

MARIA CAROLINA DE SALES RIBEIRO

**STUDY OF THE PATHOGENICITY OF
NOCARDIA SPP. BY EXPERIMENTAL INFECTION
IN MEAGRE, *Argyrosomus regius* (Asso, 1801)**

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Universidade Lusófona de Humanidades e Tecnologias

Faculdade de Medicina Veterinária

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Dissertação apresentada para a obtenção do grau
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*The important thing is not to stop questioning.
Curiosity has its own reason for existing.*

Albert Einstein

Para a minha família.

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RESUMO

A Nocardiose, causada por *Nocardia* spp. em peixes, foi primeiramente identificada por Rucker (1949) como *Streptomyces salmonicida* no salmão vermelho (*Oncorhynchus nerka*). Muitos casos desta doença têm sido descritos desde então, um pouco por todo o mundo, afetando um amplo leque de espécies. A Nocardiose é causada por uma bactéria Gram-positiva, álcool-ácido resistente, aeróbica e filamentosa. Sinais típicos de doença manifestam-se por anorexia, emaciação, inatividade, ascite, descoloração e úlceras da pele, brânquias e músculos, bem como granulomas no rim, baço, fígado, coração, olhos, cérebro, músculo e pele.

Neste trabalho, diferentes doses (10^1 to 10^8 CFU/mL) de *Nocardia* spp. foram inoculadas por via intraperitoneal em 160 corvinas, *Argyrosomus regius* (Asso, 1801) distribuídas por 8 tanques, cada um com diferente dose. Um nono tanque foi ainda adicionado como grupo controlo. A mortalidade cumulativa atingiu 6% sendo que todos os animais encontrados sem vida pertenciam aos tanques onde as doses inoculadas eram superiores. Os sinais clínicos observados traduziram-se em anorexia e emaciação e foram identificados apenas nos animais provenientes dos tanques com maior dose da bactéria inoculada (T1-T3).

Amostras de rim, baço e fígado foram retiradas e analisadas histológica e bacteriologicamente. Os estudos histológicos corados com Hematoxilina-Eosina revelaram lesões típicas granulomatosas no rim (23%), no fígado (11%) e baço (2%). Foi ainda observado que o número de granulomas identificados por órgão parece sofrer influência da dose inoculada, sendo que o maior número de granulomas por órgão foi encontrado em T1 e T2. O recurso a técnicas de coloração especiais como Gram e Ziehl-Neelsen não se revelou frutífero, tendo em conta que não permitiu a observação da bactéria em nenhuma das amostras. *Nocardia* spp. foi isolada nos mesmos órgãos acima descritos através de cultivo em meio Agar Sangue com 1.5% de Cloreto de Sódio, Brain Heart Infusion com 1.5% de Cloreto de Sódio e Yeast Extract-Malt Extract. No entanto, a percentagem revelou-se oposta à observada em histologia, fígado (10%), baço (5%) e rim (4%). O facto de a *Nocardia* ser uma bactéria difícil de isolar em meio de cultivo pode explicar a discrepância entre os resultados obtidos de histologia e bacteriologia. Foi também verificado que a incidência de granulomas parece ser afetada pelo tempo, enquanto o isolamento da bactéria está mais dependente da dose. Estes resultados

demonstram a evolução da Nocardiose numa infeção experimental de *Nocardia* spp. em corvina.

PALAVRAS-CHAVE: Corvina · *Argyrosomus regius* · *Nocardia* spp. · Granulomas · Patologia de Peixes · Aquacultura

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INTRODUÇÃO

O desenvolvimento da aquacultura tem levado ao surgimento de inúmeras doenças responsáveis por sérios problemas económicos e ecológicos (Murray & Peeler, 2005; Rigos & Troisi, 2005; Lafferty *et al.*, 2015).

O presente trabalho foca-se no estudo da *Nocardia* spp. em corvinas, *Argyrosomus regius* (Asso, 1801) de produção. Embora ainda negligenciada, a *Nocardia* spp. causa Nocardiose, uma doença responsável por grandes perdas em aquacultura, dada a sua semelhança com outra doença muito conhecida, a Micobacteriose. Tendo em conta esta situação, até ao momento, pouca informação é conhecida sobre a forma como a doença pode afetar os animais. Por esta razão, é imperativo reforçar a informação já conhecida com novos estudos. O presente trabalho enquadra-se nesta problemática, tendo como objetivo principal a compreensão dos mecanismos desta doença com o intuito de fornecer informação para o desenvolvimento de estratégias de controlo e prevenção, bem como possíveis tratamentos.

1. AQUACULTURA

A Pesca tem sido uma das principais atividades durante séculos para um vasto número de culturas. Contudo, nos últimos anos tem vindo a sofrer um decréscimo e a maioria das principais áreas de captura atingiu já o seu máximo potencial de exploração (Figura 1) (Food and Agriculture Organization of the United Nations [FAO], 2014).

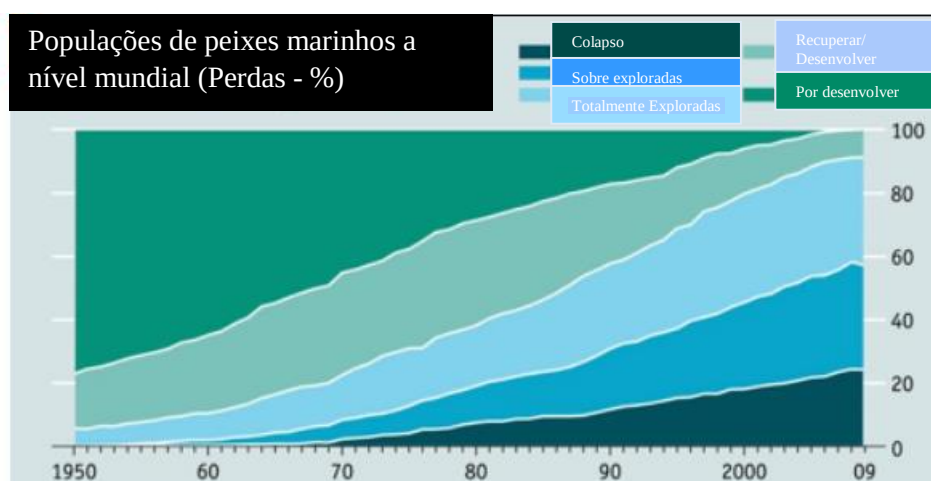


Figura 1. Perdas nas populações de peixes marinhos a nível mundial (%) (Froese *et al.*, 2012).

Esta situação tem-se verificado problemática tendo em conta que, num curto período de tempo, a produção associada à captura na natureza não terá capacidade para dar resposta à crescente demanda por peixe ou outros alimentos de origem marinha. Assim, com o objetivo de se desenvolverem alternativas sustentáveis à pesca tradicional, tem crescido o interesse pela aquacultura (FAO, 2014a).

De acordo com a FAO (1988), a aquacultura traduz-se no cultivo de organismos aquáticos, desde peixes, moluscos, crustáceos ou plantas, e implica intervenção direta no processo de produção de modo a otimizar a produção (FAO, 1988; 2014a). Esta prática inclui tanto as espécies marinhas como as fluviais, e pode ser posta em prática em terra, em instalações apropriadas ou em oceanos, mares e rios. Além disso pode ser feita em sistema extensivo, em lagoas, lagos ou zonas costeiras; semi-intensivo, em lagoas ou lagos; e intensivo, em tanques ou jaulas. Nesta perspetiva, as pesquisas mais recentes têm visado a melhoria dos sistemas de produção bem como da qualidade do peixe produzido, aliadas à crescente preocupação ambiental (Asociación Empresarial de Productores de Cultivos Marinos de España [APROMAR] & Asociación Española de Productores de Acuicultura Continental [ESACUA], 2013; FAO, 2014a).

A aquacultura tem sido nos últimos anos um dos sectores de maior crescimento da indústria alimentar, representado 50% da produção global de peixe, continuando a crescer e a diversificar de modo a dar resposta à crescente procura por produtos ricos em proteínas, que sejam de qualidade e seguros para a saúde (Abellán & Basurco, 1999).

O maior contributo para este crescimento tem vindo da Ásia, África e América do Sul. A Ásia por si contribui com 89% da produção total de aquacultura, sendo a China o seu maior produtor (60%). América do Norte, Europa e Oceânia têm sofrido uma estagnação nos últimos anos, em especial devido à competição com outros países onde os custos de produção são significativamente inferiores (FAO, 2012; 2014a).

Também o padrão de trocas tem sido revertido em favor dos países em desenvolvimento, apesar dos países desenvolvidos continuarem a dominar com uma margem cada vez menor. A China é o maior exportador mundial, apesar de as suas importações serem cada vez mais significativas e a Noruega é o maior exportador Europeu. O Japão é o principal importador mundial, sendo a Espanha e a França os principais importadores Europeus (Figura 2) (Váradi *et al.*, 2011; FAO, 2014a).

Na Europa, com um mercado ainda muito dependente das importações (FAO, 2014a), 85% do total da sua produção é assegurada por apenas 6 países, a saber: Espanha, França, Itália, Alemanha, Reino Unido e Portugal.

	2002	2012	APR
	(US\$ millions)		(Percentage)
Exportadores			
China	4 485	18 228	15.1
Norway	3 569	8 912	9.6
Thailand	3 698	8 079	8.1
Viet Nam	2 037	6 278	11.9
United States of America	3 260	5 753	5.8
Importadores			
Japan	13 646	17 991	2.8
United States of America	10 634	17 561	5.1
China	2 198	7 441	13.0
Spain	3 853	6 428	5.3
France	3 207	6 064	6.6

Figura 2. Lista dos 5 principais exportadores e importadores (FAO, 2014a).

2. CORVINA (*Argyrosomus regius*)

Argyrosomus regius (Asso, 1801) é vulgarmente conhecido pelo nome de corvina. Esta espécie pertence ao Filo *Chordata*, Classe *Actionopterygii*, Subclasse *Teleostei*, Ordem *Perciformes* e Família *Sciaenidae* (Pollard *et al.*, 2011; García-Mesa, 2012).

A corvina tem um corpo longo prateado com traços de bronze na região dorsal. Possui uma linha lateral que se estende até à barbatana caudal. Além desta, tem ainda duas barbatanas dorsais, uma peitoral, uma pélvica e outra anal (Figura 3). A sua boca está em posição terminal e no interior apresenta pequenos dentes organizados em séries de diferentes tamanhos que não se estendem até ao palato; a porção caudal da maxila tem uma conformação mais alargada enquanto a porção cranial da maxila é alongada. O ouvido interno possui 3 pares de otolitos, sagitta, lapillus e asteriscus com função vestibular e auditiva. Estes animais podem atingir cerca de 2 metros de comprimento e 50 kg de peso (Secor *et al.*, 1991; Popper & Lu, 2000; Campana, 2004; FAO, 2005; Cárdenas, 2010; Ictioterm, 2013).

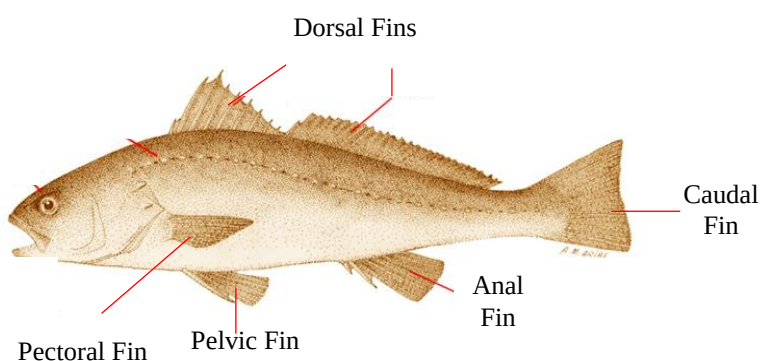


Figura 3. Características morfológicas da corvina (Arias, 2001).

2.1. Corvina Selvagem

Esta espécie distribui-se amplamente pelo Mar Mediterrâneo, apesar de ser menos comum ao largo das costas italianas e gregas. A região da baía de Dakar, no Senegal é, até à data, o ponto mais a sul onde se pode encontrar (Nelson, 2006; Haffray *et al.*, 2012; FAO, 2014a).

É uma espécie eurialina e euritérmica, o que significa que tem a capacidade de se adaptar a um amplo intervalo de salinidade e temperatura (Cárdenas, 2012; Lavié *et al.*, 2008). Além disso, pode ser encontrada junto ao fundo das plataformas continentais ou perto da superfície, dada a sua natureza semi-bentónica (Nathanailides, 2013).

As fêmeas atingem a diferenciação sexual antes dos machos, sendo que as oogónias podem ser observadas aos 9 meses, enquanto as espermatogónias só surgem após os 11 meses. No entanto, a maturação sexual só ocorre por volta dos 3 anos em machos e dos 2 em fêmeas (Cárdenas, 2012; Schiavone *et al.*, 2012).

A corvina é uma espécie gonocórica com uma organização ovárica assíncrona. Isto significa que é uma espécie que possui os sexos separados e oócitos em diferentes fases de desenvolvimento em simultâneo, respetivamente. Um detalhe importante desta espécie é que tanto a espermiacção como a ejaculação podem ser sincronizadas com a época de desova das fêmeas através de comunicações por feromonas. A fertilização externa garante assim o sucesso reprodutivo e uma maior variabilidade genética.

Além disso, podem fertilizar ovos de diferentes fêmeas, assim como as fêmeas podem produzir ovos de diferentes machos, garantindo assim o sucesso reprodutivo e uma maior variabilidade genética (Petersson & Järvi, 2001; Mylonas *et al.*, 2004; 2010).

São caracterizadas como sendo uma espécie anádroma, dado que aquando da fase reprodutiva migram da água salgada para a água doce para proceder à desova. Este processo ocorre entre Abril e Maio, quando a temperatura da água atinge os 16-17°C. Nesta fase os machos produzem um som típico, possivelmente para atrair as fêmeas, por associação de um músculo com a bexiga-natatória, que se encontra hipertrofiada como resultado de um aumento de testosterona. No final do Verão, os adultos deixam os estuários para se alimentarem junto à costa, permanecendo junto à superfície até ao início do Outono e descendo a águas mais profundas durante o Inverno (Quero, 1985; Connaughton *et al.*, 2002; Quéméner *et al.*, 2002; FAO, 2005; Lagardère & Mariani, 2006; Cárdenas, 2010; Soares, 2015). No final do Verão que os juvenis deixam os estuários e passam entre 2 a 3 anos em zonas costeiras antes de migrarem para o mar; para estes, a temperatura ótima de crescimento varia entre os 17 e os

21°C, sendo a temperatura da água o fator que mais contribui para as migrações (FAO, 2005; Cárdenas, 2010; Soares *et al.*, 2015).

As corvinas adultas alimentam-se basicamente de pequenos peixes (Famílias *Cupleidae* e *Mugilidae*), crustáceos, equinodermes, moluscos e poliquetas, enquanto os jovens têm uma dieta mais limitada à base de misidáceos e camarão (Jiménez *et al.*, 2005; Cárdenas, 2010; Soares *et al.*, 2015).

Dado o seu grande tamanho, comportamento de grupo e a produção de sons típicos durante a época reprodutiva, a corvina é um alvo fácil de localizar e capturar. Estas características associadas à poluição ambiental, a degradação das áreas de desova e transferências de patologias entre animais de cultivo e selvagens, têm contribuído para o declínio da sua população selvagem (Marshall *et al.* 2003; Sadovy & Cheung, 2003; Gil *et al.*, 2013).

2.2. Corvina em Aquacultura

A história da corvina em aquacultura é bastante recente. Foi pela primeira produzida em 1997, em França, e quase simultaneamente em Itália, tendo vindo a aumentar desde então. Espanha entrou no mercado em 2004, seguida pela Grécia e Turquia, estendendo-se atualmente a outros países mediterrânicos (Monfort, 2010).

Várias razões estão na origem da introdução e subsequente crescimento de produção desta espécie em aquacultura, designadamente: a fácil capacidade de adaptação a diferentes meios (euritérmica e eurialina), o que facilita a sua sobrevivência em diferentes sistemas e ambientes de cultivo; a grande capacidade de crescimento, especialmente em zonas temperadas (~1 Kg/ano); o baixo índice de conversão alimentar (0.9-1.2); uma criação de larvas relativamente fácil de recriar em cultivo, bem como uma elevada resistência a fatores de stress. Além destes fatores, possui ainda um elevado valor nutricional, garantindo uma carne com reduzido teor em gordura saturada e elevado em gordura insaturada com um excelente sabor e textura, características que facilitam uma boa aceitação por parte dos consumidores (Quéméner, 2002; FAO, 2005; Jiménez *et al.*, 2005; Lavié *et al.*, 2008; Piccolo *et al.*, 2008; Cárdenas, 2010; Monfort, 2010; Roo *et al.*, 2010; Mittakos *et al.*, 2012; Duncan *et al.*, 2013; Millán-Cubillo *et al.*, 2016).

O interesse no cultivo de corvina não decorre apenas de uma perspectiva comercial, visando igualmente a conservação da espécie, dada a sua vulnerabilidade em meio natural pelas razões já referidas no ponto anterior (Sadovy & Cheung, 2003; Jimenez *et al.*, 2005).

No entanto, a produção de corvina defronta-se com alguns obstáculos que dificultam a sua expansão no mercado da aquacultura como o facto de ser uma espécie ainda pouco conhecida pelo consumidor e, portanto, ainda com pouca visibilidade e de, em alguns países, ter uma imagem depreciativa, como acontece em França, onde “maigre” (=corvina) significa delgado (Battaglene & Cobcroft, 2007; Monfort, 2010).

Outro importante bottleneck na produção de corvina em cativeiro tem sido o desenvolvimento de um protocolo de reprodução eficaz. Uma disfunção comum associada às fêmeas é a falha na maturação de oócitos e consequente ovulação. Fêmeas com este tipo de disfunção passam por uma vitelogénese normal mas, no início da época reprodutiva, os oócitos não iniciam a maturação e sofrem atresia. No entanto, estas alterações surgem muitas vezes associadas a disfunções masculinas, como o reduzido volume de esperma ou da sua qualidade (Zohar & Mylonas, 2001; Mylonas *et al.*, 2010; Duncan *et al.*, 2013; Fernandez-Palacios *et al.*, 2014). Tratamentos hormonais são atualmente usados como terapia para ultrapassar estes problemas e assegurar a reprodução em cativeiro. Agonistas da hormona libertadora de Gonadotropina (GnRHa) são os mais usados, podendo ser administrados através de injeções líquidas ou implantes que libertam a hormona durante um maior período de tempo. Para a sua utilização é importante ter em conta o estado de desenvolvimento ovário e os níveis de stress por manipulação, bem como a dose de hormona a administrar. Fatores igualmente importantes para conseguir uma reprodução eficiente incluem condições que promovam o bem-estar, nomeadamente, adequada temperatura da água, salinidade, tamanho do tanque, volume da água, densidade animal, entre outros (Zohar & Mylonas, 2001; Ibarra & Duncan, 2007; Mañanos *et al.*, 2008; Mylonas *et al.*, 2010; Duncan *et al.*, 2012, 2013; Pastor *et al.*, 2013; Fernández-Palacios *et al.*, 2014; Mylonas *et al.*, 2015).

A alimentação da corvina em aquacultura é feita à base de proteínas de origem animal, tendência que se procura inverter devido aos elevados custos, baixa disponibilidade e restrições alimentares associadas. Os novos ingredientes incluem produtos de origem vegetal, como soja, ervilhas, milho, entre outros (Glencross *et al.*, 2007; Tacon & Metian, 2008;).

O facto de ter sido recentemente introduzida em aquacultura, leva a que ainda haja um conhecimento limitado no que respeita às doenças que afetam a corvina. A FAO (2005), descreve casos de amiloodiniose (*Amyloodinium ocellatum*), doença causada por um parasita protozoário que se fixa às brânquias por estruturas rizóides e causa irritação, excesso de

produção de muco e asfixia. O parasita *Gyrodactylus* sp. é outro parasita, um trematode, que à semelhança do anterior se fixa às barbatanas e brânquias e origina uma maior produção de muco bem como pontos hemorrágicos na pele. A Vibriose (*Vibrio anguillarum*), uma doença bastante comum, é responsável pela perda de apetite e vermelhidão na zona envolvente à boca (FAO, 2005).

3. NOCARDIA

O género *Nocardia* (Trevisan, 1889) pertence ao Filo *Actinobacteria*, Classe *Actinobacteria* (Stackebrandt, Rainey & Ward-Rainey, 1997), Ordem *Corynebacteriales* (Buchanan, 1917; Zhi, Li & Stackebrandt, 2009) e Família *Nocardiaceae* (Castellani & Chalmers, 1919; Zhi, Li & Stackebrandt, 2009).

A *Nocardia* é caracterizada como uma bactéria Gram-positiva a Gram-variável; aeróbica, tendo em conta que cresce na presença de oxigénio; catalase-positiva e ácido-álcool resistente, pela sua capacidade de resistir à descoloração com corantes álcool-ácidos. A sua morfologia é variável, podendo formar ramos filamentosos ou fragmentados irregulares, dependendo da fase de crescimento. Pode ainda formar hifas (Beaman *et al.*, 1988; Goodfellow & Maldonado, 2006; Lewis & Chinabut, 2011).

As vias pela qual esta doença é transmitida ainda não são claras. Na maioria dos casos, envolve a cavidade oral, no entanto esta via não tem sido verificada em laboratório como a causa primária de infecção. Outra hipótese apontada reside na contaminação através do alimento, principalmente por dietas não pasteurizadas que contêm produtos animais de origem marinha (Bransden *et al.*, 2000; Lewis & Chinabut, 2011).

Esta bactéria tem o potencial de causar doenças supurativas ou granulomatosas, nomeadamente, Actinomicetoma e Nocardiose, respetivamente.

Actinomicetoma é uma doença inflamatória localizada crónica, responsável por alterações a nível dos tecidos subcutâneos e, em alguns casos, dos ossos. Pode surgir como um pequeno nódulo que gradualmente aumenta de tamanho e se torna purulento, podendo necrosar (Beaman and Beaman, 1994; Brown-Elliott *et al.*, 2006).

É, no entanto, a Nocardiose o tema central deste trabalho. A Nocardiose está definida como uma doença granulomatosa crónica sistémica, que afeta igualmente peixes de água doce e de água salgada. É responsável por anorexia, emaciação, inatividade, descoloração da pele, ascite, ulceração da pele, necrose focal nos músculos e brânquias e granulomas, sinal mais

característico, que podem afetar rim, fígado, baço, coração, cérebro, pele, músculo, entre outros. Estes granulomas podem ser observados histologicamente apresentando tipicamente um centro necrótico rodeado por numerosas células epitelioides e tecido conectivo fibroso. Podem, ocasionalmente, ser observadas colónias de bactérias (Chen *et al.*, 2000; Austin & Austin, 2007; Lewis & Chinabut, 2011; Goodfellow & Maldonado, 2012).

A *Nocardia* encontra-se amplamente distribuída em habitats aquáticos e terrestres e existem casos da sua presença descritos por todo o mundo. Até ao momento, apenas 3 espécies foram identificadas em peixes - *N. asteroides* (anteriormente conhecida como *N. farcinica*), *N. seriolae* (anteriormente conhecida como *N. kampachi*) e *N. salmonicida*. (Austin & Austin, 2007; Cornwell *et al.*, 2011; Goodfellow & Maldonado, 2012; Buller, 2014)

A seguinte tabela (Tabela 1) dá conta de alguns dos casos publicados desde que esta bactéria foi, pela primeira vez, isolada em peixes.

Tabela 1. Casos descritos de Nocardiose em Peixes.

Scientific Name	Location	Reference
<i>Oncorhynchus nerka</i>	EUA	Rucker, 1949 (como <i>Streptomyces salmonicida</i>)
<i>Paracheirodon innesi</i>	Argentina	Valdez & Conroy, 1963
<i>Oncorhynchus mykiss</i>	EUA	Snieszko <i>et al.</i> , 1964
<i>Salvelinus fontinalis</i>	Canadá	Campbell & MacKelvie, 1967
<i>Seriola quinqueradiata</i>	Japão	Kubota <i>et al.</i> , 1968
<i>Seriola purpurascens</i>		
<i>Oncorhynchus tshawytscha</i>	EUA	Wolke & Meade 1974
<i>Channa maculata</i>	Tailândia	Hsu <i>et al.</i> , 1987
<i>Paralichthys olivaceus</i>	Japão	Kudo <i>et al.</i> , 1988
<i>Channa maculata</i>	China	Chen, 1992
<i>Micropterus salmoides</i>		
<i>Salmo salar</i>	Austrália	Brandsen <i>et al.</i> , 2000
<i>Lateolabrax japonicus</i>	Tailândia	Chen <i>et al.</i> , 2000
<i>Scophthalmus maximus</i>	Portugal	Dos Santos <i>et al.</i> , 2002
<i>Larimichthys crocea</i>	China	Wang <i>et al.</i> , 2005
<i>Seriola quinqueradiata</i>	Japão	Itano <i>et al.</i> , 2006
<i>Channa argus</i>	China	Wang <i>et al.</i> , 2007

<i>Epinephelus</i> spp.	Tailândia	Shimahara <i>et al.</i> , 2009
<i>Mugil cephalus</i>		
<i>Morone saxatilis</i>		
<i>Lutjanus erythropterus</i>		
<i>Lateolabrax japonicus</i>		
<i>Micropterus salmoides</i>		
<i>Terapon jarbua</i>		
<i>Trachinotus ovatus</i>		
<i>Scatophagus argus</i>		
<i>Terapon jarbua</i>	Tailândia	Wang <i>et al.</i> , 2009
<i>Cynoscion regalis</i>	EUA	Cornwell <i>et al.</i> , 2011
<i>Clarias batrachus</i>	Índia	Hassan & Qureshi, 2014
<i>Argyrosomus regius</i>	Grécia	Elkesh <i>et al.</i> , 2013
<i>Cynoscion regalis</i>	China	Xia <i>et al.</i> , 2015

O diagnóstico desta infeção bem como a identificação da espécie podem ser um desafio em clínica. Uma diferenciação positiva só pode ser obtida pelo isolamento e identificação do agente causal. Para identificar o género, um esfregaço direto pode ser suficiente para revelar a morfologia típica das células de *Nocardia*, normalmente organizadas em filamentos, podendo estes estar associados a células polimorfonucleadas. O facto de ser uma bactéria ácido-álcool resistente indica que a utilização de corantes como Ziehl-Neelsen (ZN) e Fite-Faraco são uma mais-valia para a sua observação. O cultivo da bactéria em meio apropriado, nomeadamente Brain Heart Infusion, Yeast Extract-Malt Extract (YEME) podem ser igualmente úteis na deteção de *Nocardia* spp.. As colónias resultantes apresentam uma relativamente rápida taxa de crescimento, de 3 a 7 dias e exibem diversos aspetos, de suaves a rugosas, convexas e irregulares, bem como uma vasta gama de cores desde bege, castanho, laranja, rosa, vermelho e amarelo (Gordon and Mihm, 1962; Orchard *et al.*, 1977; Maldonado *et al.*, 2000; Brown-Elliott *et al.*, 2006; Lewis & Chinabut, 2011; Goodfellow & Maldonado, 2012; Brown-Elliott *et al.*, 2015). Outros métodos de diagnóstico incluem os bioquímicos, atualmente pouco usados dada a sua baixa especificidade, e os moleculares, sendo os últimos os mais utilizados dada a sua capacidade de detetar género e espécie. Incluem PCR e REA de diferentes genes (hsp65 ou 16S rRNA) e sequenciação genética (secA1 gene, 16S rRNA gene, HSP gene, gyrB gene, rpoB gene, Multilocus sequencing). Além destes, a análise proteómica é também uma ferramenta a ter em conta (Conville *et al.* 2000; Conville *et al.* 2006; Brown-Elliott *et al.*, 2015).

Nenhum tratamento absolutamente efetivo foi, até à data desta tese, descrito. Tentativas de desenvolver vacinas foram feitas em *Seriola quinqueradiata*. Estes foram imunizados com *Nocardia seriolae* viva atenuada e apresentaram um aumento do número de linfócitos e granulócitos em 72 horas. Vacinas vivas com espécies semelhantes de *Nocardia* também resultaram no aumento de alguns níveis de resposta imune protetiva em relação à *N. seriolae*. Antibióticos têm também sido sugeridos para o tratamento de Nocardiose, entre eles Trimetoprim e Sulfametoxazole em associação com Cloreto de Benzalcónio com a duração de 3 semanas. Esta opção resultou num decréscimo das mortalidades. No entanto, o elevado custo que acarreta a sua aplicação não é visto como compensatório para a indústria (Chen 1992; Itano *et al.*, 2002; Itano *et al.*, 2006; Lewis & Chinabut, 2006).

