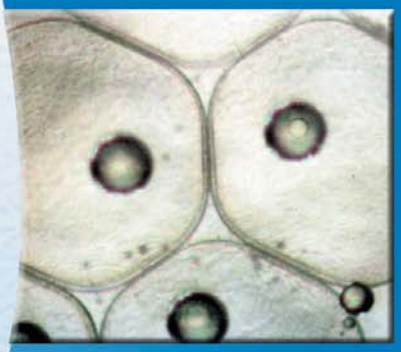
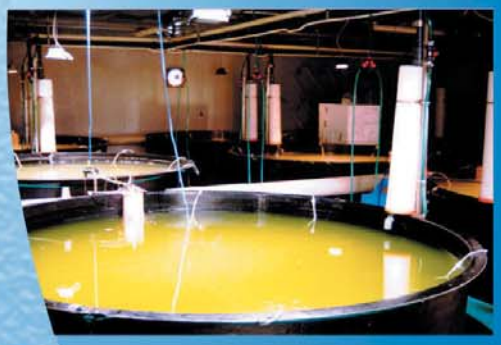
Manual on Hatchery Production of Seabass and Gilthead Seabream

Volume 1





Food and Agriculture Organization of the United Nations

Manual on Hatchery Production of Seabass and Gilthead Seabream

Volume 1

by

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PREPARATION OF THIS DOCUMENT

This is the first of two volumes of a Manual on hatchery production of seabass and gilthead seabream. It is part of the programme of publications of the Inland Water Resources and Aquaculture Service. The manual has been written based on the direct experience of technicians and managers of commercial hatcheries operating in the Mediterranean. It is intended to assist both technicians entering this field as well as investors interested in evaluating the complexity of hatchery production of seabass and gilthead seabream.

The manual has been prepared by the authors under the overall coordination of Mario Pedini, Senior Adviser (Aquaculture Development), and with the collaboration of numerous colleagues contributing comments to sections of the manual, ideas, and who assisted in its finalization. The contributions to this volume of Massimo Caggiano (Ittica Ugento Spa), Pepino Candreva (INVE Aquaculture), Béatrice Châtain (IFREMER), Licinio Corbari (Ittica Ugento Spa), Brigitte Loix (STM Aquaculture Srl) and Marlène Dehasque (INVE Technologies) are greatly appreciated. The assistance in the editorial work and final presentation and graphics given by André Coche, Rine Sola, Magda Morales, José Castilla and Emanuela D'Antoni has also been invaluable.

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ABSTRACT

Seabass and gilthead seabream are the two marine fish species, which have characterized the development of marine aquaculture in the Mediterranean basin in the last two decades. The substantial increase in production levels of these two high value species has been possible thanks to the progressive improvement in the technologies involved in the production of fry in hatcheries. As a result of this technological progress more than one hundred hatcheries have been built in the Mediterranean basin, working on these and other similar species. At present the farmed production of these two species that is derived from hatchery produced fry is far greater than the supply coming from the wild.

The development of these techniques, based originally on Japanese hatchery techniques has followed its own evolution and has resulted in what could be called a Mediterranean hatchery technology that is still evolving to provide higher quality animals and to reduce cost of production. This is a dynamic sector but it can be judged that it has reached a level of maturity that merits the production of a manual for hatchery personnel. The preparation of the manual has taken several years, also due to the progress of the sector that led to substantial revisions of sections. This has lengthened its preparation beyond what was originally expected. The manual is not intended to be the final word on hatchery production but rather a publication on how the industry produces in the late nineties. We preferred to include proven procedures rather than orient this publication to research on hatchery produced fry, as there is plenty of academic literature on this subject.

The manual has been divided in two volumes, with this first one divided in three parts. The first part dealing with the historical background which has led to the present status of this practice, and a discussion of the main factors that affect fish seed production. The second main section of the first volume has been devoted to the life history and biology of the species that enter in the production cycle of seabass and gilthead seabream fry. This includes also a short section on microalgae, rotifers and brine shrimp. The third main section of the first volume, which is the longer one, deals with hatchery production procedures, from broodstock management to production of live feed, egg management, larval rearing weaning, fry transport, and diseases.

The second volume will include sections on architecture and design of the hatchery systems, engineering aspects, financial aspects of hatchery operation and will provide some example of real cases of different commercial hatcheries that in recent years have approached hatchery production of these two species.

Key words: marine aquaculture, fry production, Mediterranean aquaculture, seabass, gilthead seabream.

CONTENTS

SUMMARY & INTRODUCTION

By far and large only two fish species, the gilthead seabream (*Sparus aurata*) and the seabass (*Dicentrarchus labrax*), account for the bulk of marine aquaculture production in the Mediterranean Sea. As a direct consequence, the greatest share of the Mediterranean hatchery output is formed by these two species.

This manual deals with mass production of gilthead seabream and seabass fry for the aquaculture industry. It focuses only on well-established practices that have reached a standardised industrialisation and can be considered as reliable. In some cases, import difficulties, shortage of adequate funding and unavailability of technical assistance and maintenance services, could limit the adoption of the latest and more capital intensive techniques that are described in this manual. These constraints may be overcome by the adoption of less recent technologies in sections of the hatchery, that are simpler and more labour intensive. When available, these methods are also briefly described in this manual. New developments such as genetic manipulations and hybridization with other species are not covered because they have not yet reached a commercial level. The manual has attempted to provide information on what are current practices in commercial hatcheries and the case study section of the second volume is included to show that different commercial hatcheries may establish different protocols for production that suit better the conditions of their sites or the markets they are supplying. The case study section in particular is not supposed to be up to date as the preparation of the manual has taken considerable time, but is included to show different approaches to production organization.

The mentioning of commercial products does not imply an endorsement by the authors. They are mentioned in the text, when they are very common products, only as a matter of information.

PART 1

HISTORICAL BACKGROUND AND PRESENT SITUATION

HISTORICAL BACKGROUND AND PRESENT SITUATION

1.1- HISTORICAL BACKGROUND

The fishery of wild fish fry in the Mediterranean

Over a long time, marine fish rearing in the Mediterranean region has been exclusively based on collection of wild juveniles from the sea. This has been practised for various extensive culture systems, taking advantage of the natural trophic migration of juveniles from the sea into coastal lagoons.

The Egyptian "hosha" is one of the simplest extensive systems used for the exploitation of wild stocks while the "vallicoltura", in north-eastern Italy, is probably the most specialized model of lagoon management for extensive fish rearing. In this case, wild fry are kept in the lagoon by placing fish-barriers (called "lavorieri") along the channels that link the lagoon to the sea. The special design of these fixed traps allows fish to enter the lagoon and impedes their reverse migration to the sea. The "lavorieri" are also used to harvest fish once they have reached marketable size. This culture system requires highly skilled personnel for lagoon management and an intensive use of juveniles from the sea.

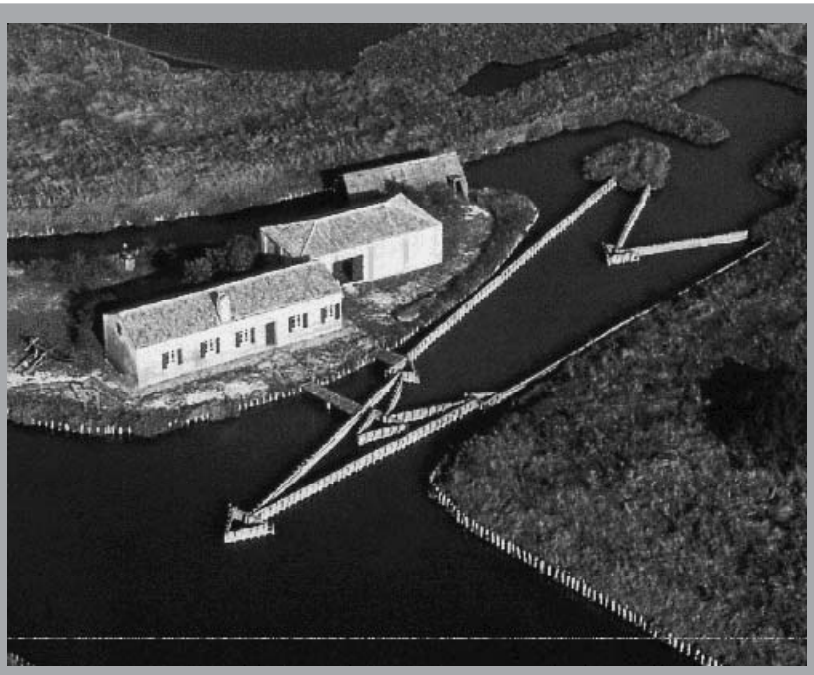


Fig. 1 - Traditional 'lavoriero' (photo G. Marcoaldi Dove,March, 1998)

The recent history of valliculture is a clear example of one of the main problems concerning the use of wild fry in aquaculture: the risk of a progressive decrease of natural stocks, limiting the expansion and the continuation of the activity itself.

Fish seed demand from controlled reproduction

Up to the sixties, the procurement of "seed" (wild fry and fingerlings) for "valli" owners did not represent a major problem. Wild seed was abundant and its migration into the lagoons was mainly regulated by climatic conditions. During the following decades, wild fry stocks drastically decreased for several reasons: over-fishing, change of coastal environmental conditions, and pollution of freshwater sources flowing into the sea.

Fry availability was drastically reduced and this situation caused a series of events:

- a stronger competition among farmers for fish seed, which led to higher costs;
- an increased waste of early fry due to the new demand for younger fish, less resistant to manipulation and transport;
- a higher fishing effort with over exploitation of far away fishing grounds located in places such as Greece, Turkey and the Mediterranean coast of Africa;
- an increased sensitivity on the side of local governments leading them to either limit or ban fry fishing in order to preserve this natural resource for local fishermen and farmers.

By the end of the seventies, the "valli" and all other rearing facilities in the north Mediterranean countries, were facing a very difficult situation. Reduced availability of wild fry did not allow appropriate stocking rates. Even under improved management, the survival rate from fry to adult for seabass and mullets did not exceed 5 to 10 percent. A strong decrease of the production was foreseeable.

In addition, the need for an alternative source of fry was made more acute by the high demand for fry coming from certain producers, which during the same period were successful in developing in Italy intensive rearing pracices for seabass and gilthead seabream. It was soon evident that this culture system, new for the region and which involved greater investments, could not depend only on the unpredictable availability of natural fry.

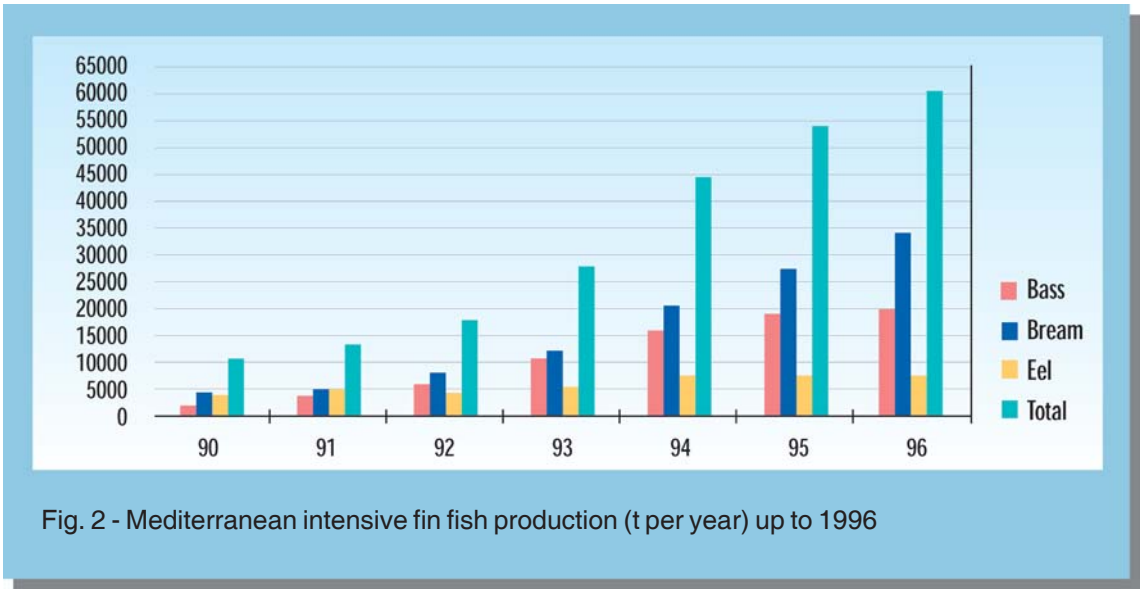


Fig. 2 - Mediterranean intensive fin fish production (t per year) up to 1996

The availability of "controlled" reproduction techniques generated a production scheme based on a reliable and programmable supply of fry, and it opened the door towards the industrialization of marine aquaculture in the Mediterranean region. This should be considered a milestone for the farming of these species in the region. Actually, the move from the research stage to an industrial production of juveniles, with its economic implications, increased the efforts to improve the reliability of these new techniques.

The first hatcheries had to face several problems such as the definition of adequate larval diets, the setting of mass culture units for synchronised production of live feed, the training of specialised personnel, and the control of diseases and larval quality.

Engineering problems arose in connection with the need to provide large quantities of seawater of suitable quality, and of its treatment including recycling systems. Very little was known on the behaviour and physiological needs of seabass and gilthead seabream larvae under intensive rearing conditions. Sudden mortality peaks or high percentages of deformities were most discouraging. The first positive results eventually started to come, based on the successful adaptation of Japanese techniques designed for similar species.

Good success at small scale with seabass reproduction was first obtained in Italy and in France in 1978-80, but large-scale production of fry was possible only a few years later in Italy, France and Croatia.

A second important step was the first positive results obtained with gilthead seabream induced breeding in Italy in 1981-82. This fish was, and still is, the more suitable species for extensive aquaculture in the Mediterranean, due to its good price, good survival rate and feeding habits, which are relatively low in the food chain. Very soon, this species also demonstrated a high adaptability to intensive rearing conditions.

Large-scale production of gilthead seabream juveniles was definitively achieved in 1988-89 in Spain, Italy and Greece. This success largely contributed to the final definition of modern Mediterranean marine fish farming, increasingly intensive, and either based on ponds or on cages for the rearing to marketable size.

Gilthead seabream and seabass are now the two most important species produced in Mediterranean hatcheries.

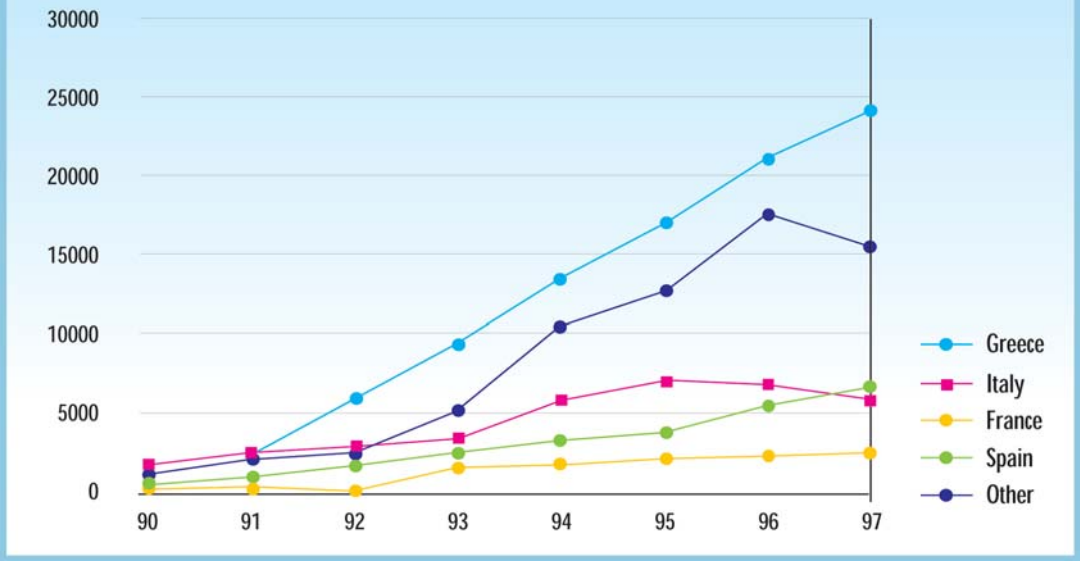


Fig. 3 - Evolution of seabass & gilthead seabream production (1 per year) in the different countries during last years.

Technological evolution of Mediterranean fish breeding

The development of seabass and gilthead seabream breeding in the Mediterranean region has been characterized by a quick move from the pilot phase to reliable zootechnical practices. In Italy and Croatia, the first industrial seabass hatcheries reached a stable production only at the beginning of the 1980s. But by the end of the decade there were already 27 seabass and gilthead seabream hatcheries in operation. At present there are more than hundred breeding centers distributed between Cyprus, France, Greece, Israel, Italy, Malta, Morocco, Spain, Tunisia, Turkey and Croatia.

The rapid evolution of aquaculture technologies has been helped by the concern of governments to push aquaculture development as a way to reduce the existing gap between supply and demand for fisheries products, and in addition because being high value species the good prospects for high profits has attracted investors.

Aquaculture production of both species has shown a rapid increase, as shown in Fig.3. At present gilthead seabream is still farmed both under intensive and extensive conditions, while seabass is mainly produced in intensive farms due to its strong predatory behaviour.

The positive production trend of recent years shown by the Mediterranean hatcheries concerns both quantity and quality. More fry of better quality with higher survival rates are produced. This has been made possible by important progress made in the control of environmental parameters and the improved knowledge of larval and fry behaviour and physiological needs. Studies carried out on the "rearing environment" greatly improved hatchery design and engineering details as well as production management.

Optimization and control of the major environmental parameters have been one of the first improvements. Studies on early larval stages have been carried out concerning temperature and salinity effects, dissolved oxygen requirements, light intensity preferred levels and photoperiod.

Since the natural reproduction of these species takes place during winter, hatcheries which could not rely on wells with temperate water had to heat the water. In order to minimise heating costs during wintertime semi-closed water recirculation system were adopted in many cases with great attention to the control and management of water quality parameters. Great attention was given to acute and chronic effects of products derived from metabolic processes such as ammonia, nitrites and nitrates, and to the toxicity of chemicals such as heavy metals.

Significant success was achieved in water quality management by improving the efficiency of biological filtration and the separation of organic matter through the use of mechanical filters and skimmers.

In the seventies, considerable emphasis was given to the study of gonadal maturation and to the use of human chorionic gonadotropin (HCG) to induce spawning in the two species. The risks linked to excessive hormonal dosages on broodstock, which would affect later larval survival and viability, were clearly understood. The use of hormones acting at an early stage of ovarian maturation (such as the LH-releasing hormones) was also studied.

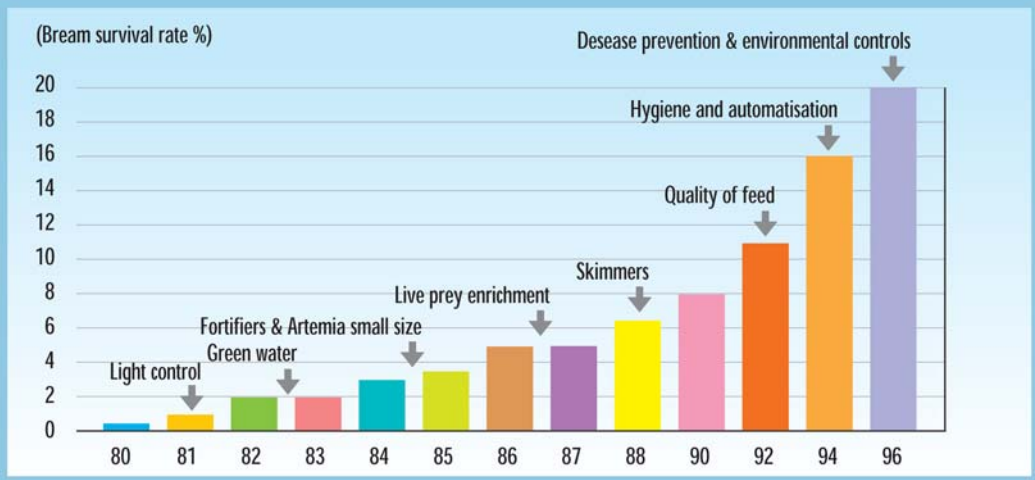


Fig. 4 - Main factors influencing seabass and gilthead seabream fry rearing performances (1980-96)

The importance of an adequate broodstock selection and management, in order to improve egg and larval quality, was progressively highlighted. Moreover, the manipulation of temperature and photoperiod provided an opportunity to prolong the spawning season.

Feeding physiology became one of the most studied subjects and the identification of feeding limiting factors was considered one of the decisive steps towards the improvement of larval survival. In France, the understanding of larval feeding behaviour resulted in the elimination of rotifers from the diet of seabass, with the consequent reduction in hatchery investment and operating costs. It also resulted in the search and selection of smaller size and more productive rotifers strains for the first feeding of gilthead seabream larvae, a basic step to

improve its larval rearing. This work was conducted both by laboratories and private enterprises.

Larval pathology also became an important research subject. Attention was focused on determining the causes of the main diseases, physiological or infectious, both environmentally induced and transmitted. Methods to prevent their diffusion in the hatcheries were also found.

Comprehensive studies were carried out on the natural behaviour of farmed species. Increasing attention was also devoted to understanding larval behaviour under rearing conditions.

1.2- PRESENT SITUATION

Vertical integration: the hatchery as a part of the "Farming System"

At the beginning, the hatchery was considered as something separate from a traditional fish farm due to its high technical requirements, to the considerable investment costs requirements and to its not sufficiently reliable output. A certain distrust about the quality of hatchery-produced fish was widespread among farmers. But the hatchery alone was also rarely viable from an economic standpoint due to the high production costs and to the unpredictability of production and market prices of fry and fingerlings.

Due to several new factors, which are listed below, in few years the situation changed completely and at present most of the Mediterranean farmed production of seabass and gilthead seabream is based on the use of artificially produced fish seed. These factors were:

- the increasing level of standardisation of the techniques for artificial reproduction;
- the continuous improvement of fry quality;
- the improved availability of skilled personnel for hatchery management and operation;
- the increasing demand by farm managers for a reliable source of healthy fry in order to better plan farm stocking and production;
- the greater economic importance of the aquaculture sector, with better access to regional, state and supranational incentives and subsidies.

At present, hatcheries are usually integrated in the production cycle of marine fish farms, but they also exist as autonomous entities, economically viable. Production targets in the hatcheries are now established according to the needs of the associated farm or farms. Hence the increasingly strong link between market demand and production strategy adopted by each hatchery.

The Hatchery Manager should rather be a skilled industrial manager than a good biologist, as it used to be in the past. He should have a good practical sense, combined with a good knowledge of mechanics, engineering and chemistry, and should be familiar with the biology of the farmed species. Such "special skills" to co-ordinate hatchery management are confirmed by the fact that in the largest farms the responsibility for hatchery operation is frequently assigned to a person who is not requested to be directly involved in the production process.

Production strategies

The hatchery is proportionally the more expensive component of a marine fish farm. Therefore, farmers aim at making the most profitable use possible of their hatcheries.

Since Mediterranean aquaculture has based its development mainly on winter spawning species, the problem faced from the beginning was to avoid long periods of inactivity (the entire summer), during which personnel and facilities maintenance costs were not covered by production. The extension of the production period throughout most of the year has been mainly achieved by adopting three main different strategies:

- the production of different species (multispecific hatcheries);
- the extension throughout the year of the spawning period of a single species (monospecific hatcheries);
- multipurpose hatchery, which is a combination of both systems, multispecific with a prolonged spawning period.

Multispecific hatcheries

The production strategy adopted by multispecific hatcheries is based on the use of different species with partially overlapping spawning seasons. A carefully planned exploitation of their natural maturation periods allows the production of two or more species over an extended period, usually from October to June under Mediterranean conditions. This strategy has been the most commonly adopted in the Mediterranean and since the beginning, after the first years dealing only with seabass, both seabass and gilthead seabream have been reproduced at the same hatchery. Even if it spawns in the same season, gilthead seabream shows a more extended breeding season and this allows a combined used of the hatchery facilities with seabass.

Frequently, this strategy is applied in farms with hatcheries producing fry for their own internal use only. Fry sales to other producers represent a secondary or occasional activity and only concern fish produced in excess of the farm own requirements. The hatchery would produce at full capacity only during part of the year and the personnel is alternatively employed during winter in the hatchery and during summer in the outdoors rearing facilities.

At present, a widely adopted solution is to exploit the first spawning of gilthead seabream in November/December, and then to spawn seabass from January to March in combination with gilthead seabream. Moreover, the possibility to anticipate/delay reproduction allows also multispecific hatcheries to start a little earlier in autumn to obtain viable larvae in October and later to extend the spawning until April. About four months is the time normally required to produce 2 g fry starting from viable larvae, at a water temperature of 18 to 20°C. This strategy would therefore allow the use of hatchery facilities for nine to ten months per year.

This production strategy can also be attractive because:

- by exploiting the natural spawning periods, the hormonal treatment of broodstock, if applied, is limited to fully ripe fish just to induce spawning at a specific time according to the production planning. Allowing natural gonadal maturation usually results in a better larval quality and improved survival rates;
- fry produced this way are ready for stocking both in extensive and intensive farms, during spring and summer. This period coincides with the most suitable timing since the whole on-growing period (16 to 22 months) would only include one winter and two warm seasons (April to October) and water temperatures would be more favourable.

In order to extend the hatchery working season many attempts have been made to select fish species that spawn in springtime. Among the Sparids, sharpsnout seabream *(Puntazzo puntazzo)* and white seabream

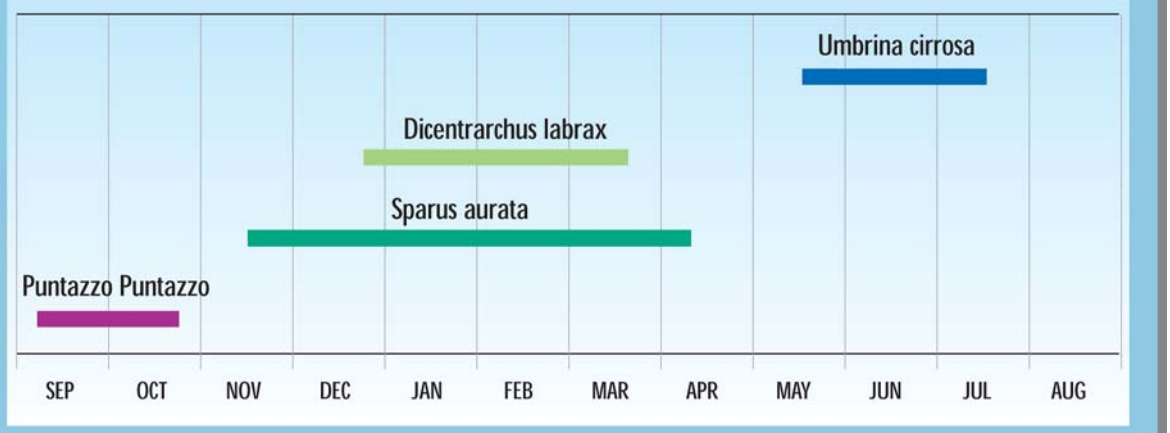


Fig. 5 - Tentative yearly production schedule for a multispecific hatchery

(Diplodus sargus sargus) have given promising results and are now produced by some farms. On the contrary striped seabream *(Lithognathus mormyrus)* was abandoned due to the long time it requires to reach a good marketable size.

The introduced red seabream or madai *(Chrysophrys major)* has been successfully reproduced in Italy and Croatia for several years. Unfortunately it has not been well accepted by the Mediterranean market due to its unfamiliar colours. Successful breeding trials have also been carried out with sole *(Solea vulgaris),* which reproduces at the end of the winter but no commercial development has followed since this species did not prove suitable for intensive farming. Some other interesting species have been recently considered, such as the common dentex *(Dentex dentex)* and the shi drum *(Umbrina cirrosa)*.

An additional step toward the optimization of use of hatchery facilities was taken at the beginning of the eighties, when the rearing of tiger shrimp (*Penaeus japonicus)* in multispecific Mediterranean fish hatcheries was added to the production schedule.

The advantage of *Penaeus japonicus* is that it has a short rearing cycle requiring 3 to 5 months to reach marketable size from postlarvae. This means that juveniles stocked in May/June could be marketed from August to October. The combination of *Penaeus japonicus*, seabass and gilthead seabream represents therefore an alternative for Mediterranean multispecific hatcheries.

Actually, this production strategy is not widely practised because *Penaeus japonicus* is mainly farmed under extensive conditions and therefore space availability tends to restrict this practice. On the other hand, shrimps are appreciated commodity in the Mediterranean markets and in appropriate locations, their high economic value can justify the additional investment required for their production.

Monospecific hatcheries

Monospecific hatcheries focus all their production efforts on a single species, usually seabass or gilthead seabream, whose maturation period and spawning is extended well beyond its natural period. In some cases they are also reared with a very small percentage of the other species (less than 15%). This strategy is applied by the large commercial hatcheries, where most of the production is sold to farmers, and where ongrowing, if at all practised, represents a marginal activity.

Water temperature and photoperiod are the most important environmental factors driving sexual maturation in fish, thus ensuring that offspring will find the most favourable environmental conditions. By acting on these two parameters in such a way as to simulate different seasonal conditions, it becomes possible to delay or anticipate the gonadal maturation and the subsequent spawning.

This technique requires rearing units designed for this purpose, completely separated from the rest of the hatchery, in which light and water temperature are controlled either manually or with the help of computers or other special devices.

Since gilthead seabream and seabass spawn in wintertime, a delay in their maturation cycle can be obtained reproducing a thermo-photoperiod that mimics summer conditions and which is longer than the natural one. Anticipating winter conditions can, on the other hand, lead to an early gonadal development.

This production strategy requires a good knowledge of the species reproductive physiology and significant resources have to be devoted to a large broodstock holding and conditioning unit. The advantage of having large quantities of fully weaned fingerlings at the onset of the stocking season, as well as well beyond the natural fry season, represents a major economic advantage for the hatchery. A manipulated maturation process also requires a more careful management of breeders to avoid larval batches of low quality.

Actually, management constrains do not permit a continuous production using this process. The hatchery units require a summer pause to be adequately disinfected and prepared for the coming production season, and the hatchery personnel should also be given their holidays. In the long run, this production strategy is less frequently adopted. On the other hand, the manipulation of the spawning season for seabass and gilthead seabream is more and more frequently applied in multispecific hatcheries to complete their annual production programme by slightly modifying thermo-photoperiod of one or more of the farmed species.

Multipurpose hatcheries

By combining the use of several species and maturation shifting, multipurpose hatcheries represent the most versatile model for a hatchery. Due to their high investment and running costs only large farms or groups of aquaculture enterprises can usually afford them.

1.3- MAIN FACTORS AFFECTING FISH SEED PRODUCTION

A production model could be defined as an orderly sequence of operations, which leads to the achievement of the planned production target. Mediterranean hatcheries follow various production models, which depend on three main groups of factors:

- the adopted technology;
- the characteristics of the site where the installation is located;
- the target market.

Technologies

Due to its dependence on the aquatic environment, fish farming faces additional problems if compared with other land-based animal husbandry practices. From a management point of view, dealing with organisms in the water requires more complicate zootechnologies than for land based animals. The updating of hatchery techniques is probably the most indicative one, due to the important investments that are required. Modern marine fish farms cannot operate without a strictly reliable and efficient production programme and hatcheries are particularly involved in this modernization process, because a failure in their production can jeopardize the success of the entire farm.

A successful hatchery relies on efficient hardware (facilities and equipment) that should be matched by an excellent software (updated know-how, standardization of procedures, and dedicated and skilled personnel). A rational, well-engineered construction employing only affordable, corrosion-free and non-toxic materials is a pre-requisite for any marine fish breeding operation. Production efficiency can also be improved by the introduction of mechanised processes, which reduce errors and personnel costs, and by the adoption of automatic, controls with alarms.

On the other hand, the use of the more advanced and highly sophisticated technology may or may not be possible and will also be influenced by the local socio-economic conditions. Each country has its own level of technological development, equipment, know-how and human resources to which the hatchery design has to be rationally adapted.

As an example, the use of advanced technical equipment that can greatly simplify working procedures, but that requires having specialised service and maintenance technicians constantly available may or may not be possible depending on the local situation. Moreover, some imported equipment supplies may turn out to be too expensive in countries where high import duties are applied. In addition, the absence in some countries of financial incentives such as favourable credit conditions, may be a major deterrent for the adoption of advanced technical solutions, whose high investment cost should be paid by the private investor.

Environmental conditions

Factors related to environmental characteristics of the site exert a strong influence when selecting the hatchery model. Apart from the quality of seawater, which can be controlled, within certain limits, by means of screens, filters, settling and stocking tanks, sterilization equipment, etc., the other main influencing factors are air and water temperatures.

In northern Italy and along the French Mediterranean coast, seabass reaches marketable size (250 - 350 g) in 20 to 24 months and gilthead seabream require 16 to 20 months to reach a commercial size of 250-300 g. Along the African Mediterranean coast the period needed to reach these sizes is reduced to 14 to 15 months for seabass, while gilthead seabream is ready to be marketed within a year. In the northern Mediterranean winters are usually so cold that without appropriate facilities, such as wintering ponds, greenhouses, water heating systems, etc., the whole fish production can be easily wiped out. In any case even without mortalities both species stop growing during the winter period.

These severe winter conditions force farmers to stock their grow-out facilities as early as possible to avoid passing two winters before reaching marketable size. That factor alone concentrates the demand for fry on April and May, with resulting negative consequences both for the fry producers, that are forced to pack production in a short time, and also for the buyers, which are affected by the shortage of offer and by the high prices. In sites with milder climatic conditions, fry demand can be spread over a much longer period of time and the hatchery can optimize its production strategy over more crops.

On the positive side, the difficulties faced in the coldest climatic zones encourage aquafarmers to develop new production models, thus accelerating the technical development of the sector. The use of water recirculating systems, with their high technological content, is just one of such examples. Efforts made to achieve complete independence from external conditions have also resulted in technological improvements in the equipment used. These range from mechanical and biological filters, heaters and sterilizers, to the recent development of computerized systems for the control and management of environmental parameters such as oxygen, pH, nitrite and nitrates.

Marketing

The market drives the hatchery output. Output is directly linked to it by the fry price variations during the year, and indirectly through the effect that the whole fishery market demand has on fish farming and consequently on the hatcheries output as well.

The duration of the marketing season for fish fry and fingerlings affects commercial hatcheries when planning their production targets. A short season means that the largest part of the production has to be concentrated in a short period of time, which implies a reduced number of cycles and requires large indoor facilities.

On the contrary, an extended fry stocking season, possible in milder climatic conditions, allows a simpler and smaller hatchery with the same production level obtained through more cycles. Moreover, the weaning and fry temporary holding sections can be built outdoors under cheaper and lighter shelters. In this scenario, a broodstock conditioning section, to extend the spawning season, may also make sense economically.

Increased capacity to deliver fry over long distances enlarges markets and contributes to reduce monopolistic trends. As a consequence, prices and, most important, quality of fry originating from different hatcheries, can be compared. The progressive establishment of a true Mediterranean fry market is resulting in increasingly uniform prices. It is also imposing quality standards, which force the farms to adopt the most efficient technologies in order to optimize production.

A parallel market for hatcheries is provided by the demand for fertilised eggs and/or newly hatched larvae by other hatcheries. Small hatcheries cannot always afford a large spawning sector, whereas in some cases hatcheries either can run out of eggs due to some technical failure or may want to boost their production with delayed cycles. Although widely adopted, this practice does not always guarantee the same fry quality due to transport and handling stress. Even with the strictest precautions, this practise is also not totally free from risk, and pathogens could be introduced in the receiving hatchery through contaminated batches of eggs/larvae.

Concerning the influence that fish market trends have on hatchery activities, it should be pointed out that an important difference exists between the market of different aquaculture products, such as trout and salmon or oysters and mussels, and the market of marine species like seabass and gilthead seabream which is also linked to the total volume of the production. In the case of seabass and gilthead seabream the competition between aquaculture and the other fishery alternatives is still important.

Another effect, largely related to the former one, is that the market offers the possibility of and concurrently imposes the need for diversification of production. Besides the technical difficulties, which limit the reproduction of new species, it is again the market itself that selects the species towards which efforts for the implementation of new “production lines” should be addressed.

PART 2

BIOLOGY AND LIFE HISTORY

BIOLOGY AND LIFE HISTORY

2.1- SEABREAM GILTHEAD

The gilthead seabream *Sparus aurata* (Linnaeus, 1758) is a perciform fish, belonging to the family Sparidae and to the genus Sparus.

LOCAL NAMES		
	Albanian	koceja, spalc
	Arabic	denis (Egypt),ourata, warka (Tunisia), zerika (Morocco)
	English	sea bream, gilthead seabream
	French	dorade, daurade
	German	goldbrasse
	Greek	tsipoúra
	Italian	orata
	Portuguese	dourada, doirada
	Spanish	dorada
	Turkish	çipura

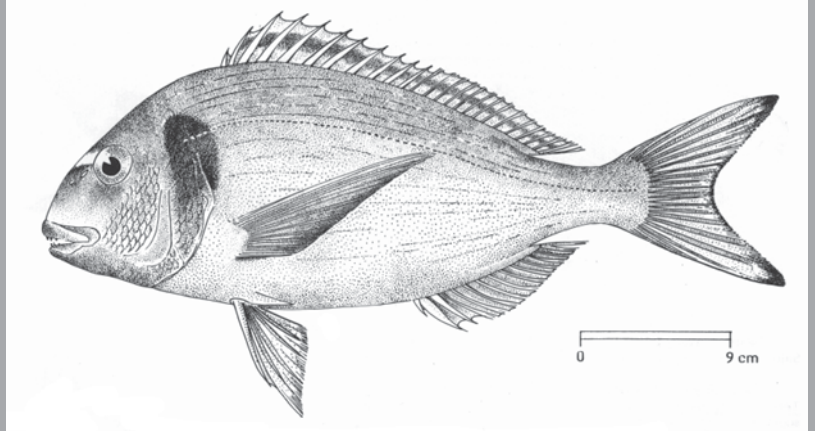


Fig. 6 Adult specimen of gilthead seabream

FAMILY DESCRIPTION

Family Sparidae

Percoid fish with body oblong, usually deep and compressed. Scales cycloid or weakly ctenoid. Head large, often with a steep upper profile. Snout and supraorbital area scaleless. Mouth small, with the upper jaw reaching no further than the middle part of the eye. Preoperculum scaled, without spines on margin. Jaw teeth usually differentiated into conical, incisor or canine teeth in front and molar-like teeth behind. Palatines bones usually toothless.

One single unnotched dorsal fin with 10 to 15 stout spines and 9 to 17 rays. The spiny anterior part has the same length of the posterior part with rays. Anal fins with 3 stout spines and 7 to 16 rays; pectoral fins usually long and pointed; ventral fins with axillary scales; caudal fin emarginate or forked. A single continuous lateral line. Colours vary greatly, from silver to reddish to almost black.

Almost all Sparidae are demersal, and are found in relatively shallow waters, often in rocky areas; the young fish generally live in shallower waters than the adults; fry and fingerling school together, while adults usually show a solitary behaviour.

The eggs are pelagic, spherical (with a diameter of around 1 mm) and have an oil drop.

Many sparids are hermaphroditic: when reaching sexual maturity there could be a majority of males (protandric hermaphroditism) or of females (protogynic hermaphroditism). Sparids are carnivorous fish and feed mainly on molluscs and other benthic organisms, which they break with their strong teeth.

Due to their excellent meat, many representatives of this family have a high commercial value. Sparids are divided amongst many genera and a large number of species living in all tropical and temperate seas, including exceptionally cold and brackish waters. In the Mediterranean eleven genera represent the family: *Dentex, Sparus, Diplodus, Pagellus, Pagrus, Lithognatus, Spondyliosoma, Oblada, Crenidens, Boops* and *Sarpa.*

DISTINCTIVE CHARACTERISTICS OF THE SPECIES

The genus *Sparus* is characterized by molariform teeth and 75-85 scales along the lateral line. *Sparus aurata* is its only species of this genus in the Mediterranean.

Biology

Morphology

The gilthead seabream presents a body with an oval shape, very high and laterally compressed. The head profile is convex with small eyes. The cheeks are covered with scales and the pre-opercular bone is scaleless. The mouth has the mandible shorter than the maxilla. Both jaws show canine (4-6) and molariform teeth, in 2-4 series in the upper jaw and 3-4 series, of which 1-2 are notably bigger, in the lower jaw.

The gill rakers are short, 11-13 on the first branchial arch and 7-8 on the lower part. The lateral line has 75-85 scales. The dorsal fin presents 11 hard and 13 soft rays, the anal fin 3 hard and 11-12 soft rays. The pectoral fins are long and pointed, while the ventral ones are shorter. The caudal fin has pointed lobes. All the vertebra present parapophises and sessile ribs are absent.

The gilthead seabream colour is silver-grey with a big dark spot at the beginning of the lateral line that covers also the upper part of the opercular bone. A gold and a black band is found between the eyes, the golden one always narrow in the central part. The dorsal fin is blue-grey with a median black line. The caudal fin is grey-greenish white with black tips.

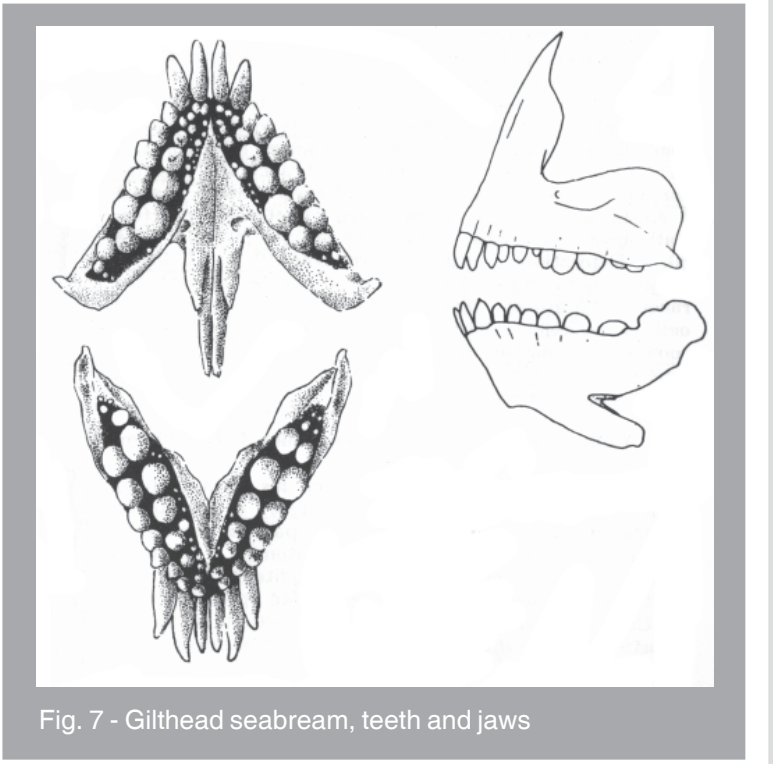


Fig. 7 - Gilthead seabream, teeth and jaws

Geographic distribution

S.aurata is common in the Mediterranean Sea, it is present along the Eastern Atlantic coasts from Great Britain to Senegal, and is rare in the Black Sea. Due to its euryhaline and

eurythermal habits, the species is found in both marine and brackishwater environments such as coastal lagoons and estuarine areas, in particular during the initial stages of its life cycle. Born in the sea during wintertime, the fingerlings typically migrate in early spring towards protected coastal waters in search for abundant food and milder temperatures (trophic migration). Very sensitive to low temperatures (lower lethal limit is 4°C), in late autumn they return to the open sea, where the adult fish breed.

The gilthead seabream is usually found on rocky and seaweed bottoms, but it is also frequently caught on sandy grounds. Young fish remain at low depth (up to 30 m), whereas adults can reach deeper waters (maximum depth of 150 m).

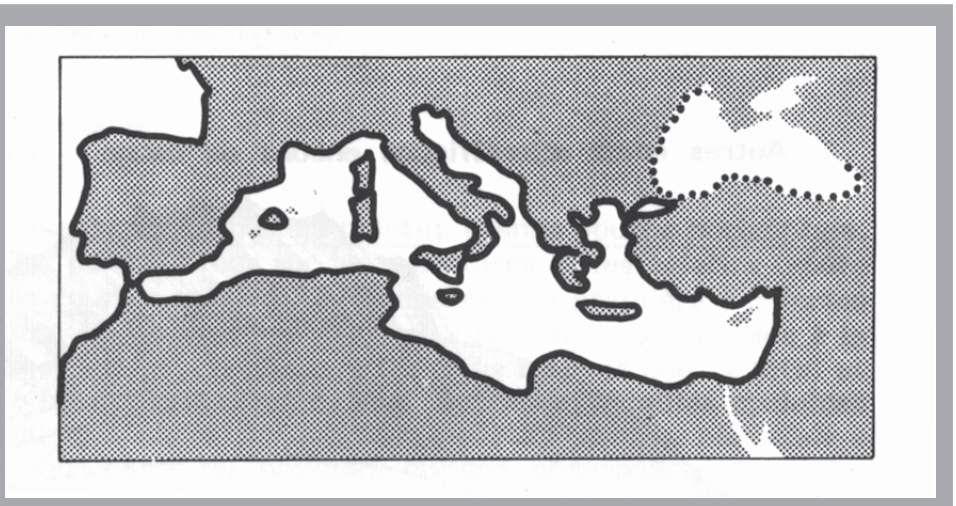


Fig. 8 - Gilthead seabream geographic distribution

Reproduction

The gilthead seabream is a protandric hermaphrodite with a breeding season ranging from October to December. The gilthead seabream is a functional male in the first two years and at sizes over 30 cm become females. After spawning, the eggs, which are spherical and transparent, have a diameter of slightly less than one mm. and present a single large oil droplet.

Fishery

The gilthead seabream is fished with traditional and sporting equipment, and sometimes with semi-professional systems (Spain, Sicily, Egypt and Cyprus), trawl nets, bottom set longlines, hand lines are also commonly used. The gilthead seabream is regularly present on the markets in Adriatic, Greece, Turkey and Maghreb. It is commercialised fresh, refrigerated and frozen.

2.2- SEABASS

The seabass, *Dicentrarchus labrax*, Linnaeus 1758, is a Perciform fish, belonging to the Moronidae family and to the genus Dicentrarchus.

LOCAL NAMES		
	Albanian	lavraku; levreku
	Arabic	karous
	English	European seabass; bass; sea perch;
	French	bar; loup; loubine; perche de mer; barreau
	Greek	lavraki
	German	wolfsbarsch; seebarsch; meerbarsch
	Italian	spigola; branzino
	Spanish	lubina; robaliza; róbalo; magallón
	Turkish	levrek

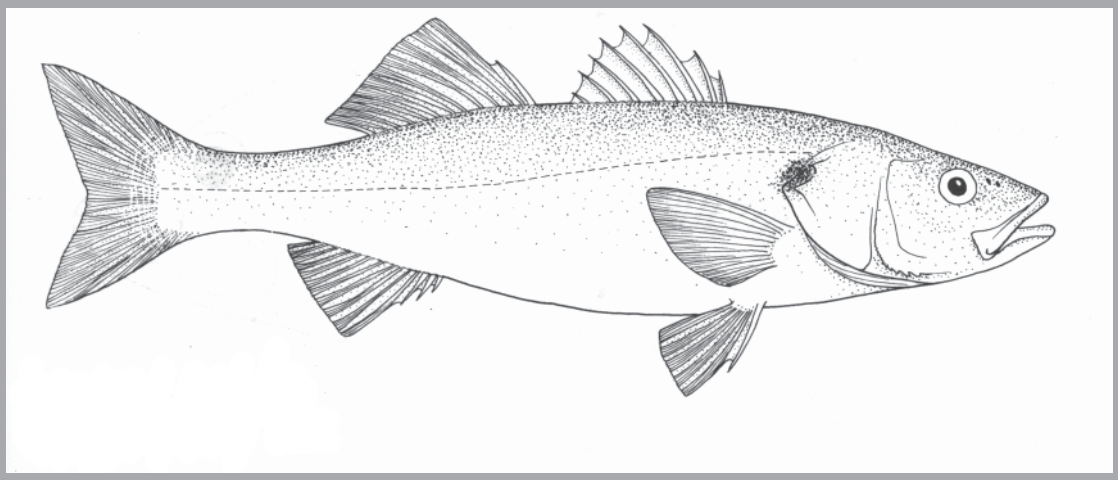


Fig. 9 - Adult specimen of seabass

FAMILY DESCRIPTION

Family Moronidae

Perciform fish of elongated body. Operculum with two flat spines; terminal mouth slightly protractile; end of the maxillary visible, not gliding under the sub-orbital bone; small teeth on the jaws and vomer, without canine teeth. Two dorsal fins separated, the first one with 8-10 spines, the second with one spine and 11-14 soft rays; anal fin with three spines and 10-12 soft rays; base of the pelvic fins without scales; caudal fin moderately forked. Large caudal peduncle. Lateral line complete, not continuing on the caudal fin. Small scales, around 55-80 on the lateral line in the Mediterranean species. Colour generally silvery; one species with small black spots; lower fins sometimes yellowish when fish are alive.

Medium to large size fish (till one meter total length) of temperated and cold regions. The two species of the Mediterranean inhabit coastal and brakish waters and are occasionally found in rivers.

Two species occur in the Mediterranean Sea: *D.labrax* (L.) and *D.punctatus* (Bloch), the latter with only a marginal interest in artisanal fishery along the southern Mediterranean coasts. They can be identified on the following characters:

presence on the back and sides of dark, permanent spots in adult	*D. punctatus*
vomerine teeth extend over the vomer like an arrow point	*D. punctatus*
vomerine teeth only on the anterior part, like a V	*D. labrax*
diameter of the eye is smaller than the interorbital space	*D. labrax*
while larger	*D. punctatus*

Biology

Morphology

The seabass, *Dicentrarchus labrax* has a silvery elongated body, with two clearly differentiated dorsal fins and a rather high tail. The opercular bone has two flat spines and a range of spines are visible in the lower part of the preopercular bone, pointing in the direction of the mouth. The vomer presents teeth with a crescent shape. This species has cycloid scales in the interorbital region. The lateral line is visible as a dark line with 62-80 cycloid scales.

The first dorsal fin has 8-10 spiny rays, and the second dorsal fin 12-13 rays of which the first is spiny. The anal fin has 3 spiny rays and 10-12 soft rays.

The colour is dark grey on the back, passing to grey-silver on the sides, while it is white-silver on the abdomen. Specimens from the sea show a much clearer colour than fish from lagoons and estuarine environments. On the opercular bone there is a dark spot. The juveniles show a livery with little dark spots, mainly on the front or only on the head, which disappear with age. The maximum size is over 1 m with a weight of over 12 kg.

The seabass is a eurythermal and euryhaline species and can survive at temperatures between 2 and 32°C, with a limited territoriality related to their search for food and to reproduction. Outside the spawning period, the seabass can be found anywhere food is available. Maturation and spawning need more specific environmental conditions (temperature, photoperiod, salinity) which determine variation in spawning period.

Feeding

Seabass is a predator consuming small fish and a large variety of invertebrates. In spite of variations associated with differences in latitude, bass hunts at any time of the day. The feeding behaviour is related to size. Juveniles feed mainly on small Crustaceans (Amphipoda, Mysidacea, Isopoda) and small fish (about 1/4 of the diet), like Atherina and Gobius. In fish larger than 20 cm, shrimps and crabs begin to be common preys.

Geographic distribution

D.labrax is common in the Mediterranean Sea, the Black Sea and along the Eastern Atlantic coasts from Great Britain to Senegal. With a tolerance to salinity and temperature fluctuations greater than the gilthead seabream, this species is found in marine to slightly brackish environments such as coastal lagoons and estuarine areas. In particular during the first stages of its life cycle displays the same behaviour of gilthead seabream. Much less sensitive to low temperatures, some fish may overwinter in coastal lagoons instead of returning to the open sea.

Reproduction

Fig. 10 -European seabass geographic distribution

In seabass sexes are separate: the female shows a deeper body with a longer pointed head and greater pre-dorsal and pre-anal lengths. Sure sex confirmation is however possible only during the spawning season by checking the presence of sperm by squeezing gently the males and by observing the protrusion of the anus and genital papilla in the females.

Sexual maturity takes place earlier in males and earlier in Southern populations. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in the Atlantic populations. Unlike gilthead seabream, 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.

After being released, the eggs acquire their characteristic spherical shape, with a size that varies according to latitude:

Place	diameter (mm)
Great Britain	1,2 - 1,5
Mediterranean	1,15 - 1,2

Fisheries

Seabass is fished both by artisanal and sport fishermen. The quantities caught in the Mediterranean are relatively small which linked to high appreciation for the species in the Mediterranean markets makes it a high value species. The gear used to catch seabass include beach and purse seines, trawl nets, trammel nets and longlines, as well as rod and line. It is regularly present in the Mediterranean markets but it is scarce in the eastern Mediterranean basin and it is rare in the Black Sea.

2.3- LIVE FOOD FOR MARINE FISH LARVAE

The timely and adequate supply of high quality live food is still essential in the rearing of the early larval stages of many marine organisms. As of today the use of some micro-organisms as first feeding of seabass or gilthead seabream larvae is still mandatory.

