% fishmeal (PP) and increasing levels of P in the diets with 14% fishmeal (PP+medium P, PP+high P). Meagre juveniles of 2 g average weight (n=600) were used for the feeding trial. The feeding trial lasted 3 months (August- November 2016). At the end of the feeding trial samples were taken for granuloma evaluation, histology and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in **Table 1.1**.

Main result: Plant proteins in the diets of meagre were found to negatively affect SG while P supplementation in the PP diets did not affect the overall condition but had a positive effect in the liver of the fish.

Trial 4. The combined effect of vitamins E, C and carotenoids in the development of SG (FCPCT)

Six experimental diets were prepared by adding different levels of vitamin E, C and astaxanthin. Meagre juveniles of 79 g average weight (n= 900) were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain). The feeding trial lasted for 135 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in Table 2. The score was organ dependent, because the number of granulomas in each organ was variable.

Table 1.2. Severity score of granulomas in liver, kidney and heart

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Main result: The combination of a high dietary content of the antioxidants vitamin E and C increased the incidence and number of fish with lower severity of SG.

Trial 5. The effect of Se, Mn and Se in the development of SG. (FCPCT)

Five isolipidic and isoproteic fish meal and fish oil-based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se. Meagre juveniles of 15 g average weight (n= 2100) were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain). The feeding trial lasted for 90 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in Table 2.

Main result: The addition of target minerals did not ameliorate the granuloma incidence or severity, but recommended levels of minerals are: 40 $mg \cdot kg^{-1}$ of Mn, 200 $mg \cdot kg^{-1}$ of Zn, and 1.5 $mg \cdot kg^{-1}$ of Se.

General conclusions and recommendations for SG in meagre

- Nocardiosis is present in Greece, most probably in a confined geographical region; however it is not the cause of SG.
- Vitamin D_3 supplementation did not affect the development of the SG,
- High P content in the diet seems to improve the condition
- Plant protein replacement affects negatively the progression of the SG.
- High dietary content of the antioxidants vitamin E and C increased the incidence and number of fish with lower severity of SG
- The addition of Se, Mn and Se did not ameliorate the granuloma incidence or severity.

Taken together the improvement of SG by change in the diet with the absence of pathogens in SG-affected population we believe that the metabolic hypothesis is more

probable. The occurrence of only a single case of nocardiosis with different characteristics enforces this hypothesis.

However, the aetiology is still unknown and other nutritional metabolic factors have to be tested.

Considering all the above results, our recommendations for prevention of SG in meagre are:

- A combined diet with high percentage of fishmeal (60%) and high dietary content of P ($15 g kg^{-1}$) and antioxidants vitamins E and C.
- Since there is no data available about the reversibility of SG we recommend to start feeding with this diet when the fish weight is about 2g.

2. CHRONIC ULCERATIVE DERMATOPATHY

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Co-funded by the Seventh Framework Programme of the European Union

Chronic Ulcerative Dermatopathy (CUD)

The lateral line is a mechanosensory system found in all fishes and in the larvae of aquatic amphibians, which is used for the detection of water movements and/or pressure fluctuations. The receptors of the lateral line that detect water flow are called neuromasts and they are distributed on the head, the trunk and the tail of the fish. Neuromasts can be either superficial in the skin or enclosed in the fluid-filled canals of the lateral line that open to the environment through a series of pores. A schematic appearance of the lateral line system, the canals and the neuromasts is presented in **Figure 2.1**. It has been demonstrated that the lateral line canals develop through a bone remodeling process with the implication of both osteoblasts for bone apposition and osteoclasts for bone resorption.

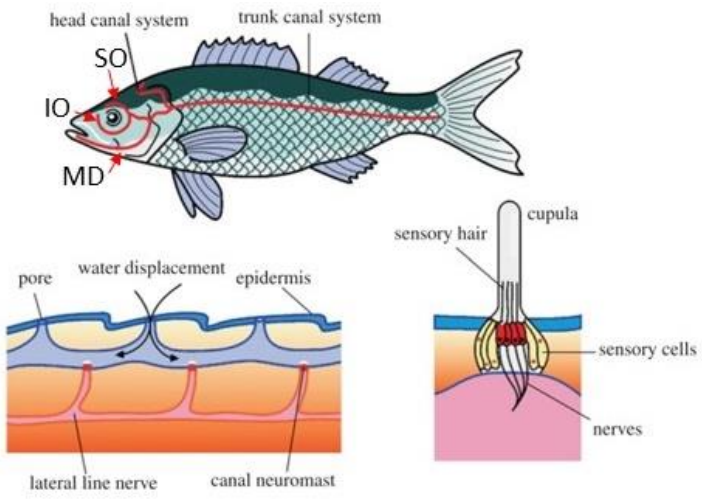


Figure 2.1. Lateral line system in fish. Structure of lateral line canal and of a neuromast. SO: supraorbital canal, IO: infraorbital canal, MD; mandibular canal (from Dagamseh et al., 2013 with modifications).

Chronic Ulcerative Dermatopathy (CUD) is a newly described condition affecting the lateral line canals of many cultured fishes both freshwater and marine. It has been described in the Australian freshwater fish Murray cod, *Maccullochella peelii peelii* in sites supplied by groundwater (Baily et al., 2005; Schultz et al., 2011, 2008). The disease results in focal erosion, ulceration and loss of epidermis around the lateral line canals of the head and the trunk, and fin erosion. It has been associated with reduced growth rates, increased mortalities and significant reduction of marketability due to the severe disfigurement of the affected fish (Baily et al., 2005; Schultz et al., 2008). The same condition was also reported for goldfish *Carassius auratus* after exposure to freshwater groundwater (Baily et al., 2005). Concerning marine species, CUD was reported to affect the sharpsnout sea bream, *Diplodus puntazzo*, after culture in saline groundwater (Pantelis Katharios et al., 2011). For the sharpsnout seabream, the authors suggested that there is an indication of osteoclastic enzymatic activity in the affected fish. The enzymes implicated in bone remodeling of the lateral line canals are the tartrate resistance acid phosphatase (TRAP) and cathepsin K for

bone resorption, and vATPase for bone apposition. Both for Murray cod and sharpsnout seabream, the authors reported that the lesions resolve if fish are transferred to natural freshwater and seawater respectively and they could not associate the disease with any infectious agent. The final conclusion of both studies was that the development of the disease is correlated with the use of groundwater sources. However, the aetiology is still unknown since they could not establish the exact component of the water which results to the development of the disease (Baily et al., 2005; Pantelis Katharios et al., 2011; Schultz et al., 2011, 2008). A similar condition under the term 'lateral line depigmentation' has been reported in channel catfish. In this case the authors concluded that the causative agent for development of the disease was the exposure of fish to chronic nutritional stress by 12 months of fasting (Corrales et al., 2009).

Meagre is one of the sensitive CUD fish species. The disease affects 100% of the population and results in ulceration of the skin overlying the lateral line canals, however is not associated with mortalities (Rigos and Katharios, 2010). The aim of this study was to describe the disease in meagre using histology and SEM and to investigate osteoclast activity using molecular markers. Through this study, the final goal was to investigate the aetiology of the disease and suggest preventive measures.

Two parallel rearing trials of meagre in borehole and natural seawater were conducted in order to study the development of CUD. Eggs produced in the facilities HCMR, Crete, Greece were used for the rearing trial, which was performed in duplicate 40-m^3 tanks. The rearing trial lasted from 1-56 days post hatching (dph). Every day, measurements of pH, CO_2, O_2 and T were made in the two water sources.

At the end of the rearing trial all the fish reared in borehole water had visible lesions associated with CUD in comparison with the fish reared in natural sea water (**Figure 2.2).**

Figure 2.2. Meagre reared in natural seawater (left) and borehole water (right). All fish reared in borehole water had visible lesions on the head associated with CUD.

The average length and weight of the fish of the different water sources at the end of the rearing trial (56 dph) are presented in **Figure 3**. The growth performance of the fish was not affected by the different source of water ($p>0.05$)

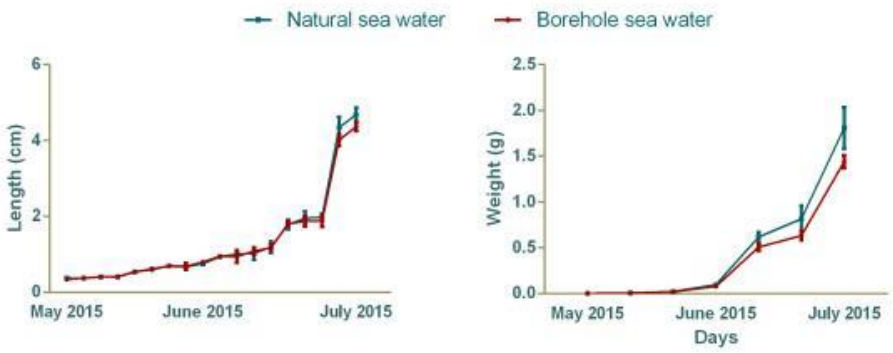


Figure 2.3. Average length and weight of meagre reared in borehole and natural seawater. The values are mean±SD.

The transfer from borehole water to natural sea water of CUD affected meagre led to almost full recovery of the lesions within 5 months (**Figure 2.4**).

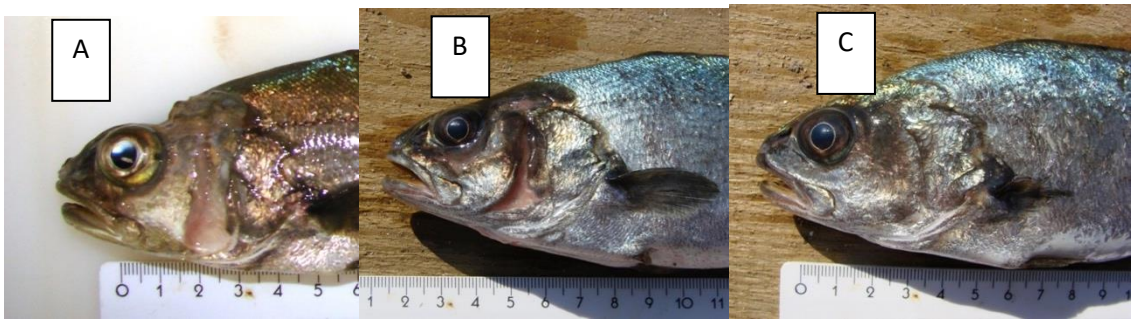


Figure 2.4. A. Nine-month-old meagre reared solely in borehole seawater. **B.** Nine-month-old meagre transferred to natural seawater for 5 months, with partial resolution of the lesions. **C.** Nine-month-old meagre transferred to natural seawater for 5 months, with complete resolution of the lesions.

The results of the studies in DIVERSIFY indicated that CUD in meagre is induced by the use of borehole water, which is in agreement with the conclusions of Baily et al. (2005) and Schultz et al. (2008) for Murray cod and of Katharios et al. (2011) for sharpsnout seabream. Furthermore, another similarity with Murray cod and sharpsnout seabream is that the lesions resolve if fish are transferred to natural seawater. The results from histology and SEM confirmed that the lesions were limited to the lateral line organ in the head.

