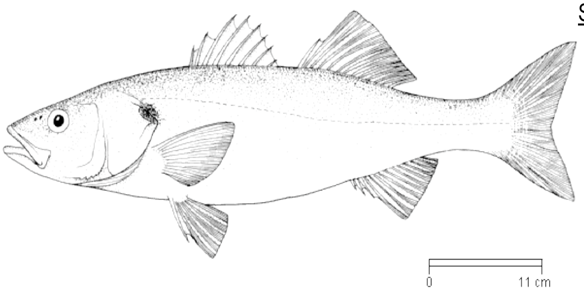
# 2. LITERATURE REVIEW

## 2.1. BIOLOGY AND LIFE HISTORY

### 2.1.1. Taxonomy

The sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), is a Perciform teleost fish, belonging to the Moronidae family and to the genus *Dicentrarchus* (Barnabé, 1990; Castilho, 1998; Bromage & Ronald 1995; Moretti *et al.*, 1999) – Figure 2.1.



Kingdom: Animalia
Phylum: Chordata
Subphylum: Vertebrata
Superclass: Osteichthyes
Class: Actinopterygii
Subclass: Neopterygii
Infraclass: Teleostei
Superorder: Acanthopterygii
Order: Perciformes
Suborder: Percoidei
Family: Moronidae
Genus: *Dicentrarchus* (Gill, 1860)
Species: *Dicentrarchus labrax* (Linnaeus, 1758)

**Figure 2.1. –** Scientific illustration of an adult specimen of *Dicentrarchus labrax* after Bagni (2005), and the accepted taxonomic hierarchy (Retrieved [4th March, 2006 at 16:27h], from the Integrated Taxonomic System (ITIS) – http://www.itis.usda.gov).

*Perca labrax*, a scientific name which is no longer recognised, was originally used by Linnaeus (1758) to classify the sea bass species. Mitchill (1814) placed *P. labrax* in the genus Morone. Some years later, another specialist (Gill, 1860), divided the previous genus into a distinct new one – *Dicentrarchus* (Retrieved [4th March, 2006 at 16:27h], from the ITIS – http://www.itis.usda.gov).

Although, in the past the European sea bass had been placed under several genuses (e.g. *Perca*, *Morone* and *Dicentrarchus*) by various authors, Dicentrarchus is at present the recognized and commonly accepted generic name of this species.

The sea bass has also been known by numerous synonymous designations, namely, *Sciaena diacantha*, *Centropomus lupus*, *Centropomus mullus*, *Perca enlongata*, *Perca* sinuosa, *Labrax lupus*, *Labrax linnei*, *Roccus labrax*, *Morone labrax* and *Dicentrarchus lupus*, proposed by various authors who collected the fish specimen from different areas. For example, Lacepéde (1802) stated that sea bass occurred in the Adriatic Sea, but named it as *Centropomus lupus* (Lloris, 2002). The vernacular names of this species are listed in Table 2.1.

**Table 2.1. –** List of the common local names, in the six worldwide most important languages (Lloris, 2002; Souto & Villanueva, 2004).

| | |
|---|---|
| English | European sea bass, Common bass, Sea perch |
| French | Bar, Loup, Loubine, Perche de mer, Barreau |
| German | Wolfsbarsch, Seebarsch, Meerbarsch |
| Italian | Spigola, Branzino |
| Portuguese | Robalo, Robalo-legítimo, Robaliza |
| Spanish | Llubina, Robaliza, Róbalo, Magallón |

### 2.1.2. Morphometric Characters

The sea bass, *Dicentrarchus labrax* has an elongated body, somewhat compressed and lean, with two clearly differentiated dorsal fins and a rather forked caudal fin (Moretti *et al.*, 1999; Souto & Villanueva, 2004; Bagni, 2005) – Figure 2.2. The opercular bone has two flat spines and a range of forward-directed spines are visible in the lower part of the preopercular bone (Moretti *et al.*, 1999).

It has a slightly protractile terminal mouth. The end of the maxillary is visible, and not gliding under the sub-orbital bone. Small teeth are present on the jaws and vomer, although no canine teeth are present. Vomerine teeth with a crescent shape are only found on the anterior part without a backward extension on the midline of the roof of the mouth (Lloris, 2002). The eye diameter is smaller than the inter-orbital space (Moretti *et al.*, 1999).

**Figure 2.2. –** Adult specimen of an European sea bass (@Croceta, F.) - Retrieved [18th March, 2006 at 00:42h], from Fish Base – http://www.fishbase.org.

This species has two separated dorsal fins: the first with 8 to 10 spiny rays, and the second fin with 12 to 13 soft rays. The anal fin has 3 spiny rays and 10 to 12 soft rays (Lloris, 2002; Souto & Villanueva, 2004; Bagni, 2005). It has cycloid scales in the inter-orbital region. The lateral line is visible as a dark line with 62-80 cycloid scales, but not extending on to the caudal fin (Bagni, 2005).

This species cycloid scales in the inter-orbital region. The lateral line, which does not extend on to the caudal fin, is visible as a dark line with 62-80 cycloid scales (Bagni, 2005). The base of the pelvic fins has no scales (Moretti *et al.*, 1999).

