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Black Sea turbot

A comprehensive production manual

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Preparation of this document

This manual has been prepared by the General Fisheries Commission for the Mediterranean (GFCM) of the Food and Agriculture Organization of the United Nations (FAO) to address priorities identified by Mediterranean and Black Sea countries in the context of existing international commitments and regional strategies; in particular, Target 3 of the GFCM 2030 Strategy for sustainable fisheries and aquaculture in the Mediterranean and the Black Sea which aims to enhance the sustainability and resilience of the aquaculture sector in the region. The manual contributes to the objective set out in this strategy to maximize technology and information systems in the aquaculture sector by providing detailed information about the entire Black Sea turbot (*Scophthalmus maximus*) rearing process and is foreseen to support the continued aquaculture training activities carried out at the Central Fisheries Research Institute (SUMAE) in Trabzon, Türkiye.

The manual was developed by Black Sea turbot aquaculture experts, namely İlhan Aydin and Ahmet Faruk Yesilsu, under the expert guidance and overall coordination of Houssam Hamza, GFCM Aquaculture Officer, based on practical applications and scientific investigations carried out at SUMAE.

The editing, graphics, layout and publishing were coordinated by Dominique Bourdenet, GFCM Knowledge Management Officer, with the assistance of Alexandria Schutte, GFCM Knowledge Management Specialist, and Ysé Bendjeddou, GFCM Publications Coordinator. Alex Chepstow-Lusty served as technical editor and José Luis Castilla managed the graphic design and layout.

Abstract

In the Mediterranean and the Black Sea, aquaculture plays a major role in achieving food security and can deliver many socioeconomic benefits and reduce dependence on potentially overexploited wild stocks, provided it is managed properly. Given the importance of this sector and the growing demand for aquatic products, it is vital that aquaculture in the region be both sustainable and productive.

In this regard, the General Fisheries Commission for the Mediterranean (GFCM) has identified, among the priorities of its GFCM 2030 Strategy for sustainable fisheries and aquaculture in the Mediterranean and the Black Sea, the need to promote practices supporting the sustainability of the sector and to maximize the sharing of technology and information in order to give producers the necessary tools and information on aquaculture production.

This manual aims to support this endeavour by presenting detailed information about the rearing practices of Black Sea turbot (*Scophthalmus maximus*). It is divided into five parts. The first section provides an introduction into the status of the turbot aquaculture sector, as well as the necessary environmental conditions for optimal production. The second section describes the production of live food for turbot larvae beginning with microalgae, followed by rotifer and *Artemia* production. The third section details the management of broodstock, followed by the procedures for optimal larval and juvenile rearing in the fourth section. With a view to capitalizing on and promoting innovative technologies, the final section presents recent applications of biotechnologies to turbot production, namely, cryopreservation, triploidy and all-female stock production.

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Abbreviations and acronyms

atm	standard atmosphere
D	day post-hatching
DO	dissolved oxygen
FAO	Food and Agriculture Organization of the United Nations
FCE	feed conversion efficiency
FRP	fibre reinforced polymer
GFCM	General Fisheries Commission for the Mediterranean
HCG	human chorionic gonadotropin
HUFA	highly unsaturated fatty acids
ICES	International Council for the Exploration of the Sea
JICA	Japan International Cooperation Agency
LHRH	luteinizing hormone-releasing hormone analogue
NASCO	North Atlantic Salmon Conservation Organization
PVC	polyvinyl chloride
ppm	parts per million
psu	practical salinity unit
ppt	parts per thousand
rpm	revolutions per minute
SUMAE	Central Fisheries Research Institute (Türkiye)
TL	total length
UV	ultraviolet
WSPG	white salmon pituitary gland

1. Turbot production overview

1.1 HISTORICAL BACKGROUND

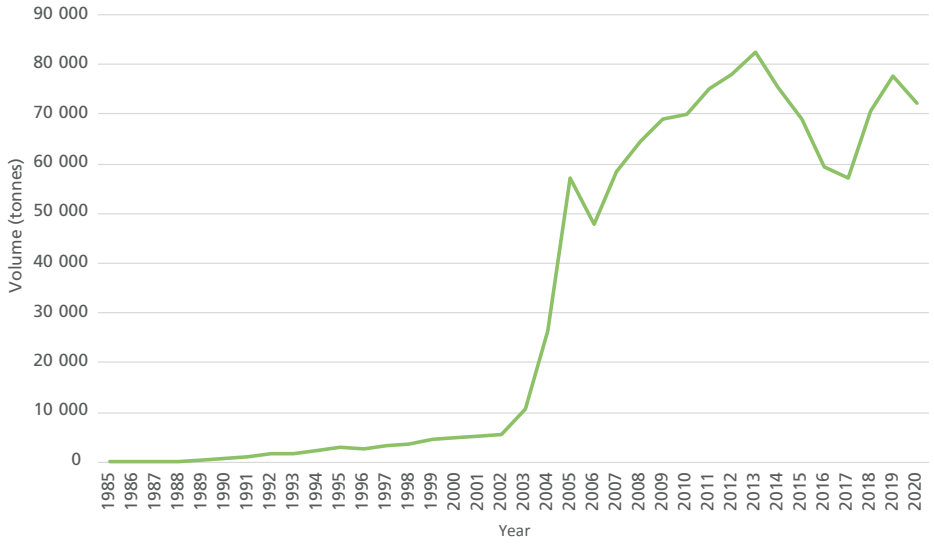
The earliest available information on turbot aquaculture stems from the use of the stripping method to obtain batches of eggs at the beginning of the twentieth century (Aydın *et al*., 2020). Roughly 70 years later, the pool of information from the field grew, following studies that took place in Scotland and France, and subsequently in Spain a decade later (Aydın, 2011). Thanks to this increase in knowledge, European turbot aquaculture production increased from 40 tonnes in 1985 to 10 116 tonnes in 2016, and in fact exceeded the production from fisheries in 2005 (Aydın, 2011; FAO, 2019) (Figure 1). In recent years, in addition to Spain and France, countries including Chile, China, Denmark, Germany, Iceland, Ireland, Italy, the Kindgom of the Netherlands, Norway and Portugal, have all been carrying out turbot aquaculture (FAO, 2019).

Although turbot aquaculture was first practised at the beginning of the twentieth century, the production of Black Sea turbot (*Scophthalmus maximus*) did not begin until 1990 (Maslova, 2002). Subsequently, in 1997, the Central Fisheries Research Institute (SUMAE; Trabzon, Türkiye) began developing Black Sea turbot aquaculture production techniques in collaboration with the Japan International Cooperation Agency (JICA), which had previously produced a manual detailing the production of the species (Çiftçi *et al*., 2002). Since then, Black Sea turbot aquaculture studies have continued successfully for over 20 years.

Today, rearing systems and methods differ by country and farm. However, turbot farming is mostly carried out in land-based systems, where water is supplied from the sea either at a suitable temperature or at an unsuitable temperature and later heated at the fish farm (Plate 1).

Following production, specifically at SUMAE, some of the Black Sea turbot juveniles are given to the private sector for trial purposes, while others are used in restocking programmes to support wild stocks of Black Sea turbot (Aydın, 2011) (Plate 2).

FIGURE 1
Global volume of turbot production, 1985–2020



Source: FAO. 2022. Statistics Query Panel: global aquaculture production quantity (1950–2020). In: *FAO Fisheries and Aquaculture*. Rome. Cited 15 November 2022. www.fao.org/fishery/statistics-query/en/aquaculture/aquaculture_quantity

PLATE 1
Black Sea turbot farm water supply infrastructure

PLATE 2
Black Sea turbot restocking activity near the Trabzon, Türkiye shore

1.2 ENVIRONMENTAL CONDITIONS

The effects of environmental factors are very important for turbot aquaculture and some factors, such as temperature, salinity and light, act directly on receptors related to growth rates. In addition, parameters such as dissolved oxygen (DO), ammonia, pH and CO_2 affect the growth below or above a certain threshold value (Person-Le Ruyet, 2002) (Table 1).

TABLE 1
Optimal environmental parameters for Black Sea turbot growth

Parameter	Optimum range
Temperature	16–19 °C
Salinity	15.0–33.5 ppt
Light	12 light:12 dark
Oxygen	6–9 mg/litre
Ammonia	< 2–3 mg/litre

Source: elaborated by the authors.

1.2.1 Temperature

As with other fish species, temperature is one of the most important factors for the production of turbot as it directly influences the growth rate (Árnason *et al*., 2009). The optimal thermal range for maximum growth is 16–22 °C for 10 g turbot and 16–19 °C for 40–50 g turbot (Person-Le Ruyet, 2002). The optimal growth temperature declines as body weight increases (22.5 °C, 20.8 °C, 19.1 °C and 17.5 °C for 1 g, 10 g, 100 g and 1 000 g turbot, respectively) (Árnason *et al*., 2009).

1.2.2 Salinity

Marine fish expend energy for the metabolic activities of ionic and osmotic regulation (Imsland *et al*., 2001). According to Imsland *et al*. (2001), for juvenile turbot, growth (initial weight of 14 g), food consumption and feed conversion efficiency (FCE) were highest and lowest at salinities of 15 parts per thousand (ppt) and 33.5 ppt, respectively. Similarly, it was reported that the weight gain for 3 g turbot in water with 19 ppt salinity was 8 percent higher compared with 35 ppt salinity (Person Le Ruyet, 2002).

While turbot can tolerate 10 ppt salinity, they cannot survive at 6 ppt and their tolerance to high salinities is unknown. According to Imsland *et al*. (2001), the ideal temperature–salinity combination for growth is 21.88 ºC and 18.5 ppt and the ideal combination for FCE is 18.38 ºC and 19.0 ppt.

1.2.3 Light

Light includes a complex of intensity, photoperiods and colour spectrum (Person-Le Ruyet, 2002). In a study conducted by Stefánsson, FitzGerald and Cross (2002), turbot (45 g) were reared under three photoperiod regimes of varying light (l) and dark (d) ratios (8 l:16 d, 12 l:12 d and 20 l:4 d) for 297 days. During this time, the growth rate of fish under the 12 l:12 d treatment was the highest.

1.2.4 Oxygen

Dissolved oxygen is a major decisive factor for turbot farming. The minimal DO level required for maximal growth is 6 mg/litre. Feed intake stops at 3 mg/litre oxygen level, and the 0.75–1.3 mg/litre range is lethal (Person-Le Ruyet, 2002).

Under satiation feeding, turbot growth rates are 30 percent lower at 4.5 mg/litre DO, and 39 percent lower at 3.2 mg/litre DO, compared to fish maintained at the normal DO level. Furthermore, at these DO levels, feed conversion ratios are 11 percent and 19 percent higher, respectively, compared to fish maintained at the normal DO level (Person-Le Ruyet, 2002).

No biological risks have been reported from using oxygen-supersaturated water. Indeed, rearing turbot in water between 147 percent and 223 percent saturation levels has had no significant effects on feed intake, feed conversion and growth (Person-Le Ruyet, 2002).

1.2.5 Ammonia

Ammonia is excreted by the gills of turbot into the water in which they are reared and is toxic in its un-ionized form. The ammonia threshold concentration for acceptable growth over three months is 5–6 mg/litre total ammonia or 0.2 mg/litre NH_3 at pH 7.5. However, safe levels of total ammonia concentrations are considered to be less than 2–3 mg/litre (Person-Le Ruyet, 2002).

2. Live food production

2.1 MICROALGAL CULTURE

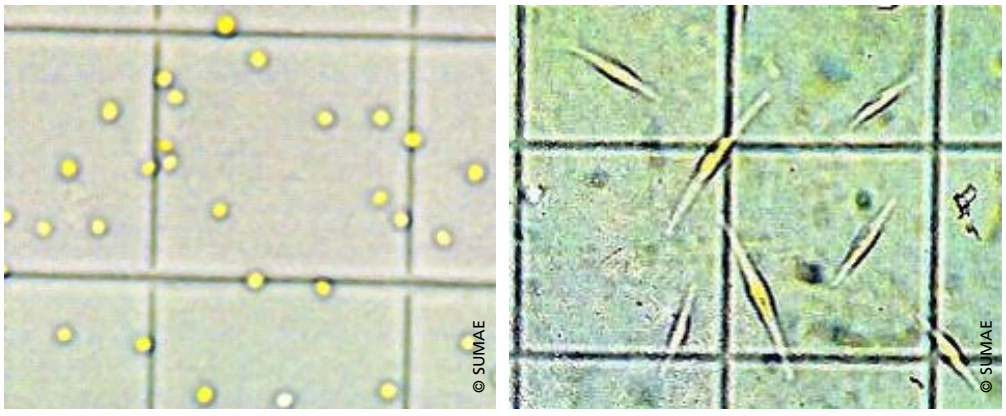
A myriad of microalgal species act as the primary producers at the base of the aquatic food chain. As such, they are used as a major food source for rotifers and *Artemia*, which are subsequently used as a source of nutrition for fish larvae. In addition, as microalgae consume toxic substances such as ammonia and pesticides, they may be directly added to the rearing water containing fish larvae in order to improve the water quality. This is known as the green water technique in aquaculture.

Of the many types of algae in the sea, only a few can be cultured in controlled conditions and exhibit suitable growth. There are several ways of selecting productive strains that are well adapted to the prevailing conditions. The general criteria considered in the selection process include growth rates, biochemical composition, temperature tolerance, and resistance to mechanical and physiological stress. The selection may be carried out in the laboratory with subsequent tests in outdoor ponds or in the actual production ponds by manipulating growth conditions (i.e. nutrient concentrations, pH and pond depth).

During the first turbot rearing attempts undertaken at SUMAE, *Nannochloropsis* sp. and *Phaeodactylum* sp. were procured from Japan to be used as starter inoculums and live food sources (Plate 3).

Microalgal cell culture involves growing these organisms under controlled conditions within specific water, light, nutrient and temperature ranges. The temperature values, for instance, for *Nannochlosopsis oculata* and *Phaeodactylum tricornutum* are 18–20 °C and 12–15 °C, respectively. Under optimal conditions, the density of *Nannochloropsis* sp. and *Phaeodactylum* sp. can reach 20 million cells/ml and 2 million cells/ml, respectively, one week after inoculation. Sizes range from a few micrometres to roughly 40 µm (Table 2).

PLATE 3
Microalgae in Thoma chamber



Notes: *Nannochloropsis* sp. (left); *Phaeodactylum* sp. (right) (magnification:10 × 40).

TABLE 2
Features of *Nannochlosopsis oculata* and *Phaeodactylum tricornutum*

Species	Colour	Size (µm)	Culture scale	Shape
Nannochlosopsis oculata	Green	3–5	Mass	Round
Phaeodactylum tricornutum	Brown	20–40	Mass	Bean-shaped

Source: elaborated by the authors.

