New species for EU aquaculture

Deliverable Report

Deliverable No:	D24.12	**Delivery Month:**	55
Deliverable Title	Determination of Efficacy of Vaccination of Meagre against *Vibrio anguillarum*		
WP No:	24	**WP Lead beneficiary:**	P1. HCMR
WP Title:	Fish health - meagre		
Task No:	24.6	**Task Lead beneficiary:**	P5. UNIABDN
Task Title:	Monitor specific immune responses		
Other beneficiaries:	P3. IRTA		
Status:	Delivered	**Expected month:**	55
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Lead Scientist preparing the Deliverable: Andree K.B. (IRTA)

Other Scientists participating: Secombes C.J. (UNIABDN), Milne D.J. (UNIABDN), Gisbert E. (IRTA), Estevez A. (IRTA)

Objective: To determine the efficacy of species-specific vaccination against *Vibrio anguillarum* for application in commercial rearing of meagre.

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1. Introduction

The objective of this deliverable was to document the efficacy of a vaccine against pathogens of significance for commercial aquaculture of meagre *Argyrosomus regius*. The original bacterium for this study was a *Nocardia* species that was hypothesized to be relevant for the systemic granulomatosis affecting meagre, but since the association of *Nocardia* to this disease has not been proven during the progress of this project we have chosen and had approved (see *Deviations* below) a different pathogen for study: *Vibrio anguillarum*. There are reports of nodavirus and monogeneans infecting meagre, but among bacteria *Vibro anguillarum*

and *Photobacterium damselae* are the pathogens that have been reported (Andree et al. 2015; Soares, et al. 2018). This DL has focused on *V. anguillarum* as it is a pathogen with broad host-range and likely to be of concern for intensive rearing facilities of meagre. The vaccine administered during this Task was provided by Acuipharma. It is a trivalent vaccine effective against *V. anguillarum, V. harveyi* and *Photobacterium damselase*. Since meagre represents a species relatively new to aquaculture no vaccine preparations have been formally approved and licensed for use with this species. As a multivalent vaccine it will also have broader applicability in a commercial production setting. During the course of this work tissues relevant to the immune response were collected for analysis of gene expression. The tissue samples were collected from fish pre- and post-vaccination and injection with *V. anguillarum* and immune gene expression will be evaluated for select target genes. Gene expression data will be presented later for completion of D24.13.

2. Materials and Methods

Fish Culture and Experimental Challenge

Juvenile meagre (n= 3000; mean weight= 5.4g) were provided by Alevines del Sureste (Andromeda Group) in Murcia, and transported by road to IRTA (P3) facilities in early May, 2018. Immediately on arrival low numbers of mortalities were observed and fish health screening was performed. All fish were given 3 consecutive prophylactic formalin baths 3 days apart after the observed mortalities were recorded and afterwards held in 1000 L circular tanks to acclimatize to the ambient water conditions of the facilities. Once acclimatized, again 10 fish were selected for examination of parasite infestation and kidney biopsies. Briefly, a branchial arch from each fish was removed and mounted on a slide together with scrapings from the lateral skin of the fish, mounted under a coverslip, for observation at 40X magnification to look for accumulations of bacteria, any signs of gill damage or parasites. The body of the animal was then cleaned with alcohol, opened aseptically with sterile scissors and forceps to examine the viscera and collect a kidney biopsy that was used for inoculating onto solid agar culture media (TSA+ 2.5% NaCL and TCBS). Agar plates were incubated at 23ºC and checked at 24 and 48 hours for bacterial growth.

By June when fish had reached a mean weight of 11.5 gm, they were given a bath vaccination of a trivalent vaccine *Vibrio anguillarum*, *Vibrio harveyi* and *Photobacterium damsela* (Acuipharma, lot# 5.22/17) following the manufacturer's instructions. Briefly, meagre (n= 250) were immersed in a vaccine suspension diluted 1:10 with ambient water in groups of 60 for one minute per group. Control fish (n= 250) were treated in a likewise manner using ambient water as a mock vaccination to provide similar handling-stress treatment. After four weeks, the fish were given a booster in a similar manner. Following the booster, the fish were held for another three weeks before challenge with injections of live bacteria near the end of July. Biometry of the fish 5 days prior to challenge found the mean weight of fish was 32.57 gm.