The colour is dark grey on the back, passing to grey-silver on the sides, while it is white-silver on the abdomen. Specimen from the sea show, a much clearer colour than fish from lagoons and estuarine environments (Lloris, 2002). On the upper side of the opercular bone there is a dark spot (Souto & Villanueva, 2004).

The juveniles show a silvery body with little dark spots, mainly on the front or only on the head, which disappear with age (Pickett & Pawson, 1994; Moretti *et al.*, 1999; Souto & Villanueva, 2004). However, a small number of young bass do not show this marking (Barnabé, 1990).

Its life span is about 30 years, although it tends to be shorter in the Mediterranean (Souto & Villanueva, 2004). The maximum length is over 1 m with a weight of over 12 kg (Moretti *et al.*, 1999).

### 2.1.3. Major Behavioural Characteristics

At an early age European sea bass form shoals, even with individuals from other species (Souto & Villanueva, 2004). The shoals may vary from a few dozen individuals to many thousands, according to the strength of the year class and local conditions. Research suggests that bass may remain in distinct groups for several years at a time, and they may retain a shoaling habit throughout most of their lives, although adults appear to be less gregarious (Pickett & Pawson, 1994; Souto & Villanueva, 2004).

When sea bass are threatened, they either retreat rapidly or adopt a typical defensive posture, making themselves seem larger and presenting as many sharp spines as possible. There is little evidence of aggressive behavior between European sea bass of similar size, although they may be territorial when occupying summer feeding areas (Pickett & Pawson, 1994).

European sea bass are strong swimmers and their swimming power and speed increases with size. Such swimming power is obtained from their large caudal fin, the sea bass propell themselves forward with bursts of three or four flicks of the tail, while all the other fins are flattened against the body to reduce drag. They can sustain a high average swimming speed while migrating (Pickett & Pawson, 1994).

Typical flashing behavior occurs when fish in a shoal, resting near the bottom, suddenly move forward, while turning on one side and appear to rub one of the flanks on the substrate. There are two possible explanations for this behavior: it is either intended to dieg up small buried crustacean, on which they feed, or it is an attempt to get rid of ectoparasites (Pickett & Pawson, 1994).

### 2.1.4. Feeding Habits

Sea bass are voracious predators, feeding on a wide range of prey which include small pelagic fish and a large number of invertebrates (Lloris, 2002; Bagni, 2005). In spite of variations associated with differences in latitude, sea bass hunts at any time of the day and tend to feed on whatever prey species are seasonally abundant in a particular location (Arias, 1980 *in* Castilho, 1998; Moretti *et al.*, 1999).

The feeding behaviour is related to size, juveniles feed mainly on small Crustaceans (Amphipoda, Mysidacea, Isopoda) and small fish like Atherina and Gobius (about ¼ of the diet). In over 20 cm, shrimps and crabs begin to be common preys (Moretti *et al.*, 1999).

European sea bass are opportunistic predators and are known to attack prey species quite violently (Pickett & Pawson, 1994). Its hunting and capture behaviour undergoes different several phases (location of prey, orientation toward the prey, aiming attack, seizing and ingestion), while using visual (prey movement) and tactile cues (prey which is too solid is regurgitated) - Barnabé, 1990.

### 2.1.5. Sexual Maturity and Spawning

In sea bass sexes are distinct: the female showing a deeper body with a longer and pointed head and greater pre-dorsal and pre-anal lengths. Sex confirmation is however only possible during the spawning season by checking the presence of sperm (squeezing gently the males) and by observing the protrusion of the anus and genital papilla in the females (Moretti *et al.*, 1999).

Sexual maturity occurs earlier in males and earlier in the southern populations (Moretti *et al.*, 1999). In the Mediterranean, first sexual maturity generally occurs between 2 and 4 years of age, while in the

Atlantic it occurs a little later (males between 4-7 years and females between 5-8 years) – Souto & Villanueva, 2004.

Adult sea bass reproduce sexually by using external fertilization (Pickett & Pawson, 1994). Sea bass is a multiple spawner, meaning that the ovaries contain more than one group of synchronous developing oocytes (Carrillo *et al.*, 1995; Mayer *et al.*, 1990 *in* Castilho, 1998; Tylar & Sumpter, 1996).

Whatever the latitude there is only one annual breeding season (Barnabé, 1990). Spawning tends to be in winter in the Mediterranean population (December to March), and up to June in the Atlantic populations (Souto & Villanueva, 2004; Bagni, 2005), although management of captive broodstock spawning time has been altered by induction of ovulation, via photoperiod manipulation or hormonal treatments, fertilisation in spawning tanks and incubation in an open-water circulation system (Bagni, 2005).

Female gonads complete their maturation at the same time, and eggs are released all together in a short time, usually at night. For hatchery purposes, spent females have to be replaced by new breeders as soon as new batches of eggs are required (Moretti *et al.*, 1999).

### 2.1.6. Eggs and Embryos

During the spawning season, each mature female may produce between a quarter and half a million eggs per kilogram of her own body weight (Pickett & Pawson, 1994; Bromage & Ronald, 1995). Sea bass spawn small pelagic eggs with no parental care exercised over them afterwards (Barnabé, 1990). They acquire their characteristic spherical shape, with a size that varies according to latitude (1.2-1.5 mm diameter in Great Britain; 1.15-1.2 mm in the Mediterranean) – (Pillay, 1990; Moretti *et al.*, 1999). Egg diameter also varies according to the origin of the parents, captive fish producing larger eggs than fish in their natural environment (Barnabé, 1990).

