Deliverable Report

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Objective: Isolation and characterization of *Nocardia* from infected meagre: The deliverable is a report on the findings on *Nocardia* infection in cage-cultured meagre in Greece and Spain and includes the microbiological, biochemical and genetic characterization of the *Nocardia* isolates, together with the epidemiological data obtained from the affected fish farms towards the development of a *Nocardia* vaccine based on the most appropriate and virulent strain.

Introduction

One of the main pathological problems of meagre (*Argyrosomus regius*) aquaculture is Systemic Granulomatosis (SG) (Katharios et al., 2011), which is investigated intensively within the DIVERSIFY project. In 2013, there was a report suggesting that the causative agent of SG 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 kingfish and greater amberjack (*S. lalandi* and *S. dumerili*, respectively) (Shimahara et al., 2008). Histopathology of *Nocardia* spp.-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 P1. 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 a threat to the sustainability of meagre aquaculture. Therefore, the aim of this task was to monitor meagre from various locations in Greece and try to identify and isolate *Nocardia* spp., or other granuloma-associated pathogens and to assess whether these bacteria and fungi represent an actual hazard for the species.

Materials and Methods

Fish samples

During the first years of the DIVERSIFY project we have 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. A summary of the samplings is presented in **Table 1**. Several samples have been obtained earlier and analyses have been performed during the DIVERSIFY project.

Table 1. Samples processed for *Nocardia* spp. 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	

Tissue sampling

Kidney, liver, spleen, heart, brain, ascitic fluid (if present) were sampled from almost all fish. Tissues used for molecular analysis were preserved at -20^{o}C. Tissues used for histology were preserved in 10% buffered formalin (PBF).

Histopathology

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.

Bacterial isolation

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 25^{o}C and were observed for more than three weeks. Isolation of fungi was attempted using the Sabouraud Dextrose Agar (SDA).

Molecular identification of pathogens

DNA was extracted from tissues using QIAGEN DNAEasy Blood and Tissue kit according to manufacturer's instructions and from bacteria in culture using the 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 the 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 samples 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 the 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 1.5 min, and final extension at 72^{o}C for 10 min. The PCR conditions for the rest of the genes/pathogens were in accordance to the authors' instructions. Characteristics of the primer pairs used for the PCR reactions are presented in **Table 2**.

Sequencing was performed using an ABI3730xl sequencer (AppliedBiosystems) according to the protocol BigDye Terminators 3.1 (AppliedBiosystems).

Table 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		

Results

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* spp. DNA from all isolates was extracted and the 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** contains the information regarding the bacterial strains identified.

Table 3. Identification of the bacterial isolates based on 16s rRNA sequencing

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

In addition to the bacteria isolated in solid media, PCR analysis was performed directly on SG-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* spp. infection due to the distinct morphology of these lesions (**Figures 1-3**). PCR for *Nocardia* spp. was positive in 4 out of the 6 different organs examined, including skin, heart, kidney and liver from both individuals.

Figure 1. Severe ulceration of the skin of cultured meagre *Argyrosomus regius*.

Figure 2. Nodular morphology of the dermal lesions, appearance alarming for *Nocardia* spp. infection in meagre.

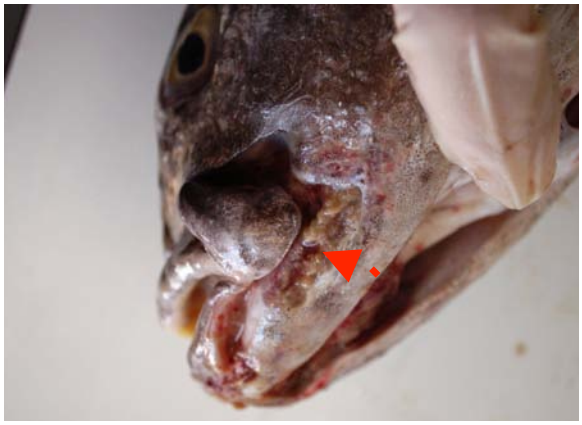

Figure 3. Nodular morphology of the dermal lesions, appearance alarming for *Nocardia* spp. infection in meagre.

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 4**).

