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Aquaculture production: current limits and contribution to innovation on fish health management

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ABSTRACT

The general objective of this thesis was to assess the main threats, in terms of diseases, disorders and pollutants, which occur in the breeding of some fish species, both marine and freshwater, and provide an innovative contribution on the health management of farmed fish through the use of immunostimulant food additives and the application of nanomaterials for water treatment.

Finfish aquaculture production has seen rapid growth in terms of both production volume and economic performance over the past decades. Aquaculture is today a key supplier of seafood. As the scale of production increases, the industry is likely to face emerging biological, economic and social challenges that can affect the ability to maintain ethically healthy, productive and eco-friendly fish production. It is therefore important that the industry aspires to monitor and control the effects of these challenges to avoid also increasing potential problems during production upscaling.

In this perspective, a fish-health monitoring survey was conducted on gilthead sea bream (*Sparus aurata*) reared in an intensive Italian fish farm and on common carp (*Cyprinus carpio*) and pike perch (*Sander lucioperca*) produced in a semi-intensive Croatian fish farm, each lasting six months. The purpose of these activities was to detect the main problems afflicting the two companies under study. The results of the survey showed that infectious diseases represent the main threat in both farms. In particular, Nodavirus and *Vibrio* sp. represented the main causes of mortality and heavy economic losses in gilthead sea bream farming; parasites such as *Ichthyophthirius multifilis* and *Dactylogyrus* spp. greatly affected the breeding of common carp with an occurrence frequency of 22.7% and 33.4% respectively. The entry of *Mycobacterium marinum* caused a mass mortality of pike perch specimens reared in recirculating aquaculture systems. Deformities represented the second threatening factor in both farms, with an onset of 4.5% and 3.01% in gilthead sea bream and common carp, respectively. Finally, pollutant investigations in water samples from the Italian farm were found to comply with the requirements of the legislative decrees concerning "Environmental standards". However, lower concentrations of microplastics, classified as emerging contaminants, were found in the gastrointestinal tract of farmed gilthead sea bream (0.48 items/specimen) and common carp (0.11 items/specimen) than in their wild counterpart.

As outbreaks of infectious disease in farmed fish emerged as the main hurdles for the development of aquaculture, and considering the limitations and side effects of several veterinary therapies commonly used in aquaculture, the interest of researchers has shifted to development of alternative strategies, such as immunostimulants, for the prevention and control of infectious diseases. In the present research, we investigated the effects of dietary Imoviral on immune-related gene expression at spleen level in gilthead sea bream infected with *Vibrio anguillarum*. Fish were fed two diets, with or without Imoviral supplementation. The trial lasted for 4 weeks, then fish were divided into five groups; the second and fourth groups were intra-peritoneally injected with phosphate buffer saline (PBS) while the first and third groups were challenged with a sublethal dose of *Vibrio anguillarum*. The fifth group was the control group. At 1 h, 24 h, 72 h and 168 h post challenge, immune-related gene expressions were evaluated. The results indicated that administration of Imoviral could significantly improve the immune response in fish fed supplemented diets, confirming the beneficial effects of Imoviral as a natural alternative immunostimulant for gilthead sea bream aquaculture.

Pollutants, such as heavy metal or pesticides, in the aquatic environment undoubtedly represent one of the main problems that society faces today, representing also one of the greatest threats for aquaculture production. Therefore, in the last few decades, the development of efficient and ecologically-friendly methods to remove contaminants from water has become of relevant importance. In this context, nanotechnology has gained a lot of attention in recent decades due to the unique physical properties of nanoscale materials, which are particularly efficient in the field of remediation. It is, however, important to verify that the materials used for the remediation of pollution are not themselves another pollutant after being used. In this study, we have investigated the toxicity of photocatalysts molecularly imprinted titanium dioxide (TiO_2), also N-doped, for selective removal of the o-phenylphenol (OPP) fungicide from water. The toxicity of the nanoparticles was established by a zebrafish embryo toxicity test, an alternative method of animal test. Zebrafish embryos were exposed to: TiO_2 bare, TiO_2 sol-gel, molecularly imprinted (MI) $MITiO_2$/o-p.p. and molecularly imprinted $MITiO_2$/o-p.p. N-doped at 0.8%, 1.2% and 4%. Moreover, other larvae were exposed to molecularly imprinted $MITiO_2$/o-p.p. N-doped at 0.8%, 1.2% and 4% with the addition of the OPP fungicide (1x10-4 mg/ml) in the ratio of 1:1. As exposure biomarkers, we evaluated the expression of metallothioneins 1 and Heat Shock Proteins 70 by immunohistochemistry

analysis. The results obtained from the toxicity test showed that neither mortality nor sublethal effects were induced by the different nanoparticles tested. The zebrafish exposed to imprinted nanoparticles with OPP fungicide (1:1) died one hour after exposure and therefore the immunohistochemical analysis showed no response to the biomarkers tested.

DECLARATION

I hereby declare that the results presented are to the best of my knowledge correct, and that this thesis represents my own original work, carried out during the designated research project period, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work. I therefore had a major role in the study design and execution of the experiments, in sampling, in the acquisition of most data, their analysis and interpretation. The thesis project (2017-2020) was a joint effort of several research teams from different institutes, mainly: the Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences, the Department of Veterinary Science and the Department of Mathematical and Computational Sciences, Physical Science and Earth Science, University of Messina; the Department for Biology and Pathology of Fish and Bees Faculty of Veterinary Medicine University of Zagreb; the Department of Biological, Geological and Environmental Sciences, University of Catania, the Istituto Zooprofilattico Sperimentale delle Venezie and Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. Moreover, permission has been obtained from the journals to use the data to support the thesis. I am responsible for any eventual plagiarism; the thesis was verified with the software Plagiarism Checker X 2019, showing a percentage lower than 20%.

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CONTENTS

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1. INTRODUCTION

1.1 World aquaculture production

Aquaculture (the farming of aquatic organisms) is one of the fastest-growing sectors in world food production and for the last decade has supplied one-third of seafood consumed worldwide [1]. Global fish production reached around 171 million tons in 2016, where aquaculture production accounted for 47% of the total [2]. Static fishery production since the late 1980s has failed to meet the ever-increasing demand for fish products from consumers. Therefore, aquaculture reacted by intensifying and diversifying the range of production, raising an increasingly large number of species, and finally becoming responsible for the continuous and impressive growth of the supply of fish for human consumption (Fig. 1). Indeed, world total marine catch dropped to 79.3 million tons in 2016, with a decrease of almost 2 million tons over the previous year. On the contrary, aquaculture continues to grow faster than other important food production sectors, recording an average annual growth of 5.8% (2000-2016) and a global production of 110.2 million tons of which 80 million tons concerned the production of food fish in 2016. Between 1961 and 2016, the annual increase in global consumption of fish (3.2%) outpaced population growth (1.6%). In per capita terms, fish consumption increased from 9 kg to 20.2 kg, with an average of approximately 1.5% per year. In 2015, fish represented around 17% of the animal protein consumed by the global population.

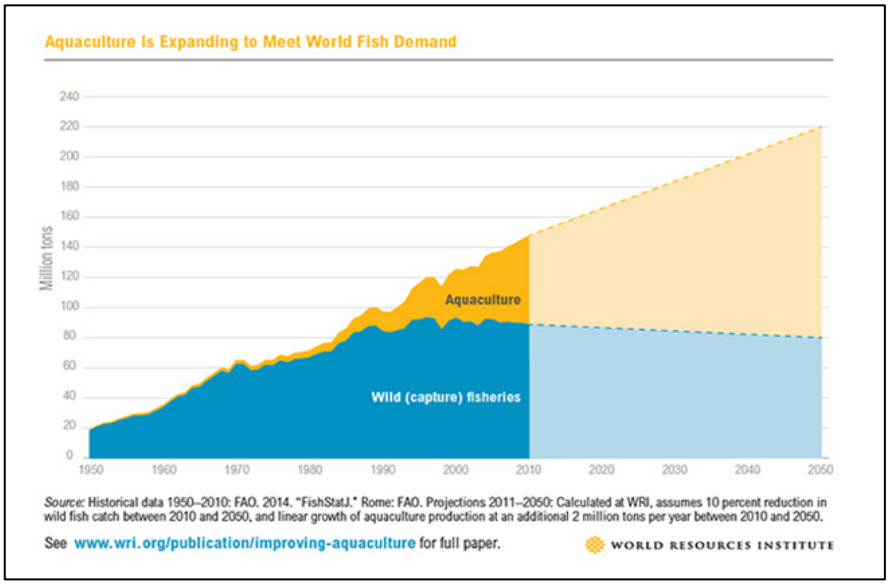

Figure 1. Current production and production estimates for fisheries and aquaculture

The world aquaculture landscape is mainly dominated by the Pacific-Asian area, which provides 89.4% of aquaculture food fish production, of which China alone provides 61.5% of total production (Fig. 2) [2].

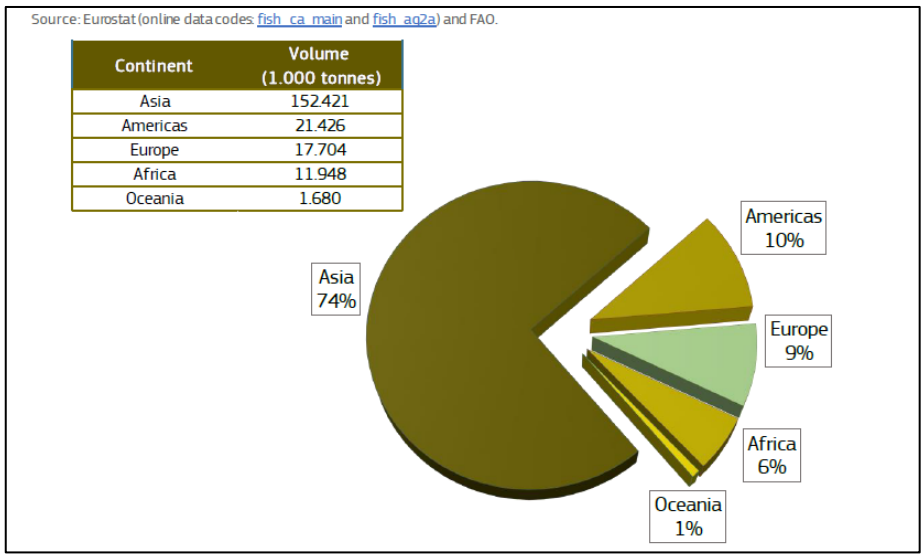

Figure 2. World catches and aquaculture production in 2017

1.2 European aquaculture production

In Europe, during 2017 the volumes of both fishery and aquaculture production were larger than in 2016, providing 9% of world production and 4% of global farmed production [3]. Since 2013, aquaculture production has been higher than catches and 21% of production originates from aquaculture in Europe. In 2017, aquaculture production in the EU reached a ten-year peak of 1.37 million tons, with a value of €5.06 billion, recording a 10-year growth of 11%. Compared to 2016, the volumes produced increased by 5% (+67,172 tons) and their overall value increased by 15% (+662 million euros). Five member States account for three quarters of EU aquaculture output volume and value. Spain produced the highest live weight of aquaculture production in 2017, corresponding to 23% of total EU production; followed by the United Kingdom, with a production of 16%, France with 14% and finally Italy and Greece, each of which supply 11% of the total EU production (Fig. 3).

Source: EUMOFA, based on Eurostat (online data code: fish_aq2a) and FAO data. More details on the sources used can be found in the Methodological background.

Member State	2008	2016	2017	2017/2016	2017/2008
Spain	252	287	315	+10%	+25%
United Kingdom	180	194	222	+14%	+24%
France	238	182	189	+4%	-20%
Italy	158	157	156	-1%	-1%
Greece	94	123	126	+2%	+34%

Figure 3. Volume of aquaculture production in the EU top-5 producers (1.000 tonnes)

Aquaculture in the EU is highly specialized at regional and country levels and is strongly influenced by geography and natural habitat of species. European aquaculture is strongly characterized by specialized productions from some member states: gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in Greece (53% and 51% of European production, respectively), mussels in Spain (31%), oysters in France (86%), clams in Italy (82%) and salmon in the United Kingdom (89%) [3]. In terms of economic value, all groups of products bred in the EU reached a ten-year peak in 2017 (compared to 2016: salmonids + 18%; bivalves + 20%; freshwater fish + 19%). However, from the point of view of volumes, the composition of the species produced has not undergone significant variations (Fig. 4).

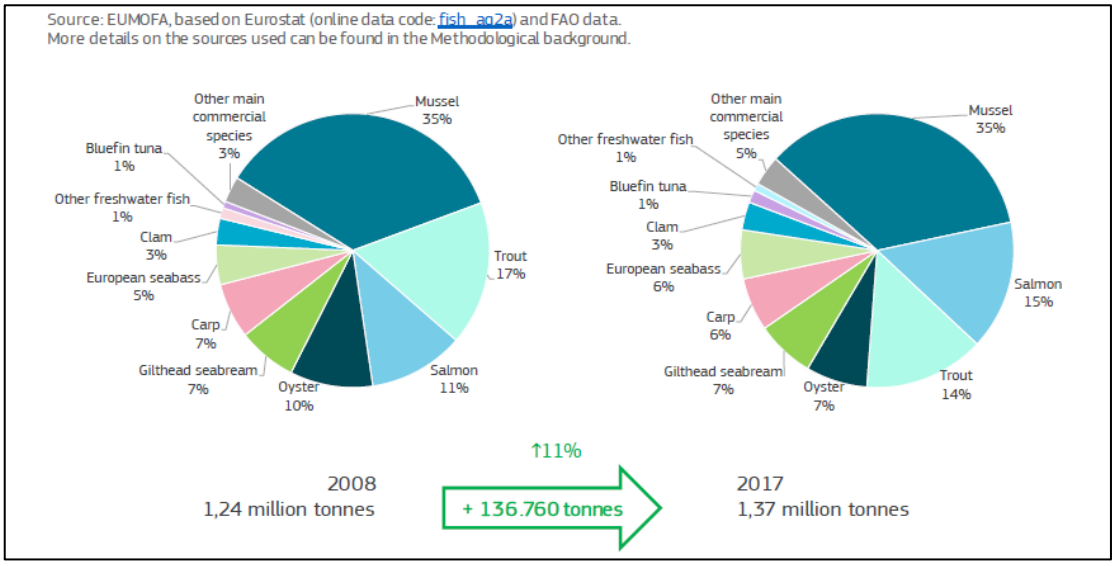

Figure 4. Composition in volume of EU aquaculture production by main commercial species

Finfish production represents more than half of EU aquaculture production (51.2%) and more than two thirds of its value (70.4%). More than 40% of the value of EU farm production is

represented by salmonids. In 2017, Atlantic salmon (*Salmo salar*) represented more than 25%, followed by trout (14%), oysters (10%), sea bass (10%), gilthead sea bream (10%), and mussels (8%). Finally, even if they are not part of the main groups produced in Europe, "new species" deserve attention, such as the meagre and the Atlantic sole. The production of these species in the EU developed around 2017, with an increase of 24% and 23% respectively, compared to 2016. Both species are mainly bred in Spain, where production covers 64% and 71% respectively of the whole European production. Other important producing countries are Greece and Croatia for meagre and France for Atlantic sole [3].

1.2.1 Current status of aquaculture in Italy

Aquaculture in Italy is based on a long tradition and history. It is characterized by a high level of specialization and know-how, high degree of industrialization and large-scale production. The sector includes both the breeding of marine and freshwater species. Traditional extensive aquaculture is carried out in the "*valli*" brackish lagoons, especially in the northern regions. Modern agricultural techniques are used for the production of marine species, including inshore intensive farms and mariculture through cage systems. As for shellfish farming, cultivation on ropes and bags (mussels) or directly on the intertidal substrate (clams) is common [4].

In 2016, the total production of Italian aquaculture reached 157,109 tons (Fig. 5). However, production declined in 2017 due to the contraction of mussel production. This was determined by a combination of factors including climate change, water conditions and nutrient sources [3]. In contrast, the value of Italian aquaculture increased by 40% over the same year.

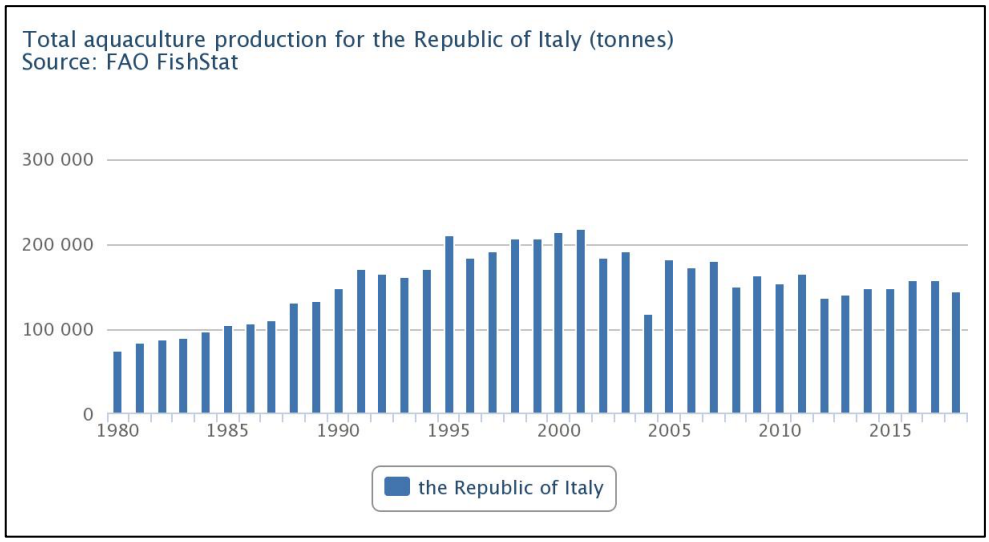

Figure 5. Italian aquaculture production (2018)

Shellfish farming contributes to 63% of total aquaculture production, of which Mediterranean mussels (63,700) and clams (36,500) represent the main species produced. Italy is in fact the main producer of clams in Europe; in 2017 production of approximately 37,157 tons covered 82% of total European production [4].

Freshwater finfish farming is particularly developed in the northern and central Italian regions, representing the most important production segment both in terms of volume and value, contributing to more than 27% of Italian aquaculture production. This amounted to 41,243 tonnes in 2016, the main species of which were rainbow trout (*Oncorhynchus mykiss*) (36,800 tonnes), followed by European eel (*Anguilla Anguilla*) (1,250 tonnes).

Marine fish species represent the third segment of Italian farming production in terms of volume and value. The production of marine fish species amounted to 15,744 tons in 2016, the main species of which were gilthead sea bream (7,600 tons) and sea bass (6,800 tons) [5]. Small productions were also registered for sharpsnout sea bream (*Diplodus puntazzo*), shi drum (*Umbrina cirrosa*) and meagre (*Argyrosomus regius*). The new candidate species for Italian aquaculture are Mediterranean yellowtail (*Seriola dumerilii*) and dover sole (*Solea solea*), for which incubation and development technologies are still being perfected [3].

The current trend in the development of Italian aquaculture is the growing production of marine species, both molluscs and fish. The growth in aquaculture production is mainly due to the control of production techniques for the breeding of gilthead sea bream and sea bass and to the application of new agricultural technologies. The Italian aquaculture sector must overcome numerous limitations, for example competition with other gilthead

sea bream and sea bass producing countries (Greece and Spain) or complicated bureaucratic procedures for starting operations. A further fundamental constraint is the lack of a close relationship between aquaculture and public research, which plays a key role in terms of innovation [4].

1.2.2 Current status of aquaculture in Croatia

The coastal areas and inland waters of Croatia present ideal conditions for the development of aquaculture, making it an economic activity with a long tradition and a role of primary importance in the fishing sector. Croatian aquaculture production reached 16,000 tons in 2017, representing 18.6% of total fish production, and recorded a slight increase in 2018 with a total of 19,681 tons (Fig. 6).