From the physicochemical analysis of the two water sources it is noteworthy that the pH was lower and CO_2 higher in borehole water in comparison with natural seawater. Katharios et al. (2011) hypothesized that borehole water which is rich in CO_2, as indicated also by the lower pH compared to the pH of natural seawater, increases the enzymatic activity of the osteoclasts. The CO_2 activates the osteoclasts, which are in close proximity with the environment, such as the osteoclasts of the lateral line canals. In this scenario there would be an environmentally induced imbalance between osteoclasts (bone resorbing cells) and osteoblasts (bone depositing cells) that would cause the lesions seen in the fish, located exclusively in the lateral line canals. Based on these results, we performed a second rearing trial in order to investigate whether CO_2 in borehole water is the aetiological agent that causes the development of CUD lesions. In this trial, we used 2 parallel rearing tanks supplied with natural sea water. In one of these tanks we adjusted the pH to 7.4 by infusing CO_2. We cultured meagre from eggs to 60 dph. The lack of lesions in the head and the trunk of the fish following visual examination in this study, suggests that neither pH nor CO_2 are the factors affecting the development of CUD lesions.

Eisler and Gardner (1973) found that copper alone or in combination with zinc or cadmium damages the epithelium of canals in the head of mummichog (*Fundulus heteroclitus*). The facilities and the water sources we used for this trial were the same that Katharios et al. (2011) used for the study of CUD in sharpsnout sea bream. From the heavy metal analysis of water samples, they found that borehole water had higher concentrations of copper, lead, nickel and zinc than natural seawater, however these levels were within the acceptable limits for marine aquaculture and much lower than the toxic limits. Our results from the metal analysis of the head of meagre reared in the two different water sources showed that the concentration of copper was significantly higher in the head of meagre reared in borehole water than in the head of meagre reared in natural sea water. However, concentrations of all metals where comparable to published data from other farmed and wild fish species where lesions are absent (Alasalvar et al., 2002; Kalantzi et al., 2016, 2013; Zotos and Vouzanidou, 2012). Nevertheless, metal toxicity as a causative factor for the development of CUD cannot be ruled out because of the lower pH of the borehole water and the longer exposure times of the fish.

Furthermore, another interesting similarity between CUD-affected meagre and CUD-affected Murray cod is the presence of the enigmatic rodlet cells. Schultz et al. (2014) found a significantly greater number of rodlet cells in the gills, kidneys and intestines of CUD-affected Murray cod and assumed that it was a response to a toxicant in the groundwater. In this task we didn't examine the soft tissues of meagre. However, in trials to investigate the causes of systemic granulomatosis we used meagre reared in borehole water with visible lesions associated with CUD. As we have described in deliverables 24.1, 24.2 and 24.5, rodlet cells in meagre are present in large numbers, aligned like epithelial cells in the peritoneal membranes, liver, pancreas, intestine and kidney. In both meagre and Murray cod, no pathogens were identified in any tissue, so the secretory nature or rodlet

cells might be connected to defense mechanisms of fish against a toxicant in the water. However, this hypothesis cannot be fully supported since no data exist on the presence of these cells in normal (not affected by either systemic granulomatosis or CUD) or wild meagre.

Although the disease is directly associated with the use of borehole water, the causative agent is still unknown for meagre, as well as for Murray cod and sharpsnout seabream. For all species the lesions resolve when the fish are transferred to natural freshwater or seawater (Baily et al., 2005; Pantelis Katharios et al., 2011). Furthermore for Murray cod, Schultz et al. (2011) found that the retention of groundwater into a vegetated earthen pond or in a tank containing biofilms growing on an artificial macrophyte for 72 h prevents the development of CUD. Thus, it is recommended to avoid borehole seawater for the rearing of meagre if natural sea water sources are available and to pay careful attention to the source of the water used. Alternatively, the residence time of meagre in borehole water should be reduced to the minimum necessary, and fish should be moved to natural seawater (*e.g.* in sea cages) as soon as possible once the nursery phase is completed, in order to allow the tissue regeneration process to complete before marketing the fish.

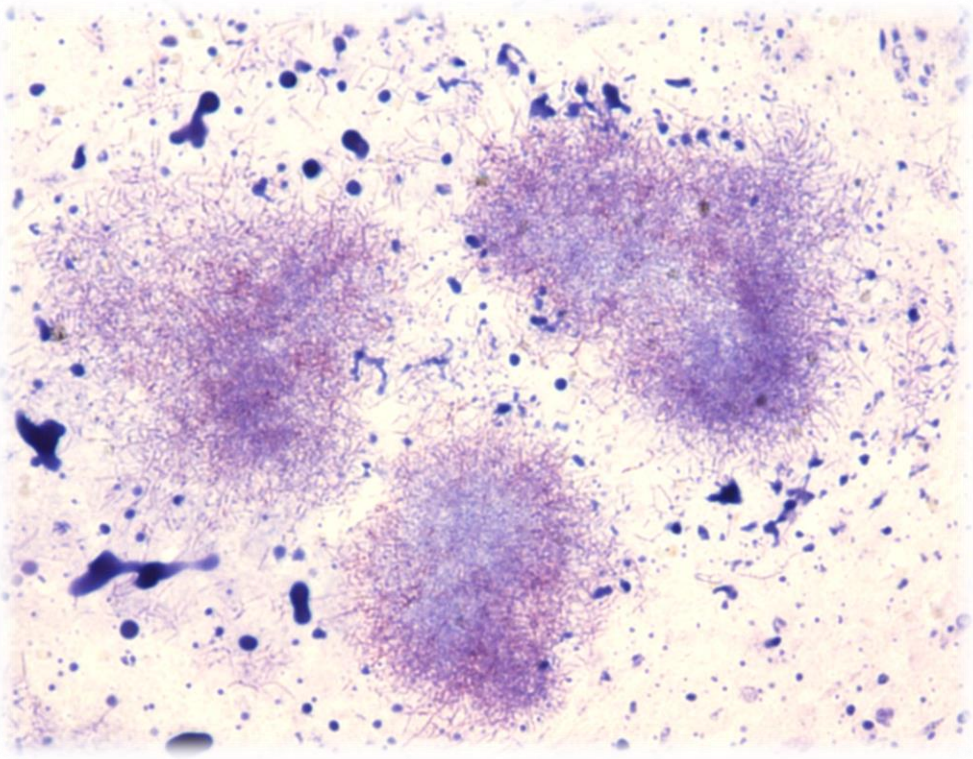
PART B

INFECTIOUS DISEASES

3. NOCARDIA AND OTHER BACTERIA

Pantelis Katharios (HCMR), Maria Ioanna Tsertou (HCMR), Maria Smyrli (HCMR)

Contributed: Constantina Kokkari (HCMR)



Results presented in this chapter has been published in

Tsertou, M.I., Smyrli, M., Kokkari, C., Antonopoulou, E. and Katharios, P., 2018. The aetiology of systemic granulomatosis in meagre (Argyrosomus regius): The “Nocardia” hypothesis. *Aquaculture Reports*, *12*, pp.5-11

Nocardia and other bacteria

One of the main pathological problems of meagre (*Argyrosomus regius*) aquaculture is Systemic Granulomatosis (SG) (P Katharios et al., 2011), which is investigated intensively within the DIVERSIFY project. In 2013, there was a report suggesting that the causative agent of granulomatosis in meagre was *Nocardia* sp. (Elkesh et al., 2013), a genus of actinobacteria related to severe epizootics in fish (Chen et al., 2000; Cornwell et al., 2011; Kudo et al., 1988; Vu-Khac et al., 2016). The clinical signs of nocardiosis include skin ulcers, small white to yellow nodules in the gills and the internal organs, while fish present anorexia and lethargy. Mortality is generally low in the range of 1-17% (Chen et al., 2000; Cornwell et al., 2011; Elkesh et al., 2013) and chronic, while mass mortalities have been reported in the Japanese industry of cultured yellowtail and greater amberjack (*Seriola lalandi* and *Seriola dumerili*, respectively) (Shimahara et al., 2008). Histopathology of Nocardia-infected fish usually reveals chronic lesions in the form of granulomas. These granulomas are aggregations of macrophages differentiating into epithelioid cells that initially demarcate bacterial colonies and as inflammation progress necrotic areas.

There are several bacterial and fungal pathogens that can result in granulomatous lesions in fish. These include the acid-fast bacteria, *Mycobacterium* spp. and *Nocardia* spp., as well as the Mesomycetozoan *Ichthyophonus hoferi*. In most of the cases, especially when the disease is as severe as in the case of SG in meagre, the pathogens are readily identifiable with histology even without the aid of specialized staining techniques. We have been monitoring SG in meagre in HCMR stocks, for more than 5 years without being able to correlate it with any of the abovementioned pathogens. However, we acknowledge that Nocardiosis is a serious disease that may pose threat to the sustainability of meagre aquaculture. Therefore, one of the aims of DIVERSIFY project was to monitor meagres from various places in Greece and try to identify and isolate *Nocardia* spp., or other granulomas-associated pathogens and to assess whether these bacteria and fungi represent an actual hazard for the species.

Fish samples

During the first years of the project we examined a large number of fish of varying sizes using both microbiological but also molecular techniques.

Fish have been collected from various localities. Healthy, moribund and fish exhibiting disease signs were sampled belonging to a range of developmental stages. Summary of the samplings is presented in **Table 3.1**. Several samples have been obtained earlier and analyses have been performed during the DIVERSIFY project.

Table 3.1. Samples processed for Nocardia isolation.

Sampling Date	Locality	# fish	Mean W (g)	Mean L (cm)
11/9/2013	HCMR	20	1	
7/10/2013	HCMR	20	2	
20/2/2014	HCMR	1	6735	
16/3/2014	HCMR	20	2	
10/4/1014	Galaxidi	1	307,7	29,5
29/9/2014	Souda	20		
5/5/2015	Atalanti	2	2,445	
10/8/2015	HCMR	9	4	6
15/10/2015	Siteia	10	200	
20/10/2015	Siteia	10	200	
26/10/2015	Siteia	10	200	
27/10/2015	Leros	5	518	37,5
26/2/2016	HCMR	1	breeder	breeder
1/3/2016	Souda	4		
11/3/2016	Galaxidi	8	471,5	33
6/4/2016	Galaxidi	7	39,4	15
1/6/2016	Astakos	2	1500	

Kidney, liver, spleen, heart, brain, ascetic fluid (if present) were sampled from almost all fish. Tissues used for molecular analysis were preserved in -20ºC. Tissues used for histology were preserved in 10% buffered formalin (PBF). The PBF preserved samples were dehydrated in gradually increased ethanol solutions (70-96%) and then embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections of 4 μm were obtained with a microtome (RM 2245, Leica, Germany). After drying, slides were stained with methylene blue/azure II/basic fuchsin, Ziehl-Neelsen (acid-fast bacteria) and Grocott stains (fungi) and examined under a light microscope. For the isolation of *Nocardia* spp. general (BHI 2% NaCl and BHI 0,5% NaCl) and selective for Mycobacteria, also recommended for *Nocardia* spp. (*Löwenstein-Jensen,* L-J) solid media were used. Cultures were performed mainly from the kidney, skin, but also lesions, like abscesses and homogenized granulomas, using aseptic techniques. Plates were incubated at 25ºC and were observed for more than three weeks. Isolation of fungi was attempted using the Sabouraud Dextrose Agar (SDA). DNA was extracted from tissues using QIAGEN DNAEasy Blood and Tissue kit according to manufacturer's instructions and from bacteria in culture using boiling extraction method. For the detection of *Nocardia* spp. from tissues and growth on culture media, the genus specific primer pair NG1-NG2 was used to amplify

a fragment of 16S rRNA gene. *Nocardia seriolae* NCIMB 13256 was used as positive control. In case of tissue samples, nested PCR was applied using as template for the primer pair NG1-NG2 the product of the universal primers for 16S rRNA gene 27f-1492R.

For the detection of *Mycobacterium* spp. the genus specific primer pair 246-1522 was used to amplify a fragment of 16S rRNA gene. Spleen tissue sample retrieved from European seabass (*Dicentrarchus labrax*) infected by *Mycobacterium marinum* was used as positive control.