Algal cell count is monitored under a microscope using a Thoma cell counting chamber (1 mm × 1 mm × 0.1 mm) and carried out twice a week for mass cultures (Plate 4). The counting chamber consists of large, medium and small squares. Five medium squares, made up of 25 small squares, are chosen at random and all cells are counted in a wide line (Figure 2). Subsequently, the density of the algal cells/ml (P) is calculated as follows:

$$P = \frac{(B1 + B2 + B3 + B4 + B5)}{5} \times 16 \times 10\,000$$

PLATE 4
Thoma chamber preparation

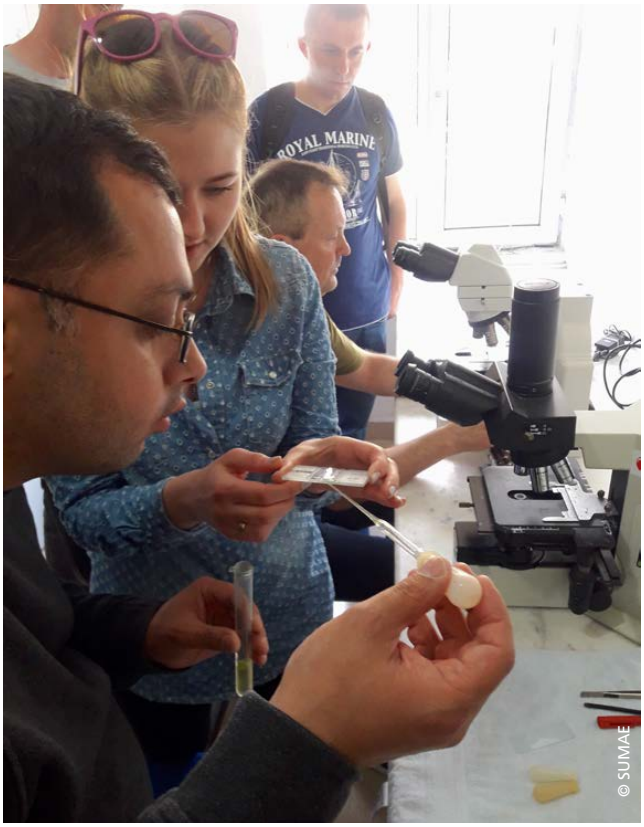
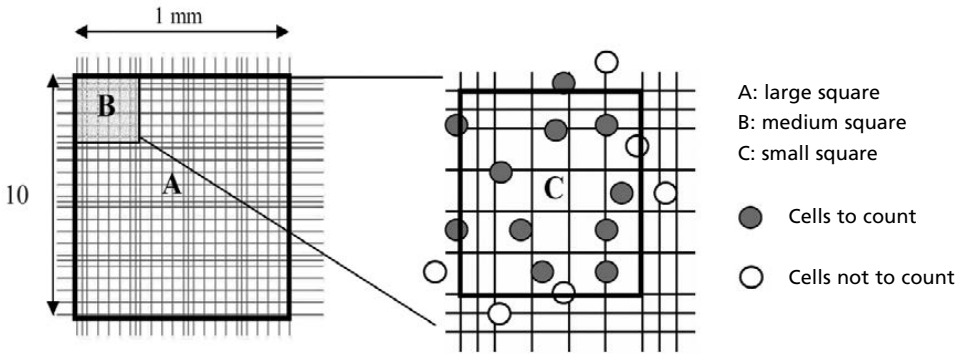


FIGURE 2
Counting algal cells on a hemacytometer



Source: elaborated by the authors.

2.1.1 Algal stock culture maintenance

All algal culture systems require a set of stock cultures (usually of about 10 ml) to provide a reservoir of algae. Moreover, it is necessary to re-isolate the algal stocks using the streak plate technique, as follows, in order to obtain pure cultures (Phang and Chu, 1999):

- Mix algal stock culture with sterile sea water.
- Put into four centrifuge tubes.
- Centrifuge at 5 000 revolutions per minute (rpm) for five minutes (Plate 5).
- Mix the residue with an equal volume of sterile seawater using a vortex.
- Centrifuge again at 5 000 rpm for five minutes.
- Repeat this process at least three times to ensure the microalgae are free from bacteria.
- Inoculate the microalgae using a loop on the agar plate (Plate 6).
- Allow microalgal cells to grow.
- Re-inoculate microalgal cells on new agar plates.
- Repeat this process until it is free from contamination.
- Put single cells into sterile Walne stock solution or F2 medium in tubes and vials.
- Allow microalgae to grow for future use.

PLATE 5
Microalgal centrifugation

PLATE 6
Microalgal streaks on an agar plate

Essentially, using the streak plate technique, washed microalgae are applied as streaks using a sterile loop on agar plates in axenic conditions and kept for at least seven days to grow. Repeated streak-plating can be carried out to build up single colonies from earlier streaked agar plates and to ensure they are free from bacteria. From previously streaked agar plates, single colonies can be picked up using a loop and allowed to grow in tubes and vials (Plate 7).

PLATE 7
Microalgal re-isolation

Culture medium ingredients for small-scale culture are made from pure grade chemicals based on Walne stock solution (Annex 1) or F2 stock solution (Annex 2), which are otherwise difficult to prepare, and used as an enrichment medium for algal culture propagation. To promote stable algal production, purified algal stock culture is used since the growth of mass algal cultures depends on the quality of the test tube culture. Uncontaminated stock culture is determined by the transparency of the medium, non-existence of algal flocks, rapid growth of the cell number and homogeneous sizes of algal cells.

Following selection of the stock culture, the excess clean stocks are kept in a refrigerator at a temperature of 2–4 °C and low illumination (< 1 000 lux) (Plate 8), in order to increase efficiency during the production season. The preserved stock in the refrigerator should be mixed once a week and can be kept for six months, provided that the necessary conditions are met (Plate 8).

PLATE 8
Algal stock culture refrigerator

PLATE 9
Algal stock subculturing

Algal cultures in test tubes should be subcultured frequently (preferably monthly), although this period could be longer (over six months) for algal cultures on petri plates. Subculturing involves inoculating some cells from an old stock culture into a fresh culture medium, so that the cells can continue to grow, divide and remain healthy (Plate 9).

2.1.2 Phytoplankton laboratory and materials preparation

In the phytoplankton laboratory, all the necessary equipment and materials should be organized before use (Plate 10). Small-scale culturing is carried out in laboratory conditions and continues from 10-ml test tubes to 5-litre flasks for both the maintenance and re-isolation process.

The laboratory should be isolated to maintain sterility and to minimize contamination risks, while unauthorized personnel should not be allowed to enter. The room should be cleaned and disinfected with a chlorine solution every week and kept at 18–22 °C in order to maintain suitable growth conditions for algae. A photoperiod of 16 hours of light and 8 hours of darkness should be supplied using daylight-type fluorescent lamps (2 000–3 000 lux). Glassware materials made from borosilicate should be soaked in neutral phosphorus-free detergent for one day, and then rinsed well with tap water to remove any soapy residue. After that, the glassware should be kept in 0.1 N HCl solution for two hours and rinsed again with tap water and subsequently with distilled water to prevent any chemical residue. After drying, the necks of glassware should be covered with aluminium foil in order to maintain their sterility. Finally, the glassware should be sterilized at 160–180 °C for 2 hours or autoclaved at 121 °C and 2 atm for 15 minutes.

The seawater to be used in the laboratory during all the processes should be filtered through a glass fibre filter and sterilized in an autoclave. In addition, seawater and the culture medium should be sterilized separately in order to prevent the deposition of minerals. They can subsequently be mixed under sterilized conditions.

PLATE 10
Phytoplankton laboratory

2.1.3 Scaled-up algal culture

All processes (except the test tube culture) should be carried out in a batch culture system to minimize the risk of contamination during the scale-up phase. Furthermore, the starter culture should only be used once.

Small-scale algal culture

Algal culture in test tubes

At this stage, two to three drops of pure algae are inoculated using a sterile pustule pipette into 20 test tubes, each containing 10 ml of stock medium. A clean uncontaminated stock culture is confirmed by its transparency, non-existence of algal flocks, rapid cell growth and uniform cell size under microscopic observation. Inoculated cultures are left for 10 to 15 days before being transferred to a 200-ml conical flask. Newly inoculated test tubes are shaken daily to resuspend the cells and to prevent any cell accumulation at the bottom. Additional aeration is not necessary after selection of the stock culture. Surplus clean stocks are kept in a refrigerator for six months at a temperature of 4 °C for possible future requirements (Plate 11).

Algal culture in 200-millilitre flasks

The best of the propagated algal test tube cultures are inoculated into 200-ml conical flasks along with 175 ml of sterilized culture solution. The necks of the conical flasks are closed with silicone plugs (Plate 12).

Newly inoculated cultures are shaken twice a day for four to seven days to re-suspend the algal cells and prevent them from settling (Plate 13).

PLATE 11
Test tube culture (10 millilitres) and propagation of algae on agar plate

PLATE 12
Algal flask culture (175 millilitres)

PLATE 13
Algal flask culture (200 millilitres)

PLATE 14
Algal flask culture (5 litres)

Algal culture in 5-litre flasks and 1 000-litre tanks

Glass tubing and a silicone plug are assembled on a 5-litre flask filled with 3.5 litres of culture medium (Plate 14). Cotton is inserted into the neck of the flask to minimize contamination from the air, and vigorous aeration (6 litres/minute) is supplied to agitate the culture medium. A single 200-ml flask culture is then inoculated into the 5-litre flask. After four to seven days, the algal cell density in the 5-litre flask increases by about ten times compared to the initial inoculation density.

This algal phase is carried out in the greenhouse, which is separated from the other hatchery facilities (Plate 15). To avoid contamination, independent equipment is soaked in chlorinated freshwater containing 300 parts per million (ppm) of liquid bleach with an active chlorine concentration of 12 percent.

For culture volumes from 100 litres to 1 000 litres, polycarbonate transparent culture tanks are filled with filtered and chlorinated seawater containing 300 ppm of liquid bleach for overnight sterilization. The following morning, the seawater is

PLATE 15
Algal culture in greenhouse

neutralized with a sodium thiosulphate solution for several minutes. The aeration is set to moderate during the sterilization period and adjusted to 8 litres/minute for 100-litre cultures and 22 litres/minute for 1 000-litre cultures.

Following aeration, an agricultural grade fertilizer is added into the tanks and an algal starter culture from the 5-litre flask is transferred to a 100-litre culture tank. Similarly, the entire algal population of the 100-litre culture tank is inoculated into a 1 000-litre culture tank. In this phase, the inoculation density of *N. oculata* is generally 2–5 million cells/ml, which reaches a target density of 30–60 million cells/ml in one week.

The doses of chemicals applied for seawater sterilization, neutralization and fertilization to enable the culture of *N. oculata* are summarized in Table 3.

TABLE 3
Chemical doses for *Nannochlosopsis oculata* mass culture

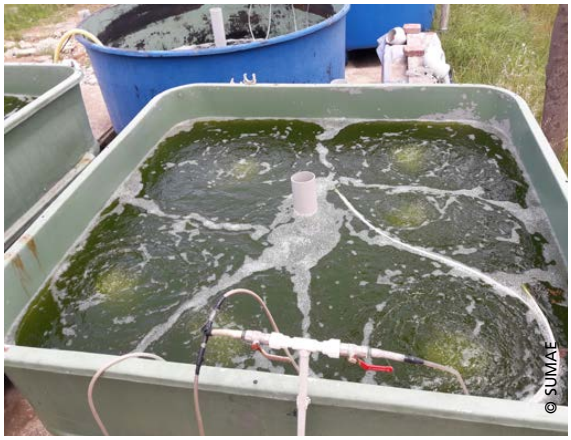
	Tank capacity		
	100 L	**1 000 L**	**3 000 L**
Initial water volume	100 L	900 L	2 000 L
Bleach (12% sodium hypochlorite)	20 ml	180 ml	400 ml
Sodium thiosulphate solution (10%)	2 ml	18 ml	40 ml
Inoculation	5 L	100 L	1 000 L
21-0-0 (ammonium sulphate)	10 g	100 g	300 g
42-44-0 (ammonium phosphate)	0.8 g	8 g	24 g
46-0-0 (urea)	10 g	100 g	300 g
Fe-EDTA	0.5 g	5 g	15 g
Fertilization interval	Every three days at optimum temperature		

Source: elaborated by the authors.

Mass algal culture

Mass algal culture is carried out in 2 m^3, 4 m^3 and 6 m^3 fibre reinforced polymer (FRP) outdoor tanks. These tanks are placed in sunny locations and those that are rectangular in shape are installed in a west to east direction to promote algal growth. For tanks with a volume of 2–4 tonnes, 6–8 air stones are used to agitate the algae; however, more vigorous aeration is required for larger volumes and a garden hose with several air stones is used for tanks greater than 6 tonnes. In these cases, the garden hose (1.5 cm in

PLATE 16
Tank bottom with air stones and a garden hose



diameter), is fixed on the tank bottom, 30 cm away from the tank wall with 1 mm holes drilled 10 cm apart. Likewise, several air stones can be installed in the tanks (Plate 16).

Algal cultures in 1 m^3 transparent tanks are inoculated into 2 m^3 FRP tanks. In the *Nannochloropsis* sp. culture, the initial water depth is determined based on the volume and density of the inoculated algae, which should be between 5 and 10 cells/ml. When the density reaches 20 million cells/ml, sterilized seawater is added to refresh the culture until a density of 10 million cells/ml is attained. Repeated SUMAE tests have found that this process results in a maximum water depth of around 40 cm in the winter, and 80 cm in other seasons. Moreover, as the interval and dosage of fertilization affect algal growth in mass culture tanks, one-third of the standard amount of fertilizer should be applied every three days in the winter.

Once cultured algae reach their peak density (20 million cells/ml), they can be transferred to temperature-controlled tanks before being concentrated and fed to rotifers. Transfer of the algae from one tank to another one can be carried out using a spiral hose attached to a submersible pump. The algae are filtered through a 45-µm plankton bag net attached to the end of the hose.

2.1.4 Contamination countermeasures

Each culture tank should be visually inspected daily, in addition to microscopic observation. If sticky green bubbles are noticed on the water's surface or if there is dirt on the culture tank's walls, rotifer or protozoan contamination can be suspected (Plate 17). In these cases, a 1 ml sample is examined under a stereo microscope. If there is

PLATE 17
Signs of contamination on the surface and wall of the tank

Notes: Protozoa (left); bacteria (right).

no evidence of contamination, a 5-ml sample is concentrated using a 45-µm plankton net and observed under the microscope. In the case of rotifer contamination, liquid bleach is added to the culture tank at a concentration of 15 ppm for 30 minutes, which is then neutralized with sodium thiosulphate (1.5 ppm). Rotifer eggs may survive this treatment, so it is then repeated the following day to kill any newly hatched rotifers.

When undesirable protozoans are observed, liquid bleach is added to the culture water at a concentration of 50 ppm for 30 minutes and is then neutralized with sodium thiosulphate (5 ppm).

2.1.5 Algae concentration

To minimize labour during the production season, provide off-season stock and create an emergency source for mass culture, some or all of the harvested algae are concentrated through a polyethylene hollow fibre membrane (0.01 µm in opening diameter) (Plate 18).

Concentrated algae are preserved either in liquid at 4 °C with aeration for up to several weeks or in frozen form at temperatures below -20 °C, which can be used for more than a year without significant deterioration in quality.

PLATE 18
Microalgal storage

Notes: Biomass concentrating machine (left); algae preserved in plastic bottles (centre); algae preserved in algal bags (frozen and liquid) (right).