Fig. 11 - Culture upscaling: small volumes

The rotifer *Brachionus plicatilis* is an excellent first food for larval stages of marine fish because of its small size, slow swimming speed, its habit of staying suspended in the water column, the possibility to rear it at high densities (a density of 800–1,000 rotifers/ml is now quite common in well run hatcheries), its high fertility rate, and the broad tolerance to salinity. Moreover, the rotifer can act as a vector to transfer specific nutritional factors and drugs to fish larvae.

While artemia is immediately (but not cheaply) available on the market as canned dry cysts (their resting eggs), the production of large quantities of rotifers (several billions per day in case of large hatcheries) still requires the production in parallel of marine microalgae on which rotifers feed.

The biology and life history of these organisms, microalgae, rotifers and artemia is briefly explained below, whereas the design of their production units and the description of the production methods are dealt with other sections of the manual.

Microalgae

Microalgae, also called in this manual phytoplankton and algae, are unicellular eukaryotic planktonic algae belonging to several taxonomic groups. Below are quoted the most common microalgae cultured in in the context of marine finfish reproduction. In aquaculture phytoplankton is directly used to feed molluscs and crustacean larvae and indirectly to culture the zooplankton on which the first feeding of marine fish larvae is based.

The benefits of microalgae in the larval rearing of marine fish are not longer limited to their original role to feed rotifers, which could now be largely replaced by artificial diets and baker's yeast *Saccaromyces cerevisiae* in their final steps of mass culture. It is now accepted that fish larvae benefit indirectly from the presence of selected phytoplankton species in their tanks during the first rearing days, where they works both as immunological stimulus and as a conditioner of water quality, limiting the development of bacteria and reducing N and P loads.

In past years many microalgae species have been tested in the Mediterranean hatcheries but only a few species are now routinely mass-produced. Their selection was driven by these criteria:

- a high nutritional value for both fish larvae and rotifers;
- absence of toxicity for their predators;
- suitable dimensions and digestibility, to be easily accepted by rotifers;
- a good capacity to adapt to standardised mass rearing conditions;
- a high rate of reproduction in artificial environments;
- reliability and affordability of their mass production.

The most commonly cultured algae in the Mediterranean region are the following:

Bacillariophyceae (Diatoms):	*Chaetoceros calcitrans* *Skeletonema costatum*
Haptophyceae	*Isochrysis galbana* *Isochrysis sp.* (Tahitian strain) *Pavlova (Monochrysis) lutheri*
Chrysophyceae	*Tetraselmis (Platymonas) suecica* *Tetraselmis (Platymonas) chuii* *Tetraselmis (Platymonas) tetrathele*
Chlorophyceae	*Dunaliella tertiolecta* *Chlorella sp.*
Eustigmatophyceae	*Nannochloropsis gaditana* *Nannochloropsis oculata*

Due to their high degree of variability, strains from different natural algal populations, although identified under the same species, may differ considerably in their nutritional characteristics and other biological aspects.

The live food production unit of the hatchery usually gets pure microalgae strains of the species selected from either other hatcheries or from specialised institutions such as CCAP (the Culture Collection of Algae and Protozoa of the Dunstaffnage Marine Laboratory, P.O.Box 3, Oban, Argyll, PA34 4AD, Scotland, UK).

Basically, microalgae can be cultures following three different methods: continuous, semi-continuous and batch culture schemes. The hatchery staff must always pay attention to keep algal density, or algal growing rate high enough to reduce chances for development of competitors or of contaminating organisms. Most algal productions in Mediterranean hatcheries centres are based on monospecific batch culture where algae from stock cultures are upscaled in successively larger containers until they are harvested. For commercial hatcheries, this method is considered more reliable than the semi-continuous or continuous culture methods due to the more limited risk of culture crashes, an easier standardisation of culture procedures and a reduced investment if compared with the other two methods.

Fig. 12 - Continuous culture: the bio-fence

Batch culture method:

As a matter of routine, axenic conditions are reserved for pure strains, back-up cultures and small culture volumes up to 20 l carboys, which are kept in air-conditioned, dedicated rooms. Subsequent upscaling

to larger volumes takes place in plastic bags, tanks and ponds, placed both indoors and outdoors according to local climatic conditions. The design of a phytoplankton production unit and the standardised culture procedures are described in other sections of the manual.

In principle, culture conditions and the composition of the liquid medium differ according to algal species or even strains. The most commonly used algae, can be cultured well using the same standardised medium and procedures that are described in detail in the chapter on microalgae mass culture.

The most relevant parameters influencing algal growth are: temperature, nutrients, light, pH and turbulence. The standard culture conditions in Mediterranean hatcheries can be summarized as follows:

- temperature : between 18° and 24°C;
- salinity: between 20 and 35 ppt;
- light: from 1 000 to 2 000 lux for smaller volumes up to 10 000 lux for larger tanks, where light penetration is limited;
- pH: normally increased up to 9 by the photosynthetic activity of the algae themselves, it is kept at a lower value (8-8.5) by the addition of acid or carbonic gas (CO_2).

The culture medium is usually prepared with micro-filtered and UV-treated seawater enriched with nitrogen and phosphorous as major elements. Addition of oligoelements includes salts of sodium, calcium, potassium, magnesium and various metals (Zn, Fe, Mn, Cu, Mo; plus Si for diatoms). Vitamins, such as B_{12}, biotin and thiamine, are also added.

The bio-chemical composition of microalgae varies also according to culture conditions. Their content of aminoacids, vitamins and essential fatty acids (PUFA and HUFA) is affected by environmental factors such as water temperature, salinity and light intensity.

Mass production of microalgae requires plenty of space, skilled personnel and time. Cheaper and easier alternatives have been investigated for years. At present, rotifers are mainly fed on artificial diets and few times with baker's yeast, thus keeping the need for algae to a minimum. Phytoplankton, however, remains a key factor for the batch scale cultures of rotifers in small volumes and, most important, for the larval rearing of gilthead seabream and other species such as molluscs and shrimps.

Biology of rotifers

Taxonomy		
	PHYLUM:	Nemathelminthes or Aschelminthes
	CLASS:	Rotatoria
	ORDER:	Monogononta
	FAMILY:	Brachionidae

Morphology

Rotifers are among the smallest filter-feeding metazoans. Composed of a fixed number of about 1,000 cells, their growth is obtained by plasma increase, not by cellular division. They filter small particles by means of a ciliated annular organ, the corona, located in the anterior part of the body. The corona is also used for its whirling locomotion, hence the name of the Class Rotatoria. Whereas many species spend their life span attached to a substrate by means of their retractile foot, *Brachionus plicatilis* that is the main species cultured for finfish larval rearing world-wide, is a planktonic, unattached rotifer.

The epidermis contains a densely packed layer of keratin-like proteins called lorica. The rotifer's body is differentiated into three distinct parts: head, trunk and foot.

- The head carries the ring organ or corona, which is easily identifiable by its ring of cilia. The retractile corona assures locomotion and a whirling water movement which facilitates the ingestion of small food particles (mainly algae and detritus).
- The trunk contains the digestive tract, consisting in a mastax that grinds the ingested particles, the oesophagus, the stomach with gastric glands and the intestine. The excretory system consists of paired protonephridia with terminal cells (cyrtocytes), the duct and the bladder. The protonephridia expel liquid excreta and play an important role in osmotic regulation. The genital organ is unpaired (*Monogononta*) or paired in the *Seisonidea* and *Bdelloidea*. The joint external opening for bladder and oviduct is called the cloaca.
- The foot is a ring-type retractile structure without segmentation, ending in one or four toes bearing pedal glands that secrete an adhesive substance in crawling and sessile rotifers.

Two different morphotypes of *B. plicatilis* exist: the small (S) type and the large (L) type. They differ in their lorica length: 130 to 340 µm (average 239 µm) for the L-type and 100 to 210 µm (average 160 µm) for the S-type. There are also differences in weight, shape of occipital spines and optimal growth temperatures (L-type rotifers have a wider temperature range while S-type rotifers have a higher temperature resistance). S-type rotifers are suitable as first food for fish larvae with a mouth opening smaller than 100 µm at first feeding, such as gilthead seabream, groupers, and rabbitfish.

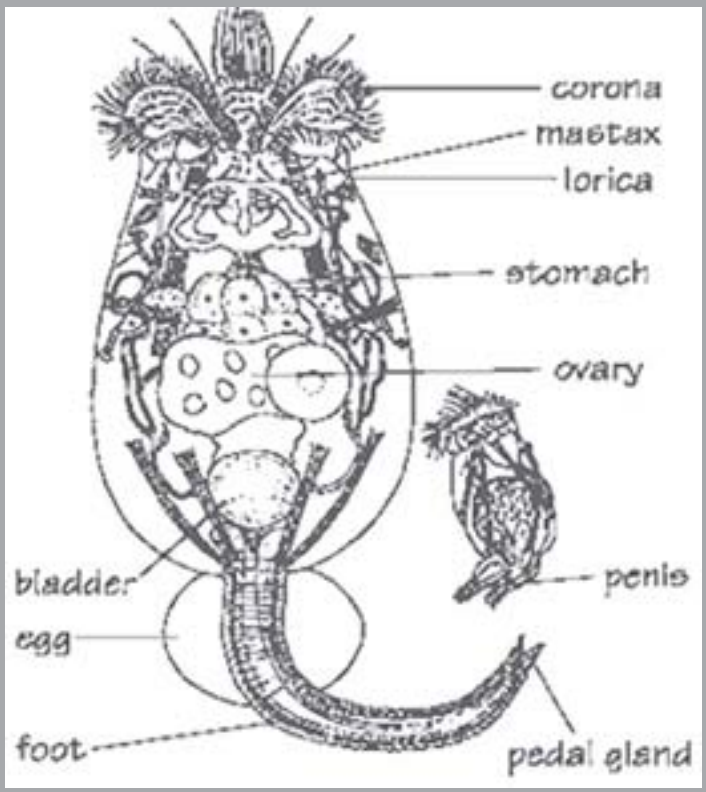


Fig. 13 - *Brachionus plicatilis*: female and male (modified from Koste, 1980)

Life history

The life span of rotifers is measured in days and depends on culture temperature, but in a controlled environment and at 25°C, it has been estimated to range around 7 days. At this temperature, larvae become adults after 0.5 to 1.5 days and then females start to lay eggs approximately every four hours. It is believed that a female can produce ten generations of offspring. The reproductive activity of *Brachionus* is also influenced by temperature as illustrated below.

Effect of temperature on reproductive activity of *Brachionus plicatilis* (after Rutner-Kolisko 1972)

Temperature (°C)	15	20	25
Embryonic development (days)	1.3	1.0	0.6
First spawning of females (days)	3.0	1.9	1.3
Interval between spawnings (hours)	7.0	5.3	4.0
Life span (days)	15	10	7
Number of eggs per female during its lifetime	23	23	20

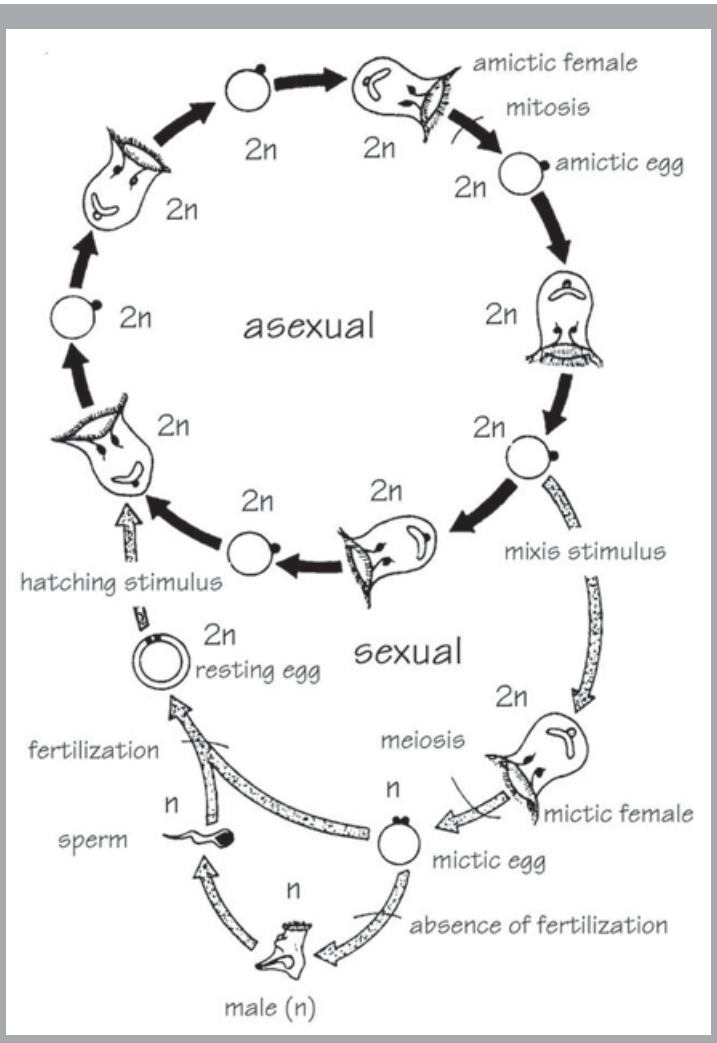


Fig. 14 - Parthenogenetical and sexual cycle of *Brachionus plicatilis* (modified from Hoff and Snell, 1987)

The reproduction of *B. plicatilis* can be either sexual (called mictic reproduction) or asexual (amictic or parthenogenetic reproduction). Only the latter is adopted for rotifer mass culture due to its faster rate and also due to the absence of males, which are useless as fish feed not having a functional digestive tract.

In the amictic reproduction the offspring are clones genetically identical to their mothers, i.e. all newly born rotifers are diploid females. Such multiplication can go on for months in a population kept in proper rearing conditions. Depending on environmental conditions, each female may produce about 20 amictic eggs.

Males are only produced after a sudden change in the environment, when females produces haploid (n chromosomes) eggs. Males and females breed, and the result is mictic resting eggs, analogous to *Artemia* cysts, which will hatch amictic females again.

Food

Rotifers are filter feeders, accepting small particles up to 30 µm in size including bacteria, algae, yeast and protozoa.

Saccaromyces cerevisiae, the common baker's yeast, is a common staple in the rotifer mass production process. Since in itself has no nutritional value for rotifers, it is believed that the bacteria associated with the yeast represent the true food. To improve rotifer culture and upgrade the nutritional value for fish larvae (in case of yeast-fed rotifers), during mass culture and before their harvest they are fed with special feeds and integrators.

Mass culture parameters and conditions

Rotifer mass culture is critical for larval rearing of gilthead seabream, which strictly depends for the first feeding from live and small preys that should be available in large quantities. What follows in the column marked as preferable range, gives a reasonable example of mass culture conditions to be maintained in order to develop rotifer cultures properly.

Parameter	Acceptable range	Preferable range
Temperature (°C)	20-30	25-27
Salinity (ppt)	1-60	18-25
Dissolved oxygen (ppm)	> 4	5-7
NH^{3}/NH^{4+} (mg/l)	6-10	-
NH^{3} (mg/l)	-	< 1
pH	5-10	7.5 - 8.5
Light (lux)	-	2000

Aeration is a critical element in rotifer culture with yeast and/or artificial diets. A proper balance must be maintained between:

- adequate dissolved oxygen level, i.e., at least 4 ppm;
- sufficient turbulence to keep rotifers and food in suspension;
- a culture medium without excessive turbulence, which causes stress and re-suspends bottom sediments (flocks).

Biology of the brine shrimp, *Artemia*

Taxonomy		
	PHYLUM:	Arthropoda
	CLASS:	Crustacea
	ORDER:	Anostraca
	FAMILY:	Artemidae
	GENUS:	*Artemia*

As the mouth size of marine fish larvae increases with age, rotifers are gradually replaced by the larger freshly hatched nauplii of the brine shrimp *Artemia salina*. Due to the larger size of its mouth at first feeding, seabass fry can accept artemia as a first prey, thus making rotifer supply not compulsory. Later on in the rearing process, and before weaning rations are given, larger *Artemia* metanauplii stages replace nauplii for larger fry.

The brine shrimp, a small crustacean living in salt ponds, represents an excellent prey for old or large fry due to its nutritional value and mobility in water, which makes it a perfect prey for the young fish. Moreover its easy and short production cycle and the widespread commercial availability of selected batches are added bonuses.

Artemia salina was considered for a long time as the only species belonging to the genus. But because of the substantial differences observed among the various *Artemia* strains found around the world, a global revision of the taxonomy of the genus is being considered even if it still remains unresolved. The differences between strains concern genetics, reproductive behaviour and physiology.

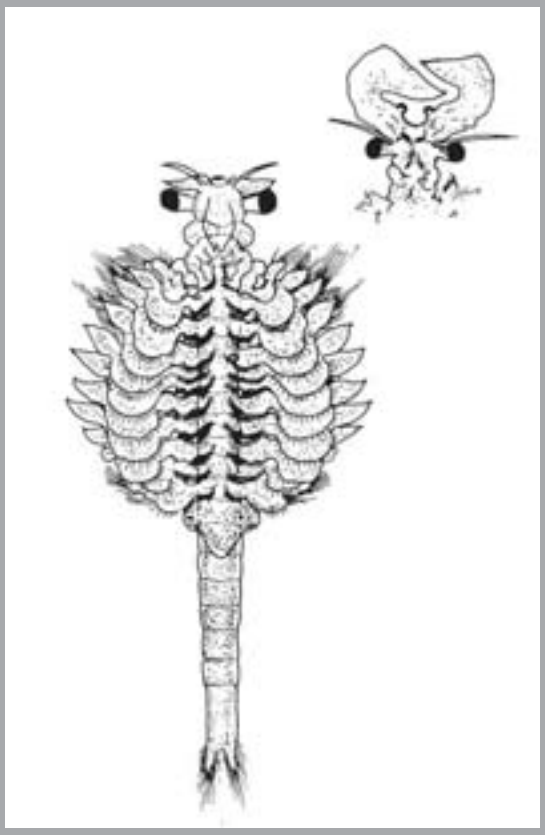


Fig. 15- Artemia salina adults, on the right, head of male

At present, a solution commonly adopted to simplify the taxonomy of the genus, is to define the species on the basis of its place of origin (Barigozzi, 1980).

Morphology and natural history

Brine shrimp most surprising characteristic is its ability to live in extremely hostile environments, such as salt lakes and man-made brine ponds throughout the world. They show a remarkable capacity to stand severe environmental conditions such as a water salinity values over 200 ppt, thus avoiding predators against which they are defenceless. Its body permeability to salts is very low when compared with other micro-crustacea. *Artemia* absorbs water from the medium and eliminates salt by defecation. In this way it can keep the correct osmotic blood values in spite of the hyperhaline environment in which it lives.

From an aquacultural point of view, the most important biological characteristic of *Artemia* is that it produces resistant cysts, containing embryos in diapause ("dormant stage"). Cysts are formed when environmental conditions become intolerable; their function is to protect the embryos against dehydration and against excessively high temperature and salinity values. Cysts preserve the inert embryonic life stage (gastrula stage) as long as they are kept dry or under anoxic conditions.

After hatching, the *Artemia* larva goes through about 15 molts with an initial size ranging from 400 to 500 µm. The first two larval stages, nauplii instar I, rich in yolk, and instar II, which has to be enriched with special integrators, are the most commonly used in fish fry feeding.

Under optimal conditions brine shrimp reach the 10-mm long adult stage in eight days and can live for several months, reproducing at rate of up to 300 nauplii or cysts every four days. The adults are characterized by an elongated body with two stalked complex eyes, a linear digestive tract, sensorial antennulae and 11 pairs of functional thoracopods. Males are easily recognizable for their pair of large muscular claspers (the 2nd pair of antennae) in the head region. Females bear the brood pouch or uterus behind the 11th pair of thoracopods.

Fig. 16 - Dehydrated artemia cysts

Parthenogenetic and bisexual *Artemia* strains exist, where ovoviviparous and oviparous reproduction alternates. Cysts' hatching gives ovoviviparous offspring; eggs are retained inside the uterus until embryonic development is fully completed (4-5 days) and free swimming nauplii are then released. Change from ovoviviparous to oviparous reproduction seems to be induced by under nourishment or even by an inadequate food quality, rather than by other abiotic factors.

Salt lakes and brine ponds with *Artemia* populations are found all over the world. At certain times of the year, large quantities of minuscule brown particles (200 to 300 m in diameter) float at the water surface and are brought ashore by wind and waves. This apparently inert brown powder is actually made of dry cysts of brine shrimp, which remain in diapause as long as they are kept dry.

Food

As a non-selective filter feeding, brine shrimp feed on particulate matter of biological origin, bacteria and algae of suitable size. While freshly hatched nauplii of selected batches represent an appropriate food for fish fry; older larval stages have to be given special feeds to upgrade their nutritional value for fish larvae.

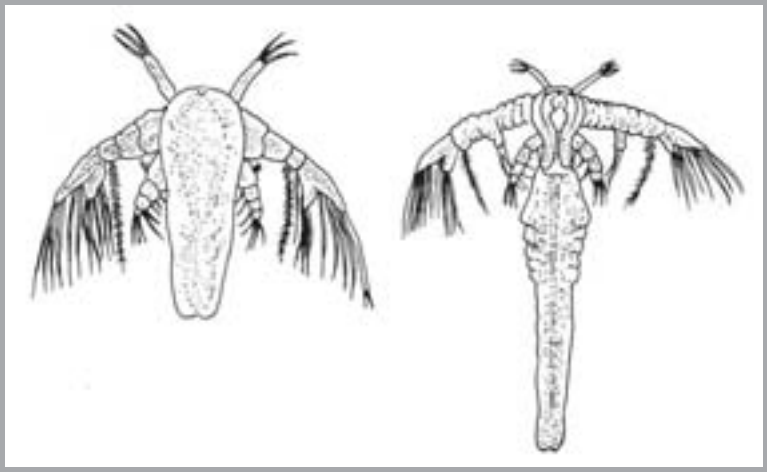


Fig. 17 -Nauplius and metanauplius

Rearing methods

Artemia rearing for aquaculture purposes follows two ways, nauplian production and biomass production. Nauplii and metanauplii are easily produced from hatching cysts in dedicated units. Cyst incubation takes approximately 24 hours at 30°C, in salty water under strong light and with aeration. Fully enriched metanauplii would require an additional 12-24 hours of rearing.

Biomass production takes a few days and adult artemia are harvested. These can be either immediately fed to fry, or be frozen to be used later. In recent years, however, this process has been progressively replaced with new and reliable artificial diets.

Artemia use in aquaculture

The use of *Artemia* nauplii as live food for the rearing of fish and crustacean larval stages, has been one of the most important steps in the development of marine aquaculture. With the exclusion of the rotifer *Brachionus plicatilis*, *Artemia* has almost entirely replaced the mass rearing of other live-food zooplankters that were tested earlier on by many researchers and producers but which were discarded for reasons of technical complexity or because they culture was economically unfeasible.

Fig. 18 - Harvesting of floating cysts with special boat

The production of resistant cysts has been the real advantage offered by *Artemia*. Cysts can be stored for years and live nauplii can be obtained after 24 to 36 h incubation. A reserve of "stored live food" has been the important guarantee needed in hatcheries, where it has simplified the planning of production activities.

The resistance to anoxic conditions of *Artemia* cysts made possible their storage in sealed containers, simplifying marketing and transport. Moreover, *Artemia* nauplii were found be an excellent food for fish and crustacean larval stages, even when used alone.

Even if *Artemia* cysts still remain an expensive product and if their market availability shows fluctuations, important progress has been made during the last years:

- additional sources of *Artemia* cysts have been found in all the continents and each strain has been classified with regard to quality (in nutritional terms) and hatching efficiency;
- hatching procedures have been standardized and new techniques have been introduced, such as cyst decapsulation, with a consequent improvement of cysts hatching efficiency and of the quality of newly hatched nauplii.

Fig. 19 - Harvesting of cysts by hand

Nutritional value of A*rtemia*

For the larval rearing of marine fish and crustacean species, *Artemia* nauplii and rotifers are the most commonly used live food preys. The advantages of *Artemia* are multiple: off the shelf and regularly available dormant cysts, they are easy to produce, are visible as prey and are highly palatable to the larvae

A major constraint, however, in the use of *Artemia* as a food organism for marine fish larvae is its variable nutritional quality. This problem can be circumvented with the use of enrichment techniques adding essential fatty acids (HUFA's). As *Artemia* is a non-selective filter feeder, it filters all particles of suitable size from its surrounding environment and special feeds can be given to improve its nutritional value. Enriched nauplii produce better performances in fish larvae in terms of growth and survival. Besides enrichment with HUFA's, other nutrients such as vitamins, as well as prophylactic and therapeutic drugs, can also be passed to the fish larvae via the *Artemia* nauplii acting as carriers.

According to the desired HUFA's levels in *Artemia,* different enrichment products and enrichment periods can be used. Maximum levels of approximately 60 mg/g DW are obtained with a 24-h enrichment with Super Selco. In all these cases, the final DHA/EPA ratio in *Artemia* is less than 1. For more sensitive species that need a higher DHA/EPA ratio, enrichment with DHA Selco is advisable; this results in a total HUFA's enrichment level similar to that obtained with Selco, though the DHA/EPA ratio exceeds 1.5.

PART 3

HATCHERY PRODUCTION PROCEDURES

HATCHERY PRODUCTION PROCEDURES

3.1- BROODSTOCK MANAGEMENT

Establishing the broodstock

To work properly and with adequate safety margins, any hatchery should secure a reliable and sufficient supply of good quality fish eggs. With this aim in mind, most Mediterranean hatcheries have established their own broodstock units, where breeders of different age groups of the species involved are kept under long term stocking conditions.

However, timing, amount and quality of the eggs produced by these units do not always fit hatchery requirements, resulting sometimes in poor final outputs in terms of viable fry.

The importance of a properly dimensioned and managed broodstock unit is now fully acknowledged and its relatively high running costs are fully justified.

In the management of a fish broodstock unit the main issues to be considered refer essentially to:

- stock dimensioning;
- stock collection;
- adaptation to captivity and long-term stocking conditions;
- sexual maturation and spawning according to the production schedule;
- renewal of the old breeders.

Stock dimensioning

To properly determine the dimension of the broodstock unit, different parameters have to be taken into consideration. First, the seasonal production target for the hatchery has to be set. In particular the desired number of fry, their size and species and delivery timing should be planned. To this purpose, it is important to set the average survival rate from larva to weaned fry. Second, the reproductive characteristics of the species, such as sexual cycle pattern, egg productivity and latency period (see Table 3.1) play a key role in stock dimensioning. A third important aspect is represented by the possibility to introduce a year-round supply of eggs, which requires the manipulation of photoperiod and temperature to obtain out-of-season maturation and spawning.

As described in Part 2 of the manual, both seabass and gilthead seabream are seasonal breeders spawning in winter and early spring, with some differences in timing according to the specific location and the species. But whereas seabass is a synchronous gonochoric species, gilthead seabream is a proterandrous hermafroditic species where males undergo sex reversal to became females. They also differ from seabass females in being sequential spawners, i.e. they can lay 20,000-80,000 eggs every day for a period of up to four months, moreover, in captivity sex reversal is conditioned by social and hormonal factors which may lead to unfavourable sex ratio if not properly considered in establishing groups of broodstock.

For practical purposes the following table presents some conservative parameters to be considered as guidelines when dimensioning the broodstock unit.

Species	Egg productivity as No.eggs per kg b.w.	Egg productivity as % of b.w.	Fertilization rate (%)	Hatching rate (%)	Viable larvae (No. Per kg per yr)[1/]	Latency (months)[2/]
Seabass	300,000	20-25	90-95	75-85	220,000	6
Gilthead seabream	800,000	50-80	90-95	70-80	560,000	12

Table 3.1 - Conservative propagation parameters under hatchery conditions for seabass and gilthead seabream

[1/] Average number of viable two days old larvae that can be expected yearly per kilogram of healthy female
[2/] Time between the introduction of brood fish in the hatchery and their first spawning .

Although these parameters are already conservative, to face any possible egg shortage during the spawning season, a safety margin in stock size is recommended. A practical way for planning purposes is therefore to consider the following average female fecundity per season:

seabass : 120,000 2-days old larvae per kg b.w.
gilthead seabream: 350,000 2-days old larvae per kg b.w.

Table 3.2 gives further information on size at sexual maturity and optimal size for spawning to be considered in broodstock dimensioning.

Species	Sex	Size at first maturity		Optimal size and age		Reform
		L or W	age (years)	wild kg (years)	farmed kg (years)	kg (years)
seabass	M	23-26 cm	2-3[1/]	0.6 (2-4)	0.7 (3-4)	> 1.5 (6)
		28-30 cm	2[2/]			
	F	31-33 cm	4-5[1/]	1-1.5 (5-8)	1.5-2 (6-8)	> 3 (9)
		37-40 cm	3[2/]			
gilthead seabream	M	100-300 g	1-2	0.3–0.5(1-2)	0.3-0.5 (2-3)	-
	F	>600 g	>3	0.8-1 (3-5)	1-1.5 (4-6)	> 2 (8)

Table 3.2 Size at first sexual maturity and optimal size for spawning
[1/] Tunisia [2/] Mediterranean France

In seabass, as fecundity and egg quality improve after the first spawning , the optimal age for female parent fish is between 5 and 8 years , whereas for males this range is lowered to 2-4 years.

When considering gilthead seabream, five age groups should be included in the broodstock, from age 1 (young males) to age 5 (old females). With this species additional precautions should be adopted. Sex reversal, which takes place at the end of the spawning season, approx. from May to September, is socially determined. In particular, the presence of young fish (potential males) during this period increases the number of older fish that become females. On the other hand, the presence of older females will inhibit sex reversal in younger fish which will remain functional males.

Furthermore, as in old fish the quality of eggs decreases, the farmer should annually replace the oldest age groups of both species with younger ones either from the wild or farmed.

A further aspect has to be considered in case hormones are extensively used to induce spawning. An improper use may induce female fish to not respond properly to repeated hormonal injections. See further for more details.

Origin, capture and handling of broodstock

Parent fish can come either from a farm or from the wild. Usually, wild fish are preferred since farmed animals could present some problems such as consanguinity or poor general conditions due to an unhealthy rearing environment. On the other hand, the use of farmed animals gives the possibility to select breeders on the basis of observed characteristics of the animals (fast growth, commercially preferred pigmentation and shape, domestication, etc.).

Wild fish have to be conditioned to captivity, which generates stress that will inhibit their sexual maturation for a certain time (latency period). It is safer to consider that new broodstock should be kept in the farm for at least six months before being used as breeders.

Fig. 20 Broodstock harvesting (photo STM Aquatrade)

The main selection criteria to identify adult fish as suitable breeders are not scientifically stated, but follow the common sense. With the advance of genetic studies, more specific criteria will probably become available related to faster growth rate and stronger disease resistance. For the time being, the following empirical selection criteria for breeders have a proven record to work for hatchery purposes:

- normal body shape and colour;
- absence of skeletal deformities;
- overall healthy status (absence of large wounds, haemorrhages, infections, parasites and necroses);
- normal behaviour such as a quick response to food distribution, fast swimming, controlled buoyancy, etc.;
- the largest size within its age group ;
- the best growth and food conversion rate within its age group .

In capturing and handling breeders, stress should always be minimised. This can be partly achieved by selecting suitable gear. The best fishing gear for wild fish is the fish barrier, a fixed trap placed at the outlet of coastal lagoons. As fish enter the trap following their migratory instinct, they can be captured without much stress.

The seine net used in ponds and tanks gives also good results but it has to be handled very carefully, harvesting only a small number of fish per haul. To avoid losses of scales, the use of knotless 15-mm stretched mesh nylon nets, as well as cotton gloves, is recommended.

Trammel nets and monofilament gill nets should be avoided because they may cause unrecoverable skin damages.

Healthy adult fish can also be captured by hook and line, but care should be taken to reject all fish with major injuries in their mouth, gut or, even worse, gills, as well as those which have lost too many scales when hauled on board.

To spare additional and potentially fatal stress to recently caught fish, the temporary holding and transport containers should have the following characteristics:

- be heat insulated;
- be filled with sea water coming from where the fish have been caught;
- be round in shape or square with rounded corners to avoid skin abrasions and mechanical shocks;
- be large enough to allow the fish a fair degree of movement.

Additional precautions are:

- keep fish density inversely proportional to transport time and water temperature;
- maintain oxygen saturation around 100%;
- take the animals to the hatchery as soon as possible: speed and care are always recommended when handling gilthead seabream and seabass breeders.

For fish transport in which oxygen is provided, consider the following:

- for short transport under favourable climatic conditions, fish density should not exceed 30 kg/m^3;
- for long transport , limit density to 10-15 kg/m^3 and use anaesthetics (at a level of sedation only);
- always monitor dissolved oxygen saturation levels during transport; safe values ranging from 85 to 120%.

Selection and quarantine treatment

Upon arrival at the hatchery, fish are anaesthetised and checked using the selection criteria mentioned above. Selected fish are then weighed, their sex is checked and they are immediately transferred to already prepared “quarantine tanks” to receive their first prophylactic treatment, as indicated in the quarantine protocol (Annex 1). The treatment is given as soon as fish enter the hatchery facilities: this limits the risk of introducing parasites or bacterial diseases and facilitates their recovery from handling stress. Fish not selected are discarded or sold.

The most commonly used anaesthetics for fish are: MS 222 (@20-50 ppm), 2-phenoxyethanol (@200-300 ppm) and quinaldine dissolved in acetone (@3-5 ppm). Excessive manipulation and sudden changes in water temperature and salinity have to be avoided.. Always operate gently when getting hold of brood fish, raising the fish with both hands palms up from the lower body (one below the head, the other below the anus). Never touch them with dirty or dry hands, wash them and dip them in the holding container water to have your skin well wet prior to touch the fish. The use of cotton gloves to handle fish is recommended.

The quarantine protocol (Annex 1) is designed to eliminate possible external parasites, as well as to seal wounds and abrasions caused by fishing, handling and the parasites themselves. The treatment usually followed in hatcheries is a sequence of medicated baths: first formalin (water solution with 37-40% by weight of formaldehyde, HCHO) and malachite green (zinc free oxalate or aniline green), followed by a second quick immersion in fresh water to end with a third bath in furanic antibiotics. This treatment is repeated four times every three other days. The entire procedure takes a couple of weeks. No additional specific treatment is required as fish are assumed to be healthy.

Quarantine tanks should have a flow-through water circulation, round or rounded shape, small size (4 to 6 m^3) and a smooth inner surface to allow for rapid cleaning, easier harvesting and reduced use of chemicals for treatment baths. The recommended building materials are: FRP, PVC, PE or plastic-lined concrete tanks.

It is mandatory that quarantine facilities do not come into contact with other rearing units through effluents or shared equipment. They must be completely isolated from the other farm facilities to prevent possible spread of parasites and diseases, sometimes tolerated by adult fish, but often deadly for their larval stages. Thus, the effluents from quarantine tanks should be treated to remove pathogens. After the transfer of brood fish, these tanks should be drained and thoroughly disinfected with a 500 ppm hypochlorite (NaOCl) solution.

Stocking facilities

After quarantine, parent fish are moved either into larger tanks where they remain for a couple of months until full recovery, or directly into the long-term holding facilities. Lowering salinity down to 20 ppt for a few days helps the recovery of weak animals. In any case, the weight of each female fish is recorded to estimate its potential egg output (see Table 3.1). For gilthead seabream weight gives also an acceptable estimate of the broodstock sex ratio (see Table 3.2).

Long term stocking facilities exist in a variety of designs and capacities. When land area is not a constrain, earthen ponds stocked at low density (up to 0,5 kg/m^3) represent a reliable and easy to manage solution. They usually measure up to 500 m^2 in size with an average water depth of 1.5 m and have a rational water exchange with inlet and outlet systems through loosely screened monks to allow small fish and crustaceans, which may represent an additional source of food, to enter the pond. The outlet offers the possibility to empty the pond

Fig. 21 Traditional earthen pond for broodstock long-term holding (photo STM Aquatrade)

by gravity. During the hottest months, a shelter should cover at least 10% of the pond area to provide some protection against the sun and a quiet place to rest. If necessary, protection against fish-eating birds should also be contemplated. Another cheap solution to stock broodstock is given by floating cages, provided that suitable sheltered coves are available. Fish control is however less easy than in land-based facilities.

More frequently, and depending from the design of the hatchery, stocking facilities are smaller tanks of 4-20 m^3 capacity either round, or square with rounded corners,: They are made of concrete, FRP or PVC-lined, and the complete control of environmental conditions favours a higher fish density up to 2-5 kg/m^3. Such tanks are also utilised to produce out-of-season spawnings (see below).

Dissolved oxygen levels in water should be maintained at near saturation with an adequate water renewal and aeration supply. Values below 50% saturation, as well as abrupt changes in other water quality parameters should be avoided. During gonadal maturation, water salinity has to be kept above 35 ppt to complete oogenesis.

Routine controls are necessary for proper management and include checking water quality parameters (salinity, temperature, dissolved oxygen, pH, etc.) and fish stock condition (general behaviour, feeding activity, diseases symptoms, prophylactic treatments, etc.). See Annex 2 for a model of the forms to be used for tank files with main daily controls and operations.

Feeding broodstock

Although studies on the effects of diets on the reproductive capacity of seabass and gilthead seabream are far from being complete, it is generally acknowledged that a diet rich in vitamins, poly-unsaturated fatty acids (n-3 PUFA) and other micro-nutrients is essential in obtaining viable eggs and larvae.

For practical purposes, two distinct feeding regimes are applied : a maintenance diet after spawning till the onset of the next ovogenesis period, some three to four months before the next spawning season, and a boosted diet thereafter to provide the essential nutritional requirements for proper gametogenesis (Annex 3).

Maintenance diet

The maintenance diet should keep spent fish or new fish breeders in good health untill the onset of the gametogenesis. It should therefore be rich and varied both in quality and quantity, and should be assessed by regular controls. It should preferably be as close as possible to the fish natural diet, including fatty and lean fish, crustaceans and molluscs. According to availability from the local fishery and suppliers, trash fish may represent a cheap solution, paying due attention to the increased pollution load in the stocking facilities. To keep their original quality, trash fish should be purchased fresh and then cleaned, minced and deep frozen immediately. This process also lowers the risk of parasitic infection .

It is advisable to get broodstock used to pelletized dry feed in order to have an alternative to fresh food at hand in case of shortage. Moreover, dry feed is useful when drugs or other feed integrators have to be supplied to the fish. The use of automatic feeders is only possible with dry pellets.

Even if the choice between fresh food and dry feed depends on several factors such as their availability, cost, use of feeding equipment and management, it is advisable to use both feed types in order to benefit from a broader range of possibilities. A practical solution envisages the distribution of pellets 6 days a week supplemented with moist food twice a week, and with no feeding one day a week, typically on Sunday. This pattern reduces the workload to manageable proportions, and still provides fish with a proper diet. The daily feeding rate usually ranges between 0.7% and 1.4% of body weight in seabass and 1 to 1.5% in gilthead seabream, both adjusted in line with water temperature and physiological status of the fish (Annex 3).

From a management point of view, a feeding schedule should be prepared at regular intervals based on periodical controls of fish weight. Feeding by hand is recommended because it would be possible to prevent food leftovers, which may rapidly deteriorate water quality, and to observe the behaviour of broodfish. Food is usually given once a day, early morning or late afternoon. Water renewal in the tank is adjusted to keep DO levels at

saturation and ammonium nitrogen below 1 ppm. Pollution caused by feeding fresh food can be controlled by using a flow-through water system and frequent cleaning. When broodstock is kept in a system using water recirculation, a mechanical filter to remove suspended solids is frequently added to improve the performance of the biofilter.

Breeding period diet

During gametogenesis female fish require a food richer than usual in proteins and lipids to produce the vitellogenin, which is progressively stored as yolk in the oocytes. As the sole source of food for the developing embryo and the early larval stage until feeding on live preys starts, yolk quality and quantity are key factors for a successful reproduction.

Both dry pellets and moist food are employed in this period. Dry pellets should include all the nutritional elements which are acknowledged to be essential in the development of viable larvae, such as the poly-unsaturated fatty acids (n-3 PUFA), in particular EHA (20:5 ω 3) and DHA (20:6 ω 3), which have to be supplied with food, as they cannot be produced by fish metabolism . In case of poor diets, the perivisceral fat of the females, rich in saturated fatty acids, is utilised for yolk production, resulting in poor egg quality and reduced larval viability.

Commercially available integrators are now widely adopted by modern hatcheries to boost the quality of pelleted feeds formulated only to grow fish. As a better alternative, specially formulated pellets or natural enrichment components such as squid oil are fed to the breeders. As a general rule, during this period the distribution of moist food should replace most of dry feed, if possible, due to its superior nutritional value.

Feed distribution follows the pattern mentioned above. A difference between the two species comes from their different spawning characteristics. As a syncrhonous spawner, seabass is not fed during its brief spawning period, but gilthead seabream, with its sequential emissions lasting several weeks, should always be.

Egg production

Gametogenesis

The natural process of sexual maturation in both species is briefly described below. By acting on photo and thermo-periods, it is also possible to obtain viable larvae almost throughout the year. A method to delay spawning is described at the end of the chapter.

Under natural rearing conditions, and with variations linked to the geographical location, the sexual resting period (only oogonies present in the female gonads) lasts from the end of the spawning season till early autumn when gametogenesis starts. This process is regulated by a complex hormonal and environmental pattern, which has been better investigated in seabass.

Species	Period (length)	Temperature (°C)	Hours of light (hours)	Duration (days)
Seabass	Sept. to Jan. (3 months)	20-8	14-8.5	16-95 mean: 74
Gilthead seabream	Sept. to Dec. (3-4 months)	20-9	14-8.5	30-154 mean: 107

Table 3.3 – Gametogenesis: environmental parameters and duration

Both species may start ovogenesis in water with a salinity lower than 35 ppt, such as in the case of estuaries and coastal lagoons, but the final steps leading to maturation (exogenic vitellogenesis and meiotic divisions) require full seawater (= 35 ppt). Temperature has an effect on the speed of vitellogenesis and acts as a minimum/maximum threshold for the spawning.

Spermatogenesis in seabass and gilthead seabream males usually takes place under captivity conditions and even at salinity levels lower than full seawater. Spermiation starts one or two months in advance of the female spawning period and males may remain active throughout the entire spawning season.

a) Stage 1: oogonia
b) Stage 2: young oocytes
c) Stage 3: individualization of oocytes and appearance of follicular cells
d) Stage 4: differentiation of oocyte cytoplasm
e) Stage 5: separation of three areas in cytoplasm
f) Stage 6: early signs of vitellogenesis and start of membrane differentiation

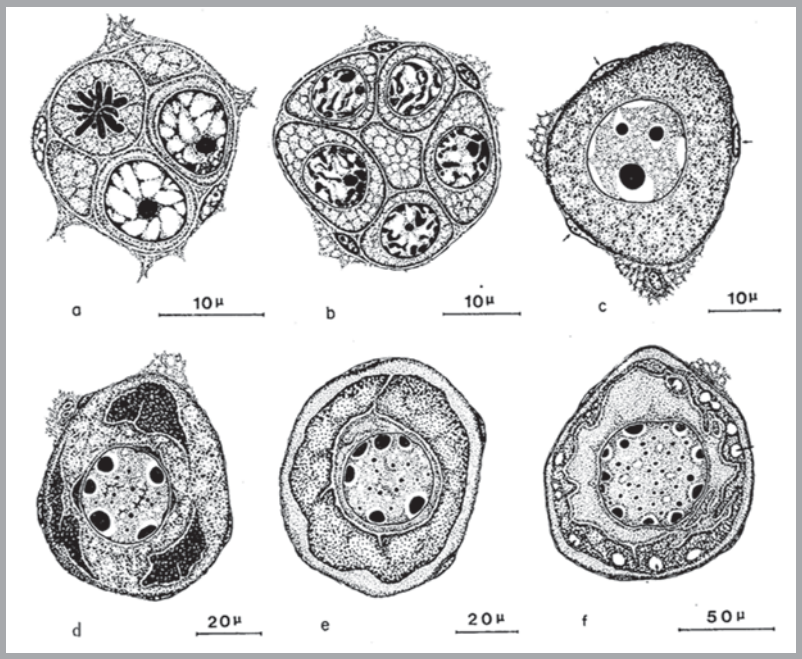


Fig. 22.00 Ovarian development in *Dicentrarchus labrax*

g) Stage 7: organization of yolk globules in perinuclear layer
h) Stage 8: a second type ot yolk globules are evident and the zona radiata appears
i) Stage 9: distribution of the two types of yolk globules
l) Stage 10: a third type of yolk globule appear
m) Stage 11: end of vitellogenesis and polar migration of the nucleus
n) Stage 12: the oocyte with oil droplet (G.h.) homogeneous yolk (V.) and cortical granules (G.c.).

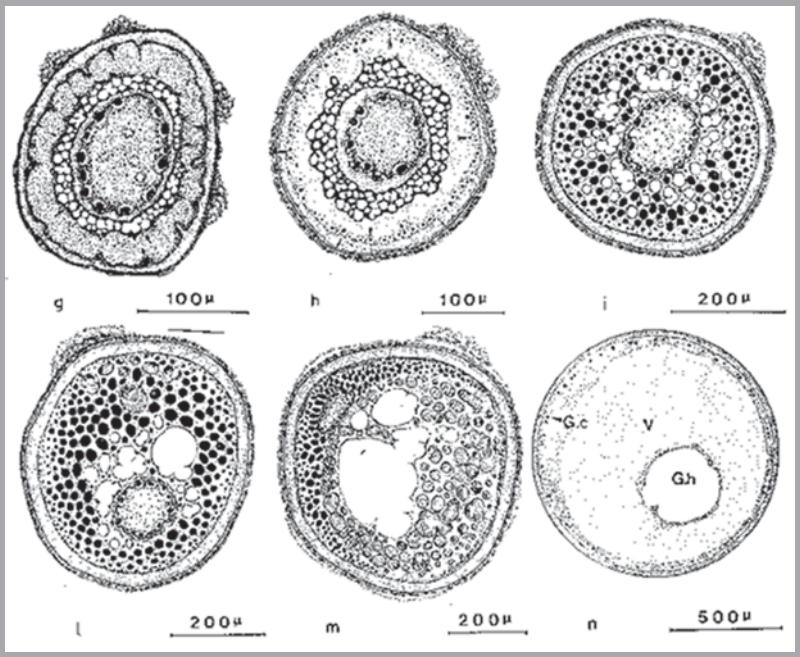


Fig. 22.00 Ovarian development in *Dicentrarchus labrax*

Species	Optimum range	Min. temp.	Max. temp.	Gametogenesis blocked
Seabass	13-15	9-10	18	21-22
Gilthead seabream	15-17	13-14	20	24

Table 3.4 - Spawning: temperature range and limits (°C)

Stocking broodstock in the spawning tanks

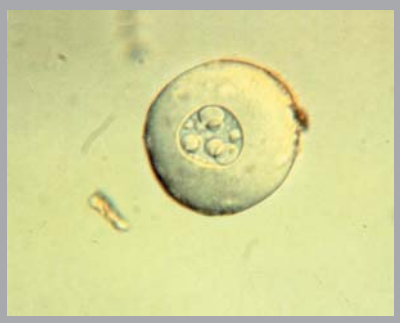
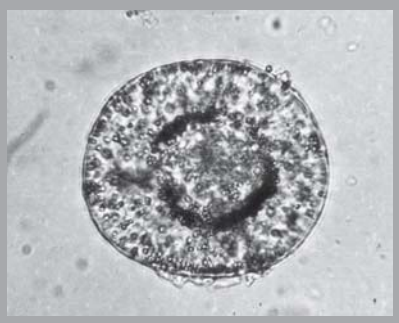
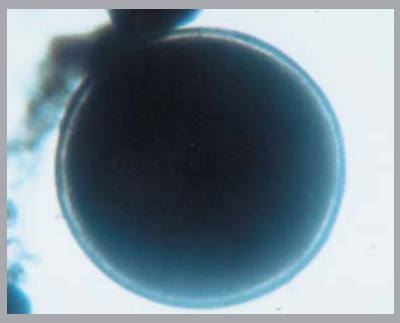
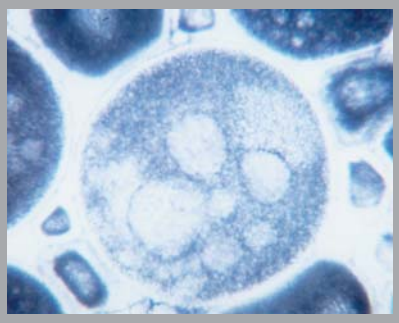
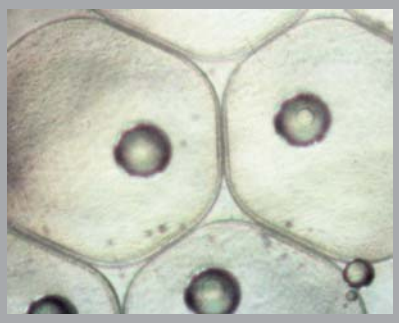
At the onset of the spawning season it is necessary to move selected batches of breeders from their long term holding facilities to the spawning tanks, where they can be better treated and their performance can be easily monitored.

The selection of suitable fish should take into account their health status, sex and maturation stage. Sex ratio in the spawning tanks is kept at two males per female. Whereas males are choosen when they release sperm spontaneously or on stripping, the females maturation stage has to be ascertained by extracting oocytes from the ovary with the use of a catheter: only females with oocytes in the late-vitellogenic stage, i.e. with a diameter larger than 650 μm in seabass and 500 μm in gilthead seabream, are selected.

Ovarian controls start in September and last up to March in gilthead seabream and from December through February in seabass. In case of necessity, wild breeders of both species caught and stocked during the same spawning season could also be utilised, provided that their ovarian development matches the above mentioned requirements and provided also that they receive a complete quarantine treatment. However, due to fishing and handling stress, in the case of these breeders egg output can be erratic as ovarian atresia may follow.

For an ovarian biopsy, proceed as follows:

1. All equipment has to be ready, cleaned and sterilized in advance.
2. Fish are not fed 24 hours before the control;Close water inlet and lower the water level; place a net barrier to divide the tank into two compartments, keeping all fish being concentrated in one of them;
3. Add anaesthetic in sufficient quantity to obtain a light sedation (for type of drug and dosage see previous section); On a tray prepare one glass slide per female, a couple of flexible sterile catheters (1.2 mm internal diameter or 8 to 10 Berchet unit), a Pasteur pipette, cover slides and alcohol;
4. Take one female at a time from the tank (males have already been selected and released into other side of the net barrier);Introduce the sterile catheter into the urogenital papilla and the oviduct, up to the ovary for a few cm; then suck carefully a small sample of oocytes up into the catheter and place the sample on a slide;
5. After sampling, release the animal into the spawning tank, where recovery from sedation will take place; Put a few drops of sea water on the sample and a cover slide, and examine under the microscope at 5 and 10 magnifications. Using a micrometric eyepiece, measure the diameter of the bigger oocytes and record egg measurements and final destination of the fish;
6. Place the catheter in alcohol and sample the next female in the same way with the other catheter. Repeat sampling alternating catheters;Handle fish with care (use cotton gloves) and do not damage the urogenital papilla area when introducing the catheter.
7. Check oxygen levels frequently


1 previtellogenic oocyte
2 oocyte at the lipid vesicle stage
3 oocyte at the tertiary yolk stage
4 oocyte at the migratory nucleus stage
5 mature oocytes
6 overripe oocyte
7 atretic follicles

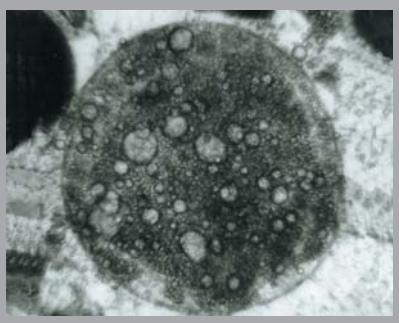


Fig. 22.01-2-3-4-5-6-7 Follicular stages observed in ovarian biopsies of gilthead sea bream (photos A. Francescon and A. Barbaro)

Induced spawning

Egg release can be obtained either by natural spawning or inducing it by hormonal treatment. Natural spawning of both species is not uncommon to achieve in confined environments and its unpredictability in terms of both timing and output is a serious obstacle for proper planning of commercial fingerling production . Induced breeding represents therefore the almost obliged choice of most commercial hatcheries. The hormonal treatment is intended to trigger the last phases in egg maturation, i.e. a strong egg hydratation followed by their release. However, if eggs have not yet reached the late-vitellogenic (or post-vitellogenic) stage, the treatment does not work, hence the need for an ovarian biopsy.The human chorionic gonadotropin (hCG) was widely used for both species at a dosage of 800-1,000 IU per kg of body weight in seabass and 250 IU in gilthead seabream. This dosage is usually delivered in two injections in the dorsal muscles, 6 hours apart. The hormone is diluted in a 0.9% sterile saline solution as 2,000 IU per 1 ml. The hormone is easily available, and comes along with vials of saline solution. However, hCG treatment presents some serius drawbacks: not all females respond to it, egg quality may be below acceptable standards (hatching rate below 80%), being a large molecule provokes an immunization reaction , and as a result of it fish treated with this hormone cannot respond when treated again the following season. Finally it is less effective in inducing out-of-season spawning. HCG has therefore been successfully replaced by an analogue of the luteinizing hormone-releasing hormone [LH-RHa des-Gly10 (D-Ala6) LH-RH ethylamide, acetate salt]. Its small molecule (10 peptides) acts on the pituitary gland to induce the release of gonadotropins which, in turn, act on the gonads. Almost 100% of injected fish spawn eggs whose quality usually matches that of natural spawnings. No immunization effect is reported in fish treated with LH-Rha because of the small size of the decapeptide.