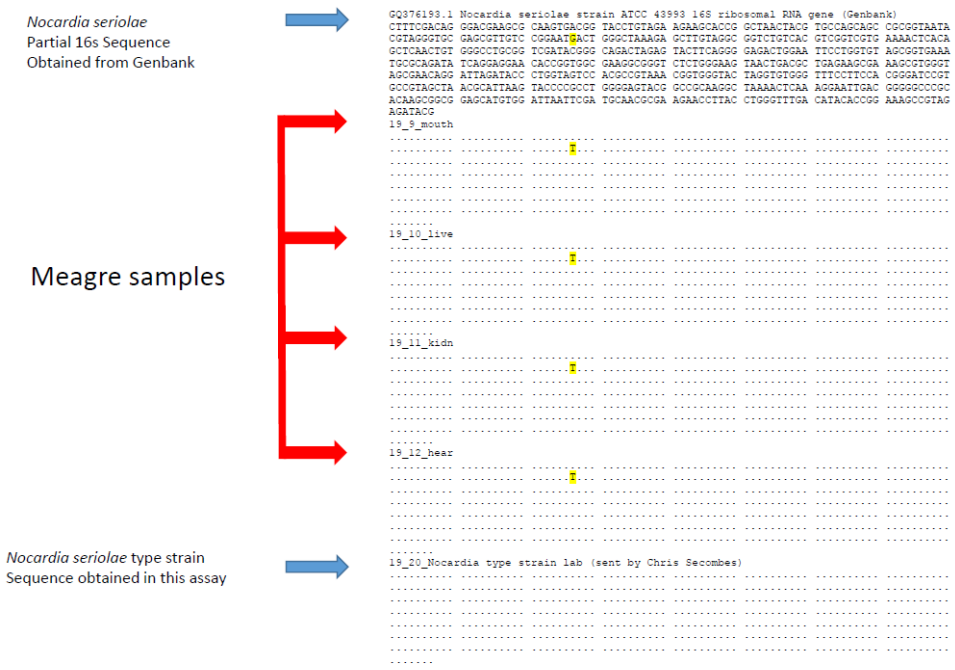

Figure 4. Multiple alignment of the partial 16s sequences of the meagre samples with *Nocardia seriolae* type strain retrieved from Genbank and sequenced by us as positive control of the assay. There is a single nucleotide mutation at position 107, possibly a result of the geographic distance of the isolates.

Histological analysis of the *Nocardia*-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* spp. 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 5-10)**. Typical granulomas were also present in all tissues examined (**Figure 10)**. The morphology of these granulomas followed the pattern described earlier (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.

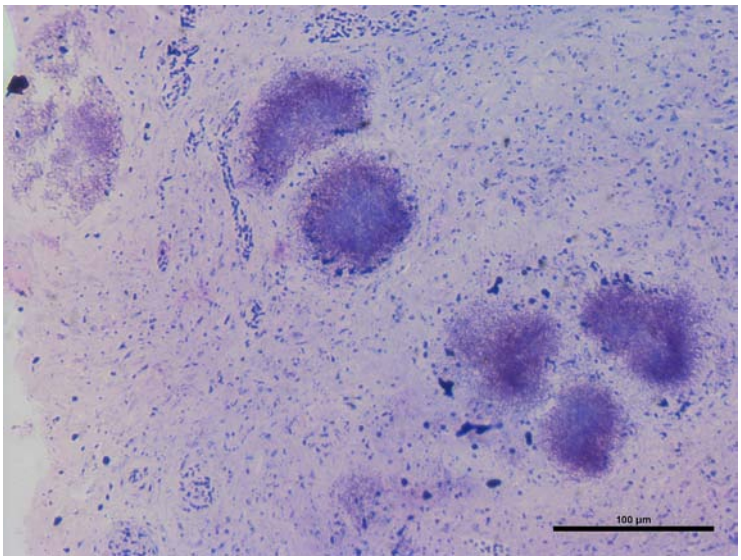

Figure 5. Histological section of a dermal lesion of a *Nocardia*-positive meagre. There are several bacterial colonies, which have elicited a moderate host response. Ziehl-Neelsen stain.