For the bacterial challenge, a strain of *V. anguillarum* previously established as being virulent for meagre (strain IRTA 15-1 provided by HIPRA and used with their permission) was recovered from culture stocks frozen at -80ºC. To estimate the LD_{50} dosage of *V. anguillarum,* a bacterial suspension was prepared from 24 hour cultures grown on TSA media. The bacterial suspension was prepared from colonies that were scraped from the solid media using a sterile bacterial inoculating loop and placed in a tube of sterile PBS, then vigorously vortexed to obtain a homogeneous suspension. The optical density of the suspension was measured using a wavelength of 550 nm on a turbidometer/colorimeter (Dinko Instruments, D-100) and the density of suspension was adjusted until an OD_{550} of ~0.6 was obtained (0.598). Serial 10-fold dilutions of this suspension were prepared in sterile PBS and 100 uL of each suspension spread onto TSA plates and incubated overnight. Colony counts from three replicate platings of each dilution provided the actual number

of colony forming units (CFUs) in each suspension that had been injected into fish the previous day. Dilutions of 10^8, 10^7, 10^6, 10^5, and 10^4 were used for injecting juvenile meagre of ~11.5 gms.

For the challenge, one half of the vaccinated fish and one half of the PBS-treated fish were injected with *V. anguillarum* resulting in four treatment groups (**Fig. 1**). Meagre were injected intraperitoneally with 100µl of either PBS as a control, or with *V. anguillarum* at a dose of 3.3 X 10^8 CFU/mL (OD_{550}= 0.621). Injected fish were distributed into 100 L tanks connected to a recirculation module (IRTAmar®) (n= 15 fish /tank) (**Fig. 2**). Ambient water conditions were 7.4 mg/L dissolved oxygen and a temperature of 21.4 ºC. Fish were fed twice daily with Biomar Intro Plus MT (1.5 mm pellet size) and Intro Plus MT (1.9 mm pellet size) when first they were installed at IRTA facilities. As the juveniles increased in size the food was switched to Skretting Optibream L2 (2mm pellet size) until the end of the experiment. Once the meagre had been injected with *V. anguillarum*, all tanks were inspected four times daily at 3 hour intervals to collect any dead fish. Differential mortality was calculated for evaluating the efficacy of vaccination with the trivalent vaccine (Acuipharma). During the first two days post-challenge all fish in one replicate tank (#22) of the vaccinated challenged fish (VAC-VB) died due to hypoxia caused by a plumbing problem. Therefore, one replicate tank was removed from all treatment groups.

At the end of the experiment, necropsies were performed on 6 fish from each Vibrio-injected treatment group to check for carrier status of the remaining fish. Kidney smears inoculated onto TSA (2.5% NaCl) solid media were incubated at 23ºC for 48 hours. Positive colonies were screened using *V. anguillarum*-specific PCR (Hong et al. 2007).