The native form of this hormone is not used because of its low bioactivity, due to the deactivation by specific peptidases, which cannot deactivate the analogue form. Its dosage and response are given in Table 3.5. In seabass LH-Rha is delivered with two intraperitoneal injections 4-6 hours apart at 5 and 10 mg/kg b.w. respectively. In gilthead seabream hormonal dosages are much lower. The hormone is dissolved in a 0.9% sterile saline solution as 5 µg per 1 ml.

Species	Min.oocyte diameter (µm)	HCG (IU/kg b.w.)	LH-RHa (µg/kg b.w.)	No. of Injections (hours)	Latency (hours)
Seabass	700	800-1,000	-	2 (6)	48-72
	650	-	5-10	2 (4-6)	72
Gilthead seabream	500	100-250	1	2 (4-6)	48

Table 3.5 – Induced spawning. Minimum oocyte size and hormonal treatments.

Stocking facilities for spawning

The spawning unit should be kept separated from the main hatchery building to avoid disturbance to the spawners and any possible risk of disease contamination. However, for economic reasons it is usual to keep the spawning fish inside the hatchery in a dedicated area. Spawning tanks usually have a water capacity of at least 5 m^3. They should preferably be round shaped and 1 m deep. Shape and depth should be such as to provide easy access to fish and to facilitate routine operations such as cleaning, fish replacement, feeding, egg collection and controls. Very often spawning tanks are made of FRP or in epoxy-painted reinforced concrete.

As a general rule, water circulation is a flow-through system, with heated and filtered sea water to keep the desired rearing conditions. Water temperature is kept within the limits of the optimal spawning range avoiding abrupt changes when moving fish from outdoor facilities. Salinity should be above 35 ppt to improve buoyancy of fertilised eggs.Optimal stocking rate is 2 to 3 kg/m^3, with a maximum acceptable load of 5 kg/m^3. Sex ratio is two males per female. See annex 4 for the cleaning operations and annex 5 for the daily work plan in the spawning tanks.

Out-of-season spawning

When fertilised eggs are required outside the natural spawning period, out-of-season sexual maturation is obtained through environmental phase shifting of the gametogenesis by manipulation of photoperiod and temperature. The tecnique is successfully applied to both seabass and gilthead seabream and out-of-season spawning is now a current practice in many large Mediterranean hatcheries.

Different methods can be applied for out of season spawning:

- fish are kept under compressed photoperiods and temperatures cycles (the commonest);
- fish under constant day length are exposed to brief periods of long or short days;
- fish live in different 12-month long natural cycles, but shifted by three months each.

The following description refers to the last phase shifting method as it gives fertilised eggs year round. The hatchery management can decide on the periods of egg production according to its marketing and/or farming needs.

The broodstock is divided in four groups including both males and females: three groups are exposed to environmental regimes that are shifted by 3, 6 and 9 months respectively compared to the natural environmental regime, which is left for the fourth group. In this way, the hatchery will have a group of fish ready to spawn on each season: in winter the parent fish exposed to natural environmental conditions, in spring, summer and autumn the other three groups. Shifting should start when fish are still in the resting phase of their sexual cycle.

If breeders are properly managed, eggs produced out of season with shifted cycles do not differ significantly in quality and quantity from the in-season eggs.

An out of phase maturation unit requires specific facilities :

- an indipendent sector equipped with tanks suitable for long term stocking, where light and water temperature conditions can be set independently from the natural cycle;
- a timer-controlled lighting, preferably equipped with a dimmer to avoid abrupt changes in light intensity (and to create a twilight effect);
- a water heating/cooling system (usually heater and heat-pump);
- a computerized control of temperature and photoperiod.

3.2 - PRODUCTION OF LIVE FEEDS

Introduction

The early life stages of seabass and gilthead seabream are zooplankton-feeders, i.e. they prey on small free living planktonic animals. As no artificial larval diet can at present totally fulfil their nutritional requirements, their successful rearing still depends on an adequate supply of high quality live feeds, usually in the form of rotifers (fed on unicellular algae) and brine shrimp (*Artemia spp).*

This chapter describes the equipment and operation to mass produce these organisms, whose biology has been presented in Part 2. The design of the hatchery sections for production of live feed is described in the Design and Engineering part of this manual.

The technology for phytoplankton and zooplankton mass production has become very reliable and the production of live feeds is part of the standard working procedures in Mediterranean hatcheries. The efficiency of this part of the hatchery mainly depends on the implementation of standard procedures by well trained staff.

As live preys for first postlarval stages of seabass and gilthead seabream, two small animals are extensively used:

- all-female (amictic) populations of the rotifer *Brachionus plicatilis* (60 - 350 µm in length);
- larval stages (nauplii and metanauplii) of a small crustacean, the brine shrimp *Artemia spp.* (length: 400 - 800µm).

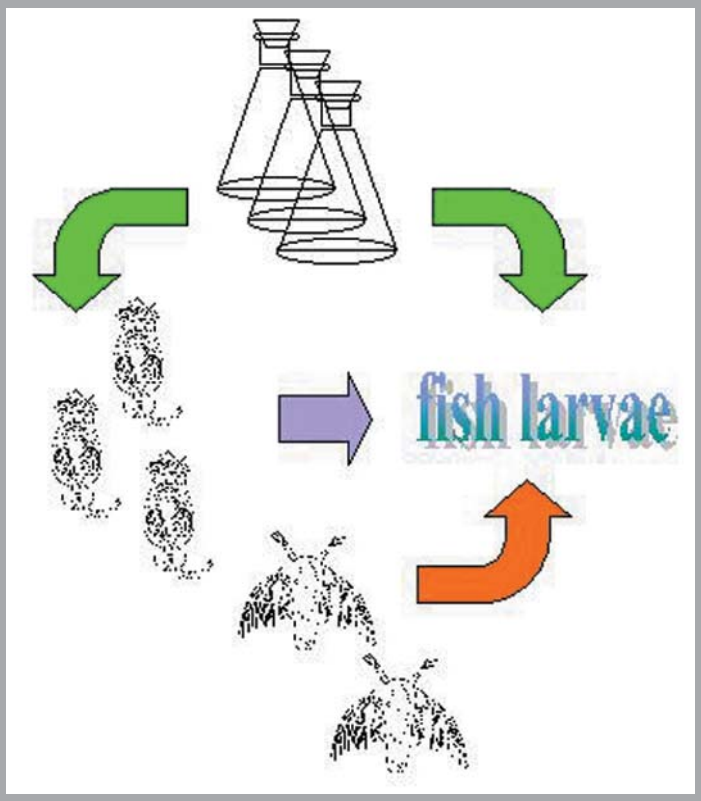


Fig. 23.00 A sort of simplified trophic chain is established in the hatchery

In farms dealing with seabass only, live feed production is often limited to the hatching of *Artemia* nauplii, which are obtained by incubating their dry resting eggs (cysts). The mouth of the seabass at first feeding is large enough to gulp brine shrimp nauplii and these larvae do not require the smaller rotifers as first feed, as it is in the case of gilthead seabream. The hatcheries working on both species, or on gilthead seabream alone, have to produce rotifers as well as microalgae.

As said in previous sections of the manual microalgae are now used not only for rotifer production (see below), but also to improve water quality in the larval tanks, creating the so called "green water", which is used during the initial rearing phases.

In case of rotifer mass production, clear advantages of this organism are given by its fast reproductive rate and by the high densities that can be reached in the rearing facilities, up to 1,000 rotifers per ml and over. The daily increase of their populations ranges between 50 and 150%, depending on the production technique chosen and the nutritional value of their diet. Their main drawback is that to culture them microalgae are needed as food, at least in the initial steps. However, for their final production process in large volumes (see below) there are now good artificial feeds which can replace algae, whose mass production remains unavoidable at least in gilthead seabream production for the "greenwater.

On the other hand, the production of *Artemia* is greatly facilitated by the availability of dry resting eggs, which can be purchased from specialised suppliers. If properly canned and stored, brine shrimp cysts can remain viable for years.

As rotifers and nauplii are produced to fulfil the needs of the larval rearing unit, they have to be available at given times, in pre-set quantities and with their nutritional quality intact. To achieve this, the design and operation of the culture systems should pay special attention to the following points:

- adequate dimensioning of the production facilities, including additional space for back-up cultures as a precaution against culture collapses;
- proper daily renewal and up-scaling of the cultures (standard operating procedures);
- maintenance of strict hygienic conditions in the culture environment (cleaning procedures);
- close control of culture conditions (monitoring procedures).

Mass culture of microalgae

Mass production of phytoplankton for rotifers and "green water" in most Mediterranean hatcheries is limited to a few species such as: *Chlorella sp*, *Isochrysis galbana*, *Pavlova lutheri*, *Nannochloropsis oculata* and *N.gaditana*, *Dunaliella tertiolecta* and *Tetraselmis suecica*. These species have been selected on the basis of their size, nutritional value, culture easiness and absence of negative side effects, such as toxicity. Their nutritional value shows a great variability not only among different species, but also in genetically different populations of the same species (strains). For hatchery purposes, the species to be cultured should both fit well the local rearing conditions and have a high nutritional value for rotifers. The increasing availability of nutritional boosters as enrichment diets for both rotifers and brine shrimps, has made this choice easier.

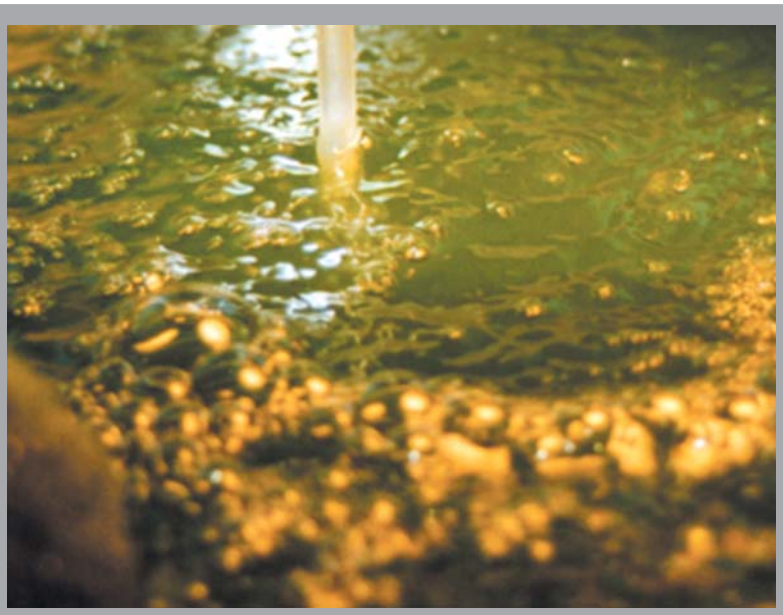


Fig. 23.01 Mass culture of microalgae (photo STM Aquatrade)

Population dynamics

Microalgae population dynamics can be described by different phases:

- the lag-phase, where, just after the inoculum, the cells increase in size, but not in number, and begin to absorb the nutriens supplied with the culture medium;
- the log-phase (or esponential phase), where cells reproduce very fast and population growth is exponential;
- the transitional phase (or declining growth phase), where growth rate slows down;
- the stationary phase, where cells remains constant in number and reproduction is balanced by death;
- the decline phase, where cell number decreases since death rate exceeds growth.

It is advisable to harvest phytoplanktonic organisms during their log phase, since in the new culture they will grow more rapidly and will yield a more viable population.

Mass production systems

For aquaculture purposes, microalgae are mass produced in three main ways: (i) batch (or discontinuous or multistep back-up system) culture, (ii) semi-continuous culture, and (iii) continuous culture.

In the batch culture a small axenic stock culture produces a series of cultures of increasing volume where the algal population of each culture vessel is entirely harvested at or near its peak density, i.e. while still conserving

a good growth potential, to be used either as inoculum for other culture vessels, or to feed rotifers or be used in fish larval tanks. It typically makes use of small (few liters) to medium size (500 liters) containers, and it is kept indoor and under strictly controlled, if not properly axenic, conditions. It is considered by many authors the easiest and most reliable method of algal production, provided that the working protocol is strictly enforced. Algal quality is less erratic than in the semi-continuous method, even if the latter is more productive for any given volume.

In the semi-continuous system the algal population, when mature, is partially harvested at intervals. The harvested culture volume is replaced by fresh medium to keep growth going on. This culture is adopted to produce large amounts of algae and frequently uses large outdoor tanks. Their main drawbacks are: (i) the unpredictable duration, (ii) the risk of contamination by other organisms as competitors (other microalgal species), contaminants (bacteria) and predators (ciliate protozoa feeding on the algae), as well as (iii) the building up of metabolites, which can affect quality.

The continuous system is a steady-state continuous flow culture in which the rate of growth is governed by the rate of supply of the limiting factor. It is a balanced axenic system where the algal population is harvested and fertilised continuously. This method, though the most efficient over extended periods, produces limited amounts of high quality cells and requires complex equipment as well as advanced management. A relatively recent development of this system is represented by the photo-bioreactor, a continuous culture device that increases the density of cultured microalgae to very high levels under predictable environmental and microbiological conditions.

Fig. 23.02 Old fashioned unit using artificial light for algae mass culture (photo M.Caggiano)

The microalgae produced can be concentrated to a dense liquid suspension by centrifugation, and can then be stored for more than one month in the refrigerator, still giving excellent viability when used. A new industry is now appearing, whose concentrated algal products can also fulfil the hatchery needs, saving the time-consuming and expensive production of microalgae in the hatchery.

The system described below is the batch culture, by far the most widely adopted method by Mediterranean hatcheries. Before its description, additional instructions are given concerning facilities, the preparation of the culture medium, and the equipment required.

Mass culture facilities for microalgae

Algae are cultured in a dedicated sector of the live feeds production section, which is made of three working areas inside the hatchery building: a lab for duplicating small cultures, a conditioned room to maintain small culture vessels and pure strains and finally a large area for the mass cultures in PE bags or, less frequently, tanks. In the warmest Mediterranean areas, a light greenhouse can replace the latter.

Small volume cultures are kept in vessels ranging from 20-ml test tubes up to 18 l carboys. They can be made of borosilicate glass, polycarbonate, PET or any other material able to stand a sterilization process. These vessels are placed on glass shelves lightened by fluorescent tubes and equipped with a CO_2 enriched air distribution system.

Hot-extruded tubular PE film is utilised for larger volumes bags. The film is usually 0.25 mm thick and its stretched width ranges from 45 to 95 cm. Two bag designs are widely adopted in Mediterranean hatcheries: the smaller suspended bag and the larger one placed within a steel wire cylindrical frame. The first type has a capacity of 60 l (single) to 150 l (double or U-shaped), whereas the latter, that stands on a saddle-like GRP base to improve circulation, can contain up to 450 l. Their top is closed by a plastic cover to prevent contamination.

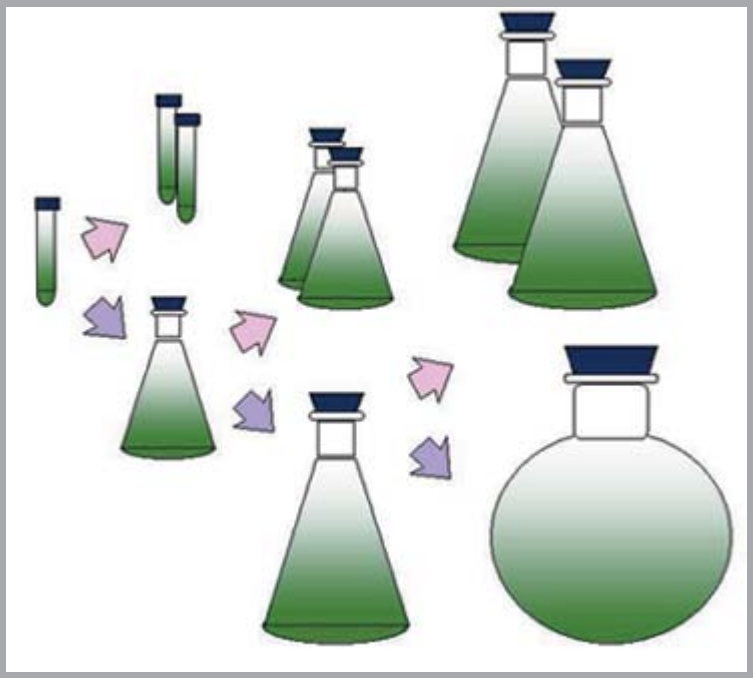


Fig. 24.00 A typical scheme of a batch type production

All units are equipped with artificial lights, usually fluorescent tubes, an aeration system, often with an additional source of carbon dioxide, and stands for the culture vessels, i.e. light shelves for small volumes and metal racks or wired frames for PE bags.

The unit also stores the special equipment to process pre-treated seawater, such as fine filters and sterilizers, as well as a laboratory where nutrients and glassware are prepared and stored, and where the necessary monitoring operations are performed. Standard cleaning procedures have to be strictly followed to maintain proper hygienic conditions (see Annex 6).

Preparation of the culture medium

In planktonic mass production, seawater is the medium of all culture vessels. The use of other mediums such as agar is limited to the preservation of pure algal strains. Ideally, seawater should be free from pathogens and pollutants. With this aim in mind, seawater is treated to remove suspended solids, contaminants and organisms and to improve its original parameters to fit the quality standards set for proper growth of microalgae. These methods are outlined below, while the description of related technical equipment can be found in the Engineering part of the manual.

Mechanical filtration

The most common systems of seawater mechanical treatment (applicable to all culture volumes: strains, small volumes and mass cultures) for microalgae production (which also apply to rotifers and brine shrimps cultures), are described below.

Raw seawater is first pre-treated to remove the bulk of suspended solids and contaminant organisms. Different methods are followed, but the most commonly used is a combination of settling and sand filtration. If properly dimensioned, a settling tank is a useful device. Not only it improves the water quality at no cost by decanting its suspended matter, but also provides a reservoir of seawater to tackle unpredictable problems in seawater supply, such as a damaged main pump station or a temporary contamination of seawater (oil spill, river plume, exceptional storm). Settled water is then filtrated through a sand or bag filter that retains particles as small as

Fig. 24.01 Mechanical sand filter for the water inlet (photo STM Aquatrade)

50, 10 or 5µm depending from the sector of destination. At this point filtered water can be used for most purposes in the hatchery, provided for some sectors like pytozooplankton it undergoes UV sterilisation before use.

In the live feeds sector a higher degree of filtration is required and pre-treated water is further micro-filtered to a size of 5 µm for large culture volumes and down to 1 µm for small volumes and strain cultures. For this finer filtering polyethylene wire cartridges, bag filters or diatomaceous earth filters are used. Such a fine filtration should even remove bacteria and other micro-organisms, but in reality the filtering capacity is not absolute and cannot totally guarantee such results, in particular under hatchery working conditions. It is therefore recommended to proceed with the final step, sterilization of filtered seawater.

Sterilization

Different methods of water sterilization have been developed. The following description refers to the most common methods adopted for hatcheries. The choice is based on local availability of equipment and service and depends also on the amounts of water to sterilize, which are related to the size of the hatchery.

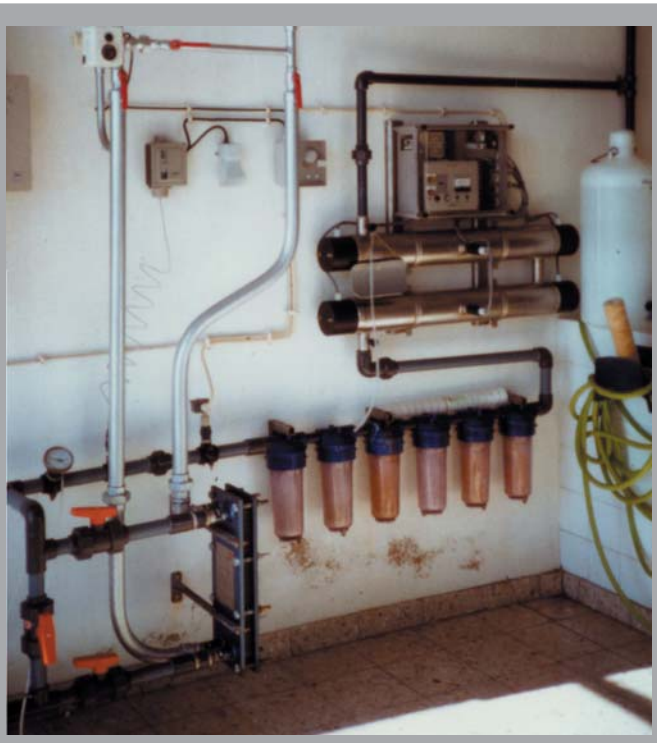


Fig. 24.02 Compact water treatment unit for zooplancton (photo STM Aquatrade)

UV light sterilisation (applicable to all culture volumes)

UV light with a wave length of 265 nm (short wave UV or UV-C) has a strong germicidal effect based on its capacity to break the DNA helix. It is produced by special high or low pressure mercury vapour lamps whose germicidal capacity depends on several factors such as their power, seawater transmittance (transparency to UV), type and quantity of micro-organisms to be destroyed, degree of purification required, water flow (contact time) and temperature. Seawater to be sterilized flows through one or more sealed chambers where it is irradiated by one or more lamps placed inside quartz tubes (transparent to UV light). The thickness of the water film inside the irradiation chamber should be such as to allow the maximum sterilization effect . If the power of UV lamps and chamber design are properly dimensioned, the contact time between water and quartz tubes is only of few seconds.

For practical purposes, and under normal hatchery conditions, an intensity of at least 40 mJ/cm^2 ,provided at the end of the life span of the lamps, removes 99% of most unwanted micro-organisms for fish farming from the treated water volume. With its auxiliary equipment (manual or automatic wipers, UV sensors and stabilizers, computer-aided control), this method is very effective and manageable and fully justifies its cost.

Chlorine sterilization (applicable to all culture volumes)

Active chlorine is a strong oxidizing agent, commercially available as liquid bleach (sodium hypochlorite or NaOCl) and as bleaching powder ($CaOCl_2$). The percentage of active chlorine in these chemicals should always

be checked in advance as it changes widely according to the producer: commercial grade NaOCl usually contains 5-15% active chlorine, while $CaOCl_2$ contains 60-70%. Annex 7 describes how to prepare hypochlorite solutions, to assess their active chlorine content and the residual chlorine in treated water, as well as the methods to sterilize seawater and deactivate residual chlorine.

Independently from the method employed, a final dosage of 5 to 10 ppm of active chlorine is used to sterilize seawater. The contact time between water and chlorine should be at least one hour, after which any residual chlorine must be neutralised with sodium thiosulphate, $Na_2S_2O_3$ (see Annex 7 for details). This technique is now widely used as final sterilisation step of water in larger vessel and of the culture equipment (air and oxygen tubing and diffusers, detritus traps, submersible water heaters)

Fig. 24.03 Chlorine container for laboratory use (photo STM Aquatrade)

Use of an autoclave, or wet vapour sterilization (applicable to small volume cultures)

With this method, applicable only up to 5-6 l volumes depending on the autoclave size, seawater is sterilised together with the culture vessels, usually made of Pyrex® glass, due to their resistance to heat. The autoclave should work at 120°C under a 2 atm pressure. Sterilization time ranges from 10 min (100-ml flasks) to 20 min (200-ml flasks) and 30 min (up to 5-6 l vessels). The neck of each container has to be covered with a loosened aluminium foil stopper to let vapour out during the sterilization.

Dry vapour sterilization (applicable to small volume cultures)

This method replicates the previous one, but the autoclave is replaced by an oven. As it works in dry vapour at ambient pressure, glass vessels filled with seawater are heated at 160-170°C for 2 to 3 hours. Dimensions and stoppers of vessels to be sterilized are the same ones used for wet vapour sterilization.

Enrichment

The exponential growth of the microalgal populations is regulated by four most important parameters: light, pH, turbulence and nutrients. Whereas the first three can be easily adjusted specific nutrients have to be added to the culture medium in proper quantities.

The main nutrients required are nitrogen (N) and phosphorus (P), followed by trace minerals, vitamins and chelating agents. Nutrient solutions are prepared in advance according the type of microalgae cultivated. With the exception of N and P solution, the other nutrients are stored as primary stock solutions, which are used to prepare the working solutions according to the day-by-day production schedule. Aseptic conditions have to be maintained in the preparation of the enrichment solutions. The vitamin solution cannot be sterilised because heating will deactivate the vitamins. The microalgae selected for the reproduction of seabass and gilthead seabream require the following fertilizers that refer to the enrichment medium Guillard f/2.

Primary stock solutions

Trace elements and vitamins are first prepared as concentrated primary stock solutions: in this way, if properly stored, they may last several months. Trace elements are prepared as four different solutions, each stored, like vitamins, in a separate container. To prepare one litre of each stock solution, the following quantities (in grams) are required.

Trace element stock solutions

Solution A: $ZnSO_4 \cdot H_2O$ (30g) + $CuSO_4 \cdot 5H_2O$ (25g) + $CoSO_4 \cdot 7H_2O$ (30g) + $MnSO_4 \cdot H_2O$ (20g)

Solution B: $FeCl_3 \cdot 6H_2O$ (50g)

Solution C: $Na_2MoO_4 \cdot 2H_2O$ (25g)

Solution D: $EDTA \cdot 2H_2O$ (50g)

To prepare the solution put the components, according to the proportions indicated above, into one 1-l graduated Pyrex® bottle and fill with distilled water (DW) to the mark. Deionized water can also be employed if distilled water is not available. When the components are fully dissolved, store at ambient temperature, avoiding exposure to direct light.

Vitamins stock solutions

B12 Cyanocobalamin (0.1 g)

B1 Thiamin (10 g)

H Biotin (0.1 g)

Place the indicated quantity of each vitamin into a sterilized 1-l graduated Pyrex® bottle filled to the required volume with sterilized DW. When fully dissolved, store in refrigerator and keep away from light. The B_{12} solution should preferably be stored in a dark or aluminium wrapped bottle.

Warning: do not sterilize any vitamin solution.

Working solutions

The working solutions represent the way to add the nutrients, trace elements and vitamins directly to the seawater medium. Two working solutions are prepared by diluting the above mentioned stock solutions, whereas the third solution is prepared directly from industrial grade chemical salts of N, P and K.

Fig. 24.04 Working solutions ready to use (photo STM Aquatrade)

Mineral salts working solution

Mineral salts solution: $NaNO_3$ (300g) + KH_2PO_4 (30g) + NH_4Cl (20 g)

Put the salts into one 1-l screw-capped oven-resistant glass bottle and fill with DW to the mark. If not available, deionized water can also be employed. When fully dissolved, sterilize either in autoclave or oven. Store at ambient temperature, avoiding direct light. Due to the comparatively higher requirements for the mineral salts stocking solution, quantities in excess of one liter are usually prepared at one time. Use 5 to 10-l glass autoclavable vessels, then store in 5- or 10-l plastic carboys with bottom tap.

Use: 1 ml per litre of seawater medium.

Trace elements working solution

One liter of trace element working solution is prepared according to the sequence and quantities oulined below:

Solution D (100 ml) + Solution A (10 ml) + Solution B (10 ml) + Solution C (10 ml).

Use only sterile glassware: a 100-ml cylinder and three 10-ml pipettes (one per solution). Mix the four solutions into a 1-l screw-capped oven-resistant glass bottle and fill with DW or deionized water and sterilise either in autoclave or oven. Store at ambient temperature, avoiding direct light.

Use: 1 ml per litre of seawater medium.

Vitamins working solution

Mix the following amounts of vitamins stock solutions: solution B12 (10 ml) + solution B1(10 ml) + solution H (10 ml).

Use only sterile pipettes, one per vitamin. Dilute the mix to one litre volume with sterilized distilled water. Pour in a dark sterilized bottle (or wrapped in aluminium foil) and store in the refrigerator just before use.

Use: 1 ml per litre of seawater medium.

Warning: do not sterilize any vitamin solution

Culture equipment sterilization

As for water medium and nutrients, also the equipment should be kept clean and disinfected to prevent contamination.

Small glassware such as volumetric pipettes, Petri dishes, Pasteur pipettes and beakers, are sterilized either in an autoclave or in an oven. Bigger glass containers as Erlenmayer flask, balloons and carboys are sterilized after being refilled with seawater. Pipettes are divided by volume capacity and stored into capped metal containers or wrapped in aluminium foil. Polyethylene (PE) bags are considered sterile as they are obtained by hot extrusion and do not need special treatments. Tanks, plastic jugs for nutrients and piping for pump transfer are disinfected with hypochlorite.

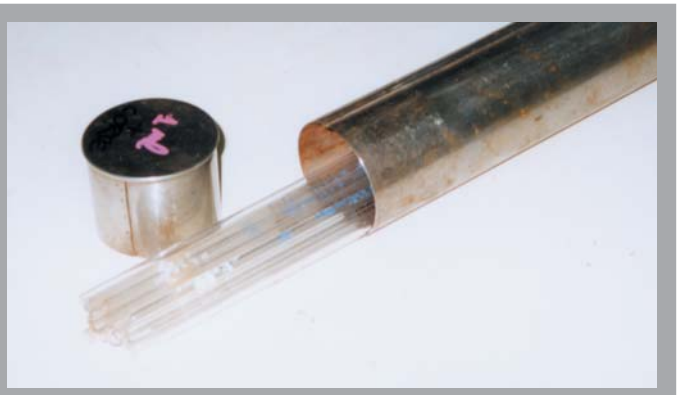


Fig. 24.05 Pipette container used with oven sterilization (photo STM Aquatrade)

All up-scaling tools and consumables that may be in contact with algae (aluminium foil, platinum needles, necks of tubes, flasks and balloons) are sterilized by flame using a Bunsen burner. On the glassware neck, flaming should lasts until all water drops have evaporated.

Sterilized hydrophobic cotton is commonly used as disposable stoppers for all glass containers. For its sterilization, it is packed in aluminium foil and sterilized in autoclave or oven. Cotton stoppers are disposed of after use. Hygiene and cleaning procedures in the live feed production sector are outlined in Annex 6.

Enrichment of culture vessels

This section describes how to proceed with the fertilization of the different culture vessels. All working nutrient solutions are diluted at 1 ml per litre of seawater medium. Pure strain cultures in test tubes, in marine agar and their initial small volumes are fertilized with half dosage (0.5 ml/l).

With small vessels, this operation should be carried out in a dedicated room kept clean and equipped with all the necessary tools, glassware and consumables. To reduce the risk of contamination, a UV-light ceiling lamp can be installed to provide germicidal irradiation when the room is not in use.

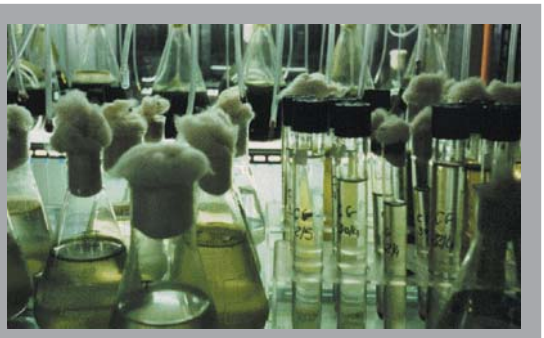


Fig. 24.06 Phytoplanktron strain cultures (photo STM Aquatrade)

Enrichment procedure for small vessels (up to 20 l capacity):

1. prepare on the bench the required vessels containing sterilized water, nutrient working solution and the necessary tools;
2. carefully loosen their aluminium foil caps;
3. heat each flask neck and the inner side of the aluminium foil caps with a burner; close again;
4. heat opening and screw cap of each bottle containing stock solution in the same way;
5. take a sterile pipette from its sealed container (if in a metal case, open and close it at the flame);
6. with the pipette place one nutrient solution at a time in each flask, using a new pipette for each solution;
7. repeat the flaming of necks and aluminium caps on the enriched flasks.

Enrichment procedure for polyethylene bags (up to 500 l capacity):

1 The bags are filled with the same treated and UV-light sterilized seawater utilised for small vessels, the only difference being that the bags are not sterilized. Due to the larger amount of nutrients involved, use clean and sterilized graduated cylinders to fertilize bags.
2 Cut a 20 cm-long slit on the upper rim (empty part) of the bag;
3 add 1 ml per liter of each solution.

When cutting the bag, bear in mind the final culture level, i.e. after the addition of the inoculum.

Fig. 24.07-8 Flask sterilization and enrichment (photo STM Aquatrade)

Batch culture of microalgae

Mass culture of phytoplankton starts from pure strains of selected species and proceeds by upscaling from small (0.5 l) up to large volumes in PE bags (450l) or tanks (1 000 l and up). In finfish breeding, where large amounts of microalgae are not required, the last upscaling step is the PE bags. See annex 8 for a typical example of microalgae upscaling protocol, and annex 9 for the daily workplan and culture file for microalgae.

Pure strain culture

The culture of pure strains of the selected microalgal species is the starting point of the mass production process. Strain quality is therefore essential for any successful production process. A good selection of pure strains of different algae should always be kept in a dedicated facility in the hatchery. This practice also allows the selection of the most suitable strains under local conditions and at a given time.

Pure guaranteed strains of algae, as well as of rotifers, are normally available from a few laboratories and institutions in the Mediterranean region and Europe .It is strongly recommended to regularly renew their lines, not only in case of culture crashes, but also to control the often unavoidable contamination or decline in quality. Strains from other hatcheries should better be avoided because of their possible decline in quality and the associated risk of introducing contaminated strains.

The algal pure strains are kept under standard controlled environment in conditioned rooms or in especially designed incubators, in which routine work can be performed under strict hygienic control. The pure strain cultures are usually kept in small glass containers, such as 10 to 25 ml test tubes or 100-ml glass Erlenmeyer flasks, closed by a sterile stopper (screw cap or a folded aluminium foil).

Pure-strains cultures should be maintained at a steady or resting stage, i.e. under environmental conditions which allow them to reproduce, but not to increase exponentially in number. In this way, their sexual reproduction is fostered, thus increasing their genetic variability, and the growth of unwanted organisms such as other algal species, bacteria and ciliate protozoa is prevented. Culture parameters are therefore kept below the values adopted for mass production. In particular, only half dose of nutrients is used, water temperature is kept at around 14-16°C, light intensity ranges from 300 (test tubes) to 1 000 lux (flasks) and no aeration and carbon dioxide are provided.

Under routine conditions, strain cultures are usually renewed every month. In the replication process, an inoculum of 0.1-0.2 ml (from test tubes) or 0.5 to 1 ml (from Erlenmeyer flasks) is taken from the best old culture which is free of contamination, to inoculate three new vessels of the same size to start a new strain. The old culture is then either utilised for upscaling, or is discarded. Strain culture vessels should be stirred at least once a day by hand, paying attention not to stir bottom debris up.

Warning: in the management of pure algal strains quality is essential: get rid of any tube or flask which is found to be contaminated by bacteria, fungi, ciliates, nematodes or different algal species.

Fig. 24.09 Pure strains kept under controlled conditions (photo STM Aquatrade)

Protocol for test tubes replication

The steps to duplicate algal strains at test tube level are described in the following paragraphs. All required equipment and consumables have to be well cleaned and sterilised in advance.

Choose the test tubes that show the best algal populations at naked eye and check under the microscope a sample taken from each of them for contaminant organisms (use a new sterile pipette for each sampling). Then keep only the uncontaminated cultures and put them on a stand avoiding any stirring.

For each selected test tube:

1 prepare four sterilized test tubes with cap on a stand;
2 prepare at least 50 ml of sterilized seawater medium, enriched with half dose of the standard nutrients mix (see above);
3 heat necks and caps of all test tubes (new and old vessels) by means of a manually operated burner and let them cool;
4 fill each new test tube with 10 ml of seawater medium taken with a graduated pipette;
5 with a sterile 1 ml-pipette take 0.5 ml of mature culture from near the surface of the selected test tube;
6 inoculate 0.1 ml of the old culture into each new test tube; be careful to make the drops fall freely into the culture medium without touching the tube walls; with one hand open and close the tube cap; with the other hand handle the pipette; do not mix or agitate the tubes;
7 place the used pipettes into the rinser cylinder;
8 after inoculation, heat the upper part of each new test tube thoroughly and cover with its cap, previously flamed; as an alternative, use sterilized hydrofobic cotton or flamed aluminium foil as stopper;
9 discard the old culture, if not needed for other replicas, and clean the empty tube following the usual cleaning routine for glassware (annex 6);
10 write date and algal species on all new test tubes with a waterproof marker;
11 place the newly inoculated test tubes on a rack in a shelf reserved to pure strain cultures.

Protocol for purification of algal strains

Even under axenic conditions, pure strains can be contaminated by other algal species or micro-organisms: in such cases before up-scaling the cultures the microalgal populations have to be purified before being used as inoculum.

Two methods to purify contaminated algal cultures are of common use: successive dilutions of the original contaminated culture, and picking up of single cells from the original culture. Both techniques are also applied when new algal species are isolated from the wild.

Dilution method

A simple technique to purify a contaminated strain is to proceed with repeated sub-cultures obtained by progressive dilutions of the original sample. Dilution rates can be very high, depending on the number of the sub-cultures. In the procedure described below a dilution rate of 10^{-10} is reached. The same nutrient quality and environmental condition of the initial culture are used.

Example of dilution:

1. prepare 10 sterile test tubes with caps on a tube rack;
2. sterilize 500 m l of seawater in an Erlenmeyer flask, then fertilize it with half dose of usual mix of nutrients when cool;
3. using a sterile 10-ml pipette add 9 ml of enriched seawater to each tube, close loosely with flamed caps and number them 1 to 10;
4. put the test tube containing the strain to be diluted in the rack, remove the cap and flame its neck;
5. using a 1-ml sterile pipette take 1 ml and add it to the tube No. 1, then stir gently;
6. using a new sterile 1 ml-pipette repeat the previous step by taking 1-ml inoculum from tube No.1 and inoculate it into tube No. 2;
7. repeat the same procedure with the remaining tubes, each time pipetting 1 ml from the previous tube (gently stirred) into the next one; flame necks and caps and let them cool;
8. keep under controlled environmental conditions.

When cell growth reappears, check samples of the tubes under the microscope and get rid of the tubes that are still contaminated, typically the initial ones, and keep only the purified cultures, usually in the more diluted tubes. Repeat the process using the last dilutions if necessary, and in any case at least every three months to always have a safe amount of purified cultures ready at hand.

Picking up method

Two systems which produce single cells are described below: the agar plate method and the capillary method:

Agar plate method

A solid medium can grant more stable conditions for the growth of the desired species. This technique needs some sterile equipment like Petri dish and platinum hooks.

Example:

1. prepare the solid medium by adding 1.5 g of agar powder to 100 ml of sterile fertilised seawater;
2. heat the medium using a Bunsen burner, stirring with a sterile glass rod to dissolve the agar, and pour it on 3 to 5 sterilised Petri dishes;
3. allow to cool and solidify;
4. using a sterilized 1 ml-pipette take 0.1 ml from the initial contaminated strain culture and drop it into each Petri dish;
5. spread the sample over the agar with a sterile platinum hook and incubate at desired environmental conditions placing the dish upside down, so that water drops will not form on the lid and then fall on the culture;
6. once algal colonies are observed, take a sample by means of a sterile platinum hook and check under the microscope
7. monospecific colonies should be kept in agar for successive replication or might be up-scaled by replication (i.e. a small portion of the colony together with some agar, using a sterile hook) on a 50-ml or 500-ml Erlenmeyer flask to continue in the liquid medium.

Capillary method:

This technique follows the dilution method, but the inoculum is obtained by selecting single cells of the desired species by means of a capillary pipette handled under a microscope.

The isolation or purification of cultured strains should be repeated as many times as required to produce contaminant-free cultures.

Upscaling culture conditions

As indicated above the upscaling of microalgae production starts from small containers (0.5 ml) and proceeds through various steps up to the mass production in PE bags (up to 450l). Each step involves an increase of the culture volume: when mature (i.e.: in log phase), the algal population of a smaller volume is sacrificed to replicate the same vessel and to inoculate larger vessels.

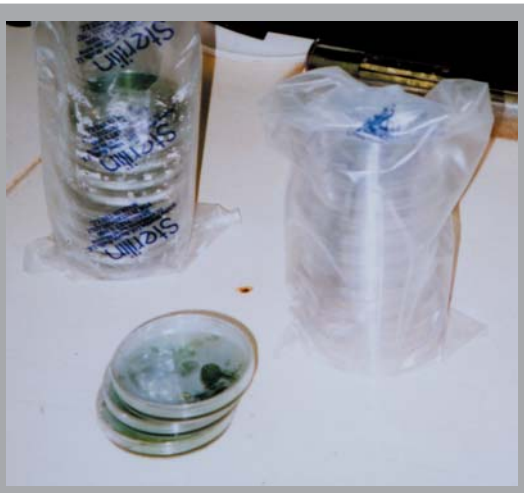


Fig. 24.10 Petri dish with culture solid medium (photo STM Aquatrade)

Small-medium size (0.5 to 10 l) cultures of microalgae are usually kept in borosilicate-glass containers with large necks, such as Erlenmeyer flasks or other flat bottom round flasks (balloons) and carboys. The flasks are usually closed with a stopper made of sterilised cotton or of plastic which can stand sterilization in autoclave.

All these cultures are provided with a proper medium to support algal growth (treated and fertilized seawater), good aeration supplemented with carbon dioxide (CO_2) as an additional carbon source, and a strong light. In this way it is possible to reach quickly the log-phase growth. Aseptic conditions should be maintained to avoid contamination and culture crashes.

For practical purposes the main parameters for the cultures are usually the same for the different species of algae mentioned in the sections above:

temperature	20 ± 2°C
salinity	25 to 30ppt
light intensity	4 000 to 8 000 lux
aeration	50 to 100% of the culture volume per minute
carbon dioxide	2 % of air volume

Fig. 25.01-2 Algae flask replication (photo STM Aquatrade)

Temperature

For mass culturing purposes, the optimal temperature for the above mentioned algal species ranges between 20 and 24°C. Generally speaking, temperatures lower than 16°C and above 27°C will slow down growth rates, whereas those above 30°C are normally lethal. That makes room conditioning necessary as artificial lights and insufflated air from the aeration system can raise temperature dangerously.

Low temperatures are used for pure strains only, where growth should be kept as slow as possible. As low temperatures also affect bacterial growth, non-axenic cultures should be maintained at the lowest possible temperature consistent with a good growth, to prevent bacterial growth.

Salinity

In the selected species salinity does not represent a limiting factor within a range of 15 to 40 ppt.

Light

Light is the source of energy for photosynthesis and therefore in mass cultures algae are usually kept in continuous light. Fluorescent tubes are the commonest choice for providing light due to their low power consumption, low installation cost and limited heat production compared to bulb lamps. Spectral quality suitable for algal growth is provided by “Cool white” and “Daylight” models, often installed in equal numbers. The commonest tube size is 42” with waterproof contacts. See Part 4 Engineering for their installation in the hatchery’s algal unit.

Fig. 25.03 Lighted table used for mid size culture up scaling (photo STM Aquatrade)

Even if each algae species has its own preferred light intensity for best growth, for practical purposes intensity in mass cultures is kept in the range of 2 500-8 000 lux, while higher intensity is used on the small volumes shelves (5 000-8 000 lux) because of their higher cell density. Large volumes (bag cultures) can also be illuminated by natural daylight, entering from sufficiently large windows or from a continuous glass wall, provided that the cultures are not directly exposed to external contamination.

Aeration

Aeration is used to maintain the culture in turbulent state, preventing settling of cells and exposing all cells to light. It also supplies carbon dioxide, which is fixed by the algae during photosynthesis, and provides essential pH stabilization.

Transparent PVC tubing, of 6 mm internal diameter, are commonly utilized to deliver air to the culture vessels. In small vessels this tubing is connected to glass pipes which fit the stopper and reach the bottom of the vessel, while in PE bags they are simply forced into a small hole near their bottom (the water pressure keep the holes sealed), whereas in tanks a weight keep them submersed.

Aeration should be moderate in the first two days of culture, then should be increased adjusting it according to culture growth. For this purpose screw clamps or cheaper plastic needle aquarium valves are required to adjust the air flow. If aeration produces foam, it is an early warning of culture troubles.

Carbon dioxide

As its normal content in air is low (approx. 0.03%), carbon dioxide is often supplied at 2% by volume to optimize culture growth. Commercial grade CO_2 bottles are utilised, and the gas is injected into the main air pipe via a dispensing vessel in which gas bubbling reveals the gas flow. Since carbon dioxide is heavier than air, to prevent stratification the pipe makes some ups and downs after the point where it is injected.

Depending on the algal species, the CO_2 supply, and the volume of inoculum, working cultures normally reach their log-phase in 5 to 7 days. At this point, cultures can be utlized either to start new cultures, to be fed to rotifers or to be used as “green water” in fish tanks..

The volume of the algal inoculum is usually 15 to 20% of the new volume. Smaller or larger inocula could be used to decrease or increase growth rate. In culture up-scaling, the new vessels have to be inoculated with a sufficiently high microalgae density in order to ensure a rapid growth and to limit the risk of contamination with different algae or other micro-organisms (protozoa, nematodes, fungi, etc.) .

Scaling up protocol

The following section describes in detail the operation to replicate different volumes.

Inoculation of flasks from test tube

Follow the same procedure as previously described for pure strains. Each 0.1-l flask will receive 50 ml of enriched medium and 0.5 ml of inoculum. At this stage, no aeration is required. When mature, each small flask will inoculate a new 2-l flask.

Fig. 25.04 Alternative use of olives containers for algae culture (photo Ittica Mediterranea)

Fig. 25.05 Very small PE bags of phytoplankton at Ittica Ugento (photo STM Aquatrade)

Fig. 25.06 Medium and large size bags (photo STM Aquatrade)

Inoculation of 2 and 6-l flasks from another 2 or 6-l flask:

1. prepare the necessary amount of new flasks filled with sterilised seawater, as well as all equipment required for the operation (pipettes, nutrients, cotton stoppers, aluminium foil, glass tubing, etc.);
2. select the mature culture that will be used as inoculum, checking a sample under the microscope for contaminants;
3. remove its cap and flame its neck; then close with a flamed aluminium foil stopper and let it cool;
4. in the meanwhile, add the fertilizing working solutions to each new flask, at a rate of 1 ml/l; using a new sterile pipette for each solution;

5. flame their necks and aluminium caps thoroughly;
6. when cool, remove the stopper and pour some algal culture of the old flask into the new vessels at a rate of about 10% of the receiving volume, avoiding to wet their neck with the inoculum, then gently shake flasks to mix the new culture;
7. flame thoroughly their necks, introduce the sterilized glass tubing for aeration and close tightly with sterilised cotton stopper (or any other type of sterile cap);
8. write date and algal species on the new flask;
9. place the flasks on the lighted shelf and connect to the air delivery system, adjusting its flow to a gentle bubbling;
10. after on hour, check all new vessels for a proper air bubbling.

Use only the upper layer of the old culture, leaving dead cells and debris in the flask used to inoculate the new ones. The size of the inoculum for small volumes is only 10% of the new volume because of the high cell density. Remenber to get rid of every contaminated flask. The above mentioned procedure applies to the upscaling of the other medium size vessels.

Fig. 25.07-08 PE bags inoculation with the help of an self-priming pump (photo STM Aquatrade)

Inoculation of a PE bag from a flask or from another bag

1. prepare the bag, either suspended to a rack or placing it inside a cylindrical frame made of wire;
2. fill the bag with sterilized water ; leaving enough free space for the volume to be inoculated; wait a couple of hours to check for possible leaks; if found, seal them or replace the bag;
3. introduce nutrients and inoculum;
4. fit two PVC tubes for aeration to the bottom of the bag, connect them to an air line and adjust the air flow to a gentle bubbling;
5. add the nutrients using a graduated cylinder at the usual rate of 1 ml/l of each working solution;
6. select a mature culture that will be used as inoculum, either from a large flask or from another bag, and check a sample under the microscope for contaminants;
7. in case a flask is used as inoculum, remove its cap and flame its neck; then close with a flamed aluminium foil stopper and let it cool;
8. then, remove the stopper and pour the algal culture from the flask into the bag. The

inoculum should be about 10% of the receiving volume;

9. if the inoculum comes from another bag, with a self-priming plastic pump transfer the inoculum from the old bag into into the new one. The volume to be transferred should be about 15-20% of the receiving volume (a bigger inoculum is necessary to compensate for a less dense culture);
10. on each bag mark date, algal species, origin of the inoculum (bag or flask) and the bag serial number.

To reduce the risk of contamination, smaller bags are usually inoculated from flasks, whereas larger bags are inoculated from smaller bags.

Fig. 25.09-10-11 Large volumes cultures (photo STM Aquatrade)

Monitoring algal populations

A regular check of microalgae cultures is essential to prevent crashes and to keep high quality standards. The main parameters to be monitored are: colour, density, pH and contaminant levels. As an example, a change in colour to opaque grey and a pH level lower than 7.5 may indicate a high degree of bacterial contamination. A lighter colour than normal may reveal insufficient nutrients or poor lighting. However, during the peak production season in the hatcheries a close monitoring of algal cultures can hardly be assured, due to the chronic lack of staff and time. Mass cultures are normally checked at naked eye by experienced staff and strict controls are usually restricted to pure strains and small vessels.

To partly by-pass this problem, the staff can do a good preliminary job outside the peak production season, when they are not so absorbed by production and there is some more time. Then test culture cycles can be closely monitored, the algal culture growth can be followed daily by counting the number of cells per ml with a haemocytometer (see below), and their average growth curves can be plotted against values obtained with a colorimeter. During more busy days, a colorimetric monitoring (comparing values against the test curves) gives a fast and reliable indication of algal cultures growth.

These test cultures are useful for a number of other reasons as it is possible:

- to determine the growth curve for each species of algae under local conditions;
- to devise criteria for quick identification of possible troubles (eg.; presence of foam, sedimentation pattern, changes in colour, etc.);
- to determine optimal utilization time, i.e. the age at which the algal population reaches the peak of the log-phase;
- to adjust environmental conditions to maximise production;
- to control possible contaminants and try countermeasures.

Counting microalgae

A Neubauer haemocytometer can be used for counting microalgae cells with diameters ranging from 2 to 20 µm and at densities up to 500 million cells/ml.

This device consists of a thick rectangular slide with an H-shaped trough delimiting two counting areas. With its special cover slip in place, each area forms a chamber 0.1 mm deep. The total area of each chamber is 9 mm^2. Each chamber is divided into 1 mm^2 squares: the four corner squares are subdivided into 16 smaller squares, whereas the central one is subdivided into 25 smaller squares, 0.2 x 0.2 mm, each with an area of 0.04 mm^2.

To count cells in a sample, proceed as follows:

1. take a 5-ml sample from each culture and place each sample in a separate test tube. Add one drop of Lugol solution and mix well;
2. prepare clean Neubauer slides and covers;
3. put one drop of well mixed algal suspension on each Neubauer chamber and cover with its cover slip;
4. check under low magnification that the algal cells are evenly distributed: avoid the presence of air bubbles, over flowing, underfilling and uneven distribution of cells. Allow the cells to settle for 5 minutes before counting;
5. start counting at the top left square and count only those cells which lie within or touch the boundary lines chosen according one of the two possibilities: either left and bottom boundary lines or right and top boundary lines;
6. for cells larger than 6 µm and not too dense, make a total count in each of the four corner squares and in the central square; then repeat this count in the second chamber;
7. for minute cells and denser populations, count the cells in 5 smaller squares in the larger central square, then repeat this count in the second chamber;
8. calculate average cell density as follows: if all cells are counted in individual blocks each with an area of 1 mm^2 and a volume of 0.1 mm^3, the average cell density expressed as cells/ml is given by the total count divided by the number of blocks and multiplied by 10 000;
9. if all cells in ten (five in upper chamber + five in lower chamber) smaller squares (volume = 0.004 mm^3) in the central block are counted, the average cell density is the total count multiplied by 4 000 000 and divided by 10, i.e. the total count multiplied by 400 000;
10. record the count for each sample and introduce it in the algae population growth curve prepared for each culture container.

Mass culture of rotifers

For the breeding of many marine finfish species the rotifer *Brachionus plicatilis* is, up to now, the only live feed that can be used in their very early larval stages. While not a compulsory choice in seabass feeding, its mass production is required for the successful breeding of gilthead seabream (and of other Sparids, grouper and grey mullet), whose small mouth cannot accept larger preys at the onset of larval feeding. Among other valuable characteristics as live feed for fish, *B.plicatilis* was also chosen due to the relative easiness to culture it in large scale.

Two main strains are used. The so-called small strain (S-type) and a large strain (L-type), 50% bigger in dry weight than the S-type. The average length of the lorica in the adult S-type rotifers is 130 µm, whereas it is 240 µm in the L-type. The two strains also show different temperature and salinity tolerances. In the last years mainly S-type rotifers are reared in the hatcheries.

