Universitat Autònoma de Barcelona

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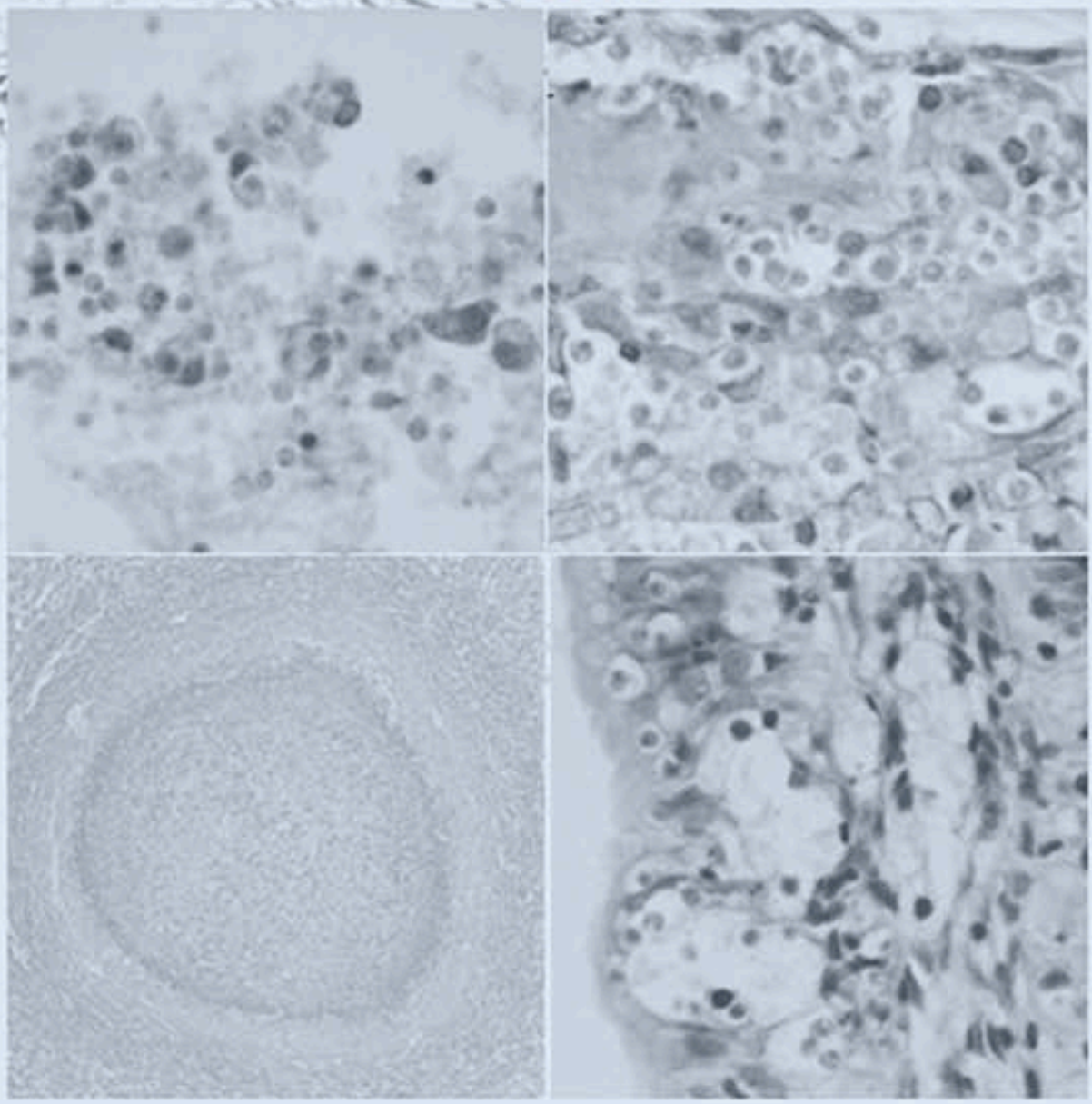
Tesis Doctoral



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Diciembre 2012

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Contributions to the knowledge of a new disease caused by an amoeba in ongrowing Senegalese sole, *Solea senegalensis (Kaup 1858)*

Tesis Doctoral

Memoria de tesis doctoral presentada por
María Constenla Matalobos
para optar al grado de Doctora en Acuicultura,
realizada bajo la codirección del **Dr. Francesc Padrós i Bover**
de la Universitat Autònoma de Barcelona
y del **Dr. Oswaldo Palenzuela Ruiz**
del Instituto de Acuicultura de Torre la Sal (CSIC)

La presente tesis doctoral está adscrita al doctorado de Acuicultura.

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Impossible is just a big word thrown around by small men who find it easier to live in the world they've been given, than to explore the power they have to change it. Impossible is not a fact, it's an opinion. Impossible is not a declaration, it's a dare. Impossible is potencial. Impossible is temporary. Impossible is Nothing.

'The soul of a butterfly'
Muhammad Ali.

A mis padres
y a Edu

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protocolo? tengo que poner un autoclave…en fin..muchas gracias por estar siempre ahí. Pero también le agradezco al resto de grupos, a Oli, Gabi, Diana, David… por acogerme desde el principio como una más. Además, en este apartado de Castellón, me gustaría hacer un agradecimiento especial a mi familia castellonense, especialmente a Quique e Itziar, Lucía e Inma, por abrirme las puertas de su casa y dejarme ser una más de la familia. Sin duda mis múltiples estancias en Castellón no hubiesen sido lo mismo sin vosotras/os: los zumitos recién exprimidos por la mañana, las nuevas y exquisitas recetas de comida, los conciertillos, las cañitas (aunque cada vez tuviésemos menos tiempo para el ocio), gracias por no cerrarme la puerta al llegar tarde de currar, por esperarme con una sonrisa e incluso con la cena hecha!!! Siempre acompañado de muy buena música e, incluso en ocasiones, de conciertos en vivo y en "petit comité" de los niños del swing ;)

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Por todo esto y mucho más, a cada uno de vosotr@s,

¡¡GRACIAS INFINITAS!!

Abstract:

A previously undescribed pathological condition is affecting the culture of *Solea senegalensis* in some farms of the Atlantic coast of Spain. This condition is characterised by the presence of external protuberances in the skin of the affected fish. These lesions correspond to nodules in the muscular tissue showing an abscess-like aspect. Similar lesions were found in kidney, heart, liver and the digestive tract, which leads us to define this pathology as a systemic disease. Histological sections of these nodules revealed the presence of a large core formed mainly of necrotic tissue surrounded with fibroblasts and macrophages. Round-shaped plasmodial organisms were found in the external layer of the nodules and usually inside macrophages or fibroblasts. These organisms were also observed in the intestinal mucosa and submucosa, without causing apparent lesions. This organisms are correspond to a new amoeba species, that belongs to the family Entamoebidae (Phylum Amebozoa, Infraphylum Archamoeba), and we tentatively describe it as a new species in the genus *Endolimax*, *Endolimax piscium* n. sp*. E. piscium* presents round to ovoid trophozoites (<5 μm) with a high degree of intracellular simplification. No mitochondria were observed but mitosome-like organelles were present. In order to establish reliable diagnostic techniques for the recognition of *E. piscium*, specific in Situ Hybridization (ISH) and Polymerase Chain Reaction (PCR) tests have been developed and evaluated using the histological examination (a combination of conventional histological technique in muscle samples and ISH in intestine samples) as gold standard to compare them. As a result, all evaluated techniques obtain quite high quality indicators. The ISH technique was the most specific and sensitive and it was useful as a reference confirmatory method in intestine samples, not only to confirm positives but also to discard negatives, diagnosed as doubtful by conventional histology. PCR technique is a fast and reliable routine method, but still needs further optimization of the sampling methodology. The preliminary results of epidemiological screening for the amoebiasis at the different farms suggest that once disease has manifested in a farm, it is quite probable that asymptomatic fish also present parasites within their intestine, although not necessarily presenting lesions. The route that these organisms use to breach through the

intestinal barrier to infect other organs and spread systemically throughout the fish, causing serious lesions especially in muscle is still not completely understood. However preliminary studies point out the connective tissue as a preferential site where the parasites are observed so it would have some role of this dissemination, although haematogenous route of dissemination should not be discard. In addition, an experimental infection by cohabitation between healthy and diseased fish was designed whereby horizontal transmission of the parasite was confirmed, although it appears to be slow and with a long prepatent period. *E. piscium* stages were also detected from water samples from the cohabitating tank, which supports the hypothesis that transmission occurs through water.

Resumen:

Una nueva enfermedad afecta al cultivo de *Solea senegalensis* en algunas granjas de la costa atlántica de España. Esta patología se caracteriza por la presencia de protuberancias que se evidencian a través de la piel de los peces afectados. Estas lesiones se corresponden con nódulos en el tejido muscular, que muestran un aspecto de absceso. Lesiones similares a éstas también se han detectado en riñón, corazón, hígado y tracto digestivo, lo que nos permite definir la enfermedad como sistémica. Las secciones histológicas de los nódulos revelaron un extenso núcleo compuesto mayoritariamente por tejido necrótico rodeado por fibroblastos y macrófagos. Además, organismos plasmodiales de morfología esférica se encontraron en la capa externa de estos nódulos, normalmente en el interior de macrófagos o fibroblastos. Estos organismos también se observaron en la mucosa y submucosa intestinal, sin causar lesiones aparentes. En este trabajo se ha podido identificar a estos organismos como una nueva especie de ameba perteneciente a la familia Entamoebidae (Phylum Amebozoa, Infraphylum Archamoeba), y se describe tentativamente como una nueva especie del género *Endolimax*, *Endolimax piscium* n. sp. *E. piscium* presenta trofozoitos redondeados (<5 μm) con un alto grado de simplificación intracelular, sin mitocondrias en el citoplasma pero con unos orgánulos compatibles con mitosomas. Con el fin de establecer técnicas fiables de diagnóstico para el reconocimiento de este parásito, se han desarrollado y evaluado técnicas específicas de hibridación *in situ* (ISH) y de reacción en cadena de la polimerasa (PCR), utilizando el examen histológico (una combinación de técnica histológica convencional en muestras de músculo e ISH en muestras de intestino) como prueba estándar para compararlos. Como resultado, todas las técnicas evaluadas obtuvieron altos indicadores de calidad. La técnica de ISH fue la más específica y sensible y se encontró particularmente útil como método de referencia de confirmación en muestras de intestino, no sólo para confirmar los positivos, sino también para descartar negativos, diagnosticados como dudosos por histología convencional. La técnica de PCR resulta ser un método rápido y fiable de diagnóstico, pero todavía necesita una mayor optimización metodológica de muestreo. Los resultados preliminares de la evaluación epidemiológica de la amebiasis en las

diferentes granjas analizadas, sugieren que una vez la enfermedad se manifiesta en la granja, es muy probable que los peces asintomáticos también presenten parásitos en el intestino, aunque no necesariamente presenten lesiones. La ruta que estos organismos utilizan para atravesar la barrera intestinal, llegar a otros órganos y diseminarse sistémicamente en el interior del pez, causando graves lesiones**,** especialmente en el músculo, todavía no se conoce con exactitud**.** Sin embargo los estudios preliminares señalan al tejido conectivo como tejido diana, por lo que se cree que pueda tener un papel importante en esta difusión, aunque la vía hematógena no puede ser descartada. Además, se ha diseñado una infección experimental por cohabitación entre peces sanos y enfermos, mediante la cual se ha podido confirmar la transmisión horizontal del parásito, aunque ésta parece ser lenta y con un período prepatente largo. También se ha detectado *E. piscium* a partir de muestras de agua tomadas en el tanque donde se alojaban los peces enfermos, lo que apoya la hipótesis de que la transmisión pueda producirse e a través del agua.

CHAPTER I: INTRODUCTION

1.1. Aquaculture:

Aquaculture is defined as the cultivation of aquatic organisms (including fish, molluscs, crustaceans and aquatic plants), which implies some sort of intervention in the rearing process to enhance production (such as regular stocking, feeding, protection from predators, etc.) and also implies individual or corporate ownership of the stock being cultivated (FAO, Food and Agriculture Organization of the United Nations).

Overall global capture fisheries production continues to remain stable at about 90 million tonnes, however world production of fish from aquaculture has continued to grow substantially over the past decade (Fig. 1.1). Farmed food fish (including finfishes, crustaceans, molluscs, amphibians, aquatic reptiles -except crocodiles- and other aquatic animals) reached 59.9 million tones in 2010, up by 7.5 percent from 55.7 million tonnes in 2009, and compared with 32.4 million tones in 2000 (FAO 2012). Actually, aquaculture is the food production sector with the fastest growth (8.8% per year since 1950 (FAO 2012)) and, nowadays, accounts for almost half (45.6 percent) of food fish in the world (including fish, crustaceans, algae, molluscs and other invertebrates) compared with 33.8 percent in 2000 (FAO 2010). Asia continues to dominate the aquaculture sector, accounting for 89 percent of global production by volume in 2010, with China alone contributing 60 percent of global production volume (FAO 2012).

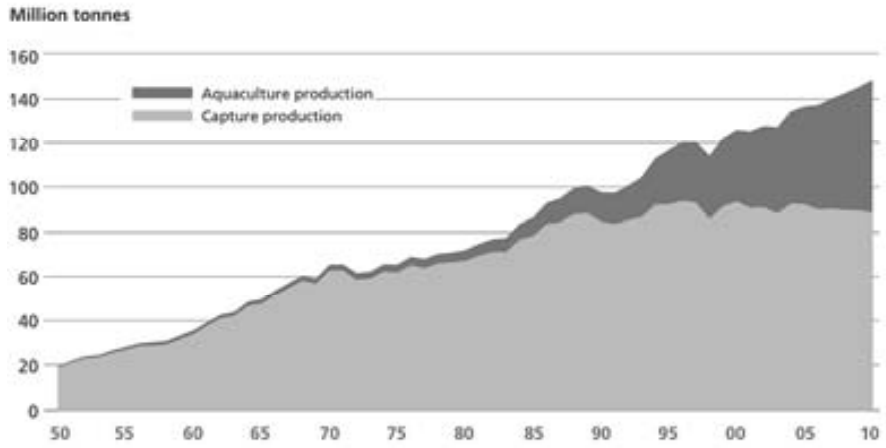

Figure 1.1: World capture fisheries and aquaculture production between 1950 and 2010. FAO 2012.

Aquaculture has been the engine driving growth of total fish production as its contribution to total world fish production increased from 20.9 percent in 1995 to 40.3 percent in 2010 (FAO 2012). Therefore, due to the stagnation of global capture fisheries and a growing human population, aquaculture is seen as the activity with the greatest potential to meet the growing demand for healthy food while respecting water environment. With the growth of aquaculture production in the last decade, the sector has a growing role to play in addressing food security by improving the supply and consumption of fish and other marine and freshwater products (whose common sources are rich in protein, essential fatty acids, vitamins and minerals) and in reducing poverty and enhancing economic and social development, by generating more income and employment and trade opportunities (FAO 2012).

In 2010 Europe produced 2.5 million tonnes of aquaculture products of which 1.2 million were in the European Union (EU), according to FAO statistics (2012). However, the total aquatic production suffered a slight decrease from the previous year, because aquaculture production, although increasing, has been insufficient to offset the declining fisheries capture in the last two decades (Fig. 1.2). Europe has developed an innovative new economic activity through modern systems of fish production, led by companies with strong scientific and technological support, while maintaining parallel traditional aquaculture systems better adapted to ecosystems (APROMAR 2012). Regarding Europe, aquaculture production of Norway grew from 151 000 tonnes in 1990 to more than one million tonnes in 2010 thanks to the farming of Atlantic salmon in marine cages. But focusing on the Member States of the European Union (EU), Spain has the greatest volume of aquaculture production, with 252,351 tonnes in 2010 (20.0% of EU total), followed by France and the United Kingdom (UK). However, when considering the value of production, France is the main producer. Concerning fish production, UK is the EU Member State with increased fish production from aquaculture in 2010, both in weight, 169,571 tons (26.7%), and value, 594 million euros (25.6% of total). Greece is the second largest producer

and Spain the third with 59,484 tons (9.4%), and 208 million euros (9.0%) (FAO 2010).

Nevertheless, there are significant challenges to be overcome by aquaculture to open wide the doors of the future: diversification of species, optimization of the sources of ingredients for their feed, technological advances to adapt their farms to tough marine conditions or the control of health of cultivated species.

Marine aquaculture in Spain is a growing industry in which production has been concentrated in three main species: gilthead seabream (*Sparus aurata)*, seabass (*Dicentrarchus labrax)* and turbot (*Psetta maxima,* synn: *Scophthalmus maximus*), the main flatfish cultured species.

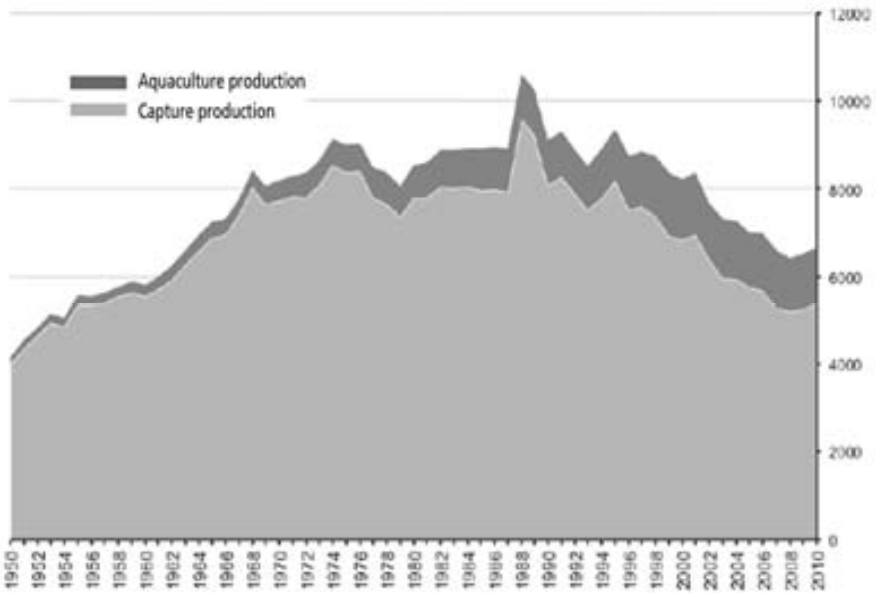

Figure 1.2: Evolution of the total aquatic production (aquaculture and fisheries, in thousand tonnes) of the 27 Member States of the European Union between 1950 and 2010. APROMAR 2012

1.2. Flatfishes:

1.2.1. Basic concepts

Flatfishes are laterally compressed fishes with both eyes present on the same side of the head in juveniles and adults (post-metamorphic individuals). All flatfishes begin life as pelagic, bilaterally symmetrical fishes, but during the larval development, they undergo a dramatic ontogenetic metamorphosis resulting in a benthic lifestyle. Munroe (2005a) documented 716 species of flatfish divided into 123 different genera. Their distribution occurs in the world's entire ocean, represented by a large number of species and genera. In some regions their populations are sufficiently large to constitute major fishery resources (Munroe, 2005a). The first articles describing research on flatfish appeared at the end of the nineteenth century (such as Petersen, 1894; Holt, 1895) and were stimulated by a concern for the state of their fisheries and why catches fluctuated.

1.2.2. Aquaculture

Flatfishes are among the most popular and valuable fishes used for human consumption (Imsland, 2010), supporting fisheries around the world with the high price of many species. These features make them attractive candidates for both stock enhancement and intensive cultivation (Howell and Yamashita, 2005). According to Imsland (2010), the most important species of flatfish for commercial fisheries and for aquaculture are representatives of the families: (1) Scophthalmidae, including turbot (*Psetta maxima*); (2) Pleuronectidae, including Atlantic halibut (*Hippoglossus hipoglossus*); (3) Rhombosoliedae, including greenback flounder (*Rhombosolea tapirina*); (4) Paralichthyidae, including Japanese flounder or bastard halibut (*Paralichthys olivaceus*), a species that has been one of the main targets for aquaculture-related activities in Japan and elsewhere in Asia, and including other species that are proving attractive to aquaculturists particularly in North America (Howell and Yamashita, 2005); (5) and Solidae, including soles (*Solea solea* and *Solea senegalensis*). However, the

family Cynoglossidae, including tongue sole (Cynoglossus semilaevis), may also be included because of its high economic value in the aquaculture industry in northern China.

Flatfish production increased from 26,300 tonnes in 2000 to 148,800 tonnes in 2008, being China and Spain the leading producers (FAO 2010). The major species concerned to these data are turbot, bastard halibut, and tongue sole, species which have been widely commercialised, like Japanese flounder (Howell and Yamashita, 2005). However, other species, including Atlantic halibut and soles, are on the verge of commercialization whereas others, such as winter flounder, are still only on the test trial stage (Imsland, 2010).

1.2.2.1. Sole Aquaculture

Sole farming involves two species: common sole and Senegalese sole (*S. solea* and *S. senegalensis*, respectively*).* Both species are very similar in shape and biology (Dinis et al., 1999), but *S. solea* is very highly appreciated in markets from Western and Northern Europe whereas *S. senegalensis* in Southern Europe. Although thirty years ago, sole was already considered one of the most interesting and promising species for marine fish farming in Europe (Howell, 1997), the success of the culture of sole has varied among the countries due to the high number of problems encountered during the tuning of the culture system, both from the standpoint of the fish with respect to its acclimation, feeding and reproduction, as well as the stability of their new environment (salinity, temperature, etc.), parasites and pathologies problems. In the last years, commercial fisheries in many countries in Europe have sole as one of the main species, but landings show strong fluctuations between years and seasons (FAO, 2004). Moreover, there is a considerable pressure on exploitation due to overfishing, which makes a continuous supply of sole in the future uncertain, based only on fisheries. For this reason, and the good acceptance of its meat quality in the market, several countries, including Spain, Chile, Ecuador, Peru and Mexico, have developed sole breeding in captivity for its development and commercialization (JACUMAR 2008). Furthermore, scientific and technical

interests in Europe have recently focused on high-value native species whose life cycle can be reproduced in captivity (Arellano and Sarasquete, 2005) and, nowadays, recirculation technology and advances in feed technology have fuelled a renewed interest in sole as an aquaculture species (Howell, 1997; Dinis et al., 1999).

1.2.2.2. Current situation of sole culture in Spain:

In the past, the Senegalese sole farming has been traditionally performed extensively in earthen ponds in the South of Spain and Portugal, usually originating from wild stocks. Large-scale production of *S. senegalensis* larvae in hatcheries has been seriously limited for years, due to limited success at obtaining eggs from the captive breeders that are kept in some facilities (Anguis and Cañavate, 2005). However, in the late nineties its reproduction in captivity was achieved, rebounding interest in this species. In 2003, Olmedo pointed to the apparently good growth aptitude of Senegalese sole under intensive farming, which was encouraging for the development of systems for hatchery-produced juveniles. Several studies have been carried out to optimize the culture conditions of this specie and make it profitable and sustainable in every one of its phases (e.g., at University of Algarve, Instituo de Investigación y Formación Agraria y Pesquera (IFAPA), or Centro Tecnológico Gallego de Acuicultura (CETGA)). The farming intensification of the Senegalese sole may noticeably contribute to its aquaculture development from the traditionally extensive exploitation regimes used in southern Atlantic coast of Spain and Portugal (Dinis, 1992). In this sense, the aquaculture cluster of Galicia has managed to create and implement a recirculation technology for production of sole (*Solea senegalensis*) that is already being implemented in some partners of the Cluster.

Spain is the world pioneer in the production and marketing of sole. In fact there is a national plan to analyse the factors that affect the growing industry of Senegalese sole (*Solea senegalensis*) (JACUMAR). In 2007, there were 43 facilities in the unfolding activity related to the rearing of sole using earthen ponds, tanks or estuaries and distributed in Andalucía, Galicia and the

Comunidad Valenciana (JACUMAR). In 2008 there were 316 tonnes from Galicia, Canarias and Andalucia awaiting the final launch of its production. However several technical and administrative issues have been slowing the production and declining production values can be observed in the years 2009 and 2011. In fact, during the year 2009 only 188 tonnes were produced, of the 421 tonnes planned (APROMAR 2009 and 2010). Regarding regions, Galicia is the leading producer of sole since 2007, and has been increasing its lead from the other regions during the following years (table 1.1).

Table 1.1: Production data of cultured Senegalese sole in different regions from Spain (t). APROMAR 2009 and 2012.

	2004	2005	2006	2007	2008	2009	2010	2011
Andalucía	31	20	55	24	1	10	4	0
Canarias	0	0	0	6	15	28	30	31
Galicia	27	20	25	30	300	150	170	79
Murcia	17	20	0	0	0	0	0	0
Total	75	60	80	60	316	188	204	110

1.3. *Solea senegalensis*:

1.3.1. Basic concepts

The family Soleidae belongs to the Order Pleuronectiformes, commonly known as Flatfishes. It is a diverse family of dextral flatfish, most of them small to medium size and distributed worldwide in a variety of marine and estuarine habitats and some freshwater species (Munroe, 2005a), but they are conspicuously absent in the western Atlantic, and rare in the eastern Pacific (Munroe, 2005b). In the eastern Atlantic, soles occur from off southern Iceland and in continental waters off Europe from southern Norway to Spain and

Portugal, throughout the Mediterranean Sea with a few species occurring in the Black Sea, and from North to South Africa (Munroe, 2005b). Soleidae is composed for 7 genera and 14 species. *Solea senegalensis* belongs to the genus Solea, whose molecular genetics revealed four sister lineages that correspond to four species *S. solea*, *S. senegalensis*, *S. kleinii* and *S. lascaris* (Tinti and Piccinetti, 2000). This study agrees with the morphological features of soles proposed by Ben-Tuvia (1990), who used the number of vertebrae, number of fin rays, shape of the blind side forenostril or shape and coloration of the dark spot on the pectoral fin to differentiate the species.

S. solea and *S. senegalensis* are closely related and very similar in shape and biology (Dinis et al., 1999) although they can be distinguished by the numerous blue spots carried in the ocular side or by the dark coloration of the ocular pectoral fin of *S. senegalensis* (Quéro et al., 1986; Bauchot, 1987) and by some additional meristic features such as shorter distances of snout to edge, greater eye diameter or longer pectoral fins (Ben-Tuvia, 1990). According to current scientific opinion, the species are so similar that experimental results yielded for one species can be applied directly to the other species (Imsland, 2010). Regarding their geographical distribution, *S. solea* extends throughout the Mediterranean and Atlantic Ocean, from Scandinavia to Senegal, while *S. senegalensis* has a more limited distribution in the eastern Atlantic Ocean, between the Gulf of Biscay and the coasts of Senegal, and less frequent in Western Mediterranean (Fishbase 2012).

1.3.2. Morphological identification

Figure 1.3. Adult specimen of *Solea senegalensis.*

S. senegalensis is a flatfish with an oval and asymmetric body, with both eyes on the right side (Fig. 1.3). The pectoral fin has a black interradial membrane on the eye side, which allows differentiating to the common sole (Quéro et al., 1986; Bauchot, 1987; Ben-Tuvia, 1990). The dorsal and anal fins extend nearly the entire body, but they are clearly separated from the caudal fin. Its snout is round and the mouth is small and semicircular, located on the bottom edge of the right eye. The ocular side is brown pigmented, varying between light and dark shades with small concentric patches of different hue to merge into the environment. The adults usually measure about 45 cm and have separate sexes without differentiable external characters.

1.3.3. Biology:

S. senegalensis is a strictly bottom-dewlling species that lives in sandy bottoms between 12 and 65 m depth, but it can reach up to 100 m, where it remains buried most of the day becoming active during the night (Kruuk, 1963; Quéro et al., 1986). It localizes the preys by mechanoreception due to cephalic neuromasts located on the blind side. It feeds mainly on sedentary polychaetes, crustaceans, molluscs and echinoderms (Abellan and Basurco, 1999; Cabral 2000; Sá et al., 2003). Active growth takes place from February until August, with females larger than males. It is a gonochoristic species and the first female maturation happens at three years of age, when fish are about 25-30 cm in length (Dinis et al., 1999). They spawn near the coast during spring (March-April–June) and autumn, being the spring spawn better in terms of quantity and quality (Dinis et al., 1999). Males produce sperm during the year, although a high proportion occurs in spring and decreases in the summer months (Garcia-Lopez et al., 2006). The female can lay one million pelagic eggs per kg body weight (Arellano and Sarasquete, 2005) of a size around 1 mm (Dinis et al., 1999). The larvae hatch in two or three days, depending on the temperature (Dinis and Reis, 1995). The larvae are also pelagic, but when they reach 4 mm of standard length (Imsland et al., 2004), the eye migration starts, initiating the metamorphosis. The metamorphosis of Senegalese sole is composed of a series of changes: eye

migration from left to right side and concomitant bending of the urostile and torsion of the digestive tract (Padrós et al., 2011). They complete the metamorphosis when larvae are approximately 8 mm standard length, between day 36 and 40 (Imsland et al., 2004). In terms of behaviour, sole abandons pelagic life to become benthic, spending almost all the time at the sea bottom.

1.3.4 Culture of *Solea senegalensis*:

Culture features of Senegalese sole are represented in fig. 1.4. Broodstock are usually obtained from coastal lagoons or estuaries or maintained in specific facilities. The quality of the broodstock, methods of capture and conditioning (environmental factors and nutrition) have been found to be important for obtaining viable gametes (Dinis et al., 1999). Although nowadays, there are five centres that have first generation (F1) broodstock of cultured sole: El Toruño IFAPA (Cadiz, Andalucia), IEO (Santander, Cantabria) and IEO (Vigo, Galicia), IATS (Castellón, Valencia) and IRTA (Tarragona, Catalunya) (JACUMAR). Fertilized eggs can be obtained by three different methods: (1) spontaneously, (2) by controlling light and water temperature to induce adult sole reproduction, or (3) by in vitro fertilization, usually aided by injection of hormones in adult flatfish to obtain gametes.

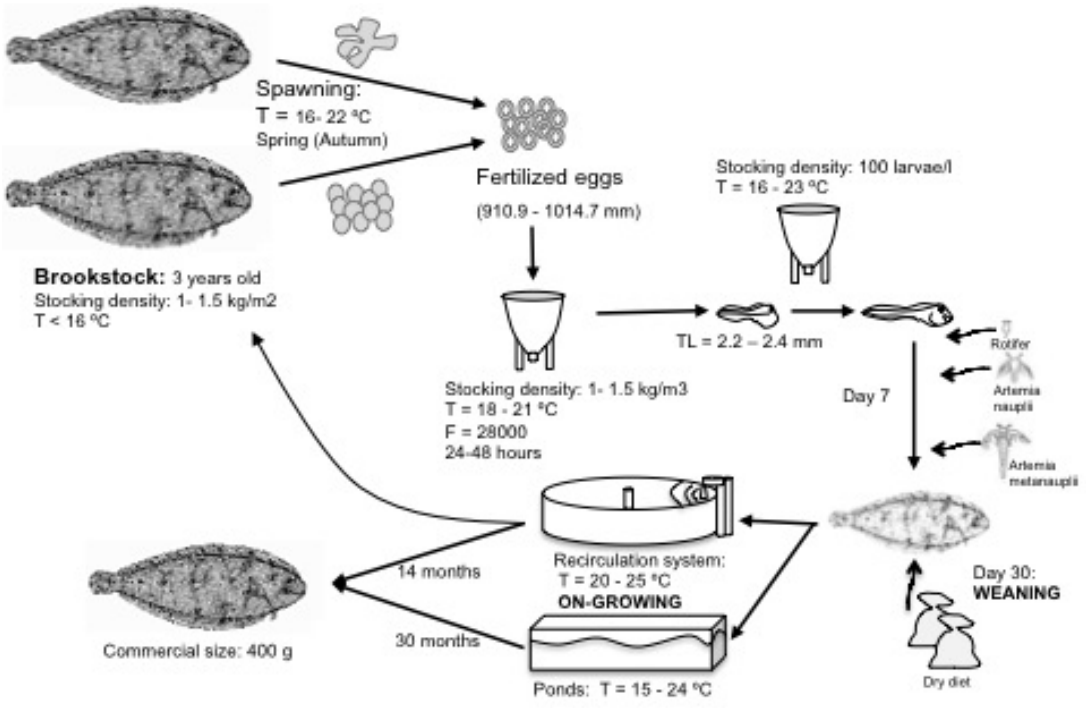

Figure 1.4: Production cycle of *S. senegalensis*.

The thermoperiod manipulation in Senegalese sole allows to obtain natural spawns and the reproductive behaviour of the sole has shown to be greatly influenced by sexual courtship and by the action of pheromones as critical factors in the lack or absence of fertilization. Under normal conditions of light and temperature, and maintaining a sex ratio 1:1 or 2:1 (male: female), two periods of spawning are described, one in spring (February-June) with temperatures between 12 and 23 º C and one in autumn (October-November). Natural spawning of *S. senegalensis* in captivity can be achieved successfully (Dinis et al., 1999), at temperatures between 16-22 ºC (Dinis et al., 1999; Anguis and Cañavate, 2005) and feeding broodstock on squid and supplemented with polychaetes. Recommended stocking density in maturation tanks is around 1- 1.5 kg/m2 and temperature should be kept above 16 ºC, as emission of eggs stops below that temperature. Salinity should be kept constant around 30-35 ‰ and the fish reared under simulated natural photoperiod. The mean diameter of the eggs is between 910.9 - 1014.7 mm. for sole eggs collected in Spanish and Portugal hatcheries (Dinis et al., 1999; Mourente and Vasquez, 1996).

Once obtained fertile eggs, incubation of eggs is performed in cylindro-conical tanks using temperatures between 18 and 21ºC (Cañavate and Fernandez-Díaz, 1999; Dinis et al., 1999, Fernandez-Díaz et al., 2001; Morais et al., 2004) during 24 and 48 hours, and salinity usually about 36 gl^{-1} (Cañavate et al., 2006). Henceforth, pelagic larvae must be cultivated and fed to reach the juvenile stage, which is achieved by complete metamorphosis. Water temperature plays an important role during the larval development. Dionisio et al. (2012) pointed out that best results regarding both growth and larval quality (incidence of deformities) were achieved with a temperature regime of 18 ºC during egg incubation followed by 21 ºC during larval development. In the first stage, larvae are fed by rotifers enriched with fish oils to improve their nutritional quality. From around day 7 starts they feeding live Artemia, until the fish are weaned (Dinis et al., 1999; Cañavate, 2005). Although some studies showed that feeding larvae with *Artemia nauplii* from the first feeding did not affect survival, growth or amino composition (Aragao, 1999). From day 30 of life of the larva, it begins the phase of "weaning" which is the replacement of live food by a micro dry diet formulated with fish meal, flour "krill" (planktonic crustaceans), soybeans, squid, fish oil, vitamins and minerals (Dinis et al., 1992). However other authors pointed to 43 days for the weaning (Cañavate and Fernandez-Diaz, 1999), or even 6 months (Flos et al. 1995). A poor diet during the larval stage or along the metamorphosis, may cause serious problems of mortality, malformations, malpigmentactions and diseases (Soares et al., 2002; Padrós et al., 2003).

The fattening of sole can be performed either in tanks on land and floating or submerged cages in the sea. The on-growing perform in an annual temperature between 15 and 24ªC in ponds (Dinis et al., 1999) feeding on dry commercial food. The optimum physiological temperature for this species is located at 20℃ (Garcia-Garcia et al., 2004). In recirculation facilities, the optimum rearing conditions with respect to growth rates and feed conversion ratios are approximately 20–25℃ (Imsland et al., 2004), altho ugh they are close to the range of temperatures that enhance the development of diseases in sole (Cañavate, 2005). From hatching until the larvae have a market size may take

approximately 30 months in extensive conditions and 14 months in intensively.