Tem sido defendido que a melhor forma de controlo desta doença é feita através da redução de stress ambiental, promovendo um bom manejo e cuidados com o bem-estar dos animais (Lewis & Chinabut, 2006).

A transmissão de Nocardiose para humanos ocorre tipicamente por ingestão acidental de água contaminada ou através do contacto tópico em caso de feridas na pele (Haenan *et al.*, 2013).

Os granulomas encontrados em peixes podem ter outras origens que não a *Nocardia*, nomeadamente, corpos estranhos, *Mycobacterium* spp., *Renibacterium salmoninarum*, *Francisella* sp.; parasitas e fungos, particularmente *Ichthyophonus hoferi* e *Aphanomyces*; deficiência em ácido ascórbico bem como outros desequilíbrios nutricionais (Elkesh *et al.*, 2013).

4. OBJETIVOS

Os objetivos do presente trabalho prendem-se com a determinação dos efeitos da *Nocardia* ssp. inoculada por via intraperitoneal numa população de corvinas (*Argyrosomus regius*), por um período de 2 meses, de modo a avaliar os resultados da infecção tendo em conta duas variáveis: tempo e dose.

MATERIAL E MÉTODOS

Para este estudo foram utilizadas 160 corvinas (*Argyrosomus regius*) jovens de produção interna, com um peso médio inicial de 69.50 g, separadas em grupos de 20 por 8 tanques. Cada tanque representava uma dose diferente inoculada de *Nocardia* spp., de 10^8 a 10^1 cfu/mL, respetivamente. Acrescentou-se ainda um nono tanque, onde foram colocados mais 20 animais sem qualquer bactéria inoculada, funcionando estes como grupo controlo.

Todos os animais foram supervisionados diariamente durante os 64 dias que durou a experiência, de forma a poder determinar atempadamente qualquer alteração.

É importante mencionar que alguns detalhes, nomeadamente em relação às estirpes utilizadas, não são mencionados neste trabalho pelo facto de se encontrarem em fase de submissão para revista científica.

1. RECOLHA DE AMOSTRAS

O presente trabalho teve a duração de 64 dias e incluiu uma recolha de amostras feita em três diferentes momentos: 30, 37 e 51 dias após a inoculação. Em cada uma das vezes, 4 peixes foram recolhidos de cada tanque, colocados num recipiente com uma dose letal de óleo de cravo e, em seguida, após a confirmação da morte de cada um, colocados individualmente em sacos com gelo identificados com o número do animal e tanque e transportados para o laboratório. No laboratório, cada animal foi pesado e os valores foram anotados numa tabela. Após a pesagem, foram procuradas lesões macroscópicas. Por fim, procedeu-se à necropsia. Foram removidos o rim, fígado e baço e o mesmo procedimento de deteção de lesões macroscópicas foi posto em prática. Cada órgão foi então cortado em dois pedaços, um para avaliação histológica e outro para avaliação bacteriológica.

Dos 10 animais encontrados mortos apenas 8 foram analisados, recorrendo aos igualmente ao procedimentos previamente referidos.

2. ANÁLISE HISTOLÓGICA

A análise histológica foi feita de acordo com os procedimentos normais de processamento de amostras. Estas foram posteriormente coradas com Hematoxilina-Eosina, Gram e Ziehl-Neelsen.

As amostras foram observadas e avaliadas para a presença de granulomas e o número de granulomas por órgão.

3. ANÁLISE BACTERIOLÓGICA

As amostras previamente recolhidas foram colocadas em Agar Sangue com 1.5% de Cloreto de Sódio, Brain Heart Infusion com 1.5% de Cloreto de Sódio e Yeast Extract-Malt Extract através de técnica de sementeiras por esgotamento em 3 estrias. Foram posteriormente coradas recorrendo à coloração de Gram.

4. ANÁLISE ESTATÍSTICA

Tal como anteriormente descrito no ponto 1 do presente capítulo, todos os animais analisados foram pesados e o seu peso foi registado. Aquando do momento de pesagem, foi observado que havia uma aparente discrepância entre os pesos das Corvinas dos tanques onde a dose inoculada era superior em relação aos restantes. De modo a determinar se havia de facto uma relação entre estas observações, foi feita uma análise estatística recorrendo ao uso de um *software* apropriado, o SPSS. A análise estatística incluiu os testes ANOVA (one-way), Post Hoc (Tukey HSD) e ANOVA fatorial (two-way).

RESULTADOS

1. SINAIS CLÍNICOS

Durante os primeiros 30 dias após a inoculação nenhuma alteração quer de origem física quer de origem comportamental foi observada. No entanto, após este período, notou-se que, no momento da alimentação, os animais dos tanques onde as doses inoculadas eram superiores (T1-T3) não mostravam interesse pelo alimento, mantendo-se no fundo do tanque, ao contrário dos restantes animais (T4-T9) que nadavam com determinação até à superfície competindo pela sua refeição. Esta situação foi analisada conjuntamente com as observações e os dados obtidos no momento da pesagem de modo a determinar se, de facto, havia um efeito real neste comportamento, tendo em conta que uma discrepância entre os pesos dos animais provenientes dos tanques com maior dose de bactéria (T1-T3) foi observada, aparentando estes ter menor peso e tamanho em comparação com os restantes (T4-T9).

De modo a confirmar estas observações, foi realizada uma análise estatística para avaliar se as variações de peso teriam alguma relação com a dose inoculada ou o tempo após a inoculação (Figura 4). Esta análise permitiu clarificar a importância destas duas variáveis, tendo revelado que havia de facto uma interferência da dose no comportamento de anorexia e emaciação dos peixes provenientes dos tanques com maior dose de bactéria inoculada (T1-T3). No entanto, estes resultados são apenas relativos aos 51 dias após a inoculação, não se aplicando às datas anteriores. Este resultado mostra ainda que o tempo é também um fator preponderante nesta experiência, tendo em conta que os sinais clínicos previamente descritos se tornaram mais visíveis quanto maior o tempo passado após a inoculação.

Não foram observados outros sinais de doença além dos já descritos acima.

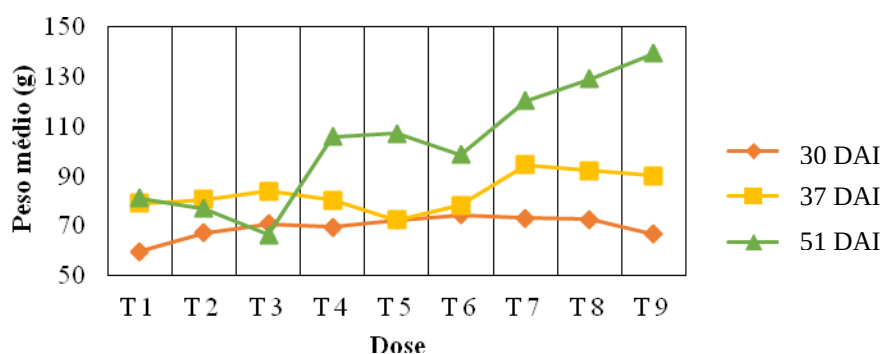


Figura 4. Efeito da dose e do tempo no peso (médio).

2. ANÁLISE HISTOLÓGICA

A análise histológica revelou granulomas em rim, fígado e baço. Diferentes fases de desenvolvimento destes foram observadas (Figura 5-8), bem como diferentes padrões. Verificou-se que os granulomas começam a formar-se pela agregação de macrófagos que tentam combater o agente invasor e culminam com um conjunto de diferentes células com um centro necrótico rodeado por macrófagos, células epitelioides, células inflamatórias e tecido fibroso. Não foram observadas bactérias com nenhuma das colorações utilizadas (Hematoxilina-Eosina, Gram e Ziehl-Neelsen).

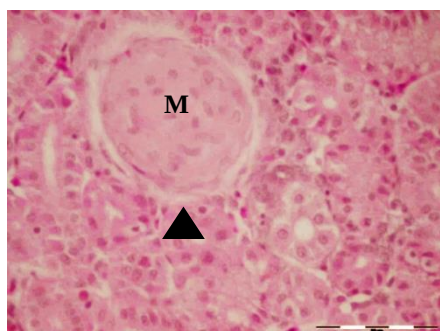


Figura 5. Rim com granuloma em formação (▲) composto por agregado de macrófagos (M) (50µm) (40x).

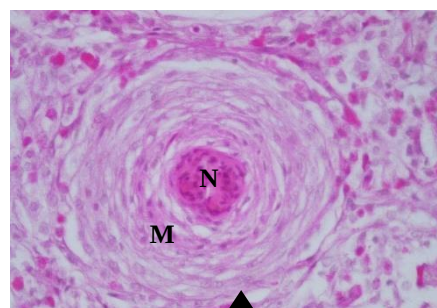


Figura 6. Fígado com granuloma (▲) com centro necrótico (N) rodeado por múltiplas lâminas de macrófagos (M) (50µm) (40x).

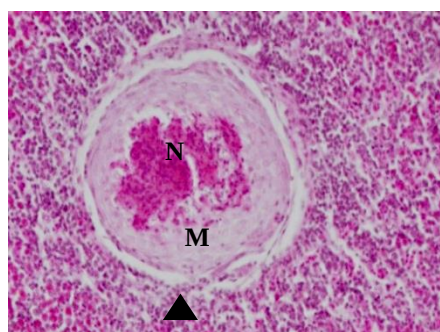


Figura 7. Rim com granuloma (▲) com centro necrótico (N) rodeado por camadas de macrófagos (M) (50µm) (20x).

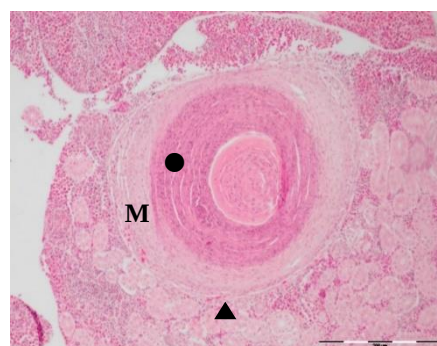


Figure 8. Rim com granuloma (▲) com centro composto por material laminar (●) rodeado por macrófagos (M) (200µm) (10x).

Foi também avaliada a relação entre o número de granulomas encontrados em cada órgão com a dose administrada ou o tempo após a inoculação. Os resultados obtidos indicaram que em 80% dos casos apenas um granuloma foi observado, em 10% entre 2 a 4 granulomas e os restantes 10% para um número de granulomas superior a 4. 88% dos casos,

em que mais do que um granuloma foi encontrado estão associados a animais inoculados com doses superiores da bactéria (T1-T3), o que leva a concluir que poderá haver uma relação com a dose. Inversamente, nenhuma conclusão foi retirada em relação ao tempo.

2.1. Incidência de Granulomas

Granulomas microscópicos foram observados em 28% dos animais analisados. A importância do tempo parece ser real neste caso, tendo em conta que a percentagem de granulomas aumentou ao longo da experiência, de 22% nos 30 dias após a inoculação para 36%, 51 dias após a inoculação. Nenhuma relação foi encontrada com a dose administrada.

O rim foi o órgão mais afetado com uma incidência de granulomas de 23%, seguido pelo fígado (11%) e pelo baço (3%). A relação com o tempo decorrido após a inoculação é também evidente na avaliação das incidências por órgão, sendo os órgãos mais afetados com granulomas após os 51 dias de inoculação. A dose, à semelhança do que foi descrito no parágrafo anterior, também não parece ter um papel decisivo nestas observações.

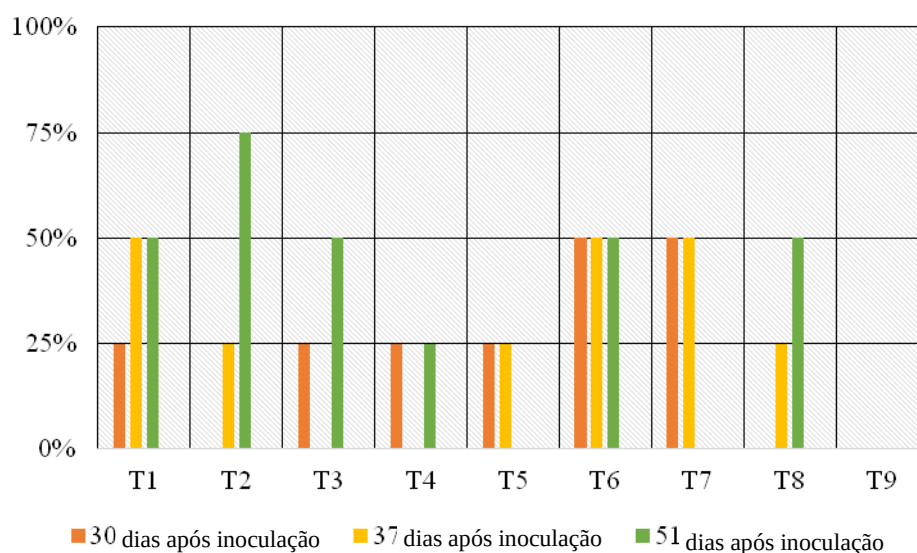


Figura 9. Incidência de Granulomas pelos diferentes órgãos.

3. ANÁLISE BACTERIOLÓGICA

Foram observadas colônias 3 a 5 dias após o seu cultivo em meio. A sua morfologia era de um conglomerado de bacilos filamentosos que, após algum tempo, sofria alterações para simples bacilos. As colônias eram de cor amarela passando a brancas, secas e ásperas com o efeito do tempo.

3.1. Incidência de *Nocardia* spp.

18% dos animais analisados apresentaram crescimento de colônias de *Nocardia* spp.. Ao contrário do que se verificou na análise histológica, a incidência de *Nocardia* spp. não parece ter qualquer relação com o tempo; no entanto, a dose é um aparente fator a ter em conta, no sentido em que grande parte das colônias isoladas tiveram origem nos animais dos T1 e T2, onde a dose inoculada é superior.

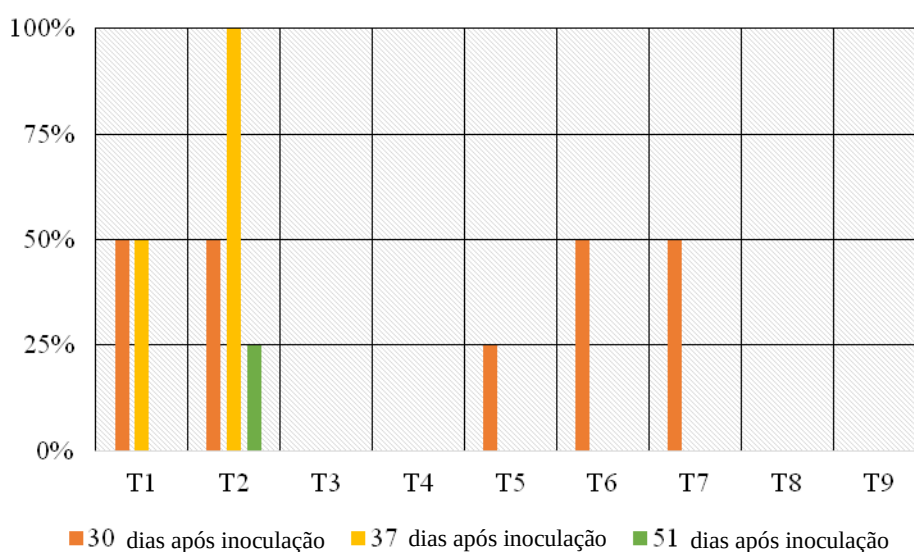


Figura 10. Incidência de *Nocardia* spp. em meio de cultivo.

Contrariamente aos resultados observados em histologia, o fígado foi o órgão onde se detetou uma maior incidência de bactéria (10%), seguido pelo baço (4%) e rim (4%). Contrariamente ao que foi descrito no parágrafo anterior, neste caso, não foi tempo mas a

dose o fator relacionado com a incidência. Sendo que as colônias de *Nocardia* spp. cresceram em meios pertencentes aos animais dos tanques com maior dose de inoculado (T1-T3).

4. ANÁLISE DE MORTALIDADE

As mortalidades descritas no presente ponto dizem respeito a animais encontrados mortos ao longo desta experiência, sendo por isso o dia da morte aqui referenciado como o dia em que o animal foi encontrado morto.

A primeira morte foi detetada 40 dias após a inoculação e ocorreu no T2; aos 42 dias após a inoculação, 6 animais foram encontrados mortos, 2 provenientes do T1 e 4 provenientes do T2; aos 56 dias após a inoculação, outra morte foi registrada no T2; aos 56 e 58 dias após a inoculação, 1 peixe foi encontrado morto em cada um dos dias, com origem no T3.

A mortalidade relativa ao T1 foi de 10%, no T2 atingiu os 30% e no T3, à semelhança do T1, foi de 10%.

A mortalidade total foi de 6%, com um total de 10 animais mortos em 180 utilizados neste trabalho.

A análise histológica dos animais encontrados mortos revelou que apenas em 38% dos casos se verificou a presença de granulomas. Em relação à análise bacteriológica, apenas 25% dos animais avaliados apresentou crescimento de *Nocardia* spp.. É importante notar que 75% dos animais analisados revelaram um crescimento de outras bactérias.

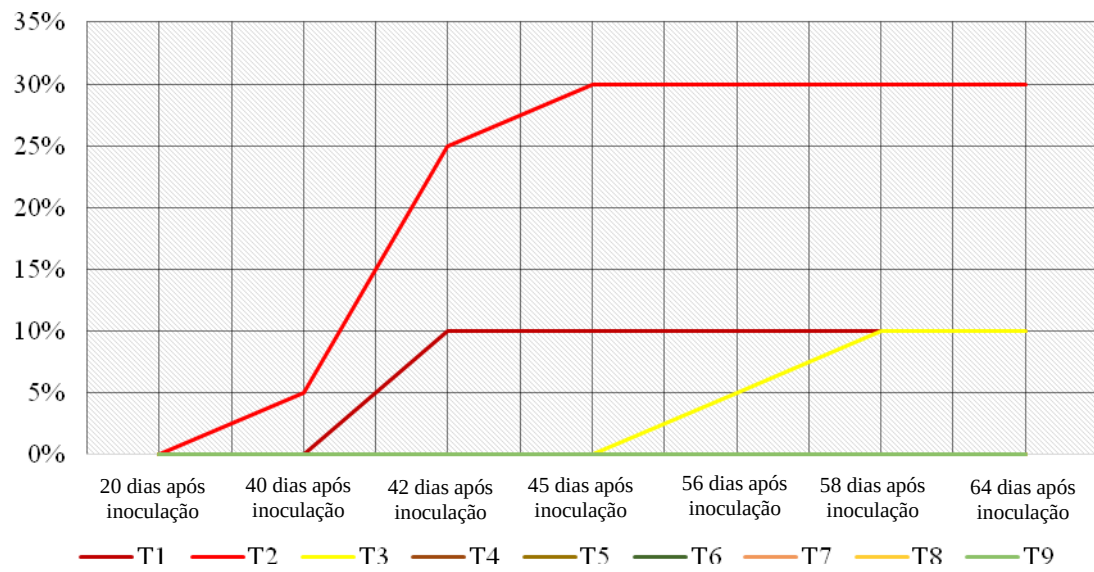


Figura 11. Mortalidade Cumulativa

DISCUSSÃO

1. SINAIS CLÍNICOS

O objetivo deste trabalho é determinar como a *Nocardia* spp. afeta uma população de Corvinas (*Argyrosomus regius*) durante um período de tempo e a diferentes doses de bactéria. Para tal, esta foi intraperitonealmente injetada em doses de 10^1 to 10^8 cfu/ml. Snieszko *et al.* (1964) mostraram que a transmissão de *Nocardia asteroides* por via oral entre animais não era efetiva, mas que a transmissão por injeção seria mais apropriada. Kusuda e Nakagawa (1978) descreveram uma transmissão positiva da doença através de injeção ou por contacto da *N. seriolae* com lesões na pele. Chen *et al.* (1992) transmitiram *N. asteroides* com sucesso através de uma injeção intramuscular. Estes resultados demonstram que as vias pela qual a infecção é mais provável são a injeção muscular ou por contacto direto com uma lesão na epiderme, deixando a transmissão oral como uma via menos provável de causar a doença (Chen *et al.*, 1992; Buller, 2014).

Inúmeros sinais clínicos associados à Nocardiose em peixes têm sido descritos, nomeadamente letargia, anorexia, emaciação, edema, granulomas com distribuição focal ou multifocal no rim, fígado, baço, coração, cérebro, mesentério, pele e músculo, sendo que estes dois últimos podem ulcerar. Snieszko *et al.* (1964) reportou um caso de invasão do cérebro e Chen *et al.* (1992) consideraram que esta migração poderia ser responsável por alterações comportamentais, como natação lateral ou circular. Wang *et al.* (2005) demonstraram que um animal infetado por via experimental era igualmente afetado em comparação com um animal infetado por via natural (Austin & Austin 1987; Chen *et al.*, 1992; Chen *et al.*, 2000; Wang *et al.*, 2005; Plumb & Hanson, 2011).

No presente trabalho, além de anorexia e alterações no crescimento, não se observaram quaisquer dos sinais clínicos dos acima descritos por outros autores. A anorexia foi observada após os 30 dias de inoculação nos animais provenientes dos tanques onde a dose inoculada foi superior (T1-T3). Esta situação tornou-se mais proeminente com o avanço do tempo, quando discrepâncias se verificaram entre os pesos dos animais dos tanques com maior dose de bactéria inoculada e os animais dos tanques com menos dose, sendo que os primeiros aparentavam menor peso. Esta situação permite inferir que a dose inoculada desempenha um papel na progressão da doença, bem como o tempo, no sentido em que nos 51 dias após a inoculação os sinais clínicos se tornaram mais óbvios. Estas observações foram confirmadas apenas para o último momento de amostragem (51 dias após inoculação) com recurso a um software de análise estatística que demonstrou que os animais dos T1-T3 eram mais leves, com uma significativa margem, em comparação com os restantes (T4-T9).

O facto de nenhum outro sinal clínico ter sido identificado, de entre os que foram descritos por outros autores, pode ser justificado pelo facto de a Nocardiose ser uma doença crónica de progressão lenta, podendo levar até 3 meses e apresentar sinais visíveis. Além disso, Sheppard sugeriu que esta doença tem início como uma infecção silenciosa, pela sua capacidade de se multiplicar nos tecidos sem revelar sinais clínicos, podendo por isso permanecer indetetável por vários meses (Chen *et al.*, 1992; Plumb & Hanson, 2011). Heuschmann-Brunner (1965) declarou que a *Nocardia* era apenas patogénica para os animais sob certas circunstâncias, atribuindo relevância ao stress como uma fator preponderante na conversão de uma infecção de baixa intensidade numa infecção aguda. Kusuda e Nakagawa (1978) consideraram também que a progressão desta doença está intimamente relacionada com o stress ambiental, atribuindo às boas práticas de manejo uma importância extrema no controlo desta condição (Campbell & MacKelvie, 1967; Chen *et al.*, 1992; Sheppard, 2005; Lewis & Chinabut, 2006; Plumb & Hanson, 2011).

2. ANÁLISE HISTOLÓGICA

28% dos animais amostrados neste trabalho apresentaram granulomas visíveis microscopicamente. É importante desde já referir que o facto de não terem sido observados granulomas em outros animais não significa que estes não estivessem presentes, dado que a amostra de tecido analisada não correspondia à totalidade do órgão assim como o corte observado correspondia apenas a uma pequena parte da porção recolhida para estudo histológico.

Foram observados granulomas em rim (23%), fígado (11%) e baço (3%), os únicos órgãos avaliados. Diferentes padrões e fases de crescimento destes foram encontrados. Os granulomas surgem como uma resposta inflamatória, em que o agente patogénico é envolvido pelos tecidos infetados, como demonstrado nas fases iniciais da formação do granuloma, com a presença de macrófagos e células epitelioides que posteriormente progridem para granulomas já completamente desenvolvidos com um centro necrótico, que se vai expandindo, rodeado por macrófagos, células epitelioides e tecido fibroso. Estes resultados vão de encontro ao estudo de Cornwell *et al.* (2011), que descrevia a presença de um ou múltiplos nódulos granulomatosos no rim, fígado e baço. Jacobson (2007) refere também a evolução pela qual passam os granulomas, tal como observado neste trabalho.

A incidência de granulomas pelos diferentes órgãos parece estar intimamente relacionada com o tempo que decorreu desde a inoculação, tendo em conta que 37 dias após a inoculação se verificaram 22% de granulomas nos animais analisados e 51 dias após a inoculação 36%. A dose parece não desempenhar um papel claro nesta análise. Não é conhecida, no entanto, a razão pela qual esta bactéria tem maior tropismo para o rim em comparação com os restantes órgãos amostrados.

Ao contrário do que foi descrito por Chen *et al.* (2000), Labrie *et al.* (2008) e Elkesh *et al.* (2013), nenhuma bactéria foi observada com a coloração de rotina Hematoxilina-Eosina. O mesmo padrão se verificou para as colorações especiais Gram e Ziehl-Neelsen que não permitiram a identificação de bactérias nos granulomas. De acordo com Wolke e Stround (1978), a *Nocardia* apenas mostra uma reação positiva com a coloração de Fite-Faraco, específica para bactérias álcool-ácido resistentes. No entanto, Labrie *et al.* (2008) declararam que haviam observado a bactéria no centro da lesão granulomatosa pelo método de Gram e Chen *et al.* (1992) e Elkesh *et al.* (2013) observaram igualmente a bactéria aquando da utilização da coloração de Ziehl-Neelsen (Chinabut; 1999; Chen *et al.*, 2000; Jacobson, 2007; Labrie *et al.*, 2008; Elkesh *et al.*, 2013).

Foi ainda avaliada uma suposta relação entre o número de granulomas encontrados em cada órgão e a dose inoculada ou o tempo após a inoculação. O tempo não se mostrou relevante para esta análise, ao contrário da dose. O maior número de granulomas encontrado num órgão teve, em 88% dos casos, origem em animais provenientes de tanques onde a dose inoculada era superior (T1 e T2).

3. ANÁLISE BACTERIOLÓGICA

Neste estudo, colónias *Nocardia* spp. foram isoladas do rim (4%), fígado (10%) e baço (4%) em 15% dos animais analisados em meio Agar Sangue, Brain Heart Infusion e Yeast Extract-Malt Extract (YEME) a 25°C. Inicialmente sob a forma de aglomerados de bacilos filamentosos que posteriormente progrediam para uma forma menos típica de simples bacilos. A cor variava entre o amarelo e o branco, podendo aparentar um aspeto seco e espesso.

A análise bacteriológica revelou que, ao contrário do que foi observado na análise histológica, o tempo parece não participar de forma ativa na identificação da bactéria em meio de cultura. No entanto, há uma aparente relação entre a dose e a incidência de *Nocardia* spp.,

no sentido em que a bactéria foi isolada com maior percentagem (79%) em animais provenientes de tanques onde a dose inoculada foi superior.

Estes resultados demonstram que não existe uma relação aparente entre a presença de granulomas microscópicos e o isolamento da bactéria em meio de cultivo. É, no entanto, de ter em conta que a *Nocardia* é uma bactéria particularmente difícil de crescer em meio de cultivo e que o meio escolhido pode ter um papel preponderante. Além disso, o facto de a bactéria não ter crescido como esperado não significa que ela não esteja de todo presente. Uma teoria proposta, é a de que a bactéria pode ter migrado para outra parte do corpo como uma resposta imunológica do animal, ou que pode estar inativa, reativando-se quando as circunstâncias lhe forem favoráveis.

Labrie *et al.* (2008) reportaram que em 20 dos 45 casos estudados não houve isolamento da bactéria pelos meios de cultivo escolhidos, apesar de esses 20 casos serem prováveis infecções com base nos sinais clínicos e esfregaços realizados. Esta situação levou a sugerir que ainda que uma infecção esteja presente no organismo, existe apenas 50% de probabilidade do agente patogénico em causa ser isolado. Cornwell *et al.* (2011) também experienciaram insucesso num isolamento bacteriano e declararam que o meio de Lowenstein Jensen é o recomendado para o diagnóstico de *Nocardia* (Labrie *et al.*, 2008; Cornwell *et al.*, 2011; Elkesh *et al.*, 2013).