Eggs present 1-2 fat drops that fuse about 12 hours after laying (Souto & Villanueva, 2004). Viable eggs are transparent, only the oil droplet(s) show a slight yellowish tinge when observed under the microscope (Barnabé, 1990).

The incubation period naturally depends on temperature and varies from 166 to 47 hours at temperatures ranging from 11-19ºC, but in experimental conditions the optimum temperature is believed to be 13ºC – Barnabé (1976), Pillay (1990).

### 2.1.7. Larvae

New hatched larvae have a total length of around 4 mm (Pillay, 1990; Bromage & Ronald, 1995). The yolk sac that characterizes the early phase is almost ½ of the whole body length. Two or more oil droplets are present (Moretti *et al.*, 1999).

The morphology of larvae is very different from that of the adult (Figure 2.3) – Barnabé, 1990. Larvae development lasts for about 40 days at 19ºC (Souto & Villanueva, 2004).

Soon after hatching the eyes are not pigmented, the mouth is still closed and the digestive tract is still incomplete. During this period the larvae survive on the reserves of their yolk sac (Moretti *et al.*, 1999). The pectoral fins develop two days after hatching. Within the following three to four days the body pigmentation increases, the mouth opens and the yolk sac size progressively reduces its volume. The swimbladder starts inflating when the larvae yolk sac is completely reabsorbed and the oil droplets only partly (García Hernández *et al.,* 2001).

**Figure 2.3. –** *D.labrax* larvae, 2 days after hatching at 21ºC (@Ueberschaer, B.) - Retrieved [17th March, 2006 at 20:32h], from Fish Base – http://www.fishbase.org

By day 16, the swimbladder primary inflation is completed, the oil droplets of the yolk sac having, by then, been completely reabsorbed. Extensive body pigmentation is observed. Between days 20 and 35 the caudal and anal fins, the stomach, and the teeth develop. The swimbladder expands progressively to its final shape. Between days 40 and 45 the dorsal and ventral fins develop. Scales appear at 70-80 days (Moretti *et al.*, 1999; Deplano *et al.*, 1991).

The larval motion is a sort of passive floating, with sudden and infrequent body movements, without assuming any clear posture. Typically, they slowly sink (head first) and after a few seconds, swim upwards, for two to three seconds. Larval fish showing a different pattern (being totally passive or hyperactive), reveal poor viability (Moretti *et al.*, 1999).

## 2.2. OVERALL DISTRIBUTION

### 2.2.1. Geographic Distribution

This coastal species is discontinuously distributed around the Atlantic (zones ASE-542ANE-533), from 30º N (Moroccan coast) to 55º N (Irish sea, North sea, Baltic) – Figure 2.4. It is also present in all of the Mediterranean coast (zone ASE-511) – Barnabé, 1990; Whitehead *et al.*, 1986 *in* Castilho, 1998.

### 2.2.2. Ecological Distribution

The European sea bass is Eurythermic (5-28°C) and Euryhaline (3 mg.L$^{-1}$ to full strength sea water). Being able to tolerate a wide range of temperature and salinity, it is often found in coastal inshore waters, and also in estuaries and brackish water lagoons (Pillay, 1990; Chervinski, 1979 *in* Castilho, 1998; Bagni, 2005). They are quite ubiquitous, as they enter costal inlets and river mouths, venturing

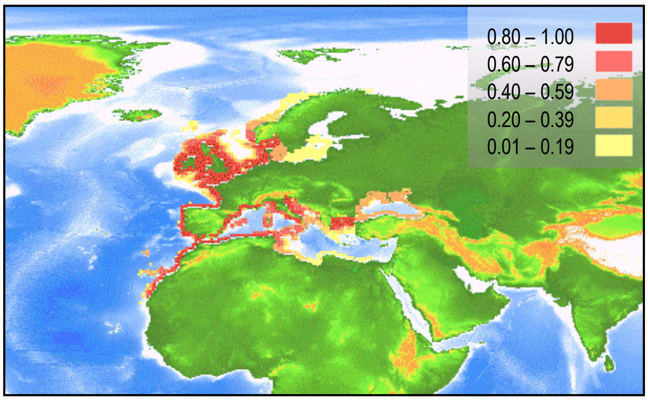


**Figure 2.4. –** European sea bass geographical distribution and respective occurrence probability – Retrieved [9th August, 2006 at 01:15h], from Fish Base – http://www.fishbase.org.

upstream for several kilometres (Barnabé, 1990). Not being particularly sensitive to low temperature, some fish may stay in coastal lagoons over winter instead of returning to the open sea (Lloris, 2002). This characteristic, according to some authors, opens up possibilities for freshwater cultivation (Castilho, 1998).

With demersal behaviour, it often inhabits littoral zones on various kinds of bottoms, near the surface to depths down to 100 m (Lloris, 2002).

Moving around a limited territorial extend, the bass behaviour patterns can be assumed as being related to their search for food and reproduction. Outside the spawning period, they can be found anywhere that food is available. Maturation and spawning seem to demand more specific environmental conditions (temperature, photoperiod, salinity, oxygen), which also determine the geographical distribution (Barnabé, 1990; Moretti *et al.*, 1999).