1.3.5. Pathologies affecting *Solea senegalensis*.

1.3.5.1 Literature review:

In last years, one of the main factors that hampered the production or fattening of Senegalese sole was the increasing number and intensity of diseases (Padrós et al., 2003; Toranzo et al., 2003). Almost all opportunistic diseases that are already known to affect other species can also affect sole. These episodes of diseases can be facilitated by poor hygienic conditions of the tanks, or when the temperature exceeds 22 ℃ values (Cañavate, 2005). The main pathological problems registered in sole culture are summarised in table 1.2.

Table 1.2. Pathogens and pathological problems affecting *Solea senegalensis*.

	Pathogen/Pathology	References
Virus	Birnavirus	Rodriguez et al., 1997
	Lymphocystis virus	Toranzo et al., 2003; Padrós et al., 2003; Alonso et al., 2005
	Virus de la septicemia hemorrágica viral (VHSV)	Toranzo et al., 2003
	Nodavirus	Starkey et al., 2001; Thiéry et al., 2004; Cutrín et al., 2007; Olveira et al., 2009; Hodneland et al., 2011
Bacteria	*Edwardsiella tarda*	Castro et al., 2012
	Photobacterium damsela ssp. *piscicida*	Zorrilla et al., 1999
	Tenacibaculum maritimum, T. discolour and T. soleae	Cepeda and Santos, 2002; Avendaño-Herrera et al., 2004; Piñeiro-Vidal et al., 2008a; Piñeiro-Vidal et al., 2008b
	Vibrio harveyi; V. parahaemolyticus; V. anguillarum	Moriñigo et al., 2001; Zorrilla et al., 2003; Rico et al., 2008
	Aeromona salmonicida sbsp. *salmonicida)*	Magariños et al., 2011
	Edwardsiella tarda	Castro et al., 2012
Protists	Flagellates (*Amyloodinium, Cryptobia and Cryptocaryon)*	Padrós et al., 2003
	Amoeba-like	Constenla and Padrós, 2010
	Microsporidia *(Tetramicra*	Padrós et al., 2003
Metazoa	Myxozoa *(Enteromyxum scophthalmi)*	Palenzuela et al., 2007
	Digenean Trematoda	Padrós et al., 2003
	Hirudinea *(Hemibdella solea)*	Dinis et al., 1999
Non-infectious pathologies	Cell necrosis of the adipose tissue syndrome	Padrós et al., 2003
	Nefrocalcinosis	Padrós et al., 2003
	Pigmentation abnormalities	Dinis et al., 1999
	Malformations	Gavaia et al., 2002 and 2009; Fernández et al., 2009; Cardeira et al., 2012

The main pathological problems are bacterial diseases, mainly Photobacteriosis (formerly Pasteurellosis) and Tenacibaculosis (formerly flexibacteriosis. Photobacteriosis, caused by *Photobacterium damsela* ssp. *piscicida*, results in high losses in the aquaculture industry since provokes massive mortalities in cultures of several marine fish species such as Gilthead seabream (Toranzo et al., 1991) or Seabass (Balebona et al. 1992) but also in flatfish Japanese flounder (Fukuda et al., 1996). This disease has also been recorded in farmed Senegalese sole of southwest Spain (Zorrilla et al., 1999). Since then, sole farms mainly in the south area of Spain have suffered mortalities caused by this disease, becoming one of the most threatening to the cultivation of this valuable fish species (Magariños et al., 2003). In most cases, peracute mortalities without apparent lesions are the most typical expression found mainly in juveniles. However, in subacute and chronic cases, external lesions of infected fish only included unspecific symptoms such as dark skin coloration and swelling of the abdominal cavity. Internally, affected specimens showed paleness of liver and kidney and the development of whitish granulomas of 1- 2 mm diameter in the spleen. The Gram-staining of samples of affected fishes revealed the presence of Gram-negative bacilli showing a pronounced bipolar staining (Zorrilla et al., 1999). This disease particularly affects *S. senegalensis* at temperatures above 18 º C and usually triggers severe acute cases in which mortality can be extremely high (Padrós et al., 2003). Another determining factor in the development and maintenance of the disease in the facility is the cohabitation with other susceptible species such as Seabream and Seabass. However, nowadays sole are reared alone in specific facilities with controlled ambience, reducing the risk of an outbreak.

Tenacibaculosis, which is mainly caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimum*), is considered a serious disease in marine aquaculture (Rigos et Katharios, 2010) because it causes significant mortality on fish farms in many countries limiting the culture of economically important marine fish species (Santos et al., 1999). The presence of this pathogen in sole in Europe was first described in Scotland from a Dover sole (*Solea solea*) suffering

from “black patch necrosis” (BPN) (Bernardet et al., 1990), probably the most important economic devastating problem in early culture attempts on this species because of its high incidence (McVicar and White, 1979; 1982). Although this disease was reported to be highly infectious, it seemed to be both prevented and controlled by providing a sand substrate in the rearing tanks (McVicar and White, 1982). The high incidence BPN and other skin lesions in flatfish species in the absence of sand or smooth textures (Ottesen and Strand, 1996; Ottesen et al., 2007) was a problem to be considered not only from an economical point of view, due to reduced survival and growth and the added cost of antibiotics, but also from a consumer standpoint. However, in the sole farming, the use of sand as a substrate has been considered as a serious obstacle to maintaining a hygienic environment (Howell, 1997). However, keeping these fish in hard-bottomed tanks without sand involves a series of harmful consequences. In this sense, Reig et al. (2010) concluded that sole seems to prefer light sand whatever the texture, and rough plastic whatever the colour. Some years later, *Tenacibaculum maritimum* was also isolated in Spain from turbot (*Psetta maxima*) (Alsina and Blanch, 1993; Pazos et al., 1993) and from salmon (*Salmo salar*) (Pazos et al., 1993). Cepeda and Santos (2002) isolated for the first time *T. maritimum* from Senegalese sole in southwest Spain causing almost 100% of mortality. Affecting sole showed several external signs including eroded mouth, rotten fins and skin lesions. Internally, specimens showed paleness of internal organs. The bacterial pathogen was also isolated from the same species by Avendano-Herrera et al. (2005). Other *Tenacibaculum* species such as *T. discolor* and *T. soleae* were also isolated from diseased Senegalese sole (Piñeiro-Vidal et al., 2008 a and b), showing the typical signs observed in fish affected by *T. maritimum*. Recently, Vilar et al. (2012) decribed a severe ulcerative disease in cultivated Senegalese sole associated with *T. maritimum*. Grossly, the affected fish from this study showed total loss of epidermis and dermis and extensive necrosis of the muscle layers. Mild to moderate inflammatory response characterised by macrophages and hyaline degenerated muscle cells were described histologically

Vibrioses affecting Senegalese sole are usually detected as secondary

infections associated with an initial Tenacibaculosis, but often can also be primary infections and its pathogenesis is still unclear (Padrós et al., 2003). *Vibrio harveyi* and *V. parahaemolyticus* are pathogenic bacteria which were described in a natural outbreak of farmed *S. senegalensis* by Zorrilla et al. (2003) causing moderate mortalities in the south of Spain. Main external signs of the disease were skin ulcers and haemorrhagic areas near the fins and mouth (Zorrilla et al., 2003). Rico et al. (2008) also characterised strains of *V. harveyi* from diseased-farmed Senegalese sole in Spain from 2000 to 2004. Reports of this bacterium on disease outbreaks in aquaculture are continuously growing (Rigos and Katharios, 2010).

In 1992, an outbreak of furunculosis by *Aeromonas salmonicida* was diagnosed for the first time in turbot reared in floating cages located in northwest of Spain, causing significant economic losses (Toranzo and Barja, 1992) and is still a significant problem in some turbot farms. Recently, Magariños et al. (2012) reported *A. salmonicida* subspecies *salmonicida* as the causative agent of a 'typical' furunculosis in cultured sole in a marine farm in Galicia with a recirculation system. Affected fish showed haemorrhagic areas at the base of the dorsal and ventral fins and, in some cases, ulcerative lesions on the ventral surface. Internally, peritoneal cavities were completely filled with ascitic fluid and livers were extremely pale and showed petechiae. These soles were grown in a farm were also cultured turbot, which pointed to a crossed infection of this bacterium from one fish species to another as the source of the outbreak.

As viral diseases, Birnavirus and Lymphocystis virus were also detected from cultured sole (Rodríguez et al., 1997; Alonso et al., 2005; Cano et al., 2010). The birnavirus was described as the causative agent for 100% mortality in wild Senegalese sole broodstock introduced and stored into a culture facility in southwest Spain. External signs of the disease were dark coloration, hyperactivity and uncoordinated swimming behaviour (Rodríguez et al., 1997). The characterisation of this virus indicated its similarity to infectious pancreatic necrosis virus (IPNV). IPNV had been described in Atlantic farmed turbot displaying muscular haemorrhages and a severe necrosis of the haemotopoietic

tissue of the kidney (Castric et al., 1987). However, birnavirus and IPNV are a widely distributed group of virus, present in many different aquatic organisms so its role of a primary pathogen in sole species does not to be so clear. Lymphocystis disease is caused by an iridovirus with a worldwide geographical distribution that involves a chronic disease characterised by papilloma-like lesions typically on the skin, fins and tail (Walker and Hill, 1980) and has also been isolated from Senegalese sole (Toranzo et al., 2003; Alonso et al., 2005). Although no reports of VHS outbreaks has been reported for the time being, López-Vazquez et al. (2011) demonstrated the susceptibility of S. senegalensis to a viral haemorrhagic septicaemia virus (VHSV) strains isolated from wild Greenland halibut, *Reinhardtius hippoglossoides,* and farmed turbot. In that study, Senegalese soles were infected by an intraperitoneal injection, immersion or cohabitation, and affected fishes showed haemorrhaging of the ventral area and ascitic fluid in the body cavity. In wild fish stocks, which were held as future breeders, the presence of intracytoplasmic inclusion bodies in erythrocytes was detected causing low but continuous mortality (Padrós et al., 2003). To this point not been able to demonstrate the presence of viral particles in them, although not ruling out the existence of a process similar to VEN (Viral erythrocytic necrosis) or similar processes described in other species. Betanodaviruses are the aetiological agents of the disease known as viral nervous necrosis or viral encephalopathy and retinopathy, a devastating neuropathological condition that affects marine fish worldwide (Munday et al., 2002). These viruses have also been detected in sole (Starkey et al., 2001; Thiéry et al., 2004; Cutrín et al., 2007; Olveira et al., 2009; Hodneland et al., 2011). In this later study, fish were obtained from an outbreak and showed disease signals such as abnormal swimming behaviour and moderate to high mortalities.

In contrast, there is limited information about parasitic diseases affecting cultured Senegalese sole. Ectoparasites such as the leech *Hemibdella solea* (Hirudinae) have been identified on broodstock of Senegalese sole in the Virginia Institute of Marine Science, (Gloucester Point, USA) but it does not seem to adversely affect the fish and can be controlled with low salinity (Dinis et al.,

1999). This leech has also been reported on *common sole* (Baynes et al., 1993) from the wild. Some sporadic infections by protist parasites like flagellates or ciliates (*Amyloodinium, Cryptobia and Cryptocaryon)* have been described (Padrós et al., 2003) usually in cases where sole were reared in tanks of ponds. Albeit they are infrequently, in cases of massive parasitosis can also eventually lead to high mortalities, especially on soles grown at high temperatures and recirculation. Palenzuela et al. (2007) described an infection by *Enteromyxum scophthalmi* in sole cohabiting with infected turbot, and other Myxozoa such as *E. leei* and *Ceratomyxa* sp. have also been observed in sole (Palenzuela, pers. comm.). Likewise, other internal parasites, the presence of mixosporidia in renal tubules, some Tetramicra xenomas and digenea trematode larvae encysted in the musculature, have occasionally been observed (Padrós et al., 2003).

Pathologies of apparently non-infectious origin have also been described by Padrós et al. (2003): cell necrosis of the adipose tissue syndrome related to lipid peroxidation and/or excessive exposure to sunlight, characterised by yellowish areas at the base of dorsal and anal fins, which correspond to a necrosis of the subdermal adipose tissue, and can be associated to secondary bacterial infections; kidney damage due to the deposition of minerals in the tubular lumen, similar to the processes of nephrocalcinosis; bacterial enteropathy, possibly associated with the administration of contaminated live food in larval stages and juveniles; as well as the usual mortality associated with maladjustment problems to inert diet. Pigmentation abnormalities as well as some morphological malformations associated with the migration of the eye are common problems associated with the cultivation of flatfishes. These abnormalities determine the external morphology of the fish, its growth and survival rate, which impact in lower market value (Estévez and Kanazawa, 1986; Takeuchi et al., 1998; Gavaia et al., 2002; Hamre et al., 2005). Hypomelanosis or pseudo-albinism, characterized by white patches or areas devoid of normal pigmentation on the ocular surface of the skin, is common in both wild and hatchery reared flatfish. The blind side may display hypermelanosis in the form of dark spots, known as ambicoloration of the skin. Malpigmentation has been reported in other cultured

marine flatfish (e.g., Bowers, 1966; Roberts et al., 1972; Seikai et al., 1987) but also in *S. senegalensis* (Dinis, 1999). Skeletal abnormalities commonly affect Senegalese sole in intensive rearing conditions (Gavaia et al., 2002 and 2009; Fernández et al., 2009; Cardeira et al., 2012). The aetiology of these abnormalities is not well understood, although nutritional causes, such as deficiency of vitamin A, genetic and environmental factors are suggested (Gavaia et al., 2002 and 2009; Fernandez et al., 2009).

3.5.2. Current situation:

During the last years, Senegalese sole, *S. senegalensis*, is becoming one of the most promising species of marine fish farming in Spain. However, as has been explained in this introduction, its development on an industrial scale is still compromised by pathologies. Bacterial diseases, Tenacibaculosis and Photobacteriosis, are still affecting the currently sole farming causing serious mortalities, especially Tenacibaculosis. There are numerous studies on vaccinations against these diseases (e.g. Romalde et al., 2005; Arijo et al., 2006), and recent studies on probiotics to control Photobacteriosis seem to have encouraging results (Garcia de la Banda et al., 2012). Vibriosis is still affecting sole, not only as secondary infections but also associated with intestinal bacterial infections. Granulomas in the kidney and spleen were also observed in this fish related to acid-resistant bacteria, Ziehl-Neelsen-positive stain, very compatible with *Mycobacterium* spp. (Padrós, per. obs.) including a new potential hazard for this species. The nutrition and feeding play an important rule and, although progress has been made on this problem, the developing of protocols for fattening with specific diets for this species still need improvements. The ethological features are also important in sole, especially because of its tendency to bury. Together with the lack of culture systems specially designed for these species, such as the use of sandy soils and, in some cases, deficient management and hygiene conditions, may favour the emergence of bacterial infections.

Occasional outbreaks of Nodavirus are sometimes detected in sole farms, but the

typical nervous clinical signs and high mortalities on these fish are usually less common than in other species (Hodneland et al., 2011). Some outbreaks were detected in F1 fish, highlighting the importance of detecting wild broodstock carriers. Risk of horizontal transmission from other cultured species in cohabitation could also exist due to the high susceptibility of soles to most of the diseases (Zarza, pers. com.). Currently there are no efficient treatments or commercial vaccines for nodavirus. The recommendation is that affected stocks were removed and sacrificed when the virus is detected. However, there are some promising results from oral, bath and injection vaccination in grouper *Epinephelus septemfasciatus* (Lin et al., 2007; Kai et al., 2008; Nishizawa et al., 2009; Yamashita et al., 2009).

Chronic proliferative mucoid inflammation in gills, with epithelial hyperplasia and fusion of lamellae in the apical region of some filaments has been observed in some Senegalese sole. These lesions were associated with the presence of amoeboid organisms similar to *Neoparamoeba* sp. (Padrós, per. obs.).

Since 2008, lethargic fishes with sporadic and erratic swimming and showing protuberances on the skin surface have been referred to the Servei de Diagnóstic de Peixos (SDPP) of Universitat Autònoma de Barcelona for histopathological examination. Although the condition was not associated to high mortalities, an apparently high morbidity was noted. The histological examination of the nodules in muscle revealed the presence of chronic inflammatory areas related to spherical amoebic-like organisms (see results Chapter IV). The fact that similar lesions had never been described before in Senegalese sole, and the peculiar characteristics of the organisms associated with, led to approach further characterization of the condition framed in this Ph.D. thesis.

1.4. Amoebae in fish:

Amoebae are unicellular protists whose main feature is the emission of pseudopodia to move and feed. From an ecological point of view, there are free-living amoebas and parasitic species. The free-living naked amoebae are highly variable and diverse eukaryotic organisms, which are distributed ubiquitously in soil and aquatic habitats. Among these free-living amoebae, the amphizoic species are able to invade host tissues and cause disease (Page, 1974) and, contrasting with true parasites, they can complete their life cycles in the environment without an animal host (Rocha-Azevedo et al., 2009). The amphizoic amoebae are the most important in health fish (Dyková, 2008). Acording to Adl et al. (2005), amoeboid protists found in fish belong to two taxonomic super-groups: Amebozoa and Excavata (Fig. 1.4). Amoeba parasitizing fish also include two main groups of organisms: specific endocommensals and free-living amphizoic amoebae (Dyková, 2008).

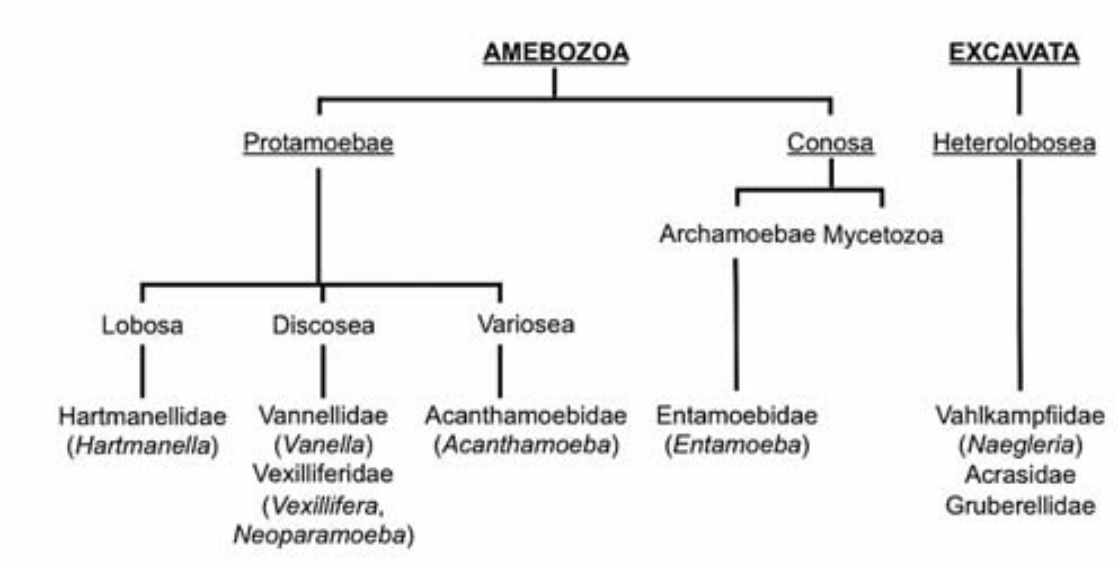

Figure 1.4: Scheme of the main amphizoic and endocommensal amoeboid protists that affect fish.

Several species in these groups have been recognized as pathogens, of invertebrates and vertebrates, including humans (Dyková and Lom, 2004). Amoebae pathogenic to humans have been well documented over the years (Martinez, 1985; Visvesvara and Stehr-Green, 1990; Visvesvara et al., 1993;

John, 1993; Martinez and Visvesvara 1997; De Jonckheere, 2002). The most commonly studied genera are *Acanthamoeba* Volkonsky, 1931, *Naegleria* Alexeieff, 1912 and *Balamuthia* Visvesvara et al., 1993. *Naegleria fowleri* is an Heterolobosean protist that causes Primary Amoebic Meningoencephalitis (PAM) in humans, an acute, fulminant and fatal disease (Rocha-Azevedo et al., 2009). Dyková and co-workers (2001) isolated different strains of *Naegleira* from different organs of freshwater fishes, showing their ability for a endozoic way of life and underlining its pathogenic potential for fish. *Acanthamoeba* spp. is one of the most commonly isolated amoebae in environmental samples and they can cause Granulomatous Amoebic Encephalitis (GAE), cutaneous acanthamoebiasis and Amoebic Keratitis (AK) in humans (Rocha-Azevedo et al., 2009). These species are also able to colonize organs of freshwater fishes as facultative parasites (Taylor, 1977; Dyková et al., 1999). Although these amphizoic amoebae can cause granulomas in different organs of the host, they are typically isolated from normal organs, like other amoebae genera such as *Vexillifera*, *Vannella, Protacanthamoeba* or *Hartmanella* (Dyková et al., 1998; 2005a and 2005b). Water supplies, swimming pools, freshwater ponds, lakes and thermally polluted waters have been recognized as a source of human infections. In this sense, the attention of researchers to the prevalence of free-living amoebae in water habitats has been limited. Dyková and colleagues have been working with this type of amoeba by advertising the need to study them in fish living in these aquatic environments, aspects that have been neglected for a long period of time (Dyková and Lom, 2004).

In marine fishes, Neoparamoeba sp. (formerly Paramoeba sp. (Dyková et al., 2000)) is an amphizoic amoeba described as the causative agent of amoebic gill disease (AGD) in salmon, *Salmo salar* (Kent et al. 1988) and *Oncorhynchus kisutch* (Roubal et al., 1989), turbot, *Psetta maxima* (Dyková et al., 2002), and other marine fishes. This free-living amoeba attaches to the gills and cause a severe hyperplastic tissue reaction and eventual death (Munday et al., 1990). Other amoebae of the genera Vanella, Platyamoeba and Flabellula have been

isolated from the gills of both asymptomatic and diseased fish with AGD (Dyková, 2008).

Systemic granulomatous infection was described in goldfish, *Carassius auratus*, due to an amoeba-like organism (Voelker et al., 1977; Lom and Dykovà, 1992; Steinhagen et al., 1993). Quite recently, a similar pathological condition was described in Tench (Palíková et al., 2012). In this latter study, a species related to the family Archamoebae has been suggested. Amoebae belonging to the class Archamoebae, contrary to free-living amphizoic amoebae, are characterized by the lack of mitochondria (Cavalier-Smith, 1998), probably related to their adaptation to parasitism. Among these parasitic amoebae species it can be highlighted the genus *Entamoeba,* which includes several species parasites of vertebrates (eg. Simitch, 1938, Rodhain and Van Hoof, 1947, Noble 1954 and 1955), including fish (Lom and Dyková, 1992), and even invertebrates (Keilin, 1917; Neal, 1966). *E. salpae* Chen, 1955, *E. gadi* Bullock 1966, and *E. molae* Noble and Noble, 1966 were described from marine fish and *E. pimelodi da Cunha and Penido, 1926* and *E. ctenopharyngodoni* Chen 1955 from freshwater fish. *Iodamoeba* and *Endolimax* are also endocommensal or facultative parasitic organisms in the intestinal tract of humans and other vertebrates. Species of *Endolimax* have been reported for a variety of vertebrate hosts (Wenyon and O'Connor, 1917; Brug, 1920; Chiang, 1925; McFall, 1926; Hegner, 1926; Lucas, 1927; Gutierrez–Ballesteros and Wenrich, 1950), and even from invertebrate hosts (Kirby, 1927). Excluding *Entamoeba*, most members of Archamoeba are not well studied, probably due to the relatively minor clinical importance and the difficulties associated with their laboratory cultivation (Silberman et al., 1999). Entamoebae species are characterised by two stages in their life cycle (Silberman et al., 1999): a trophozoite stage (vegetative and infectious form, which is the responsible for the disease) and a cyst (a form of resistance, which is the responsible for transmission). Although most of them are considered endocommensals, some of them are pathogenic, being *E. histolytica* affecting humans the species most studied. Apart of *E. histolytica* that can cause arthritis, dysentery, colitis and liver abscess (Espinosa and Martinez-Palomo,

2000), other archamoebae species are able to cause diseases in their hosts. For example, gastrointestinal disorders have been reported associated with diverse species of *Entamoeba* (Jetter et al., 1997; Hall et al., 2012), but also by *Endolimax nana* (Stauffer et al., 1974). Furthermore, *E. nana* has been described to be involved in skin processes (Veraldi et al., 1991) and rheumatoid arthritis (Burnstein and Liakos, 1983). *Iodamoeba buetschlii* has also been reported to be able to cause brain granuloma (Arava, et al., 2010).

CHAPTER II. OBJECTIVES

The main objectives considered during the planning and development of the present work were:

1. To characterise the main histopathological alterations in different organs and tissues associated to a new pathological condition affecting farmed Senegalese sole, *Solea senegalensis*, (CHAPTER IV).
2. To carry out a morphological description of the aetiological agent (an amoebic organism) responsible of this new disease (CHAPTER IV and V).
3. To approach the molecular characterisation and taxonomic identification of the parasite causing amoebiasis in *S. senegalensis* (CHAPTER V).
4. To develop and evaluate the performance of specific diagnostic techniques for the recognition of *E. Piscium* using In Situ Hybridization and Polymerase Chain Reaction techniques (CHAPTER VI).
5. To assess the distribution of the parasite within the host and to obtain preliminary epidemiological data on the distribution of the disease in different sole farms (CHAPTER VI).
6. To study the horizontal transmission and the establishment of *E. piscium* in healthy fish, during experimental infections using a cohabitation model (CHAPTER VII).

CHAPTER III: MATERIALS & METHODS

3.1. Disclaimer

3.2. Source material:

3.2.1 Material from clinical cases received in Servei de Diagnostic en Peixos (SDPP)

3.2.2 Samples of symptomatic fish from different farms where the disease was detected

3.2.2. Asymptomatic fish sampled randomly from different sole farms

3.2.4. Samples from experimental infections

3.3. Post-mortem techniques

3.3.1. Histological techniques:

3.3.1.1. Optic microscopy

3.3.1.2. *Transmission electron microscopy (TEM):*

3.3.2. Molecular techniques

3.3.1.1. Extraction material

3.3.1.2. Amplification and purification of DNA samples

3.1. Disclaimer

Due to confidentiality agreements with the customers, we regret we cannot indicate the origin or the geographical location of the farms where samples were obtained or shipped to the *Servei de Diagnostic en Peixos* (SDPP) of Universitat Autònoma de Barcelona.

3.2. Source material

The biological material used in the present work was obtained from sole specimens from the following sources:

- Material from clinical cases received in SDPP.
- Samples from fish in different farms where the disease was detected with visible lumps on the muscle (henceforth called symptomatic fish).
- Randomly sampled fish from different sole farms without external lesions (henceforth called asymptomatic sole).
- Samples of experimental infections.

3.2.1. Material from clinical cases received in Servei de Diagnòstic en Peixos (SDPP):

Between 2008 and 2011, a total of 42 samples containing organs or whole symptomatic fish (table 3.1), fixed in 10% buffered formalin, were referred to the SDPP of Universitat Autònoma de Barcelona for histopathological examination. These samples include pieces of muscular tissue and internal organs (heart, liver, spleen, stomach, intestine, kidney and gonads) or fish fixed “in toto” at the same farms. In most cases, bumps on the body and lesions in the internal organs were recorder by the staff of the farms.

Table 3.1: Cases related to outbreaks of symptomatic soles remitted to SDPP between the years 2008-2011. N: number of fish

Case number	Date	N	Remitted samples
189/08	04/07/08	2	Muscle, gills and internal organs
216/08	18/07/08	1	Muscle, gills and internal organs
217/08	18/07/08	1	Muscle, gills and internal organs
218/08	18/07/08	1	Muscle and intestine
282/08	09/09/08	1	Muscle and internal organs
288/08	09/09/08	1	Muscle and internal organs
289/08	09/09/08	1	Muscle and internal organs
361/08	02/12/08	1	Muscle, gills and internal organs
16/09	08/09/08	1	Muscle and internal organs
17/09	08/09/08	1	Muscle and internal organs
18/09	11/09/08	1	Muscle and internal organs
20/09	29/09/08	1	Muscle and internal organs
21/09	30/09/08	1	Muscle and internal organs
96/09	23/01/09	1	Muscle and internal organs
97/09	27/01/09	1	Muscle and internal organs
119/09	15/04/09	5	Entire fish
120/09	15/04/09	1	Entire fish
122/09	15/04/09	1	Muscle and internal organs
183/09	17/04/09	1	Internal organs
258/09	19/08/09	2	Entire fish
56/10	03/02/10	1	Muscle and internal organs
62/10	03/02/10	1	Muscle and internal organs
328/10	16/09/10	1	Muscle, gills and internal organs
329/10	16/09/10	1	Muscle, gills and internal organs
331/10	16/09/10	1	Muscle, gills and internal organs
335/10	16/09/10	1	Internal organs
339/10	16/09/10	1	Muscle, gills and internal organs
352/10	16/09/10	1	Internal organs
186/11	25/08/11	5	Muscle and internal organs
187/11	25/08/11	2	Muscle and internal organs

3.2.2. Samples of symptomatic fish from different farms where the disease was detected:

Symptomatic Senegalese soles were sampled in different facilities where outbreaks of the disease were detected. These samples were taken in 2011 (October, November, December) and 2012 (February) (Table 3.2). Fish were kept on ice and immediately killed by spinal severance, measured and weighted. Samples of muscle, liver, spleen, kidney, digestive tract (stomach, anterior intestine and posterior intestine), heart and gills were dissected and fixed in 10% buffered formalin for histopathological studies. Parallel subsamples of muscle and intestine were also preserved in 90% ethanol for molecular analyses. Some subsamples of muscle and intestine were also fixed in glutaraldehyde to electron microscopic analyses.

Table 3.2: Samples related to symptomatic soles in different facilities between the years 2011 and 2012. N: number of fish; MTL: Mean Total Length (cm); MTW: Mean Total Weight (g); SD: Standard Deviation.

Sample	Farm	Date	N	MTW ± SD	MTL ± SD
5	3	05/10/11	30	34,47 ± 19.72	14,44 ± 1.86
6	4	15/11/11	12	145,33 ± 54.40	22,75 ± 2.49
9	4	05/02/12	10	159,6 ± 59.51	23,72 ± 3.23

3.2.3. Asymptomatic fish sampled randomly from different sole farms:

During 2009 to 2012 (Table 3.3) a survey was performed in apparent uninfected soles (asymptomatic sole) from different sole facilities. In some of these farms the infection was previously recorded but in other farms was never recorded. In any case, all the asymptomatic fish were stratified random probability sampled from tanks without any signs of disease. After the capture, fish were kept on ice and immediately killed by spinal severance, measured and weighted. Fish were dissected to find potential macroscopical lesions and samples of muscle, liver, spleen, kidney, digestive tract, heart and gills were excised and fixed in 10% buffered formalin for histopathological studies. In the year 2011, parallel subsamples of muscle and intestine preserved in 90% ethanol were also obtained for molecular studies

Table 3.3: Random samples related to asymptomatic soles in different facilities between the years 2009-2011. N: number of fish; MTL: Mean Total Length (cm); MTW: Mean Total Weight (g); SD: Standard Deviation.

Sample	Farm	Date	N	MTW ± SD	MTL ± SD
1	1	20/03/09	15	-	-
2	2	15/04/09	15	41.13 ± 10.17	15.23 ±1.16
3	1	19/08/09	15	28.87 ± 5.62	13.33 ±1.01
4	2	28/06/10	10	32.70 ± 9.99	12.65 ± 1.31
4	2	28/06/10	10	68.56 ± 5.03	17.95 ± 0.83
5	3	05/10/11	10	52.00 ± 23.28	15.19 ± 1.41
6	4	15/11/11	15	55.53 ± 22.36	16.43 ± 2.51
7	2	16/11/11	20	107.88 ± 19.67	19.58 ± 1.16
8	3	10/12/11	20	17.23 ± 6.33	11.39 ± 1.37
9	4	05/02/12	10	74.4 ± 31.09	18.25 ± 2.30
10	5	24/04/12	20	-	-

3.2.4. Samples from experimental infections:

Experimental infections were carried out at the Estación de Ciencias Mariñas de Toralla (ECIMAT), Universidad de Vigo, from April to August 2012, performed by cohabitation between symptomatic and healthy fish.

The characteristics and conditions of the experimental infection are detailed in the corresponding chapter in order to make it more comprehensive (Chapter VII).

Table 3.4: Different samplings performed during the experimental infections between the April and August of 2012. MTL: Mean Total Length (cm); MTW: Mean Total Weight (g); N: number of fish; p.e.: post-exposure; SD: Standard Deviation; T: Temperature.

Sampling	Time (days p.e.)	Water T (Cº)	Fish Group	N	MTW ± SD	MTL ± SD
0	0	13,8	Initial Control	25	12.44 ± 4.36	10.40 ± 2.36
			Donor	10	128.19 ± 67.19	21.90 ±3.12
1	15	13,8	Receiver	13	12.75 ± 4.27	10.70 ±1.04
			Donor	5	185.40 ± 81.56	24.50 ± 2.98
2	21	15,5	Receiver	14	13.10 ± 4.82	9.79 ± 1.30
			Donor	5	152.38 ± 45.35	23.60 ± 2.10
3	30	16,4	Receiver	15	10.10 ± 2.68	9.63 ± 2.13
			Donor	5	145.95 ± 76.29	22.90 ± 2.97
4	60	17,2	Receiver	15	9.11 ± 3.19	10.27 ± 1.16
			Donor	5	141.37 ± 34.52	23.20 ± 2.39
5	120	20	Receiver	14	9.30 ± 4.41	10.42 ± 1.38
			Donor	10	161.53 ± 51.92	24.95 ± 2.82
			Control	13	14.47 ± 6.14	13.39 ± 4.42

3.3. Post-mortem techniques:

3.3.1. Histological techniques:

3.3.1.1. Optic microscopy:

Samples fixed in 10% buffered formalin were processed by routine histology (Table 9.1; Annex 9.1.1). Sections of 4μm were performed using a microtome Microm HM330. They were stained with haematoxylin and eosin (H&E) stain. Some of these sections were stained with Giemsa, Gram stains and Periodic Acid Schiff (PAS). The protocols of dehydration, paraffin embedding and staining with H&E, gram, Giemsa and PAS are described in Table 9.2,9.3, 9.4 and 9.5, respectively (Annex 9.1.1)

All histological preparations were observed in Olympus BSH2. The images were captured by the camera ProgRes C3 and the program ProgRes (Capture Pro 2.7 (Jenoptik, Germany).

3.3.1.2. Transmission electron microscopy (TEM):

Small pieces of muscular lesions were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide (Table 9.6; Annex 9.1.2). After the postfixation, they were embedded in Eponate 12™ resin (Ted Pella Inc., Redding, CA, USA) and polymerized at 60℃ for 48 h (Table 9.6; Annex 9.1.2). Semi-thin sections (1 μm) were obtained with a Leica ultracut UCT microtome (Leica Microsystems GmbH, Wetzlar, Germany). Ultra-thin sections (70 nm) were cut with a diamond knife (45°, Diatome, Biel, Switzerland), mounted on copper grids and stained with uranyl acetate and Reynolds solutions solutions (Table 9.7; Annex 9.1.2). Sections were observed with a Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) equipped with a Gatan ES500W Erlangshen CCD Camera.

3.3.2. Molecular techniques:

3.3.2.1. Extraction material:

Samples preserved in 90% ethanol were extracted using a silica-based commercial kit (High Pure PCR Template Preparation Kit, Roche). A piece of 5 mm of tissue fish was introduced in a nuclease-free 1.5 ml microcentrifuge tube and continued with the extraction protocol. Cells are lysed during the incubation of proteinase K in the presence of a chaotropic salt, which immediately inactivates all nucleases (the addition of ARNase increase the efficacy of this process). Cellular nucleic acids bind selectively to glass fibers and the following washes are used to remove the contaminating cellular components. Finally, low salt elution releases the nucleic acids from the silica fiber.