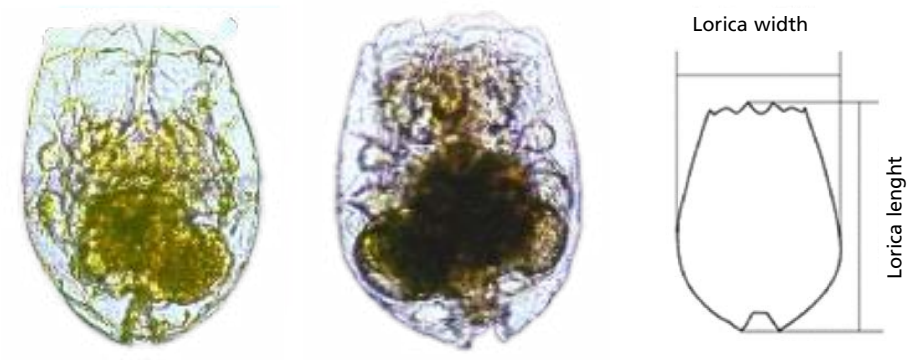
2.2 ROTIFER CULTURE

Rotifers are a relatively small phylum of aquatic or semi-aquatic invertebrates and consist of about 2 000 species. Only two species of rotifers are used in aquaculture: *Brachionus plicatilis* (large type) and *Brachionus rotundiformis* (small type). They have different morphological characteristics: the lorica length of *B. plicatilis* ranges from 130 µm to 340 µm (mean 240 µm) and that of *B. rotundiformis* ranges from 100 µm to 210 µm (mean 160 µm) (Figure 3).

The species also differ in their optimal growth temperature range; *B. plicatilis* grows well between 20 °C and 25 °C, while *B. rotundiformis* favours temperatures between 25 °C and 30 °C.

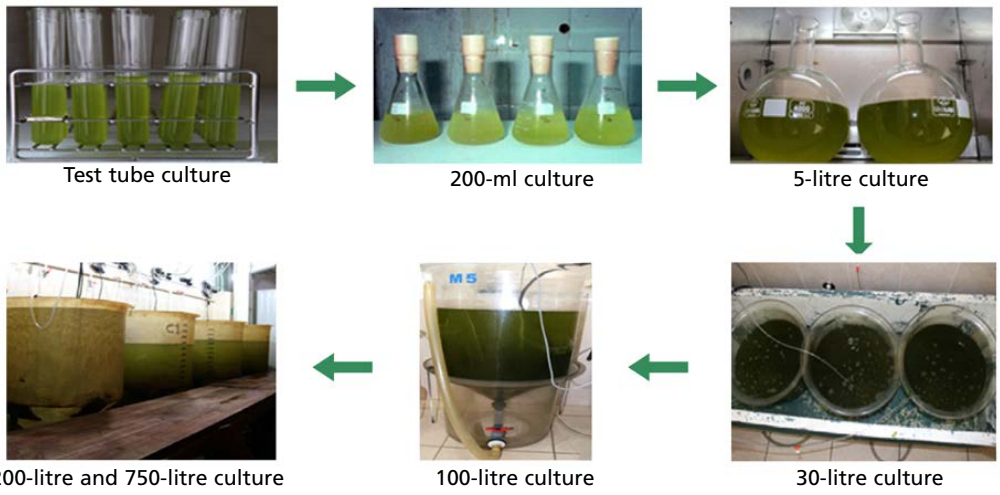
In aquaculture, the small-type rotifers are preferred for the first feeding of fish larvae with small mouth openings (i.e. with mouth openings at the first feeding of less than 100 µm). However, in turbot culture, it is essential to use both small- and large-type rotifers as food for early larval stages, due to the range in mouth width size of the fish larvae.

FIGURE 3
Rotifers



Notes: Brachionus plicatilis (left); *Brachionus rotundiformis* (centre).
Source: elaborated by the authors.

FIGURE 4
Rotifer culture workflow



Source: elaborated by the authors.

The process of culturing rotifers (*B. rotundiformis*) consists of six scaling-up phases and a mass culture phase (Figure 4). The details of the process are described in the following sections.

2.2.1 Scaled-up rotifer culture

Small-scale rotifer culture

The culture of rotifers in test tubes, 200-ml flasks and 5-litre flasks is carried out in incubators at a temperature of about 22 °C (Plate 19). In this phase, rotifers do not need supplemental aeration and are fed *Nannochloropsis* sp. cultured under laboratory conditions.

Rotifer stock culture in test tubes

Rotifers are inoculated into a test tube at an initial density of 1 rotifer/ml along with *Nannochloropsis* sp. at a density of approximately 2 million cells/ml. One week later,

PLATE 19
Onsite training class on 200-millilitre rotifer flask culture

the number of rotifers increases to 50 rotifers/ml without supplemental algae. A small portion of the rotifers in the test tube is then transferred to new test tubes for stock sustainability, while the remaining rotifers are used for scaling-up the culture.

Rotifer culture in 200-millilitre flasks
Before the rotifers are transferred, 20 ml of fresh *Nannochloropsis* sp. are added to a sterilized 200-ml conical flask. The rotifers are then gently aspirated from a test tube using a sterilized Pasteur pipette, without disturbing the accumulated dirt at the bottom, and inoculated into the flask. Fresh algae are added to the flask at regular intervals until the culture volume reaches 150 ml. It takes approximately one week for the density of rotifers to reach 150 rotifers/ml.

Rotifer culture in 5-litre flasks
The rotifers in the 200-ml flask are gently transferred to the 5-litre flask. About one-third of the culture medium from the bottom of the 200-ml conical flask is discarded to avoid contamination. After inoculation into the 5-litre flask, the rotifers are fed with 200 ml of fresh *Nannochloropsis* sp. An additional 200–500 ml of fresh *Nannochloropsis* sp. with a density of 50 million cells/ml are added daily to supply enough food to the rotifers until the culture volume reaches 4 litres. In one week, the density of rotifers increases from 50 to 150 rotifers/ml. No aeration is needed during this phase.

Medium-scale rotifer culture
In this phase, 30-litre, 200-litre and 750-litre tanks are used for medium-scale culture. These tanks are set up in suitable areas in order to prevent sudden changes in environmental conditions, such as pH and temperature. To further stabilize conditions, the temperature is maintained at 22 °C using an electric heating system. To trap faecal matter, dead rotifers and waste, an air cleaning filter sheet is left in suspension in the rearing water. During this phase, fresh algae, cultured in the greenhouse, are used as food and moderate aeration is supplied.

Rotifer culture in 30-litre tanks

To begin, 30-litre culture tanks, having been sterilized with chlorine, are set up in a water bath. Five litres of fresh *Nannochloropsis* sp. (20 million cells/ml) are then transferred into each 30-litre tank with moderate aeration (3.5 litres/minute), and on the following day, rotifers from a 5-litre flask are inoculated into each tank. It takes approximately two days for the algae in the tanks to be consumed and during this time the colour of the water changes from a translucent light green to a transparent light green. Until the culture volume reaches 30 litres, an additional 3–6 litres of fresh *Nannochloropsis* sp. are added to the tank every day. In about a week, the density of rotifers increases from 75 to 200 rotifers/ml.

Rotifer culture in 200-litre and 750-litre tanks

Initially, the 200-litre or 750-litre tank is sterilized with a chlorine solution. One day prior to rotifer inoculation, 30 percent of the tank volume is filled up with fresh *Nannochloropsis* sp. (7.5 million cells/ml) and the aeration is adjusted to 4 litres/minute in the 200-litre tank and 6 litres/minute in the 750-litre tank.

During this culture phase, the tank is frequently examined for protozoans and the rotifers are rinsed well to remove any protozoans that are present, as they compete with rotifers for food. Prior to inoculation into the 200-litre tank, a 30-litre tank with rotifers sits for 10 minutes without aeration to allow large particles of sediment in the water column to settle before inoculation. The same process is followed prior to inoculation of rotifers from a 200-litre tank into the 750-litre tank. Then, in both cases, the rotifers are carefully concentrated in a submerged plankton net (mesh size of 60 µm) with a hose by siphoning until the water level drops to 5–10 cm above the tank bed. Subsequently, the concentrated rotifers are washed with sterilized heated seawater.

During inoculation, the rotifer density is 200–500 rotifers/ml. Algae in the tank are consumed in one day and the water colour turns to a transparent light green. Using a peristaltic pump, 20 percent of fresh *Nannochloropsis* sp. (20 million cells/ml) are continuously added to maintain a translucent light green colour of the water. Dried baker's yeast is also added to feed the increasing population of rotifers at a maximum rate of 0.4 g/10^6 rotifers. In one week, the density of rotifers increases to over 500 rotifers/ml.

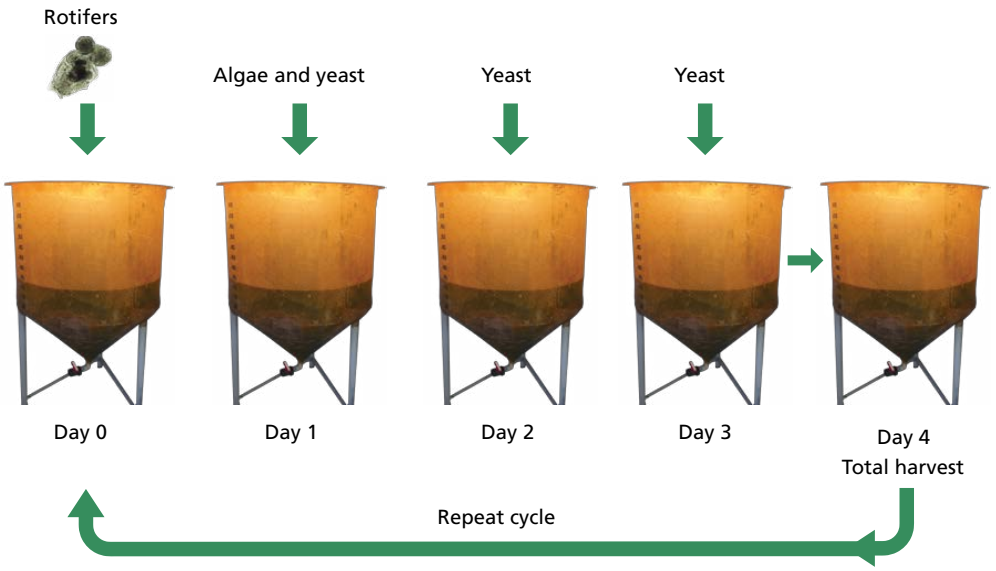
Mass rotifer culture

Two standard culture systems are usually used in the mass culture of rotifers: the batch culture system and the exploitation culture system, also known as the semi-continuous culture system.

The major difference between the two systems is in terms of harvesting. In the batch culture system, all the cultured rotifers in the tank are harvested, with a portion being used to inoculate the other culture tanks. In contrast, in the exploitation culture system, the rotifers are partially harvested from the same tank several times and after each harvest, the volume of harvested medium is replaced with fresh algae.

In the batch culture system, 750-litre tanks are used and the temperature is maintained between 20 °C and 25 °C. A four-day batch culture system is applied to *B. rotundiformis*. On day 0, the tanks are filled with 250 litres of algae (20 million cells/ml), and 250 litres of sterilized seawater. Rotifers are then inoculated at a density of 100 rotifers/ml with aeration at 7.5 litres/minute. On day 1, an additional 250 litres of algae are added to the tank and dry baker's yeast, which is first homogenized in tap water, is used to feed the rotifers (0.2 g/10^6 rotifers). The same amount of homogenized baker's yeast is fed to the rotifers on days 2 and 3. All the rotifers in the tank are harvested on day 4 and one-third of the harvested rotifers is inoculated into a newly prepared tank as described on day 0 (Figure 5).

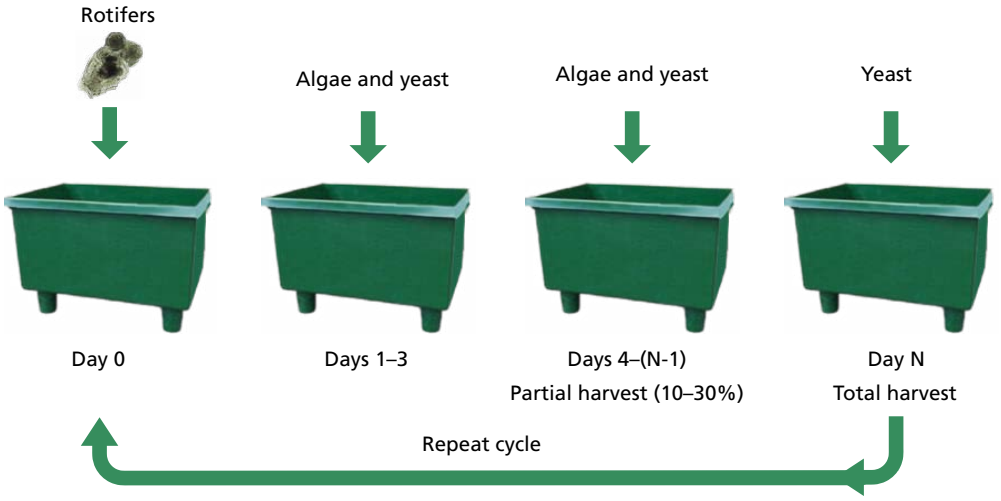
FIGURE 5
Four-day batch culture system



Source: elaborated by the authors.

The exploitation culture system is used for 3 400 litre FRP rectangular tanks at a temperature of approximately 20 °C. On day 0, the tanks are filled with 750 litres of fresh *Nannochloropsis* sp. (20 million cells/ml) and 750 litres of sterilized seawater. Rotifers are then inoculated at a density of 50 to 100 rotifers/ml. On days 1 and 2, in the morning, an additional 750 litres of algae are added to each tank, and in the afternoon, the rotifers are fed with homogenized dry baker's yeast (0.2 g/10^6 rotifers). On day 3, 400 litres of algae are added to each tank and dry baker's yeast is fed to the rotifers in the morning and afternoon. From day 4 to day N-1 (the day before the final harvest), a partial harvest is carried out and the tank is refilled with the same volume of algae as harvested. The growth of rotifers is maintained using the combination of dry baker's yeast and algae. After total harvest on day N, 35 percent of the harvested rotifers is inoculated into a newly prepared tank as described on day 0 and the cycle is repeated (Figure 6).

FIGURE 6
Exploitation culture system



Source: elaborated by the authors.

2.2.2 Rotifer counting method

To determine the density, fertility and general culture conditions, samples from each tank are observed under a stereo microscope at 40 times magnification (Plate 20). More specifically, a sample of at least 1 ml is spread on the zooplankton-counting chamber and initially examined for contaminants such as ciliates, nematodes and protozoans. A drop of Lugol's iodine or formalin is then added to the sample to kill any rotifers and protozoans – instead of Lugol's iodine, iodine gargle can also be used. Under the microscope and using a multiple-key hand counter, the total number of rotifers, rotifers with eggs, eggs, and protozoans are counted. To estimate the fertility rate, the number of rotifers with eggs is divided by the total number of rotifers. A one day old culture has up to a 30 to 40 percent fertility rate and this gradually decreases with the duration of the culture. Generally, a culture with less than a 10 percent fertility rate has difficulty with population recovery.