For the detection of *Ichthyophonus hoferi,* the species-specific primer pair Ich7f-Ich6R was used *to amplify a fragment of 18S rRNA gene*.

PCR reactions were performed in a Bio-Rad MJ Mini Personal Thermal Cycler. PCR conditions for the amplification of the bacterial 16S rRNA gene using universal primers were the following: denaturation at 94^{o}C for 3 min, 30 cycles at 94^{o}C for 1 min, annealing for 1 min, extension at 72^{o}C for 1,30 min, and final extension at 72^{o}C for 10 min. The PCR conditions for the rest of genes/pathogens were in accordance to the authors' instructions. Characteristics of the primer pairs used for the PCR reactions are presented in **Table 3.2**. Sequencing was performed using ABI3730xl sequencer (AppliedBiosystems) according to the protocol BigDye Terminators 3.1 (AppliedBiosystems).

Table 3.2. Characteristics of the primer pairs used for the PCR reactions.

Pathogen	Gene	Primer	Primer's sequence (5'-3')	Product size (bp)	Reference
Nocardia spp.	16S	NG1	ACCGACCACAAGGGG	596	(Laurent et al., 1999)
		NG2	GGTTGTAACCTCTTCGA		
Universal Bacterial	16S	Bac27F	AGAGTTTGATCMTGGCTCAG	1450	(Lane, 1991)
		1492R	TACGGYTACCTTGTTACGACTT		
Mycobacterium spp.	16S	246	AGAGTTTGATCCTGGCTCAG	1400	(Böddinghaus et al., 1990)
		1522	AAGGAGGTGATCCAGCCGCA		
I. hoferi	18S	Ich 7F	GCTCTTAATTGAGTGTCTAC	370	(Whipps et al., 2006)
		Ich 6R	CATAAGGTGCTAATGGTGTC		

In most of the cases examined, no bacterial growth was observed on the solid media used. Bacteria were isolated from 7 fish from 4 different localities. In total we purified approximately 25 isolates from various organs including the kidney, skin but also lesions, like abscesses and homogenized granulomas. None of the isolated bacteria had phenotypes consistent to Nocardia. DNA from all isolates was extracted and 16s rRNA gene was sequenced using the universal primers set. Sequencing confirmed that none of the isolates belonged to the *Nocardia* genus. Moreover, none of the identified bacteria have been reported as causative agents of disease and they are more likely environmental strains. **Table 3.3** contains the information regarding the bacterial strains identified.

Table 3.3. Identification of the bacterial isolates based on 16s rRNA sequencing

Code	Tissue	Isolation Medium	Blast ID
14.1	Kidney	BHIA 2% NaCl	*Micrococcus luteus*
14.2	Kidney	BHIA 2% NaCl	*Pseudomonas oryzihabitans*
14.3	Kidney	TSA 2%	*Micrococcus luteus*
14.4	Kidney	L-J	*Shewanella putrefaciens*
14.5	Kidney	L-J	*Stenotrophomonas maltophilia*
14.7	Kidney	L-J	*Vibrio gigantis*
14.8	Skin	BHIA 0.5% NaCl	*Staphylococcus epidermidis*
14.9	Kidney	L-J	*Pseudomonas aeruginosa*
14.10	Operculum	BHIA 0.5% NaCl	*Novosphingobium panipatense*
15.1	Brain	BHIA 2% NaCl	*Micrococcus luteus*
15.2	kidney	BHIA 0.5% NaCl	*Pseudomonas oryzihabitans*
15.3	kidney	SDA	*Pseudomonas* sp.
15.4	Kidney	L-J	*Pseudomonas aeruginosa*
15.5	Kidney	L-J	*Shewanella baltica*
23	Kidney	L-J	*Bacillus cereus*
27.2	Kidney	L-J	*Pseudomonas aeruginosa*
28.4	Kidney	L-J	*Pseudomonas aeruginosa*
30.9	Kidney	L-J	*Pseudomonas aeruginosa*

In addition to the bacteria isolated in solid media, PCR analysis was performed directly on granulomatosis-affected tissues and organs using specific primers against the suspected pathogens, *Nocardia* spp., *Mycobacterium* spp., and *Ichthyophonus hoferi*.

All samples examined with this method were negative for all 3 pathogens surveyed, except 2 fish that we received in June 2016 from a commercial fish farm located in Astakos, West Greece. These fish had severe dermal lesions and ulceration of the skin and considered suspicious for Nocardia infection due to the distinct morphology of these lesions (**Figures 3.1-3.3**). PCR for Nocardia was positive in 4 out of the 6 different organs examined, including skin, heart, kidney and liver from both individuals.

Figure 3.1. Severe ulceration of the skin of cultured meagre.

Figure 3.2. Nodular morphology of the dermal lesions, appearance alarming for Nocardia infection.

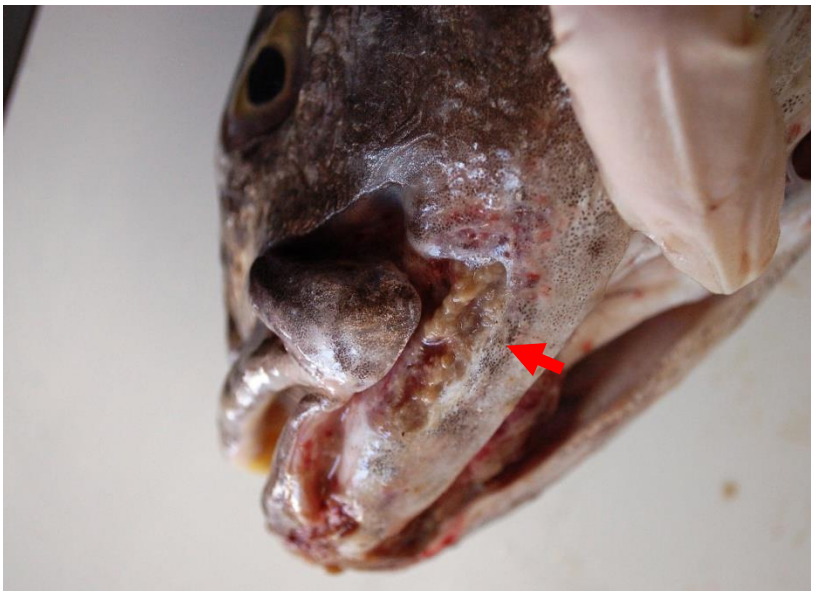


Figure 3.3. Nodular morphology of the dermal lesions, appearance alarming for Nocardia infection.

Positive PCR samples from both species were sequenced and compared against GenBank sequences using BLAST algorithms. The analysis showed 100% identity with *Nocardia seriolae*.

DNA was also extracted from the *Nocardia seriolae* type strain of our collection (kindly offered by Prof. Secombes) followed by the same procedure described above. Four sequences obtained from the meagre samples, and 2 sequences of *Nocardia seriolae* type strain (one retrieved from the Genbank and one sequenced by us as positive control) were aligned and compared using ClustalW in MEGA6. The results indicated that the novel sequences differ at only one nucleotide at position 107 over a range of 567 nucleotides (**Figure 3.4**).

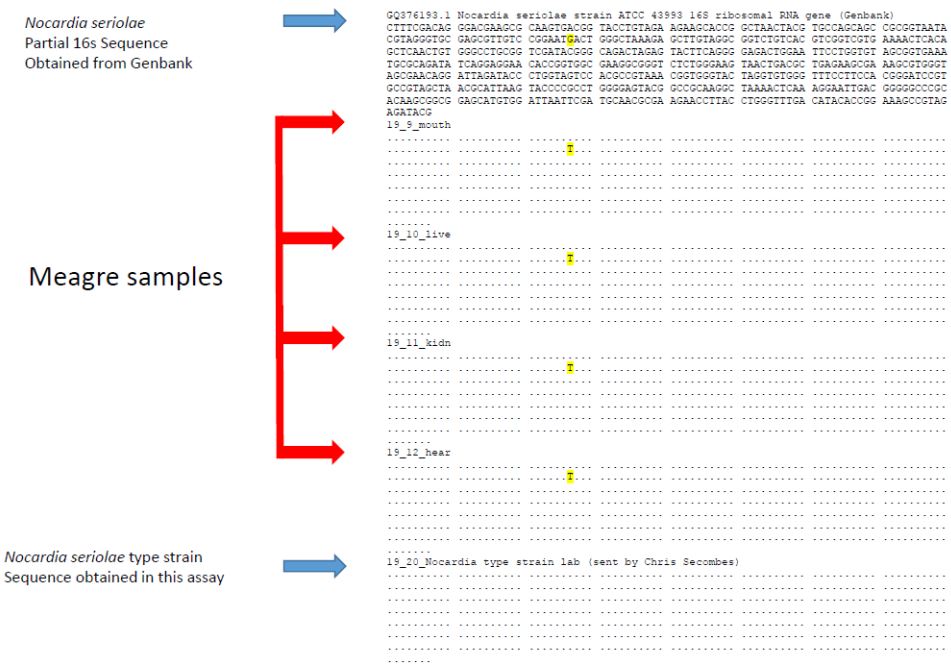


Figure 3.4. Multiple alignment of the partial 16s sequences of the meagre samples with *Nocardia seriolae* type strain retrieved from Genbank and sequenced by us as positive control of the assay. There is a single nucleotide mutation at position 107, possibly a result of the geographic distance of the isolates.

Histological analysis of the Nocardia-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* in meagre (Elkesh et al., 2013). The bacterial colonies were located in the skin lesions and in the heart while no bacteria could be found in the other tissues examined. Ziehl-Neelsen stain was weakly positive in the colonies located in the skin lesions and negative in the colonies located in the heart. The bacterial colonies were not demarcated by a granulomatous formation (**Figure 3.5-3.10)**. Typical granulomas were also present in all tissues examined (**Figure 3.10)**. The morphology of these granulomas followed the pattern described earlier (P Katharios et al., 2011), consisting of a central necrotic amorphous area surrounded by a multilamellar layer of epithelioid cells and fibrous tissue. In these granulomas, no bacteria could be seen.

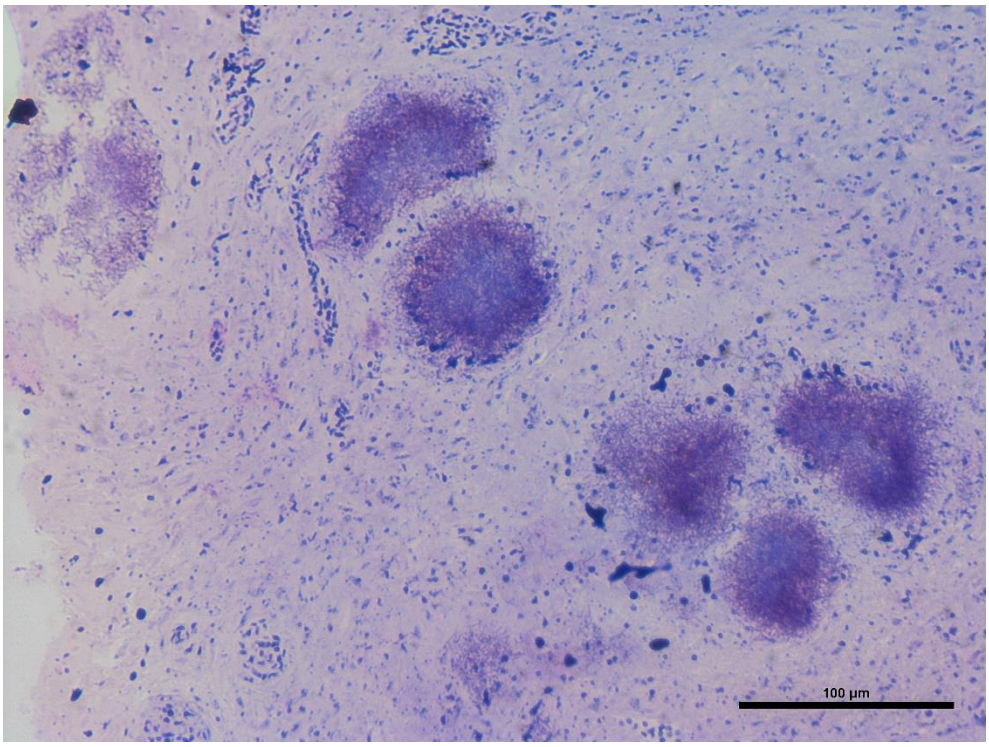


Figure 3.5. Histological section of a dermal lesion of a Nocardia-positive meagre. There are several bacterial colonies, which have elicited a moderate host response. Ziehl-Neelsen stain.