Water samples were fixed in acetone. The acetone dissolves the cellulose ester filters and, after a pre-treatment detailed in Table 9.8 (Annex 9.2), extraction is continued with the protocol for the isolation of tissue fish samples explained above.

3.3.2.2. Amplification and purification of DNA samples: Amplification products were analysed on 1% TAE agarose gels. When necessary, bands of interests were excised from agarose gels, purified with a clean-up kit (PureLink, Quick gel Extraction and PCR Purification Combo Kit, Invitrogen) following the manufacture's instructions. An area of the agarose gel containing the desired DNA fragment were excised using a clean disinfected scalpel. The gel slice containing the DNA fragment were weight using a scale sensitive to 0.001 g and placed into a nuclease-free 1.5 ml microcentrifuge tube. Solubilisation buffer, allowing efficient extraction of the DNA fragment, dissolves the excised gel pieces. Then, DNA is purified by a series of washes and centrifugations.

For cloning, fresh PCR products were ligated into a plasmid vector (PCR4-TOPO, Invitrogen Paisley, UK), by which was used to transform competent *E. coli* using a kit (TOPO-TA for sequencing, Invitrogen), following the manufacture's instructions. The PCR product is ligated to the vector at the 3 'end. The vector contains a lethal gen to *E. coli* but, by binding with a PCR product, its expression is inhibited. Thus, cells that do not contain the

recombinant vector die on plates. This system allows only the growth of cells with the PCR fragment inserted.

CHAPTER IV. HISTOPATHOLOGICAL CHARACTERISATION OF A NEW PATHOLOGY AFFECTING CULTURED SENEGALESE SOLE

4.1. Introduction:

Since 2008, a previously undescribed pathological condition is affecting the culture of *Solea senegalensis* in some farms of the European Atlantic coast. This condition is characterised by low mortality but an apparently high morbidity. It appears to be a seasonal problem, mainly emerging with the rise of temperature in spring and summer, but it can also appear in cold seasons. Affected fish are usually juveniles between 20 and 40 g, although larger fish, up to 250 g can be affected. Diseased fish usually display a lethargic behaviour with sporadic and erratic swimming. Some of these fish show protuberances arising from the skin surface and in some cases these lesions can be ulcerated. These lumps appear to progress quite slowly according to the observations of the farms staff. Preliminary attempts to identify the origin of the problem using basic diagnostic techniques, such as scrapings, imprints, and microbiology from the lesions were unsuccessful. The absence of clear evidences of the aetiology of the disease, lead the affected fish farms to submit samples for histopathological study to the SDPP, in order to get a deeper approach about the aetiology of the problem. Histopathology is a widely used tool as reliable technique that allows recognising a varied range of diseases. It is also particularly useful as a first step in the diagnosis of a disease of unknown origin, since most parasite groups can be identified in stained sections (Bruno et al., 2006).

This study aims to describe the main macroscopical lesions associated to this new disease in Senegalese sole as well as to characterise the histopathological alterations observed in the different organs. Moreover, a histological description of the causative agent and its presumptive identification are also elucidated.

4.2. Materials and Methods

4.2.1. Source material:

A total of 42 samples from different fish farms in the north-Atlantic area were sent to the “Servei de Diagnòstic Patológic de Peixos” of Universitat Autònoma of Barcelona between 2008 and 2011 for their histopathological evaluation (see table 3.1, Chapter III (M&M)). Farm staff drew blood from the caudal vein in 5 of

these fish and blood smears were stained with a quick stain (Diff-quick®), following the manufacture's instructions. Fish were dissected, and samples of different organs or entire fish (see table 3.1, Chapter III (M&M)) were fixed in 10% formalin buffer.

Three groups of 15 apparently asymptomatic Senegalese sole were sampled randomly in two affected facilities at the same area in March, April and August of 2009 and two groups of 10 fish also in July 2010 (see table 3.3 of Chapter III (M&M)). Fish were immediately killed by spinal severance, measured and weighed, and they were fixed "in toto" in 10% formalin buffer.

4.2.2. Post-mortem techniques:

Histological techniques: Samples were processed by routine histology (see M&M) and stained with haematoxylin and eosin (H&E) stain. Some of these sections were stained with Giemsa, Gram, or Periodic Acid Schiff (PAS) stains. The protocols of dehydration, paraffin embedding and staining are described in Annex 9.1 (Chapter IX).

4.2.3. Rates determination:

The presence or absence of the parasite in the different organs was studied. An individual or a tissue was considered infected when some stage of the parasite was found within the tissue studied. This allowed obtaining a prevalence value (P) of infection, defined as the proportion between the numbers of infected individuals (n) of the total number of tested individuals (N).

$$P = (n/N)*100$$

4.3. Results

Macroscopical observations from affected fish included protuberances in the skeletal muscle, often noticeably from the skin surface (Fig 4.1A). When these lumps were small, they were usually found along the entire base of the dorsal fin and / or ventral fin, and they were observed both on the ocular (Fig. 4.1B) and the blind side (Fig. 4.1C). Typically, they did not present in a specific location, but

they could be found throughout the muscle and sometimes reach a considerable size (Fig. 4.1A). In severe cases, the lesion may occupy much of the muscle volume and, in some cases, could ulcerate outwards (Fig. 4.1D). Lumps were also observed protruding from the visceral cavity (Fig. 4.1E).

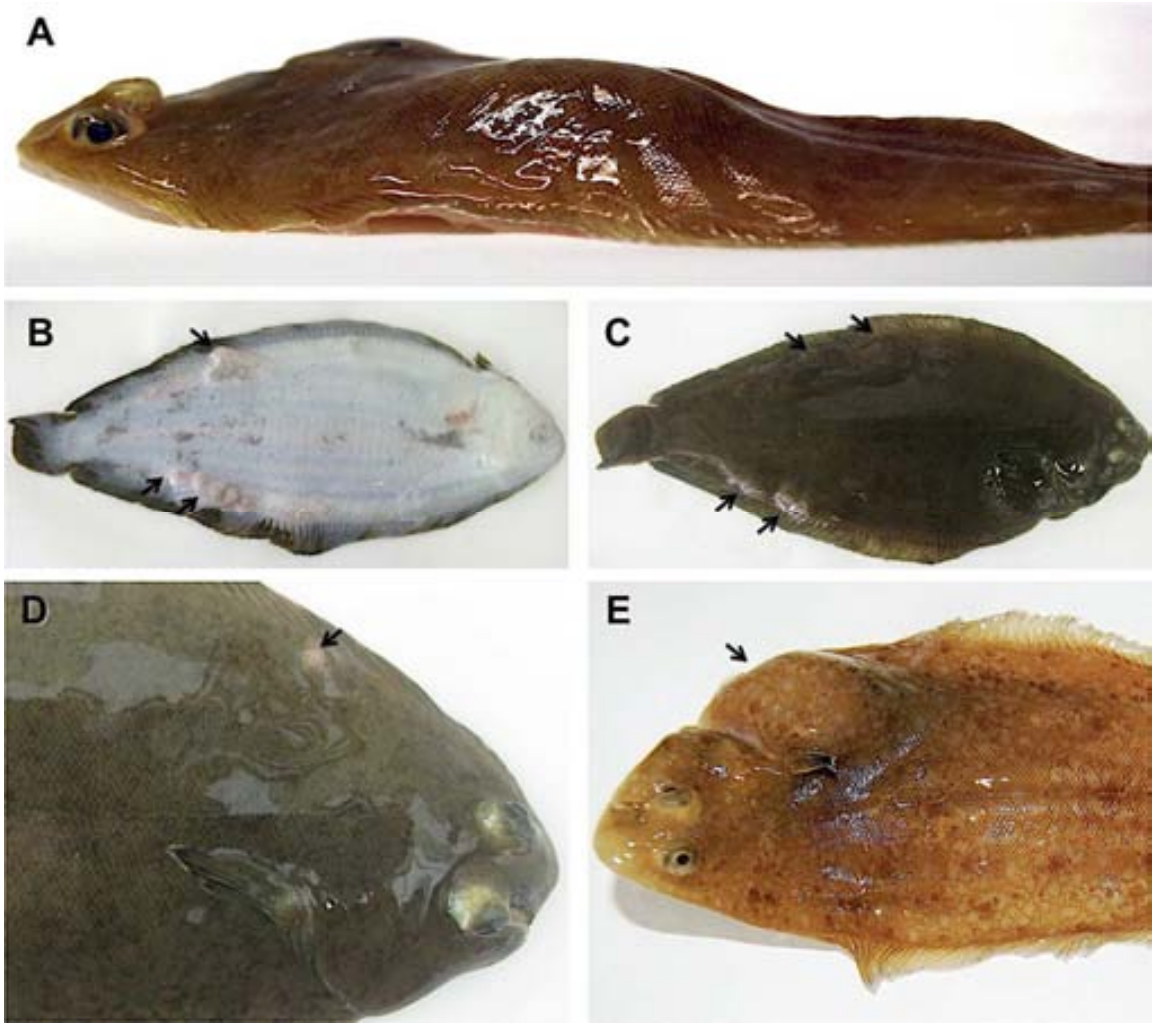

Figure 4.1: Affected Senegalese sole with macroscopical lesions in different areas: A, Senegalese sole with lumps in the muscle that are evident on the skin surface; B-C, Sole blind (B) and ocular (C) sides where small lumps (arrows) along the entire base of the dorsal and ventral fins are observed; D, ulcerated lesion (arrow) on the ocular side; E, *S. senegalensis* with a large lump can be noticed at the visceral cavity.

Figure 4.2: Liquefactive lesions in different organs of Senegalese sole: A, Delimited lesion in muscle. Note the soft and liquefied consistency; B, Small lesions with purulent aspect within muscle, distributed following a miliary pattern, can be observed; C, Small nodules protruding throughout the spine and protruding into the visceral cavity; D, Visceral cavity with an extensive lesion in liver, which is ruptured and draining outside; E, Multiple whitish confluent lesions in a heart; F, Visceral cavity where small nodules (arrow) within the intestine can be observed.

Macroscopic lesions consisted on nodules with an abscess-like aspect, with a soft and liquefied consistency (Fig. 4.2A). Sometimes, although no lumps were observed at the surface in some fish, when dissected, small granulomas of liquefied material were noticed between the muscle fibers following a miliary distribution (Fig. 4.2B). Occasionally, granulomas were found throughout the spine and penetrating into the visceral cavity (Fig. 4.2C).

Lesions were not only found in muscle but also in other organs such as: liver (Fig. 4.2D), digestive tract, heart (Fig. 4.2E), kidney, and ovary of affected fish. In the digestive tract, they appeared to have a harder consistency (Fig. 4.2F).

Histological sections of the nodules revealed the presence of chronic inflammatory areas displaying a relatively common pattern in all of the affected tissues, although some differences between tissues and organs could also be found. These inflammatory areas presented a large core of homogeneous necrotic tissue surrounded by fibroblasts and macrophages. The presence of granulocytes in these lesions was usually low. No organised fibrous capsule was detected in the majority of the samples. The width and extent of the layer formed by macrophages and fibroblasts seemed to be tissue-dependent, usually more intense in the kidney, moderate in the digestive tract and heart and low in the liver and muscle.

The inflammatory response in muscle was usually weak, with some infiltration of inflammatory cells between muscle fibres (Fig. 4.3A). However, in occasions, when injuries were more severe and lesions were occupying much of the muscle mass, a thicker layer of fibroblasts could be seen around them (Fig. 4.3B). Between the external inflammatory reaction and the necrotic tissue, a zone with abundant macrophages harbouring spherical, plasmodial-like organisms was observed in almost all cases (Fig. 4.3C&D). In some samples, extensive necrosis and diffuse inflammatory areas were also found in muscle (without a clear granulomatous response) with multiple plasmodial organisms between the muscle fibres (Fig. 4.3E&F). Sometimes, similar organisms were observed within subcutaneous tissue and close to the vertebral column. These organisms, measuring about 2 μm in diameter, were gram-negative (Fig. 4.4A) and showed one single nucleus and one or two basophilic bodies. The nuclei appeared dark blue in Giemsa stains (Fig. 4.4B) and their cytoplasm was characterised by the presence of PAS-positive granules (Fig. 4.4C&D). They were usually observed

within the cytoplasm of macrophages or in extracellular position.

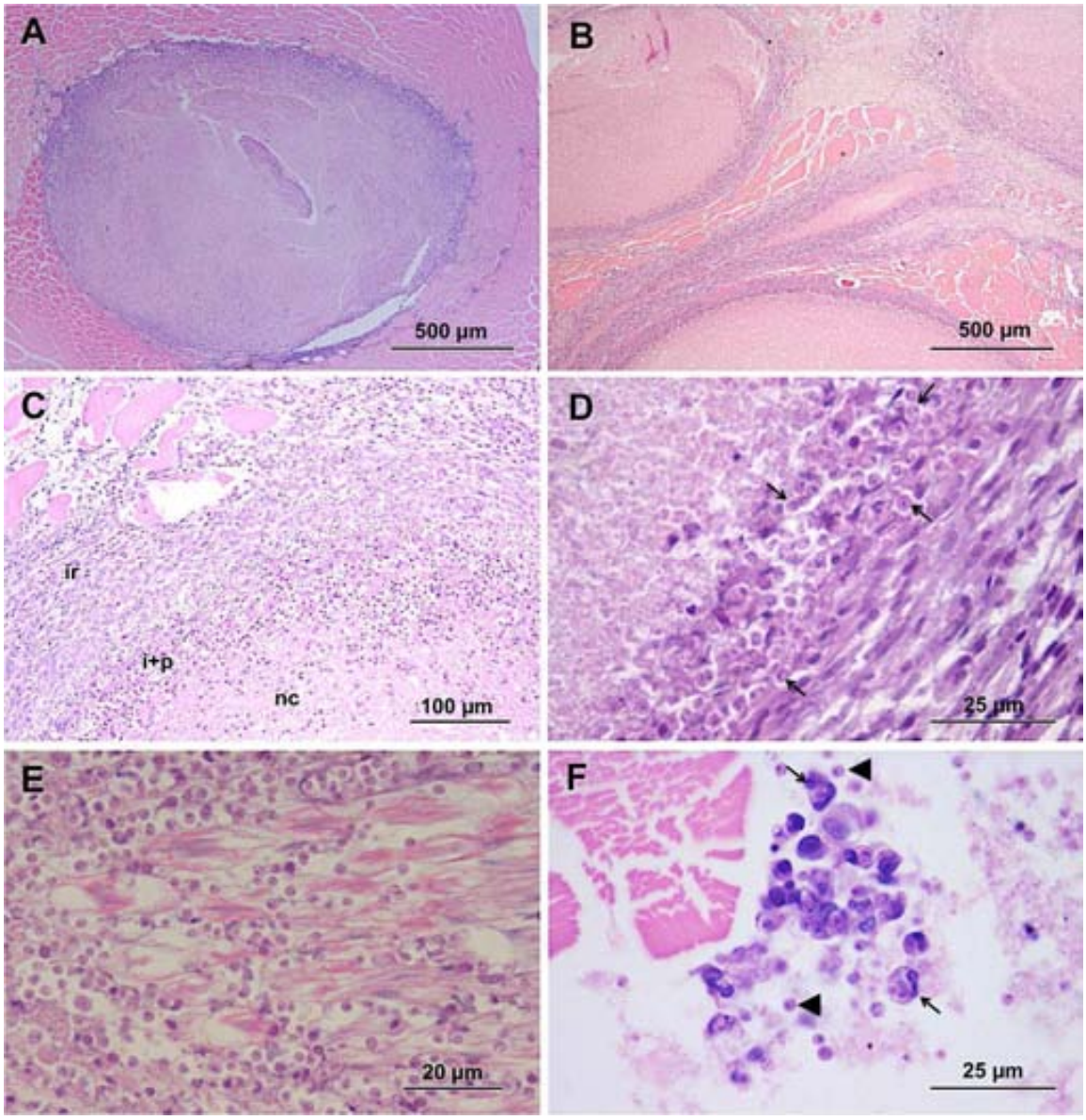

Figure 4.3: Histological lesions in muscle of Senegalese sole (Stain: H&E): A, granulomatous inflammatory response in muscle with a necrotic core and an external inflammatory layer surrounding it; B, extensive inflammatory areas in muscle with a large band of inflammatory reaction (*); C, external part of the lesion where several layers are visible: (nc) necrotic core, (i+p) inflammatory area with parasites and (ir) external inflammatory reaction, D, detail of the external part of the lesion. Notice the round organisms (arrows) inside macrophages in the periphery of a lesion in muscle. E, extensive necrosis with inflammatory infiltrate (macrophages) and multiple plasmodial organisms between muscle fibres are observed; F, parasitic organisms within macrophages among muscle fibres (arrows). Notice also the presence of extracellular organisms (arrowhead).

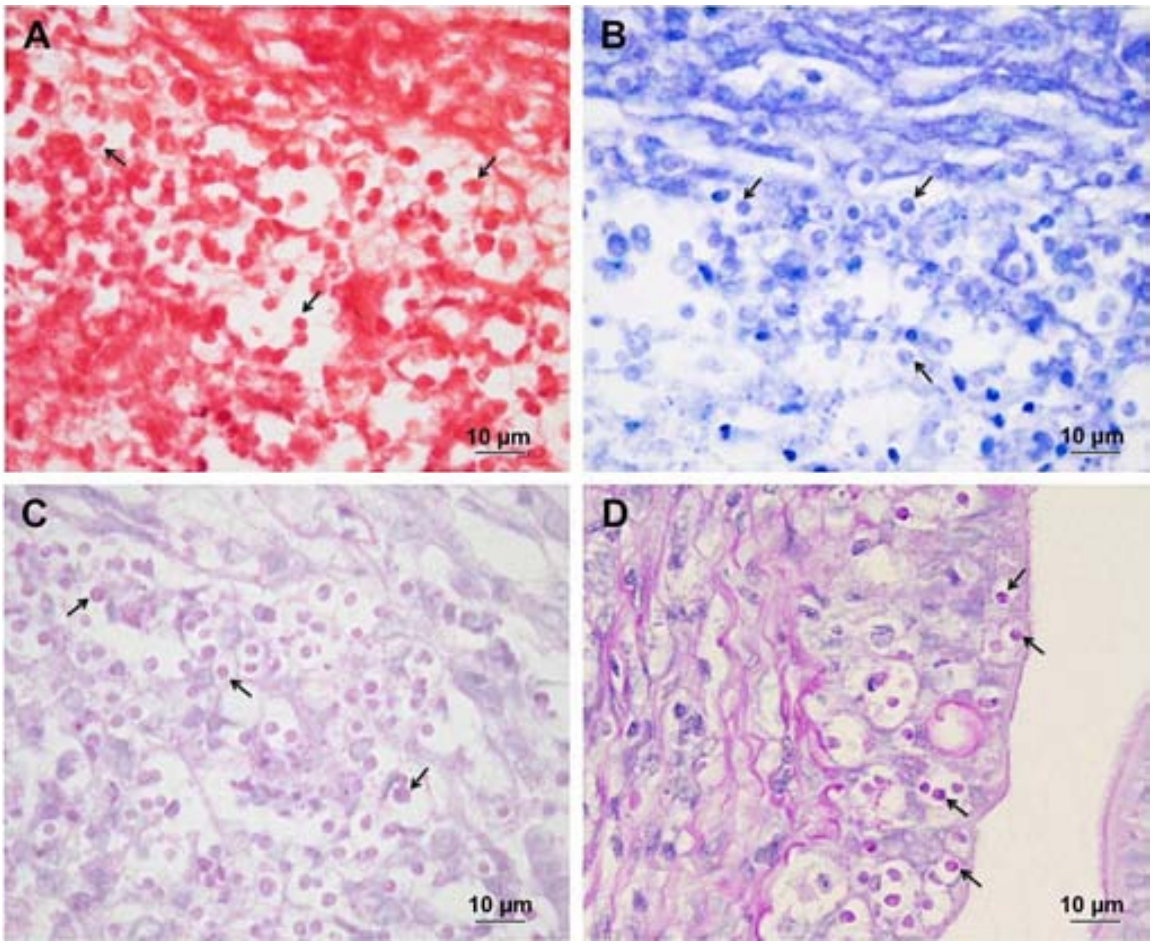

Figure 4.4: Detail of the parasitic organisms in sections using different stains: A-C, Detail of the parasites (arrows) surrounding a granuloma in muscle stained with Gram (A), Giemsa (B) and with PAS stain (C); D, parasites (arrows) with intense PAS-positive granules within the cytoplasm, located inside phagocytic cells or parasitophorous vacuoles within the intestinal mucosa (PAS stain).

The heart was the second most affected organ, in terms of prevalence of lesions (Table 4.1). Confluent lesions in the heart were located mainly at the pericardium (Fig. 4.5A), but also within the myocardium (Fig. 4.5B) and very extensive lesions, invading virtually the entire organ, were also frequently observed (Fig. 4.5C). As in muscle, multiple organisms were observed surrounding the granulomas, not only within macrophages but also extracellular organisms (Fig. 4.5D), as observed in the muscle.

Table 4.1: Prevalence of the histological lesions (PL) and the organisms within the intestine (without lesion) (PO) of the symptomatic and asymptomatic sampled soles. N: number of fish; Ms: muscle; Lv: liver; Ht: heart; Kd: kidney; Ov: ovary; In: intestine.

Date	N	PL (%)						PO (%)
		Ms	Lv	Ht	Kd	Ov	In	In
Symptomatic fish (sent to SDPP)								
2008 -2011	42	65,85	26,83	36,59	9,76	7,32	31,7	63,41
Asymptomatic fish sampled								
20/03/09	15	0,00	6,67	13,33	6,67	0,00	60,0	80,00
15/04/09	15	0,00	0,00	0,00	0,00	0,00	6,67	6,67
19/08/09	10	0,00	0,00	0,00	0,00	0,00	0,00	13,33
28/06/10	10	0,00	0,00	0,00	0,00	0,00	0,00	10,00
28/06/10	10	10,00	0,00	0,00	10,00	0,00	0,00	60,00

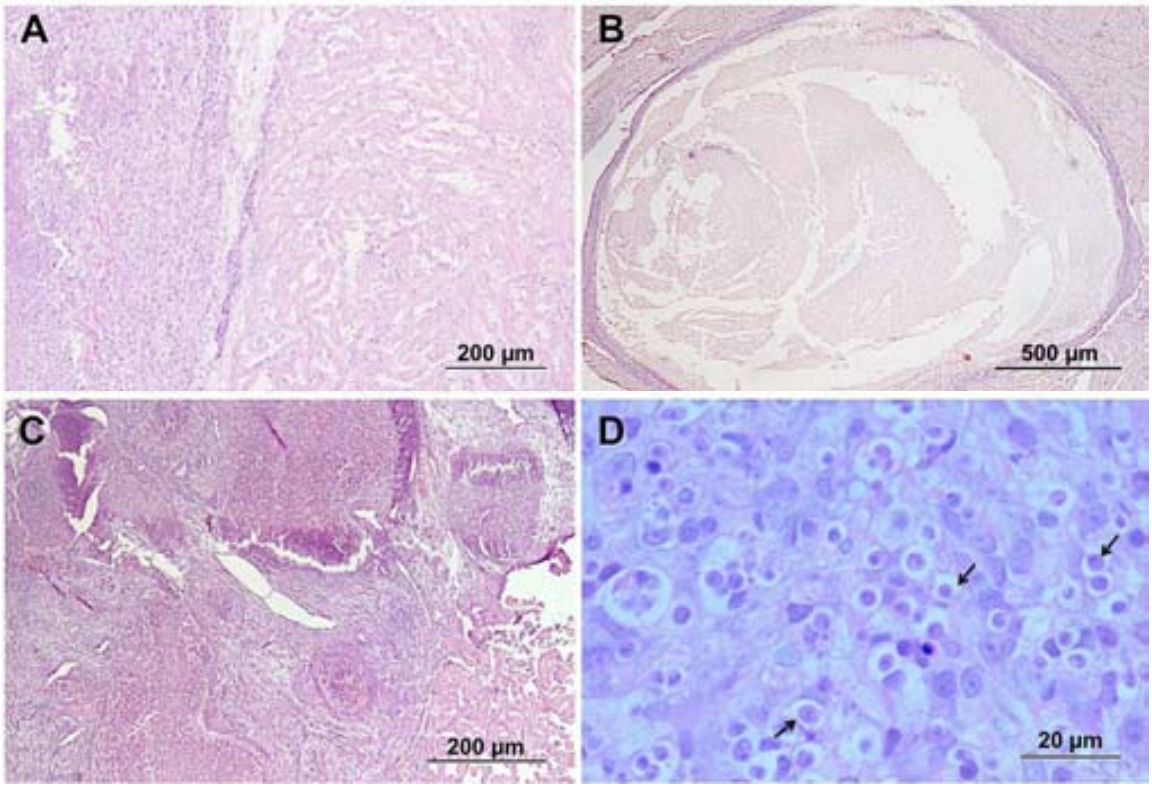

Fig. 4.5: Histological lesions in heart of Senegalese sole (Stain: H&E): A, granuloma in pericardium, B, granulomatous inflammatory reaction in miocardium with a necrotic core and external inflammatory reaction; C, extensive lesions with multiple granulomas, invading virtually the entire organ; D, organisms within macrophages (arrow) and extracellular organisms among heart tissue (arrowheads).

Granulomas located within the liver parenchyma had the same granulomatous features explained above, but in some cases, an exudative response was found in the external layer of the inflammatory lesion (Fig. 4.6A). In some cases, granulomas with parasites and intense inflammatory reaction were observed in the perivisceral serosa, even causing the destruction of the transversum septum and adhesions between liver and heart (Fig. 4.6B). Occasionally, extensive necrosis and diffuse inflammatory areas were also found in liver, with multiple plasmodial organisms within the hepatic parenchyma (Fig. 4.6C). Sporadically, clusters of microorganisms could be observed in this tissue, without apparent inflammatory reaction, in what could be interpreted as the early steps of a granuloma formation, sometimes related to blood vessels (Fig. 4.6D). However, blood samples had in all cases a normal appearance and no plasmodial organisms were observed.

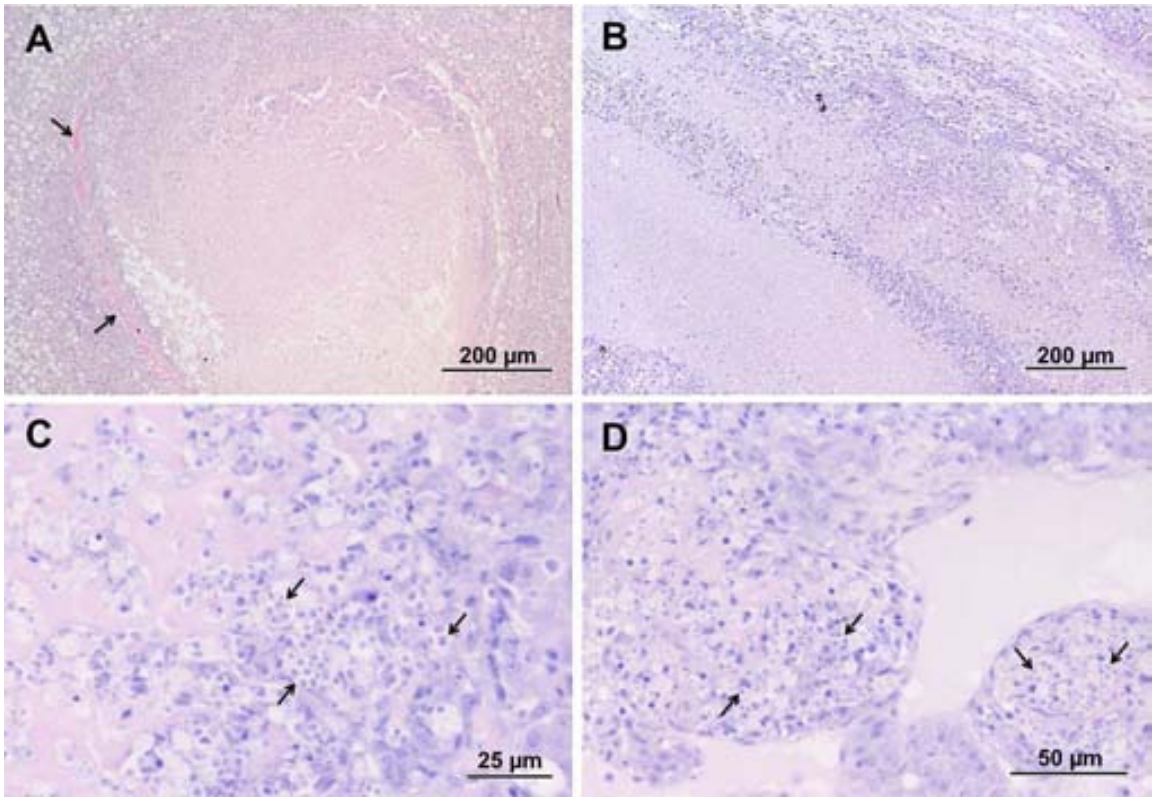

Fig. 4.6: Histological lesions in liver of Senegalese sole (Stain: H&E): A, granuloma in liver. Notice the exudative response in the periphery of the lesion (arrows); B, granulomas in the connective tissue between the liver and the heart; C, extensive necrotic lesion in liver with multiple plasmodial organisms within the liver parenchyma (arrows) and exudative response; D, Groups of organisms (arrows) in the vicinity of the blood vessels in liver.

In the kidney, the inflammatory reaction consisted of several layers of inflammatory cells and calcified material inside the necrotic core (Fig. 4.7A). In these cases, the detection of the parasites surrounding the lesion was difficult. Commonly, the more severe the inflammatory reaction associated with the granuloma was, the more difficult that resulted the detection of the parasites.

Nodules located in the submucosa of the intestine were also observed, often associated with a moderate inflammatory reaction (Fig. 4.7B&C).

The muscle was the highest prevalence tissue affected among symptomatic soles, followed by the heart, the intestine and the liver (Table 4.1). The kidney and the ovary were the organs presenting the lowest prevalence of lesions (Table 4.1). No lesions were found in spleen.

In addition, similar parasitic organisms were also observed in the mucosal epithelium of the digestive tract, inside phagocytic cells or apparently in parasitophorous vacuoles within the enterocytes (Fig. 4.7D). In these cases, they could be found isolated or, more often, grouped together in small clusters, and usually no inflammatory response was observed surrounding them. These amoeboid organisms were also sometimes observed at the submucosa, frequently in association with clusters of macrophages. Smaller basophilic cell formations (Fig. 4.7E) were occasionally observed. Infected intestines showed different degrees of damage, usually correlating with the infection intensity. Only in the most intense cases, the intestinal mucosa was clearly affected, showing a vacuolated epithelium (Fig. 4.7F) and an undulating aspect (Fig. 4.7G). In some of these cases there was an increase in the normal gut thickness with areas where the presence of lipid droplets within enterocytes was clearly different from the normal pattern. The presence of macrophages at the submucosa, and other inflammatory cells like lymphocytes in both mucosal and submucosal layer, were observed in association with the presence of these amoeboid organisms. In the most severe lesions, destruction of intestinal mucosa and atrophy of the intestinal villous were observed (Fig. 4.7H).

Organisms in the intestinal epithelium were detected in 63,41% of the symptomatic soles. Remarkably, the same organisms were also detected in the intestinal epithelium of asymptomatic fish, without causing apparent lesions. The prevalence of these organisms in asymptomatic fish varied between 6% and 80% (table 4.1), depending on the farm sampled. Furthermore, small granulomas

present in some organs, such as heart and muscle, were also detected from asymptomatic fish when examined by histology (Table 4.1).

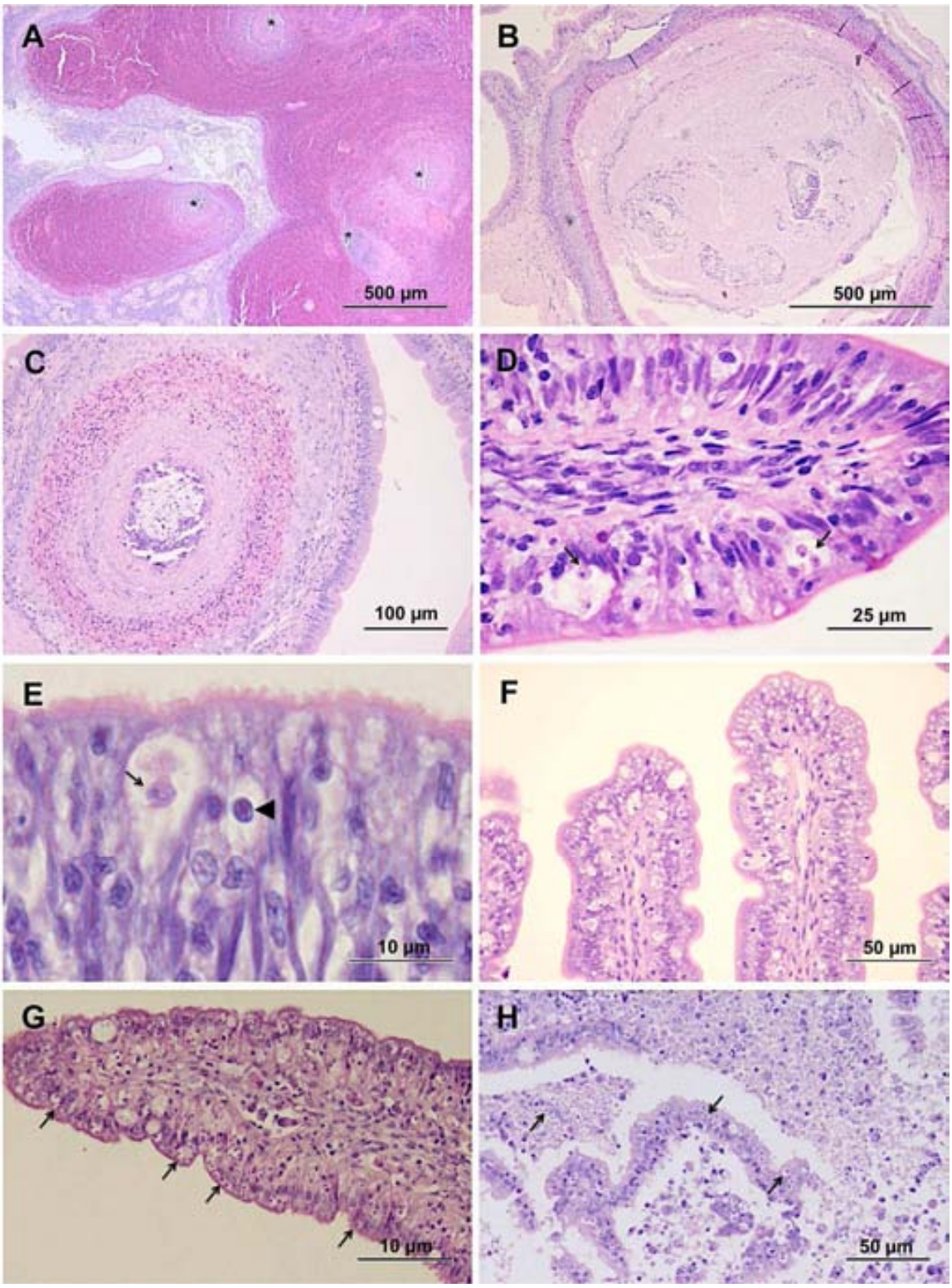

Figure 4.7: Histological lesions in kidney and intestine of Senegalese sole (Stain: H&E): A, granulomatous inflammatory reaction in kidney with multiple layers of inflammatory infiltrate and fibroblasts. Notice the calcified necrotic cores (*), B, granuloma in the intestine submucosa; C, early granuloma within the intestine submucosa; D, organisms inside phagocytic cells or parasitophorous vacuoles within the intestinal mucosa (arrows), E, organisms inside phagocytic cells or parasitophorous vacuoles within the intestinal mucosa (arrow) and smaller basophilic organism (arrowhead), F, vacuolated intestine with parasitic organisms within the epithelium; G, several organisms (arrows) within the intestinal epithelium. Notice the undulating aspect of the intestinal mucosa; H, destruction of intestinal mucosa associated with parasitic organisms (arrows).