## 2.3. PRESENT SITUATION AND TRENDS OF EUROPEAN SEA BASS CULTURE

Aquaculture has had a crucial role in providing human food since ancient times. However, it was only in recent years that a high development rate has been reached (Souto & Villanueva, 2004).

World Aquaculture has grown tremendously, from a production of less than one million ton in the early 1950's, to 59.4 million ton by 2004, being considered as probably the fastest growing food-producing sector (FAO, 2006). It accounts presently for almost 50 percent of the world's food fish production and is regarded as having the greatest potential to meet the growing demand for aquatic food (Subasinghe, 2006).

### 2.3.1. Global Aquaculture Production

The European Sea bass, *Dicentrarchus labrax*, is one of the most commercially important fish specie, which has characterized the development of marine aquaculture in the Mediterranean basin, during the last three decades (Conides & Hunter, 2000; Moretti *et al.*, 2005). It is considered to be the first marine fish specie, in the Mediterranean region, to be successfully cultured from egg up to a commercial market scale (Barnabé, 1990; Filic *et al.*, 1987).

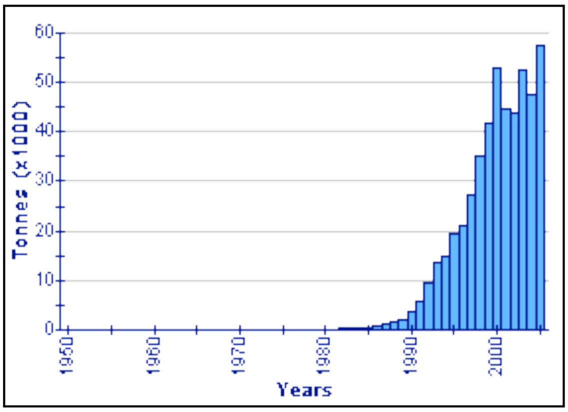


**Figure 2.5. –** Global Aquaculture production for *D.labrax* (FAO statistics *in* Bagni, 2005).

Note: this chart excludes the production of Turkey.

In 1983, the global aquaculture production of *D.labrax* was estimated to be 300 ton, where Spain, Greece and France, being the three countries responsible for the initial impetus given to this industrial production (Figure 2.5).

By 1990, production had increased to 3.819 ton, with Greece, Spain and Italy, dominating the industry, and being responsible for nearly 80% of all production (Retrieved [2nd August, 2006 at 19:43h], from FEAP – http://www.feap.info).

Five years later, production had increased five-fold to over 19.475 ton. This figure continued to rise, and by year 2000, the combined country production exceeded 52.800 ton. However in the following years up to 2005, when global production of farmed European sea bass approached an output of 57.550 tons, there has been a slow down in expansion of the growth rate (FAO statistics *in* Bagni, 2005) – Figure 2.5.

Since then production has steadily increased. Although this year's figures have not yet been made available by all the countries involved, partial figures already released point to a further increase in production volumes, with probably even higher volumes, maybe topping the 80.000 ton mark (Catarci, 2007).

While, in the past, limited production took place in extensive pond systems, nowadays most of the production comes from cages farms (Prickett, 1998). The current production leader is Greece, with nearly 50% of all production, the other important producing countries being Turkey (20%), Italy (12%) and Spain (10%) – Retrieved [2nd August, 2006 at 19:43h], from FEAP – http://www.feap.info.

### 2.3.2. Historical Background and Main Issues

One of the biggest success stories in European aquaculture has been the Mediterranean sea bass industry, which in less than 15 years has grown from an annual production of a few thousand ton, in to more than 60.000 ton today (Eurofish retrieved [31st August, 2006 at 22:28h], from http://www.eurofish.dk).

The historical background from such rearing activity shows that, its industrial evolution can be promptly divided into four phases (Stephanis, 2000 *in* Theodorou, 2002):

*RandD (1965 to 1979)*

After several trials, in the early 1970's, wild-caught juveniles could finally be on-grown up to market size, at reasonable densities, in a variety of systems (Prickett, 1998). Culture conditions were first established in Italy and France, quickly extending to Spain and the U.K., and by 1980, was almost Mediterranean wide (Theodorou, 2002).

*Predevelopment (1980 to 1990)*

Research began to focus primarily on problems related to large scale hatchery production of juveniles, considered the major "block" to the development of the industry. Towards the end of the decade, successful spawning of broodstocks "out of season", using hormone injection techniques, was achieved. The first larval protocols for sea bass were established (Prickett, 1998).

Larval quality was extremely variable and there was a high incidence of spinal deformities, non functional swimbladder and malfunction of yolk sac absorption, which hindered production (Chatain, 1991 *in* Castilho, 1998).

*<u>Development</u> (1990 to 1994)*

Phase in which some important discoveries allowed the removal of the last bottleneck in the production of large numbers of healthy juveniles. Once fry production problems were solved, particularly the problem of swimbladder inflation[1], the industry rapidly expanded (Theodorou, 2002).

With an under-saturated market prepared to pay high prices, the industry boomed during the early 1990's, a period that could be described as the "producer-led" phase of the industry's history, where demand considerably exceeded supply (Barnabé, 1990; Prickett, 1998).

