I. INTRODUCTION

The present report describes the activities developed during the internship of the Aquaculture and Fisheries Master, of the University of Algarve in Faro, from January to June 2013, and with scientific orientation by Dr. Maria Teresa Dinis (UALG), Dr. Alicia Estévez Toranzo (Instituto de Acuicultura de Santiago de Compostela) and Dr. Diogo Rosado (SAFIESTELA). The internship was realized in a Sole Hatchery SAFIESTELA – Sustainable Aquaculture Investments, which belongs to Sea8 group.

The main objective of this internship was the gathering of theoretical and practical information in the areas of Biology, Icthyopathology and Prophylaxis on the production of *Solea senegalensis*.

This thesis will begin with the description of the local where the internship took place, followed by a bibliographic review about the importance of sole aquaculture, and about the culture of soles. Later chapters will address the major diseases that affect sole and on the experimental work it will be described the materials and methods for bacteriological analysis of water and for parasitological and bacteriological analysis of sole.

1.1. CHARACTERIZATION OF THE LOCAL OF INTERNSHIP

The SAFIESTELA sole hatchery is located in Rio Alto, Estela, Póvoa de Varzim, at approximately 500m far from the coast line. SAFIESTELA has an expected production of 1,2 to 1,5 million juveniles per year. The farm is contained in a building with an area of about 5300m^2. It comprises an hatchery, a nursery and ongrowing sectors, along with a machine room and support services.

The soles are produced on an intensive system in indoor tanks with recirculation system. The adult breeders were located in four isolated rooms each one corresponding to the different seasons, therefore with different water temperatures and photoperiod. This way permits the control of the egg postures with the objective of having new sole larvae available all year. Juveniles are kept in raceway tanks (figure 1), to be pre-fattened, this way permits larger volume of production in the same area. These juveniles are then transferred, when they reach

the weight of around 40g to 50g, to the fattening unit Aquacria belonging to the same group Sea8, in Torreira. Aquacria has an annual production of 400 to 500 tonnes of Senegalese Sole per year.

FIGURE 1 – Raceway tanks with *S. senegalensis* (photo from Safiestela, 2013).

1.2. AQUACULTURE OF *SOLEA SENEGALENSIS*

Through the last decades, marine fish aquaculture in Southern Europe has been mostly restricted to gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Consequently, market saturation and decrease of the market price has taken place, requiring supplementary scientific and technological efforts for the improvement of culture technologies for new potential species (Agulleiro *et al.*, 2006).

One of the fish species more promising for aquaculture farming is the Sole. On-growing methods for sole, which comprise both the common sole (*Solea solea*) and Senegal sole (*Solea senegalensis*), have enhanced in the last twenty years. Studies demonstrated that Senegal sole may have a superior growth in captivity than common sole and, as a result, may be better appropriate for market (Agulleiro *et al.*, 2006).

There are two main species in sole aquaculture. *Solea solea* (Linnaeus, 1758) and *S. senegalensis* (Kaup, 1858), both belong to the *Soleidae* family. The world distribution of *S. senegalensis* is shown in figure 2 (Le François *et al.*, 2010).

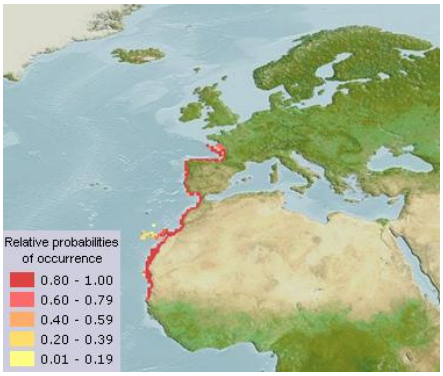

FIGURE 2 – World distribution of *S. Senegalensis* (http://www.fishbase.us/summary/Solea-senegalensis.html).

Solea senegalensis is generally mistaken with *Solea solea* (= *S. vulgaris*), the most frequent species in North Atlantic waters, although it also coexists with *S. senegalensis* in the eastern Atlantic and the Mediterranean. Both present a strong similarity. Externally they differ in the interradial membrane of the pectoral fin of the ocular side, black on *S. senegalensis* at its rear half (Figure 3) (Agulleiro *et al.*, 2006).

FIGURE 3 – Black interradial membrane of the pectoral fin (photo from Safiestela, 2013).

The adults generally measure 30-35 cm in the Mediterranean and up to 60 cm in the Atlantic. The sole is a benthic marine species, which inhabits sandy bottoms and mimetize them, reaching a depth of 100 meters. While the larvae are diurnal and visual feeders, the demersal stages are nocturnal, becoming active at dusk and burying in the substrate throughout the day (Stickney, 2000).

It feeds on benthic invertebrate organisms such as annelids, larvae of polychaetes, molluscs, and small crustacea. Sole has a weakly developed stomach, contrasting to those of more predatory flatfish, such as turbot and halibut. An important result of this condition is that they require feeding frequently, being incapable to gather their dietary energy requirements with reasonably large,

infrequent meals. It is a gonochoristic fish devoid of secondary sexual characteristics. Although during the breeding season females experience a significant increase in the ovaries, and sometimes a colour change may be noticeable in the ventral side (Stickney, 2000; Agulleiro *et al.*, 2006).

Although these species are closely related and almost identical, there are, however, vast differences in market values across the Europe. Typically, *S. solea* is much appreciated and more expensive in Western and Northern Europe, while *S. senegalensis* is cheaper. The contrary occurs in Southern Europe (Le François *et al.*, 2010). However these differences in the market value of sole do not occur in Portugal. Thirty years ago, sole was already considered one of the most attractive and capable species for marine fish aquaculture in Europe. However, the species never achieved a favourable outcome, because technological (weaning strategies and feeds) and disease problems troubled the development of commercial sole culture. Some of the main problems initially found on sole farming were the low growth rates in juveniles associated with the apparent greater susceptibility to diseases, especially photobacteriosis (formerly pasteurellosis), vibriosis, tenacibaculosis and viral diseases. The most important was a disease known as black patch necrosis, which will be described later in this work, on the chapter 2.1.2.1. (Le François *et al.*, 2010). This problems have been partly solved over time by improving diets and by studying stress due to intensive farming densities, although is still necessary and urgent to develop vaccines and prophylactic methods against their typical pathogens (Agulleiro *et al.*, 2006). An additional limitation of sole farming in Western and Northern Europe used to be water temperature control. Even the more northern of the two species (*S. solea*) demands moderately warm water (approximately 20°C) for optimal growth rates in the juvenile and on-growing stage. Consequently, appropriate sites were unusual. Currently, recirculation technology is completely established and generously available. This signifies that optimal growth conditions for sole may be done all year round, even in temperate areas. This factor, together with recent advances in feed technology surrounding weaning and on-growing, have incited an improved concern in sole as an aquaculture species (Le François *et al.*, 2010).

Natural spawns of viable eggs have already occurred from wild Senegal sole previously acclimatized to captivity, and optimized weaning methods for the larvae

are now being improved. Consequently, Senegal sole is actually a significant focus of research for flatfish aquaculture in Europe, mainly in Spain and Portugal. Nevertheless, before achieving a technology for mass production of Senegal sole able to be transferred to the industry, numerous features of its culture must to be solved and enhanced (Agulleiro *et al.*, 2006).

1.3. OBJECTIVES

This study was conducted with the objective of identifying any biotic and/or abiotic factors capable of causing diseases and hence decreasing production levels, by processing various types of laboratory samples. For that end is also needed to hold a different approach to current and potential techniques for the analysis of water samples and for bacteriological and parasitological analysis of the sole. The final goals have been to describe the most accurate way to perform a necropsy of the sole, in order to collect the necessary samples and the best approach to establish biosecurity measures with the aim of preventing the occurrence of these diseases.

II. STATE OF THE ART

2.1. FACTORS WITH IMPORTANCE ON AQUACULTURE FISH HEALTH

There is still few information about sole pathologies in farming conditions. The description of the principal pathologies is one of the main targets for correct farming and, or breeding of this specie. The farming conditions of flatfish have issues directly related to the pathologies detected till now.

The close relationship between the fish and the substrate establish that the farming density is related to area and not to volume. The direct and continuous contact of the fish with the substrate indicates that the skin is one of the main target organs and the primary access for infections. Besides that, the typical mimicry of the Pleuronectiformes makes them particularly susceptible to stress in conditions of absence of substrate and/or intense lighting. The presence of a thin layer of sand improves their adaptation by facilitating their mimicry and preventing the formation of small deformations in the tanks that could damage the skin of the fish. However, the presence of sand in the bottom of the tanks may constitute serious problems for maintenance and cleaning, increasing, for instance, the risk for bacterial growth. In any case, the absence of a natural substrate didn't avoid the development of the farming of this and other flatfish species (Dinis *et al.*, 2007).

The clinical signs (external or internal) that each pathogenic agent provokes on fish, depend of the host specie, the age and the state or phase of the disease (acute or chronic). Sometimes there is no correlation between external and internal symptoms.

It is important to differentiate two large groups of pathologies, the infectious and the non-infectious. The first are caused by microorganisms or parasites that have the capacity of enter and multiply in the host, while the others are caused by the remaining external and internal agents. The infectious pathologies can be transmitted easy and rapidly, known as contagious diseases while the non-transmissible are called non contagious.

2.1.1. ABIOTIC FACTORS

In aquaculture the most part of epizootic diseases that occur are due to bad farming conditions which cause immunossupression and allow the presence of opportunistic organisms. The type of infection, the nature of the condition and the degree of mortality is directly related to the environmental parameters and to the innate host resistance. There are numerous factors that favour the occurrence of infections and other kind of pathologies. Environmental factors are very important to the welfare of fish. There are physical agents as temperature, radiant energy (UV), pressure, mechanical and electrical traumas. Abnormal oxygenation of the water and incorrect feeding procedures are, more often than we think, the main origin of pathological occurrences. For instance, the exponential growth of phytoplankton in earthponds can cause sudden and intense hypoxia, which can give rise to rapid mortality. Most often this hypoxia is limited, chronic and nocturnal (due to the interruption of photosynthesis), causing loss of appetite, growth retardation and increased sensitivity to infections. Food shortages are frequent, especially in essential fatty acids and trace elements. Dietary deficiencies in vitamin C, for example, cause malformations and vertebral fractures. Specifically in turbot, may cause granulomatous hypertyrosinemia characterized by the presence of reactive nodules in most tissues. Pigmentary changes are also usually associated with dietary deficiencies in the early stages of fish farming, something very common in flatfishes. The loss of pigmentation is not a disease, although it causes less profits because the unpigmented fish is discarded for market (figure 4). In all the fish the transport, the handling, the temperature, salinity, oxygen and pH variations cause stress which doesn´t always evolve favourably (Barnabé, 1996).

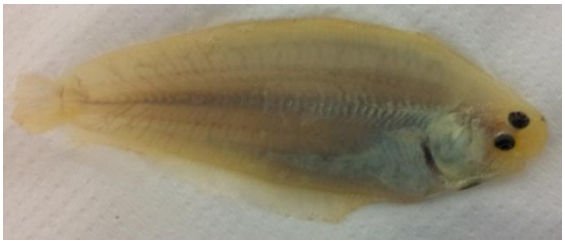

FIGURE 4 – Unpigmented *Solea senegalensis* juvenile (photo from Safiestela, 2013).

Self-contamination, that is, the expulsion for the water and degradation of the waste products in the form of ammonia (NH_3) and nitrite, under certain conditions, may become excessive. The suspended solids are deposited on the gills, bridging or irritating them which causes a choking hazard. NH_3 disturbs the chemical equilibrium of the blood, decreases the oxygen affinity by the haemoglobin and increases the gill permeability, which limits the capacity of osmorregulation. The nitrite ion has methemoglobinizant ability, which affects the breathing capability (Barnabé, 1996).

Contamination may also have external origin, by contaminants as pesticides, hydrocarbons, disinfectants, among others. The pathogenic mechanisms depend on the nature and concentration of the product concerned, such as cholinesterase inhibition by the organophosphates, change of the ion transport in the gills and cell lysis by various metals, particularly zinc (Zn^{2+}) and copper (Cu^{2+}), or anaemic processes triggered by hydrocarbons (Barnabé, 1996).

2.1.1.1. PATHOLOGY OF NON INFECTIOUS ORIGIN

Gas Bubble Disease is a non-infectious pathology that can occur in sole, predominantly in the earlier stages of lifecycle. Supersaturation of atmospheric gases in water is frequent due to several natural and man-induced processes. The exposure of aquatic animals to gas supersaturation may lead to the development of gas bubbles on body surfaces or within the vascular system and tissues. This condition is known as gas bubble disease or trauma (Stickney, 2000).

Gas supersaturation (ΔP) occurs when the total pressure of gases dissolved in water is superior than the ambient atmospheric pressure. This may happen when water is pumped up from a deep (more than 90m) well, given that such water is frequently supersaturated with nitrogen and/or carbon dioxide (Noga, 2010). Gas supersaturation can be caused by several physical and biological processes. Some of the mechanisms that can produce gas supersaturation are, for instance, heating of waters, ice formation, mixing of waters of different temperatures, air entrainment, photosynthesis, pressure changes, physiological processes, and bacterial action. In a specific situation numerous mechanisms may be involved. Many surface waters, groundwaters, and springs can be naturally supersaturated at some stage across the

year (Stickney, 2000). Fish that have been transported by air may also develop gas bubble disease, however it rarely occurs (Noga, 2010).

In deep culture systems, fish that swim near the surface succumb more rapidly due to the difference in hydrostatic pressure. Therefore, fish in hatcheries are particularly vulnerable to gas bubble disease because they cannot escape to lower depths (Noga, 2010).

The majority of gas emboli are produced by excess nitrogen, because oxygen is absorbed metabolically and thus less probable to form persistent bubbles. Nevertheless, very high oxygen concentrations are dangerous. Intensive culture systems that utilize liquid oxygen to enhance fish carrying capacity can accidentally overdose the fish (Noga, 2010).

In the case of fish breathe supersaturated water before it reaches equilibrium, the excess gas may leave solution in the bloodstream, forming emboli in various tissues and resulting in gas bubble disease. Histopathology of gas bubble disease has been reported to include edema of the gill secondary lamellae, with complementary degeneration of the overlying epithelium. Other lesions consist of edema and embolic disruption of buccal and intestinal mucosa, and also vacuolar degeneration of the renal tubular epithelium. Lesions can also take place in the liver and muscle (Noga, 2010). Formation of bubbles (figure 5) in the vascular system may result in petechial haemorrhaging, restricted blood flow, necrosis, and death. Subcutaneous emphysema (presence of gas bubbles within skin tissues) is usually found on fins and tail, inside the mouth and operculum, and on the body surface. The formation of gas bubbles and consequential inflammatory reaction provides an ideal environment for opportunistic secondary bacterial invaders. Emphysema of tissue in the mouth can also lead to the blockage of respiratory water flow and death by asphyxiation (Stickney, 2000). Tissue haemorrhage and brain damage may lead to death, however the mechanism of tissue damage is still uncertain. The severity of the damage is determined by the number of emboli formed and by which tissues are affected. Behavioural abnormalities related to the target organs (for instance, hyperactivity, erratic swimming) may be present (Noga, 2010).

Acute gas super saturation (ΔP >50 – 200 mm Hg) can cause high mortality rates (up to 100%) in few minutes. Eggs float to the surface, and larvae or fry may show hyperinflation of the swim bladder, cranial swelling, exophthalmos, gas bubbles

in the eyes, swollen gill lamellae, pneumoperitoneum, or gas bubbles in the yolk sac (Noga, 2010).

Low super saturation levels (ΔP <76 mm Hg or <110% saturation at sea level) are related with chronic low (typically < 5%) mortality rates, hyperinflation of the swim bladder, and extravascular emboli in the gastrointestinal tract and mouth. Low level super saturation rarely produces well visible lesions, therefore fish must be closely examined. Secondary effects as unusually high mortalities, skeletal deformities and opportunistic infections are most evident (Noga, 2010).

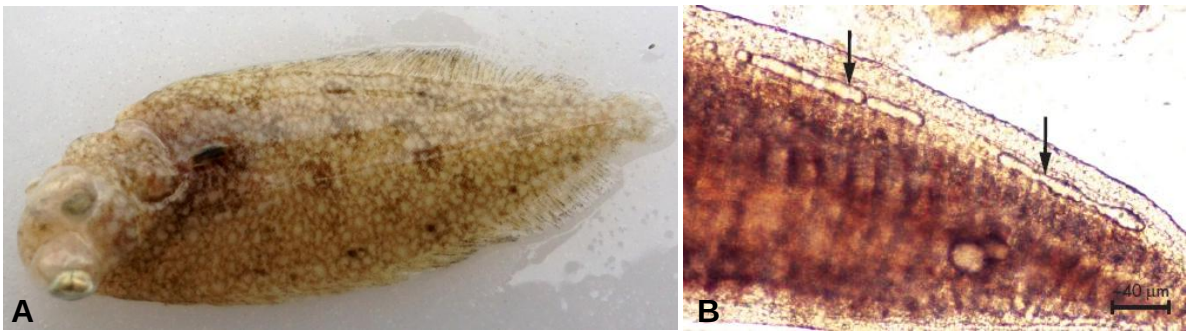

FIGURE 5 – A. Gas bubbles in the eyes of a Senegalese sole (photo from Safiestela, 2013); **B.** Gas emboli (arrows) in the gills of an European seabass (adapted from Noga, 2010).

