New species for EU aquaculture

Deliverable Report

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Deliverable Title	Diagnostics protocol for Systemic Granulomatosis, causes and solutions in meagre		
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WP Title:	Fish health - meagre		
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Task Title:	Systemic Granulomatosis		
Other beneficiaries:	P2. FCPCT		
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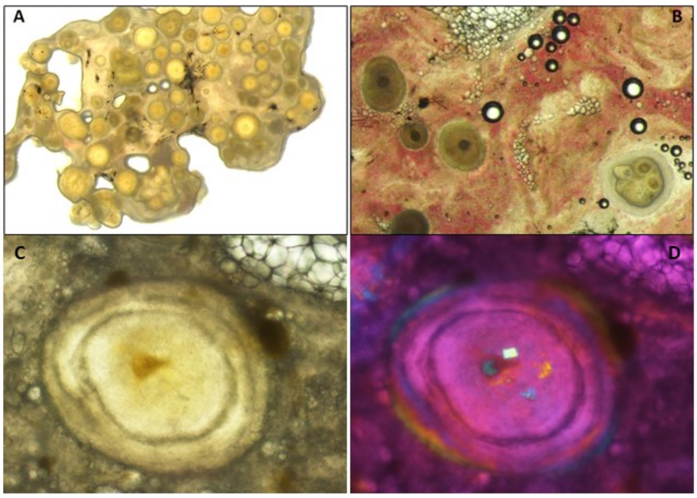
Objective: Diagnostics protocol for Systemic Granulomatosis, causes and solutions in meagre: Systemic granulomatosis is the most significant disease affecting almost every cultured meagre. The disease is characterized by multiple visceral granulomas that progressively develop extensive tissue calcification. This deliverable contains the pathological assessment performed by two partners, HCMR and FCPCT on various fish samples derived from the nutritional experiments performed in the previous task of the WP. The assessment will be based on visual inspection, histopathology, electron microscopy and on the assessment of selected blood biochemical parameters.



Introduction

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; 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; Katharios et al., 2011) similar to systemic granulomas observed in other cultured fish species (Balouet and Baoudin Laurencin, 1986; Baudin Laurencin and Messager, 1991; Dunbar and Herman, 1971; Good et al., 2015; Paperna, 1987).

Through various tasks in Work Package 24 – Fish health–meagre (WP24) we tried to test these two hypotheses; for this we have run 5 feeding trials to identify potential nutritional causes of SG (Task 24.1) and monitored meagre populations farmed in various locations in Greece over the past 3 years in order to isolate and identify *Nocardia* spp., or other granuloma-associated pathogens (Task 24.4).

The aim of this deliverable is to provide a diagnostic protocol for SG supported mainly on the macroscopic and microscopic results from various tasks.

Macroscopic and microscopic examination of SG

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 most by SG. In heavily affected fish with many visible granulomas, a 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 and 2**).

Figure 1. A,B. External lesions on the eye and the tail fin of meagre related to SG. **C,D.** Dystrophic calcification of the liver and the kidney.

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 3**). Using polarized light, crystal structures are seen in the center of the granulomas (**Figure 3C, D**). The characteristic "onion-like" appearance was also evident in photos from scanning electron microscopy (**Figure 4).**