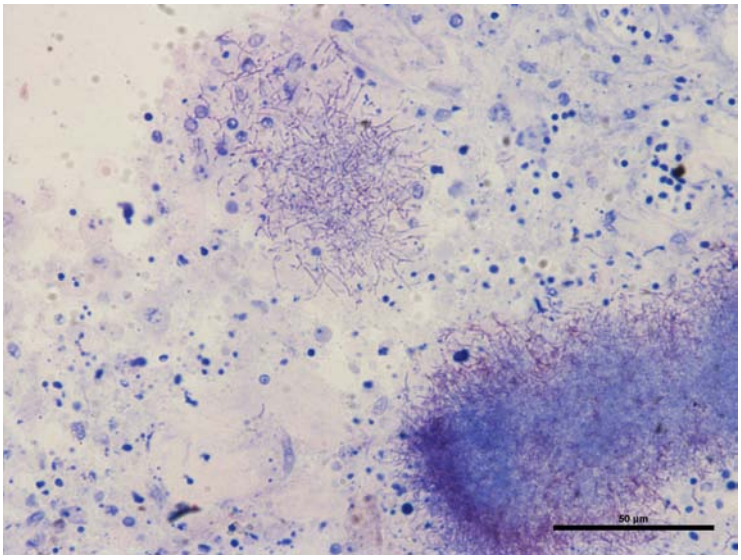

Figure 6. Higher magnification of the bacterial colonies from dermal lesions. Red staining indicates acid-fast positive bacteria. Note the filamentous branching morphology of the bacteria.

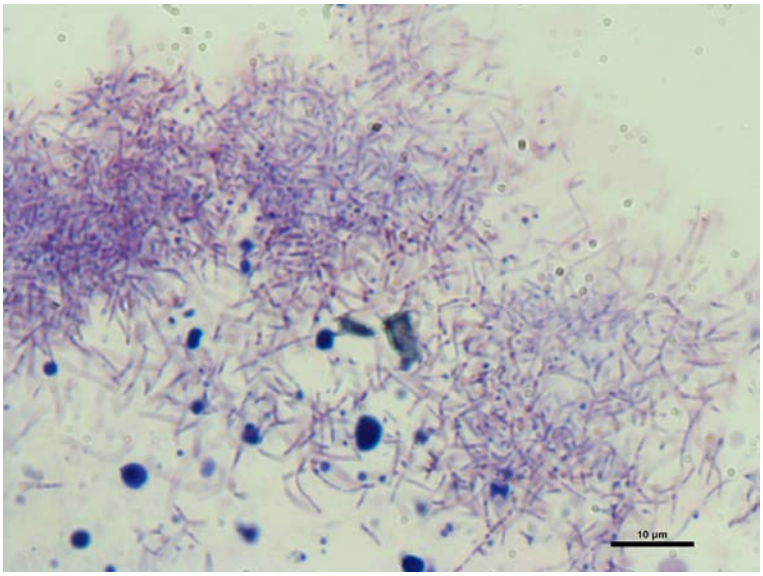

Figure 7. An x100 magnification of the bacterial colony of a dermal lesion showing the morphological characteristics of the bacteria which are consistent with the descriptions of *Nocardia* spp. in other fish species.

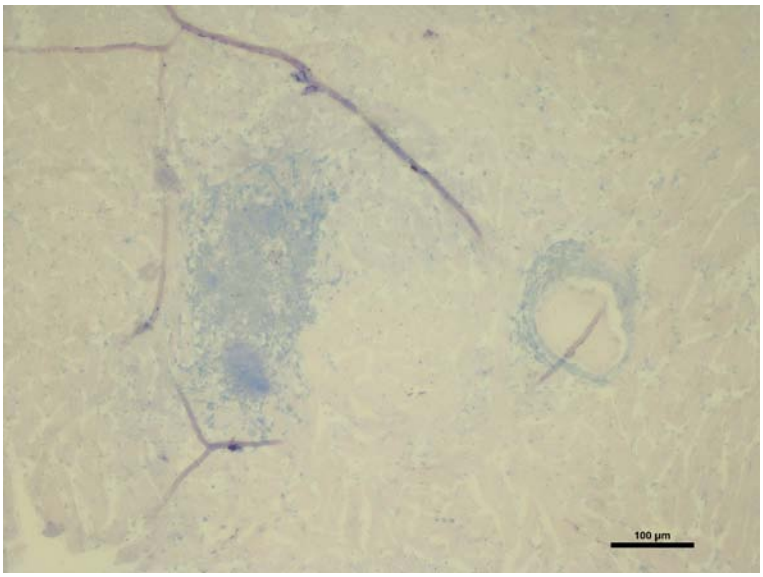

Figure 8. Heart histological section of a *Nocardia*-positive meagre. There are two different lesions standing out; on the left is a *Nocardia* spp. colony, on the right a non-bacterial granuloma. Ziehl-Neelsen stain.