The presence of gas emboli is pathognomonic for gas bubble disease. Holding fish up to a light source is helpful to observe emboli. Bubbles can be squeezed from fin or gill clips while the fish is held submerged, confirming the diagnosis. Putrefaction in dead fish should not be mistaken with gas bubble disease. Determination of gas super saturation is based on the measurement of the total concentration of dissolved gas in the water source. Nevertheless some gases may be present in harmful concentrations without occurrence of gas bubble disease. Measurement of excess gases can be done with a saturometer. Generally, levels of approximately 110% saturation are considered unsafe for fish. However, this value varies according with the species and age of the fish. Eggs are usually more tolerant (Noga, 2010).

Treatment of gas bubble disease requires eradicate the excess gas in the water source. This can include first aerating the water source in a reservoir in order to allow it to equilibrate with air, or the use of degasification columns which allow the elimination of excess of gas (Noga, 2010). The monitoring of ΔP of influent waters

and at key points in the hatchery may be helpful for the identification of problems and to permit correct adjustments before major mortality occurs (Stickney, 2000).

To prevent the cases of gas bubble disease in Safiestela, that were rare, a Venturi system was installed at the exit of the protein skimmer.

2.1.2. BIOTIC FACTORS

Like any other animal, living in the wild or in farming conditions, the sole is susceptible to parasites (ectoparasites and/or endoparasites) and diseases of bacterial, fungal or viral origin. There are a considerable number of pathogens naturally present in water, which, if the fish do not find in better health, lead to a rapid morbidity and are usually fatal (Stickney, 2000).

2.1.2.1. PATHOLOGY OF BACTERIAL ORIGIN

Unlike other fish, such as seabream or salmon, in which the incidence of disease and the types of bacterial pathogens have been well documented, data regarding the isolation and characterization of pathogenic bacteria from sole is scarce. Identification of pathogenic micro-organisms is essential for future research on vaccine improvement, as well as for other means of controlling and treating pathologies (Zorrilla *et al.*, 2003).

Some bacterial diseases are resolved naturally dissipating after some time if the fish presents a good immunity status. These diseases tend to occur during the different stages of the life cycle of the fish, for instance due to damage caused by the tanks, which cause lesions in the skin, and therefore a route of entry for pathogens. In intensive cultivation systems the risk of infection is high, due to the conditioning of fish, and there are high levels of close proximity between them. The main causative agents of bacterial diseases in *Solea senegalensis* are *Photobacterium damselae* subsp. *piscicida*, causing photobacteriosis, *Tenacibaculum maritimum*, agent of tenacibaculosis (formerly flexibacteriosis) and different species of the genus *Vibrio*, causing septicaemia, commonly known as vibriosis.

Photobacterium damselae subsp. *piscicida* (formerly known as *Pasteurella piscicida*), a halophilic, Gram negative, nonmotile, bipolar coccobacillus is the aetiological agent of pasteurellosis, a bacterial septicaemia also referred to as pseudotuberculosis (Rajan *et al.*, 2003; Romalde, 2002; Toranzo *et al.*, 2005).

Until 1990 there were no cases of pasteurellosis noted in Europe. Toranzo, (1991) described the first outbreak of this pathology in the northwestern region of Spain, affecting juvenile gilthead seabream (*Sparus aurata*). Few time later, outbreaks of pasteurellosis also occurred in south-western Spain, Portugal, France, Italy, Israel and Greece, mainly in populations of seabream and seabass, becoming one of the major limiting factor in the culture of this fish species in the Mediterranean. Actually it affects a wide variety of marine fish (table I), like yellowtail (great economical impact in Japan), Atlantic bluefin tuna and *Solea senegalensis* (Romalde, 2002; Magariños *et al.*., 2003; Toanzo *et al.*., 2005 Austin & Austin, 2007).

Table I – Isolations of *Photobacterium damsela* subsp. *piscicida* from fish (adapted from Woo & Bruno, 1999).

Fish		Citation
White perch	*Roccus americanus*	Snieszko *et al.*. (1964)
Striped bass	*Morone saxatilis*	Snieszko *et al.*. (1964)
Menhaden	*Brevoortia tyrannus*	Lewis *et al.*. (1970)
Striped mullet	*Mugil cephalus*	Lewis *et al.*. (1970)
Yellowtail	*Seriola quinqueradiata*	Kimura and Kitao (1971)
Ayu	*Plecoglossus altivelis*	Kusuda and Miura (1972)
Black seabream	*Mylio macrocephalus*	Ohnishi *et al.*. (1982)
Red seabream	*Acanthopagrus schlegeli*	Yasunaga *et al.*. (1983)
Oval file fish	*Navodan modestus*	Yasunaga *et al.*. (1983)
Snakehead fish	*Channa maculata*	Tung *et al.*. (1985)
Red grouper	*Epinephelus okaara*	Ueki *et al.*. (1990)
Gilthead seabream	*Sparus aurata*	Toranzo *et al.*. (1991)
Sole	*Solea solea*	Baudin Laurencin *et al.*. (1991)
Mullet	*Mugil cephalus*	Baudin Laurencin *et al.*. (1991)
Seabass	*Dicentrarchus labrax*	Baudin Laurencin *et al.*. (1991)
Atherine	*Atherina boyeri*	Ceschia *et al.*. (1991)
Seabream	*Pagrus pagrus*	Ceschia *et al.*. (1991)
Atlantic salmon	*Salmo salar*	Speilberg *et al.*. (1991)

The pathology is influenced by climatic changes and the age of fish may also be considered (Mancuso, 2012).

Pasteurellosis is characterized by a bacteraemia/septicaemia that takes one of the two following forms. In the acute form, few clinical signs are present. There may be some slight haemorrhages around the gill covers or the bases of the fins, or there can be abnormal skin pigmentation (darker areas) and can present splenomegaly and nephromegaly. Histologically, there is acute necrosis of spleen, liver, and pancreas with no inflammation. Erratic swimming behaviour can also be observed. In the chronic form, they present granulomatous-like deposits in the target organs (spleen and kidney) (figure 6), which led to the use of the misleading pathological term ‘‘pseudotuberculosis’’. These deposits comprise many greyish-white bacterial colonies of 0,5-1,0mm in size which incite a chronic inflammatory response. Purulent material may accumulate in the abdominal cavity and hemorrhagic septicaemia may occur (Mancuso, 2012; Austin & Austin, 2007; Buller, 2004; Noga, 2010).

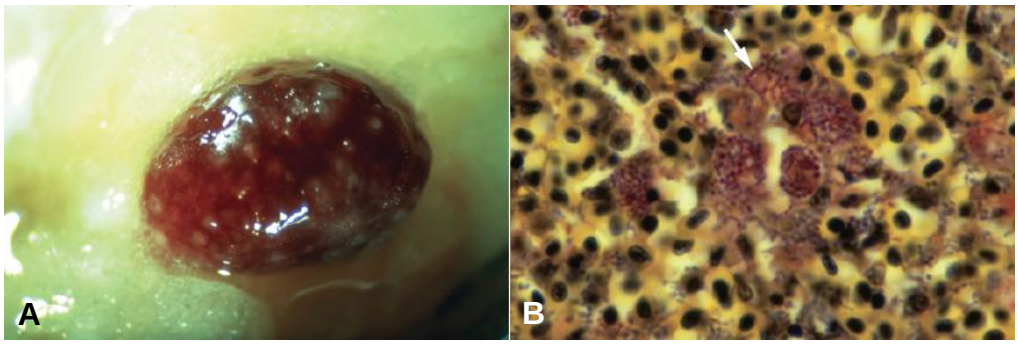

FIGURE 6 – A. Spleen of gilthead seabream with multiple white foci caused by *P. damselae* subsp. *piscicida*. **B**. Histological section of spleen from gilthead seabream showing phagocytes containing numerous bacteria (arrow) (adapted from Noga, 2010).

This bacterium may be able to survive in water and sediment for one month at 20ºC, demonstrate the capacity to entry in a viable but non cultivable state and may be transmitted to fish through water, using the skin as route of entry, so horizontal transmission from fish to fish within a culture unit is the most probable method of spread throughout epizootics. Outbreaks on fish farms are explosive and are characterized by sudden reduction in feeding response and rapid onset of mortality. Outbreaks of photobacteriosis are associated with water temperatures of 18–25 °C and salinities of 5–25 ppt. (Mancuso, 2012; Stickney, 2000).

Because of the fast onset of disease, medicated feeds are usually not administered early enough in the infection to be effective. If timely application is achieved, the pathogen responds well to treatment with oxytetracycline, oxolinic acid, ampicillin, amoxicillin, and florfenicol medicated feeds. However control of the pathology via vaccination is required, as the bacterium rapidly becomes resistant to antibiotics (Woo & Bruno, 1999; Stickney, 2000). One study of the use of a divalent vaccine, prepared with formalized whole cells and extracellular products of *Photobacterium damselae* subsp. *piscicida* and *Vibrio harveyi* virulent strains, indicates that two prolonged immersions of 5-10g fish (*Solea senegalensis*) in this divalent bacterin at a one month interval gave high levels of protection (Arijo *et al.*., 2005).

The potential exchange of pathogens between wild and farmed fish is expected and the development of rapid identification methods for bacterial disease has been suggested to limit damages (Mancuso, 2012).

Isolation and phenotypic characterization of isolates *P. damselae* subsp. *piscicida* can be isolated from internal organs of diseased fish after 2 to 4 days of incubation at 22 ºC by using media such as trypticase soy agar (TSA) and blood agar, both supplemented with 1-2%NaCl, or marine agar 2216E. The presumptive diagnosis is based on the isolation of a gram-negative, non-motile bipolar rod that is oxidase and catalase positive, fermentative without gas production, sensitive to the vibriostatic agent O/129, and has strict salt requirements (table II). *P. damselae* subsp. *piscicida* can grow at temperatures from 15 to 32.5ºC, although its optimum growth temperature is 22,5 to 30ºC (Romalde, 2002).

Table II – Characteristics of isolates of *P. damselae subsp. piscicida* (adapted from Buller, 2004).

Gram stain	**–**	**Phenylalanine deamination**	**–**
Bipolar staining	+	Gluconate utilization	–
Cell morphology	Short rods	D-Tartrate	–
Motility	–	Gelatinase	–
Growth on nutrient agar	+	Caseinase	–
In nutrient broth	+	Lipase	+
In peptone water	+	Phospholipase	+
On heart-infusion agar	+	Amylase	–

On BHI agar	+	Urease	–
On SS agar	–	Acid production from:	
On MacConkey agar	–	Glucose	+
On Endo agar	–	Mannose	+
Growth at 5ºC	–	Galactose	+
10ºC	–	Fructose	+
15ºC	+	Maltose	–
25ºC	+	Sucrose	–
30ºC	+	Arabinose	–
37ºC	–	Amygdaline	–
Growth in 0% NaCl	–	Melibiose	–
Growth in 0.5% NaCl	+	Mannitol	–
Growth in 3% NaCl	+	Inositol	–
Growth in 5% NaCl	–	Sorbitol	–
Cytochrome oxidase	+	Glycerol	–
Catalase	+	Xylose	–
Methyl red	+	Lactose	–
Voges–Proskaur	+	Trehalose	–
Indole production	–	Raffinose	–
Nitrate production	–	Cellobiose	–
Ammonium production	–	Dextrin	–
Citrate production	–	Inulin	–
H2S	–	Glycogen	–
O/F	F	Adonitol	–
Gas from glucose	–	Inositol	–
Arginine dihydrolase	+	Dulcitol	–
Lysine decarboxylase	–	Erythritol	–
Ornithine decarboxylase	–	Salicin	–
Tryptophan deaminase	–	Aesculin	–
Galactosidase (ONPG)	–		

Vibriosis is a pathology caused by infection with one of numerous members of the genus *Vibrio*, as well as the related genera *Moritella* and *Photobacterium* (family *Vibrionaceae*). Vibriosis is one of the most important pathologies of marine fish.

Probably almost all marine and brackish water fish are susceptible to at least one species (Noga, 2010).

Originally it was thought that scavenger fish, which fed around the farms, were the natural reservoir of this pathogen. However there are facts that vibrios are generally present in foods of healthy farmed and wild fish (Woo & Bruno, 1999). Vibrios are usually facultative pathogens that can easily survive and multiply in the environment, being frequently isolated from the mucosal surfaces and internal organs of asymptomatic fish, as well as from invertebrates, sediments, and the water column. Environmental prevalence is directly proportional to organic pollution of water and high salinity levels (Noga, 2010).

One of the main predisposing risk factor for most types of vibriosis is high temperature, turning it a summer disease in most, but not all cases (for instance, cold water vibriosis, winter ulcer). High population density and other stress factors can also increase outbreaks. Different strains vary significantly in virulence, and some strains can cause pathology with no predisposing stress associated. Some vibrios produce hemolysins (which can cause anaemia) and proteases (which could cause muscle damage) (Noga, 2010).

The vibrios more frequently isolated from *Solea senegalensis* belong to the species *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio pelagius* (Woo & Bruno, 1999).

Vibrio anguillarum is a Gram negative curved bacilli and is the most common fish-pathogenic *Vibrio*. The characteristic clinical signs of this kind of vibriosis include red spots on the ventral and lateral areas of the fish and swollen and dark skin lesions that ulcerate, releasing blood exudates. In some cases there are also corneal lesions, characterized by an initial opacity, followed by ulceration and evulsion of the orbital contents. However, in acute and severe epizootics, the course of the infection is fast, and most of the infected fish die with no clinical signs observed. Internally this pathology is characterized by a hemorrhagic septicaemia and the number of leucocytes is decreased. Systemic infections frequently localize in iron rich filtering organs, such as spleen and kidney. The intestine becomes distended and filled with a clear, viscous liquid. *V. anguillarum* was observed in large numbers in the blood and haemopoietic tissues. The disease is more severe in the descending gastrointestinal tract and rectum than in the anterior region, due to a pH gradient,

which is alkaline in the rectum and becomes acidic towards the anterior gastrointestinal tract. It has been demonstrated that *V. anguillarum* cannot grow in an acidic medium (Noga, 2010; Woo & Bruno, 1999). Table III lists the vibriosis outbreaks in cultured fish, molluscs and crustacea caused by *V. anguillarum* in different countries.

Table III – Vibriosis outbreaks caused by *Vibrio anguillarum* (adapted from Woo & Bruno, 1999).

FISH	COUNTRIES
Pacific Salmon:	
Oncorhynchus kisutch;	U.S.A., Japan, Spain;
O. keta, O. nerka, O. gorbuscha;	Canada;
O. masou, O. rhodurus;	Japan;
O. tshawytscha;	U.S.A., Canada;
Atlantic Salmon (*Salmo salar*);	Norway;
Trout:	
Oncorhynchus mykiss;	U.S.A., Japan, Italy, Norway, Denmark, Spain;
Salmo trutta;	Scotland;
Turbot (*Scophthalmus maximus*);	Scotland, Spain;
Striped Bass (*Morone saxatilis*);	U.S.A.;
Winter Flounder (*Pseudopleuronectes americanus*);	U.S.A.;
Cod (*Gadus morhua*);	Norway, Denmark;
Red Seabream (*Pagrus major*);	Japan;
European Eel (*Anguilla anguilla*);	Norway;
Japanese Eel (*Anguilla japonica*);	Japan;
Saith (*Pollachius virens*);	Norway;
Gilthead Seabream (*Sparus aurata*);	Israel;
Sea Mullet (*Mugil cephalus*);	Scotland;
Seriola (*Seriola quinqueradiata*);	Japan;
Channel Catfish (*Ictalurus punctatus*);	U.S.A.;
Milkfish (*Chanos chanos*);	Taiwan;
Ayu (*Plecoglossus altivelis*);	Japan;

Tilapia (*Oreochromis aureus*);	Kuwait;
European Oyster (*Ostrea edulis*);	U.S.A., U.K., Spain;
Japanese Oyster (*Crassostrea virginica*);	U.S.A.;
Clam (*Mercenaria mercenaria*);	U.S.A.;
Lobster (*Homarus americanus*);	U.S.A.;
Shrimp (*Penaeus sp.*);	U.S.A..

Vibrio harveyi (= *Vibrio carchariae* = *Vibrio trachuri*), this agent has been related with outbreaks affecting farmed Senegalese sole with moderate mortalities. The following symptoms were observed in diseased specimens: dark skin, haemorrhagic areas in fins and mouth, septicaemia, and occasionally, ulcers on the skin surface. Internally, diseased fish accumulated fluids in the peritoneal cavity and haemorrhagic livers have been found in some specimens (Rico *et al.*, 2008; Zorrilla *et al.*, 2003). It was initially isolated from a sandbar shark and lemon sharks. It was also isolated from Japanese horse mackerel (*Trachurus japonicas*), summer flounder (*Paralichthys dentatus*), Senegalese sole (*Solea senegalensis*), red drum (*Sciaenops ocellatus*), coioides grouper (*Epinephelus coioides*), spotted grouper (*Epinephelus* spp.), silvery black porgy (*Acanthopagrus cuvieri*), snook (*Centropomus undecimalis*), jack crevalle (*Caranx hippos*), milkfish (*Chanos chanos*) and marine sunfish (*Mola mola*) (Noga, 2010).