4.4. Discussion

The type of lesions described in the present work has not been previously described in sole. The analysis of the lumps observed in the muscle and in other organs reveals a granulomatous inflammatory reaction with a conspicuous liquefied necrotic core. Although this inflammatory reaction was similar in all affected tissue, differences among them were observed. In order to interpret this observation the effect of the time for the organisation of the inflammatory reaction should also be taken into account. Although these lesions can be very extensive, even in vital organs, they are almost always observed well circumscribed, preserving the integrity of the rest of the organ. This fact can partly explain the low mortality as well as the chronic course associated to this disease.

Granulomas are considered as circumscribed inflammatory processes associated to chronic course (Schulz and Trautwein, 1985). The cellular composition of granulomas and the number of the different involved cells depend on the causative agent. However, they share in common the concentric arrangement of the phagocytic cell layers surrounding the causative agent (Schulz and Trautwein, 1985). Most of them tend to a peripheral encapsulation with an outer concentric layer of connective tissue. Necrosis is the most common tendency in the inner layer, and it is considered abscess when this necrosis involves neutrophil granulocytes. (Schulz and Trautwein, 1985). In the case of fish, the definition of abscesses is also limited to circumscribed lesions composed

by polymorphonuclear cells surrounding areas of liquefactive necrosis (Reimschuessel, 2008). In the case of sole lesions, the main inflammatory cells are macrophages, and that fact suggests they could be granulomatous inflammation rather than abscesses. The granulomatous inflammation in fishes is mainly characterised by focal accumulations of macrophages surrounded by fibroblasts with a central core of necrotic debris (Reimschuessel, 2008). There are three types of necrosis: (1) the coagulation necrosis, characterised by maintaining cell structures moderately; (2) the liquefactive necrosis, characterised by a total loss of cellular structures; and (3) the caseous necrosis, which is a combination of the former. About the lesions described in the present work, the necrosis observed within the granulomatous responses corresponds to the liquefactive type, since there is not recognizable cell structure. Similar granulomatous inflammatory reactions are observed in the responses of fish to foreign bodies. One example of this process occurs in European smelt *Osmerus eperlanus*, where food-induced granulomas were described in the oral cavity surrounding cuticular fragments of dietary amphipods (Anders and Möller, 1987).

However, in the present pathology, the lesional profile indicates the existence of a widespread process with apparently infectious-parasitic origin, and the main pathogens to consider in this pathology are summarised in table 4.2.

Endoparasitic interactions with the host change, from the initial penetration of host tissues to multiplication and senescence of the parasites. This process can be rapid resulting in acute infections with potentially fatal outcomes or more chronic, with low or no mortality (Feist and Longshaw, 2008). The severity of the pathology often depends on the immune system of the host, the efficiency of the parasite and the importance of the affected organ and its function. Micobacteria are Ziehl-Neelsen positive bacteria that usually cause a sub-acute to chronic disease in both wild and captive fish (Chinabut, 1999). Micobacteriosis is characterised specially by the formation of granulomas in various organs (Ashburner, 1977; Beckwith and Malsberger, 1980; Gómez et al., 1993; Roberts, 2001; Zerihun et al., 2011), although asymptomatic infections may also occur (Colorni, 1992). In Senegalese sole, micobacteria causing myositis and focal necrosis in muscle and granulomatous reaction within internal organs have also been observed (Padrós per. obs). In these cases, the lesions caused by these bacteria are morphologically similar to those described in the present work,

nevertheless they may be distinguished by the presence in the later of a peripheral layer of plasmodial organisms around the lesions and the absence of Zielh-Neelsen positive bacteria.

Table 4.2: Main pathogens causing systemic granulomatous inflammatory response in fish. (References are indicated in the text).

	Parasite	Lesion	Differential diagnosis
Bacteria	Mycobacterium	Systemic granulomas	Zielh-Neelsen positive
Protist Flagellates	*Cryptobia iubilans* *Hexamita* spp.	Granulomatous gastritis and necrosis in various organs Granulomas in different organs	Trophozoites with 1 or many flagella and a kinetoplast within a mitochondrion
Apicomplexa	*Haemogregarine spp.*	Nodular lesions with clusters of macrophages	Organisms in white blood cells
	Cryptosporidium spp.	Intestine epithelium	Clusters of oocytes Extracytoplasmic stages lining the intestinal epithelium
Ciliates		Granulomas in different tissues and/or intestinal desquamation and ulceration extensive	Presence of cilia on the surface Macronucleus
Amoebae	Amphizoic amoebae (eg. *Hartmanella* sp.) *Entamoeba* sp.	Systemic and chronic granulomatous inflammatory response Granuloma in visceral organs and/or endocomensals in the intestine in intestine	Rounded shape Small nucleus with central nucleolus. Pas positive
X-cells		Pseudotumours	Extracellular organisms with size >20µm in diameter
DRIP	*Dermocystidium* sp.	Systemic nodular infection	
Microsporidia	eg. *Pleistophora* sp., *Tetramycra* sp. *Loma salmonae* *Ovipleistophora mirandellae*	Xenomas, myoliquefaction and severe inflammatory response in muscle Pericarditis and necrosis of musculature Oocyte atresia and interstitial inflammation	Spore formers
Metazoa Myxozoa	*Kudoa* sp. Enteromyxum sp.	Liquefacted response in muscle Intraepithelial stages	Spores formers and “cell-in-cell” stages

Helminth infections can also usually induce granulomatous inflammatory response by the host, attempting to encapsulate the parasite, irrespective of the organ infected (Sharp et al., 1992; Valtonen et al., 1994; Torres et al., 2002; Dezfuli et al., 2007). In advanced stages of encapsulation, the parasitic structures are difficult to distinguish inside. However, since parasite cells are observed surrounding the lesions, and considering their size and morphology, the main groups to consider in this pathology are protozoans of the phylum Sarcomastigophora such as Amoebae or Flagellates, members of the phyla Apicomplexa, Microspora, Mesomycetozoa, the so-called X cells, or metazoans belonging to phylum Myxozoa. These organisms include stages with plasmodial or amoeboid aspect in their developmental cycles and they may cause chronic inflammatory, necrotic or granulomatous responses in different organs. Some groups of parasites have unique features that allow their differential diagnosis by classical techniques such as histology; however, many of these features may not be easily identified in histology sections, which are typically 3 to 5 microns thick. In addition, the level of taxonomic detail available varies among the major groups (Bruno et al., 2006).

Granulomatous gastritis and infiltration of macrophages in the stomach and intestine, as well as necrosis of the liver and spleen, were described in systemic infections by the flagellate Cryptobia iubilans in cichlid fish (Yanong et al., 2004), and in salmonid species (Bahmanrokh and Woo, 2001). In general, hexamitid infections in the intestine are usually chronic, triggering the development of whitish granulomas in different organs (Woo, 2006). The trophozoites of these parasites present a single or many flagella, a kinetoplast (an organelle consisting of an agglomeration of DNA fibres within the mitochondrion) and most have a single nucleus (Dyková and Lom, 2007; Alvarez-Pellitero, 2008). The kinetoplast is often visible in preparations stained by Giemsa (Bruno et al., 2006). In our case, the apparent lack of flagella or kinetoplast in the observed organisms does not support the involvement of this group of parasites in the aetiology of the pathological condition.

Apicomplexans protozoan parasites are equipped with a special set of organelles, the apical complex, only visible by transmission electron microscopy, which is used for penetration into the host cells. Haemogregarines have been described in several studies parasitizing insects and molluscs, but also in fish

such as *Solea solea* (Laveran and Mesnil, 1901) or turbot (Kirmse, 1978) within the enterocytes and leucocytes. Nodular lesions with accumulations of macrophages and a liquefied core were described in turbot, *Psetta maxima,* related to the hematozoan apicomplexa *Haemogregarine sachai* (Kirmse, 1980) and affecting white blood cells. Similar lesions were also found in Atlantic mackerel, *Scomber scombrus,* (McLean and Davies, 1990) and in Gilthead seabream, *Sparus aurata,* (Paperna, 1979), also related to haemogregarines. Apicomplexans infecting fish blood cells are usually observed and identified in Giemsa-stained blood smears an in tissue sections (Bruno et al., 2006). However, in our studies we did not find parasites in white blood cells of affected fish. The parasitic cells, with an apparently intracytoplasmic location, may resemble *Cryptosporidium* oocysts due to their size and spherical morphology, but in this case they do not arise in the typical clusters (Alvarez-Pellitero et al., 2004). In addition, the extracytoplasmic stages lining the epithelium (Alvarez-Pellitero et al., 2004) have never been found in sole and they can be ruled out in the differential diagnosis.

X-cells are protozoan parasites observed in fish pseudotumours (Miwa et al*.,* 2004). They have been frequently described in flatfish species (Kent *et al.* 1988, Miwa et al., 2004) and share some characteristics of amoebic organisms (Watermann, 1982). In tissue sections X-cells can be observed as extensive clusters of polygonal cells with a large nuclei and a prominent nucleolus (Miwa et al., 2004; Freeman, 2009). Nevertheless, granulomatous lesions or abscesses-like lesions are not characteristic of these parasites. Furthermore, X-cells have not been reported as intracellular (Freeman, 2009) and the size, sometimes more than 20µm in diameter (Ito et al. 1976), is much larger than the organisms found in this work.

Dermocystidium sp. (Mesomycetozoa) infections in fish are occasionally characterised by a systemic nodular infection involving viscera (Allen et al. 1968, McVicar and Wootten, 1980; Landsberg and Paperna, 1992; Bruno, 2001) and they share some characteristics with the lesions observed in this study. However, features of this group are the presence of spores or thick-walled plasmodia (Bruno, 2001; Feist et al., 2001; Pekkarinen et al., 2003; Zhang and Wang, 2005), which were not found in infected sole.

Microsporidia are strictly intracellular spore-forming parasites in a wide variety

of hosts. They can manifest as xenomas, characteristic structures of these parasites, or as systemic infections similar to those found in sole. Examples of the later one are *Loma salmonae*, causing pericarditis and necrosis of musculature among other lesions (Hauck, 1984); *Pleistophora* or *Tetramicra*, which cause infections in muscle that result in myoliquefaction and severe inflammatory responses (Matthews and Matthews, 1980); or *Ovipleistophora mirandellae, which* causes oocyte atresia and interstitial inflammation (Wiklund et al., 1996). However, no characteristic features of Microsporidia such as spores were found related to the lesions of Senegalese sole.

Histological changes caused by parasite ciliates in severe cases can include intestinal desquamation and extensive ulceration, and the formation of granulomas in the deep tissues (Bruno et al., 2006). These parasites are ovoid with cilia on the surface and a macronucleus in the interior, features not shared by parasitic organisms found in the present study.

The histopathological alterations caused by some myxozoan infections are large and often depend on the stage of infection and host susceptibility. Some *Kudoa* spp. are known to cause a softened muscle texture after death of the fish. Although the vegetative stages may not show definitive diagnostic features, characteristics visible in routine sections, such as size, morphology, location in the host and host reaction, can provide clues for identification (Bruno et al., 2006). No characteristic features of Myxozoa such as “cell-in-cell” stages (Lom and Dyková 1999, Dyková and Lom, 2007) or spore formation were observed associated with lesions in Senegalese sole.

Amoebic infections characterised by granulomatous lesions have been described in goldfish (Voelker et al., 1977; Lom and Dyková, 1992; Steinhagen et al., 1993). Dyková and colleagues (1996) described a systemic chronic granulomatous inflammatory response associated to Amoeba-like organisms in goldfish, *Carassius auratus*. These organisms were also detected by light microscope in the periphery of the granulomas inside a parasitophorous vacuole. Recently, Palíková and co-workers (2012) described a similar pathology in Tenca, *Tinca tinca,* related to amoebae of the class Archamoeba. Other amphizoic amoebae, such as Hartmannella (Dyková et al., 1997), have been found associated with granulomatous changes in various fish species. Identification of amoebae in tissue sections can be difficult because frequently

they do not retain their characteristics after fixation and staining (Hoffman, 1999), but they usually appear as cells of variable, mostly rounded shape with relatively small nuclei with a central nucleolus (Dyková and Lom, 2007), and some of them can be PAS-positive (Bruno et al., 2006).

The granulomatous lesion in different organs and especially the presence of similar parasitic organisms in the intestine of symptomatic and asymptomatic fishes may suggest the possibility that these organisms could be endocommensals in the digestive tract, and under specific conditions, these parasites can break the gastrointestinal barrier and extend to other parts of the body of the host. However only the severest cases could develop into the death of the fish. In this sense, endocommensal amoebae belonging to the genus *Entamoeba* have been described from the digestive tract of various fish species (Dyková, 2008). Most Entamoebids have two stages in their life cycles: trophozoites and cyst stages (Silberman et al., 1999). The smaller basophilic cell formations within the digestive tract of Senegalese soles could correspond to developing forms of these amoeba-like organisms. Although this disease does not appear to produce significant clinical effects or mortality, damage to the intestinal epithelium may contribute to cause poor feeding, weakness, and increased susceptibility to other infections or interactions with other pathogens.

According to the histopathological studies, our observations strongly suggest Amoebae as the putative etiological agent of sole infections, however the final identification of these organisms should be made after a more accurate study of the morphological characteristics of the organism and also by a detailed molecular characterisation (see Chapter V).

CHAPTER V. *ENDOLIMAX PISCIUM* SP. NOV. (AMOEBOZOA), CAUSATIVE AGENT OF SYSTEMIC GRANULOMATOUS DISEASE OF CULTURED SOLE (*SOLEA SENEGALENSIS)*

5.1 Introduction:

Recently, systemic inflammatory lesions were described in cultured Senegalese sole, *Solea senegalensis* (Constenla and Padrós, 2010). These lesions were characterised by lumps in the muscle, often noticeable evident from the skin surface, which make the fish unmarketable. The condition was found to respond to the presence of large numbers of minute spherical, plasmodial protozoans at the periphery of granulomatous lesions, which were most evident in the skeletal muscle, but also present in the digestive tract, liver, heart and kidney (see CHAPTER IV). The differential diagnosis based on histopathological studies in this previous work, initially pointed to a presumptive parasitosis due to amoeba, the so-called “X-cells” (Freeman, 2009), or stages from an unknown amitochondriate organism. Although no morphological unambiguous characters were found to confirm the etiology of the disease, most of the presumptive data suggested that amoeba could be the causative agent.

Amoebic infections involving granulomatous inflammatory lesions and abscesses can affect different animal and human organs, especially the liver and the brain (Candreviotis, 1977; Visvesvara et al., 1993; Riestra-Castaneda et al., 1997). However, systemic amoebiasis have seldom being reported in fish and the amoebae involved have not been fully characterized (Nash et al., 1988). This notwithstanding, a systemic granulomatous infection by a possibly related amoeba-like organism was described in goldfish, *Carassius auratus* (Voelker et al., 1977; Lom and DykovÁ, 1992; Steinhagen et al., 1993) and recently also in Tench, *Tinca tinca* L. (Palíková et al., 2012). Ultrastructurally, amoeba-like organisms found in goldfish and in tench show a vesicular nucleus with a single and central nucleolus, features also observed in other amoebae (Ludvik and Shipstone, 1970; Dyková et al., 1997; Kudryavtsev and Hausmann, 2007). These parasites especially have similarities with the genus *Entomaeba (*Archamoebae*),* which is characterised by a nucleus with a small central nucleolus and chromatin bodies on the periphery, a reduced Golgi apparatus and lack of mitochondria.

Although a taxonomical hotchpotch for many years, recent studies have narrowed Amoebozoa to a diverse, but phylogenetically congruent clade grouping classical amoeboid Lobosa, slime moulds (Mycetozoa), and Archamoeba (Cavalier-Smith et al., 2004; Nikolaev et al., 2006; Minge et al., 2009). Archamoebae includes amitochondriate, endocommensal or facultative parasitic organisms such as *Entamoeba* and *Endolimax*, reported from a wide variety of vertebrate hosts. Whereas *Entamoeba* spp. are better

known due to their clinical importance in humans, *Endolimax nana* is the only species in this genus for which comprehensive data, including genetic information are available, despite the existence of multiple reports of isolates and putative species descriptions in vertebrates and invertebrates (Table 5.1). This paucity of information maybe due to the relatively minor clinical importance of this taxon (Silberman et al., 1999), their pleomorphism and lack of distinct morphological characters and the difficulties associated with their laboratory cultivation. In a recent study, *Iodamoeba,* considered the last genus of obligate parasitic human protists without proper phylogenetic characterization, was placed as a sister taxa to *E. nana*, although a striking intrageneric diversity was reported (Stensvold et al., 2012).

The aims of this study were the identification and description of the organism parasitizing Senegalese sole. Molecular characterization and phylogenetic analyses, as well as an ultrastructural study of the organism were carried out.

Table 5.1: Described species of the genus *Endolimax* and its respective hosts

Species	**Host**	**References**
E. nana	Human	Weynon and O'Connor, 1917
E. kueneni	Monkey	Brug, 1920
E. reynoldsi	Common swift	McFall, 1926
E. caviae	Guinea pig	Hegner, 1926
E. janisae	Domestic fowl	Hegner, 1926
E. termitis	Termite	Kirby, 1927
E. blattae	Cockroaches	Lucas, 1927
E. gildemeisteri	Human	Momcilo, 1936
E. leptocoridis	Hemiptera	Kay, 1940
E. suggrandis	Termite	Henderson, 1941
E. clevelandi	Turtle	Gutierrez-Ballesteros and Wenrich, 1950
E. tayassusi	Pig	Mello et al., 1951

5.2. Materials and methods:

5.2.1. Source material:

In the course of parasitological surveys at different sole farms located in NW Spain, animals displaying obvious body bumps and inflammatory lesions in the muscle were selected (Table. 3.2, Chapter III (M&M)). Affected regions were removed and preserved in 90% ethanol. Parallel subsamples were fixed in 10% neutral-buffered formalin and processed for paraffin-embedding and routine histopathological examination, in order to confirm the nature of the lesions prior to attempting further molecular work.

5.2.2. DNA isolation, cloning and sequencing:

Granulomatous lesions in ethanol-preserved skeletal muscle were excised under a binocular scope and Genomic DNA was extracted using a silica-based commercial kit (Roche Applied Science, Barcelona, Spain) (see section 3.3.2.1., Chapter III (M&M). Control DNA was also extracted from healthy juvenile sole. Different sets of primers targeting eukaryotic SSU rDNA were assayed (Table 5.2). All PCRs were carried out in 50 ul volumes containing 1x Taq buffer 2.5mM $MgCl_2$, 0.2mM each deoxyribonucleotide triphosphate (dNTP), 1 U Taq DNA polymerase and 25 pmol of each primer. Cycling conditions consisted on an initial denaturation (2-3 min 94ºC) and 35x amplification cycles (94ºC / 1 min, 55ºC / 1 min 72ºC / 1 min) followed by a final, 8min incubation at 72ºC. Reactions using primers 18S-EUK581-F and 18S-EUK1134-R (Bower et al., 2004) consisted of 40 cycles with a shorter (30s) annealing time.

Amplification products were analysed on TAE agarose gels and amplicons were cloned or used directly for automated sequencing. When necessary, bands of interests were excised from agarose gels, purified with a clean-up kit (Invitrogen, Paisley, UK) and sequenced (see section 3.3.2.2., Chapter III (M&M)). For cloning, fresh PCR products were ligated into a plasmid vector (PCR4-TOPO, Invitrogen Paisley, UK), which was used to transform competent *E. coli*. Transformants were selected on LB-agar plates and plasmids were purified from o/n cultures in liquid media. The presence of the inserts of the expected size was confirmed by restriction digestion analysis with *Eco*RI enzyme (Fig. 5.1). Both strands of cloned products were sequenced using M13F and M13R primers, and additional walking primers s1, sx, r1 and r2 (table 5.2) designed for the purpose.

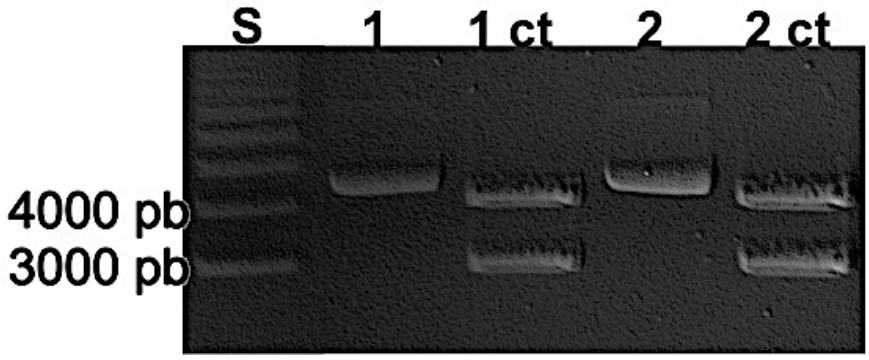

Figure 5.1: Ethidium bromide-stained agarose gel with restriction digestion analysis (with EcoRI enzyme) of purified plasmids. S: Molecular weight standards (1Kb ladder); 1 and 1 ct: plasmid vector PCR4-TOPO with the insert *E. piscium* Clone PM1 before and after digestion with the enzyme, respectively; 2 and 2 ct: plasmid vector PCR4-TOPO with the insert *E. piscium* Clone PM2 before and after digestion with the enzyme, respectively.

Table 5.2: List of primers used for primary amplification and sequencing of the parasite causing systemic granulomatous disease in Senegalese sole.

Primer name	Sequence 5'- 3'	References
18(a)	CCGAATTCGTCGACAACCTGGTTGATGCTG	Medlin et al, 1988
18B*	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC	Medlin et al, 1988
MM18Sr (*)	CGGTACTAGCGACGGGCG	Palenzuela et al., 2002
MM18Sf	CTGGTTGATTCTGCCAGTGGTC	Palenzuela et al., 2000
18S-EUK581-F	GTGCCAGCAGCCGCG	Bower et al, 2004
18S-EUK1134-R(*)	TTTAAGTTTCAGCCTTGCG	Bower et al. 2004
280f	ATCCATCAGCCATCGACGC	Freeman 2009
1300r(*)	TCGTCCGATCCTCAGTCGG	Freeman 2009
sx	ACAGAGCCAAGGTTCAGTGA	This study
s1	GCCCATTCTAACAGACACAAC	This study
r1(*)	GAAGAGGTTGTTGCCGAGAAG	This study
r2(*)	TCCCACACTTGTTCGTATG	This study

* Reverse primers

5.2.3. Phylogenetic analysis:

DNA sequences were assembled and edited using MacVector software package (Rastogi, 2000). Homologous positions presenting differences between contigs were detected and verified by eye inspection of the electropherograms. Consensus sequences were used as queries to the NCBI GenBank database using Blastn (Altschul et al., 1990) to identify the closest organisms. The final consensus sequence was inserted in an alignment of 2091 sequences available (November 2010) under the category "Amoebozoa" in the SSU_ r104 database release by SILVA (Pruesse et al., 2007: http://www.arb-silva.de). The alignment was refined by eye under ARB software (Ludwig et al., 2004) according to secondary structure criteria and the dataset was then pruned to the closest relevant taxa. Unambiguously aligned positions were sampled for phylogenetic inference, using different methods and substitution models with MEGA v.5.0 software (Tamura et al., 2011). Bayesian phylogenetic inference was conducted with MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Hueslsenbeck, 2003), under the EPoS framework (Griebel et al., 2008).

5.2.4. Transmission *electron microscopy (TEM):*

Small pieces of muscular lesions of symptomatic Senegalese sole (17/09 and 122/09, see table 3.1, Chapter III (M&M)) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide. Posteriorly, they were processed for transmission electron microscopy analysis (see section 3.3.1.2., Chapter III (M&M)). Small pieces of intestines of symptomatic fish (one fish from farm 3 and other from farm 4, see table 3.2.)) were also processed. Some sections were mounted on gold grids and stained by the Thiéry reaction for carbohydrates (Thiéry, 1967) and OTO stain for lipids (Seligman et al., 1966). Parasites cells and subcellular structures were measured from micrographs and dimensions are given as the mean ± S.D (n=26 cells and 16 mitosomes).

5.3. Results

5.3.1. Obtaining of the parasites:

Samples of *Solea senegalensis* from different farms examined, which presented macroscopical lesions compatible with the systemic parasitic granulomatous disease, were chosen. Histopathological examination of these samples demonstrated the presence of small (2-4 μm diameter), inconspicuous protozoans at the periphery of granulomatous lesions and abscesses. The anatomopathological findings were identical to those described previously in detail (Constenla and Padrós, 2010).

5.3.2. SSU rDNA sequence and phylogeny:

Universal primers 18SA and 18SB (Medlin et al., 1988) did not amplify any PCR product selectively from infected samples. The sequences obtained by this approach corresponded to the host's SSU (Fig. 5.2A). Attempts with other primers sets, suggested for X-cell organisms, were also unsuccessful as no amplification was achieved. Reactions using universal eukaryotic primers described by Bower et al. (2004) yielded an amplicon differentially present in parasitized samples (Fig. 5.2B). This band was excised from the gels and sequenced. The 853 basepairs (bps) sequence obtained matched a short segment (191 bps) of *Mastigamoeba simplex* and *Endolimax nana* as the closest organisms in Blastn searches (85-91% ID, respectively). The reactions with universal subterminal eukaryotic primers MM18Sf & MM18Sr (Palenzuela et al., 2002) also amplified differentially a ~3 kb product (Fig. 5.2C) from parasitized samples, from which two clean, partial sequences could be obtained (one with each amplification primer). These sequences also matched fragments of *Endolimax nana* and other Archeamoebae as the highest scoring hits in Blast searches. With these segments of the organism SSU rDNA, new primers were designed in combination with the universal subterminal MM18S primers, and the resulting PCR products (Fig. 5.2 D&E) were cloned and sequenced entirely. No variability was detected in the sequences from several PCR amplicons and cloned products, and the final consensus assembly comprised 2875 bps. It has been deposited in GenBank as *Endolimax piscium*. The most significant matches in Blastn searches in GenBank (lowest E-values) were SSU rDNA of *Endolimax nana*, *Iodamoeba spp.* and other Archamoebae. However, the matches were limited to 2 short segments, ~350bps and ~280 bps showing roughly 88% and 92% pairwise identity, respectively. Comparing the entire range of the alignment, the most similar sequence, from *E. nana*, only reached 60% pairwise identity.

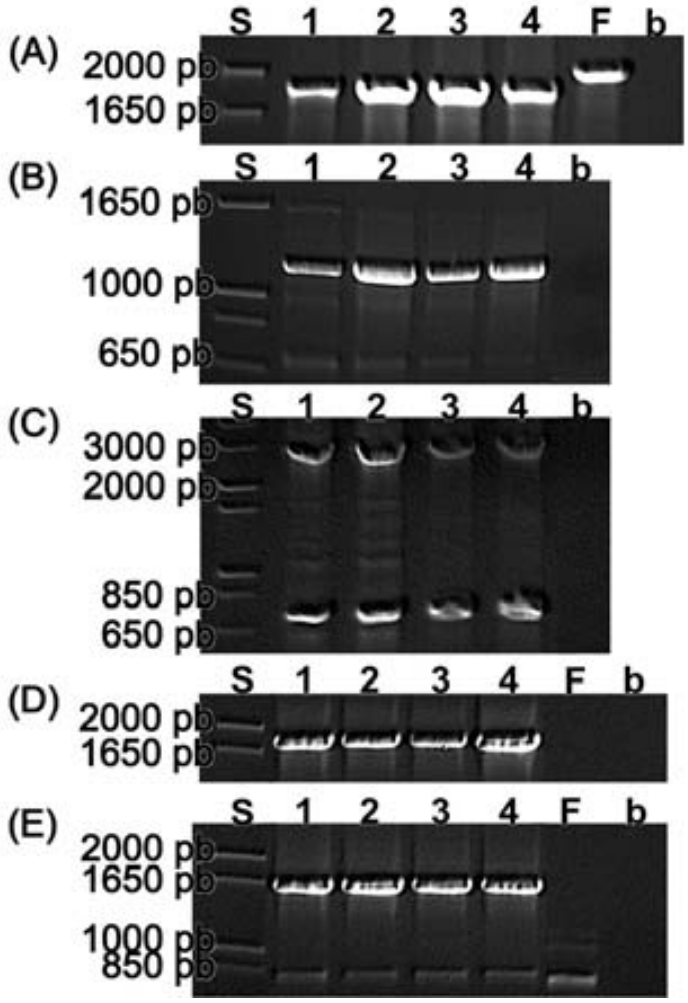

Figure 5.2: Ethidium bromide-stained agarose gel with PCR products generated from muscle of symptomatic soles using different set of primers: (A), Medlin et al. (1988) universal primers; (B), Bower et al. (2004) universal non-metazoan primers; (C), MM18Sf and MM18Sr of Palenzuela et al. (2002) universal subterminal primers; (D), a combination of MM18Sf primer of Palenzuela et al. (2002) with the designed primer r1; and (E), a combination of MM18Sr primer of Palenzuela et al. (2002) with the designed primer sx. S: Molecular weight standards (1Kb ladder); 1-4: different affected muscle of Senegalese soles; F: healthy (control) Senegalese sole; b: blank

As a result, the organism was identified as a new archamoeba whose closest know relative is *Endolimax nana*, and is tentatively described as a new species in this genus. Phylogenetic trees constructed with different inference methods and models of substitution agreed on the clustering of *E. piscium* with *E. nana* (Fig. 5.3A). *Endolimax* spp. were resolved as sister group with *Iodamoeba* spp. genotypes and this clade grouping both parasitic amoebae lineages was always robustly supported (0.98-1.00). The *Endolimax* + *Iodamoeba* clade branched as sister to a clade of free-living Archamoebae (*Mastigamoeba spp.* and *Mastigella commutan*), although the later excluded *Mastigamoeba simplex* whose position was somewhat unstable. The overall topology of the Archamoebae cladograms was quite robust and consistent using different inference methods, but these affected the bootstrap support for the branching of *M. simplex* and *E. nana* relative to their closest neighbours. In most cases *M. simplex* branched off basal to *Endolimax* + *Iodamoeba* clade with moderate (0.7-0.8) support, but the use of more conservative alignment masks (i.e. disregarding more alignment positions of dubious homology for some sequences), lowered this value. In addition, support for the monophyly of *Endolimax* spp. was lower on ML-inferred trees than on Distance or Parsimony-based analyses, and it even disappeared using the most stringent alignment sampling masks with ML and Bayesian inference methods. In these cases, *E. nana*, *E. piscium* and *Iodamoeba* spp. were resolved either as independent branches from a multifurcating node (Fig. 5.3B) or as a weakly supported node grouping *E. nana* + *Iodamoeba* spp., from which *E. piscium* branched off at basal position (Fig. 5.3C).

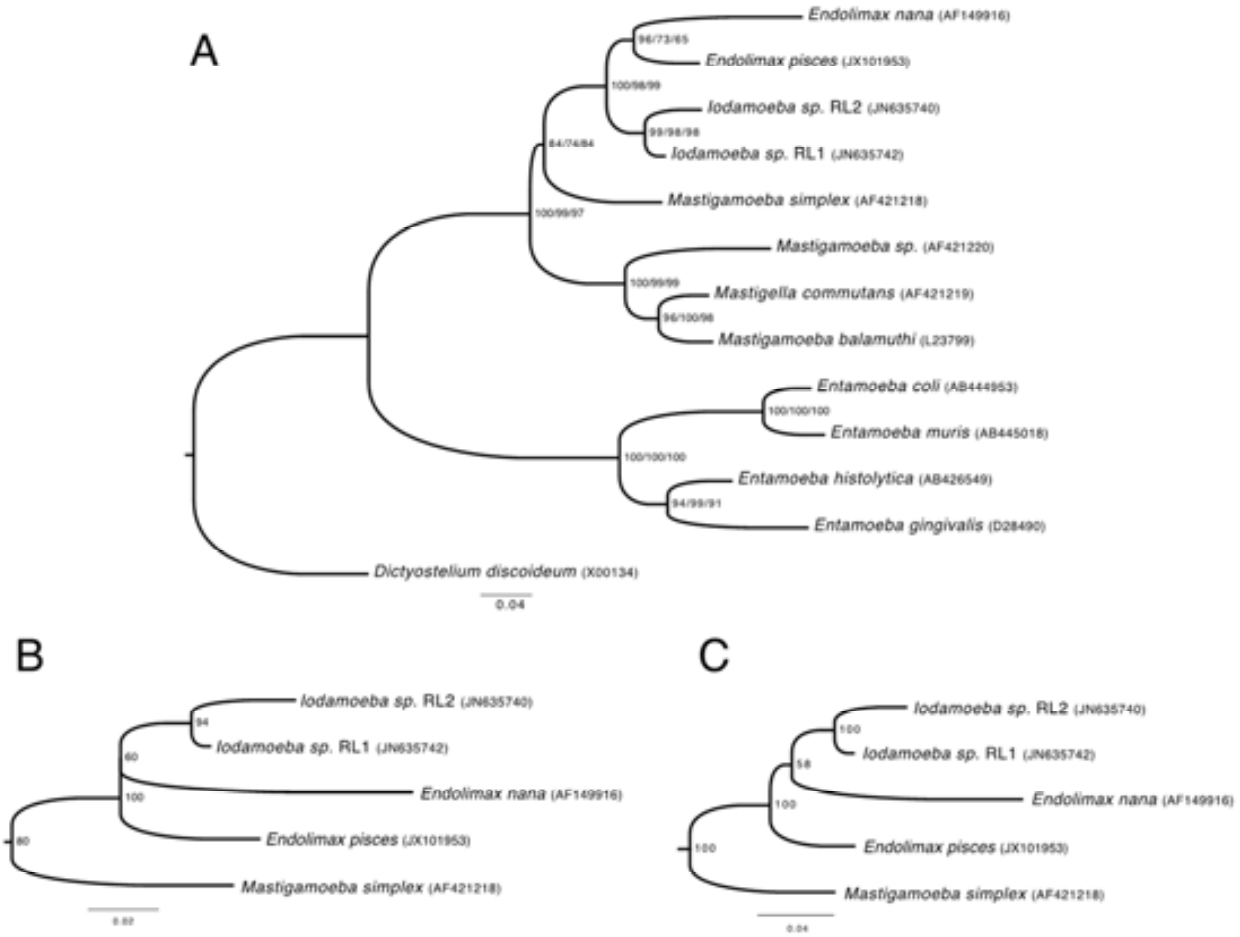

Figure 5.3: SSU rDNA gene-based phylogeny of *Endolimax piscium* and its closest amoebozoan relatives. The topology was inferred using three alignments sampling masks disregarding variable numbers of positions of dubious homology, and multiple reconstruction methods and models of nucleotide substitution: A, “Relaxed” dataset (1,493 sites). Numbers at nodes represent bootstrap values after 500 resamplings, determined by Distance methods (Tajima-Nei model with a gamma distribution parameter G=0.36, determined from the dataset) / Maximum Parsimony (with Close-Neighbour Interchange on Random Trees) / and Maximum Likelihood (GTR Model with 4 gamma categories); B, Very stringent alignment sampling mask (1,350 sites) using the Maximum Likelihood method with the General Time-Reversible (GTR) model and 4 gamma categories. C, Stringent alignment sampling mask (1,410 sites): numbers at nodes represent posterior probabilities resulting from a Bayesian analysis using the GTR model.