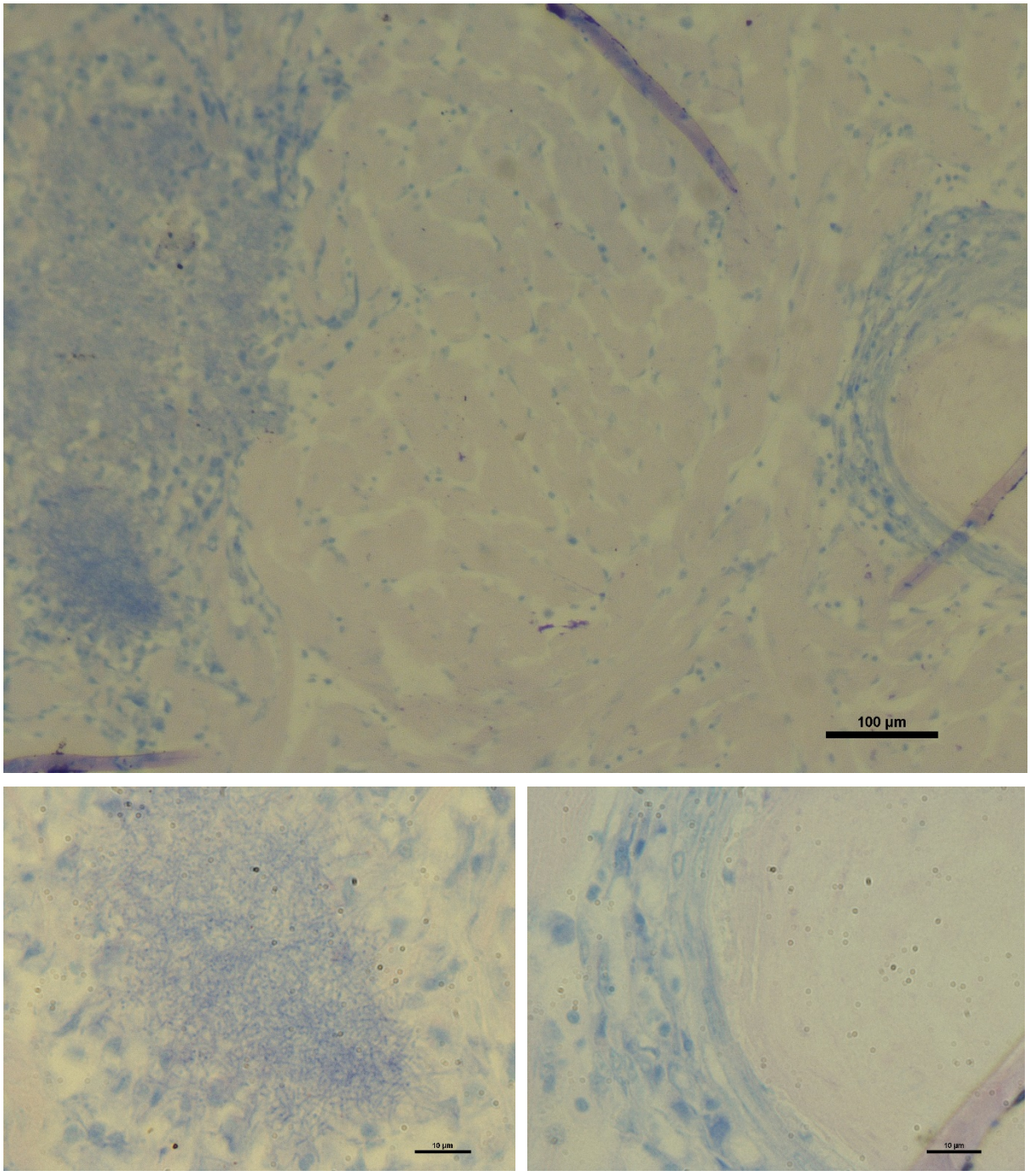

Figure 9. Upper picture. Higher magnification of the heart of a *Nocardia*-positive meagre (*Argyrosomus regius*) (**Fig. 8**) showing the differences of the two lesions. Lower left: x100 magnification of the bacterial lesion showing the distinct morphology of the filamentous bacteria. Lower right: x100 magnification of the granulomatous lesion where no bacteria can be seen. Ziehl-Neelsen stain.