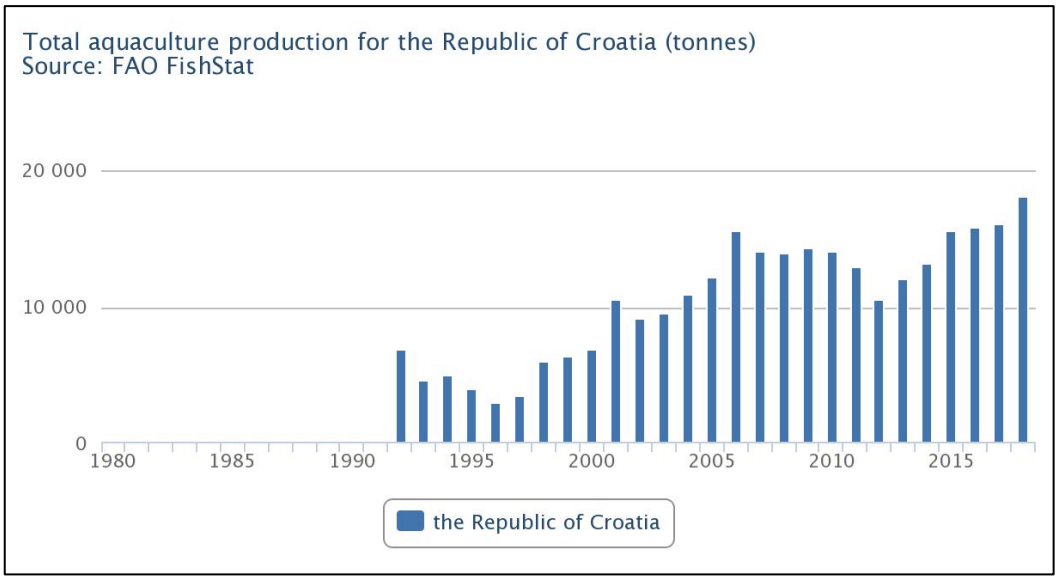

Figure 6. Aquaculture production in Croatia (2018)

The breeding of aquatic organisms in the Croatian Republic includes both marine and freshwater aquaculture. Croatia was a pioneer of commercial marine aquaculture, with one of the first and largest hatcheries for sea bass in the early 1980s. Marine aquaculture has gradually increased over the years, passing from a production of 12,043 tons in 2015 to 16,782 tons in 2018. The marine species most produced in 2018 included sea bass (6,220 tons), gilthead sea bream (5,591 tons), meagre (808 tons) and Atlantic bluefin tuna (*Thunnus thynnus*) (3,227 tons).

With regards to shellfish farming, *Mytilus galloprovincialis* and *Ostrea edulis* are the most important species, with a production of 882 tons in 2018 [6].

The breeding of freshwater fish species peaked in 2015-2016, with an average production of 4,433 tons. However, this declined in the following two years, falling to 2,899 tons in 2018. The breeding of freshwater species mainly concerns warmwater fish species (Cyprinidae) and cold-water fish species (Salmonidae). Warmwater species include common carp (*Cyprinus carpio*) (1,959 tons), grass carp (*Ctenopharyngodon idella*) (141 tons), bighead carp (*Hypophthalmichthys nobilis*) (301 tons), silver carp (*Hypophthalmichthys molitrix*) (36 tons), European catfish (*Silurus glanis*) (23 tons), pike (*Esox lucius*) (7 tons) and pike perch (*Sander lucioperca*) (7 tons) [6].

Among the cold-water fish species rainbow trout (*Oncorhynchus mykiss*) and sea trout (*Salmo trutta*) represent the most important species both in terms of volume and value, with a total production of 370 tons (336 and 34 tons respectively) in 2018.

To improve and develop sustainable aquaculture, the Republic of Croatia has adopted a multiannual national strategic plan for aquaculture 2014-2020 (NSPA). The general objectives are aimed at the development of aquaculture through social and commercial improvement, increased national consumption, increased employment in the aquaculture sector, while promoting the development of local communities, simplifying administrative procedures, increasing competitiveness by creating fair competition on the market and finally increasing interactions with the scientific community [6].

1.3 Aquaculture and production systems

Aquaculture is defined as the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants, in fresh, brackish and marine waters. This implies different levels of human intervention in the rearing process to improve production, such as regular stocking, feeding and protection from predators [7]. From a legal point of view, aquaculture takes the form of an activity of breeding or cultivation, differentiating itself from the simple "capture", resulting therefore attributable to agriculture, whether it is carried out inland or on marine waters [8]. National and international aquaculture systems, in terms of technical and structural characteristics of farms, are divided into three types: extensive, semi-intensive and intensive; the latter in turn is divided into in-shore and off-shore plants (Fig 7a, b, c, d).

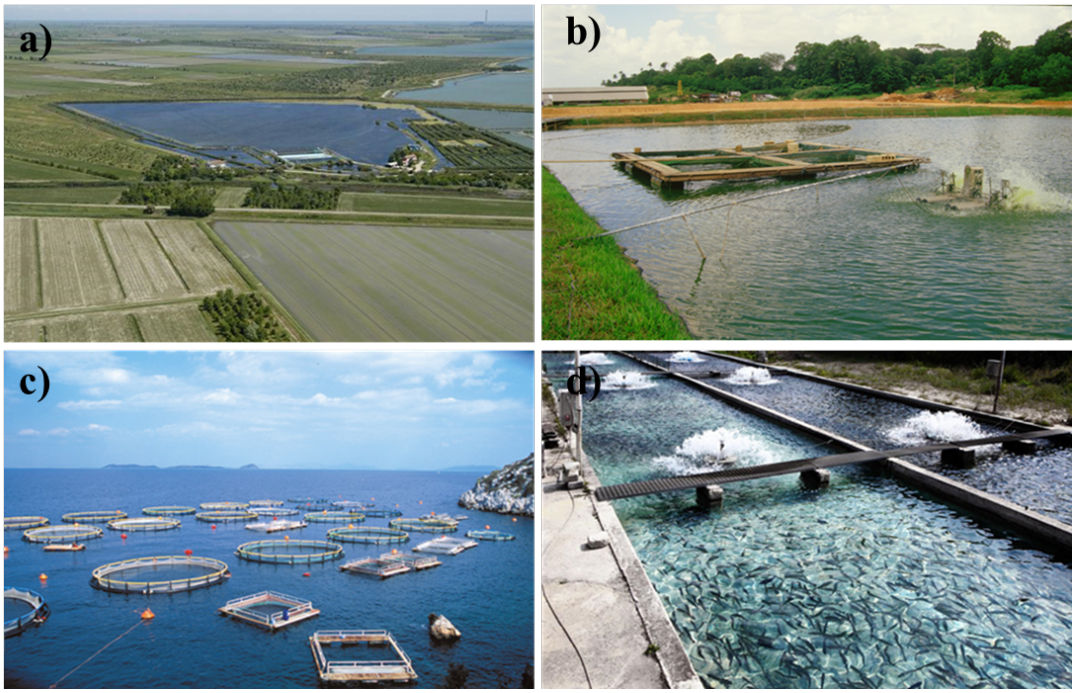

Figure 7. Production systems: a) extensive aquaculture; b) semi-intensive aquaculture, c) off-shore intensive aquaculture, d) in-shore intensive aquaculture

The extensive systems exploit certain morphological characteristics of areas that allow to build a farm without making structural changes to the environment, both environmental and ecological. In this type of aquaculture, the diet of farmed organisms is based exclusively on the trophic potential of the environment in which they are raised. Human activities in extensive aquaculture are limited to sowing fry and controlling predators. These farms often persist in *valli*, lagoons or coastal ponds. Despite the low human contribution and the contained costs, this system can provide good results in terms of fish production with a low or almost zero impact on the environment [9]. The semi-intensive systems also located in relatively closed natural areas, foresees some human interventions during the growth of farmed species. The food source no longer derives exclusively from the environment but is supplemented by an artificial diet (commercial pellets or other food sources). Another aspect concerns artificial reproduction; these systems may be equipped with a hatchery, thus providing controlled reproduction of farmed species through artificial propagation. In addition, in some cases the culture water is oxygenated and the wastewater treated [7]. Intensive farms, unlike those previously described, are based on a high concentration of biomass in restricted environments and on total control of the life cycle of the organisms reared, their feeding, the breeding waters and of all the aspects concerning production. This can be carried out in ground tanks (in-shore) and/or in sea cages (off-shore). The latter

system described is the most profitable and widespread on the national and international scene. Clearly, such a system entails a series of repercussions on the various intrinsic aspects of a fish farm such as fish pathology, nutrition, genetics, management, economics, etc. The type of aquaculture described above is framed in a strictly industrial and business perspective, defined as "industrial aquaculture" [10]. It is from this type of aquaculture that the highest values recorded in terms of production and sale of fish species in recent years derive [11].

1.3.1 Gilthead sea bream (*Sparus aurata*) production in Italy

Gilthead sea bream is the most produced marine fish species in Italy (7,600 tons in 2016) [5]. There are different production systems for sea bream ranging from extensive polyculture (*valliculture*) or semi-intensive production in earth ponds, to highly intensive land-based systems (raceway or tanks), inshore and offshore cage.

Until the late 1970s, fish farming in the Mediterranean was based exclusively on the collection of juveniles in nature, for breeding in extensive and semi-intensive systems. In Italy, gilthead sea bream was traditionally cultivated in the lagoons of the northern Adriatic (known as "*valli*") in large systems in which juveniles, together with mullets, were caught in spring and stored in the *valli* [12]. At the end of the year, fish were stored in overwintering ponds, and then released again into the *valli* during the spring.

Since the 1980s, the development of intensive breeding techniques and the scarce availability of wild fry has led to a sharp increase in the demand for fry, thus stimulating the development of techniques for egg, larvae and fry production [13]. Italy was among the countries where the first production units and the first breeding programs for this species appeared. Since the early 1990s, the standardization of these techniques has allowed the industrialization of aquaculture.

Intensive breeding is characterized by high stocking density, controlled conditions of water quality, light intensity, photoperiod and feeding. The development of aquaculture on a commercial scale requires, first of all, control of the reproductive function and the production of eggs of high fecundity and quality. Usually each hatchery has its own broodstock unit, where breeders of various age groups, from 1-year-old males to 5-year-old females, are kept in long term storage conditions. Breeders can come from a farm or from the wild.

At the start of the breeding season, the selected breeding lots are transferred from their long-term position to the breeding tanks. To ensure a good fertilization rate, since females

can lay about 20,000–30,000 kg m-1 eggs for a period of 3-4 months, the ratio of males and females is normally maintained at 3: 1.

The gilthead sea bream broodstock can be conditioned by environmental manipulation to prolong or modify the reproduction time. Fish are stored in tanks equipped with a water heating/cooling system and computerized control of temperature and light intensity. Sexual maturation is achieved by exposing the broodstock to photoperiod and water temperature conditions that occur during the natural deposition period. Broodstock are mostly fed with commercial feed, sometimes with squid [14,15] which seem to have a strong influence on fertility.

The rearing methods of sea bream larvae are essentially attributable to 2 systems: i) “clear water” and ii) “green water” technique.

The first method, called "clear water", is the one most used in commercial hatcheries [16,17]. This system is based on the use of small or medium-sized tanks (3-6 m^3) into which newly hatched eggs or larvae are introduced. Phyto- and zooplankton, necessary for the nutritional needs of the larvae, are grown separately and added daily to the larval breeding tanks, thus keeping the concentration constant and controlled. Between the third and fourth day post-hatching (dph), the larvae begin exogenous feeding until they complete metamorphosis. During the first 40-50 days of breeding, live food is administered, first rotifers (*Brachionus plicatilis*) and subsequently Artemia nauplii (*Artemia* spp.); they progressively move on to weaning with inert food (commercial feed). Phytoplankton is used as a dietary supplement for rotifers and introduced into breeding tanks with the dual purpose of keeping the trophic chain active (phytoplankton, zooplankton, larvae) and improving the environmental conditions of breeding larvae, filtering the light that may be too intense, especially in the first days of life of the larvae [18,19].

The second method called "green waters" system, in addition to using larger volumes (20-50 m3), is essentially based on the introduction of larvae or embryonic eggs into tanks where a culture of phyto and zooplankton was previously triggered, in order to reproduce conditions similar to natural ones [20]. In the early stages of breeding (15-30 days) no water exchange is carried out, so as to keep the previously triggered trophic chain active. The "clear water" methodology offers a higher level of standardization of the production process, greater modularity of the plants and, finally, significant productions to be destined for sale. The "green water" methodology allows to operate at a lower technological level and therefore with lower investment costs. However, the latter

has several limitations including slow development and dependence on the season of the endogenous food chain, causing food shortages for larvae, rapid deterioration of the food chain after 20-25 days, and a high mortality rate [21]. Juveniles usually remain in the hatchery until they reach about 5g in weight, after which they are transferred to on-growing structures. If on-growing is practiced in open sea conditions, the pre on-growing period is prolonged until the fish reach a weight of 10-30 g, depending on the management strategy of the farm. During this phase, several procedures are commonly applied including grading, vaccination and quality control.

Grading is carried out when the reared populations have an irregular distribution of dimensions within the same batch; this could lead to breeding difficulties and cannibalism. Therefore, uniformity of size is important.

Malformations during the incubation process are different and can lead to significant economic losses. Usually these include skeletal, opercular, spinal deformities and the absence of a swim bladder. The identification of malformed individuals usually occurs in the hatchery and is a manual process.

Transporting fish from hatchery to growth facilities represents the end of the incubation phase and the beginning of on-growing. This phase can take place in land-based system (inshore) or in marine cages (offshore). Gilthead sea bream is mainly cultivated intensively in marine cages with an average density of 15–25 kg m^3 with a food conversion ratio (FCR) of 1.5–2. The cultivation period varies according to the location and temperature of the water, but usually lasts for 18-24 months so that a specimen reaches 400 g from the hatched larvae. Commercial size can vary from 250 g to 1.5 kg [22].

Commercial diets are normally extruded pellets with a percentage of 45-50% of proteins and about 20% of lipid content. As with most carnivorous marine fish, the main raw materials used in feed are fishmeal and fish oil.

The sea bream industry could now be described as a sector oriented towards its mature phase, but which still needs more efficient production systems and new technologies to further minimize the limits that still persist, promoting closer interaction with the scientific community. Furthermore, new technologies should be geared towards sustainable aquaculture and take into account the need to minimize the potential impact of sea bream aquaculture practices in coastal areas [20].

1.3.2 Common carp (*Cyprinus carpio*) production in Croatia

Common carp is the main freshwater fish species produced in Croatia (1,959 tons in 2018), representing more than 70% of the total national cyprinid production [6]. Carp aquaculture is traditionally carried out on large farms through polyculture semi-intensive production systems, the majority of which are located near large river basins in the continental part of Croatia. The production cycle of semi-intensive carp breeding lasts two or three years and consists of five fundamental processes [23]:

1) Hatchery propagation. Reproductive cycle of the species is completely programmed, and each phase is completed in hatchery, under controlled conditions. It is a practice normally conducted in the spring months (May-June) in conjunction with the natural reproductive period of the species, when the water temperature exceeds 18 ° C. The technique uses hormonal stimulation of the broodstock. In the hatchery, hormone administration, stripping and artificial fertilization of the eggs take place. In this phase fertilized eggs and larvae are incubated at 23 ° C for 6 days.
2) Early fry rearing, which starts with the stocking of feeding fry and ends with one-month-old advanced fry. The larvae are transferred from the hatchery to ponds of about 1-2 ha in size. Preparation of the ponds starts two weeks before stocking is planned, with the cleaning of the pond bottom and spreading of lime, and then fertilized to promote the growth of zooplanktonic organisms, necessary for the feeding of the larvae.
3) Fingerling rearing, which starts with the stocking of one-month-old fish and ends with the harvesting of fingerlings. The fry on-grow for the rest of the summer in larger ponds.
4) On-growing period from the stocking of fingerlings until the end of the second season. After preparation, the fish are transported to larger ponds (size 5-10 ha). Given the high stocking density, it is necessary to add additional feed, usually represented by many varieties of cereals.
5) Production of marketable size fish, from the stocking of two-summer-old fish until the end of the third season (pond size > 10 ha) (except in the case of a two-year growing cycle).

1.3.3 Pike perch (*Sander lucioperca*) production in Croatia

Pike perch is a carnivorous species, native to the European and Asian basin, characterized by an important cultivation tradition and with a high economic value (Fig. 8). The breeding of this species was initiated in the nineteenth century, along with carp farming,

typically cultivated in earth ponds, in central and eastern Europe. It was typically considered an "additional" fish and therefore produced in insignificant quantities. Currently, the main pike perch producing countries are the Czech Republic, Denmark, Hungary, Romania, Tunisia and Ukraine, although production also appears to be growing in Poland and the Netherlands [24].

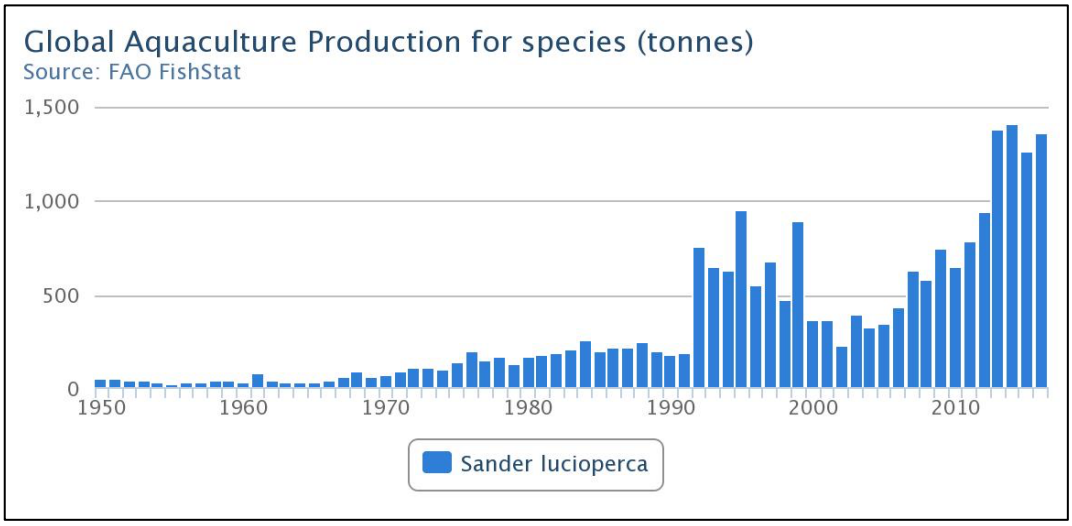

Figure 8. Pike perch global production (2015)

In Croatia, pike perch production represents 0.24% of farmed freshwater fish, producing 7 tons in 2018 [6]. The species is mostly bred in polyculture with carp, by means of semi-intensive systems in earthen ponds, in which the species reproduces naturally.

However, extensive and semi-intensive production, in conjunction with the contraction of catches, has failed to satisfy the increased global demand for this species by fishermen and consumers. The fall in catches was certainly linked to environmental problems such as pollution, habitat loss and overfishing. These factors therefore reduced the number of pike perch in natural waters. With regard to traditional production, this is severely limited to the seasonality of production, and therefore unable to supply fish throughout the year [25]. To overcome these problems, numerous European research projects have been launched to develop reproduction technologies and techniques under controlled conditions. At the beginning of the 21st century, the first aquaculture farms that produced pike perch using recirculating aquaculture systems (RAS) were established in Western Europe and by the end of the first decade there were around 10 of these plants [24,26].