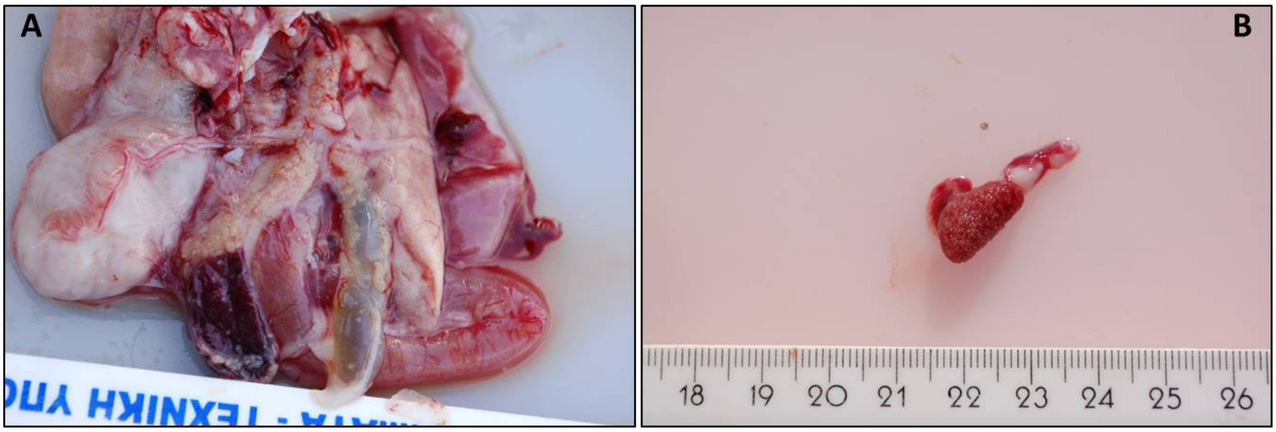


Figure 2. **A**. Multiple granulomas in the soft tissues of meagre. **B**. Heart of meagre fully covered by granulomas.

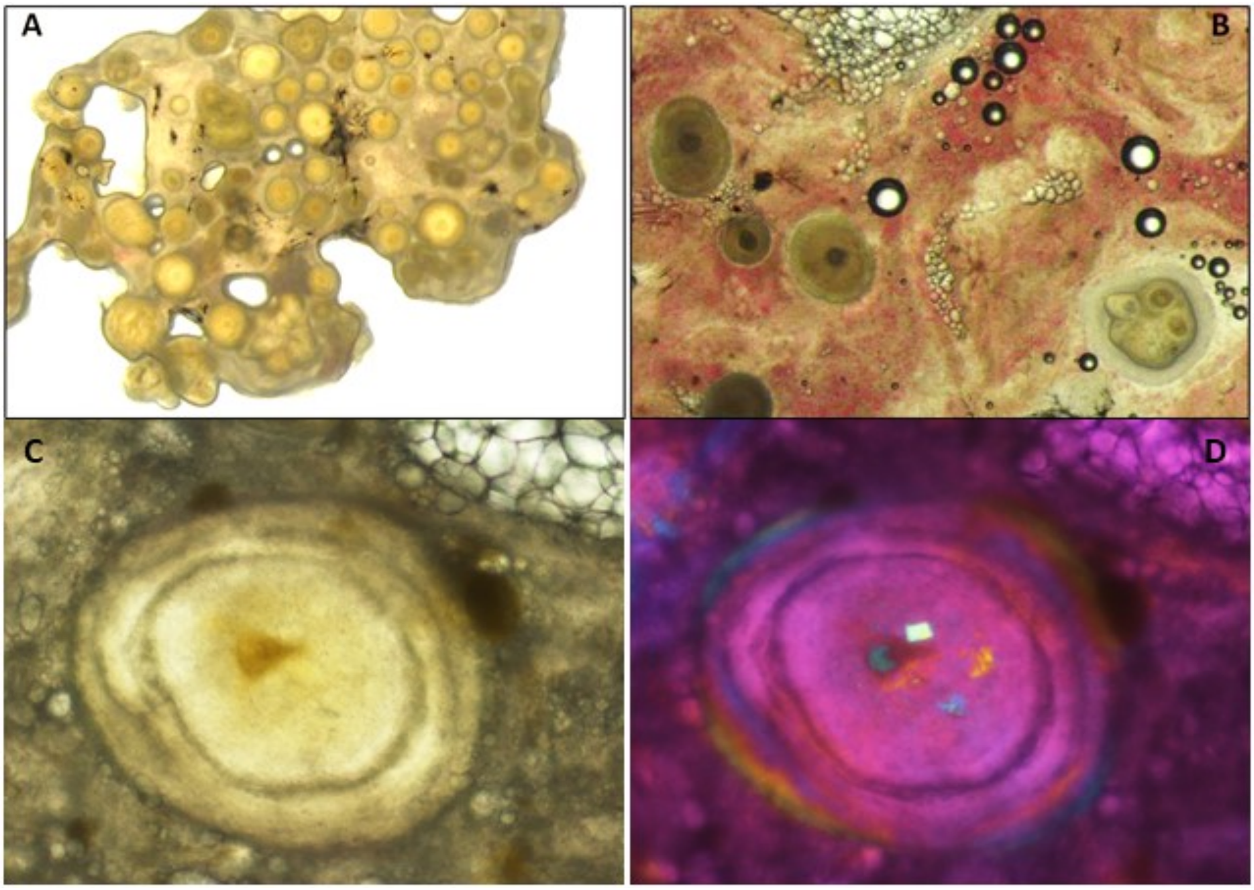


Figure 3. Fresh squash preparation from liver (**A**) and kidney (**B**) with granulomas (A: stereoscope x1, B: stereoscope x2.5). **C.** Granuloma under light microscopy. **D.** The same granuloma under polarized light showing crystal structures in the center.