4. ANÁLISE DE MORTALIDADE

Foram encontrados animais mortos aos 40, 42, 45, 56 e 58 dias após a inoculação. O T1 apresentou uma mortalidade de 10%, o T2 de 30% e o T3 de 10%. Todas as mortalidades tiveram origem em animais provenientes dos tanques onde a dose inoculada foi superior (T1-T3). Estes resultados sugerem que a dose de bactéria inoculada pode ter um papel ativo na mortalidade dos animais.

A mortalidade total deste trabalho foi de 6% com 10 animais mortos de entre os 180 que integraram este trabalho.

Os animais mortos foram analisados igualmente por meios histológicos e bacteriológicos. Destes, 38% apresentaram desenvolvimento de granulomas e 25% crescimento de *Nocardia* spp. em meio de cultivo. É significativo referir que 75% destes animais tiveram crescimento de outras bactérias que não a *Nocardia*. Chinabut (1999) referiu que a presença de agentes patogénicos adicionais pode contribuir para a mortalidade de

animais previamente infetados com *Nocardia*. Fatores extrínsecos e intrínsecos individuais podem ainda contribuir para a mortalidade de determinados indivíduos que coabitavam com outros aparentemente saudáveis (Chinabut; 1999; Cornwell *et al.*, 2011; Plumb & Hanson, 2011).

CONCLUSÃO

Torna-se imprescindível o conhecimento dos mecanismos patológicos da Nocardiose, doença infecciosa que afeta inúmeras espécies de peixes por todo o mundo. O seu estudo tem-se revelado um desafio, pelo facto, entre outros, de poder ser facilmente confundido com outra doença bastante comum, a Micobacteriose, causada por *Mycobacterium* spp. Como consequência, estudos sobre a relevância e os efeitos da *Nocardia* spp. em peixes têm sido menosprezados nas últimas décadas. No entanto, com o recente desenvolvimento da aquacultura e a crescente preocupação com a saúde pública e interesse dos consumidores em produtos seguros e de qualidade, bem como a promulgação de legislação cada vez mais rigorosa nesse sentido, a necessidade de conhecer melhor esta doença tornou-se indispensável.

O presente trabalho teve como objetivo aumentar o curto leque de informação já conhecida em relação aos diferentes aspetos da Nocardiose, através do estudo dos efeitos ao longo do tempo de uma infecção experimental com diferentes doses de *Nocardia* spp. (10^1 - 10^8 cfu/mL) numa população de corvinas (*Argyrosomus regius*).

Os únicos sinais clínicos observados durante o período em que decorreu este trabalho foram anorexia e emaciação. Os animais que apresentaram estes sinais pertenciam aos tanques onde a dose de bactéria inoculada foi superior (T1-T3), tornando-se esta situação mais evidente aos 51 dias após a inoculação. Com isto, pode concluir-se que os sinais clínicos poderão estar dependentes quer da dose inoculada quer do tempo.

A análise histológica revelou granulomas microscópicos, associados à infecção experimental de *Nocardia* spp. em 28% dos 108 animais amostrados. Esta situação pode ser justificada pelo facto de a Nocardiose ser uma doença crónica de progressão lenta, podendo levar até 3 meses a manifestar sinais visíveis. Outro aspeto importante de referir é que apenas uma pequena porção dos órgãos está representada nestas observações, podendo essa porção não ser representativa do estado geral do órgão. Foram observados granulomas em diferentes fases de desenvolvimento, desde agregados de macrófagos com células epitelioides a complexos com centro necrótico rodeado por macrófagos e tecido fibroso. O rim foi o órgão mais afetado (23%), seguido pelo fígado (11%) e baço (3%). A incidência dos granulomas observados parece ter uma relação direta com o tempo, no sentido em que, aos 30 dias após a inoculação, 22% dos animais amostrados apresentavam granulomas visíveis e aos 51 dias após a inoculação se verificou a presença de granulomas em 33% dos animais. Ao contrário, a dose não parece interferir neste estudo.

O número de granulomas visualizado por órgão foi variável. No entanto, 80% dos órgãos amostrados revelou a presença de apenas um granuloma e apenas 20% apresentaram

mais do que um. O efeito do tempo parece ser menos relevante neste caso. No entanto, a dose inoculada parece desempenhar um importante papel, tendo em conta que os órgãos em que mais do que um granuloma foi observado eram originários de animais inoculados com as doses mais elevadas de bactéria (T1 e T2).

Apesar da ocorrência de granulomas e de a bactéria ter sido experimentalmente inoculada, não foi observada nenhuma bactéria nos cortes histológicos corados com hematoxilina-eosina. O mesmo padrão se verificou aquando da utilização de colorações especiais como Gram e Ziehl-Neelsen.

Os resultados da análise bacteriológica provaram que não existe uma relação com a observação histológica. Notou-se um crescimento de colónias de *Nocardia* spp. em 25% dos animais amostrados. No entanto, ao contrário do que foi observado na análise histológica, a incidência da bactéria nos órgãos é diferente, com o fígado (10%) a ser o mais afetado, seguido pelo baço (5%) e rim (4%). Outra diferença diz respeito à relação com o tempo, que aqui parece não existir. No entanto, a dose parece ser determinante para o crescimento destas bactérias em meio de cultivo, tendo em conta que foram os órgãos pertencentes aos animais inoculados com doses superiores de bactéria que verificaram maior crescimento (T1-T3). É, no entanto, de notar que o facto de não ter sido observado crescimento de bactérias nos meios de cultivo utilizados não significa que elas não estivessem presentes.

A Mortalidade neste trabalho atingiu os 6%. A influência da *Nocardia* spp. não é clara, pois alguns animais não revelaram sinais de Nocardiose nos estudos histológicos e bacteriológicos. Os animais encontrados mortos tinham proveniência dos T1-T3, onde as doses inoculadas foram superiores, levando a pensar que uma relação entre a mortalidade e a dose de bactéria é possível. No entanto, o tempo parece não ser um fator determinante. 75% dos animais mortos amostrados revelaram ainda a presença de outras bactérias.

Estes resultados visam demonstrar a forma como uma infecção de *Nocardia* spp. progride com o tempo e a dose bacteriana em corvinas. Tem assim, de certa forma, o intuito de renovar o interesse do estudo desta tão importante doença no sentido de a conhecer melhor e desenvolver formas eficazes de diagnóstico, tratamento e controlo de modo a garantir melhores condições de saúde e bem-estar para os animais bem como para as pessoas que com eles contactam e reduzir as perdas associadas.

Os potenciais de desenvolvimento da aquacultura são imensos, levantado igualmente problemas e desafios complexos, que reclamam uma investigação de âmbito pluridisciplinar articulado, de forma poder assegurar-se, no futuro, de forma equilibrada, a rentabilidade

económica com a sustentabilidade ambiental e a qualidade do produto. A aquacultura será, no futuro próximo, um setor de grande peso económico e social quer do ponto de vista do aumento e diversificação da produção, quer no plano da conservação da diversidade biológica de ambientes naturais.

Impõe-se, pois, o desenvolvimento de ações de pesquisa e inovação que potenciem o desenvolvimento deste setor produtivo, associando a universidade ao setor produtivo, de forma a assegurar-se o desenvolvimento tecnológico do setor. Mas impõe-se também uma abordagem pluri e interdisciplinar envolvendo várias áreas do conhecimento científico. Foi nesta perspetiva que procurei desenvolver o presente trabalho, esperando com o mesmo contribuir para o desenvolvimento da aquacultura e para o alargamento da intervenção dos profissionais de veterinária.

ABSTRACT

Nocardiosis caused by *Nocardia* spp. in fish was first identified by Rucker (1949) as *Streptomyces salmonicida* in sockeye salmon (*Oncorhynchus nerka*). Since then several cases of the disease have been reported all over the world in different species, caused by this Gram-positive, partially acid-fast, aerobic and filamentous bacteria. Typical signs of the disease include severe emaciation, inactivity, granulomas in gills, spleen, kidney and liver with skin discoloration, multiple ulcer and/or nodule formation, cachexia and in some cases ascites.

The present work aimed at the characterization of nocardiosis in meagre. *Nocardia* spp. was inoculated via intraperitoneal injection in 160 meagres, *Argyrosomus regius* (Asso, 1801), distributed along 8 tanks with different inoculation doses (10^1 to 10^8 cfu/ml). The cumulative mortality was 6% and all dead fish belonged to tanks with higher doses (T1-T3). Anorexia and emaciation were observed in animals from tanks inoculated with higher doses (T1-T3).

Samples of liver, spleen and kidney were taken from all animals for microbiological and histological analysis. Histological studies showed typical granulomatous lesions in kidney (23%), liver (11%) and spleen (3%). However, special staining of lesions did not show the presence of the bacteria. A possible relation between the number of granulomas per organ and the dose inoculated was also perceived, with the most affected organs belonging to animals inoculated with higher doses of bacteria (T1 and T2). Nonetheless, *Nocardia* spp. was isolated from the same organs using Blood Agar with 1.5% of Sodium Chloride, Brain Heart Infusion with 1.5% Sodium Chloride and Yeast Extract-Malt Extract medium, although a different incidence was observed in liver (10%), spleen (5%) and kidney (4%). The fact that *Nocardia* is a difficult bacteria to isolate may explain to this discrepancy between histological and bacterial results. It was also observed that incidence of granulomas seems to be time related, while the isolation of the bacteria is dose related. Together, these results describe the pathological evolution of an experimental *Nocardia* infection in meagre.

KEY WORDS: Meagre · *Argyrosomus regius* · *Nocardia* spp. · Granulomas · Fish Pathology · Aquaculture

LIST OF SYMBOLS AND ABBREVIATIONS

% - Percentage

> - Greater than

< - Less than

°C – Degree Celsius

APROMAR - Asociación Empresarial de Productores de Cultivos Marinos de España

BA – Blood Agar

BHI – Brain Heart Infusion

CEE - Central and Eastern Europe

CFU/mL – Colony-forming unit per millimetre

DPI – Days post inoculation

EC – European Commission

EEZ – Exclusive Economic Zone

ESACUA - Asociación Nacional de Acuicultura Continental

EU – European Union

EU-27 – European Union with 27 member states

EU-28 - European Union with 28 member states

FAO - Food and Agriculture Organization of the United Nations

FEAP - Federation of European Aquaculture Producers

g – gram

GIA – Grupo de Investigación en Acuicultura

GnRH α - Gonadotropin Releasing-Hormone Agonist

ISA – Infectious Salmon Anemia

IUCN - International Union for Conservation of Nature

LAC – Latin America and Caribbean

LH – Luteinizing Hormone

MALDI-TOF - Matrix-assisted laser desorption ionization–time of flight

MED – Mediterranean

NaCl – Sodium Chloride

NE - North Europe

NEU - non-European Union

OM – Oocyte maturation

PBS - Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PCTM - Parque Científico Tecnológico Marino

POP - Persistent Organic Pollutants

PPM - Parts per million

PUFA - Polyunsaturated fatty acids

REA - Restriction Endonuclease Analysis

rRNA - Ribosomal ribonucleic acid

T# - Tank

USA – United States of America

UK – United Kingdom

YEME – Yeast Extract-Malt Extract

ZN – Ziehl-Neelsen

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INTRODUCTION

The development of aquaculture has led to the emergence of numerous diseases responsible for serious economic and ecological problems (Murray & Peeler, 2005; Rigos & Troisi, 2005; Lafferty *et al.*, 2015).

This work will focus on the study of *Nocardia* spp. in meagres (*Argyrosomus regius*) from an aquaculture facility. *Nocardia* spp. causes Nocardiosis, a disease responsible for great losses in aquaculture, but it is still overlooked given its resemblance with another much known disease affecting fishes, Mycobacteriosis. Given this situation, to date there is little data on the degree to which these disease may impact affected animals. Hence, it is imperative to reinforce the limited data already known with new studies. Help understanding the disease providing useful information for the development of control and prevention strategies as well as treatments is the main goal of this thesis.

1. AQUACULTURE

Fishing has been one of the main human activities for centuries and across several cultures as fish is considered to be a vital source of food for people given its high content of high-quality protein. It is a particularly important source of protein in regions where livestock is relatively limited. Fish supplies account for 17% in Africa and 26% in Asia, while Europe and North America account for less than 10% (Food and Agriculture Organization of the United Nations [FAO], 2009a).

However, in recent years there has been a global decrease in capture fisheries. From 1990 to 1997, fish consumption augmented 31% while the supply from marine capture fisheries increased by only 9%. This situation resulted in the intensification of the pressure on the harvesters, which has translated into increased pressures on, and over-fishing of, many commercial fisheries. According to an article published by Froese *et al.* (2012), in 2009 25% of wild world fish stocks collapsed, 35% was over explored, 30% fully explored and 10% recovering, with no more undeveloped populations (Figure 1). Over half of the known ocean fisheries are completely exploited and most of the main fishing areas have reached their maximum potential. This is problematic, since the production of fish is going to be, in a short period of time, unable to meet the increasing global demand for fish and other marine foods. For this reason, it is of extreme importance the development of a new sustainable alternative to fish production – Aquaculture (FAO, 1988a, 2014a; Froese *et al.*, 2012).

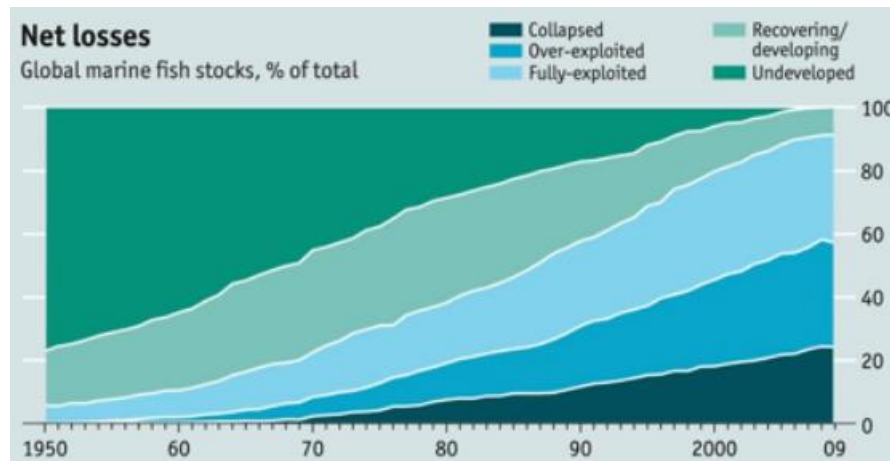


Figure 1. Net Losses – Global Marine Fish Stocks (% of total) (Froese *et al.*, 2012).

According to FAO (1988a), aquaculture is the farming of aquatic organisms, comprising fish, molluscs, crustaceans or aquatic plants and encloses both marine and freshwater species ranging from land-based (inland aquaculture) to open-ocean (mariculture) production. It involves a direct form of intervention in the rearing process in order to enhance production and implies either individual or corporate ownership of the stock being cultivated. Farming systems and technologies include extensive culture, a system that takes advantage of the natural food sources and conditions, and can be done in ponds, lagoons and coastal areas; semi-intensive farming, that depends largely on natural food yet the use of supplementary feed to complement natural food can be applied, it can be done in ponds and lagoons; and intensive culture in flow through systems, recirculating aquaculture systems and sea cages, both in sheltered and more exposed zones, and implies higher densities of organism as also regular feeding added to the system, either fresh, wild, marine or freshwater fish, or on formulated diets, usually in dry pelleted form (FAO, 1988b, 1998, 2014a; Váradi *et al.*, 2011; Asociación Empresarial de Productores de Cultivos Marinos de España [APROMAR] & Asociación Española de Productores de Acuicultura Continental [ESACUA], 2013).

Aquaculture has been, for the past twenty years, one of the fastest growing food-producing sector, accounting for nearly 50% of the world's food fish ¹production. This is due to the combined effects of diminishing wild fisheries, increasing world population and changes in consumer preferences associated with increasing affluence and a positive health image

¹ Food fish in this context includes finfishes, crustaceans, mollusks, amphibians, freshwater turtles and other aquatic animals produced for human consumption (FAO, 2014).

associated with seafood. The sector continues to intensify and diversify by introducing new species and improving its systems and practices as demands for different protein-rich food sources and safe quality products increases (Basurco & Abellán, 1999; Frankic & Hershner, 2003; FAO, 2014a).

Its growth brings major benefits as cultured seafood can diminish pressure on overexploited wild stocks and stocked organisms may augment depleted stocks. Besides, aquaculture often improves natural production and species diversity. It can also allow to provide fish products of improved quality and safety and the creation of employment and business opportunity (FAO, 1988b; Diana, 2009).

On the negative side, the impact of aquaculture facilities and infrastructure may distress the local fauna and flora, including threatened species; the effluents from aquaculture may cause eutrophication; farm escaped organisms may become invasive in areas where they are nonnative; exotic species in aquaculture with the potential to bring new pathogen agents can cause its spread out to a new environment; and the use of two to five times more fish protein (fish meal and fish oil) to feed the farmed animals than is produced in the form of farmed fish may contribute to an exaggerated pressure on existing fisheries as stocks of some ocean fish exploited for fish meal are now over-fished, situation that could be changed by introduction of a more meaningful number of herbivorous fishes or alternative diets for the carnivorous fishes with substitutes for fish-derived feed ingredients (Naylor, 2000; Diana, 2009).

In order to overcome this problems, a strong focus on upgrading the efficiency of production systems and resource utilization through management and integration or more technological solutions are being developed (Bostock *et al.*, 2009; Váradi *et al.*, 2011).

1.1. World Aquaculture

The growth importance of aquaculture in total fish supply has remained uninterrupted. Its contribution to the total fish production raised from 13% in 1990 to 42% in 2012 (FAO, 2010, 2014a).

World aquaculture production is still growing, even if at a slower rate, owing to an increasing demand for food fish among most producing countries. According to the statistics collected by FAO (2014), world aquaculture production of food fish expanded at an average annual rate of 6% in the period 2000–2012, a reduced growth rate when compared with periods

of 1980–1990 (11%) and 1990–2000 (10%). Yet, it more than doubled between the periods of 2000 and 2012 from 32.4 million tonnes to 66.6 million tonnes (FAO, 2010, 2014a).

Aquaculture output by some major producers (Figure 2), such as United States of America (USA), Spain, France, Italy and Japan has fallen in recent years especially concerning finfish production (FAO, 2014a).

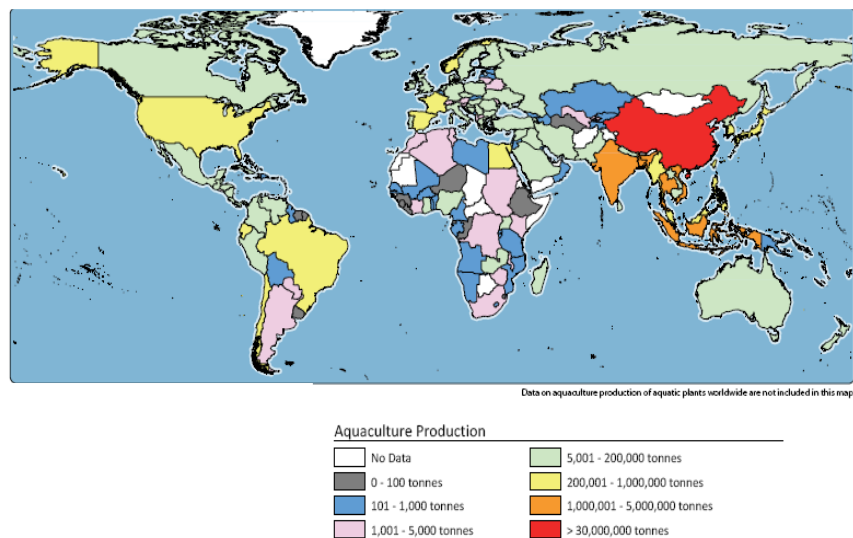


Figure 2. Aquaculture production of aquatic animals for human consumption in 2009 (tonnes) (FAO, 2010).

In USA the decline in the finfish sector resulted mostly from high feed costs and strong competition from imported Asian catfish, the predominant specie produced by USA. Other obstacles were related with lack of freshwater resources and supportive regulatory process for marine aquaculture (FAO, 2011a; Olin, 2012).

As for Japan, one of the world's top producers, the decline in its productivity has been occurring over the past decades as a consequence of different situations, namely an earthquake that struck on 2011, devastating a very important region for the industry of fisheries that accounted for 10% of Japan's total fisheries production. The impact caused by this accident resulted not only in a reduction in fisheries production but also in the discharge of radioactive materials into the atmosphere and nearby sea, provoking public concern and consequently affecting the consumption of fishery products in Japan due to fears of contamination of fishery products. Before that, Japan was already facing problems in aquaculture production, mainly due to a decreasing activity in distant waters after the establishment of the Exclusive Economic Zone (EEZ) regime; insufficient conservation measures for some species in Japan's EEZ as well as a lack of international cooperation for controlling fish stocks; a rise of fuel prices; a reduction

in profits from fishing companies and decrease of fishery enterprises and employees; a decline on per capita fish consumption in favor of meat consumption; and a decrease of fish prices as a consequence of the increasing imports (World Fishing & Aquaculture, 2006, 2013; Popescu & Ogushi, 2013).

Overall, this situation has been encouraging production expansion in other countries with a strong focus on export-oriented species. The greatest progresses in world aquaculture have been occurring from 2000-2012 in developing countries of Asia, Africa and Latin America (FAO, 2014a). Growth rates were relatively higher in Africa, reaching 12% in 2012, with Egypt being the biggest producer. Africa accounts for 2% of the total global aquaculture production, with the greatest contribution from North African countries (1.6%). These rates were due to significant increases in demand associated with a decrease in capture fisheries. Besides, the implementation of fresh approaches to technology development as also as the emergence of successful large-scale tilapia cultures directed at the export market both contributed for this growth (Jamu & Brummett, 2004; FAO, 2014a).

Latin America and the Caribbean (LAC) accounted for less than 4% of the total global aquaculture production, yet its annual growth reached 10% in 2012 with major contributions from Chile and Brazil. LAC has been showing a significant development for the past 20 years. The implementation of modern production systems and the use of new technologies have permitted a more effective management of productive resources, allowing the sector to contribute significantly to food supply, employment and foreign currency. However outstanding this growth, LAC aquaculture is limited to only four species – salmon/trout, shrimp, tilapia and mussels. Chile is the most important supplier of the USA and Japan (FAO, 2001, 2014a; Wurman, 2010).

Asia itself accounted for 89% of world aquaculture production by volume, with the biggest contribution coming from China, corresponding to more than 60% of total Asian production. The great majority of Asian aquaculture production is a consequence of small scale, individual holdings clustered together based on family business. This system generate synergies making it easier for dissemination of technologies and best management practices. Besides, its expansion in both inland and marine waters associated with a greater intensity of production and development of improved technologies contribute too to this great volume of production (Silpachai, 2001; De Sena, 2010; FAO, 2014a). Excluding China, the expansion in farmed food fish production in Asia recorded an annual growth rate of 8% with main production coming from Viet Nam, Bangladesh and Thailand, in East Asia; India in South Asia; and Iran and

Turkey in West Asia (Silpachai, 2001). China, being the single largest aquaculture producer saw its annual growth rate fell to 6% (FAO, 2014a). The growth trend in Asia is particularly strong for freshwater finfish culture. The progress of Chinese aquaculture over the past years has undergone a few problems, as lack of natural resources, with a shortage of freshwater and land in a country with a growing population occur. Besides, the deterioration of the water quality in lakes, reservoirs, rivers and coastal areas affect greatly the development of aquaculture and the safety for animals and consumers (Silpachai, 2001; De Sena, 2010; FAO, 2009, 2011b, 2014a).

Despite aquaculture being continuously developing and intensifying in almost all regions of the world, in Sub-Saharan Africa the same development is not occurring. This situation is a consequence of the fact that in Sub-Saharan Africa most places practice a rural, secondary and part-time aquaculture in small farms with small fresh water ponds. Production systems applied are generally extensive to semi-intensive with a limited income, as product is mostly consumed directly or sold locally. Besides, one of the major difficulties to the development of aquaculture in this region is the lack of locally produced high-quality fish feed that would allow a balanced diet for a better growth and attainment of market size within the shortest possible time. For aquaculture to thrive it is absolutely necessary to improve local production of fish feed so that a reduction of costs, as feed accounts for 60% of the total costs of fish production, and a better quality product can be achieved. Besides, poor infrastructures such as road, telecommunications and electricity; lack of inputs such as feeds or chemicals; political instability; lack of policies to guide aquaculture development; unfavorable investment policies; absence of relations between farmers; cash market limitations; poor quality/quantity on the extension services; and lack of research/technology development represent other major constraints to commercial aquaculture in sub-Saharan Africa (Jamu & Brummett, 2004; Gabriel *et al.*, 2007; FAO, 2012, 2014a).

The role of fishery trade varies among countries. Since 2002, China has been the largest exporter, but its imports are also growing, having become the world's third-largest importing country by 2011, after the USA and Japan. Norway follows as the second major exporter with a diversity of products (Figure 3) (FAO, 2014a).

Japan and USA are the largest importers of fish and fishery products.

	2002	2012	APR
	(US\$ millions)		(Percentage)
EXPORTERS			
China	4 485	18 228	15.1
Norway	3 569	8 912	9.6
Thailand	3 698	8 079	8.1
Viet Nam	2 037	6 278	11.9
United States of America	3 260	5 753	5.8
IMPORTERS			
Japan	13 646	17 991	2.8
United States of America	10 634	17 561	5.1
China	2 198	7 441	13.0
Spain	3 853	6 428	5.3
France	3 207	6 064	6.6

Figure 3. Leading exporters and importers in world aquaculture trade (FAO, 2014a).

In the past ten years, international trade patterns have been changing as a number of emerging countries became of growing importance to the world's exporters namely, Brazil, Mexico, the Russian Federation, and Egypt. The share of developing countries in fisheries trade has increased, representing, in 2012, 54% of total fishery export. Developed countries, on the other side, dominate world imports of fish and fishery products, although their part has decreased recently as a result of the descending pressure experienced by international prices of fish and fishery products for human consumption, in particular of farmed species, and their reduced demand in many as a consequence of the economic contraction still affecting consumer confidence (FAO, 2014a).

1.2. European Aquaculture

Aquaculture accounts for about 20% of fish production in Europe. Quality of European Union (EU) seafood should constitute a major competitive advantage for EU aquaculture; yet it is stagnating by contrast with increasing rates of aquaculture production worldwide. Between 2001 and 2008, EU aquaculture reached only 0.5% growth compared to 7.6% for all non-EU countries combined. Currently, EU farmed production is equivalent to 2% of global aquaculture production. Even though its production has been declining, the EU preserves great reliance on its wild fisheries, most of which is consumed within the EU (Bostock *et al.*, 2009; European Parliamentary Research Service, 2014).

Three regions are grouped for the 27 member states of the EU-27 based on their main supply characteristics: Central and Eastern Europe (CEE), North Europe (NE) and

Mediterranean (MED). A fourth group was added, including European countries which although non-EU (NEU) members, have noteworthy aquaculture and fisheries sectors, comprising particularly Norway, the largest aquaculture producer in Europe, and Iceland (Bostock *et al.*, 2009).

The NE and MED have extensive coastlines making it easier for them to dominate production with superior incomes. On the other hand, CEE region is by contrast, dominated by inland aquaculture and generates lower incomes (Bostock *et al.*, 2009).

MED includes the production from 7 countries of the Mediterranean region. They accounted for 65% of total EU-27 production in 2006, with the largest share. Sea bream, sea bass, oysters, clams and mussels represent 90.5% of total income. Spain, France, Italy and Portugal represent the countries with the greatest falls in traditional mussel and trout sectors. On the other hand, Greece saw its production grow (Bostock *et al.*, 2009).

The NE region accounted for 28% of EU-27 volume in 2006, being the second largest of the three regions. It is also responsible for the highest profits, due to salmon production in the region. However, production has declined as a result of falling salmon production in United Kingdom (UK) and Ireland due to an outbreak of Infectious Salmon Anaemia (ISA) in 2004. Rainbow trout production is present in all 8 countries from NE region, yet, it has also suffered a significant decline due to competition from larger Norwegian sector and changing consumer habits. Denmark is the largest producer of rainbow trout in the whole EU-27. The third most important NE sector, mussel, fell too as a consequence of the collapse of the North Sea bottom-culture dredge-fisheries in Holland and Germany, where total production also declined. In UK, Ireland and Sweden this was compensated with production in more environmentally friendly and effective systems (Bostock *et al.*, 2009).