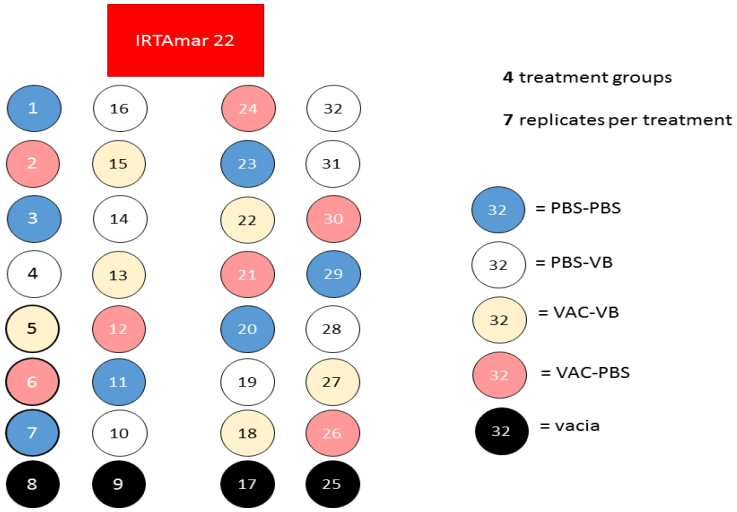


Figure 1. Schematic of tank arrangement for different treatments in RAS module. Abbreviations: PBS-PBS= mock vaccination with PBS and mock challenge with PBS; PBS-VB= mock vaccination with PBS followed by injection with *V. anguillarum*; VAC-VB= bath vaccination against *V. anguillarum* followed by injection with *V. anguillarum*; VAC-PBS= bath vaccination against *V. anguillarum* followed by injection with PBS. Tank numbers are shown inside circles.

Identification of Bacterial Contamination

From each TSA (2.5% NaCl) plate containing inoculum from two different fish, a bacterial colony that had originated from a culture-positive fish was collected from the agar media using sterile toothpicks. Seven isolates were purified by re-streaking on new individual TSA plates. A single colony from these new plates was used for preparing genomic DNA to be used for 16S rDNA PCR. Primers used for 16S rDNA

amplifications were previously described (Suzuki et al., 1996). Amplified products were purified (PCR Purification Kit, Qiagen) and the purified products sent for sequencing (Sistemas Genomicos, Valencia, Spain) to obtain approximately 500 bp of unambiguous sequence. The sequence obtained was compared to the database of NCBI using the BLAST utility.

Figure 2. Experimental set up using IRTAmar® RAS for meagre challenge with *Vibrio anguillarum*.

Sample Collection

To prepare the fish for sample collections (tissue, blood and mucus) they were euthanized by immersion in ambient water containing an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich). Tissue samples (gills, mid-gut, head kidney and spleen) were collected before vaccination, just prior to bacterial challenge, and at 24 and 48 hours after bacterial injections were administered. In addition, baseline samples of blood and skin mucus were collected prior to vaccination and three weeks after the booster (prior to *Vibrio* injection). Blood was collected into heparinized tubes and the serum was separated by centrifugation at 5000 rpm for 10 min and 4ºC, then transferred to clean 1.5 mL Eppendorf tubes and frozen at -20ºC. Mucus was collected by gently scraping the flank of the fish with a plastic spatula and collecting the mucus in a clean petri dish; afterwards this was pipetted into 2 mL Eppendorf tubes and put on ice until later storage in -20ºC.

The gills, mid-gut, head kidney and spleen that were dissected were placed immediately in RNAlater and kept at 4ºC for 24 hrs before storage at -80ºC until processing. Once these samples are processed to purify RNA (RNeasy Mini Kit, Qiagen) and the RNA converted to cDNA (Quantitect Reverse Transcription Kit, Qiagen), they will be conserved at -80 ºC for qPCR analysis to enable a quantitative comparison of gene expression between pre- and post-vaccination, and challenge samples. This will be done using the primers previously described (Campoverde et al., 2017; Milne et al., 2017; Milne et al., 2018), and reported in D 24.13.

3. Results

Establishing Health Status of the Fish lot Used for Experimentation

Upon arrival of the meagre juveniles at IRTA facilities, low numbers of mortalities were collected daily (1-4 per day). No external signs of disease were evident, but internally there was some accumulation of ascites fluid and liver tissue appeared pale. The kidney smears from 7 of 10 (70%) apparently healthy fish that had been plated onto TCBS and TSA (2.5% NaCl) agar plates to check for septicemia showed growth on TSA media after 24 hours at 23 ºC. The seven culture-positive fish had slight to moderate growth of a monoculture of small raised circular colonies that were opaque and beige in color on TSA media (**Fig. 3**). No growth appeared on TCBS media. All of the meagre were given prophylactic baths in formalin to prevent the problems with ciliate infections that occurred the previous year and led to the delay of completion of this deliverable. After the third formalin bath 10 fish were sampled randomly to check their gills and skin scrapings visually by light microscopy, and also to dissect and remove kidney biopsies for spreading onto TSA and TCBS agar plates again. From these later samples no pathologies were detected in any fish and no bacterial growth was observed on the agar media.