Pike perch production throughout the year (out of season reproduction method) is feasible only in RAS production systems, in which it is possible to control the

reproduction cycle by regulating the temperature of the water, photoperiod and hormonal stimulation. After the sex products are obtained, the eggs are dry fertilized (1-2 ml semen/100 eggs) and adhesiveness is removed from the eggs using an aqueous tannin solution (0.5-1.0 g/litre). Following the incubation period, the larvae are raised in circulation tanks of 0.5-1.5 m^3. For the first two weeks larvae are fed with *Artemia nauplii* and commercial feed. The initial stocking density varies from 20 to 50 larvae/litre and water temperature is 20 ° C [24].

The nursery phase generally lasts from 8 to 10 weeks. Juvenile pike perch (0.2-10 g) are bred in 2-5 m^3 RAS tanks. The temperature ranges from 22-24 ° C. Stocking density is 10 larvae/litre. The feed ratio is 10-12% of biomass. Juveniles destined for intensive on-growing are maintained until they reach a minimum of 15 g [24].

On-growing in a recirculation system is still under development. During this phase, juveniles of 15-30g are stocked. In the initial stages (body weight 15-100 g) 2-5 m^3 tanks are used and the stock is kept at 10-30 kg/m^3. For the final phase, tanks of 20-30 m^3 are used, in which the fish are bred up to 1 kg, with a breeding density of maximum 80 kg/m^3. Fish are sorted firstly at 100-150 g, secondly at 200-250 g and finally when they reach 500-600 g. The optimum temperature for perch growth is around 27-28 ° C, although rapid growth rates can be observed as early as 23 ° C. RAS grow-out feeds are high in protein (42-50%) and low in lipid (8-14%). Feed is delivered by automatic feeders (as in the previous phases), at least three times a day [24].

As an alternative to exclusively extensive or intensive breeding methods, a mixed type of hatchery production, extensive/intensive RAS, can be used. In this type of system, the larvae are bred in earth ponds where natural or semi-natural reproduction of the perch has been conducted. After 6-8 weeks of breeding, the fry are collected and stored in RAS [24].

In Croatia, there is currently only one fish farm equipped with a RAS system in which the breeding of the pike perch is carried out. Generally, the methods for pike perch cultivation are still under study and perfection. Breeding methods still have severe limitations related to the high sensitivity of the species to stress, O_2 levels, mechanical injury and its need for a relatively large space, probably due to the predatory nature of the species. In fact, in small spaces it is easier to observe cannibalism phenomena [27]. The domestication process of this species can induce anatomical, physiological and molecular changes, thus influencing a variety of biological functions. Currently the major bottlenecks for the further expansion of the pike perch fish culture include: 1) high

sensitivity of the species to stress factors, management and breeding practices that cause high and sudden mortality; 2) low larval survival rate; 3) high incidence of deformity; 4) little information on the genetic structure and variability of the species; 5) high costs of rearing larvae in RAS.

However, the species is believed to offer good prospects for European aquaculture, and significant progress in the technological development of artificial propagation, incubation, nursing and feeding have been made in recent years [24]. Furthermore, the high protein content and the low lipid content of its meat make this species one of the best candidates, with the highest diversification potential of European internal aquaculture [28].

1.4 Main economic problems related to gilthead sea bream and carp farming

The main causes of economic loss and damage to aquaculture production are essentially attributable to infectious diseases that cause high mortality rates of farmed species, malformations that make the product unsaleable or greatly reduce its commercial value, poor management and maintenance of fish stocks and finally the accidental entry of pollutants into culture waters which can cause direct damage to farmed organisms or exacerbate the damage caused by pre-existing pathologies.

1.4.1 Infectious diseases

Infectious diseases, especially of viral and bacterial origin, today represent one of the major unresolved problems in modern aquaculture, with consequent serious economic losses. Infectious diseases that are particularly threatening, as they resist or do not respond to therapies, are restricted to a limited geographical area or are characterized by high socio-economic importance as they involve species of international economic interest, must be reported to the Office International des Epizooties (see list on OIE, www.oie.int). However, some diseases that cannot be notified to the OIE could cause significant damage to fish production, generating a high mortality rate for farmed species.

1.4.1.1 Viral diseases

Among the viral diseases affecting Mediterranean aquaculture, Nodavirosis and the sporadic onset of Lymphocystis should be noted.

According to the Federation of European Aquaculture Producers, gilthead sea bream (*Sparus aurata*) represent one of the most important farmed fish species in Mediterranean aquaculture (FEAP 2016; FAO 2018). Despite its economic value,

there are no specific provisions in the current EU legislation on disease surveillance and control on this species (2006/88/EC), it is simply listed as a vector species for Viral haemorrhagic septicaemia according to EU regulation (1251/2008/EC) and the European Food Safety Authority (EFSA) (2007). However, a survey conducted by the European Union Reference Laboratory (EURL) for Fish Disease showed that viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN) [29] represents the most dangerous infectious disease affecting sea bream and sea bass breeding in Mediterranean aquaculture [30]. The nervous necrosis virus (NNV, Nodaviridae family, Betanodavirus genus), aetiological agent of VER, is certainly one of the most devasting infectious agents in marine aquaculture, affecting a wide range of wild fish species and responsible for massive mortalities in a large number of economically relevant farmed fish species [31,32]. Betanodaviruses have been classified into four genotypes: barfin flounder nervous necrosis virus (BFNNV), redspotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV) and tiger puffer nervous necrosis virus (TPNNV) Temperature is important for the distribution of these various subtypes: 15-20°C for the BFNNV genotype; 25-30°C for RGNNV; 20-25°C for SJNNV; 20°C for TPNNV. RGNNV genotype seems to be the most widely diffused genotype and it is the most commonly detected in Mediterranean area and highly infectious for sea bass. Disease episodes generally occur when the water temperature reaches 18-20 ° C and symptoms are evident above 22-25 ° C. Affected fish typically suffer from several neurological disorders, characterized by intensive brain, spinal cord and retina granular layer vacuolisations, resulting in abnormal swimming behaviour, lethargy and darkening of fish colour [33]. VER is an endemic disease in the Mediterranean basin and the occurrence of the disease has been widely reported both in wild and farmed fish from this area [32]. European sea bass is one of the most susceptible species to Betanodavirus, especially at larval and juvenile stages and often associated with a mortality rate of up to 100%. On the contrary, gilthead sea bream is generally considered less susceptible and is a potential asymptomatic vector of the infection, for most of the NNV strains [34,35]. Although only few sporadic mortality events associated to Nodavirus infection in gilthead sea bream have been reported [36], this species can suffer mortalities when infected with some NNV reassortant strains [37,38].

Although it does not cause high mortality, the possible onset of Lymphocystis deserves attention. Lymphocystis is a chronic, self-limiting viral disease that affects more than 125 species of teleost worldwide, including sea bream [39–41]. The causative agent of this pathology is a DNA virus, belonging to the family Iridovirus, genus *Lympocystivirus*, which replicates in the cytoplasm of the affected cells. Transmission generally occurs through contact with infected individuals; trauma to the skin due to handling, excessive population density or aggressive behaviour can promote development and rapid transmission of the infection.

Lymphocystivirus replicates at the level of skin fibroblasts with the formation of hyperplastic cells which, however, do not undergo mitosis; the lesions, affecting the skin or fins, consist of nodular masses similar to warts that can range from 0.3 mm to exceed 2 mm in diameter [41]. Injuries rarely involve internal organs. Being a self-limiting pathology, these lesions tend to heal spontaneously leaving scars at the epithelial level as the only consequence [42].

Although this pathology does not cause high mortality, the probability of developing secondary bacterial or fungal infections should not be underestimated; the lesions, moreover, can however have different consequences depending on their location such as, breathing difficulties if located in the gills, or feeding difficulty if in the peri-buccal area [43]. Furthermore, the presence of scars at the level of the skin significantly affects the marketability of the product resulting in significant economic losses for the affected farm [44].

Regarding carp farming, spring viremia of carp (SVC) and Koi herpesvirus disease (KHVD) represent the viral pathologies that most affect production.

SVC, caused by *Rhabdovirus carpio*, also named spring viremia of carp virus, is a disease typically with an acute course, which can infect several freshwater fish species, but which mainly affects common carp (*Cyprinus carpio*) [45,46]. This disease has proven to be of great economic importance, causing serious losses in carp pond fisheries of the central and eastern part of Europe in the past [46]. When clinical disease appears, mortality can vary from 30 to 70%, spreading horizontally during the winter, when host immunity is suppressed. In spring, as soon as temperatures begin to approach 10 ° C, fish develop clinical signs of SVC.

Fish of all ages are susceptible to disease, but the more severe form affects juveniles. Affected fish show both external and internal signs, such as exophthalmia, abdominal distension, haemorrhage in different tissues and organs, peritonitis and ascites [45].

KHVD is a serious emerging disease caused by *Cyprinid herpesvirus-3* (CyHV-3) [47]. The disease is relatively host-specific, causing mass mortality in the common carp and its ornamental subspecies, koi carp [48]. The virus was first identified in Israel and the United States [49], but has since been reported in an increasing number of countries in Asia and Europe. The rapid spread of KHVD is probably due to the increased trade in ornamental fish [50]. KHVD is highly contagious, it can be transmitted by water or direct contact and the affected populations can reach 100% mortality [51]. Outbreaks generally occur in spring or summer, when the temperature is between 18 and 28 ° C. Affected fish typically show branchial damage (pale, swollen and mottled). Other symptoms include abnormal coloration, skin lesions, enophthalmia and dyspnoea; sometimes even anorexia and irregular swimming. The lack of clinical signs in a latent state and the persistence of the virus for long periods represents one of the major problems in the spread of the disease [52].

1.4.1.2 Bacterial diseases

Bacterial diseases provoke significant damage to aquaculture [53].

Among the most economically significant bacterial disease, vibriosis is the most important and most found, especially in the mariculture industry. It can affect a large number of cultured and wild fish [54]. Being a widespread disease caused by several species belonging to the genus Vibrio, it is responsible for disease, mortality and serious economic losses in aquaculture production; *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus* and *V. anguillarum* are the most frequently isolated [55]. Although vibrio species are ubiquitous organisms in marine and estuarine environments, they represent a constant threat to any sensitive host under predisposing environmental conditions [56,57]. *Vibrio alginolyticus* is frequently involved in epizootic outbreaks in cultured *Sparus aurata* in Mediterranean aquaculture [58]. Disease caused by *V. alginolyticus* in sea bream is classified as a typical bacterial septicaemia provoking haemorrhaging

septicaemia, fluid accumulation in the peritoneal cavity, dark skin, exophthalmos and ulcers on the skin surface in some cases [59]. Recovered for the first time in a marine fish tank, this bacterial agent is generally described as a secondary invader, constituting an opportunistic organism of already damaged tissue or a weak pathogen of stressed fish [55,60]. However, it has also been reported as a pathogen of several marine animals and humans [61].

Several studies reported *V. alginolyticus* as causal agent of vibriosis outbreaks in reared sea bream, especially during early life stages, from different geographical areas [62–64]. Infected larvae showed abdominal swelling and a consequent high mortality rate. Generally, abdominal changes tend to take two typical forms: 1) the intestine appears dilated with a thin wall, without peristaltic movement, full of undigested prey or still alive, however the larvae maintain their appetite and their feeding behaviour; 2) the intestine does not show any distension or rather moderate distension, it appears empty and the larvae appear anorectic and lethargic. Both types of alterations are characteristic of larval enteropathy (LE), previously described as distended gut syndrome (DGS). *V. alginolyticus* alone or in association with other bacteria pathogens, is considered primarily responsible for LE [65].

Photobacteriosis is one of the "classic" bacteriosis that often occurs in Mediterranean hatcheries, causing outbreaks with high mortality, with consequent large financial losses in European and Mediterranean aquaculture. It is a chronic to subacute systemic infectious disease of marine and brackish water fish caused by *Photobacterium damselae* subsp. *piscicida* with features including white granulomatous lesions in internal organs. Pasteurellosis, which appeared in Italy in 1991 [66], causes serious epizootic diseases in seabass and sea bream farms especially in hot seasons, when the water temperature reaches 22-25 ° C, or when salinity drops below 25-30 ‰ following prolonged periods of rain. Favourable and aggravating conditions of the infection are high population density, low concentration of dissolved oxygen in the water, high degree of pollution and concomitant presence of opportunistic pathogens. The most likely route of transmission seems to be direct contact through the gill epithelium; however, oral contagion through food is also considered possible [67]. Fish pasteurellosis can essentially be considered a septicemic disease with both acute and chronic

manifestations. The acute infection mainly affects juveniles (between 1 and 40 g of body weight) and generally leads to a very high mortality rate (80-90%). In the acute form, the fish display ataxic swimming, anorexia and lethargy, the body appears hyperpigmented and diffuse haemorrhages appear at the base of the fins and on the sides. At a visceral level, multifocal necrotizing lesions and haemorrhages can be detected mainly on the liver, spleen and kidney. The branchial epithelium has areas of necrosis and dyscrinia, the cause of respiratory failure. Chronic infection most frequently affects adult subjects where mortality drops to around 30%. In this form, characteristic whitish nodules, normally between 0.5 and 3.5 mm in diameter, located in the kidneys and spleen and smaller in size, can also be observed at liver level [68].

In carp farming, the most common bacterial diseases are carp erythrodermatitis and columnaris disease.

Carp erythrodermatitis (CE) is a common bacterial disease caused by the atypical strains of *Aeromonas salmonicida* (*Aeromonas salmonicida* subsp. *achromogenes*), which affects several freshwater fish species, but mainly common carp [69,70]. Unlike its typical counterpart, *Aeromonas salmonicida* subsp. *salmonicida*, generally associated with systemic furunculosis disease, the atypical strain causes localized infections at the skin level, resulting in skin ulceration. CE has been described as an acute to chronic contagious skin disease that varies in morbidity and mortality [71,72]. The infection often begins at the epidermal injury site which then becomes a haemorrhagic inflammatory process between the epidermis and the dermis. This red inflammatory zone gradually extends as the infection spreads. Rupture of the tissue leads to the formation of a central ulcer, which can occur in any position on the surface of the body. Secondary infections by fungi or other bacteria are common [55]. In some cases, CE can also cause generalized septicaemia and death. Unlike furunculosis, which usually occurs only when the water temperature exceeds 16 °C, CE can occur at all water temperatures [55].

Flavobacterium columnare is the causative agent of columnaris disease. It was first isolated in Europe from cultured rainbow trout in Mugla, Turkey; later it was recognised to have worldwide distribution in a wide range of freshwater fish,

including carp [73–76]. The gill is usually the main damage site in both young and adult fish. Gill injuries are usually made up of yellowish areas of necrosis. Extending erosion can completely destroy the gill filament [77]. On the body, small initial lesions appear as areas of pale discoloration at the base of the dorsal fin or occasionally at the base of the pelvic fin and lead to deterioration of the fins. These areas increase in size and can cover up to 20-25% of the total surface of the fish. Often, the skin is completely eroded, exposing the underlying muscle. It is common for fish to die within 48 hours of the appearance of skin discoloration [77,78].

It cannot be ignored that some diseases, such as mycobacteriosis, although not frequent in fish farming such as those described above, can be transmitted to humans, constituting a safety problem for workers (fish tank granuloma).

Mycobacteriosis is an infectious disease, affecting a large number of fish species, typically aquarium fish. More than 120 species of mycobacteria [79] have been recognized, many of which cause chronic progressive disease, mycobacteriosis, in fish. The species belonging to the genus *Mycobacterium* can be classified into two large groups: those that cause tuberculosis in humans and animals, the so-called mycobacterium tuberculosis complex (MTBC), with the exception of *Mycobacterium leprae* [80], and the non-tuberculous species (NTM), which are universal living organisms in the environment, causing a wide range of mycobacterial infections in animals and humans [81]. *M. marinum*, *M. salmoniphilum*, *M. fortuitum*, *M. chelonae* and *M. abscessus* have been recognized as pathogens for fish [87, 88]. More than 167 fish species have been found to be sensitive to mycobacteriosis [83].

Mycobacteriosis caused by *Mycobacterium marinum* was first reported by Aronson in 1926 [84] in marine fish kept in the Philadelphia Aquarium. Subsequently, mycobacteriosis has been reported in many marine and freshwater fish species worldwide [55]. All fish species should be considered susceptible [85]. Disease outbreaks have been described in a number of cultured species such as striped bass (*Morone saxatilis*) [86], turbot (*Scophthalmus maximus*) [87], African catfish (*Clarias gariepinus*) [88], yellowtail (*Seriola quinqueradiata*) [89], and meagre (*Argyrosomus regius*) [90]. Most of these outbreaks have been

associated with high mortality and significant economic losses. For example, in striped bass mortality was around 50% [91] and in African catfish 5% [88]. *M. marinum* outbreaks in cultured fish appear to be related to high stocking density [90,91], poor diet [92] and water quality [83]. Such stressors could reduce immune function [83] and affect the progression [93] and severity of the disease [92]. Granulomatous inflammation [94] is the main pathological lesion of mycobacteriosis. A central area of necrosis in granulomas is surrounded by epithelial cells, macrophages and fibrous connective tissue [95]. Granulomas are found mainly in the liver, kidneys and spleen.

1.4.1.3 Parasitic diseases

Farmed fish can be affected by a wide variety of parasites. The concept of parasitism is rather extensive; in its wide meaning, all living organisms which live in, feed from, and damage other organisms, are considered parasites. However, in its practical sense, only organisms belonging to protozoans and metazoans are considered real parasites. The main groups of fish disease caused by parasites include:

- Diseases caused by protozoans: flagellates, ciliates, apicomplexans;
- Diseases caused by myxosporeans;
- Diseases caused by parasitic worms (helminths): monogeneans, digeneans, cestodes, nematodes, acanthocephalans, leeches;
- Diseases caused by parasitic larval molluscs;
- Diseases caused by crustaceans.

Some parasites live their entire lives in or on the same host, while others have more complex life cycles. Besides the final host in which they mature, they may have one or more intermediate hosts, in which they grow during their subsequent developmental stages. Of these hosts, the main or final host is that organism in which they reach sexual maturity.

Parasitoses mostly reported in Mediterranean farms are attributable to *Trichodina* spp., *Amyloodinium ocellatum*, *Cryptocaryion irritans*, *Diplectanum aequanse* and endoparasites of the genus *Sphaerospora* (e.g. *S. sparis*, *S. sparidarum*, *S. dicentrarchi*) [96].

In carp farms the most commonly found parasites, under dense rearing conditions of aquaculture are represented by *Ichthyophthirius multifilis*, *Sphaerospora dykovae*, *Diplostomum spathaceum* and also members of the monogenean orders Dactylogyridea and Gyrodactylidea [97].

Some of these parasitic diseases can have a more impacting effect when particular conditions are present in the farm, such as poor water quality, inadequate forms of prevention, inadequate management or excessive stressors. Anthropogenic environmental change is increasingly implicated in outbreaks of parasite disease. Factors such as increasing temperatures, environmental pollution or the introduction of new species can all unbalance parasite-host interactions. The cumulative effect of multiple environmental stressors may be particularly damaging, with negative implications for immune function and animal health. For example, *I. multifiliis* and the marine *C. irritans* can devastate farmed and wild fish, causing the parasitic white spot disease although, in controlled breeding conditions they tend to be less harmful as they are easier to control. Problems associated with protists in aquaculture arise because they can transmit without the need for an intermediate host. Thus, environmental manipulation on farms to decrease stress in host animals or increase water quality may reduce the lethal outcome of infection [94].