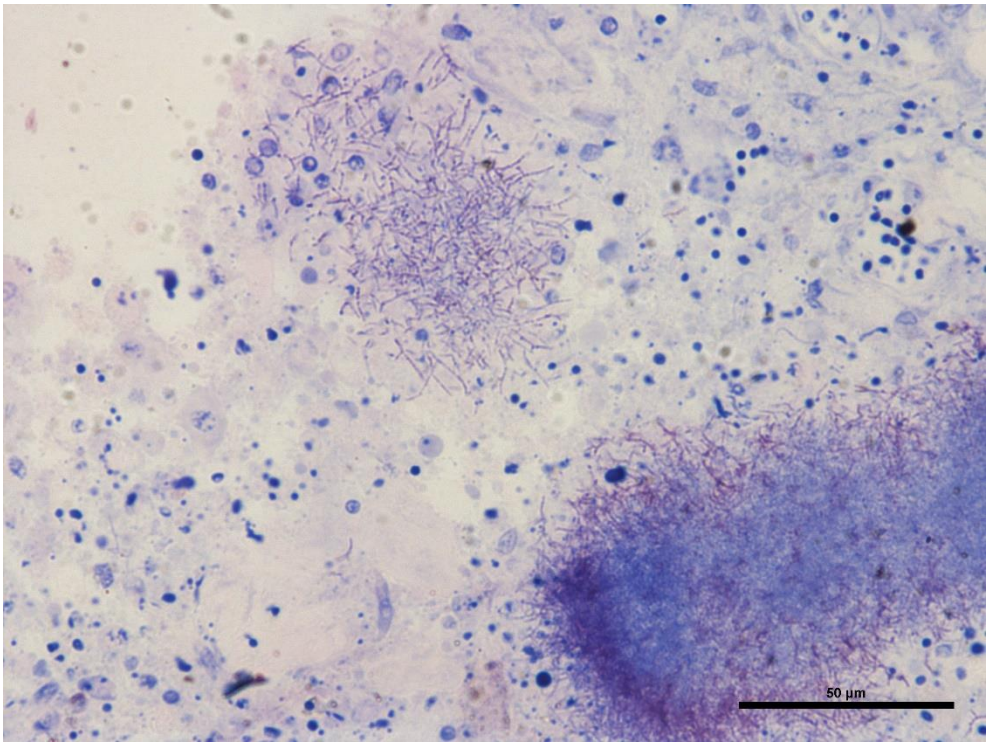


Figure 3.6. Higher magnification of the bacterial colonies from dermal lesions. Red staining indicates acid-fast positive bacteria. Note the filamentous branching morphology of the bacteria.

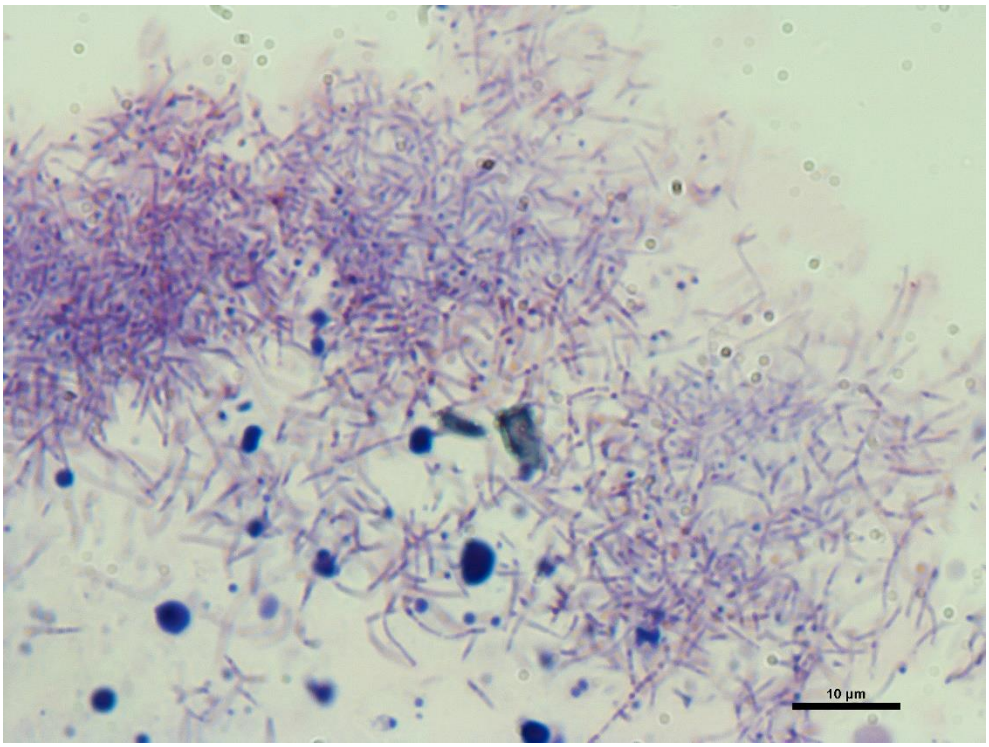


Figure 3.7. x100 magnification of the bacterial colony of a dermal lesion showing the morphological characteristics of the bacteria which are consistent with the descriptions of Nocardia in other fish species.

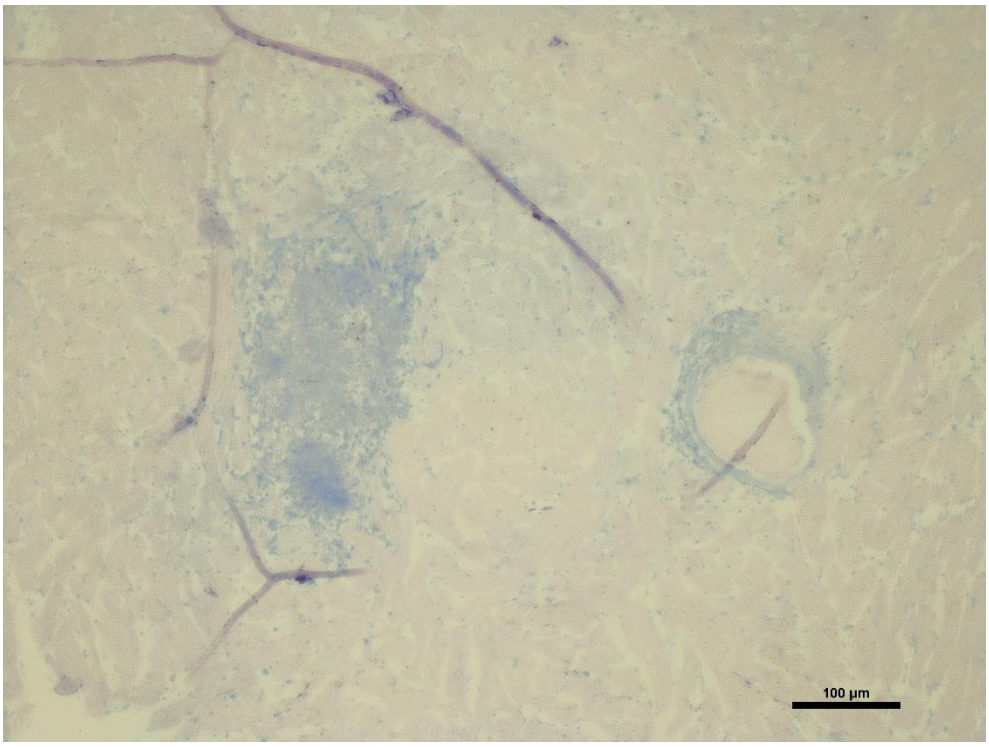


Figure 3.8. Heart histological section of a Nocardia-positive meagre. There are two different lesions standing out; on the left is a Nocardia colony, on the right a non-bacterial granuloma. Ziehl-Neelsen stain.

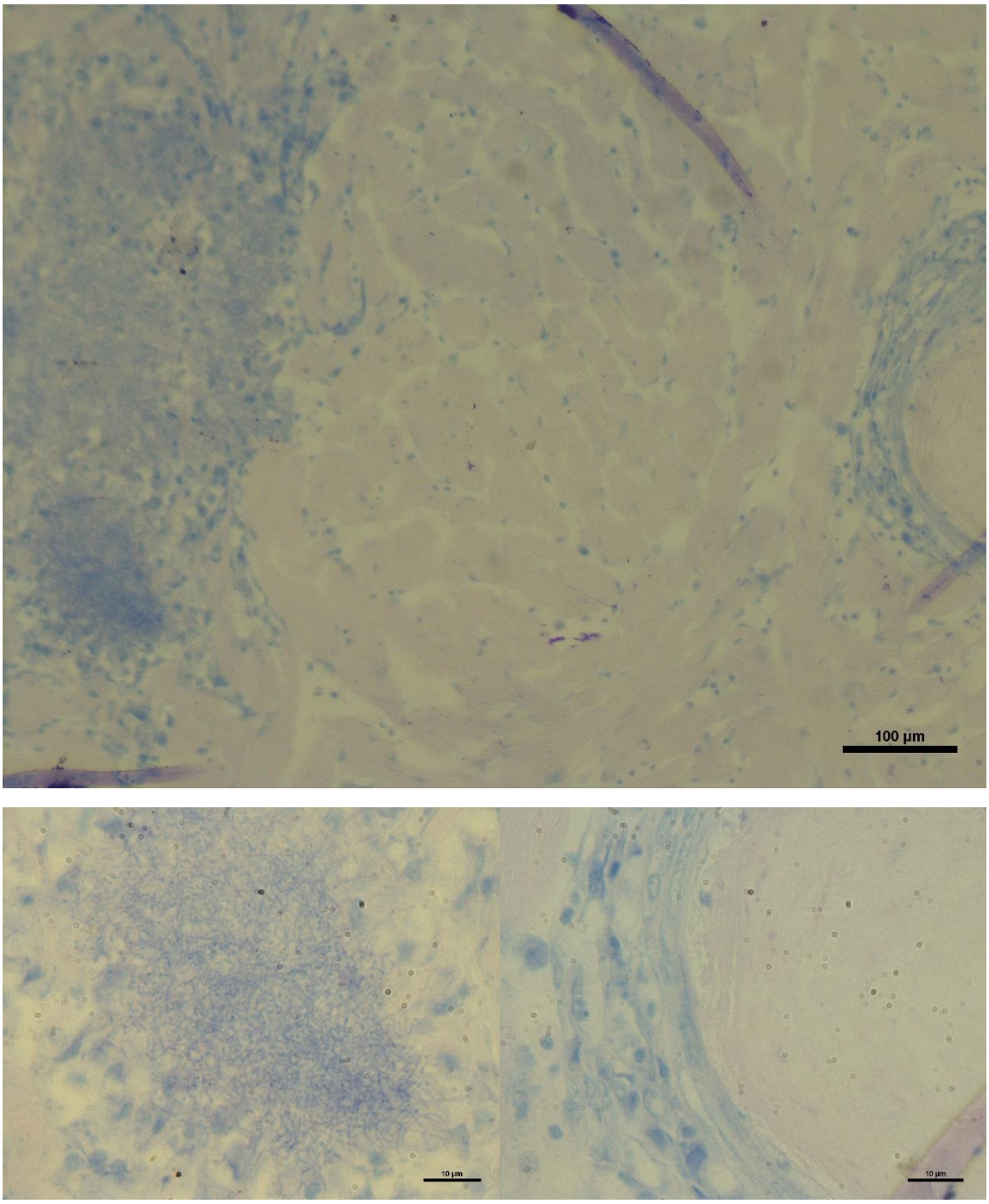


Figure 3.9. Upper picture. Higher magnification of the previous picture showing the differences of the two lesions. Lower left: x100 magnification of the bacterial lesion showing the distinct morphology of the filamentous bacteria. Lower right: x100 magnification of the granulomatous lesion where no bacteria can be seen. Ziehl-Neelsen stain.