Vibrio pelagius was related with an epidemic in larval and juvenile cultured turbot in Spain with water temperatures approximately at 12 – 15 °C. Diseased fish showed skin lesions and a systemic infection (Noga, 2010).

Vibrio parahaemolyticus was isolated from an outbreak in February 2001, causing moderate mortalities (near 20%) in populations of Senegalese sole farmed in the south of Spain. The major external signs observed were skin ulcers and haemorrhagic areas close to the fins (figure 7) and mouth (Zorrilla *et al.*, 2003).

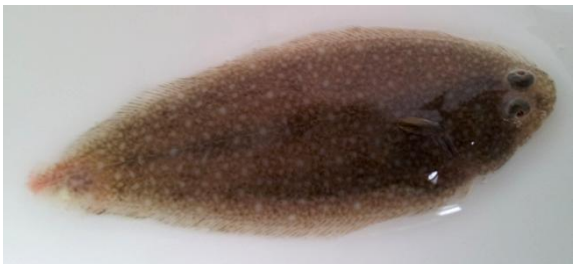

FIGURE 7 – Lesions in the caudal fin of a sole, caused by *Vibrio spp.* (photo from Safiestela, 2013).

Vibriosis is a classical example of a pathology caused by stress. The losses caused by vibriosis are highly dependent on the severity of the environmental stress that provoked the outbreak, varying from acute to chronic. Exposure to copper (> 30 µ g/ml) or iron (> 10 µ g/ml) also increases susceptibility to vibriosis. Oxytetracycline, nitrofurans, potentiated sulfonamides, and oxolinic acid have been administered successfully, however resistance to these drugs can occur, particularly in *V. anguillarum* and *V. salmonicida*. Commercial bacterins, available for certain vibrios provide good protection for populations at risk. Reducing stress levels is essential for a long term management (Noga, 2010).

For definitive diagnosis of vibriosis identification of the bacterium in target tissues is required (usually through biochemical tests of a culture), with correlated clinical signs. Isolation in a mixed culture from normal colonization sites, as skin or gastrointestinal tract, on fish may not necessarily specify that the vibrio is the agent responsible for the pathology. It is important to be certain that this is the primary infectious cause of the problem because vibrios can be secondary invaders. Kidney is the best organ for isolation, however lesions should also be sampled (Noga, 2010).

Tenacibaculum maritimum, formerly known as *Flexibacter maritimus*, a Gram-negative and filamentous bacterium, which has been described as the etiological agent of tenacibaculosis in marine fish. Tenacibaculosis is an ulcerative pathology, that affects a large number of marine fish species globally and has massive economic significance to aquaculture producers. Problems related with epizootics include high mortality rates, increased susceptibility to secondary infections and huge waste of money for treatment solutions. Since 1990, outbreaks of tenacibaculosis started to occur in Spain and Portugal, mainly in turbot *Scophthalmus maximus*

populations, Atlantic salmon *Salmo salar* and, later, in sole,in gilthead seabream and in seabass (Avendaño-Herrera *et al.*, 2006; Stickney, 2000).

The major clinical signs related with this disease are characteristic gross lesions on the body surface of fish such as ulcers (figure 8), necrosis, eroded mouth, frayed fins and tail rots, and occasionally necrosis on the gills and eyes. This pathology was known as salt water columnaris disease, gliding bacterial disease of sea fish, bacterial stomatitis, eroded mouth syndrome and black patch necrosis (Avendaño-Herrera *et al.*, 2006).

FIGURE 8 – External symptoms of tenacibaculosis (black patches and ulcers) (in Dinis *et al.*, 2007).

The pathogen shows a lack of strict host specificity and can be isolated from sediment, the surface of tanks and from water cultures that have been exposed to infected stocks. The primary sites of infection with *T. maritimum* are body surfaces like the head, mouth, fins, and flanks. This pathogen attaches strongly to the epidermis and mucus of fish where there aren't physiological methods to inhibit the growth of this bacterium. This location of the bacteria within the mucus layer propose that *T. maritimum* could be part of the autochthonous bacterial populations of the fish skin, and consequently the pathogen can remain in the aquatic environment for a long time, using fish mucus as a reservoir (Avendaño-Herrera *et al.*, 2006).

Some authors have observed differences in the susceptibility of some fish species (such as red and black seabream, sole, seabass, salmonids and turbot) to tenacibaculosis according to fish age. Therefore, while fish with body weights ranging from 2 to 80 g show the highest incidence and a more severe form of the disease, fish weighting more than 100 g appear to be resistant. This might be because of the apparently superior susceptibility of smaller fish to *T. maritimum*, where the severe damage of the affected tissues could evolve from early stages to advanced ulcerative lesions within a few days. Temperature also influences incidence and severity of the disease, which increases at higher temperatures (above 15°C) and salinities (30 to

35‰) as well as with low water quality. Despite this, winter outbreaks of tenacibaculosis have also been reported. The disease is also influenced by a large variety of environmental conditions (such as stress, excess of UV radiation, absence of sand substrate on the tank), management factors (such as high density and poor feeding) and host-related factors (skin surface state). In these unfavourable conditions, the systemic disease became more prevalent, demonstrating that *Tenacibaculum maritimum* has strong virulence mechanisms (Avendaño-Herrera *et al.*, 2006).

The presumptive diagnosis of tenacibaculosis is actually based on the clinical signs of the affected fish, predominantly gross external lesions, as well as in the microscopic examination of innumerous long, thin, rod-shaped bacteria in wet mounts or Gram preparations obtained from gills or skin lesions of these symptomatic fish. Nevertheless, the absence of visible *Tenacibaculum maritimum* in early lesions and the relative high incidence of secondary bacterial infections such as those caused by *Vibrio* spp. and saprophytic organisms, mostly ciliated protozoans such as *Trichodina* and *Uronema* spp., turns visualization difficult and increases the possibility of misdiagnosis (Avendaño-Herrera et al., 2006).

Dot blot assays and immunoblot analysis of lipopolysaccharides exposed the existence of antigenic diversity in *Tenacibaculum maritimum* and verified that at least 3 major O-serogroups apparently related to the host species can be detected. Therefore, the majority of *T. maritimum* isolated from sole in the northwest of Spain and all gilthead seabream isolates belonged to serotype O1, while all strains isolated from sole in Portugal and southern Spain constituted a serotype (O3), different from those strains isolated from turbot (serotype O2) (Avendaño-Herrera et al., 2006).

Among the drugs administered to turbot and sole cultures (tetracycline, enrofloxacin, flumequine and potentiated sulfonamides), enrofloxacin proved to be the best pharmaceutical compound for controlling *Tenacibaculum maritimum* outbreaks, although the rapid emergence of resistant strains has already been described. Variable results were also obtained accordingly to the route of administration (oral medication and/or bath treatment). When infection occurs, fish do not take the medicated feed, and they become anorexic immediately post-infection, therefore, bath treatment appears to be more effective than the oral treatment (Avendaño-Herrera *et al.*, 2006).

Other prophylactic treatments can be used as an alternative to drugs, such as surface-acting disinfectants administered by immersion. Routine formalin treatments (30 to 40 ppm bath for 6 hours) demonstrated to be effective in the control of tenacibaculosis in Dover sole. However, gill problems related with the extended use of this compound were observed and some hatchery managers questioned about its environmental impact. In addition, this compound is expensive and difficult to utilize and store. Hydrogen peroxide (H_2O_2) has recently demonstrated its effective control of various external pathogens to fish. It can be used at a concentration of 240 ppm for tenacibaculosis prevention, as a general disinfection method for treating water culture and the surface of tanks before the introduction of fish (Avendaño-Herrera *et al.*, 2006).

The appearance of outbreaks can also be avoided or the occurrence of the disease can be reduced by controlling fish densities, decreasing stress conditions, and avoiding overfeeding. Sand substrate has been added to tanks to prevent the incidence of tenacibaculosis and has treated an established disease situation in Dover sole showing black patches necrosis. Although all these measures help prevent the disease, modifying husbandry parameters will probably engage to related technical problems. Therefore, immunoprophylaxis have become the best way to prevent tenacibaculosis (Avendaño-Herrera *et al.*, 2006; Stickney, 2000).

Actually, only one bacterin is commercially available to prevent turbot mortalities caused by *Tenacibaculum maritimum*. The regular use of this vaccine in some turbot farms has reduced the incidence of tenacibaculosis. However, the vaccine developed for turbot may not be effective in preventing the tenacibaculosis in other fish species. As result, a new tenacibaculosis bacterin specific for cultured sole is currently being developed and evaluated (Avendaño-Herrera *et al.*, 2006).

2.1.2.2. PATHOLOGY OF VIRAL ORIGIN

Fish nodaviruses belong to the family *Nodaviridae* and genus *Betanodavirus*. The family *Nodaviridae* includes small (25–30 nm), non-enveloped, icosahedral single-stranded RNA viruses, arranged into two genera, *Alphanodavirus* and *Betanodavirus*, which infect a wide range of insects and fish, respectively. Betanodaviruses are the aetiological agents of the disease named as Viral Nervous Necrosis or Viral Encephalopathy and Retinopathy (VER), a devastating neuropathological condition that affects more than 38 species, belonging to 21 families of 10 different orders of marine fish worldwide (Oliveira *et al.*, 2009).

Betanodaviruses have been classified into four types, designated Striped Jack (*Caranx vinctus*) nervous necrosis virus (SJNNV), Tiger puffer (*Takifugu rubripes*) nervous necrosis virus (TPNNV), red-spotted grouper (*Epinephelus coioides*) nervous necrosis virus (RGNNV) and barfin flounder (*Verasper moseri*) nervous necrosis virus (BFNNV). These types display a different ability to infect fish species. Therefore, RGNNV exhibits the broadest host range and infects a variety of warm-water fish species, BFNNV is restricted to cold-water marine fish species and TPNNV causes disease in only one species. Regarding to the SJNNV type, although for several years it was considered to be restricted to a few species common in Japanese waters, in latest years it has been observed in Senegalese sole, as well as gilthead seabream and seabass cultured in the Iberian Peninsula (Oliveira *et al.*, 2009).

The disease generally occurs in larvae and/or juveniles causing high mortality rates and seems to be a function of the stress induced by the density and intensity of culture (Cutrín *et al.*, 2007). The majority of the infections that take place with water temperatures between 25-28ºC causing severe mortalities, while between 20-23ºC ceases or decreases. Some authors reported outbreaks at 4-15ºC (Hodneland *et al.*, 2011).

Studies on the transmission mechanisms of nodavirus in fish strongly propose both horizontal (by water, cohabitation, asymptomatic carriers, and live food) and vertical (ovaries and sperm) routes. Horizontal transmission has so far been considered the primary mechanism for the transmission of virus to naive hosts. A screening survey of apparently healthy wild invertebrates from the Korean Peninsula

observed the presence of betanodaviruses in crabs, shrimps and Mediterranean mussel, which may represent a reservoir for nodavirus infection in cultured fish. It has been demonstrated that nodavirus can be transmitted from experimentally infected broodstock of European seabass, *Dicentrarchus labrax*, to larval offspring, and a study from Asian seabass, *Lates calcarifer*, strongly suggests a vertical transmission mode. Transmission of nodavirus between different fish species is also possible, and information about potential cross infections is important in marine fish-farming management. Subclinically infected fish might act as hidden nodavirus replication sites (Hodneland *et al.*, 2011).

Live fish and eggs trade is relevant in the dissemination of nodaviruses to different geographical areas and commercial exchange is regarded as the major responsible for the dissemination of nodaviruses within the natural range of affected fish species. However, neither of these conditions provides a reasonable explanation for the emergence of SJNNV strains in the Iberian Peninsula. SJNNV might have been present for a long period in wild Senegalese sole remaining unnoticed until intensive rearing of this fish species, using wild fish as broodstock, in Spain and Portugal (Cutrín *et al.*, 2007).

The major target organ of nodaviruses is the central nervous system (CNS) including brain, spinal cord and nuclear layers of the eye retina. Clinical signs of VER include anorexia, lethargy, changes in pigmentation (pale-grey), neurological signs as uncoordinated or erratic swimming behaviour (circling) causing that the affected fish get unable to retain an upright position, and mortality rates up to 100%. Usually, VER is diagnosed histologically by the observation of extensive areas of necrosis and vacuolization in brain and retina combined with positive immunohistochemistry (IHC) for nodavirus. Cells of optic tectum, cerebellum, tegmentum, vagal lobes, medulla oblongata and spinal cord present enlarged basophilic cells with a rounded shape, inclusion bodies and cytoplasmatic vacuolization. Larvae show heavy necrosis of the brain whereas adults show higher concentrations of the virus in the brain but with lesser necrosis of the tissue (OIE, 2013).

Some disinfection methods have been described, as the fast inactivation of SBNN at 60ºC over a period of 30 minutes, which suggests that standard heat sterilization procedures such as autoclaving at 121ºC for 15 minutes at 15psi would guarantee total destruction of the virus. At a more practical level, it appears that

treatment of non heat-labile equipment and hatchery water by heating to 60ºC or above for not less than 30min would make all contaminating virus inactive (Frerichs *et al.*, 2000).

UV irradiation treatment of hatchery water may be affected by the particulate nature of the sea water impeding light penetration. Viral inactivation under hatchery water-flow conditions demonstrated to be practicable. UV irradiation treatment of SBNN infected clarified culture fluid at 440 µW/cm^2 showed to reduce the virus titre by 99,9%. However, the undetectable residual virus may still be sufficient to cause infection in vivo and it would be unwise to ignore this possibility when proposing UV irradiation as the unique method of disinfecting hatchery water supplies. It has been reported that UV-irradiated viable virus becomes more vulnerable to other disinfectant methods. Some reports say that virus is also susceptible at pH 12, ozone at 0,1 µg/ml and to sodium hypochlorite, calcium hypochlorite, benzalkonium chloride and iodine. Is not inactivated by formalin, ethanol, methanol, ether or chloroform. Dosages of ozone of 4 to 10 (concentration x time) seem to be sufficient to disinfect (Frerichs *et al.*, 2000).

Numerous methods for VNNV detection in fish samples have been reported, such as indirect fluorescent antibody test (IFAT), immunohistochemistry, ELISA and the combination of cell culture and IFAT. Although amplification-based techniques have demonstrated to be powerful diagnostic tools for nodavirus detection, conventional PCR is unable to determine the number of copies of viral template, which is a fundamental parameter of the viral disease. Subsequently, real time PCR has become a necessary tool in infectious pathogen diagnosis due to its high sensitivity and specificity (Cherif *et al.*, 2011).

2.1.2.3. PATHOLOGY OF FUNGAL ORIGIN

Ichthyophoniasis, due to infection with *Ichthyophonus hoferi*, is the most important disease caused by fungi affecting Senegalese Sole and other hosts, as various marine and freshwater crustaceans, elasmobranch and teleost fish, amphibians, reptiles and piscivorous birds. This disease is known to be of economic

significance, in both cultivated fish and wild fisheries, and to have a wide host and geographical distribution (Dinis *et al.*, 2007; Woo & Bruno, 1999).

Some authors proposed that *I. hoferi* might be a secondary invader, masking a possible primary bacterial agent, responsible for the debilitating condition in the host. The extensive incidence of *I. hoferi* in diseased fish, the simplicity of establishing experimental infections and of sequentially maintaining these in different species of fish, the isolation of infective stages and the development of typical pathology, suggest a major role of this infective agent in the disease course (Woo & Bruno, 1999).

The complexity to detect *Ichthyophonus* from gross signs is closely connected to the level of infection and pathogenicity recorded. Like most disease conditions with high level of pathogenicity, several non-specific signs may accompany the advanced stages of infection with *I. hoferi*. These signs include behavioural changes and changes related with organ failure, such as lethargy, emaciation, colour abnormalities, fluid accumulation, nervous disorders and an increase of mortality rates. Such changes are obvious in groups of captive fish but are also discernible in wild populations. To distinguish *Ichthyophonus* from other granuloma-inducing conditions, it is suggested that the field diagnosis should be supported by some evidence of the characteristic germination of the parasite after the death of the host. When infected fish are examined internally, it is frequent for gross white or cream-coloured nodular lesions, which release a typical odour, 1 to 5 mm in size to be visible throughout most tissues, although, for Senegalese sole, muscle is the tissue most severely infected. Apparently the main route of infection of a fish with *I. hoferi*, confirmed by experimental transmission, is through the intestine. Therefore captive fish might get infected through feeding of contaminated fresh food (Dinis *et al.*, 2007; Woo & Bruno, 1999).

The widespread natural reservoir of infection in marine and estuarine waters and the relative simplicity of horizontal experimental transmission of the disease between most species of fish suggest that wild and farmed fish stocks in these areas should be at risk for the infection. Spores demonstrated to be able to survive for almost 2 years in sea water and to be fully viable after 6 months in sterile sea water. Some authors also showed that the parasite can grow well in the temperature range

of 0 to 25°C and between pH levels of 3 and 7, but was unable to tolerate salinities above 4% NaCl (Woo & Bruno, 1999).

Fresh squash preparations of infected organs generally show the presence of the usually spherical resting stage (spore) which varies in size from 10 to 250 mm in diameter. These are typically surrounded by varying amounts of host granulomatous reaction tissues, and the employ of phase contrast microscopy is desirable to facilitate distinction of them from other similar structures present in the body of fish (Woo & Bruno, 1999).