1.4.2 Deformities

Developmental deformity studies in fish have been widely described and sometimes associated with particular causative factors. Malformations observed in fish have commonly been associated with physical, chemical, environmental, genetic and infectious organisms. Some have also been attributed to specific nutritional deficiencies. Developmental anomalies are reported in both wild and farmed fish species [98–103]. In nature, they could be a consequence of the teratogenic effects of some xenobiotics or toxins produced by other marine organisms, changes in radiation, temperature or salinity. However, these deformities occur much less frequently than in farmed fish [104–107]. It would seem that fish affected by deformity have difficulty surviving in natural conditions, undergoing strong selective pressure [108]. On the contrary, farmed fish suffer a high incidence of deformities, representing an important problem in fish production.

Skeletal deformities represent one of the bottlenecks of intensive aquaculture. Despite the fact that progress in the field of nutrition, larval incubation methodologies, and increased knowledge of diseases have all improved the survival and quality of fry, skeletal deformities continue to be a current problem. Malformation can occur at all life stages, although in general, the effects of exposure to suboptimal conditions tend to be much more severe in early development than in old age. Even very slight changes can have a significant impact on larvae and juveniles leading to mortality or severe deformation. Fish that have not yet undergone skeletal ossification are particularly sensitive to negative influences and this can have serious economic consequences for farmers, due to mortality, reduced growth, deformities and non-marketability of the final product. Consequently, a greater knowledge of skeletal system development of farmed species could represent an important contribution to the optimization of larval rearing. Indeed, knowing when the skeletal structures involved in swimming and feeding have completed their development could provide useful information for identifying potentially critical stages of growth.

Skeletal anomalies are economically important for various reasons. First of all, they require manual sorting; fry with deformities are selected and eliminated. Generally, hatcheries have a malformation rate of about 5%; up to 15-50% of gilthead sea bream fry affected by deformity are eliminated from the production cycle at the end of the hatchery phase [98]. The methods currently used in hatcheries to eliminate deformed juveniles can be different. For example, individuals with uninflated swimming bladders are easily sorted, as they do not float when they are anesthetized. In the case of skeletal deformities, obviously only individuals with externally detectable anomalies can be identified for killing by manual sorting after anaesthesia. Nonetheless, the sorting method does not eliminate the presence of deformities in the on-growing phase, this is because serious skeletal anomalies can initially be masked, since they often begin with slight aberrations of the internal elements, which then develop into more serious anomalies that influence the shape of the adult's external body [98,99,109–111]. Therefore, further sorting is necessary before the product can be marketed. This obviously requires a large workforce and a high percentage of fish produced is lost. Secondly, deformities reduce fish performance, such as swimming capacity, feed conversion ratio, growth rate, survival and increased susceptibility to stress and pathogens [99,107,112]. Furthermore, a fish that presents anomalies, in shape or colour with respect to its wild counterpart, induces distrust in the consumer and this prevents marketing.

Skeletal deformities of varying degrees and severity are very common in farmed fish. These generally include spinal column, head and fin deformities.

Spinal deformities are well documented and include scoliosis (lateral curvature), lordosis (V-shaped dorsoventral curvature of the sagittal plane including the spine and spinal cord) and kyphosis (∧-shaped curvature) of the vertebral column. In some cases, these can present as a combination of all three deformities [113]. Scoliosis is one of the most frequent malformations found in fish and has been extensively studied. It is a debilitating problem that can cause mortality and, if this happens with high frequency, a decrease in the commercial value of fish. The partial or unilateral failure of the formation of the vertebral body can give rise to incomplete or wedge-shaped vertebrae. Lordosis is often found together with scoliosis; it is generally attributed to genetic causes or even to the speed of current [113]. In some cases, lordosis is associated with the lack of functionality of the swim bladder, attributed to compensatory mechanisms due to the lack of good buoyancy, leading to a progressive torsion of the spine. In general, the survival of lordotic larvae is higher than that of other deformed larvae, probably because, unlike other coarser deformities, lordosis in larvae does not totally interfere with swimming and feeding behaviour [112]. Severe spinal deformities have always led to anomalies over a relatively wide range of vertebrae, which can appear fused and deformed mainly in the region of curvature of the maximum axis. Vertebra deformities can sometimes be present regardless of the presence of axis deviation [114,115]. Other anomalies include incomplete dorsal fusion of the vertebrae around the spinal cord called spina bifida, segmentation errors that can give rise to fused vertebrae [116].

Cephalic deformities include different classes of malformations:

1) Anomalies of branchial coverage. These are deformations, unilateral (generally) or bilateral, of the bones of the opercular complex which consist of reduction, twisting or bending of the operculum, suboperculum and sometimes the preoperculum, which can sometimes occur in association with the alteration of the gill arches [117]. Shortened opercular plates, together with lordosis, are the most frequently observed deformities in bred sea bream [117,118].

2) Pugheadedness; deformity affecting the skull, jaws and eyes, causing a reduction in the frontal skull and upper jaw bones.

3) The cross bite; deformity in which the lower jaw is tilted off-centre or moved laterally, so that it appears folded or crossed and not oriented parallel to the upper jaw.

4) Lower jaw reduction.

Other deformities that can be found in farmed fish are borne by the caudal, pectoral and dorsal fins which can present malformations, such as the total or partial absence of the anatomical portion [119] or, in some cases, reduction in size [120]. When these types of deformities are very serious, they can induce skeletal deformities due to the unnatural movement of the body, especially if this occurs during development.

Other anomalies may involve fish shape, pigmentation and scales. The shape is the first visual criterion for the recognition of fish species and the main commercial reference. Anomalies of the shape are in fact the main causes of unsalability of the product. Pigmentation anomalies are another important factor in the marketability of the fish product. They may involve partial or total albinism, ambulatoryization and xanthochroism [121]. Finally, scale anomalies can lead to the absence or loss of scales [122], reduction or increase in size [123,124] and abnormal scale distribution [100].

Multiple factors have been suggested as possible causes of developmental anomalies:

Mechanical factors and technopathies. Manipulation of larvae and fry during transport operations, anaesthesia and overcrowding can contribute to the alteration of anatomical structures. For example, it has been reported that poor current management can cause intense muscle activity in the juvenile's caudal portion, causing an excessive load on the spine, which is still developing, and therefore malformations develop, determined by intense activity muscle of the caudal portion, [109]. However, these anomalies do not seem to excessively reduce performance in terms of growth and resistance to stress and fish infections.

Nutritional factors. These can determine the onset of spinal pathologies such as deficiency of ascorbic acid, tryptophan, phospholipids and excess of vitamin D and A in food. Nutritional deficiencies are often associated with scoliosis in fish [125]. Vitamin C, for example, contributes to the development of the spine, and a deficiency has been implicated in a series of vertebral deformations [111], as well as in the weakening of the cartilage of the gills which can lead to distortion of gills filaments [126].

Environmental factors. Spinal changes can be the result of sudden changes in environmental factors such as salinity, temperature and light [127], and pH shock [128]. For example, thermal shock, if it occurs during a critical early development phase, can cause 100% incidence of scoliosis in exposed embryos. Apparently high temperatures cause asynchronous organ development leading to morphological abnormalities [129]

Toxic substances. Spinal deformities can be caused by exposure, even at relatively low concentrations, to some toxic materials. Pollutants such as zinc, chlorine,

organophosphates and carbamates can be associated with various vertebral anomalies [94]. Heavy metals and pesticides can cause an interruption of vertebral development with consequent curvature of the vertebral column [130]. Cadmium and zinc can alter the biochemical composition of bones by interfering with mineralization and muscle activity [131]. Deformed vertebrae and fins are often observed in fish that have high levels of heavy metals in the liver and muscles [132]. Some pesticides can cause cranial anomalies in embryos, exposed during a critical phase of development [133].

Pathogens. Several pathogens, such as metacercariae (*Apophallus* sp.), myxozoan (*Myxobolus* sp.) and *Flavobacterium psychrophilum* have been associated with skeletal deformities in fish [134,135].

Genetic factors. Spinal deformations, although rare, can have hereditary causes [111]. In sea bream, high levels of consanguinity between reproducers can be responsible for the development of vertebral anomalies [134].

1.4.3 Technopathies

Production related infectious diseases and disorders refer to a wide range of conditions, with often multifactorial origin and therefore require a multidisciplinary approach in management. The main factors affecting the welfare of farmed fish relate to the production system, production cycle, farming and management practices and the control of infectious diseases. While an extensive production system requires minimal human intervention, semi-intensive and intensive production systems require sophisticated management practices at all stages of the production cycle, i.e. management of culture water, broodstock management, larval growth, nutrition and feeding. During regular breeding procedures, fish are exposed to various ailments that could compromise their well-being. Potential stressors can be detected within the production cycle; these include cleaning, handling and manipulation, crowding and confinement, sexing of the broodstock, food distribution methods, transport between farm units, prophylactic measures, use of chemicals and anaesthetics. All these factors can cause physical injury, morphological changes and stress, and compromise both the well-being and quality of the final product [20]

Poor water quality is a detrimental factor for any aquatic species; therefore, the management of culture water represents one of the fundamental factors for the success of aquaculture production. Several water parameters such as oxygen, pH, temperature, salinity or conductivity, and ammonia, can have a strong influence on the growth of fish

species [136,137]. For example, fish excrete highly toxic ammonia and exposure to it represents a dangerous problem in fish farming, which translates into a significant increase in oxygen consumption and difficulty in breathing [20]. In some species that are particularly sensitive to ammonia concentrations, such as *Sparus aurata*, this can lead to mortality, while long-term exposure leads to reduced growth [136]. Other species, such as *Cyprinus carpio*, are more tolerant to variations in ion concentrations in the water, as well as to fluctuations in oxygen levels. However, low oxygen levels can affect the preparation of broodstock for propagation, affecting or preventing maturation. For the control of oxygen levels, in semi-intensive systems, the management of vegetation in ponds is of fundamental importance. A large biomass of plants or algae can alter the oxygen balance inside the pond, allowing a high volume of dissolved oxygen and an efficient absorption of carbon dioxide during the day, but causing a sharp drop in oxygen levels during the night [138]. In complex systems, in terms of technology, such as recirculation systems, water treatment is based on solid filtration; nitrification and denitrification for the removal of ammonia and nitrates respectively, exchange of gas for the supply of oxygen and removal of carbon dioxide by stripping.

Another fundamental aspect for the productive success of the farm concerns broodstock management. This allows the establishment of selective breeding programs that exploit the inheritance of some traits for the optimization of the production cycle of a specific fish species. Breeding selection procedures are fundamental to obtain better performance in growth, shape, disease and stress resistance, egg production, percentage of hatching and survival rate [20]. Successful fish propagation, in more or less complex systems, can only be obtained by properly preparing the broodstock. The reproduction process requires high demands in terms of fish body resources and therefore the breeder must create and maintain the necessary conditions for gamete development and maturation. The time required to prepare broodstock for spawning is species-specific, as is stock density or management of the male and female population for hermaphrodite species [139].

Regarding larvae management, growth can be influenced by numerous factors, among which the main ones are temperature and food. In semi-intensive systems the survival of larvae is highly dependent on weather and preparation of the ponds. In carp farms, preparation begins with cleaning the bottom of the pond and spreading quicklime, to discourage the survival of pathogens. Subsequently this is fertilized to promote the growth of zooplantonic organisms, essential for the nutritional needs of the larvae [138].

In marine intensive systems other important factors for larval growth include oxygen, salinity, turbidity and light (intensity and photoperiod). The optimal requirements are generally species-specific and change during larval ontogeny. In general, temperature has deeper effects during early life stage. There is a positive correlation between increase in temperature and the growth and development of many marine fish species [140]. Osmotic stress associated with high salinity can also have harmful effects, reducing inflation of the swim bladder and affecting growth [141]. As visual feeders, different marine species need light to feed and growth can be stimulated by longer photoperiods and hence feeding duration [142].

Microbial management of water and live feeds through water treatment, use of disinfectants, probiotics and immunostimulants, can also have important influences on the growth of larvae.

In aquaculture, the use of disinfectants is allowed only for the disinfection of equipment to reduce the transmission of pathogens. The main disinfectants used in aquaculture include formaldehyde, chlorine derivatives and iodophor acting against viruses, bacteria and fungi, caustic soda only against virus, phenols against bacteria, quaternary ammonium salts against bacteria and fungi and finally chloramine that is a natural non-toxic disinfectant. Vaccination is key to large-scale commercial fish farming and is undoubtedly one of the main reasons for the excellent results obtained in farmed fish.

The future development of the European aquaculture industry will surely depend on a greater scientific knowledge of the biology and genetics of fish species, the influence of environmental factors on growth and an increase in health monitoring procedures to ensure an ever better quality of final product [143].

1.4.4 Pollution

Pollutants represent a possible indirect cause of economic loss in the aquaculture industry. Fish diseases are known to result from biotic factors, such as pathogens, and from abiotic causes such as contaminants [55]. These factors are closely related to the quality of the water, which represents the most important of the parameters to manage for the success of aquaculture production.

1.4.4.1 Metallic and non-metallic contaminants

Aquatic animals are healthier and grow better when environmental conditions fall within certain ranges, which define water quality as "good". Culture water must be

managed during production to ensure good growth, reduce stress and the mortality rate of farmed species. There are various types of pollutants, the presence of which causes a reduction in the quality of the aquatic environment and consequently of fish produced. Substances such as heavy metals and pesticides have attracted public attention in aquatic and aquaculture ecosystems, contributing to one of the major problems of water pollution, which can seriously damage the environment and cause risks to aquatic organisms and human health [144]. Pollution of surface and underground waters, coming from industrial, urban and agricultural sources, allows these substances to be transported by the outflow and river waters, to then reach the coastal environment. Due to their toxicity, persistence and tendency to accumulate in water and sediments, pollutants such as heavy metals, persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), can become seriously harmful to all living organisms [145]. Various studies have investigated heavy metal concentrations in both wild and cultured fish [146–148]. There is evidence of significant correlation between increased heavy metal pollutants and increased mortality in aquaculture species [149,150]. Fish can absorb heavy metals from surrounding water or food through gills, intestinal absorption or skin; these are transported by the blood reaching and accumulating in different organs and tissues [151]. In general, heavy metals can affect various metabolic processes during the development of fish and in particular during the embryonic stages, resulting in a slowdown in growth, morphological and functional deformities and, in severe cases, death of the most sensitive organisms [132]. Heavy metal exposure has recurrently been associated with deformities in a wide variety of fish species. For example, cadmium and zinc appear to have a strong correlation with spinal deformities [152–154]. Physiological injuries such as fertility decrease and other abnormalities of the reproductive and immune system can also be related to exposure to heavy metals [155]. By acting as endocrine disruptors, metals such as cadmium can raise the levels of thyroid hormone and inhibit estrogen receptors [156,157]. There is also evidence that metal ions can cause oxidative stress [158], genotoxic effects and alterations in the sensory organs of fish [159,160]. Moreover, it has been observed that the presence of heavy metals can increase the susceptibility of aquatic organisms to various pathogens such as hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis (IPNV), *Vibrio* spp., *Saprolegnia* spp. [149,150,161,162].

Further sources of contamination in fish farms include PCBs and PAHs, two classes of hydrophobic organic chemicals characterized by a strong tendency to bioaccumulation [163]. Both classes of contaminants show toxicological effects related to cancer, endocrine disruption and neurobehavioural changes [164,165]. There is evidence that exposure of fish embryos to PAHs can cause spinal, jaw and skull deformities [166], cardiac dysfunction and oedema [167,168]. Furthermore, both PAHs and PCBs can cause oxidative stress in fish [158].

Therefore, it is clear that the incidence of pathologies presents an interesting correlation with pollution, which requires considerable control measures, comparable to the many efforts dedicated to the control of disease in fish farms**.**

1.4.4.2 Emerging contaminants: Microplastic

Plastic pollution has become a global problem. Since large-scale industrial production started in the early 1900s, the production of modern plastic has undergone a substantial increase. World plastic production was estimated to have reached 322 million tons in 2015 [169] . Plastic is now involved in many aspects of daily life and in the industrial sector, such as packaging, building and construction, automotive, electronics, medical, cosmetics, textile industry, agriculture and many others [170]. The term “plastic” describes a wide range of polymeric materials that are moulded under specific conditions of temperature and pressure, which have different properties depending on the final product required. In short, it includes an extended family of synthetic or semi-synthetic polymers, and copolymers corresponding to the mix of 2 or more polymers. Furthermore, depending on the desired final product, different additives can be added to the polymers to improve performance. These additives can include plasticizers, antioxidants, lubricants, colorants, etc. Although these manufacturing processes represent an evolution in the plastic industry, they add a layer of complexity to the disposal and recycling of modern plastics. Inadequate management of plastic waste has generated a heavy environmental impact, culminating in ever increasing contamination of freshwater, estuarine and marine environments [171,172].

Plastic items directly produced measuring less than 5 mm are defined "microplastics" or "primary microplastics". Alternatively, microplastics that may result from fragmentation or degradation of larger plastic particles are indicated as "secondary microplastics". Microplastics, coming from terrestrial or marine sources, can enter the

aquatic environment through different pathways, reaching all the matrices of this environment [173–176].

In the marine environment, plastic waste represents the most abundant litter category, which can amount to more than 80% of the debris reported [177]. Both sea and land-based activities are responsible for the continued input of plastic, making it a ubiquitous pollutant. Once released into the sea, depending on their physical properties and environmental conditions, microplastics can colonize all compartments of the marine environment: coastlines, water surface, water column, seafloor and biota [178–181]. Their ultimate destination in the environment is mainly influenced by the density of the polymers: polymers with a density higher than that of water (> 1.027 g / cm3) will tend to settle on the bottom; while low density polymers will tend to float in the water column [178,182].

However, some processes such as biofouling, colonization by organisms on plastic surfaces, or even degradation and fragmentation processes can change the density of microplastics and consequently their distribution within the marine environment [183,184]. Microplastic distribution can subsequently be influenced by environmental factors, such as winds, surface currents, turbulent flows, tides, waves, sea storms [185].

The presence of microplastics has also been reported in fresh water environments (lakes, rivers, estuaries), resulting in a wide variability and spatial distribution [172,186,187]. In general, as for the marine environment, sediments show greater plastic waste accumulation than their aquatic counterpart. Lake environments located near urban and industrial centres represent one of the main plastic waste storage sites, although, in some riverine systems, this relationship is not so evident and could be the result of flow dynamics and flooding [188].

Microplastics in aquaculture

Proportionally to the ever-increasing demand for seafood, aquaculture has changed and developed rapidly, using increasingly innovative technologies. The sector expansion and the use of modern materials for the construction and maintenance of culture systems, have advanced in parallel with the development of the plastic industry, through the production of synthetic polymers that have characterized the last 50 years. The production of modern plastics has strongly influenced the development of fishing and aquaculture. The replacement of natural materials with those of a synthetic or semi-synthetic nature (cheaper, durable and handier) has certainly

allowed an advancement of the techniques used in this sector [189]. In this context, plastic materials have found numerous applications: lines, cages, nets, boat construction (including paints), worker protection devices, transport, transfer and packaging activities. Unfortunately, part of these materials could become marine litter. Lost materials, deriving from fishing and aquaculture, are regularly reported in investigations of plastic debris on beaches, waters and sediments. Plastics and microplastics sampled on the North Sea coast, and in Korean waters, have been widely attributed to shipping and fishing activity [190–192]. As regards the fishing and aquaculture sector, abandoned, lost or otherwise discarded fishing gear (ALDFG) is believed to be the main source of plastic waste released into the marine environment [7]. This would appear to consist mainly of gillnets, trawls, handlines and longlines. The loss of these structures could be due to a number of reasons, such as rope wear, severe weather conditions, accidents or conflicts with other maritime users [193]. However, to date there are no estimates, globally or regionally, of the contribution by fisheries and aquaculture to the amount of plastic waste in the aquatic environment [169].