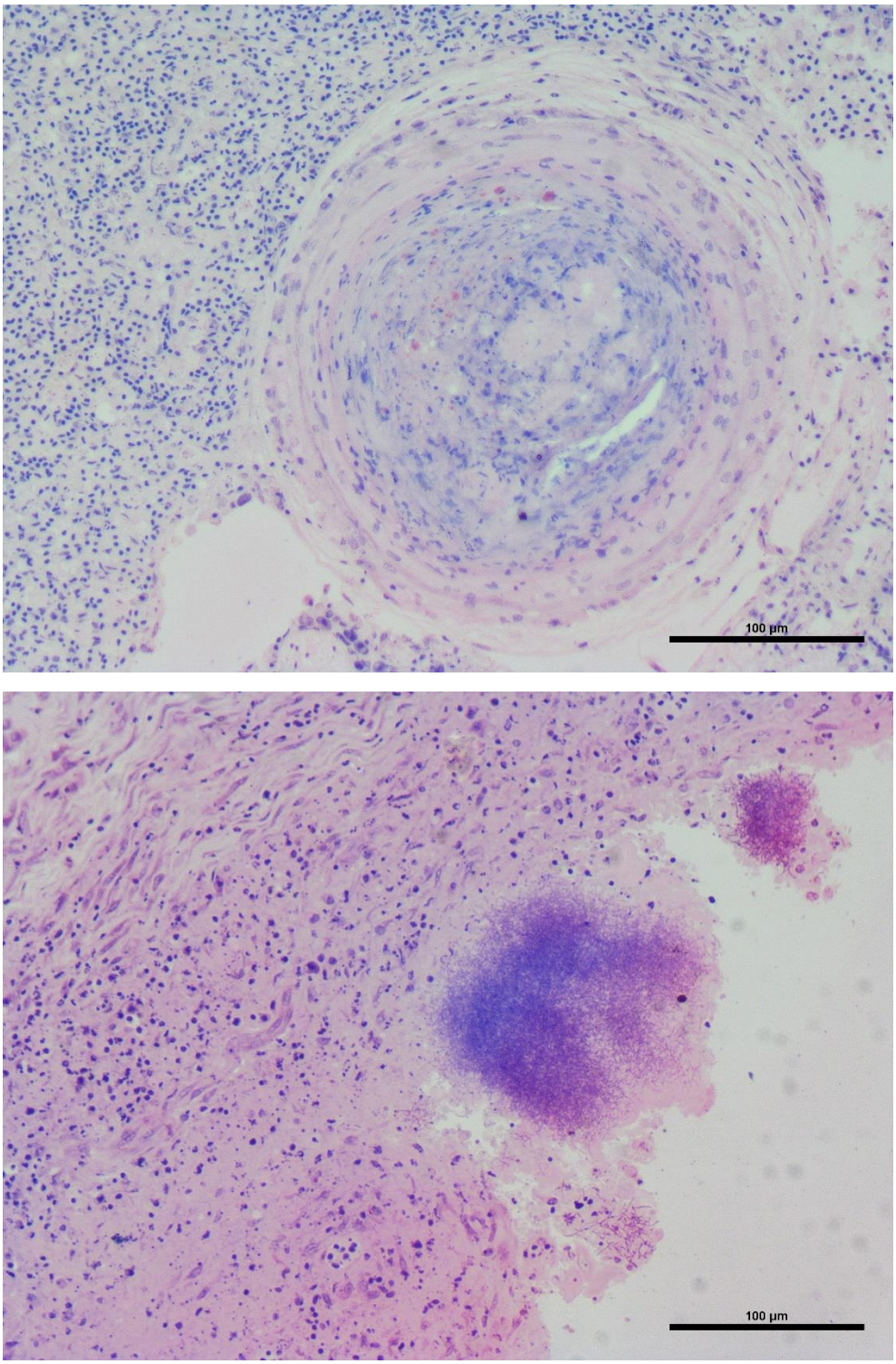


Figure 3.10. Comparison of a non-bacterial granuloma in the spleen (up) and a bacterial lesion (down) in the skin from the same, Nocardia-positive meagre.

Another interesting case was from the island of Leros. Five fish exhibiting epidermal lesions were sampled. All exhibited abscesses and granulomas in the kidney to different extend (**Figure 3.11**). Several mixed bacterial colonies were obtained in L-J. PCR from bacterial growth on L-J was negative for *Nocardia* spp. but gave a positive result for presence of Mycobacteria. *Histopathological examination* of spleen, kidney and liver *revealed that all samples were negative for Nocardia spp., however,* the fish had granulomatous lesions of bacterial origin in all organs. The bacteria were faintly acid fast, very small and coccoid in shape (**Figure 3.12)**.

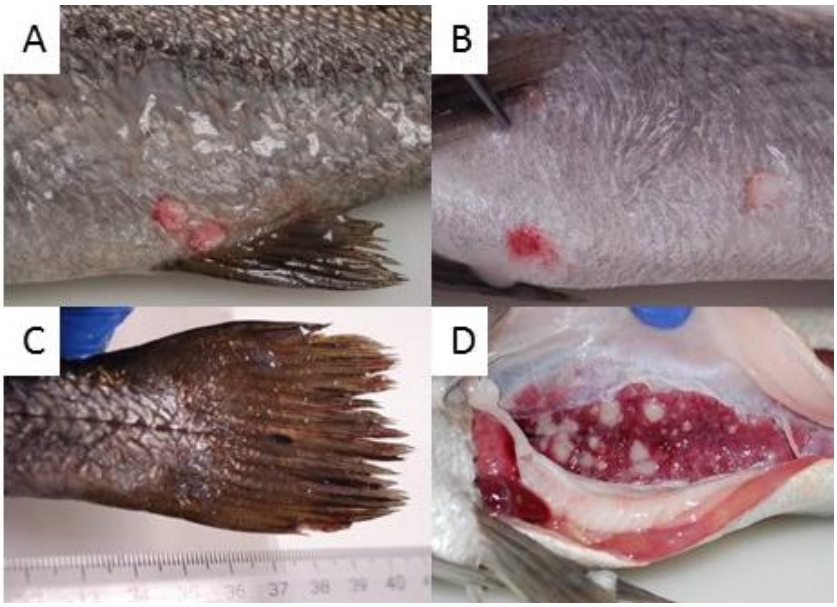


Figure 3.11. Fish exhibited granulomatous lesions on the skin (A and B), fin erosion (C) and abscesses and granulomas in the kidney resembling mycobacterial infection.

Subsequent recultures from the initial isolation were done in order to isolate putative Mycobacteria with no success. In addition, the positive PCR products from the initial mix isolation, were sequenced and compared against the GenBank sequences using BLAST algorithms. The analysis showed similarity with *Pseudomonas* sp. and not with *Mycobacterium*, possibly a cross reaction of the probes used.

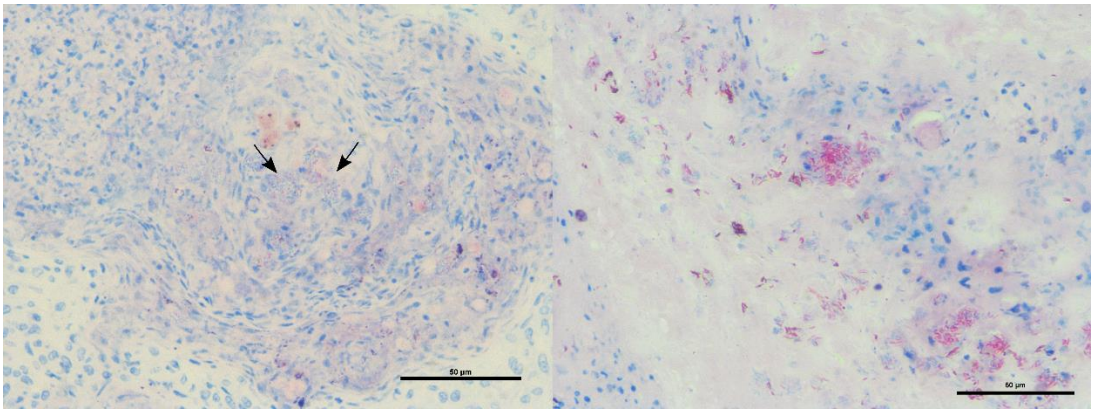


Figure 3.12. Left: Granuloma in the spleen of meagre from Leros. The granuloma contains "pockets" of small coccoid bacteria (arrows), which are stained faintly red with Ziehl-Neelsen. Right: Spleen granuloma from seabass, *Dicentrarchus labrax*, with confirmed infection by *Mycobacterium marinum*. This sample was used as positive control for this assay. Note how the acid-fast bacteria are stained vividly red and stand out in the section.

Figure **3.13** shows the phylogenetic analysis of the bacteria, which were either isolated in pure cultures in vitro, or were sequenced directly from the granuloma-bearing tissues.