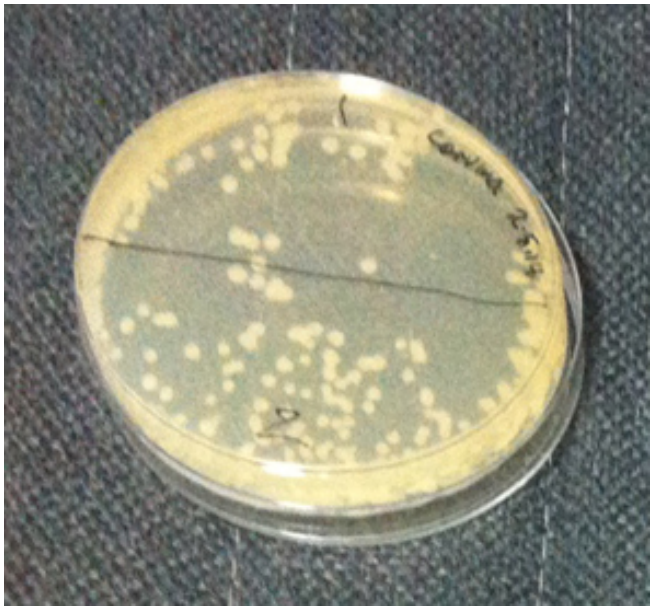


Figure 3. An example of colonies isolated from 2/6 meagre juveniles after arrival at IRTA facilities. 16S rDNA sequencing identified this bacteria as *P. fluorescens*. A seventh fish also had septicemia caused by *Staphylococcus equorum*.

Identification of Bacterial Contaminants

Bacterial 16S rDNA primers were used to amplify ribosomal DNA sequences from the pure cultures obtained from the first fish screening. The obtained sequence was the same from 6/7 fish (**Fig. 4**) and when analyzed using BLAST was a 99% match to *P. fluorescens* and *Pseudomonas koreensis*. A seventh culture-positive fish was found to be infected with *Staphylococcus equorum* (Jeong et al., 2017). During the acclimatization period (3 weeks) there were no further mortalities.