The CEE contains the largest number of countries – 10. Only 3 countries account for the 75% of total production in this region, namely Hungary, Poland and the Czech Republic. It is by far the minor producer in volume (7%) and value of the EU-27. Besides, it has a low variability concerning the species produced, relying almost entirely on freshwater aquaculture with carps (72%) and rainbow trout (25%). A small-high value niche market has been growing too e.g. sturgeon in Bulgaria, Slovenia and Baltic States; Barramundi in Bulgaria; Koi-carp in Czech Republic and Hungary; and Crayfish in Estonia and Bulgaria (Bostock *et al.*, 2009).

Only eight countries in EU produced 81% of the total aquaculture value in 2006. All located in NE and MED regions, except for Germany. In terms of value of production, France is the leading producer (21%), followed by the UK (19%) and Spain (12%). Nevertheless, in

terms of volume the main producers from EU are Spain (20%), France (17%), UK (16%) and Italy (12%) (European Parliament, 2015).

There are three main species in value-terms produced in European Union, salmonids, sea bream/sea bass and bivalve shellfish. Salmon and trout were the most relevant cultured finfish species, followed by sea bream and sea bass. The main shellfish group comprised mussels, oysters and clams (Bostock *et al.*, 2009).

EU represents only 13% of the global population, yet the region accounts for 15% of the world consumption of fish and fishery products, being particularly dependent of external supplies. In the internal trade, Denmark, Sweden and Greece were the biggest intra-community exporters. Denmark and Sweden imported significant amounts of Norwegian salmon for processing and re-export. Greece exported mostly sea bream and sea bass as well as mussels. Most of the sea bass or sea bream trade occurred with other Mediterranean countries. France and Italy are the biggest intra-community importers, mainly of salmon and mussels. Globally, biggest imports of salmon come from Norway, with smaller contributions from Chile and China. However, most of the value-added processing occurs within the EU in Denmark and Sweden. Imports of pangasius catfish and tilapia from Southeast Asia have growth quickly, a situation that rises awareness with sustainability. Mussels were the third largest import with a great part originating from Chile. Another group with significant volume were sea bass and sea bream originating mainly from Turkey and Croatia. The most exported products in EU was salmon, with USA and Russia being the two largest importing countries; mussels, with a significant growth due to importations from Russia, Croatia and USA; and Eels, sea bass and sea bream with Russia also showing interest in importing oysters and trout (Bostock *et al.*, 2009; Váradi *et al.*, 2011; FAO, 2014a).

Several strengths and limitations are still present in the European Aquaculture as shown in the Table 1 below:

Table 1. Strengths and Limitation in European Aquaculture (Bostock *et al.*, 2009)

Factors	Strengths	Limitations
Legal and administrative factors	EU harmonization reducing costs for international business.	Lack of effective national strategies in U.E. countries. Weak or unsuccessful community strategies. Mismatches between producers need and funded research programs. Bureaucracy especially concerning licensing.
Environmental aspects	A good environmental condition is necessary in most aquaculture practices. Regulations to assure a control in aquaculture discharges.	Dependence of most aquaculture systems on environmental services with little concern on pollution.
Availability of production sites	Ratio of appropriate places or freshwater resources to land are or population is higher in Europe.	Restricted availability of new sites given environmental protection or competition with more valuable industries.
Food safety	Good health image associated with seafood products and growing concern over sustainability of capture-based fisheries.	Questionable quality of aquaculture products.
Animals health and welfare	Strong legislation to diminish the introduction and spread of fish diseases. Harmonized pharmaceutical legislation providing a larger market to encourage development. Access to diagnosis even if still a bit limited way.	Limited ranged of medicines and vaccines. Insufficient diagnostic methods available that would allow a short time detection of diseases. Lack of knowledge on pathogens and their mechanisms in new cultured species.
Third countries competition and market issues	Closeness to the world's largest seafood market. Largest market for value added qualities is close. Purchasing power.	Flexible requirements for imported products. Lack of industry and market studies.
Fish oil and fish meal availability	Advances in diet formulation reducing the quantity of fishmeal and fish oil.	High dependency on imported fish meal and fish oil due to enormous contamination of Persistent Organic Pollutants (POP) in European supplies. Excessive use of fish oil and fish meal in inland aquaculture.
Technological issues	Technological competence, particularly in reproduction.	Few diversity on cultured species.

	Capacity of high level research.	Risky nature of aquaculture industry can detain investors and researchers.
Production costs	Investments in appropriate technology are improving economies of scale.	High production costs.
Public image of aquaculture	Source of high quality protein. Substitutes marine resources over exploited.	Negative environmental and social impact.
Others	Aquaculture is gaining more visibility, increasing possibilities for investments.	Limited access to credit for many small and medium-sized enterprises. Lack of innovation in a few sub-sectors. Lack of market information concerning products and its origins.

1.3. Spanish Aquaculture

Spain has a vast coast with almost 8,000 km of coastline and a very diverse topography and climate that provide physical, chemical and environmental characteristics necessary for the development of marine aquaculture. It also has numerous fluvial resources, lakes and reservoirs where conditions are ideal for the development of inland aquaculture (FAO, 2014b).

Modern aquaculture is quite recent in Spain, counting only with 60 years of history (Figure 4). It began in the forties with mussels and reached an unprecedented success, paving the way for a diversification of the sector. In the sixties, the development of aquaculture was mainly based in inland aquaculture, with particular relevance for Trout, and Spain occupied a dominant position in world production of aquatic products. By the early eighties Spanish aquaculture was based on a familiar and very traditional small business. However, later in the eighties a great development in marine aquaculture, boosted by the entrance of Spain in European Union, was observed. Yet, numerous limitations were still present, as lack of expertise, lack of scientific and technological knowledge, poor coordination between administrations and the absence of a strong financial structure. The introduction of new technologies and greater industrialization of the sector came with the nineties, when new species such as turbot in northern Spain and sea bream and sea bass in the south and east of

Spain and the Canary Islands were added. In addition, mussel farming was greatly improved by industrialization of the sector.

Currently, the sector has a solid entrepreneurial with good potential prospects. In this line, it is important to keep in mind that consumers have great expectations, mainly due to a change in dietary habits and a crescent demand for quality and safe products (Vela-Vallejo *et al.*, 2007; FAO, 2014b).

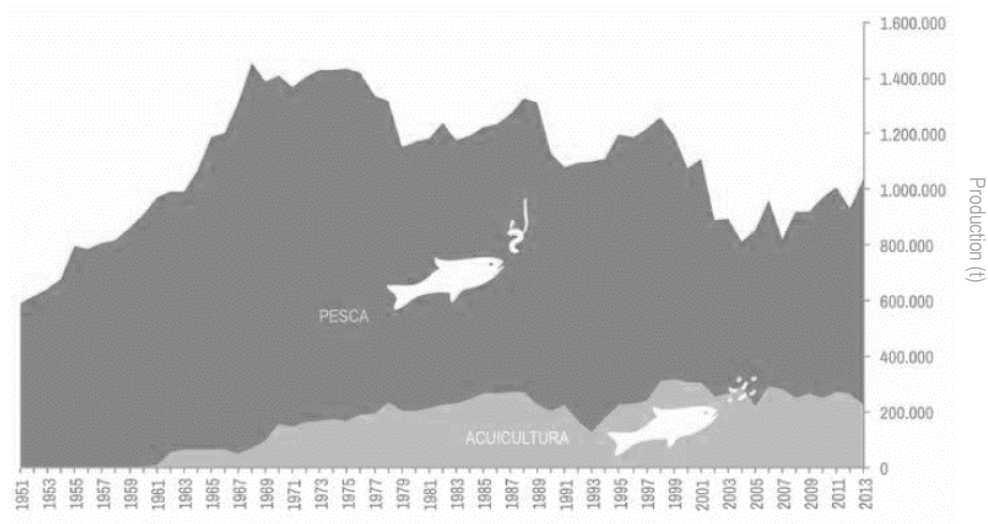


Figure 4. Total Aquatic Production (t) in Spain between 1951 and 2013 (APROMAR, 2015).

Marine aquaculture dominates the sector with a production of 96% while freshwater aquaculture only accounts for 4%. The main type of facilities applied in Spanish aquaculture are rafts for mussels, bottom cultures for other mollusc cultures such as clams, ponds for extensive cultures in inland zones, and cages for main finfish species (Ecoaqua, 2015).

In the Cantabrian coast and the northwest region, with relatively cold waters, the marine production of mussel predominates in rafts in the Galician estuaries, and inland production of turbot with contribution from oceanic seawater. Other prominent species are oysters, clams and cockles. Species with secondary relevance include scallops, salmon and octopus. The Autonomous Region that assures almost the totality of this productions is Galicia. The Mediterranean and South Atlantic area, characterized by their warmer waters, have developed mainly sea bream and sea bass, both inland as also in floating cages in the South Atlantic. Other produced species include oysters, clams and mussels. Bluefin tuna, octopus, sea bream and flounder are gaining importance in this sector too. Valencia and Andalusia lead aquaculture production in these regions. It is remarkable the production in Andalusia of sea

bream and sea bass. Bluefin tuna production in cages in Murcia is also a relevant specie. Eel culture has great potential in Valencia and in the South area. And meagre is also representative in this region. Canary Islands produce sea bream and sea bass in floating nurseries and their temperate waters all over the year provide a good opportunity for this production. In Balearic Islands, sea bream is the main specie produced (Vela-Vallejo *et al.*, 2007).

In regard to the continental production, main species include rainbow trout produced in culture tanks, intensive type, mainly on Galicia. Tench is also produced, growing in lakes and reservoirs in Extremadura (Vela-Vallejo *et al.*, 2007).

In Spain, finfish aquaculture species account for 27% of aquaculture production and the most prolific (Figure 5) are the european sea bass, *Dicentrarchus labrax* (Linnaeus, 1758); gilthead sea bream, *Sparus aurata* (Linnaeus, 1758), rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) and Meagre, *Argyrosomus regius* (Asso, 1801), a new specie recently introduced, as represented in Figure 5. Shellfish production accounts for 73% of production due to Mediterranean mussel (APROMAR, 2015; Ecoaqua, 2015).

PRODUCTION (tons)		YEAR									
COUNTRY	SPECIES	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
SPAIN	Sea Bass	5.492	8.930	10.480	9.840	13.840	12.495	14.370	14.270	14.700	17.376
	Sea Bream	15.577	20.220	22.320	23.930	23.690	20.360	16.930	19.430	16.800	16.230
	Portion Rainbow Trout	25.000	24.000	20.000	20.000	20.000	18.000	18.000	14.400	15.000	13.000
	Turbot	4.275	5.815	6.080	7.870	8.320	6.910	7.760	7.970	6.810	7.808
	Large Rainbow Trout	1.500	2.000	2.000	2.000	1.500	1.500	1.500	1.600	1.600	2.600
	Meagre	273	385	810	1.300	1.660	3.250	2.880	1.640	90	1.090
	Sole	60	80	60	55	180	204	110	194	313	786
	European eel	405	328	360	470	510	446	402	350	315	366
	Sturgeons nei	102	104	183	370	166	35	40	66	66	100
	SPAIN Total	52.684	61.862	62.293	65.835	69.866	63.200	61.992	59.920	55.694	59.356

Figure 5. Spain Production (t) between 2005-2014 (Federation of European Aquaculture Producers [FEAP], 2015).

European sea bass is a suitable specie given the fact that its reproduction in captivity is fully controlled. Gilthead sea bream is bred in marine water and it is a very appropriate specie for extensive aquaculture in the Mediterranean, mainly due to their good market price, high survival rate and favourable feeding habits. The rainbow trout, named after the rainbow-colored spots on its skin, is one of the main species bred in freshwater due to its favourable characteristics; it can occupy many different habitats, moving from freshwater to saltwater (Catadromous), or staying permanently in lakes, making it tolerable to a wide range of environments and handling procedures (European Commission, 2015a, b; APROMAR-ESACUA, 2013).

Even though it is one of the big producers in volume, Spain highly relies on imports predominantly of crustaceans, molluscs and cephalopods. Meanwhile, it is also a large exporter,

especially to EU. Spain has the second largest fish and seafood consumption per capita (42.9 per kg) in Europe following Portugal (61.1 kg per capita) (Eurofish, 2014).

2. MEAGRE, *Argyrosomus regius* (Asso, 1801)

This work is focused on a particular fish - *Argyrosomus regius* (Asso, 1801) - vulgarly known as meagre. This specie belongs to Phylum *Chordata*, Infraphylum *Gnathostomata*, Class *Actionopterygii*, Infraclass *Teleostei*, Order *Perciformes* and Family *Sciaenidae*. This family has a worldwide distribution with a large number of species (311), and genera (68) (Pollard *et al.*, 2011; García-Mesa, 2012).

Meagre is a euryhaline and eurithermic specie, being able to adapt to a wide range of salinity (5-42 g/L) and temperature (2-38°C) values, the later having an optimal range between 22°C and 24°C. These features are responsible for their wide distribution. Meagres can be commonly found in temperate waters from Atlantic Ocean and Mediterranean Sea, even though they are not very common in Italian and Greek waters (Figure 6). Besides, their semi-benthic nature, allows them to live with a depth range between 15–300 m, both close to the bottom of the continental shelf or near the surface. It may also be found in estuaries and coastal lakes (Sasaki, 1989; Jiménez *et al.*, 2005, Nelson, 2006; Lavié *et al.*, 2008; Cárdenas, 2012; Haffray *et al.*, 2012; Nathanailides, 2013).

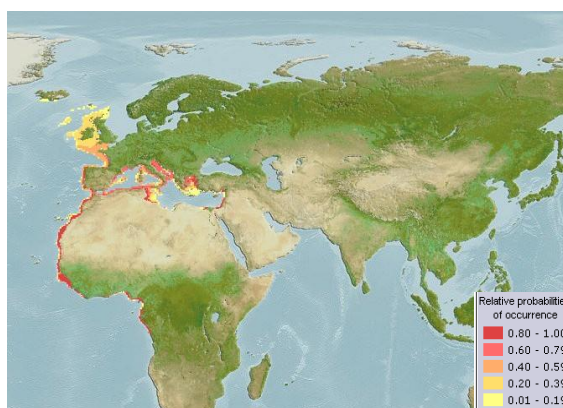


Figure 6. Distribution of Meagre (*Argyrosomus regius*) (Aquamaps, 2013).

— Anatomy

Meagre has a silver-grey elongated body with bronze traits dorsally. The lateral line is evident, extending onto caudal fin. This fin has the particularity of having a rounded edge in young fishes and straight or shaped soft S in older adults. Its dorsal fin is composed of two fins, being the second, with one hard ray and twenty seven soft rays, much longer than the first one, with nine hard rays. Anal fin has two hard rays and seven soft rays (Figure 7). Its head is big with quite small eyes and a fairly big mouth in terminal position with a yellow-gold cavity and small teeth arranged in series of different sizes. The palate does not possess teeth and the back of the maxilla is enlarged while the front is elongated (FAO, 2005; Cárdenas, 2010; Ictioterm, 2013).

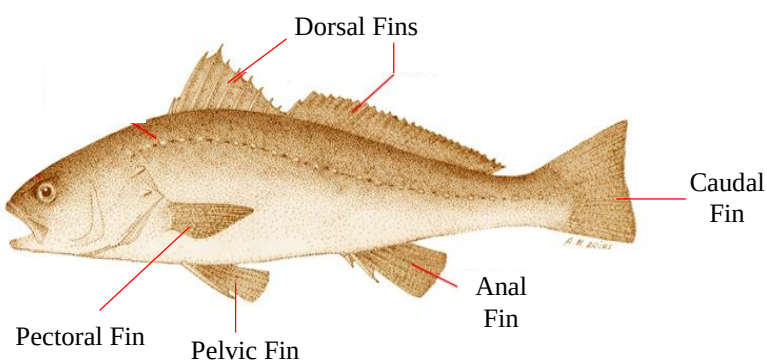


Figure 7. Morphological characteristics of Meagre (Arias, 2001).

Its inner ear has three pairs of otolithic organs, utricle (lapilli), lagena (astersci), and saccule (sagittae). Apparently otoliths have a dual function, vestibular and auditory. In meagre otoliths are particularly large, which according to some authors, should provide higher sensitivity to lower frequencies. (Secor *et al.*, 1991; Popper & Lu, 2000; Campana, 2004; FAO, 2005).

The gastrointestinal tract is relatively short, as typical in carnivorous fishes. Meagres can reach up to 2 m in length and 50 kg in weight (FAO, 2005; García-Mesa, 2012).

2.1. Wild Meagre

— Reproduction

In females, gonadal differentiation occurs earlier than in males. While primary oögonia can be detected in 9-month-old females, primary spermatogonia cannot be detected in males younger than 11-12 months. Female meagre reaches puberty at 3 years old and males at 2 years old (Schiavone *et al.*, 2012)

According to seasonal histological examinations, meagre is a gonochoristic species that exhibits an asynchronous or group-synchronous ovarian developmental organization. This means that they have separate sexes and their ovary has oocytes in all stages of development all the time, with no predominant population, respectively. Thus, meagre appears to be a multiple spawner, releasing eggs in batches over relatively extended periods of time (Zohar *et al.*, 1995; Mylonas *et al.*, 2004, 2010). Wild populations are reported to have larger spawning periods of 6-8 months, from late winter to mid-summer (Murua and Saborido-Rey, 2003; Prista *et al.*, 2009; Mylonas *et al.*, 2010; González-Quirós *et al.* 2011; Cárdenas, 2012; Schiavone *et al.*, 2012; Gil *et al.*, 2013).

The reproductive cycle of meagre is separated into two major phases. Proliferation, growth and differentiation of the gametes constitute the first phase (spermatogenesis and vitellogenesis), while the second phase comprises maturation and preparation of the oocytes and spermatozoa for release (spermiation and oocyte maturation) (Mylonas *et al.*, 2010).

An important detail from meagre is that both spermiation and ejaculation can be synchronized with female spawning via pheromonal communications. Males also have a long period of spermiation which covers the spawning season of females by a few months. Females may spawn with more than one male, either in a single event or multiple times. This behaviour guarantee reproductive success and genetic variability (Petersson & Järvi, 2001; Mylonas *et al.*, 2010).

As an anadromous species, meagre migrates from salt water to fresh water in order to spawn. In the reproductive season, that usually starts in the beginning of spring, when water temperature rounds 16-17 °C. Adults form large groups and enter the mouths of estuaries to spawn. During this period, probably to become appealing for females, males produce a typically deep sound by a close association of the sonic muscle with a hypertrophied swim bladder, as a result of a rise of testosterone levels (Quero, 1985; Connaughton *et al.*, 2002; FAO, 2005;

Lagardère & Mariani, 2006; Cárdenas, 2010; Soares, 2015). From mid-June until the end of July they leave estuaries to feed along the coast, remaining in superficial water until the beginning of autumn and returning to deeper waters during the winter (Quéméner *et al.*, 2002; FAO, 2005).

Planktonic eggs are spawned in open water and hatched for 48 hours (Cárdenas, 2010; Haffray *et al.*, 2012; García-Mesa, 2012). At the end of summer, when water temperature decreases to 13°-14°C, juveniles leave estuaries and spend two to three years in coastal waters before migrating to offshore feeding areas. Growth is mainly achieved during summer, with an optimal temperature range reported to be between 17-21 °C and with an acceptable range of 14-23 °C. Indeed, the most determining factor for these reproductive migrations is water temperature (FAO, 2005; Cárdenas, 2010; Soares *et al.*, 2015).

The most relevant known areas for spawning are the estuary of the river Gironde, Bay of Biscay (France), river Tagus (Portugal) and river Guadiana (Southern Spain and Portugal) (Quéméner *et al.*, 2002; Gonzalez-Quiros *et al.*, 2011).

— Feeding Habits

Meagre feeding habits in adults are based on smaller fish species (*Cupleidae* and *Mugilidae* families) polychaetes, crustaceans, echinoderms and molluscs (Jiménez *et al.*, 2005; Soares *et al.*, 2015). However, juveniles have a less diversified diet, based only on Mysidacea and shrimps (Cárdenas, 2010). Peak feeding occurs in the summer when water temperature is higher and is substantially reduced when sea temperatures drop below 13-15 °C (FAO, 2005).

— Vulnerabilities

Meagre is a particularly vulnerable specie given its large size, aggregating behaviour and production of sounds during spawning season. These features attract a lot of attention, making it easier to find their location and capture them. Besides, fishing pressure, environmental pollution, degradation of spawning areas and transfer of disease from cultivated animals to wild populations are leading to a severe decline in wild populations of meagre worldwide (Sadovy & Cheung, 2003; Gil *et al.*, 2013).

By now, it is listed in International Union for Conservation of Nature (IUCN) as a Least Concern species, not being listed on the Red List of endangered species because of scarce available data (Marshall *et al.* 2003; Pollard *et al.*, 2011).

Nonetheless, in some places, such as the Balearic Islands, it is considered as a critically endangered species (Mayol *et al.*, 2000; Pollard *et al.*, 2011; Millán-Cubillo *et al.*, 2016).

2.2. Farmed Meagre

The history of meagre in aquaculture is quite recent. It was first produced in 1997 in a commercial hatchery in France and since then its production has been increasing. Farming started almost simultaneously in Italy, with Spain entering into the market in 2004, followed by Greece and Turkey and spreading to other Mediterranean countries. By 2010, European meagre aquaculture production was mainly practiced in Spain, accounting for 84% of the European production, with smaller productions from France, Portugal, Italy, Greece, Cyprus and Croatia (Monfort, 2010; Cárdenas, 2012; Diversify, 2014).

Many reasons have been pointed in favour of Meagre in aquaculture. The fact that it is a eurythermic and euryhaline specie, which makes it possible to survive and be cultured in different types of systems and environments with different ranges of temperature and salinity makes the adaptation to captivity easier. Besides, meagres have great growth rates (~1 Kg/year) especially in high temperature zones; a low feed conversion ratio of 0.9-1.2; a relatively easy larval rearing; high resilience against stress factors; low mortality from diseases; high nutritional value, providing a low fat meat with high polyunsaturated fat content; and an excellent taste and firm texture (Quémener, 2002; FAO, 2005; Jiménez *et al.*, 2005; Lavié *et al.*, 2008; Piccolo *et al.*, 2008; Cárdenas, 2009; Cárdenas, 2010; Monfort, 2010; Roo *et al.*, 2010; Mittakos *et al.*, 2012; Duncan *et al.*, 2013; Millán-Cubillo *et al.*, 2016).

However, interest in meagre aquaculture lies not only in commercial terms, but also in conservation of natural resources. The vulnerability of this specie is very high due to its high market value, since its geographical distribution is highly explored, and it can be easily captured during spawning season. Moreover, spawning in estuaries is itself a threat to the maintenance of the species due to degradation and pollution of many of these coastal areas (Sadovy & Cheung, 2003; Jimenez *et al.*, 2005). To protect it, its high adaptability to farming conditions is

vital in the sense that can safeguard the growth and reproduction process of this species (Millán-Cubillo *et al.*, 2016).

Nevertheless, meagre production has its weaknesses, as this specie is still a barely known one to consumers. In some countries, its name may carry a depreciative image, as in France, where it is called “maigre” meaning slim. The small amount of farmed meagre available in the market makes its visibility quite limited, which associated with a poor knowledge of the product among consumers, brings difficulties for independent fishmongers and supermarkets sellers to promote it. However, as it occurs with other new species, the most important bottleneck of meagre production is related to reproduction and larval rearing, not only in relation to larval survival success, but also related to the elevated fry production cost (Battaglene & Cobcroft, 2007; Monfort, 2010).

In order to optimize meagre production, it is necessary to find an equilibrium between maximum culture profitability and minimum stress exposure in specimens. Stress is responsible for an increase of plasma catecholamines and cortisol levels, leading to an increase of metabolic rates which in turn causes a degradation of muscle proteins and also interferes with the reproductive and immune system. One of the main origins of stress is overcrowded facilities. However, according to some reports, the relation between density and stress is not linear, since negative effects on growth rates have been reported in facilities with low densities for Japanese meagre. This situation may be explained by the gregarious nature of meagre, where grouping in shoals prevents stressful situations. Pastor *et al.* (2002) reported higher growth rates for growth in cages. Also, Millán-Cubillo *et al.* (2016) reported higher growth rates, both in terms of body mass and body length, for higher stocking densities. Water temperature is also another stress factor. According to Lavié *et al.* (2008), juveniles in high temperature show greater metabolic activity and better growth (Mommensen *et al.*, 1999; Pastor *et al.*, 2013; Lavié *et al.*, 2008; Millán-Cubillo *et al.*, 2016).

— **Reproduction**

Although the production of meagre has increased rapidly over the last few years, reproduction in captivity is still a bottleneck for its development in aquaculture and an ideal rearing protocol has yet to be determined (Zohar & Mylonas, 2001; Mylonas *et al.*, 2010; Duncan *et al.*, 2013).

The most common dysfunction affecting females is the failure of oocyte maturation (OM), and hence ovulation. Females with this type of dysfunction go through a normal vitellogenesis, but in the beginning of the spawning season the developing oocytes fail to initiate ovulation and instead undergo atresia. However, these dysfunctions are often associated with male dysfunctions. Reduced sperm volume and diminished quality are the most common reproductive dysfunctions in males (Zohar & Mylonas, 2001; Mylonas *et al.*, 2010; Fernández-Palacios *et al.*, 2014).

Hormonal treatment has been used as a therapy to overcome these reproductive dysfunctions and ensure spawning in captivity. Currently, it is being used a gonadotropin releasing-hormone agonist (GnRHa) that stimulate the pituitary and the secretion of luteinizing hormone (LH) for the consequent oocyte maturation, spermiation and spawning. This hormone can be successfully used via liquid injections or controlled-release delivery systems (implants) that release GnRHa for a prolonged period of time (Zohar & Mylonas, 2001; Duncan *et al.*, 2012, 2013; Fernández-Palacios *et al.*, 2014; Mylonas *et al.*, 2015).

A few features to be considered in the development of a GnRHa induced spawning protocol are stage of ovarian development, levels of stress concerning husbandry and manipulations to administer the GnRHa, as also as GnRHa dose (Ibarra & Duncan, 2007; Mañanos *et al.*, 2008; Mylonas *et al.*, 2010; Fernandez-Palacios *et al.*, 2014).

Mylonas *et al.* (2013) reported the first documented spontaneous spawning of meagre. Wild meagre usually spawn near the Deltas of large rivers around the Mediterranean Sea and Eastern Atlantic Ocean, so it is conceivable that this condition might be related to lower water salinity at the time of spawning. It was also observed by Mylonas *et al.* (2013) that females keep their vitellogenic oocytes without substantial atresia for a period of at least 2 months, around April-June, and these oocytes could be induced to undergo maturation, ovulation and spawning at any time during this “post-vitellogenesis” period (Gil *et al.*, 2013; Mylonas *et al.*, 2013, Shabana *et al.*, 2013).

Rearing conditions are equally important to achieve an effective reproduction in captivity, such as water temperature, salinity, tank size, water volume, stocking density, etc. (Pastor *et al.*, 2013)

— Feeding Habits

Meagre is a carnivorous fish which implies great amounts of protein to grow. As for lipids in general, it requires longer-chain n-3 and n-6 polyunsaturated fatty acids (PUFA) for optimal growth and health (Chatzifotis *et al.*, 2012).

Fish meal and fish oil have been the main proteins for meagres over the last years. However, rising prices, growing demand, restricted availability and fluctuations in the supply of these ingredients, volatility of the market, and restrictions on the use of several animal derived proteins in fish feed formulations, have made this source of protein an uncertain one (Tacon & Metian, 2008).

In order to overcome that, alternatives to fishmeal and fish oil have been proposed with new ingredients from either plant origin or terrestrial animal origin. Soy bean, lupine, canola raps, peas, corn, wheat, bioproteins, protein concentrates and oils, rendered meat meals, blood meals and poultry meals are a few options.

According to Velazco-Vargas *et al.* (2013), the optimal soybean inclusion in diets for meagre is 27%. Yet, in some cases, soy bean was being used as the only plant protein ingredient, resulting in nutritional imbalances. To overcome this situation, studies have been done to find other alternatives, namely, processed soy bean, the addition of crystalline amino acids, phosphorus and taurine, mixtures of different plant ingredients as well as the use of technological processes for the deactivation or removal of endogenous antinutritional factors contained in the soy bean (Glencross *et al.*, 2007; Chatzifotis *et al.*, 2008; Velazco-Vargas *et al.*, 2012).