Interaction of microplastics with aquatic organisms

The ubiquitous presence of microplastics in the aquatic environment has raised concerns about the interaction with biota, both from an ecological point of view and about the potential contamination of the human food supply [194,195]. Microplastics present in the aquatic environment are directly exposed to organisms and ingestion seems to be the most likely of the interactions between them. Microplastics uptake by organisms can be direct or indirect. Given their size, they may be indistinguishable from natural prey and be accidentally ingested according to the feeding behaviour of aquatic species. This phenomenon has been described as "primary ingestion of microplastics". In addition, microplastics previously ingested by prey can be an indirect source of contamination for predators, through trophic transfer, causing bioaccumulation and biomagnification phenomena. In this case we will talk about "secondary ingestion of microplastics" [196]. Although there is currently no direct evidence of trophic transfer of plastic debris in wild populations, the presence of microplastics has been observed in several groups of organisms, including zooplankton, invertebrates, bivalves [197–200] and pelagic and benthic fish [201–203]. Over 200 aquatic species showed contamination by microplastics of which

more than 50% were represented by species of commercial interest (GESAMP 2016; Kershaw Peter J 2016).

Several reports have documented the presence of microplastics in the digestive tracts of wild caught fish from different geographical areas, such as the Mediterranean Sea [194,205–209], the North Sea [210], the Baltic Sea [211], and the North Eastern Atlantic [202]. An increasing number of studies have targeted species of commercial interest such as *Pagellus* spp. [212], *Zeus faber, Lepidopus caudatus* [213], *Merluccius merluccius* [214] *Mullus barbatus* [215], *Xiphias gladius, Thunnus thynnus* and *Thunnus alalunga* [196]. Recently plastic particles have also been reported in wild fish larvae, showing that they can contaminate aquatic organisms at all life stages [216,217]. Although it is clear that microplastics can be ingested by many species of significant economic importance, still little is known about the impact of this debris on the health of aquatic organisms and consequently on consumption. Some laboratory investigations have provided evidence of damage caused by the ingestion of plastic microparticles affecting marine organisms, such as lesions affecting the anatomical structures, physiological and neuronal injuries, energy deficiencies, reduction of food activities and finally death [218–221]). In addition, it has been observed that microplastics can act as carriers in the transport of pollutants such PCBs, PAHs, heavy metals and pathogens [222,223]. However, although the presence of microplastics has been investigated in many commercial species, few studies have verified the occurrence of plastic microparticles in farmed fish species [224] whose emission source could be represented by the surrounding environment or from the fish farm itself.

1.5 Innovation in aquaculture fish health management

1.5.1 Immunostimulants in aquaculture

As mentioned above, world aquaculture has grown rapidly in terms of production over the past few decades. However, the rapid development of aquaculture and the increased fish demand have led to the intensification of fish culture, magnifying stressors and increasing the risk of infectious diseases [225]. Factors including overcrowding, handling, poor water quality and malnutrition can contribute to physiological changes in fish, such as stress or immunosuppression, and increases susceptibility to infections [226–228]. Infectious disease outbreaks in farmed fish have emerged as the main limit for the development of aquaculture, often causing high levels of mortality and partial or total

loss of production. To avoid economic losses due to health failings, several veterinary medicines are commonly used in aquaculture to prevent or control epidemics, such as antibiotics and vaccines.

Unfortunately, treatment with antibiotics is not an effective and sustainable solution. It is often very expensive, and prolonged use of antibiotics could also lead to many collateral effects such as antibiotic resistance, immunosuppression, environmental pollution, and the accumulation of chemical residues in fish tissues which can be potentially harmful to public health. [226,229].

Vaccination is a valid prophylactic treatment for infectious diseases in fish culture, however the production of effective formulations for several pathogens is often hampered by high production costs and by the antigenic heterogeneity of microbial strains [230,231].

Given these problems, in recent years the interest of researchers, pharmaceutical companies and farmers has shifted towards the development of alternative strategies for the management of infectious diseases in aquaculture, which can strengthen the immune responses (immunocompetence) of fish and consequently resistance to pathogens. Among these strategies, the application of immunostimulants in aquaculture has emerged as one of the most promising alternatives in the prevention and control of infectious diseases [225,232,233].

An immunostimulant is defined as a natural or synthetic compound, with diverse origin and function, that modulates the immune system and increases host resistance by enhancing the non-specific defence mechanism. [234,235]. In practice, immunostimulants are promising dietary supplements to potentially aid in disease control of several organisms, including aquatic organisms, and increase disease resistance by causing up-regulation of host defence mechanisms against pathogens in the environment.

Different aquaculture needs have given rise to different methods of administration of immunostimulants: injection, immersion, and oral. Oral administration is the simplest method, is economically suitable for extensive and intensive aquaculture, is not stressful and allows mass administration regardless of the size of the fish, but obviously, it can only be administered with an artificial diet. Immunostimulants that are effective in fish laboratory diets act within the non-specific immune system at different levels. Following administration, responses that are regularly reported include macrophage activation, and an increase in phagocytosis by neutrophils and monocytes, in the number of lymphocytes, in serum immunoglobulins, and in lysozyme [236,237].

1.5.1.1 Use of plant and fungal extracts as immunostimulants in aquaculture

According to Sakai [238], immunostimulants can be catalogued according to their sources: bacteria, yeast, fungi, plants, algae derivatives, animal derivatives, nutritional factors, and hormones/cytokines. However, the use of hormones, vitamins, and chemicals is often not recommended as they can cause collateral effects in fish and leave potentially dangerous residues for consumers.

On the contrary, natural products such as plant or fungi and their extracts can represent a promising complementary approach to vaccination and traditional drugs, since they provide a useful source of biologically active secondary metabolites, but at the same time are easily available, cheap and eco-friendly [239].

Among the various classes of immunostimulants used in aquaculture practices, b-glucan represents one of the most promising immunostimulants. β-glucans are naturally occurring polysaccharides, the structural component of which is glucose, linked by b-glycosidic bonds. In nature, β-glucans are widespread in the cell wall of many plants, yeast (*Saccharomyces genus*), algae such as *Laminaria* sp*.,* and various species of mushrooms such as Shiitake (*Lentinus edodes*), Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*) [240].

Several studies have shown that β-glucan is a potent immunostimulant to improve immune status and to control fish culture diseases [232,241–244]. Many investigations have been conducted on different commercial fish species such as Atlantic salmon [245], rainbow trout [246], African catfish (*Clarias gariepinus*) (Yoshida et al. 1995), sea bass (Bagni et al. 2000; Bonaldo et al. 2007) and gilthead sea bream [250]. These demonstrated the positive effect of β-glucan on growth [251], survival, resistance against pathogens [252,253], antibody production [254,255], and on immunized gene expression [256,257]. It has been observed that β-glucan plays an important role in the activation of both innate and acquired immune responses. In innate immunity, the responses stimulated by β-glucans not only act in defence against microorganisms but also by completing the activation and action of acquired immunity [238]. β-glucans are responsible for a multitude of actions that protect and improve the immune system by providing excellent resistance to any possible health aggressors, thanks to their ability to bind directly with macrophages and other white blood cells (neutrophils and natural killer cells), and to activate them by receptor bonds [258,259]. When β- glucan receptors are involved with beta 1,3 / 1,6 glucans, all immune functions improve, including phagocytosis, the release of some cytokines,

IL-1, IL-6, interferons, and antigen processing. These cytokines stimulate the formation of new white blood cells, thus providing immunity to the β-glucan binding receptors present in all vertebrates ranging from fish to humans [240].

As for β-glucan, the use of medicinal plants and/or plants in aquaculture has attracted a lot of attention globally and has become the subject of numerous scientific studies. Indeed, many plants and their extracts can act as immunostimulants [260,261].

The bioactive portions of plants include a variety of compounds such as alkaloids, tannins, flavonoids, coumarins, saponins, quinones, terpenoids, steroids, simarubalidans, melicianins, limonoids, lactones and lignans [262]. The content of secondary biomolecules depends on specific plant factors such as weather conditions, soil characteristics, age of plants and harvest time, as well as on the method of extraction of these principles. These characteristics influence the properties of the plants giving them peculiar antibiotic, antiviral, anticancer and immunostimulant activities [263–265].

Several of these dietary additives have proven to be useful in improving immune status, food efficiency and growth performance in fish and crustaceans [250]. For example, acetone extracts of four plants *Cynodon dactylon, Aegle marmelos, Withania somnifera* and *Zingiber officinale* were screened for their inhibitory activity against different fish *Vibrio* pathogens, such as *V. alginolyticus, V. parahaemolyticus, V. mimicus, V. campbelli, V. vulnificus, V. harveyi* and *Photobacterium damselae subsp. piscicida.* These extracts, mixed with fish feed in the proper ratio, produced an enhancement in leucocrit, phagocytic and lysozyme activities in *Oreochromis mossambicus* fed with an experimental diet [266]. *Trigonella foenum graecu* and *Jasonia glutinosa* in gilthead sea bream improve immune and oxidative status and growth performance [267,268]. Other herbal plant extracts such as *Azadirachta indica,* and *Picrorhiza kurooa* have shown antiviral and immunostimulant characteristics in *Penaeus modon* specimens affected by white spot syndrome virus (WSSV) [269]. In *Litopenaeus vannamei,* the methanolic extracts of *Aegle marmelos, Tinospora cordifolia* and *Eclipta alba* have shown similar antiviral action against WSSV [270]. *L. vannamei* fed on the powder and extract of *Sargassum hemiphyllum var. chinense* increased immunity and resistance against *Vibrio alginolyticus* infection [271]. The powder of *Echinacea pupurea, Uncaria tomentosa* and *Ocimum sanctum* exerted greater immunity and resistance to WSSV infection in *L. vannamei* [272]. *U. tomentosa* has been used as a medicinal plant in humans for a long time ago

and its immunostimulant activity appears to be due to quinovic acid glycosides and oxide-pentacyclic alkaloids; several studies have shown that *Uncaria* stimulates the immune defences and induces greater reactivity of immunocompetent cells and natural killer cells [273–275].

The studies mentioned so far indicate that diets enriched with plant extracts have beneficial effects on the health of fish, improving the immune system and that they could therefore play an important role in the prevention of disease outbreak in aquaculture systems. However, in most cases the mechanisms responsible for fish immune response are still unknown or poorly understood. Hence it would be interesting to investigate more in depth on the synergistic effect of several extracts of mixed herbs, thus determining whether the observed bioactivity is due to isolated molecules or is rather a consequence of a more effective synergistic effect between different molecules contained in the extracts [276–278].

The study of immune response in fish species is proving increasingly important today to characterize adaptations of the innate and adaptive immune system in the recognition of self and non-self. Knowing the expression of certain genes that characterize an immune response specific to a specific antigen can help understand the defence mechanisms of the host under consideration and can start the production of foods and supplements that can amplify host defences against that specific pathogen, to eradicate it in fish species that can be bred in aquaculture. The study of the immune system of teleosts is a necessary requirement to create new effective vaccines against pathogens ever more found in fish species. The use of substances of natural origin with antibiotic and immunostimulant effects is essential in a modern, eco-sustainable aquaculture sector, along with increasing resistance levels and improving the quality of the meat, through the use of medicated feed, of farmable fish species bringing a diversification of the offer necessary to cover a demand that is becoming higher and more difficult to meet each year.

1.5.2 Application of nanotechnology in aquaculture

Nanotechnology is an emerging, evolving sector that is playing an important role in promoting global economic growth and, by 2024, the global nanotechnology market is expected to exceed US $ 125 billion [279]. The rapid progress of nanosciences and nanotechnologies in recent years has opened new horizons for many industrial sectors such as electronics, energy, the biomedical industry, including agriculture and related

sectors. Nanotechnology has been defined by the National Nanotechnology Initiative (NNI) of the United States as "understanding and controlling matter at dimensions between about 1 and 100 nm, where unique phenomena allow new applications". In this size range, particles show new physical and chemical properties that can be used in diverse scientific and productive fields, as their smaller size and increasing number of surface atoms gives them specific biological, optical, magnetic and electric properties [280].

Several applications of nanotechnology for aquaculture production are under development. A variety of different nanomaterials, such as inorganic, carbon-based and polymer-based materials, have found application in the aquaculture industry such as growth, genetics, reproduction, immunity, disease control, water and wastewater treatment (Fig. 9) [281]. With a long history of adopting new technologies, the highly integrated fish farming industry may be among the best for incorporating and commercializing nanotechnology products [282].

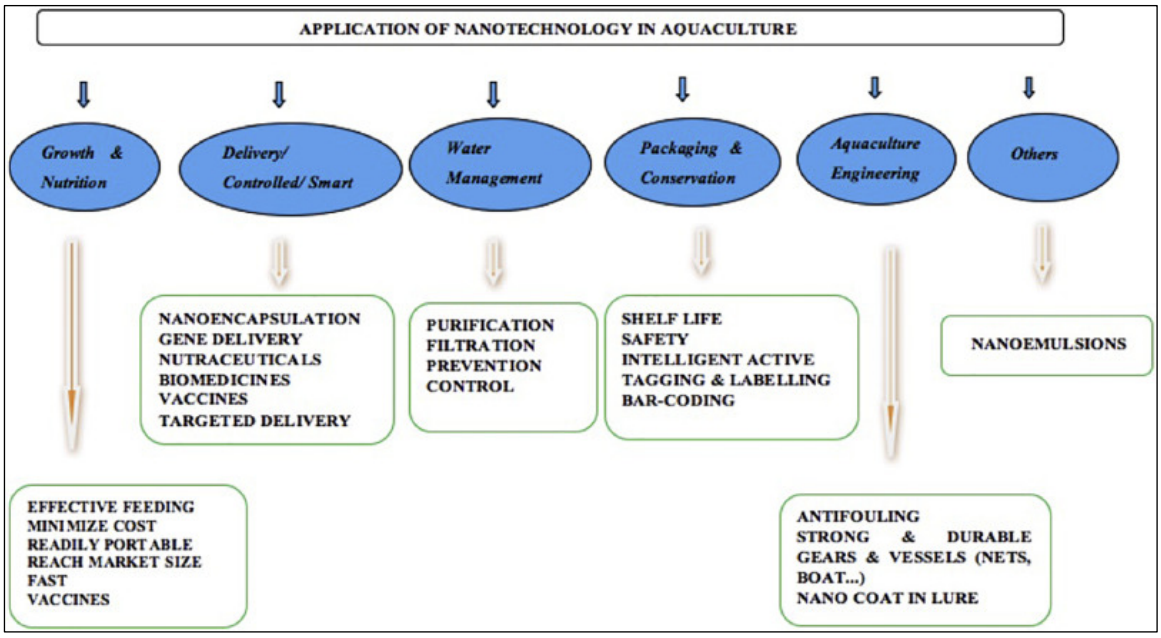

Figure 9. Applications of nanotechnology in several areas for aquaculture enhancement.

1.5.2.1 Use of titanium dioxide in water treatment

Aquatic pollution is undoubtedly one of the main problems that society faces today, representing also one of the greatest threats for aquaculture production (see section 1.4.4). The physicochemical properties of water in aquaculture ponds can be

influenced by various parameters such as soil composition, environmental pollution, and food waste [281,283], while in coastal or open-sea cages, water quality is generally influenced by the natural environment. Heavy metals, pesticides, fertilizers, oil spills, toxic gases, industrial effluents and sewage are just a few examples of the many contaminants involved. Therefore, in recent decades, the development of efficient, ecologically-friendly methods to remove contaminants from water has become of relevant importance, since pollution is increasing drastically [284]. Nanotechnology has gained a lot of attention in the past few decades due to the unique physical properties of nanoscale materials. Nanomaterials offer the potential to harness a unique surface chemistry, which allows them to be functionalized or grafted with functional groups that can target specific molecules of interest (pollutants) for more efficient remediation. It is only important to verify that the materials used for the remediation of pollution are not themselves another pollutant after being used [285].

A variety of different functional nanomaterials and nanocomposites can be used for the environmental remediation of different pollutants through absorption, adsorption, chemical reactions, photocatalysis and filtration processes [285] (Fig. 10).

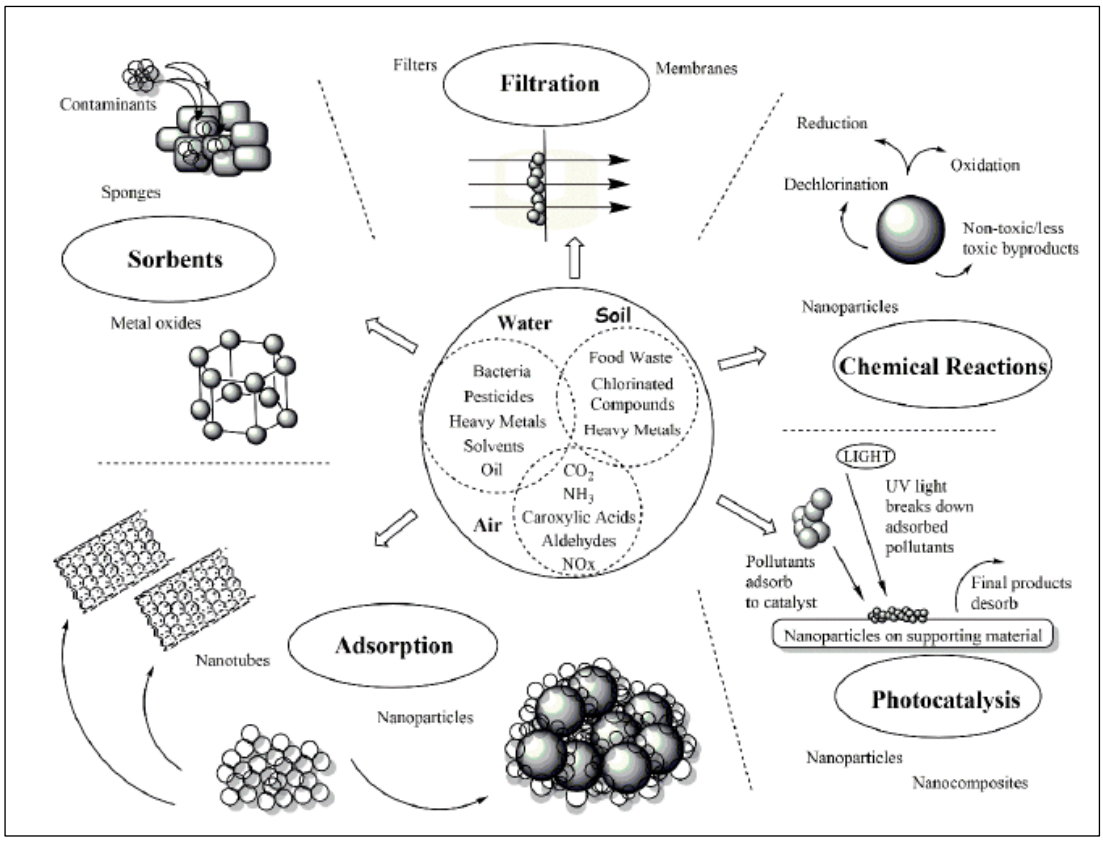

Figure 10. Environmental remediation approaches.

Over the last few decades, a great deal of interest has been focused on the photodegradation of organic compounds present in water and wastewaters with the application of TiO_2 as a photocatalyst. TiO_2 is one of the most widely studied materials for applications in pollutant degradation and photolysis of water, due to its excellent photocatalytic reactivity, high chemical stability, non-toxicity, biocompatibility, and low cost [286].

TiO_2 is a semiconductor oxide with high reactivity for which it can be chemically activated by sunlight. It, in fact, through the direct absorption of incident photons, can participate in surface photochemical processes.

This strong photocatalytic activity, due to its chemical and physical characteristics, has been the object of numerous studies [287,288]. In particular, TiO_2 shows the most effective catalytic activity compared to other catalysts used in the degradation of many contaminants of interest.