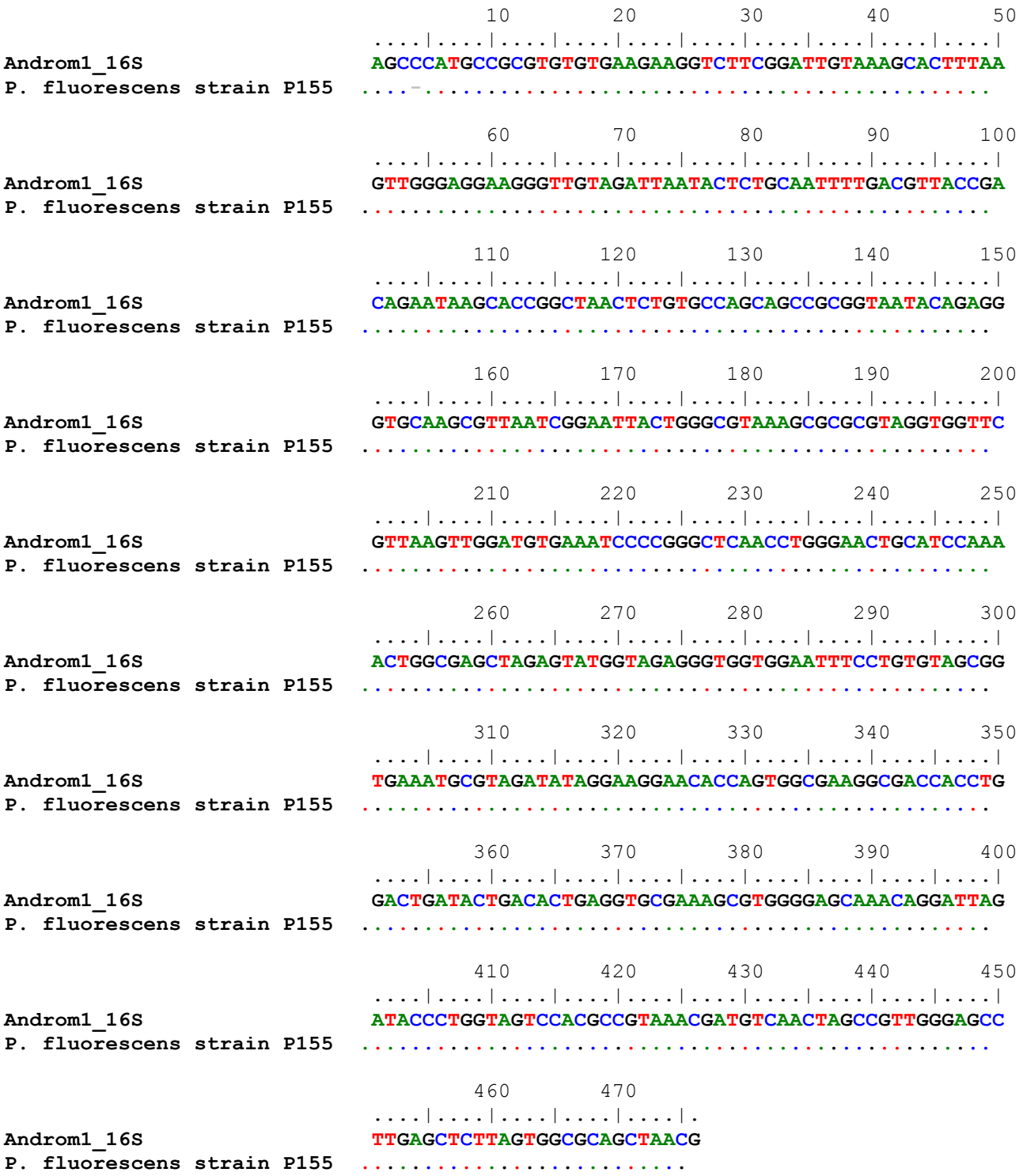


Figure 4. Alignment of the 16S rDNA sequence obtained from bacteria isolated from kidney smears of juvenile meagre with a GenBank accession for *P. fluorescens*. Dots (·) signify identity between the sequence obtained from the bacterial isolate from meagre juveniles and that of *P. fluorescens* accession #MH518308.1. Dashes (-) signify a gap in the alignment of the two sequences.