It was previously reported that fish oil can be replaced to 50% by vegetal oils without affecting the production, well-being or nutritional quality of the fish (Halwart *et al.*, 2007; Hardy, 2010).

Nowadays, meagres are being fed on pelleted diets raised on feeds used for sea bass and sea bream, as there are no optimized diets for meagre yet (Moura, 2013).

— Diseases

There is still few available data regarding meagre diseases, although it appears that it is quite resistant to bacterial diseases affecting other marine species. Some of the diseases reported are described below in Table 2.

Table 2. Pathogenic agents reported to affect Meagre

Pathogenic Agents	Organism	Consequences	References
<i>Gyrodactylus</i> sp. (the skin fluke)	Parasite	Attaches to fins and gills. Flashing, rubbing, and panting. Lethargy. Clamped fins. Excess mucus production. Secondary cutaneous ulcerations. Scale loss. Mortality in severe infestations.	FAO, 2005 Roberts <i>et al.</i> , 2009 Read <i>et al.</i> , 2007 Soares <i>et al.</i> , 2012a
<i>Diplectanum</i> sp.		Attaches to the gill lamellae. Hyperplasia of the epithelium and mucous cells. Deformation and fusion of the secondary lamellae. Lethargy. Anorexia. Asphyxia.	Toksen <i>et al.</i> , 2010 Soares <i>et al.</i> , 2012a
<i>Lamellodiscus</i> sp.		Attaches to gills. Destruction, fusion and hyperplasia of the gill filaments.	Katharios <i>et al.</i> , 2006 Soares <i>et al.</i> , 2012a
<i>Sciaenocotyle pancerii</i>		Attaches to gills. Lethargic swimming. Emaciation. Gill anaemia. Mortality.	Merella <i>et al.</i> , 2009 Soares <i>et al.</i> , 2012a
<i>Caligus</i> sp.		Skin lesions. Growth retardation. Mortality.	Toksen <i>et al.</i> , 2010 Soares <i>et al.</i> , 2012a Nagasawa, 2013
Myxozoans		Lethargy. Loss of appetite. Emaciation. Gills thickened with nodules. Poor growth.	Read <i>et al.</i> , 2007 Roberts <i>et al.</i> , 2009 Soares <i>et al.</i> , 2012a
<i>Trichodina</i> sp.		Emaciation. Lethargy. Excessive production of mucus. Skin erosion and dark skin. Irregular opercula. Head-up, swimming near surface. Chronic morbidity. Mortality.	Read <i>et al.</i> , 2007 Roberts <i>et al.</i> , 2009 Toksen <i>et al.</i> , 2010 Soares <i>et al.</i> , 2012a
<i>Amyloodinium ocellatum</i>		Attaches to gills. Irritation. Asphyxia. Hypermucosis.	FAO, 2005 Toksen <i>et al.</i> , 2010 Soares <i>et al.</i> , 2012b

<i>Vibrio anguillarum</i>	Bacteria	Loss of appetite. Reddening of fins and areas around the vent and mouth.	FAO, 2005 Roberts <i>et al.</i> , 2009 Soares <i>et al.</i> , 2012a
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3. Nocardia

The genus *Nocardia* (Trevisan, 1889) belongs to the Phylum *Actinobacteria*, Class *Actinobacteria* (Stackebrandt, Rainey & Ward-Rainey, 1997), Order *Corynebacteriales* (Buchanan, 1917, emend. Zhi, Li & Stackebrandt, 2009), and Family *Nocardiaceae* (Castellani & Chalmers, 1919, emend. Zhi, Li & Stackebrandt 2009) (Goodfellow & Maldonado, 2006).

3.1. Morphology

Genus *Nocardia* is characterized as a non-motile Gram-positive to Gram-variable bacteria. It is an aerobic actinomycete as it grows in the presence of oxygen; tests positive for the catalase enzyme and, at some stages of the growth cycle, is able to resist decolorization with acid alcohol dyes, being for that reason described as acid-alcohol-fast (Goodfellow & Maldonado, 2006).

Its morphology can alternate from a branched filamentous cell to a fragmented, irregularly shaped, pleomorphic or coccobacillary cell, depending on the phase of growth (Beaman *et al.*, 1988; Lewis & Chinabut, 2011)

Nocardia forms aerial hyphae which fragment into coccoid and rod-like elements as shown by Locci (1976). The development and stability of aerial and substrate mycelia changes with organisms as also with the conditions used to cultivate them (Goodfellow & Maldonado, 2006, 2012).

3.2. Transmission of the disease

The routes by which infection takes place are not known. Most cases of Nocardiosis in fish involve the oral cavity. However, oral exposure has not been recognized experimentally as a primary route of infection. A possible infection through contaminated feed was also suggested. Bransden *et al.* (2000) proposed that it may be transmitted through diets containing

unpasteurized marine fish or infected fish products. Moreover, the hypothesis that phyloplane flora contaminated with a high proportion of Gram-positive bacteria could introduce pathogens into fish tanks via falling leaf litter of overhanging trees was also considered (Bransden *et al.*, 2000; Lewis & Chinabut, 2011).

3.3. Pathogenesis

Nocardia spp. has the potential to cause suppurative or granulomatous diseases, termed actinomycetoma and nocardiosis, respectively (Beaman & Beaman, 1994; Brown-Elliott *et al.*, 2006).

Actinomycetoma is a localized chronic, granulomatous and progressive inflammatory disease that involves subcutaneous tissue and sometimes the bones. It often starts as a nodule that gradually increases in size and may become purulent and necrotic over time (Beaman & Beaman, 1994; Brown-Elliott *et al.*, 2006).

Nocardiosis, however is the main concern in this work. It is defined as a systemic chronic granulomatous disease affecting a variety of both freshwater and marine fish species. Symptoms described include severe emaciation and cachexia, anorexia, lethargy, skin discoloration and ulceration and, the most characteristic sign, granulomas in the internal organs such as kidney, heart, spleen, liver, digestive tracts, brain and eyes, and also on the gills, skin and muscle. They can be present with a focal or more diffuse distribution, and vary in size and constitution. Fishes can be infected in all ages (Austin & Austin, 2007; Lewis & Chinabut, 2011; Goodfellow & Maldonado, 2012).

Histologically, granulomatous inflammation may develop variable patterns. The typical granulomatous foci consists of necrotic tissue debris with some bacterial clusters in the center, surrounded by epithelioid cells and fibrous connective tissue forming clear fibrous capsules (Chen *et al.*, 2000; Labrie *et al.*, 2008; Elkesh *et al.*, 2013). Elkesh *et al.* (2013) noted that a diffuse granuloma pattern with more granulomas having necrotic centers and bacteria present were mostly seen in the skin and muscle, while granulomas in the liver, spleen, kidney, gut and mesentery were more multifocal and irregular in shape, but with some regular, well-developed, focal granulomas (Chen *et al.*, 2000; Labrie *et al.*, 2008; Lewis & Chinabut, 2011; Elkesh *et al.*; 2013).

Granulomas also undergo different phases of development. Young granulomas show macrophages and epithelioid cells but no multinucleated giant cells or connective tissue, while developed granulomas show a more typical form, with a necrotic center surrounded by peripheral cellular zone with numerous epithelioid cells, histiocytes, lymphocytes and a small number of multinucleated giant cells and fibrous connective tissue forming fibrous capsules. Colonies of acid-fast bacteria if visible lie within and at the periphery of the granulomas (Chen *et al.*, 2000; Labrie *et al.*, 2008; Elkesh *et al.*; 2013).

Studies addressing the mechanisms involved in pathogenicity of *Nocardia* showed that virulent strains of *Nocardia asteroides* were facultative intracellular pathogens that grew in a variety of cells. Its virulence appears to be related with numerous factors, such as the stage of the growth cycle, its ability to inhibit phagosome-lysosome fusion, neutralize phagosomal acidification, resist oxidative killing mechanisms of phagocytes, and alter lysosomal enzymes within phagocytes (Goodfellow & Maldonado, 2012).

3.4. Diagnostic methods

Diagnose a nocardial infection and identify its specie can be a challenge in clinical laboratories. Positive differentiation can only be made by isolation and identification of the causative agent (Brown-Elliott *et al.*, 2015).

— Identification to Genus Level

Direct smear is a method that may reveal the organism morphology, showing typical *Nocardia* cells. Normally, they are observed microscopically as beaded gram-positive, thin, branching, filamentous organisms. They can be often seen in association with polymorphonuclear leukocytes and may be detected intracellularly in macrophages and mononuclear cells. Optimal smears should be prepared using two slides, one for Gram stain and a second for the modified Kinyoun stain. Gram staining is the most sensitive method to visualize and recognize *Nocardia* species in clinical samples. With the modified Kinyoun acid-fast stain using weak acid (1% sulfuric acid) for decolorization, *Nocardia* spp. are often visualized as partially acid-fast filamentous. However, difficulties in the preparation and interpretation of the modified acid-fast stain make it an unreliable test. For that reason, it should be used only to

confirm the acid fastness of organisms detected by the Gram stain (Brown-Elliott *et al.*, 2006, 2015).

Colonies growing in culture may also provide evidence for the presence of a member from the genus *Nocardia*, as it usually produce abundant aerial hyphae that give the colonies their characteristic velvety or powdery appearance. Nocardioform bacteria grow on standard agar media used to cultivate filamentous actinomycetes such as glucose-yeast extract, brain heart infusion, modified Bennett's or Sauton's media Sabouraud glucose and yeast extract-malt extract agars, and on Mueller–Hinton II medium supplemented with glucose. Colonies can present smooth to rough, convex to irregular shapes with filamentous margins. They show a wide range of colours, from beige to brown, buff, orange, pink, red or yellow. They also have a relatively rapid growth rate, from 3 to 7 days, and most strains grow well from 20 up to 45°C (Gordon & Mihm, 1962; Maldonado *et al.*, 2000; Goodfellow & Maldonado, 2012; Brown-Elliott *et al.*, 2015).

Histopathological examination of infected organs should reveal the characteristic granulomas. They apparently start forming by the aggregation of macrophages converting posteriorly in a developed granuloma with a necrotic center, a peripheral cellular zone of numerous histiocytes, lymphocytes and a few multinucleated giant cells, all of which circumscribed by a fibrous capsule. A filamentous branch-forming bacteria might be observed within and at the periphery of granulomas. Nevertheless, the fact that *Nocardia* has a high content of structural lipids (particularly mycolic acid), a typical characteristic of alcohol-acid fast bacteria, hinders the penetration of the tissue by most of the dyes applied, affecting their efficiency. To overcome this situation, special stains for alcohol-acid organisms can be used, as Ziehl-Neelsen (ZN) and Fite-Faraco. Results with both of this dyes show a blue background and acid-fast bacteria stained red (Orchard *et al.*, 1977; Brown-Elliott *et al.*, 2006; Lewis & Chinabut, 2011; Brown-Elliott *et al.*, 2015).

— Biochemical identification

By tradition, *Nocardia* species were identified using a battery of biochemical tests, including hydrolysis of adenine, casein, tyrosine, xanthine, and hypoxanthine. However, with the current increase in the number of recognized *Nocardia* species, biochemical identification methods have been outpaced by molecular techniques concerning genera (Brown-Elliott *et al.*, 2015).

— Molecular Identification

PCR paired with restriction endonuclease analysis (REA) of the hsp65 or 16S rRNA gene was described as a useful technique for the identification of a limited number of *Nocardia* species. Nevertheless, because the recognition sites are relatively short and because of the increasing number of clinically relevant *Nocardia* species, this method is no longer sufficient for species identification (Brown-Elliott *et al.*, 2015).

Gene sequencing has been the gold standard for the identification of *Nocardia* to the species level. The secA1 gene, 16S rRNA gene, HSP gene, gyrB gene, rpoB gene are the most used. Besides, a Multilocus sequencing, with several genes being used, is also an accurate tool for the identification of *Nocardia* species (Conville *et al.* 2000; Conville *et al.* 2006; Brown-Elliott *et al.*, 2015).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry allows identification of isolates based on protein profiling. Results from individual isolates are compared to spectra from databases and rated to provide identifications (Brown-Elliott *et al.*, 2015).

3.5. Control and treatment

Any absolute effective treatment has been reported for fish Nocardiosis. Yet, attempts to develop vaccines against Nocardiosis have been made. Yellowtails immunized with attenuated live *Nocardia seriolae* showed a rise in the number of active lymphocytes and granulocytes within 72 hours. Live vaccine, with *N. seriolae* related species (*N. soli*, *N. fluminea* and *N. uniformis*) also showed some levels of protective immune response to *N. seriolae* in yellowtail (Itano *et al.*, 2006).

A number of antibiotics have also been suggested for treating Nocardiosis. They include trimethoprim and sulphamethoxazole (40 ppm in feed for 3 weeks) with a concurrent application of benzalkonium chloride (2 ppm in water). These treatments appeared to decrease the mortality rates of the infected fish. But, given that they are not entirely effective, from an economic point of view it might be hard to justify their use. Yet, Labrie *et al.* (2008) have suggested recently that this disease can affect marketable fish (over 100 g) and can therefore

cause significant loss. However, the fact that the abundant use of some drugs might be implicated in the increasing occurrence of antibiotic resistance amongst *N. seriolae* isolates may also contribute to strengthen the opposition concerning antibiotherapy as a way to treat Nocardiosis (Chen, 1992; Labrie *et al.*, 2008; Lewis & Chinabut, 2006).

It has been defended that the best way to control the advance of Nocardiosis in fish is probably through a reduction of environmental stress, providing good husbandry in the culture system, avoiding overcrowding, high water temperature or inadequate nutrients (Lewis & Chinabut, 2006).

Nocardia infections similarity of clinical presentation and histological findings to *Mycobacterium* spp. added to the difficulty of isolation suggests that the incidence of nocardiosis in fish may be underreported (Chinabut, 1999; Lewis & Chinabut, 2006).

Nocardia transmission to humans occurs typically through accidental ingestion of contaminated tank water or through contact with skin wounds. As a way to prevent the transmission it is important to wash hands and arms with warm water and soap after handling fish and contacting tank water; wear gloves specially if skin cuts are present, report injuries or illnesses, and always warn the physician about current research (Haenan *et al.*, 2013).

3.6. Geographical distribution and host range

Nocardiae are widely distributed in aquatic and terrestrial habitats, notably in soils, marine sediments and wastewater systems (Austin & Austin, 2007; Goodfellow & Maldonado, 2012; Buller, 2014)

There are reports of *Nocardia* fish infections from all over the world. Until now, only three species were reported to be associated with fish Nocardiosis, these being *N. asteroides* (formerly called *N. farcinica*), *N. seriolae* (formerly called *N. kampachi*) and *N. salmonicida*. These species have been isolated from the skin, oral cavity, brain, gills, spleen, heart and kidney of fish (Cornwell *et al.*, 2011).

Table 3 presents different cases of Nocardiosis reported since the first time it was misdiagnosed as *Streptomyces salmonicida* in 1949 by Rucker until now.

Table 3. Cases of Fish Nocardiosis

Scientific Name	Common Name	Location	Reference
<i>Oncorhynchus nerka</i>	sockeye salmon	USA	Rucker, 1949 (as <i>Streptomyces salmonicida</i>)
<i>Paracheirodon innesi</i>	neon tetra	Argentina	Valdez & Conroy, 1963
<i>Oncorhynchus mykiss</i>	rainbow trout	USA	Snieszko <i>et al.</i> , 1964
<i>Salvelinus fontinalis</i>	brook trout	Canada	Campbell & MacKelvie, 1967
<i>Seriola quinqueradiata</i>	japanese amberjack	Japan	Kubota <i>et al.</i> , 1968
<i>Seriola purpurascens</i>	greater amberjack		
<i>Oncorhynchus tshawytscha</i>	chinook salmon	USA	Wolke & Meade 1974
<i>Channa maculate</i>	blotched snakehead	Taiwan	Hsu <i>et al.</i> , 1987
<i>Paralichthys olivaceus</i>	japanese flounder	Japan	Kudo <i>et al.</i> , 1988
<i>Channa maculate</i>	blotched snakehead	China	Chen, 1992
<i>Micropterus salmoides</i>	largemouth bass		
<i>Salmo salar</i>	atlantic salmon	Australia	Brandsen <i>et al.</i> , 2000
<i>Lateolabrax japonicas</i>	japanese seabass	Taiwan	Chen <i>et al.</i> , 2000
<i>Scophthalmus maximus</i>	turbot	Portugal	Dos Santos <i>et al.</i> , 2002
<i>Larimichthys crocea</i>	large yellow croaker	China	Wang <i>et al.</i> , 2005
<i>Seriola quinqueradiata</i>	japanese amberjack	Japan	Itano <i>et al.</i> , 2006
<i>Channa argus</i>	snakehead	China	Wang <i>et al.</i> , 2007
<i>Epinephelus spp.</i>	grouper	Taiwan	Shimahara <i>et al.</i> , 2009
<i>Mugil cephalus</i>	common grey mullet		
<i>Morone saxatilis</i>	striped bass		
<i>Lutjanus erythropterus</i>	crimson snapper		
<i>Lateolabrax japonicus</i>	japanese sea bass		
<i>Micropterus salmoides</i>	largemouth bass		
<i>Terapon jarbua</i>	tiger perch		
<i>Trachinotus ovatus</i>	pompano		
<i>Scatophagus argus</i>	spotted scat		
<i>Terapon jarbua</i>	tiger perch	Taiwan	Wang <i>et al.</i> , 2009
<i>Cynoscion regalis</i>	common weakfish	USA	Cornwell <i>et al.</i> , 2011
<i>Clarias batrachus</i>	-	India	Hassan & Qureshi, 2014
<i>Argyrosomus regius</i>	meagre	Greece	Elkesh <i>et al.</i> , 2013
<i>Cynoscion regalis</i>	common weakfish	China	Xia <i>et al.</i> , 2015

3.7. Differential Diagnosis

Granulomas found in fish with Nocardiosis have also been reported associated with other causes. Foreign bodies; *Mycobacterium* spp., *Renibacterium salmoninarum*, *Francisella* sp.; parasites and fungi, particularly *Ichthyophonus hoferi* and *Aphanomyces*; ascorbic acid deficiency; and *Penicillium digitatum*, a plant pathogen responsible for disease in citrus fruits under Mediterranean climatic conditions (Elkesh *et al.*, 2013).

Yet, only the most relevant for the context of this work will be approach, as are Mycobacteriosis and Nutritional Imbalance.

— Mycobacteriosis

It may be a challenge to distinguish an infection caused by *Mycobacterium* and *Nocardia*. It is difficult to define from largely histological reports the genus to which an acid-fast pathogen belongs. Nevertheless, *Nocardia* spp. Can be differentiated from *Mycobacterium* spp. by the production of aerial hyphae when cultured on solid media (Francis-Floyd & Yanong, 2002; Plumb & Hanson, 2011).

Mycobacteria reported to infect fish include *M. marinum*, *M. shottsii*, *M. pseudoshottsii*, *M. ulcerans*, *M. abscessus*; *M. avium*; *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. montefiorensis*; *M. neoaurum*; *M. poriferae*; *M. salmoniphilum*, *M. smegmatis*, *M. scrofulaceum*, *M. simiae*, *M. stomatopiae* and *M. triplex* (Buller, 2014).

The most common method of transmission is through ingestion of infected material or through open wounds in the skin. It is also suspected that vertical transmission may occur through egg or sperm (Buller, 2014).

It is considered as a slow-progression type of disease and for this reason younger fish infected with mycobacteriosis show no external signs. Emaciation, poor growth, retarded sexual maturation or diminished reproductive performance may be the only clinical signs of mycobacteriosis (Francis-Floyd & Yanong, 2002). However, as fish age or become stressed, the infection propagates and a chronic progressive disease develops, resulting in emaciation, skeletal deformities, chronic non healing shallow to deep ulcers or fin erosion, inflammation of the skin, exophthalmia. Infected animals may become sluggish and bloated, refuse to eat, and necrosis of fin and tail as well as scale loss. Internally, gray-white granulomas develop in the

liver, kidney, spleen, heart, and muscles. Edema, as well as peritonitis, may result from their growth (Francis-Floyd & Yanong, 2002; Noga, 2010; Buller, 2014).

Mycobacteriosis is described to have low to moderate mortality rates, resulting in significant financial loss since there is still no cure (Francis-Floyd & Yanong, 2002).

Mycobacteria acts as a zoonosis, being able to infect humans. Even though transmission from infected fish to humans is infrequent, it is important to be aware of potential occurrences (Francis-Floyd & Yanong, 2002; Baumgartner, 2011).

— Nutritional Imbalance

Among noninfectious pathologies, visceral granulomatosis and systemic granulomatosis were described (Good *et al.*, 2015).

These diseases are characterized by multiple systemic or visceral granulomas that present as a metastatic calcification with a gritty texture caused by the presence of calcium salts. Microscopically, granulomatous nodules comprise a central area of mineralization (with calcium or phosphate concretions) and/or necrosis surrounded by concentric aggregates of epithelioid macrophages, fibrous connective tissue and lymphocytes (Herman, 1996; Good *et al.*, 2015).

Granulomas may be present in association with skeletal muscle, kidney, brain, gill, heart, spleen and within the submucosa and smooth muscle of the gastrointestinal tract (Good *et al.*, 2015).

The exact cause of systemic granuloma remains unknown. However, it is recognized that even though a specific cause has not yet been determined, diet is likely to play an important role in the development of the disease. Herman (1996) suggested that while water chemistry may have some influence on the occurrence of visceral granulomas, a nutritional imbalance of dietary calcium, phosphorus, magnesium and vitamin C or nutritional inadequacy (plant ingredients, tyrosine, etc.) is more likely to be the basic cause (Herman, 1996; Diversify, 2015a, b; Good *et al.*, 2015;).

According to the observations of Paperna (1987), in gilthead sea bream (*Sparus aurata*), the presence of these granulomatous lesions appears to be related to hypertyrosinaemia. Messenger *et al.* (1986) experimentally induced granulomatosis hypertyrosinemia in turbot fed diets lacking ascorbic acid. Coustans *et al.* (1990) confirmed the effect of ascorbic acid

deficiency and showed that the condition is aggravated by hypovitaminosis of B group vitamins. Tyrosine catabolism is reduced leading to deposition of crystalline tyrosine and subsequent granulomatosis (Herman, 1996; Good *et al.*, 2015).

This pathology is not associated with high mortality rates. However, both prevalence and intensity are high, which constitutes a major bottleneck for further expansion of meagre production as this condition may lead to reduced growth and physiological performance, affecting the final product and making it unacceptable for the consumer (Diversify, 2015a, b).

4. OBJECTIVES

The aim of this work was to determine the effect of different doses of *Nocardia* spp. inoculated via intraperitoneal injection in a population of meagre (*Argyrosomus regius*) for a period of time (2 months) in order to characterize the effects of the infection according this two variables: time and dose.

MATERIAL AND METHODS

The experiments described in this study lasted 64 days and included 3 sampling points: 30, 37 e 51 days post inoculation. They were partially performed in Parque Científico Tecnológico Marino (PCTM) (Taliarte, Gran Canaria) of the Aquaculture Research Group (GIA) and in the Faculty of Veterinary, University of Las Palmas Gran Canaria and were made respecting the rules of the bioethics committee of this same university. It is important to mention that some details are not disclose in the present work as manuscript is currently being submitted to a peer reviewed journal.

In this study, a total of 160 healthy juvenile meagres (*Argyrosomus regius*), produced in Parque Científico Tecnológico Marino (PCTM) (Taliarte, Gran Canaria), with an average initial weight of 69.50 g, were previously inoculated via intraperitoneal injection with 1 mL of bacterial suspension of *Nocardia* spp. with different concentrations, ranging from 10^1 to 10^8 CFU/mL. Animals were separated in groups of twenty and placed in 8 tanks (T) according to the injected doses. In T1 were placed the animals inoculated with the higher dose, decreasing gradually until T8. Another tank (T9), with twenty more fishes was added, and phosphate-buffered saline (PBS) was administered intraperitoneally to those animals, as control group.

The animals were fed with a daily commercial diet and supervised in order to observe kept during the challenge time in a biosecurity room with proper conditions of temperature, water salinity, tank size, water volume and stocking density, and controlled and supervised according to in-house standard procedures for any clinically relevant changes or deaths.

1. CLINICAL SIGNS AND GROSS PATHOLOGY

All animals were fed with pelleted commercial diets and observed every day to evaluate any behavioral or physical changes, particularly the ones present in Nocardiosis, such skin ulcers or granulomas, anorexia, emaciation or mobility changes.

2. SAMPLE COLLECTION AND WEIGHT EVALUATION

Sample collection was performed at three different times – 30, 37 and 51 days post inoculation (dpi) - and only included 108 out of the 180 inoculated. Four fishes from each one

of the nine tanks were collected and euthanized and necropsies were carried out at the laboratory.

Every fish sampled was weighted and classified according to its tank and order of necropsy and the results were recorded in a table (Appendix I). They were carefully observed for macroscopic lesions, such as granulomas or ulcers.

Sampling consisted on the removal of spleen, liver and kidney. Each organ was rigorously analyzed from a global perspective, when still attached to the body, and then independently when detached. Spleen, liver and kidney were then cut in two pieces and further analyzed through histological and microbiological methods.

Ten fishes were found dead. Yet, only eight of them were evaluated with the same methods as the ones described above.

3. HISTOLOGICAL STUDIES

I. Fixation: Proper fixation of tissues for histological observation is extremely important. The fixative minimizes the loss or enzymatic degradation of cellular and extracellular molecules, so that the-postmortem activities of decay, putrefaction (microorganisms attack) or autolysis (enzyme attack) are prevented. A fixative also enhances differences in refractive indexes of different tissue elements, increasing the visibility or the contrast between them (Carson & Hladik, 2009; Rhodes, 2013). The fixative of choice was a chemical additive noncoagulant - 10% neutral buffered formalin (Pancreac) - where the organs were immersed. Several cuts were performed in the bigger organs, so that the fixative could better penetrate the tissue. Samples remained in the fixative solution for 48 hours.

II. Cutting in of Tissue and placing it in cassettes: Tissues were then cut into small pieces, and placed in cassettes without touching its sides, then dipped in formalin to avoid organs deterioration.

III. Tissue Processing: This part consisted in placing the samples in an automated tissue processor (Figure 8), where dehydration, clearing and infiltration of tissue occurred, with the goal of providing a matrix that can support the tissues during sectioning.



Figure 8. Automated tissue processor.

○ **Dehydration:** It is necessary to prepare the tissue for embedding in a nonaqueous medium such as paraffin. Paraffin is hydrophobic and that is the reason why most of the water in a specimen must be removed before it can be infiltrated with it (Carson & Hladik, 2009; Spencer & Bancroft, 2013). Dehydration must be done slowly. For this reason, specimens are subject to a process of gradual increase in concentration of dehydrating agents (Carson & Hladik, 2009; Mescher, 2013; Spencer & Bancroft, 2013). The dehydrating agent used in this experiment was ethanol. It was used in graded concentrations being the tissue first immersed in 70% ethanol solution, followed by immersion in 96% and 100% solutions. In this work the protocol of dehydration consisted in the following steps (Table 4):

Table 4. Protocol of Dehydration

1) 70% ethanol	3 hours
2) 96% ethanol	2 hours
3) 96% ethanol	2 hours
4) 96% ethanol	1 hour
5) 100% ethanol	1 hour
6) 100% ethanol	2 hours
7) 100% ethanol	2 hours

○ **Clearing:** The main purpose of a clearing agent is to remove the alcohol used for dehydration and to make the tissue receptive to the infiltration medium, so it has to be fully miscible with both ethanol and paraffin wax. Another important function is to

remove a considerable amount of fat from the tissue which would act as a barrier to wax infiltration (Carson & Hladik, 2009; Ross & Pawlina, 2011; Mescher, 2013). Xylene was the clearing agent of choice because is miscible with most organic solvents and paraffin wax (Carson & Hladik, 2009; Spencer & Bancroft, 2013). The clearing procedure is shown below (Table 5):

Table 5. Protocol of Clearing

1) Xylene	30 minutes
2) Xylene	30 minutes
3) Xylene	1 hour

○ **Infiltration:** After dehydration and clearing, the tissue was infiltrated with a supporting medium [paraffin wax (Q Path)], generally referred to as an embedding medium, which held the cells and intercellular structures in their proper positions while thin sections were cut (Carson & Hladik, 2009; Mescher, 2013). The infiltration sequence is presented in Table 6:

Table 6. Protocol of Infiltration

1) Paraffin	3 hours
2) Paraffin	7 hours

IV. Embedding: Comprised the embedding of processed specimens in Paraffin wax, a medium that provides external support during microtomy. It was dispensed into a suitably sized mold. The tissue was then oriented in the mold and a cassette was attached, producing a flat block face with parallel sides. The mold was placed on a small cooling area to allow the paraffin to solidify (Carson & Hladik, 2009; Spencer & Bancroft, 2013).