*<u>D. Maturation</u> (1995 to present days)*

As the output of sea bass kept growing, costs were driven down and market prices declined by more than two-thirds, between 1990 and 2002 (from US$ 16/kg to about US$ 4-5/kg) – Bagni, 2005. The rapid saturation of the market and the parallel rapid price decline (60-70 percent in ten years) are attributed to the much smaller traditional market for these species, the lack of diversified products, and limited market development and promotion (Chatain *et al.*, 1996; Lloris, 2002).

The substantial drop in prices of these species was in turn responsible for the general fall in total production between 2000 and 2002 (Figure 2.5) – Bagni, 2005. However, the decline in prices (due to supply exceeding demand) is opening new markets and expanding existing ones (Moretti *et al.*, 2005).

In recent years, several analysts have voiced their concern about the continued rapid expansion of the industry and its lack of focus on the market needs. Still, the overall development for the sea bass industry has so far been positive (Franz, 2006).

The year 2005 was, in many ways, a very stable year for the industry. Increased consolidation on the production side brought down average costs, allowing at least the large and medium-sized producers, in particular, to operate profitably at current prices (Lem, 2006).

Presently, the main European markets are quite strong. With a good match between supply and demand, prices in the past have been in most cases, quite adequate for producers (Franz, 2006; Lem, 2006). Even so, there are still two main constraints to the cost effective production of "table fish" (popular portion sizes: 350-450 g), namely, the generalised slow growth of this species and the high proportion of males in many fish farm populations (males grow slower than females) – Carrilho *et al.*, 1995 *in* Castilho, 1998.

Given the "considerable level of maturity" that this dynamic sector has reached, it is now very improbable that we shall see again such extreme boom and burst cycles, as was the case in the past (Lem, 2006; Moretti *et al.*, 2005). Future prospects will depend on further improvements in productivity (broodstock genetic selection, disease control and improved nutrition), product diversification and new

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[1] Analysis of this swimbladder inflation problem showed the main reason to be the presence of a surface film on the rearing tanks, which prevented the larvae from gulping air. Ultimately the problem was solved by using the Japanese system of blowing air across the water surface using a skimmer (Prickett, 1998).

aquaculture strategies, such as concentrating the production around large companies, or groups of companies (with associated economies of scale lowering production costs) – Lloris, 2002.

### 2.3.3. Expansion through Research, Exploitation and Governmental Support

Aquaculture has been practiced for centuries, but the industry has only grown dramatically in the last half century. It has now matured into a major industry in many parts of the world, with numerous training centres and rapid technology transfers to every corner of the globe (Davenport *et al.*, 2003).

Both private and international entrepreneurship have played an important part in this development, helped by a strong incentive policy from the European Union (e.g. European funds have been provided for research programs) – Chatain *et al.*, 1996

The initial stimulus given the industry was the formation of MEDRAP (Mediterranean Regional Aquaculture Programme), which coordinated information and ran seminars on the emerging technologies, which helped to attract the necessary attention of the private sector. Fuelled by European Union grants (between 40 to 60%), for capital investment in aquaculture projects, several enterprises as well as individuals rapidly invested in the industry (Prickett, 1998).

In countries from the Middle East and the North of Africa, production has been sporadic and weak. This is attributed to weak sector support from Government (cf. the structural funds of the Financial Instrument for Fisheries Guidance of the European Union – provision of financial assistance for capital investment) and lack of material/service support (i.e. feed companies, technical and managerial expertise).

The purpose of research, on the other hand, is to better understand the mechanisms of disease processes, in order to provide more effective means of disease management through accurate diagnosis, prevention, control and treatment. In addition, research is often required to establish the cause of emergent diseases or to confirm the diagnosis of known diseases (NACA/FAO, 2001).

## 2.4. Disease as a Primary Constraint – Alternative Prophylactic and Prevention Approaches

Aquaculture development has been hindered by recurrent disease outbreaks, as several pathogenic microorganisms are normal inhabitants of the aquatic environment (Austin & Austin, 1999; Davenport *et al.*, 2003; Pillay, 2004). Disease is increasingly recognized as a significant primary constraint to aquaculture production, and is responsible for severely affecting both economic and socio-economical development of the sea bass industry worldwide (Chatain *et al.*, 1996; Verschuere *et al.*, 2000a).

### 2.4.1. The Potential Drawbacks Using Antibiotics

Modern aquaculture has experienced severe disease problems, owing to the lack of control of the microbriota in the rearing systems. Prophylaxis is an inherent part of any intensive animal production, however in the aquatic environment, the intimate relationship between bacteria and their host and the frequent use of open production systems adds to the problem (Olafsen, 2001).

Conventional prevention and control approaches, often rely on the use of vaccines and antimicrobial disinfectant compounds (Austin & Austin, 1993 *in* Austin *et al.*, 1995, Gómez *et al.*, 2007). Fortunately, vaccines and other health control means have so far kept most diseases under relative control, although various organisms may not respond so well to vaccines, and new diseases or variants of known ones, are a constant challenge to the industry (Olafsen, 2001).