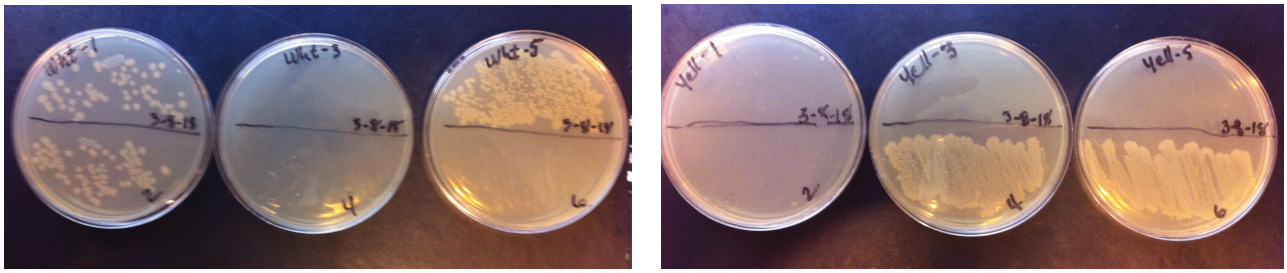
Cumulative Mortality

To estimate the dosage for challenge, a previous challenge had been performed with meagre of ~11 gm. During this prior challenge with multiple doses only the suspension of 8 X 10^8 CFU/mL caused mortalities (100%). This information was used for preparing the bacterial suspension for the final challenge experiment. Meagre of 32.57 (±11.23) gm were used in this second experiment, but the final CFU count was somewhat lower (3.3 x 10^8 CFU/mL) even though the OD_{550} was slightly higher (0.621). Meagre began dying 48 hours post-injection. Of the seven replicate treatment tanks only six were used due to technical problems. No fish from any of the control treatments died during the course of the experiment. The vaccine was effective in stimulating immune memory for providing protective immunity. The experiment was concluded after no mortalities had been observed for one week in the PBS-VB treatment tanks. Total mortality results for all treatment groups is shown below (**Table 1**).

Table 1. Total mortality recorded during ten days post-injection using 3.3 X 10^8 CFU/mL of *V. anguillarum*. Mean weight of those that died is shown.

Treatment	Total Mortality	Mean Weight (SD)
PBS-PBS	0%	-
PBS-VB	17.5%	27.01 (4.8)
VAC-VB	0%	-
VAC-PBS	0%	-

At the end of the experiment 10 dpi with *V. anguillarum*, necropsies were performed on 6 fish from each Vibrio-injected treatment group to check for carrier status of the remaining fish. From the PBS-VB treatment group 3/6 were positive for culture, while from the VAC-VB group 2/6 were positive for growth on TSA (2.5% NaCl) media. All cultures appeared to be pure with consistent colony morphology on each plate. Isolated colonies were collected using sterile toothpicks and used for DNA extraction. Following previously published protocols (Hong et al. 2007), the purified DNA tested positive as *V. anguillarum*.



a) **b)**

Figure 5. a) TSA (2.5% NaCl) plates from six fish from the non-vaccinated treatment group that had been euthanized for bacterial screening at the end of the experiment. **b)** TSA (2.5% NaCl) plates from six fish from the vaccinated treatment group that had been euthanized for bacterial screening at the end of the experiment.

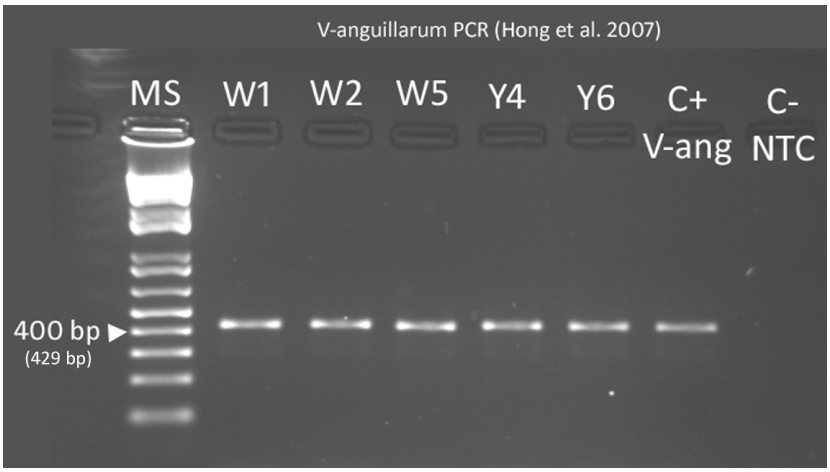


Figure 6. PCR screening of bacteria recovered from kidney smears at the end of the experiment. W1, W2, W5 are DNA isolated from bacteria collected from kidney smears of fish from the PBS-VB treatment group (tanks labeled White). Y4 and Y6 are DNA isolated from bacteria collected from kidney smears of fish from the VAC-VB treatment group (tanks labeled Yellow). C+ = genomic DNA from *V. anguillarum*. C- = No Template Control (NTC). MS = Molecular Weight Standard. Expected amplicon size for a positive result is shown in parentheses below 400 bp marker.