PLATE 20
Magnified rotifer culture sample

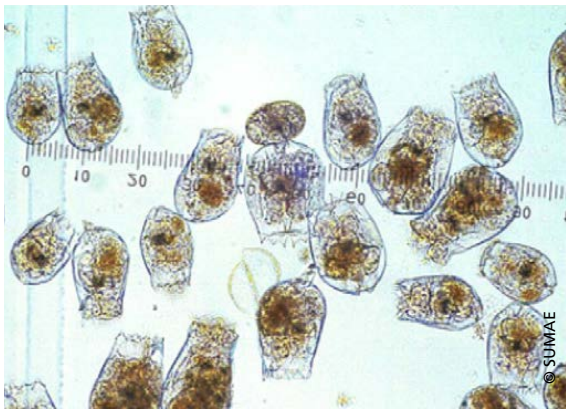
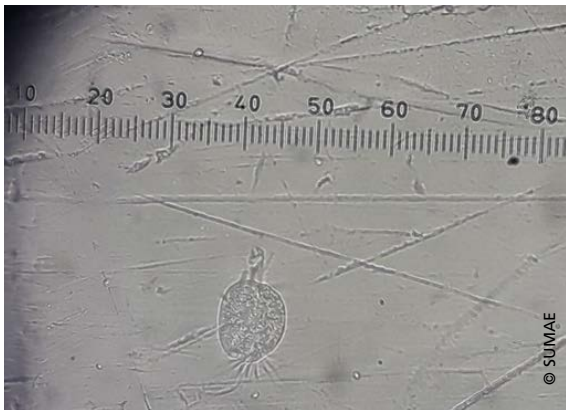


PLATE 21
Protozoan parasite of rotifer (*Euplotes* sp.)



2.2.3 Rotifer harvesting

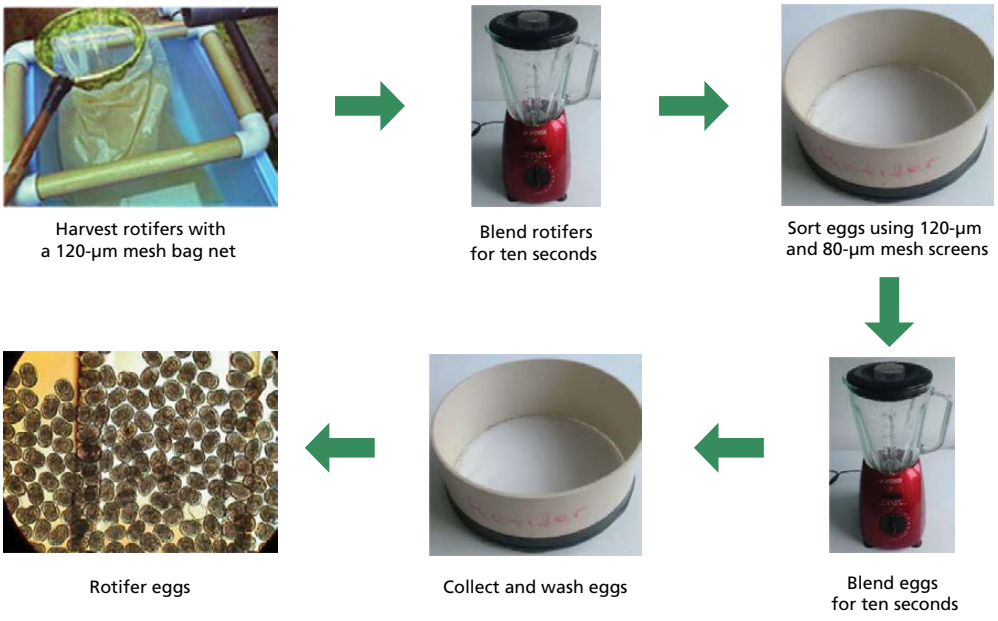
Sterile seawater is first used to clean the drainage pipes on the tanks. The harvesting protocol is then initiated after blocking the air inlet, which leads solid particles in the water column to sink and be removed via the drainage pipes. Rotifers are subsequently harvested using a 60-µm nylon bag net. During harvest, close monitoring and occasional gentle agitation of the bag net are important to prevent clogging and overflowing. Special attention should also be given to avoid cohesion of rotifers by air bubbles, which could otherwise lead to high mortality.

2.2.4 Egg mass isolation technique

In turbot culture, ciliates are one of the most serious parasites, causing high mortality of larvae and adult fish. For this reason, the presence of ciliates in the rotifer culture should be checked daily via microscopy (Plate 21). When their density reaches 20–40 ciliates/ml, they pose a great risk to rotifer culture. If such a level of ciliate contamination is observed in the culture tanks, it is recommended to renew the culture. The egg mass isolation technique can be effective to recover the culture in the short term.

The following procedure is carried out to separate rotifers from their eggs (Figure 7). Approximately 200 litres of rotifers are concentrated through a 120-µm harvesting bag net and placed in a blender (700 watts). Rotifers are homogenized for ten seconds in order to crush them and separate the eggs from the bodies. Subsequently, the homogenized rotifers are separated by passing them through two layers of filter screens. The upper mesh size (120 µm) retains the rotifers, while the lower mesh (80 µm) collects the eggs. Next, the eggs are rinsed well with sterilized seawater. At this point, few rotifers are left alive and able to contaminate the eggs. Nevertheless, all the rotifers are killed for safety and then the eggs caught by the 80-µm mesh are homogenized once again for 10 seconds. Afterwards, the separated eggs are washed

FIGURE 7
Egg isolation technique



Source: elaborated by the authors.

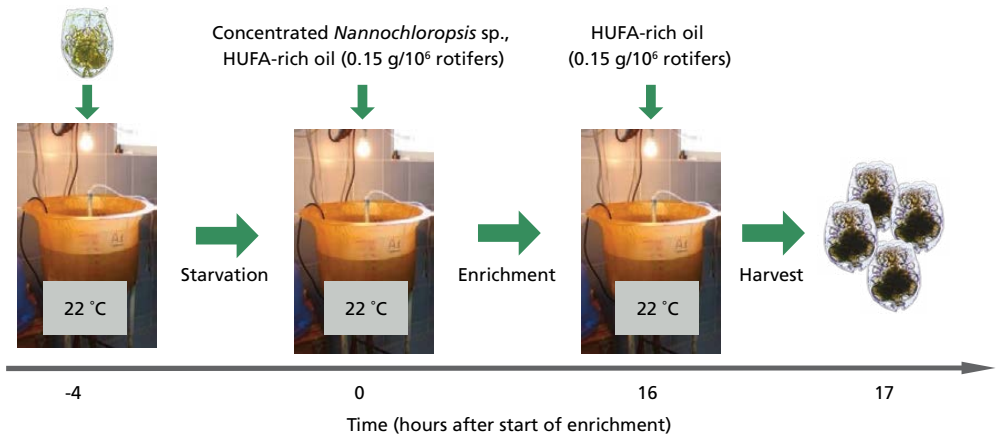
once more with sterilized seawater. To incubate the isolated eggs, 100-litre cylindro-conical tanks containing seawater and fresh algae with chlorine dioxide are used.

2.2.5 Rotifer enrichment

The nutritional value of mass cultured rotifers is sufficient for the fish larvae, especially in terms of highly unsaturated fatty acids (HUFA). However, the occurrence of ocular malpigmentation in flatfish larvae is heavily tied to the quality of the food organisms; therefore, the rotifers must be enriched. Larval survival rates were below 5 percent when docosahexaenoic acid and eicosapentaenoic acid were used. As such, it is preferred to use a suitable enrichment medium. Furthermore, algal concentrate, which is used for rotifer feeding, is a very good source of eicosapentaenoic acid for fish larvae.

The enrichment procedure conducted for rotifers is shown in Figure 8. The concentrated rotifers are transferred to a 100-litre cylindro-conical tank filled with sterilized seawater. The rotifers are stocked at a density below 500 rotifers/ml and are

FIGURE 8
***Brachionus rotundiformis* enrichment**



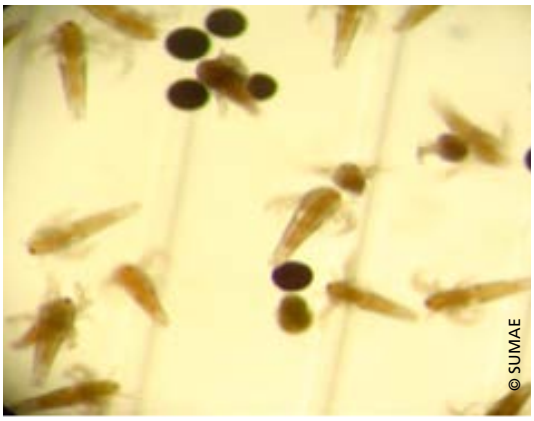
Source: elaborated by the authors.

kept in the tank without any supplemental feed, but with moderate aeration (7.5 litres/minute).

After four hours of starvation, the enrichment medium for rotifers – concentrated *Nannochloropsis* sp. and commercial HUFA-rich oil (0.15 g/10^6 rotifers) – is added to the tank. Aeration is strictly controlled to avoid cohesion of rotifers with the oil and air bubbles. The water temperature is maintained at 22 °C with an electric heater.

Sixteen hours after the start of enrichment, the same amount of HUFA-rich oil (0.15 g/10^6 rotifers) is again added. An hour later, the rotifers are harvested to feed the fish larvae.

PLATE 22
***Artemia* nauplii**



2.3 *ARTEMIA* CULTURE

Artemia, a genus of brine shrimp, has a broad tolerance to changing environmental conditions and can survive in a wide range of temperatures (6–37 °C), salinities (15–35 ppt) and pH levels (8–8.5) although its optimal reproduction temperature is about 25 °C. The use of *Artemia* as live food for aquaculture is very important, as it is rich in digestible protein and of a suitable size to be consumed by the larvae of a large number of fish species (Gamsiz and Alpbaz, 2006). The most common species found in the world and the most widely used as live feed is *Artemia franciscana*.

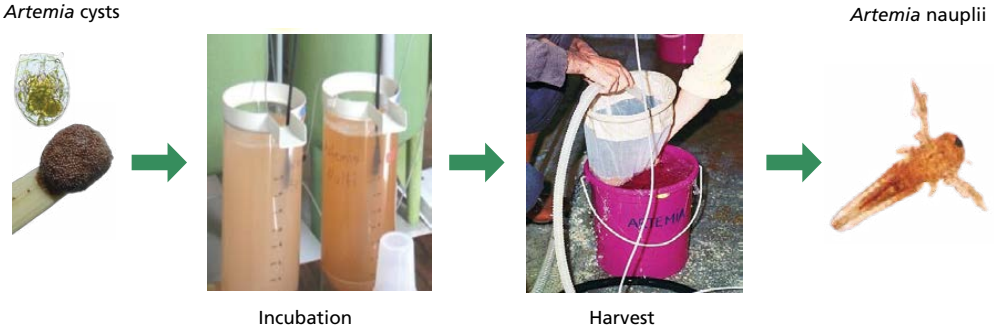
In turbot aquaculture, commercial *Artemia* cysts are used. The nauplii, the first crustacean larval stage, hatch in saline water approximately 24 hours after incubation (Plate 22). In turbot seed production, *Artemia* nauplii provide the secondary food organisms for fish larvae following the rotifer feeding phase.

2.3.1 *Artemia* incubation

The following incubation procedure is employed to minimize the microbial load on *Artemia* nauplii (Figure 9).

A cylindro-conical tank is filled with seawater and aerated by an air stone. The seawater in the tank is sterilized and moderately aerated overnight. The following morning, after ensuring that the tank water is equal to its initial level and adjusting it if not, the cysts are introduced into the tank at a maximum quantity of 1 g/litre. After the addition of 400 ppm chlorine dioxide, the cysts are incubated for 22 hours

FIGURE 9
***Artemia* incubation**



Source: elaborated by the authors.

with vigorous aeration. Prior to harvest, aeration is stopped and the tank is left for ten minutes to allow unhatched cysts to settle and empty shells to float. Subsequently, stagnant water and unhatched cysts are drained away. Nauplii are then concentrated using a 114-µm nylon bag net, rinsed with tap water and the empty shells are removed.

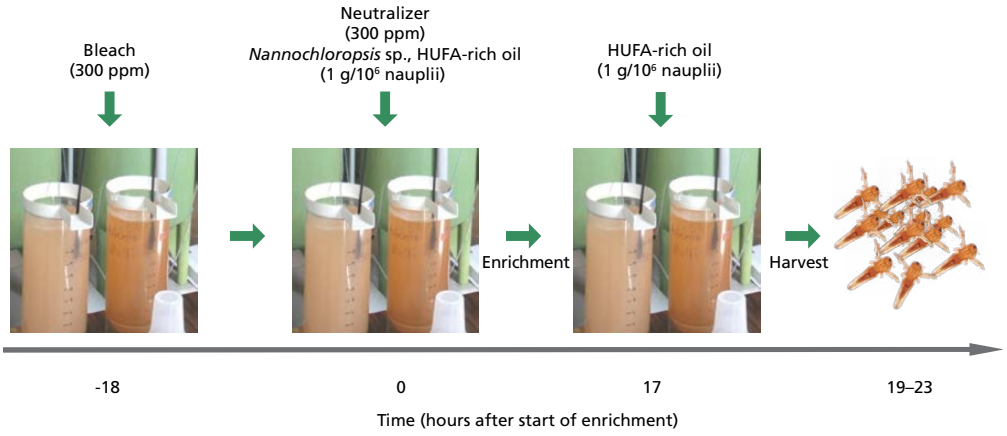
2.3.2 *Artemia* enrichment

Long-chain polyunsaturated fatty acids are essential in turbot culture, especially during early life stages, and are usually provided along with the feed. Thus, the enrichment of *Artemia* nauplii is a common way to increase feed quality. Enrichment mediums may consist of phospholipids containing n-3 fatty acids, which provide vitamins and essential components to support nutrition, immunity and collagen tissue development, therefore ensuring the highest vitality of live food and subsequently fish larvae.

To begin the enrichment process, nauplii are transferred into the tank filled with neutralized seawater, and *Nannochloropsis* sp. microalgae and commercial HUFA-rich oil (1 g/10^6 nauplii) are then supplied to the tank. Enrichment continues until the desired HUFA levels are reached, which usually occurs in 12 to 24 hours.

Seventeen hours after the start of enrichment, the same quantity of HUFA-rich oil (1 g/10^6 nauplii) is again given to the nauplii (Figure 10). After enriching the nauplii for a total of at least 19 hours, they are harvested, rinsed with fresh water and then distributed to the larval tanks.

FIGURE 10
***Artemia* enrichment**



Source: elaborated by the authors.

3. Broodstock management

3.1 BROODSTOCK MANAGEMENT AND REPRODUCTION

3.1.1 Wild-caught broodstock management

It is possible to obtain eggs and sperm for the seed production of turbot using both wild-caught and pond-reared broodstock (Annex 3). Initially, however, eggs are collected from wild spawners until the hatchery-bred broodstock mature.

To achieve successful turbot production, it is crucial that they be high-quality fertilized eggs. In this regard, broodstock management is extremely important.

Furthermore, hormone application and artificial insemination are essential techniques for ensuring the effective maturation of gametes (eggs and spermatozoa).

Wild capture and transportation

Migration

The turbot is a typical demersal fish that lives on sandy, muddy-sandy or gravel bottoms. In the Black Sea, turbot can be found at depths of up to 120 m and migrate towards the shoreline (25–50 m) to spawn in the spring. Spawning takes place in batches over ten weeks between March and June, depending on the water temperature. During this period, the surface water temperature in the eastern Black Sea (Trabzon, Türkiye shore) can increase from 10.2 °C in March to 16.9 °C in May.