Germination of ingested spores in the host intestine typically involves the development of tubular protrusions known as germination tubes or hyphae, which usually become branched (figure 9) (Woo & Bruno, 1999).

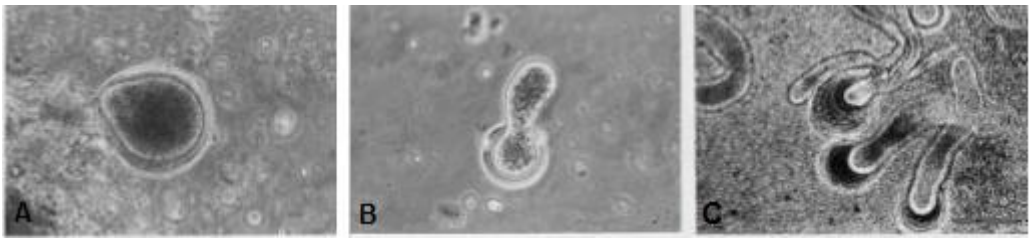

FIGURE 9 – A, B. Beginning of germination of *Ichthyophonus hoferi* spherical bodies in the Intestine; **C.** Development of branched germination tubes (hyphae) (adapted from Woo & Bruno, 1999).

2.1.2.4. PATHOLOGY OF PARASITOLOGICAL ORIGIN

Amyloodinium ocellatum is the major dinoflagellate that affects fish, causing amyloodiniosis, also known as marine velvet disease, marine *Oodinium* disease and oodiniosis. It causes severe morbidity and mortality in brackish and marine warm-water fish at aquaculture facilities globally and is often considered the most important pathogen of marine fish. Outbreaks can occur extremely fast, reaching 100% mortality rate in just a few days. *A. ocellatum* is one of a very small number of fish parasites that can infect both elasmobranchs and teleosts and almost all fish that live inside its ecological range are vulnerable to infection. *A. ocellatum* has continued to be one of the most serious impediments to warm water mariculture, with well over 100 species known to be susceptible (Woo, 2006; Woo & Buchmann, 2012).

A. ocellatum is a warm-water parasite and is more pathogenic at higher temperatures, therefore, in more temperate regions, only becomes a problem in warmer months. The optimal temperature for tomont division and sporulation ranges from 23 to 27ºC, accordingly to studies of environmental tolerances made with Red Sea isolates. Conclusion of tomont division is limited to 16–30ºC and infections do not occur at less than 17ºC. Temperature also influences salinity tolerance, which diminishes as one deviates further from the optimal temperature range (Woo, 2006).

A. ocellatum has a direct, although triphasic life cycle. The parasites feed as stationary trophozoites (trophonts) on the epithelial surfaces of the skin and gills. Trophonts remain attached to the fish by root-like structures (rhizoids) that firmly adhere the parasite to the epithelium. After reaching maturity, the trophont detaches from the host, forming a reproductive 'cyst' or tomont in the substrate. This tomont divides, forming up to several dozen free-swimming individuals (dinospores) that can then infect a new host (figure 10) (Woo & Buchmann, 2012).

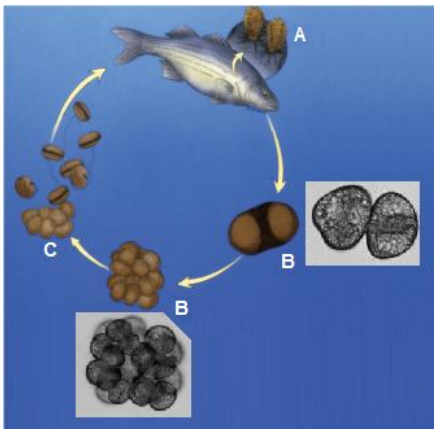

FIGURE 10 – *Amyloodinium ocellatum* life cycle; **A** - trophont; **B** - tomont; **C** – dinospore; (adapted from Woo, 2006; Woo & Buchmann, 2012).

The main lesion caused by *A. ocellatum* is the damage of epithelial cells of the skin and gills and the clinical signs are directly proportional to epithelial injure. A single trophont can feed on multiple epithelial cells at the same time. Despite the fact that it has not been documented, the cause of death is possibly due to osmotic imbalance in the majority of cases, although secondary infections may also take place (Woo & Buchmann, 2012).

The major clinical signs of amyloodiniosis include anorexia, depression, dyspnea (gasping) and pruritis. Generally the gills are the primary site of infestation,

however severe infestations may also affect the skin, fins and eyes. Severely infested skin may show a dusty appearance ('velvet disease'), although this might not be a frequent finding and fish often die without evident gross skin lesions. Young fish seem to be most susceptible, while there are still few knowledge in this area. Trophonts may also take place on the pseudobranch and in the branchial cavity and nasal passages. Although tomonts have been described in the oesophagus, stomach and intestines, possibly they developed in a different place and were subsequently swallowed. The hydrodynamic forces applied on the trophont, which projects into the water when the fish swims, may exacerbate damage. Trophonts can also move slowly back and forth while attached, also improving host cell fragmentation (Woo, 2006; Woo & Buchmann, 2012).

Mild infestations, with one or two trophonts per gill filament, cause slight pathology. However, serious infestations, with up to 200 trophonts per gill filament, cause severe gill hyperplasia, inflammation, haemorrhage and necrosis. Death is usually associated to anoxia and can take place within 12 hours in the case of a severe infestation. In contrast, acute mortalities are occasionally associated with apparently mild infestations, suggesting that hypoxia might not always be the cause of death (Noga, 2010; Woo & Buchmann, 2012).

For typical diagnosis of *Amyloodinium* spp., parasites are observed on infested tissues under a microscope (Woo & Buchmann, 2012).

Management decisions are established according on the species of fish infected, the proposed use of these fish, and constraints that may be imposed by the type of system they are kept in. Generally, treatment implicate eliminating the free-swimming dinospores before they can attach to a new fish host, or removing, or killing the trophonts from the fish, consequently breaking the life cycle (Francis-Floyd & Floyd, 2011).

The free-swimming dinospore is sensitive to chemotherapy (Table IV), however trophonts and tomonts are reasonably resistant, making eradication complicated. For instance, tomonts tolerate copper concentrations that are over ten times the levels that are toxic to dinospores. Even when tomonts are inhibited from dividing, they can regularly resume the division when returned to untreated water. Therefore periodic examination for re-infestation after treatment is desirable. Copper is most widely used for treatment. The free copper ion is the active component and

free copper must be kept at 0.12–0.15 mg/l for 10–14 days to control epidemics. Superior concentrations of free copper should be avoided because they are toxic to fish. Copper levels required to treat amyloodiniosis are also toxic to most invertebrates and algae (Woo, 2006).

Chloroquine has no effect on tomont division, but kills dinospores immediately upon their excystment. However, chloroquine is very expensive and is improbable to be approved for food fish (Woo, 2006).

Because of the multi-stage life cycle, multiple treatments or extended treatments (more than 10 to 14 days), often followed by transference of the fish to an uncontaminated tank, will be essential to a correct control of an outbreak of *A. ocellatum* (Francis-Floyd & Floyd, 2011).

Table IV –Treatments reported to be effective for treating *A. ocellatum* (adapted from Woo & Buchmann, 2012).

TREATMENT	DOSAGE AND TIME	COMMENTS
Copper	0.12-0.15 mg/l for 10-14 days	Must maintain within this range
Chloroquine	5-10 mg/l for 10 days	Single dose exposure
Hyposalinity	0-10 ppt salinity for 10-14 days 0 ppt salinity for 5 min; repeat every 3 days x 3 times 0 ppt salinity for up to 5 min	Isolates vary in salinity tolerance, but fresh water is usually needed to treat; Must remove fish to uncontaminated system after each treatment to prevent reinfestation by detached trophonts; Must remove fish to uncontaminated system to prevent reinfestation by detached trophonts
Hydrogen peroxide	75 mg/l for 30 min; repeat after 6 days and then transfer to uncontaminated tank	Must remove fish to uncontaminated system within 24 h after last treatment to prevent reinfestation by detached trophonts; no trophonts detected after 2 weeks
Formalin	100-200 mg/l for 6-9 h 50 mg/l for 1 h; repeat after 15 days 4 mg/l for 7 h; repeat after 15 days	Must place fish in an uncontaminated system to prevent reinfestation by detached tomonts; Reported to control a natural outbreak;
Lasalocid	1 mg/l for 24 h	Requires water-soluble form

Hypothermia	Temperature <15°C	Isolates vary in temperature tolerance

For preventing the risk of introduction of infectious dinospores, into an aquaculture system, disinfection of incoming water should be done, for instance by ultraviolet irradiation, or ozone, which may be particularly useful in re-circulating systems. Ageing of water beyond the survival time of dinospores and quarantine of new fish for at least 20 days are additional measures that may decrease, but not eradicate, the risk of parasite introduction (Francis-Floyd & Floyd, 2011; Woo, 2006).

A. ocellatum is easily observed by microscopic examination of infected tissue, visible at total magnification of 40X and 100X with a light microscope. Gill or skin biopsies expose the parasite attached to tissue (figure 11). *A. ocellatum* is dark brown in colour, ovoid to pear-shaped, and non-motile. Trophonts can be up to 350 µm, but smaller sizes of <150 µm are more typical. It is not unusual to have organisms of different sizes visible in one field (Francis-Floyd & Floyd, 2011).

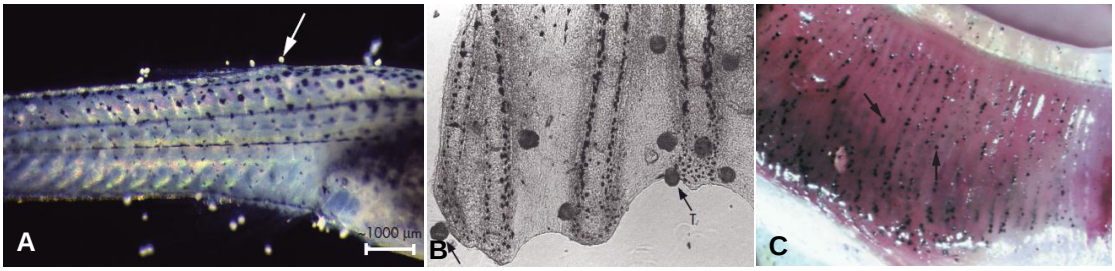

FIGURE 11 – Trophonts of *A. ocellatum* attached to the surface of the skin (**A, B**) and in the gill filaments stained with Lugol's iodine (**C**) (adapted from Noga, 2010).

Infections can be identified by microscopic examinations of wet mounts from skin scrapings, gills, brain squash or body cavity fluid (when ascitis is present) (Woo & Buchmann, 2012). Even when skin lesions are present, skin scrapings might not detect the organisms in wet mounts if they are located deep in the tissues. Therefore, scraping deep into the muscle, preparing wet mounts of internal, or examining tissues histologically must also be done for a precise diagnosis (Noga, 2010).

Scuticociliatosis, is a disease caused by marine holotrich ciliates of the subclass Scuticociliatia, ciliates that can cause skin and gill lesions and systemic infections. Numerous scuticociliates are facultative parasites (are free-living protozoa

and can live in the absence of a host) of aquatic animals and histophagous members have showed to be a problem in commercial and ornamental fisheries. Several genera, including *Anophryoides*, *Mesanophrys*, *Miamiensis*, *Philasterides*, *Pseudocohnilembus*, *Tetrahymena* and *Uronema* have been isolated from diseased organisms (Noga, 2010; Woo & Buchmann, 2012).

Scuticociliates inhabit eutrophic marine coastal waters. Lately, it has become a severe problem in Japanese flounder culture in Asia (China, Japan and Korea), and also with turbot and European seabass culture in the Mediterranean. There are also reports of the disease in southern bluefin tuna in Australia (Noga, 2010). Mortality is predominantly high for flatfishes (like the olive flounder and turbot) and, consequently, is of great economic significance. Flatfishes might be more predisposed to the disease due to their aggregation behaviour and consequently more skin-to-skin contact, which may increase direct transmission because the ciliate occurs in great numbers in skin ulcers and fin lesions. Additionally, scuticociliate density is superior at the bottom of the tank than in the water column. Therefore, sedentary, benthic fish are more exposed to infection (Woo & Buchmann, 2012).

Uronema marinum is the most frequent scuticociliate in tropical marine fish, which seems to have an extensive host range and can infect fish over a large range of temperature (8 – 28°C) and salinity (20 – 31 ppt). The closely related and morphologically comparable *Miamiensis avidus, Uronema marinum*, *Philasterides dicentrarchi* and *Pseudocohnilembus persalinus* were associated with scuticociliatosis in farmed Japanese flounder (Noga, 2010).

Philasterides dicentrarchi has also been reported to cause systemic infection in European seabass and turbot. Recent studies reported that there is morphological evidence that *Miamiensis avidus* and *Philasterides dicentrarchi* are the same species (Noga, 2010).

Outbreaks of the disease in food fish mainly affect younger fish, including fry and juveniles. Differently from typical ectoparasitic protozoa, scuticociliates regularly invade internal organs and cause deep ulcers. Muscle, peritoneal cavity, kidney, pancreas, liver, urinary bladder, spinal cord and brain may also be affected. Frequently there is slight inflammatory response. Fish typically develop white skin foci, which develop to areas of depigmentation and ulceration. Some fish may show

no external signs, except lethargy. There can be skin hemorrhage and/or necrosis and gill aneurysms. Once established in a host, death is rapid (Noga, 2010). *Miamensis avidus* is extremely histophagous in olive flounder and turbot and it causes severe haemorrhages and ulcers on skin muscles, fins and jaws. *M. avidus* probably enters the hosts via the body or branchial surfaces, particularly if there are lesions present on these regions, and then spread via blood vessels and lymphatic channels to different internal organs (Woo & Buchmann, 2012).

Experimental *Philasterides dicentrarchi* infections in farmed turbot using different routes of infection were analysed. Intraperitoneal, periorbital and intramuscular inoculations resulted in systemic infections and elevated fish mortality. Infection by immersion was just successful after artificial abrasion of gills and opercula, suggesting that lesions in the skin or the gills are entry routes into fish under culture conditions. Similarly, experimental infection in olive flounder by immersion was only achieved after abrasion of the gills and muscles. Despite these factors, other reports demonstrated 60-100% mortality via immersion infection without any artificial abrasion or other treatment before infection (Woo & Buchmann, 2012).

Recognizing a protozoan as a scuticociliate can be simply done by morphological criteria, however, species identification of scuticociliates usually required the preparation of silver-stained mounts of individual cells which allow observation of the cilia pattern on the cell. There is a severe number of highly similar ciliates included in this disease, so new diagnosis methods have been developed, as gene probes which allow the fast and specific identification of infections, and some can also determine if more than one scuticociliate species is involved in an epidemic (Noga, 2010). Mitochondrial cytochrome C oxidase subunit 1 (cox1) gene and the internal transcribed spacer genes may facilitate to differentiate between species (Woo & Buchmann, 2012).

Live ciliates are ovoid to pyriform or elongate in shape (20-50 µm in length and 15-25 µm in width), with variations as a result of species fixative condition and feeding status, and actively moving by caudal cilia (figure 12). They might contain food vacuoles filled with blood cells and/or cellular debris (Woo & Buchmann, 2012).

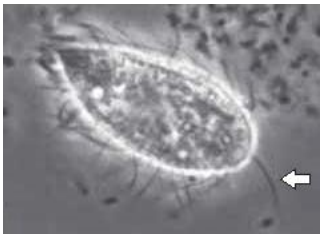

FIGURE 12 – Scuticociliate with caudal cilium at the posterior end (pointed arrow) observed under phase contrast microscope (adapted from Woo & Buchmann, 2012).

The scuticociliates are complicated to exterminate because they are able to survive in nutrient-rich water and bottom sediments, and also in internal organs of infected fish where the chemical treatment is not very efficient. It has been reported that early stages of *Uronema marinum* infection, in tropical marine fish, can be controlled by a freshwater bath, followed by prolonged immersion in formalin. Some studies also show that advanced lesions have responded to methylene blue or nitrofurazone (Noga, 2010). Farmers utilize formalin, hydrogen peroxide or sodium chloride in combination with antibiotics (for example, oxytetracycline, gentamycine and tetracycline) to eliminate the ciliate and to prevent secondary bacterial infections by skin lesions (Woo & Buchmann, 2012).

In Japanese flounder, a 200 ppm formalin bath for 2 hours is recommended once a day for six days. However, in all fish species, systemic or deep muscle infections have a weak prognosis. Scuticociliates prove to be relatively resistant to UV, compared with fish pathogenic viruses or bacteria, however they can be successfully eliminated from a contaminated water supply by using a high intensity treatment ($3{,}0 \times 10^5$ µW · sec/cm^2) (Noga, 2010).

Nowadays, the majority of protozoan infections are controlled using chemotherapy. However, its employ is being constrained as there are rising concerns over food safety and environmental pollution. Vaccination is an interesting alternative to chemotherapy, particularly when the ciliate is located in internal organs. It has been reported that fish that survived scuticociliate epizootics acquired disease resistance and appear to have specific antibodies against the pathogen. As a result, vaccination might be a possible option (Woo & Buchmann, 2012).