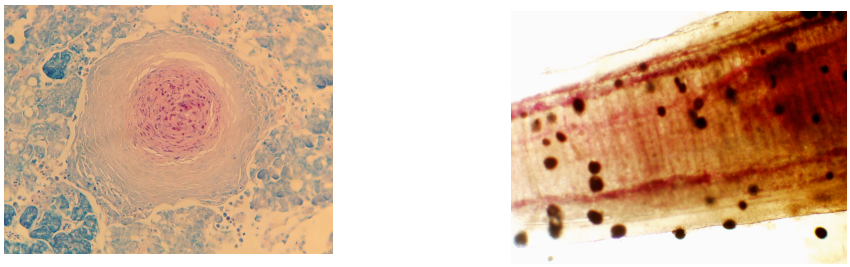
4. Discussion

In a previous attempt to complete this deliverable in 2017 using a different lot of meagre, health examinations had found significant numbers of very small granulomas as has been described for this species in significant detail in other project Deliverables (**Fig. 7a**). However, in none of the granulomas observed using Ziehl-Neelsen stain for detection of acid-fast-bacteria were there seen any bacteria. The fish had appeared healthy and no septicemia was detected. There was apparently a low abundance of ciliates infesting the population and during the incubation period post-vaccination the parasite population expanded to lethal proportions (**Fig. 7b**) killing all fish in the experiment before the actual bacterial challenge could be initiated.

Apart from ciliate infection, another complicating factor that became evident upon performing necropsy examinations at the end of the challenge experiment in 2017 was the adhesions that had formed among the mesenteries surrounding the gut in the peritoneal cavity. These adhesions may have had an adverse effect on the digestion, or appetence of the fish. Many of the fish had stopped eating and the guts of the fish were nearly empty in spite of there being administered sufficient food. Presence of food in the gut seemed to correlate with reduced adhesions. Whether the lack of feeding was due to the ciliate infection or the adhesions of the gut is an open question. These adhesions had been seen previously when using this particular injectable vaccine with seabass (unpublished observations), so it was decided to use a bath, or dip, vaccine for the following year when this work was to be repeated.

Due to the problem with an infestation of ciliates in 2017, the work was repeated in 2018. During the health exams of the new lot of meagre juveniles provided by Alevines del Sureste (Andromeda Group) for 2018, there was observed septicemia in significant numbers of fish that was only mildly virulent. Mono-cultures were obtained on TSA agar media from kidney smears of 70% of the fish tested (**Fig. 3**). 16S rDNA PCR was used for identification of the bacteria. The obtained sequences were the same from 6/7 fish and when analyzed using BLAST was a 99% match to accessions in GenBank: *Pseudomonas fluorescens* and *P. koreensis*. The former species can be a pathogen of fish (Li et al., 2015), but the latter is an isolate from

fermented foods. A seventh positive culture obtained was a different bacterial species, but also a potential pathogen of fish: *Staphylococcus equorum* (Jeong et al., 2017). After three formalin baths and a period of acclimatization, the mortalities ceased and no septicemia could be detected after three weeks. The etiological agent of the septicemia was not highly virulent and after fish had recovered from transport and handling stress they appeared to eliminate the septicemia on their own without further intervention. We proceeded with the bath vaccination at this point and no further complications were encountered. During the experiment, mortalities were collected and weighed. The experiment was terminated after no mortalities had been observed for one week.



a) **b)**

Figure 7. a) Histological sample of liver tissue collected from meagre prior to the vaccination and challenge in 2017. Granuloma visualized with Ziehl-Neelsen stain for acid fast bacteria. While granulomas were evident, as described for this fish species in DL 24.4, no bacteria were visible or isolated from tissues of apparently healthy fish. **b)** An abrupt epizootic of a ciliate parasite killed most of the fish in 2017 three weeks after vaccination.