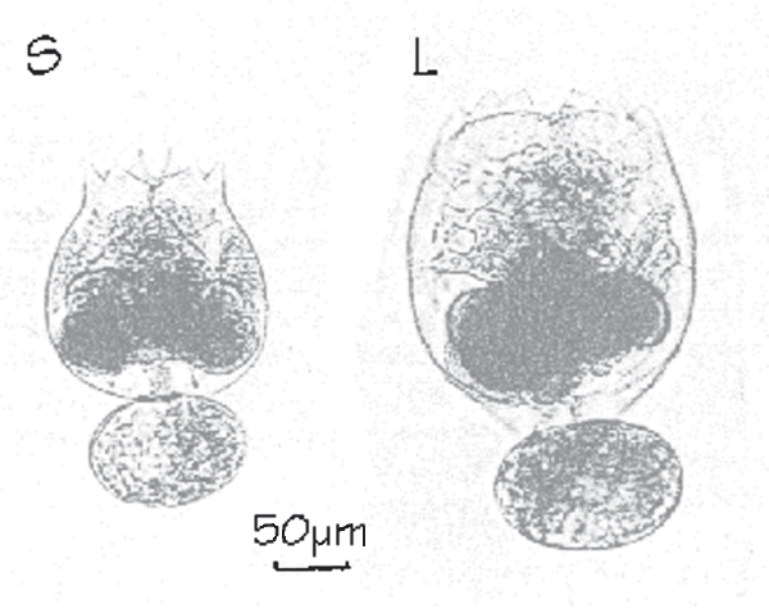


Fig. 26 *Brachionus rotundiformis* and *Brachionus plicatilis* (modified from Fu et al.)

Population dynamics

Rotifers can reproduce both sexually (mictic reproduction) and asexually (amictic reproduction), according to the environmental conditions. Usually amictic, rotifers may turn to sexual reproduction when sudden changes in salinity or temperature take place. Then, they produce large resting eggs, similar to brine shrimp cysts. However, in hatcheries is the asexual reproduction that provides the large amounts of animals required for the early feeding of fish larvae. Rotifer population dynamics under mass rearing conditions follow different phases, mimicking those of microalgae:

- the lag-phase, when, just after the inoculum, rotifers begin to consume the phytoplankton of their culture medium and the number of both egg-bearing individuals as well as the quantity of amictic eggs increases;
- the log-phase (or exponential phase), where rotifers reproduce very fast and population growth is exponential;
- the transitional phase (or declining growth), where growth rate slows down and egg-bearing rotifers become rarer;
- the decline phase, where almost only old rotifers without eggs are found and their number decreases rapidly as death rate exceeds growth rate.

The quality of the rotifer population to start new cultures is even more important than in the case of microalgae. To be used as inoculum, the rotifer population must still be in the middle of its log-phase with at least 20% fertility rate (measured as percentage of eggs over total rotifers. Populations in their last declining phase, characterised by limited motility, scarce repletion and absence of egg-bearing animals, should always be discarded. With a proper inoculum and under optimal rearing conditions, a rotifer population should reach its harvesting density within 4 to 5 days.

Under hatchery conditions, rotifer populations can reach the following densities:

in flasks and bags, after 5 to 7 days:
- S-type rotifers, 500 to 700 ind/ml
- L-type rotifers, 150 to 250 ind/ml

in tanks, after 4 to 6 days:
- S-type rotifers, 1000 and more ind/ml
- L-type rotifers, 400 ind/ml

Mass production systems

As this microscopic animal is a filter feeder, its nutritional value strictly depends on its food. In hatcheries, the species is first cultured on microalgae, following the same scale-up protocol described for microalgae, then its final mass production is achieved in large tanks where artificial diets are fed to rapidly increasing numbers, improving at the same time their nutritional value.

Fig. 27 Medium and large volumes cultivated in the same room (photo STM Aquatrade)

The rotifer *B.plicatilis* is a rather sturdy species able to tolerate a wide range of salinity, temperature and ammonia levels. It can also use several food sources, provided that particle size remains within a 2-20 µm range. Obviously, the highest growth rate is achieved under more restricted environmental parameters, and is closely related to the selected rotifer strain and feeding provided,. In particular, good yields are obtained with high dissolved oxygen levels, temperature at 25°C, pH at 7.5-8.5, salinity in a 20-30 ppt range, less than 1 mg/l free ammonia (NH_3), and moderate turbulence. Light is required only when rotifers are fed microalgae.

Dissolved oxygen levels

During the upscaling phase in algal vessels, the oxygen requirements of rotifers are fulfilled by microalgae photosynthetic activity. In mass culture, artificial diets, enrichment boosters, high rotifer densities and metabolic products, contribute to deplete oxygen in the culture medium. To keep its levels within safe margins, i.e. above 80% saturation, a strong aeration is supplied, linked to an emergency oxygen delivery system. See section below for technical details.

Temperature

In mass culture, a temperature as high as 30°C is acceptable, but as bacterial and other contaminants would also increase at these levels, a more manageable 20-25°C range is commonly adopted.

pH

An acceptable range is pH 5-9, but the buffer capacity of seawater keeps it within a narrower range. A low pH also influences the balance between the toxic un-ionized ammonia and the ionized form (NH_4^+). The lower pH reduces the fraction of toxic NH_3.

Salinity

The acceptable salinity range for this rotifer species is quite broad: 1-60 ppt. However there are differences between the two strains, the optimal salinity for the S-type strain being 18-20 ppt, whereas L-type grows better at 30 ppt.

Turbulence

A moderate to strong turbulence is required to keep food particles and rotifers in suspension. The aeration system should provide enough water circulation, but the position of the air diffusers should be adjusted in such a way to avoid stirring and re-suspension of bottom sediments: airstones are hung 15 cm above the bottom, both along the periphery and in the centre of the tanks.

Mass culture facilities for rotifers

Inside the hatchery building, phytoplankton shares the same culture facilities with rotifers, which have a specific section with large tanks for their final mass production. In Mediterranean hatcheries, these tanks have a volume ranging from 1 to 10 m^3. These tanks are made of FRP, PE or PVC-lined concrete. As water circulation and particles sedimentation are important in rotifer culture (see below), a widely adopted design is the round tank with conical bottom. This section for mass production of rotifers is equipped with an air distribution system, air conditioning and seawater heaters to keep high water temperatures. Special filtering devices to harvest and rinse rotifers are also available.

As mass culture of rotifers does not require light (except for the phases in which algae are used as food), a normal lighting system is installed. Treated seawater (filtered and sterilized) is provided by the same source that feeds the algal section.

As in microalgae production, hygiene is important and requires the strict implementation of standard cleaning procedures (see Annex 6).

Fig. 28 Large rotifers tanks for mass culture (photo STM Aquatrade)

Preparation of the culture medium

The same procedures and precautions described in the algal production section apply to rotifer culture. The only enrichment added to the rotifer culture medium, be it either a log-phase algal culture or treated seawater plus artificial diet, is represented by the addition of vitamin B_{12} (cyanocobalamin) as a fertility booster for the rotifers. Its dosage is usually 100 ml of B_{12} stock solution per m^3 of rotifer culture in tanks, whereas small vessels and bags are fertilized at the rate of 1 ml/litre. In both cases the vitamin is added together with the inoculum.

To prepare the stock solution put 0.1 g of vitamin B_{12} into a sterilised 1-l graduated Pyrex bottle and fill with sterilised DW to the mark. When fully dissolved, store in the refrigerator. Warning: never sterilise vitamin solutions.

Pure strain cultures

The first phases of rotifer cultures (strains and small volume cultures) are performed in the same conditioned room where microalgae are kept, but on separate stands to avoid the risk of possible contamination. The same propagation techniques described for microalgae strains are used for rotifer strains. As for microalgae, culture conditions discourage exponential growth. Rotifer strain inoculums are kept at 1 or 2 individuals per ml in a low-density algal culture and no vitamin is added. Both test tubes and small flasks are used to maintain rotifer strain cultures.

Upscaling rotifer cultures

A clean culture of egg-rich rotifers from a 0.5-l flask is usually inoculated directly into a 5 to 10-l flask, by-passing the 2-l flask stage. The inoculum should provide an initial density of 10 to 20 rotifers per ml. A larger inoculum results in a faster pace of population growth. Rotifers should always be inoculated in algae cultures which have not yet reached their peak growth (that is their log-phase). Environmental conditions should be the same ones at which algae are kept. However, aeration can be reduced to diminish the amount of foam and bottom sediments produced by the metabolic activity of rotifers.

Vitamin B_{12} is routinely added to all new vessels, at 1 ml of stock solution per litre of algal culture. Mature rotifer cultures from small vessels (5 liters or larger) are poured into bags with an algal population that has not yet entered its full log phase. Five to ten % inoculum is used at this stage, i.e. one 5-liter vessel is used to inoculate one 100-l bag, following the rule of the new culture starting at 30-50 rotifers/ml. Using a sterile volumetric cylinder, add 1 ml of the Vitamin B_{12} per litre of culture; mark the date on the bag surface, together with the origin of the inoculum and the serial number to facilitate its handling and record keeping.

If the population is growing normally and remains free from contaminants, the horizontal culture method is also frequently used for rotifer culture upscaling. In this case, a good bag makes it possible to inoculate a group of new bags with a initial density of at least 30-50 rotifers/ml.

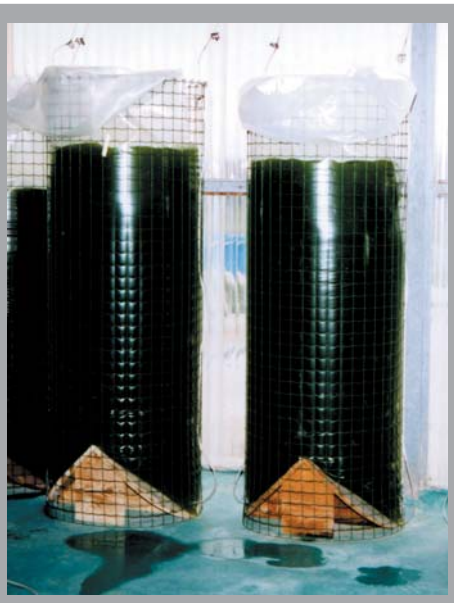


Fig. 29 Mature algae bags ready to be inoculate with rotifers (photo STM Aquatrade)

In the same way as it happens in microalgae cultures, also rotifer cultures are subject to occasional failures (culture crashes). A culture crashes either when rotifers do not multiply as foreseen, or when the entire culture dies abruptly. The possible causes are described below in the Monitoring section. As a precaution against these problems, always maintain a certain number of culture bags in excess of those required by the production schedule. They could be useful to replace quickly a crashed culture at any stage.

Mass culture

Rotifer mass culture is carried out in large tanks as mentioned above. Because of the very high density achieved (up to 1.000 individuals per ml or more), rearing procedures and protocols to maintain strict hygienic conditions (see Annex 6) have to be rigorously applied. Such routine procedures are described below.

There are basically two main mass rearing methods for rotifers: the older technique that is based on algae and baker's yeast as food for the rotifers, and the more recent one that uses an artificial diet, the Culture Selco® produced by INVE SA of Belgium (or similar). Both are described below.

Tank preparation

Before starting a new production cycle, normally after the harvest of the previous rotifer culture, prepare the tank as follows:

1. rinse with tap water to eliminate the bulk of organic debris;
2. wash it thoroughly with brush and detergent and rinse it again;
3. wash or spray the tank walls with 500 ppm active chlorine solution (Annex 6); after a couple of hours, drain the tank and rinse it well until the chlorine smell is gone;
4. let the tank dry and fill it with sterilized heated water only when needed; as an alternative possibility, fill the tank with seawater and sterilize with hypochlorite, then neutralize the residual chlorine with sodium thiosulphate (see method in Annex 7).

Repeat the procedure for the equipment to be used in the tanks: aeration tubing, drain valves and suspended traps (see below). A practical procedure is to assemble all small equipment in the new tank, fill with SW and sterilize with hypochlorite: the equipment will be disinfected as a consequence.

Fig. 30.00 The classic cylindroconical rotifers tank (photo STM Aquatrade)

Inoculation

To be used as inoculum, the rotifer population must still be in the middle of its log-phase with at least 20% fertility rate (measured as percentage of eggs over total rotifers). Never use rotifers which have already reached the last phase characterised by limited motility, scarce repletion and no egg presence.

In algae/yeast fed tanks, the initial density of inoculum, one of the most important factors in rotifer culture, should be kept at least at 100 animals/ml, with an optimal density of 150-200 animals/ml. High density cultures with artificial feeding need up to 500 rotifers per ml (see below). With an initial density of 200 animals/ml, rotifer density should reach its peak within four to six days at 25°C.

Tank inoculum may come from large rotifer bags (vertical upscaling), or from other tanks (horizontal upscaling). One day before being harvested to be used as inoculum, rotifers in the tanks should be fed a fertility booster such as Protein Selco® by INVE SA. In order to reduce contamination and culture crash risks, horizontal upscaling should be limited to 7-8 cycles, after which the inoculum should be again taken from large bags.

Feeding

As previously indicated two feeding methods are most widely adopted: 1) a combination of algae and baker's yeast and 2) a totally artificial diet. For its reliability and higher output, the latter is progressively replacing the first method.

Fig. 30.01 Large containers with hypochlorite solution are used to keep cleaned small equipment (photo STM Aquatrade)

Mass culture with algae/yeast as food

The initial method of mass culturing rotifers in Mediterranean hatcheries, makes use of a common and easily available food staple, the bakers' yeast *Saccaromyces cerevisiae*. It is a labour and cost sparing food, which has no nutritional value for rotifers that feed on bacteria associated with the yeast.

Compared to the artificial diets, this method has a lower yield and requires more time, typically one extra day. Density at harvest rarely exceeds 450 rotifers/ml with an average daily increase ranging from 19 to 33%. In addition, rotifers should be enriched with high levels of (n-3) HUFA and vitamins. A major constraint of this method is the absolute necessity to improve the otherwise very poor nutritional quality of yeast-fed rotifers before their distribution to fish larvae. The enrichment procedure, which takes place the day before harvesting the rotifers is explained below.

Protocol:

1. fill the tank with sterilized sea water diluted with tap water to obtain a 20 ppt salinity; check the chlorine content of tap water and, if present, neutralize it with an excess of sodium thiosulphate (see Annex 7). Take care to leave enough space for the algal cultures to be supplied as food (about 30% of the tank volume).

2. place the air diffusers and switch on the aeration;
3. place the traps for ciliates and impurities;
4. select the most suitable rotifer bags to be used for inoculum, checking for contaminants and using only clean batches;
5. filter the selected batch or batches (see below for procedure);
6. inoculate the tank to achieve an initial density of 150-200 rotifers/ml. This is considered day 0;
7. add algal culture as 20% of tank volume to provide rotifers with their initial food; as usual, the algae should be in their log-phase and from non contaminated cultures, even if of different species.
8. the next day (day 1) fill the remaining 10% volume with algal culture;
9. on the tank file, record all information on the culture growth, food distributed and environmental parameters monitored (see below for details and Annex 9 for a file sample);
10. from day 1 on, feed with bakers' yeast at the following rates, according to the recorded rotifer density.

Rotifer density (No./ml)	Daily feeding rate (g yeast/million rotifers)
less than 50	3
50 to 100	2
more than 100	1

Fig. 30.02-03 Rotifers mass culture performed in very large heated tanks at Ittica Mediterranea (photo STM Aquatrade)

The daily amount of fresh yeast is divided into 4 equal rations fed at 2 and 8 am, 2 and 10 pm (the last two rations are given by the night watchman). Each yeast ration is taken from weighed out of the yeast cake kept in the refrigerator and placed in a plastic bucket filled with tap water at a concentration of 100 g/l. A kitchen or better an industrial blender helps to separate yeast cells and keep them suspended in water. Feed immediately and discharge any leftover. Prepare fresh for every meal.

Mass culture with Culture Selco® as food

A different technique based on a compound feed has been developed a few years ago by the Belgian Company INVE SA. The product, named Culture Selco® (CS) is a dry and complete rotifer diet that does not require algae and is also effective as enrichment medium. Particle size (5 to 7 µm) and physical characteristics ensure an optimal uptake by rotifers. The feed composition includes proteins (>35%), lipids (>15%, of which 23% are PUFA), carbohydrates (30%), carotenoids and other micronutrients as minerals and vitamins A, D3, F and C.

The average daily production of rotifers fed on CS ranges consistently from 45 to 60% of the initial rotifer density. In addition rotifers are enriched with high levels of the essential (n-3) PUFA and vitamins.

This diet has made rotifer mass culturing reliable and predictable, and has consistently reduced the need for algal cultures and their associated labour and facilities requirements. New rotifer cultures can be easily started from old ones, thanks to their enhanced fertility.

Feeding

The following schedule gives the CS daily feeding rate (DFR), expressed as grams of CS per million rotifers. The day before harvest rotifers are enriched with other artificial diets made by INVE, Protein Selco® or DHA Protein Selco® (see Enrichment below for details).

steps	age days	rot./ml	DFR (g/million rotifers)
inoculation	0	200	0.55
	1	250	0.44
	2	350	0.34
enrichment [a/]	3	450	0.31
harvest	4	600	

[a/] Replace CS with Protein or DHA Protein Selco®

Fig. 30.04 An industrial blender for yeast or Culture Selco® suspension (photo STM Aquatrade)

To prepare CS suspend the amount required for a single meal in tap water, up to 50 g CS/l, and mix vigorously for 3 minutes (use a kitchen or better an industrial blender). Mixing or shaking by hand or using a magnetic stirrer is not sufficient to separate the CS cells. Remember that cell agglomerates left in the feed suspension cannot be ingested by rotifers because of their large size. Take care not to overfeed as uneaten feed can also quickly spoil water quality. Feed the daily amount in four to six meals evenly distributed over the 24-h period. In case it would be necessary, the feed suspension can be stored at a temperatures below 8°C, and the amount needed for the whole day can be prepared at one time. The feeding ration can thus be distributed from the stored suspension at each meal.

Fig. 30.05-6 CS preparation and distribution to the tanks (photo STM Aquatrade)

Before transferring rotifers to a new tank, place about 25% of the first day's food ration in the tank, so that the feed would be already evenly spread when the rotifers enter the medium. Add a few drops of silicon based antifoam agent, such as Rhodorsil Antimousse AM 70414 by Caldic Belgium NV.

Aeration

Aerating the rotifer culture is essential to provide oxygen, to keep rotifers and food cells suspended and to optimise tank cleanliness. The aeration rate and the air diffusers positioning should be carefully adjusted. A reasonably strong aeration is detected by an evenly spread water turbulence at water surface without large air bubbles.

Number and positioning of air diffusers depend on tank shape and volume. In all cases, however, they should be suspended at about 15 cm above the tank bottom to prevent re-suspension of sediments. As to the number of diffusers, a reliable rule of thumb is to keep a distance of approximately 60 cm among them.

Fig. 30.07 CS distribution by gravity to the tanks at Ittica Ugento (photo STM Aquatrade)

Cleaning cultures

In round tanks with conical bottom, bottom sediments are removed by letting them first settle for 10 15 minutes in absence of aeration (take care to check DO levels during the operation). Then the bottom valve is opened for a few seconds and it is closed when the outflowing water is again clean. Repeat the operation twice a day, in the morning and evening.

Complete the cleaning procedure by removing any greasy layer that may be formed at the water meniscus with a sponge or a paper cloth. Never dip your hands in water.

The rotifer tank water can be rapidly polluted by particulate matter, faeces and flocks of uneaten food. Their removal may prevent an excessive bacterial development and will increase the oxygen available for rotifers. As the continuous aeration of the water volume prevents in part the settlement of particles, their removal is achieved by means of “particle traps”. These devices consist of floating mats of coarse sponge-like material, such as the housing polishing mat Scotch Brite™. In the case of a 3,000-l tank, three pieces measuring 15 x 100 cm are hung vertically in the water (by means for example of wooden stick laid across the tank top rim), and are kept vertical by a terminal weight. The water circulation pushes particulate matter against the trap, where they adhere and clog the mat. These mats are also an ideal substrate for *Vorticella*, a sessile ciliate that competes with rotifers for food. To be effective, the traps must be cleaned at least twice a day.

To clean particle traps proceed as follows:

1. take them out of the tank carefully, avoiding dripping of trapped material back into the water;
2. remove all particles with a high pressure jet of tap water;
3. dip the trap in a 500 ppm hypochlorite solution for one hour to disinfect;
4. rinse well with tap water, dip in a thiosulphate solution to neutralize residual chlorine and place again in the tank.

Fig. 31.01-2 Scotch Brite™ after one night of work and during cleaning procedures (photo STM Aquatrade)

Harvest

At harvest, rotifers are filtered and rinsed before being fed to fish or utilized as inoculum for new tanks. For this purpose, a double submerged filter is used. The inner filter has a mesh size of 250-300 mm to retain larger particles, flocks of agglomerated food particles and ciliates which would rapidly clog the finer filter. The outer filter has a 50 mm filter mesh. Its capacity should be large enough to keep safely the whole rotifer population for the time needed to complete harvest and rinsing. Both filters are placed inside a large wheeled container full of water to avoid pressure build-up from the outgoing water that would smear rotifers against the net. A gentle air bubbling along the inner side of the filter helps to keep the filter free from clogging.

Harvesting and rinsing protocol for round-conical tanks with central drain:

1. prepare the harvesting device, always clean and disinfected with hypochlorite;
2. inject pure oxygen into the tank to be harvested for 10 to 15 minutes to have a supersaturated medium (at 10 ppm DO) in which rotifers could safely stand filtering operation;
3. switch off aeration for 10 - 15 minutes and purge the bottom sediments;
4. into the central drain fit a PVC pipe with holes at 15 cm from the drain; wait 5 minutes;
5. fix a flex hose to the bottom drainage valve and place the other end into the harvesting device;
6. open the valve and start filtering;
7. regulate the water flow so that the filter does not clog and the culture water does not overflow; do not exceed 100 l/minute;
8. stop water flow and clean the clogged pre-filter whenever necessary to avoid overflow of rotifer concentrated water;
9. while harvesting, check for possible loss of rotifers through the net by sampling some filtered water with a beaker or a Petri dish;
10. at the end of filtration, close the valve and rinse for 10 to 15 minutes the rotifers with filtered sterilized seawater at the same temperature as the tank of origin.

Fig. 32.01-2-3-4 Harvesting procedures of rotifers mass cultures (photo STM Aquatrade)

High density rotifer culture

Recent advances in mass culture technique yield higher rotifer densities. As an example, the procedure described below shows how to produce one billion rotifers daily with a battery of five 1-m^3 round tanks with conical bottom.

Protocol:

Inoculum: 500 rotifers/ml, harvesting at 1 000 rotifers/ml in four days.

Prepare five tanks following the above mentioned procedure.

Environmental parameters

- oxygen: keep 7-9 ppm with pure oxygen integration, preferably controlled by an automatic monitoring device
- pH:7.5-8.5 add 10% hydrochloric acid when needed
- TAN Total Ammonia Nitrogen:< 6 mg/l
- NH_3:< 1 mg/l
- aeration: reasonably strong. Hang three airstones placed at 15 cm above the bottom along the tank wall for air injection, and place one in the centre at 20 cm from the cone tip for oxygen injection
- particle traps: place four mats along the tank wall.

Cleaning operations (three times a day):

1. remove traps, air tubing and diffusers and wash carefully with tap water;
2. stop aeration for 10 minutes and purge the tank cone through its bottom valve;
3. put back in place clean and disinfected traps, air tubing and diffusers.

Feeding schedule (distributed at 2 and 8 am, 2 and 10 pm)

steps	age days	rot./ml	DFR (g/million rotifers)
Inoculation	0	500	0.3430 [a/]
	1	750	0.2857
	2	900	0.2857
Enrichment [b/]	3	1200	0.2857
Harvest [c/]	4	1500	

[a/] Of which 50% Protein Selco® and 50% Culture Selco®: Calculate the daily amount, distribute before the inoculum 100 g of it (100 ppm in a 1-m^3 tank), then divide the remaining quantity into three rations, using the same proportion for the whole day 0, at 4 and 10 pm and 2 am.

[b/] Replace Culture Selco® by Protein or DHA Protein Selco®

[c/] 1/3 of the harvest is used to inoculate other tanks, and 2/3 for fish larvae feeding (direct or after 12 hours-enrichment in a new tank).

Enrichment

Rotifers have a limited nutritional value for marine finfish larvae. In the past their nutritional value was upgraded by an enriching process before their harvest through feeding them with microalgae rich in PUFA and vitamins such as *Chlorella, Nannochloropsis* and *Isochrysis*.

At present, the enrichment is provided by specially formulated artificial diets like the above mentioned Selcoâ products. This oil emulsion gives excellent results in terms of high levels of EPA, DHA and vitamin C, which was not possible with the only use of algae. Moreover, labour, time, investment and running costs are spared. Rotifers can be enriched either in their mass culture tanks or after harvesting by placing them in dedicated enrichment tanks.

The first method produces an enrichment of the tissues, as it is continuous along the entire culture period. The acquired fatty acids reserves are more stable and are less exposed to a rapid decrease in nutritional value during starvation. This method also saves time and reduces handling losses.

The second system is a short term enrichment or, rather, a gut enrichment. It implies the harvesting and rinsing of the rotifers and the preparation of a separate enrichment tank.

Enrichment with algae:

- use selected algae as specified above;
- enrich for at least 8 h;
- maximum rotifer density: 500/ml;
- microalgae density: *Isochrysis* 5 million cells/ml; *Chlorella* sp. o *Nannochloropsis* spp. 12 million cells/ml;
- resulting average total PUFA content of enriched rotifers: ± 7 mg/g dry weight

Enrichment with Selco® products or other similar products:

- follow the instructions provided by the manufacturer;
- enrichment should take between 6 to 12 hours;
- maintain the oxygen level at or above 4 ppm throughout the entire procedure;
- dry products can be used directly in the production tank, whereas oily products are only fed in enrichment tanks;
- use an antifoam product during enrichment to prevent rotifers losses by foam aggregation;
- during the enrichment process check frequently rotifer mortality and dissolved oxygen content; the latter should be kept above 80% saturation, with addition of pure oxygen if necessary.

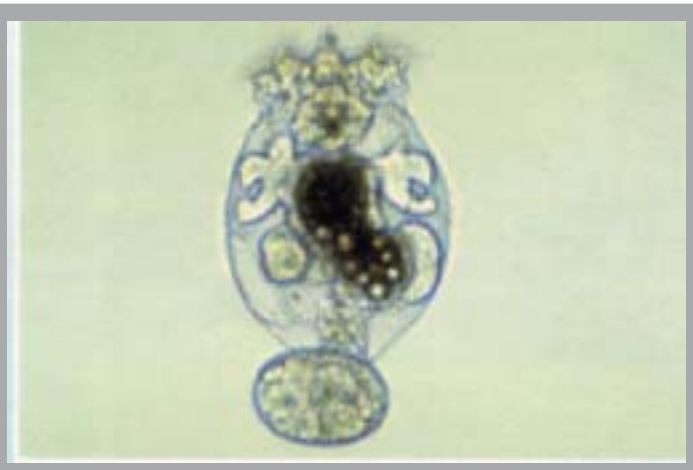


Fig. 33.00 Enriched rotifer (photo INVE Aquaculture)

The content of nutrients decreases rapidly in rotifers that are not immediately consumed by fish larvae. In starving rotifers the total PUFA loss reaches 60% after 6h at 18°C. Even in green water, i.e. with microalgae, this loss remains important (about 40% after 6 h). To prevent this degradation in nutritional quality, enriched rotifers not immediately fed to fish should be stored in containers at low temperature as follows:

- storage time should not exceed 14 hours;
- temperature should be kept between 5 and 10°C by means of insulated tanks and blue ice or ice bags.
- rotifer density should not exceed 2 500 to 3 000 ind/ml;
- oxygen level should be kept at or above 4 ppm.

Monitoring rotifer populations

Check all rotifer cultures daily for both quantitative and qualitative evaluations. From each vessel, flask, bag and tank, take a 1 ml sample and observe under the stereoscopic microscope.

Measure the following parameters:
quantitative parameters:

- total number of rotifers per ml
- total number of eggs per ml
- fertility as percent of total eggs over the total rotifers

qualitative parameters:

- average number of eggs per individual (estimate)
- repletion (presence of food in the stomach, note 0 for empty, + for medium full, ++ when full)
- motility (++ active, + slow, 0 absent)
- filtration (activity of the ciliated corona)

Fig. 33.01-2 Rotifers populations are daily checked and counted (photo STM Aquatrade)

In addition the following qualitative parameters of the culture should be checked:

- presence of foam at the culture surface or sediment on the wall and bottom of the container
- presence of other micro-organisms, such as protozoa, fungi, bacterial flocks, etc. (identification and frequency, note 0 when clean, + for medium contamination, ++ large contamination)

All these information must be recorded in the individual file of each culture. All daily monitoring procedures for the rotifers sector are given in Annex 10.

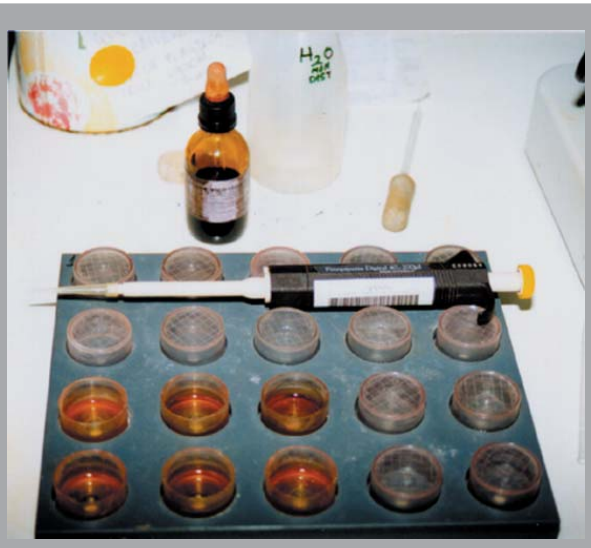


Fig. 34.01-2 Micropipette and microscope used to evaluate population parameters (photo STM Aquatrade)

Production of the brine shrimp *Artemia*

Artemia cyst strains

Having a larger size than rotifers, the larval stages of a small crustacean, the brine shrimp *Artemia salina* are used as the second (after rotifers), and last live food fed to fish larvae before their weaning on artificial feed. Strictly speaking, *Artemia* is not cultivated in the hatchery as is the case for algae and rotifers, but their larval stages are obtained by incubating and hatching their resting eggs, available as dry storable cysts, which can be purchased from specialised suppliers. The first *Artemia* larval forms, the nauplii, which are also the smallest and richest in yolk, are followed by a larger metanauplius, whose nutritional value has to be boosted by feeding them special enrichment diets 12 to 24 hours before being offered to fish.

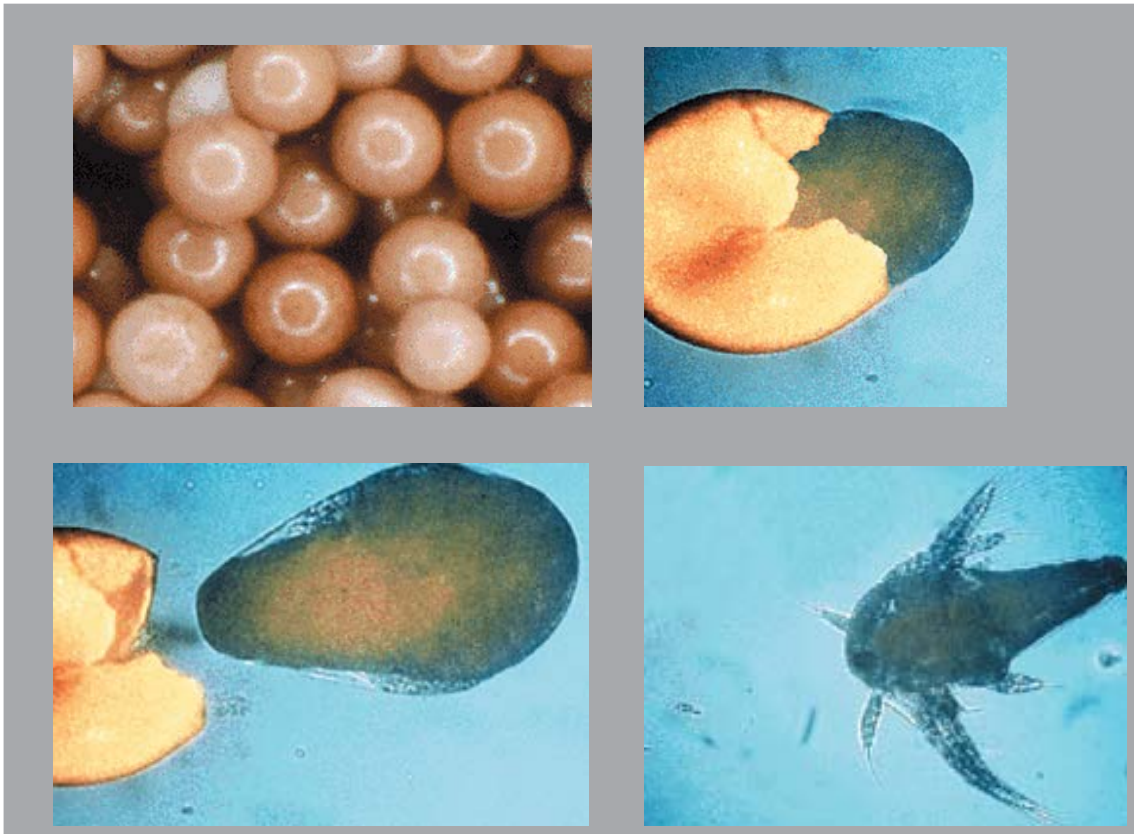


Fig. 35.01-2-3-4 Various *Artemia* stages (photo INVE Aquaculture)

Many types of brine shrimp cysts from different locations in the world are marketed: they differ in naupliar size, important in gilthead seabream first feeding, in nutritional value and in hatching characteristics. For practical purposes they can be categorized as follows.

- Cysts giving small Instar I-nauplii (with a length of around 430 µm at yolk- sac stage) with high levels of the essential highly unsaturated fatty acids (n-3 HUFA). An example is the AF strain of the Belgian producer INVE. Such nauplii allow an early switch from rotifers to *Artemia* in larval feeding.
- Cysts producing medium-sized Instar I-nauplii (around 480 µm) with high levels of n-3 HUFA, such as the AF 480 strain of INVE. These nauplii are useful to switch from the smaller nauplii to the bigger metanauplii.
- Cysts hatching large Instar I-nauplii (around 520 µm) with low levels of n-3 HUFA, such as the EG strain of INVE. These cysts, which are the commonest and cheaper, are widely used to produce metanauplii which represent the bulk of live feeds in larval fish rearing. Their enrichment is mandatory to increase their essential fatty acids content to meet the nutritional requirements of young fish.

		Length (µm)	Width (µm)
EG	24 h	± 630	± 186
AF	16 h	± 430	± 162
	24 h	± 500	± 172
AF 480	16 h	± 480	± 173
	24 h	± 520	± 182
BE 480	16 h	± 480	± 173
	24 h	± 520	± 182

Table 3.6 - Biometrical analysis parameters of some *Artemia* nauplii strains

Two additional parameters characterize the *Artemia* batches: the number of cyst per gram and their hatching rate (the number of nauplii produced per gram of cysts). The best strains can give about 290 000 - 300 000 nauplii per gram of cyst hatched, with a hatching rate close to 95%. In a hatchery the use of good quality cysts allows a synchronization of the production cycle on a 24-h period, with the harvest of freshly hatched nauplii coinciding with the start of the incubation of new batches. Basic information on *Artemia* biology and life history is given in Part 2 of this manual.

Disinfection and decapsulation of brine shrimp cysts

Artemia cyst shells are usually contaminated with bacteria, spores of fungi and other microorganisms. Fish larvae can be infected when untreated empty shells, unhatched cysts or cyst hatching medium residues are introduced into the larval rearing tank.

Before incubation cysts should therefore be disinfected. This process also improves hatching by reducing the bacterial load of the hatching medium. Disinfection is done by keeping the cysts for a few minutes in a hypochlorite solution at a maximum density of 50 g/litre. This product is easily available as commercial grade bleach. The duration of the treatment varies according to the active chlorine concentration of the disinfecting solution. Typical duration is :

- 1 minute in a 10 000 ppm solution,
- 20 minutes in a 200 ppm solution.

As in commercial bleach the chlorine content may range from 5 to 15%, it is mandatory to check the actual chlorine concentration in the bleach that is going to be used. This can be done either by titration or by determination of the refractive index. The titration method is explained in Annex 7.

The following example shows how to disinfect one kg of cysts in a 200 ppm hypochlorite solution obtained from a household bleach with 5% active chlorine:

1 one kg of cysts needs 20 l of fresh water for the disinfecting solution;
2 if this solution is going to be used for a 20 minutes bath you will need 20 l x 200 mg/l = 4 000 mg = 4 g active chlorine;
3 the quantity of 5% bleach required to give 4 g active chlorine is:
(1 000/50) x 4 = 80 ml
4 pour 80 ml of 5% bleach in 20 l of fresh water;
5 add one kg of cysts; place an airstone for continuous aeration to keep cysts in suspension, and keep the cyst in the solution for 20 minutes;
6 harvest cysts on a sieve (125 µm mesh size) and rinse thoroughly with plenty of tap water;
7 transfer the rinsed cysts to the incubation tank.

A more effective way to obtain completely contaminant-free cysts is decapsulation, which implies the elimination of the cysts thick external layer, the chorion, by chemical oxidation. This process, which requires greater attention, has additional advantages. As they spend less energy to hatch after the removal of the chorion, the hatching nauplii have better nutritional value. Moreover, fish do not risk to suffocate by gulping empty or unhatched cysts offered together with the nauplii, as it may happen when using disinfected cysts.

The decapsulation process consists in four steps: hydration, treatment in a chlorine solution, washing and deactivation of the residual chlorine. The example described below refers to the decapsulation procedure of one kg of cysts.

The hydration, a necessary step as the complete removal of the chorion may only happen when cysts are spherical, proceeds as follows:

1. water volume required: around 6 l per kg (maximum amount: 200 g/l); both fresh and sea water can be used; water temperature should be between 20 and 25°C;
2. duration: 45 minutes;
3. aeration: sufficiently strong to keep cysts in constant suspension; use an open end pipe in a 10 l bucket;
4. collect the hydrated cysts on a sieve and treat them immediately with the decapsulation solution.

The decapsulation solution requires a source of hypochlorite, usually liquid bleach (NaOCl), and an alkaline product to increase pH level of the decapsulation solution above pH10. Usually technical grade caustic soda (sodium hydroxide NaOH) is utilized . The first product is added at 0.5 g active chlorine per gram of cysts, and the second as 0.15 g of sodium hydroxide per gram of cysts. For hydrated cysts the procedure is as follows (figures refers to one Kg of cysts):

1. prepare 0.5 g Cl x 1 000 g cysts = 500 g of active chlorine, equal to 3 33 l of a 15% bleach;
2. prepare 0.15 g NaOH x 1 000 g cysts = 150 g of NaOH, equal to 0.375 l of a 40% NaOH solution;
3. put the bleach and NaOH in a suitable container (e.g.: a 20 l plastic bucket) and fill with seawater to 14 litres (14 – 3.33 – 0.375 = about 10.3 l of seawater)
4. provide a strong aeration and eventually if available add antifoam;
5. place the hydrated cysts in the bucket;
6. control the temperature: it should remain within 25°-30°C. In case of higher temperatures, add ice to prevent that it reaches 40°C which are lethal for the cysts;
7. verify cyst colour changes. The change in cyst colour confirms that decapsulation is in progress: the cyst colour shifts from dark brown to grey and finally to orange, which is the colour of the nauplius body seen by transparency through its outer cuticular membrane, left exposed by the dissolution of the chorion. The process usually lasts 5 to 15 minutes.
8. using a pipette or a graduated cylinder, check floatability: non decapsulated cysts will float and decapsulated cysts will sink; as soon as all cysts have turned orange, stop the process by harvesting them on a sieve and rinse thoroughly with plenty of tap water and rinse well until no more chlorine smell is noticed;
9. the residual hypochlorite adsorbed by the decapsulated cysts has to be neutralised by dipping them in a 0.1% solution of sodium thiosulfate ($Na_2S_2O_3 . 5H_2O$) for 5 minutes;
10. then, after a final rinsing, they are transferred to the incubation tank.

Warnings

Decapsulation is an exothermic reaction: during the process the temperature will raise by approximately 12°C, from 20 to about 32°C.

The chemicals used for this process are toxic to humans and must be handled properly. Wear gloves and protective eyeglasses.

Check if the local legislation requires any special permission for (or bans) the use of any of these products.

Always check the chlorine content of the bleach used, to optimize the entire procedure and give constant results. See Annex 7 for detailed description of procedure and reagents

Fig. 36.01-2-3-4 *Artemia* industrial decapsulation (photo INVE Aquaculture)

Besides incubation, the decapsulated cysts can be either stored or, unless not commonly, be fed directly to fish. For short-term storage, up to one week, keep in the refrigerator at a temperature of 0 - 4°C. Decapsulated cysts that have to be stored for a longer period have to be dehydrated in a saturated brine solution as follows:

- per each kg of decapsulated cysts prepare a brine solution by dissolving 1.7 kg kitchen salt (fine crystals) in fresh water to reach a final volume of 6 l (solubility after 1 h is ± 270 ppt),
- aerate with an open pipe for 12 h,
- place the decapsulated cysts in the brine solution overnight or for minimum 3 hours at room temperature,
- drain cysts on a 125 µm sieve, transfer them to a plastic container topped with fresh brine and store in a refrigerator or a freezer.

As the water content of cysts should be about 16-20% after this process, maximum hatchability is guaranteed for a storage period of only a few months.

In an emergency, they can be directly offered to fish larvae, provided that water circulation and aeration can keep them in suspension for a sufficient time to be ingested by fish. As the decapsulated cysts are 50% smaller than newly hatched nauplii, they may be used as initial food for small fish larvae.

Fig. 37.01 *Artemia* incubation tanks of 1 m3 (photo STM Aquatrade)

Incubation

As soon as the dry cysts are placed in water to be hydrated, the embryos inside the cyst start their metabolic activity. The free swimming nauplius, called instar I, hatches after about 20 to 24 hours if incubated under optimal conditions as follows:

- tank design: round with conical bottom, white gel-coated inside with a semi-transparent window near the cone tip for harvesting; a drain with a valve is installed in the cone tip;
- filtered and sterilized seawater, 35 ppt salinity;
- water temperature 28-30°C;
- strong aeration to provide a vigorous water agitation, to keep cysts in suspension: air is provided through the open end of a 1/2 inch PVC pipe placed close to the tank bottom;
- dissolved oxygen level above 4 ppm;
- pH over 8; if needed, add sodium bicarbonate ($NaHCO_3$) at the rate of about one gram (previously dissolved) per litre;
- a strong illumination of 2 000 lux at the water surface during the first incubation hours; light can be provided by two neon tubes (2 x 58 W) placed just above the tank rim;
- cyst density for incubation: 2.5 g per litre

Harvesting of nauplii

Artemia should be harvested when at the energy-rich instar I larval stage, just after hatching. This occurs in about 22 h at 28°C. To assess the proper time, sample the incubation medium with a 5-ml glass pipette and check for nauplii and umbrella stages (embrios still attached to their cyst) under the stereomicroscope.

To harvest nauplii, proceed in the following way:

1 Purge the bottom tip of unhatched cysts that has sunk by opening the drain valve for a few seconds;
2 Fit a flexible hose at the drain valve and place its open end into a container full of seawater equipped with a central filter.

3 Stop aeration, switch off the light above the tank, cover its top with a light-proof lid and place a light source at the window on the tank bottom: empty cyst shells will float, unhatched cysts will sink, whereas freshly hatched nauplii will concentrate at the tip of the conical bottom, attracted by light because of their positive phototactism. To attract the nauplii use a 150 W bulb lamp placed in a waterproof holder;

4 Wait 10 minutes, the time the nauplii will separate from their empty shells and start to harvest the tank, either fully or partially, by draining it into the filter . After another 10 minutes, a second harvest may be carried out if all nauplii were not harvested earlier. Do not drain the tank completely to keep floating empty shells in it. Adjust the water flow to avoid clogging the screen/water overflowing . Suggested maximum water flow is 100 l/min. If the water level in the filter stands higher than the water level in the tank, it would also be possible to harvest nauplii using a small submersible pump. When filtering, check with a glass beaker for possible losses of nauplii that may escape the filter .

5 During the settling time monitor the oxygen level, which should not drop below 2 ppm, as well as excessive crowding nauplii above the tip of the conical bottom. If necessary prior to harvest inject pure oxygen into the culture tank for 10 to 15 minutes to raise the dissolved oxygen content up to 10 ppm.

6 Rinse the nauplii thoroughly (for about 15 minutes), preferably with fresh water, to wash out the hatching debris.

7 Put the rinsed nauplii in a temporary container filled with a known volume of filtered and sterilised seawater and count them (see the procedure below).

8 Transfer nauplii to the storage tank filled with sterilised sea water and adjust the water volume to give a maximum density of 4 million nauplii per litre; provide a moderate aeration from the cone tip and add blue ice (or sealed ice bags ice) to keep water temperature at 5 to 10°C or better use a cold storage refrigerated tank or a freezer when available. At higher temperatures their nutritional value decreases rapidly.

9 If the nauplii have to be enriched, transfer them to a previously prepared enrichment tank.

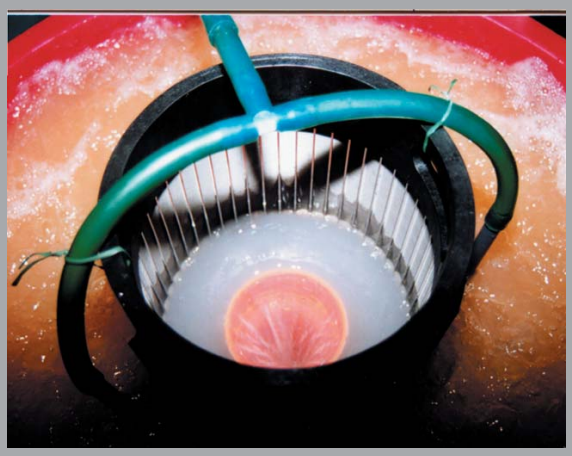


Fig. 38.01-2-3-4 *Artemia* harvesting (photo STM Aquatrade)

Counting and evaluating *Artemia* nauplii

To assess the hatching results and to feed the larval rearing tanks at the established densities you have to count the *Artemia* nauplii.

Three methods are described below, one for high nauplii densities, such as after harvesting and in a cold storage tank, one more common for counting the nauplii when they are in the incubation tank and the other for low nauplii densities, similar to those which can be found in fish tanks.

Counting high density nauplii samples:

- take a 10-ml sample of the population to be estimated;
- dilute the nauplii concentration by adding 90 ml of sea water to obtain a total sample volume of 100 ml;
- take three sub-samples with a 1-ml pipette, avoiding to suck air bubbles;
- transfer each sub-sample to a Petri dish;
- add a few drops of the fixative staining solution Lugol (see Annex 7) to each of the Petri dishes and wait until all nauplii are immobile and deeply stained;
- add water so as to distribute the nauplii over the whole surface of the three Petri dishes;
- put each Petri dish on a grid and count the nauplii present in each 1 ml sub-sample;
- calculate the average number of nauplii per ml by dividing the sum of the three counts by three;
- the total nauplii density per litre is given by the average number of nauplii per ml multiplied by 10 to give the number per undiluted ml and finally by 1 000 to get the final density per liter.

Counting nauplii samples from the incubation tank:

- take with a test tube a 50-ml sample of the population to be estimated;
- take three sub-samples with an automatic 0.1-ml pipette;
- transfer each sub-sample to a 3 cm-wide Petri dish, whose bottom has been subdivided in a 5 mm-grid;
- add a few drops of the fixative staining solution Lugol (see Annex 7) to each of the Petri dishes and wait until all nauplii are immobile and deeply stained;
- add water so as to evenly distribute the nauplii over the whole surface of the three Petri dishes;
- count the nauplii present in each 0.1 ml sample;
- calculate the average number of nauplii per ml by dividing the sum of the three counts by three;
- the total nauplii density per litre is given by the average number of nauplii per ml multiplied by 10 to give the number per ml and finally by 1 000 to get the final density per liter.

Counting low densities nauplii samples:

- take three 50 or 100 ml samples, using a graduated cylinder which has been cut at the 50 or 100 ml mark;
- transfer each sample to a large Petri dish;
- add several drops of Lugol (see box below) to each of them and wait until all nauplii are immobile and deeply stained;
- put each Petri dish on a grid and count the nauplii present in each sample;
- calculate the average number of nauplii present in each sample;
- calculate the nauplii density per litre by multiplying this count either by 20 (50 ml sample) or by 10 (100 ml sample).

The two main criteria to evaluate hatching results are:

- hatching rate: the number of nauplii hatched per 100 cysts; good batches have a hatching rate around 90-95%;
- hatching efficiency: the number of nauplii produced per gram of cysts; top quality cysts yield about 300 000 nauplii/g.

Enrichment and storage

Older larval stages of brine shrimp, the metanauplii, are used typically as feed for growing fish post-larvae. However, their poor nutritional value has to be boosted by dedicated enrichment diets that are rich in essential n-3 HUFA. Such diets can only be given when *Artemia* feeds actively, which is during its larval stages of instar II and instar III. First feeding in brine shrimps actually coincides with their moult into the second Instar stage.

Fig. 39.01 Enriched *Artemia* (photo INVE Aquaculture)

The best results are obtained when the hatching time and the moulting pace are exactly known. The beginning of the enrichment can easily be determined by observation. After the first 18 hours small samples are taken every hour and checked under the microscope. The appearance of second instar stage is easily detected since it is larger than the first instar and presents a gastro-intestinal tract. Enrichment will begin as soon as the first instar II stages appear.

The duration of the enrichment process as well as the type of product to be used depend on the HUFA content that is desired. Full enrichment takes 24 hours and two doses of enrichment emulsion, at time zero and 12 h later. Short-term enrichment takes 12 h and only the initial dose.

Optimal enrichment conditions

- for water quality and container design, see previous section; initial nauplii density: between 150,000 and 300,000 nauplii/l;
- vigorous water agitation to keep the nauplii in suspension and pure oxygen to keep dissolved oxygen above 4 ppm throughout the enrichment period: use one open PVC pipe for air and a micro-bubbles diffuser for oxygen;
- lighting: not required.

Prepare the enrichment meal as specified by the producer, and make sure to prepare a new enrichment emulsion for each meal. At the end of the enrichment time harvest the metanauplii as usual, rinsing them thoroughly with seawater until no oily emulsion is noticed in the outflowing water.

Fig.40.01-2 *Artemia* cold storage tank (photo STM Aquatrade)

Cold storage of enriched Artemia nauplii

Enriched metanauplii rapidly loose their nutritional value at room temperature, as it happens to rotifers, unless they are stored in cold seawater (4 to 10°C). Keep density below 4 millions per litre. Equipment and procedure are the same described for cold storage of nauplii.

Annex 11 shows examples of daily procedures and recording files for Artemia sector.

3.3- FISH EGG MANAGEMENT

Fertilized seabass and gilthead seabream eggs float in water with 35 to 37 ppt salinity. With lower salinity egg buoyancy decreases and a strong aeration is advisable to prevent their sinking to the bottom, which would pose a big risk in terms of physical stress and bacteriological contamination.

Because egg quality represents a crucial factor for hatchery success and for production of fry of good quality, any stress to fertilised eggs should be avoided, such as:

- physical shock during egg collection, transport, disinfection, weighing and incubation;
- thermal and salinity shocks. It is recommended to maintain in the incubation tanks the same environmental conditions at which eggs have been exposed during spawning. The same precaution should be adopted when packing eggs for sale.

Before dealing in detail with egg management and larval rearing of seabass and gilthead seabream, the following paragraphs give a brief description of the development stages from egg to juvenile.

What follows takes place as close as possible to spawning temperature, characteristic of each species, up to completion of swimbladder primary inflation and at 17-18°C afterwards. Larval length indicated is equivalent to total length, age in days starts from the hatching day, considered day zero.

Gilthead seabream eggs and larvae development

- Gilthead seabream eggs are spherical with a diameter range of 0.94 - 0.99 mm and have a single large oil droplet. Hatching starts approximately 48 hours after spawning at 16 - 17 °C (common range) and the newly hatched larvae have a total length of around 3 mm.
- At hatching the yolk sac occupies approximately 1/3 of the larvae length. The eyes are not pigmented, the mouth is closed and the larvae have very few melanophores on their body. The pectoral fins develop two days after hatching.
- Within the following three to four days the body pigmentation increases, the mouth opens and as the yolk sac is consumed its volume is progressively (more than 50 %) reduced. The larvae length is around 3,8 mm and the eyes are completely pigmented. The swimbladder starts inflating when larvae reach 4 mm, at which length the yolk sac is completely reabsorbed.
- By day 15 (at 5 mm length), the swimbladder primary inflation is completed; by this time the cauda fin develops and extensive body pigmentation appears.
- By day 20 the anal fin and the stomach develop. By the day 35 a complete pigmentation of visceral organs appears.
- Between day 45 and day 50, the dorsal and ventral fins develop and scales appear. The swimbladder expands progressively to its definitive size.