Numerous trichodinid species infect marine or freshwater fish, including *Trichodina*, *Trichodinella*, *Tripartiella*, *Dipartiella*, *Paratrichodina*, *Hemitrichodina*, and *Vauchomia* species (Noga, 2010). Ectozoic (live on the surface of an animal) species utilize their host as a suitable substrate upon which they glide and to which they temporarily adhere. They feed on waterborne particles, bacteria, and detritus particles from the fish surface. Trichodinids never occur in large numbers on healthy fish and they also demonstrate over-dispersion under natural conditions (Woo, 2006). All trichodinids have an identical morphology (figure 13). All clinically relevant species affect the skin and/or gills. Some species infect the urinary bladder, oviducts, or gastrointestinal tract, but they are not confirmed pathogens. The majority of trichodinids show little host specificity. Generally, the larger (> 90 µm), skin-dwelling trichodinids have a wide host range, while smaller (< 30 µm), gill-dwelling parasites have a tendency to infect one or a few fish species. Several species infect both skin and gills (Noga, 2010). Some authors reported that in cases of serious infestations, ectozoic trichodinids may also occur in the rectum and cloaca (Woo, 2006).

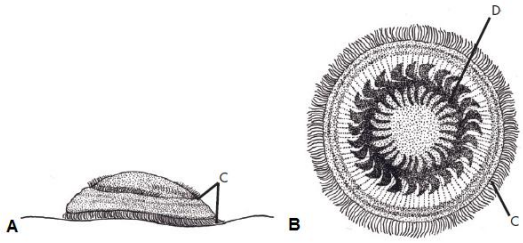

FIGURE 13 – Diagram of a typical trichodinid life cycle; **A** – side view; **B** – top view; C – cilia; D – denticle (adapted from Noga, 2010).

Trichodinosis is typically a relatively mild disease that normally presents as chronic morbidity or mortality, however in some cases, can cause considerable losses, particularly in young fish (Noga, 2010). In a tightly attached *Trichodina*, the rim of the border membrane "bites" into the surfaces of epithelial cells, and the surface it encircles is forcibly vaulted as by a sucker, probably causing irritation to the fish (Woo, 2006). Seriously infected fish are anorexic, lose condition, and usually experience low level (approximately 1% per week) mortality. Nevertheless mortalities can be much higher, mainly in young fish. Secondary bacterial infections can severely increase mortality rates. Trichodinid infestations are seen essentially in fish that are debilitated due to other conditions, as poor nutrition, overcrowding, stress,

another disease or in fish larvae or young fry. Some trichodinids showed to be able to survive off the host for 1-2 days (Noga, 2010).

Severely infested fish may show a greyish-blue colour, produced by excessive mucus secretion and peeled epithelia, and frayed fins. The excessive epithelial growth seems to be a protective reaction, however, at the same time, trichodinids feed on it. Debilitated fish are lethargic, swimming just beneath the water surface or near the water edge, and become anorexic. It has been reported a case of *Trichodina* spp. infestation on young farmed turbot resulting in approximately 26% of weight loss over a 12 month period (Woo, 2006).

Trichodinids are easily eliminated through formalin bath or prolonged immersion, potassium permanganate prolonged immersion, acetic acid bath (freshwater only), salt bath (freshwater only), freshwater bath (marine only) or copper prolonged immersion. Fish will frequently recover spontaneously if water quality is improved. Some trichodinid species are able to infect both freshwater and marine fish, but practically all common pathogens are limited to either fresh or saltwater environments (Noga, 2010).

Entobdella soleae is a monogean skin parasite of *Solea solea*, found mostly in the eastern Atlantic Ocean from Norway to Senegal and in the Mediterranean Sea. *E. soleae* uses its pharynx as its feeding organ and possesses glands in the pharynx that secrete proteolytic enzymes to digest the epidermis of the flatfish. Common sole, which is generally only slightly infected under natural conditions, can show dense populations of *Entobdella soleae* when in captivity conditions (Woo, 2006).

This trematode shows high host specificity with studies demonstrating that when experimentally transferred to wrong hosts it seemed to become detached after 24 to 30 hours, even though it was able to survive on glass for 2 to 6 days (Rohde, 2005).

Some authors observed large capsalid populations grazing on farmed fish, which injured and eroded epithelium faster than it could be healed. On the contrary, wild fish are able to support smaller natural capsalid populations and parasite mobility may extend injuries. In farmed fish, lesions may aggravate from epithelial irritation from flashing, host health degradation disturbing the immune system and secondary infection (by bacteria, viruses, fungi) of capsalid-inflicted traumas (Woo &

Buchmann, 2012). A severe increase of *E. soleae* populations on the skin of sole kept in captivity with several hundred worms present on hosts can be lethal if left untreated (Woo, 2006).

Eggs of *E. soleae* are tetrahedral and have a stalk with adhesive material for attachment to sand grains or other materials in the environment. Egg development takes 27 days at 13–17°C. The oncomiracidium escapes via an operculum and the hatching process may be induced by host mucus or light. As control measures repeated formaldehyde bath treatments can cure the infection (Woo, 2006).

The adult form of *Entobdella soleae* has a length of about 2–5 mm and a width of 1–2.5 mm. The worm is dorsoventrally flattened and has four small eyes (figure 14). A characteristic feature of the organism is its unique prohaptor, an anterior structure with two adhesive pads (Woo, 2006).

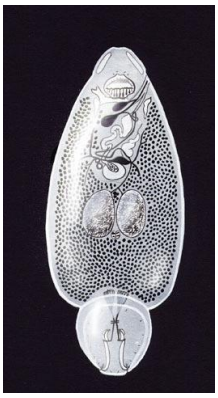

FIGURE 14 – Diagram of adult/sexual mature *Entobdella soleae* (adapted from http://animaldiversity.ummz.umich.edu/accounts/Entobdella_soleae).

2.2. PATHOGENS ASSOCIATED TO LIVE FOOD

One of the main problems in the early rearing of marine fish is the susceptibility of the fish larvae to parasitological and microbial infections. It is assumed that the live food, which is used to feed the first stages of fish lifecycle, can be a significant source of potentially pathogenic parasites and bacteria, which are easily transferred through the food chain to the predator larvae.

According to FAO, Halotricha and Hypotricha ciliates, such as *Uronema* sp. and *Euplotes* sp., are undesirable in intensive cultures as they compete for feed with the rotifers, besides they can be harmful for the fish. The appearance of these ciliates

is generally due to sub-optimal rearing conditions, which induces minus performing rotifers and superior chances for competition. Ciliates generate metabolic wastes which raise the $NO2^{-}$-N level in the water and cause a decline in pH. Nevertheless, they demonstrate a positive effect in clearing the culture tank from bacteria and detritus. The addition of a low formalin concentration to the algal culture tank, 24 hours before rotifer inoculation can notably decrease protozoan contamination. Screening and cleaning of the rotifers by using phytoplankton filters (net mesh <50µm), or a scrub sponge on the rotifers tank helps to clean particles in the water and diminish the number of ciliates or other small contaminants (Lavens & Sorgeloos, 1996).

Vibrio sp. acts as the major bacterial flora in *Artemia* cyst hatching solutions. At large cyst densities and high incubation temperatures through hatching, bacterial growth can be significant and hatching solutions might become turbid, which could also result in reduced hatching rates. As a result, if commercially disinfected cysts are not used, it is suggested to apply routinely a disinfection procedure by using hypochlorite. Nevertheless this treatment might not destroy all germs present in the alveolar and cortical layer of the outer shell. Total sterilization can be accomplished by cyst decapsulation (Lavens & Sorgeloos, 1996).

2.3. BIOSECURITY

Biosecurity involves the practices, procedures and policies used to prevent the introduction of pathogenic agents (as bacteria, viruses, fungi and parasites) and/or invasive species (mussels for instance) and their spread in aquaculture facilities. It is difficult to predict when disease might occur, however the routine use of biosecurity measures can reduce the risk of introduction and economic impact of these diseases.

Determining the Biosecurity measures needed for each facility involve the identification of risk areas and the determination of the necessary preventive measures to obtain the greatest cost-benefit. Most preventive procedures are inexpensive when compared to the potential loss that can occur from fish deaths or decreased production. A large part of them are rapidly implemented (as foot dips, disinfection buckets for material) (figure 15). One important step to obtain a good

health status for the aquaculture unit is to train the employees on hygienic fish handling and fish disease prevention methods.

FIGURE 15 – Disinfection buckets for material (photo from Safiestela, 2013).

Fish diseases can be spread by direct contact, water sources, fomites, ingestion (oral), and vectors. Direct contact is one of the common routes of disease transmission in aquaculture. This involves the transfer of disease causing agents through direct contact with infected fish. Entry may occur through the skin, open wounds, mucous membranes, or gills.

Contamination by water sources is due to urine, faeces, reproductive fluids and mucus of infected fish. A few fish pathogens as *Ichthyophthirius multifilis* have been found to spread via aerosols, sprays or splashes between tanks.

Fomites are inanimate objects that can transfer pathogens between rearing areas or production sites. These items become contaminated following contact with infected fish or contaminated water sources. Examples include equipment such as nets, buckets, siphon hoses, footwear, clothing, etc...

Vectors are living creatures, such as fish-preying birds or rodents, which can spread pathogens by carrying them on their body or by dropping fish or fish parts at different locations. Some parasites use these vectors as hosts to be able to complete their life cycle. Humans can also be vectors transferring pathogens to fish during handling. Pathogenic agents may also be transmitted orally by consumption of contaminated feed, or cannibalism of death or ill fish.

The movement of fish onto, within or off the farm is the greatest risk factor for disease introduction and spread in aquaculture. Quarantine by isolating new arrivals before adding them to current stock minimizes the risk of disease transfer to resident fish.

Surface water sources should be avoided as they present greater potential for carrying fish pathogens.

Regular veterinary checks should be undertaken and fish vaccination programmes and disease treatments introduced when suitable.

Many pathogenic agents can survive in the environment for long periods, for this reason all the equipment utilised needs to be cleaned and chemically disinfected before being used. Disinfectants should be appropriate and applied using the proper concentration, application method and contact time (table V). Many disinfectants can be lethal to fish so rinsing and/or neutralization (for instance with sodium thiosulfate for chlorine products) are necessary to remove any toxic residues.

Table V – Chemical disinfectants used in aquaculture (adapted from Manual of Diagnostic Tests for Aquatic Animals, OIE 2012).

Product	Concentration	Uses and Precautions
Virkon® Aquatic	1:100 – 1:200 (1% - 0,5%) for 1 minute	Equipment, pumps, protective clothes, foot dips, buckets, bins, harvesting equipment.
Sodium hypochlorite (bleach – 5,25% NaClO solution)	200-500ppm	Nets, boots, clothing. Surfaces must be clean before application; easily inactivated by organic debris. May be corrosive. Highly toxic for aquatic animals; Allow to inactivate for several days or neutralize with Sodium thiosulfate after 3 hours.
Iodine (iodophors)	200ppm for a few seconds	Nets, boots, clothing. Surfaces must be clean before application; easily inactivated by organic debris. Highly toxic for aquatic animals.
Benzalkonium chloride	250ppm	Plastic surfaces. Can be toxic to fish.
Alcohol (ethanol)	70%	Hand sanitizing

III. EXPERIMENTAL WORK

3.1. INTRODUCTION AND OBJECTIVES

The present work was made with the objective of identify any possible biotic or abiotic factors, able to cause pathologies levels, through laboratorial research of different kinds of samples, and to implement specific biosecurity measures to the aquaculture unit, in order to minimize the risk of diseases.

3.2. MATERIAL AND METHODS

3.2.1. SAMPLING

3.2.1.1. WATER SAMPLES

Daily, several physico-chemical parameters of water were analyzed from different locations, including the hatchery, breeding and weaning tanks. The parameters encompassed evaluation of temperature, pH, oxygen and salinity.

The sampling for bacteriological examination of water was carried out wearing gloves and the water was collected in sterile containers with a minimum capacity of 120 mL, to avoid contamination by external factors. After closing the container, this was identified with its collection site. The water analysis need to be performed soon after collection.

3.2.1.2. FISH SAMPLES

To be able to realize bacterial and parasitic analysis, it was necessary to perform a necropsy to the sole. Therefore the internal organs of the fish can be seen, through a systematic dissection. The fish selected for carrying out necropsy should show signs of external lesion, and should preferably be moribund or dead recently, since the autolysis process is fast in fish due to its composition rich in unsaturated fatty acids. Several methods of euthanasia can be utilized, although the most recommendable is to sectioning the spine in the caudal portion of the operculum, with

caution of not damage near organs (figure 16). This process should be done with a sterilized blade to minimize the risk of contamination.

FIGURE 16 – Sole euthanasia (photo from Safiestela, 2013).

The material needed for the dissection was composed of a dissection base (made of hard material, non-absorbent, easy to clean and disinfect), surgical scissors (blunt tip and fine tip), surgical tweezers, a scalpel and an alcohol lamp.

One of the most important steps before the beginning of the necropsy is the external observation of the fish, giving particular attention to morphology, changes in colour, presence of skin lesions, presence of ectoparasites, erosion of fins, alterations in the eyes (exophthalmia, haemorrhages in the eye might indicate septicaemia).

The first stage of the necropsy, after the euthanasia, consists in the extraction of the operculum, exposing the gills (figure 17).

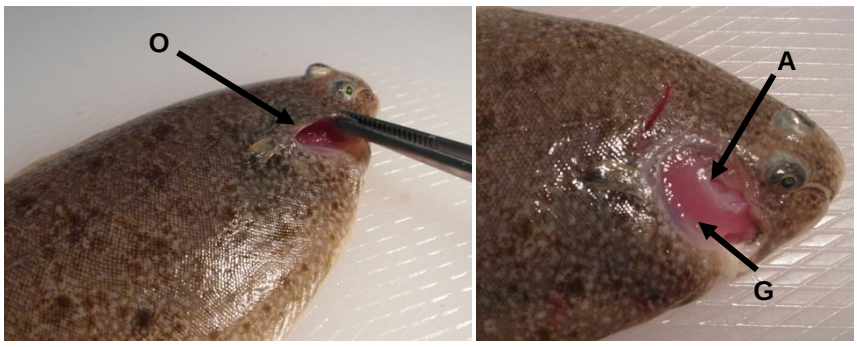

FIGURE 17 – Senegalese sole necropsy. **Legend: O** – operculum; **A** – gill arch; **G** – gills (photo from Safiestela, 2013).

The second stage consists in the extraction of the gills and the visualization of the heart (figure 18).

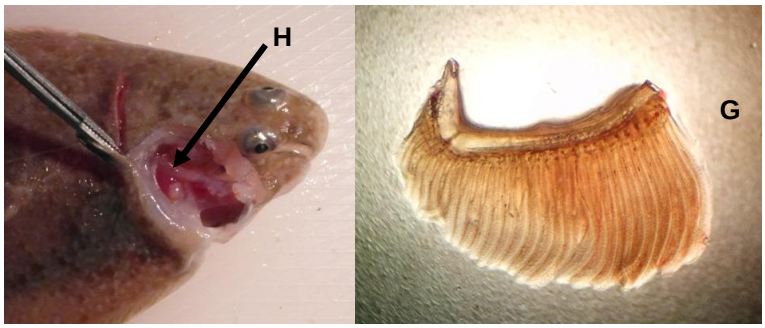

FIGURE 18 – Senegalese sole necropsy. **Legend: H** – heart; **G** – gill observed at stereoscope (photo from Safiestela, 2013).

The third step consists in the exposition of the abdominal cavity, through an incision with a bisturi (a surgical scissor may be helpful as well) from the anus till the operculum, as represented with the yellow line (figure 19).

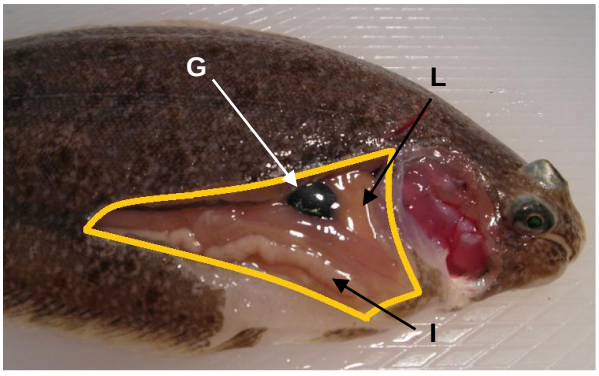

FIGURE 19 – Senegalese sole necropsy. **Legend:** the yellow line represents the incision line; **G** – gallbladder; **I** – intestine; **L** – liver (photo from Safiestela, 2013).

The fourth stage consists in the extraction of the guts. For this process is necessary to cut the distal portion of the rectum and the esophagus just behind the operculum (figure 20).

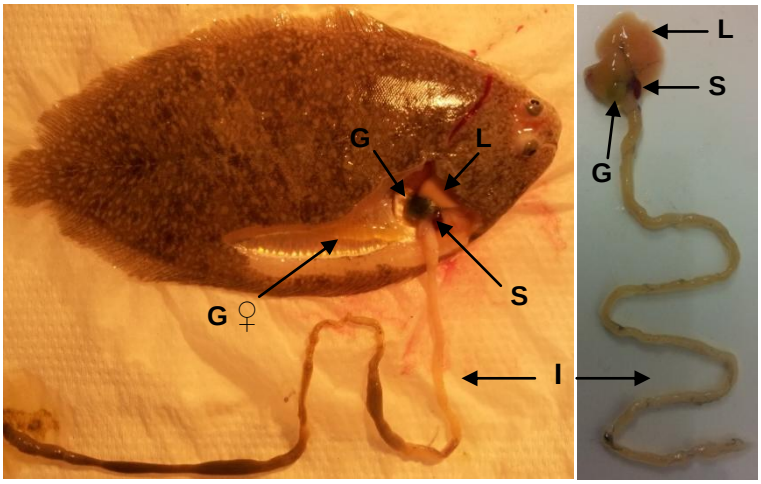

FIGURE 20 – Senegalese sole necropsy. **Legend: G** – gallbladder; **G** ♀ – female gonad; **I** – intestine; **L** – liver; **S** – spleen; (photo from Safiestela, 2013).