Photocatalysis is defined as the acceleration of the speed of a photoreaction due to the presence of a catalyst. The oxidation of most hydrocarbons would proceed rather slowly in the absence of catalytic active substances.

A photocatalyst decreases the activation energy of a reaction. A heterogeneous photocatalytic system consists of semiconductor particles (photocatalyst), which are in close contact with a liquid or gas reaction medium. Exposure of the catalyst to light generates an excited state capable of starting chain processes such as redox reaction molecular transformations. These materials have the rare property of attracting rather than rejecting water. This feature is called super hydrophilicity. Water remains flat on the surface instead of forming droplets. If lighting is interrupted the super hydrophilic behaviour remains for about two days. Moreover, the UV lighting of TiO_2 leads to the formation of powerful agents with the ability to oxidize and decompose many types of bacteria and organic and inorganic materials.

Different methodologies have been investigated to enhance the selectivity degradation of the organic contaminants of TiO_2 [289,290], but an innovative and effective method can be obtained by the molecular imprinting (MI) process. The synthesis method consists of the interaction of template molecules onto an organic or inorganic matrix during preparation; subsequent removal of the imprinted template leads to forming a cavity complementary to the template. The combination between molecular imprinting and photocatalysis allows selective photodegradation; in particular, the molecular imprinting process promotes a selective interaction between TiO_2 and the dangerous contaminant, whereas the photocatalytic process, activated by TiO_2, efficiently degrades the specific pollutant [291].

1.6 Aim of the present thesis

The research activities carried out in this thesis are part of the National Operational Program for Research and Innovation 2014-2020 (CCI 2014IT16M2OP005) "Innovative doctorates with industrial characterization". This project aimed to bring research and industry closer together, allowing research to provide support to the needs of the company by encouraging a practical/empirical approach rather than basic research.

The general purpose of this project was to highlight the main problems that occur in the breeding of some target fish species, with special attention to the hatchery, and to provide a contribution to aquaculture research through innovative applications.

The research activity was divided into five objectives:

Research objective 1: Identification of the main diseases and disorders as causes of mass mortality during the production of gilthead sea bream (*Sparus aurata*) by means of a survey carried out in an Italian fish farm.

Research objective 2: Identification of the main diseases and disorders as causes of mass mortality during the production of common carp (*Cyprinus carpio*) and pike perch (*Sander lucioperca*) by means of a survey carried out in a Croatian fish farm.

Research objective 3: Microplastic (MP) as an emerging issue in aquaculture: investigation in gilthead sea bream and common carp. MP contents in the gastrointestinal tract (GIT) of *Sparus aurata* and *Cyprinus carpio* specimens were assessed. The study aimed to estimate the MP load that occurred in both species at different production stages (larvae, fry and adults), to highlight any significant differences in the micro-items uptake by the two target species and, consequently, if there are tangible differences in the levels of contamination in the farms examined. Furthermore, by means of polymer identification, identify potential sources of pollution.

Research objective 4: Evaluation of the immunostimulant activity of Imoviral in *Sparus aurata*. This study aimed to evaluate the efficacy of the immunostimulant activity of the Imoviral complex in *Sparus aurata* fingerlings, through the study of some immune-related gene expression involved in the acute phase response. The Imoviral complex, commercially available for human use, is a mixture of exclusively natural extracts such as Uncaria (*Uncaria tomentosa*), Shiitake (*Lentinula edodes*), Beta-glucan, and Blackcurrant (*Ribes nigrum*), whose immunostimulant properties of individual extracts have already been demonstrated on some organisms. The study aims to evaluate the organism immune response following the administration of the experimental dietary additive, highlighting any

differences with the specimens fed only with commercial feed and in uninfected and infected animals with *Vibrio anguillarum*.

Research objective 5: Zebrafish embryo toxicity test (ZFET) to demonstrate the preferential removal of fungicides from water by molecular imprinting with TiO_2 photocatalysts.

This study aimed to evaluate, for the first time, the preferential photodegradation of one fungicide OPP, using molecularly imprinted TiO_2 catalysts synthesized with the sol-gel technique. For many years, OPP and its salts have been used world-wide as active ingredients in broad spectrum fungicides used in wood preservation and as surface biocides. Furthermore, they are used as fungicides to protect materials such as textiles. They are also used for inhibition of mould growth on citrus fruits. Toxicity of the samples was evaluated by the (ZFET). ZFET is a short term test, carried out on fertilized eggs of zebrafish; it represents an effective alternative to acute test with adult fish and moreover, is an excellent test to evaluate toxicity of micro/nanoparticles. Metallothioneins 1 (MTs1) and Heat Shock Proteins 70 (HSP 70) were analysed as biomarkers of exposure.

2. MATERIALS AND METHODS

2.1 Fish health monitoring at an Italian fish farm

2.1.1 Italian fish farm

Data on farmed marine fish are the result of a survey performed at an Italian fish farm located in Italy from March to August 2018. By means of an intensive production system, the farm produces and raises sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). The breeding of other species (such as *Argyrosomus regius*, *Seriola dumerili*, and *Haliotis* spp.) is still in an experimental phase, but the commercial line is not currently active. The source farm is structured as a "full operation" farm, with commercial hatchery and offshore cage technology. With the addition of packaging and offices, the company has a total of around 100 employees. The hatchery alone is self-sufficient as 75% of production is sold to other fish farms throughout the Mediterranean basin, with significant revenues. The remaining 25% is used by the same company to be transferred and fattened in the off-shore plant at sea, which in turn produces commercial-sized fish, which are then packaged and ready for sale in the dedicated department.

The fish farm consists of hatchery production, which includes:

1) Broodstock culture: *Sparus aurata* and *Dicentrarchus labrax*;

2) Phyto-zooplankton culture: phytoplankton culture (*Dunaliella* sp. and *Nanochloropsis* sp.); zooplankton cultures, including rotifers (*Branchionus* sp.) and Artemia sp. (regularly hatched from cysts);

3) Nursery: equipped with temperature-controlled tanks and controlled lighting;

4) Weaning: where the larvae are fed until reaching 1 gr body weight;

5) Pre-on-growing: where the fish are fed with commercial feed consisting of fish meal, up to 30 gr; and then transferred for the on-growing phase.

On-growing takes place using cage culture technology. The fish farm is equipped with about 40 circular floating cages organized in modules of 6, with a capacity of about 500,000 fry, in which these remain until they reach the commercial size.

With regard to annual production, in 2020 the farm achieved a fry production of 34.8 million and 5.9 million gilthead sea bream and sea bass, respectively, while 1,110 tons of sea bream and 600 tons of sea bass, market size, were produced in 2020.

2.1.2 Samples monitored

Gilthead sea bream. Fish samples were monitored for 6 months by regular bi-monthly checks.

The samples, coming from the different production phases of the farm, were collected when appropriate, or in the presence of behavioural anomalies, disorders or evident injuries. In this case, a pool of 5 fish samples was taken from each affected tank or cage and prepared for subsequent analyses. A total of 600 larvae, 240 fry and 240 adult specimens were examined. Fish were euthanized on-site by an overdose of tricaine methanesulfonate (MS-222, Sigma). Immediately thereafter, all fish were grossly examined and necropsied.

During March 2018, 19-26-day-old larvae exhibited lethargy, abnormal swimming, loss of balance, but also an unusual distended abdomen. Samples of larvae were sent to Istituto Zooprofilattico Sperimentale delle Venezie and Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna for virologic and bacteriological analyses, using cultural and molecular methods respectively, as previously described by Toffan et al [310]. A total of 400 specimens were collected from 4 different rearing tanks, euthanized with an overdose of MS-222 (Sigma-Aldrich) and transferred to the Centre for Experimental Fish Pathology of Sicily (CISS), University of Messina, for a complete histopathological examination. Water parameters were also collected.

Phyto-Zooplankton. Aliquots of 15 ml of algae, rotifers and brine shrimp samples were collected for examination during the same period. External examination of algae, rotifers and brine shrimp as well as parasitological test, were carried out under DM6 B microscope (Leica, USA).

Water samples. Water monitoring was carried out in the Italian fish farm to monitor water quality and bacteriological pollution, the trend of ion concentrations, as well as any pollutants such as heavy metals, PCBs, organochlorine pesticides and PAHs (Tab. 1). Samplings were carried out in the months of March, April, May and June in conjunction with regular fish health assessment and the study of deformities, to identify any possible correlation among the factors. Water parameters, such as temperature, salinity and pH were also collected when necessary. The samples analysed included both culture water and water from 21 boreholes and from the sea, which supply the farm. Two litres of sample from each sampling site were collected using sterile bottles and transported in thermal bags (4 ° C) to the laboratories of the University of Messina.

2.1.3 Histological examination

All examined fish were fixed in 10% buffered formalin solution, dehydrated in alcohol, diaphanized in xylene and embedded in paraffin wax. Subsequently, 5µm thick sections

were stained with haematoxylin and eosin. Images were acquired by a DM6 B microscope (Leica, USA).

2.1.4 Parasitological examination of fish specimens

Larvae, fry and adult samples were regularly examined every two weeks, during the period of permanence in the farm. After a careful external parasitological examination of the skin, fins, gills, eyes and mouth, the sampled fish were examined internally. Precisely oesophagus, stomach, intestine, gonads and swim bladder of the specimens were removed and carefully examined. Depending on the size of the parasite, these were examined under a stereomicroscope or under an optical microscope for identification according to standard procedures.

2.1.5 Deformity occurrence investigation in gilthead sea bream

Sea bream fry were monitored with an average frequency of four days a month, during March, April and May 2018. A total of 11,128 samples were examined grossly for morphological abnormalities in the field. The data were collected in the context of manual sorting activities that normally take place in the farm hatchery. Fry with notable morphological deformities were counted and recorded. A contingency table was constructed for all the deformity categories found, to highlight any significant differences in frequency occurrence between the detected anomalies and any monthly differences.

2.1.6 Microbiological analysis of water samples

Enumeration of *E. coli* and total coliform bacteria was carried out using ISO 9308-1 method based on membrane filtration. Briefly, 100 ml of water sample was filtered through cellulose nitrate membranes (Sartorius) 47 mm in diameter and 0.45μm in porosity. Subsequently, the membranes were placed in medium Tergitol TTC nutrient pad (Sartorius) and incubated at 37 ° C for 18-24 h. After the incubation period, the colonies were counted and those yellow/orange in colour that had a yellow halo (presumed *E. coli*) were subjected to further confirmatory tests such as oxidase, indole, methyl red, Voges-Proskauer and motility tests.

Enumeration of intestinal enterococci was performed using ISO 7899-2 method based on membrane filtration. Also in this case, 100 ml of sample was filtered on a membrane (as previously described). This was placed on a medium Azide nutrient pad (Sartorius) and incubated at 37 ° C for 40-48h. After the incubation phase, colonies that showed a typical red/dark brown colour were counted. Identification was confirmed on Bile Esculin Azide agar (BEA) (DIFCO) culture medium, in which *Streptococcus* colonies turn black.

All samples were analysed in triplicate.

2.1.7 Determination of inorganic anions in water samples

The water samples were filtered on a 0.20 μm PTFE filter and directly analysed by means of an ion chromatography system (ICS-3000, Dionex Corp., Sunnyvale, CA, USA), equipped with autosampler, gradient pump, self-regenerating suppressor for anions, conductivity cell, ion exchange column (Dionex IonPac ™ AS19-HC, 4 mm × 250 mm; particle size 13 μm) equipped with pre-column (Dionex IonPac ™ AG19-HC, 4mm × 50 mm; particle size 13 uM); deionized water and an aqueous solution of sodium hydroxide (60 mmol / L) were used as eluents. The gradient was as follows: 0–10 min, 10 mM NaOH; 10–30 min, 10–58 mM NaOH. Detector temperature was set at 25 ° C, with a current of 50 mA.

Identification of the anions was carried out by comparing the retention times of the chromatographic peaks of each sample with those of the commercial standards, analysed under the same conditions; the anions were quantized by calibration lines.

2.1.8 Determination of mineral elements in water samples

Nitric acid 60% (w/v), hydrogen peroxide 30% (w/v), water and standard solutions at 1000 mg/L in HNO_3, at 5% of each element studied used for the construction of the calibration lines, were purchased from Merck (Darmstadt, Germany), choosing those with the highest degree of purity available and free of any contaminants for ICP analysis. All glassware used in the preparation of samples and in subsequent analyses was washed keeping it immersed for a whole night in a solution of 10% HNO_3 (w/v), then rinsed with deionized water. The water samples were analysed as such without pre-treatment, diluting with 10% HNO_3 (w/v) in a 1:50 ratio. The samples thus obtained were subjected to instrumental analysis using the Agilent 7500cx ICP-MS (Agilent Technologies, Santa Clara, CA).

Argon and helium of purity> 99.999% were used as gas. By suitable dilutions with highly pure deionized water with electrical resistivity ~ 18 MΩ, from the standard starting solutions of 1000 mg/L of each metal, mixtures were prepared for each element with 5 concentration levels (0.5, 1.0, 5.0, 10.0, 20.0 μg / L), which were used as an external standard for the construction of the calibration lines. Linearity (R2) was always > 0.989, the detection limits (LOD) were between 0.007 mg/L and 0.07 mg/L and the quantification limits (LOQ) were between 0.021 mg/L and 0.132 mg/L.

2.1.9 Determination of pesticide and polychlorobiphenyl residues in water samples

The method [292] is based on gas chromatographic determination of active substances, after they have been extracted from the water with solid phase extraction technique (SPE) with cartridges, consisting of silica linked to chains with 18 carbon atoms.

The water sample (500-1000 mL) is added to methanol (0.5 mL per 100 mL of water).

The solution thus obtained is passed through a 500 mg C-18 cartridge, washed and conditioned by passing, in succession, 5 mL of ethyl acetate, 5 mL of methanol, 10 mL of pure water, finally leaving a head of a few mm, to a speed of about 500 mL/hour.

After the complete passage of the sample, the cartridge is washed with 10 mL of pure water; most of the residual water is eliminated by means of nitrogen suction. The cartridge is eluted with 3-5 mL of ethyl acetate. The collected eluate is carefully evaporated until dry in a rotary evaporator and the residue was taken up with 0.050 mL of internal standard (bromophos-methyl at a concentration of 1 mg/L) and with 0.950 mL n-hexane.

2.1.10 Determination of polycyclic aromatic hydrocarbon residues in water samples

60 mL of dichloromethane are added to a litre of water in a separating funnel, the mixture is vigorously stirred, separation of the phases occurs and the underlying organic phase is recovered in a flask. Extraction of the water is carried out successively 2 times with 60 mL of dichloromethane each, bringing the phases together. The organic extract collected is filtered on anhydrous sodium sulphate to eliminate any traces of water, it is then reduced to a volume of 0.950 ml by means of nitrogen flow and added to 0.050 mL of internal standard (bromophos-methyl at a concentration of 1 mg/L), to reach a final volume of 1 ml [292].

2.1.11 Simultaneous analysis HRGC-MS/MS

Simultaneous analysis of residues of pesticides, PCBs and PAHs (Tab. 1) was carried out using an HRGC-MS/MS system (GCMS-TQ 8030 Shimadzu), equipped with a ZB-5MS capillary column (5% biphenyl-95% methyl polysiloxane) (30 m × 0.25 mm; 0.25 μm film thickness). Helium was used as a carrier gas at a constant flow of 0.68 mL/min. The injector temperature was maintained at 250 ° C and the injections were carried out in splitless mode. Interface and ion source temperatures were maintained at 290 ° C and 230 ° C, respectively. The oven temperature was set as follows: 60 ° C (1 min), from 60 to 150 ° C with an increase of 15 ° C/min, from 150 to 270 ° C with an increase of 10 ° C/min, from 270 to 300 ° C (2 min) with an increase of 2 ° C/min. The mass spectrometer

was set in EI (Electronic Impact) mode (70 eV), from 40 to 800 amu, 1 spec/s. The mass spectra were acquired in MRM (Multiple Reaction Monitoring) mode, using argon as a collision gas at a pressure of 200 kPa. Each compound is characterized by the retention time and two MRM transitions, one used for quantification and the second as confirmation. The analytical method was validated according to the guidelines of the European Union, in terms of linearity, LOD, LOQ, recovery and repeatability.

LOD values varied between 0.03 and 5.20 µg/L, those of LOQ between 0.10 and 18.0 µg/L; the values of R2 were always shown to be > 0.901. The recovery values were > 80% and repeatability, expressed as RSD%, was < 6%.

Table 1. Contaminants analysed and related chemical classes. *PCB: polychlorinated biphenyls; PAH: polycyclic aromatic hydrocarbons; OCP: organochlorine pesticides.*

Metals	PCB	PAH	OCP
B	**PCB 28**	**Acenaphthylene**	**Aldrin**
P	**PCB 52**	**Anthracene**	**Dieldrin**
Mn	**PCB 77**	**Benzo[g,h,i]perylene**	**2,4'-DDD**
Fe	**PCB 81**	**Benzo[b]fluoranthene**	**2,4'-DDE**
Cu	**PCB 101**	**Benzo[k]fluoranthene**	**2,4'-DDT**
Zn	**PCB 105**	**Benzo[a]pyrene**	**4,4'-DDD**
Cr	**PCB 114**	**Benzo[a]anthracene**	**4,4'-DDT**
Co	**PCB118**	**Chrysene**	**4,4'-DDE**
Ni	**PCB 123**	**Dibenzo[a,h]anthracene**	**Eldrin**
As	**PCB 126**	**Fluoranthene**	**HBC**
Cd	**PCB 138**	**Indeno (1,2,3-cd) pyrene**	**α-HCH**
Pb	**PCB 153**	**Naphtalene**	**β-HCH**
Hg	**PCB 156**	**Phenanthrene**	**γ-HCH**
	PCB 157	**Pyrene**	**heptachlor**
	PCB 167		**Heptachlor epoxy**
	PCB 169		**Methoxychlor**
	PCB 180		**Mirex**
	PCB 189		**oxychlordane**
			cis-Chlordane
			trans-Chlordane
			trans-nonachlor
			cis- nonachlor

2.2 Fish health monitoring at a Croatian fish farm

2.2.1 Croatian fish farm

Data on farmed warmwater fish are a result of a health monitoring program performed at a fish farm in Croatia from May to November 2019. The farm had semi-intensive farming with a polyculture stocking structure in which common carp (*Cyprinus carpio*) is a primary species. In this carp pond polyculture, common carp was reared together with other cyprinids such as tench (*Tinca tinca*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*) and bream (*Abramis brama*). Besides cyprinids, some predatory fish such as European catfish (*Silurus glanis*), pike (*Esox lucius*) and pikeperch (*Sander lucioperca*) were reared together with common carp. Predatory fish are used in polyculture to control the population of common carp competitors (Prussian carp, Pseudorasbora etc.) that may accidentally enter the pond and to control unwanted common carp reproduction.

In the farm studied, common carp were fed naturally available food and food organisms produced by fertilization of the pond (zooplankton, benthic organisms, detritus etc.) as well as supplementary feed. Fine mixed feeds in the form of a hard dough (ingredients: fish meal, poultry by-product meal, soybean meal, wheat flour, corn flour, vitamin and mineral premixes) and cereals (particularly corn, wheat and barley) were used as supplementary feed.

The source farm is structured as a “full operation” farm with commercial hatchery and production of all fish age-groups from fry, advanced fry, fingerlings to marketable size fish. Annual production per hectare is variable, depending on environmental factors. With fertilization and supplemental feeding, yields range from 500 to 700 kilograms of market-size fish per hectare per year.

Additionally, the enterprise used an enclosed aquaculture facility with recirculating system for commercial farming of pikeperch. The recirculating aquaculture system (RAS) was supplied by borehole fresh water. Standing stock in RAS was approximately 6,000 fish, and farming was based on juvenile pikeperch purchased from private hatcheries. Fish in RAS were fed a commercial pelleted diet.