Capture

Turbot are caught by commercial fishers with gillnets (mesh size: 20 cm; length: 1 000 m; and height: 1.4 m). As this process is stressful for fish, which can in turn affect the condition of the fish and the quality of the eggs, gillnets should not be left in the sea for a long time (ideally less than three to seven days). Alternatively, trawls can be used to capture brood fish in order to reduce the time fish spend in the net and help prevent the deterioration of their physical condition and their seed. Once caught, brood fish should be transferred immediately into containers with aeration (oxygenation using a diffuser) in the fishing boat and kept there until transfer to the hatchery (Plate 23).

Transportation

Fibreglass tanks with dimensions of 1 m × 1 m × 0.5 m are used to transport brood fish (Plate 24). Once each tank is filled with seawater, aeration is provided. Since the turbot is a calm and relatively inactive fish species, there is no need to use an anaesthetic during transportation. Approximately 3–6 brood fish/m^3, ranging from 2–7 kg each, can be stocked in the tank. Long-distance transportation of turbot is possible when the tank is aerated with pure oxygen (8–10 mg/litre). Nevertheless, it is important that the captured breeders are transported to the hatchery as soon as possible.

PLATE 23

Onboard containers for wild Black Sea turbot capture

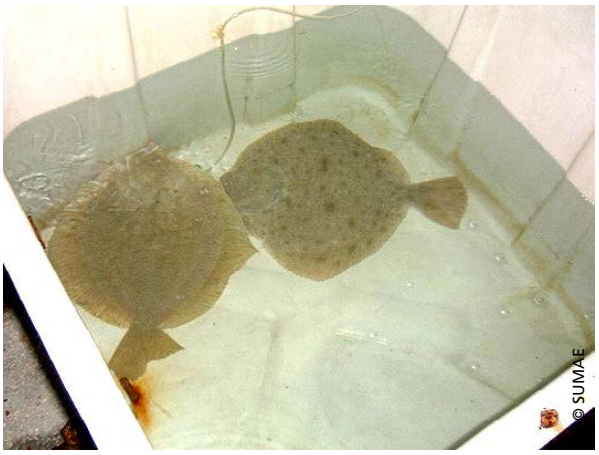


PLATE 24
Fibreglass brood fish transportation tank

PLATE 25
Black Sea turbot brood fish visual inspection

3.1.2 Broodstock selection

An initial examination of the fish is necessary in order to select those of good quality that can be used in production and to minimize disease infection risks from nature to the hatchery (Plate 25).

Upon arrival at the hatchery, each fish is placed in a separate container with roughly 50 litres of seawater and water exchange of 4 litres/minute with one air stone. It is at this stage that the selection of brood fish is carried out. If necessary, clove oil can be used in quantities of 180–220 mg/litre as an anaesthetic during selection (Aydın *et al.*, 2015).

The criteria used in the selection of broodstock are as follows:

- whether the fish is healthy or not – there should be no wounds, bleeding or lesions on the skin and fins, or redness in the mouth;
- whether the fish is morphologically normal or not – the body form should be asymmetric and almost round, with both eyes on the left side of the head, the blind side (right) a whitish colour, and the eye side generally a grey–brownish colour with dark spots; and
- whether the fish is injured or not – especially those injuries caused by gillnet damage along the margin of the genital pore.

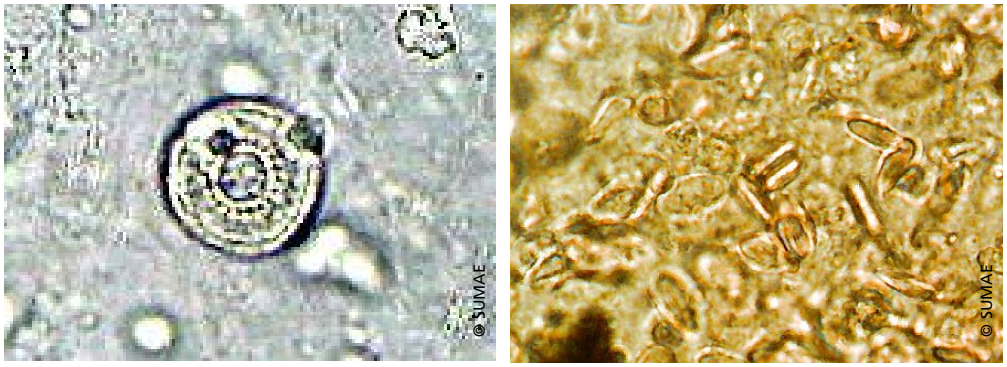
Disease diagnosis and prevention

Brood fish candidates from nature should be visually inspected for parasitic species such as trematodes and nematodes. These parasites are visibly large and can be found on the skin, gills and fins. In addition, a mucous sample from the gills and skin should be examined using a microscope as some species of protozoa such as *Trichodina* spp., *Ichthyobodo* spp. (Plate 26) and those in the subclass Scuticociliatia are common in turbot.

The selected fish are placed in quarantine tanks (1 m × 2 m × 0.5 m) for 24 hours at a stocking density of 4–8 kg/m^3 for general observation and disease treatment, after which they are transferred into broodstock tanks.

The selection and use of antimicrobial drugs are also extremely important in preventing disease transmission to the hatchery. For this purpose, each fish is treated using a one-hour long formaldehyde (100 ppm) bath followed by a one-hour long

PLATE 26
Parasites



Notes: *Trichodina* sp. (left); *Ichthyobodo* sp. (right).

furazolidone (20 ppm) bath each in a 40-litre plastic basin filled with seawater (Plate 27). During treatment, gentle aeration is provided at a rate of 0.6 litres/minute.

PLATE 27
Antimicrobial treatment for a Black Sea turbot brood fish

3.1.3 Fish selection

Sex identification

During sex identification, fish with swollen and slightly saggy abdomens are designated as female and those with flat, yet hard abdomens are designated as male. In the cases where gender cannot be determined from abdominal morphology, the illumination method is used (Plate 28). For this method, the fish is held approximately 15 cm away from a light box with a warm white light source (1 200 lumen) and the gonadal region is examined.

PLATE 28
Black Sea turbot sex identification

Notes: Control under light (left); female (top right); male (bottom right).

PLATE 29
Sperm stripping of a male Black Sea turbot

PLATE 30
Gonadal tissue sampling: cannulation method

Sexual maturity determination

For males, a gentle pressure is applied a few times to determine sexual maturity, starting from the area just prior to the pelvic fins and ending near the urogenital pore. If the fish is mature, milt usually extrudes from the urogenital pore (Plate 29). If it does not, these fish are either immature or have already extruded the milt and they cannot be used to fertilize the eggs. Instead, these fish may be used for fertilization in the following years.

For females, eggs can be obtained by hand stripping or the cannulation method. In mature females, when the abdominal area is gently pressed, the eggs easily extrude from the genital pore. In these cases, the fish should be stripped immediately. In non-mature fish, even if the abdomen is pressed strongly, the eggs may not extrude. In these cases, a gonad (oocyte) sample can be taken with a cannula. The cannulation is performed using a thin 50 cm long polyethylene tube (0.7 mm or 1.5 mm in diameter). The tube is gently placed into the genital tract (Plate 30) and the oocyte samples are siphoned into the tube, and subsequently the cannula is removed. The oocyte samples are poured into small bottles filled with Ringer's solution for turbot and examined under a microscope.

Maturity evaluation: gonadal sample analysis

For males, sperm activity is examined under a microscope at 100 times magnification. Initially, one drop of seawater is placed on a glass slide and a small amount of milt is added and observed under the microscope. Normal viability of sperm is characterized by whirl-like movement for 20 minutes after the mixing of seawater with the milt. If sperm motility is 70 percent and above, it is considered to be good quality sperm and can be used in artificial insemination.

For females, a small amount of eggs are transferred onto a glass slide to measure their diameters. Measurements can be carried out under a microscope at 40 times magnification (Plate 31). If the fish has oocytes greater than 400 µm in mean diameter (n = 100), it can be used for spawning. Those with oocytes of less than 400 µm in mean diameter (n = 100) will not be used and are kept as broodstock.

3.1.4 Stocking to maturation tank

Spawning tank

The maturation tank is made of FRP and has dimensions of 1 m × 2 m × 0.5 m (Plate 32). Light intensity in the room in which the tank is located is controlled at around 100 lux using fluorescent bulbs; however, during the daytime the room is subjected to ambient conditions. An adequate water exchange rate is approximately 900 percent in a day and aeration is 4 litres/minute with one air stone per cubic metre. During the spawning period, the temperature is controlled at 14 °C.

PLATE 31
Egg sample measurement using a microscope

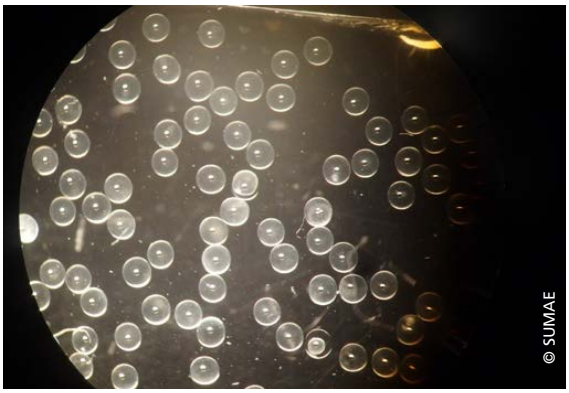


PLATE 32
Maturation tanks for artificial insemination

3.1.5 Broodstock stocking and management

The spawners are stocked at two to four fish (2–7 kg/individual) per cubic metre in a maturation tank. Overstocking should be avoided to prevent overcrowding stress on the broodstock. The light intensity is kept between 400 lux and 600 lux, with the duration of the light phase corresponding to the local number of daylight hours. The broodstock are fed with commercial pellets or raw fish (whiting, horse mackerel, etc.) to satiation three times a week.

3.1.6 Induced spawning and artificial insemination

Hormone preparation

During turbot production, the application of hormones to female turbot can initiate or accelerate the last stages (oocyte diameter > 400 µm) of the maturation of eggs. For male turbot, which often produce very small quantities of milt and can be difficult to strip, hormone implants or injections can foster an increase in sperm volume.

For males, human chorionic gonadotropin (HCG) and white salmon pituitary gland (WSPG) are mixed well and homogenized in a ceramic bowl. The hormone preparation is injected into the male fish as soon as possible.

PLATE 33
Luteinizing hormone-releasing hormone analogue pellet preparation

For females, 5 mg of luteinizing hormone-releasing hormone analogue (LHRHa) and 1 ml of 60 percent ethanol are mixed in a ceramic bowl, and 625 mg of cholesterol is added and mixed well (Crim, Peter and Van Der Kraak, 1987). The following day, 125 mg of cacao butter is added and mixed thoroughly. Using pellet moulds, 30 mg of this preparation is made into individual pellets (Plate 33). Each pellet contains approximately 200 µg LHRHa hormone and is stored in a glass bottle that is kept in a freezer at 20 °C until time for use.

Hormonal treatment

Only mature males with milt and maturing females with oocytes greater than 0.4 mm in diameter are used for hormonal treatments.

Dosage

For males, the optimal dosage is 500 IU HCG and 7 mg WSPG/kg of fish weight. A single treatment is sufficient and the response time is around one week.

For females, the optimal dosage is 100 µg pelleted LHRHa/kg of fish weight. A single treatment is sufficient and the response time is around 24 hours.

Administration method

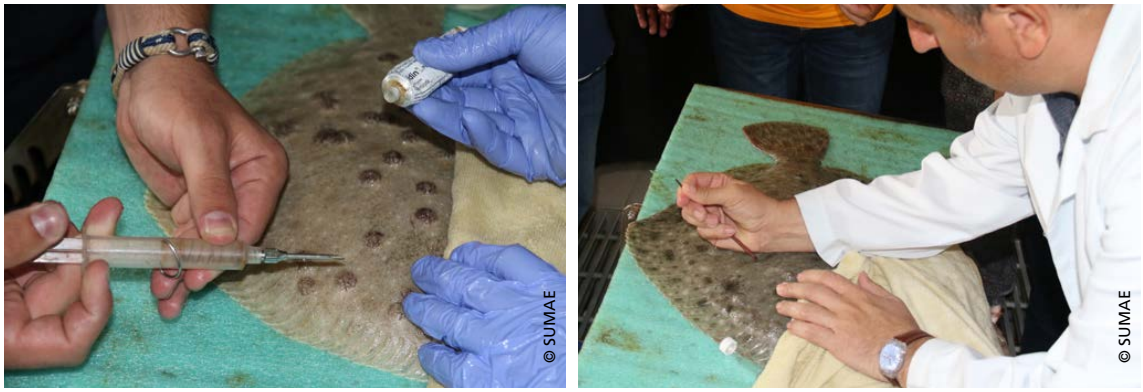
For males, injection is carried out using a 10 ml syringe with an 18-gauge needle into the dorsal muscle near the dorsal fin (Plate 34).

For females, LHRHa in pellet form is implanted into the muscle near the dorsal fin using a metal tube (Plate 34).

3.1.7 Artificial insemination

Eggs are stripped by applying gentle abdominal pressing towards the genital pore (Plate 35). If blood accompanies the stripped eggs, egg collection must be stopped. It is necessary to compress the abdomen of the female at regular intervals in order to observe if ovulation is occurring and then eggs can be collected daily after the first ovulation. This collection period can continue for 7–15 days. On average, the relative fecundity is approximately 826 000 eggs/kg of fish (Plate 35).

PLATE 34
Hormone administration



Notes: Injection into a male (left); implant application for a female (right).

Both dry and wet fertilization methods can be used in the artificial insemination of turbot eggs. For fertilization with the dry method, eggs are stripped into a dry plastic container. Semen is added and gently mixed with the stripped eggs using a glass rod. The optimum amount of semen is 1 ml/400 g of eggs. There are approximately 900 eggs/g. Afterwards, a small quantity of seawater is added for effective fertilization. Eggs are kept for approximately ten minutes in this mixing container to allow sperm to fertilize the oocytes (Plate 36). In contrast, the wet fertilization method involves stripping the

PLATE 35
Artificial insemination process

Notes: Egg stripping (top); egg batch collection (middle); egg evaluation under the stereo microscope (bottom).

PLATE 36
Dry artificial fertilization: mixing the gametes

PLATE 37
Seawater filtration system

eggs into a container with seawater, rather than into an empty container, and adding semen in the same manner as described above.

3.2 INCUBATION

Careful management of egg incubation is extremely important so as not to degrade the quality of eggs. This includes the cleaning and disinfection of hatchery materials, use of ultraviolet (UV)-treated filtered seawater and daily monitoring of the physicochemical parameters during the entire incubation period (Plate 37).

3.2.1 Egg disinfection

To prevent microbial (bacterial diseases: *Vibrio* spp., *Streptococcus* spp., *Aeromonas salmonicida* subsp. *Salmonicida*; and parasitic diseases: scuticociliatosis) or viral (viral haemorrhagic septicaemia virus) contaminations, the eggs are disinfected with an iodine solution as described below.

Ten minutes after insemination, eggs are collected using nylon nets (mesh size: 220 µm) and rinsed with seawater heated to 14 °C to remove remaining sperm, body fluid and mucus (Plate 38). The eggs are then transferred to buckets and immersed in 50 ppm povidone-iodine for five minutes (Plate 38) before being gently rinsed with heated seawater to remove the solution. Finally, the eggs are transferred to an incubation tank which is mildly aerated at approximately 0.6 litres/minute.