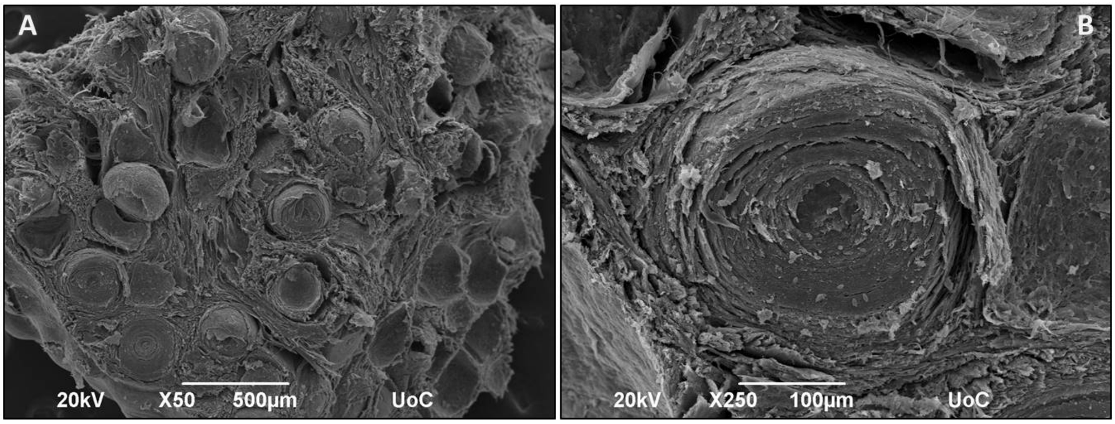


Figure 4. A. SEM microphotograph of the heart covered by granulomas. **B.** SEM microphotograph of a heart granuloma with "onion-like" appearance.

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 5 and 6**).

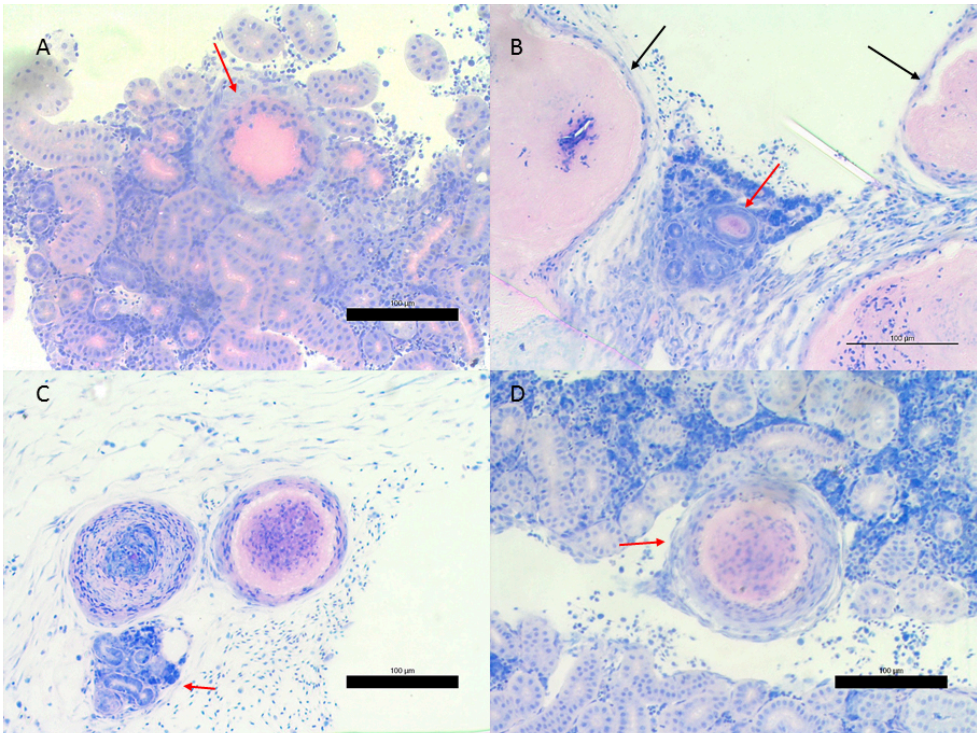


Figure 5. A. Immature granuloma in the kidney of meagre. There is an amorphous, acellular area, which is surrounded by inflammatory cells. B. An immature granuloma in the kidney (red arrow) in a small area of a kidney with normal appearance. In this particular fish there was extensive caseous necrosis 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.