The fifth step consists in the extraction of the kidney, located parallel to the dorsal spine, covered by the peritoneum (figure 21).

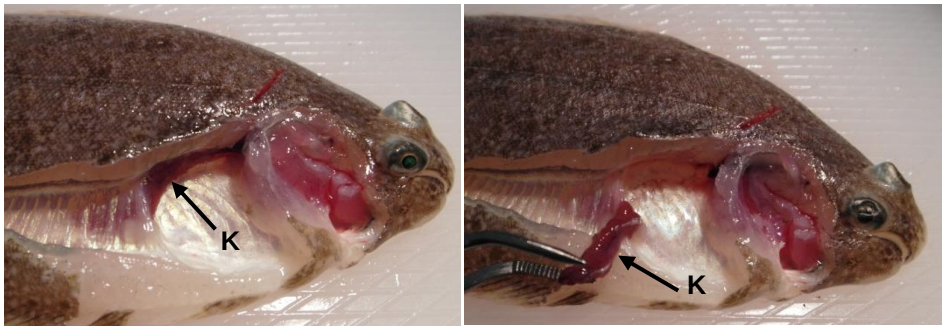

FIGURE 21 – Senegalese sole necropsy. **Legend: K** – kidney (photo from Safiestela, 2013).

The sixth stage consists in the observation of the gonads to determine the sex of the fish (figure 22). In smaller fish the gonads are still bad developed and are very difficult or impossible to visualize.

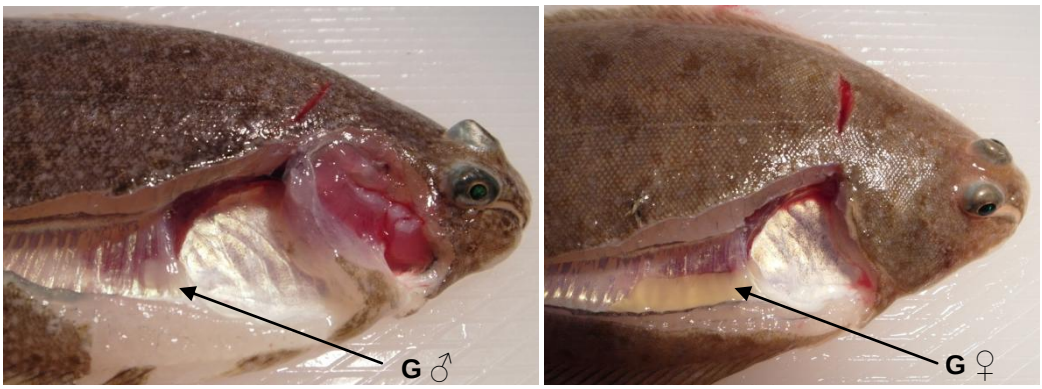

FIGURE 22 – Senegalese sole necropsy. **Legend: G** ♂ - male gonad; **G** ♀ - female gonad (photo from Safiestela, 2013).

The last step consists in brain exposition, and extraction for instance for parasite observation, as some parasites like *Philasterides* spp. might reach the brain (figure 23).

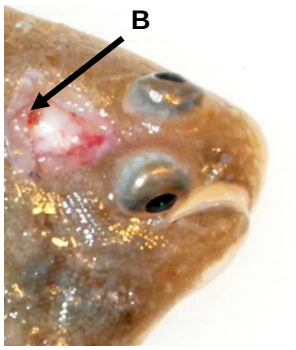

FIGURE 23 – Senegalese sole necropsy. **Legend: B** – brain (photo from Safiestela, 2013).

All the fish analysed according to a protocol and are registered in a checklist, including information as the number of the tank where the fish came from, date, sex, which alterations were observed externally and internally, if present, and which were the results of diagnostic procedures performed, this protocol and checklist can be seen in Annex I and Annex II, respectively.

3.2.1.3. LIVE FOOD SAMPLES

The sampling for ciliate observation and counting in the rotifers cultivation tank was carried out wearing gloves and the sample was collected with a sterile pipette to a sterile volumetric flask with 1L capacity. For keeping the temperature of the sample

equal to the temperature of the rotifers cultivation tank, a thermostat was placed in a bucket full of water, in order to perform a water bath. One air stone was placed inside the volumetric flask to provide oxygen to the rotifers and to avoid stratification (figure 24). Formalin and Hydrogen peroxide were added with the help of a micropipette.

FIGURE 24 – Rotifer sample in a water bath (photo from Safiestela, 2013).

3.2.2. ANALYTICAL METHODS

3.2.2.1. BACTERIOLOGICAL ANALYSIS OF WATER

The samples for bacteriological analysis of the water from the production plant could be examined by **viable count method**. The principle behind this method is that all viable cells, under suitable growth conditions, multiply and each cell forms a colony. Therefore the number of colonies is equal to the number of viable cells present in the original sample. This method can be realized through two different techniques, **plate count** or **membrane filter**.

In the **plate count technique** a measured amount of diluted bacterial suspension was introduced into a petri plate, after which the agar medium was added. Then the agar medium was immediately mixed with the inoculum by rotating the plate. Dilution procedure influences overall counting process (figure 25). After the solidification of the medium the plates were incubated at 37ºC for 24 h in an inverted position. Total number of colonies is counted and this number multiplied by the dilution factor to be able to determine the concentration of cells in the original

sample. For instance, if a plate containing a 1/1000000 dilution of the original ml of sample shows 150 colonies, then 150 represents 1/1000000 the number of CFUs present in the original ml. Therefore the number of CFUs per ml in the original sample is found by multiplying 150 x 1000000 as shown in the formula below:

Number of CFUs per ml of sample = Number of colonies (20-200 plate) X Dilution factor of the plate counted.
In the case of the example above, 150 x 1000000 = 150000000 CFUs per ml.

Counting plates should have 20-200 colonies at least. Since the enumeration of microorganisms involves the use of extremely small dilutions and extremely large numbers of cells, scientific notation is routinely used in calculations. Great care must be taken during dilution and plating to avoid errors. Even just one error in dilution can have large effects on the final numbers. The rate at which bacteria give rise to an observable colony can also vary. If too short an incubation time is used, some colonies may be missed. The temperature of incubation and medium conditions must also be optimized to achieve the largest colonies possible so that they are easily counted.

This method is highly sensitive, being able to count extremely high or very low viable populations. However it has certain disadvantages, for instance, if the suspension contains different microbial species, then all of them may not grow on the medium used and under the specified conditions of growth. If the suspension is not homogeneous and contains aggregates of cells, the resulting colony count will be lower than the actual number of microorganisms, because aggregate cells produce a single colony.

Plates with more than 200 colonies could not be counted and were designated too many to count (TMTC). Plates with fewer than 20 colonies were designated too few to count (TFTC).

In the spread-plate technique some of the highest dilutions (lowest bacterial density) were then taken and spread with a sterile glass rod onto a solid medium that would support the growth of the bacteria. It is important that the liquid spread onto the plate soaked into the agar. This prevented left over liquid on the surface from

causing colonies to run together and the need for dry plates restricted the volume to 0,1 mL or less.

Procedure:

1. Prepare assay tubes containing 9 mL of sterile marine water;
2. Sterilize by autoclaving for 20 minutes at 120 °C and 1 atmosphere;
3. Prepare a dense suspension of the bacteria in a tube containing sterile marine water;
4. Shake the test tube on a vortex mixer (about 5 seconds);
5. With a sterile 1 ml pipette or by using an automatic pipette with sterile tips, aseptically remove 1 ml of the microbial suspension;
6. Enter the volume of 1ml of the suspension in the first tube dilution (10^{-1});
7. Mix the suspension by shaking in vortex;
8. With a new sterile pipette to withdraw 1 ml of the 10^{-1} dilution and transferred to another tube to obtain 10^{-2} dilution;
9. Continue this procedure until the 10^{-6} dilution;
10. Select the last three dilutions of the suspension of the bacteria 10^{-4},10^{-5} and 10^{-6};
11. Shake vigorously each of the test tubes containing the dilution prior to inoculation into solid medium;
12. Three half-open petri dishes and put 0.1 mL of each three dilutions in the medium;
13. Dip the spreader in the glass with pure alcohol and pass it in the flame of a Bunsen burner or lamp. Let the flame extinguish the burner and wait about 30 seconds until it cools down;
14. Passing over the surface of the spreader means by rotating the Petri dishes carefully. After some time, we note a certain resistance in moving the cap, indicating that the inoculum was absorbed by the medium;
15. All the inoculated petri plates should be incubated at approximately 25ºC for 24 to 48 hours.

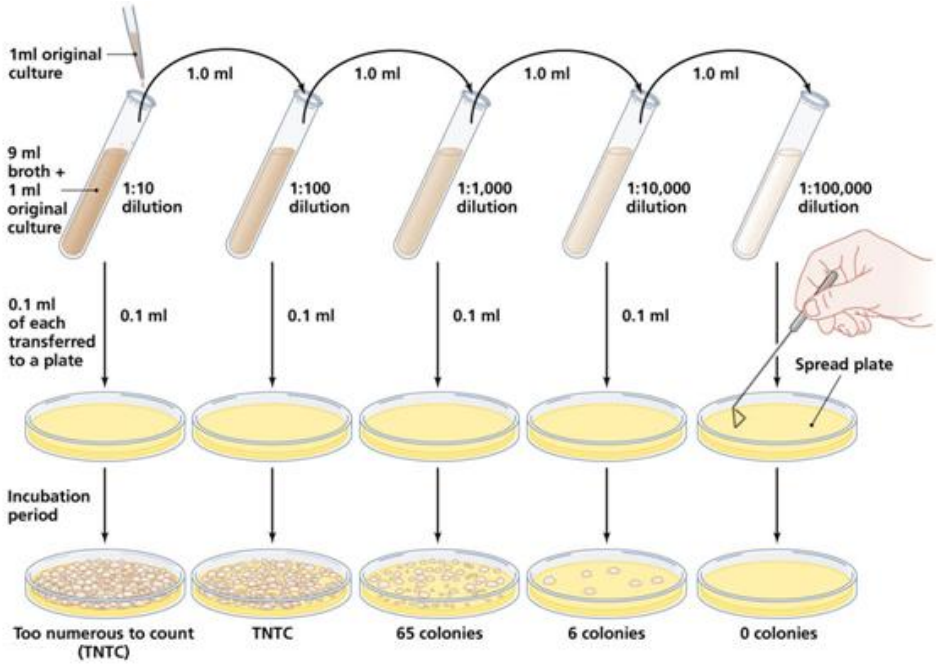

FIGURE 25 – Representation of the viable plate count method (source: http://academic.pgcc.edu/~kroberts/Lecture/Chapter%206/counting.html).

Membrane filter technique (figure 26), water is drawn through a thin filter with pores of 0,22 or 0,45 µm which is used for filtering out most part of the bacteria, as the major part of them present a diameter superior than 0,45 µm, with the exception of cocci. Bacteria were retained on the filter, which was then placed on a suitable nutrient medium. Each bacterium trapped on the filter developed into a colony, therefore the bacterial colonies growing on the medium could be counted. When a selective or differential medium was used, desired colonies had a distinctive appearance.

This method presents several advantages over the plate count, such as high degree of accuracy, allowing to analyse larger sample volumes, especially when the number of organisms is very low.

The sample container was shaken to homogenize the sample. The mouth and lid of the container were sterilized by flaming. The material used for the membrane filtration method consisted of a Kitasato, comprising a funnel (cylindrical or conical, with variable capacity), a support, a glass cup connected to a water hose or a vacuum pump and a porous plate which stands on the support, whose function is to serve as a basis for the membrane filter. The filter membranes are usually formed from cellulose esters. Finally, were also needed tweezers and an alcohol lamp.

Procedure:

1. Flame the upper part of support, for approximately 3 seconds;
2. Place a sterile membrane on the porous plate using a flame sterilized tweezer, properly cooled in order to not damage the membrane;
3. Place the sterile funnel on the support, adapting it to the fixator device;
4. Shake gently the sample container and pouring the water sample in the reservoir, under sterile conditions, until it reaches level;
5. Connect the vacuum pump to start filtering the liquid;
6. Disconnect the vacuum pump when the funnel is empty;
7. Remove the funnel;
8. Collect the membrane with a tweezer, previously sterilized and cooled, by pinching the membrane at the periphery;
9. Place the membrane in a petri dish with culture medium indicated, taking care to put the gridded part upwards;
10. Place the petri dish properly identified with the date, time and place of collection, sample size, and the culture medium utilized, in the incubator with the plate upside down, at temperatures between 22 and 24 °C.

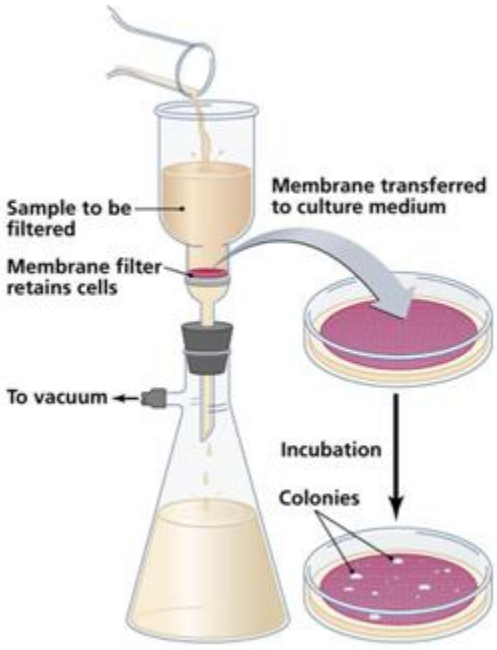

FIGURE 26 – Representation of the membrane filter system (source: http://academic.pgcc.edu/~kroberts/Lecture/Chapter%206/counting.html).

The bacteria dispersed in the solid medium originated isolated colonies which could be counted directly. Knowing the volume of the cultivated sample ables to express the final result of the counting in function of a volume of water, taking as unit x colonies per ml, or y colonies per 100 ml.

All the plates were properly identified before placing them in the incubator (25-30°C). It was essential that the number of colonies developed on the plates was not too great because some cells could not form colonies and the counting would not be correct. However, it must not be too small in order to derail the statistical significance of the count.

Generally, the mediums used for bacteriological analysis of the water were TCBS (thiosulphate agar, citrate, bile and sucrose) and MA medium (marine agar). The first culture medium is used for selective differentiation of species of the genus Vibrio. The MA medium is used for isolation and cultivation of total heterotrophic marine bacteria.

3.2.2.2. PARASITOLOGICAL ANALYSIS OF THE SOLE

To be able to observe and identify the parasites present in the sole several methods of rapid diagnostic were made, as for instance, scraping of skin and gills, fresh smears of blood and mucus, *squash* of internal organs, muscle and brain.

Latex gloves were used when handling fish for disease diagnosis. Sole skin is not keratinized and as a result is more vulnerable to iatrogenic damage. Latex or nitrile gloves are soft and slippery when wet, decreasing possible skin injure and preventing the loss of ectoparasites when handling the fish. Also, some zoonotic pathogens can be contracted by handling infected fish.

After the visual examination, the skin and gills were biopsied to search for pathogens. Skin biopsies generally can be taken from any fish larger than 25 mm, and gill biopsies generally can be taken from any fish larger than 50 mm (Noga, 2010). These techniques are helpful because many of the diseases that affect fish are restricted to the skin or gills.

Immediately before performing any biopsies, a drop of seawater was added to a slide for every biopsy that was going to be performed on the sole. Water from the tank where the fish come from can be used. The biopsy should be positioned without delay in the water drop to prevent any organisms in the sample from drying out and consequently dying.

Skin biopsy is the most helpful method available for diagnosing diseases in fish since the skin is a primary target organ for a large number of common infectious agents. Two main methods were used to achieve skin biopsies: skin scraping and fin clipping. Skin scraping was performed by smoothly scraping along the side of the body or fins with the use of a spatula or a scalpel. Scraping should be done in a cranial to caudal direction. A suitable scraping should always have mucus and numerous epithelial cells, a few scales can also be present, as well as a few blood cells (Noga, 2010).

Scrapings should be taken where evident lesions are present. The smaller wounds should be analyzed carefully since older lesions are frequently overgrown by opportunistic bacteria or water molds.

The principal edge of a lesion should always be examined because is the area that is most likely to contain the responsible pathogen(s). In order to determine the first etiological agent, it may require sampling sites other than evident lesions, to determine which pathogens are present and also examining other fish from the same group. In the cases of fish without visible lesions, scrapings behind the pectoral or pelvic fins might detect parasites since these areas are frequently chosen by parasites. When pathogens were not detected by wet mount, bacterial culture of lesions was recommended.

The wet mount of the skin scrape (and all other skin and gill biopsies) should be examined as soon as possible, since many parasites, particularly the protozoa, will die soon after being removed from their hosts. Most parasites are difficult to identify when dead.