Seabass eggs and larvae development

1	Seabass egg (1/2 hour after fertilization)
2	First cell division after 1 h 30 min.
3	Egg in stage 4 cells after 2 hours
4	Six blastomers stage after 2 h. 30 min.
5-6	Eggs in morula stage (4 h. 30 min.)
7	Upper view of the blastodisk (9 hours)
8	Egg 22 hours after fertilization
9	Yolk being englobed by the blastodisk after 28 h.
10	Same after 34 hours
11	After 40 hours the embrionic axis is visible
12	Same as above but lateral view
13-15	Evolution of the embrio after 45h., 50 hours and 57 hours.
16	Pigmentation oppears after 69 of incubation.
17	Segmentation visible in middle part of the embryo (80 h.)
18	Same as previous but seen on the side. Note expansion of head area
19	Segmentation continues towards the tail (85 h.)
20	The embryo increases in length (95 h)
21	Lateral view of embryo at 97 hous. The primordial fin is forming
22	The embryo bends inside the egg and the primordial fin is formed (104 h.)
23-28	Hatching process sequence, after 110 h. The chorion is dissolved by an enzyme secreted by glands on the head of the embryo. Hatching will take between 1 and 2 hour at 15° C.

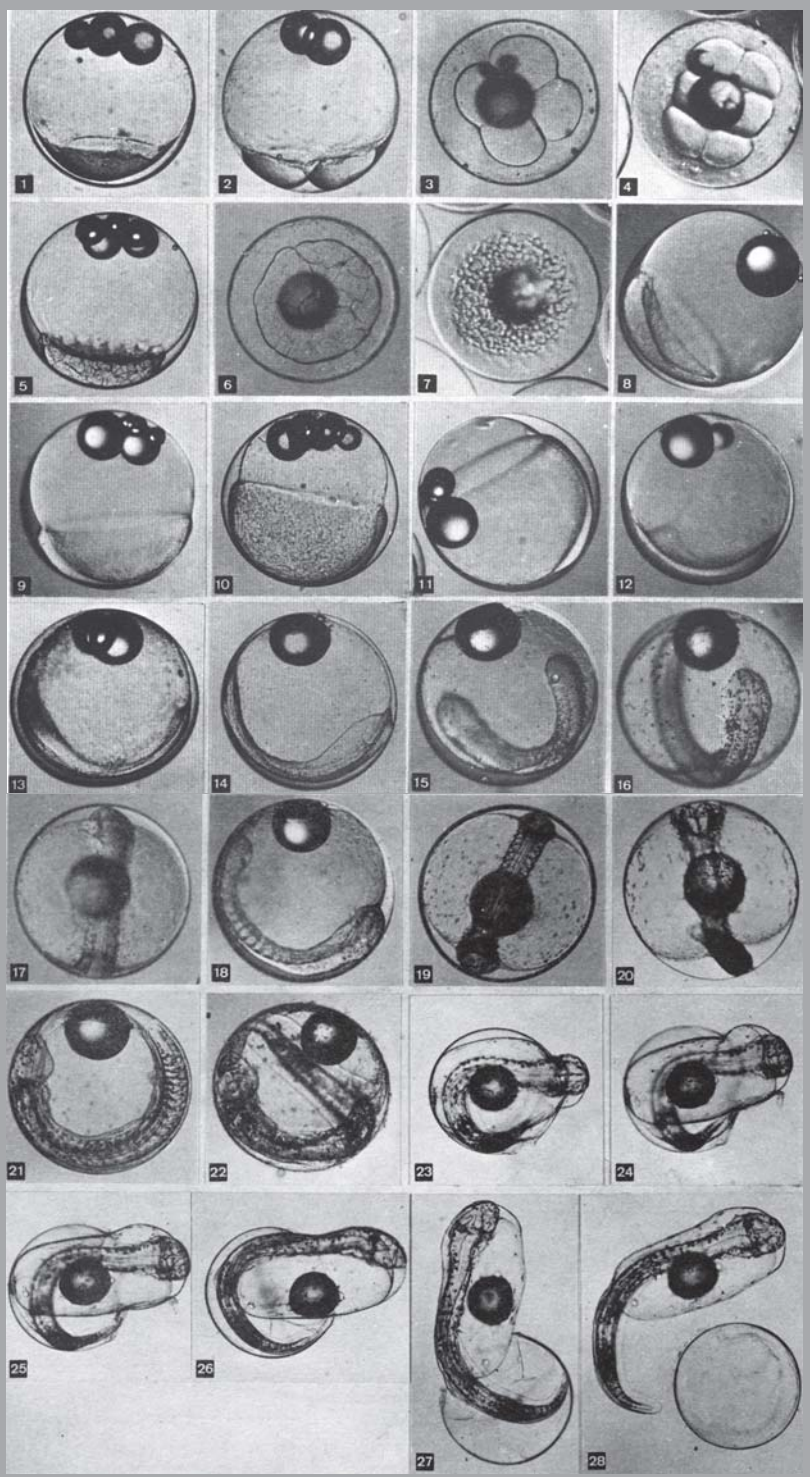


Fig.41.01 Seabass eggs and larvae development (photo Barnabè)

- Eggs are spherical with a diameter range of 1.1 - 1.25 mm. Hatching starts approximately 72 hours after spawning at 13 - 14 °C (common range) and newly hatched larvae have a total length of around 4 mm. The yolk sac is almost 1/2 of the whole body length and the oil droplets are smaller than in seabream and frequently two or more are present. Eyes are not pigmented, the mouth is closed and larvae have evident melanophores on their body. 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 (more than 60 %) its volume. The length is around 5 mm and the eyes are now completely pigmented. The swimbladder starts inflating when larvae reach 5,5 mm, at which stage the yolk sac is completely reabsorbed and the oil droplets only partly.
- By the day 16 (6,5 mm length) the swimbladder primary inflation is completed; by this time the oil droplets of the yolk-sac have 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.

Egg harvest

As fertilised seabass and gilthead seabream eggs float in full seawater, most Mediterranean hatcheries have adopted the automatic egg collectors described below. If well dimensioned and properly placed, these devices harvest only the floating eggs, while the dead or unfertilised ones sink to the bottom. A few important precautions should be taken into consideration. The presence of eggs in the collectors should be checked rather frequently in the case of seabass, as its spawning produces a large amount of eggs in a very short time and there is risk of clogging the collectors or of mechanical stress to the eggs. Due to the waste produced by spawners in their tanks, egg collectors have to be kept properly cleaned and should be replaced at least daily with sterilised ones. The water flow should be adjusted so as to gently transfer eggs from the spawning tank into the egg collector without harmful mechanical shocks.

The overflow collector is placed outside the spawning tank. It consists in a screened container that receives the water of the spawning tank by overflow and that is placed inside another container. The collector is usually a PVC cylinder with large lateral and bottom openings screened by a 400 µm mesh size nylon net . A flexible hose connects the spawning tank with the open top of the screened cylinder, assuring that the surface water from the spawning tank flows into the egg collector. The water level inside the collector tank is maintained by another overflow outlet in a way that it is only few centimetres below the level in the spawning tank. Eggs retained by the screen are kept floating by water flow and a gentle aeration.

The airlift collector is a device placed inside the spawning tank. It is basically a screened box or bucket equipped with floaters and small airlifts. These airlifts are PVC pipes that transfer the surface water of the spawning tank into the collector by means of an air flow. They are connected to the low-pressure air distribution system of the hatchery, and their flow is adjusted to gently lift the floating eggs, sparing them from any mechanical stress.

To take out the eggs from both types of collectors, the aeration and water flow have to be stopped. Viable eggs are allowed to float freely in still water. In this way a first separation between sinking dead eggs and viable ones takes place. To minimise the presence of poor-quality eggs, which usually float deeper in the water, it is advisable to collect only the eggs found at the water surface, after a period of settling without aeration, that in any case should not exceed 10 minutes to avoid risk of anoxia. Then with a 1-l jar scoop the eggs from the

water surface and place them directly in a temporary stocking vessel (that could be a bucket or a conical container). This vessel should have been previously filled with sterile water at the same temperature and salinity and should be provided with a gentle aeration through a fine air stone. A floating layer of eggs thicker than one cm should be avoided. A thicker layer may reduce oxygen supply to the eggs, leading to possible anoxia after a short time. When in the temporary container, eggs must be thoroughly examined to assess their quality, number and development stage.

Table 3.7 Comparative development of seabass and gilthead seabream larvae and postlarvae

	Seabass		Gilthead seabream	
Age	**Tot. length**	**Observations**	**Tot. Lenght**	**Observations**
Days	mm		mm	
1	4	Hatching	3	Hatching
2	4,5	Appears pectoral fins	3,5	Appears pectoral fins
3			3,8	Exotrophy starts
4				Eyes pigmentation
				60% of yolk sac reabsorbed
				40% of drop reabsorbed oil droplet
5	5	Exotrophy starts	4	Primary swimbladder inflation
		Eyes pigmentation		100% of yolk sac reabsorbed
		60% of yolk sac reabsorbed		70% of oil droplet reabsorbed
		40% of oil droplets reabsorbed		
7	5,5	Primary swimbladder inflation		
		100% of yolk sac reabsorbed		
		60% of oil droplets reabsorbed		
15			5	End of primary swimbladder inflation
				100% of oil droplet reabsorbed
				Caudal fin
16	6,5	End of primary swimbladder inflation		
		100% of oil droplet reabsorbed		
17			7	Anal fin
20	8	Caudal fin	7,5	Stomach starts developing
25	12	Anal fin		
35	14	Teeth		
		Stomach starts developing		
40	15	Second dorsal fin		
45	20	First dorsal and ventral fins	11	Second dorsal fin
50			15	First dorsal and ventral fins
60-70			20	Scales
70-80	30	Scales		
		Definitive morphology		
90			30	Definitive morphology

Quality controls

A reliable egg control quality needs usually just a few dozens of eggs, which are placed under a microscope (10 to 100x magnifications), or a transmitted-light stereomicroscope. With a pipette they should be taken from the floating egg layer in the temporary container, and should be placed on a watch-glass or on a Petri dish, making sure that the eggs form a single layer.

Check for the following egg characteristics:

- absence or occasional presence of dead (opaque, whitish) or unfertilised (transparent, but without evidence of cell divisions) eggs in the sample (less than 5% of the batch normally),
- regular rounded shape and size (diameter in gilthead seabream: 980-990 µm, in seabass: 1 000 -1 250 µm), regular cell division that can be observed only in the first blastomers;
- regular shape of vitellus (it should occupy the egg volume entirely, without perivitelline space),
- crystal clear transparency (no superficial spots and dark areas),
- proper number and size of oil droplets (in seabass from 1 to 6 of a 400-440 µm diameter; in gilthead seabream one droplet with a diameter of 230-240 µm),
- absence of parasites or associated micro-organisms on the chorion surface.

An early estimate of irregular or aborted eggs can be made at this stage. Any irregular egg will soon develop into an abortive embryo or an abnormal larva. Spots on the external chorion account for physical or bacterial damages. Sometimes, the vitellus colour is yellowish, apparently due to broodstock husbandry/feeding regimes. In such case, eggs with a yellowish vitellus are considered to be normal.

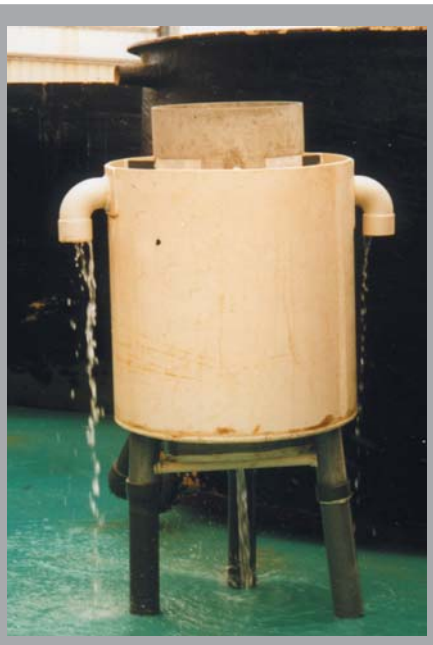


Fig.42.01 Overflow collector (photo STM Aquatrade)

As a general rule, good egg batches have usually less than 10% abnormal eggs. Any batch containing more than 20% abnormal eggs should be discarded.

A batch of eggs with an abnormality rate between 10 and 20% may be accepted only if there is a severe egg shortage, or when eggs are imported from other hatcheries. In this case, the management has to take into account a higher mortality rate at hatching and during the early larval stages. Otherwise, such poor egg batches should be replaced as soon as possible by better ones.

Dead and unfertilized eggs are thus discarded twice, first in the collector where only floating eggs are scooped up, and then in the temporary container before counting and disinfection. But it is also advisable to select only good eggs. Poor-quality eggs usually float deeper in the water. Collect only the eggs found at the water surface, after a repeated decantation without aeration.

In case of evidence of severe infestation by parasites or undesirable micro-organisms (ciliates, flagellates, nematodes, etc.) the eggs should be discarded to avoid contamination risks in the larval rearing sector.

Weighing, disinfecting and counting eggs

Prior to stocking eggs, either into the hatching facilities or directly into the larval rearing tanks, three more steps are required: weighing, estimation of their quantity and disinfection.

Even a rough estimate of the egg numbers allows the person responsible of the larval rearing sector to properly plan the stocking of the larval tanks, to optimise production routines and to coordinate the work of related sectors (live feeds and weaning). It does also allow a proper evaluation of the final survival rate to be expected.

The procedure to weigh the eggs consists in dividing them in many sub-samples, taking each of them out of the temporary container in a plastic filter removing quickly the excess of water, and weighing them on a balance calibrated for the egg filter tare. They are immediately returned to the temporary container (or disinfected, see below). During the collection of the eggs for this operation, only floating, viable eggs are picked. Dead and unfertilized eggs are thus discarded twice, a first time in the collector and then on this occasion.

Egg disinfection is the very first effective barrier against transmission of fish diseases, and is therefore highly recommended for all batches of eggs, both those produced in the hatchery and those brought from other hatcheries. This important operation is usually conducted just after the weighing, when the filter containing the egg sub-sample is dipped in the disinfecting bath for a short period of time prior to being put into the incubation tank. The most commonly used egg disinfectants are Penicillin G, Streptomycin sulphate and active iodine. Even if these antibiotics are commonly used, due to the undesirable side-effects they have and the risks to induce bacterial resistance, active iodine is suggested as the preferred disinfectant .

Active substance	Dosage	Time	Usage
PENICILLIN G	80 I.U./ml	1 min	500 mg / 10 l of sea water for 100-200 g of eggs at a time
STREPTOMYCIN-SO_4	50 µg/ml	1 min	500 mg / 10 l of sea water for 100-200 g of eggs at a time
ACTIVE IODINE	50 ppm/litre	10 min	8 litres for : $1x10^6$ seabass eggs or $1.5x10^6$ gilthead seabream eggs

Table 3.8 Disinfectants used for seabass and gilthead seabream eggs

The assessment of egg numbers can be made in two ways: by relating number to weight or by counting. In the first case the total egg weight is divided by the average individual egg weight, assessed from a small sample. The second method contemplates counting the eggs present in a few 1-l sub-samples and multiplying the average value by the total tank volume. While the water taken with the egg samples biases the first method, the latter requires a uniform egg distribution in the tank to be statistically correct. This method can also be applied to count freshly hatched larvae, which gives a better estimate of the initial population.

The protocol to weigh, count and disinfect eggs is given in Annex 12.

Incubation of eggs

Egg incubation can take place either in dedicated incubation tanks or directly in the larval rearing tanks. The latter choice has some drawbacks that fully justify the inclusion of a separated hatching sector as the ideal solution. After hatching, only the hatched larvae are moved to the clean larval tanks, whereas the hatching facilities are easily emptied, washed, disinfected and refilled for the next egg batch. Moreover, in this way the management of egg batches with poor hatching rates is facilitated by the smaller size of the hatching facilities.

Egg incubation in dedicated facilities

To incubate eggs, plastic or fiberglass round tanks with a conical bottom and a 100 to 250 l capacity represent the most common technical solution adopted by Mediterranean hatcheries. The cylindro-conical shape gives a good water circulation pattern, provided that a central aeration source is placed near the tip of the conical bottom, and a better separation of not viable eggs and hatching debris. Their inner surface is smooth to prevent any damage to eggs and newly hatched larvae.

Before being stocked with eggs, these tanks are carefully cleaned and disinfected with a hypochlorite solution (following the same procedure used for rotifer or artemia tanks), including the inlet/outlet pipe system and the submerged aeration devices (diffusers and tubes). Each tank is then filled with filtered and sterilized sea water, taking care to check that the temperature and salinity are the same as in the spawning tank from which the

eggs originate. The incubation tanks should be part of a flow through (open) water system, i.e. the outlet water should not re-enter the tank, even if filtered, in order to eliminate the hatching by-products and also potentially dangerous micro-organisms, frequently associated with eggs (see previous chapter). Water exchange rate depends on egg density and has to be adjusted so as to provide enough dissolved oxygen (at 100% of saturation) to the floating eggs without crowding them against the outlet screen.

Fig. 43.00 Fiberglass cylindroconical tank used for eggs incubation (photo STM Aquatrade)

The outlet is usually screened with a removable 400-µm mesh nylon net. A continuous aeration creates a gentle current all around the screen to prevent its being clogged by eggs or larvae pushed against the net by the outflowing water. Besides that, additional aeration is provided from the bottom of the tank mainly to keep eggs and larvae in suspension and to avoid water stratification. The photoperiod is the same one applied to the larval rearing sector. Recommended stocking density ranges from 10 000 to 15 000 eggs per litre. Water turnover is maintained at one total renewal per hour during the incubation period, a rate that doubles during and shortly after the hatching time.

Egg incubation in the larval rearing tanks

When eggs are incubated directly into the larval rearing tanks, two methods can be followed:

1. direct stocking of eggs into the rearing tank, where they occupy the entire water volume;
2. egg stocking into screened floating containers, placed inside the rearing tank.

The first method requires a density not exceeding 200 eggs/litre, it has an easy follow-up and does not need additional equipment, but it is not free from drawbacks. After hatching, the tank bottom must be carefully cleaned using a siphon to remove hatching debris, as they represent a good substrate for bacterial growth. This cleaning process is a time consuming matter, a hard to complete procedure that also causes the loss of many larvae. Moreover, if incubation or hatching is unsuccessful, the entire larval tank has to be emptied, washed, disinfected and refilled. In addition to this waste of time, one should also consider the few days lost when the tank was occupied by the batch of eggs discarded.

Fig.43.01 Large incubation facilities in Nuova Azzurro hatchery (photo STM Aquatrade)

The second method allows a better control over the incubation process and could be seen as a partial adaptation of the above mentioned incubation tanks. However, the inconvenients of the first method are not entirely eliminated.

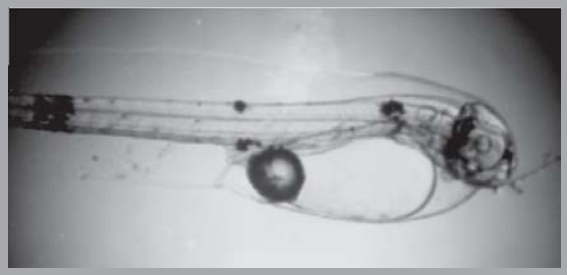
A floating incubator can be easily made with a 30-l screened plastic bucket. A 400 µm mesh size nylon net is glued to side and bottom openings. Buoyancy is provided by sealed LDPE pipes or by pieces of polystyrene foam. The incubator is equipped with an aeration device made with a couple of fine diffusers connected to the aeration system to keep the eggs moving gently in the water. Inside each bucket water renewal is assured by tank water passing through the mesh. If needed, it can be increased by a pair of airlifts attached to the outside

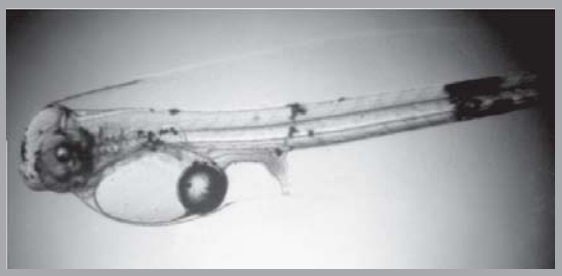
of the bucket. Water circulation inside the larval rearing tank should be adjusted to provide a gentle current through all incubators.

Once hatched, the yolk-sac larvae are easily counted and then released into the same rearing tank, by gently tilting the floating bucket, having stopped the aeration to facilitate their release. In this way the hatching debris remain inside the incubating bucket and can be then disposed of easily. Egg density is maintained at 6 000 to 10 000 eggs/litre, and though still linked to the water exchange rate inside the bucket, it far exceeds the density foreseen for the same rearing tank in absence of incubators.

Incubation lasts for about 50 h at 18°C and 36 h at 22°C.This time varies also according to the egg development stage at stocking (see Annex 13).

Fig.43.02 An outdated hatchery layout were eggs incubates directly in the tanks (photo M. Caggiano)





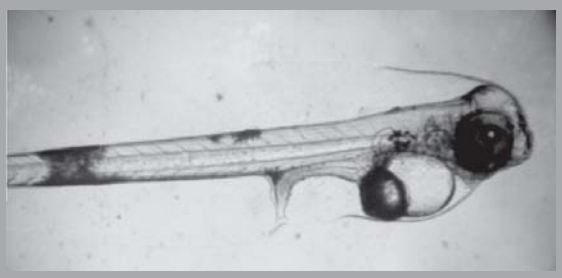


Fig.44.01-2-3 Sea bream larvae development within 12 and 36 hours (photo STM Aquatrade)

Hatching

A few hours before the estimated hatching time, water renovation in the incubation tank should be increased to two complete exchanges per hour, paying attention to avoid the clogging of the outlet filter by the eggs.

As soon as hatching begins, environmental parameters should be reset according to the indications contained in Annex 13. Care should be taken to flush out or remove hatching debris and by-products. Amongst the latter of particular importance are proteolithic enzymes, active during egg opening, which may damage the freshly hatched larvae, not protected any more by the chorion.

One day after hatching the viability of young larvae is assessed. In the good batches it remains above 80%. If possible, discard egg batches with a viability rate lower than 80%.

Viability of newly hatched larvae

Before being stocked in the larval rearing tanks, the newly hatched larvae are checked to assess their viability and condition. The evaluation criteria listed below refer to the shape of the larva and to its behaviour.

From each batch, at least 10 to 20 specimens are sampled (the procedure is described in detail in Annex 14), and are then placed on a watch glass in a water drop and observed under the stereomicroscope at low magnification (5 and 10x). They are checked for normality in respect of:

- overall shape and dimensions;
- integrity of the larval (primordial) fin, that should not present malformations and/or erosions;
- absence of external parasites;
- disposition of internal organs;
- proper pigmentation.

In their very first days, the larvae of both species show a typical behaviour and its observation contributes to evaluate their viability. Examination of this behaviour can take place directly in the hatching tank, as well as on a sample of at least 50 larvae taken in a transparent container.

The larval motion is a sort of passive floating with sudden, and infrequent body movements without assuming any clear posture. Typically they sink slowly, head first, and then, every few seconds, swim upwards for two to three seconds. A difference in buoyancy can be noticed between gilthead seabream and seabass larvae, the latter being more buoyant due to their bigger oil droplet in the yolk sac. See Annex 15 for an example of recording data sheet for observations.

Larval fish showing a different pattern (being totally passive or hyperactive), reveal poor viability and should better be eliminated. Such different behaviour should also not be dismissed as being only the result of a poor larval quality, in fact it may also point out the presence of a toxic pollutant in the water circuit. If the same abnormal pattern is observed in several batches of larvae produced one after another, it is probably a sign of pollution problems.

Larval transfer to the rearing facilities

The larvae hatched in the incubation tanks are easily transferred to the larval rearing tanks. To make this operation easier and faster, it is advisable to stock the incubator with enough eggs so as to get the right amount of larvae to stock one larval rearing tank. In this way the larvae produced by an incubator are transferred into one larval rearing tank.

For the transfer proceed as follows:

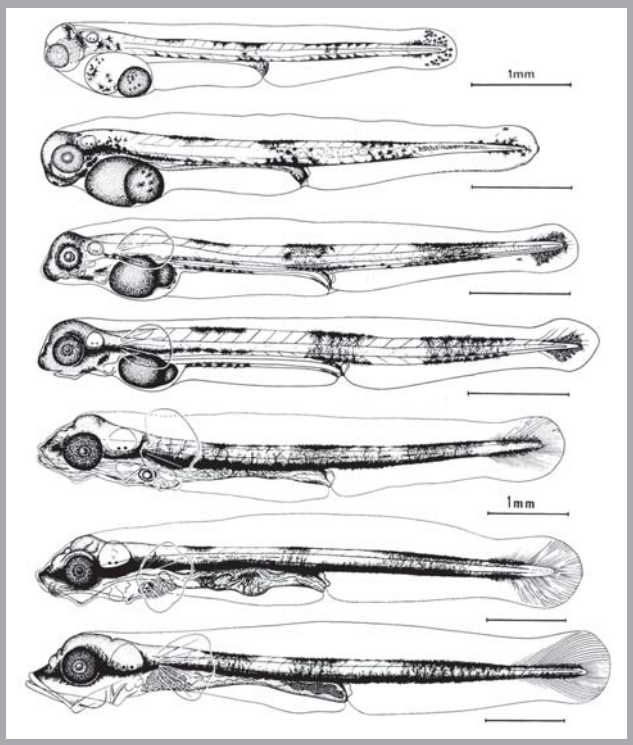
1. stop water exchange and aeration in the incubator;
2. after a few minutes (5 to 10 maximum), drain the settled hatching debris through the bottom valve;
3. restart a gentle aeration and lower the water level keeping the outlet screen in place;
4. harvest the larvae either via the bottom valve into several partially filled buckets (avoiding any mechanical stress, minimising the difference between inner water level and water level in the bucket), or using a 5 litres jug directly dipped in the incubator. Mechanical stress should be minimised by avoiding splashing.

No special operation is needed when eggs are incubated directly in the larval rearing tanks, except that hatching debris must be carefully siphoned out as soon as the hatching ends and the aeration and water exchange are adjusted to the new situation.

Starting soon after hatching, seabass larvae school in dense clouds at the water surface because of the early development of a continuous swimming activity. Gilthead seabream larvae, on the contrary, have a more pronounced tendency to sink, and their almost complete passivity accounts for their more uniform dispersion in the water body.

Such irregular swimming pattern evolves progressively towards the more active and continuous movements characterizing first-feeding larvae, coinciding with the appearance of fully developed visual and digestive organs. As a matter of fact, and at similar temperature, seabass larvae develop a correct predatory position earlier than gilthead seabream larvae. Due to their being relative passive, significant samples can be easily collected to carry out quantitative analyses to determine, for example, their total number and the percentage of deformities present in the batch. Abnormal larvae often show irregular swimming, an irregular shape or a twisting of the body.

Soon after hatching the eyes are not yet 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.



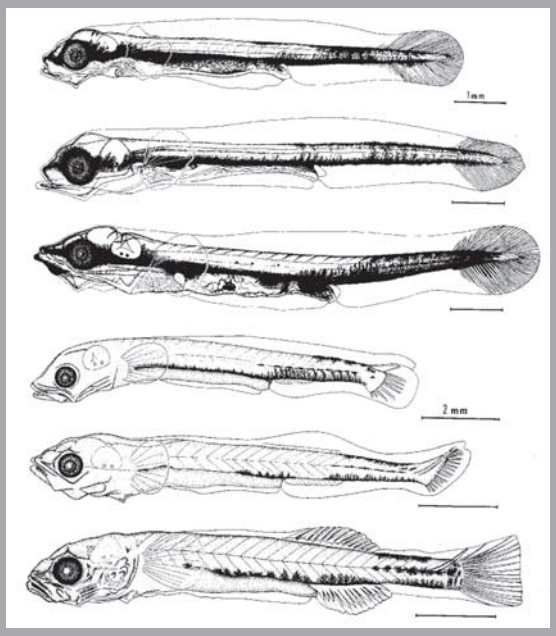


Fig.45.01-2 Seabass larval development (from Barnabè)

3.4 - LARVAL REARING

The larval rearing of gilthead seabream and seabass is a typical intensive rearing technology ideally involving complete control over the environmental parameters and fish population. Two different protocols are acknowledged. The first takes place in a lighted environment using rotifers as first feeding and adding microalgae in the gilthead seabream initial culture. The second protocol, frequently referred to as the “French technique”, is applied for seabass larval rearing and is characterised by a dark environment during the first days after hatching, and by the use of small newly hatched brine shrimp nauplii as first food.

Because it is more widely adopted and it is the only applicable technology for both species, the following description refers to the first protocol. It discusses in detail the working procedures concerning the management of the abiotic (water system, water chemistry and hygiene) as well as biotic factors (husbandry of fish larvae,

live feeds and rearing environment). In this manual the term larval rearing refers to both the larval phase, i.e. when the yolk is the only energy source, and the post-larval phase, i.e. when feed is provided as external energy source .

Layout of the larval rearing system

The first four to six weeks of the life of young seabass and gilthead seabream are spent in a specific larval rearing unit of the hatchery . The most common equipment consists in a number of round fibreglass tanks of an individual capacity of 6-10 m^3. Seawater is either recirculated through a biofilter in the case of a semi-open recirculation system, or is just pumped to the tank and discharged after use in the open systems.

Fig.46.01-2 Old and modern tanks for larval rearing units (photo STM Aquatrade)

As seabass and gilthead seabream reproduction takes place during the winter season, heating the water in the larval rearing tanks accelerates the growth rate. In some cases, where wells with higher temperature than seawater can be used, the heating requirements are reduced. A light hanged over each tank provides the necessary illumination to allow visual predation. Since live feed is mostly distributed by hand, automatic feeders are employed only during the last culture days before the transfer to the weaning section. Additional equipment include: water sterilizers, small insulated tanks for the short term stocking of live feed, tank cleaning devices (siphons, brushes, disinfection tanks for equipment, etc.) and manual or automatic devices to control main environmental parameters.

Preparing the larval rearing system

In advance of the onset of the reproduction season, the entire larval rearing system (piping, tanks, filters, sterilizers, air system, equipment, etc.) has to be properly set . Everything should be thoroughly cleaned with detergent and disinfected with a hypochlorite solution (500 ppm of active chlorine). A practical way to do it for tanks and piping is to fill them with the hypochlorite solution and keep the solution in circulation or standing (depending on the circuit layout) for a few days. It is also mandatory to perform these cleaning operations at the end of the rearing season, to avoid dirt drying and sticking.

During the production season, after a batch of fry has been transferred to the weaning sector, tanks and their outlet pipes should be similarly disinfected with hypochlorite. The outlet pipe system should be dismantled and kept in a hypochlorite solution overnight (further details are given in annex 16). If the system includes a biofilter, it has to start working at least 30 days before any larval rearing is started in order to allow a proper colonization of the filter medium by nitrifying bacteria. The disinfection of the biofilter components has to take place well in advance.

All consumables must be ordered well in advance and be at hand before production starts. A typical example of poor management is given by delays and mistakes in ordering items, which can quickly jeopardize the work of the hatchery. Some items such as special batches of brine shrimp cysts, food integrators and drugs may also run out of stock during the rearing season. The search for new suppliers is often a time-consuming and expensive activity. This recommendation also applies to having available an adequate stock of spare parts, in particular for pumps, sterilizers and piping, where a quick servicing can save the production of an entire season.

Well before the first stocking, the entire rearing system has to be assembled and tested running at full capacity. Any trouble with equipment must be solved either by fixing or by replacing it.

Fig.46.03 Hypoclorite disinfection tank (photo STM Aquatrade)

Environmental parameters for larval rearing

Since the larval and postlarval rearing conditions of gilthead seabream and seabass differ to some extent, the table below compares the two species for different parameters.

A comprehensive summary of the evolution of the main environmental parameters in the rearing system is also given in Annex 13. As a general rule, since fish mortality is highest during the first month, the greatest attention is required at that time.

Table 3.9 – Environmental parameters in the larval and post-larval rearing of seabass and gilthead seabream.

Gilthead seabream	Seabass
Water temperature Same as spawning temperature at incubation up to yolk sac resorption (-2 to +6 days). Slowly increasing (0.5◦C/day) to reach 18-20◦C, the choice depending on management considerations and period of the year. Particular care has to be given to maintaining water temperature during the first 25 days, when water renewal is nil or very limited. Fluctuation should never exceed 0.5◦C in 24 h.	*Water temperature* Same as spawning temperature at incubation up to yolk sac resorption (-2 to +6 days). Slowly increasing (0.5◦C/day) to reach 18◦C within the complete swim-bladder inflation. Then increased to 20◦C by the 15th day. Fluctuation should never exceed 0.5◦C in 24 h.
Air temperature Fluctuation within 1◦C. That should preserve water temperature especially at night. Special attention should be paid when water renewal is nil or very limited.	*Air temperature* Fluctuation within 1◦C That should preserve water temperature especially at night. Special attention should be paid when water renewal is reduced.
Salinity Usually the same salinity at spawning (35-38 ppt = full seawater). A lower salinity down to 25-30 ppt during first feeding may enhance survival rate, but at a cost: - at least two separate hydraulic circuits are needed - live food settling is increased - a change in salinity can increase stress.	*Salinity* Same.
Photoperiod 16 hours light, 8 hours dark when temperature remains below 21◦C. Above 21°C increase to 20h L / 4h D.	*Photoperiod* 16 hours light, 8 hours dark when temperature remains below 21◦C. Above 21◦C increase to 20h L / 4h D.
Light intensity 1,000 to 3,000 lux at water surface till age 25 days, thereafter 500 to 1,000 lux until metamorphosis. During on/off operations use a 10-min twilight effect by means of a dimmer switch driven by a timer. Halogen lamps are advisable for light quality and cost effectiveness.	*Light intensity* 500 lux at water surface. The alternative "French method" foresees complete darkness during a short initial period of 5-7 days.

Bottom aeration A very slow fine bubbling of 0.1 l/min during first feeding. Gently, but constantly increased from 15th day on up to 0.6 l/min, related to larval activity, surface dirt and distribution of live feeds. If eggs are stocked directly into the larval tanks, a rather strong air flow keeps eggs suspended, and is reduced at hatching to be completely stopped at the end of hatching to allow debris settling and removal.	*Bottom aeration* Same pattern, but twice as much flow due to the sturdy larval stage of bass.
Water renewal None during first feeding light periods, moderate at dark (0.5 to 1 total tank renewal). Increasing steadily up to metamorphosis to 10 total tank renewals at night. Regular water quality monitoring should confirm or adjust renewal to the actual need. If eggs are stocked directly into the larval tanks, 1-2 renewals/day during incubation, and 3 to 6 during hatching (check water temperature).	*Water renewal* Same.
Dissolved oxygen (DO) DO saturation should remain between a minimum of 80% and a maximum of 100%. Adjust aeration, water renewal, phytoplankton daily ration and bottom cleaning accordingly.	*Dissolved oxygen* Same.
Total Ammonia Nitrogen (TAN) It should not represent a major problem in the larval unit due to its scarce total biomass. In any case keep it below 0.5 ppm.	*Total Ammonia Nitrogen (TAN)* Same.
Screen mesh Use a 500 µm filter mesh when you want to discard uneaten rotifers and brine shrimp (as their nutritional value is lost if they remain there overnight). To keep them in the tank, if a water renewal is needed during feeding, replace with a 100 µm filter. If only artemia nauplii are fed, mesh size can be increased to 250 µm. Change every time it is clogged or near to clogging, in particular just before dark. If eggs are stocked directly into the larval tanks, use a 400µm filter during incubation and hatching.	*Screen mesh* Same.

Table 3.9- Continued

Photoperiod

At a water temperature of 18°C, a photoperiod of 16h light - 8h darkness is provided to extend the predatory feeding and to increase larval growth. Higher temperatures require a longer light time to match increased larval activity and growth rate.

To automatically set light intensity and photoperiod, a dimmer switch controlled by a timer should be installed. The twilight effect should last 10 to 15 minutes to prevent stress induced by abrupt changes of light intensity at on/off.

Light

For gilthead seabream in their early post-larval development stage, light intensity is critical to start a proper predatory activity. It should be at least 800 lux in the less lit areas of the tank surface, with an optimal range of 1 000 to 3 000 lux. In case of seabass, the light intensity at first feeding may be as low as 100 lux, with an optimal value of 500 lux.

In both cases direct solar light has to be avoided because it would be too strong and would create an interference with the artificial environment. Instead, incandescence bulb lamps or daylight halogen lamps are hung over the rearing tanks at a suitable distance from the water surface to provide the required light intensity.

Aeration

Tank aeration maintains fish postlarvae and live feed in suspension and allows a proper mixing of the tank water. Aeration should be adjusted to avoid a stressful turbulence to which post-larvae are most sensitive in particular at two critical stages: during the first feeding, and during the formation of the swimbladder. Excessive water movement may prevent predatory activity of fish postlarvae, and may make more difficult the gulping of an air bubble at the water surface that is necessary to activate the inflation of their swimbladder.

According to tank shape, water depth and inlet/outlet position, the aeration should create a slow up-welling current for proper vertical mixing of the entire water mass. In round tanks with conical bottom, this is obtained with a central fine airstone placed at 20 cm from the cone tip. In this way dirt and debris can settle and accumulate to facilitate cleaning.

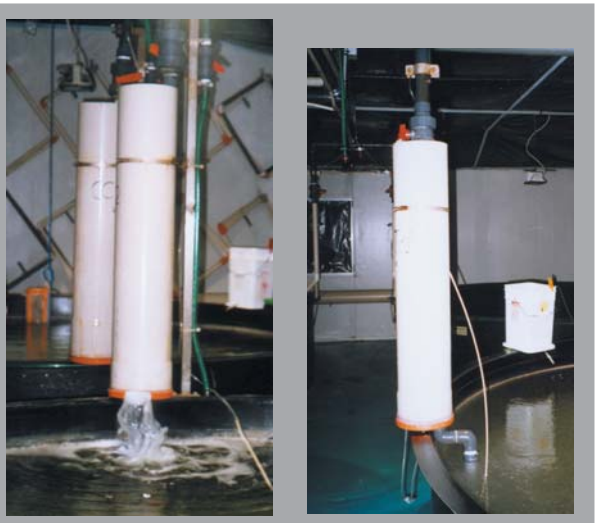


Fig.47.01-2 Open and submerged water inlet for the larval rearing tank (photo STM Aquatrade)

A common device to inject air from a low-pressure aeration pipeline is the transparent PVC 6 mm-hose to which a standard plastic needle-valve is fitted. Diffusers range from the standard carborundum porous stone to textile porous hose..

Water flow

The water inlet has to be placed carefully in order to avoid dangerous strong currents (more than 10cm sec^{-1}) that can trigger spinal deformities and impede the onset of predatory behaviour. As a general rule, the water inlet should be at the tank periphery and positioned in a way that would avoid tangential currents. A current velocity at the periphery of not more than 2cm sec^{-1} is suggested.

Dissolved oxygen

Oxygen is provided to fish through the water renewal and, to a limited extent at least in the larval rearing, through aeration. Since renewal rates during the first stages of larval culture are rather low, it is important to monitor dissolved oxygen content and to adjust it whenever it falls below 80% saturation.

The addition of microalgae at high density helps in stabilising DO concentrations during the period in which lights are on and water renewal is absent or reduced. As a rule, even if it becomes truly significant during weaning, the DO levels should be checked after feeding, when DO demand peaks, and after a prolonged period without water renewal. In presence of excessive amounts of microalgae coupled with absence of water renewal at night, a check of the DO level during and towards the end of the dark period is advisable, since algae are net oxygen consumers at night. Normal practice is to renew water in the larval tanks at night, so as to remove catabolites and to introduce oxygen when is most needed (final part of the light period).

Fig.47.03 Stationary probe for O_2 monitoring in the larval rearing tank (photo STM Aquatrade)

A practical and effective way, though expensive, to ensure high DO levels is to add pure oxygen to the water in the tanks. This can be done either by injecting pure oxygen into the water piping system, or by bubbling it inside the tanks. The first way is more efficient since it prolongs the time in which oxygen and water are in contact before reaching the tanks, thus reducing oxygen losses. In a simpler but less efficient way, pure oxygen is directly injected into each tank through one or more fine air stones. Pure oxygen injected through additional diffusers must follow the same rule of avoiding potentially harmful water currents originated from the upward movement of oxygen bubbles.

In any case, it is recommended to install an emergency oxygen supply to overcome the risk of anoxic conditions that may occur unexpectedly . An oxygen supply can also be helpful when peaks of biomass are present during the last stages of fry rearing.

Outlet filters

The management of outlet removable screens follows the various feeding regimes. When water renewal takes place at dark, as fish larvae are visual predators they will not consume significant amounts of live preys at night during darkness. Rotifers and artemia nauplii lose nutritional value in a night and therefore can be eliminated during the water exchange. In this case a filter mesh of 500 µm is used to discharge them when water is renewed . When for any reason water has to be renewed also during the period in which lights are on , live feed has to be kept in the tanks for the fish which will be preying actively. In this case a 100 µm filter for rotifers and a 250 µm screen for artemia nauplii are used.

Outlet screens must be changed before they become completely clogged, and in any case should always be replaced with 500 µm screens just before dark. Check the filter material for dead larvae as this could be an early warning of possible troubles. It is recommended to have a set of outlet filters of different mesh sizes for every tank to save time during filter replacement .

Fig.48.01-2 Two different shapes of outlet screen for larval rearing tank (photo STM Aquatrade)

After use screens should be cleaned by removing dirt with a water jet, then by a placing them for half-an-hour in a 500-ppm active chlorine bath followed by a thorough rinsing with freshwater. Screens should be stored in a place where they can dry out of the range of tank spray and should be at hand during replacement. If eggs are stocked directly into the larval rearing tanks, use a 400 µm filter during incubation and hatching.

Fig.48.03-4-5-6 Larval rearing screen outlet changing, washing, disinfecting and stocking (photo STM Aquatrade)

Feeding seabass and gilthead seabream post-larvae

After being stocked in the tanks (150 to 250 larvae per liter should be a correct density), the larvae will continue their development during some days relying only on their yolk sac reserves. Depending on the rearing temperature they will start feeding on living micro-organisms in three to four days from hatching.

First feeding

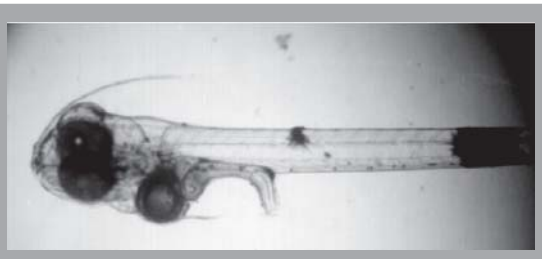
At hatching, fish larvae are not yet completely formed and, among other things, lack functional eyes and mouth. Moreover they do not have an active swimming behaviour. In the first three to six days after hatching, and depending on water temperature, the fish larva therefore relies only on its yolk sac reserves as food source. At the end of this period the young fish has developed functional eyes, which are recognisable by their dark colour, its mouth has opened and the digestive tract, though still primitive, can now assimilate food. Then, its swimming behaviour becomes active and the animal is thus able to keep a horizontal position. At this stage the post-larval stage begins and the young fish starts feeding on live preys, such as rotifers or brine shrimp nauplii (the latter only a first feed in the case of seabass). These live feeds are supplied on time to the larval tank (see annex 17 and 18 for feeding regime of seabass and gilthead seabream).

The onset of the first larval feeding is a crucial step in the young fish life: if something goes wrong, starvation quickly kills a weakened animal. Starvation is actually a major cause of larval mortality. Therefore it is important to help them to overcome this delicate and key phase. The main actions to be taken are the following:

- Maintain the larval population uniformly dispersed in the water column with the help of a gentle aeration, avoiding any mechanical o physical stress until the larvae reach the proper combination

of size, organ development and behaviour.

- Water temperature, a crucial factor for a correct organ development, should be kept within ±0.2°C from the chosen value and close to the spawning/hatching temperature during the first week and after that the temperature increase should be kept within some fractions of a degree per day until the final rearing temperature is reached.



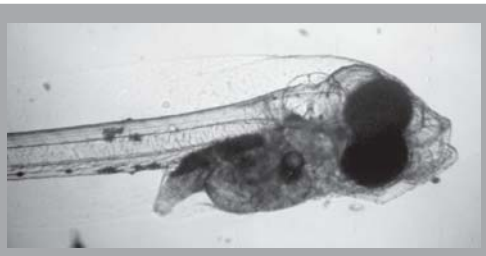


Fig.49.01-2 Gilthead seabream larvae: before first feeding and with functional digestive tract (photo STM Aquatrade)

- At least during the first ten days after feeding has started a high prey density increases the chance for the fish to approach and gulp some rotifers or artemia nauplii, improving significantly their survival possibilities. Prey density at this stage should be kept at 5 to 10 rotifers/ml and regularly checked throughout the period when the lights are on to adjust subsequent distribution accordingly.
- Take into consideration the differences between seabass and gilthead seabream larvae in feeding behaviour, food ingestion and food assimilation: large fluctuations of prey density are to be expected in seabass populations due to their faster and greater ingestion rate than in the case of gilthead seabream, which are less active predators. See Annexes 17 and 18 for feeding protocols.
- Prey size at first feeding is of crucial importance for gilthead seabream. Because of their very small mouth, during the very first days they can only ingest preys smaller than 100 µm. Thus a ration composed almost exclusively of rotifers of a small-size-strain (50-100 µm juvenile and adult respectively) or of the juvenile rotifers of a medium-size-strain (70-130 µm) is essential. Later on, bigger rotifers (150-250 µm) can also be fed.

Fig.50.01 Manual live feed distribution (photo STM Aquatrade)

- The proper management of first feeding requires a close monitoring of the larval population: daily samples from each tank have to be collected and examined under the microscope to assess repletion rates (percentage of larvae having preys in their stomach) and number of prey per fish (see below for a detailed description of the sampling procedure). At the end of the first week of feeding, in a healthy population almost 100% of sampled fish should show some preys in their digestive tract, their quantity being directly linked to the mouth size. Table 12 gives the

indicative figures of repletion rates and the number of ingested preys for both species. Annex 19 gives an example of a larval quality control form.

- Record also all feeding data on dedicated tables, (see Annex 20 for an example of tank file, Annex 21 for larval rearing unit daily feed distribution schedule, and Annex 22 for daily quantities of live food to be distributed during the week).
- To check all sector operations use a larval rearing sector daily workplan sheet as indicated in Annex 23.

Fig.50.02 Larval rearing tank during *Artemia* nauplii feeding (photo STM Aquatrade)

Age	Repletion rate	Actual ingested rotifer/ARTEMIA	
	%	Gilthead seabream	Seabass
3rd day	20-40	2-3	2-4
4th-5th day	70-90	4-8	7-12
6th-8th day	100	10-15	15-20
9th day onwards	100	up to 50	up to 100

Table 3.10 – Repletion rate and number of ingested preys per larva at first feeding of gilthead seabream and seabass larvae.

Transition from live feed to artificial food

Feeding on live prey usually lasts 40 to 50 days, according to water temperature, species, rearing protocol and the opinion of the larval unit manager. The sooner the fish move to an artificial diet, the better in terms of savings on labour, overall costs and time. With this aim in mind, the timing of first dry food supply has been continuously anticipated in recent years, thanks also to new more elaborated artificial diets, including vitamins and immunostimulants, which fit better the larval requirements in terms of composition, size, buoyancy, and flavour. Special processing techniques can now blend high quality ingredients into micro-particles showing excellent stability in water, a slow sinking rate and attracting fish larvae better.

Before these advanced diets were available, fish were weaned on freshly prepared wet diets, formulated according to the nutritional requirements of other fish species. Special attention had to be given to the quality of the raw materials and integrators, as well as to their processing. A detailed description of such a wet diet (moist feed) and an example of feeding rates are presented in Annex 24.

The main difficulty encountered when trying to feed an artificial diet for the first time is how to stimulate fish to accept it, which is a time-consuming task since they are used to live preys. This delicate transition is indeed one of the main sources of larval mortality . In practice, at the beginning inert feed is distributed daily in very

small quantities to accustom fish to a new flavour. To incentivate its consumption , it is advisable to start the distribution of inert feed in the morning, after the night starvation, and well before live feed is offered. Once dry food is accepted by most fish, it can be distributed by automatic feeders. To induce feed ingestion by the rest of the fish population , brine shrimp can be dropped close to the automatic feeder. The slow water movement will attract fish just below the feeder. When feeders are employed, distribution can be automatically set with a timer. In any case it is recommended to watch closely the ingestion rate. Artificial feed leftovers have to be avoided not only because they represent a loss of money, but mainly because they can severely pollute the water in the tank.

Fig.51.01 Belt feeder and the *Artemia* nauplii dispenser during gilthead sea bream weaning (photo STM Aquatrade)

From a management point of view, feeding fish with an artificial diet involves a series of changes in the rearing environment (see Annex 13):

- aeration should be slightly increased to keep the inert feed in suspension;
- water renewal should be gradually increased to remove faeces and uneaten food particles, as well as to dilute ammonia and to supply more oxygen, which is related to an increased metabolic requirement;
- the frequency in cleaning the bottom of the tank should be increased to remove more debris and keep associated bacteria to a minimum.

In larval rearing cannibalism is not yet a major problem as it is in the weaning unit, but towards the end of the production cycle it may require attention, in particular when fish of different sizes and stage of metamorphosis coexist. The problem tends to be more serious with seabass. In presence of cannibalism, the supply of both artificial and live feeds should be better calibrated and its frequency should be increased. Another possible solution is to dilute the population in two or more tanks or introduce microalgae again, which would reduce the visibility and in consequence the more aggressive behaviour. This subject is extensively treated in the description of the weaning sector below.

Feeding protocol

As already indicated , the diet of seabass and gilthead seabream post-larvae in their first weeks of life is represented by small animals, rotifers and brine shrimp larval stages, whose biology, culture methods and nutritional value have been previously discussed in the manual. Microalgae are also provided to feed rotifers and improve the overall quality of the rearing environment. This section will deal with their handling and distribution to fish.

The feeding protocols for gilthead seabream and seabass are given in Annexes 17 and 18 respectively. In both cases, the protocol is based on the following assumptions:

- initial density of 150-200 post-larvae/litre;
- water temperature of 18°C;
- salinity of 35-37 ppt;
- photoperiod of 16 h light and 8 h darkness;
- all quantities mentioned are referred to a culture volume of 1 000 liters in order to adjust easily the feeding ration to tanks of different capacity;
- the feed quantities at different ages are only indicative as the actual rearing conditions change continuously from tank to tank.

Gilthead seabream aged 3 to 7 days receive a daily amount of 20 million rotifers per tank together with 40 liters of mature algal culture (at $12x10^6$ cell/ml) . From day 8 to day 12 the amount of rotifers is increased by 20% to 24 millions and to 28 millions from day 13 to 16, whereas the algal supplement remains at 40 litres.

From day 17 the first brine shrimp nauplii are fed to the postlarvae: they should be of a particularly small strain in order to facilitate their gulping by the still small-mouthed fish. The Artemia AF cyst are an example of such strains. The amount used ranges from 0.1 to 0.5 millions. At the beginning, frequent controls on fish are recommended to check is they are accepting the new food item. Together with the first nauplii, the rotifer ration is increased to 32 millions, whereas microalgal supplements are progressively reduced to 20 litres. At this time the first artificial feed (of a very small size, 80-200 µm), is also distributed. The quantity offered is limited to 1-3 g, but its function at this stage is mainly to start getting postlarvae accustomed to this new taste.

From day 20, algae are further reduced to 10 litres, rotifers quantities begin to decrease (to 20 millions), being replaced by an increased amount of artemia AF (0.5-1 millions) and, for the first time, also artificially enriched artemia metanauplii, produced with cheaper artemia strains (0.3-0.6 millions are offered). Inert feed is also gradually increased to 10 g.

From day 24 to day 27 algae are progressively eliminated, rotifers decrease to 10 millions, whereas artemia AF increases to 1.5 millions and artemia EG or RH to 3 millions. Artificial feed is also increased to 15 g.

From day 28 the distribution of algae, rotifers and artemia AF nauplii is suspended and only artemia EG or RH (10 millions) and inert feed (15-20 g) are fed to fish, whose average weight should now be about 5 mg.

From day 34 to day 39 fish are given more EG or RH artemia (12 millions) and 20 g of inert feed of 80-200 µm size, plus 10 g of the larger 150-300 µm size.

From day 40 to day 43, when metamorphosis from post-larval to juvenile shape (fry) has started , the distribution of the 80-200 µm inert feed ceases, and it is replaced by more EG or RH artemia (up to 16 millions) and an additional 20 g of 150-300 µm feed . From this point the fish are ready to be moved to the weaning sector.

In **seabass**, whose post-larvae are much larger than in the case of gilthead seabream, feeding with brine shrimp starts two weeks earlier. Fish aged 3 to 7 days receive a daily amount of 20 millions rotifers, 2 millions small size brine shrimp nauplii (cysts Artemia AF or BE) and 40 liters of mature algal culture, the latter one decreasing gradually till day 23.

From day 8 to 12 the amount of rotifers is increased to 25 millions and AF or BE artemia nauplii to 3 millions.

From day 13 to 16, rotifers decrease to 15 millions, and AF or BE artemia nauplii ration is increased to 4 millions.

Fig.52.01 High concentration of gilthead seabream around *Artemia* nauplii dispenser (photo STM Aquatrade)

From day 17 a few grams (between 1-3 g) of artificial feed of very small size (80-200 µm) are distributed together with 10 million rotifers for the smaller part of the fish population with 6 million of AF or BE artemia nauplii plus the first 2 millions of EG or RH nauplii for the larger fish.

From day 20, the enriched brine shrimps of large size(EG or RH) offered are 14 million, rotifers decrease to 5 millions and inert feed is gradually increased to 10 g.

From day 24 microalgae and rotifer distribution ceases, the EG or RH artemia nauplii ration increases to 16 million and the inert feed to 10 -15 g.