5.3.3. Ultrastructural observations:

Since no parasites were detected in intestines fixed in glutaraldehyde, the ultrastructural study of the parasites was made from parasites located only in muscle samples.

This ultrastructural study confirmed macrophages as the most common location of these organisms in muscle lesions. Within the cytoplasm of the macrophages, a single organism or groups of two, three or more organisms were detected (Fig. 5.4A), although extracellular stages were also observed (Fig. 5.4A). In occasion, parasites within macrophages showed a degenerated appearance (Fig. 4B). Parasite stages were mostly round to ovoid in shape, measuring 3.33 µm ± 0.47 x 2.78 µm ± 0.42 (Fig. 5.5A). They contained one vesicular nucleus (1.18 µm ± 0.07 µm in diameter) with a large central round nucleolus (diameter 0.54 µm ± 0.04) (Fig. 5.5 B&C), filling roughly half of the nuclei surface. Small aggregates of heterochromatin associated with the nucleolus and peripheral chromatin were usually observed (Fig. 5.5B). The double-layered nuclear membrane, similar to that of the plasma membrane, was noticed which presented conspicuous pores (Fig. 5.5B).

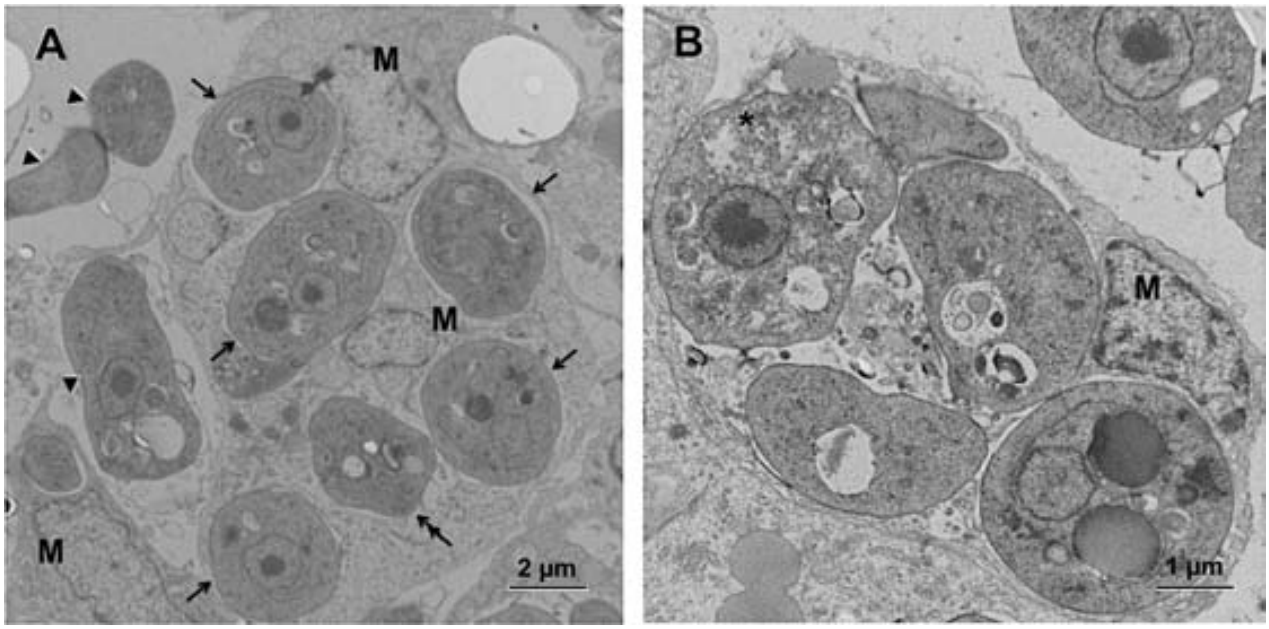

Figure 5.4: Transmission electron micrographs of parasites in muscle of Senegalese sole: A, Parasites (arrows) within macrophages (M) in a sample of a granulomatous lesion. Notice also the presence of extracellular parasites (arrowheads); B, Parasitic organisms within macrophages (M), with a degenerated appearance (asterisk).

The cytoplasm of the parasites contained small glycogen granules, often forming aggregates (fig. 5.5C), and a variety of intracytoplasmatic structures such as some single-membrane bound vesicles, putative digestive vacuoles containing particulate material, myelinic figures or products of lysosomal action (Fig. 5.5A). No mitochondria were observed although double membrane-bounded, electron-dense organelles with no apparent cristae, interpreted as mitosomes, were frequently observed within the cytoplasm (Fig. 5.5D). These organelles were present in variable numbers per cell, normally one to three in 70 ηm-thick TEM slices. They were rounded to ovoid, 157.73 ηm ± 46.77 x 107.71 ηm ± 20.3. Elongated vesicles resembling dictyosomes of Golgi apparatus cisternae were sometimes observed (Fig. 5.5E). Structures appearing as long rods or whirls of a bilayered membrane were commonly observed extending across the parasites or, sometimes, beside the nuclear membrane (Figs. 5.5 A&E). Vacuole-like structures surrounded by an electron-lucent, wide concentric aureole were sometimes observed within the parasites cytoplasm (Fig. 5.5F). The parasites mostly presented a regular smooth surface but amoeboid stages presenting a more irregular shape with invaginations/evaginations could also be identified (Fig. 5.5C). Filopodia-like structures were detected in the surface of some trophozoites as slender cytoplasmatic projections (Fig. 5.5G) and they were commonly observed sectioned transversally around the parasites (Fig. 5.5F). Round to elongated electron-dense bodies (0.7µm – 0.3µm) were found associated with the external layer of the plasma membrane (Fig. 5.5H). No evident cysts could be detected in any of the samples.

5.3.4. Description of the Species:

Type species: *Endolimax piscium n. sp.*

Type host: *Solea senegalensis* Kaup, 1858

Locality: the parasite was detected in cultured Senegalese soles from fish farms located at different sites in NW Spain (Atlantic Ocean).

Location in the host: Systemic. Parasites were localized as a compact layer at the periphery of granulomatous lesions in different tissues: muscle, liver, kidney, heart, intestine and ovary. Parasites were also found within the intestine epithelium.

Material deposited: Histological sections (sole tissues containing conspicuous granulomatous lesions and abscesses surrounded by *E. piscium* cells) have been deposited at the Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain with accession numbers: MNCN 33.04/1 (Holotype) and MNCN 33.04/2 – MNCN 33.04/3

(paratypes). Partial SSU rDNA sequences have been deposited in Genbank: *E. piscium* clone MALEN3 (accession no. JX101953), □ *E. piscium* Clone PM1 (accession no. JX101944) and *E. piscium* Clone PM2 (accession no. JX101955).

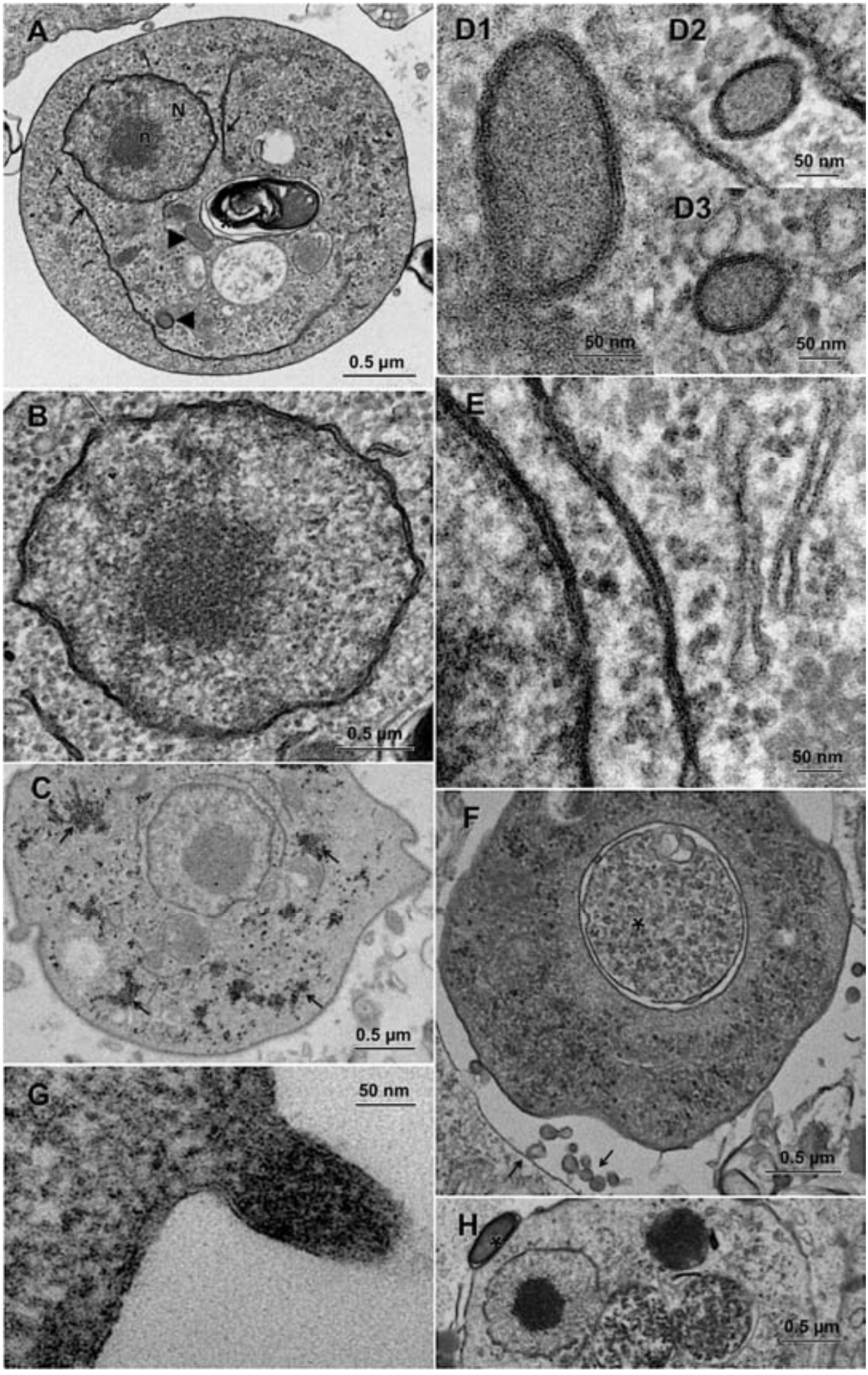
A
N
n
0.5 µm
D1
D2
50 nm
D3
50 nm
50 nm
B
0.5 µm
E
50 nm
C
0.5 µm
F
0.5 µm
G
50 nm
H
0.5 µm

Figure 5.5: Transmission electron micrographs of *E. piscium* trophozoites: A: Amoeba cell displaying a nucleus (N) with a single nucleolus (n), myelin figures (*), whirls of bilayered single membrane extending across the parasites (arrows) and mitosome (arrowheads); B: Detail of the nucleus with a large central karyosome. Note the presence of nuclear pores (arrows) and peripheral chromatin (*); C: Presence of carbohydrates as small glycogen granules often forming aggregates over the whole surface of the trophozoite stage, demonstrated by the Thiéry stain; D: Detail of various mitosomes: note the electron-dense, homogeneous matrix without cristae and double membrane around them; E: Elongated vesicles resembling dictyosomes close to the nucleus; F: Vacuole-like structures within the parasites cytoplasm (*), containing a withered matrix and an electro-lucent concentric aureole. Note several filopodia sectioned transversally at the periphery of the amoeba (arrows) G: Detail of one of these filopodia-like structure emerging at the surface of a trophozoite; H: Elongated electron dense body associated with the plasma membrane (*).

5.4. Discussion

5.4.1. Sequence and phylogeny:

The combined results of the ultrastructural and genetic study of the parasite *Endolimax piscium* causing systemic granulomatous in sole allowed its unambiguous identification as an amitochondriate Archamoeba related to *Endolimax nana* and *Iodamoeba spp*., enteric commensals and parasitic species in humans and other mammals. The SSU rDNA sequence fragment obtained was 2875 bps, and its total length, inferred from the alignments with other mastigamoebids, is estimated > 3120 bps. This is the second-longest known amoebozoan SSUrDNA, after that of the aberrant amoeboflagelate *Pelomyxa palustris* (3502 bps) (Milyutina et al., 2001). Even though amoebozoans are characterised by SSU rDNAs longer than most eukaryotes and archamoebids represent an extreme to this tendency (Nikolaev et al., 2006), *E. piscium* sequence is 20% longer than its most similar, *E. nana*. The overall topology of the Archamoebae cladograms is quite robust and consistent using different phylogenetic inference methods, placing Iodamoebae, *Endolimax spp.* closer to Mastigamoebae than to Entamoebae and thus supporting different events of adaptation to parasitic lifestyles or, alternatively, a re-adaptation of Mastigamoebae to free-living niches. This topology is congruous with previous molecular phylogeny studies of related amebozoans (Cavalier-Smith et al., 2004; Nikolaev et al., 2006; Tekle et al., 2008; Lahr et al., 2011; Stensvol et al., 2012). Although *E. piscium* grouped together with *E. nana*, there is much variation between their rDNA sequences, with only 60% pairwise identity along the alignment of the available common segment. Interestingly, the recent molecular characterization of *Iodamoeba* spp. showed two different rDNA lineages with 31% divergence among them and further substantial diversity within each lineage (Stensvold et al., 2012). These values seem strikingly heterogeneous for congeneric species, although amoebozoans are known examples of high rDNA heterogeneity and evolution rate. Thus, some instability on the branching of *E. piscium* relative to *E. nana* and *Iodamoeba* spp. was noticed that was particularly patent when bayesian or maximum likelihood inference methods and, specifically, when stringent alignment sampling masks were used. A very similar instability was reported to affect the placement and monophyly of Iodamoeba genotypes with these reconstruction methods, even though they are firmly resolved by distance or MP-based methods and despite the fact that multiple synapomorphic motifs are apparent by eye inspection of the alignments, as previously pointed out (Stensvold et al., 2012). Species of *Endolimax* have been reported for multiple vertebrate hosts (Table 5.2),

however a single isolate of *E. nana* from monkey was still now the only species in this genus with some genetic data available (Silberman et al., 1999). Furthermore, no species of *Endolimax* has been described in fish to date. Since the placement and relative support for *E. piscium* branching in phylogenetic analyses is influenced by the inference method, we conservatively describe the new archamoeba in the genus *Endolimax* but the significant genetic distance with the closest *Endolimax* and *Iodamoeba* genotypes, as well as the putative discovery of additional piscine genotypes, could support a higher-level taxon corresponding to a distinct lineage of parasitic archamoeba.

5.4.2. Morphology:

Cavalier-Smith (1998) in his "Revised six kingdom system of life" grouped entamoebids, *E. nana*, and *Mastigamoeba* spp. in the subphylum Conosa (infraphylum Archamoebae), in spite of their great phenotypic diversity. Members of these linages share features such as the absence of mitochondria and an apparently simplified intracellular organization (Martinez-Palomo, 1986; Simpson et al., 1997). Whereas mastigamoebae are generally free-living and have flagellated cells, known *Endolimax* and *Entamoeba* are commensals or parasites with amoeboid trophozoites displaying locomotion by pseudopodia (Silberman et al., 1999). *Endolimax* nana and *Iodamoeba* develop cyst stages, which are also present in most *Entamoeba* spp. Even though these archamoebae are regarded as marginally pathogenic to vertebrates, the role of different *Entamoeba* spp, in gastrointestinal disorders of humans is well known (Jetter et al., 1997; Heredia, et al., 2012). In 90% of cases these amoebic infections are asymptomatic and self-limited (Haque et al., 2003), with trophozoites remaining in intestine lumen as commensals and some encysting for the perpetuation of the cycle through fecal-oral spread (Mortimer and Chadee, 2010). However, *E. histolytica* is able to play a pathogenic phenotype disrupting the mucosal barrier, entering the portal circulation and dispersing to soft organs, generally producing abscesses (Espinosa-Cantellano and Martinez-Palomo, 2000; Mortimer and Chadee, 2010). *Endolimax nana* and *Iodamoeba* spp. have also been characterized as occasional causes of gastrointestinal disorders (Stauffer, et al., 1974) cutaneous processes (Veraldi, et al., 1991), reumathoid arthritis (Burnstein and Liakos, 1983) and brain granuloma (Arava, et al., 2010). *Endolimax piscium* is usually found as causative agent of systemic granulomas and abscesses in Senegalese sole viscera, but it can also be detected in the intestinal epithelium of asymptomatic fish (Constenla and Padrós, 2010). However, even in these cases, the amoebae appear

always in intraepithelial locations and occasionally with some degree of host response associated (see Chapter IV). Although the whole transmission and developmental cycle of *E. piscium* is yet unknown, the data available so far suggest a primary parasitic, rather than commensal behaviour in this piscine host. Contaminated water sources like swimming pools, freshwater ponds, lakes, or drinking supplies are recognized sources of human and animal amoebiases, but the occurrence of amoebae in the fish that inhabit aquatic environments has been neglected for a long period of time (Dyková and Lom, 2004).

Species of *Endolimax* have been classical described as intestinal commensal amitochondrial amoebas with eruptive pseudopodia, lacking cilia, centrioles, contractile vacuoles or intracellular crystals (Cavalier-Smith et al., 2004). Previous studies about other Endolimax spp. (see table 2.2) are dated and incomplete descriptions on morphological grounds, what make difficult their comparison due to the lack of characters. In any case, the trophozoites of *Endolimax* spp, have been reported to measure between 5 µm to 14 µm depending on the species, and to contain one vesicular nucleus (1.5 µm - 6 µm) with a large karyosome. According to this description, *E. piscium* appears to be the smallest species of the genus, with trophozoites smaller than 5 µm. Our ultrastructural analysis of *E. piscium* revealed some similarities with *Entamoeba* spp. stages, such as the absence of mitochondria, Golgi apparatus and rough endoplasmic reticulum (Ludvik and Shipstone, 1970; Martinez-Palomo, 1986). However, structures resembling isolated dictyosomes were observed in some *E. piscium* cells. Ultrastructural data of *E. nana* or *Iodamoeba* are quite scarce (Zaman et al., 1998; 2000). In *E. nana* cysts, Zaman (2000) pointed out the existence of tubular structures made up of a double row of ribosome-like particles with a single membrane running between them. Since they have not been described from any other intestinal amoeba, they were suggested to be species-specific characters. Our material from Senegalese sole did not include any cyst, but we observed similar, conspicuous double membranous layer, not associated with ribosomes and resembling nuclear membrane. Since they were not observed in all stages and they frequently extended across the entire parasite, they could be structures related with the cell cycle or proliferation.

The lack of mitochondria in *E. piscium* is consistent with its phylogenetic affinities. In Archamoebae, this absence is a derived condition, either by loss of mitochondria or by conversion of these organelles in mitosomes (Tovar et al., 1999), as adaptation to an anaerobic environment (Cavalier-Smith, 2002; Cavalier-Smith et al., 2004). Mitosomes are mitochondrion-related organelles found in a range of unicellular eukaryotic organisms

that inhabit oxygen-poor environments, usually parasites invading digestive tract or various cell tissue, and including Archamoebae (Cavalier-Smith, 1991; Tachezy and Smíd, 2008). Mitosomes in *Entamoeba histolytica* and in *Mastigamoeba balamuthi*, as well as other intestinal parasites such as *Criptosporidium*, were described as ovoid to elongate double-membrane organelles, with electron-dense material (Tachezy and Smíd, 2008). These organelles bear a strong resemblance to the structures found in *E. piscium* cells, with two tightly opposing membranes without intermembrane space. The size and number of mitosomes varied in different cells (Tachezy and Smíd, 2008). Based on TEM studies, they measure about 0.5 μm to 2.0 μm in *E. hystolitica* (Tovar et al., 1999; Ghosh et al., 2000), but more precise studies by confocal microscopy images revealed a estimated sizes of 0.5 μm (León-Avila and Tovar, 2004), and of 0.1 to 0.2 μm in *M. balamuthi* (Gill et al., 2007). In TEM sections, *E. piscium* mitosome-like organelles appear considerable smaller than those described for *Entamoeba*, which can be due to the minute size of this parasite or simply reflect the methodological inaccuracy. It must be stressed that even though the morphological evidence strongly suggests the presence of mitosomes in *E. piscium*, further biochemical and genetic studies would be required to confirm the nature of these double-membrane organelles.

The vacuole-like structures surrounded by a bright concentric zone are very similar to those described in systemic granuloma agent affecting Goldfish (Paperna and Kim, 1996; Dykova et al., 1996) and interpreted as endocytotic channels. Unfortunately, no invagination of the organism's body wall was observed in this work to verify this possibility, since *E. piscium* cells appeared rather smooth and only presented some slender filopodia-like structures in the sectioned material. Form these reports, the parasite from goldfish seems quite similar to this new *E. piscium* from Senegalese sole. Further characterization of that parasite, and eventually of other piscine parasitic archamoebae, might contribute to clarify their relationships with *E. nana* and to interpret the large genetic distance observed between *Endolimax* species.

CHAPTER VI: DEVELOPMENT OF TECHNIQUES FOR THE DIAGNOSIS OF *E. PISCIUM* (ARCHAMOEBAE) AND THEIR PRELIMINARY APPLICATION IN EPIDEMIOLOGY.

6.1. Introduction:

6.1.1. *E. piscium* diagnosis

Endolimax piscium is the causative agent of systemic amoebiasis in Senegalese sole. This amoeba causes a granulomatous inflammatory reaction in different organs of the host. So far, the only procedure available to diagnose *E. piscium* is based on conventional histology techniques. In histological sections, *E. piscium* cells appear at the periphery of the lesions, but this detection is hampered by the degree of inflammation associated to the development of granulomas. Besides granulomatous lesions in various organs, unicellular parasites were also found within the intestinal epithelium, without apparent lesions, both in symptomatic and asymptomatic fish (see Chapter IV). These organisms, due to their size and general morphology, are presumptively identified as stages of *E. piscium*. However, at this location, degenerate or apoptotic host cells are often difficult to differentiate from the parasites due to their minute size and their inconspicuous morphology. In addition, histological techniques are time-consuming and have limited throughput because the sample processing time, and the examination by a skilled pathologist requires several days before assessing a diagnosis.

Diagnostic methods bases on the detection of parasite's nucleic acids can circumvent some of the limitations of morphology-based techniques. Since DNA does not change during the parasite cycle, choosing such diagnostic targets can dramatically improve specificity of the detection. In addition, some molecular methods can offer higher throughput and enhanced sensitivity than most conventional morphology-based techniques under many diagnostic scenarios (Palenzuela and Bartholomew, 2002a). In order to establish reliable diagnostic techniques for the recognition of *E. piscium*, *in situ* Hybridization and Polymerase Chain Reaction tests have been developed and evaluated. The performances of the new techniques were compared with conventional histology. Furthermore, the different techniques were used in combination to further understand the distribution of the parasite within the host, and to obtain a preliminary epidemiological image of this amoebiasis in different sole farms.

6.1.2 *In Situ* Hybridisation (ISH)

ISH is a method involving hybridisation of labelled DNA or RNA probes with the complementary sequences in cells or tissues. As routinely used for diagnostics in parasitology, this technique allows the detection of parasites directly in the host tissues,

thus combining the specificity and sensitivity of DNA detection techniques, and the ability to study the parasites in their biological context.

Studies with ISH in fish parasites are particularly useful to detect different stages and to describe the invasion and progression of parasites within the host tissues. Fluorescence ISH has been successfully used to detect *Acanthamoeba* strains (Dyková et al., 1999) and *Hartamanella vermiformis*, either as trophozoites attached to slides or in histological lesions from freshwater fishes (Dyková et al., 2005b). Recently, this technique has been developed for amoebae parasitizing gills in rainbow trout to complete the identification of the aetiological agents diagnostic (Dyková et al., 2010). Similar ISH techniques have also been used and proved useful to detect different stages of parasites such as Myxozoa: *Myxobolus cerebralis* in rainbow trout, *Oncorhynchus mykiss,* and *M. spirosulcatus* in yellowtail, *Seriola quinqueradiata,* (Antonio et al., 1998; Yokoyama et al., 2010), *Ceratomyxa shasta* in salmonids and other fishes (Palenzuela and Bartolomew, 2002; Bjork and Bartolomew, 2010), *Sphaerosphora truttae* in salmon, *Salmo salar* (Holzer et al. 2003) and *Kudoa* sp. also in salmonids (Jones et al., 2003; Young and Jones, 2005); or Microsporidia: *Tetramicra brevifilum* in turbot, *Psetta maxima* (Leiro et al., 2001), or *Glugea plecoglossi*, *Loma salmonae* and *Nucleospora salmonis* in salmonids (Lee et al., 2000; Sanchez et al., 2001; Yokoyama et al., 2010).

6.1.3 Polymerase Chain Reaction (PCR)

Although ISH may improve the throughput in the diagnosis due to easier interpretation of slides, it is still time-consuming and has relatively high cost per slide. PCR-based detection methods are a good alternative method for diagnostics due to their speed, low cost and very high throughput. They are particularly useful for rapid screening and testing for presence / absence of parasites in a wide diversity of samples. Furthermore, due to the amplificative nature of PCR-based methods, they are extraordinarily sensitive and may allow the detection of few, or just one single microorganism per volume of sample analysed.

In amoebae, although conventional histological or immunofluorescent-stained (IIF) tissue sections were the main means of diagnosis of human amoebiasis, their morphology or imprecise symptomatology caused mistakes in pathologist identification (Rocha-Azevedo et al., 2009). Due to the importance that these diseases have been acquiring in humans, and especially the rapidly fatal outcome of some of them, specific and rapid diagnosis techniques have been developed. PCR assays in fresh tissue as well as in formalin-fixed paraffin-embedded tissue have been reported for the diagnosis of *Naegleria* sp. (Schild et

al., 2007) infections, acanthamebiasis (MacLean et al., 2007; Yagi et al., 2007) and balamuthiasis (Tavares et al., 2006; Yagi et al., 2005). This alternative method enhances the sensitivity of the pathology diagnosis and reduces the time needed to produce results. Regarding parasitic amoeba from the human digestive tract, the development of molecular tools, including PCR and real-time PCR, has led to major advances in the accuracy of diagnosis during recent years. Regarding parasitic amoeba from the human digestive tract, a variety of conventional PCR-based techniques are currently known, which allow differentiation of the *Entamoeba* spp. based on genetic diversity introduced 18S rDNA gene (Fotedar et al., 2007). In fish, the molecular characterisation of the clones based on the small subunit (SSU) rRNA gene sequences, is used for phylogenetic analysis of some amoebae such as *Naegleria* (Dyková et al., 2001). Moreover, PCR- based assays are performed for *Neoparamoeba perurans* providing a simple and flexible tool for the diagnosis of AGD by this amoeba with high specificity and sensitivity (Young et al., 2008).

6.2. Materials and methods:

6.2.1. Source material

A total of 52 fish displaying signs of *E. piscium* infection were sampled in some facilities where outbreaks of the disease were detected during 2011 and 2012 (see table 3.2, Chapter III (M&M)). Moreover, 160 apparently asymptomatic Senegalese sole were randomly sampled between 2009 and 2012 from fish farms in the north-Atlantic area of Spain (table 3.3, Chapter III (M&M)). Fish dissection procedures and sample fixation and preservation are explained in Section 3.2.2 and 3.2.3, Chapter III (M&M).

6.2.2. Post-mortem techniques

6.2.2.1. Conventional histology:

Histological techniques procedures are detailed in section 3.3.1.1, Chapter III (M&M). Staining of choice for all histological sections was H&E stain.

6.2.2.2. Diagnosis by ISH

Probes: In Situ Hybridisation probes design was aided by software packages OLIGO-7 (Rychlik, 2007) and PRIMER PREMIER (Premier Biosoft, CA, USA), choosing variable regions of *E. piscium* small subunit ribosomal RNA gene (SSU rDNA). The probes consisted

in oligonucleotides (20-22-mers) reproducing the sequence of the Crick's (negative) DNA strand, in order to hybridise with Watson's (positive) DNA strand and with RNA. This design allows labelling of both the nuclear rDNA and cytoplasmatic rRNA, resulting in maximum intensity of the signal. Higher stringency parameters were selected. The specificity of the probes was checked against the host rDNA sequence and oligonucleotides showing homology were rejected. The resulting oligonucleotides (Table 6.1) were synthetized and labelled with a 5'-end non-fluorescent Digoxigenin (DIG) label, followed by HPLC purification, from a commercial supplier (Metabion, Munich, Germany). A 100-pmol·μl^{-1} solution of each DIG-labelled probe was prepared and they were mixed in equimolar amounts.

Table 6.1. Oligonucleotide rDNA probes used for the detection of *E. piscium* by in situ hybridisation.

Probe	Sequence
1575L24 Amsolea	5'- GTTGCCGAGAAGCCAAAATCCTGT -3'
1999L24 Amsolea	5'- TTCGATTTCTCTTCTAGGTGCTGG -3'

Pre-treatment of the sections: Paraffin sections were obtained as described in section 3.3.1.1 of Chapter III (M&M) and they were mounted onto electrostatically charged glass slides (Starfrost-plus, KnittelGläser, Germany). Slides were dewaxed with 3 changes of xylene for 10 minutes each, hydrated through sequential incubations in decreasing concentration alcohol solutions (100%, 90% and 70% ethanol) for 5 minutes each, and equilibrated with a Tris-$CaCl_2$-bath for 10 minutes. Deparaffinised tissue sections were permeabilised by treatment with 20 $\mu g \cdot ml^{-1}$ of proteinase K in Tris-$CaCl_2$ (Table 9.9, Chapter IX (Annex)) (30 minutes at 30ºC).

Hybridisation: After two washes (10 minutes each) with 2x saline sodium citrate buffer (SSC) (table 9.9, Annex)) at room temperature, the sections were denatured at 95℃ in hybridisation buffer (table 9.9, Chapter IX (Annex)) containing the mixture of labelled probes at 1:1000 dilution. The slides were covered with the hybridisation buffer and placed on a hot plate during 10 minutes. To avoid the formation of large bubbles that could interfere in the process, after 2-3 minutes the tissues were covered with nuclease-free plastic coverslips (HybriSlip™, Sigma). Finally, the slides were removed from the hot plate and they were allowed to hybridise overnight at 30℃ in a moist chamber.

Stringency washes: Unbound probes were washed off by double successive stringency washes in decreasing concentrations of SSC buffer as follows: (2x, 1x and 0,25x). Washes

were carried out in an orbital shaker at 30ºC. The last wash was carried out with mild agitation.

Immunological detection: The slides were equilibrated in a bath with " Genius 1" buffer (table 9.9, Chapter IX (Annex)) for 10 minutes, and then covered with blocking solution (table 9.9, Annex) and incubated in a humid chamber (saturated with Genius 1), for one hour at room temperature. Immunological detection was then carried out using an AP-conjugated goat anti-digoxigenin antibody (1:500 dilution in blocking solution), which was incubated for 2 hours at room temperature. The antibody was drained and slides were washed in Genius 1 and Genius 3 (table 9.9, Chapter IX (Annex)) (10 minutes in each, at room temperature). Subsequently, slices were covered with nitroblue tetrazolium/5-bromo-4chloro-indolyl-phosphatase (NBT/BCIP) (Roche Diagnostics) substrate solution (table 9.9 Chapter IX (Annex)) and incubated overnight at room temperature.

Counterstain and mounting: The colour reaction was stopped with a wash of Genius 1 for 10 minutes. The slides were immersed in Light Green stain (1%) for 2 minutes followed by an acetone bath containing 0,05% acetic acid for one minute. They were washed with distilled water, dehydrated in increasing concentrations of alcohols (70%, 90% and 100% ethanol) and immersed in xylene. Finally, the slides were mounted in permanent medium (Vecta mount) and they were air-dried until observed at the microscope.

6.2.2.3. Diagnosis by PCR:

Primers: PCR primers design was aided by software packages OLIGO-7 (Rychlik, 2007) and PRIMER PREMIER (Premier Biosoft, CA, USA), choosing variable regions of *E. piscium* small subunit ribosomal RNA gene (SSU rDNA). Oligonucleotides (19-22-mers) were searched using high stringency parameters. The specificity of the probes was checked against the host rDNA sequence and oligonucleotides showing homology were rejected. The highest scoring oligonucleotides (Table 6.2) were ordered from a commercial supplier (Life Technologies, Madrid, Spain). A 100-pmol·μl^{-1} solution of each primer was prepared and both primers of each set were mixed in equimolar amounts.

Table 6.2. rDNA probes of *E. piscium* used for PCR diagnostics.

	Probe	Sequence
Set of primer A	Ep1579fL20	5'- GATTTTGGCTTCTCGGCAAC -3'
	Ep2017rL22	5'- GAAGCCCAAACACTTATTCGAT-3'
Set of primer B	Ep1306fL22	5'- GCAAGCAGACCAGAGACCACAA-3'
	Ep1573rL22	5'- TCCCTCCGAAGAAAGACCCAGT-3'

DNA isolation: Samples of skeletal muscle and intestine were taken and preserved in 90% ethanol. Small portions (approximately 50-100mg) were briefly hydrated in TE buffer and genomic DNA was extracted using a silica-based commercial kit (Roche Applied Science, Barcelona, Spain) (see section 3.3.2.1, Chapter 3 (M&M)).

PCR: All reactions were carried out in 20 µl volumes and the concentrations of the different components are listed in Table 6.3. Cycling conditions consisted on an initial denaturation (2 min 94ºC) and 35x amplification cycles (94ºC / 30 s, annealing temperature / 20 s, 72ºC / 20 s). The annealing temperature was 57ºC for the primers set A and 60ªC for the set B. Amplification products were analysed on TAE agarose gels (1%), containing 0.1 µg/ml ethidium bromide.

Table 6.3: Components for the PCR reaction.

Component	Concentration	Volume
Buffer (10x)	1x	2.00
ClMg (50mM)	2.5mM	0.80
dNTP (5mM)	0.2mM	0.16
Primer (50µm)	0.5µM	0.20
Taq (5u/µl)	1u	0.20
HPLC Water		15.59

Precision of the technique: The precision of PCR was assessed using *E. piscium* oligonucleotides in muscle samples at different distances from the lesions (into the lesion, at 1 cm and over 3 cm).

6.2.3. Evaluation of diagnostic techniques

Three diagnostic methods for *E. piscium* were compared: Conventional histology (CH), In situ Hybridisation (ISH) and Polymerase Chain Reaction (PCR).

6.2.3.1. Gold standard and accuracy of the techniques:

The sensitivity and specificity of each technique was tested comparing them with the overall condition of each fish, as estimated by a Gold Standard Technique (GST). This GST was established by a combination of direct techniques (CH and ISH). In general, conventional histology was considered more appropriate and reliable proof in muscle samples, whereas ISH resulted in enhanced diagnostic abilities in intestine samples. The evaluation of the methods was assessed using the program winEpi (de Blas et al. 2006). (Available online at http://www.winepi.net/).