Gill biopsy is also a helpful diagnostic tool in fish medicine. Gill biopsy was done by inserting the tip of a pair of fine scissors into the gill chamber. The scissors were then gently opened, lifting the operculum until the gill arches could be seen. The tips of several primary lamellae were then cut and transferred to a slide, then a coverslip was applied. Just the tips of the lamellae should be cut and bleeding should

be minimal. Gill hyperplasia and hypertrophy can result from the feeding activity of protozoa such as *Trichodina, Chilodonella,* or *Ichthyobodo*. Some parasites, such as *Ichthyophthirius* and *Amyloodinium*, provoke focal hyperplasia at their attachment sites (Noga, 2010).

Gills are extremely vascularized, as a result lamellar biopsy can also be used to examine the blood in fish that are too small to be bled by conventional means (puncture of caudal vein or heart), allowing the detection of hemoparasites or other pathogens.

The surface of the body and all organs (gills, intestine, gallbladder, liver, spleen and kidney) were examined macroscopically and microscopically for visible parasite infection. Fresh smears of gills and skin were prepared and examined for the presence of Protozoans.

After euthanasia, the fish was placed in lateral recumbency, and a longitudinal incision was made along the ventral midline from the anal opening to just ventral to the gill chamber. This incision should extend from the posterior peritoneal cavity into the pericardial sac. Then latitudinal incisions at both ends of this previous incision were realized to be able to extend to the dorsal aspect of the body cavity and to expose the viscera by reflecting the body wall dorsally, exposing the viscera.In the cases were peritoneal fluid was present (for instance when fish presented ascitis), smears were realized.

The intestines, gallbladder, liver, spleen, swim bladder, gonads, and heart were identified and examined. The braincase was accessed by using a pair of sharp scissors to reflect the dorsal cranium anteriorly. After visual inspection, fine scissors and forceps were used to remove the brain in total. Direct smears of various tissues were stained for bacteria.

It is often useful to make tissue squashes, especially of kidney, spleen, liver, or any lesions. Small fish can be squashed whole or the entire viscera can be removed and squashed. To make a tissue squash, a small (approximately 8 mm) piece of tissue was excised and placed on a slide with a drop of saline water. Then the edge of a plastic coverslip was placed near the tissue, and then gently squashed (Noga, 2010).

In the case of specific search for some parasites, such as belonging to the genus *Philasterides*, beyond the samples taken from the skin and gills, samples from brain tissue were also taken, and then examined by the squash method.

3.2.2.3. BACTERIOLOGICAL ANALYSIS OF SOLE

Several diagnostic methods may be performed to search and identify bacterial pathogens, including bacterial culture using different culture media, Gram staining, antibiograms and tests such as oxidase, catalase, indole, mobility, indole, methyl red, Voges-Proskauer, amylase and Esculin hydrolysis. In Annex III is described a table for bacterial identification.

To proceed to the bacterial culture it was necessary to collect samples of various anatomical regions. Generally, the sampling started from the exterior to the interior, along with the dissection of the fish. The sampling was performed with a loop sterilized by heat and the whole process of sample collection and inoculation was performed near a flame, to ensure maximum asepsis. The loop must be sterilized before and after each contact with the sample or medium. At least five fish from each tank were analyzed, in order to increase the sensitivity of the test.

The first collection site was the dermis, samples were usually collected from areas showing lesions. Then a sample was collected from the liver. Subsequently a sample was collected from the spleen, and finally, a sample of the kidney. In smaller fish, besides of the dermis sampling, the kidney was the only organ sampled due to the little size of the organs and because the cranial portion of kidney is one of the target locations for bacterial isolation. The easiest way to reach the kidney in small fish was by an incision, with a sterile blade, caudal to the operculum and bending the head of the fish, as seen on the figure 27.

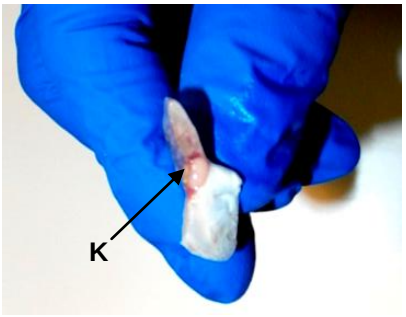

FIGURE 27 – Kidney access for bacterial sampling, on smaller fish. **Legend: K** – kidney (photo from Safiestela, 2013).

These samples were inoculated by streaking, using plates with different culture media, which were identified with the date of inoculation of the sample, the number of the tank (from which the fish were collected), the organ collected, and the culture medium used. Then the plates were placed in the incubator at temperatures around 25°C for 24 to 72 hours. The culture media used were the medium TCBS, the FMM medium and the TSA medium (trypticase soy agar) containing 1% NaCl. To be able to observe bacterial growth at different temperatures and salinity levels some TSA plates were maintained also at 4ºC and 37ºC, and with different salinities of 3, 5, 8 and 10%.

The TSA medium is a recommended medium to isolate aerobic or facultative anaerobic microorganisms. The FMM medium (*Flexibacter maritimus* medium) is a specific medium to isolate *Tenacibaculum maritimum*, as this bacterium requires the seawater salts (Buller, 2004).

When required **antibiograms** were performed, in order to test the sensitivity to various antibiotics, such as oxytetracycline (EO-30), novobiocin (NV-30), florfenicol (FFC-30), trimethoprim-sulfamethoxazole (SXT) and a vibriostatic agent (0/129). Generally, it was collected a portion of the colony in TSA with a swab, which was then spread into the MHA (Mueller-Hinton Agar) medium. Later it was performed the measurement of inhibition zones of antibiotics (figure 28). In Annex IV are described the diameters of the inhibition zones.

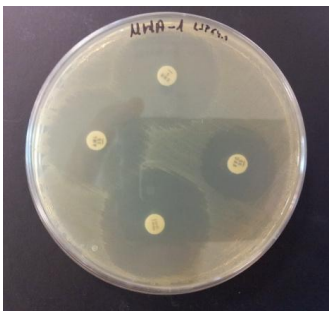

FIGURE 28 – Antibiogram (Source: photo courtesy of Instituto de Acuicultura - Universidade de Santiago de Compostela).

The **oxidase test** is based on the intracellular production of the cytochrome C oxidase enzyme by the bacterium. This test distinguishes enterobacteriaceae (oxidase negative) from other common fish pathogens such us *Vibrio*, *Aeromonas*

and *Pseudomonas* species. The original colour of the compound used in the oxidase test is transparent or slightly bluish. If the oxidase test is positive, a strong blue colour is produced immediately at the site of inoculation of the bacteria. If it is negative there is no change in the colour of the paper at the site of inoculation of bacteria (figure 29).

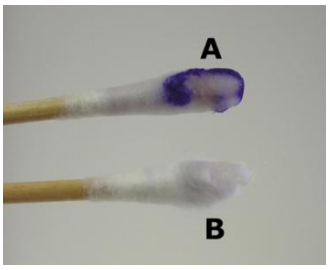

FIGURE 29 – Interpretation of the oxidase test. **Legend: A –** Positive; **B –** Negative (Source: http://www.rci.rutgers.edu/microlab/CLASSINFO/IMAGESCI/enzymes.htm).

The **catalase test** is useful to differentiate the aerobic and facultative anaerobic bacteria from aerotolerant bacteria such us Streptococcus or Lactobacillus species which are catalase negative. Catalase is an enzyme which decomposes hydrogen peroxide (H_2O_2) into water and oxygen. If the test of catalase presents a positive result shows the immediate presence of bubbles because the production of effervescence indicates the conversion of H_2O_2 to H_2O and O_2 in gas form. If the result is negative there's absence of bubbles or effervescence (figure 30).

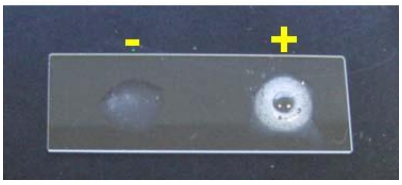

FIGURE 30 – Interpretation of the catalase test. **Legend: A –** Positive; **B –** Negative (Source: http://ftp.ccccd.edu/dcain/ccccd%20micro/catalase_test.htm).

The **indole test** determines the ability of one microorganism to degrade tryptophan (present in almost all proteins) with formation of indole. Tryptophan is an essential amino acid which can be oxidized by the enzyme activities of certain bacteria. The conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. As the ability to hydrolyze tryptophan with the production of indole (it is not used and accumulates in the medium), is not a characteristic of all

organisms, it serves as a biochemical marker. There are microorganisms that do not metabolize, or metabolize completely, tryptophan, without producing indole.

The cultures that produce a reddish ring on the surface of the medium after addition of the reagent are indole positive. The persistence of the yellow colour of the reagent demonstrates that the tryptophan substrate was not hydrolyzed into indole and indicates a negative reaction (figure 31).

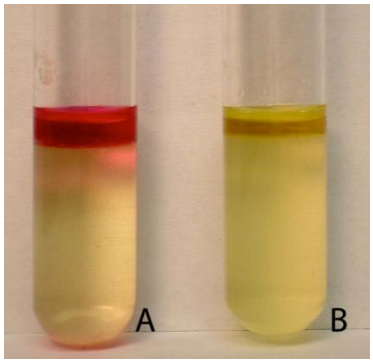

FIGURE 31 – Interpretation of the indole test. **Legend: A –** Positive; **B –** Negative (Source: http://germsandworms.wordpress.com/2013/03/08/imvic-indole/).

Microscopically to assess the **motility** of live bacteria in aqueous solution we used the technique between slide and coverslip. Despite being simple and easy to perform, is associated with the facility of forming convection currents and with rapid desiccation of the sample. For its achievement a portion of a colony was removed with the aid of a loop, properly sterilized, and placed in a drop of saline solution on a slide with subsequent placement of a coverslip over the suspension obtained, avoiding the formation of air bubbles so as to be observed under a microscope. It should be noted that sometimes static bacteria when in an aqueous medium appear to move awkwardly and erratic. These movements are due to Brownian motions arising from the random movement of water molecules against bacteria and should therefore not be confused with the proper motions of the bacteria.

The technique of **Gram staining** is a differential staining technique, as it differentiates the retention capacity of some dyes in various bacteria. In some bacteria Gram stain resist decolouration for a longer period of time than other.

In Gram staining the primary dye, gentian violet, is applied to a bacterial smear. Then the mordant, Lugol's solution, a substance that enhances the reaction between the cell and the dye is applied. In third place a differentiator is used (alcohol-

acetone solution) to decolorize certain cells. Gram negative bacteria decolours faster than Gram positive. The action time of the differentiator is essential for obtaining good colouring. After washing with water the bacteria which have decolorized in the previous step are stained with the dye of contrasting colour pink, diluted Ziehl fuchsin or safranin. In the end, the cells stained purple are designated positive and the ones stained pink, Gram negative.

The Gram technique is a fast and easy way of evaluating the structure of the bacterial cell wall. The cell walls of Gram positive bacteria are structurally different from Gram negative bacteria. Gram-positive cells have a thicker layer of peptidoglycan, whereas Gram negative bacteria have a thinner layer and present an outer membrane. The chemical nature of the bacterial cell wall is the predominant factor in the outcome of the Gram stain. Old cells or the ones which have been subjected to physicochemical factors, which in turn compromise the integrity of the cell wall, affect the result of staining.

KOH (potassium hydroxide) test is a secondary test for identification of Gram+ or Gram- cultures. 3% KOH dissolves cell walls of Gram- (they present a thin layer of peptidoglycan), but does not affect Gram + cell walls. The dissolution of G- cell wall lyses the cell and spills its contents, including the DNA. DNA is very viscous, and with a large enough cell mass, the DNA strands can be seen sticking to/dragging from a loop when touched. G+ cells are not lysed, so there is no free DNA, and consequently no viscosity will be observed. This test requires a large amount of cells. To perform this test a drop of 3% KOH was placed on a slide, and then with the help of a toothpick, a generous amount of bacteria was transfered to the drop of KOH. After stirring and raise and lower the toothpick (metal loops can give false results) off slide bacteria were viscous and formed a mucoid string in 15 sec if they were Gram- (figure 32), otherwise if the bacteria did not become viscous or form mucoid strings in 15 sec, they were Gram+.

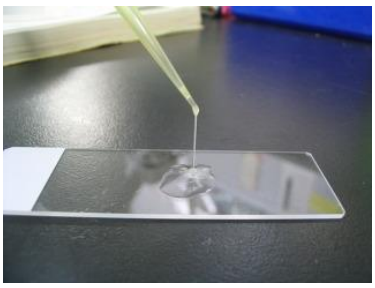

FIGURE 32 – Identification of a Gram- bacteria with the KOH test (Source: http://cfile21.uf.tistory.com/image).

The **Methyl Red** (MR) test was used to identify mixed acid fermenting bacteria that yield a stable acid end product. The **Voges-Proskauer** (VP) test was used to identify bacteria capable of 2,3 butanediol fermentation following mixed-acid fermentation. MRVP media contains glucose, peptone and phosphate buffer. Many organisms can overcome the buffering capacity of the media by producing large quantities of a stable acid end product, therefore lowering the pH. Acid produciton is detected using the pH indicator methyl red (red pH<4,4 or yellow pH>6). In order to insure that the acid was stable, the indicators were added a minimum of 48 hours after inoculation of the broth test media. Some organisms do not produce stable acid end products and, instead, further metabolize acids to more neutral end products as 2,3 butanediol. The reagents used, however, don't test for 2,3 butanediol but, rather, its precursor acetoin.

To perform the **Methyl red** test for acid production from dextrose, 1ml of the broth culture was removed and placed into a glass test-tube. Then 5 drops of methyl red indicator were added and the result was observed instantly, red colour (positive test) or yellow colour (negative test) (figure 33). If negative, the rest of the sterile broth culture could be incubated for 24-48 hours and the test repeated.

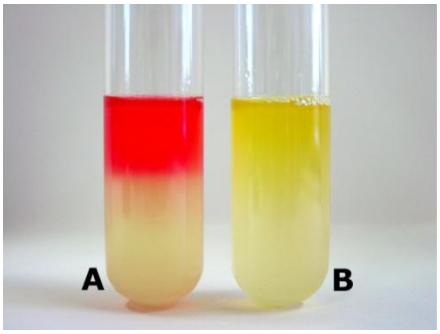

FIGURE 33 – Interpretation of the methyl red test. **Legend: A –** Positive; **B –** Negative (Source: http://rci.rutgers.edu/~microlab/CLASSINFO/IMAGESCI/methylred.htm).

To perform the **Voges-Proskauer** test for the production of acetylmethylcarbinol from dextrose, 1ml of the broth culture was removed and placed into a glass test-tube. Then 15 drops of 5% alpha-naphthol were added in absolute ethyl alcohol and 10 drops of 40% KOH. The contents could not be mixed after the addition of test reagents. A positive test was indicated by the development of a red colour at the surface layer in the tube after approximately 15 to 30 minutes.

Amylase is an exoenzyme that hydrolyzes starch into mono and dissacharide subunits. After incubation, the plate was flooded with iodine. Iodine binds to starch but not to its breakdown products, so a clear zone appeared around the streak of organism that produced amylase (figure 34).

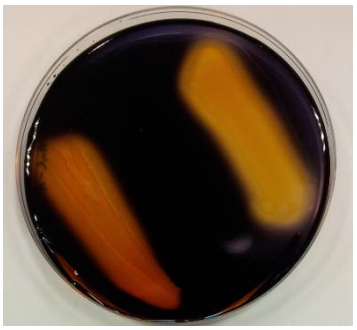

FIGURE 34 – Amylase production (Source: photo courtesy of Instituto de Acuicultura - Universidade de Santiago de Compostela).

Esculin hydrolysis, esculin is a glucoside composed by glucose and esculin (6,7-dihydroxycoumarin). The hydrolysis was observed by the formation of black zones around the bacterial growth due to reduction of iron salts with esculin (figure 35). This test was used to isolate the bacteria with the ability to hydrolyze esculin.

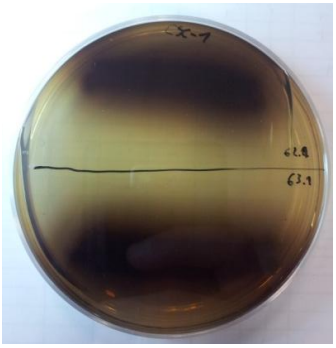

FIGURE 35 – Esculin hydrolysis (Source: photo courtesy of Instituto de Acuicultura - Universidade de Santiago de Compostela).

3.3. RESULTS

According to the defined stage protocol, some of the results obtained during the internship may not be divulged. Therefore, some of the results presented here were reported and described by other authors, listed in the bibliography.

3.3.1. BACTERIOLOGICAL RESULTS

Throughout the days passed in the Instituto de Acuicultura at the Universidade de Santiago de Compostela several fish and water samples were analyzed. The most part of the results didn't show any significant bacterial presence, however some fish samples revealed the presence of *Vibrio anguillarum, V. Harveyi, V. parahaemolyticus* and *Tenacibaculum maritimum*.