Furthermore, there is a growing concern with the widespread and indiscriminate use of antibiotics (Chatain *et al.*, 1996). Earlier reports referred three primary drawbacks: (i) the high cost involved, (ii) the fact that few antibiotics are registered for legal use, and far more problematic, (iii) the proof that antibiotic solutions may rapidly promote the selection and dissemination of antibiotic-resistant bacteria in livestock and throughout aquaculture facilities, either following alterations of the existing genome or by transfer of genetic material between cells through plasmidic or bacteriophages (Towner, 1995 *in* Gomez-Gil *et al.*, 2000; Lagat, 2001; Cabello, 2006; Poletto, 2006). For this reason, there is an urgent need to control the microbiota in aquaculture facilities by using alternative approaches (Marques *et al.*, 2005).

### 2.4.2. Alternative Methods

Owing to the problem of antibiotic resistance and subsequent reluctance to their use (Vine *et al.*, 2006), several promising alternative approaches have already been proposed (e.g. vaccines, prebiotics, probiotics, immunostimulants, quorum sensing analysis, antimicrobial peptides, etc.), either to enhance the host organism resistance or to maintain a healthy microbial environment in aquaculture systems (Dhert *et al.*, 1998; Skjemo & Vadstein, 1999; Olafsen, 2004; Marques *et al.*, 2006a).

One method that is gaining wide acceptance within the aquaculture industry, is the introduction of desirable beneficial microorganism strains (Chatain *et al.*, 1996; Gomez-Gil *et al.*, 2000; Vine *et al.*, 2006). There is increasing evidence that microflora manipulation, or addition of probionts, may improve health conditions, enhance larval survival and prevent the proliferation and colonisation of opportunistic and/or pathogenic bacteria in aquaculture rearing systems (Table 2.5).

#### 2.4.2.1. Probiotics

The word probiotics, derived from the Greek “pros bios” meaning “for life” (Vine *et al.*, 2006), is currently used to describe a microbial formulation responsible for biocontrol or bioremediation (Farzanfar, 2006).

The definition of probiotics has, evolved over time (Holzapfel *et al.*, 1998). Lily & Stillwell (1965) had originally proposed to use the term, to describe the compounds produced by one protozoan, which had stimulated the growth of another (Fuller 1989; Farzanfar, 2006; Vine *et al.*, 2006). The scope of such definition, was further expanded by Sperti in the early 1970s, to include tissue extracts that stimulated microbial growth (Xu, 1998).

Later on, in an attempt to improve such definition, Fuller (1989) redefined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". In 1974, this same definition was once again modified, to describe "animal feed supplements which had a beneficial effect on the host animal, by affecting its gut flora" (Parker, 1974 *in* Vungot, 1997).

It was not until 1999, that Gatesoupe provided a definition more appropriate to aquaculture purposes: "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to kept alive, with the aim of improving health". Gram *et al.* (1999 *in* Gomez-Gil *et al.*, 2000) broadened the definition still further, by removing the restriction to the improvement of the intestine: "a live microbial supplement which beneficially affects the host animal by improving its microbial balance".

Based on this last definition, probiotics may include microbial adjuncts that (i) suppress pathogens from proliferating in the intestinal tract, on the superficial structures and in the culture environment, through production of inhibitory compounds, and by promoting competition for adhesion sites and for nutrients; (ii) secure optimal use of the feed, by aiding in its digestion with active enzymes, or by enhancing its nutritional value; (iii) improve water quality from its environment surroundings, both as antibacterial agent and as a nutritional factor; (iv) be a source of macro and micronutrients; or (v) stimulate the immune system of the host by increasing production of systematic antibody levels and macrophage activity (Vungot, 1997; Xu, 1998; Verschuere *et al.*, 2000a; Lagat, 2001; Marques, 2005; Farzanfar, 2006; Tinh, 2007).

It is generally accepted, that bacteria delivering essential nutrients to the host (single-cell protein), being neither active in the host, nor interacting with other bacteria, within the environment of the host, or with the host itself are not included in the definition (Verschuere *et al.*, 2000a). Yet, current studies have revealed that inactivated cells when applied as feed additives, have been beneficial in controlling microbial infections (e.g. Irianto *et al.*, 2003).

This raises an interesting paradox question, concerning whether or not inactivated cells should be regarded as probiotics, immunostimulants or as oral vaccines, notwithstanding the fact that there is clear benefit in the use of dead cells. Moreover, cell inactivation may eliminate the risk of using as live probiotics, several representatives from pathogenic taxa, e.g. *A. hydrophila*, which are known to cause fish diseases (Austin & Austin 1999 *in* Irianto *et al.*, 2003).

Although probiotics application in aquaculture seems highly promising (e.g. Gatesoupe, 1991); Douillet & Langdon, 1994; Erasmus *et al.*, 1997; Riquelme *et al.*, 1997; Gibson, 1998; Ringø & Vadstein, 1998; Rombaut *et al.*, 1999; Ruiz-Ponte *et al.*, 1999; Sakai, 1999; Verschuere, 2000a; Gomez-Gil *et al.*, 2000; Verney-Jeffreys *et al.*, 2003; Venkat *et al.*, 2004; Vine *et al.*, 2006; etc.), mostly through pragmatic approaches (Vine *et al.*, 2004), implementation should proceed cautiously since the overabundance of beneficial organisms in the culture system, could have unpredictable detrimental effects. Successful implementation of such alternative strategies must be based on a thorough understanding of the mechanisms involved and their putative effects (Marques, 2005). This will have to rely, on a better insight into the complex interactions between the cultured organisms and the various microbacterial communities that constitute the conventional microbiota (Olafsen, 2004).