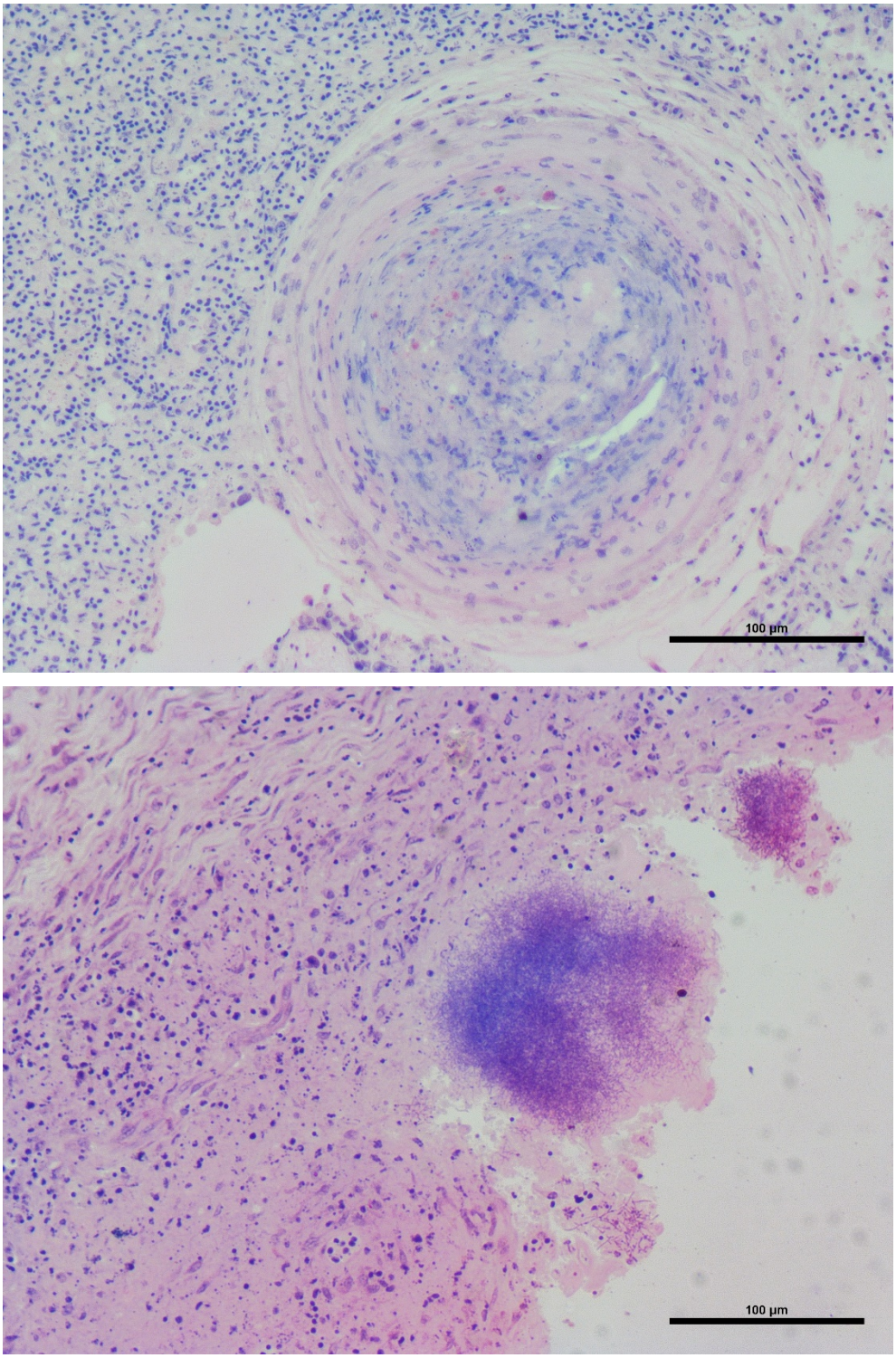

Figure 10. Comparison of a non-bacterial granuloma in the spleen (above) and a bacterial lesion (below) in the skin from the same, *Nocardia*-positive meagre.