From day 28 the distribution of artemia is increased to 20 millions and inert feed stays at 10-15 g, although but 10 g of 150-300 µm feed are added.

From day 34 the fish get the same previous amount of artemia metanauplii. Distribution of the 80-200 µm inert feed particles ceases and 20 g of 150-300 µm are fed.

From day 40, when metamorphosis from post-larval to juvenile (fry stage) is almost completed, the artemia metanauplii ration is decreased (down to 16 millions) and the 150-300 µm inert feed is increased to 20 g. From now on fish will be ready to move to the weaning sector.

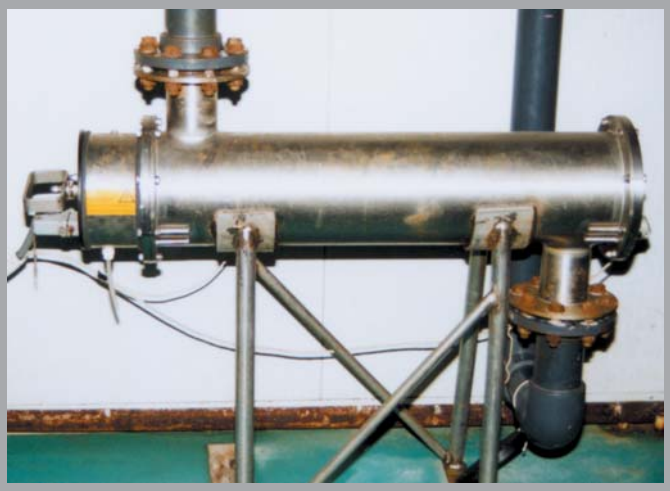


Fig.52.02-3 UV lamp and titanium plate exchanger are frequently used for temperature and bacterial growth stabilisation of the whole larval rearing unit. (photo STM Aquatrade)

Daily distribution of live feed

Rotifers and brine shrimp are distributed by hand into the areas of the tank surface where larval density is lower. Feed is distributed three times per day, starting as soon as the lights have been switched on in the morning until four hours before the artificial sunset, in late evening. A quick distribution of the first ration in the morning is recommended to stop the forced starvation, which takes place during darkness.

The daily ration should be distributed every 6 hours in the following way (time is indicative):

- 50% at 08.00 h;
- 25% at 14.00 h;
- 25% at 20.00 h.

A prey density check is highly recommended before the second and the third distributions to adjust the concentration and thus avoid situations of over or under feeding. Use a 1-ml pipette to take two to three samples at different places in the tank and count the number of rotifers or artemia nauplii at naked eye or with the help of a portable lens.

From an operating point of view, the head of the larval sector prepares a feeding schedule for the day with the quantities to be distributed to each tank for the three meals. Rotifers and brine shrimp are quoted in millions: the worker has therefore to convert this figure in liters of stocked culture, according to the density marked on each stocking tank.

The live feeds should be distributed by keeping the jar above the water surface at the centre of the tank, and gently pouring its content over the rising air bubbles, to obtain an optimal dispersion. The operator has to take the highest care to avoid any splashing, waves or current the could induce stress to larvae.

First feeding with rotifers has already been discussed. Shifting from rotifers to brine shrimp is done progressively, feeding artemia nauplii from small size strains to gradually adapt the growing larva to the new food. The worker should check the ingestion of *Artemia* by regularly sampling larvae with a transparent beaker and looking at their digestive tract, which assumes a pale orange coloration when nauplii are ingested. See Annexes 21 and 22 for examples of record-keeping forms to help daily and weekly management of live food production and distribution.

When rotifers are fed to the fish larvae , microalgae are added daily to the rearing tanks to obtain a final density of 500 000 to 800 000 cells/litre. Because of their high PUFA content, preferable species are *Nannochloropsis oculata, N.gaditana* and *Isochrysis galbana* (Tahitian strain), the latter being particularly rich in the essential DHA.

Apart from maintaining the rotifer high nutritional value, their other positive effects in the intensive rearing environment are thought to be a certain bacteriostatic capacity and a shading effect that reduces the larval aggressive behaviour. Larval culture in clear water is also feasible, but it gives lower average results in terms of survival and size homogeneity.

In large hatcheries, where algal volumes to be distributed are important, a more practical way is given by the use of centrifugal reversible pumps connected to a stocking tank placed above the larval tanks. The required daily volume of algal culture is pumped into the tank and then easily distributed to the rearing tanks by gravity.

Fig.52.04 Wine reversible pump used to transfer live food (photo STM Aquatrade)

Notice: before distributing the microalgae, the tank cleaning must have been completed in all tanks as water turbidity prevents from seeing the bottom.

Daily storage of live feed

As soon as produced, live feed is stored in the daily stocking tanks of the larval rearing unit, to be available for distribution during the hours when the lights are on. The most practical tanks are PRF rounded containers with a conical bottom equipped with a drainage ball valve. Their capacity ranges from 100 to 500 litres although larger tanks are also used , according to the larval unit size. A central strong aeration obtained with a coarse air stone maintains live feed in suspension and keeps oxygen levels within safe margins. It is in any case advisable to install an emergency supply of pure oxygen as not only the nutritional value of dead rotifers and brine shrimps is low, but also the risk that they can spoil the rearing environment is very high.

To prevent the loss of their nutritional value, live feed is kept at low temperatures (5-10°C) which reduce the metabolic rate. For this purpose, a complete heat insulation is applied to the tank sides, bottom and removable

top. Styrofoam or polyurethane mats and bubble plastic foils are commonly used. Sealed ice bags or blue ice packs are then introduced in the tank and regularly replaced when melted. To avoid contamination, sealed ice bags should be discarded after use, whereas blue ice packs should be disinfected in a 500 ppm hypochlorite solution before reuse. Remember to control the presence of punctures and to thoroughly rinse them with freshwater before freezing.

An alternative and more appropriate option is to stock the concentrated cultures in buckets into a big freezer, whose thermostat has been set to a temperature range of 0-10 °C. The lid of the freezer is drilled to fit the air or pure oxygen hose and supply the highly dense populations with the necessary oxygenation and water circulation. As already mentioned the best cold storage is obtained using a refrigerated tank.

Maximum daily stocking density for live feed is as follows:

- rotifers: 10 millions/litre;
- artemia nauplii: 5 millions/litre;

Remember to clearly mark the stocking density on each tank, otherwise the staff in the larval rearing unit will not be able to distribute the live feed properly.

At the end of the day any live feed leftover can be concentrated by filtering, then packed in sealed plastic bags, named and dated and deep frozen. These leftovers can be used as an emergency supply in case of a production failure or delay, bearing in mind their generally reduced nutritional value.

Hygiene in the larval rearing environment

Due to the relatively high water temperature and density of living organisms, hygienic conditions in the larval rearing tanks can deteriorate rapidly. Moreover the quantity of organic matter creates a favourable environment for bacterial growth, often harmful to the delicate larval fish. A complete control of the hatchery hygienic conditions should therefore be contemplated and duly enforced. The health status of marine fish larvae depends mainly on a well-planned prevention including the treatment of the raw seawater and the compliance with strict cleaning routines, as detailed in Annex 16. Table 11 below gives a minimum daily planning for larval rearing controls during the day. The procedures check list is given in Annex 23.

Hour	Feeding	Water control[1]	System Control [2]	Biological control	Various works
08.00	1	T	WAF	Rotifer/Artemia quality	Light switched on, Change screens
09.00				Larval behaviour	Clean make-up cartridges [3] Clean UV/by-pass Siphon tank bottom
09.30		T-DO			
11.00				Larval behaviour	
11.30				Rotifer/Artemia quantity	
12.00		T-DO			
12.30			WAF		
14.00	2	T-DO	WAF	Rotifer/Artemia quantity	Siphon tank bottom
15.00 17.00				Feeding Rotifer/Artemia quantity	
17.30		T-DO	WAF		
18.00 20.00	3	T-DO	WAF	Larval behaviour Larval behaviour	 Change screens
24.00					Switch off light

Table 3.11- Daily schedule of controls

[1/]T-DO = Temperature + Dissolved Oxygen measurements
[2/]WAF = Water renewal + Aeration + Filters overall controls
[3/]In case a semi-closed system is present

Monitoring and controls

As soon as the larval population develops an active behaviour and starts feeding, the following controls should be performed to monitor its health status:

- quantitative evaluation of the feeding performance;
- qualitative evaluation of the preying activity;
- qualitative evaluation of stress;
- control of swim bladder development.

Quantitative evaluation of feeding performance

Presence and quantity of ingested food gives such a clear indication of fish health status to become one of the most important controls to be carried out during the first ten days of larval rearing. To correctly estimate the preying activity, a sample containing 30 to 50 fish from each tank population should be examined under the microscope every day. This figure can be doubled in the very first days of feeding. See Table 11 above for feeding patterns of seabass and gilthead seabream post-larvae.

Larval feeding examination of each tank:

- Pipette the larvae on a slide with the minimum water possible. Group them together.
- Remove excess water with the pipette and put under the microscope without a cover lid.
- Take a first look under the microscope and look for body deformities not caused by handling.
- Cover with the glass lid, remove excess water with filter paper and observe the gut content at x100 magnifications
- Look for whole rotifers or for their components such as mastax, lorica, or eggs.
- Look for other ingested material, if any, and internal/external parasites.
- Look for the presence of calculi in the urethra and in the urinary bladder.
- Record all findings on a specific form (Annex 19).

Try not to damage the larvae with the pipette. Remember that an excessive pressure on the cover slide may result in the expulsion of eaten rotifers through the anus of the larvae. Morphological condition assess should be made as quickly as possible since heat irradiated by microscope lamp makes the larval body to shrink in a minute.

The presence of ingested rotifers can be easily recognized by the presence of their masticatory parts (mastax), left undigested in the larval gut. Under a 100x magnification they clearly appear amongst rotifer's eggs and other debris. In practice, only the number of mastax found is recorded to estimate the total number of rotifers eaten. The number of preys per larva ranges from 2-3 (early feeding) to over 50.

To check the ingestion rate of brine shrimp nauplii, it is sufficient to visually estimate the percentage of repleted larvae (carefully sampled in a 100-ml transparent beaker and checked for their reddish-coloured digestive tracts as the deep orange nauplii are visible through the transparent larval skin).

Observations on prey quantity should be integrated with an assessment of fish behaviour, which can be done easily and in a quicker way (see below the section on evaluation of stress).

Qualitative evaluation of stress

Stress in fish larvae induces both morphological and behavioural changes that can be detected by the hatchery operator in order to improve culture conditions or to replace as soon as possible a poor larval batch. The main criteria for stressed fish larvae are:

- starvation;
- calculosis;
- abnormally passive behaviour;
- absence of "schooling" (in the first two weeks of life);
- frenzied crowding at the tank meniscus.

Starvation is the more obvious indication that something has gone wrong. It is a general response to stress and therefore it is impossible to link this deadly condition to a single possible cause. With the sole exception of acute intoxication, all rearing parameters, alone or more probably in association, may stop larval feeding. As a starved fish will not survive for long, it is also important to monitor the onset of first feeding (see above).

There is some scientific evidence that a direct correlation exists between environment-induced stress and the appearance of calculi in the urinary system of gilthead seabream and seabass larvae. Although there is no confirmed correlation between calculi and death, they are often associated to starvation and consequently are present at a higher rate in dead larvae. The early appearance of calculi in a larval population is therefore considered as a stress indicator.

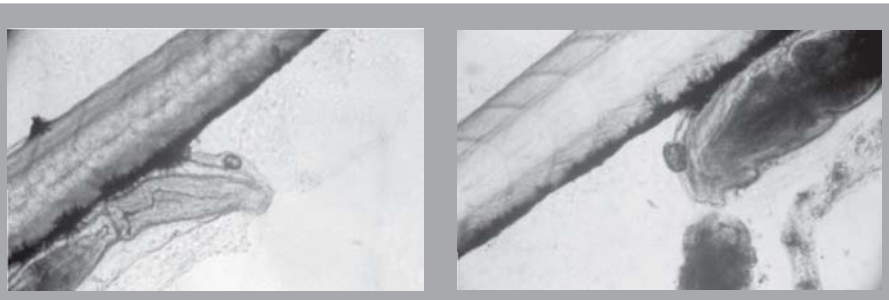


Fig.53.01-2 Gilthead seabream calculi (photo STM Aquatrade)

Calculosis can be easily detected by examining the lower part of the larval urinary duct (urethra and urinary bladder) under a microscope at 100 magnifications. This condition becomes evident by the appearance of a single stone-like corpuscle or a chain of smaller ones, reddish or grey in colour. Sometimes, they completely obstruct the urethra. The count of renal calculi may be done when the repletion rate is being evaluated and can be recorded in the same sheet. When a large calculosis, say in more than 40% of the fish examined, is observed, it can be interpreted as a sign of poor rearing conditions which typically will result in a low survival rate. In this case environmental and feeding parameters such as the following should be checked:

- excessive water currents due to wrong aeration or water inflow;
- disproportionate prey size;
- insufficient light intensity;
- dangerous levels of some water quality parameters, such as dissolved oxygen and TAN content.

Another stress revealing parameter is represented by the presence of a large amount of post-larval fish that do not actively swim and attack preys. At the mercy of water currents, their passive behaviour prevents an efficient hunting of live prey. Such abnormal conditions can be easily detected because the artificial lights reflects in their eyes in a typical and specific way when these animals turn passively upside-down: small glimmering points appear throughout the rearing tank. This condition may lead to the loss of a consistent part of the population. An unhealthy rearing environment, together with a possible congenital factor, are considered as the most probable causes of this distress.

By comparison, a healthy fish displays the following normal preying behaviour:

- brief to continuous swimming looking around for something to eat;
- pointing a prey and approaching it;
- further observation and final decision;
- attack preparation by tail bending;
- attack by tail straightening up and capture of prey;
- prey gulping;
- further searching activity.

Larval schooling starts at an age of 5 days and ends approximately between the 10th and the 15th day. It is believed that schooling indicates a healthy population. The so-formed larval shoals result from:

- water currents induced by aeration and water inflow;
- tank shape and size and water depth;
- active counter-current swimming of the larvae, as a reaction to an external stimulus.

In each tank there are one or more shoals, typically in the calmest places of the tank, slowly moving around. Samples to control the state of the population are taken inside these shoals.

A particular type of behaviour, an erratic swimming at the water meniscus, should also be considered as a possible response to stress. This syndrome is characterized by a frenzied activity of larvae, which seem to be attracted by the water meniscus where they get stuck, beating the tank wall head-on or being shaken by spasmodic head-up movements. This affects their feeding rate and consequently their survival.

Apparently only a small part of the population shows these symptoms for a long time, thus suggesting a possible chronic stress or unsuitable rearing conditions. Therefore, massive mortality will occur only in populations that exhibit the meniscus-stress syndrome to a large extent. Usually, this syndrome accounts for part of the "normal" larvae mortality when their age is between 10 and 30 days.

If measures cannot be taken to counter the stress inducing factors, the larval section manager should consider the opportunity to eliminate the compromised population and quickly start a new batch.

Control of swim bladder development

At 18°C, the swim bladder formation starts between day three and four. The first evidence of swim bladder inflation is noticed between days five and seven, looking as a small air bubble inside a tissue vesicle that becomes clearly visible under the microscope (at 40x magnification). A few days later, a second bubble develops and joins the first one to form a almost spherical body that will gradually expand to become an elongated vesicle.

Many authors agree that the initial activation of the swim bladder relies on the gulping of air at the water surface. A temporary connection between the swim bladder area and the mouth (typical of physoclist teleost fish) makes this process possible. This active air swallowing is apparently crucial for a proper swim bladder development : if this air gulping cannot take place, the swim bladder will not form , as the conduct remains open only for a few days. The absence or the incomplete filling of an active swim bladder has severe consequences for the fish, causing a deformed backbone. This, linked to limited or negative buoyancy and an abnormal swimming behaviour, reduces the feeding rate and slows growth. Even if the deformed fish reaches marketable size, its price will be well below that of normal fish. Therefore an early and correct determination of the percentage of swim bladder inflation is vital to decide how to proceed with the rearing programme.

Amongst the factors interfering with normal swim bladder activation the following are considered to be important:

- physical obstacles at the air/water interface;
- physio-morphological abnormalities in newly hatched larvae;
- early disease outbreaks;
- insufficient and irregular feeding;
- unsuitable water quality.

Any obstacle at the air/water interface that prevents larval fish to gulp air is responsible for a poor rate of swim bladder inflation. The presence of an oily layer originating from rotifers being fed an enrichment diet is considered particularly dangerous.

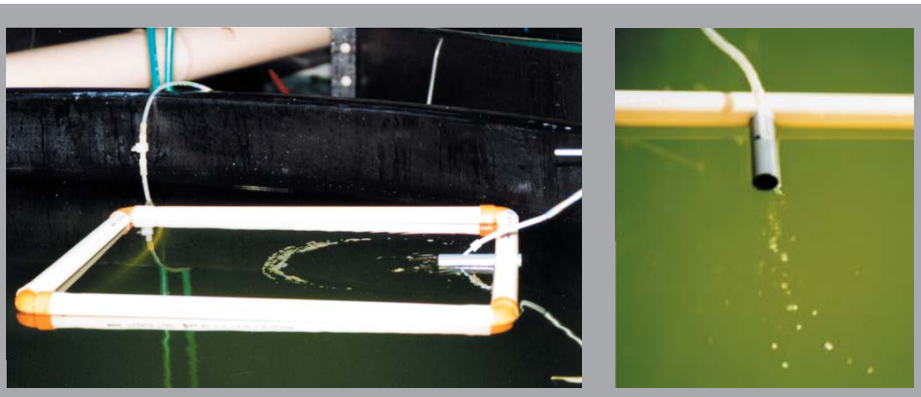


Fig.54.01-2 Surface skimmers (photo STM Aquatrade)

The introduction of “surface skimmers” has overcome this problem. These floating devices are traps that continuously remove any floating debris and grease by blowing air at low pressure tangentially to the water surface. Skimmers are periodically cleaned (at least three times per day) with either a beaker or a using a soft paper foil.

Fig.55.01 Heavy loaded skimmer before cleaning (photo STM Aquatrade)

In the early larval stages the swim bladder can be easily observed under a microscope at 20 to 40 magnifications, thanks to the transparency of the larval body and the brightness of the gas bubble, whose surface reflects light as a mirror. Monitoring should be made after complete filling of the swim bladder on samples aged 15 to 20 days.

The procedure for swim bladder inflation control in each tank is as follows:

1. Sample 30 to 50 larvae per tank or use the sample taken for checking first feeding;
2. Pipette the larvae on a slide with as less water as possible. Group them together.
3. Cover with the glass lid, remove excess water with filter paper and observe them at 20 and 40 magnifications
4. Look for the presence of the swim bladder.
5. Record all findings on a dedicated form (Annex 19).

Fig.56.01-2 Cleaning procedure for surface skimmers (photo STM Aquatrade)

3.5- WEANING

The rearing system

Weaning takes place in a dedicated section of the hatchery where the metamorphosed fish (about 45 days old) will grow until a size of 2-3 grams. At this stage they are named fingerlings or juveniles, and must have assumed the adult aspect.

The layout of the weaning sector recalls that of an enlarged larval sector equipped with a number of larger round or rectangular tanks. Their capacity is typically between ten and twenty-five m^3 since a larger size become more difficult to handle due to the many operations of grading and thinning out of the fish population. Because fish biomass becomes important at this stage as it can reach up to 20 kg/m^3, the weaning stage represents a true intensive rearing system. This is also conceptually far from the larval rearing stage. It can also be considered an intermediate step between the culture of small delicate post-larvae and the much stronger juveniles.

Where environmental conditions are favourable, i.e. in particular for temperature and water quality, a simple flow-through water system is sufficient to feed the unit, provided that the incoming water goes through of coarse filtration (50-100 µm) and passes through UV-light sterilizers. Cold winters or poor environmental conditions require a semi-closed system, in which water is partially recirculated, heated and passed through a biofilter. The latter is a more complex and costly situation, whose management requires additional expertise.

Heating and equipment do not differ significantly from their analogues in the larval rearing section. Light is provided by neon daylight tubes, controlled by a timer. One tube of 58 W for each 10 to 20 m^3 tank is considered appropriate. The mesh size in the tank outlet screens should increase to 1, 2 and 3- mm according to fish size. At least one complete set of filters is needed and, to be on the safe side some additional screens should be kept as spare parts. Outlet screens are changed every evening or even more frequently if they are quickly clogged by suspended solids or others debris. They should be flushed with tap water and soaked in hypochlorite solution overnight.

Fig.57.01-02 Modern weaning units with round and rectangular tanks (photo STM Aquatrade)

Water circulation is a key parameter for keeping good rearing conditions: good circulation eliminates dead zones and water stratification, helps in accumulating faeces and debris close to the outlet, provides current for a healthy swimming activity, distributes oxygen evenly in the water and attracts fish under the automatic feeders. An optimal water circulation is related to the shape of the tank and to its water depth. The position of the water inlets and of the aeration should be adjusted accordingly.

High fish densities can be maintained only if pure oxygen is added, thus contributing to reduce the water exchange rate and therefore pumping costs. However, if oxygen bottles are used, they are comparatively expensive and their management and replacement with new ones is not so efficient, in particular in case of a big hatchery. In cases where large oxygen demand has to be supplied, it may be advisable to use liquid oxygen instead. Its storage in large containers assures long term autonomy. Its consumption can be optimised by a dedicated water circuit, under pressure, supplying supersaturated water to the rearing units with higher efficiency (above 70% solubility) and reduced oxygen waste. Moreover this specific circuit can also be linked to a computer-assisted monitoring and regulating system that, through a feedback regulation, can automatically adjust oxygen levels to the actual rearing conditions and demand, with significant savings and an increase in safety and optimisation of growth performances.

Preparation of the weaning unit

Before any rearing activity can start, the entire system (tanks, water pipes, recirculating pumps and sumps) should undergo the same preparation process which has been previously described for the larval rearing unit. The same procedure also applies whenever a harvested tank has to be restocked.

If a biofilter is installed, its conditioning process should start at least one month before the first production cycle starts. Annex 25 describes the more important cleaning procedures needed in the weaning unit.

Fig.58.01 Small liquid oxygen reservoir sized for hatchery need (photo STM Aquatrade)

Fry culture

Rearing parameters

After metamorphosis, fish fry are more tolerant to small environmental variations than post-larval fish, but the rearing parameters in the weaning/nursery sector still require close monitoring. The values for the major environmental parameters in the weaning unit are described in Annex 13.

A complete control of water quality should be carried out if possible every day, whereas the main chemical and physical parameters such as dissolved oxygen, pH and temperature should be frequently monitored during the day.

- Temperature: should be kept within an 18-22°C range. Higher values result in better growth, but also increase bacterial growth and fish metabolic rate, which in turn increase oxygen consumption and total ammonia nitrogen production. In addition high temperature ranges also induces negative forms of fish behaviour such as cannibalism. Without adequate solutions to keep key environmental parameters within safe margins, such as, for example, the possibility to increase water renewal and oxygen supply, to raise rearing temperature usually is equivalent to increasing management problems.

- Salinity: better feed conversion and survival rates can be achieved by using slightly brackish water at 20-25 ppt salinity, but because of the large quantity of fresh water required to lower salinity in full strength seawater, this operation becomes economically feasible only when a natural brackish-water supply or a fresh water well with adequate capacity is readily at hand.

- Total Ammonia Nitrogen (TAN): it is expressed as ppm of ammonia nitrogen (the sum of NH_3 plus NH_4^+ expressed as -N) and should preferably remain below 1 ppm and never exceed 2 ppm. The toxic fraction of TAN, the Unionised Ammonia Nitrogen (UAN, expressed as ppm of $N\text{-}NH_3$) can be calculated from appropriate tables, knowing the pH and salinity of water sample. Pure oxygen injection can partly convert toxic unionised ammonia $N\text{-}NH_3$ into non toxic nitrites (NO_2^-)and nitrates (NO_3^-). Decreasing water pH values is also effective in reducing the fraction of toxic ammonia.

- Dissolved oxygen (DO): the relevant biomass of the fry in the weaning tanks and their high metabolic rates creates a very high demand for oxygen. DO values should be kept as close to saturation as possible, a safe range being 80-100% saturation measured at the water outlets. Oxygen supply should also be able to cope with the daily fluctuations that are associated with feeding. Modern extruded pellets require higher DO levels in the water (100-120% saturation) than traditional pellets. DO levels should be checked all day round and should be plotted by a computerised monitoring system. In case that this ideal solution could not be implemented, DO levels should be checked every hour for a couple of days in order to be able to plot its evolution in the 24 hours and identify the peak demand times. Then, it would be enough to check DO at least during these peak times and whenever fish behaviour seems to be altered. For practical purposes this means after the morning first feeding, twice in the afternoon and twice in the evening. At night, and in particular at the beginning of the weaning period, a few measurements are advisable, although this is a less critical period because at night fish activity is greatly reduced.

- Photoperiod should be shortened by two hours in relation to the larval sector, i.e. 14 hours light (8 – 22h) and 10 hours dark, to avoid excessive activity in late hours that may increase pollution and cannibalism. At 80-90 days of age the natural photoperiod can be set.

- Light intensity should be lower than in larval rearing tanks and can be set at 1,000 lux. As the twilight effect is considered not so relevant, the elimination of a dimmer switch allows the stallation of common daylight neon tubes. A timer to switch on/off automatically may beuseful, but it is not strictly necessary.

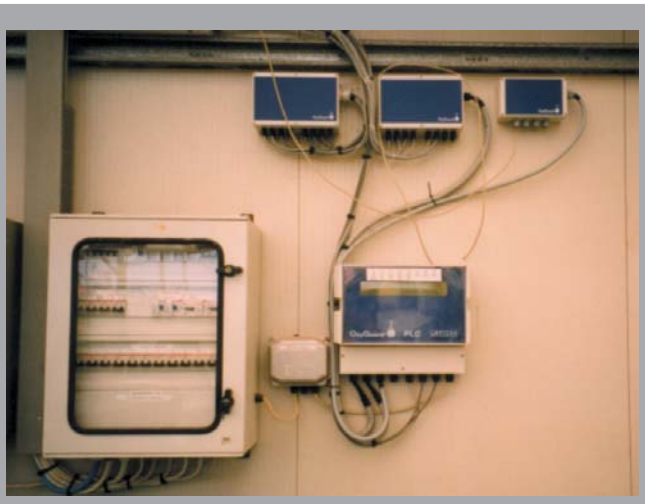


Fig.59.01 Oxygen monitoring devices are often installed to check automatically DO level in the weaning sector (photo STM Aquatrade)

Fig.60.01 Hand made outlet screen with 2 mm plastic net (photo STM Aquatrade)

- Water renewal rates should be increased with respect to those of the larval rearing unit, as the fish biomass increases considerably and dry pellets are provided in large amounts. A peak renewal rate of at least one complete tank volume every two hours should be foreseen in the design of the weaning sector. However this rate may vary according to the water system used: the less efficient recirculating semi-open circuits should contemplate a higher renewal rate (one volume per hour) than in the case of the flow-through circuit.

- Bottom aeration during weaning it is no longer intended to keep fish dispersed in the water, but its importance should not be underestimated. A properly placed aeration does not only add oxygen to the water, but contributes to create optimal hydrodynamic conditions. Square or rectangular tanks require a particularly careful placement of the aeration to prevent the formation of dead corners, where uneaten feed and faeces would accumulate and spoil the water quality.

- Screen mesh size should be set at 1 mm at stocking, to be replaced as soon as possible with 2 and 3 mm screens to stand the increased water flow and the associated debris.

Transferring fish from larval to weaning section

Seabass and gilthead seabream can be moved to the weaning section at about 45 days of age when the rearing water temperature is 18°C. To anticipate the transfer is not advisable because the young postlarval stages are very fragile when their metamorphosis is in progress.

As the transfer operation is clearly very stressful to fish, a few precautions should be adopted:

- prepare all equipment, well cleaned and disinfected, the day before,
- train staff beforehand: everybody should know what to do, when and why,
- if possible, organize the transfer early in the morning,
- choose harvesting and transporting methods which are gentle with the young fish (see below),
- three-four days in advance fish should receive an increased amount of vitamin C (up to 10,000 mg/kg of feed) with their diet, as it has anti-stress properties
- do not feed fish before transfer,
- clean thoroughly the bottom of the larval tank to avoid polluting the transfer medium,
- receiving water quality parameters should match those of the larval rearing tank in terms of salinity and temperature,
- have an emergency oxygen supply at hand in case of some unforeseeable delay,
- try to never touch fish, and never let them jump or stay out of the water,
- never concentrate too many fish in a small container,
- never let dirty water enter the weaning tanks, and avoid that fry containers touch the floor before being immersed in the receiving weaning tank;
- try to keep the period in which fish are without aeration or water renewal to the shortest possible,
- feed fish as soon as possible when placed in the new tank in order to avoid cannibalism,
- immediately after transfer feed plenty of live feeds (artemia metanauplii) to favour a prompt recovery.

Preventive bacteriostatic treatments before and after transfer are sometimes applied to reduce the risk of disease outbreak on fish stressed by the transfer. However, since this practice can induce the appearance of drug resistant bacterial strains, it is recommended to replace this practice in favour of an anti-stress diet and a carefully managed operation.

Fig.61.01 Gilthead seabream fry ready for transfer in larger tanks (photo STM Aquatrade)

In some cases where it would be necessary to minimize the risk of bacterial infections caused by excessive handling, or by weak larvae, a preventive treatment lasting five days is advisable. In this case the larval tanks can be treated with Furazolidone, at a concentration of 30 ppm during two to three hours, a treatment which is repeated for three days before harvesting and two days after. The larvae should not be fed during the treatment. In order to apply the treatment:

1. dissolve the required amount of Furazolidone in a beaker (some drops of formalin will help);
2. increase DO levels in the tank to 130% saturation and then stop water exchange for the time of the treatment;
3. distribute evenly the Furazolidone solution;
4. once the time established for the treatment has expired, flush out the water, renewing it on a flow-through basis during a suitable time to eliminate the product;
5. use a skimmer to retain floating Furazolidone foam and clean the tank walls with a sponge.

Depending on the design of the larval tank, fry can be harvested either by netting them or by draining the tank through the bottom outlet while concentrating fish in a screened container (a procedure similar to the harvest of rotifers and brine shrimps, already described). The key points in the last case are the position and the dimensions of the tank outlet, as it should be sufficiently large (at least 1½“) and should be placed at a minimum distance of 40 cm above the floor.

Fry transfer from in-floor larval tanks:

1. fill an adequate number of weaning tanks with water at the same temperature and salinity as that of the larval tanks; the volume of the weaning tanks will be related to the desired final fry

density. Make sure that the weaning tanks have an adequate water inlet, proper lighting and aeration and a 1000-µm water outlet screen;

2. place a soft nylon seine net with 2-mm knotless mesh inside the tank where fry have to be transferred and gently encircle part of the fry stock. Do not trap too many fish at once to avoid excessive overcrowding;
3. close the net and lift its two ends to the water surface creating a sort of bag hanging from the tank rim. Adjust the net to keep fish inside a submersed pouch;
4. dip a plastic bucket in the pouch and gently fill it with water and fry in a way that the fish do not remain exposed to air;
5. pour the bucket directly into the weaning tank or, if it is far away, into a wheeled and aerated plastic container which will be used for the transfer;
6. repeat steps 3 to 6 till completion of the harvest;
7. the very last fish which escaped capture with the seine net, typically the strongest animals, can be collected through the bottom drain or by means of a dip net
8. empty and clean the empty tank before it gets completely dry.

Fig.62.01-02-03-04-05-06-07 Harvesting steps from in floor fibreglass tanks (photo STM Aquatrade)

Larval transfer for tanks placed above the floor (typically FRP round tanks with conical bottom):

1. repeat steps 1 and 2 of the previous protocol;
2. fit in the weaning tanks the outlet screen of 1 mm;
3. dip the harvesting filter inside a large wheeled container placed near the tank to be harvested; inside it place a diffuser connected to an oxygen bottle or to the liquid oxygen distribution line;
4. connect a flexible hose, not collapsible, to the drain placed at the tank bottom and place the opposite end into the harvesting filter; to work properly, the drain must have a PVC ball valve of the same diameter of the hose;
5. siphon the bottom dirt out;
6. stop water inflow in the tank, but keep the aeration on;
7. place a screened siphon into the tank and start draining the water into the filter container; the screen in the siphon will prevent fish from escaping;
8. once the water level inside the tank has reaches the upper level of the conical bottom, open the drain valve and the fry will move with the water into the filter container through the hose;
9. always keep the end of the hose in the filter container under water, and avoid a strong outflowing current, if necessary adjust the differences in water level to reduce the current;
10. as soon as the larval tank is empty, flush the hose with water taken from the water inlet valve of the tank to help the last fish to get out of it;
11. close the valve and disconnect from the bottom, lift it with the attached hose over the filter level and re-open it so as to drop the last fry into the filter. A bucket with some water placed under the larval tank bottom will ensure that no fish will fall on the floor;
12. dip the filter container into the tank and let fish get out. If the filter is too small to contain all the fry of the larval tank, repeat the procedure for a phased harvest;
13. clean the empty filter tank before it gets dry.

Feeding

Feeding procedures in the weaning section differs from those of the larval rearing unit . Main changes are the end of the live feed supply and the setting up of a truly intensive rearing system based on automatic distribution of dry feed. The feeding protocol that follows applies to both species and is based on the following assumptions:

- initial fry density 10-20 fish/litre;
- water temperature of 18°C;
- salinity range 35-37 ppt;
- feed quantities refer to those supplied to one m^3 of rearing tank volume.

Refer to Annex 17 and Annex 18 for an indicative feeding regime during weaning, considering that in any case figures refer to a specific diet. Quantities should be adjusted during the first days of weaning according to fish behaviour and feed demand.

Feeding live-food

Artemia is supplied to minimize stress and cannibalism, usually associated with the early weaning stages. At an initial fry density of 10-20 fish/litre 4,000 metanauplii/litre are distributed three times a day at 11, 16 and 21 hours.

Feeding moist food

Even if today there is a tendency to replace moist food with by more advanced dry feeds, it still remains a useful resource to supply additional nutritional integrators and, in some cases, drugs at low cost. It can as well

replace dry feed in an emergency. In this case its ration is determined according to fish size: for fry up to 1 g it is 25% of the total biomass (wet weight), between 1 and 10 g it is gradually reduced to 5%. As its distribution is not mandatory but supplementary, it is not normally included in the feeding protocols (Annexes 17 and 18). If distributed as supplement (5% of biomass), the quoted dry feed rations should be reduced by 20%.

Fig.63.01 Belt feeders are frequently used for the distribution of small crumble (photo STM Aquatrade)

Moist feed should be prepared fresh every day and should be totally consumed within the same day. If distributed as supplement of dry feed they should be given in three rations at 15, 17 and 19 hours. This pattern may change according to local conditions, in particular when moist feed is also a vehicle to deliver drugs.

The composition and texture should ensure its stability when placed in water, as well as assure its buoyancy for a better control of feed uptake by fish. For this purpose it can be smeared on a framed mosquito net that is kept submerged just below the water surface. Preparation and distribution have already been discussed in the previous section and can also be found in Annex 24.

Feeding dry feed

Strictly speaking, weaning (in the sense of shifting from live to artificial feed) commences during the larval rearing. The young fish actually receive the first feeding with inert feed at the very early age of 17-19 days, but it is much later, after the transfer into the weaning sector, that dry compounded feed become their only nutritional source. Live feed distribution is discontinued when they reach an age of sixty days.

A strict control of feeding behaviour is necessary to prevent both under and overfeeding. Underfeeding triggers cannibalism and broadens the range of fish sizes. Overfeeding causes health problems and rapidly increases water ammonia levels in the tanks. Feeding is first determined theoretically and is later fine-tuned depending on fish behaviour and growth performance. It is then adjusted to the actual biomass after assessing the average individual weight and the number of fry in the tanks. This is normally done in connection with grading and thinning out operation

Fig.63.02 Demand feeders are used with bigger size pellets (photo STM Aquatrade)

Feed distribution

Feeding should be carried out at regular intervals, spreading feed over the whole water surface with a plastic spoon and always checking the actual feed consumption rate, the presence of any feed leftover on the tank bottom, and the general fish behaviour. To reduce pollution and cannibalism, as well as to better adapt fish to dry feed, 60% of the dry feed daily ration should be distributed in the morning at 8, 9, 10 and 12 hours, the remaining 40% in the afternoon and early evening at 14, 16, 18 and 20 hours. To avoid mistakes and to keep records of the feeding rates, each tank should have its own plastic container to hold the daily ration of dry feed. Any feed leftover has to be noted down on the tank file (see Annex 26). The same staff should be responsible for feeding fish in order to acquire experience in detecting fish behaviour and needs.

Management of the weaning section

The management of the weaning section requires the same procedures indicated for the larval rearing unit, with special attention for new characteristics of older fish such as cannibalism, pollution, greater DO needs and fluctuation, and disease outbreaks.

Staff

The weaning unit should be staffed with a head of the unit and with sufficient skilled workers to cover the whole period of light hours. The personnel should also be recruited according to the increased workload as fry are transferred from the larval unit to this sector. Typically, staff moves gradually from the larval rearing section to the weaning section at the end of three or four larval production cycles. The degree of automation can also marginally affect staff numbers.

As an example, a weaning sector with an overall capacity of four-five million fry to be produced in three cycles is adequately staffed by a team of six workers plus the unit head. The same watchman in charge of the larval unit takes care of the night controls.

Staff of the weaning section should be well trained and should always know what to do, when and why. Working protocols have to be distributed to all workers, and written instructions for the work to be carried out the next day must be prepared by the unit head. Every duty has to be performed properly and has to be recorded in its file. Nothing has to be hurriedly done, and everything has to be carefully planned.

Fig.63.03 Large weaning/pre-growing unit in Ittica Mediterranea (photo STM Aquatrade)

Daily operations

Weaning requires the same carefully planned and implemented working protocol as set for the larval rearing sector. Annex 27 summarises the activities to be performed during working hours. The use of all consumables should be recorded on a specific file and their replacement must be ordered well in advance. Proper hygienic conditions are mandatory (see below), as well as a complete separation among the sections of the hatchery to avoid possible contamination.

Control of environmental and biological parameters

The weaning procedures require a close monitoring of environmental conditions (abiotic parameters) and of the fish population (biological parameters). The first ones, to be checked as a routine daily, have already been described before and their frequency is indicated in Annex 27. The biological parameters are detailed below in terms of their operating procedures and frequency of monitoring.

As weaning fry are much sturdier than post-larval stages, they can be periodically sampled and checked for a closer control on the population.

These observations should focus on:

- fish behaviour,
- growth and food conversion rate (leading to grading),
- deformity rate (selection),
- swim bladder presence
- mortality and final survival rate.

Fig.64.01 Fry sampling nets (photo STM Aquatrade)

Fish behaviour

As a general rule, any trouble with the environmental conditions in the tanks directly affects fish behaviour before the onset of unequivocal signs of stress such as a large mortality. A routine watching by experienced personnel immediately reveals if something is going wrong. A healthy fish displays the following signs of normal behaviour:

- complete control of the swimming activity
- successful feeding/preying activity (even some cannibalism),
- fast response to sudden stimuli (typically a hand waved over the tank),
- proper colour (silver grey instead of black),
- mass concentration under feeders and artemia buckets,
- all water volume occupied by actively swimming fish,
- absence of mass concentration at the water inlet (which may reveal oxygen deficiencies).

Whereas observation of fish behaviour has to take place on a daily basis, other controls require a representative sample of the population. To avoid excessive stress to fish and the bias of inaccurate samples, these controls usually take place when weaning tanks are periodically harvested to grade and thin out their fish population. Moreover, as counting and weighing are done in each size batch, accuracy is higher.

Fig.64.02 Healthy gilthead sea bream population (photo STM Aquatrade)

As a rule, these periodical checks of the fish population should take place at 80, 100 and 120 days of age. Till 80 days of age 100 fish represent a suitable sample, whereas in older populations a larger sample of 200 fish is suggested to cover the population variability. Fry must be anaesthetised as described below before checks are conducted, in order to avoid mortality and pathologies.

Controlling growth and deformity rate

Weaning growing performance should be assessed fortnightly, and possibly in coincidence with fish grading. If that is not possible, weight and length are measured on a limited sample of the population as follows:

1. prepare one 1-l beaker filled with 300 ml of water from the rearing tank. Put it on the balance and tare;
2. harvest some fish by means of a hand-net and place them in the beaker, trying to avoid adding water to the beaker in the operation (the use of a tea strainer may help);
3. for greater accuracy, the amount of water added could be calculated in advance by weighing the difference between a wet and a dry strainer, and this difference can then be subtracted from the final weight;
4. weigh the beaker with the fry and record the weight;
5. return fish to a bucket and count them;
6. repeat the previous steps for every new batch taken with the hand-net from the tank till a sample of 100 fish is obtained;
7. calculate the average individual weight by dividing the sum of weights by the total number of fish;

To measure body length and perform other biological controls proceed as follows:

1. anaesthetise with 2-phenoxyethanol the sampled animals (200 to 400 ppm solution depending on fish size), place them on a clean glass and measure length (in mm) with a small piece of millimetric paper placed under the glass;
2 .for each individual record its total length (TL) and any morphological abnormality (see below for details);
3. by placing a strong light source below the glass, the presence of the swim bladder can be easily detected in seabass, while it is more difficult to visualize for gilthead seabream;
4. anaesthetised animals should be returned as quickly as possible to a bucket with clean aerated seawater, but they should be returned to the rearing tank only when they have totally recovered, to avoid aggression by the other fish.

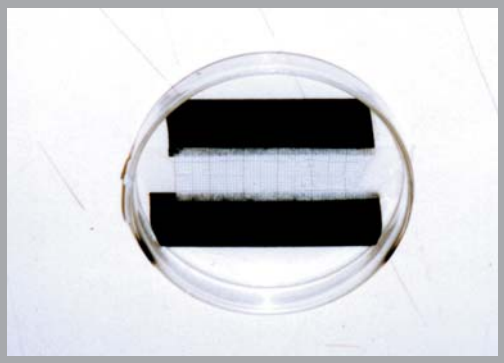


Fig.64.03 Hand made device for an accurate measurement of small fry (photo STM Aquatrade)

This procedure is fast and does not harm the sampled fish if properly done. However, the hand net does not offer a completely representative sample of the population, as the biggest sizes usually remain close to the bottom. A better solution is sampling the fish obtained by means of a small seine net hauled through half tank.

Fry grading

Even when coming from the same egg batch, post-larval fish and fry of both species do not exhibit a uniform growth pattern. As a result, small fish co-exist with fish twice as big in the same tank. The aggressive behaviour of larger fish and the unnatural crowding condition of the rearing tanks quickly result in cannibalism. In seabass populations it develops in a typical way: at the beginning larger fish gulp smaller specimens from their tail, leaving their heads protruding out of the predator mouth ("double-headed fish"), later on, with the full development of the scales, the gulping sequence is reversed from head to tail, as in the adult fish. Gilthead seabream does not gulp, but bites their unlucky mates first in the eyes then in the belly and in the caudal fin.

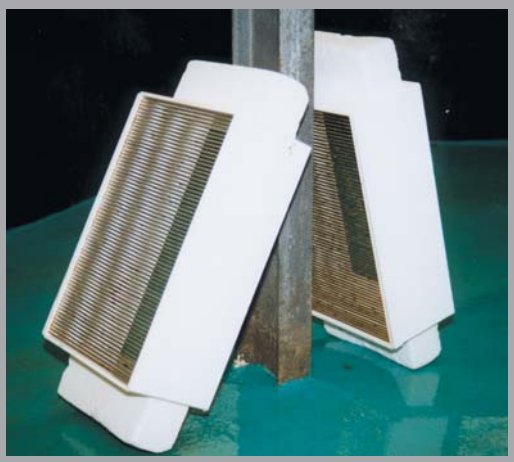


Fig.65.01-02 Small and large fry graders (photo STM Aquatrade)

If left uncontrolled, cannibalism may become a major cause of mortality. Reducing its incidence can be achieved by diminishing light levels over the tank, by reducing water transparency with the addition of microalgae and by increasing the food ration both in terms of quantity and frequency. The best solution is, however, to grade frequently fish into homogeneous size groups.

Fry grading is achieved by letting them pass through a series of sorters of different calibre. A common model is represented by a floating PVC tray (length 45 x width 20 x height 15 cm), whose base is equipped with a grid of stainless steel bars whose distance is calibrated. When floating in the tank, the base remains submerged.

The space between bars is set according to the fry thickness at various ages. A complete set of graders, covering the various ages, should then include the following measures: 2 - 2.5 - 3 - 3.5 - 4 - 4.5 – 5 and 6 mm. To accelerate the grading operation, it is advisable to use three boxes per graded size.

An acceptable size variability of the sorted batch should remain within 10%, i.e. the difference in weight between the smallest and the largest fry should not be more than 10%. When the periodical population weight check shows a larger variability, a grading should be performed. To select the adequate sorters, a preliminary test should be made with a sample of about 100 fish and the size dispersion should be determined after grading.

Grading operations require the following equipment:

- three new weaning tanks ready to host fish,
- at least two graders of the two selected sizes,
- three floating cages placed in the tank to be sorted,
- a couple of hand nets,
- plastic buckets.

The small floating cages used to keep for a short while the graded fish during the operation are simply made with a floating square frame (a PVC pipe of 50 mm diameter) to which a net bag of soft mosquito net is glued (70x70 cm, 80 cm deep).

The grading operation proceeds as follows:

- harvest all fish and keep them in a floating cage (or in a submersed bag made with the harvesting seine net as described in the harvesting procedure for fry transfer),
- place the empty floating cages in the tank and place the larger grader stacked on top of the smallest one, with the piled graders inside a floating cage,
- collect the trapped fish with the hand net and pour gently into the piled sorters,
- move gently the graders to help fish moving through the bars,
- place the fish remaining in the upper grader into one cage or directly into the receiving tank,
- repeat for the underlying grader with the intermediate sized animals,
- the smallest fish which have not been retained by both graders concentrate into the cage from which are then moved to their tank,
- repeat the above mentioned procedure till all fish have been graded,
- when handling fish adopt the same precautions adopted for their transfer.

Sorting fry with skeletal deformities

A significantly higher incidence of anatomical abnormalities may be observed in seabass and gilthead seabream produced at industrial hatcheries than in wild caught animals. Deformed specimens should not be utilised due to their slow growth rate, and also because of they are prone to get diseases and have poor marketability. Moreover, they compete for food and space with healthy fish. Therefore they should be detected as early as possible and eliminated.

Skeletal deformities in seabass and gilthead seabream fry typically affect snout, opercula and backbone. The snout may show a deformed mouth in a variety of shapes, with deformities affecting both upper and lower jaw, as well as cranial bones. One or both opercula may not develop completely, leaving part of the gills exposed. The most severe deformities affect the backbone in the form of kyphosis, lordosis or a mix of them. Swimbladder in part of such animals is missing, and its absence is probably at the origin of such deformity, although this is not, however, the only possible cause. In most cases swimbladder is present and the cause of spinal deformity has to be researched in other directions. Such skeletal deformities have been associated to nutritional deficiencies, gas supersaturation in the water, something possible when heating is used, genetic disorders and generally unsuitable rearing conditions (abnormally strong water currents in the rearing tank). Of all

deformities, the less damaging one is that of the opercula, for which an incidence rate below 10%, if it is the only deformity present, is usually considered acceptable. The presence of other deformities should not be accepted, and batches affected by several deformities have to be screened and animals that carry the deformities must be eliminated.

Fig.66.01-02-03 Gilthead seabream grading (photo STM Aquatrade)

A visual examination is the most common method to detect abnormal fry. Not only their shape, but also thei unusual swimming behaviour may reveal an abnormal development. Other methods include an analysis of morphometry, stereoscopic observations and soft X-rays, the latter usually carried out on fingerlings being sold, as a proof of their quality. A major drawback is that skeletal deformities will fully develop to be visually identified only when fish are over 0.5 g in size.

A search for deformities is routine work on the fry samples collected to assess size. If it is desired to return the animals to the tank the control can be easily carried out on anaesthetised fish. A careful examination should be done on both sides of each fish. All findings must be recorded on a dedicated file for each tank population.

If the percentage of deformities in a given fish population exceeds the quality standards set above, the deformed animals should be sorted out. The only effective technique to remove such fry is to sort them by hand. To reduce the workload, fish should first be checked for the presence of swim-bladder (see below) and then the selection should be carried out only on the fraction with swim-bladder.

In any case, the entire population has to be fished out and anaesthetized. It is then spread over a smooth surface, such as a PVC or stainless steel table maintained constantly wet, where fry are sorted by hand and the deformed fish are discarded. Only small groups of 2-300 fish should be caught and anaesthetized to avoid a long permanence in the tub where they are treated and on the sorting table, which could be damaging.

Fig.67.01-02-03 Fry quality sorting (photo STM Aquatrade)

Swim-bladder control

The importance of a proper swim-bladder development and the precautions to be adopted for its activation in young post-larvae have already been mentioned (see previous section). However, a variable percentage remains usually without a functional swim bladder. The importance of this organ for a normal development and growth (see above) requires the sorting and elimination of those specimens without swim-bladder. As handling represents a considerable stress for fish, they should be sorted only in coincidence with other grading or measuring controls.

Marine fish fry without a functional swim-bladder can be easily separated from normal fish. The method to separate them is based in the difference in buoyancy in hypersaline water. Anaesthetized animals with functional swim-bladder will float while the others will sink to the bottom. The procedure requires one fry sieve, three 50 l plastic tubs, buckets and the usual harvesting tools such as a fry seine net and hand nets. The sieve can easily made with a 40 cm long piece of a 20 cm PVC pipe with mosquito nylon net glued on one bottom.

Proceed as follows:

1. fill one tub with tank water and dissolve enough sea salt to obtain a salinity of 50 ppt; place in it the fry sieve (with an aeration line inside);
2. water supply in the other two tubs should flow through them. To avoid changes in environmental parameters water should come from the weaning circuit;
3. add 10 to 20 ml of 2-phenoxyethanol to the first tub (200 to 400 ppm solution depending from fish size) and mix well;
4. harvest the fish population as usual and keep it inside the net at the water surface;
5. with the hand net take 100 to 200 fish at time and place them into the screened cylinder;
6. wait a couple of minutes until they separate completely and the entire water surface is covered with floating fry;
7. collect carefully all floating fry with a hand net and stock them in the other two large tubs, ensuring that water is renewed, until complete recovery from the anaesthesia;
8. take the sieve out and discard the fish without swim bladder that have sunk to the bottom of the sieve;
9. repeat this procedure until the whole population of the tank has been checked. The use of several sieves speeds up sorting, provided that enough staff is available to take care.

Remarks:

- before starting the procedure, test anaesthetic at different concentrations with a dozen fish to determine the optimal concentration under your particular working conditions (as the effect will vary according to water quality and fish size);
- check DO frequently and in case supply pure oxygen to maintain DO levels above 80% saturation;
- check the effectiveness of the separation by examining a few fry of each haul by transparency to verify the presence of a normal swim-bladder;
- use the recovery tubs alternatively and never mix recovered fish with anaesthetised ones as the first will attack those that have not yet recovered;
- when treating fry older than 70 days, increase water salinity up to 60 ppt in the tub where the anaesthetic is being used to speed up separation;

Cleaning

The same cleaning procedures described for the larval rearing section apply here. Due to the much larger organic load and higher water temperature, the sanitary conditions of the rearing environment require a close monitoring and thorough cleaning. Every day, when siphoning the entire tank bottom, the presence and the number of dead fish has to be recorded on the tank file. A complete description of the cleaning routines in the weaning sector is given in Annex 25.

Fig.68.01 Well cleaned biofiltration unit (photo STM Aquatrade)

Hygiene and sanitary conditions in the rearing environment

When dealing with rearing fishes at high density, the danger of a disease outbreak is always present. This danger is greater when fish of various age groups are reared close to each other, thus increasing the danger of spreading diseases. Hatchery staff should be made aware of such danger and be fully trained, both mentally and technically, to maintain rigorous hygiene criteria as a matter of routine. The following general rules can be adopted to minimise contamination in a modern industrial hatchery:

- identify clearly four major sectors for: live feed, larval rearing, fry weaning and broodstock;
- do not exchange or mix water, equipment and instruments among these sectors and apply this rule strictly;
- establish a routine for daily and weekly cleaning procedures to ensure that all equipment and material are kept under the best hygienic conditions;
- adopt disinfecting procedures and identify a disinfectant sufficiently efficient for the cleaning routines;
- select equipment and instruments and establish working plans which can coexist with the rule of maximum hygiene in the simplest way.

If in spite of these preventive measures, disease symptoms are noticed in one of the hatchery sectors, then it becomes urgent:

- to be able to make an early diagnosis identifying symptoms and their origin;
- to initiate rapidly the appropriate therapeutic treatment to cure the fish showing the symptoms

.

An efficient veterinary service and a regular sanitary survey are therefore essential to ensure the success of intensive hatchery operations.

A number of chemical treatments are now commonly applied and have become standards as routine practices during the rearing period:

- treatments of broodstock against bacteriosis and parasites;
- disinfection of fertilised eggs;
- prevention of stress-related diseases after handling.

The last one is particularly relevant in the case of transfer and grading of fry. Since these are operations periodically involving all individuals reared in the hatchery, it is of extreme importance to standardize them into routine practices which prevent the occurrence of stress-induced bacteriosis. A record after each treatement (Annex 28) must be kept. The suggested routine practice is to treat all groups of fish being transferred from larval to weaning tanks and from weaning to pre-growing tanks, as well as all fish being graded.