Vibrio anguillarum was identified by different diagnostic methods that gave the following characteristics:

- Gram-negative;
- Curved rod (figure 36);
- Facultative anaerobe;
- Grows rapidly at 25–30°C in rich media, such as brain-heart, trypticase soy broth (TSA) or agar containing 1.5% sodium chloride (NaCl), colonies with 2mm can be observed in 24 hours;
- Forms yellow colonies in thiosulfate-citrate-bile salts-sucrose agar (TCBS), which has a green colour, due to saccharose fermentation (figure 37);
- On solid medium, it produces circular, cream-coloured colonies;
- Oxidase positive;
- Sensitive to the vibriostatic agent O/129.

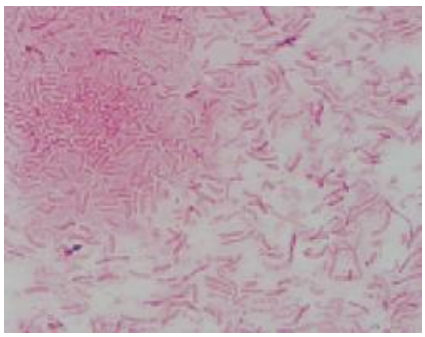

FIGURE 36 – *Vibrio anguillarum* Gram stained (Buller, 2004).

FIGURE 37 – Culture of *Vibrio anguillarum* in TCBS medium (Source: photo courtesy of Instituto de Acuicultura - Universidade de Santiago de Compostela).

The principal differences between *Vibrio harveyi* and *V. parahaemolyticus* strains can be seen in the figures 38 and 39, as the production of yellow colonies on TCBS medium only by *V. harveyi* due to fermentation of the sucrose, and in table VI.

Table VI – Biochemical and morphological characterization of strains of *Vibrio harveyi* and *V. parahaemolyticus* (adapted from Zorrilla, 2003).

	Vibrio harveyi	***V. parahaemolyticus***
Growth on TCBS	Yellow colonies	Green colonies
Gram stain	-	-
Swarming	+	-
Cytochrome oxidase	+	+
O/129 sensitivity	+	+
Growth at (% NaCl):		
0%	-	-
6%	+	+
8%	+	+
Growth at (ºC):		
4ºC	-	-
22ºC	+	+
37ºC	+	+
ONPG test	-	-
Arginine dihydrolase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+

Citrate	-	+
Production of H_2S	-	-
Urease	+	-
Tryptophan deaminase	-	-
Indol	+	+
Voges-Proskauer test	-	-
Gelatin liquefaction	+	+
Acid from:		
Glucose	+	+
Mannose	+	+
Mannitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	+	-
Melobiose	+	-
Amydaline	+	+
Arabinose	-	+
Lactose	-	-
Hydrolysis of:		
Casein	+	+
Starch	+	+
Tween 80	+	+
Lipase (egg yolk)	+	+
Hydrolysis of aesculine	-	-

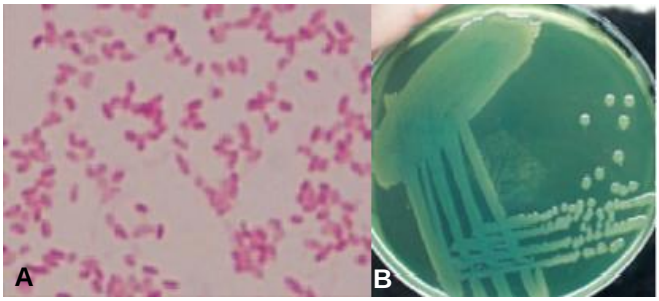

FIGURE 38 – **A.** *V. parahaemolyticus*, Gram stain**; B.** *V. parahaemolyticus* on TCBS, 24h (adapted from Buller, 2004).

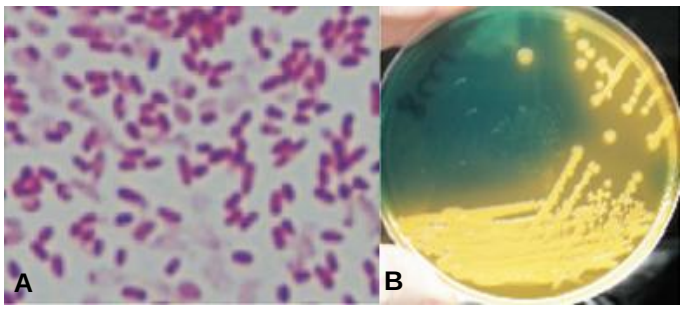

FIGURE 39 – **A.** *V. harveyi*, Gram stain**; B.** *V. harveyi* on TCBS, 48h (adapted from Buller, 2004).

Tenacibaculum maritimum was identified by different diagnostic methods that gave the following characteristics:

- Gram-negative;
- Rods, occasionally form filaments of up to 100 µm in length;
- Strictly aerobic;
- Mesophilic and growth occurs at temperatures from 14,6 to 34,3°C, with an optimum growth temperature of 30°C, and at pH levels from 6 to 9 during 2 to 5 days;
- Does not grow on media prepared only with NaCl, it has to be cultured on a non-selective oligothrophic media containing at least 30% of seawater;
- *Flexibacter maritimus* medium (FMM) proved to be one of the most appropriate mediums;
- Forms pale yellow, flat, thin colonies;
- Gliding motility on wet surfaces is a characteristic feature of all isolates;
- On solid medium, it produces circular, cream-coloured colonies;
- Catalase and oxidase are produced, but not indole;
- Methyl red test is negative;
- Casein, gelatin, tributyrin and tyrosine are degraded, but not aesculin, agar, cellulose, chitin, starch or urea;
- Resistant to the vibriostatic agent O/129.

Because isolation of the bacterium from diseased fish is not always successful, the definitive diagnosis must be supported by the isolation of colonies on appropriate specific media, followed by the determination of at least a limited number of morphological and biochemical characteristics, or by the use of molecular biology

diagnostic methods useful for identifying different strains of *Vibrio* and *Tenacibaculum*, as PCR and Dot blot technique.

3.3.2. PARASITOLOGICAL RESULTS

All the soles collected in Safiestela for necropsy were submitted to skin biopsies by skin scrapping and fin clipping for parasitological investigation. Few parasites were observed, mainly ciliates as Trichodinids.

Trichodinids frequently exhibit a characteristic scooting motion on tissue surfaces. All trichodinids are treated similarly, so is not necessary to identify the genus (which requires silver staining of fixed samples). Their disc-shaped cell has a ciliated spiral on the upper side and a sucker-like, reinforced adhesive disc (figure 40) on the lower side, which ables them to temporarily attach to the substrate (Rohde, 2005).

FIGURE 40 – Wet mount of a typical trichodinid (adapted from Noga, 2010).

The observation of few trichodinids (for instance, 1 per 100X field of view) on a skin or gill biopsy is insignificant. However, due to their tenuous attachment to the tissues, they may be easily lost during fixation (Noga, 2010).

3.3.3. LIVE FOOD RESULTS

Elimination of parasitic ciliates in the cultivation of Rotifers through administration of **Formalin.** For this study we used a 1L volumetric flask with the culture of rotifers to which formalin was administered in the following quantities:

- **50ppm** (0,05ml - 500µl) - 20 minutes after the administration of formalin both rotifers and ciliates were death;
- **10ppm** (0,01ml - 100µl) - 20 minutes after the administration of formalin rotifers had reduced motility and ciliates were death;
- **5ppm** (0,005ml - 50µl) - 20 minutes after the administration of formalin rotifers had reduced motility and the ciliates which were still alive presented very reduced motility (just some cilia beating were observed);
- **1ppm** (0,001ml - 10µl) - 15 hours after the administration of formalin both rotifers and ciliates presented motility.

Elimination of parasitic ciliates in the cultivation of Rotifers through administration of **Hydrogen Peroxide (H_2O_2)**. For this study we used a 1L volumetric flask with the culture of rotifers to which H_2O_2 was administered in the following quantities:

- **20ppm** (0,02ml - 200µl) - 20 minutes after the administration of H_2O_2 rotifers had reduced motility and the ciliates still alive had reduced motility (only some of the cilia were beating);
- **10ppm** (0,01ml - 100µl) - 20 minutes after the administration of H_2O_2 both rotifers and ciliates presented reduced motility;

  - 40 minutes after the administration of H_2O_2 rotifers presented reduced motility and some ciliates still alive presented motility;

  - 15 hours after the administration of H_2O_2 both rotifers and ciliates were death.

3.4. DISCUSSION

At present, the bacteriological analysis of the production water in large aquaculture units is accomplished by membrane filtration method, since this method has advantages that lead to quicker analysis and higher volumes of sample

analyzed. This method of analysis is influenced by the microbial burden present in the sample, for instance the greater the microbial load present in the sample, the smaller the required volume to be filtered. As disadvantages presents a lot of steps and materials needed, making the process somewhat lengthy.

Nowadays, the proofs for parasitological analysis of sole are vast, allowing a rapid diagnosis, simple and reliable and require few materials. On this type of analysis is important to make a representative collection of samples from different tissues and different anatomical regions. In parasitological analysis results describe the presence or absence of agents and, being present, can be identified by morphological characteristics. Finally, according to Noga (2010), Rohde (2005), Woo (2006) and Woo & Buchmann (2012) parasites found more often in sole aquaculture include the dinoflagellate *Amyloodinium ocellatum,* scuticociliates as *Uronema marinum*, *Philasterides dicentrarchi*, trichodinids as *Trichodina* spp., monogenean as *Entobdella soleae.* During this stage few parasites were observed, some trichodinids adhered to the gills and some scuticociliates were present in the rotifers tanks, without being necessary to apply therapeutically measures.

For the bacteriological analysis of sole produced in aquaculture there are various diagnostic methods available which allow search and identification of various bacterial agents. The means employed include bacterial cultures in different media, staining techniques, antibiograms, rapid biochemical tests (oxidase, catalase and indole), assessment of mobility, indole, methyl red, Voges-Proskauer, amylase and Esculin hydrolysis. In general, these methods are easy to perform, without neglecting the importance of asepsis in every step on the techniques used, as well as in all material, to ensure the absence of external contamination.

According to Austin & Austin (2007), Romalde (2002), Stickney (2000), Toranzo et al. (2005), Woo & Bruno (1999), *Photobacterium damselae* subsp. *piscicida* (formerly known as *Pasteurella piscicida*), *Tenacibaculum maritimum* and *Vibrio anguillarum* are the most common pathogens found in cultured soles, due to its wide range of hosts, resistance to environmental factors and several predisposing factors. The few cases of vibriosis and tenacibaculosis identified at Safiestela were not significant and did not require the use of any antibiotic. Fish that showed any kind of sintomatology were promptly discarded and analysed.

As said by Dinis (2007) and Woo & Bruno 1999), ichthyophoniasis, due to infection with *Ichthyophonus hoferi*, is the most important disease caused by fungi affecting Senegalese Sole.

According to Cutrín (2007) the most important viral disease that affects Senegalese sole is Viral Nervous Necrosis or Viral Encephalopathy and Retinopathy (VER), a devastating neuropathological condition caused by Striped Jack (*Caranx vinctus*) nervous necrosis virus (SJNNV), a type of nodaviruses belonging to the family *Nodaviridae* and genus *Betanodavirus*.

The experiment to determine the elimination of parasitic ciliates in the cultivation of rotifers by administration of formalin and H_2O_2, showed that the resistance of the ciliates to this chemicals is very similar to the resistance demonstrated by the rotifers. The administration of formalin at 10ppm seems to be the most efficient method for the elimination of ciliates without eliminating the rotifers, however rotifers presented reduced motility 20 minutes after the administration of formalin and their motility is one of the key factors for stimulating fish larvae to prey and feed. The method used for the better elimination of ciliates was the repeated washing of the rotifers, by several purges of the tank. The aerators were located a few inches upper from the bottom of the cilindro-conical tanks, so the waste sediments could accumulate at the bottom near to the drain and consequently enhancing the efficacy of the purge.

The main factors that cause bacterial, fungal, viral or parasitological infections are the abiotic factors, including temperature fluctuations, water quality, overcrowding and handling of the fish. These are factors which may cause changes in the immune system, facilitating the occurrence of infectious diseases. Thus, there must be a strict biosecurity plan to control the quality of water and in the hygio-sanitary state of fish in fish farms by parasitological and bacteriological analysis of water and fish, and of maintaining records in order to facilitate traceability and to avoid mistakes that might cause the existence of pathologies and the commitment of public health.

Some of the biosecurity measures implemented were:

- ✓ The employees were trained on hygienic fish handling and fish disease prevention methods;
- ✓ The application of a pest management control, predominantly against rodents;

- ✓ The use of disposable gloves, however this issue is debatable. In some cases the persons that use gloves have less care than the ones who don't, because they forget to disinfect/change the gloves in different situations, while the persons who do not wear them wash and disinfect the hands more often. On another point of view the absence of gloves may increase the occurrence of zoonotic diseases;
- ✓ Site specific protective clothing was provided for visitors, as they might have been previously in another aquaculture unit;
- ✓ The vehicles used for fish transportation were roughly disinfected at the entrance and at the exit of the unit, to prevent the cross contamination between aquaculture facilities;
- ✓ Eggs were obtained from certified disease-free broodstock;
- ✓ The breeders caught from the sea were isolated from the other fish, with an independent water circulation system and they were screened for nodaviruses and several species of bacteria;
- ✓ Quarantine area had dedicated equipment to avoid fomite transfer to resident fish;
- ✓ The water inlet was located near the coast and beneath the sand, this way allows to use the sand bed as a mechanical filter;
- ✓ Fish production tanks and raceways were disinfected between each lot of fish;
- ✓ Construction materials were nonporous and easy to clean and disinfect, wood was not utilized as it is unsuitable;
- ✓ Aerosols were minimized by using tank covers or by placing barriers between tanks;
- ✓ Foot dips were placed near the entrance to animal areas (figure 41);
- ✓ Each area (breeders, hatchery, weaning, fattening) had its own specific gear and disinfection facilities were sited between the areas.

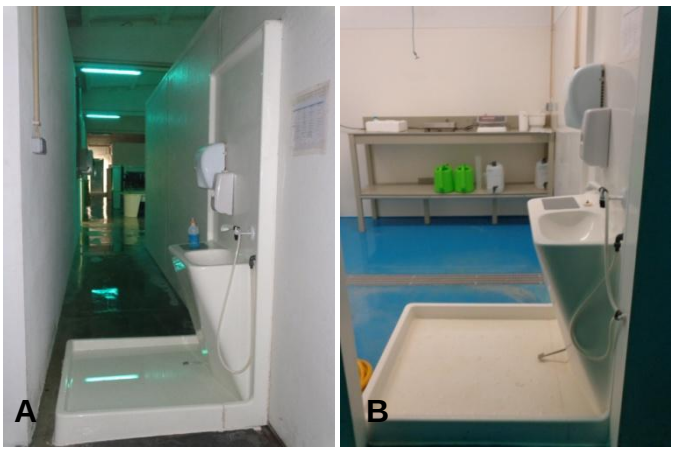

FIGURE 41 – Foot dips at the entrance of the weaning (**A**) and of the egg room (**B**) (photo from Safiestela, 2013).

Optimum fish health is essential for disease prevention, this can be achieved by minimizing stress, therefore appropriate water quality parameters and proper nutrition were maintained, by removing potential infectious disease sources (as dead or moribund fish), and monitoring and maintaining fish production and health records.

It is also important that this strict control is able to ensure a quality end product coupled with a competitive price, so that it becomes an appealing alternative compared to fish from the extractive fishing.

3.5. CONCLUSION

Due to global population growth and increasing consumption of fish per capita, the global demand for fish products more than tripled between 1961 and nowadays. Currently, fish products constitute one of the most important sources of animal protein in the world. Aquaculture and extractive fishing are two complementary activities that must respond to this rising need for fish products. The production of extractive fishing reached peak levels in the late 1980s, indicating an excessive exploitation of natural marine resources, so future increases in fish products can only come from aquaculture. Aquaculture has become an important socio-economic activity, offering new opportunities in the regions where it is implemented, due to the creation of jobs, the more effective use of natural resources and the promotion of local and international trade.

In general, aquaculture has the potential to contribute to an increase in fish stocks that are declining or endangered. This role may be important in the growth of species for recreational, commercial fisheries, and the preservation of wildlife species and fisheries and maritime traditions. Despite the advantages they offer, there are also some disadvantages, as it can become harmful by antibiotic residues or organic material released into the water, by introduction of non-endemic species in local ecosystems, in case of leakage, and the large amounts of fish required to provide food for the fish farming.

In Portugal, the marine fish farming is held traditionally in earth ponds (under extensive/semi-intensive regime), sustaining its activity in the production, almost exclusive, of seabream and seabass, similar to what occurs in the countries of southern Europe, with increased aquaculture production.

In order to increase competitiveness and profitable aquaculture activities, it is essential to diversify the offer of complementary or alternative species. The sole, is of high commercial value, from the standpoint of livestock, one of the most promising species for aquaculture. This is due to characteristics such as physical strength and resistance to adverse conditions coupled with a favourable growth rate.

The bacteriological and parasitological analysis of the water and of the fish are crucial to identify the pathogens and to be able to fight infections. Better than applying a treatment is to prevent the risk of disease, so the role of biosecurity is essential for a correct production performance and for guarantee high level of quality of the fish. Only in this way it can generate a new competitive market area, profitable and with socio-economic gain.

The main objectives of the internship were met and it was a very enriching experience, allowing me to acquire theoretical and practical knowledge in an area which Portugal should invest and improve, being a country with a very old maritime tradition and culture.

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