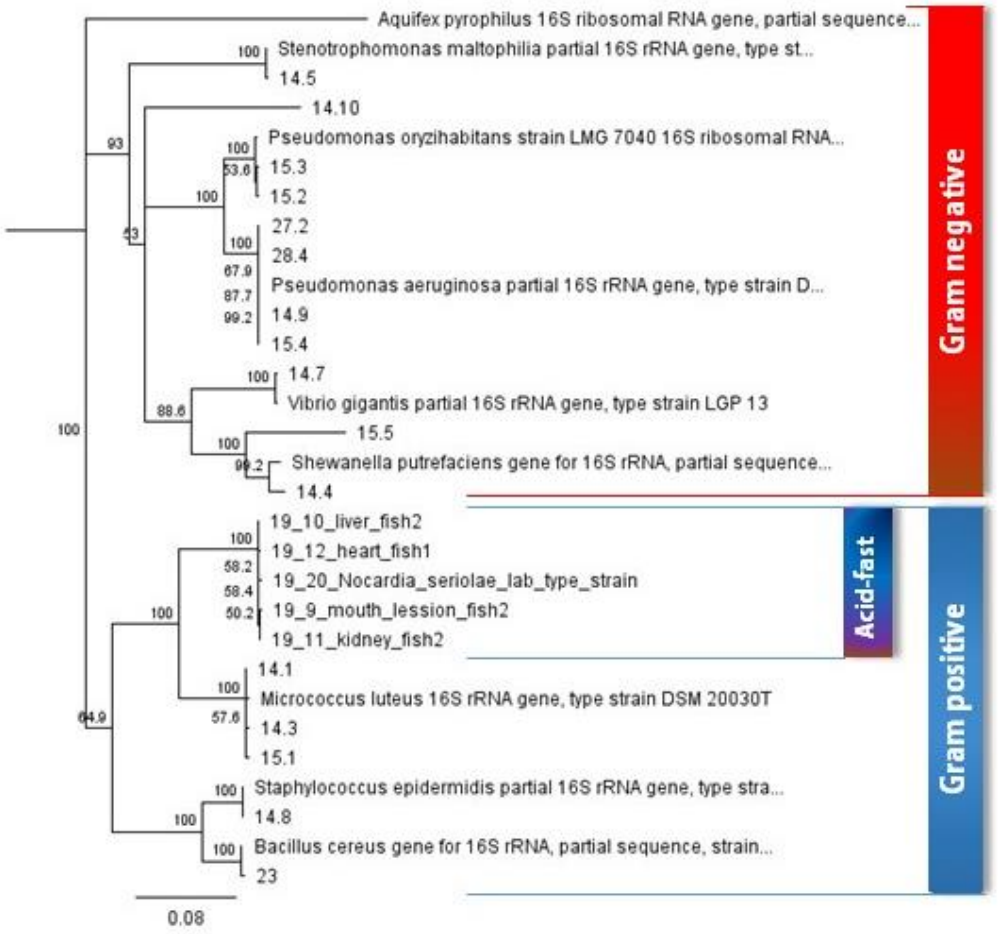


Figure 3.13. Phylogenetic analysis of the bacteria identified from the granulomatosis-affected meagres (information about the strains are included in Table XX). *Aquifex pyrophilus* was used as an outgroup. Partial 16s rRNA sequences of the type strains of the closest species of bacteria following BLAST search were retrieved from GenBank and used in the analysis. Phylogenetic analysis was performed in Geneious 9.1, using the Neighbor-Joining method with 1000 bootstraps.

Following extensive samplings covering a more than 3-year period, we have identified only one single case of nocardiosis in cultured meagre from a fish farm in West Greece. The affected fish were more than 2-years old and were very cachectic in their appearance. The most prominent characteristics of the nocardia-affected fish were the skin ulcers and the nodular lesions around the mouth. These findings are in accordance with the description of nocardiosis in meagre (Elkesh et al., 2013) and in other fish species (Chen et al., 2000; Cornwell et al., 2011; Kudo et al., 1988; Vu-Khac et al., 2016). The simultaneous presence

of the granulomas are very confusing and can be misleading since *Nocardia* can induce granulomatous lesions. SG affects almost all cultured meagre to a bigger or lesser extent, however this is the only case where Nocardia was detected. Fish from all other areas examined were negative for Nocardia using molecular tools for detection, including the fish from HCMR hatchery who develop the lesions without having been exposed to the open sea and are exclusively reared in borehole water. In addition, where Nocardia is the cause of disease, its traits are readily visible both macroscopically but also microscopically in histological sections as shown here. Therefore, the hypothesis that SG is of non-infectious aetiology is enforced. Nevertheless, Nocardia can be considered a serious threat if it becomes widespread, not only to meagre but also to other cultured species known to be susceptible such as greater amberjack. Until now, this is the second report of Nocardia in Greece. It comes from the same geographic location and it has affected again cultured meagre. It could be speculated that this might be an endemic problem, however it should be closely monitored, since Nocardia is hard to eradicate and on top of the morbidity it could lead to a deterioration of the product quality.

From the bacteriological survey conducted in this task, it seems that meagre is a species which is not prone to bacterial infections. We have not seen any serious epizootic related to bacteria during this survey. This is a surprising fact since, severely affected fish from SG would be considered compromised and more susceptible to disease. The bacteria isolated and identified here, are more likely common inhabitants of seawater and can be considered accidental findings without any true clinical significance. The most interesting isolate is *Micrococcus luteus*. This bacterium has been isolated from several individuals coming from different places; it has also been isolated from meagre's brain. Moreover, this bacterium was the most commonly isolated species in HCMR facilities exclusively from meagre before the start of DIVERSIFY project. *Micrococcus* spp. are usually considered environmental contaminants (Konar and Das, 2013), however in our lab it has been recovered only from meagre and no other fish. It has been considered as an occasional fish pathogen (Austin and Austin, 2007) being able to induce mortality in rainbow trout challenged with 10^5 cfu, but it has also been used as a probiotic supplement in Nile tilapia offering protection against *Aeromonas hydrophila* (Abd El-Rhman et al., 2009). Apart from its common presence in various samples examined, interestingly, *Micrococcus luteus* belongs to the order of Actinobacteria, which also contains *Nocardia* spp., also evident in the phylogenetic analysis presented here.

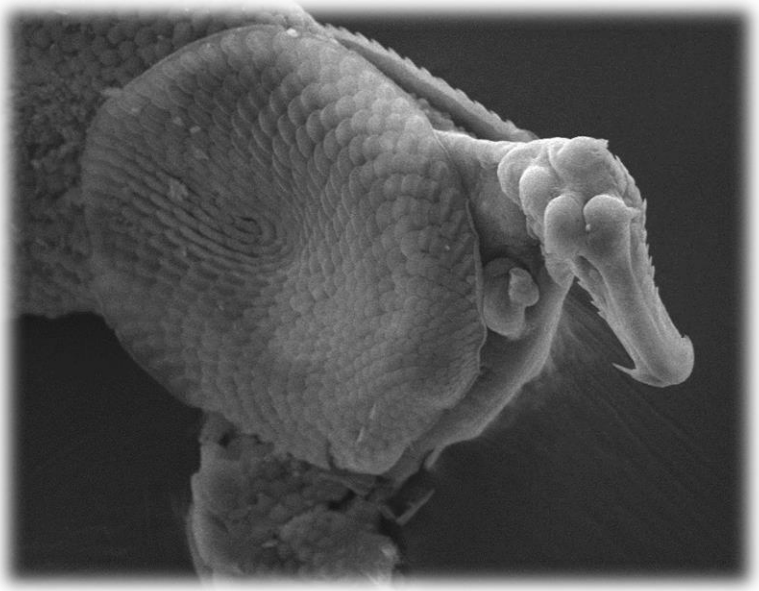
Other unpublished reports from Greece regarding bacterial pathogens of meagre suggest that the fish could be susceptible to vibriosis caused by *Vibrio anguillarum* and photobacteriosis caused by *Photobacterium damselae* subsp. *damselae*. Photobacteriosis caused by *Photobacterium damselae* subs *damselae* has been reported to be responsible to accumulative mortality of 80% in Spain (Labella et al., 2011). Mycobacteriosis has also been reported from Turkey (Avsever et al., 2014; Timur et al., 2015).

The conclusions of this task are that nocardiosis is present in Greece, most probably in a confined geographical region, however it is not the cause of SG. Generally, the species does not seem to be very susceptible to common bacterial infections, however there are sporadic reports suggesting that several pathogens may become problematic in the future. Vibriosis is expected to affect meagre culture in the future especially as this intensifies with time. Vaccination has reduced significantly the incidences of vibriosis in other established species, such as seabass and seabream, therefore emphasis should be given in developing and testing of vaccines against this disease of major importance.

4. DIPLECTANUM SCIAENAE

Pantelis Katharios (HCMR)

Contributed: Maria Ioanna Tsertou (HCMR), Karl Andree (IRTA), Ana Roque (IRTA), Neil Duncan, (IRTA), Enric Gisbert (INRA), Alicia Estevez (IRTA)



Results presented in this chapter has been published in

Andree, K.B., Roque, A., Duncan, N., Gisbert, E., Estevez, A., Tsertou, M.I. and Katharios, P., 2015. Diplectanum sciaenae (Van Beneden & Hesse, 1863)(Monogenea) infecting meagre, Argyrosomus regius (Asso, 1801) broodstock in Catalonia, Spain. A case report. *Veterinary Parasitology: Regional Studies and Reports*, *1*, pp.75-79.

Diplectanum sciaenae

Recurrent infestation of captive meagre, *Argyrosomus regius* by a monogenean parasite has been observed in the broodstock facilities of IRTA in Catalonia, Spain over the past years. Following handling procedures related with hormonal treatment for spawning induction, one fish died. *Post mortem* examination and detailed microscopical examination using light microscopy, SEM and histology revealed hyperinfection by *Diplectanum sciaenae* (Monogenea, Diplectanidae) which caused significant gill pathology. In the present chapter we provide detailed description of the parasite and the pathology caused to its host.

A broodstock of meagre, *Argyrosomus regius* was brought into the facilities of IRTA (Catalonia, Spain) in November 2008. Fish were given a formalin bath (1 hour at 0.01 mL L^{-1}) each week for 3 weeks and were fed 5 mg kg^{-1} of praziquantel (Duncan et al. 2013). In May 2009, there was an outbreak of an unidentified diplectanid monogenean that resulted in mortality of 6 breeders. Subsequently, each spring, 2 formalin baths were administered to the broodstock as described and no further outbreaks were observed until the present report. Monogeneans are highly host specific parasites and this applies also to species of the *Diplectanum* genus. Up to date, 3 monogeneans have been described in wild meagre; *Diplectanum sciaenae, D. bocqueti* and *D. dollfusi* (Oliver, 1980).

Following certain handling procedures related to hormonal treatment one breeder died. The breeder in question was a 13.7 kg male captured at the coast of Algarve, Portugal. The fish had undergone a series of handling procedures including anesthesia, GnRHa injections, sperm extraction and transfer between tanks during May and June 2015. The fish was examined post-mortem and gills were found to be heavily infested (>300 parasites per gill arch) by the monogenean parasite. Gill samples were dissected and preserved in 5% buffered formalin in order to identify the parasite and assess the pathology causing to its host. Parasites and gill samples were also fixed in 2.5% glutaraldehyde in sodium cacodylate buffer for Scanning Electron Microscopy (SEM) observation. In addition, the dead fish showed overinflation of the swimbladder which has been observed to cause mortalities of meagre breeders in the absence of any infection (Duncan et al. 2013).

We have made thorough microscopical observation of the parasite and the infected gills including light microscopy, histology and Scanning Electron Microscopy in order to identify the parasite to species level and to assess the pathology related with the infestation. Samples for histology and SEM were processed as described previously (Katharios et al., 2013). SEM observations were made at the Electron Microscopy Laboratory of the University of Crete while parasites were photographed and measured (n=20) from formalin preserved samples using a Nikon Eclipse 50i microscope equipped with a digital camera and Image Analysis System.

Body length of the parasite was 457.83±10.50 μm and width was 228.11±1.39 μm. One of the main characteristic of the *Diplectamum* genus is the presence of two

squamodiscs (Bikhovski, 1961) supplementing the attaching armature of the parasite **(Figure 4.1A, 4.1D, 4.2A and 4.2B)**. They were located on the upper edge of the haptor, one at the ventral and one at the dorsal side. They were equipped with a large number of small chitinous rodlets (figure 1D) arranged in concentric rows and covered with scales (figure 2B and 2C). Squamodiscs had a diameter of 159.63±7.96 μm and consisted of 40 rows of rodlets. The parasite's hamuli were located at the posterior part of the body on the haptor (figure 1A). Total width of haptor was 280.32±9.78 μm. There was a chitinous plate consisting of three linked parts (transverse bar) connecting the hamuli **(Figure 4.1C)**.

A sharply curved sclerotized canal was evident at the prostatic reservoir **(Figure 1B and 1E)**, which together with a short penis with spiral ending are diagnostic features of *Diplectanum sciaenae* (Oliver, 1980). Tegument with plate-like scales covering half of the body including squamodisc and up to the level of ovary **(Figure 4.2A, 4.2C and 4.2D)**.