Another interesting case was from the island of Leros (Greece). Five fish exhibiting epidermal lesions were sampled. All exhibited abscesses and granulomas in the kidney to different extents (**Figure 11**). Several mixed bacterial colonies were obtained in L-J. The 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 12)**.

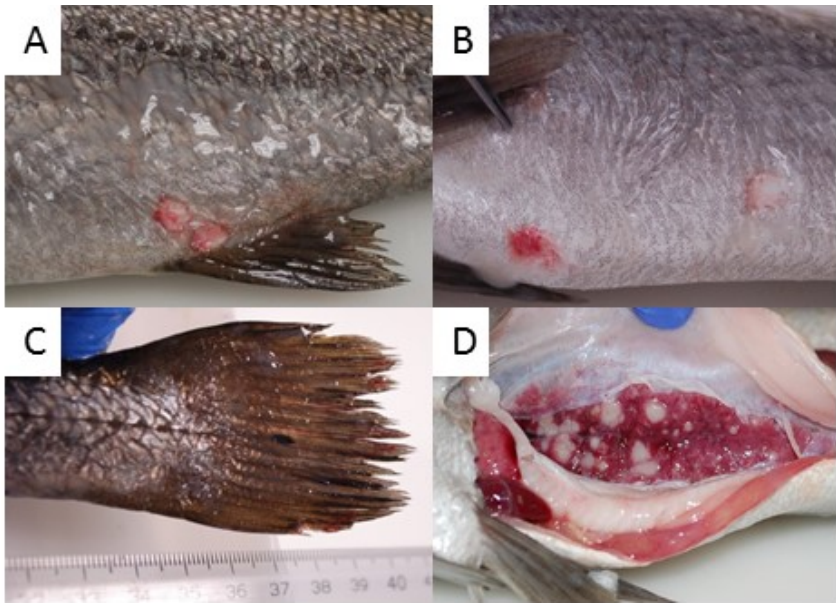

Figure 11. Fish exhibited granulomatous lesions on the skin (A and B), fin erosion (C) and abscesses and granulomas in the kidney resembling mycobacterial infection.

Subsequent re-cultures 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* sp., possibly a cross reaction of the probes used.

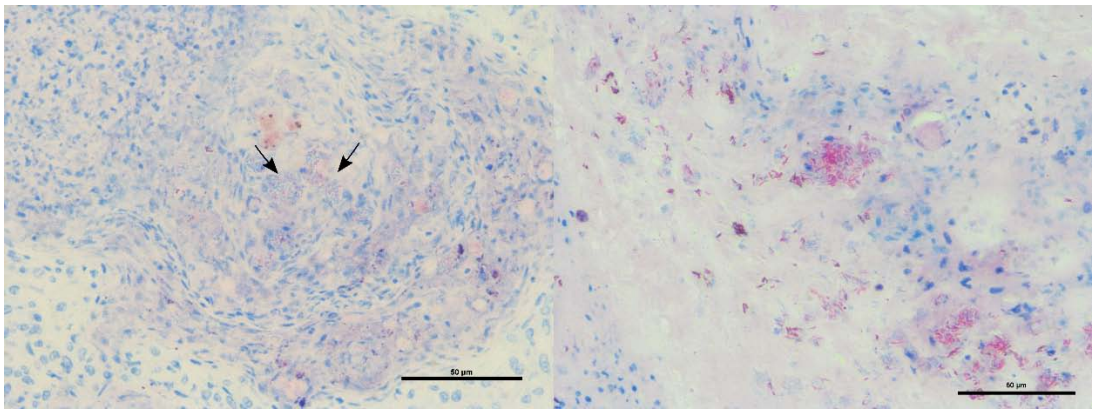

Figure 12. Left: Granuloma in the spleen of meagre from Leros (Greece). The granuloma contains "pockets" of small coccoid bacteria (arrows), which are stained faintly red with Ziehl-Neelsen. Right: Spleen granuloma from European seabass, *Dicentrarchus labrax*, with confirmed infection by *Mycobacterium 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 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.