2.2.2 Common carp (*Cyprinus carpio*) health monitoring

Fry, one-year old and two-year old specimens were regularly examined every two weeks. All examined fish (796 specimens) were randomly collected for regular health assessment, whereas moribund fish were sampled when appropriate. All fish were grossly examined and necropsied.

2.2.2.1 Bacteriological examination

Flavobacterium columnare detection. Wet mounts of skin and gill scraping were examined for the presence of "columns" containing flavobacteria. Samples from skin or internal organs (spleen and/or kidneys) were immediately streaked on general-purpose media tryptone soya agar (TSA) and 5% sheep blood agar. For the isolation of *Flavobacterium columnare*, samples were streaked on tryptone-yeast extract-salt medium or tryptone yeast extract glucose agar, supplemented with neomycin sulphate at 4 mg/l [293]. All plates were incubated at 22-25 °C for up to five days. Individual colonies were subcultured onto fresh plates to obtain pure cultures and for further phenotypic characterization. Isolates were identified to the species or genus level by colony morphology, Gram-stain and phenotypic properties according to standard procedures [55].

Aeromonas spp. detection. Atypical *Aeromonas salmonicida*, in pure culture or in mixed infection with *Aeromonas* spp., was isolated from the edge of ulcers of common carp specimens affected by carp erytrodermatitis (CE). Isolated strains were grown on blood agar (5% sheep blood agar – 5ml of defibrinated sheep blood plus 95 ml Columbia agar or TSA). *Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas caviae* and *Pseudomonas fluorescens* were isolated in pure or mixed cultures from spleen and kidneys of moribund common carp showing signs of haemorrhagic septicaemia. In the case of CE, in the presence of typical clinical symptoms (ulcers), the identification was presumptive, combining the suspected clinical case with colony morphology, Gram stain, motility test and biochemical tests (i.e. API).

2.2.2.2 Parasitological examination

Wet mounts from skin and gill were examined for the presence of ectoparasites. Following necropsy, all specimens were screened for the presence of swim-bladder inflammation due to *Sphaerospora* infection, and the presence of any other parasitic infections including cestode and coccidian infections. For the presence of coccidian infections, fresh preparations of mucus and intestinal scrapings were analysed. When parasites were found, morphological species discrimination was conducted.

2.2.2.3 Deformity occurrence and skeletal development

The presence of skeletal system deformities was recorded as presence/absence to quantify the number of affected specimens. Considering the absence of existing data

in literature on the development of the skeletal system in common carp, we tested, parallel to the survey, the hypothesis on the existence of a trade-off mechanism between the ossification process and growth on the intraspecific level. Briefly, two females, marked “A” and “B”, were selected and hand stripped to obtain eggs. Eggs were fertilized and incubated in separate plastic bowls and incubation jars, respectively, until hatching. Larvae were fed according to standard fish farm procedures (see section 2.2.1). Offspring from both groups (A and B) were sampled at multiple time points: 5, 17, 22, 24, 26 and 29 days post-hatching (DPH). Every sample contained 8 randomly selected specimens. After sampling, fish were euthanized by immersion in the buffered solution of MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and measured. Standard length (SL) was measured and used for correlation with DPH. For both groups, mean and standard deviation (SD) was calculated. Differences between groups were analysed with the Mann – Whitney U test with significance level $P<0.05$ and $P<0.01$. Statistical analysis was performed using STATISTICA v.13.5 (Statistica, Inc., 2018).

To evaluate skeletal system development, whole specimens were fixed in 10% neutral buffered formalin, and stained for bone and cartilage according to Dingerkus and Uhler [294]. This double staining method allowed simultaneous detection of cartilaginous elements (alcian blue) as well as calcified structures (alizarin red). Stained specimens were visualized and photographed under Olympus SZX7 stereoscopic microscope. Clearly visible bony elements and onset of ossification process is described: frontal, parietal, supraoccipital, parasphenoid, basisphenoid, basioccipital, premaxillary, maxillary, dentary, articular, quadrate, hyomandibular and ceratohyal bone, opercle, cleithrum, abdominal and caudal vertebrae, dorsal, caudal, anal, pelvic and pectoral fin.

2.2.3 Pike perch (*Sander lucioperca*)

Five specimens of moribund pikeperch farmed in the RAS were collected from a single rearing tank due to increased mortality rate at the farm (6%). To collect additional data about farming, an interview-based survey was carried out. Water parameters were also collected (T, pH and dissolved oxygen). Moribund fish were measured and then transferred to a Laboratory for Fish Diseases at the Faculty of Veterinary Medicine, University of Zagreb. Once in the laboratory, fish were euthanized with an overdose of MS-222 (Sigma-Aldrich), examined and necropsied.

2.2.3.1 Histological examination

For histological examination, spleen and kidney from pike perch samples were fixed in a 10% neutral buffered formalin. Fixed material was embedded in paraffin and 5 μm serial sections were prepared. Sections were stained with haematoxylin and eosin (H&E), periodic acid-Schiff reaction (PAS) and Ziehl-Neelsen method (ZN). Selected sections were also stained with Masson's trichrome staining for collagen and by the von Kossa/van Gieson method to demonstrate mineralized tissue. Imprints and sections were analysed by light microscopy using an Olympus BX41.

2.2.3.2 Bacteriological examination

Mycobacterium spp. detection. Samples from kidney were inoculated on TSA and 5% sheep blood agar, and subsequently incubated at 25°C for 7 days. Isolation of acid-fast bacteria was performed at the National/Supranational Reference Laboratory for tuberculosis (Croatia). Samples were processed according to standard procedures as previously described by Pfyffer et al. [295]. Culture was performed using BACTEC MGIT 960 system (BD, Sparks, MD, USA) and additionally Löwenstein-Jensen medium for six weeks at 25°C. Species identification was carried out by molecular methods, using GenoType Mycobacterium CM and GenoType Mycobacterium AS reverse hybridisation assays (GenoType© CM/AS; Hain Lifescience, Nehren, Germany).

2.3 Microplastic occurrence investigation in gilthead sea bream and common carp

2.3.1 Fish samples

Cultured specimens of larvae, fry and adults belonging to the species *Sparus aurata* and *Cyprinus carpio*, were collected from two fish farms, located in Italy and Croatia, respectively. Fry (60 for each species) and adult (20 for each species) samples were weighed and measured directly in the field, and subsequently transported at 4 ° C to the laboratory. Larvae (700 and 795 of gilthead sea bream and common carp respectively) were collected and placed directly in sterile glass containers, while all the other specimens were wrapped with aluminium foil during transport operations. The gilthead sea bream specimens were analysed at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, while the common carp samples were processed at the Department for Biology and Pathology of Fish and Bees, Faculty of Veterinary Medicine, University of Zagreb.

2.3.2 Microplastic extraction protocol

Once in the laboratory, the samples were washed with deionized water to eliminate any external contamination. The larvae were counted, the gastrointestinal tracts (GITs) of fry and adults were extracted. GITs of the fry for both species were digested in pools of 5 samples. The intestine and hepatopancreas of adult common carp specimens were separated and treated individually. All samples were processed adopting a modified version of the chemical digestion protocol previously suggested by Savoca et al. [216]. Briefly, samples were placed in a conical glass flask. After adding a calculated quantity of 10% KOH solution (minimum ratio 1:5 w/v), the flask was covered with aluminium foil. To remove the organic matter, the flasks were placed in an oscillation incubator to be continuously stirred at 50°C for 48 h. Each sample was then put into a graduated glass cylinder and hypersaline NaCl solution (15%) was added to obtain separation of the two phases by density. The supernatant was collected in a glass beaker, and doubly filtered through a glass fibre membrane with 1.5 and 0.7 mm pore size and 47 mm diameter (Whatman GF/F, UK) using a vacuum system (Millipore). After filtration procedures the membranes were placed in sterile Petri glass dishes for subsequent observations under the stereomicroscope (Leica M205C) to isolate plastic debris. The isolated samples were recorded and categorized based on their shape (fibres and fragment), size and colour.

2.3.3 Contamination prevention

Workspaces and tools were cleaned from any particles according to Bottari et al. [213]. All materials used for dissection, during different steps of extraction and analysis were rigorously cleaned with ethanol and filtered deionized water. The same prevention measures for sample contamination were adopted during the digestion procedures. In addition, deionized water, potassium peroxide, and hypersaline solution were always pre-filtered (0.45 mm filter). Only sterilized glass items were used for all the assays. Fish dissection and digestion protocols were performed in a clean air flow cabinet to exclude external contamination from fibres, which might represent a major source of contamination. Filter paper in Petri dishes exposed to the laboratory air was used as control blank during the analysis of each membrane under stereomicroscope and run through the entire laboratory procedure [205].

2.3.4 Microplastic identification

The chemical composition of the isolated samples was identified by micro-infrared spectroscopy (μ-FT-IR). Prior to each measurement, a microscopic image of each sample

was taken. μ-FT-IR spectra were recorded using a Bruker FTIR LUMOS microscope equipped with a liquid nitrogen cooled 64 × 64 detector. Infrared spectra were recorded in transmission method in the range 4000–900 cm−1 with a resolution of 4 cm−1. Background and baselines of recorded spectra were calculated and, if necessary, subtracted to the spectra, with Origin 9.0 software. To identify the polymers, the obtained spectra were compared with the multiple libraries provided by the Knowitall FTIR library. Only spectra matched over 80% with the standard database were accepted. To identify the natural, artificial and synthetic textile materials in the fibre samples, the spectral data collected by Peets and collaborators [296] were used. In this way, we were able to distinguish different kinds of single- and two-component mixed textiles.

2.4 Evaluation of the immunostimulant activity of Imoviral in *Sparus aurata*

2.4.1 Fish maintenance and experimental diet

One hundred gilthead sea bream (*Sparus aurata*) specimens ranging between 12-15 g in body weight, were obtained from the Acqua Azzurra fish farm and sent to the Centre for Experimental Fish Pathology (Centro di Ittiopatologia Sperimentale della Sicilia – CISS), Department of Veterinary Sciences, University of Messina, Italy. CISS has been accredited since 2006 for use and since 2010 for production of aquatic organisms for experimental research (DM n°39/ March/2006). In the laboratory, fish were randomly assigned and kept in 10 tanks of 110 L in volume, with strictly water-controlled conditions: Temperature 20-22°C, salinity 35‰, pH 8 and dissolved oxygen (DO) 7ppm.

Experimental feed was prepared by adding to the basic ingredients of the diet. Uniform distribution of the ingredients in the feed was obtained by first mixing the pellet with water by means of a Heating magnetic stirrer (VELPA-Scientifica) at 70 ° C and 4 Stirrer, until a soft, moist consistency compound was obtained and, subsequently, by means of adding the ingredients and mixing at 7 Stirrer for 30 minutes. The mixture obtained was dried at room temperature for 2 hours and then ground and sieved to produce a crumble of dimensions consistent with the feed of gilthead sea bream.

The specimens were fed twice a day with commercial pellet during an acclimatization period of 20 days, prior to the experimental trial. Following the acclimatization phase, 40 specimens (4 tanks) were fed with Imoviral powder added to regular commercial feed (25 mg/10 gr of pellets) (treatment). The remaining 60 fish (6 tanks) were instead fed with commercial feed (control). The whole feeding trial lasted 4 weeks.

2.4.2 Bacterial challenge

The pathogenic bacterium *Vibrio anguillarum* (serotype O1), was kindly furnished by the Istituto Zooprofilattico Sperimentale delle Venezie. Before the experimental challenge, the virulence of the pathogenic strain was increased by serial in vivo infections in gilthead sea bream specimens. Briefly, fish were infected with a dose of 0.1 ml of bacterial suspension in saline solution (10^8 cell/ml) and monitored for 3 days. After that, the blood taken from the caudal vein was spread on Marine agar (MA, Difco) and incubated at 24°C for 24h-48h. The procedure was carried out 3 consecutive times. The sub-lethal dose selected for bacterial infection was 10^5 cell/ml.

At the end of experimental feeding phase, fish were treated as 5 experimental groups, of which each group contained 20 fish stocked in duplicate 100L tanks. The description of the experimental groups is shown below:

Group 1 (IVS): fish fed with Imoviral added to commercial feed, to which a dose of 0.1 ml 24h broth from virulent *V. anguillarum* was administered, by intraperitoneal injection (IP).

Group 2 (IPS): fish fed with Imoviral added to commercial feed, to which a dose of 0.1 ml sterile saline solution was administered as control by IP.

Subgroup 3 (PVS): fish fed exclusively with commercial feed, to which a dose of 0.1 ml 24h broth from virulent *V. anguillarum* was administered by IP.

Group 4 (PPS): fish fed with commercial feed, to which a dose of 0.1 ml sterile saline solution was administered as control by IP.

Group 5 (CTRL): fish fed with commercial pellet that were not injected.

After the experimental infection, 5 fish from each experimental group were randomly collected from both tanks (replicates) and sacrificed by an overdose of anaesthetic (500mg/l, MS-222 buffered with $NaHCO_3$) for each of the following time points 1, 24, 72 and 168 hpi (hours post injection) and organs collected during necropsy. After that, organs were partially frozen at -80°C and fixed in 10% formalin. Each spleen sample was used for subsequent molecular analyses.

2.4.3 RNA extraction and cDNA synthesis

Total RNA was isolated from spleen tissues of gilthead sea bream, collected at each time point. Tissue homogenization and RNA extraction were performed using TissueLyser II (Qiagen) and RNeasy Plus Mini Kit (Qiagen), according to manufacturer's instructions. RNA integrity was evaluated on 1% (w/v) agarose gel and concentration and purity verified using Nanodrop spectophotometer (Thermo Scientific). cDNA synthesis from 1

µg total RNA was performed by QuantiTect reverse transcription Kit (Qiagen), after gDNA wipe-out buffer treatment, as suggested in manufacturer's instructions.

2.4.4 Gene expression analyses by real-time PCR (qPCR)

Analysis of the acute phase immune response in treated sea bream was carried out by qRT-PCR with the aim of evaluating the modulation of a set of immune-related genes. The screening included analysis of pro-inflammatory response (*Interleukin 1 beta* [*il-1β*]; *tumour necrosis factor alfa* [*tnf-α*], and two important antimicrobial peptides (AMPs), β-defensin and Hepcidin, involved in the innate immune response against microbial invasion. Gene expression quantification was performed using the Rotor-Gene Q 2plex Hrm thermocycler (Qiagen) with SYBR Green chemistry (Qiagen). In each reaction, fifteen-fold diluted cDNA samples were run in duplicate together with no template and minus reverse transcriptase controls. PCR efficiency was determined as detailed by Fernandes et al. [297]. Four different reference genes (*Elongation factor 1 alfa* [*ef1-α*]; *Glyceraldehyde 3-phosphate dehydrogenase*[*gap-dh*]; *beta actin* [*β-act*]; *Ribosomal RNA 18S* [*18S*]) were assessed and the normalization factor from the two most stable genes (calculated by geNorm software, http://medgen.ugent.be/~jvdesomp/genorm/), *β-act* and *18S*, was used to correct the raw target gene data as described by Giannetto et al. [298]. Specificity of the reactions was confirmed from single-peak melting curves. Specific primer sets for each gene are detailed in Table 2.

Table 2. Primer sequences, amplicons size (bp), qPCR efficiencies (E%), and correlation coefficients (R2) of the calibration

Primer	Forward primer sequence	Reverse primer sequence	Size (bp)	E (%)	R2
tnf-α	CTCACACCTCTCAGCCACAG	CAGTTTGTCGCCTCTGTTCA	186	109	0.99
il-1β	CTGCTCAACATCTTGCTGGA	TCGGACTAAGTGCCTCTGCT	135	90	0.99
hep	GCCATCGTGCTCACCTTTAT	CTGCTGCCATACCCCATCTT	152	98	0.99
def	AGGGCAATGATCCAGAAATG	CCGTGATGACCAACGATGTA	97	96	0.99
ef1-α	CTGTCAAGGAAATCCGTCGT	TGACCTGAGCGTTGAAGTTG	87	102	0,98
gap-dh	AGCCACTCCTCCATCTTTGA	TGCTGTAGCCGAACTCATTG	97	105	0.99
β-act	CACCGCAAATGCTTCTAACA	CTGAAGCCATCCCAATGAGT	149	100	0.95
18S	GACAAATCGCTCCACCAACT	CCTGCGGCTTAATTTGACTC	134	103	0.99

2.4.5 Statistical analysis

Gene expression data were analysed using two-way analysis of variance (Two-way ANOVA), followed by performing a post-hoc Tukey's Honestly Significant Difference

(HSD) test, to determine eventual significant differences in gene expression in response to dietary treatments at different time points. The significance level was set at $P < 0.05$. Statistical analysis was conducted using Sigmaplot V.12.

2.5 Zebrafish embryo toxicity test to demonstrate the preferential removal of fungicides from water by molecular imprinting with TiO_2 photocatalysts

2.5.1 Catalyst preparation

Different types of TiO_2-based materials were used: The commercial TiO_2 was provided by Degussa AG (TiO2 P25 Degussa), whereas the sol-gel technique was used for the as-prepared samples [291]. In particular, a TiO_2 sample (called TiO_2 sol-gel, Fig. 11A) was synthetized mixing 2 ml of titanium butoxide with 0.5 ml of acetic acid and 1.5 ml of ethanol (first solution). The mixture was stirred for 10 minutes and subsequently a solution containing 2 ml ethanol and 2 ml demineralized water (second solution) was added. The as-obtained slurry was stirred for 3 h at room temperature and aged for 24 h. Finally, the resultant wet gel was dried at 100°C for 12 h and calcined in air at 500°C for 6 h. With the same methodology molecularly imprinted TiO_2 samples were synthetized utilizing the OPP fungicide (Fig. 11B). In this case, a stoichiometry amount of OPP required to obtain a molar ratio of 5:1 respect to the TiO_2 and fungicide was dissolved until solubilization in the first solution containing acetic acid and ethanol [291], and the same procedure described above was employed. Finally, to obtain N-doped molecularly imprinted TiO_2 materials a proper amount of ammonium nitrate, as nitrogen precursors, was added dropwise in the second solution containing demineralized water and ethanol. The samples were prepared to obtain different atomic concentration of N (0.8%, 1.2% and 4%) (Fig11C).

The morphology of the samples was determined by scanning electron microscopy (SEM), with a field emission Zeiss Supra 25 microscope.

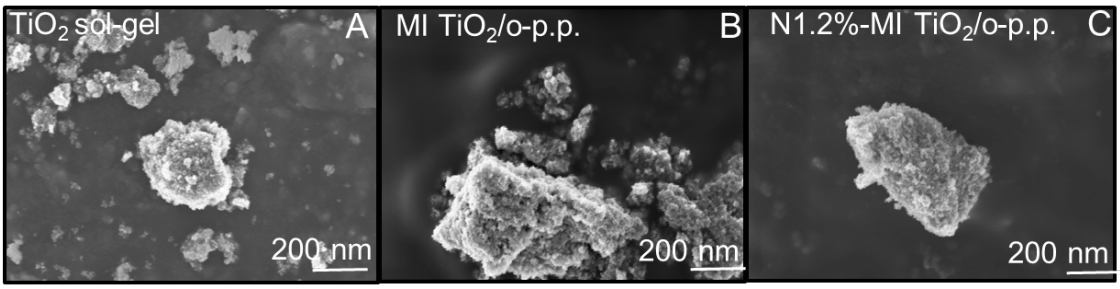

Figure 11. The SEM images of (a) "TiO_2 sol-gel", (b) "Molecularly imprinted (MI) TiO_2/o-p.p.", (c) "N1.2%-MI TiO_2/o-p.p.", chosen as representative samples, are reported.