V. Sectioning Tissue: The tissues were sectioned with a rotary microtome in 4 µm slices (Figure 9).



Figure 9. Rotary Microtome.

VI. Flotation bath and Placing Sections on Slides: A thermostatically controlled water bath at 45 °C was used for floating of tissue ribbons after sectioning. Ribbons were stretched gently and placed on the flotation bath (Figure 10) then paraffin sections were picked up on glass slides.



Figure 10. Ribbons on the floatation bath.

VII. Drying: Samples were then placed in an incubator to dry at 100°C (Figure 11).



Figure 11. Incubator

VIII. Staining

○ **Hematoxylin and Eosin:** The hematoxylin and eosin stain (H&E) stains a large number of different tissue structures. Hematoxylin stains the cell nuclei blueblack, showing good intranuclear detail, while eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange, and red. Harris's hematoxylin was the one used for this work (Carson & Hladik, 2009; Bancroft & Layton, 2013). In the present work we followed the staining procedure shown in Table 7 (Figure 12 and 13) below:

Table 7. Protocol of staining Hematoxylin and Eosin

1) Xylene	2 minutes
2) Xylene	2 minutes
3) Absolute alcohol	2 minutes
4) Absolute alcohol	2 minutes
5) Alcohol 70%	2 minutes
6) Distilled water	2 minutes
7) Distilled water	2 minutes
8) Distilled water	2 minutes
9) Hematoxylin (RAL Diagnostics)	15 minutes
10) Tap water	20-30 seconds
11) Hydrochloric acid 1 % in 70% alcohol	3-4 dips
12) Tap Water	10-15 dips
13) Ammonia water	15 dips or until blue
14) Tap Water	10 minutes
15) Alcohol 96%	1 minute
16) Eosin-phloxine (DC pancreac)	5 minutes
17) Alcohol 96%	2 minutes
18) Alcohol 96%	2 minutes
19) Absolute alcohol	2 minutes
20) Absolute alcohol	2 minutes
21) Xylene	2 minutes
22) Xylene	2 minutes
23) Xylene	2 minutes
24) Mounting with DPX	



Figure 12. Image illustrating the staining procedure.



Figure 13. Final results of Hematoxylin and Eosin staining.

○ **Gram Staining:** The Gram stain is used for detection and identification of bacteria. Gram-positive bacteria differentiates from Gram-negative for its distinctive thick layer of peptidoglycan in its cell wall. This characteristic allow Gram-positive bacteria to retain the crystal violet and become blue when observed unlike Gram-negative bacteria that are unable to absorb it, staining red because of the safranin added. However, results are unreliable in the absence of positive controls (Bartlett, 2013). This worked followed the gram-staining protocol shown below (Table 8):

Table 8. Protocol of Gram staining

1) Xylene	2 minutes
2) Xylene	2 minutes
3) Absolute alcohol	2 minutes
4) Absolute alcohol	2 minutes
5) Alcohol 70%	2 minutes
6) Distilled water	2 minutes
7) Distilled water	2 minutes
8) Distilled water	2 minutes
9) Crystal Violet 2% (Fluka Analytical)	1 minute Until it is clean
10) Tap water	1 minute
11) Lugol's iodine (DC pancreac)	Until it is clean
12) Tap Water	15 seconds or until gets colorless
13) Acetone/ethanol	Until it is clean
14) Tap Water	1 minutes
15) Safranin (Rhone-Poulenc)	Until it is clean
16) Tap Water	Gram + (blue) / Gram – (red)
17) Dry and mounting with DPX	

○ **Ziehl-Neelsen Modified for acid-fast bacteria:** The lipoid capsule of *Nocardia* spp., an acid-fast organisms, takes up carbol-fuchsin, soluble in the lipids of the cell wall, and repels decolorization with mineral acid. Ziehl-Neelsen staining is enhanced by the phenol and alcohol, and both of these chemicals also assist in dissolving the basic fuchsin (Carson & Hladik, 2009). Table 9 shows the steps to achieve a Ziehl-Neelsen staining.

Table 9. Protocol of Ziehl-Neelsen staining

1) Xylene	2 minutes
2) Xylene	2 minutes
3) Absolute alcohol	2 minutes
4) Absolute alcohol	2 minutes
5) Alcohol 70%	2 minutes
6) <i>Distilled water</i>	2 minutes
7) <i>Distilled water</i>	2 minutes
8) <i>Distilled water</i>	2 minutes
9) Carbol Fuchsin (Fluka Analytical)	30 minutes Until it is clean
10) Tap water	5 minutes
11) Acid Alcohol	5 minutes
12) Tap Water	1 minute
13) Brilliant Green (Sigma)	Until it is clean
14) <i>Distilled water</i>	2 minutes
15) Alcohol 96%	2 minutes
16) Alcohol 96%	2 minutes
17) Absolute alcohol	2 minutes
18) Absolute alcohol	2 minutes
19) Xylene	2 minutes
20) Xylene	2 minutes
21) Xylene	
22) Mounting with DPX	

IX. Mounting: Sections were mounted with DPX, a synthetic mounting medium. Coverslips were applied to preserve the stained section of tissue and to allow a better microscopic examination.

X. Observation: Every organ sampled was observed microscopically. In organs where granulomas were observed they were classified according its intensity in order to evaluate a possible correlation between the number of granulomas per organ and the bacterial dose inoculated or time.

4. BACTERIAL STUDIES

I. Culture in Blood Agar with 1.5% of Sodium Chloride, Brain Heart Infusion with 1.5% Sodium Chloride and Yeast Extract-Malt Extract medium: Samples of liver, kidney and spleen from each animal were swabbed on Blood Agar [5% desfibrinated sheep blood (v/v)] supplemented with 1.5% sodium chloride (DSBA+1.5%NaCl), Brain Heart Infusion with 1.5% Sodium Chloride (BHIA + 1.5% NaCl) and Yeast Extract-Malt Extract (YEME) medium (Pronadisa/Conda laboratories). Samples were aseptically taken and fixed and placed in each one of the mediums. They were posteriorly incubated for a period of 3 weeks at 25°C, with daily observations. After 3-5 days it was already possible to evaluate them.

II. Gram Staining: They were subsequently subjected to the Gram stain protocol (Table 10).

Table 10. Protocol of Gram staining in Bacteriology.

1) Smear Preparation	-Drop of water and a monolayer of organisms in a glass slide.	
2) Smear Fixation	-Pass smear two or three times through a flame or for a few seconds.	
3) Gram Staining	-Crystal violet	1 minutes
	-Wash off briefly with tap water.	
	-Drain.	
	-Lugol's iodine solution (mordant)	1 minutes
	-Wash off with tap water.	
	-Drain.	
	-95% alcohol	15 seconds
	-Wash off with tap water.	
	-Drain.	
	-Safranin solution	30 seconds
	-Wash off with tap water.	
	-Drain and dry.	

5. STATISTICAL ANALYSIS

As previously described in the section 2 of this chapter, every fish sampled in this experiment was weighted and had its weight recorded before being necropsied.

To determine whether there was effectively a correlation between animal weight and dose inoculated or time, a statistical analysis was necessary. SPSS was the software used for it.

This statistical analysis include the following different tests shown below:

I. Analysis of Variance (ANOVA): A one-way ANOVA was the first test applied. This test compares the means between groups. In the present case, it calculated the mean fish weight of every tank (T1-T9) and compared these values for the three sampling points (30, 37 and 51 dpi).

II. Post hoc test (Tukey HSD): An ANOVA test can prove whether there is an overall difference between groups, but do not specify which groups differ. In cases where a significant ANOVA result is present, a Post hoc test must be performed. The purpose of Tukey's HSD test is to determine which groups among the sample have significant differences. In this work it was only used to compare tank mean weights for the last sampling moment (51 dpi), where the one-way ANOVA test showed to be significant.

III. Factorial ANOVA (two-way): This test allowed to understand whether the two independent variables (time or dose) or their interaction (time + dose) are statistically significant, *i.e.* if they play a role both individually and together in fish weight disparity between different tanks.

RESULTS

1. CLINICAL SIGNS AND GROSS PATHOLOGY

From the beginning of this experiment all animals were monitored on a daily basis so that any change could be noted and corrected if possible.

For the first 30 dpi any physical or behavioral variation was observed. This situation changed a few days after the first sampling moment when fishes from T1-T3 appeared to show anorexia, being reluctant to feed and not showing interest in catching food when it was administrated, as normally observed until then. This behavior got worse over time, and clear size differences became evident between animals from T1-T3 and T4-T9. In contrast, fishes from tanks where the inoculated doses were lower (T4-T8) still maintained great interest in food.

The fact that only animals inoculated with higher doses of bacteria (T1-T3) showed signs of anorexia points to a correlation between the dose of *Nocardia* spp. injected and the progression of the clinical signs. Another fact consistent with this observation was that, when collected and weighted, fishes from T1-T3 were smaller and lighter when compared to animals from T4-T9. A statistical analysis of the recorded fish weights was made in order to assess if there was in fact a correlation between weight variation and dose or time.

Using SPSS, a software for statistical analysis, a one-way ANOVA was performed. This test gathered the weights of fishes from each one of the 9 tanks and calculated a mean with a 95% confidence interval for every tank concerning the 3 different moments of sampling. The aim of this step was to make the comparison of values for every tank at 30, 37 and 51 dpi easier and to establish if weight variation between T1-T9 were significant. For 30 and 37 dpi the ANOVA test showed, with a significance of 0.37 and 0.28 ($p>0.05$) for 30 and 37 dpi, respectively, that there was no statistically significant variation between tanks for this two sampling times, meaning that weight discrepancies found between these two moments were not relevant. In contrast, the results obtained concerning to 51 dpi revealed a statistically significant variation of weight between tanks ($p<0.05$) (Appendix II, Table 1).

The ANOVA test previously described showed that there was in fact a significant difference between groups for the last sampling (51 dpi), but it did not specify which groups differed. Therefore, a Tukey's HSD test was used to clarify that, comparing groups of tanks and possibly evaluating a significant difference of weight between them (Appendix II, Table 2). Results allowed to group tanks in terms of most and least affected. T1-T3 were the most affected concerning weight lost, consistent with the abovementioned observations, followed by T4-T6

group, and T7-T9 that were clearly the least affected. Moreover, the difference between the mean weight of T9 and T1 was 58g.

To understand if time or dose were significant for these observations, either independently or together, a two-way ANOVA test was used. This test (Appendix II, Table 3) revealed that the independent variables, dose of *Nocardia* spp. inoculated ($F(8,81)=7,846$, $p=0,000$) and time ($F(2,81)=61,600$, $p=0,000$), are significant for weight variation (dependent variable), but it is mostly their combination (time + dose) that is significant ($F(16,81)=4,494$, $p=0,000$). These results of the ANOVA test suggest that higher doses of *Nocardia* spp. tend to affect animals at a higher scale, as represented in Figure 14, leading them to develop premature signs of disease, such as anorexia and emaciation, and also interfering with their growth. No other signs of disease were observed, as they appeared to maintain their normal mobility and behavior, and no visible physical changes related with *Nocardia* or any other disease were noticed.

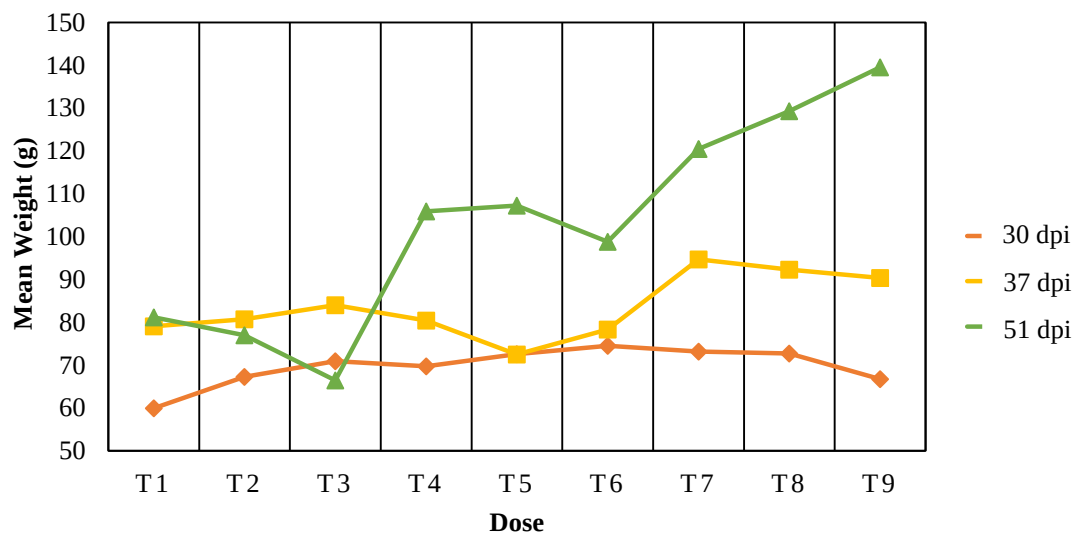


Figure 14. Effect of Dose and Time in Fish Weight (means).

2. HISTOPATHOLOGY

2.1. Histopathology with Hematoxylin-Eosin

Histologically, a granulomatous inflammation was observed. In most cases a single granuloma was found per organ, yet in a few cases two or more granulomas were seen with a focal or more diffuse distribution. A varying pattern of granuloma development was observed. It was also possible to note the different phases of development that a granuloma undergoes, from an initial phase when macrophages start to accumulate and then aggregate (Figures 15-17), trying to fight the infectious agent; to a more complex granuloma with a necrotic center surrounded by a layer of macrophages and epithelioid cells (Figures 18-23). Noticeably, no bacteria were found at the center of the necrotic zone. The remaining areas of the affected organs were undamaged.

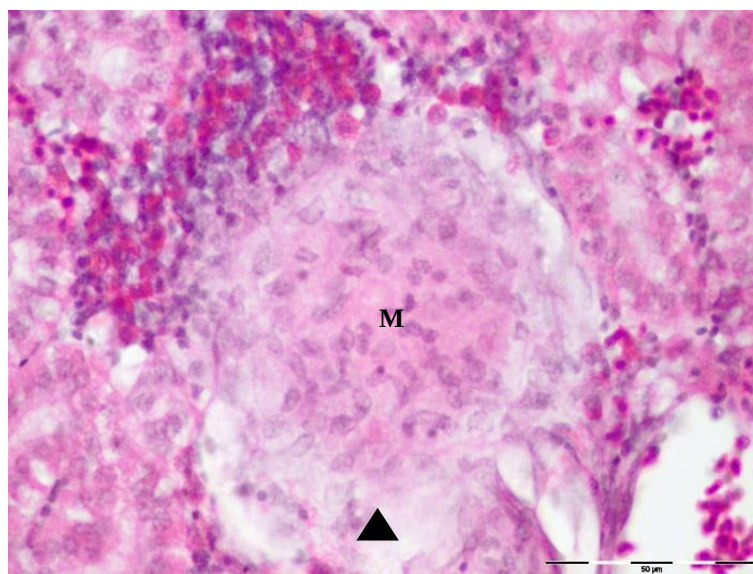


Figure 15. Kidney with a forming granuloma (▲) composed by an aggregate of macrophages (M) (50μm) (40x).

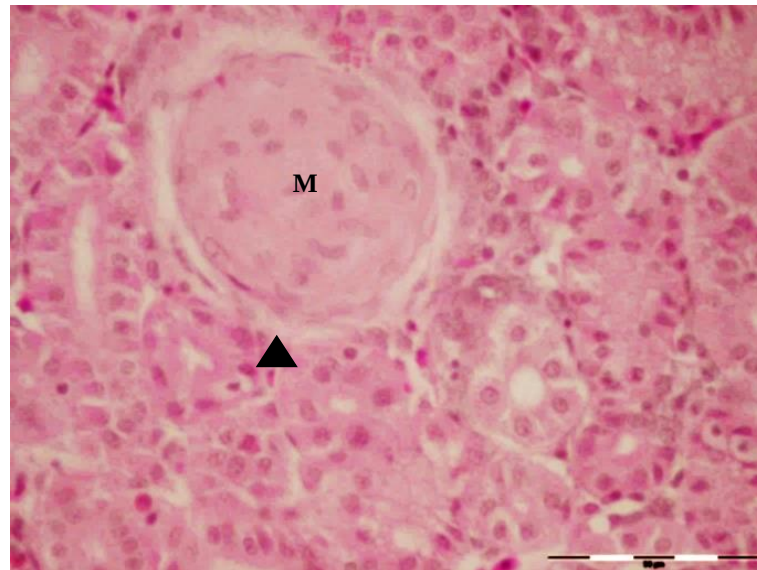


Figure 16. Kidney with a forming granuloma (▲) composed by a now organized aggregate of macrophages (M) (50µm) (40x).

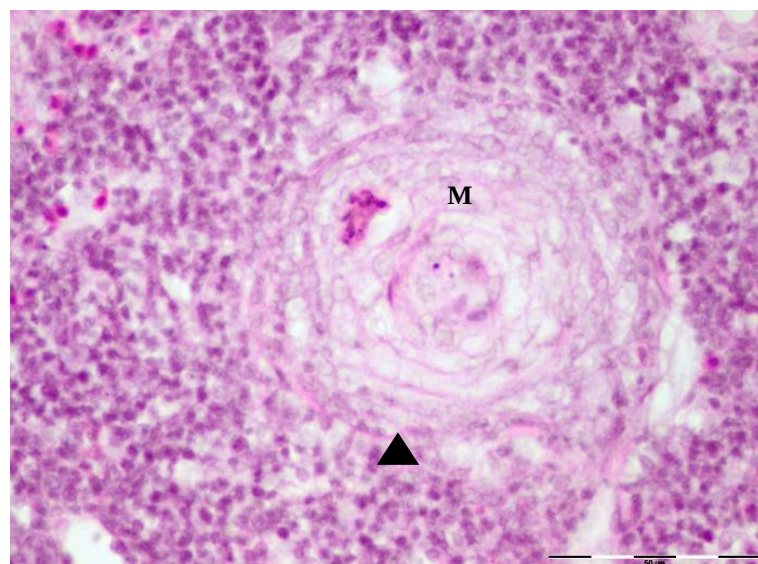


Figure 17. Kidney with a forming granuloma (▲) composed by macrophages (M) arranged in layers (50µm) (40x).

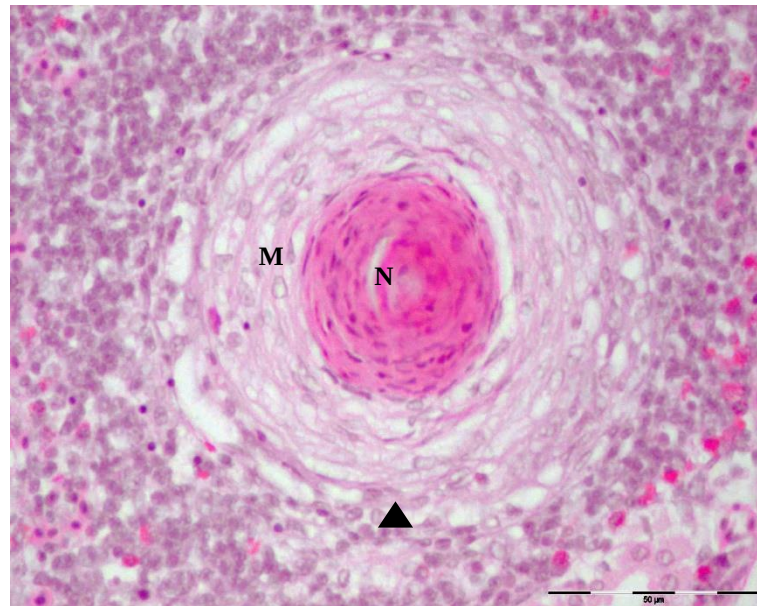


Figure 18. Kidney with a marked granuloma (▲) with a necrotic center (N) surrounded by layers of macrophages (50μm) (M) (40x).

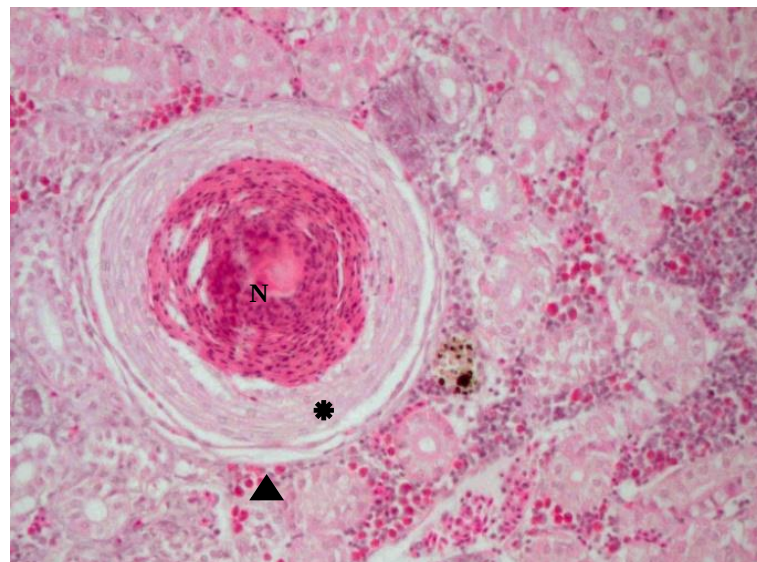


Figure 19. Kidney with a marked granuloma (▲) with a necrotic center (N) and an external layer of fibrocytes (*) (50μm) (40x).

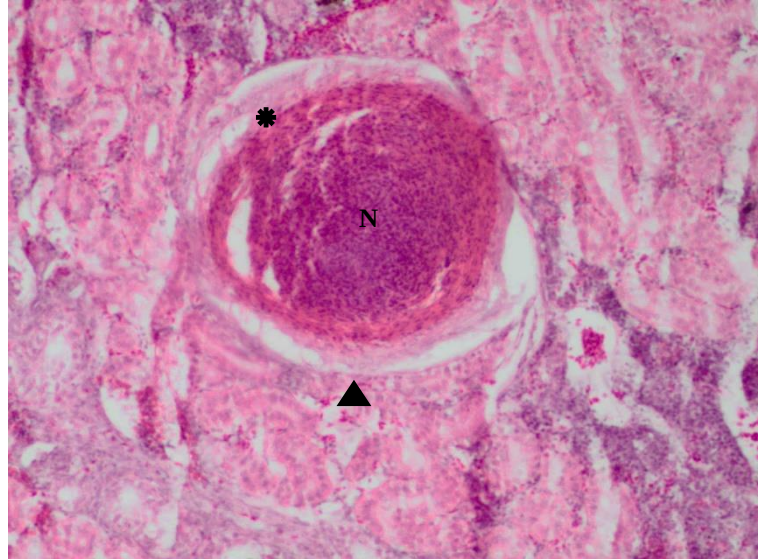


Figure 20. Kidney with a marked granuloma (▲) with a large necrotic center (N) surrounded by a layer of Fibrocytes (*) (200µm) (20x).

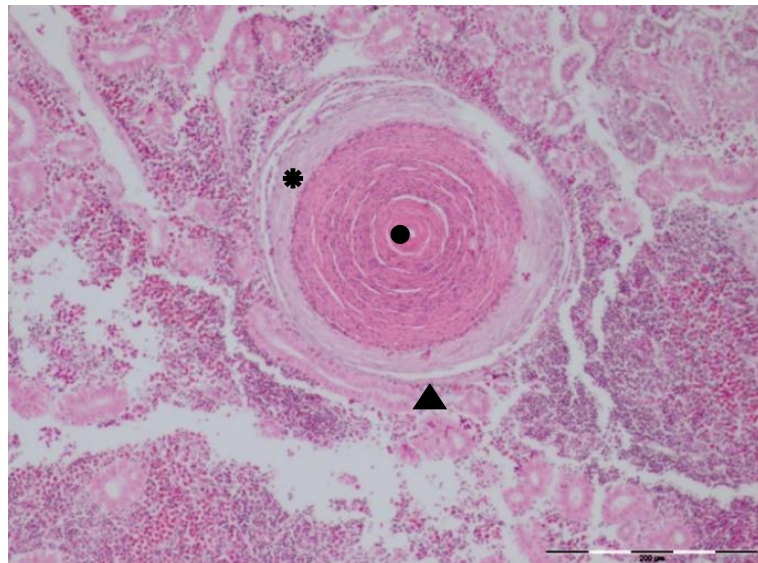


Figure 21. Kidney with a marked granuloma (▲) with a center composed of laminar material (●) surrounded by a layer of Fibrocytes (*) (200µm) (20x).

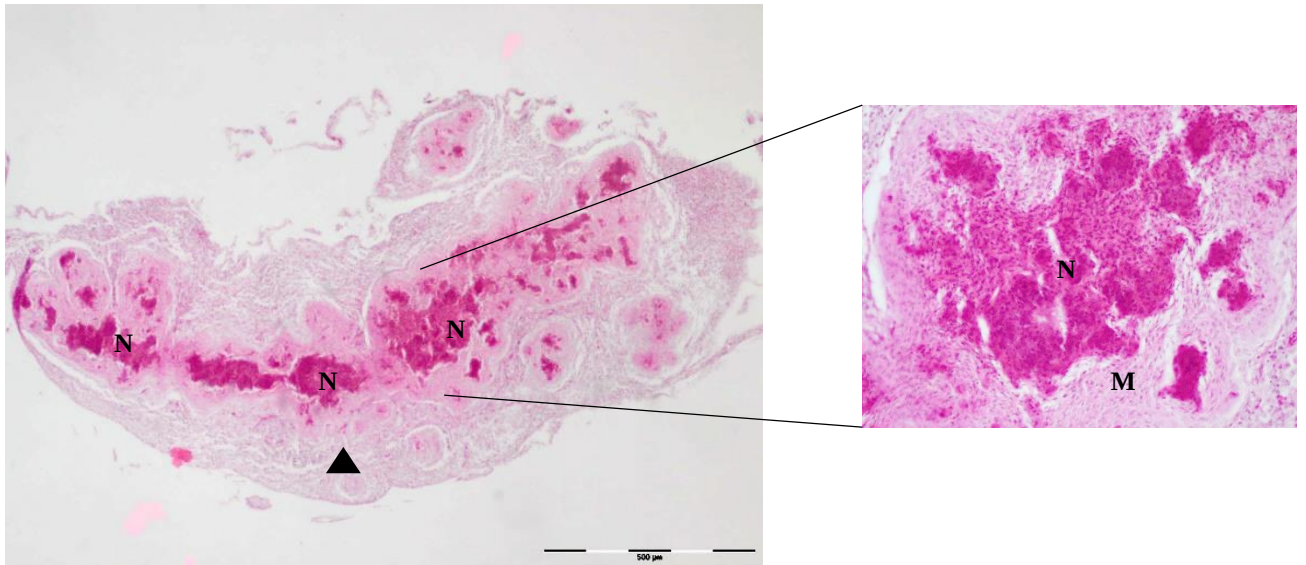


Figure 22. Spleen with multiple granulomas (▲) with a marked necrotic center (N) surrounded by a Macrophages (M) (500μm) (4x).

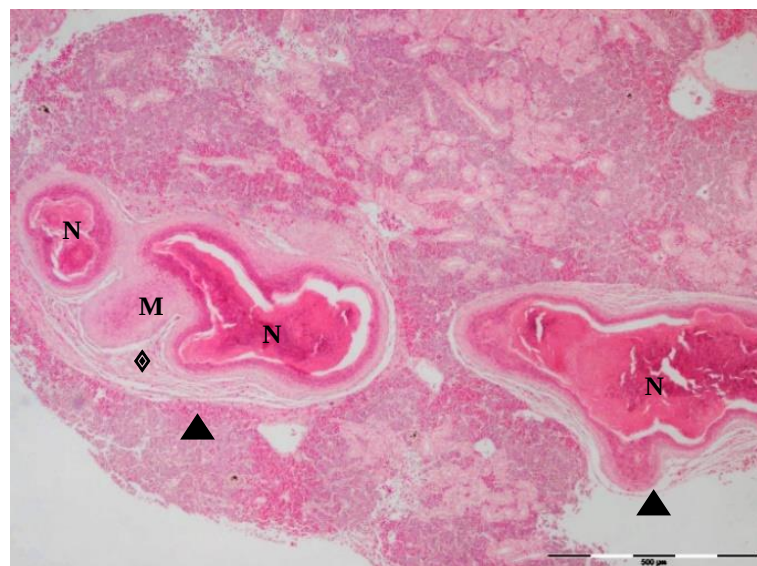


Figure 23. Kidney with multiple granulomas (▲) with a marked necrotic center (N) surrounded by Macrophages (M) and Connective Tissue (◇) (500μm) (4x).