A key strategy to study these host-microbial relations, in detail, is to first define the animal performance in the presence of well characterized microbial community (under germ-free or gnotobiotic conditions), and then evaluate the effects of adding a single or a defined population of microbes (Gordon & Pesti, 1971; Douillet & Langdon, 1994).

## 2.5. GNOTOBIOTIC AQUATIC ANIMAL AS A VALUABLE RESEARCH TOOL, TO INVESTIGATE HOST-MICROBIAL INTERACTIONS

### 2.5.1. Gnotobiotic Terminology and Criteria

The terminology of gnotobiotic experimentation now in use, derives primarily from an article of Reyniers *et al.* 1949 (*in* Gordon & Pesti, 1971).

While gnotobiotics and "germ-free" animals were being developed at the University of Notre Dame, a committee was nominated and a series of meetings were held, to devise a proper nomenclature (Trextler & Orcutt, 1999), since most of the terms then in use, were quite misleading (Mickelsen, 1962). The term "germ-free" was occasionally confused with "pathogen-free", and the only scientific term then available, "axenic", did not lend itself to broader applications (i.e., germ-free animals associated with one or more organisms) – Woostman, 1996.

Somehow, the term gnotobiotic, from the Greek "γνωσισ" (gnosis), meaning knowledge, and "βιοσ" (bios), meaning life (Gustafsson, 1959 *in* Falk *et al.*, 1998) was proposed. Thus, its meaning could be gleaned from its original root words (Trextler & Orcutt, 1999).

The committee agreed that a gnotobiotic animal (GN), or gnotobiote, is an animal or strain, in which all life forms, therein present, are fully defined by accepted current methodology (Heneghan, 1973; Woostman, 1996). Gnotobiotes containing no other apparent life forms other than their own protoplasm, should be referred as "germ-free" (GF) or "axenic" organism (Woostemann, 1996) and depending on the number of microorganisms species deliberately seeded into a GF animal, it may be classified as mono/dixenic or polyxenic (Hem, 2001).

Gnotobiotics, using the Greek suffix –"ics" meaning "pertaining to", was proposed for the name of the science itself, as used in other well known fields such as mathematics, genetics and so on. The use of the Greek suffix –"ology", meaning a branch of learning, to form gnotobiotology was considered a bit long, while gnotobiology was initially rejected because the roots of the word did not provide an obvious clue as to its meaning (Trextler & Orcutt, 1999). However, T. D. Luckey, who had participated in the meetings, used it in his monograph (1963 – Germfree and gnotobiology), and the term became widely used thereafter (Trextler & Orcutt, 1999).

### 2.5.2. Gnotobiology Value

When reviewing the gnotobiology field, the first thing that one notices is that the potential importance of GF organisms and the concept of gnotobiotic experimentation, were first recognized more than 100 years ago (Falk *et al.*, 1998). In historical terms, the need for a better defined animal model free of microbial associates, was already obvious to Louis Pasteur in 1885 (Woostman, 1996). This is unquestionably correct, in a microbial sense; however, the recognition of the necessity to work with "pure" systems in biological experimentation, from which Pasteur appears to have drawn his analogy, can be traced further back to the research of Boussingault (1838 *in* Gordon & Pesti, 1971).

Despite Pasteur's doubts about the feasibility of developing such a model, not more than 10 years had elapsed, before the first GN animal was achieved. However, it took more than 50 years[2], from the time the first GN organism was achieved, until the zoological community worldwide, offered defined proof that breeding organisms in the germ-free state, to successive generations, was possible (Woostman, 1996).

This general consent, besides settling in a define manner the general initial disbelief, made the gnotobiotic animal available as a practical tool for research, for the first time. Of the various experiments in the aquatic field which were reported at the time, the most flourishing one, was Drs. James Baker and Malcolm Ferguson's[3], both from the Rockefeller Institute for Medical Research, who in 1942, raised a group of gnotobiotic Mexican platyfish, *Platypoecttus maculates*, from birth to full maturity (Johnson, 1944).

#### 2.5.2.1. Gnotobiology Potentials and Limitations

The ability to raise aquatic bacteria-free animals provides considerable potential as an experimental tool for a better understanding of the mechanisms involved in host-microbial relationships, since (i) it portrays the host with a known microbiological status; (ii) studies can be realized to determine how communities are assembled, assess how different members (probiotics and prebiotics, immunostimulants, pathogenic or parasitic agents) impact on the microbial community function and host biology, and ascertain the extent of redundancy or modularity within a microbiota; (iii) it allows an

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[2] The years between 1900 and 1950, saw major developments in the science of nutrition, and the development of the GN animal model followed closely those endeavours (Woostman, 1996).

[3] BAKER, J.A., FERGUSON, M.S. (1942). Growth of Platyfish (*Platypoecilus maculatus*) free from bacteria and other microorganisms, *Proc. Soc. Exp. Biol. Med.*, **51:**116–119.

reasonable control of most of the variables involved, making the experimental system designs more accurate and the results highly reproducible; (iv) in combination with different methodologies it may be applied to a wide range of studies (e.g. nutritional requirements, immune reactions, metabolic functions and responses, etc.) – Gordon & Pesti (1971), Pleasants (1973 *in* Marques, 2005), Trust (1974), Marques *et al.* (2006a), Falk *et al.* (1998), Rawls *et al.* (2006), Tinh (2007).