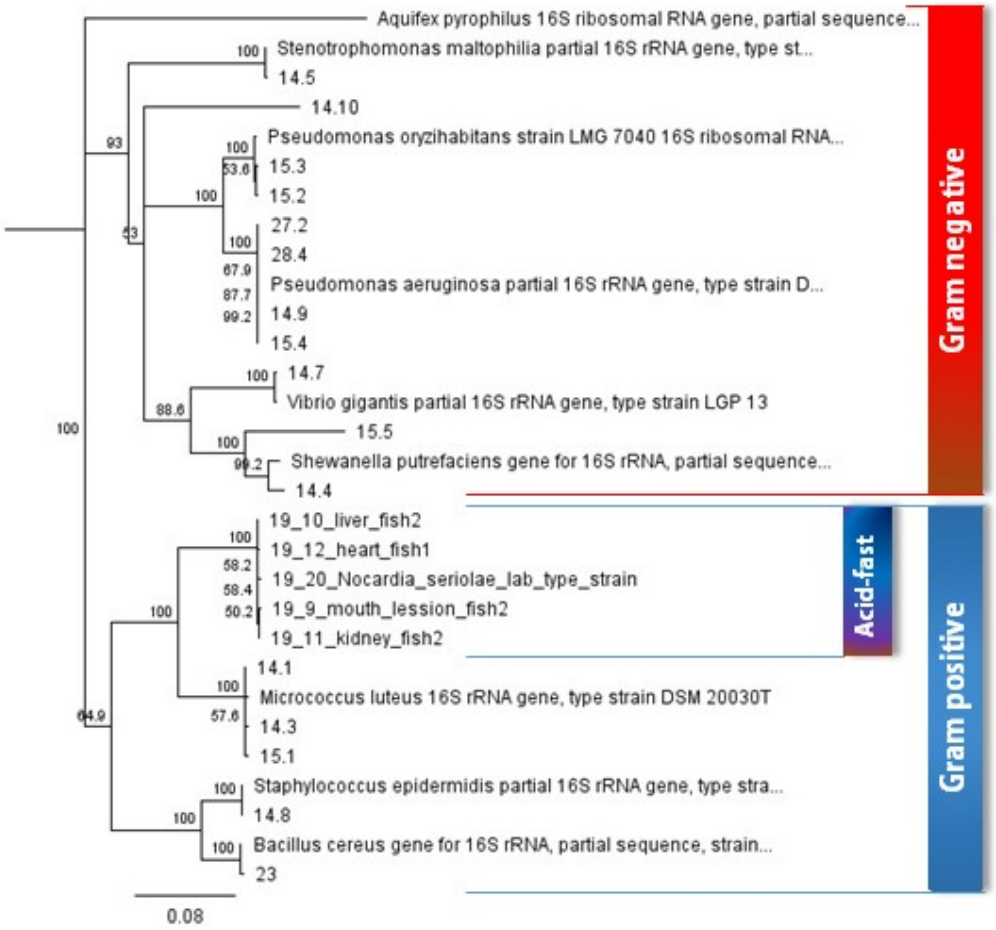

Figure 13. Phylogenetic analysis of the bacteria identified from the granulomatosis-affected meagre (information about the strains are included in Table 3). *Aquifex pyrophilus* was used as an outgroup. Partial 16s rRNA sequences of the type strains of the closest species of bacteria following BLAST search were retrieved from GenBank and used in the analysis. Phylogenetic analysis was performed in Geneious 9.1, using the Neighbor-Joining method with 1,000 bootstraps.

Discussion

The main target of this task was to identify and subsequently isolate *Nocardia* sp. from SG-affected meagre. 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* spp. can induce granulomatous lesions. SG affects almost all cultured meagre to a greater or lesser extent, however this is the only case where *Nocardia* sp. was detected. Fish from all other areas examined were negative for *Nocardia* spp. using molecular tools for detection, including the fish from the HCMR hatchery which develop the lesions without having been exposed to the open sea and are exclusively reared in borehole water. In addition, where *Nocardia* spp. 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* spp. 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* spp. in Greece. It comes from the same geographic location and it has again affected cultured meagre. It could be speculated that this might be an endemic problem, however it should be closely monitored, since *Nocardia* spp. 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 brain. Moreover, this bacterium was the most commonly isolated species at the P1. HCMR facilities exclusively from meagre before the start of the 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 for cumulative mortalities of 80% in Spain (Labella et al., 2011). Mycobacteriosis has also been reported in 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 incidence of vibriosis in other established species, such as European seabass and gilthead seabream (*Sparus aurata*), therefore emphasis should be given in developing and testing of vaccines against this disease of major importance.

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Deviations: There was no deviation from the proposed DOW.

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