The best chemicals for treatment appear to be those belonging to the family of the furanics, such as nitrofurazone, furazolidone and furaltadone. The treatment consists in a 6-h bath at 30 to 50 ppm, repeated for three days before and two days after fish handling.

In case of evidence of lesions caused by the handling operations the antibiotics of the quinolones family are quite effective, such as flumequine and oxolinic acid. The treatment consists either in a 1 hour bath at 2 to 50 ppm repeated for two days or in administration through treated feed at doses of 12 to 50 mg/kg fish for seven days. Since these antibiotics are actually the most efficient therapeutics for some of the most dangerous and widely distributed bacterial infections (vibriosis and pasteurellosis), they should never be used for prophylaxys, but only for a therapeutical action against a detected pathogen infection.

In Annex 29, a number of chemicals are listed with their most common dosages. A more complete list of diseases affecting cultured seabass and gilthead seabream can be found in Annex 30.

Note: All chemicals for prophylaxys and therapy should only be handled by officially appointed personnel following the advice of a veterinarian; the choice of chemical and its dosage for treatment should strictly abide to current national regulations.

3.6- FRY TRANSPORT

When a size of 2 to 5 g is reached, weaned fry leave the hatchery to be stocked in the fattening facilities, either pre-growing tanks or floating cages. The transport within the farm where the hatchery is located is very short and does not require special equipment. Fish are usually stocked in round tanks mounted on a tractor-trailer or a small lorry and are moved to their final destination. An oxygen supply is required for safety reasons.

Transport becomes a more complicated matter when fish are sold or when the on-growing facilities of the farm are far from the hatchery. Delivery time may reach many hours or days and the quantity of fish to be delivered may be in the order of hundred thousand or more per transport. This section deals with the transport of live fish over long distances.

Transport equipment

By far and large the most common way to transport fry is by means of open containers, with or without lid and insulation, installed on trucks. These containers are attached to other equipment, more or less complicated, such as aeration-oxygenation systems, cooling systems, automatic DO and temperature monitoring. Another way to transport fry, not frequently used anymore, is in PE bags, which are filled with 1/3 seawater and 2/3 pure oxygen. Bags are placed into insulated (polystyrene) cardboard boxes to keep the temperature at an optimal low level during transport. This method works well, but it is not practical when large amounts of fry are involved because packaging is quite expensive and time-consuming. It is typically adopted for air transport of valuable and not bulky biological material, such as shrimp post-larvae, tropical fish or fish eggs.

Vehicles

The use of trucks is the main way of transporting live fish. Conventional light trucks equipped with 2-3 round open tanks are commonly utilised over short distances and for small quantities of fry. To deliver large quantities and for long journeys, dedicated heavy truck-trailer units equipped with several rectangular closed tanks are used.

Fig.69.01 Fry transportation trucks (photo STM Aquatrade)

Tanks

Reinforced fibreglass is the preferred material to build strong, lightweight and easy to clean tanks for live fish transport. For relatively short distances (covered within 2 to 4 hours) and for limited amounts of fry (max 15 kg of biomass per cubic meter), the commonest and simplest design is the round, flat bottom tank with open top. With a volume up to 1.5 m^3, it has neither insulation nor lateral outlets. The smooth inner sides are painted in white and are gel-coated to offer the best visibility of transported fish and to prevent skin damages. A floating wooden cross with size smaller than the tank diameter is a simple device used to reduce water splashing during transport without affecting fish. A light truck can carry two to three such containers.

Over longer distances and for bulk transport, the round tanks are replaced by closed rectangular tanks with a capacity ranging from 1 600 to 2 400 l to make better use of the space available on the chassis, allowing up to13 tanks on a long vehicle, and up to 6 on a normal truck. This type of tank has white gel coated internal surfaces and has smoothed corners, a large square trapdoor on the top, closed by a screen (to prevent fish from jumping out when the lid is open) and a lid. They also have a large lateral sluice gate with a removable gutter for unloading the fish, and a bottom valve for complete drainage and a vent hole with a valve or an air scoop on the top to prevent the accumulation of toxic gases as carbon dioxide and ammonia. Insulation is provided by styrofoam or polyurethane injected in the between the walls of the tank.

Fig.70.01 Before departure (photo STM Aquatrade)

Oxygenation systems

Large volumes of oxygen will be consumed due to the high fish load and to their high oxygen requirements, which are also increased by the stress induced by the transport and the build up of ammonia due to the absence of water renewal. Additional supply of oxygen has to be foreseen in both tank models, either by aeration or by pure oxygen injection. Aeration requires the installation of an air blower driven by the lorry engine. It provides filtered air through a frame of air diffusers fixed to the tank bottom. Because of their lower efficiency and shorter life span, air stones and ceramic diffusers are being frequently replaced by new textile flat hoses which provide finer bubbling, which minimise water turbulence and that last longer. Diffuser's output is controlled via a valve placed on the tank cover. Being a less efficient system to add oxygen to water, aeration is considered a sort of emergency solution to replace the more effective oxygenation system, widely adopted in fry transport, should it break down.

Oxygenation is provided in two ways: industrial grade oxygen bottles, and liquid oxygen containers. For their handiness and low installation cost the oxygen bottles are preferred for short distance transport, while the liquid oxygen containers are the preferred choice for long distance transports thanks to their longer autonomy. Both systems employ the same fine oxygen diffusers.

Fig.71.01 Liquid oxygen bottle for fry transportation (photo STM Aquatrade)

Monitoring oxygen

During transport the crew has to monitor periodically the DO levels in the tanks. In the past this was usually done by simply checking the bubbling inside each tank and adjusting it according to the operator's experience. Recently, sophisticated pieces of equipment such as portable oxymeters, and automatic DO monitoring devices have been introduced. The latter are made of a series of probes, one per tank, linked to a display installed in the driving cab of the lorry. A manually operated selector allows the driver to know the oxygen content of the water in all tanks while driving, thus saving time. A more sophisticated version includes motor-driven needle valves to automatically adjust the oxygen flow to pre-set values.

Water quality

Transport tanks should be filled with the same water used for fry in the hatchery. The water used for refilling should be at least mechanically filtered to remove suspended solids and should be well oxygenated to reduce the fish stress during transport.

Dissolved oxygen

DO is the single most important parameter in live fish transport. The aeration system should provide enough oxygen to keep its saturation level between 150 and 200%. As oxygen consumption peaks during and in the first hours after loading, the transport water should be hyper-oxygenated before the fry transport starts and actual DO carefully regulated during the very first hours of travel.

Salinity

To reduce stress, salinity should be the same to which fish are accustomed in the hatchery, which usually is full seawater at around 35 ppt. However, since brackish water at 20-25 ppt is considered less stressful because it reduces the energy needs for osmotic regulation of transported fish, it may be useful to adapt fish to lower salinity levels a few days before transport. An additional advantage of a brackish environment is the increased capacity for oxygen solubility. A portable salinometer or a cheaper optical refractometer are common portable measuring tools to assess the salt content in water.

pH

In seawater and brackish water pH usually is not a problematic parameter. The increased level of carbon dioxide generated by respiration acidifies the transport water, but the natural buffer capacity of seawater usually compensates for it. The pH level also affects directly the balance of un-ionised ammonia (NH_3), which is highly toxic to fish, and ammonium ion (NH_4^+), which is not toxic. The lower the pH the lower the toxic fraction of ammonia nitrogen. The addition of pure oxygen contributes to oxidise the toxic form into non-toxic products.

Temperature

Temperature strongly influences oxygen consumption by fish. Low temperatures during transport are therefore preferable since they decrease fish metabolism, thus reducing respiration and the consequent need of oxygen, as well as ammonia excretion. Oxygen solubility in water is also higher at lower temperatures. Transport temperature should, however, not differ sharply from that of the hatchery environment, but could be adjusted gradually to reduce stress, thus increasing the chances for a successful transport. A gradual decrease of 1°C per hour if possible to 16° C does not harm the fry and is considered excellent for transport conditions.

Seasonal variations of temperature can be dangerous when not taken into consideration in transport over medium to long distances. In summer it is preferable to travel by night, and the opposite in winter, when diurnal transports are advisable to minimise temperature fluctuations. In any case, the insulation of the transport containers remains a necessary precaution.

Ammonia

When fish are transported in tanks without water renewal, their metabolic products accumulate in the water. Ammonia, which is excreted through the gills, is their main metabolic product. In water it exists in chemical equilibrium between the un-ionised form NH_3, and the ionised ammonium (NH_4^+). The un-ionised form is very toxic to fish, even at very low concentrations. While in closed rearing systems it is eliminated by biological filtration, which oxidises ammonia to non toxic nitrites and nitrates, in the case of transport containers this problem is addressed in the following ways:

- fish are not fed at least 24 to 48 hours prior transport to reduce their excretion rate, provided that their cannibalistic behaviour is kept under control;
- water is partially or totally renewed each 24 hours during long distance deliveries;
- temperature is kept as low as possible to reduce metabolism without affecting fish, and to reduce the percentage of the toxic un-ionised ammonia;
- the transport tank lid or the vent are left partially open to prevent the build-up of ammonia (and carbon dioxide) inside the tank.

Carbon dioxide

Produced by respiration, carbon dioxide is dangerous because it reduces the oxygen carrying capacity of fish blood, even in presence of an adequate oxygen level. It is removed by water aeration and tank ventilation: the gas leaves through a vent or an air scoop. Sealed tanks can produce a dangerous build-up of carbon dioxide. The presence of foam at the water surface also reduces the water-air gas exchange (see below).

Turbidity

The removal of suspended solids and other agents causing turbidity allows a better view of the fish and reduces the risk of gill clogging, oxygen depletion and bacterial build-up. Transport tanks should therefore be filled (or refilled) with clean, filtered water.

Foam

The presence of foam and scum on the water surface of transport tanks may result from excessive production of mucus by a large number of fish hauled over long distances. The foam interferes with oxygen and carbon dioxide transfer at the air/water interface and also makes it difficult to observe the fish. The truck crew can easily remove foam with a scoop net, but this is a time-consuming activity that may delay the journey. More effective is the partial or total water renewal at the beginning of and during the transport.

Stocking density for transport

During the transport fry require more space and consume more oxygen than similar weight of adult fish. Moreover, marine species are far more fragile than their freshwater counterparts and as a consequence, fry stocking density for transport is lower. The optimum fish density in transport containers is influenced by a number of factors, which are usually learned and calibrated by practical experience. Seabass and gilthead seabream stocking for long trips up to 2-3 days does not usually exceed 20-25 kg/m^3, while shorter deliveries (24 hours or less) allow a 50% increase, up to 30-35 kg/m^3.

As a reference, according to professionals involved in fry transport in Northern Italy, the stocking density of 2 g seabass fry should not usually exceed 30 kg/m^3 for a transport of 12-13 hours with a salinity of 25 ppt and a temperature of 20°C. If water can be renewed one or two times during transportation, this transport time can be safely doubled.

Fig.72.01 High density of gilthead seabream fry in a transportation tank (photo STM Aquatrade)

Fry handling

To prevent excessive stress, the precautions previously mentioned for fry handling indicated in the weaning section are also valid for transport operations. Due to the prolonged time of a transport, from some hours to a few days, the precautions to be adopted should be strictly adhered to.

Transport conditions

It is advisable to fill the transport containers with water of similar quality of that present in the hatchery. Often, when loading is completed, water is totally renewed in the transport tanks to remove the load of contaminants (dirty, foam, mucus, faeces) that entered the tank with the fry. Again care should be taken to use only well oxygenated water. For transport over long distances, lasting more than 24 hours, at least a complete water change is recommended. It is usually done at well-known sites where good quality seawater is easily accessible and can be pumped using equipment available in the truck.

A working protocol for water change during fry transport does not exist because, again, too many variables are to be considered. A simple rule of the thumb based on practical experience foresees at least a 50% water change every 12 hours for transports with a fry density as high as 30 kg/m^3. In absence of marine water, pure

freshwater can be added to the containers in replacement of an equal volume of original medium, provided that the resulting drop in salinity does not exceed 5 ppt per hour. The addition of ice bars can also be considered when a lower temperature is desired.

Loading

Fish to be transferred must be in good health, should be well accustomed to their rearing tanks, should be free from diseases and any possible treatment for parasites must be done well in advance. Injured or weak fish, which are easily recognisable by their dark colour, slow or irregular swimming and altered behaviour, should be removed prior to loading. If their numbers are high in the stock to be transported, this is an alarming sign indicating that something has gone wrong with that population and therefore no transport should be possible before its complete recovery.

Due to the vigorous handling to which fish fry are subjected while being loaded into the transport containers, they become hyperactive and increase their respiration rate and metabolic excretion. To minimise oxygen consumption and ammonia production, as well as to decrease the amount of faeces and regurgitated food in the transport container, fry are usually starved at least 24 hour prior to shipping. This precaution is recommended for cannibalistic species such as young gilthead seabream and seabass only if their predatory behaviour can be kept under control. This can be achieved by keeping fish under dark conditions or increasing a little turbidity in water limiting hunting. Tranquillisers or anaesthetics in low dosage can also be considered .

Controls during transport

During transport the crew periodically monitors the oxygen supply and the presence of foam and dead animals at the water surface. Other parameters are seldom considered because too often nothing can be done to fix a possible problem during the journey, in particular when it takes place far from the coast. It is however highly recommended to control water temperature, DO, pH and salinity when new water is added along the journey to avoid abrupt changes in these parameters.

A first stop is usually done soon after departure (about half an hour), to control fish behaviour and calibrate the aeration/oxygenation flow meters. Then, frequent checks made at regular intervals are strongly advisable. The use of reliable automatic DO monitoring devices saves time and provides an early warning in presence of unexpected oxygenation failures.

Unloading and precautions at point of arrival

A logical precaution at arrival is to make sure that the transport water matches as much as possible the salinity and temperature levels of that of the receiving stocking facilities. If necessary, add slowly an increasing volume of the receiving water by using buckets or pumps to acclimatise fish to the receiving water salinity and temperature values before unloading. It is very important that fry are not exposed to abrupt shocks of temperature and salinity. In case fish are badly suffering from poor transport conditions, the faster they are moved to the receiving facilities, the better.

The discharge operations are related to the type of containers utilised for transport. Fry transported in round, open tanks are harvested by means of a small seine net, and then transferred in buckets or tubs to the receiving facilities. Fish stocked in closed tanks for long distance transport cannot be fished from the top trapdoor. In this case fry are unloaded through the lateral opening where a gutter has been placed. Flexible, non-collapsible hoses may be connected to the gutter to discharge fry into facilities placed in a position that cannot be reached by the truck. To reduce the discharge speed, the water level is lowered through the screened draining valve or by a screened siphon. In this phase, the oxygen flow must be maintained to avoid critical low levels due to the temporary overcrowding.

Upon stocking, long starved fry should be immediately fed with dry feed to prevent the appearance of cannibalism. Dead fish should be carefully removed, as well as moribund specimens. Mortality has to be controlled carefully

and dead fish must be counted and noted on a daily basis. Administering antibiotics for prevention purposes is no longer recommended for the possibility to create drug resistant bacterial strains. Their use should be limited to cure actual disease outbreaks, always possible in weakened or stressed populations.

Fry counting

During weaning the number of fry has to be assessed several times to calculate their total biomass, as well as their survival rate, and to adjust feeding rations accordingly. Their number is also needed to plan sales and consequent truck loading. Both manual and automatic methods are utilised to assess the number of fish fry. Manual counting can be done in three different ways:

1. the individual count, where the whole fish population is counted one by one;
2. the estimated count, where the fish population is assessed by counting only some well chosen sub-samples;
3. the count by weight, based on the total weight divided by the unit weight obtained through sub-samples.

For its large subjectivity, visual estimation of quantities is almost completely abandoned, while automatic counter devices are being increasingly adopted by large fish farms for the opposite reason.

Individual counting

This method is reserved to fry such as gilthead seabream and to those batches of fry where there is no agreement between seller and buyer about their estimated counting. It has the advantage to be very precise, but it takes time and personnel, usually three to five counters for each tank plus a supervisor who takes note of the count .

Fig.73.01 Individual counting in a traditional “valle” before fry seeding (photo STM Aquatrade)

Fry are counted during transfer to the receiving facility or prior to their loading into the transport tanks. The equipment required is a small seine net for fishing (2 mm in size, stretched mesh), white small containers (large tea cups work well), markers for every 50 or 100 fry and aeration or oxygenation equipment of the stocking containers (more frequent water change is also required).

Counting procedure: fry are harvested and transferred to the immersed part of a net stretched over the new tank or a temporary stocking container. The personnel standing around the tank start catching small numbers of fish (5 to 10 fry a time) with their cup. After a quick assessment of their number, fish are released under the net into the tank/container. In this way fish stress is minimal and the counting procedure is fast and precise. Counting is often done with the aid of pebbles. One small pebble represents 50 or 100 fish, according to what has been agreed upon in advance. Each counter tells the supervisor to cast a pebble into a bucket when he reaches that number. When all fish have been counted, the number of pebbles multiplied by 50 or 100, plus the residual figures from each counter, gives the total number of the stock.

Counting based on sub-samples

With large amounts of fry, manual counting is limited to one or more carefully selected sub-samples. Fry are harvested and stocked in small containers, usually 10-20 l white plastic tubs, in approximately equal amounts. Then all fry in the tub or tubs , which are serving as sample for the operation, are counted and their number multiplied by the number of tubs to obtain the total amount.

A similar method would be counting by hauls using hand nets (or plastic strainers). It has the advantage to be quick, but it lacks precision, in particular when fry are not uniform in size. This method requires knowing previously the carrying capacity of the hand net, which is calculated by counting all fish caught in a few hauls. This average number is then multiplied by the number of dips required to harvest the entire population. The hand net can be replaced by a plastic strainer, provided that it is not too deep and large to damage overcrowded fish.

Counting by weight

When counting large amounts of relatively large fry (individual body weight above one gram), the counting by weight is preferable. Its advantages are the rapidity of the operation and the limitation of stress, but its precision is adversely influenced by the practical impossibility to eliminate the water coming with the scooped fry (which induces a water weight error). Of course, the more homogeneous the size of fry, the better the final result will be.

This method calculates the total number of the fry population by dividing the weight the whole population by the average individual weight measured from a sample. This can be easily done during the grading operation. Proceed as follows:

1. tare the balance with a bucket half filled with seawater to a pre-set weight, say 5 Kg;
2. harvest fry by means of a seine net (see transfer procedures above), or directly from the floating cages if already graded (see below), the latter method giving a more precise counting;
3. transfer with a hand-net a small lot of fish into the pre-weighed bucket and record the net weight displayed by the calibrated balance; try to keep the water in the hand net to a minimum;
4. repeat the previous steps until no fish is left and sum all the weights;
5. obtain the average weight by counting the fish of three hand net hauls:
6. calculate the total number of fry of each size class by dividing the total weight by the average individual weight.

Remarks

- however carefully done, this method remains only a good estimate of the actual figure, its error being inversely proportional to the size of fish; its accuracy is considered overestimated by no more than 10%;
- for a greater accuracy, the amount of water trapped in the hand-net can be pre-determined and then subtracted from the final weight;
- when counting small fry an electronic balance with 0.1-gram division and automatic tare switch is strongly recommended to better match size variability.

Fig.74.01 Digital counter with over 100 000 fry per hour of capacity (photo STM Aquatrade)

Automatic counting

The increasing production of fry of valuable species has motivated the development of faster and more accurate counting systems and as a result reliable automatic counters for fish fry are available in the market. They are based on a photocell that counts the fish passing in front of it. They are designed to keep fry always in water, swimming along a mild water current to the counter where patented nozzles separate the fingerlings to minimise counting errors. Their counting capacity is of about 30-35 000 fry/hour within a size range of 0.5 to 6 g.

Some models of automatic counting devices can also be linked to an automatic grader, and the combination permits a complete automatic selection process. The advantages of such equipment are fairly good accuracy (98-100%), rapidity and reduced disturbance and damage to fish. The personnel required to operate the counting machine is also less than that required for the more traditional counting sessions. Their rather high cost can be justified when large amounts of fry have to be counted.

3.7- FRY DISEASES: INTRODUCTION TO THEIR OBSERVATION, ANALYSIS AND FIRST TREATMENT

When the cause of fish mortality is a bacterial disease (Annex 30), any successful therapy requires the identification of the pathogen. Administering drugs without knowing the etiological agent may result in a total failure in the treatment and in the selection of drug resistant strains.

Most fish farmers are not trained to diagnose bacterial diseases. However, a fish pathologist is not often at hand, nor it is always possible to send samples to a certified diagnostic laboratory in a reasonably short time. In these cases, the advantage of being able to carry out a rapid initial screening of disease possibilities should convince the farmer of the necessity to acquire some basic knowledge and tools to perform an initial analysis by himself.

This section provides a minimal basic knowledge to observe the main bacterial diseases in farmed seabass and gilthead seabream. It should be pointed out that this section does not intend to replace the experience of a professional fish pathologist, who bears the final responsibility as the only source of reliable advice and expertise in this field. However, if properly performed, such initial investigations can provide an amount of information that often becomes essential in the decisional process of a professional that has to decide rapidly upon a suitable therapy in case of acute outbreaks associated with mass mortality.

In examining fish from an affected population, standard operating procedures must be adopted to avoid mistakes and to facilitate the exchange of information among farmers and specialists. These standard operating procedures can be summarised as follows:

- collection of environmental data (main parameters);
- observation of fish behaviour;
- *in vivo* bacteriological observation (smears of possible lesions, blood, skin, gills, spleen and kidney, either stained or fresh),

- *in vitro* bacteriological examination *(*cultures on Petri dishes of samples from spleen, anterior kidney, dorsal aorta and swim-bladder),
- identification of the pathogenic bacterium and culture of a pure strain,
- screening essays to identify which drugs are effective on the pathogen,
- selection of a suitable therapy.

In vivo tests to trace bacterial pathogens on the skin surface and in some of the internal organs, can be a quick and often decisive step to provide a first answer for many diseases. Then, the *in vitro* culture from a sample of the most infected organs may be carried out. Most bacterial colonies develop after 24 to 36 h and their examination under a microscope (x1000 with oil objective) can be helpful to prepare an antibiotic essay. In this way, it should be possible to determine the most appropriate treatment 48 hours after having observed the presence of the disease for the first time.

Minimum time required for therapeutic treatment determination

• *In vivo* examination of skin and main organs	1 h
• *In vitro* culture of sample in Petri dish	+24 h
• Antibiotic assay results	+48 h

To diagnose a disease, identify the responsible bacterial pathogen and determine the most efficient treatment for the fish proceed as follows:

1. sample at least 10 to 30 moribund fish;
2. examine their external surfaces and wounds;
3. obtain some skin and gill scrapings; examine (after staining) and culture samples *in vitro*;
4. examine main internal organs looking for: appearance and presence of lesions. In particular observe and culture *in vitro* samples of spleen, kidney, and stomach/intestine contents. Take samples for analysis of bacteria and viruses, to be performed later by a specialist;
5. examine results of *in vitro* cultures: type of colonies; *in vivo* microscopical study; staining;
6. perform an antibiotic test.

Studying lesions at the skin surface

Several opportunistic bacteria can be found in the skin surface together with pathogenic bacteria and it is very difficult to distinguish them. It is best to reduce your study to ascertain the presence of Myxobacteria (immobile bacillus, Gram negative). For this, proceed as follows:

1. scrub the lesion with a cover slide; place the latter together with the scraping over a slide; observe the presence of bacteria under a microscope (x400 and x1000 with oil immersion);
2. take off the cover slide and dry the material over a Bunsen burner. Make a Gram stain;
3. take a Pasteur pipette, break the tip and sterilize on the burner flame; let it cool;
4. obtain a first sample for *in vitro* culture:
    - using sterile tweezers, lift one margin of the skin lesion, detaching it from the muscle; introduce the pipette between lesion margin and muscle and suck some liquid;
    - place one drop on a TSA/salt (Tryptophan Soya Agar with 1.5 percent NaCl) culture medium in a Petri dish;
    - incubate at ambient temperature (20 to 25°C) for 24 to 48 h.
5. obtain a second sample for *in vitro* culture following the instructions given in the previous point;
6. in the two sample cultures, observe the growth of germ colonies for colour, size and shape.

Observing internal organs of diseased fish

Open the fish abdomen:

1. using sterile scissors, make a first cut just in front of the pectoral fin, at ventral line level;
2. cut along the ventral line, stopping just before the anus;
3. return to the first cut and continue to open in the direction of the lateral line, cutting around the pectoral fin;
4. continue to cut along the lateral line until you reach the previous ventral cut at the anus;
5. remove the piece of skin and flesh;
6. carefully observe the appearance of the swim bladder;
7. using sterile tweezers pull swim bladder out and move it forward, to give access to the kidney.

ATTENTION

- swim bladder and kidneys are sterile inside; avoid opening them and always use sterile equipment;
- do not cut open the digestive tract;
- do not open any nodule.

Then observe internal organs for abnormalities:

1.	Loss of colour(LOC)	colour intensity is far below standard for a given organ
2.	Atrophy (ATR)	significant decrease of the volume of a given organ
3.	Hypertrophy (HYP)	significant increase of the volume of a given organ
4.	Red Spots (RSP)	presence of small red spots, easily identified
5.	Redness (RED)	presence of an abnormal red stain over part of or the whole surface of an organ
6.	Haemorrhage (HAE)	presence of blood at the surface of an organ
7.	Nodule (NOD)	an identifiable mass in an organ having such a consistency and a colour that it can be clearly distinguished from that organ tissue
8.	Ascitis (ASC)	presence of liquid, usually clear, in the abdominal cavity

Look for the presence of these abnormalities according to the following example:

	Abnormality (see above)							
Location	LOC (1)	ATR (2)	HYP (3)	RSP (4)	RED (5)	HAE (6)	NOD (7)	ASC (8)
Abdominal cavity	-	-	-	-	-	-	-	•
Swim bladder	-	-	-	•	•			
Digestive tract	-	•	-	•	•	-	•	
Kidney	•	-	•	•	•	•	•	
Liver	•	•	•	•	•	•	•	
Muscle	-	-	-	•	•	-	•	
Pyloric caeca	-	-	-	•	•			
Spleen	•	•	•	-	•	•	•	

Record all the information described following as much as possible the above mentioned procedures (see Annex 31) and contact a fish pathologist to proceed further.

3.8- MORPHOANATOMIC AND MORPHOMETRIC STANDARDS

Morphoanatomic performance

The estimation of the larval rearing performance derived from morphoanatomic criteria refers to the development conformity and/or the chronology of development of specific organs. The assessment of such criteria will therefore be dependent on the existence of standards which accurately:

- describe the normal or abnormal aspect of the specific organs,
- fix the period when the observation can or should be done,
- indicate how it has to be done.

Then, the choice of the criteria depends on the importance of its consequences on the rearing performance (frequency of appearance, effects on growth, on survival, etc.). For seabass and gilthead seabream larvae, morphoanatomic quality principally refers to anomalies affecting urinary bladder, swim bladder and skeleton.

Fig.74.02 Good gilthead seabream fry and juvenile profile (photo M. Caggiano)

Urinary calculosis

Urinary calculi can be observed in the urethra or urinary bladder. They have the aspect of greyish or yellowish little stones. The colour may sometimes be reddish during the larval stage. They are essentially calcium phosphate crystals, $Ca_5(P0_4)$. Urinary calculi can be observed from hatchlings to juvenile stages. Up to a total larval length of 20 mm, calculi can be observed under the microscope. At a larger size, calculi can only be detected using soft X-rays.

Calculi are believed to be related to stress and poor management, but do not seem to be systematically lethal. The frequency of presence of calculi in the fish stocks varies from 0 to 30%. In some particular cases, it may reach 60%.

For larval rearing calculi are the earliest signal of poor population quality, due to many factors like management, water quality, scarce feed etc. For gilthead seabream when the total observation overcome 30% during the first feeding days (5 to 15) is better to empty the tank and restart the culture with a new population.

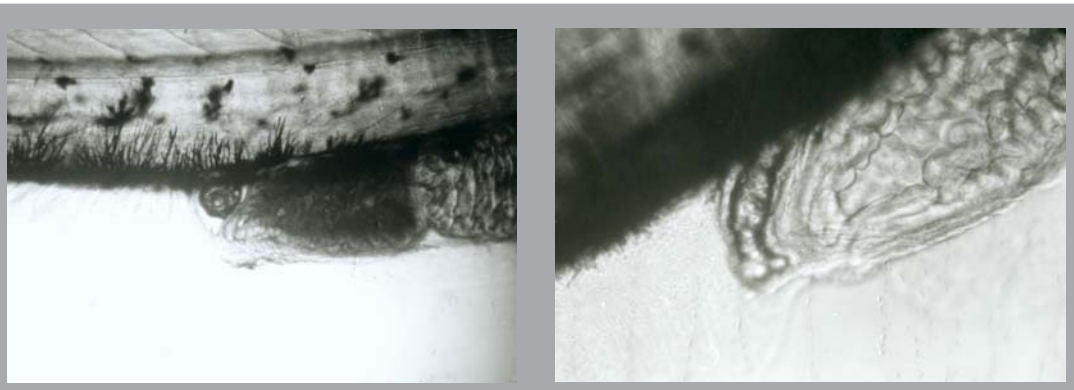


Fig.75.01-2 Very large calculi in this case larval survival is frequently very low (photo STM Aquatrade)

Swim-bladder development

The swim bladder is located between the backbone and the anterior part of the digestive tract of the fish larva. The functional organ looks like a refractive bubble. It reaches 20-30% of the total fish length in individuals longer than 40-50 mm. When the swim-bladder is not functional, it looks like a small slightly translucent vesicle whose size does not exceeds 3 to 5% of the total fish length. The initial inflation of the swim-bladder occurs in the early weeks of the larval life, when gilthead seabream larvae measure 4-5 mm and seabass about 5.5-6.5 mm. This organ can be observed by transparency under a microscope up to a larval size if 15 mm. Thereafter, soft X-rays have to be used to detect it.

The initial inflation of the swim-bladder is triggered when the larvae gulp for air at the water surface. The presence of an oily film or an excessive turbulence in the larval tank inhibit its inflation by preventing fish to reach the surface. Hence the introduction of floating skimmers that remove any surface dirt and oily films, and the adoption of a gentle water circulation to prevent excessive turbulence. Without these basic precautions, the percentage of larvae which do not develop a functional swim-bladder can reach 100%. The non-activation of the swim-bladder has serious consequences on fish, such as:

- an important delay in growth in both species, reaching 20-30% (in weight) when larvae measure around 10-15 mm (60 days old) and over 50% in 30-50 g fish;
- deformities of the backbone (lordosis) that appear at a size of about 20 mm, in both species.

Skeletal deformities

The most common skeletal deformities affecting bass and bream larvae, juveniles and adults concern jaws, gill opercula, head and backbone.

Deformities in newly hatched larvae

A fair number of abnormalities can be observed in newly hatched larvae, the most frequent ones being a form of body twisting. Affected larvae do not survive more than few hours, or few days at best, as the affected portion generally necroses. This deformity may affect from a small percentage to the totality of the population. If this percentage is above 10% it may be opportune to discard the entire batch.

The genetic origin of such anomalies is not proven, even if in trout farming it can be induced by inbreeding. On the contrary most authors believe that poor rearing conditions are a likely cause, in particular in relation to:

- nutritional deficiencies in the broodstock during ovogenesis (the most probable);
- inadequate lighting during incubation;
- excessive egg density (leading to mechanical stress and limited oxygen supply);
- handling, salinity or thermal shocks;
- pollutants in the rearing environment;
- a mix of the above mentioned causes.

Jaw and opercula deformities

Deformities can affect both the maxilla and/or the mandible, which can be either incomplete or protruding. A single operculum or both of them may be absent or be incomplete, or even be bent outwards. For larval sizes below 15-20 mm a microscope has to be used to detect them. For larger sizes they can be visually observed. Deformed jaws may be observed in larvae from hatching. Operculum deformities cannot be detected before larvae reach a length of 12 mm.

In both cases frequencies vary from 0 to 80% during the larval stage. Mandible deformities are often lethal as more than 80% of the affected larvae die, most probably due to starvation. The growth of surviving fish, although delayed, is not greatly affected (about 20% less than normal) and no additional mortality is observed later on.

On the contrary, opercula deformities severely affect growth performance (a difference of up to 60% in weight was observed in 7 months old fish) as well as they affect survival rate (over twice the mortality present in normal fish).

Backbone deformities

The most frequent skeletal deformities affect 2 to 6 vertebrae of the backbone. Scoliosis, kyphosis and fusion of several vertebrae are frequently observed, but lordosis remains the most diffused type of backbone deformity. When fish are affected, the backbone shows a typical V shape with a more o less pronounced angle. In fish without a functional swim-bladder, lordotic deformities are mainly located at the 15th vertebra (counting from the tail), and at 9th vertebra in other cases. As muscles involved in swimming act mechanically on the spine, in a fish with an abnormally developed spine they induce deformities in the area where the swim-bladder should be.

The first spinal deformities can be observed by transparency in larvae measuring around 15-20 mm, which corresponds to a stage in which bone calcification is sufficiently advanced. For larger fishes, soft X-rays have to be used.

The frequency of lordosis in the stock is directly linked to its origin:

- in fish without a functional swim-bladder it appears in both species. The percentage is equal to that of the non functional swim-bladder;
- in fish having a functional swim-bladder it may range from 0 to 100%.

The effects of lordosis on fish also vary according to the origin of the deformity:

- in 1 g seabass with a functional swim-bladder, the lordosis was associated to retarded growth (not well quantified), but no mortality was apparently induced by this deformity, whose angle decreased as fish grew, without disappearing completely.
- in fish without a functional swim-bladder, the lordosis is associated to growth delays and to the mortality previously described. These deformities are irreversible, even in case of late inflation of the swim-bladder (e.g., between 7 and 54 g in gilthead seabream).

In both cases, environmental conditions that force swimming during the nursery stage, which may be due to an excessively strong water circulation, increase lordosis frequency. In fish without a functional swim-bladder, these conditions also increase the lordosis angle.

The criteria described above are now commonly used to assess the quality of hatchery-produced fish during the rearing process. Morphoanatomic criteria are more frequently used when marketing fingerlings. Actually, the quality assessment of seabass and gilthead seabream, both as fingerling and market size fish, is based on the percentage of fish lacking a functional swim-bladder or affected by jaw, operculum or spinal deformities.

Fig.76.01 Gilthead seabream fry with incomplete operculum (photo STM Aquatrade)

Essential information available on the origin of such anomalies, which concerns both fresh water and marine species, has led

to formulate an hypothesis of a common osteogenic origin of the different skeletal anomalies at operculum, jaw or spine level. In case of lordotic deformities, the hydrodynamism of the larval rearing tanks would represent a condition that highlights this problem.

A review of osteogenesis related troubles in fish, at the origin of their skeletal deformities, showed that they are mainly induced by nutritional deficiencies or by the toxicity of some ingested elements. They affect bone texture, mainly modifying collagen metabolism or altering calcium and phosphorous fixation. Examples of toxicity-induced deformities are also numerous, involving heavy metals and pesticides, as well as an excess of some metabolites or vitamins.

At first glance, the choice among the possible causes of skeletal deformities in seabass and gilthead seabream is ample. Nevertheless, mechanisms involving heavy metal toxicity, environmental disturbances or the involvement of pathogens should be excluded because they would be too much site related when compared to the relatively large diffusion of this problem. Actually such skeletal deformities are observed in too many different farms and in different rearing conditions to believe that they are only due to some exceptional circumstances.

Fig.77.01 Lordotic gilthead seabream fry (photo STM Aquatrade)

Therefore, there should be a common factor that acts on a variety of environmental situations and rearing conditions. Some nutritional deficiency seems the more realistic hypothesis because rearing techniques (feeding sequences, preys, artificial feed, etc.) are more or less the same in most hatcheries. Looking at nutrition more in detail, a deficiency in vitamin C and/or a toxic excess of vitamin D could represent the most probable candidates. Vitamin C is one of the main agents in the collagen metabolism, an essential component of the bone tissue. Vitamin C deficiency can easily be explained by its high solubility in water.

The hypothesis of hypervitaminosis D is also attractive because it takes into account the appearance of urinary calculosis. This vitamin is present in large amounts in the fish oil used to enrich rotifers and brine shrimps for larval fish, as well as in the fish viscera, a component of artificial feeds used in the nursery stage. Tuna liver oil, for example, may contain up to 200 000 IU of vitamin D and cod liver oil up to 500 IU per gram (1 International Unit = 0.025 µg of vitamin D2 as crystalline form).

In human beings, the non-active D3 form is transformed into the active form 25-1-hydroxycolecalcipherol in the kidney. If this transformation does not take place for any pathological reason, a functional deficiency appears, even if the vitamin is abundant in the assumed food. Unfortunately, the active form of the vitamin D in fish is still unknown.

ANNEX 1

BROODSTOCK SECTION: QUARANTINE AND ROUTINE PROPHYLACTIC TREATMENTS

All fish arriving for the broodstock section should pass a quarantine.

Quarantine facilities

As a general rule, fish should be quarantined in a totally independent and separate part of the hatchery. Quarantine tanks should be first disinfected with a 500-ppm active chlorine (hypochlorite) solution during one day and should then be left to dry for seven days. Seawater should be filtered through a pressurized sand filter (with a screening capacity of at least down to 80 µm) and should possibly be sterilized using an UV system.

Quarantine procedure

The quarantine lasts a couple of weeks. During this time, the following sequence of treatments should be strictly followed, with no interruption.

Day 0. Dip each fish in a fresh water bath for 5 minutes (use 50-l tubs), then place them in the quarantine tanks and treat them with Furanics (50 ppm for 30 minutes).

Day 1. Treat with a mix of 200 ppm formalin and 0.2 ppm malachite green for 1 hour. Dip then each fish in a bath of fresh water for 5 minutes. Finally, put them back in the quarantine tanks and treat with Furanics (50 ppm for 30 minutes). Recent investigations have recommended not to use malaquite green for fish which are going for human consumption. Suitable alternatives to the products have still to be found, however.

Day 5. Repeat procedure as in Day 1.

Day 8. Repeat procedure as in Day 1.

Day 11. Repeat procedure as in Day 1.

Day 13. Repeat procedure as in Day 0.

During quarantine, fish should be closely monitored and well fed during the days without treatment. The ration to be administered is 0.5% of the body weight, distributed by hand daily in the morning.

At the end of the quarantine period breeders are transferred to stocking tanks, the quarantine tanks are drained, washed with detergent and fresh water, thoroughly disinfected with a hypochlorite solution for one day, and then let to dry for a few days.

Routine prophylactic treatments

1. Each batch of breeders should undergo a periodical bath treatment, once a month, from May to October, as follows:

Furazolidone	at 40 ppm for 1 h	repeated for 3 days
Formalin	at 200 ppm for 1 h	repeated for 2 days
plus Malachite green	at 0.2 ppm for 1 h	repeated for 2 days

This treatment should be given on the same day, at 2 to 3 h intervals.

2. Every time breeders are transferred from stocking tanks to internal spawning tanks, the following bath treatment should be applied:

Furazolidone	at 40 ppm for 1 h	repeated for 3 days
Formalin	at 200 ppm for 1 h	repeated for 2 days
plus Malachite green	at 0.2 ppm for 1 h	repeated for 2 days

This treatment should be given on the same day, at 2 to 3 h intervals.

File No. _____

Tank ____ Species _____ Females: initial number _____ av. size: _____ kg Biomass _____ kg

From tank : ___ Males: initial number _____ av. size: _____ kg Biomass _____ kg

Transferred to:

Date

Age

WATER QUALITY

T°C

Salinity (ppt)

DO (ppm)

pH

NH4+ (ppm)

FEEDING

Dry feed (kg)

Moist food (kg)

FISH MANAGEMENT

Mortality Males

Females

EGG MANAGEMENT

Viable eggs (g)

Non-viable eggs (g)

TANK MANAGEMENT

Water renewal rate (%)

Bottom cleaning

Treatments

ANNEX 3

BROODSTOCK SECTION: FEEDING REGIMES

A. FEEDING DURING OR AFTER DRUG TREATMENTS

To allow a quick recovery after harvesting or treatment, it is recommended to feed the fish with diets rich in vitamin C and unsaturated fatty acids. On the day in which the treatment is applied, either for therapeutic or prophylatic reasons, fish are not fed.

From the day following each treatment and for one week, the broodstock should be fed the usual pelleted diet with the following additional enrichment:

• Vitamin C (protected)	at 5 g/kg of feed
Cod liver oil	at 20 ml/kg of feed
or	
• Selco®	at 50 g/kg of feed
Vitamin Premix	at 10 g/kg of feed

To be effectively absorbed by fish, vitamin C should be offered as a protected and stable form, such as micro-encapsulated or as salt (e.g.: as L-ascorbyl 2-phosphate magnesium).

All the ingredients should be well mixed, using a kitchen blender, before being sprayed on or kneaded into the pellets to obtain a thorough coating of each pellet. The same enrichment diet is one of the possible options to enrich feeds to be given during oogenesis.

A third option is to prepare a moist food paste homogenising trash fish or cuttlefish, to which the same enrichment ingredients are added. Already enriched feed formulations are now being commercialised, which can replace the above-mentioned enriched diets, reducing the workload.

B. MAINTENANCE FEEDING

This refers to the feeding period going from the end of the spawning season till the onset of the vitellogenesis (approximately from May to August). The rations to be given are calculated as follows:

Pellets: 0.5% biomass/day

Fresh feed: 1.0% biomass/day, fed two days a week (usual ingredients being a combination of cuttlefish, sardines, hake/whiting).

C. ENRICHED FEEDING

During vitellogenesis (approx. from September to March) a special diet is provided:

Pellets (enriched or with special formulation):	0.5% biomass three times per week
Fresh:	1.0% biomass six times per week

Gilthead seabream: usually is fed during spawning.
Seabass: usually is not fed during spawning.

Annex 4

BROODSTOCK SECTION: CLEANING ROUTINES

Disinfecting solutions:

Hypochlorite = 500 ppm active chlorine solution

Hydrochloric acid = 10% v/v solution

Personal hygiene

- Wash your hands before starting work and whenever necessary.
- Disinfect your boots before entering the sector and when leaving it (renew the disinfecting solution in the dipping tray once a week).
- Wear cotton gloves when handling fish.
- Wear plastic gloves, protective glasses and a protective apron when handling dangerous chemicals.
- Smoking is not allowed inside the hatchery

UV-light sterilizer:

- Clean quartz tubes twice a day or whenever the UV output falls below the pre-set minimum level by means of the pre-installed wipers. In case your tubes do not have wipers, the sterilizer has to be disconnected from the electricity and water circuits, has to be disassembled and the quartz tubes should be cleaned by hand.
- Open the by-pass twice a day (morning and evening) for about 10 seconds to eliminate stagnant water. Warning: do not open the by-pass in case of disease outbreak in the hatchery.
- Clean the probe window once a month or whenever it does not work properly. Disassemble and clean with ethanol.

Working tanks

- Bottom: remove sediments by means of a mud aspirator or a siphon. Act each day to assess feed acceprance of breeders, avoiding disturbing fish as much as possible. Disinfect the tool before cleaning the next tank. Operate the siphon gently to avoid resuspending sediments. Use a disinfected siphon per tank. Store siphons in hypochlorite solution and rinse well with freshwater or seawater just before use.
- Tank inner wall and meniscus: lower water level by 10 cm and clean with a paper tissue whenever greasy deposits accumulate. Never dip hands in the water.

Empty tanks

- At the end of their use, open the bottom valve and drain completely. Remove outlet filter and pipe, air hoses and airstones.
- When still wet; scrub inner walls, bottom and all its equipment with detergent and hot water.
- Rinse and disinfect overnight with hypochlorite solution.
- Rinse with freshwater or sterilised seawater and let it dry before refilling.

Egg collectors

- Check for eggs morning and evening.
- Once emptied the collector, drain it completely and rinse with freshwater.
- Clean carefully screen and inner walls with hypochlorite solution, rinse with freshwater and connect again to spawning tank.

Outlet filters

- Replace filters routinely with clean and disinfected filters once a day, in the evening before switching the lights off.
- Close the water inlet and remove the dirty filter with care, avoiding disturbing fish and resuspending the sediments. Wash it with hot water or fresh water at high pressure and soak in hypochlorite for 30 min. Rinse thoroughly with water and store to dry.

Air hoses and diffusers

- Once a week replace with clean and disinfected sets (plastic hose + tap + stone).
- Wash with hot water and detergent to remove the greasy film, then soak in 10% hydrochloric acid Rinse thoroughly with water before use, making sure that no disinfectant remains in the hoses and stones.

Water inlet flexible hose

- Once a week, replace it with a clean one. Treat as indicated for the air hoses.

Equipment (buckets, jugs, beakers, pipettes, etc.)

- During the day: rinse thoroughly with hot water before and after each use.
- At the end of the working day: dip in hypochlorite solution.
- At night: rinse with water and store to dry (this job is usually performed by the night watchman).

Containers for disinfectant solutions

- Renew the disinfecting solution and remove bottom sediments once a week.

Floor

- Wash twice a week first with strong water jet and then with hypochlorite. Do not rinse.

Thermometer/Salinometer/Oxymeter

- Measure water samples taken from each tank; never dip the probes in the tanks. After use wash thoroughly with fresh water.

WARNING

- Never exchange material and equipment with other sections of the hatchery to prevent any risk of spreading diseases among different sections.
- Do not assume that materials and equipment you are going to use are clean. Clean them by yourself each time you need them.
- Any acid or hypochlorite residue is a deadly poison for fish, so rinse tools and equipment thoroughly before using them.

Annex 5

BROODSTOCK SECTION: DAILY WORK PLAN IN THE SPAWNING TANKS

Hour	Feeding (1)	Water controls (2)	Systems controls (3)	Fish controls (4)	Miscellaneous
08.00		T, DO, S, pH	OS, R	E, M, B	Lights on, change outlet screens, check eggs
09.00					Collect eggs, remove and clean collectors
10.00	F			F, B	
11.00					Clean tank bottom
13.00		T, DO	R	E, B	
14.00					Clean UV sterilizer
15.00					Clean floor
16.00					Place eggs collectors
17.00					Change outlet screens
18.00		T, DO, S, pH	OS, R	E, M, B	Lights off

(1) – Feeding: only for gilthead seabream spawners, for details see Annex 3.3
(2) – Water controls: T = temperature, DO = dissolved oxygen, S = salinity,
(3) – System controls: OS = outlet screens, R = water renewal rate
(4) – Fish controls: E = eggs, M = mortality, B = behaviour, F = feeding, C = cannibalism, M = mortality

ANNEX 6

LIVE FEED PRODUCTION SECTION:

CLEANING ROUTINES (ALGAE, ROTIFERS AND BRINE SHRIMP)

Disinfecting solutions:

Hypochlorite = 500 ppm active chlorine solution
Hydrochloric acid = 10% v/v solution

Personal hygiene

- Disinfect your boots before entering the sector and when leaving it.
- Wash your hands before starting work and whenever necessary, in particular in the pure-strain culture room and after having handled rotifers.
- Disinfect hands with alcohol when working in the pure-strain culture room.
- Wear plastic gloves, protective glasses and a protective apron when handling dangerous chemicals.
- Smoking is not allowed inside the hatchery

Note: to prevent the risk of contaminating the algae cultures with rotifers, which is always present, all manipulations involving algae should be completed before doing those involving rotifers. Moreover, as a basic rule, all equipment used for handling algal cultures must be kept separate from that used for rotifers.

UV-light sterilize:

- Clean quartz tubes twice a day or whenever the UV output falls below the pre-set minimum level by means of the pre-installed wipers. In case your tubes do not have wipers, the sterilizer has to be disconnected from the electricity and water circuits, has to be disassembled and the quartz tubes should be cleaned by hand.
- Open the by-pass twice a day (morning and evening) for about 10 seconds to eliminate stagnant water.
- Clean the probe window once a month or whenever it does not work properly. Disassemble and clean with ethanol.

Fine filtration devices:

- Service routinely according to the producer instructions.
- Replace filtering elements whenever the flow rate drops below a pre-set safe value.
- Use only new or disinfected filtering elements.
- Make sure to have plenty of spare parts at hand.

Equipment (buckets, jugs, beakers, pipettes, etc.)

- Always use clean and disinfected equipment
- Keep tools dipped in a 500 ppm hypochlorite solution.
- When necessary, take them out, rinse well and use.
- Self-priming plastic pumps: rinse well with sterilized seawater after each use. At the end of the working day rinse with hypochlorite solution in closed circuit for 10 minutes. Keep its

hose in the disinfecting container.
- Keep plastic air tubing in a solution of 10% HCl.
- Jugs used to deliver oily nutrient solutions for rotifer/artemia feeding/enrichment culture should be washed with soap after use and should be stored in hypochlorite solution. Rinse with sterile water before use.
- Dispose PE bags after use

Glassware

- Use only thoroughly cleaned and sterilized glassware.
- After use, rinse all glassware with tap water, and then dip it in a 10% hydrochloric acid solution for one hour to allow an easier removal of the organic residues of old cultures.
- In presence of greasy or thick deposits, wash with detergent and brush. If there is a need to avoid any mineral deposit, the last rinsing should be made with distilled water.
- Wet cleaned glassware should be either hanged on suitable racks to dry, or should be immediately filled with filtered seawater, ready for the sterilization process. Once dry and if not to be used immediately, glassware is stored with the opening sealed with an aluminium foil, away from dust.
- Pipettes and glass tubing are placed into cylindrical plastic rinsers filled with a 10% hydrochloric acid solution for at least one hour to allow an easier removal of the organic residues of old cultures. Rinsing sequence is always tap water first and distilled water later on.

Thermometer/Salinometer/Oxymeter

- Measure only water samples taken from each vessel to be analyzed, never dip the probes directly in the vessels. After use wash thoroughly with fresh water.

Mass culture tanks and equipment

- At the end of their use, open the bottom valve and drain completely. Remove air and oxygen hoses and diffusers, remove submersible heaters and floccule traps.
- When still wet rinse tank and all equipment with freswater, then scrub inner walls, bottom and all equipment with detergent and hot water.
- Rinse with freswater all equipment and dip in a 500 ppm hypochlorite solution overnight.
- Rinse tank with freswater and treat again with hypochlorite solution.
- Rinse with freshwater and let it dry before refilling.

Containers for disinfectant solutions

- Renew the disinfecting solution and remove bottom residues once a week or whenever the active chlorine content falls below the pre-established value.

Tables and light shelves

- Before starting work, disinfect all surfaces with alcohol
- At the end of the day wash with detergent, rinse with tap water and dry

Light shelves for small vessels

- Clean and disinfect all surfaces with alcohol daily

Floor and tiled walls

- Wash every day first with water jet and then with hypochlorite solution. Do not rinse.

WARNING

- Do not assume that material and equipment you are going to use are clean. Clean them by yourself each time you need them.
- Do not expose hypochlorite solutions to UV rays or mix with HCl since highly toxic chlorine gas (Cl_2) will be produced
- Any acid or hypochlorite residue is a deadly poison for fish, so make sure to have rinsed thoroughly before use.
- Never exchange material and equipment with other sections of the hatchery to prevent risk of spreading diseases.

ANNEX 7

LIVE FEED PRODUCTION SECTION: PREPARATION OF THE MOST COMMONLY USED CHEMICALS

A - Determination of active chlorine content in commercial grade bleach (hypochlorite solution)

Principle:

Active chlorine will release free iodine from potassium iodide solution at pH 8 or less. The released iodine is titrated with a standard solution of sodium thiosulphate, using starch as the indicator.

Reagents:

- acetic acid (glacial, concentrated),
- potassium iodide (KI) crystals,
- sodium thiosulphate ($Na_2S_2O_3$ x 5 H_2O, i.e. 248.18 g/mol) 0.1 N standard solution:
  - dissolve 25 g of sodium thiosulphate in 1 l of freshly boiled distilled water;
  - improve its stability by adding 0.1 g of Na_2CO_3;
  - store in a clean glass bottle in the dark,
- starch indicator solution:
  - mix 5 g starch with a little cold water and grind in a mortar, pour into 1 l of boiling distilled water, stir and let settle overnight;
  - use the clear supernatant preserved with 1.25 g salicylic acid and store in a dark bottle.

Procedure:

- dissolve 0.5 to 1 g KI in 50 ml distilled water, add 5 ml acetic acid,
- add 1 ml from sample to be checked,
- titrate away from direct sunlight: add 0.1 N thiosulphate from a burette until the yellow colour of the liberated iodine is almost disappearing, add 1 ml starch solution and titrate until the blue colour disappears.

Calculation:

- 1 ml of 0.1 N thiosulphate equals to 3.54 mg active chlorine.

B - Preparation of a disinfecting 500 ppm hypochlorite solution from commercial grade bleach

- Check the content of free chlorine in the available bleach following the above-mentioned method.
- Say the content found is 6.5% free chlorine (typical range: 5 to 15% in commercial bleach).
- A free chlorine 500 ppm solution means 500 ml of free chlorine in 1 000 000 millilitres, or 0.5 ml in 1 litre.
- 0.5 ml of free chlorine are found in 7.7 ml of 6.5% bleach (0.5/0.065);
- to prepare say 50 l of a 500 ppm hypochlorite solution add 385 ml (7.7 x 50) of 6.5% active chlorine bleach to 50 l of water.