2.2. Histopathology with special stains

Acid-fast bacteria were not identified in the nodules, especially in the center of granulomas and macrophages, when stained by the Ziehl-Neelsen (Figure 24) and Gram (Figure 25) methods. Nonetheless, the kidney, spleen and liver shown signs of being infected given the presence of granulomas and the fact that *Nocardia* spp. was previously inoculated.

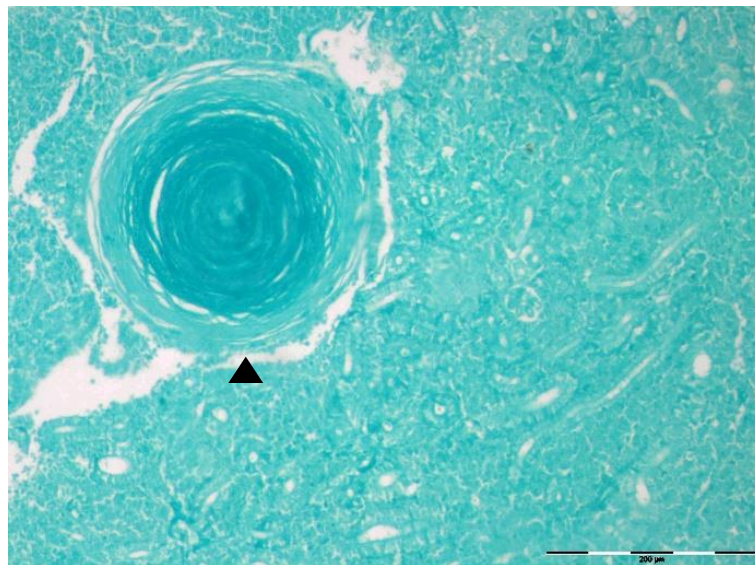


Figure 24. Kidney with granuloma (▲) stained with Ziehl-Neelsen (10x).

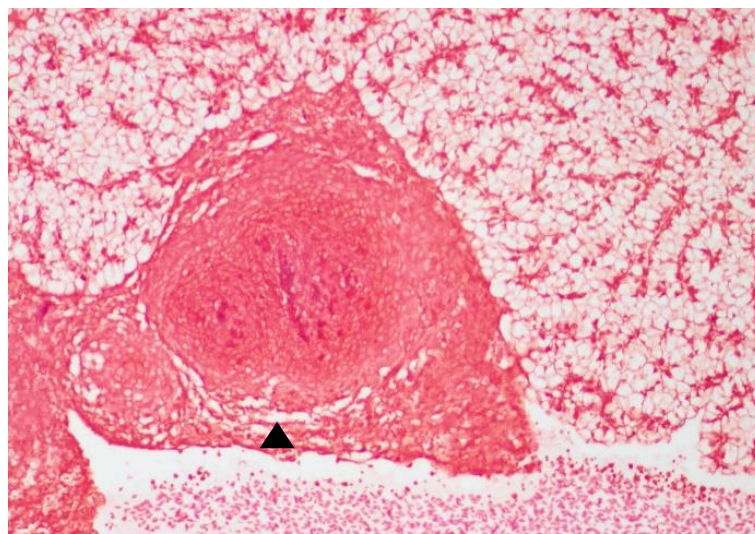


Figure 25. Liver with granuloma (▲) stained with Gram (10x).

2.3. Number of Granulomas per Organ

Granulomas were observed in several organs although the number of granulomas in each organ was variable. The intensity of the nodular lesions in all of the sampled organs is shown in Figure 26.

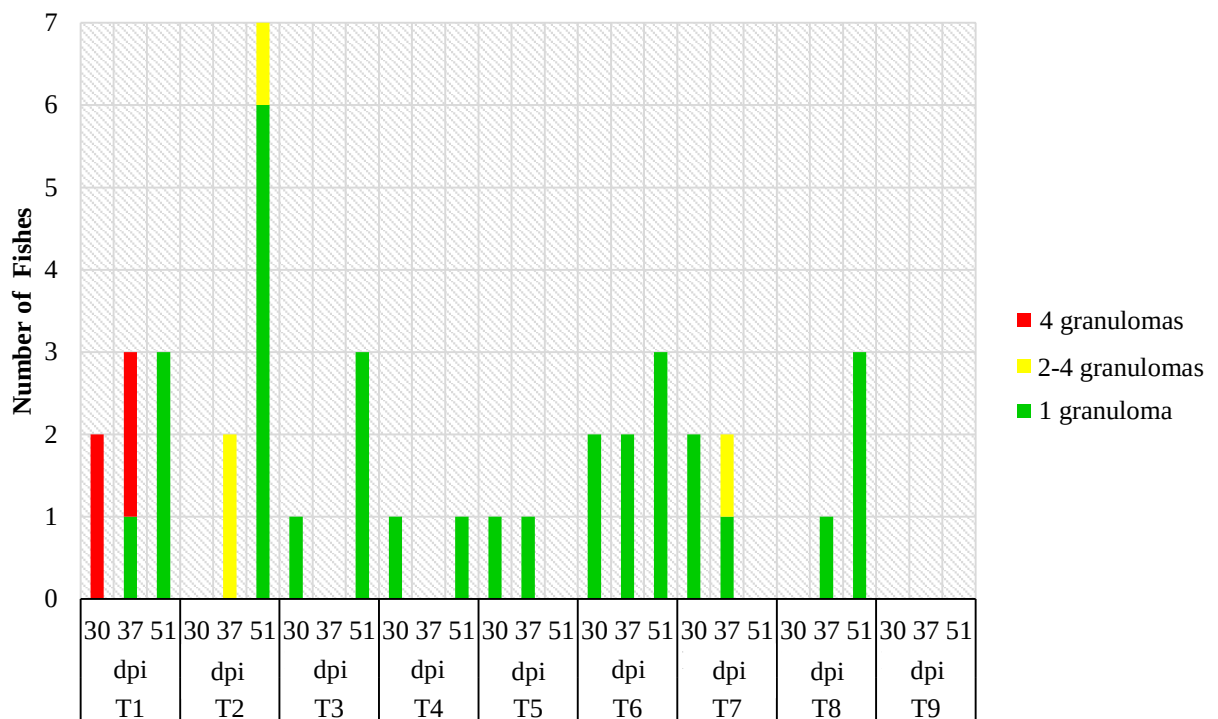


Figure 26. Number of granulomas per organ according dose and time.

Figure 26 shows that in 32 cases only one granuloma was detected, representing 80% of the total cases observed. Organs where 2 to 4 granulomas were found represented 10% as also happened with organs where more than 4 granulomas were present.

No apparent correlation between the number of granulomas and time was found. Although it is important to note that organs with more than one granuloma were mostly observed (88%) in tanks where fishes were inoculated with higher inoculation doses (T1, T2), showing that dose might be responsible for a bigger intensity of the nodular lesions.

2.4. Incidence of Granulomas

Figure 27 shows the overall incidence of granulomas, visualized by microscopy.

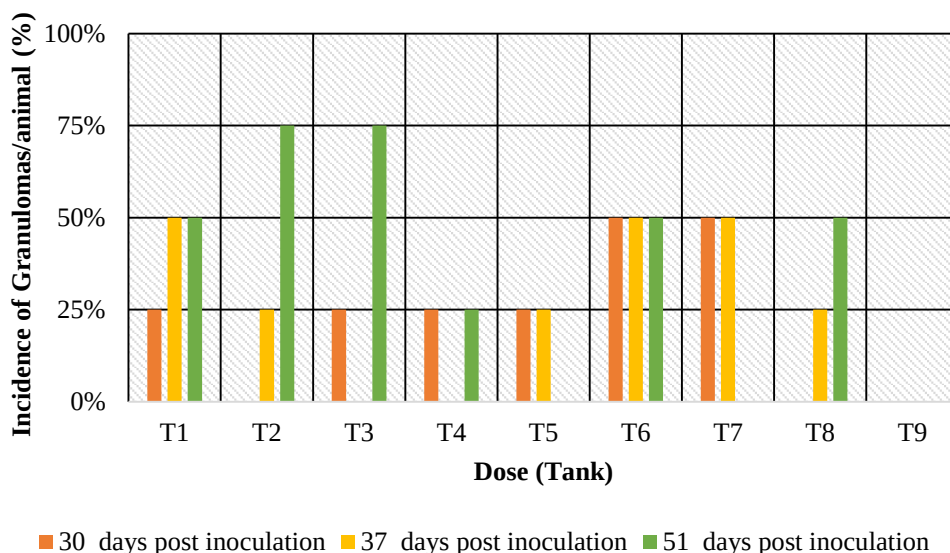


Figure 27. Incidence of Granulomas.

Microscopic granulomas were found in 28% of all sampled animals.

30 dpi the incidence of granulomas was of 22%, increasing continuously for the next two sampling moments, reaching 25% and 36% 37 and 51 dpi, respectively. These results point to an apparent time-incidence correlation, as the incidence of granulomas tends to grow over time. It was also observed that the most affected animals 30 dpi belonged to tanks where the inoculated dose was higher whereas animals from tanks with a dose below 10^6 CFU/mL (T4-T9) appeared to be most affected in the first two sampling moments.

However, no correlation was verified between granuloma's incidence and dose, as it can be shown by the similar incidence found between T1-T3 (36%) and T5-T8 with lower inoculation doses (36%).

2.5. Incidence of Granulomas in Kidney

Kidney is the most affected organ with a granuloma incidence of 23%. Figure 38 shows that the effect of time is clear, with an incidence of 19% 30 dpi and 22% and 28% in 37 and 51 dpi, respectively. This is more evident in the first three tanks with higher inoculation doses.

For tanks where the inoculated dose is above 10^6 CFU/mL (T1-T3) a 40% incidence is observed, whereas for doses below 10^3 CFU/mL it is 44%. Nonetheless, there is no clear correlation between dose and incidence, as granulomas seem to be observed with the same incidence in T1, T2 and T3, with higher doses, as in T6, T7 and T8, with the lower doses.

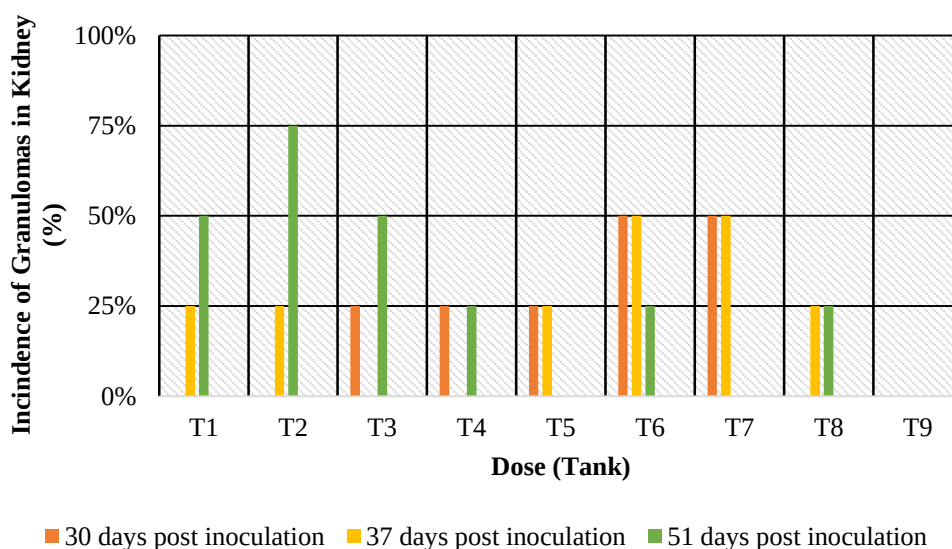


Figure 28. Incidence of Granulomas in Kidney.

2.6. Incidence of Granulomas in Liver

Liver is the second most affected organ with an incidence of 11%. There is an apparent relation with inoculation dose, as 75% of all granulomas observed in liver were from animals inoculated with doses above 10^6 CFU/mL (T1-T3), whereas for doses below 10^3 CFU/mL the incidence of granulomas was only 17%, as it can be shown in the Figure 29, with the most affected animals belonging to tanks where the inoculation dose was higher (T1-T2).

However, no granulomas were observed in T4, T5 and T7. Time was a clear relevant factor of incidence: with 3% at 30 dpi, 8% at 37 dpi 22% at 57 dpi.

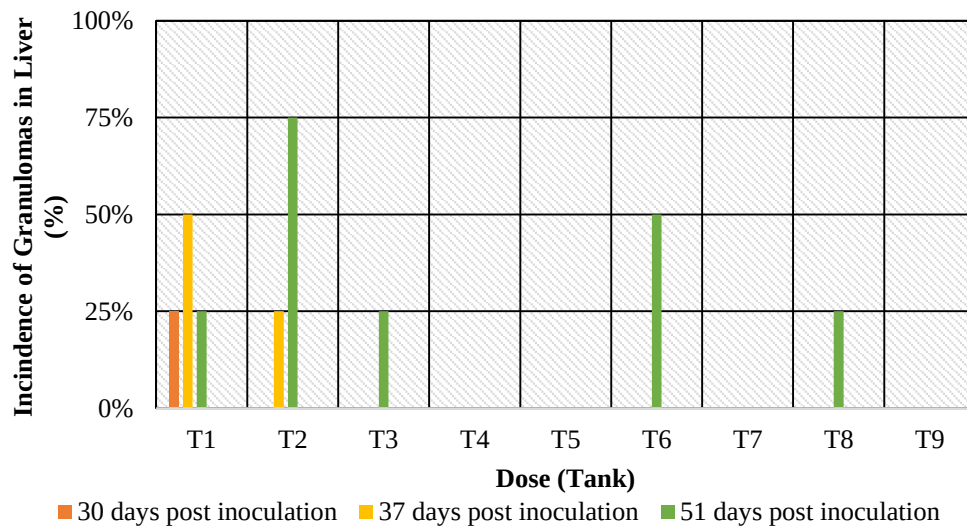


Figure 29. Incidence of Granulomas in Liver.

2.7. Incidence of Granulomas in Spleen

Spleen is apparently the least affected organ (3%). The insufficient data obtained did not allow to evaluate if a relation between incidence of granulomas and bacterial dose or time was in fact present, as it can be seen in the Figure 30.

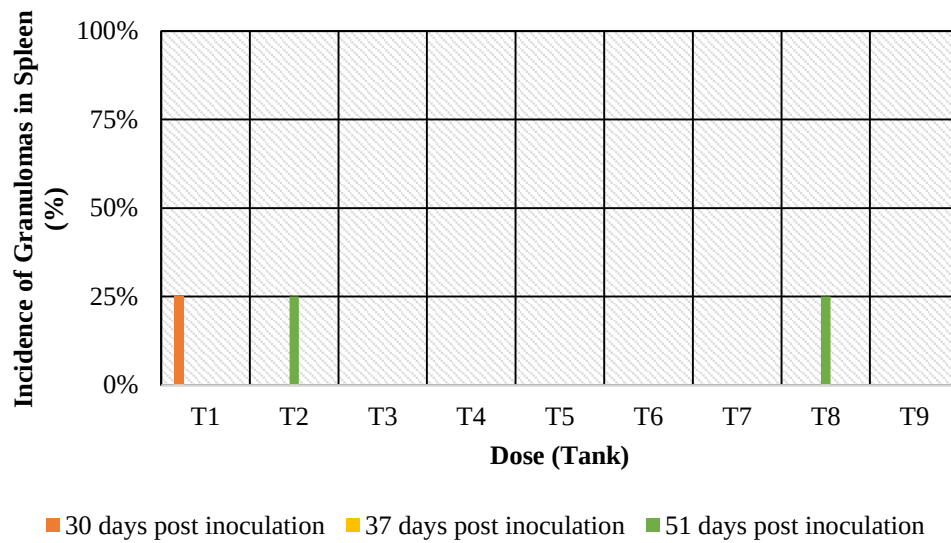


Figure 30. Incidence of Granulomas in Spleen.

3. BACTERIOLOGY

3.1. Culture Medium Results

Bacterial colonies from liver, spleen and kidney appeared 3-5 days after culture on Blood Agar with 1.5% of Sodium Chloride (BA+1.5% NaCl), Brain Heart Infusion with 1.5% Sodium Chloride (BHI + 1.5% NaCl) and Yeast Extract-Malt Extract (YEME) medium at 25°C. The bacteria were Gram-positive and first appeared as conglomerates of filamentous *bacilli* changing later into a less typical form of *Nocardia* as simple *bacilli*. Colonies were yellow, although after some time they became white, dry and rough (Figure 31).

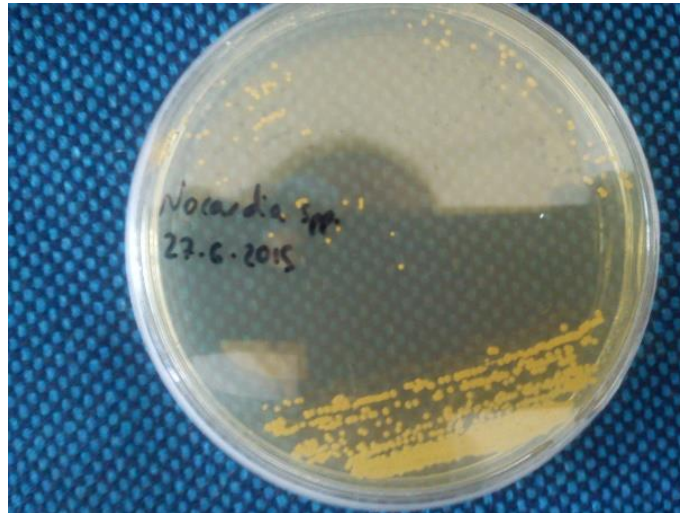
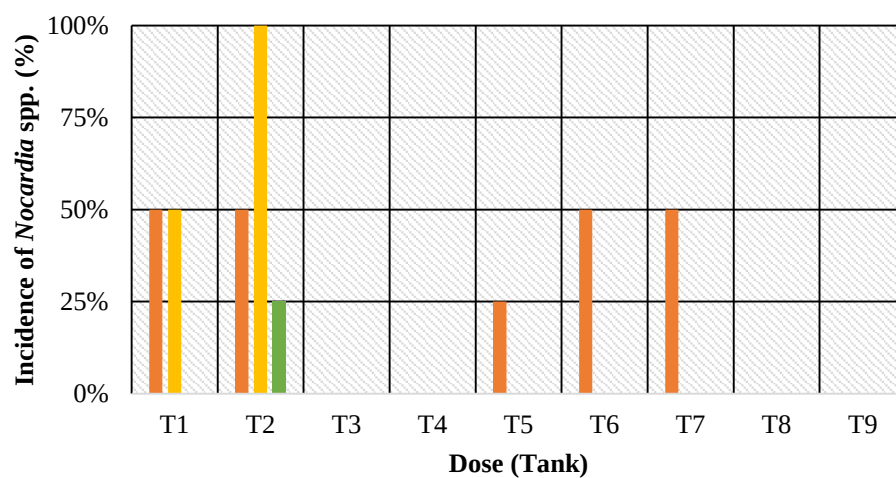


Figure 31. *Nocardia* spp. growing in a culture medium.

a. Incidence of *Nocardia* spp.

Figure 32 shows that *Nocardia* spp. was isolated at 30 dpi in both high and low inoculation dose tanks. At 37 dpi it was only detected at higher dose tanks, T1 and T2. At 51 dpi revealed a very low incidence of bacteria, even though it seems to be dependent of the dose with T1 and T2 being clearly more affected, contrasting with the overall trend verified in histology. According to the results showed below, some tanks did not show infection, such as T3, T4 and T8. No time relation appear to exist.



■ 30 days post inoculation ■ 37 days post inoculation ■ 51 days post inoculation

Figure 32. Incidence of *Nocardia* spp. in culture medium.

b. Incidence of *Nocardia* spp. in kidney

Nocardia spp. was barely identified in kidney (4%), contrasting with the situation observed in histological analysis where kidney is clearly the most affected organ. The only identification of bacteria was made 30 dpi without further observations, which again is opposite with the histological observations, where some time dependent effect was observed (Figure 33).

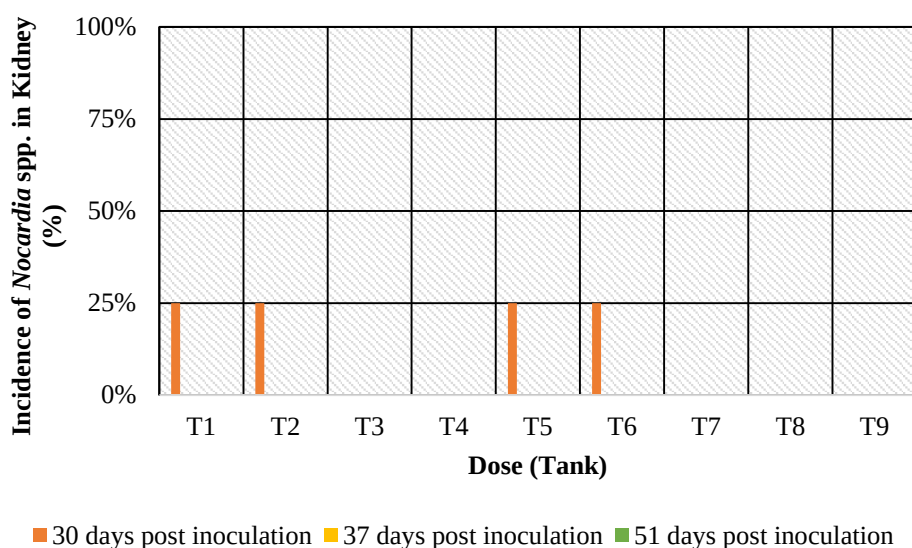


Figure 33. Incidence of *Nocardia* spp. in Kidney

c. Incidence of *Nocardia* spp. in liver

Based on microbiological evaluation, the liver is the most affected organ with an incidence of 10%. This situation contradicts the results found in histology, where kidney was clearly the most affected organ. Nonetheless, it seems that there is a relation with inoculation dose, given the incidence of *Nocardia* spp. (79%) in tanks mainly with doses above 10^6 CFU/mL (T1 and T2) (Figure 34).

Time is not a determinant factor in this case, as *Nocardia* spp. was not detected passed the 37 dpi.

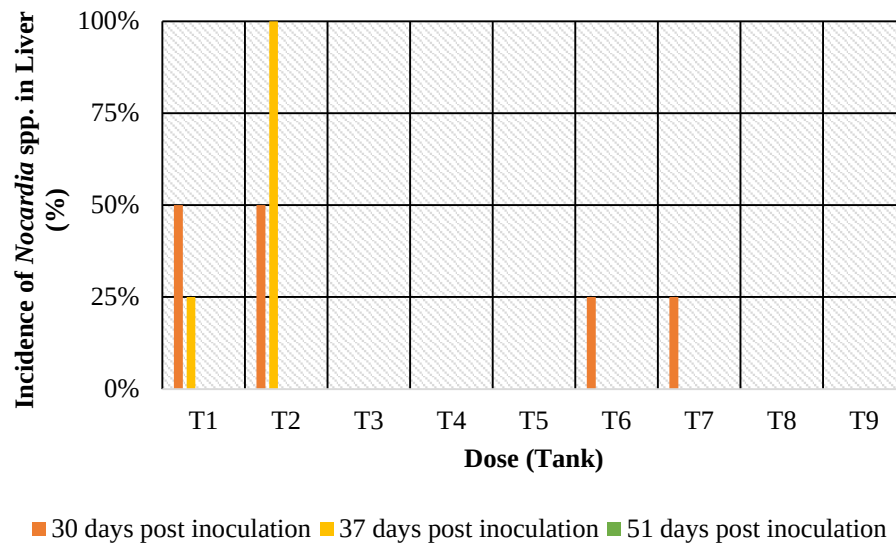


Figure 34. Incidence of *Nocardia* spp. in Liver.

5.5. Incidence of *Nocardia* spp. in spleen

The incidence of *Nocardia* spp. in spleen is low (4%) as represented in figure 35. It is spread along the three sampling times and there is no apparent time dependence. Yet, in most cases, the bacteria was isolated from animals previous from tanks with higher doses of bacteria (T1 and T3), meaning that a possible dose relation might be present.

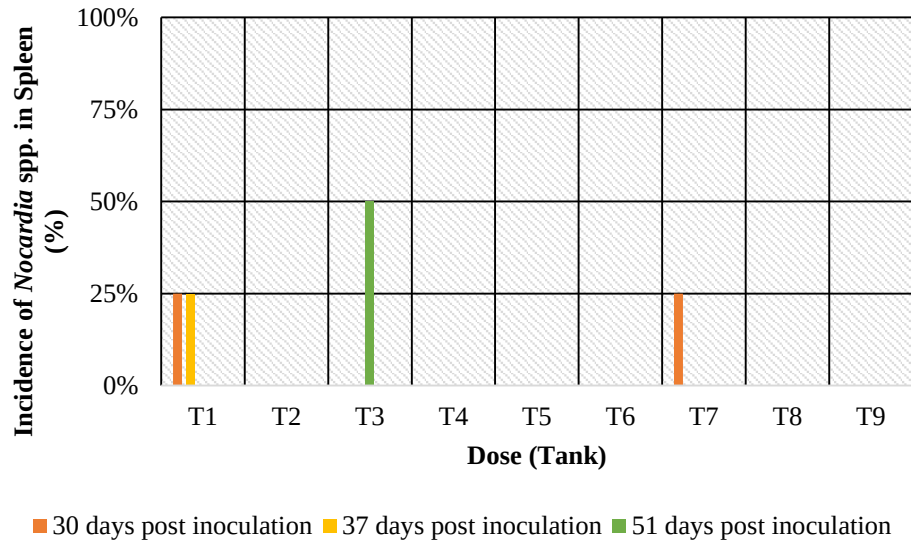


Figure 35. Incidence of *Nocardia* spp. in Spleen.

4. MORTALITY

Figure 36 represents the number of fishes found dead since the inoculation day until the end of the experiment.

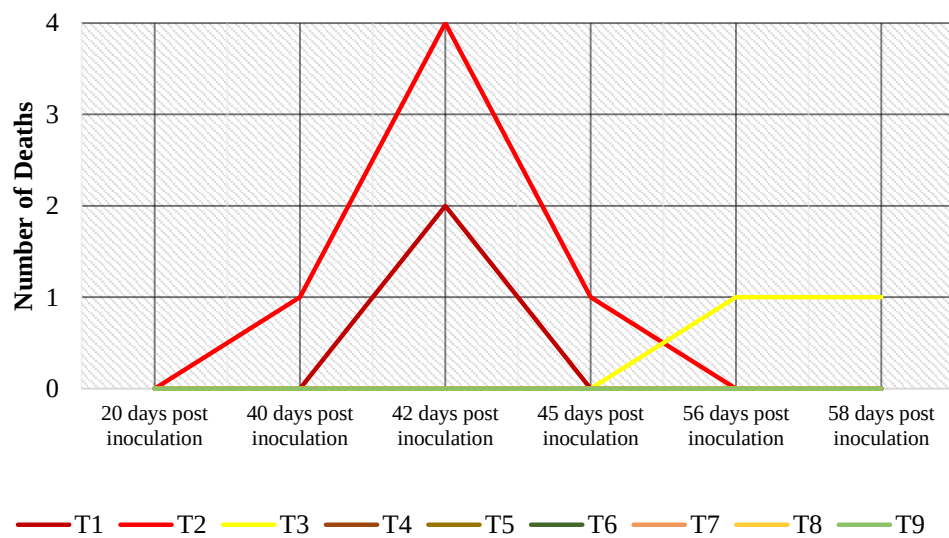


Figure 36. Number of Deaths.

A total of 10 dead fish were found. The first death occurred 40 dpi in T2 (10^7 CFU/mL), and the higher number of deaths was registered 42 dpi, being the T2 the most affected with 4 deaths, and T1 (10^8 CFU/mL) with 2 deaths. At 45 dpi one additional death was registered again in T2. 56 and 58 dpi two deaths were registered for T3.