There were six glandular organs at the cephalic region **(Figure 4.1A, 4.2F)** and 2 pairs of eyespots at the anterior part of the worm below the cephalic glandular organs **(Figure 4.1A and 4.1B)**.

Eggs were oval-shaped 75 μm long and 35 μm wide (figure 1G). Lower pole of the egg had a sprout called little foot extending outwards approximately the length of the egg. Little foot had a star-like platform (figure 1H) at its free ending helping it to anchor to substrates.

According to Oliver (1980), *Diplectanum sciaenae* is 500-1160 μm in length, 140-400 μm in width and has a haptor measuring 260-380 μm in width. Its squamodisc is 144-170 μm in diameter and consists of 31-41 rodlet rows. It is differentiated from the other two *Diplectanum* species infecting meagre through the shape of sclerotized canal of the prostatic reservoir and the size and shape of penis.

Based on the above, the parasite was identified as *Diplectanum sciaenae*. The difference in the body length may be due to shrinkage caused by formalin fixation.

The parasites had caused significant pathology at the gills of their host. Focal lamellar epithelium hyperplasia was evident and in several lesions gill lamellae were fused **(Figure 4.3A)**. In addition, there was congestion of the lamellar capilaries (also evident in SEM) located mostly at the areas where the parasites were attached **(Figure 4.2G, 4.3B and 4.3C)**. There was a lifting of epithelium, focal haemorrhages and moderate inflammation **(Figure 4.3C, 4.3D and 4.3E)**. Cross sections of *D. scianae* haptors indicated that the parasite was anchored with the hamuli at the base of gill lamellae and used the two squamodiscs to attach firmly between two adjacent lamellae **(Figure 4.3D, 4.3E and 4.3F)**. It is likely that the tegument scales may also contribute to attachment at the lamellae offering increased grip at the half of the body which is within the lamellar area. This is probably why scales are missing from the upper half which is not used for attachment and emerges out of the lamellae **(Figure 4.2H)**.

It is generally accepted that *Diplectanum* spp. are not highly pathogenic parasites, however under adverse conditions these parasites can multiply and have detrimental effect

on their hosts. In aquaculture there are several reports associating monogenean infection with increased fish mortality (Katharios, Hayward, Papandroulakis & Divanach 2006b; Katharios, Papandroulakis & Divanach 2006a; Dezfuli, Giari, Simoni, Menegatti, Shinn & Manera 2007). This is mostly because fish in aquaculture are grown in high densities and are usually under a continuous stress which has a direct effect on their immunocompetence (Faliex et al., 2008) in addition to the direct life cycle of monogeneans which in combination with the availability of host may easily result to hyperinfection. The attachment of the parasite together with the feeding mode of the monogeneans may also result to secondary bacterial infections that may complicate further the health status of the fish.

In this case, the animals had been subjected previously to certain handling stress as they were undergoing hormonal treatments for spawning, and sampling to observe gonadal maturation. Furthermore, it has been shown steroid hormones have an immunomodulatory effect in both humans (Grossman, 1985) and fish (Harris and Bird, 2000) which may have played a significant role in the manifestation of the parasitism. There is real cause for concern, due to this parasite's ability to induce respiratory distress and in cases of heavy infestations, mortality. Therefore, seasonal dietary regimes enhancing immune system coupled with antihelminthic chemotherapeutics or other preventive measures should be sought.

This is the first report of *Diplectanum scianae* infecting cultured meagre and the first report associating this parasite with fish mortality. Although actual cause of death may be a combination of factors it is highly likely that parasitic hyperinfection was an important contributing factor. This parasite has been reported in the Biscay Gulf (Oliver, 1980) while *Diplectanum* sp. (unidentified to species level) infecting cultured meagre has been reported in the Atlantic coast of Portugal (Soares et al., 2012). It has also been reported at the Eastern Mediterranean (Oliver, 1987) however recent parasitological surveys in Corsica (Ternengo et al., 2010a), Italy (Merella et al., 2009), Turkey (Toksen et al., 2007) and Greece (unpublished) have not indicated it in cultured populations. With the expansion of meagre culture in Europe, great caution should be exercised during transportation of fish stocks between farms in order to reduce risks related to the spread of the parasite.

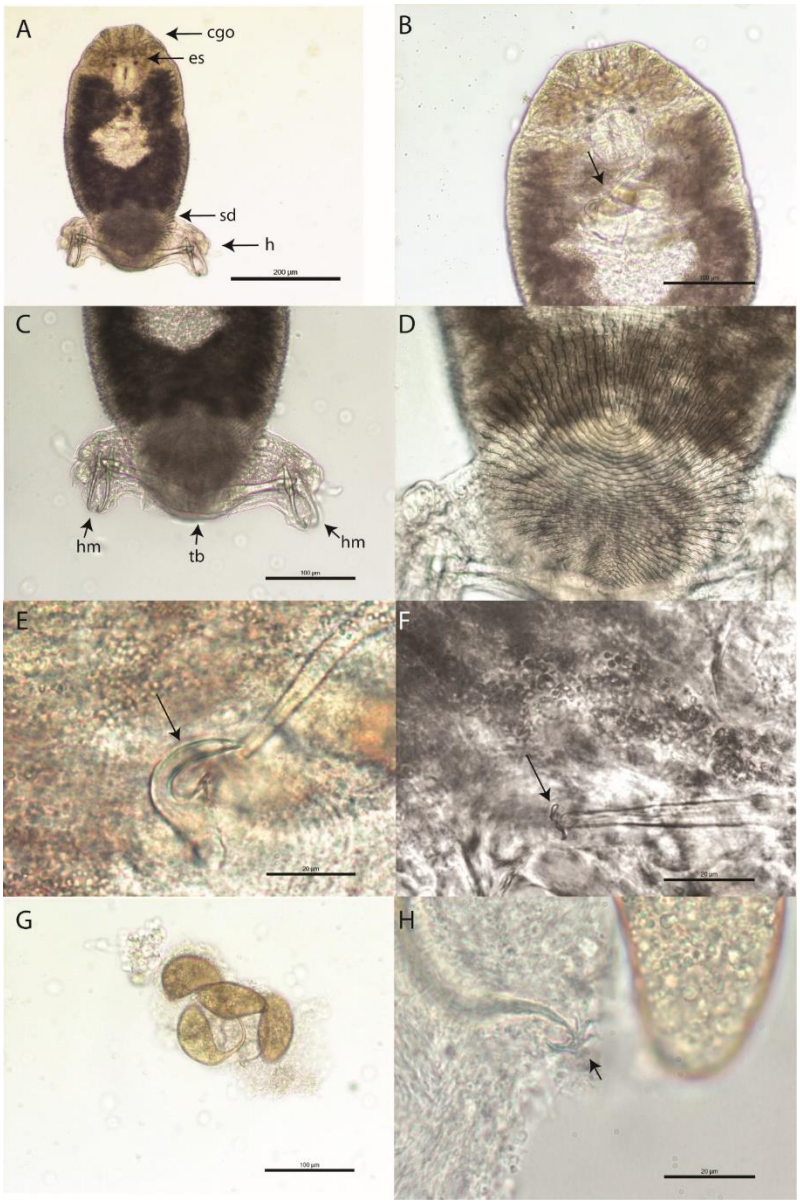


Figure 4.1. Light Microscopy**.** *A) Diplectanum sciaenae* from meagre gills, **cgo**: Cephalic glandular organs, **es:** eyespots, **sd:** squamodisc, **h:** haptor. *B)* Arrow points to the copulatory organs. *C)* Details of the haptor, **hm:** hamuli, **tb:** transverse bar. *D)* Squamodisc consisting of 40 concentric rows of rodlets. *E)* Arrow points to the sclerotized canal of the prostatic reservoir. The sharp curve is a diagnostic feature of the species. *F)* Arrow points to the end of the penis which has a spiral shape, another diagnostic feature of the species. *G)* Eggs of *Diplectanum sciaenae*. *H)* The little foot of the egg ends at anchor-like structure (arrow).

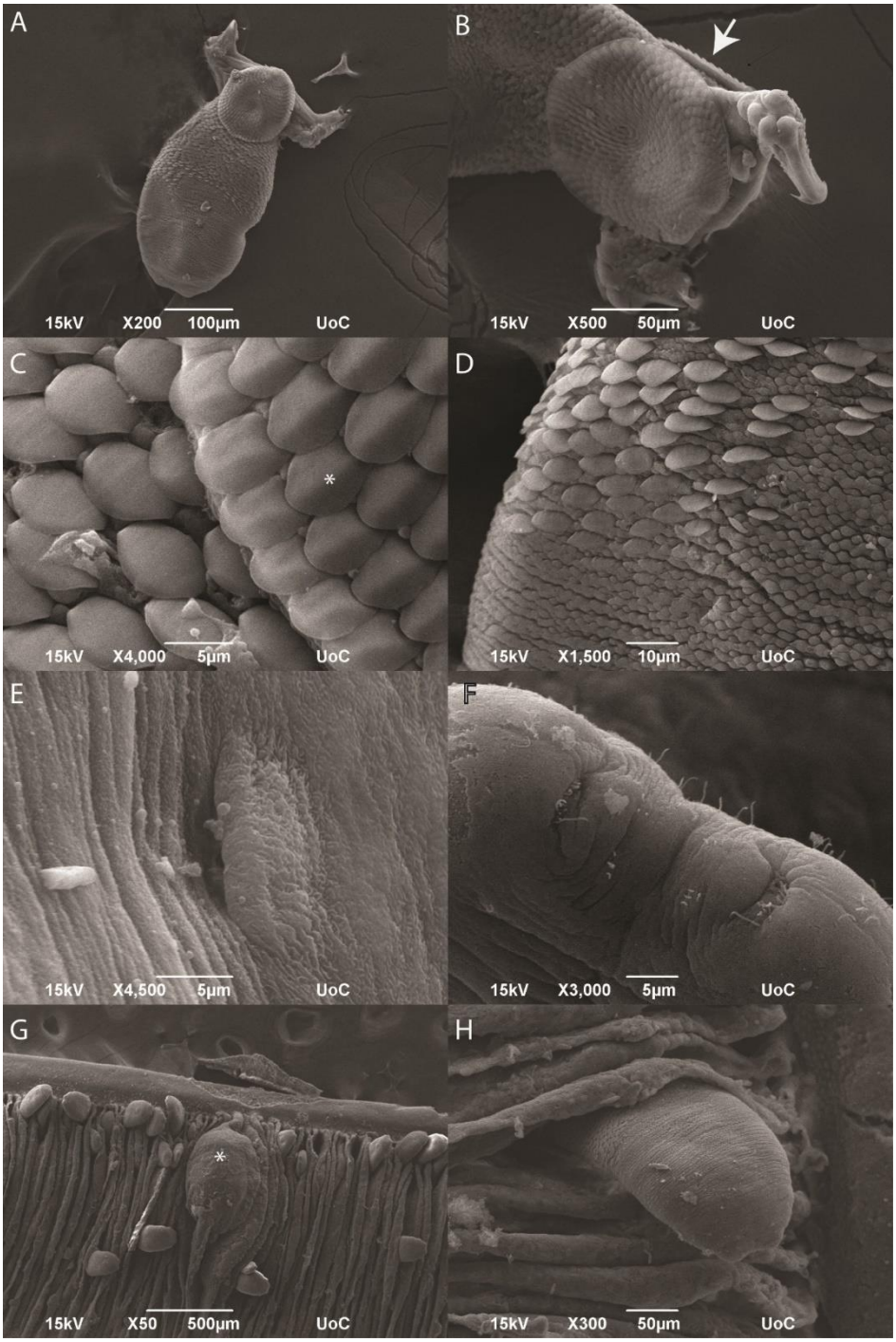


Figure 4.2. Scanning Electron Microscopy. *A) Diplectanum sciaenae*, note the scaly tegument from the squamodisc up to the midpart of the body. *B)* Squamodisc, arrow points to the second squamodisc at the back of the body. *C)* Scales of the tegument, **(*)** squamodisc area, *D)* Transition of the body between the scaly and non-scaly part. *E)* Pocket-like opening of the mouth, *F)* Cephalic area with the glandular organs, note the sensory cilia. *G)* Gill lamellae with numerous parasites, **(*)** congestion of the lamellar capillaries. *H) D. sciaenae* emerging from gill lamellae.