3.2.2 Egg incubation

The incubation tanks can vary in size, e.g. 30, 50 and 100 litres, depending on the quantity of eggs. The tanks are cylindro-conical with a central drain to which a strainer is attached. The strainer is made of polyvinyl chloride (PVC) pipe (3 cm in diameter) covered by a polyethylene net (mesh size: 8 mm) and a plankton net (mesh size: 520 µm) to prevent the passage of eggs (Plate 39). The water volume in the tank is controlled by changing the position of the drain hose.

PLATE 38
Black Sea turbot egg disinfection protocol

Notes: Cleaning (left); disinfection (right).

PLATE 39
Turbot egg incubators

The water in the incubators is filtered using a 1-µm filter and a UV sterilizer. In addition, the water exchange rate is adjusted to 2 000 percent or 20 times/day. The stocking density is approximately 2 000 eggs/litre, while the water temperature should be kept at 14 °C. Aeration is provided at a rate of approximately 0.6 litres/minute in order to suspend the eggs in the water column. However, if the aeration is too strong or the water exchange rate too high, the eggs can be damaged through contact with the tank walls. The illumination is kept at around 100 lux in the daytime.

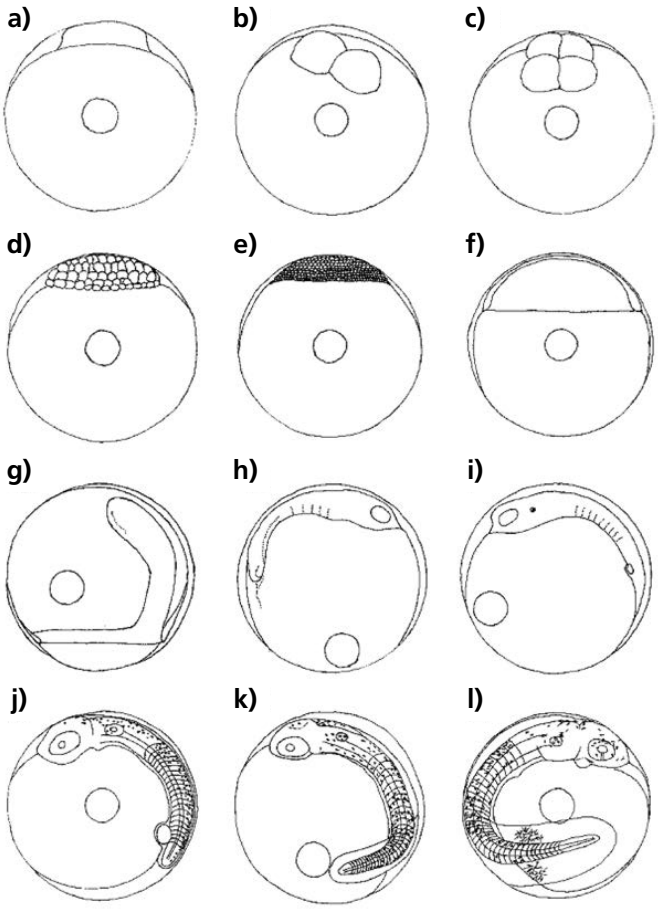
3.2.3 Egg development

The fertilized eggs are spherical, pelagic and non-adhesive, with a single oil globule and narrow perivitelline space, no special structures on the chorion and a diameter of approximately 1.08–1.21 mm. The developmental stages are shown in detail in Figure 11. Hatching occurs approximately 110 hours (64 degree-days) after fertilization at temperatures of 14 °C.

3.2.4 Fertilization rate estimation

The fertilization rate can be estimated at the four-cell stage, three hours after fertilization at 14 °C (Plate 40).

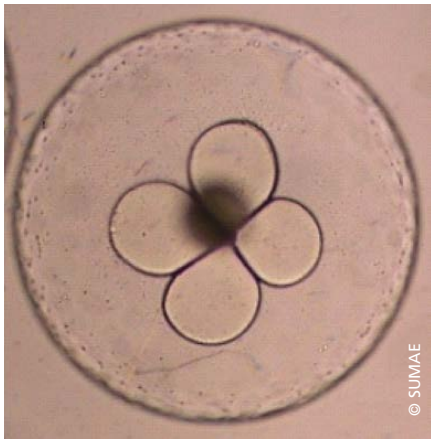
FIGURE 11
Black Sea turbot egg development



Notes: (a) blastodisc formation; (b) two-cell stage, 2.5 hours after insemination; (c) four-cell stage, 3 hours; (d) morula stage, 9.5 hours; (e) blastula stage, 12 hours; (f) gastrula stage, 26.5 hours; (g) embryo formation, 35 hours; (h) eye vesicle formation, 40 hours; Kupffer's vesicle appeared, 42.5 hours; formation of four myomeres, 43 hours; (i) blastopore closed, 47 hours; (j) melanophores appeared on the embryo, 52 hours; optic lens formation and auditory vesicle appeared, 58 hours; (k) disappearance of Kupffer's vesicle; erythrophores appeared on the embryo; oocyte formation; heart begins to beat and embryo begins to move, 64 hours; (l) just before hatching with incubation temperature at 14.3–15.3 °C, 107 hours.

Source: elaborated by the authors.

PLATE 40
Four-cell egg stage for Black Sea turbot



To estimate the fertilization rate, three 50-ml samples are taken from a gently aerated incubator. The samples are examined and counted under a microscope. Subsequently, the fertilization rate is calculated using the following equation:

$$\textit{Fertilization rate}\ (\%) = \frac{\textit{mean number of fertilized eggs}}{\textit{mean number of total eggs}} \times 100$$

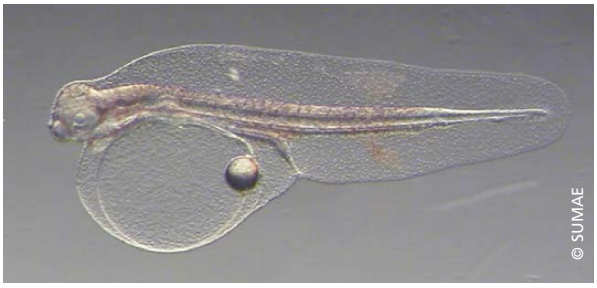
If the fertilization rate of an egg batch is less than 50 percent, incubation is stopped.

3.2.5 Incubation tank management

Whitish-coloured sunken eggs are usually considered dead, although there can still be a few viable eggs among them. In general, viable eggs float on the water surface or in the water column. To remove dead eggs, the aeration

and water supply is stopped for a few minutes. As a result, the dead eggs will settle to the bottom and can then be siphoned out to minimize bacterial and protozoan growth.

PLATE 41
Newly hatched Black Sea turbot larva



3.2.6 Hatching rate estimation

The hatching rate is estimated based on the ratio of hatched out larvae (Plate 41) and the total number of counted eggs.

To begin, three 50-ml samples are taken from a gently aerated incubator or rearing water using beakers and the number of hatched larvae in each sample is counted under a stereo microscope. The total number of hatched larvae in the tank is then calculated using the following equation:

$$\textit{Total number of hatched larvae} = \frac{\textit{mean number of larvae}}{\textit{sample volume}} \times \text{water volume of hatching tank}$$

Subsequently, the total number of eggs in an incubator is calculated using the following equation:

$$\textit{Total number of eggs} = \frac{\textit{mean number of total eggs}}{\textit{sample volume}} \times \text{water volume of incubator}$$

Finally, the hatching rate, is determined using the following equation:

$$\textit{Hatching rate} (\%) = \frac{\textit{total number of hatched larvae}}{\textit{Total number of eggs}} \times 100$$

Only batches with a hatching rate higher than 70 percent are transferred to the larval rearing tank.

4. Larval and juvenile rearing

4.1 LARVAL REARING

During metamorphosis, turbot larvae exhibit radical changes in their shape as they transform to the juvenile stage.

At the end of metamorphosis, any fish that are deformed or malpigmented will have a low commercial value, but generally they constitute only a small percent of production.

4.1.1 Morphological development

To determine the stage of morphological development, the larvae are randomly sampled from the middle of the water column using 100-ml beakers and analysed under a stereo microscope (Plate 42).

PLATE 42
Visual analysis of Black Sea turbot larvae under a stereo microscope

The morphological development stages of turbot larvae during the approximate 70-day rearing period at water temperatures of 16–19 °C are divided into three stages as follows:

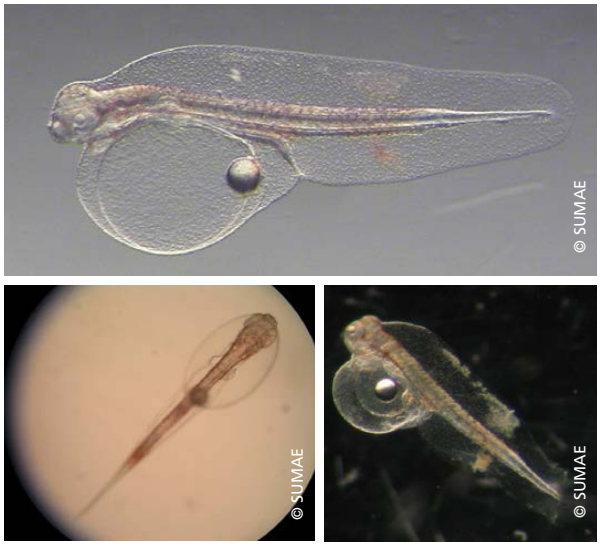
Pre-larval stage
(0–2 days post-hatching)

Larvae are symmetrical in shape, with the yolk sac and oil globule present. Newly hatched larvae are 2.6 mm in mean total length. The eyes are not pigmented, the mouth is not yet open and the anus is not yet formed (Plate 43). The larvae grow rapidly following yolk absorption.

Post-larval stage
(3–29 days post-hatching)

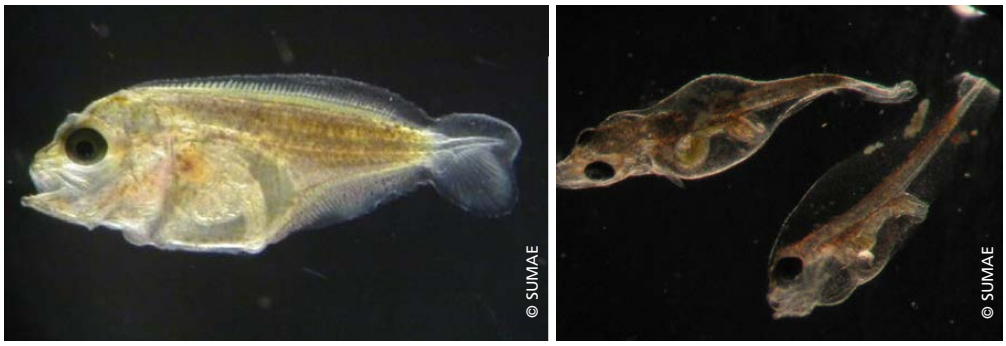
On day post-hatching (D)-3, the eyes of the larvae are pigmented and the mouth (0.15 mm in width) and anus are open (Plate 44). The

PLATE 43
Pre-larval stage (0 days post-hatching) of Black Sea turbot



Notes: Normal larvae (top and bottom left); larva with abnormal yolk sac and oil globule position (bottom right).

PLATE 44
Post-larval stage of Black Sea turbot



Notes: Normal larva (12 days post-hatching) (left); larvae with abnormal body structure and dropsy (8 days post-hatching) (right).

PLATE 45
Metamorphosis stage (30 days post-hatching) of Black Sea turbot

mouth width increases as larvae grow. At this stage, external feeding begins, though carnivorous behaviour does not occur often.

Metamorphosis stage (30–70 days post-hatching)

This stage involves the transition from larvae to juvenile fish. By D-40, fish attain 20 mm total length (TL) at a rearing temperature of 20 °C and become asymmetrical in shape. The eye begins to migrate from the blind side to the upper side (Plate 45) and fish start to settle down on the bottom of the rearing tank. By D 70, eye migration is complete and larvae are 50 mm TL.

4.1.2 Tanks and facilities

Tanks

The shape of the tanks varies and can be circular, square or a raceway. The size of the larval rearing tanks ranges from 0.2 m^3 to 8 m^3, with a depth of 0.5 m to 1 m depending on the aims (Plate 46). Discharge occurs in the middle of the tank.

PLATE 46
Larval rearing tanks

Aeration

It is recommended that many air stones are used to provide gentle aeration (2.5 litres/minute) rather than only a few ones with strong aeration. As such, 4–6 air stones/m^3 should be hung around the walls and centre of the tank.

Water quality

Water is filtered by a 50 µm sand filter, 5–20 µm cartridge and UV filters in order to obtain suitable larval rearing conditions. The rearing water is heated either by putting the heater inside the tanks or by using water that has already been heated (Plate 47).

PLATE 47
Rearing water heating method

Notes: Use of heater inside the tank (left); use of pre-heated water (right).

Illumination

It is recommended that 24-hour illumination at an intensity of 300–600 lux using fluorescent lamps or bulbs is used (Plate 48).

PLATE 48
Larval rearing tank illumination

PLATE 49
Larvae transfer

4.1.3 Stocking density and method

The initial larval stocking density is around 30 000 individuals/m^3. Before transferring newly hatched larvae to the rearing tanks, the water temperature of the rearing tank is set at the same temperature as the incubation tanks. Just prior to transfer, the undeveloped eggs and detritus are removed using a hose connected to the bottom of the incubator and the larvae are transferred gently to the rearing tanks using a bucket (Plate 49).

4.1.4 Feeding

Feeding schedule

Three types of feed are used for the fish larvae: the rotifer *Brachionus plicatilis*, the brine shrimp *Artemia* sp. and artificial diets (Table 4).

TABLE 4
Feeding regime for rearing larval Black Sea turbot

Day		3–4	5–6	7–12	13–15	16–17	18–25	26–30	31–35	36–40	n+1
Algae (cells/ml)			0.5×10^6								
Rotifer (number/ml)		2	3	4	3						
Artemia nauplii (number/ml)					0.3						
Artemia metanauplii (number/ml)						0.3	2	4	3	2	1	
Artificial diet	Size of diet particles (µm)								250	300–500	500–800	> 800
	Quantity (g/10 × 10^3 larvae)								10	15	18	

Source: elaborated by the authors.

Rotifers

From D-3 to D-15, when the mouths of the larvae have opened, enriched rotifers are given as feed. The density of rotifers in the rearing tank depends on the larval development stage and is maintained at 2 to 4 rotifers/ml, with the density checked four times per day. The green algae, *Nannochloropsis* sp., which serve as food for the rotifers, are maintained at 0.5 million cells/ml in the rearing tank.

Artemia

Between D-8 and D-17, newly hatched *Artemia* nauplii are given to the fish larvae. From D-13 to D-40, enriched *Artemia* are provided, the density of which increases from 0.3 to 1.0 individual/ml by D-40.

Artificial feed

Microdiets are initially fed to the larvae on D-26 (Table 5). However, when *Artemia* are available in the rearing water, larvae prefer live food rather than microdiets. For this reason, microdiets need to be used in adequate quantities and their quality should be carefully considered as it affects larval survival and growth rates. In addition, microdiets must meet the nutritional requirements of the larvae and when suspended in the water column for several minutes, nutrients should neither leach out immediately into the water, nor disintegrate quickly.