WARNING:

- This is a practical method, not a stoichiometric preparation.
- When using a disinfecting hypochlorite solution for a prolonged time, it becomes essential to check regularly its active chlorine content (see above).

C – Chemical methods to sterilize seawater (for algal, rotifer and artemia cultures, both strain and mass level)

- The standard disinfecting concentration for culture seawater is 10 ppm of free chlorine and the corresponding safe concentration of sodium thiosulphate to deactivate residual chlorine is 12 ppm.
- Say the content of free chlorine in the available bleach is 6.5%.
- A free chlorine 10 ppm solution means 10 ml of free chlorine in 1 000 000 millilitres, or 0.01 ml in 1 litre.
- 0.01 ml of free chlorine are found in 0.15 ml of 6.5% bleach (0.01/0.065);
- to disinfect each litre of culture water add 0.15 ml of 6.5% active chlorine bleach (final chlorine concentration: 0.01 ml/l),
- provide a gentle aeration for 30 minutes to mix well, then let stand overnight;
- add 12 mg of sodium thiosulphate to each litre of seawater (to have a 12 ppm solution, i.e. 12 mg in 1 000 000 mg) and aerate strongly for one hour,
- check for residual chlorine and use only if no blue stain develops.
- For practical purposes always prepare and keep at hand a relatively large stock of disinfected seawater, say 100 l, to be used to fill small volumes of culture.

D - Identification of active chlorine residue

The same process indicated in the section A can be repeated in a simplified way for a quick check of residual active chlorine in water. To one ml sample add 1 ml of KI solution, 1 ml of 0.1 N thiosulphate solution and 1 ml of starch indicator. If a blue colour develops it is a proof of presence of active chlorine residues.

E - Preparation of disinfecting 10% (v/v) hydrochloric acid solution

Reagents:

- commercial grade hydrochloric acid (HCl or muriatic acid)
- tap water

Procedure:

- add 100 ml of HCl to 900 ml of tap water

WARNING

- fill the vessel first with water to drive out the acid fumes
- pour the acid without splashing to avoid acid splashes
- wear protective gloves, glasses and cloths when handling concentrated acids
- avoid breathing acid fumes

F - PREPARATION OF LUGOL SOLUTION

Lugol is a fixative staining solution for easier counting of brine shrimp nauplii.

Reagents:

- solution A: dissolve 50 g potassium iodide (KI) and 25 g iodine (I_2) in 100 ml boiling water
- solution B: 25 g sodium acetate (NaOAc) dissolved in 250 ml water

Procedure:

- when solution A cools, mix the two solutions and store in a cool, dark place

Use:

- Add a few drops to each 1-ml sample of rotifers or brine shrimps

ANNEX 8

LIVE FEEDS PRODUCTION SECTION: UPSCALING PROTOCOL FOR MICROALGAE AND ROTIFERS

The following upscaling protocol is dimensioned for a daily production of 200 l of algal mature culture and rotifers, excluding mass production in large tanks. A 20% overcapacity is advisable in the first production season to make up for occasional crashes and for problems that may arise from a planning process not properly tested.

Symbols:

t = test tube (10-20 ml capacity)
E = small Erlenmeyer flask (0,5 l)
EE = large Erlenmeyer flask (2 l)
B = balloon (round flasks, 6 l)
S = small polyethylene bag (60-80 l)
SS = large polyethylene bag (450 l)
Tank = 1 000 l

General scaling-up protocol:

ALGAE

1 t → 3 t + 2 E
1 E → 1 E + 2 EE
1 EE → 2 EE + 2 B
1 B → 1 S
1 S → 5 S or 1 SS
1 SS → 5 SS

ROTIFERS

1 t → 3 t + 2 E
1 E → 1 E + 3 EE (or as an alternative 1 B)
1 EE → 2 EE + 3 B
1 B → 1 S rot. (or as an alternative 1 EE → 1 S rot.)
1 S → 1 SS rot.
2 SS → 1 tank

Production strategy - daily harvesting:

The aim is to produce the required amount of microalgae for the greenwater in larval tanks and to inoculate one new rotifer tank daily. To obtain that a certain amount of small and large bags of algae and rotifers must be inoculated daily to keep the production chain:

harvest	to inoculate	to obtain
2 rotifer SS	1 tank	1 new rotifer tank
2 rotifer S	2 algal SS	2 new rotifer SS
2 algal SS	for greenwater in larval tanks	
4 algal S	4 SS	4 new algal SS

Note: when the sector is running at full capacity, rotifer tanks can be inoculated from other top quality rotifer tanks, as well as algal large bags can be inoculated from other top quality algal large bags.

Production strategy - daily working program:

Six day a week (Monday to Saturday):

From	*obtain:*
3 EE alg.	6 EE alg.+ 6 B alg.
5 B alg.	5 S alg.
5 S alg. (or 1 SS)	5 SS alg. (2 for greenwater, 2 for rotifers and one spare)
1 EE rot. + 2 EE alg. + 2 B alg.	2 EE rot. + 2 B rot.
(or 1 EE rot.	1 S rot.)
2 B rot.	2 S rot.
2 S rot.	2 SS rot.
2 SS rot.	1 tank

ANNEX 9

LIVE FEED PRODUCTION SECTION: DAILY WORK PLAN AND CULTURE FILE FOR MICROALGAE PRODUCTION

Indicative daily work plan

Time	Systems controls (1)	Cultures controls (2)	Upscaling (3)	Harvesting (4)	Others
08.00	Gen. C. T. A. LUV			S	day plan
09.00	W	Gv, S	Gv	S, Gv	
10.00			Gv, S	Gv	
11.00			Gv, S		
12.00			S		
13.00					
14.00					
15.00	C				
16.00					next day plan
17.00	Gen				cleaning

(1) - Systems controls: Gen = general overview, W = seawater treatment, T = air conditioning, A = air/CO_2 distribution, C = consumables, LUV = lights and UV lamps
(2) - Cultures quality/quantity controls: Gv = glass vessels, S = bags
(3) - Upscaling: Gv = glass vessels , S = bags
(4) - Harvesting: Gv = glass vessels, S = bags

Microalgal culture file

Date	Age (days)	Volume (l)	Cells/ ml	Light (lux)	CO_2 (l/min)	Air (l/min)	pH	Temp. (°C)	Salinity (ppt)	Contaminants	Use	Notes

ANNEX 10

LIVE FEED PRODUCTION SECTION: DAILY WORK PLAN AND CULTURE FILE FOR ROTIFERS PRODUCTION

Indicative daily work plan

Time	System controls (1)	Cultures controls (2)	Upscaling (3)	Harvesting (4)	Feeding	Others
07.00		T		S T		Rotifer counting
08.00	Gen. T. A. SWA	T		T		day plan
09.00		Gv , S	Gv	S, Gv, T		
10.00			Gv , S, T	Gv	F	
11.00			Gv , S, T			
12.00			S, T			
13.00	T, A	Gv , S, T	T			
14.00	SWA					cleaning
15.00	C					
16.00					F	next day plan
17.00	Gen	Gv , S, T				Rotifer counting
22.00	SWA				F	
04.00					F	

(1) - System controls: Gen = general overview, T = air conditioning, A = air distribution, C = consumables, SWA = seawater analysis
(2) - Cultures controls: Gv = glass vessels, S= bags, T = tanks
(3) - Upscaling: Gv = glass vessels, S= bags, T = tanks
(4) - Harvesting: Gv = glass vessels, S= bags, T = tanks

Rotifer daily working schedule:

Enriched rotifers for fish larvae **:**
filter ____ litres of tank ____
filter ____ litres of tank ____

Inoculation from tanks:
filter ____ litres of tank ____ to inoculate tank ____
filter ____ litres of tank ____ to inoculate tank ____

Inoculation from large bags (SS):
filter ____ litres of SS ____ to inoculate tank ____
filter ____ litres of SS ____ to inoculate tank ____

Addition of algae**:**
add ____ litres of SS ____ to tank____
add ____ litres of SS ____ to tank____
Filling 20 ppt treated water to tanks::
add ____ litres of seawater to tank ____
add ____ litres of tap water to tank ____
add ____ litres of seawater to tank ____
add ____ litres of tap water to tank ____

Settling and purge of tanks
tank ____
tank ____

Discharge:
tank ____
tank ____

Rotifer flask and bag file:

Date	Age (days)	Volume (l)	Rotif./ ml	Eggs/ ml	Fertility (%)	Inoculum	Destination	Notes

Rotifer tanks - daily counting and feeding schedule:

Tank Nr.	Volume (l)	Origin	Age (days)	count1 (egg/Rot)	count2 (egg/Rot)	count3 (egg/Rot)	Density (R/ml)	Tot. Rot (million)	Egg-ratio (%)	Amount of food + type		Ciliates	Activity	Remarks

_____ litres from tank _____ to tank

_____ litres from tank _____ to tank

_____ litres from tank _____ to tank

Rotifer tank file:

Tank number: Nr. Cycle:

Originating tank:

Density at harvest originating tank:

Egg-ratio at harvest originating tank:

Date	Age (dd)	Density (R/ml)	Egg (%)	Tot. Rot. (million)	DO (ppm) am	T (°C) am	DO (ppm) pm	T (°C) pm	NH4+ (ppm)	pH	Remarks
	0										
	1										
	2										
	3										
	4										
Rotifer production in the tank:											

ANNEX 11

LIVE FEED PRODUCTION SECTION: DAILY WORK PLAN AND CULTURE FILE FOR ARTEMIA PRODUCTION

Indicative daily work plan

Time	Cultures controls (1)	System controls (2)	Incubation	Enrichment (3)	Harvesting (4)	feeding & cold storage (5)	Others
06.30	N, EM	T, DO, pH					FM counting
07.00					EM N2		N counting
07.30				N2 1st dose	N1	EM	
08.00			N2			N1	
08.00-09.30							Cleaning
11.30			N1				
14.00-16.00		T, DO, pH					tank preparation
18.00							next day plan
19.30				N2 2nd dose			
22.00		T, DO, pH					

(1) - Culture controls: N = nauplii, EM = enriched metanauplii
(2) - System controls: T = Temperature. DO = Dissolved Oxygen
(3) - Incubation: N1 = nauplii for fish larvae, N2 = nauplii for enrichment
(4) - Harvesting: N1 = nauplii for fish larvae, N2 = nauplii for enrichment, EM = enriched metanauplii for fish larvae
(5) - Feeding and cold storage: either direct feeding to larvae or store in freezer between 5 and 10°C for later feeding

Time	Cultures controls (1)	Incubation	Enrichment (2)	Harvesting (3)	Others
06.30	N, EM	S		N1	N counting
07.00		B		EM	EM counting
08.00			N2	N2	
11.00					
12.00					
13.00					next day plan
14.00					Cleaning

(1) - Culture controls: N = nauplii, EM = enriched metanauplii
(2) - Incubation: S = small vessels, B = bags
(3) - Harvesting: N1 = nauplii for fish larvae, N2 = nauplii for enrichment, EM = enriched metanauplii for fish

Artemia incubation file

INCUBATION (cysts)					
date	time	tank	litres	cysts (g)	Cyst type

Artemia hatching file

HATCHING (nauplii)					
date	time	tank	No./ml (1)	million	HE

(1) to count use a triplicate sample
HE = hatching efficiency

Artemia enrichment file

	ENRICHMENT				HARVEST		
tank	SELCO 1st Time	dose	SELCO 2nd Time	dose	No./ml (1)	million	Survival (%)

(1) to count use a triplicate sample

ANNEX 12

FISH EGGS WEIGHING, DISINFECTING AND COUNTING OPERATIONS

The following equipment is needed to weigh and disinfect eggs:

- a couple of plastic filters with a 500 μm net (a 12-cm high cylinder cut from a 20-cm diameter PVC pipe with plankton nylon net glued to one end) and with its weight (tare) marked on the external side of the filter;
- a few 10 to 15 l plastic buckets or large beakers with handle;
- a couple of plastic jars, of a capacity of one liter each;
- aeration devices for buckets (fine air diffusers, plastic hoses and air taps)
- oxygen supply emergency set (oxygen bottle, manometer, fine diffusers, plastic hoses and air taps);
- glassware to sample mix and distribute the disinfectants (pipettes, spoons, beakers, glass rods, disposable plastic gloves);
- a balance with automatic tare clearance;

Working protocol to be followed when weighing and disinfecting eggs:

1. prepare all the equipment, which should be sterilized and carefully rinsed. Prepare pencil and paper;
2. prepare the containers with sterilized seawater at the same temperature and salinity of the spawning tanks; provide a gentle aeration; prepare one container adding the selected disinfectant (see table below for dosage and use);
3. dip the filter in the egg temporary stocking container and gently scoop out at the most 150-200 g of eggs floating at the water surface;
4. take the filter with eggs out of the water, drain quickly most of the water in excess on a paper filter and weigh the filter with the eggs; record the weight on a form (if the balance has not been adjusted for the filter tare, the filter weight has to be subtracted) ;
5. quickly place the filter in a bucket containing well aerated seawater with the selected disinfectant (see table below); wait for the proper disinfection time;
6. transfer the eggs to the incubation tank and open the seawater circuit.

Note - Do not start operations if something is still not ready as time is essential in egg handling to reduce stress and risks.

Active substance	Dosage	Time	Use
Penicillin	80 I.U./ml	1 min	500 mg / 10 l of sea water for 100-200 g of eggs at a time
Streptomycin- SO_4	50 μg/ml	1 min	500 mg / 10 l of sea water for 100-200 g of eggs at a time
Active Iodine	50 ppm/litre	10 min	8 litres for: $1x10^6$ seabass eggs or $1.5x10^6$ gilthead seabream eggs

Working protocol to follow when counting eggs:

1. close the water inlet in the tank and adjust aeration to assure a uniform distribution of eggs;
2. take several samples of water and eggs by means of a volumetric pipette (tip off) or a beaker, sample size between 10 and 100 ml, according to egg density, the bigger the sample, the lesser should be the egg number. A minimum of five samples are required;
3. count the eggs in each sample, obtain the average number per unit of volume, say one liter, then multiply this number by the total water volume to obtain the total amount of eggs in the tank.

Note - Only officially appointed personnel should handle all chemicals; product choice and dosage must strictly follow national regulations

Alternative counting system (starting at step 6, see above)

1. Fill a few 30-l cylindro-conical tubs with sterilised seawater and provide with gentle aeration.
2. Using the 500 µm filter (see above for description) transfer eggs into the counting tubs and adjust water volume to a known value.
3. Take at least 5 samples per tub using a 1-ml pipette; count the eggs.
4. After disinfection, transfer to the incubation containers in clean buckets.

WARNING

- Avoid overloading the filter when weighing and do not allow eggs to dry or to stick together in a thick layer; the whole weighing process should last a few seconds only.
- Avoid any unnecessary manipulation.
- Avoid any mechanical shock to the eggs.

Stage age in days		Incubation -2/0	Hatching 0	Yolk sac 1/6	Post-larvae 7/15	 16/25	 26/50	Fry 50/60	 60/70	 70/100
Gilthead seabream										
Water temperature	°C	Spawning	Spawning	>Spawning	>Spawning	18°C	20°C	20°C	20°C	20°C
Salinity	ppt	Spawning	Spawning	35	35	35	35	30	30	30
Photoperiod	h	-	16-8	16-8	16-8	16-8	16-8	14-10	14-10	14-10
Dissolved oxygen	%sat	100	100	80-100	80-100	80-100	80-100	80-100	80-100	80-100
Tot. Ammonia-N	ppm	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<1	<0.5
Light intensity	lux	1000	3000	3000	3000	3000	1000	1000	1000	natural
Water renewal	N°/day	12	12	8	8	8	12	18	18	18
Tank volume	l	5-10000	250	6-10000	6-10000	6-10000	6-10000	10-20000	10-20000	10-20000
Screen mesh	µm	400	400	125	125	250-500	250-500	1000	2000	3000
Seabass										
Water temperature	°C	Spawning	Spawning	>Spawning	>Spawning	18°C	20°C	20°C	20°C	20°C
Salinity	ppt	Spawning	Spawning	35	35	35	35	30	30	30
Photoperiod	h	-	16-8	16-8	16-8	16-8	16-8	14-10	14-10	14-10
Dissolved oxygen	%sat	100	100	80-100	80-100	80-100	80-100	80-100	80-100	80-100
Tot. Ammonia-N	ppm	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<1	<0.5
Light intensity	lux	50	100	100	500	500	500	natural	natural	natural
Water renewal	N°/day	12	12	8	8	8	12	18	18	24
Tank volume	l	5-10000	250-500	6-10000	6-10000	6-10000	6-10000	10-20000	10-20000	10-20000
Screen mesh	µm	400	400	125	125	250-500	250-500	1000	2000	3000

ANNEX 14

LARVAL REARING SECTION: SAMPLING EGGS OR LARVAE

1. Prepare on a tray four sterilized Petri dishes and one 100 ml beaker, note-pad and pencil.
2. Take four samples of 100 ml water from any point of the larval tank at least 10 cm either from the centre or from the walls.
3. Transfer larvae of each sample to a Petri dish; record normal/abnormal larvae on a specific form.
4. Empty the Petri dishes and repeat steps (2) and (3) at least five times, in order to sample at least two liters of tank water.
5. Calculate average and multiply by 10 to obtain average density of eggs/larvae per litre.

Alternative method.

1. Prepare one 1-l glass beaker (clean and sterilized), one 100 ml glass beaker and three Petri dishes.
2. Take a 1-l sample; stir it carefully before quickly taking one 100-ml sub-sample; repeat such sub-sampling procedure twice.
3. Transfer the three sub-samples to Petri dishes and allow time to settle.
4. Count larvae or eggs.
5. Repeat (2), (3) and (4) at least five times, sampling in different tank points.
6. Obtain average-density as above.

NOTE

When sampling newly hatched larvae:

- Prior to sampling adjust aeration so as to evenly distribute the larvae in the whole tank water volume;
- Examine larvae visually in the Petri dishes when the samples have settled, over a black surface;
- To check more carefully for abnormalities prepare a sample on a slide, avoiding as much as possible physical shocks (such as filtering or repeated pipetting) in order to preserve body integrity, then observe under the microscope;
- A more practical way consists in preparing a slide directly from each 100 ml sub-sample. Examine at least 20 larvae (an optimal number would be 50) and in any case consider the whole sample to avoid any form of selection.

ANNEX 15

RECORD-KEEPING FORM: EGGS AND LARVAE QUALITY CONTROL

Year _____ Date _____ Hour _____ Spawning _____
Tank _____ Age _____ Species _____

Sample/ Sub-sample		No. Dead eggs	Larvae				Notes
			Normal	Abnormal	Dead	Total	
1	a						
	b						
	c						
2	a						
	b						
	c						
3	a						
	b						
	c						
4	a						
	b						
	c						
5	a						
	b						
	c						
6	a						
	b						
	c						
7	a						
	b						
	c						
8	a						
	b						
	c						
9	a						
	b						
	c						
10	a						
	b						
	c						
TOTAL							

Eggs Total weight (g) ..... N..... Tank volume .....

Larvae N/l ..... Total N..... Sample vol.....Sub-sample vol.....

Total % hatching ..... Total % normal .....

Temperature (°C) ..... Salinity (ppt) .....

ANNEX 16

LARVAL REARING SECTION: CLEANING ROUTINES

UV-light sterilizer:

- Clean quartz tubes twice a day, or whenever the UV output falls below the pre-set minimum level, using the pre-installed wipers. In case your UV sterilizer does not have wipers, the sterilizer has to be disconnected from the electricity and water circuits, has to be disassembled and the quartz tubes must be thoroughly cleaned by hand.
- Open the by-pass valve twice a day (in the morning and evening) for about 10 seconds in order to eliminate stagnant water. Warning: in case of disease outbreak in the hatchery do not open the by-pass.
- Clean the probe window once a month or whenever it does not work properly; disassemble and clean with ethanol.

Biofilter:

- Walls: clean with a paper tissue whenever dirt accumulates. Do not dip hands in the water.
- Pump sump bottom: remove dirt by siphoning. Operate the siphon gently to avoid resuspending settled debris. Always use a clean and disinfected siphon. Soak the siphon in 500 ppm hypochlorite solution and rinse well with fresh or seawater just before use.
- Clean the artemia trap at the inlet of the biofilter every morning, wash thoroughly and disinfect by soaking for 30 minutes in a 500 ppm hypochlorite solution before putting it back in place. Get rid of the trapped artemia.
- Common outlet gutter: clean twice per month with a brush, by-passing the mechanical filter. Clean and disinfect with 500 ppm hypochlorite at the end of each cycle.

Larval tanks in operation

- Conical bottom: remove dirt by siphoning at least half of the bottom surface per day. Operate the siphon gently to avoid resuspending settled debris. Use a disinfected siphon per tank. Do it before algae are added to the tank. Store the siphons in a 500 ppm hypochlorite solution and rinse well with fresh or seawater just before use.
- Tank inner wall and meniscus: clean with a paper tissue whenever dirt accumulates. Do not dip hands in the water.
- Bottom drain (for conical tanks only): open the bottom valve and flush out the water accumulating at the tip (approximately 5 to 10 l) twice a day in the mornings and evenings. Remember to remove any air-stone before opening the valve. In the first rearing days check the water purge for trapped larvae, later on for dead post-larvae and fry which may indicate the onset of some health problem.

Empty larval tanks

- At the end of each larval rearing cycle, open the bottom valve and drain completely. Remove the outlet filter and pipe, the air hoses and air-stones, the bottom cork and the valve.
- When still wet, scrub the inner wall, bottom and all its associated equipment with detergent and hot water.
- Rinse and disinfect overnight with a 500 ppm hypochlorite solution.
- Rinse with freshwater and let it dry in the air before refilling.

Skimmers

- Remove the floating debris and the oily film trapped by the skimmers with a paper tissue at regular intervals, approximately every four hours, and more frequently after rotifer and artemia are fed, if necessary. Special attention is required in particular during the first 10 days of larval rearing when the swim bladder gets activated.

Outlet filters

- Replace filters as a matter of routine with clean and disinfected ones twice a day, in the morning and evening, before switching the lights off. Replace more frequently during the day if there is risk of filter clogging.
- Stop water renewal and remove the dirty filter with care, avoiding to disturb the young fish and to resuspend the dirt attached to the filter. Wash it with hot water or with a high pressure fresh water jet and soak in the hypochlorite solution for 30 minutes. Rinse the filter thoroughly with water and store close to the tank (the handrail of the walkway around the tanks is a suitable place, given that they do not hamper the working activities).
- When replacing filters, be sure that no fish remain trapped inside the new filter.

Air hoses and diffusers

- Once a week replace with a clean set (plastic hose + tap + air-stone) in each tank.
- Wash with hot water and detergent to remove the greasy film, then soak in 10% hydrochloric acid. Rinse thoroughly with water before use, making sure that no acid solution remains inside the hose and stone.

Water inlet flexible hose

- Replace once a week with a clean one. Treat as indicated above for air hoses.

Equipment (buckets, jugs, beakers, pipettes, etc.)

- During the day: rinse thoroughly with hot water before and after each use.
- At the end of the working day: dip in 500 ppm hypochlorite solution.
- At night: rinse with water and store to dry (this job is usually performed by the night watchman).

Containers for disinfecting solution

- Renew the disinfecting solution and remove bottom deposits once a week.

Live feed stocking containers for daily use

- After the last meal, scrub inner wall, bottom, cap, air hoses and air-stones with detergent and hot water.
- Disinfect with 500 ppm hypochlorite solution.
- Rinse with freshwater and let them dry in the air, in order to have them ready for live feed distribution the next day.

Floor

- Wash twice a week, first with water jet and then with 500 ppm hypochlorite solution. Do not rinse,

WARNING

- Do not assume that material and equipment you are going to use are clean. Do clean them by yourself each time you need them.
- Any acid or hypochlorite residue is a deadly poison for larvae, so rinse equipment thoroughly before use.
- Do not exchange material and equipment with other sections of the hatchery, to avoid the risk of spreading diseases.
- Personal hygiene:

  - keep your hands clean;
  - dip your feet in the disinfectant basin before entering any hatchery section;
  - do not smoke inside the hatchery.

HATCHERY FEEDING REGIME FOR GILTHEAD SEABREAM

LARVAE			*Algae*	*Rotifers*	*ARTEMIA* grade		Artificial feed (particles size in micron)							REMARKS
Age (days)	Weight live (g)	Light h/d			AF	EG or RH +Selco	Larval feed 80-200	Larval feed 150-300	Larval feed 300-500	Art. feed 300-500	Art. Feed 500-800	Art. feed 800-1200	Art. Feed 1200-2000	
00-02		16												Surface skimmers
03-07		16	40	20										Swimbladder formation
08-12		16	40	24										100 % swimbladder
13-16		16	40	28										
17-19		16	20	32	0.1-0.5		1-3							
20-23		16	10	20	0.5-1.0	0.3-0.6	3-10							STARTING EG/RH Artemia with SELCO enrichment
24-27	0.003	16	10	10	1-1.5	1.5-3.0	10-15							According to the date in which mouth opens in larvae, Selco enriched Artemia can be used earlier
28-33		16				10	15-20							
34-39	0.01	16				12	20	10						
40-43		16				16		20						
44-47	0.04	16				14		20	10					The feeding regime should be adapted according to growth performance of larvae at higher temperatures (20-22°C)
48-52		16				12		20	20					
53-57		16				8		30	30					
58-59		16				4		0	70					
60-65	0.1	16							90					
66-75		16							70-50	30-60				
76-81		16							0	95-125	20-40			
82-86	0.5	16								20-40	145-160			
87-97		16								0	200-500			
98-103	1	16									300			
104-110											5-10 %			
111-120	1.5										3-5 %	2 %		
121-130												5 %		
131-150	2											3 %	2 %	
151-170													5 %	

Indicative feeding regime for gilthead seabream reared from hatching to juveniles using artemia and commercial inert diets.

Quantities should be adapted to local conditions such as rearing system, temperature, fish density, etc.

Initial density is 150-200 larvae per litre for larval rearing and 10-20 fry per litre during weaning; assuming a temperature of 18°C and salinity at 35-37 ppt.

Artemia is indicated in millions/m^3 of rearing volume/day; algae in l/ m^3 of rearing volume/day; rotifers in millions/ m^3 of rearing volume/day; compounded diets in g/m^3 rearing volume/day or otherwise in percent of fish live weight/day

HATCHERY FEEDING REGIME FOR SEABASS

LARVAE			*Algae*	*Rotifers*	*ARTEMIA* grade		Artificial feed (particles size in micron)							REMARKS
Age (days)	Weight (gram)	Light H/d			AF/BE	EG or RH +Selco	Larval feed 80-200	Larval feed 150-300	Larval feed 300-500	Art. feed 300-500	Art. feed 500-800	Art. feed 800-1200	Art. feed 1200-2000	
00-02		16												Surface skimmers
03-07		16	40	20	2									Swimbladder formation
08-12		16	40	25	3									100 % swimbladder
13-16		16	40	15	4									
17-19		16	20	10	6	2	1-3							STARTING EG/RH Artemia with SELCO enrichment
20-23		16	10	5		14	3-10							According to mouth opening of larvae, SELCO enriched Artemia can be fed earlier
24-27	0.005	16				16	10-15							
28-33		16				20	10-15	10						
34-39		16				20		20						
40-43	0.02	16				16		30						
44-47		16				14		40	10					The feeding regime should be adapted according to growth performance of larvae at higher temperatures (20-22°C)
48-52	0.07	16				12		45	20					
53-57		16				8		45	30					
58-59		16				8		30	70					
60-65		16							90					
66-75	0.3	16							70-50	40-60	10			
76-81	0.5	16							0	95-125	20-40			
82-86		16								20-40	145-160			
87-97	0.8	16								0	5-10 %			
98-103	1	16									2-3 %	2-3 %		
104-110											1%	4 %		
111-120	1.5											2-3 %	2-3 %	
121-130													5 %	
131-150	2												3-5 %	
151-170													3-5 %	

Tentative feeding regime for seabass reared from hatchling to juveniles using Artemia and dry commercial diets.

Quantities should be adapted to local conditions such as rearing system, temperature, fish density, etc.

Initial density assumed is 150-200 larvae per litre and 10-20 fry per litre during weaning; temperature 18°C and salinity 35-37 ppt.

Artemia figures refer to millions/m^3 of rearing volume/day; algae to l/ m^3 of rearing volume/day; rotifers to millions/ m^3 of rearing volume/day; compounded diets in g/m^3 rearing volume/day or otherwise in percent of fish live weight/day

ANNEX 19

LARVAL QUALITY CONTROL KEEPING FORM

TANK _____ SPECIES_____

DATE_____ AGE _____ HOUR _____

	Quality controls			Ingested live food				Remarks
Larva	Skeletal deformities	Swimbladder	Calculi	Rotifers	Artemia		Algae	
No.		Yes No		mastax	nauplii	Eggs		
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								
29								
30								
TOTAL								

Tank ___	Species _____	Egg quantity: ___ g	coming from tank ____
Incubation date _____	Hatching date _____	Hatching rate ____%	Initial larval number ____
Date			
Age			
Degrees-day			
WATER QUALITY			
T°C			
Salinity (ppt)			
DO (ppm)			
pH			
$NH4^{+}$ (ppm)			
FEEDING			
Algae (liters)			
Rotifers (million)			
rotifer/ml			
Nauplii (million)			
nauplii/ml			
Metanauplii (million)			
m.naupli/ml			
Dry feed (g)			
LARVAL QUALITY			
Repletion (%)			
Swimbladder (%)			
Mortality			
TANK MANAGEMENT			
Water renewal rate (%)			
Bottom cleaning			
Treatments			
Outlet filter			
NOTES			

Date	tank	1	2	3	4	5	6	7	8	9	10
	time										
Algae (litres)	11										
Rotifers (mill.)	8										
	14										
	20										
	total										
Nauplii (mill.)	8										
	12										
	16										
	20										
	total										
Metanauplii (mill.)	8										
	12										
	16										
	20										
	total										
dry feed 80-200 (g)											
dry feed 150-300 (g)											
moist food (g)											

ANNEX 22

DAILY QUANTITIES OF LIVE FOOD TO BE DISTRIBUTED DURING THE WEEK

WEEK from _____ to _____ Tank No. _____

Day							
	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM
Algae (litres)							
Rotifers (mill.)							
A. nauplii (mill.)							
A..m.naup. (mill.)							

WEEK from _____ to _____

Day							
	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM
Algae (litres)							
Rotifers (mill.)							
A. nauplii (mill.)							
A..m.naup. (mill.)							

WEEK from _____ to _____

Day							
	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM	AM PM
Algae (litres)							
Rotifers (mill.)							
A. nauplii (mill.)							
A..m.naup. (mill.)							

WEEK from _____ to _____

Day													
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Algae (litres)													
Rotifers (mill.)													
A. nauplii (mill.)													
A..m.naup. (mill.)													

WEEK from _____ to _____

Day													
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Algae (litres)													
Rotifers (mill.)													
A. nauplii (mill.)													
A..m.naup. (mill.)													

ANNEX 23

LARVAL REARING SECTION. DAILY WORK PLAN

Mark the appropriate cell when the action requested is completed

Time	07	08		09		10		11	12	13	14	15	16	17	18	19	20	21	22
Algae distribution								☐											
Rotifers distribution		☐									☐						☐		
Artemia nauplii distr.		☐						☐			☐						☐		
Artemia metanauplii dist.		☐						☐			☐						☐		
Artificial feed distr.	☐	☐		☐		☐		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Rotifers check					☐								☐						
Artemia check					☐								☐			☐			
Bottom cleaning	☐	☐	☐	☐	☐	☐													
Purge bottom		☐													☐				
Skimmers cleaning				☐		☐		☐	☐	☐	☐	☐	☐	☐	☐	☐		☐	
Outlet screens change	☐								☐										☐
UV wipers						☐						☐							
UV by pass						☐						☐							
T°C			☐																
S ppt			☐																
PH			☐																
DO ppm			☐			☐						☐				☐			
N-NH_4 ppm												☐		☐					

Controls in brief:

- Outlet screen: check if: of proper mesh size, not clogged, no larvae adhering on it, well screwed on its support.Central aeration: check if: working properly, not disturbing larvae, air stone 20 cm from the bottom.
- Skimmer: check if: working properly, all air holes open, no air flow so strong as to disturb larvae, effectively collecting surface dirt, close to the tank wall, central aeration not inside the skimmer, clean inner surface; clean the skimmer, note if floating dead larvae are present.
- Water surface: check if: no rotifer layer, no grease, no bacterial film.
- Water renewal: check if: hose always above the water surface when a stable water lever has been reached, flow neither disturbing larvae, nor pushing them against the filter surface.

- Larvae: check if: normal active behaviour, no larva is passively turning upside down; predatory behaviour, schooling, normal swimming against current, menisc, erratic swimming, density.
- Bottom: look for dead larvae, dirt, fungi, red bacteria spots, position of the rubber stopper, and any fallen object inside the tank.
- Light: correct position of the lamp, no shaded area in the tank.
- Water level: after stopping water exchange , it must remain constant during the day.

Annex 24

PREPARING MOIST FEED

Composition:

- 75% frozen cod (or hake) fillet
- 25% frozen squid (or cuttlefish)
- Fat-soluble poly-vitamin complex 10 g/kg moist feed
- Water-soluble poly-vitamin complex 10 g/kg moist feed
- Poly-aminoacid complex 5 g/kg moist feed
- Microencapsulated vitamin C: 2 g/kg moist feed
- Stabilizer: soya lecithin
- Antioxidant: BHT

Equipment: (if ingredients are purchased fresh)

- Grinder, capacity: 10 kg/hour
- Cutter, stainless steel, blending 10 l- container, double blade

WARNING

Fish and molluscs must be properly cleaned and must be frozen before use in order to eliminate possible parasites, if they are purchased fresh. Fish must be filleted and squid or cuttlefish must be cleaned by removing mouth, eyes, gut and bone. Wash thoroughly, then grind and store in 1 kg PE bags, which should be deep frozen. Proper deep freeze has to be quick and complete. Use flat bags and stockpile them in the freezer in such a way so that cold air could circulate between them.

Procedure

1. Fish and squid should be processed when still frozen.
2. Grind the required quantities of cod fillet and squid, adding sterile seawater if necessary to obtain a soft homogeneous cream. Check its buoyancy: if necessary add squid to increase it.
3. Place the paste in the cutter.
4. Weigh the required amount of integrators in a plastic beaker, mix well with a kitchen blender and add to the paste.
5. Mix thoroughly at high speed.
6. Put the moist paste in a disinfected bucket and distribute over the rearing tank with a disinfected spoon of known capacity. Note on the tank file the number of quantity given
7. Use one disinfected bucket and spoon at a time.
8. Clean all equipment with hot water and detergent, and then soak in 500 ppm hypochlorite solution.

Notes:

- As a practical rule, bulk moist feed should be freshly prepared every morning and stored in the freezer.
- Vitamins, aminoacids and other additives have to be added just before distribution, as they do not last long after processing.
- To prepare a medicated food drugs can be added to moist feed.
- This moist feed contains about 20% dry matter.

MOIST FEED INTEGRATORS

1.	Fat-soluble poly-vitamin complex (per kg)			
	Vitamin A protected	650 000	I.U.	Retinyl acetate
	Vitamin D3 protected	100 000	I.U.	Cholecalciferol
	Vitamin E protected	30 000	I.U.	DL-áTocopherol acetate
	Matrix:			cod liver oil up to 1000 g.
	Antioxidant:			BHT
2.	Water-soluble poly-vitamin complex (per kg)			
	Vitamin B1	3.5	g	Thyamine
	Vitamin B2	2.5	g	Riboflavin
	Vitamin B6	3.0	g	Pyridoxine
	Vitamin B12	0.005	g	Cyanocobalamin
	Vitamin PP	22.0	g	Nicotinic acid
	Vitamin K	0.600	g	Menadione Na bisulfate
	Pantothenic Acid	9.0	g	
	Pholic Acid	0.750	g	
	Biotin	0.060	g	
	Vitamin C protected	100.00	g	Ascorbic acid
	Choline	100.00	g	
	Myo-Inositol	25.00	g	
	Matrix:			lactose to 1 000 g
	Antioxidant:			Ethoxyquin
3.	Poly-aminoacid complex (per kg)			
	Tryptophan	2.5	g	
	Cystine	2.6	g	
	Leucine	14.5	g	
	Isoleucine	7.5	g	
	Arginine	13.7	g	
	Valine	10.0	g	
	Threonine	7.4	g	
	Phenylalanine	10.7	g	

ANNEX 25

WEANING SECTION: CLEANING ROUTINES

UV-light sterilizer:

- Clean quartz tubes twice a day or whenever the UV output falls below the pre-set minimum level by means of the pre-installed wipers. In case your tubes do not have wipers, the sterilizer has to be disconnected from the electricity and water circuits, has to be disassembled and the quartz tubes should be cleaned by hand. Open the by-pass twice a day (morning and evening) for about 10 seconds to eliminate stagnant water. **Warning: do not open the by-pass in case of disease outbreak in the hatchery.**
- Clean the probe window once a month or whenever it does not work properly. Disassemble and clean with ethanol.

Biofilter:

- Walls: clean with a paper tissue whenever dirt accumulates. Do not dip hands in the water
- Pump sump bottom: remove dirt by siphoning. Always use a clean and disinfected siphon. Soak the siphon in 500 ppm hypochlorite solution and rinse well with fresh or seawater just before use.
- Check the backwash of the mechanical filter at the inlet every morning and evening. Once a week wash the filter components with detergent and hot water, then disinfect by soaking for 30' in 500 ppm hypochlorite solution before replacing.
- Common outlet gutter: clean twice per month with a brush, by-passing the mechanical filter. Clean and disinfect with 500 ppm hypochlorite at the end of each cycle.

Tanks in operation

- Bottom: remove the bulk of sediment by purging twice a day through the bottom valve (open it few seconds until the outcoming water is clear). Remove the remaining dirt by siphoning the whole surface every day. Use a vacuum cleaner to suck settled debris (mud aspirator). Disinfect the siphon before moving to another tank. Store siphons in 500 ppm hypochlorite solution and rinse well with fresh or seawater just before use.
- Tank inner wall and water meniscus: clean with a paper tissue whenever dirt accumulates. To help operation lower the water level by 10 cm. Do not dip hands in the water

Empty tanks

- When no longer in use, open the bottom valve and drain completely. Remove the outlet filter and pipe, air hoses and air-stones, and valve.
- When still wet, scrub inner walls, bottom and all its equipment with detergent and hot water.
- Rinse and disinfect with a 500 ppm hypochlorite solution.
- The following morning rinse with freshwater and let it dry in the air before refilling.

Skimmers

- Remove the floating debris and the oily film trapped by the skimmers with a paper tissue whenever debris accumulate in the skimmer. Use skimmers to clean water surface especially when feeding moist food, that can pollute water.

Outlet filters

- Replace filters routinely with clean ones and disinfected filters twice a day, morning and evening before turning the lights off. Replace more frequently during the day if there is risk of clogging.
- Stop water flow and remove the dirty filter with care, trying to avoid resuspending the dirt. Wash it with hot water or fresh water at high pressure and soak in the hypochlorite solution for 30 min. Rinse thoroughly with water and store close to the tank (the handrail of the walkway around the tanks is a suitable place, if filters do not hamper work).
- When replacing filters, be sure that no fish remains trapped inside the new filter.

Air hoses and diffusers

- Once a week replace with a clean set (plastic hose + tap + air-stone) in each tank.
- Wash with hot water and detergent to remove the greasy film, then soak in 10% hydrochloric acid. Rinse thoroughly with water before use, making sure that no acid solution remains inside the hose and stone

Automatic feeders

- Remove dust and feed not distributed on a daily basis.
- Clean the container and distribution mechanism once a week, with commercial ethanol and paper tissue.

Artemia dropping buckets

- Clean every day, when artemia distribution is completed, with detergent and hot water, then soak in the hypochlorite solution for 30 minutes. Verify that delivering hose and tap are not clogged.

Equipment (buckets, jugs, beakers, pipettes, etc.)

- During the day: rinse thoroughly with hot water before and after each use.
- At the end of the working day: dip in 500 ppm hypochlorite solution.
- At night: rinse with water and store to dry (this job is usually performed by the night watchman).

Containers for disinfectant solutions

- Renew the disinfecting solution and remove bottom dirt once a week.

Live feed daily stocking containers

- After the last distribution, scrub inner walls, bottom, cap, air hoses and air-stones with detergent and hot water.
- Disinfect with 500 ppm hypochlorite solution.
- Rinse with freshwater and let them dry in the air to be ready for the next day.

Floor

- Wash twice a week first with water jet and then with 500 ppm hypochlorite solution. Do not rinse,

WARNING

- Do not assume that material and equipment you are going to use are clean. Clean them personally each time you need them. .
- Any acid or hypochlorite residue is a deadly poison for larvae, so rinse thoroughly before use.
- Do not exchange material and equipment with other sections of the hatchery to avoid risk of spreading diseases.
- Personal hygiene: 1: keep your hands cleaned, 2. Dip your feet in the hypochlorite basin before entering any hatchery section, 3. Do not smoke inside the hatchery.

Tank ____	Species _____	Initial size: _____ mg	From tank : ____
		Initial number: _____	
Transferred to:			
Date			
Age			
Degrees-day			
WATER QUALITY			
T°C			
Salinity (ppt)			
DO (ppm)			
pH			
NH4+ (ppm)			
FEEDING			
Metanauplii (million)			
Metanauplii/ml			
Dry feed 80-200 (g)			
Dry feed 150-300 (g)			
Dry feed 300-500 (g)			
Dry feed 500-800 (g)			
Dry feed 800-1200 (g)			
Dry feed 1200-2000 (g)			
Moist feed (g)			
LARVAL QUALITY			
Mortality			
Average size (mg)			
Grader size (mm)			
Size dispersion (%)			
Deformity rate (%)			
Swim-bladder rate (%)			
TANK MANAGEMENT			
W. exchange rate (%)			
Bottom cleaning			
Treatments			
NOTES			

Annex 27

WEANING SECTION: DAILY WORK PLAN

Hour	Feeding (1)	Water controls (2)	System controls (3)	Fish controls (4)	Other
08.00	DF	T, S,	general	general	lights on, check live food change outlet screens bottom purge
08.30					start bottom cleaning
09.00	DF			F, C, M	clean skimmers
09.30					
10.00	DF		R		
10.30		T, DO, S, pH		F, C	
11.00	AM				
11.30					
12.00	DF		general		
12.30		DO, N		F, C, M	finish bottom cleaning
13.00					
13.30					
14.00	DF		R		
14.30		DO		F, C	
15.00	MF				
15.30			OS		
16.00	DF	T, DO, S, pH	general		
16.30				F, C, M	
17.00	MF				
17.30		DO	OS		clean skimmers
18.00	DF				clean UV sterilizer
18.30		DO,N	R	F, C	Bottom purge
19.00	MF				change outlet screens lights off
20.00	DF	DO	OS		
21.00	AM	DO		F, C, M	
22.00		DO	general	general	

(1) – **Feeding:** DF = dry feed, MF = moist food, AM = enriched artemia metanauplii
(2) – **Water controls**: T = temperature, DO = dissolved oxygen, S = salinity, N = ammonia
(3) – **System controls**: OS = outlet screens, R = renewal rate
(4) – **Fish controls**: F = feeding, C = cannibalism, M = mortality

Controls in brief:

- Outlet screen: check if: of proper mesh size, not clogged, no fry adhering on it, well screwed on its support.Aeration: check if: working properly, not disturbing fry, air stone 20 cm from the bottom.
- Skimmer: check if: working properly, all air holes open, no air flow so strong as to disturb fry, effectively collecting surface dirt, close to the tank wall, central aeration not inside the skimmer, clean inner surface; clean the skimmer.
- Water renewal: check if: hose always above the water surface when a stable water lever has been reached, flow neither disturbing fry, nor pushing them against the filter surface.
- Fry: check if: normal active behaviour, no fry is passively turning upside down; alimentary behaviour, cannibalism, schooling, normal swimming against current, erratic swimming, density.
- Bottom: look for dead fry, dirt, fungi, red bacteria spots and any fallen object inside the tank.

ANNEX 28

RECORD-KEEPING FORM: FISH TREATMENT

TREATMENT	DOSAGE

BIOMASS	MODALITY

DATE	TANK(S)	CONCENTRATION	HOUR

PROCEDURE

NOTES

Annex 29

ANAESTHETICS AND CHEMICALS FOR DISEASE PREVENTION AND TREATMENT

	LARVAE	FRY	ADULTS
ANAESTHESIA			
2-Phenoxyethanol	-	200-400 ppm	200 ppm
MS 222	-	20 ppm	20-50 ppm
Quinaldine	-	-	3-5 ppm (in acetone)
PROPHYLAXYS			
Nitrofurazone[2/]	-	-	30-50 ppm/6 h/2 days
Furazolidone	30 ppm/1 h/3 days		
THERAPY			
Streptomycine			
Ampicillin	5 ppm/6 h[1/]		
Nitrofurazone[2/]	5 ppm/6 h[1/]		
	-	-	30-50 ppm/3-6h/2days
Flumequine	-	12 mg/kg BW/10 days 2-50 ppm/1 h/2 days	12 mg/kg BW/10 days 2-50 ppm/1 h/2 days
Oxytetracycline	-	40-160mg/kgBW/7days 50 ppm/1 h/2 days	40-160mg/kgBW/7days 50ppm/1 h/2 days
Formalin 37%	-	100-150 ppm/3 h/2 days	100-150 ppm/3 h/2 days
$CuSO_4 . 5H_2O$	-	0.5 ppm/3 h/2-3 times	2 ppm/3 h/2-3 times
Neguvon	-	-	0.05-0.5 ppm/1-3 h
Malachite green	-	-	0.2 ppm/1 h/2 days

[1/] Starting from the sixth day
[2/] Mix first with a small quantity of formalin to help suspension in water

WARNING

All chemicals for prophylaxys and therapy should be only handled by officially appointed personnel; the choice of product and its dosage should strictly take into account national regulations.

ANNEX 30

MAIN DISEASES IN FARMED GILTHEAD SEABREAM AND SEABASS

GILTHEAD SEABREAM

PATHOGEN	CLINICAL PICTURE	THERAPY
Viral infections		
Iridoviridae	Limphocystis: white-grey nodules on skin and fins	
Bacterial infections		
Vibrio spp	Acute septicemia:	Nitrofuranics
Pseudomonas spp	Loss of appetite	Tetracyclines
Pasteurella spp	Skin darkening and ulceration	Quinolones
	Internal lesions: spleen and kidney liquefaction and tumefaction	
	Haemorrhages at heart surface	
	Spleen nodules	
Gram negative	Ulcerative and necrotic lesions:	Nitrofuranics
	Dermal and sub-dermal after handling	Tetracyclines
Flexibacter spp	Myxobacterial fin and gill rot	Nitrofuranics
		Tetracyclines
	Epitheliocystis, affecting gills	Tetracyclines
Parasitic diseases		
Protozoa		
Trichodina spp	Ectoparasite on gills and skin	Formalin
Cryptocaryon spp (Ciliata)	Ectoparasite on gills and skin	Formalin
Colponema spp (Flagellata)		Formalin
Amyloodinium ocellatum (Dinoflagellata)	On gills	$CuSO_4$
Cryptobia spp	On skin and gills	
Ichthyobodo spp (Zoomastigophora)	In blood	
Kudoa spp (Myxosporidia)	On skin and gills	
Haemogregarina cystozoites (Sporozoa, Coccidia)	Kidneys and viscera	
	Kidneys, liver, spleen and intestine	
Monogeneans		
Furnestia echeneis (Diplectanidae)	On gills	Formalin
Gyrodactylus spp (Gyrodactilydae)	On skin	Formalin
Microcotyle chrysophryii (Microcotylidae)	On gills	Formalin
Digenea Trematoda	Body severely twisted	
	Attacking liver, muscles and viscera	
Nutritional diseases	Systemic granuloma	Change diet
	Nephro and urolithiasis due to tyrosinosis	Vit. C addition to diet
	Chronic inflammation of viscera	
Lipoid liver degeneration	Liver	Change to fresh diet

SEABASS

PATHOGEN	CLINICAL PICTURE	THERAPY
Viral infections		
Picornaviridae	Lesions on brain and retina Skin darkening, ataxic swimming and quick twisting movements	
Bacterial infections		
Vibrio spp	Acute septicemia:	Nitrofuranics
Pseudomonas spp	Loss of appetite	Tetracyclines
Pasteurella spp	Skin darkening and ulceration Internal lesions: spleen and kidney Liquefaction and tumefaction Haemorrhage at heart surface Spleen nodules	Quinolones
Gram negative	Ulcerative dermal necrosis of the head Subcutaneous haemorrhages (head)	Nitrofuranics Tetracyclines
Flexibacter spp	Myxobacterial fin (caudal, dorsal) and gill rot Ulcerative and necrotic lesions	Nitrofuranics Tetracyclines
Mycobacterium spp	Exophthalmus monolateral Granulomatosus spleen and kidney	
	Epitheliocystis, affecting gills	Tetracyclines
Parasitic diseases		
Protozoa		
Trichodina spp	Ectoparasite on gills and skin	Formalin
Cryptocaryon spp	Ectoparasite on gills and skin	Formalin
Scyphidia spp (Ciliata)	On gills	
Colponema spp (Flagellata)	On gills	Formalin
Amyloodinium ocellatum (Dinoflagellata)	On skin and gills	$CuSO_4$
Cryptobia spp	In blood	
Ichthyobodo spp (Zoomastigophora)	On skin and gills	
Haemogregarina cystozoites (Sporozoidae, Coccidia)	Affects kidneys, liver, spleen and Intestine	
Sphaerospora spp	Affects intestine, head and bile	
Myxidium spp	On skin and gills	
Myxobolus spp (Myxosporida)	Affects bile and gills	
Eimeria spp. (Sporozoidae)	In intestine	
Monogenea Trematoda		
Diplectanum spp (Diplectanidae)	On gills	Formalin
Gyrodactylus spp (Gyrodactylidae)	On skin	Formalin
Microcotyle labracis (Microcotylidae)	On gills	Formalin
Serranicotyle labracis	On gills	
Crustacea		
Caligus spp	In oral cavity	Neguvon
Ergasilus spp	On gills	
Lernanthropus spp	On gills	
Colobomatus labracis	On opercula	
Anellida		
Trachelobdella lubrica	On gills and in oral cavity	
Lipoid liver degeneration	Affects liver	Change to fresh diet

WARNING

All drugs/chemicals for disease prevention or treatment must be handled only by officially appointed personnel; choice and dosage should strictly follow existing national regulations.

ANNEX 31

RECORD-KEEPING FORM: FRY ANAMNESIS AND IDENTIFICATION OF A BACTERIAL DISEASE

SPECIES_____ WEIGHT_____ AGE_____ FEED_____

STEP 1 behaviour and general body status

observe fish in water and then take it out water over a smooth surface. Tick appropriate box and record any observed detail

HEALTHY	LETHARGIC	ATYPICAL SWIM	THIN	DEAD

DEFORMITY MELANISM		DECOLORATION	PIGMENTATION

STEP 2 - external lesions

observe fish over a smooth surface. Tick appropriate box and record any observed detail

Gills	INTACT	FILLED UP	ERODED
	DECOLORATED	HYPERTROPHY	RED SPOTS

Eyes	ENUCLEATED	OPAQUE	EXOPHTHALMIC

Body	REDNESS	HAEMORRHAGES	SUPERFICIAL LESIONS
	DEEP LESIONS		

Skin (observation of a skin scraping at low and high magnification)	MYXOBACTERIA		

STEP 3 - internal lesions

Proceed with a complete autopsy. Observe each organ at naked eye. Tick appropriate box and record any observed detail and associated organ

Abnormalities of internal organs	LOSS OF COLOUR:	REDNESS:
	ATROPHY:	HAEMORRHAGES:
	HYPERTROPHY:	NODULES:
	RED SPOTS	ASCITIS:

STEP 4 - IN VIVO RESEARCH FOR BACTERIA

Proceed with an in vivo examination of smashes of each organ. Tick appropriate box for presence and type of bacteria. Proceed with a Gram staining

to identify Gram+ and Gram-

Blood (dorsal aorta)	GERM	MOBILE	COCCUS	BACILL.	MYXO	GRAM
Spleen	GERM	MOBILE	COCCUS	BACILL.	MYXO	GRAM
Kidney	GERM	MOBILE	COCCUS	BACILL.	MYXO	GRAM
Stomach/intestine contents	GERM	MOBILE	COCCUS	BACILL.	MYXO	GRAM

STEP 5 - IN VITRO RESEARCH FOR BACTERIA

Take sample from each organ and lesion and proceed with an in vitro culture. Tick appropriate box for presence and type of bacteria. Proceed with a Gram staining

to identify Gram+ and Gram-

Bacteriological culture	MONOSP	MOBILE	COCCUS	BACILL.	MYXO	GRAM
	PLURISP	IDENTIFICATION				

Refer to the chapter on fish diseases for standard operating procedures.

Seabass and gilthead seabream are the two marine fish species that have characterized the development of marine aquaculture in the Mediterranean basin in the last two decades. The substantial increase in production levels of these two high-value species was made possible by the progressive improvement in technologies for fry production in hatcheries. As a result, more than 100 hatcheries have been built in the Mediterranean basin, working on these and other similar species. At present, the farmed production of these two species derived from hatchery-produced fry is far greater than the supply coming from the wild. The first volume of this manual deals with the historical background and main factors influencing fish seed production; the life history and biology of the species; and hatchery production procedures. The second volume will deal with engineering and financial aspects of hatcheries.

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