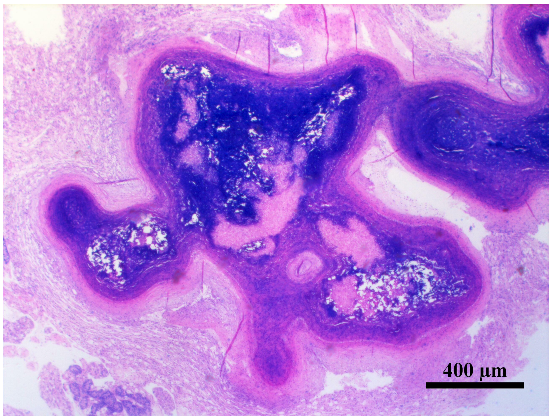


Figure 6. Dystrophic calcification in the kidney of meagre.

In some cases, mainly in livers, the initial stages of the granulomas are located at the blood vesicles resembling vasculitis (**Figure 7**). In these cases, there is also an involvement of rodlet cells. Rodlet cells are present in large numbers in all tissues. Rodlet cells are aligned like epithelial cells in the peritoneal membranes but they are also found in liver, pancreas and intestine. (**Figure 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. Under specific conditions rodlet cells expel their rodlets into the extracellular environment (Depasquale, 2014). The composition of the rodlets is not known, however it has been shown that they contain the antimicrobial peptide piscidin (Silphaduang et al., 2006). 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.

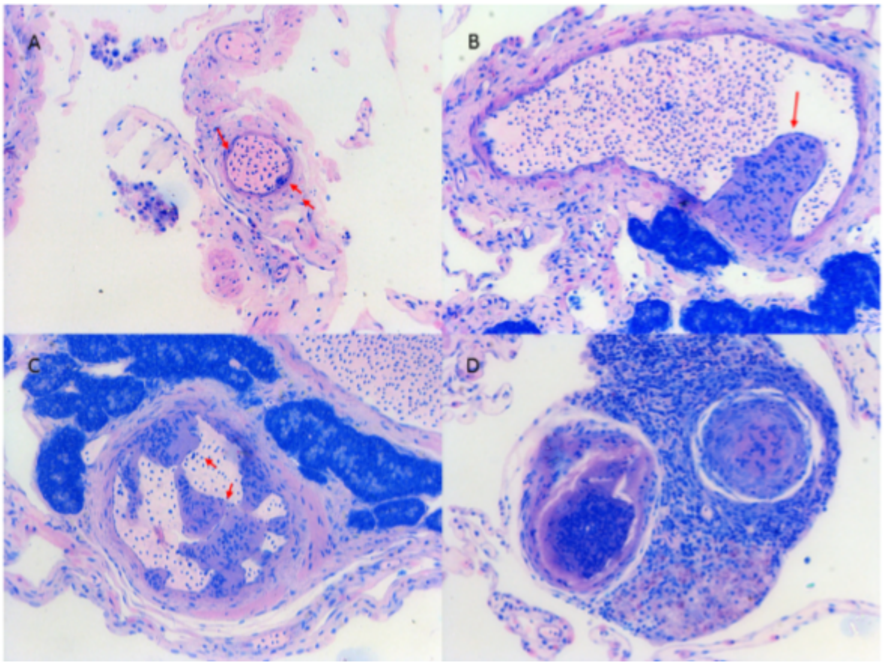


Figure 7. Blood vessel implication is evident in the manifestation of the disease. Various sections of blood vessels from the peritoneal membranes and the liver of affected fish are shown. There are specific growths composed of inflammatory cells at the endothelium of the vessels, which are indicated with red arrows **(A, B)**. In more progressed stages (**C, D**) these growths seem to block the lumen of the vessel.