TABLE 5
Artificial diet feeding rate for different sizes of Black Sea turbot larvae

Total length (mm)	Total weight (mg)	Feeding rate (%)	Size of diet particles (mm)
8–11	40–60	10	0.25
12–17	100–120	7	0.3–0.5
18–20	130–160	7	0.5–0.8

Source: elaborated by the authors.

4.1.5 Tank management

Temperature

Larvae are less tolerant to changes in water temperature during the early stages of growth and as such, the water temperature of the larval tanks should be the same as that of the incubation tank. After stocking, the water temperature is gradually increased from 15 °C to 21 °C over four days.

Water exchange

No water exchange is applied until D-3, at which time the water is changed gently at a rate of 0.4 times/day to avoid water quality deterioration, using plastic pipes submerged into the tank water. The water change rate is gradually increased to 3 times/day on D-10. The PVC drainage pipe is perforated and wrapped with a polyethylene net with an adequate mesh size (Plate 50). As the fish grow, polyethylene nets with a larger mesh size should be used.

PLATE 50
Drainage for water exchange

Bottom cleaning

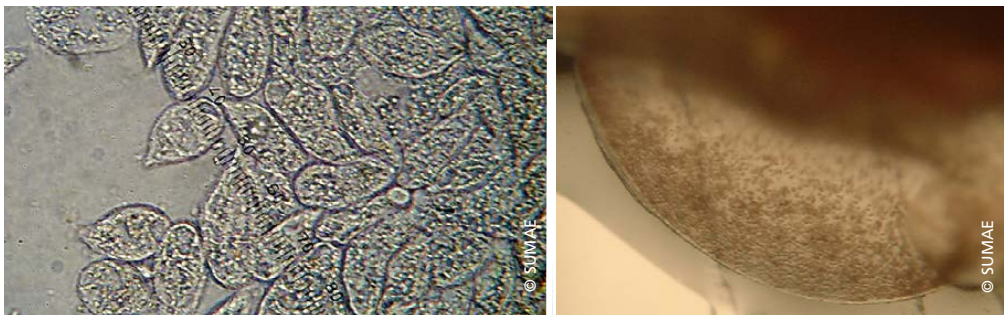
Tank bottom cleaning starts on D-10 using a cleaner to remove dead larvae, uneaten food and faecal matter. The cleaner is made of a PVC pipe (20 mm in diameter) with a suction mouth attached to one end and a flexible hose on the other. A piece of sponge is attached to the suction mouth in order to clean the bottom more effectively (Plate 51). During siphoning, aeration is stopped and care must be taken not to stir up the organic matter on the bottom or to siphon larvae swimming near the bottom. Dead or escaped larvae are collected in a plastic container.

PLATE 51
Tank siphoning equipment

4.1.6 Disease control

Larvae are highly susceptible to parasites and diseases. Pathogenic parasites, especially in the Scuticociliata subclass, are often observed in the detritus or in dead fish collected from the tank bottom. Unfortunately, scuticociliates comprise one of the most serious groups of parasites and can cause mass mortality. As detection of these issues though external observation may be difficult during the larval stage, regular microscopic examination should be carried out for the larvae and detritus collected from the tank bottom. In addition, to prevent the spread of these pathogens, routine cleaning of the tank bottom is crucial. Moreover, the water exchange rate should be increased to 4–5 times/day to improve water quality. If ciliates are detected outside or inside the larval bodies (Plate 52), or if the density of protozoans increases in the detritus, the

PLATE 52
Parasitic scuticociliate infestation



Notes: Outside of larvae (left); inside of larvae (right).

fish larvae should be immediately medicated with a 20–40 ppm formalin bath (37–40 percent) for two hours.

4.1.7 Grading

Although cannibalism is not common, fish are sorted in order to minimize any disturbance of smaller fish caused by larger ones and to enable efficient feeding. The first grading is carried out when fish reach around 20 mm TL (D-40) using a selector made of a plastic or nylon material (Plate 53). During this process, fish are scooped out with a bucket and poured into the selector, which has been set in a new tank.

PLATE 53
Black Sea turbot larvae grading equipment

4.2 JUVENILE REARING

Nursery rearing starts around D-40, although at this stage fish are still sensitive to stress from handling and transport. This stage lasts around 110 days during which fish reach approximately 100 mm TL with a survival rate of over 80 percent.

4.2.1 Tanks and facilities

Since larvae start to settle on the tank bottom at 20 mm TL, the surface area of the tank bottom becomes more important than the tank volume. The stocking density of fish is calculated based on the surface area from this phase onwards. Fish are reared in FRP tanks with a water depth of 0.5 m. The tank can be circular, square or raceway in shape, with an area of around 5–8 m^3. The tank should drain the waste in the rearing water and the detritus on the tank bottom using water circulation and perforated PVC pipes (Plate 54). The rearing tanks are aerated with 2–4 air stones/m^3 that are placed near the walls and centre of the tank. Illumination is maintained at 300–600 lux with a fluorescent lamp placed above the rearing tanks between 07.00 and 19.00 hours. Oxygen levels should not drop below 4 mg/litre.

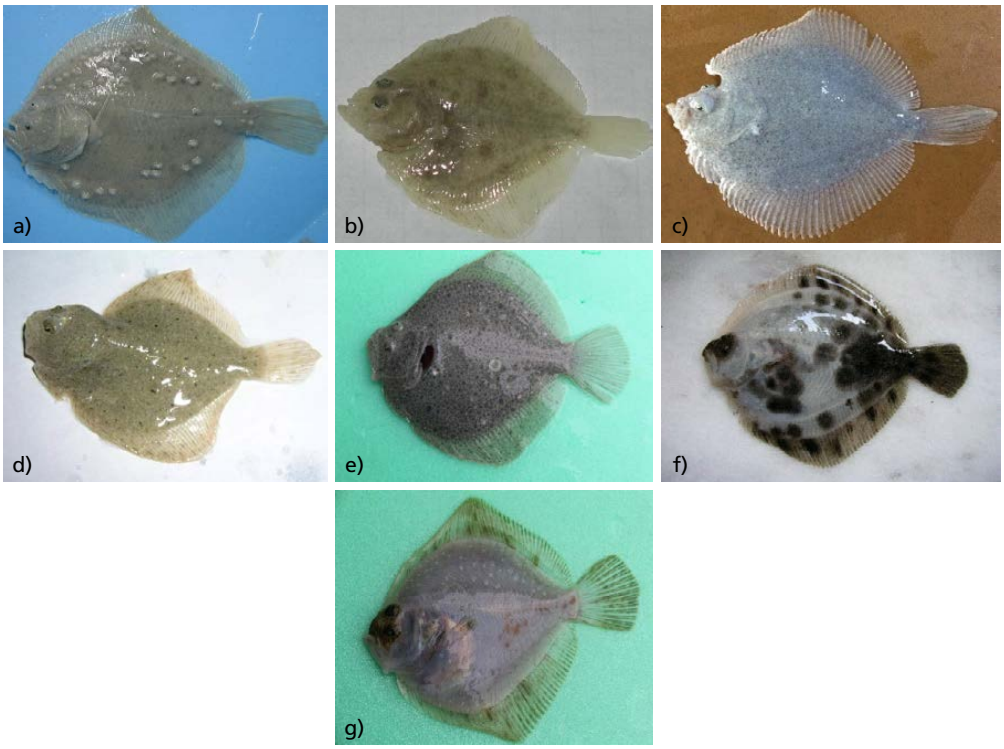
PLATE 54
Drainpipe for tank-water discharge

4.2.2 Grading and stocking

The second grading is carried out when the fish are around 80 mm TL (D-100). During grading, the exact stock number is determined, the tank is cleaned, and normal juveniles are separated from malpigmented or deformed juveniles using a small basket (Plate 55). Overall, the ratio of normal larvae is above 95 percent; however, a small number of abnormalities can occur, including of the lower jaw length, immigration eye, the body shape, gill shortage, and malpigmentation such as semi-colouring and colourlessness (Plate 56). The stocking density changes depending on the size of the fish (Table 6).

PLATE 55
Hatchery-reared Black Sea turbot selection

PLATE 56
Juvenile Black Sea turbot selection



Notes: (a) normal; (b, c, d, e) deformed; (f, g) malpigmented.

TABLE 6
Stocking density of Black Sea turbot juveniles

Total length (mm)	Stocking density (individuals/m³)
20–50	400–500
50–80	250–300
80–100	120–150

Source: elaborated by the authors.

4.2.3 Feeding

Pellets providing nutritionally complete diets with a diameter of 0.8–1 mm are used at the start of juvenile rearing. As fish grow, the pellet diameter can be gradually increased, with fish ranging from 20 to 50 mm TL being fed five times per day. When fish exceed 50 mm TL, the feeding frequency is decreased to three times per day and fed until apparent satiation. The feeding rate of juvenile turbot starts at 5 percent of body weight for 20 mm TL and gradually decreases to 2 percent of body weight for 100 mm TL. A recommended feeding regime and pellet sizes for turbot juveniles are given in Table 7. As such, juvenile turbot require high crude protein content at around 55 percent and a dietary lipid requirement below 15 percent. To achieve these dietary needs, high quality ingredients such as fishmeal and fish oil (for example, obtained from whiting) are used.

TABLE 7
Feeding rates and pellet sizes for different sizes of Black Sea turbot juveniles

Total length (mm)	Feeding rate (percent of body weight)	Size of pellet (mm)
21–24	5	0.8–1.0
25–30	5	1.0–1.2
31–35	4	1.2–1.5
36–40	3	1.5
41–55	3	2.0
56–80	3	2.0–3.0
81–100	2	3.0

Source: elaborated by the authors.

4.2.4 Tank management

Since juvenile fish are fed an artificial diet during the nursery stage and the amount of food is increased with fish growth, there is an increasing likelihood for water deterioration. Therefore, the water exchange rate is 10–15 times per day throughout the nursery phase. The temperature and salinity of the rearing water range from 18 °C to 20 °C and from 18 psu to 19 psu, respectively. It is recommended to clean the bottom of the tank twice a day.

4.2.5. Growth

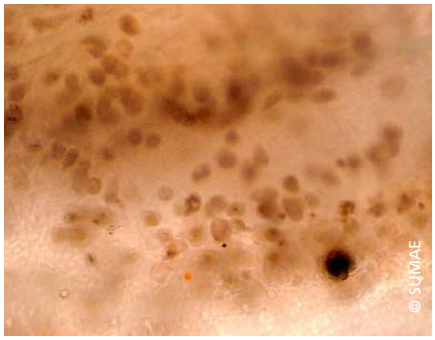
The growth of juveniles is affected by tank management, quality of feed and initial condition of the stocked juveniles. In normal conditions, juvenile fish with 20 mm TL can grow to 50 mm TL in approximately 70 days. Regular measurement of the weight or length of juveniles is highly recommended. If the growth of juveniles tends to be stagnant, the rearing conditions and health of the fish should be checked.

4.2.6 Disease control

Pathogenic parasites – Nematoda, *Trichodina* and, in particular, Scuticociliata – are often found in the detritus gathered from bottom cleaning or on dead juveniles. Therefore, protozoan density of all tanks is checked every day under a microscope. If scuticociliates are abundant in the detritus or the base of fins, around the eyes, under the skin or in the abdominal cavity of dead or live juveniles, then all juveniles in the tank are immediately treated with a bath of 100 ppm formalin and 0.5–1.0 ppm $CuSO_4 + 5H_2O$ for two hours (Plate 57). This treatment is continued for at least three days.

If there is redness on the fins and a reduction in feed intake, a bacterial disease can be suspected. In cases of bacterial infection, an antibacterial treatment is applied through

PLATE 57
Scuticociliate parasites under the skin of a Black Sea turbot



feed or injection. For example, 10 mg of florfenicol or 20 mg of enrofloxacin per kg of fish are added to the daily diets. This treatment is continued for eight to ten days. In addition, the tanks are treated with a bath of chloramine T (15 mg/tonne of water) for two hours.

4.2.7 Harvesting and transportation

Juveniles with 50 mm TL are resilient to handling. Therefore, they can be harvested using a scoop net after reducing the level of the rearing water. During this process, juveniles are graded into three groups: normal, abnormal and malpigmented. Fish smaller than 50 mm TL are put directly into the transport tank, but for those longer than 50 mm TL, it is recommended to put them into a plastic basket fixed within the tank to prevent it from overturning (Plate 58). However, fish should not be fed at least 24 hours prior to transport in order to empty their digestive system and slow their metabolism which in turn reduces waste production during transport and maintains stable water quality (Saraiva *et al.*, 2021).

PLATE 58
Plastic baskets used for transporting live Black Sea turbot

PLATE 59
Interior of live Black Sea turbot transportation vehicle

For local- or long-distance transport, larger sized tanks with oxygenation, aeration and refrigeration systems can be used (Plate 59). The temperature of the transport tank is maintained at 5 °C lower than the rearing tanks and the oxygen concentration is held at over 4 mg/litre. Transport density depends on the size of the fish. Generally, the stocking density is 5 000 juveniles/m^3 for those with 50 mm TL, and 1 500 fish/m^3 for those with 100 mm TL. Finally, upon arrival at the fish farm, the transport water temperature should be made to match that of the farm. After transport, fish should not be fed for a few days until the stress of the move subsides.

5. Biotechnological applications for Black Sea turbot

While conventional turbot production methods continue to be used, today several biotechnological methods including cryopreservation, triploidy and all-female stock production are also being implemented.

5.1 CRYOPRESERVATION

During turbot aquaculture with spontaneous spawning, both egg quality and quantity can be variable. Thus, stripping females and using artificial insemination are recommended for controlled production. Since turbot are considered to be poor sperm producers in terms of spermatozoa concentration and semen volume, the improvement in sperm handling techniques has been a prerequisite for the improvement of artificial reproduction (Cabrita, Robles and Herráez, 2008).

Semen cryopreservation (commonly called sperm banking) is a procedure used to successfully preserve sperm cells for many years. For instance, the longest reported successful storage of human sperm is 24 years (Cabrita, Robles and Herráez, 2008).

The advantages of the use of cryopreservation for aquaculture include:

- Breeders no longer need to be kept available for artificial fertilization since gametes can be stored.
- Semen collection, especially of wild fish, can be extended over multiple days before artificial fertilization is carried out in adapted facilities.
- Gametes can be shipped from the fish to distant breeding sites.
- Some pathological diagnoses can be carried out on the gametes and the results obtained before the gametes are released for use (Cabrita, Robles and Herráez, 2008).

5.1.1 Freezing and thawing protocol

Only sperm samples with high motility (> 70 percent) are used for freezing. To begin the cryopreservation process, sperm is sucked into a syringe and immediately stored at 4 °C since the percentage of motile cells significantly decreases after 5 minutes at 15 °C. Semen is then diluted at a 1:2 ratio (sperm : diluent) with a modified Mounib's extender (100 mM of $KHCO_3$, 125 mM of sucrose, 6 mM of reduced glutathione, and 10 mg/ml of bovine serum albumin; pH 7.8), supplemented with 10 percent dimethylsulphoxide. Subsequently, samples are sucked into 200 µl straws, placed on a floating tray in nitrogen vapour and, after 15 minutes, plunged into liquid nitrogen. To thaw the straws, they are placed in a water bath at 30 °C.