Deaths occurred only in animals inoculated with higher doses of bacteria (T1-T3), being T2 the most affected with 6 deaths.

The cumulative mortality of meagre inoculated with different concentrations of *Nocardia* spp. is shown in Figure 37.

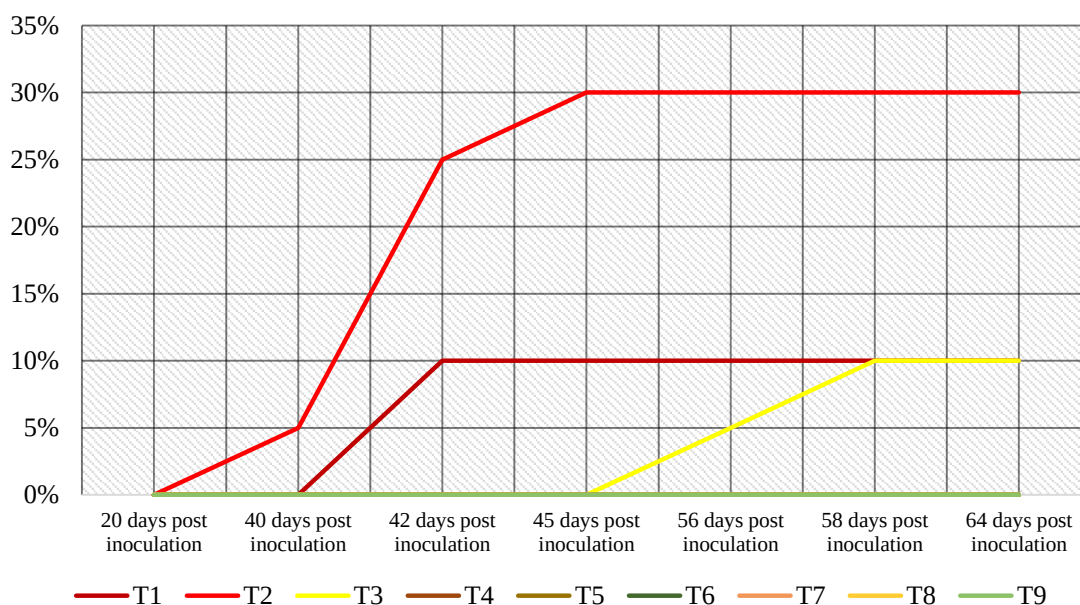


Figure 37. Cumulative Mortality.

Higher bacterial concentration (T1-T3; $>10^5$ CFU/mL) caused 6% mortality within 58 days of the inoculation. Fishes to which were injected lower concentrations of bacterial suspension showed no mortality.

Mortality of meagres at 40 dpi was of 5% in T2. At 42 dpi the mortality of T2 raised to 25% and later to 30% at 45 dpi. T1 and T3 were less affected, revealing a cumulative mortality of 10% at 42 dpi in T1 and of 10% in T3 at 58 dpi.

The histological evaluation of animals found dead revealed granulomas in kidney, liver and spleen. Only 3 fishes (38%) showed microscopic granulomas, all at 42 dpi. Two out of those three fishes belonged to tank T2 and one to tank T1 (Table 11).

Table 11. Histology of Dead Fishes.

Time of Death	Tank	Liver	Kidney	Spleen
40 dpi	T2	-	-	-
42 dpi		-	+	-
		-	-	-
		-	+	+
		-	-	-
		T1	-	-
		+	+	+
45 dpi	T2	-	-	-
56 dpi	T3	NOT SAMPLED		
58 dpi				

Bacteriological evaluation of *Nocardia* spp. was found positive in 25% of the animals sampled. Cultures from liver of dead fish at 40 and 42 dpi were positive for T1 and T2 in two animals. *Nocardia* spp. was also isolated from kidney and spleen 42 dpi from T1 (Table 12).

Table 12. Bacteriology of Dead Fishes.

Time of Death	Tank	Liver	Kidney	Spleen
40 dpi	T2	+	-	-
42 dpi		-	-	-
		-	-	-
		-	-	-
		-	-	-
		-	-	-
T1		+	+	+
	-	-	-	
45 dpi	T2	-	-	-
56 dpi	T3	NOT SAMPLED		
58 dpi				

Other bacteria were found in 63% of the dead animals sampled (Table 13).

Table 13. Other bacteria found in dead animals.

Time of Death	Tank	Other Bacteria		
		Liver	Spleen	Kidney
40 dpi	T2	-	-	-
42 dpi		-	-	+
		+	-	+
		-	-	-
		+	+	+
		+	-	-
		-	-	-
45 dpi	T2	-	-	+
56 dpi	T3	NOT SAMPLED		
58 dpi				

DISCUSSION

1. CLINICAL SIGNS AND GROSS PATHOLOGY

Nocardia spp., a Gram-positive bacteria have been reported to induce diseases in fish, namely Actinomycetoma and Nocardiosis. Nocardiosis is a disease that affects both saltwater and freshwater fish worldwide, from USA and Canada to Spain, Portugal, Greece, China, Taiwan, and Japan. It is characterized by the presence of granulomas in skin and visceral organs (Chen *et al.*, 2000).

Valdez & Conroy (1963) reported the first case of fish Nocardiosis caused by *Nocardia asteroides* in neon tetras (*Paracheirodon innesi*) kept in aquariums in Argentina. However, the first case was actually described by Rucker (1949) in sockeye salmon (*Oncorhynchus nerka*) in USA, and was originally identified as *Streptomyces salmonicida*, currently known as *Nocardia salmonicida*. Many cases were reported following since then, affecting a wide variety of species. *Nocardia seriolae*, originally reported as *Nocardia kampachi*, was also isolated in many fish species, from Japanese amberjack (*Seriola quinqueradiata*) in Japan, striped bass (*Morone saxatilis*), Japanese seabass (*Lateolabrax japonicus*), largemouth bass (*Micropterus salmoides*), large yellow croaker (*Larimichthys crocea*), tiger perch (*Terapon jarbua*) and many others species in Taiwan and China. Moreover, *Nocardia* spp. appears to affect an increasing number of cultured or wild species (Kudo *et al.*, 1988; Shimahara *et al.*, 2008; Wang *et al.*, 2009; Buller, 2014).

Three species of *Nocardia* have been described to affect fishes so far, these including *Nocardia asteroides*, *Nocardia seriola* and *Nocardia salmonicida*.

The purpose of this work was to determine how *Nocardia* spp. affects a population of meagres over a period of time and in different doses. Therefore, bacteria were intraperitoneally injected in doses ranging from 10^1 to 10^8 CFU/mL. Snieszko *et al.* (1964) showed that an experimental transmission of *Nocardia asteroides* orally from fish to fish is not effective, but transmission by injection is slightly more successful. Kusuda and Nakagawa (1978) reported positive transmission of the disease by injection or by smearing surface wounds with *N. seriolae* in Yellowtail. Chen (1992) succeeded in transmitting *N. asteroides* by intramuscular injection in Largemouth Bass. These results suggest that the route of infection is more likely through epidermal injury than orally (Chen *et al.*, 1992; Buller, 2014). Itano *et al.* (2006) proposed that immersion system is the one that closely resembles natural exposure, being for that reason more appropriated than the injection method. (Itano *et al.*, 2006).

Sindermann (1990) reported that a natural infection is likely to occur via the injured skin or gill, being the intragastric exposure to the pathogen a less effective route of infection. Itano *et al.* (2006) also suggests that the pathogen infects the host through gills, surface injury, lateral line or anus. Contaminated water or diets containing unpasteurized marine fish or fish products may be responsible for the dissemination of the bacteria in aquaculture facilities (Wang *et al.*, 2005, Itano *et al.*, 2006).

A large number of signs caused by this disease have been described and include lethargy, anorexia and emaciation, distension of the mouth or abdomen; compact focal to multifocal granulomas distributed in the spleen, liver, kidney, heart, ovary and mesenteries; ulcers and necrosis in muscle and skin. Snieszko *et al.* (1964) reported an invasion of the brain capsule by *Nocardia*, and Chen *et al.* (1992) considered this migration responsible for brain granulomas and associated behavioral changes, namely fishes that swam either in a rapid tail-chasing motion or on their sides in a circular movement. Fish may also lose scales, exhibit exophthalmia with opaque eyes and blood pools under the epithelium of the oral and operculum cavities. Wang *et al.* (2005), demonstrated that experimental infection in striped tigerfish resulted in lesions very similar to those found in naturally infected fish, indicating that signs of Nocardiosis are the same for captive or wild fish (Chen *et al.*, 1992; Chen *et al.*, 2000; Wang *et al.*, 2005; Plumb & Hanson, 2011).

However, in the present work there are no evident physical or behavioral anomalies, except for an anorexia concomitant with changes in weight and size. These changes were observed a few days after the first sampling moment (30 dpi) and appear to affect mostly fishes from tanks where inoculated doses were higher (T1-T3) becoming more prominent over time. This situation, in association with the weight discrepancies between fishes from tanks where the inoculated dose is higher and the ones where is lower, led to the possibility that dose of *Nocardia* spp. injected and time could interfere with the course of the disease. Such observations were confirmed only for the last sampling moment (51 dpi) using a statistical software that showed that fishes from T1-T3 were clearly lighter than fishes from T7-T9. No granulomas were macroscopically observed either.

These results are in line with the study of Plumb and Hanson (2011), where it is reported that whatever the source, *Nocardia* spp. produce a slowly developing chronic infection. Moreover, Sheppard (2005) suggests that it starts as a “silent infection”, given that the bacteria can multiply in the tissues with no visible outward clinical signs and can go undetected in fingerling fish for months. Chen *et al.* (1992) also stated that an infection of

Nocardia by injection requires 1–3 months to develop. Labrie *et al.* (2008), also stated that *Nocardia* tends to occur mainly in larger fish (> 100 g, up to 600 g) (Chen *et al.*, 1992; Labrie *et al.*, 2008; Plumb & Hanson, 2011).

The hypothesis of Heuschmann-Brunner (1965) that *Nocardiae* are pathogenic for fish only under certain circumstances considering the role of stress factors in converting low-grade infections into acute disease forms is also well recognized and must be considered for this discussion. Kusuda and Nakagawa (1978) considered that the advance of nocardiosis is strongly dependent of the environmental stress. A polluted environment provides an auspicious habitat for pathogenic bacteria, and thus, favors the infection. Besides, a deficient diet may also be responsible for the advance of the disease, as animals are likely to be immunologically depressed when poorly fed. Higher densities and skin lesions are also described as being responsible for a negative advance of the infection. Good husbandry can be achieved reducing the outflow of extracts from the feed, as well as avoiding overcrowding and overfeeding, keeping the environment clean as the best way to control the disease (Kusuda & Nakagawa, 1978; Chen *et al.*, 1992; Lewis & Chinabut, 2006; Plumb & Hanson, 2011).

Considering that these signs precede typical granulomas associated to Nocardiosis, they could be useful as a control for the effects of the pathology and anticipate severe situations.

This experimental study indicated that *Nocardia* spp. inoculated was pathogenic to meagres.

2. HISTOPATHOLOGY

Granulomas found in this work closely correspond to the ones described by other authors, as described in Introduction. Granulomas were observed in kidney, liver and spleen as these were the only organs sampled. None of the organs observed appeared to be macroscopically affected at the time of the sampling. These results are in agreement with the study of Cornwell *et al.* (2011), which reported the presence of single or multiple granulomatous nodules on the posterior kidney, spleen and liver. Granulomas appear as an inflammatory cell response, with the pathogen being encircled within the infected tissues as it can be seen in the early stages of development, thus explaining the presence of macrophages and epithelioid cells. Later they become more complex, containing necrotic centers, surrounded by epithelial cells, fibroblasts, or fibrous encapsulation; and finally to expansion of this centers,

as described by Jacobson (2007). The distinct types of granulomas observed may also be explained by a different pattern of granulomas independent of the phase of development, as the ones reported by Guirado and Schlesinger (2013). Wolke and Stround (1978) declared that Nocardial granulomas without the epithelioid cells, as the ones in the earliest stages of development, may be easily mistaken for Mycobacteriosis (Chinabut; 1999; Chen *et al.*, 2000; Labrie *et al.*, 2008; Cornwell *et al.*, 2011; Plumb & Hanson, 2011; Elkesh *et al.*, 2013).

Contrary to what was observed by Chen *et al.* (2000), Labrie *et al.* (2008) and Elkesh *et al.* (2013), no bacteria were observed with the routine Hematoxylin-Eosin staining. Ziehl-Neelsen and Gram stainings were used in an attempt to see the filamentous acid-fast organisms, but proved to be ineffective. According to Wolke and Stround (1978), acid-fast *Nocardia* only show a positive reaction with Fite-Faraco acid-fast stain even though Labrie *et al.* (2008) declared to have observed a Gram-variable filamentous bacteria in the center of the granuloma with Gram method and Chen *et al.* (1992) and Elkesh *et al.* (2013) noticed the presence of filamentous bacteria when stained by special stains as Ziehl-Neelsen's (Chinabut; 1999; Chen *et al.*, 2000; Labrie *et al.*, 2008; Elkesh *et al.*, 2013).

Microscopic granulomas were found in 28% of all animals and at 37 dpi all tanks had infected animals, except for T3, T4 and control. A correlation between time and incidence appears clear, as 22% of fish collected in the first sampling point presented granulomas, whereas 25% and 33% presented granulomas in the second and third sampling points, respectively. This observation is particularly evident in tanks where inoculation dose is higher (T1, T2 and T3). However, no correlation was verified between incidence and dose.

The distribution of granulomas for the different organs is not homogeneous, as kidney is the most affected organ with a granuloma incidence of 23%, followed by liver with 11% and spleen with 3%. In kidney and liver the effect of time appears to be preponderant as incidence tends to grow from 30 dpi to 51 dpi, yet there is no apparent relation with inoculation dose. In spleen, the small incidence observed does not allow to reach any conclusion.

A single granuloma per organ was found in most cases (80%), however in fewer cases (20%) two or more granulomas were seen with a focal or more diffuse distribution. No correlation between the number of granulomas and time appears to exist, as organs where more than one granuloma were observed had a disperse distribution at 30, 37 and 51 dpi. Nonetheless, inoculation dose appears to play an important role in this experiment, as organs with more than one granuloma were mostly observed (88%) in tanks where fishes were inoculated with higher doses (T1, T2).

3. BACTERIOLOGY

Aerobic and Gram-positive *Nocardia* spp. are identified uncertainly by their rapid growth rate, which ranges from 3 to 7 days, and the characteristic small, heaped and wrinkled humid to pale colonies. The morphology of *Nocardia* differs from a branched filamentous cell to a fragmented, irregularly shaped, pleomorphic or coccobacillary cell, according to the phase of growth and genus. *Nocardia* is known to be difficult to culture (Beaman *et al.*, 1988).

In this study, bacterial colonies from liver, spleen and kidney appeared 3-5 days after culture on Blood Agar with 1.5% of Sodium Chloride (BA+1.5% NaCl), Brain Heart Infusion with 1.5% Sodium Chloride (BHI + 1.5% NaCl) and Yeast Extract-Malt Extract (YEME) medium at 25°C. The bacteria were Gram-positive and first appeared as conglomerates of filamentous *bacilli* changing later into a less typical form of *Nocardia* as simple *bacilli*. Colonies were yellow, although after some time they became white, dry and rough.

Isolation of *Nocardia* spp. show that there is no correlation between incidence and time as bacteria was isolated diffusely through 30, 37 and 51 dpi. Yet seems that the incidence is bigger for animals inoculated with higher doses (T1 and T2), showing that there is a possible influence of dose on the growth of colonies, contrasting to what was found in histology. This results indicate that there is no relation between the presence of microscopic granulomas and the isolation of bacteria. It is important to have in account that *Nocardia* is a particularly difficult bacteria to grow and the agar type used may influence this situation. The fact that the bacteria did not grow as expected in the selected medium does not mean that it is not present at all. Other theories are that bacteria might have migrated for another part of the body as an immunological response from fish or that it inactivated itself for some reason being able to reactivate when circumstances are favourable. Labrie *et al.* (2008) reported that in 20 out of 45 cases, no bacteria were isolated through culture method even though those 20 cases were probable infections based on clinical signs and impression prints, suggesting that even when an infection is present in the population, there is only a 50% chance that the bacteria will be isolated. Cornwell *et al.* (2011) also experienced an unsuccessful bacterial and declare that the Lowenstein Jensen medium is the recommended medium for the diagnostic of *Nocardia* (Labrie *et al.*, 2008; Cornwell *et al.*, 2011; Elkesh *et al.*, 2013).

Liver was the most affected organ with an incidence of 10%, spleen with 5% and kidney 4%, contrasting again with the situation observed in histological analysis. Time is not a

determinant factor in this case, as *Nocardia* spp. was not detected passed the 37 dpi, except for two cases in spleen. Dose seem to be irrelevant too, given its identification in tanks with high or lower inoculation doses.

4. MORTALITY

Dead fishes were only found at the higher inoculation dose tanks (T1-T3, 10^8 - 10^6 CFU/mL). The first deaths were found 40 dpi in tank T2 (10^7 CFU/mL), and the higher number of deaths were registered 42 dpi, being the tank T2 again the most affected with 4 deaths, and tank T1 (10^8 CFU/mL) with 2 deaths. At 45 dpi one additional death was registered again in tank T2. In the final days of the experiment (56 and 58 PI) one death was registered in both days.

Cumulative mortality of meagres 40 dpi was of 5% in tank T2. At 42 dpi the mortality of tank T2 raised to 25% and later to 30% at 45 dpi. Tank T1 and Tank T3 were less affected, revealing a cumulative mortality of 10% at 42 dpi in Tank 1 and of 10% in Tank T3 at 58 dpi. The mortality rates obtained here were far lower than that achieved by other authors, in similar conditions, as shown in Table 14 below:

Table 14. Cumulative Mortalities by Experimental Intraperitoneal Infection of *Nocardia* spp.

PESO	DOSE	MORTALIDADE CUMULATIVA	DÍAS APÓS INOCULAÇÃO	REFERÊNCIAS
30g	1.1x10 ² 1.1x10 ³ 1.1x10 ⁴	40% 80% 100%	10 dias	Itano <i>et al.</i> , 2006
34-40g	1.0x10 ⁷	100%	10 dias	Chen <i>et al.</i> , 2000
55g	1.2x10 ⁶ 1.2x10 ⁷	95% 100%	5 semanas	Wang <i>et al.</i> , 2000
93g	1.2x10 ⁶ 1.5x10 ⁷ 8.5x10 ⁷	40% 80% 90%	19 dias	Labrie <i>et al.</i> , 2008

Mortality in this experiment reached 6%, with 10 out of 180 fishes dead.

Animals found dead only reveal granulomas in 3 fishes, all at 42 dpi. Two out of those three fishes belonged to tank T2 and one to tank T1. Bacteriological evaluation of *Nocardia*

spp. was found positive in cultures from liver of dead fish at 40 and 42 dpi. Positive results were also found in kidney and spleen at 42 dpi (Table 9).

It is relevant to refer that 75% of fishes found dead revealed the presence of bacteria other than *Nocardia*. Chinabut (1999) said that the presence of additional pathogens may act as a stressor factor contributing to mortality of fishes already affected with *Nocardia* (Chinabut, 1999).

In marine finfish culture, *Nocardia* infections tend to progress rapidly in the summer months when water temperatures are 24°C or superior, but mortalities do not occur until water temperatures begin to drop in the fall and early winter possibly due to declining immune responses of the host. Lan *et al.* (2008) also observed that as water temperatures cooled in the late fall the disease became more acute and mortalities increased. Cornwell *et al.* (2011) declared that *Nocardia* spp. tend to be opportunistic pathogens, and it is possible that the stress of capture and higher stocking density, coupled with the slightly elevated levels of ammonia and nitrites in the system, allowed the infection to cause mortalities in these fish. (Chinabut, 1999; Cornwell *et al.*, 2011; Plumb & Hanson, 2011).

CONCLUSION

As an infectious disease that affects many fish species all over the world, understanding the pathological mechanisms of Nocardiosis is of major concern. Its study is challenging, however, it is frequently misdiagnosed as Mycobacteriosis, a well-known disease caused by *Mycobacterium* spp.. As a consequence, studies on the relevance and effects of *Nocardia* spp. in fish have been overlooked in the past decades. Nonetheless, with the expansion of aquaculture, concerns over public health brought the need to address this spreading disease. Thus, the present work aimed at gaining new knowledge about clinical and pathological aspects of Nocardiosis, through the study of the effects over time of the experimental infection of a population of meagres with different doses of *Nocardia* spp. (10^1 - 10^8 cfu/mL).

The only clinical signs observed during this experiment were anorexia and emaciation. These signs were present in animals where inoculation doses were higher (T1-T3) and became evident 51 days PI, leading to the conclusion that clinical signs might be dose and time dependent. No other typical signs, such as macroscopic granulomas, were observed. Nonetheless, microscopic granulomas, known to be associated with the experimental infection of *Nocardia* spp., were observed in 29% of the 108 animals sampled. This may be justified by the slow development of this chronic disease, which in the case of infection by injection may take 1 to 3 months to progress.

Histologically, a variety of granuloma patterns were observed in different phases of development, from aggregates of macrophages with epithelioid cells to more complex granulomas with a necrotic center and fibrous connective tissue.

The incidence of granulomas observed histologically appears to be correlated with time. Nonetheless, dose does not appear to play a determinant role in granuloma developed. Kidney was the most affected organ.

The number of granulomas found per organ was variable, as most of the organs sampled presented only one granuloma. The effect of time appears to be less relevant in this case. On the other hand, dose plays a determinant role since cases where more than one granuloma per organ was observed corresponded to fishes from tanks inoculated with higher dose of bacteria (T1 and T2).

Despite the occurrence of granulomas bacteria was not detected neither with Hematoxylin-Eosin nor with special stains such as Gram and Ziehl-Neelsen.

Bacteriological results showed to be positive in 25% of the sampled animals for *Nocardia* spp., thus standing in contradiction to the ones obtained by histological analysis. Regarding organs incidence, liver was the most affected. Dose is the most determinant factor, since fishes inoculated with superior doses were the most affected. In opposition to the histological findings, time does not seem to be relevant for bacteriological detection. The only explanation found to justify this discrepancy was the fact that even though the bacteria was not isolated does not mean that it is not present at all.

Mortality in this experiment reached the 6%. The influence of Nocardiosis is not clear, as some dead animals did not revealed the presence of the bacteria by histological or bacteriological methods. Moreover, 75% of the dead animals sampled revealed the presence of other bacteria. Dead animals belonged to tanks inoculated with higher doses (T1-T3), although time does not appear to play a relevant role.

These results intend to demonstrate how an infection of *Nocardia* spp. progresses with time and bacterial dose in meagres.

The present work has, somehow, the aim to renew the interest in the study of such an important disease as is Nocardiosis. New studies may allow a better understanding of the disease, to develop new diagnostic methods, treatments and control measures to assure an improved health and welfare for animals, reduce losses, as well as to prevent health problems for humans direct or indirectly affected for this disease.

The potential for the development of aquaculture is tremendous. However, it also poses some problems and complex challenges. This situation may be overcome if an articulated multidisciplinary research is encouraged, so that it is able to guarantee in the future an economically viable and profitable industry, with respect for the environment and the consumers. Aquaculture shows potential to become a sector of great economic and social importance in future, either from the point of view of increasing and diversifying production means, or in terms of conservation of biological diversity in natural environments.

The development of research and innovation actions which encourage the development of this productive sector, by linking the university and industry in a multidisciplinary effort, is of extreme importance. The present work thus tried to move in this direction, with the expectation of contributing to the development of aquaculture and to extend the intervention of veterinary professionals.

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APPENDIX I

	Tank	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
30 dpi	1	4	59,9350	5,60770	2,80385	51,0119	68,8581	55,82	68,00
	2	4	67,2525	5,37726	2,68863	58,6961	75,8089	60,06	73,04
	3	4	70,9325	5,54679	2,77340	62,1063	79,7587	62,67	74,60
	4	4	69,7325	1,69583	,84791	67,0341	72,4309	68,37	72,18
	5	4	72,5750	3,51319	1,75659	66,9847	78,1653	67,90	76,30
	6	4	74,4750	11,01132	5,50566	56,9535	91,9965	59,60	86,20
	7	4	73,1300	15,68256	7,84128	48,1756	98,0844	58,23	92,46
	8	4	72,6850	9,80641	4,90321	57,0808	88,2892	61,58	81,26
	9	4	66,7700	8,71294	4,35647	52,9058	80,6342	57,78	78,64
	Total	36	69,7208	8,60168	1,43361	66,8104	72,6312	55,82	92,46
37 dpi	1	4	79,0900	19,09942	9,54971	48,6986	109,4814	62,27	101,39
	2	4	80,7150	10,11612	5,05806	64,6180	96,8120	72,30	93,01
	3	4	84,0175	10,30025	5,15013	67,6275	100,4075	68,90	92,00
	4	4	80,4175	8,41603	4,20801	67,0257	93,8093	72,80	90,83
	5	4	72,5050	4,72300	2,36150	64,9896	80,0204	66,27	77,75
	6	4	78,2875	14,28193	7,14096	55,5618	101,0132	68,68	99,44
	7	4	94,6600	8,22453	4,11226	81,5729	107,7471	87,39	105,76
	8	4	92,2875	14,18292	7,09146	69,7193	114,8557	77,84	106,72
	9	4	90,3800	18,76092	9,38046	60,5272	120,2328	71,46	107,14
	Total	36	83,5956	13,31970	2,21995	79,0888	88,1023	62,27	107,14
51 dpi	1	4	81,1975	8,60595	4,30297	67,5035	94,8915	75,72	93,89
	2	4	77,0125	9,98155	4,99077	61,1296	92,8954	70,29	91,72
	3	4	66,4600	10,06059	5,03030	50,4514	82,4686	57,14	80,63
	4	4	105,8950	9,14837	4,57418	91,3379	120,4521	96,02	116,96
	5	4	107,2450	21,35851	10,67925	73,2589	141,2311	81,79	132,40
	6	4	98,8375	12,80549	6,40275	78,4611	119,2139	80,40	109,64
	7	4	120,4950	18,59133	9,29566	90,9121	150,0779	100,29	143,84
	8	4	129,2725	25,02123	12,51062	89,4581	169,0869	95,50	155,62
	9	4	139,5375	17,02178	8,51089	112,4521	166,6229	118,75	160,05
	Total	36	102,8836	27,34111	4,55685	93,6327	112,1345	57,14	160,05

APPENDIX II

Table 1. One-way ANOVA

		ANOVA				Sig.
		Sum of Squares	df	Mean Square	F	
Peso1	Between Groups	652,752	8	81,534	1,137	,371
	Within Groups	1936,858	27	71,735		
	Total	2589,609	35			
Peso2	Between Groups	1736,208	8	217,026	1,310	,280
	Within Groups	4473,297	27	165,678		
	Total	6209,505	35			
Peso3	Between Groups	19443,138	8	2430,392	9,764	,000
	Within Groups	6720,626	27	248,912		
	Total	26163,764	35			

$p > 0.05$
Not significant

$p < 0.05$
Significant

Table 2. Post-Hoc (Tukey HSD)

Post Hoc Tests					
Homogeneous Subsets					

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4,000.

Table 3. Factorial ANOVA

Tests of Between-Subjects Effects					
Dependent Variable: Peso					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41803,780 ^a	26	1607,838	9,918	,000
Intercept	787661,280	1	787661,280	4858,856	,000
Momento	19971,682	2	9985,841	61,600	,000
Tanque	10175,626	8	1271,953	7,846	,000
Momento * Tanque	11656,472	16	728,530	4,494	,000
Error	13130,780	81	162,108		
Total	842595,840	108			
Corrected Total	54934,560	107			

a. R Squared = ,761 (Adjusted R Squared = ,684)

$p < 0.05$
Significant

APPENDIX III

Classification of Granulomas	T1			T2			T3			T4			T5		
	30	37	51	30	37	51	30	37	51	30	37	51	30	37	51
	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI
1	0	1	3	0	0	6	1	0	3	1	0	1	1	1	0
2	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0
3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0

Classification of Granulomas	T6			T7			T8			T9		
	30	37	51	30	37	51	30	37	51	30	37	51
	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI	PI
1	2	2	3	2	1	0	0	1	3	0	0	0
2	0	0	0	0	1	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0