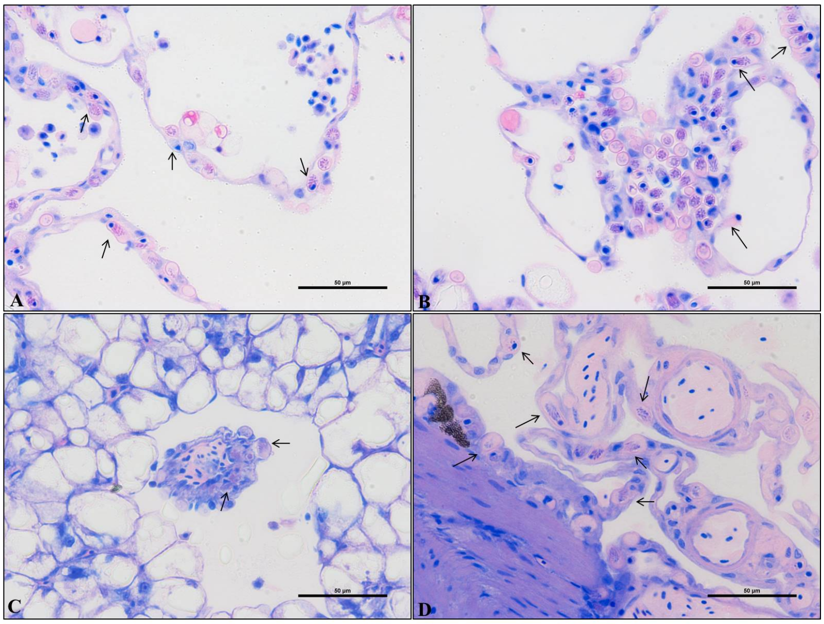


Figure 8. Rodlet cells (black arrows) aligned in the peritoneal membranes (**A, B**) and surrounding blood vessel walls (**C, D**).

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 area 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 10 A, B, C**). Typical granulomas were also present in all tissues examined. In these granulomas, no bacteria could be seen (**Figure 10D**).

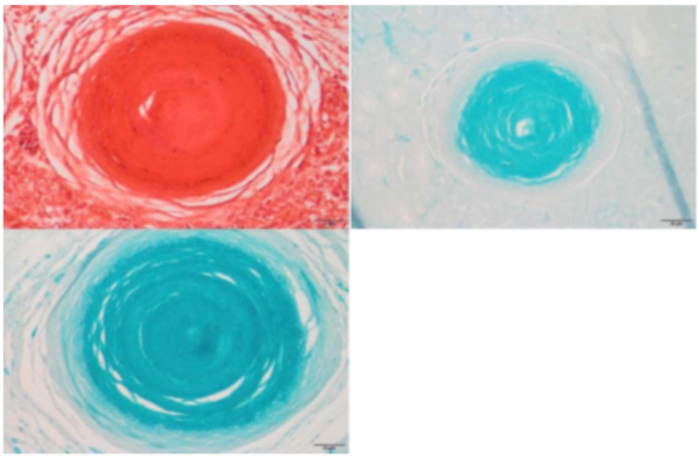


Figure 9. A. Gram stain of granuloma in liver, **(B)** Ziehl-Neelsen stain of granuloma in kidney, and **(C)** Fite Faraco stain in liver.