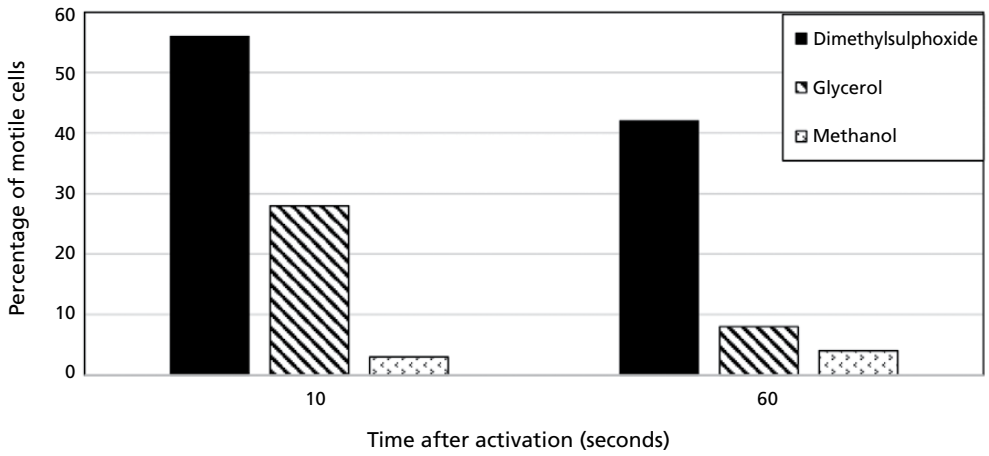
Compared to fresh sperm, 60–80 percent of thawed spermatozoa can be reactivated, although the fertilization capacity of thawed sperm decreases at low sperm to egg ratios (Figure 12). However, no differences were observed using 20 000 sperm/egg in terms of hatching and larval survival rates (Cabrita, Robles and Herráez, 2008).

PLATE 60

Black Sea turbot sperm cryopreservation process

Notes: (a) collection; (b) quality determination with computer assisted sperm analysis system; (c) dilution with extender; (d) insertion into straws; (e) cooling with nitrogen vapour; (f) plunging into liquid nitrogen.

FIGURE 12
Cryoprotectant (concentration: 10 percent) effect on thawed turbot sperm motility



Source: **Cabrita, E., Robles, V. & Herráez, P., eds.** 2008. *Methods in reproductive aquaculture: marine and freshwater species*. Boca Raton, United States of America, CRC Press.

5.2 TRIPLOIDY

Polyploidy can be defined as organisms having one or more additional sets of chromosomes. In the cell nuclei of normal (diploid) fish, there are two sets of chromosomes (2n) and in triploid fish, three sets of chromosomes (3n).

Triploidy is advantageous in fish farming for many reasons, including increased growth, carcass weight, survival rate and quality of the fish. This is due to the redirection of energy normally used for reproduction towards somatic growth as a result of reduced or inhibited gonadal development.

Since no gene transfers or manipulations have occurred, there is generally less contention regarding the consumption of triploid organisms by humans. Indeed, it has been recommended by various international organizations – North Atlantic Salmon Conservation Organization (NASCO), FAO, International Council for the Exploration of the Sea (ICES) that triploid organisms be used for aquaculture and restocking due to their sterile formation and limited genetic effect on natural populations (Aydın, 2011).

5.2.1 Triploid progeny production

Triploid progeny is commonly produced through the application of one of various methods, including pressure, chemical or temperature shocks to fertilized eggs (Figure 13). A temperature shock is most widely used as it is inexpensive and not harmful to the organisms. High temperatures are usually applied for cold water species, while a cold shock is applied for warm water species (Aydın, 2011).

In order to obtain triploid progeny in turbot, cold shock application is carried out with fertilized eggs according to the procedures set out by Piferrer *et al.* (2000, 2003) and Aydın (2008). The eggs are kept at 14 °C for 6.5 minutes after fertilization and then transferred to a vessel containing -1 °C seawater. This vessel is subsequently placed in a cooled incubator set at -2.5 °C. Twenty minutes after the temperature shock begins, the eggs are returned to normal incubated water conditions (~14 °C) (Aydın, 2011).

5.3 FEMALE MONOSEX STOCK PRODUCTION

Female turbot have a significant growth advantage over males (Imsland *et al.*, 1997), which becomes apparent as early as eight months after hatching. Differential growth rates are maintained throughout the production cycle, including during sexual

maturation with maturing females able to reach 1.8 kg in 20 months and males only able to reach 1 kg (Aydın *et al.*, 2011). Given the difference in growth rates, production of the species can be optimized by preferentially producing all-female turbot populations (Aydın *et al*., 2011).

Although triploid turbot tend to favour a greater female to male sex ratio, there is an additive genetic effect at play in the sex determination of the species (Cal *et al*., 2004). Specifically, the production of female monosex populations requires an XX (female) or XY (male) sex determination system; that is, females must be the homogametic sex (Devlin and Nagahama, 2002). Based on these results, a study conducted by Cal *et al*. (2006) found that a great starting point for artificially generating all-female stocks is through the induction of gynogenesis. In this process, freshly collected eggs are activated with sperm that is diluted in Ringer's solution at a rate of 1:10 and UV-irradiated (30 000 erg/mm). Starting 6.5 minutes after fertilization, the eggs are then subjected to a cold shock of -1 °C to 0 °C for 25 minutes (Cal *et al*., 2006).

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Glossary

Terms and definitions are provided, when possible, as in the FAO TERM portal (FAO, 2022). Additional definitions are in line with GFCM practice.

Batch culture	Suspension culture in which cells grow in a finite volume of liquid nutrient medium and follow a sigmoid pattern of growth. All cells are harvested at the same time.
Biotin	Vitamin of the B complex. It is a co-enzyme for various enzymes that catalyse the incorporation of carbon dioxide into various compounds. It is essential for the metabolism of fats.
Chloramine-T	Investigational animal drug used as an odour-controlling compound, algaecide, bactericide, germicide, parasite control medium and drinking water disinfectant.
Chorion	Embryonic membrane that encloses the fish egg.
Degree-day	Unit used in the measurement of the duration of a life cycle or a particular growth phase of an organism (e.g. fish egg incubation); calculated as the product of time and average temperature over a specific number of days.
Feed conversion efficiency	Figure used to represent the efficiency of food use. The inverse of the feed conversion ratio.
Feed conversion ratio	Ratio of the gain in the wet body weight of the fish to the amount of food distributed.
Furazolidone	Antibacterial agent.
Gynogenesis	Development of ova without paternal genetic contribution.
Homogamety	Having or producing gametes that possess one type of sex chromosome.
Juvenile	Young stage of animals, which for fish is usually between the post-larval stages up to becoming sexually mature.
Lorica	Rotifer's hard protective case or shell.
Lux	Unit of illumination equal to one lumen per square metre (UN, 2022).
Malpigmentation	Major deviation from the normal colouration of the body.
Meiosis	Process by which the diploid (2n) complement of chromosomes is reduced to the haploid (n) state during gametogenesis.
Microbial load	Number and type of microorganisms contaminating an organism or object (UDSA, 2022).
Microdiet	Prepared (dry) diet fed to larvae.
Milt	Sperm-bearing fluid; also refers to gonads from male fish, often called soft roe.
Mitosis	Division of the nucleus into two daughter nuclei with equivalent chromosome sets.

Metanauplii	Late nauplius stage of crustaceans, with more than three pairs of limbs present but no functional thoracic limbs.
Metamorphosis	Marked change of shape or structure, particularly in the transition of one developmental stage into another, as for example in crustaceans and molluscs.
Microalgae	Microscopic, motile or non-motile chlorophyll-containing plants.
Morula	Globular solid mass of blastomeres formed by the splitting of a zygote.
Nauplius	Earliest larval stage of a crustacean; it exhibits the simplest type of head region with three pairs of appendages, uniramous first antennae, biramous second antennae and mandibles. Although the nauplius larva is typical, it does not appear in all crustaceans. It is common in lower forms, but in many of the higher forms it occurs during development in the egg, and the young are hatched as differentiated and more advanced larvae.
Nematode	Elongated, cylindrical, unsegmented worm; includes a number of plant and animal parasites.
Mucus	Slimy secretion of the mucous glands containing mucin.
Oocyte	Cell, which develops into an ovum.
Pelagic	Of, relating to, living or occurring in the open seas or oceans.
Perivitelline space	Area between the yolk and chorion of an egg, where embryo expansion occurs.
Phytoplankton	Minute plants suspended in water with little or no capability of controlling their position in the water mass; frequently referred to as microalgae (the plant component of plankton).
Polyploidy	Condition of having more than two chromosome sets.
Ringer's solution	Solution of the chlorides of sodium, potassium and calcium in purified water that has the same osmotic pressure as that found in blood or tissues and can be used in physiological research and to correct dehydration.
Salinity	Expression for the concentration of soluble minerals (often restricted to salts of the alkali metals or of magnesium) and chlorides in water or soil; usually expressed as parts per thousand.
Spawning batches	Multiple spawning of an individual in a spawning season.
Stripping method	Method in which sperm or eggs are collected from fish.
Thiamine	Also known as vitamin B_1, it acts as a coenzyme in the metabolism of the body.
Triploid	Cell or organisms having three sets of chromosomes.
Ultraviolet	Non-visible electromagnetic waves, which follow at the end of the violet end of the light spectrum and have a length between 40 nm and 400 nm.
Zooplankton	The animal component of plankton.

Annex 1

Stock solution used for the culture of *Nannochloropsis* sp.

WALNE'S MEDIUM

Preparation

Stock solution	1 ml
Vitamin solution	1 ml
Sterilized aged seawater	1 000 ml

Add 1 ml of silicate solution for diatom culture.

Stock solution

$FeCl_3 \cdot 6H_2O$	0.4 g
$MnCl_2 \cdot 4H_2O$	0.2 g
H_3BO_3	16.8 g
Na_2EDTA	27.5 g
$NaH_2PO_4 \cdot 2H_2O$	10 g
$NaNO_3$	50 g
Trace metal solution	0.5 ml

Add distilled water until 500 ml is reached (use volumetric flask).

Trace metal solution

Dissolve the following metals in 200 ml beaker using a magnetic stirrer:

$ZnCl_2 \cdot 4H_2O$	2.1 g
$CoCl_2 \cdot 6H_2O$	2.0 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.9 g
$CuSO_4 \cdot 5H_2O$	2.0 g
Concentrated HCl	10.0 ml

Add distilled water until 100 ml is reached (use volumetric flask).

Vitamin solution

Vitamin B_1	50 mg

Add distilled water until 500 ml is reached (use volumetric flask).

Silicate solution (for diatom culture)

$Na_2SiO_3 \cdot 5H_2O$	40 g

Add distilled water until 500 ml is reached (use volumetric flask).

Annex 2

Stock solution used for the culture of *Nannochloropsis* sp. and *Phaeodactylum* sp.

F/2 MEDIUM (GUILLARD, 1975)

Preparation

F/2 stock solution	1 ml
F/2 trace metal solution	1 ml
F/2 vitamin solution	1 ml
Add filtered seawater until	1 000 ml reached
Autoclave	121 °C, 15 minutes

Add 1 ml of F/2 silicate solution for diatom culture.

F/2 Stock solution

$NaNO_3$	37.5 g
NaH_2PO_4	2.5 g

Add distilled water until 500 ml is reached.

F/2 Silicate solution

$Na_2SiO_3 \cdot 9H_2O$	15 g

Add distilled water until 500 ml is reached.

F/2 Trace metal solution

Na_2EDTA	2.18 g
$FeCl_3 \cdot 6H_2O$	1.575 g
$CuSO_4 \cdot 5H_2O$ solution *1	1.0 ml
$MnCl_2 \cdot 4H_2O$ solution *2	1.0 ml
$ZnCl_2 \cdot 4H_2O$ solution *3	1.0 ml
$CoCl_2 \cdot 6H_2O$ solution *4	1.0 ml
$Na_2MoO_4 \cdot 2H_2O$ solution *5	1.0 ml

Add distilled water until 500 ml reached.

Autoclave	121 °C, 15 minutes
*1 $CuSO_4 \cdot 5H_2O$ solution	2.45 g/500 ml distilled water
*2 $MnCl_2 \cdot 4H_2O$ solution	45.0 g/500 ml distilled water
*3 $ZnCl_2 \cdot 4H_2O$ solution	5.5 g/500 ml distilled water
*4 $CoCl_2 \cdot 6H_2O$ solution	2.5 g/500 ml distilled water
*5 $Na_2MoO_4 \cdot 2H_2O$ solution	1.575 g/500 ml distilled water

F/2 Vitamin solution

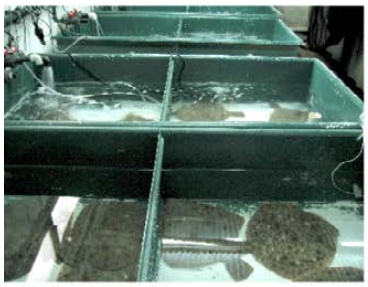
First vitamin B_{12} (VB_{12}) stock solution *1	1 ml (= VB_{12} 0.5 mg)
First biotin stock solution *1	1 ml (= biotin 0.5 mg)
Thiamine HCl	100 mg

Add distilled water until 500 ml is. reached (use volumetric flask).

Annex 3

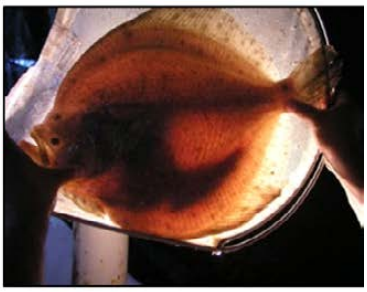
Spawning process for Black Sea turbot

Wild fish capture
(April–May)



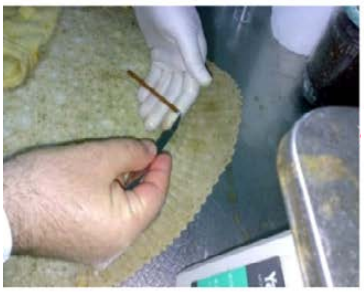
Holding in quarantine tank for one day

Broodstock selection and treatment (D-2)



Determination and evaluation of maturity (D-2)

Stocking to maturation tank
(D-2)



Hormone treatment
(D-3)

First and later strippings, artificial fertilization and egg disinfection
(D-10 to D-25)

Incubation for five days after fertilization at 14 °C

Hatching out the larvae and transfer to growing tank
(D-15 to D-30)

In the Mediterranean and the Black Sea, aquaculture plays a major role in achieving food security and can deliver many socioeconomic benefits and help to maintain or increase production of aquatic foods while ensuring wild stocks are fished within their maximum sustainable yield. Given the importance of this sector and the growing demand for aquatic products, it is vital that aquaculture in the region be both sustainable and productive.

In this regard, it is necessary to promote practices supporting the sustainability of the sector and maximize the sharing of technology and information in order to give producers all the necessary tools and information on aquaculture production. This manual aims to support this endeavour by presenting detailed information about the rearing practices of Black Sea turbot (*Scophthalmus maximus*). It provides an introduction into the status of the turbot aquaculture sector, as well as the necessary environmental conditions for optimal production. It also describes the production of live food for turbot larvae as well as the management of broodstock and the procedures for optimal larval and juvenile rearing. Finally, with a view to capitalizing on and promoting innovative technologies, it presents recent applications of biotechnologies to turbot production.

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