Furthermore, gnotobiology can help provide new insights on the etiologies of infectious diseases, acute and chronic inflammatory conditions, and help evaluate relevant appropriate methodologies for disease control (Falk *et al.*, 1998).

However, the key experimental strategy in using germ-free and gnotobiotic animal models has faced several constraints and technical limitations. Firstly, (i) the difficulty in recreating, under laboratory conditions, the exact complex variable factors affecting their normal conspecific environment; (ii) the technical complexity and the high costs involved in rearing, handling and maintaining such organisms under gnotobiotic conditions; (iii) the difficulty in assuring a complete axenity of a GF culture system, and conveniently identify all colonizing life forms; last but not least (iv) the inherent characteristics of GF organism models (Gordon & Pesti, 1971; Marques *et al.,* 2006a). Results in this scientific research field, have revealed that organisms cultured in the absence of their indigenous microbiota complement, give rise to a difference in the homeostasis, which cannot be ignored by investigators (Woostman, 1996). A range of host functions are affected by the indigenous microbiota, such as educating the immune system and the gut-associated lymphoid tissue, affecting the integrity of the intestinal mucosal barrier, modulating proliferation and differentiation of epithelial cells, and processing nutrients consumed in the diet (Gordon & Pesti, 1971; Hem & Engh, 2001a; Olafsen, 2004).

Although such discrepancies, between GF organisms and their normal conspecifics (conventional organisms), may be diminished, by simply recolonising the germ-free specimen with functionally known symbiotic microflora components, acquired from conventional organisms. Some fundamental aspects, such as the composition of the common microbial community, are still poorly understood and have not yet been determined for most of the aquatic animal species (Marques, 2005).

### 2.5.3. Procedures to Obtain and Maintain Gnotobiotic Aquatic Organisms

Several different methods have been used to obtain bacteria-free (axenic) aquatic organisms (Tables 2.2 to 2.4). Initial axenization requires elimination of all foreign organisms. This can be achieved by physical and chemical means, or their combination (Provasoli, 1977).

The ideal approach depends mainly on the organism taxonomical group and its developmental stage. So far, gnotobiotic aquatic organisms were mainly achieved by aseptic hysterectomy (e.g. Trust, 1974) or by sterile surface disinfection of conventional hatched eggs, which were further reared and continuously maintained under germ-free conditions, using strong antimicrobial chemicals (e.g. Harboe *et al.*, 1994), or efficient antibiotic solutions (e.g. Peck *et al.*, 2004). Furthermore, some authors advocated (e.g. Marques *et al.*, 2006a) the use of the “dilution method”, as a complementing technique. Larvae, new hathed, embryos, eggs, cysts, etc., may undergo a series of rinsing processes with sterile medium (filtered and autoclaved), so as to dilute any remains of unwanted chemicals and to eliminate the remaining accompanying microorganisms.

The success of culturing a gnotobiotic aquatic organism, also depends on the availability of appropriate apparatus, able to reproduce and maintain the complex environment conditions, in which experimental organisms can subsist within a gnotobiotic state (Falk *et al.*, 1998).

During the last three decades, gnotobiotic experimental apparatus have gradually become more readily available, and sterilization procedures (autoclaving, radiation, dry-sterilization or chemical disinfection) have been simplified to such an extent, that they are now accessible to anyone interested in its use (Trextler & Orcutt, 1999; Hem & Engh, 2001a). Furthermore, the availability of adequate sterilisable diets from commercial sources, together with the possibility of handling these animals under aseptic conditions, simply by using a laminar flow hood, have brought gnotobiotic experimentation within the reach of most if not all laboratories (Gordon & Pesti, 1971).

### 2.5.4. Methods to Verify the Gnotobiotic Status

The microbial status of a laboratory animal determines to a large extend, its usefulness as a research tool (Hem, 2001).

Most experimental research studies involving axenic organisms, may be restrictive to the use of two distinct approaches so as to assess the presence of foreign viable organisms: (i) conventional culture-based microbiological media (e.g. marine agar, brain heart infusion broth, zobell broth), and (ii) microscopy examination of previous stained preparations (e.g. acridine orange, DTAF[4], DAPI[5]), when microbial strains are not recoverable with the first approach (Holzapfel *et al.*, 1998; Marques, 2005). On the other hand, gnotobiotics systems require a specific range of methodologies and greater precision, as it is essential to distinguish between contaminants and the characteristic known associated microbiota (Marques *et al.*, 2006a). Furthermore, it is important to differentiate between dead and live viable strains, since it is well known that dead bacteria may still significantly influence results obtained with gnotobiotic organisms (e.g. Marques *et al.*, 2005).

Specific methodologies, such as molecular biology techniques (e.g. DGGE[6] analysis of PCR fragments generated by universal primers), fluorescent microscopy using particular staining techniques (e.g. ethidium bromide, rhodamine), or even specific culture media, in case the known microbiota, may be labelled with distinct markers (Marques, 2005). Nevertheless, parallel studies should be performed,

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[4] DTAF – 5-(4,6-dichlotriazin-2-yl) aminofluorescein staining.

[5] DAPI – 4’,6-diamidino-2-phenylindole staining.

[6] DGGE – Denaturating gradient gel for electrophoresis analysis.