The bath vaccination protocol provided good protection for the meagre juveniles with 0% mortalities in the vaccine treatments (VAC-VB; VAC-PBS). The presence of bacteria in both these groups at 10 days post-challenge could be expected, but the absence of mortalities in the vaccinated group demonstrate protective immunity was activated by the vaccine formulation. Total mortalities in the bacterial challenge treatment (PBS-VB) may have been lower (17.5%) than expected for several reasons. First, we were not able to vaccinate the fish immediately after their arrival at IRTA facilities because a portion of the population had a mild bacterial septicemia that needed to be cured before the fish could be used further. During this recovery period, there was a significant increase in size; this complicated the later work. Second, the increase in fish size from a mean weight of 11.5 gm at the time of establishing the dose to be used for the challenge, to 32.57 (±11.23) gm at the time of the actual challenge with *V. anguillarum* meant there was likely to be greater resistance to the bacterial infection. Although, the 100% mortality observed using 10^8 CFUs, versus 0% mortality when using 10^7 CFUs suggested that some increased resistance might be to the benefit of the experiment where the target was 50% mortality. Third, the rapid growth of *Argyrosomus regius* also leads to high size dispersion. The average weight of fish used during the challenge was larger than fish initially used in determining the challenge dose (11.5 gm), but there were also exceptional individuals of ~60 gms that were discarded to avoid too much bias in the final results. Some of the larger fish that were finally used may have been even more prone to resist the dose administered. This size dispersion was notable among the mortalities collected post-challenge where the mean weight of the dead fish (27.01 gm) was significantly below the mean weight of all surviving fish at the end of the experiment (35.7 gm). Most significantly, there was the septicemia from *P. fluorescens*. This appeared to affect only some fish as it was detected in 5/10 fish tested. Also, it was not highly virulent as there were only 26 mortalities from the original 3000 fish in the population. Although exposure to this bacterial species did not provide specific immunity to our challenge

organism (*V. anguillarum*), it would have provided immune priming of non-specific innate immune effectors (eg. - toll-like receptors, C3 complement, and lysozyme) that further reduced recorded mortalities. This was exemplified among the replicate Vibrio exposure (PBS-VB) tanks where there were two replicates in which no mortalities occurred. These two tanks may have been populated with fish that received this type of non-specific immune priming as there were fish among these whose weight was within the range of the fish that did die and might be expected to have succumbed to infection. Finally, there is some difficulty in getting exactly the same bacterial suspension each time inoculum is prepared. While procedures for preparing inoculum were the same each time, we obtained a suspension of $8x10^8$ CFU/mL for the first challenge with smaller fish of 11.5 gm, and achieved a bacterial density of only $3.3x10^8$ CFU/mL the second time when preparing the suspension for the actual challenge; nearly a two-fold difference. Although the dose used was not an actual LD_{50} it did provide useful information on the effectiveness of the vaccine for commercial use with meagre, *A. regius*. The relatively low mortalities recorded in this study (17.5%) were not ideal for the purposes of documenting efficacy of the vaccine, but the size dispersion of the fish and chance occurrence of bacterial septicemia closely reflect potential pitfalls of actual use of vaccines in a commercial setting. We have seen also that some injectable vaccine formulations can stimulate formation of adhesions among the mesenteries of the fish gut that needs to be considered in the formulation. The study provides some guiding metrics, in terms of the weight that would be most appropriate when performing challenge experiments with *A. regius*. Additional information will be obtained upon completion of D24.13 - Description of Immune Gene Expression Pre- and Post-immunization of Meagre.

5. References

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Deviations: This deliverable has focused on vaccine trial using *Vibrio anguillarum* as a challenge model organism. In the original text of the DoW there was supposed to be used a novel species of *Nocardia* that was expected as the cause of chronic systemic granulomatosis, but this etiology was not proven during the course of the work. Approval for this change of the DoW was obtained previously.

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