Sensitivity measures the proportion of true positives that are correctly identified:

Se = True Positives / (True Positives + False Negatives)

Specificity is the proportion of true negatives that are correctly identified:

Ep = True Negatives / (True Negatives + False Positives)

The positive predictive value PPV) is the proportion of positive test results that are true positives (such as correct diagnoses):

PPV = True Positives / (True Positives + False Positives)

The negative predictive value (NPV) is the proportion of subjects with a negative test result who are correctly diagnosed:

NPV= True Negatives / (True Negatives + False Negatives)

The Accuracy indicates proximity of measurement results to the true value:

A= (True Positives+ True Negatives) / (True Positives+ True Negatives+ False Positives+ False Negatives)

6.2.4. Further testing and application of diagnostic techniques

6.2.4.1. Parasite distribution within the host

The presence or absence of the parasite in the different organs within the fish was studied. A tissue was considered infected when some stage of the parasite was directly identified within the tissue studied, or when DNA amplification was achieved in the PCR. This allowed obtaining a prevalence value (P) of infection, defined as the proportion of tested individuals (N) that resulted infected (n).

$$P= (n/N)*100$$

In order to evaluated the infection intensity, three random semi quantitative counts of the number of parasites were performed on each intestine histological section at 100x of

magnification, and scored as: none (0), low (1) (1-5 *E. piscium* cells per observation field approximately), medium (2) (6-25 *E. piscium* cells per observation field approximately) or high (3) (more than 26 *E. piscium* cells). Mean intensity was calculated from the average intensity values of each individual fish. In addition, the infection was classified as “extensive or general infection” (if the parasite was present in all examined tissue) or “restricted or partial infection” (parasites affecting only limited areas of some of the examined tissues).

Using individual fish as replicate samples, differences in *E. piscium* prevalence within the intestine (Total prevalence (P), anterior intestine prevalence (AIP) and posterior intestine prevalence (PIP)) were tested using a generalized lineal model (GZM) (binarial model) with total weight and total length as covariables.

6.2.4.2. Epidemiology:

In order to assess the prevalence of the infection by *E.piscium* in different sole culture farms located at the north of Spain, combined studies using histopathology, ISH and PCR techniques were carried out. Two samplings methods were conducted in these farms: a “targeted”, non-probabilistic sampling directed to symptomatic fish; and a stratified random probability sampling studying normal or asymptomatic fish (Table 3.2 and 3.3, Chapter III (M&M), respectively).

6.3. Results:

6.3.1. In situ Hybridisation (ISH)

Hybridisation of the probes to paraffin-embedded sections of fish tissue infected by *E. piscium* was observed as dark purple precipitates in the tissues, revealing the labelled probe bound to *E. piscium* cells. Host tissue was counterstained in green. Negative controls showed no hybridisation signal in the sampled organs, validating the specificity of the probes labelling in parasite-infected samples. Furthermore, no significant background or nonspecific binding to the host mucous surfaces, enzymatic components or structures was observed in any of the tissues analysed.

Within the granulomatous inflammatory lesion, the ISH-positive signals were observed at sites occupied by the parasites observed with H&E-staining, thus confirming the location of the amoebae at the periphery of the granulomas, either inside macrophages or extracellularly (Fig. 6.1A). At this peripheral area of the granuloma, the parasites were

sometimes concentrated in large numbers, to the extent of being difficult to differentiate individual organisms, but a large purple band was conspicuously stained (Fig. 6.1B). Some parasites extending towards the necrotic centre of the granuloma were also labelled by the probes, sometimes less intensely and, in some cases, only at the periphery of the cells (Fig. 6.1C). In some slides, the staining signal of muscle granulomas was less intense than in other organs, and some parasites even appeared unlabelled (Fig. 6.1D). ISH signal was also positive in kidney and intestinal granulomas, where the parasites were difficult to recognize by conventional histology (see Chapter IV) due to the intense inflammatory response and by the similar aspect of some cells usually present in this tissues (Fig. 6.1E). In intestine samples, the presence of *E. piscium* cells within the epithelium was confirmed by ISH (Fig. 6.2A), which facilitated the detection of parasites even when they were present in small numbers (Fig. 6.2B).

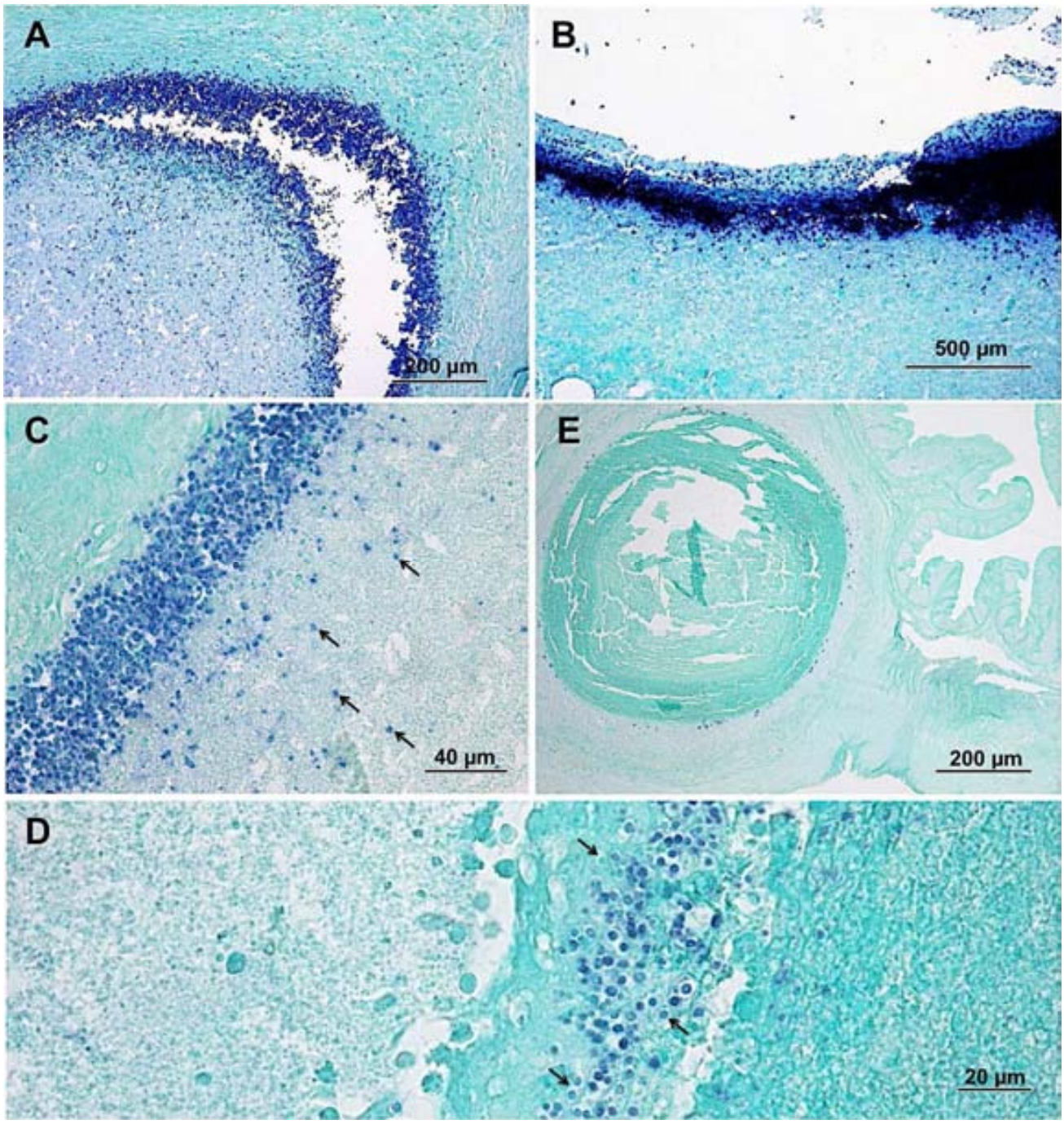

Figure 6.1: In situ hybridisation of histological sections of different affected Senegalese sole organs: A, Granulomatous inflammatory reaction in muscle with a necrotic core and an external layer of parasites (stained in blue-purple) surrounding it; B, Detail of the external part of a granuloma where an intensely stained purple layer is observed, corresponding to a high intensity of infection by *E. piscium*; C, Necrotic area of a granuloma where some parasites were detected stained with weaker intensity and, in some cases, only stained at the cells periphery (arrows); D, Granuloma in intestine with few *E. piscium* cells at the periphery (stained purple); E, *E. piscium* cells at the periphery of a muscle granuloma with a weak ISH- positive signal. Note the presence of parasites without hybridisation signal (arrows).

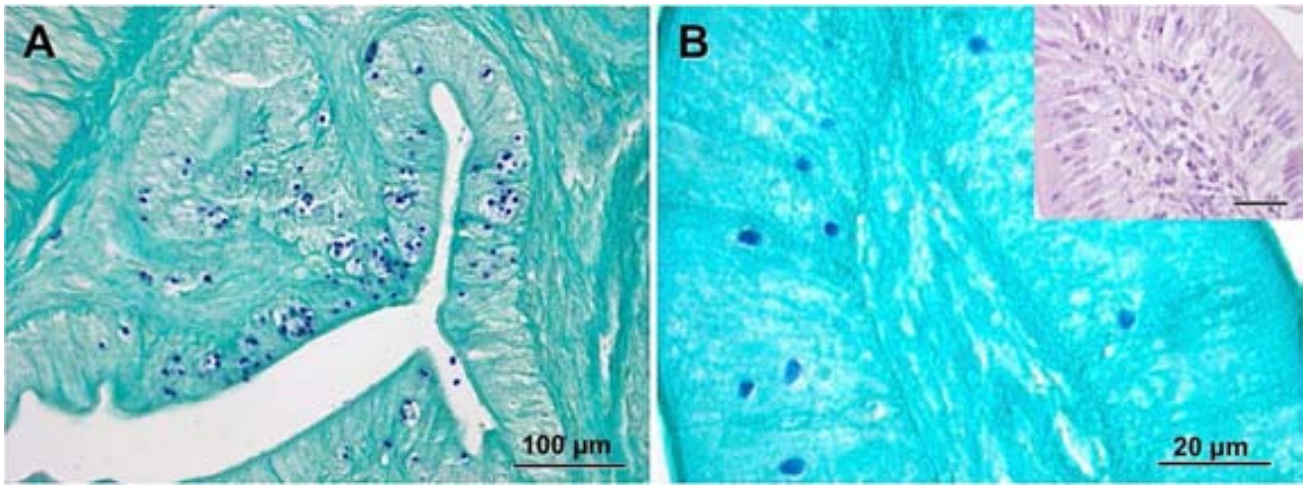

Figure 6.2: In situ hybridisation of histological sections of affected Senegalese sole intestines: Parasite cells are stained in blue/purple. A, Parasites within the epithelium; B, Small number of disperse parasites in a histological section where they were undetectable by conventional histology (inset: H&E-stained slide).

6.3.2. Diagnostic PCR

The four designed primers were used in pairs (table 6.2): the set A_amplified a 460 bp rDNA fragment region, and the set B yielded a 289 bp product (Fig. 6.3). The amplicon obtained with set A was specific to *E. piscium*, and it never amplified DNA from uninfected fish (negative controls), however a very faint band was amplified by set B (Fig. 6.3).

The precision of PCR technique was evaluated with tissue samples taken at different distances from the lesions. Variations on the detection of the parasite by PCR were observed among samples from symptomatic fish taken directly from the lesion (P = 85.19%), at 1 cm (P= 100%) and at distances larger than 3 cm from the lesions (P= 91.67%) (Fig. 6.4). Some asymptomatic fish were detected from a stock where the disease had previously identified. Although none of these fish presented macroscopical or histological lesions, PCR-positive muscle samples were detected in 54.55% of these fish.

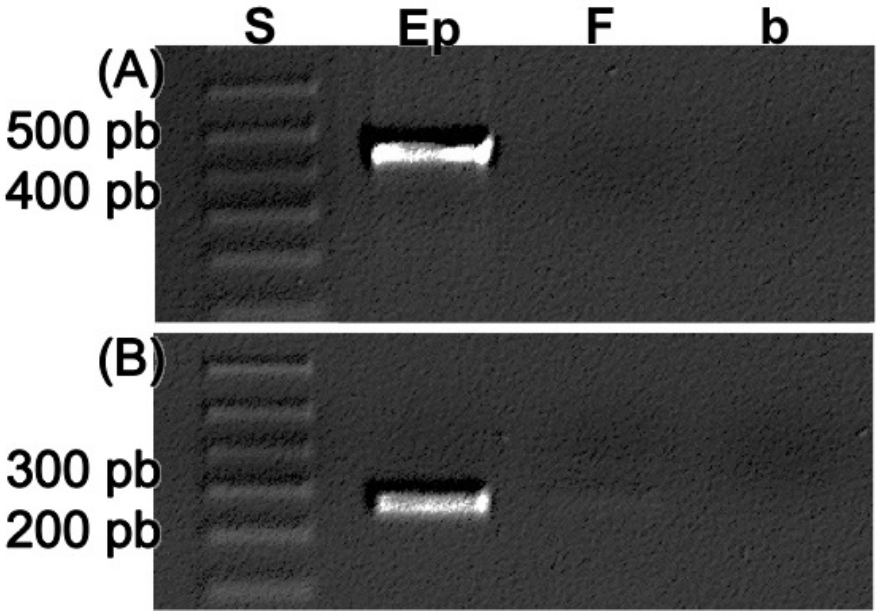

Figure 6.3: Ethidium bromide-stained agarose gel with PCR products generated using oligonucleotide probes specific for *E. piscium*: (A) Ep1579fL20 and Ep2017rL22; (B) Ep1306fL22 and Ep1573rL22. Products loaded in the different lanes are: S: Molecular weight standards (1Kb ladder). Ep: *E. piscium*- infected sole muscle. F: healthy (control) Senegalese sole; b: blank

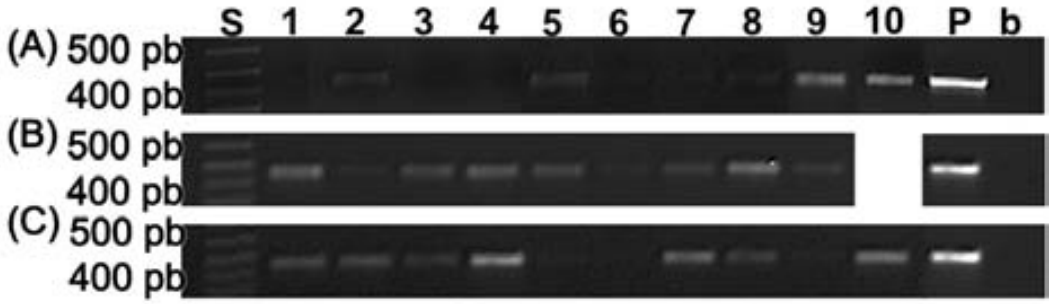

Figure 6.4: Ethidium bromide-stained agarose gel with PCR products generated from muscle of symptomatic soles using primers Ep1579fL20 and Ep2017rL22: (A) samples drawn from the lesion; (B) samples taken at 1 cm of the lesion (C) samples taken at 5 cm of the lesion. Products loaded in the different lanes are: S: Molecular weight standards (1Kb ladder); 1-10: different fishes; P: confirmed positive sample (positive control); b: blank.

6.3.3. Evaluation of diagnostic techniques

The epidemiological values of each technique are disclosed in table 6.4. ISH was the technique with the highest sensitivity and specificity in sampled intestines (Fig. 6.5). In these samples, the lowest positive predictive value was obtained with conventional histology. However, the best technique to detect *E. piscium* in muscle samples was conventional histology, although the ISH-positive predictive value remained in maximum values. In contrast, PCR yielded the lowest sensitivity values (Fig. 6.5), although positive and negative predictive values were still above 0.7. In general, both in intestine and muscle samples, the accuracy values were higher than 80% for all the diagnostic techniques tested.

Table 6.4: Comparative values among the different diagnostic techniques. CH: Conventional histology; ISH: In situ hybridisation; PCR: Polymerase chain reaction; NPV: Negative Predictive Value; PPV: Positive Predictive Value; S: Specificity; Se: Sensitivity.

	Se (%)	S (%)	PPV (%)	NPV (%)	Accuracy (%)
Intestine samples					
CH	85.1 (74.9-95.3)	76.7 (67.2-86.1)	69.1 (57.1-80.9)	89.4 (82.0-96.8)	79.8 (72.8-86.9)
ISH	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
PCR	68.6 (53.2-84.0)	96.8 (90.6-103.0)	96.0 (88.3-103.7)	73.2 (59.6-86.7)	81.8 (72.5-91.1)
Muscle samples					
CH	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
ISH	95.5 (89.3-101.6)	100 (100-100)	100 (100-100)	86.7 (69.5-103.9)	96.5 (91.7-101.3)
PCR	90.9 (83.3-98.5)	83.7 (73.3-94.0)	86.2 (77.3-95.1)	89.1 (80.1-98.1)	87.5 (81.1-93.9)

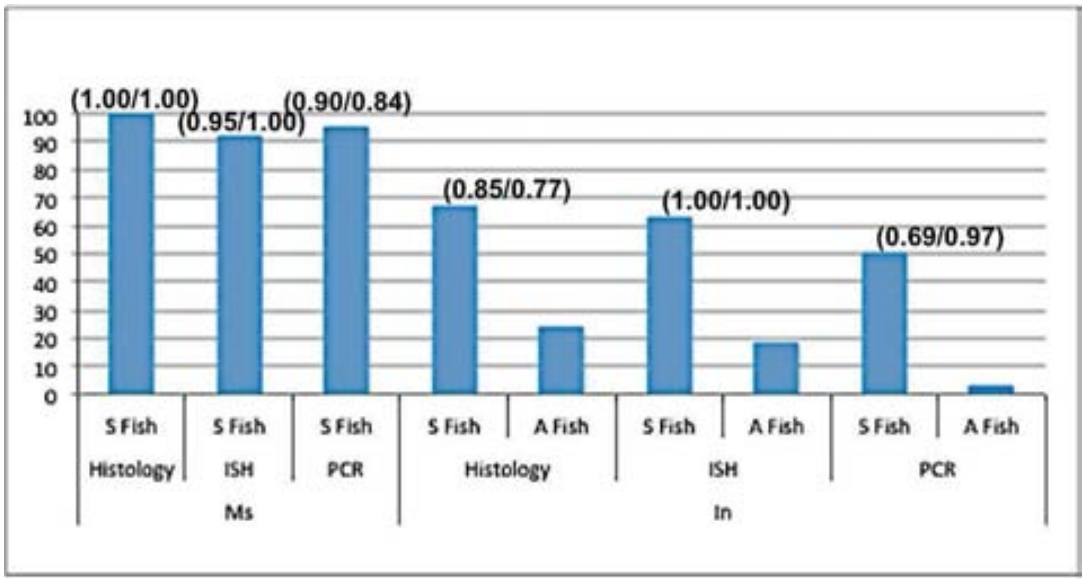

Figure 6.5: Prevalence of *E. piscium* in muscle from symptomatic fish (S Fish) and in intestine from both symptomatic and asymptomatic (A Fish) cultured sole analysed by different diagnostic techniques: Conventional histology (Histology), In situ hybridisation (ISH) and polymerase chain reaction (PCR). Mu: muscle; In: intestine. Numbers at the top represent values of sensitivity /specificity of each technique.

6.3.4. Further testing and application of diagnostic techniques.

6.3.4.1. Parasite distribution within the host:

E. piscium was detected in the periphery of the abscess-like lesions in muscle tissue of every symptomatic fish. Besides the skeletal muscle, lesions related to *E. piscium* were detected using histopathological techniques in heart and liver with high prevalence, followed by intestine and, eventually, kidney (Fig. 6.6). No lesions were found in the ovary or in the spleen. Interestingly, *E. piscium* was also detected by ISH within the stomach mucosa of one fish (Fig. 6.7A) as well as in the kidney glomeruli and parenchyma of another one (Fig. 6.7B), without apparent lesions.

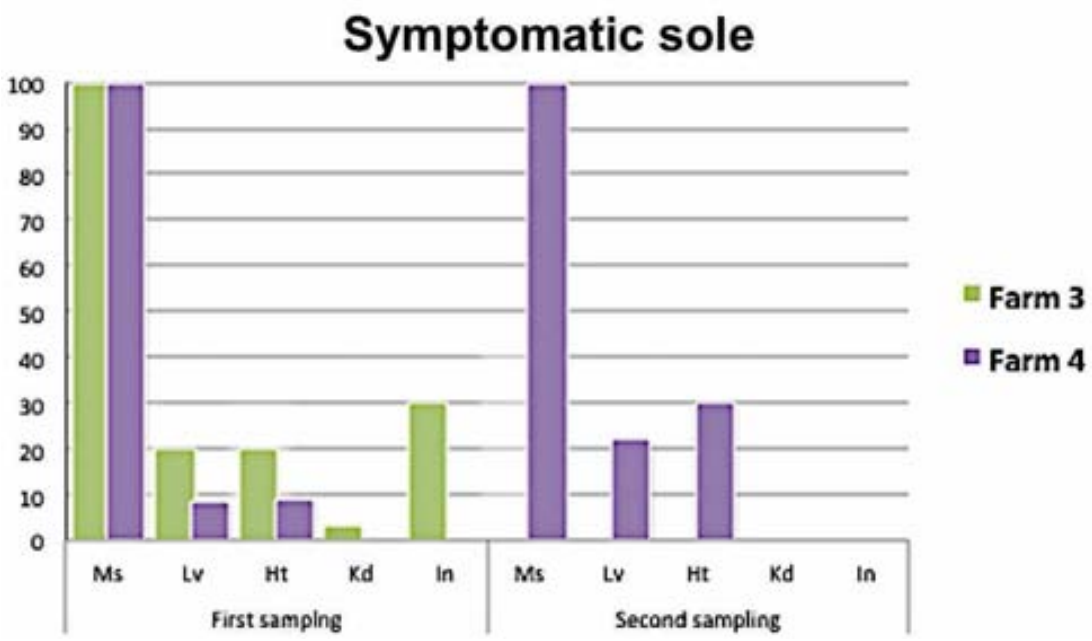

Figure 6.6: Prevalence of *E. piscium* in different organs from symptomatic cultured sole sampled at different facilities at the north-Atlantic area. Mu: muscle; Li: liver; He: heart; Ki: kidney; In: intestine.

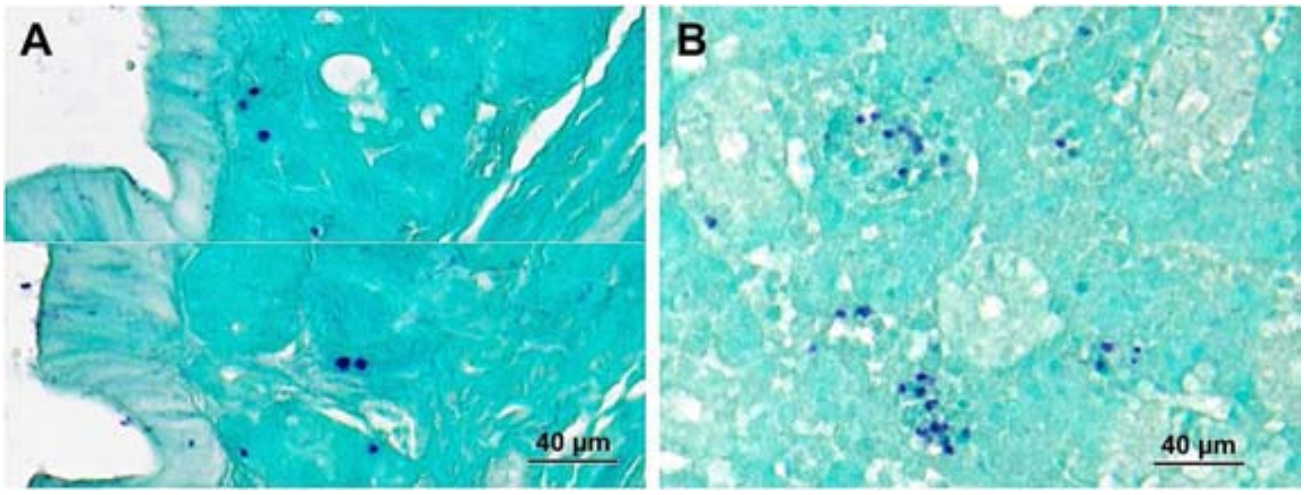

Figure 6.7: ISH of histological sections of affected Senegalese sole organs: A, Parasites within the stomach mucosa; B, *E. piscium* cells within the renal parenchyma and glomeruli without associated lesions.

Furthermore, *E. piscium* cells were detected within the intestinal epithelium from both symptomatic and asymptomatic fish. The parasites were present at different levels in the submucosa (Fig. 6.8A & 6.8B). Parasites within the intestinal epithelium appeared irregular in shape, and more variable in size. ISH-positive organisms were also detected on the border of the intestinal epithelium (Fig. 6.8C), entering or leaving it, and free in the lumen of the intestine (Fig. 6.8D). The intensity of the infections by *E. piscium* from symptomatic fish varied irregularly among each fish (MI between 1 and 2), whereas from asymptomatic soles was usually low (MI between 0-1) (Fig. 6.9). The distribution of parasites through the intestinal epithelium in infected fish was irregular, and areas with higher parasite load were usually found. Higher prevalence of parasites was usually found within the posterior part of the intestine in all samples examined, both from symptomatic (AIP=53.8%; PIP=55.0%) and asymptomatic fish (AIP=15.1%; PIP=19.5%), although no significant trends were detected ($p>0.05$, GZM).

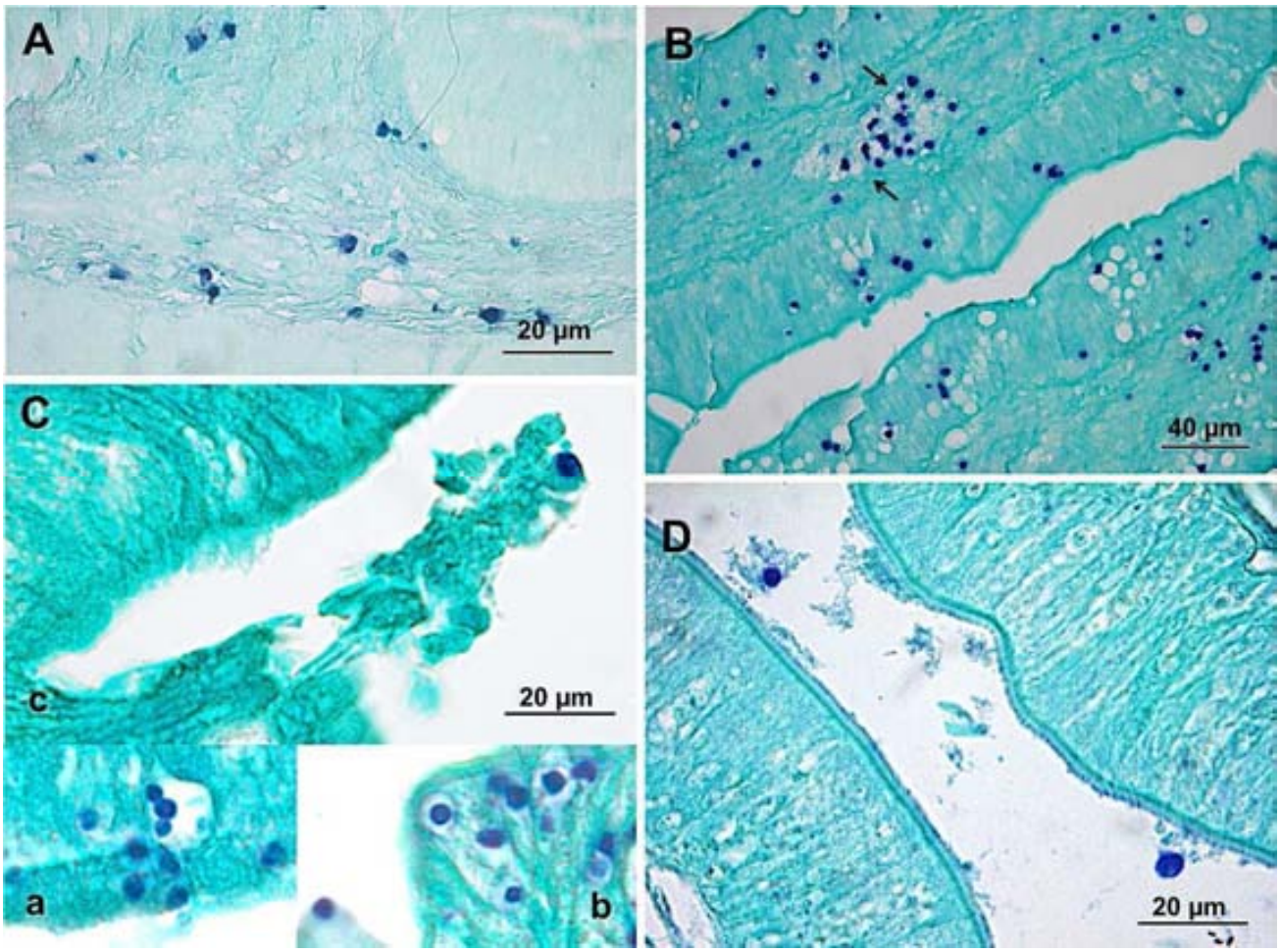

Figure 6.8. ISH of histological sections of affected Senegalese sole intestine: A, Parasites within the submucosa; B, Parasites within the submucosa in association of macrophage clusters (arrows) C, ISH-positive parasites located on the border of the epithelium (a and b) and entering or leaving it (c); D, ISH-positive parasites in the lumen.

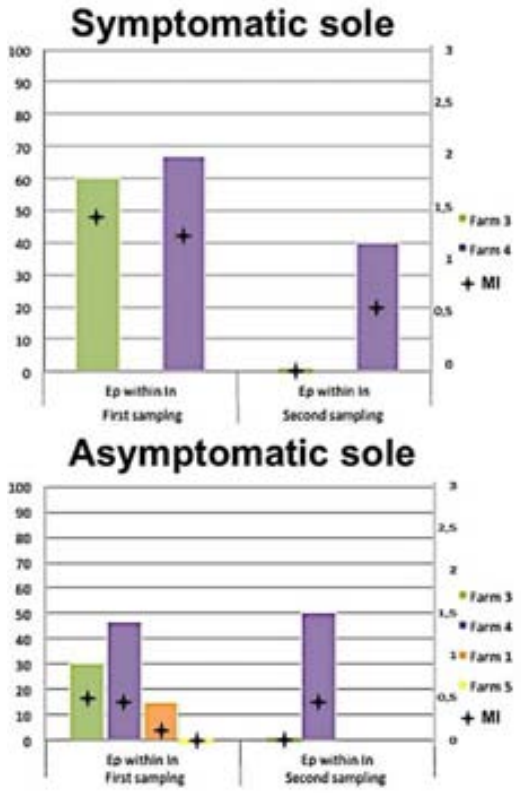

Figure 6.8: Prevalence of *E. piscium* within the intestinal epithelium from symptomatic and asymptomatic cultured soles sampled at different facilities at the north-Atlantic area. Ep: *Endolimax piscium*; In: intestine; MI: Mean Intensity. DATA are available for all farms both years: only available data are represented.

6.3.4.2. Epidemiology:

Preliminary epidemiological evaluation of the disease was studied by thorough histological examination (CH and ISH) and by PCR, on targeted samplings of symptomatic fish and random samplings of asymptomatic fish from different facilities.

Muscle lesions caused by *E. piscium* were detected in 100% of the symptomatic fish sampled in all farms (Fig. 6.6). In these fish, granulomatous lesions in the liver and heart were also detected, but with lower prevalence. In one farm only, granulomatous lesions in intestine and kidney were also detected (Fig. 6.6). Presence of *E. piscium* cells within the intestine (not associated to lesions) was also registered in symptomatic fish of all farms, with prevalence between 40 and 70% (Fig. 6.9). The prevalence of these infections with "disperse" *E. piscium* cells in the intestinal epithelium varied depending on the farms sampled. In farms where the disease had already been identified, *E. piscium* positive cases were detected in 30 to 50% of asymptomatic (randomly sampled) fish. Two months later, in one of these farms positive samples were not detected (after a sanitation period), however in the other farm the number of positive cases increased (Fig. 6.9). In two farms where no symptomatic fish had been detected, we found *E. piscium* cells within the intestine in 15% of the fish from one of the farms (Fig. 6.9), but no positive cases were detected in the other one.

6.4. Discussion:

6.4.1. In situ Hybridisation (ISH)

The ISH protocol detects *E. piscium* in different tissues of Senegalese sole. This technique proved to be an extremely useful tool for the detection of the parasite in fixed, paraffin-embedded tissues. The most important advantage of the ISH protocol over other current methods of detecting parasites is its ability to facilitate anatomic localization of the parasite in early developmental forms or in low- level infections. The hybridisation protocol developed did not alter the integrity of the tissue, allowing the detection of parasites when they were found in small numbers, when they were diagnosed as doubtful or even when they had been undetectable by conventional histology. Consequently, parasites were more easily detected by ISH than in parallel sections stained with H&E. Dyková and Lom (2004) already suggest that identifying amphizoic amoeba in histological material could contribute

significantly to disclose their pathogenicity and, in this sense, the ISH protocol developed for the detection of *E. piscium* can be extremely useful in the course of specific studies focusing on experimental infections or host-parasite relationships.

Neutral buffered 10% formalin is frequently used in the fixation of fish tissues for ISH detection of parasite DNA (Antonio et al., 1998 and 1999; Frasca et al., 1999; Morris et al., 1999) because its cross-linking activity causes limited fragmentation of DNA. Furthermore, it has been proved to maintain the restriction endonuclease patterns of methylation-sensitive enzymes (Dubeau et al., 1986) and the ability to clone the DNA (Goelz et al., 1985). However, cross-linking between DNA, RNA and proteins, induced by formalin, can decrease the accessibility of nucleic acids, particularly during prolonged fixation times, resulting in reduced signal intensity (Mostegl et al., 2011). Length of cross-linking fixation by formalin takes 24-48 hours depending on the size of the tissue sample (Werner et al., 2000; Webster et al., 2010; Mostegi et al., 2011), and it is recommended to be kept to a minimum for ISH protocols, in order to reduce masking of the targets. In our study, no differences were found in the ISH signal intensity of samples fixed for a week. In this sense, Jones et al. (2003) pointed out that the ISH signal loss, which occurred during fixation in neutral buffered formalin, was somewhat less rapid than in Davidson´s fixative, and weak ISH reactions were occasionally detected even after 28 days. In more specific studies, the signal intensity was found to be similar for variable times between 6 weeks and 1 year, depending on the pathogen and probe detection system, and the strength of the proteinase K treatment can restore the signal level to that of a 24h-fixed sample (Mostegl et al., 2011). In our protocols, less intense signal was observed in muscle lesions and some parasites even appeared unlabelled in some samples. This was probably due to the fixation of muscle samples in 15% formic acid, necessary to decalcify skin and/or bone debris. The use of 15% formic acid as a decalcification agent reduced the ISH sensitivity (Antonio et al., 1998) since it did not show consistent hybridisation signal in *M. cerebralis*-positive tissues. It has been shown that fixation in formalin at acidic pH or in the presence of formic acid causes degradation of the DNA much faster and extensively than neutral buffered formalin (Srinivasan et al., 2002). Better results in muscle tissue could be obtained by designing specific sampling and fixation protocols for this tissue directed to the use of this technique.

6.4.2. Diagnostic PCR

PCR primers set A developed in this study target specifically a 460 bps region of *E. piscium* 18S rRNA gene, providing a rapid alternative method for the diagnosis of this

amoeba. However, in this study its sensitivity and specificity was lower than in the other techniques. In muscle samples, PCR results seem to be affected by the site of the sample collection, and the technique was proved more effective in samples not taken directly from macroscopical lesions. This may be due to the presence in these lesions of dead or degraded cells, reducing the number of available targets, as well as secretions and necrotic material that can result inhibitory for the reactions. As observed microscopically in the lesions, their core is mostly filled by purulent material and scarce or no viable cells are present. However, obtaining the sample from apparently healthy tissue in the periphery of the lesion, or even further away, the accuracy of the technique substantially improved. The PCR proved a very convenient and sensitive test for fast, high-throughput screening and it allowed early detection of the parasites, although it provides little information on parasite location in specific tissues.