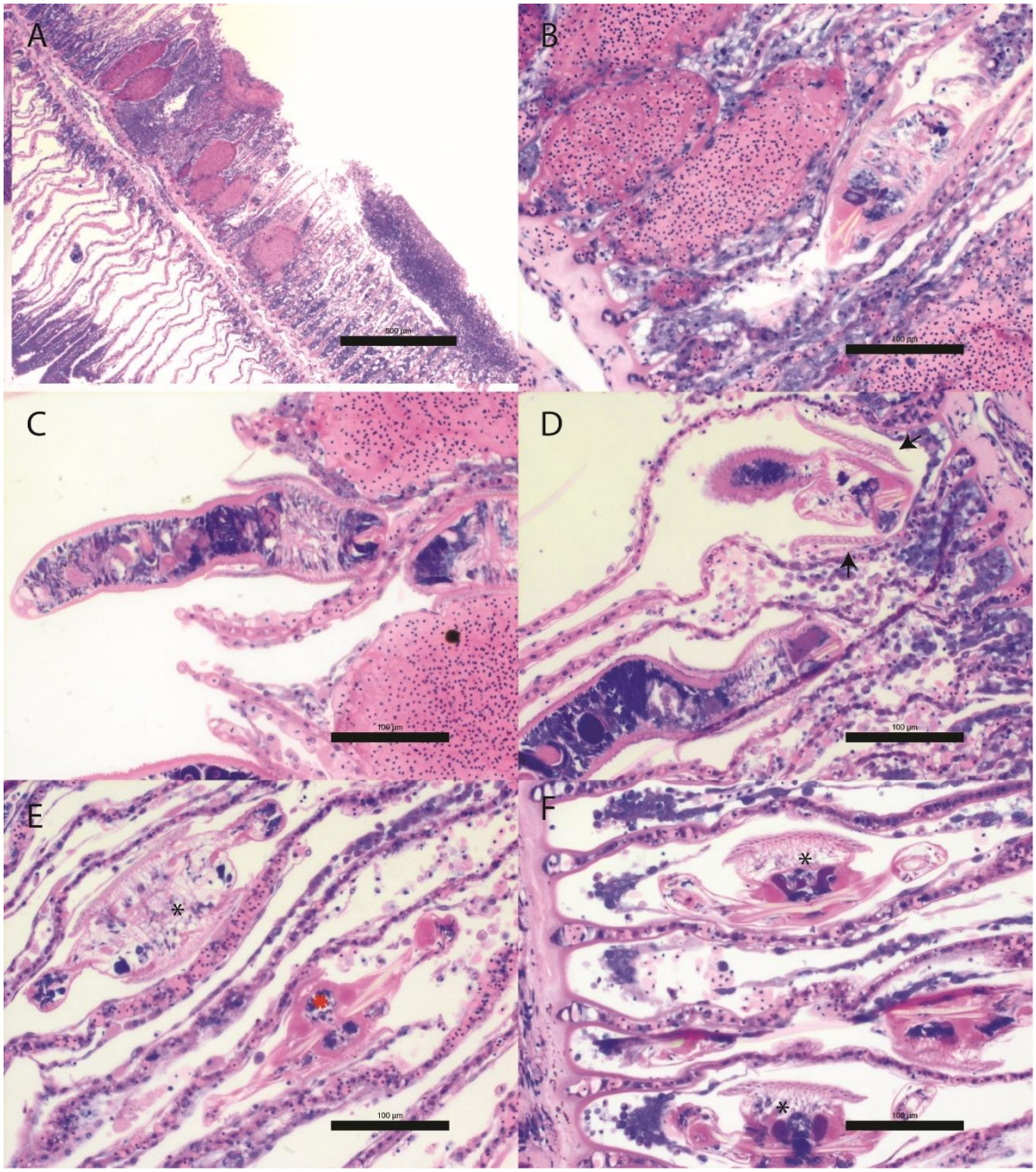


Figure 4.3. Histopathology. *A)* Epithelial hyperplasia of the secondary lamellae and congestion of the lamellar capillaries at the gills of *D. scianae* infected meagre. *B)* Higher magnification of the capillary congestion with a parasite at the adjacent lamella. *C)* Parasites attached at the gill lamellae next to capillary congestions *D)* Cross section of the parasite, arrows indicate the two squamodiscs. Note the scaly appearance of the tegument. Lifting of the epithelium is evident. *E)* and *F)* Cross section at different levels of the haptor (*) which is the attaching device of the parasite. Red asterisk indicates a haptor sectioned at the level of the transverse bar.

5. SCIAENACOTYLE PANCERI

Pantelis Katharios (HCMR)

Contributed: Maria Ioanna Tsertou (HCMR), Ana Roque (IRTA), Neil Duncan, (IRTA), Enric Gisbert (INRA)

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Sciaenacotyle panceri

In the early autumn 2005, disease outbreaks in meagre were reported in some Corsican fish farms. The outbreaks were attributed to microcotylosis and the causative agent was identified as *Sciaenacotyle panceri* **(Figure 5.1A)**. This parasite is known to occur on the gills of wild shi drum (*Umbrina cirrosa*) and wild meagre (*Argyrosomus regius)* (Ktari, 1970). The presence of the parasite in reared meagre is probably due to a transmission from wild populations (Ternengo et al., 2010b). Since the first description of the species by Sonsino in 1891, few reports mentioned the presence of *Sciaenacotyle panceri* and no pathology has been described in shi drum or in meagre. However, under mariculture conditions, as consequence of fish overstocking and direct life cycle of the monogenean, *S. panceri* caused severe pathology in fish and losses in Corsican aquaculture.

Sciaenacotyle panceri is a large gill parasite. Elongated body can reach 10 mm in length (Ktari, 1970). The monogenean possesses a strong posterior attachment organ or haptor armed with numerous clamps **(Figure 5.1B)**. As in many polyopisthocotyleans, clamps are used to grip gill lamellae **(Figure 5.1C)**. Buccal sucker is the main blood feeding organ. Dispersal rates are favored by the eggs' features. They are fusiformes and show one filament at pole. Egg filaments entangle on nets or fish gills contributing to fast infection spreading. The larvae (oncomiracidia) are able to swim and can easily find suitable hosts.

Initial pathological responses of *Argyrosomus regius* include lethargy and loss of appetite. Mucus excess, gill opacity, and haemorrhages can also be observed. Due to its blood-feeding nature, *S. panceri* causes severe anaemia and reduces fish tolerance to stressors such as treatments or handling.

The course of the disease has been described in details previously (Merella et al., 2009; Ternengo et al., 2010b). At the beginning of the infection, the parasite prevalence was very high since all the fish were parasitized. As for many species of parasites, the intensity of *S. panceri* is linked with the fish size. The largest fish harbor more parasites due to the greater gill surface for parasite attachment, greater water flow and increased gills attractiveness as consequence of physical (ventilation) and chemical (mucus) stimuli (Kearn, 1967). This can be demonstrated by the fact that larger meagres were infested with more than 250 monogeneans per gill arch.

Intensities and fish mortality have increased in the months following the first diagnosis. Unlike adult fish, juveniles continued to experience mortality until December when water temperature was lowest. Temperature is thought to drive seasonal variation of parasite intensity. This could be either due to parasite species specific preferences or due to host immune system condition which is known to be affected by changes in water

temperature. Furthermore, adult fish may have confronted a milder parasitosis in the past that may have led to higher immune tolerance to that of the juvenile fish that seem more vulnerable to the disease. The implication of the immune system to the resistance of fish to monogeneans have been studied by Kim and Cho (2000) which have suggested that specific and non-specific immune factors participate in the protection of rockfish, *Sebastes schelegeli* when immunized with *Microcotyle sebastis*.

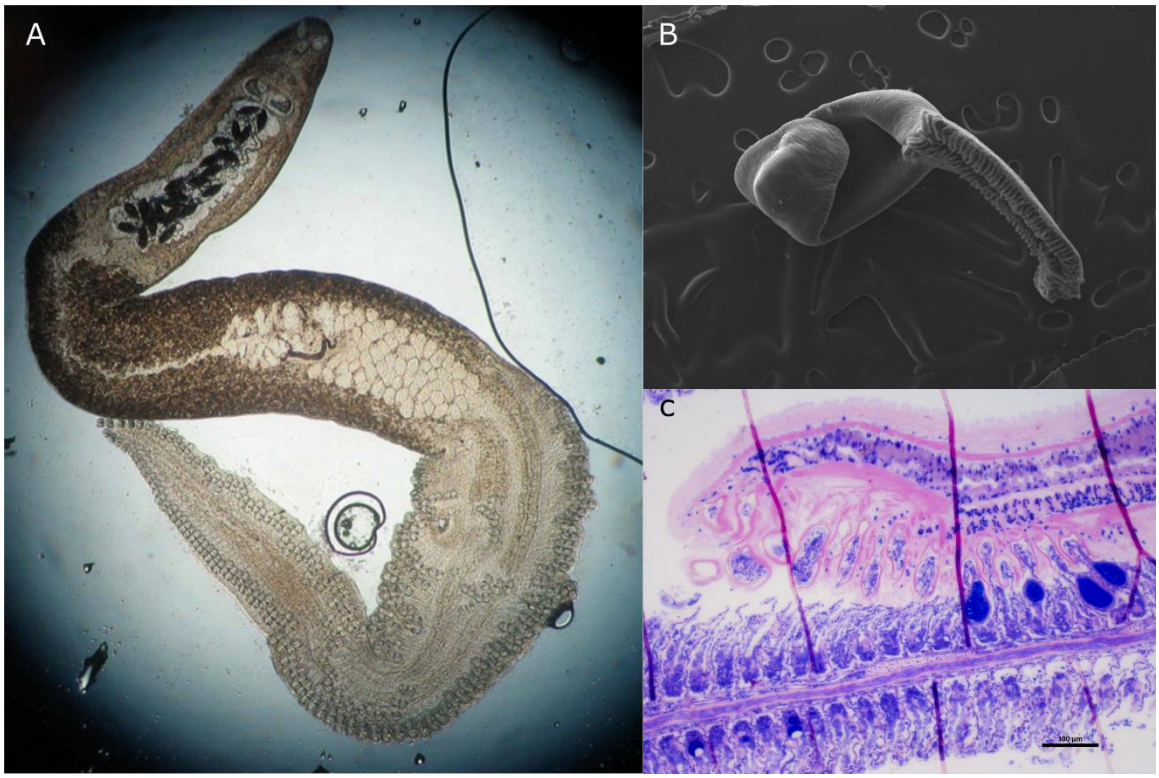

Figure 5.1A Mature specimen of *Sciaenacotyle panceri* (photo of Dr. Y Quilichini, University of Corsica as published in Fish Farming Expert 2008 by Ternengo and Katharios). **B.** SEM picture of the parasite showing the large number of clumps in the haptor, its attaching device. **C.** Histological section of meagre gills infected by *S. panceri* showing the attachment on the lamellae using the clams.

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