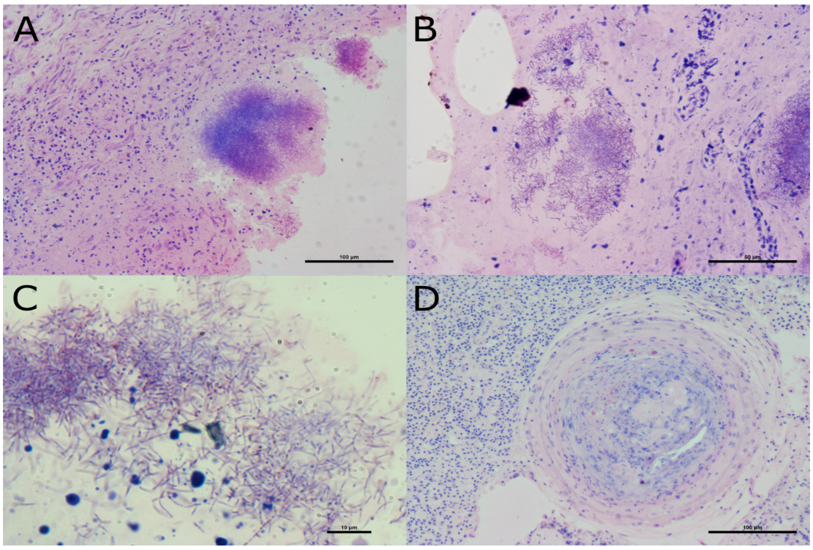


Figure 10. A, B. Histological section of a dermal lesion of a Nocardia-positive meagre (methylene blue/azure II/basic fuchsin stain). There are several bacterial colonies, which have elicited a moderate host response. **C**. Higher magnification of the bacterial colonies from dermal lesions. Note the 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 (Hall and Cash, 2012). In general, the elevation of ALT and AST concentrations may indicate hepato-cellular diseases, while the elevation of ALP may indicate cholestatic diseases of the liver (Sahoo et al., 2015). In task 24.1 (Subtask 24.1.1, Feeding trial 1) regarding the effect of Vitamin D3 in SG the results 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 11**). 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 (Maita, 2007). 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 (Peres et al., 2014, 2013), so those results may be associated with SG, but further investigation needs to be done.

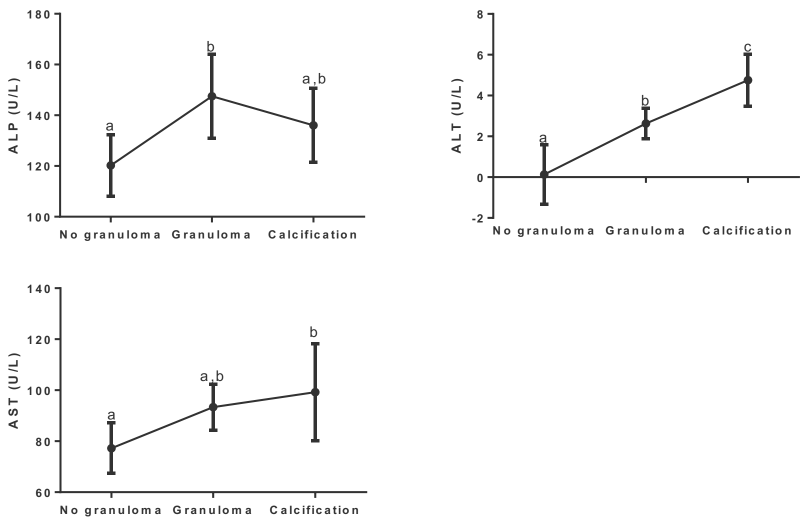


Figure 11. Mean concentrations (±SD) of ALP, ALT and AST in meagre with no granulomas, granulomas and calcification of even one tissue at the end of Vitamin D3 experiment (Task 24.1). Different letters show statistically significant differences between the three conditions (p<0.005)

Diagnostic methods

Gross pathology of heavily affected fish (**Figure 1 & 2**) includes:

- Emaciation
- Fin erosion
- Exophthalmia
- Unilateral blindness
- Granulomas of various sizes in the internal organs
- Necrotic and calcified tissues (mainly liver and kidney)

Microscopic pathology

Dissect out all internal organs and prepare wet mounts of each organ. Place a drop of saline in the center of the slide. Position sample on liquid, using tweezers. Place a cover slip slowly, avoiding air bubbles. Remove excess water with the paper tower. Squash the preparation with the butt of the tweezers. Examine the preparation using a light microscope at x10 or x20 magnification.

Fresh squash preparations of internal organs and examination under microscopy can reveal:

- The presence of granulomas with an "onion-like" appearance (**Figure 3**)
- Necrotic and calcified areas

Microscopically examination of histological sections

- Granulomas with a central necrotic amorphous area surrounded by a multilamellar layer of epitheloid cells and fibrous tissue (**Figure 5**)
- Several stages of the granuloma formation (immature granulomas, multilayer mature granulomas, areas with dystrophic calcification) (**Figure 5 & 6**)
- Special stains like Ziehl-Neelsen can reveal the presence of acid-fast bacteria like *Nocardia* sp. and *Mycobacteria* spp. that can result in different granulomatous lesions NOT related to SG (**Figure 10**)

Visual evaluation of granulomatosis

To assess fish status regarding the presence of granulomas, a semi-quantitative ordinal-scale scoring system can be used based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. After dissection and macroscopically examination of internal organs, fresh squash preparations of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney can be assessed under a stereoscope according the following scoring system. For the general state of each individual, the sum of the scores from the various tissues can be calculated.

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

Deviations

No deviations were made to the proposed task in the DOW.

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