6.4.3. Evaluation of diagnostic techniques

The diagnosis methods are not always exact because of the possibilities to detect false positive results (healthy animals detected as positives) or false negative results (diseased animals detected as negatives). These errors can be quantified by comparing the diagnostic methods available with the real situation (or the best approach possible to the real situation, which is taken as a "Gold Standard" or GS). The microscopic diagnosis is the GS for some of the intestinal amoeba species, especially human parasitic amoebas, introducing morphological characteristics that allow their differentiation (Gomila Sard et al., 2011). However, there are some amoebas whose morphological features convergence hinder their differentiation and make it necessary to use other diagnostic tools. In the case of *E. piscium* diagnosis, histological examination (a combination of CH technique in muscle samples and ISH in intestine samples) was used as GS to compare the other techniques applied. The ISH technique was the most specific and sensitive, but it was not used as a GS in muscle samples because of the signal loss observed occasionally in this tissue, due to the use of 15% formic acid as decalcifying agent (see section 6.4.1). This sensitivity loss could be improved by fixing the samples briefly in Davidson's instead of in formic acid (Antonio et al., 1998; Palenzuela and Bartholomew, 2002) or using only NBF in muscle samples devoid of skin or bone.

In intestine samples, there were many positive cases that were not detected using conventional histology due to the small size and cryptic morphology of the parasite, resulting in reduced sensitivity. Furthermore, the specificity of conventional histology was

also compromised for the same reasons, because certain host cells can be easily mistaken by amoeba. Therefore, ISH was useful as a reference confirmatory method in intestine samples, not only to confirm positives but also to give stronger confidence in the final negative diagnostic, identified as doubtful by conventional histology. The differences in prevalence of *E. piscium* estimated by the ISH or the PCR techniques were higher in intestine samples, and they were probably due mostly to differences in the amounts of tissue assayed. It must be stressed that the amount of tissue taken for PCR, and "examined" in a PCR reaction, is orders of magnitude smaller than the histology-based methods, and thus PCR is more vulnerable to sampling artefacts or to patchy distribution of parasites, despite its higher sensitivity. As the parasite is not regularly distributed over the intestine, particularly at low infestations rates, it would be expected that increasing the sample size would increase the likelihood of parasite detection within it. In fact, almost all false negative samples by PCR corresponded with low parasite intensity and discontinuous distribution over the intestine.

Although the best results were found for conventional histology and ISH, depending on the tissue analysed, in the present work all evaluated technique have quite high quality indicators. Moreover, taking into account that some procedures are susceptible to improvement as observed in the course of the techniques development and evaluation, it will be possible to increase their reliability in further studies. By improvement of the fixation procedures in muscle tissues, ISH should be an optimum reference method for the confirmation of difficult cases or when the location of parasites in their biological context is necessary. In contrast, PCR should be a fast and reliable routine method once optimized sampling methods are tested and adopted.

6.4.4. Further testing and application of diagnostic techniques

6.4.4.1. Parasite distribution within the host

The prevalence of *E. piscium* from symptomatic fish revealed the muscle and the intestine as the target organs of this disease, although the parasites were also detected in liver, heart and kidney. This leads us to define this pathology as a systemic disease, as it was pointed out in earlier results. *E. piscium* was detected with high prevalence within the intestine of symptomatic fish, but also from asymptomatic fish. The studies of Entamoebid species from the human digestive tract (Gomila Sard et al., 2011) also reveal differences in prevalence between asymptomatic and symptomatic patients, being always higher in the latter, as in the case of this study. This finding is in agreement with the behaviour of other

intestinal amoebae, which are considered endocomensal organisms of different vertebrates and invertebrates, but in some cases can cause lesion in other organs. The route that these organisms use to breach through the intestinal barrier to reach other organs is still not completely understood. In the case of *E. histolytica*, the intestinal infection varies from asymptomatic colonization to severe invasive infections (Costa et al., 2010) and may even produce liver necrosis with slow expansion (amoebic liver abscess), progressing to the diaphragm and right lung by contiguity. The haematogenous dissemination through the portal vein is the most commonly accepted route of *E. histolytica* trophozoite invasion from the large intestine to the liver (Pérez-Tamayo, 1986) and to other organs (left lung, brain and pericardium) (Costa et al., 2010). *E. piscium* stages were found not only within the intestinal epithelium and inner layers of the submucosa, but also in the pericardial membrane and the connective tissue between muscle fibres and other organs. The presence of this organism within the connective tissue suggests that this tissue may play a significant role in the dissemination within the host, possibly facilitated in some way, or associated, to host cells such as macrophages. Once the parasite arrives to an organ and induce the development of a lesion, they are located in the periphery and progress toward healthy tissue expanding the lesion. Scattered, weakly stained organisms present towards the centre of the lesion may be inactive, decaying or dead parasite bodies. Holtzer et al. (2003) describe similar remarks for *Sphaerospora truttae* stages present within macrophages. The mechanisms related to the progression of the lesion are unknown, but some authors suggested that the destruction of *E. histolytica* trophozoites could promote the consequent release of toxic products and/or enzymes that would contribute to the increase of necrosis and stimulate the development of the granuloma (Rivero-Nava et al., 2002; Costa et al., 2007). Costa et al. 2010 pointed out the induction of apoptosis by *E. histolytica*, through direct activation of the host's cells distal apoptotic machinery. However, the liver parenchyma destruction seems related to the extensive lysis of inflammatory cells through release of their cytotoxic granules rather than to the direct effect of the amebae on the hepatocytes (Tsutsumi and Martínez-Palomo, 1988; Tsutsumi et al., 1984; Martinez-Palomo et al., 1989; Shibayama et al., 1998).

6.4.4.2. Epidemiology

The preliminary results of epidemiological screening for the amoebiasis at the different farms suggest that once disease has manifested in a farm, it is quite probable that asymptomatic fish also present parasites within their intestine, although not necessarily

presenting lesions. Similarly in other amoebiases, some *Neoparamoeba* strains have been isolated from clinically healthy fish (Dyková et al., 1999), and *Entamoeba gadi* was also detected in the intestinal lumen of asymptomatic fish (Bullock, 1966). According to some authors, the endocommensal and free-living amoebae are ubiquitously distributed in soil and aquatic habitats (Dyková and Lom, 2004; Gomila-Sard et al., 2011). Therefore, *E. piscium* could invade fish through the environment water, and remain latent in farms where disease is not detected. Under certain conditions, which are currently unknown, *E. piscium* could breach the intestinal barrier and spread systemically throughout the fish, causing serious lesions especially in muscle. Presence of parasites exclusively in the intestine, without lesions or infiltration to other tissues seems to be a initial stage in the development of the disease, and it does not appear commonly in farms in which the disease has not manifested at some point. In farms suffering outbreaks, the parasites were detected in the intestine of a high proportion of apparently healthy fish, and it is presumed that most of them would develop the disease later. Consequently, early detection of the parasite in the farm should be considered a priority for the management of this disease in the culture of sole. Although the parasite does not seem to cause direct mortality, the detrimental effects in fish health and growth are presumably not neglectable, and the muscular lesions developed later can prevent the commercialisation of the fish, after an important investment in their feeding and maintenance

The parasite does not appear to be widespread in all farms, since no parasite was detected in one of them. However, the epidemiological survey conducted in this study had a limited reach. The prevalence of *Entamoeba* from human digestive tract may also vary depending on the sampling area or age classes studied (Gomila Sard et al., 2011). Interestingly, in this work the only negative farm was northernmost sampled, and the one in which smallest fish were sampled. Further studies are needed to investigate which factors influence the occurrence of this disease, as well as the transmission of these parasites among fish (see Chapter VII).

CHAPTER VII: STUDY OF THE ESTABLISHMENT OF *E. PISCIUM* IN EXPERIMENTAL INFECTIONS BY COHABITATION

7.1. Introduction:

As it has been previously described in this work and in Constenla and Padrós, (2010) *E. piscium* is an Archamoebae causing systemic disease in Senegalese sole, *Solea senegalensis*, in culture conditions. In previous chapters the pathology, the identification of the causative organism and the development of diagnostic techniques have been described, providing the necessary tools for approaching the epidemiological study of the disease. Since no effective treatments are available to control the disease until now, prophylaxis appears as the only relevant way to manage the disease. The best way to prevent the entrance and spreading of the disease into the farms, is to avoid the parasite entering the fish or the farms and to control, or at least to difficult, the dispersion of the parasite to the different rearing units in the farms. To be able to accomplish these objectives, a deep and detailed knowledge on the mechanisms of parasite transmission is paramount. The knowledge of whether horizontal transmission of this parasite exists may facilitate maintenance of infections in laboratory conditions and thus increasing the understanding of the pathogenesis of this disease. The intestinal amoeba complex (*Entamoeba-Endolimax-Iodamoeba*) has common interesting epidemiological characteristics used as a first hypothetical approach for this study. Most of them have a cosmopolitan distribution and they have an identical transmission mechanism, always related to infectious mature cysts or through a fecal-oral transmission. Most studies conducted to try to understand the transmission of these amoebae have been made from human stool analysis (Garrido-González et al., 2002; Gomila-Sard et al., 2011; Heredia et al., 2012). Among these amoebae, the most studied species is *E. histolytica* due to its pathogenicity. *Entamoeba* spp. infections are maintained by transfer of infective cysts, from fecally contaminated food or water, to a non-infected individual through ingestion (Beaver et al., 1984; Haque et al., 2003). Experimentally, amoebic liver abscess can be induced in some rodents by inoculating virulent trophozoites of *E. histolytica* through various routes (Meerovitch and Chadee, 1988). Two of the most popular transmissions methods used for in vivo studies are injecting parasites in the portal vein or directly into the liver parenchyma via intraperitoneal injection (Shibayama et

al., 1998) but this method is very dissimilar to the natural routes of infection by these organisms. Assuming a similar transfer by cysts or other infective forms through ingestion of contaminated material, in fish farming conditions, the direct transmission of intestinal pathogens would likely be by direct contact between fish (fecal-oral transmission) or by cannibalism (oral transmission). In another relatively well known enteric disease in fish by the myxosporean *Enteromyxum leei,* horizontal transmission was experimentally demonstrated in different fish species via effluent, cohabitation, and oral routes as well as by anal intubation (Diamant, 1997; Diamant and Wajsbrot, 1997; Yasuda et al., 2002 and 2005; Sitjà-Bobadilla et al., 2007; Alvarez-Pellitero et al., 2008; Estensoro et al., 2010). In this disease the prevention of contamination by infected fish, effluents, faecal material and prevention of cannibalism from dead fish is very important to reduce the risk and the impact of this disease. The Apicomplexa *Cryptosporidium molnari* is also successfully transmitted by cohabitation and by oral infections with infected stomach scrapings. (Sitjà-Bobadilla and Alvarez-Pellitero, 2003).

In order to establish whether a horizontal infection is possible and to assess the likely route of entry of the parasite within the fish, an experimental infection by cohabitation between healthy and diseased fish was designed maintaining the condition as close and similar as possible to the real ongrowing conditions.

7.2. Materials and methods:

7.2.1. Source material

A total of 155 fish were used in 2012 for experimental infection tests conducted in ECIMAT (Vigo) (table 3.4, Chapter III (M&M)). Two groups of fish were used in this study: a first group of unaffected juveniles sole considered as ‘receptors’ and a second group of symptomatic fish considered as ‘donors’. Both groups were from different farms. The group of receptors was transported from a site considered free of the parasite (with no previous cases recorded) and also confirmed by a pilot sampling and testing. The donors group was selected from a farm where the disease was detected and a subgroup was

previously tested for the presence of *E. piscium*. Furthermore, 25 receptor and 10 donor fish were sampled before starting the experiment and tested for the presence of *E. piscium* to verify that the two groups complied the necessary conditions for the experiment.

Donor fish were maintained in a 1,500 litres fiberglass cylindro-conical tank with a continuous flow of natural seawater (37.5‰ salinity), filtered with a sand filter (40 μm) and under natural photoperiod and temperature (Fig. 7.2). A smaller (400 litres) quadrangular floating perforated plastic cage was introduced into the main tank harbouring 75 receptor fish, about 10 g in weight approximately (Table 3.4, Chapter III (M&M)) (Fig. 7.1). The ratio receiver: donor was approximately 1:2 and the cohabitation remained for four months (May-August 2012). This system allowed the cohabitation between the two stocks of fish avoiding the dominance, aggressiveness and competition for food pellets between them. Fish were fed ad libitum with commercial pelleted dies according to their sizes. A smaller control tank was maintained in another isolated room with 15 fish from the same stock of the receptors, and it was kept under the same conditions of the cohabitation tank.

Figure 7.1: Chronogram of the samplings carried out during the experimental infection according to the time. Numbers in brackets correspond to the number of fish sampled from each group: A, First control sampling and beginning of the experimental infection by cohabitation; B, First sampling at 15 days of cohabitation; C, Second sampling at 21 days of cohabitation; D, Third sampling at 1 month of cohabitation; E, Fourth sampling at 2 months of cohabitation; F, Fifth sampling and last control sampling at 4 months of cohabitation; CF: Control Fish; DF: Donor Fish; RF: Receptor Fish; WT: Water temperature.

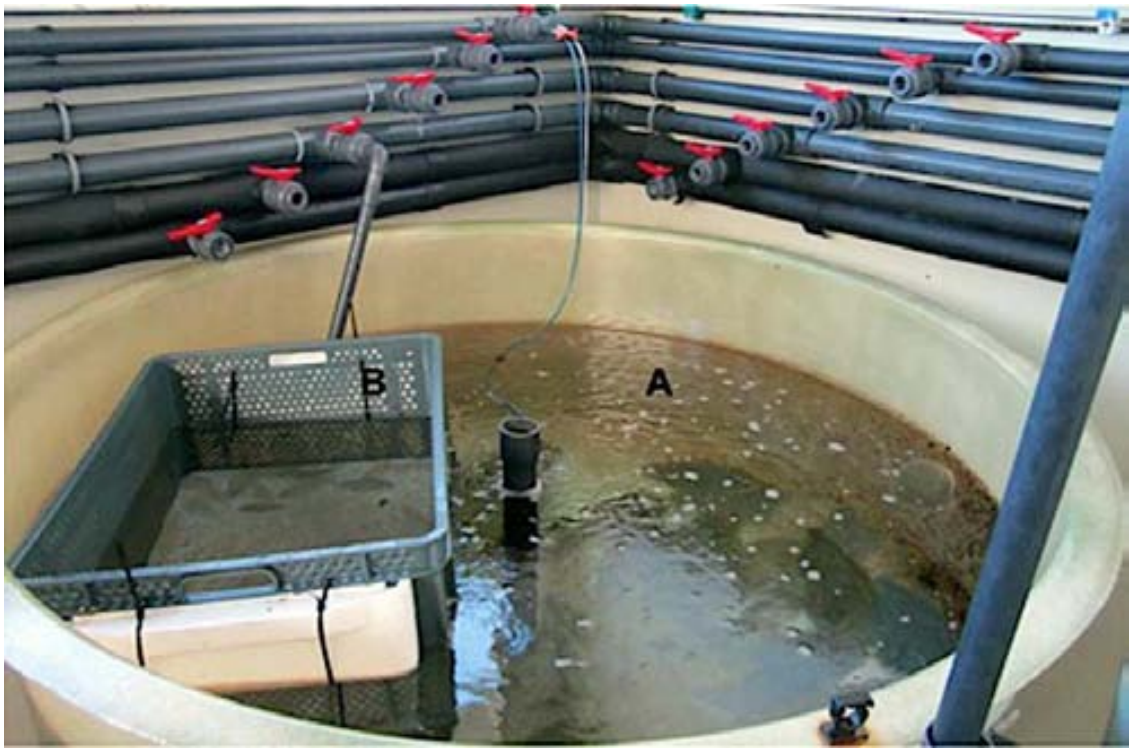

Figure. 7.2: Fiberglass 1,500 litres cylindro-conical tank containing donor fish (A), with a smaller (400 litres) quadrangular floating fiberglass tank containing receptor fish (B) during experimental infections.

Groups of 13-15 fish were sampled at different times post-exposure (p.e.) (see table 3.4, Chapter III (M&M) and Fig. 7.1). At each sampling, fish were sacrificed by an overdose of anesthetic (phenoxyethanol) previously dissolved in water, measured and weighted. Fish were necropsied in order to examine macroscopical lesions and samples of muscle, liver, spleen, kidney, digestive tract, heart and gills were dissected and fixed in 10% buffered formalin for histopathological studies. Muscle samples were taken always from the same area (medial dorsal zone). Samples of muscle and intestine were also preserved in 90% alcohol for molecular analyses.

Half a litre of water was collected from each tank at time 0, 1 month, 2 months and 4 months and filtered through cellulose ester filters (5 μm pore diameter) and preserved in acetone.

Due to budgetary and space constraints for more tanks, it was not possible to conduct the experience with the three tank replicas as originally envisaged.

7.2.2. Post-mortem techniques

7.2.2.1. Conventional histology:

Histological technique procedures are detailed in section 3.3.1.1 of the Chapter III (M&M). Staining of choice for all histological sections was H&E stain (see table 9.2, of Chapter IX (Annex 9.1)).

7.2.2.2. Diagnosis by in situ Hybridisation:

Intestines and muscle sections of each fish and some slices of other internal organs presenting lesions were processed by ISH protocol as developed previously (see section 6.2.2.2. of the previous chapter).

7.2.2.3. Diagnosis by PCR:

Fish samples preserved in 90% ethanol and water samples preserved in acetone, after a specific pre-treatment (table 9.9 of Annex 9.2), were processed for diagnosis by PCR (see section 6.2.2.3. of the previous chapter)

7.2.3. Rates determination

The presence or absence of the parasite in the different organs within the fish was studied. A tissue was considered infected when some stage of the parasite was directly identified within the tissue studied, or when DNA amplification was achieved in the PCR. This allowed obtaining a prevalence value (P) of infection, defined as the proportion of tested individuals (N) that resulted infected (n):

$$P= (n/N)*100$$

In order to evaluate the infection intensity, three random semiquantitative counts of the number of parasites were performed on each ISH positive area of histological section at 100x of magnification, and scored as: none (0), low (1) (1-5 E. piscium cells per observation field approximately), medium (2) (6-25 *E. piscium* cells per observation field approximately) or high (3) (more than 26 *E. piscium* cells) (see Fig. 7.3). Mean intensity was calculated from the average intensity values of each individual fish for both donor and receptor

groups. In addition, the infection was classified as extensive or general infection (if the parasite was present in all examined tissue) or restricted or partial infection (parasites affecting only limited areas of some examined tissues).

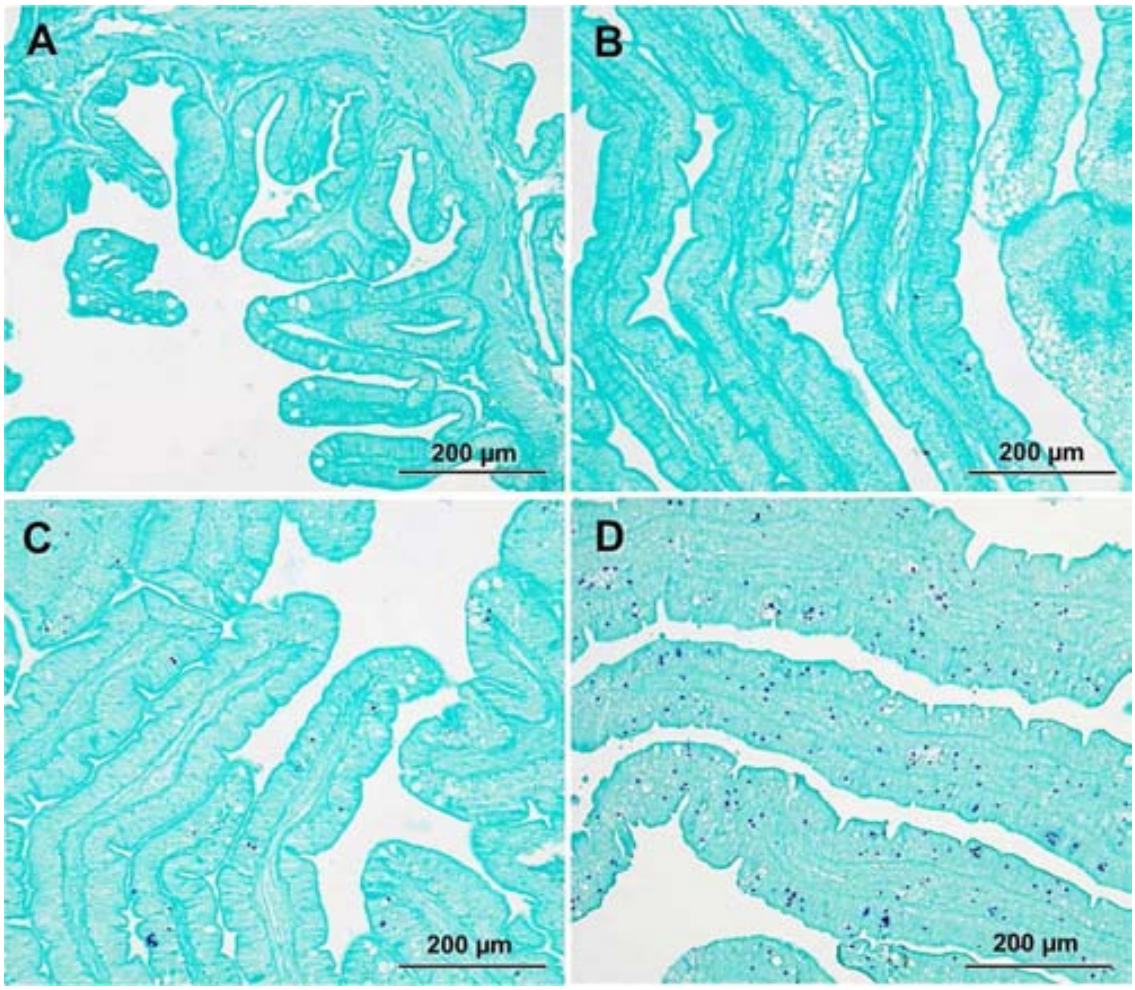

Figure 7.3: Histological sections of intestines stained with *E. piscium* specific ISH: A, ISH-negative intestine which correspond with uninfected tissue; B_D, *E. piscium* parasitized intestines (ISH-positive) displaying different degrees of intensity: low (B), medium (C) and high (D).

7.3. Results:

7.3.1. Control fish

None of the 25 control fish showed positive results in any of the diagnostic tests carried out before the start of the experimental infection.

During the experiment, two fish died but unfortunately due to advanced autolytic conditions were not acceptable for analyses and at the end of the experiment, none of the 13 fish from the control tank showed clinical signs or presented specific lesions or parasites detected by any of the techniques.

7.3.2. Receptor fish

After the introduction of the receptor fish into de experimental tank at the first 15 days of cohabitation, 7 receptor fish presented progressive erosion on the caudal fin (Fig. 7.4A) and two of them died. Light microscopy of wet mount preparation from the external lesions of these two fish revealed the presence of filamentous bacteria (Fig. 7.4B). Histopathological analysis confirmed the results, indicating a typical bacterial dermic lesion originated in the caudal fin rays and progressing towards the caudal peduncle (Fig. 7.4C). These lesions were characteristic of caudal infection by *Tenacibaculum* sp. so fish were treated accordingly (one hour bath of 50 ppm Oxytetracycline (Oxytevall^{R}) in a recirculation without biofiltration during 3 days). A positive response was detected after the treatment and no further deaths occurred. No other histopathological changes were observed in any of the receptor fish. Both ISH and molecular assays were negative to *E. piscium*.

In the third post-exposure week, 6 receptors fish were still showing lesions in the caudal fin and one of them was died. Histopathological analysis also revealed the presence of filamentous bacteria associated to necrotic and inflammatory lesions in the caudal fins similar to the observations in the previous sampling, but no other histopathological changes neither positive results by ISH and PCR against the amoebic organisms were detected.

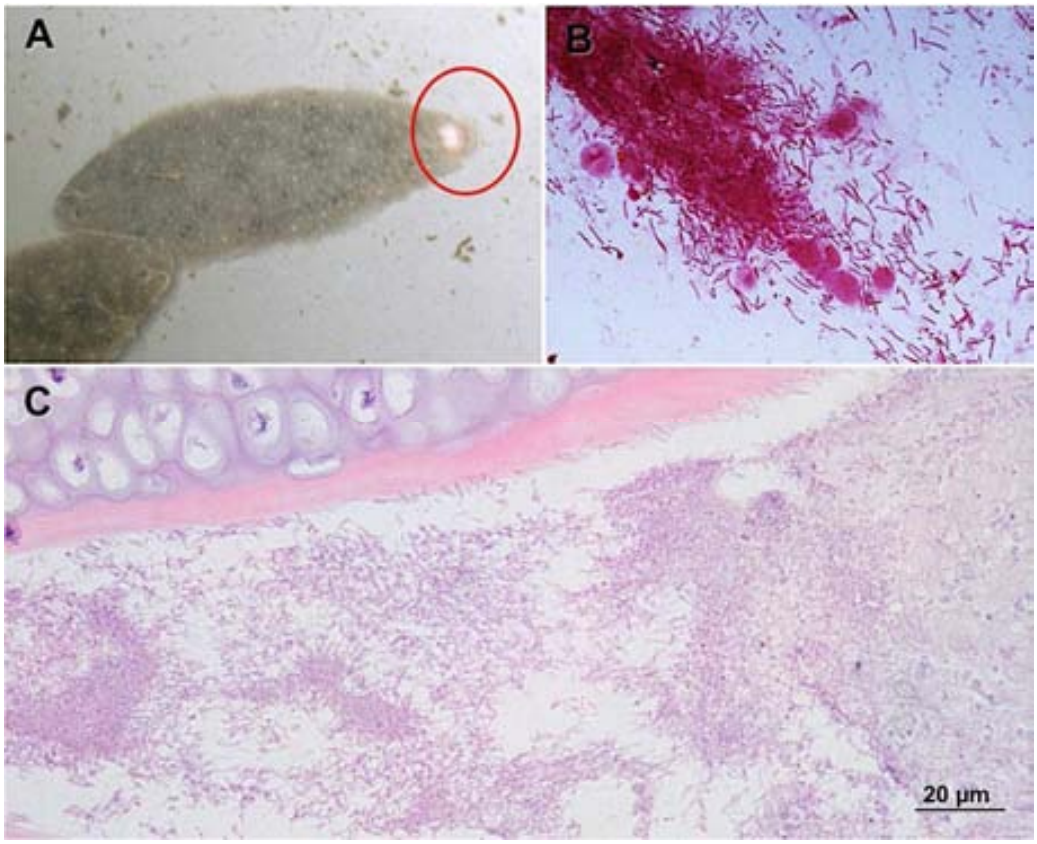

Figure 7.4: A, Receptor fish with a lesion on the caudal fin (circle); B, Light microscopy of an imprint from the external lesions showing abundant presence of filamentous bacteria; C, Histological section of the caudal fin where several filamentous bacteria are observed close to the fin rays.

After a month in cohabitation, 4 fish displayed some mild intestinal distension, however no pathological changes were detected by the histopathological study and both ISH and molecular assays were also negative to *E. piscium*.

No macroscopic alteration was observed after two months post-exposure, except for two more cases of intestinal dilatations. The histopathological study of these both intestines revealed no pathological changes, however at this time, intestines of five other fish displayed some changes compatible with amoeba-like organisms within the intestinal epithelium (Fig. 7.5A). Furthermore, some of the liver samples showed areas of necrosis. Nevertheless, ISH studies and molecular assays did not detect *E. piscium* positive signal in any of the cases (Fig. 7.5B).

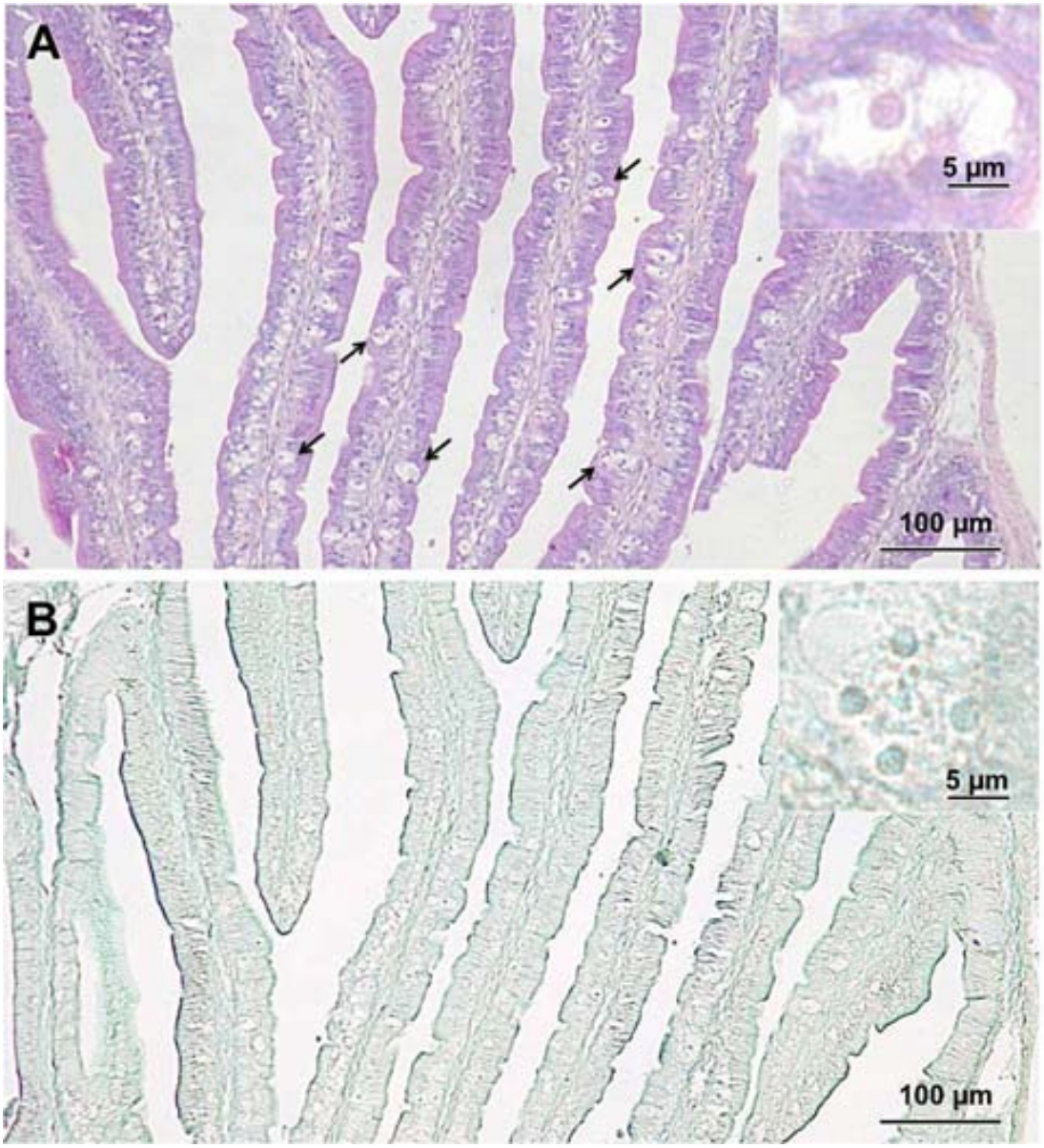

Figure 7.5: Histological sections of intestines from a receptor fish sampled at 60 days post-exposure. A: Several amoeba-like cells within the intestinal epithelium (H&E stain). Detail of these organisms; B: ISH-negative section of the same intestine. Detail of the ISH- negative amoeba-like organism.

At the end of the experiment (4 moths post-exposure), one single fish was found dead in the tank (autolytic too). From the fish sampled at this time, no macroscopical alterations were found in any of the fish. However, the histopathological study revealed the potential presence of amoeba-like organisms within the intestinal epithelium from 6/14 fish, and ISH studies confirmed *E. piscium* organisms in four of the intestines (Fig. 7.6 A&B), some of them showed also areas of necrosis within the liver, but no relationship was found between liver lesions and parasites. Molecular assays for *E. piscium* were positive in four of the 14 sampled fish (Fig. 7.7A), but one of them was not consistent with the ISH results. Moreover, infection was detected by PCR from muscle samples in three fish, (Fig. 7.7B), and two of these also presented parasites within the gut.

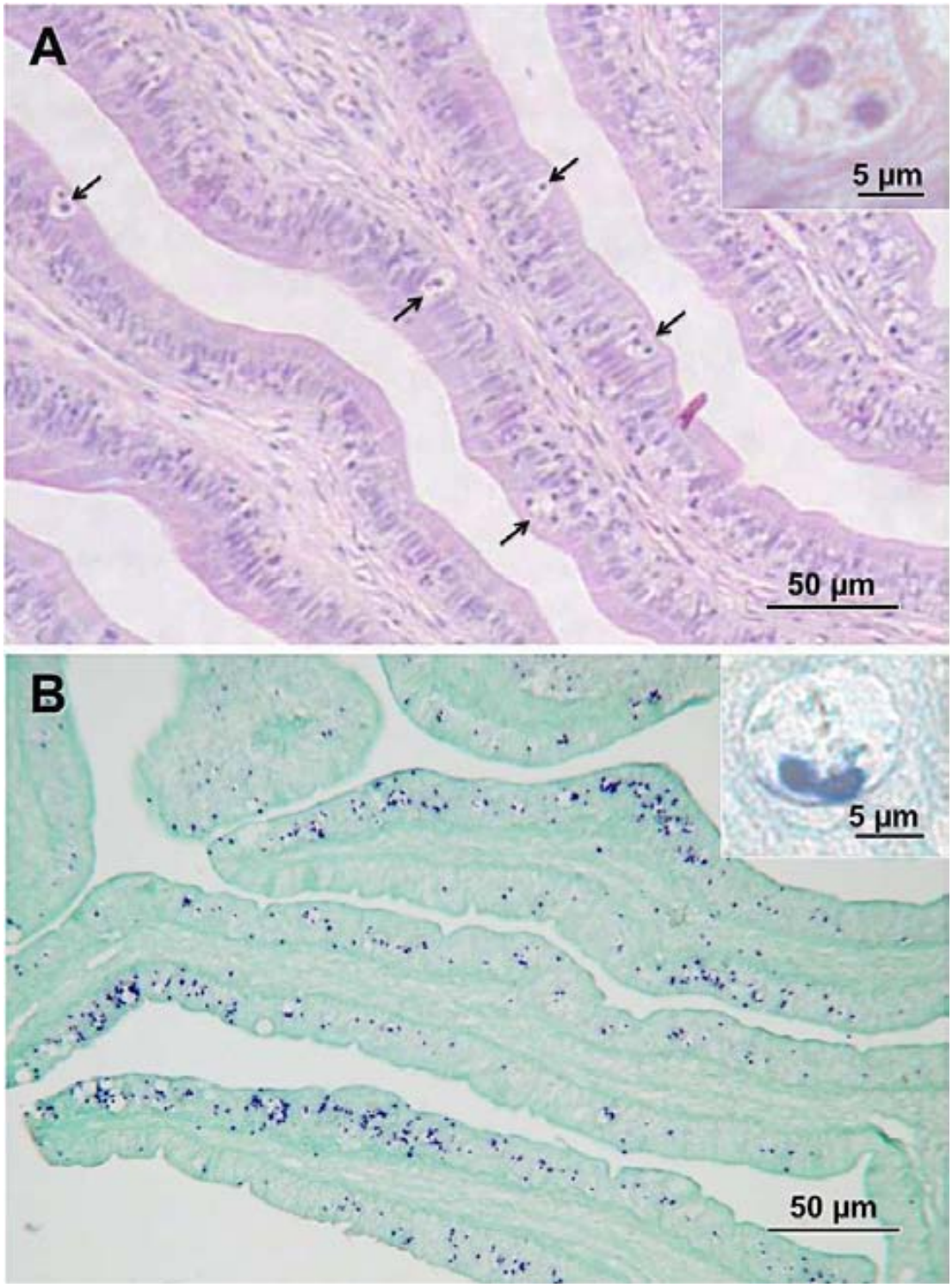

Figure 7.6: Histological sections of intestines from a receptor fish sampled at the end of the experimental infections (4 months post-exposure). A: Several amoeba-like cells within the intestinal epithelium (H&E stain). Detail of these organisms; B: *E. piscium* ISH-positive signal (purple cells) in section of the same intestine. Detail of *E. piscium* ISH- positive.

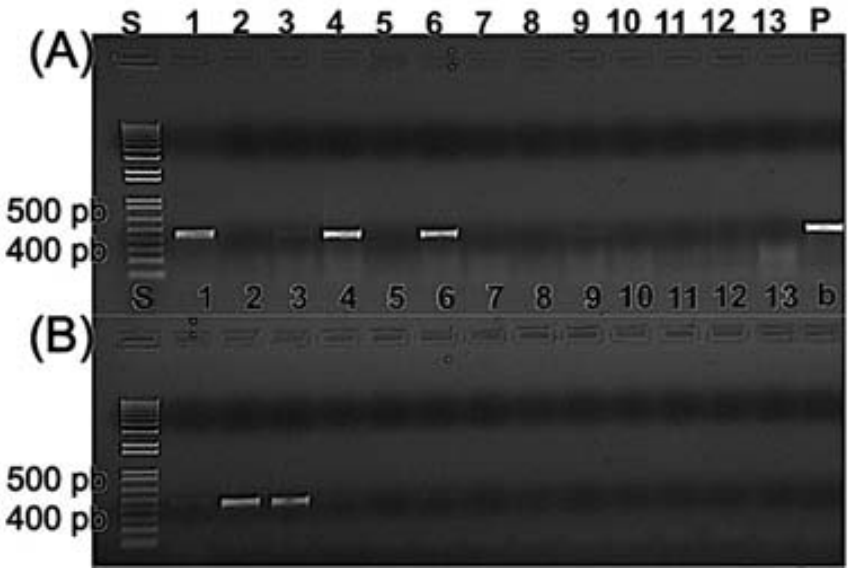

Figure 7.7: Ethidium bromide-stained agarose gel with PCR products generated using oligonucleotide probes specific for *E. piscium* (Ep1579fL20 and Ep2017rL22) in intestine (A) and muscle (B) samples from receptors fish at 4 moths post-exposure. S: Molecular weight standards (1 Kb ladder); Ep: samples of *E. piscium* infected muscle from Senegalese sole; 1-13: different fish; P: positive control; b: blank.

7.3.3. Donor fish

In order to confirm the infection by *E. piscium* in the donor fish, at the beginning of the challenge 10 fish were sampled. Five of them showed macroscopically visible muscle lesions (bumps) (Fig. 7.8A) containing material with purulent aspect under the dermis (Fig. 7.8B). 30% of the sampled fish also presented nodules in the heart (Fig. 7.8C) and in one of the fish, well-defined nodules protruding from the skin surface into the abdominal cavity were also observed (Fig. 7.8D.). The histopathological study of the different organs confirmed that these lesions were caused by *E. piscium* (Fig. 7.9 A&B) and also revealed lesions in the liver (Fig. 7.9 C&D), and kidney associated with the parasites. Virtually all fish showed *E. piscium* in the intestine (Fig. 7.9F) with a low to medium intensity (Table 7.1), in most cases with restricted

distribution (only specific areas with parasite load) as described in the previous chapter.

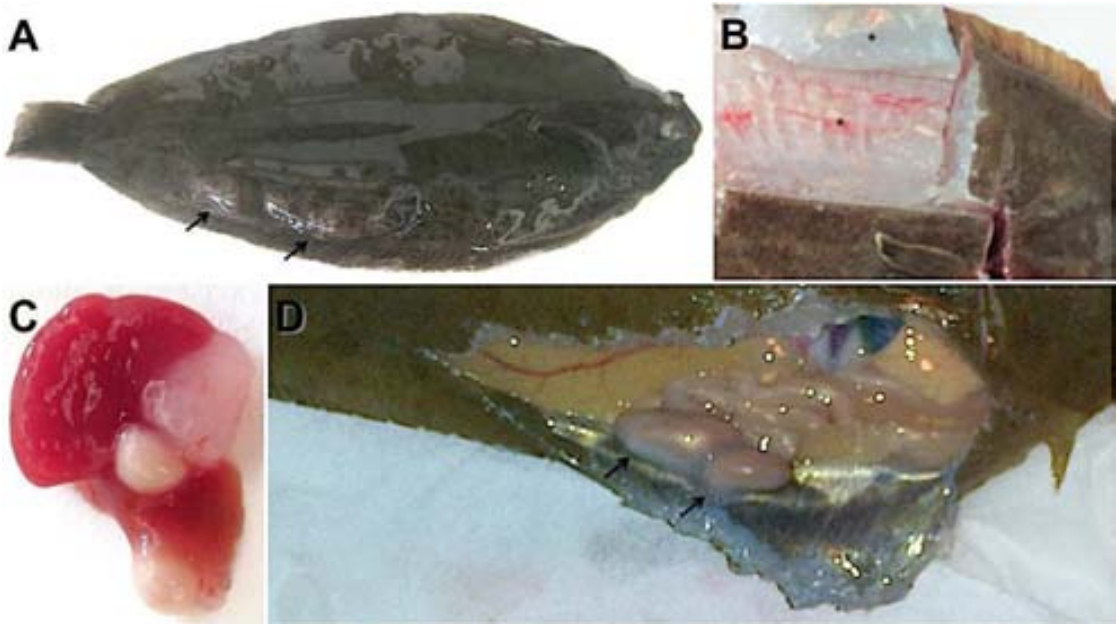

Figure 7.8: Donor fish with nodules in different organs: A, Ocular side where lumps (arrows) along the base of ventral fin are observed; B, Small lesions (*) with liquefactive aspect within the muscle; C, Two circumscribed nodules in the heart; D, well-defined nodules (arrows) protruding from the skin surface into the abdominal cavity.

In the subsequent samplings of donor fish, infection by *E. piscium* was confirmed with similar lesions to those described above. However, fluctuations were observed among samplings, being the last sampling the one in which the highest prevalence of muscle lesions were found (Fig. 7.10). Parasites within the intestinal epithelium were detected with mean prevalence of 60%. However, fluctuations were also observed among samplings, being the last sampling the one with lowest prevalence values of *E. piscium* within intestine (table 7.1). The intensity of the infection within the intestine was between low and medium in most of the cases (table 7.1). *E. piscium* was detected in less cases by PCR assays than by histological observations (table 7.1). Parasites were usually distributed as a restricted infection, but in some cases an extensive distribution was also found (table 7.1). In addition, through ISH, *E.*

piscium stages within the gill lamellar capillaries were also detected (Fig. 7.11) without an apparent lesion in the host.

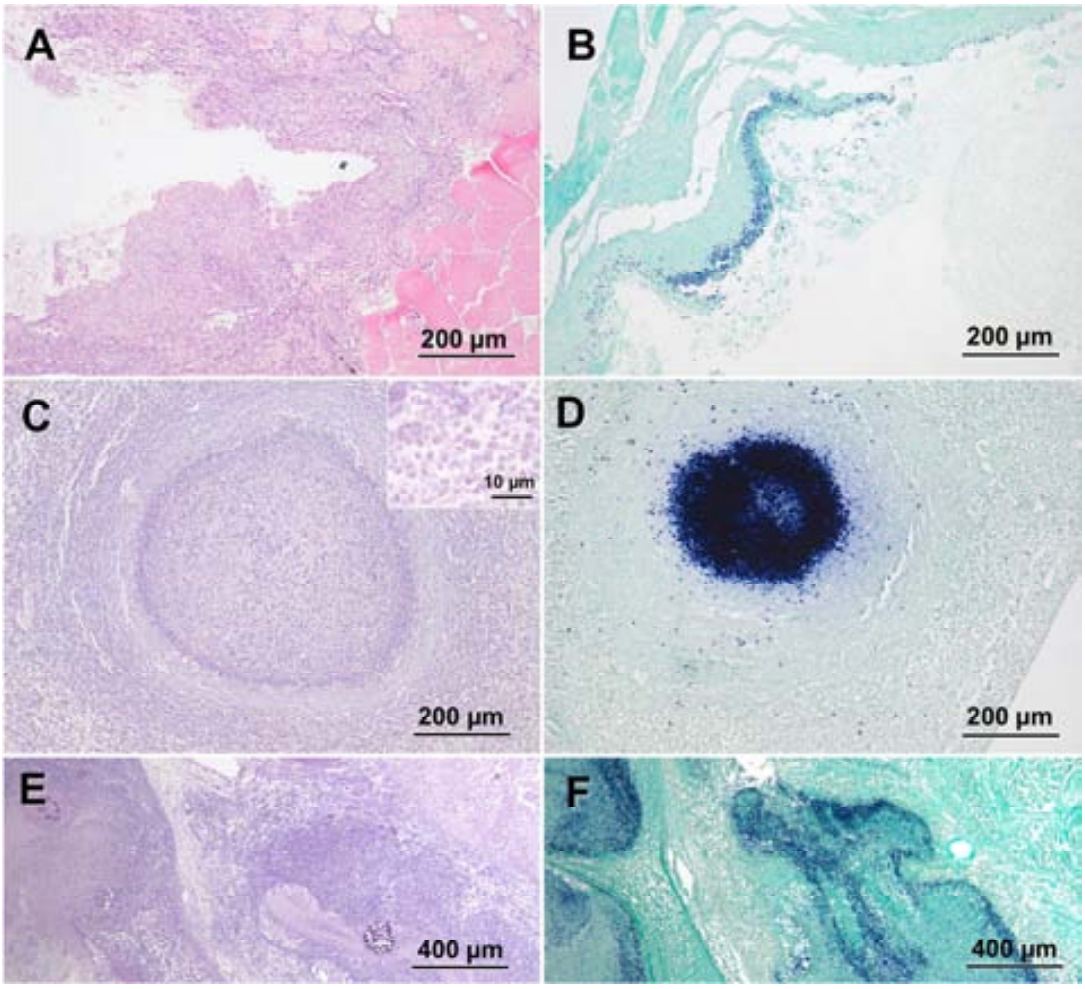

Figure 7.9: Histological sections of donor fish before the start of the experimental infections by cohabitation: A, Inflammatory reaction in muscle (H&E); B, Inflammatory reaction in muscle where *E. piscium* ISH-positive signal (purple cells) is observed; C, granulomatous inflammatory reaction in liver (H&E). Detail of parasites surrounding the lesion; D, granulomatous inflammatory reaction in liver with *E. piscium* ISH- positive signal (purple cells); E, Intense and extensive inflammatory reaction in heart, liver and transversum septum (H&E); F, *E. piscium* ISH-positive signal (purple cells) of the same anterior section.

Table 7.1: Prevalence (P), Mean Intensity (MI) and distribution (D) of *E. piscium* within intestine from donor fish at different samplings. In: intestine; p.e.: post-exposure.

Days	**P (%)**		**MI (%)**				**D (%)**	
p.e.	Histology	Molecular	0	1	2	3	Partial	Extensive
0	70	70	30	50	10	10	86	14
15	60	20	40	20	40	0	67	33
21	80	20	20	60	20	0	75	25
30	60	40	40	0	40	20	33	67
60	60	20	40	40	20	0	100	0
120	40	-	60	30	10	0	75	25

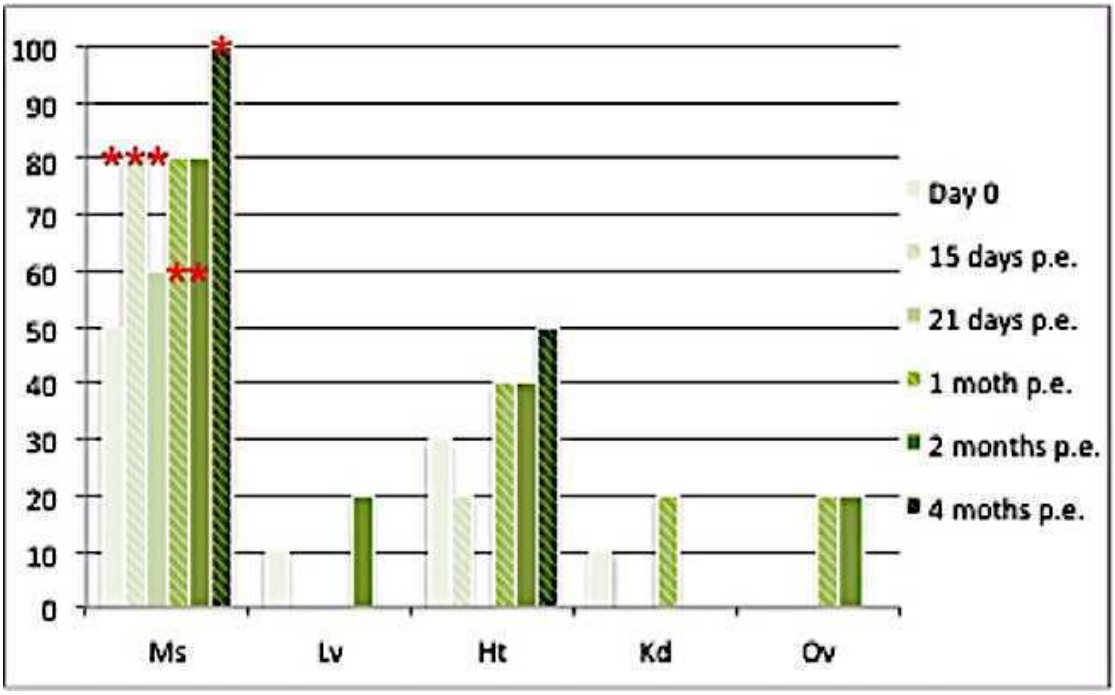

Figure 7.10: Prevalence of lesions caused by *E. piscium* in different organs from the donor fish during the experimental infections. Asterisks show the results by PCR technique. Ht: heart; Kd: kidney; Lv: liver; Ms: muscle; Ov: ovary; p.e.: post-exposure.

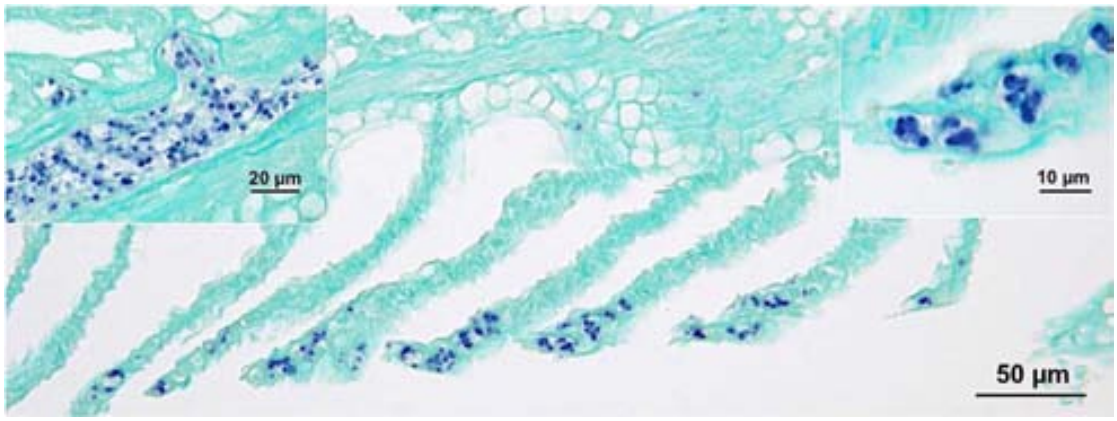

Figure 7.11: ISH-positive parasites (purple cells) within the connective tissue of the gill filament and gill lamellar capillaries, without lesions associated to their presence.

7.3.4. Water samples

None of the water samples collected in the control tank, during the experiment, were detected positive for *E. piscium* by PCR.

Water samples from the transmission tank, both at the beginning of the experiment and at months 1 and 2, were also negative. However, after 4 months post-exposure, *E. piscium* presence was detected by PCR in the water (Fig. 7.12).

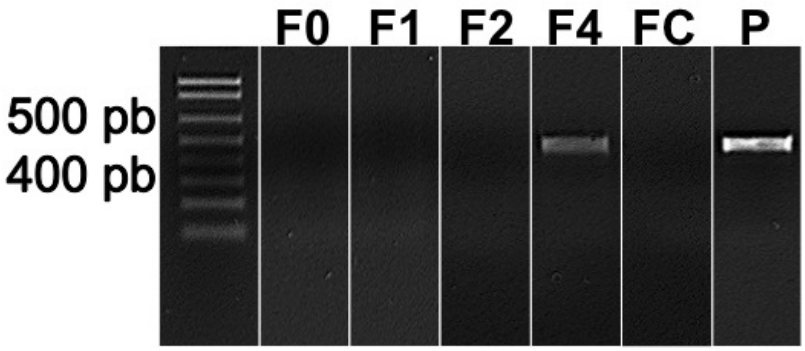

Fig. 7.12: Ethidium bromide-stained agarose gel with PCR products generated using using *E. pisces* specific oligonucleotide primers (Ep1579fL20 and Ep2017rL22) in water samples. S: Molecular weigth standards (1kb DNA ladder); F0-F4: water samples from the transmission tank at different times of the experiment: at the beginning of the experiment (F0), at 1 month post-exposure (F1), at 2 month post-exposure (F2), at the end of the experiment (4 month post-exposure) (F4); FC: water sample from the control tank at the end of the experiment (without exposure); P: positive control.

7.4. Discussion:

To the best of our knowledge, this is the first time that an experimental infection of endoparasitic amoebae is performed by cohabitation between fish. The results obtained in this study of experimental infection have yielded relevant information about the transmission of this disease in sole, since the horizontal transmission is clearly confirmed. Since receptor and donor fish were cohabiting in the same tank but they had no physical contact between them, it is strongly suggested that transmission occurs through water. Presence of *E. piscium* DNA in the water samples from the cohabitating tank was demonstrated by the PCR, further supporting this hypothesis of the horizontal transmission. In addition, *E. piscium* organisms were not detected in any of the samples from the control tank during the entire duration of the experiment (control fish or water samples). This fact supports the hypothesis that infective forms infecting receptor fish proceed from donor fish and not from other potential sources, such as normal water supply or food, as it has

been sometimes suggested.

Since positive cases were not detected in fish or water samples until four months post-exposure, a slow transmission and long prepatent period are suggested for this disease. However, no samplings were carried out between the second and the fourth month, and an earlier infection should not be disregarded. Other infections in fish by intestinal parasites appear to be faster, but timing also depends on the infection methodology used. The Coccidium *Cyptosporidium molnari* in experimental infection by cohabitation between sea bass (*Dicentrachus labrax*) and infected gilthead sea bream (*Sparus aurata*) (Sitjà-Bobadilla and Alvarez-Pellitero, 2003) is detected in the 100% of the receptor fish in less than 21 days. The mixosporean *Enteromyxum scophthalmi* is not detected in the intestine of turbot (*Scophthalmus maximus*) until 40 days post-cohabitation with donor fish, though parasites are detected at 20 days in effluent transmission and only after 2 days in orally transmitted (Redondo et al., 2004). The incubation periods of the diseases caused by free-living amoebae infecting humans are not well known, but they are usually considered tedious and several weeks or months may elapse (Visvesvara et al., 2007). Intestinal infections by *E. histolytica* can be developed months or years after a colon infection (Stanley, 2003), so according to this, the relatively extended time for the detection of the infection in this case is not so unusual for amoebic infections.

E. piscium belongs to the family Entamoebidae, which includes parasites with two stages in their life cycle: a trophozoite and a cyst (Silberman et al., 1999). The cyst is the resistant stage that allows the parasite to remain in the environment after leaving the host, until reaching new hosts to continue their life cycle. The prevalence of infection in a population is an indicator of the abundance of infective cysts and the efficiency of transmission (Knight, 1975; Walsh, 1986). The abundance of infective cysts depends on total cyst production, mechanisms of dispersion, and cyst survival in the environment. Notwithstanding its relevance for transmission, very little has been added to this field of *Entamoeba* research since the initial studies in the first half of the 20th century (Dobell, 1917 and 1928; Wenyon and O'Connor, 1917; Svensson and Linders, 1934; Lincicome, 1942). In these studies, short synchronised cycles related to Entamoeba cyst production are described, in such a way that

periods in which cysts are not detected are followed by sudden cyst production periods. In the case of *E. histolytica,* infective cysts invade the host when they are ingested through contaminated food or water. Once in the intestinal lumen, the trophozoites are released by excystation (Haque et al., 2003). Usually trophozoites remain in the lumen as commensals, multiplying and feeding on the host's microflora and nutrients. Some parasites can encyst again, resulting in the formation of mature infectious cysts in faeces and the perpetuation of life cycle (Haque et al., 2003). In the present study of *E. piscium,* it was not possible to observe differential trophozoite and cyst stages. However, and according to the information available for closest relatives to *E. piscium,* the possibility that this species generates cysts should be can be considered. If that is the case, the *E. piscium* parasitic forms detected in the water of experimental transmission tanks by PCR could correspond to the cysts, which would remain in the water and infect the receptor fish. If this were confirmed, it should be taken into account for the farms prophylaxis because some amoebae cysts can be very resistant to temperature, desiccation and disinfection treatments (Coulon et al. 2010).

In 90% of cases amoebic infections are asymptomatic and self-limited (Haque et al. 2003). Many of amoebae are opportunistic pathogens and disease outbreaks take place favoured by immunodepression or suboptimal environmental conditions (Nash et al., 1988; Noble et al., 1997). Brain lesions caused by infections by *Acanthamoeba* species, *Balamuthia* or even *Entamoeba*, among others, are usually seen in people suffering from chronic diseases, having compromised immune systems, or during outbreaks of bacterial infections such as Legionella and Mycobacteria (Visvesvara et al., 2007). Regarding *E. piscium* in fish and from the results obtained in the present studies, the intestinal mucosa appears to be the main target organ for the survival of the amoeba within the fish. However, in donor fish and in previous studies of this disease (see Chapter I and III), *E. piscium* was also found with high prevalence in muscle and in other organs causing lesions. The fact that *E. piscium* is detected only within the intestine of the receptor fish and no lesions are observed in any of their sampled organs, suggest two possible scenarios: a) the duration of the challenge (four months) is not enough for the

dissemination of the organism to other tissues and organs and; b) the triggering factor or specific conditions for the progression of the disease is necessary. In any case, a combination of both possibilities would also be possible. Until this condition or conditions are met, *E. piscium* would remain within the intestinal epithelium, in a latent form of the disease. At certain point, the dispersion of *E. piscium* throughout the fish organism would arise. In this study, temperature could have an important role as a triggering factor, since in the last month of the infections the highest values of this parameter were recorded, getting close to the discomfort range of temperatures that trigger thermic stress and enhance the development of diseases in sole (Cañavate, 2005).

In *E. histolytica*, extraintestinal dispersion of the disease can coexist simultaneously with further invasion of the intestine, but often occurs in the absence of either intestinal symptoms and trophozoites or cysts in faeces (Adams and MacLeod, 1977). Curiously, the highest prevalence of lesions in muscle detected in donor fish from the last sampling corresponds with the lowest prevalence of *E. piscium* organisms within the intestine in the same fish. However, the differences in the distribution of parasites in the donor fish during the experiment cannot be definitively taken into account because they could be due to the presence of fish with different disease developmental stages at the beginning of the experience, rather than to a chronological development of the disease during the study.

The dispersion mechanisms of *E. piscium* throughout the fish body could not be completely elucidated in our work, although the observations point to the connective tissue as a preferential site where the parasites dwell, and thus it would have a significant role on its dissemination (see previous chapter). In one donor fish examined during the transmission experiment, *E. piscium* stages were also detected in the gill capillaries. This observation, not noticed in previous studies on the pathology of the disease and other clinical cases, could indicate an haematogenous route of dissemination for the parasite. Nevertheless, *E. piscium* cells were not detected in blood vessels or haemopoietic organs such as the spleen in any other sample, neither by routine histology nor by ISH. Only in one fish with multiple lesions in different organs, a few parasites could be observed within the hematopoietic tissue

from kidney (see Chapter VI). In addition, the fish containing *E. piscium* within the gill capillaries displayed one of the highest levels of infection. According to these observations, the haematic route should not be disregarded as a parasite dispersion route, being more likely in advanced diseases.

CHAPTER VIII. CONCLUSIONS

7.1. The external lesions (bumps) observed on the skin surface of *S. senegalensis* correspond to granulomatous inflammatory reactions in the muscle, which are characterised by a liquefactive necrotic core surrounded by inflammatory cells, especially macrophages and fibroblasts.

7.2. The skeletal muscle is the most frequently affected tissue (in terms of prevalence), but lesions can also be found in internal organs such as liver, heart, intestine and, with lower prevalence, in kidney and ovary. Therefore, this disease is considered systemic.

7.3. These lesions are caused by spherical unicellular organisms measuring 2-4 µm in diameter, which are mainly located at the periphery of the lesions, between a necrotic core and the layer of inflammatory cells. These organisms are gram-negative and PAS-positive.

7.4. Parasites present one vesicular nucleus with a large central round nucleolus filling roughly half of the nuclei surface. They are amitochondriate organisms, although they contain double membrane-bounded organelles interpreted as mitosomes.

7.5. Molecular phylogenetic analysis allows the assignment of these organisms to the family Entamoebae (Phylum Amebozoa, subphylum Archamoebae) and the results reflect their relatively close relationship with *Endolimax nana* and with *Iodamoeba spp*., enteric commensals and parasitic species in humans and other mammals.

7.6. According to the present morphological and molecular studies, we propose *Endolimax piscium* as the latin binomial name of this new species.

7.7. Specific diagnostic tools for the detection of *E. piscium* were developed in this study and they presented good quality indicators in comparative tests. ISH is the technique with the highest sensitivity and specificity in intestine samples and PCR is also a fast and reliable routine method but still needs further optimization of the sampling methodology.

7.8. *E. piscium* is detected with high prevalence within the intestinal epithelium from symptomatic and asymptomatic fish and also in the intestinal lumen. Infected intestines can show different degrees of damage, usually correlating with the infection intensity.

7.9. The distribution of parasites throughout the intestinal epithelium is not homogeneous and areas with higher parasitic intensity are usually found. Although higher prevalence of parasites is usually detected within the posterior part of the intestine no significant trends could be demonstrated.

7.10. *E*. piscium is distributed evenly over different farms, and it could even be detected in farms where no symptomatic fish had been observed. For this reason, the early detection of this parasite should be considered a priority for the management of sole aquaculture.

7.11. The transmission of this parasite to healthy fish by cohabitation with diseased individuals has been demonstrated in this study. The establishment of the infection under these conditions takes several weeks and probably can be modulated by external conditions.

7.12. The connective tissue seems to be play and important role in the distribution of the parasites throughout the fish during its dissemination. No clear evidences of the main route of dissemination through the different organs and tissues are detected, although a haematogenous route of dissemination should not be completely disregarded.

7.13. Stages of the parasite can be detected in the water from diseased fish, although not in all the samplings carried out.

CHAPTER IX. ANNEXES

9.1 Histological techniques

9.1.1 Optic microscopy

Table 9.1. Processing of fixation, dehydration and tissue embedding in paraffin.

	Reactive	Washes number	Time (hours)
Fixation	10% buffered formalin*	1	1
Fixation-	10% buffered formalin: 50% Ethanol	1	2
Dehydration	50% Ethanol	1	1
	70% Ethanol	1	1
	96% Ethanol	1	1
	100% Ethanol	2	1
Dehydration-Inclusion	Xylene / Clear-Rite®	2	1
Inclusion	Paraffin (58-60ºC)	2	1
	Paraffin (58-60ºC)	1	2

*****10% buffered formalin (5L): 500 ml of concentrated formaldehyde (37-40%)

4.5 L of distilled water

100 g salt buffer pH 7.2 (phosphate)

Table 9.2. Haematoxylin and Eosin stain (H&E) protocol:

	Reactive	Washes number	Time (minutes)	Agitation
Dewaxing	Xylene	2	3	1
Hydration	100% Ethanol	1	3	1
	96% Ethanol	1	3	1
	70% Ethanol	1	3	1
	50% Ethanol	1	3	1
	Water	1	5	1
Stain	Mayer Haematoxylin	1	6	2
	Water	1	12	3
	Eosin	1	4	3
Dehydration	96% Ethanol	2	15 seconds	3
	100% Ethanol	2	3	1
Inclusion	Xylene	2	3	1

Table 9.3. Giemsa stain protocol:

	Reactive	Washes number	Time (minutes)
Dewaxing	Xylene	2	5
Hydration	100% Ethanol	2	5
	96% Ethanol	1	5
	70% Ethanol	1	5
	50% Ethanol	1	5
	Water	1	10
Stain	50% Giemsa	1	25
	Water	1	25
	0.1% Acetic Water*	1	15 seconds
Dehydration	96% Ethanol	2	10 seconds
	100% Ethanol	2	2
Inclusion	Xylene	2	5

*0.1% Acetic Water: 100 ml distilled water

4-5 drops of Acetic Water

Table 9.4. Gram stain procedure:

	Reactive	Washes number	Time (minutes)
Dewaxing	Xylene	2	5
Hydration	100% Ethanol	2	5
	96% Ethanol	1	5
	70% Ethanol	1	5
	50% Ethanol	1	5
	Water	1	10
Stain	Hucker-Conn Solution*[1]	1	30 seconds
	Water	1	10 seconds
	Weigert Iodine solution*[2]	1	20 seconds
	Water	1	5 seconds
	Acetone	2-3	5 seconds
	Water	1	5 seconds
	1% Neutral Red*[3]	1	1
	Water	1	1
Inclusion	Xylene	2	5

*[1] Hucker-Conn Solution: 80ml Distilled water
2g Crystal violet
20ml 96% ethanol
0.8g Ammonium oxalate

*[2] Weigert Iodine Solution: 100ml Distilled water
2g Potassium iodide
1g Iodine crystals

*[3] 1% Neutral Red: 100ml Distilled water
1g Neutral red

Table 9.5. Periodic acid-Schiff (PAS) stain protocol:

	Reactive	Washes number	Time (minutes)
Dewaxing	Xylene	2	5
Hydration	100% Ethanol	2	5
	96% Ethanol	1	5
	70% Ethanol	1	5
	50% Ethanol	1	5
	Water	1	10
Stain	5% Periodic acid*	1	10
	Water	1	10
	Schiff Reactive	1	30
	Water	1	5
	Mayer Haematoxylin	1	1
	Water	1	10
Dehydration	96% Ethanol	2	3
	100% Ethanol	2	3
Inclusion	Xylene	2	5

*5% Periodic acid: 5g of Periodic acid (HIO_4)
100ml distilled water

9.1.2. Transmission electron microscopy (TEM):

Table 9.6. Fixation, dehydration and inclusion protocol for TEM samples.

	Reactive	Washes number	Time (minutes)	Temperature (ºC)
Fixation	2.5% Glutaraldehyde + 0.1M Sodium cacodylate buffer	2	2 hours (at least)	4
Washes	0.1M Sodium cacodylate buffer (pH 7.4)	2-3	15	4
Post-fixation	1% Osmium tetroxide*[1]	1	2 hours	4
Washes	Distilled water	2-3	15	Room
Dehydration	50% Acetone	1	10	Room
	70% Acetone	2	10	Room
	90% Acetone	3	10	Room
	95% Acetone	3	10	Room
	100 Acetone	2	10-15	Room
	100% Acetone	1	20-30	Room
Inclusion	(3:1) 100% Acetone:Epon resin *[3]	1	90-120	Room
	(2:2) 100% Acetone:Epon resin *[3]	1	90-120	Room
	(1:3) 100% Acetone:Epon resin *[3]	1	3 hours	Room
	Epon resin *[3]	4-5	3 hours	Room
Polymerisation			48	60

*[1] 1% Osmium tetroxide (10ml): 10ml 0.1M Sodium cacodylate buffer
1g Osmium tetroxide
0.08g Potassium hexocyanoferrate

*[2] Uranyl acetate (10ml): 10 ml 70% Ethanol
0.1g Uranyl acetate

*[3] Epon resin: 24 ml Eponate 812
16 ml DDSA
10 ml MNA
1.1 ml BDMA

Table 9.7. Ultrathin sections staining with uranyl acetate protocol.

	Reactive	Time (minutes)
Counterstain	2% Uranyl acetate*[1]	30
Washes	Distilled water (4 or 5)	5 seconds
	Reynolds Solution*[2]	5-10
	Distilled water (4 or 5)	5 seconds

*[1] 2% Uranyl acetate: 10 ml distilled water
0.2 g Uranyl acetate

*[2] Reynolds solution: 50ml distilled water
1.33g Lead nitrate
1.76g Sodium citrate
(8ml 1M NaOH or until solution become transparent)

9.2. Molecular techniques

9.2.2. Extraction of parasitic DNA from water samples:

Table 9.8. Treatment protocol of samples fixed in acetone

	Reactive	Time (minutes)	Centrifugation (G)	Centrifugation time (minutes)	
Filter dissolution	1.5 ml Acetone	15	16,000	3	Discard liquid
Washes	750 µl 70% Ethanol	5	16,000	3	Discard liquid
Residue removal*		Hours			

* keep the samples in a hood to evaporate the ethanol remains.

9.2.2. ISH technique:

Table 9.9. Reagent composition for ISH procedures.

Solution	Reagent		Specific requirements
Tris-$CaCl_2$ (1l)	200 mM Tris Base	24,23 g	pH 7.2; autoclave
	2 mM $CaCl_2$	0,29 g	Store at -4ºC
Hybridisation buffer (1ml)	HPLC water	280 µl	Keep on ice until used
	20x SSC *[1]	100 µl	
	100x Denhart's solution	20 µl	
	50% Dextran sulphate*[2]	200 µl	
	10x Phosphate buffer (PBS)*[3]	100 µl	
	Denaturalised DNA	50 µl	
	Deionized formamide	250 µl	
Genius 1 (10x) *[4] (1l)	1M Tris Base	121.14 g	pH 7.5; autoclave
	1,5M NaCl	87.66 g	
Blocking solution (1ml)	2% Sheep serum	20 µl	
	0,1% Triton X-100	1 µl	
	Genius 1 (1x)	1000 µl	
Genius 3 (1l)	0,1M Tris Base	12,11 g	pH 9.5
	0,1M NaCl	5.84 g	Store at -4ºC
	50mM $MgCl_2$	10.16 g	
Substrate solution (1ml)	NBT/BCI	20 µl	
	Genius 3*[5]	1ml	

*[1] 1x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0 — pH 7.0 autoclave

*[2] 50% Dextran sulphate: 20 ml HPLC water, 10 g Dextran sulphate

*[3] PBS (10x): 8,18 g/l of NaCl (1,4M)
0,2 g/l of KCl (27mM)
1,42 g/l of Na_2HPO_4 (100mM)
0,25 g/l of KH_2PO_4 (18mM)
— pH 7,5; autoclave

*[4] Dilute 1:10 in bidistilled water before use

*[5] Genius 3 without $MgCl_2$

CHAPTER X: REFERENCES

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