A non-homogenous morphology with heterogeneous shaped particles is typical of the matrix-free sol-gel synthesis and is a common feature of all the examined samples, notwithstanding the molecular imprinting or the doping processes.

2.5.2 Zebrafish embryotoxicity test

To test any eventual toxicity of the synthesized materials, zebrafish eggs fertilized within 4 h post fertilization (hpf) were provided from the Centre of Experimental Fish Pathology of Sicily (CISS), University of Messina (Italy). Zebrafish embryos were exposed to: TiO_2 bare, TiO_2 sol-gel, molecularly imprinted TiO_2/o-p.p. and molecularly imprinted TiO_2/o-p.p. N-doped 0.8%, 1.2% and 4%. Moreover, other larvae were exposed to molecularly imprinted TiO_2/ o-p.p. N-doped 0.8%, 1.2% and 4% with the addition of the OPP fungicide (1x10-4 mg/ml) in the ratio of 1:1. Table 3, shows concentrations of the different types of TiO_2 used in the test.

The solutions were renewed and embryonic/larval mortality and hatching rate were evaluated every 24 h. According to Pecoraro et al. [299], healthy embryos were placed in 24-well culture plates (10 embryos in 5 ml solution/well). Each group had five replicate wells. Each experiment was repeated four times.

Table 3. Types and concentrations of TiO_2 used for ZFET.

	Nanoparticles (NPs)	Concentrations used
Type 1)	TiO_2 bare	1×10^{-4} mg/ml 1×10^{-5} mg/ml
Type 2)	TiO_2 sol-gel	2.32×10^{-3} mg/ml 2.32×10^{-4} mg/ml
Type 3)	Molecularly imprinted TiO_2/ o-p.p	3.8×10^{-4} mg/ml 3.8×10^{-5} mg/ml
Type 4)	Molecularly imprinted TiO_2/o-p.p N-0,8%	3.6×10^{-4} mg/ml 3.6×10^{-5} mg/ml
Type 5)	Molecularly imprinted TiO_2/o-p.p N- 1,2%	6.5×10^{-4} mg/ml 6.5×10^{-5} mg/ml
Type 6)	Molecularly imprinted TiO_2/o-p.p N- 4%	6.9×10^{-4} mg/ml 6.9×10^{-5} mg/ml

2.5.3 Immunohistochemical analysis

The immunofluorescence protocol was performed on two larvae exposed and on controls, to detect positivity to two biomarkers: metallothioneins 1 (MTs 1) and Heat Shock Protein 70 (HSP70). After washing the samples (fixed in paraformaldehyde) in PBS, permeabilization was carried out for 15 minutes in PBS-Triton X-100, which improves the penetration of the antibody. Samples were then incubated with a blocking solution to block non-specific binding sites of the antibodies for 20 minutes. The larvae, placed on the slides, are incubated overnight in a humid chamber at 4 °C with the primary antibodies: anti-mouse-MTs, anti-mouse-HSP70 (Gene Tex, 1:1000). After rinsing in PBS buffer for 10 minutes, the samples were incubated for 1 hour at 4 °C in the dark with FITC-conjugated Anti-mouse secondary antibody. The samples were washed in PBS-Tween20, each time for 5 min at room temperature, dehydrated in increasing alcohol solutions (70°, 80°, 95°) for 1 minute each, and air dried. Finally, samples were mounted with antifade solution using a coverslip and sealed with rubber cement [299]. The

observations were made with the NIKON ECLIPSE Ci fluorescence microscope and the images taken with the NIKON DS-Qi2 camera.

2.5.4 Statistical analysis

Statistical analysis was carried out with Prism Software (Graphpad Software Inc., La Jolla, CA, USA). Data were expressed as mean or standard deviation. Statistical analysis was carried out by two-way ANOVA test. A p-value of <0.05 was considered to indicate a statistically significant difference between experimental and control groups.

3. RESULTS

3.1 Fish health monitoring at an Italian fish farm

3.1.1 Infectious diseases detected in gilthead sea bream

Larvae. The reports produced by Istituto Zooprofilattico Sperimentale delle Venezie and Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna showed the presence of *Betanodavirus* (isolates identified as reassortant strains RGNNV/SJNNV) and *Vibrio alginolyticus* in larval samples examined.

From our results, no significant external lesions or parasites were observed in any samples analysed (Fig. 12).

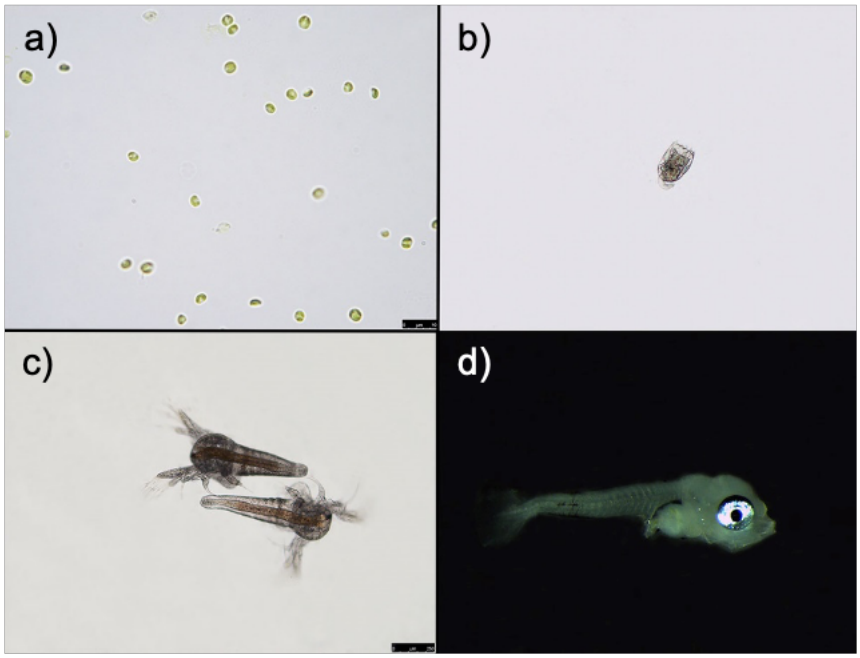

Figure 12. Microalgae (a), rotifer (b), artemia (c) and gilthead sea bream larvae (d) samples examined.

Histopathology revealed a peculiar abdominal distension in more than 60% of the larval samples (Fig. 13a), with severe mucosal degeneration (Fig. 13b).

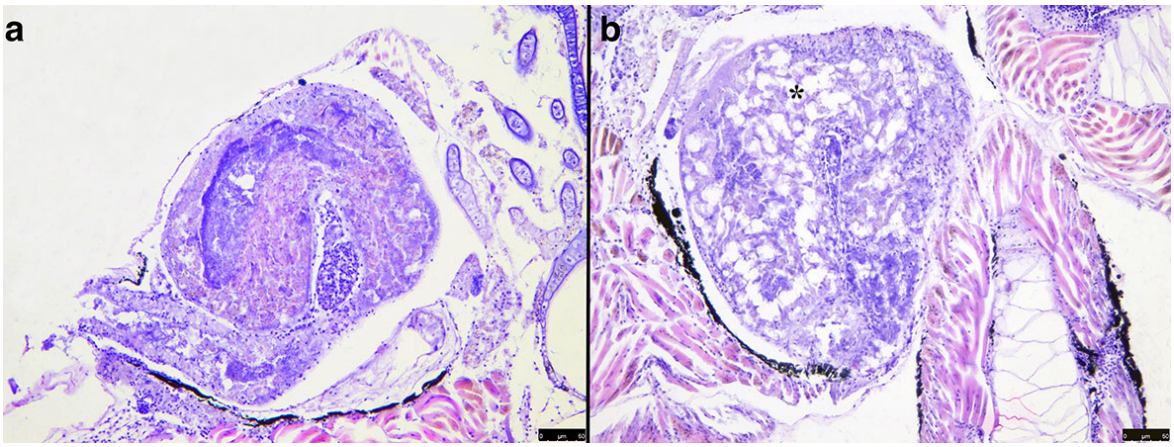

Figure 13. Histological evidence of abdominal distension with severe mucosal degeneration (*)(H&E 20x).

Mild to moderate neuronal degenerative vacuolations were observed in the brain and in the eye of larval gilthead sea bream. Multifocal empty areas 6-10µm, irregular in shape, were found in the retina and throughout the central nervous system (CNS) (Fig. 14) characterized by pyknotic nuclei and karyorrhexis phenomena. No other lesions were found in any other organs.

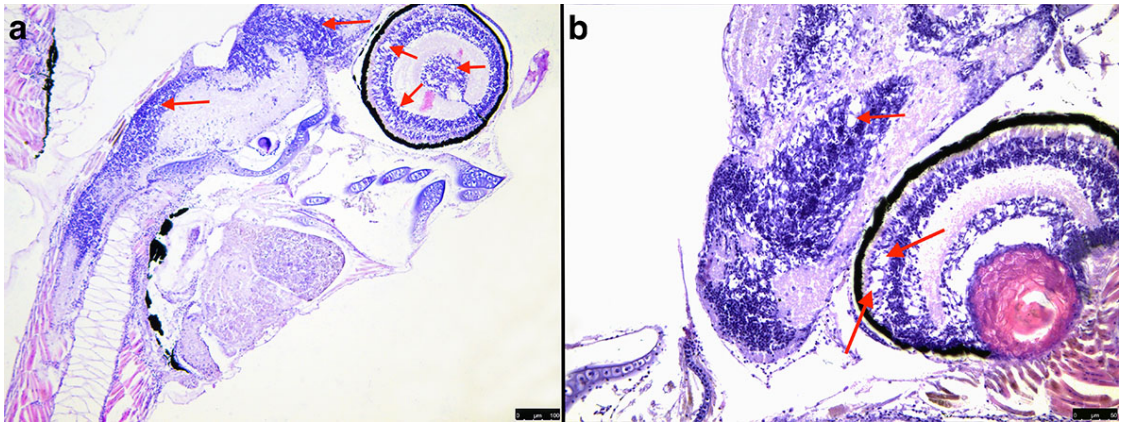

Figure 14. Histological evidence of multifocal empty areas 6-10µm, irregular in shape, found in the retina and throughout the central nervous system, (H&E 20x).

Water parameters showed temperature values between 18 and 20 °C, pH was 8 and salinity was 38‰.

Fingerlings. A total of 100 fry were analysed during the survey. The specimens were in good health and no bacterial or viral pathologies were diagnosed during the health assessment. However, in July 2018 the presence of *Cryptocaryion irritans* was detected in three tanks. Five specimens, ranging in length between 6 and 8 cm, were collected

from each tank and the diagnosis was confirmed by microscopic analysis of wet mounts of skin and gills scraping.

Adults. No infectious diseases were diagnosed in adult specimens.

3.1.2 Evaluation of deformity occurrence in reared gilthead sea bream

A total of 11,128 gilthead sea bream fry were examined; among these, 503 (4.5%) specimens showed deformity. The deformities detected included deoperculation, short nose, torsion, lordosis, prognathism and others indicated as general vertebrae deformities (of dubious identification to the naked eye) (Tab. 4). Short nose was the most frequently observed deformity (2.08%), followed by shortened operculum (1.22%) (Fig. 15). In April, the largest number of fry affected by deformity was recorded (5.5%). Significant differences were found in the frequency of occurrence of shortened operculum and prognathism during the months surveyed ($p < 0.05$) (Tab. 5). Finally, 3.45% of produced fish was eliminated from sales.

Table 4. Percentage of occurrence of different types of deformity in *Sparus aurata*

Type of deformity	N° of specimens examined	N° of specimens with deformities	Occurrence %
Deoperculation	11128	136	1.22
Pugheadness		232	2.08
Cross bite		49	0.44
Lordosis		43	0.39
Prognathism		39	0.35
Vertebrae deformity		4	0.04

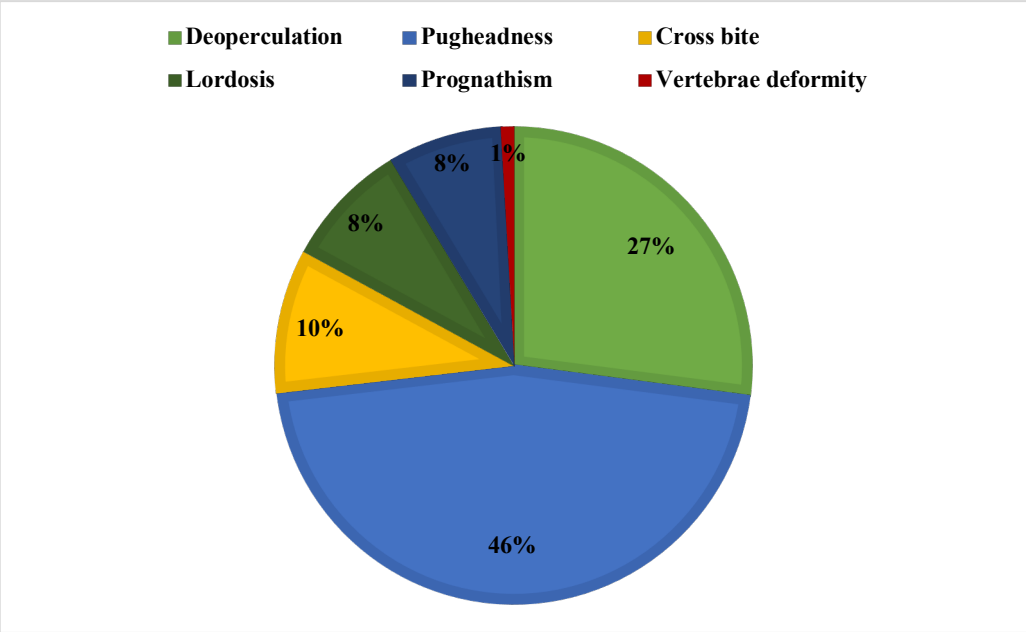

Figure 15. Deformity percentage observed in reared fry

Table 5. Monthly percentage of occurrence of different types of deformity in *Sparus aurata*

	Occurrence (%)		
Type of deformity	March	April	May
Deoperculation	0.85	1.60	1.14
Pugheadness	1.80	2.53	1.94
Cross bite	0.30	0.40	0.51
Lordosis	0.30	0.40	0.41
Prognathism	0.20	0.62	0.26
Vertebrae deformity	0.00	0.00	0.07

3.1.3 Water quality monitoring

The water from 21 boreholes that supply water to the fish farm, water entering and leaving the hatchery tanks and wastewater were monitored to detect any microbiological contamination and anthropogenic pollution.

All the samples analysed, as required by Legislative Decree 152/2006 concerning "Environmental standards", Legislative Decree 30 May 2008, n. 116, transposing Directive 2006/7 / EC "relating to the management of the quality of bathing water" and to Legislative Decree 2 February 2001, No. 31 "relating to water intended for human consumption", were found to comply with the requirements of the various legislative decrees. From the analytical data, the microbiological parameters were within standards

set by the law decrees (Tab. 6) and no chemical-physical anomalies were found due to possible anthropogenic contamination. Ion and pollutant concentrations from all water samples examined were within the ranges as shown in Appendix I, II, III, IV and V.

Table 6. Mean counts for *E. coli* and intestinal enterococci from water entering and leaving the hatchery

Sample	Fecal indicator bacteria	CFU	Reference values
Incoming water	*Escherichia coli*	0	500 n*/ 100 ml
	Intestinal enterococci	0	200 n*/100ml
Outgoing water	*Escherichia coli*	0	500 n*/ 100 ml
	Intestinal enterococci	0	200 n*/100ml

n* = number of colony forming units

3.2 Fish health monitoring at a Croatian fish farm

3.2.1 Common carp health assessment

During the survey conducted on the Croatian fish farm, a total of 796 specimens of common carp were examined, including 40 fry, 378 one-year old and 378 two-year old carp. Results of the health assessment are reported in Table 7.

In general, from the investigation it emerged that the main problem present in the farm was parasites, affecting 62.06% of analysed fish, followed by the deformities that were found in 3.01% of the specimens, while bacterial diseases were detected in only 2% of the fish examined. Finally, in 0.62% of the specimens examined, skin wounds inflicted by piscivorous birds were reported.

The bacterial diseases detected on the farm were CE (Fig. 16), columnaris disease and bacterial haemorrhagic septicaemia.

As for parasites, *Ichthyophthirius multifiliis* and *Dactylogyrus* sp. were the most frequently encountered and numerically most abundant parasites in all age groups examined (Tab. 7).

No cases of mass mortality or high mortality of common carp specimens were recorded during the survey period.

Table 7. Summary of the results obtained during common carp health assessment

Causative agent		No. Of infected specimens	% of affected fry	% of affected one-year-old specimens	% of affected two-years-old specimens
Pathogens					
Bacteria	*Aeromonas spp.*	1			0.26
	Atypical *Aeromonas salmonicida*	14			3.70
	Flavobacterium columnare	1			0.26
Protozoans	*Goussia subepithelialis*	2			0.52
	Ichthyophthirius multifiliis	181	100	23.8	13.49
	Thelohanellus nikolskii	10		2.1	1.05
	Trichodina sp.	50		2.6	10.5
Myxozoans	*Sphaerospora dykovae*	29	25	7.4	
Monogeneans	*Dactylogyrus sp.*	266	50	26.4	38.62
Cestodes	*Atractolytocestus huronensis*	49	40	7.4	1.32
	Bothriocephalus acheilognathi	26		6.3	0.52
	Khawia sinensis	7	7.5		1.05
Crustaceans	*Lernea cyprinacea*	10		0.26	2.30
Deformities		24		6.3	
Piscivorous birds		19			5.02

Figure 16. Carp erythrodermatitis of common carp caused by atipical *Aeromonas salmonicida*

3.2.2 Skeleton development and ossification in common carp

A total of 96 specimens were examined. The mean and standard deviation of body length for both groups is shown in Table 8.

Table 8. Total number, mean and standard deviation of body length (SL) in mm for A and B group.

Group	N	Standard length (mean ± SD)					
		5 DPH	17 DPH	22 DPH	24 DPH	26 DPH	29 DPH
Group A	8	5.80 ± 0.31	10.49** ± 0.48	16.24** ± 1.67	17.98** ± 2.23	21.11** ± 4.15	28.98* ± 2.27
Group B	8	5.87 ± 0.28	13.39** ± 0.44	24.67** ± 1.02	28.06** ± 2.00	28.81** ± 2.10	31.85* ± 1.55

At 5 DPH no sign of ossification was observed in either group (Fig. 17).

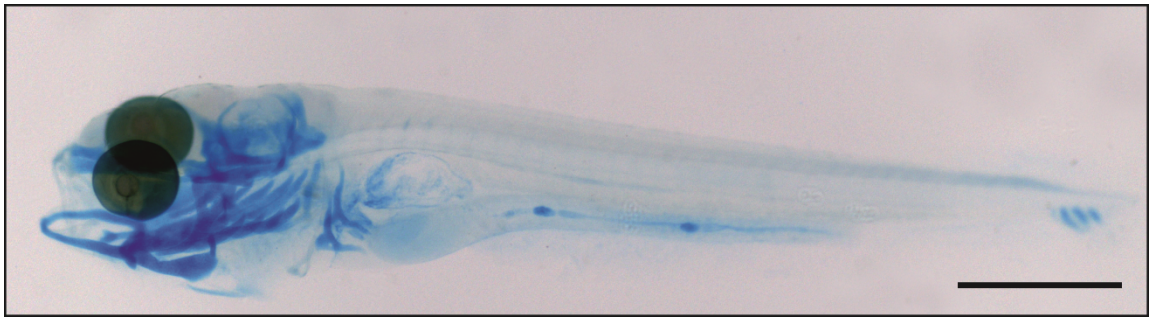

Figure 17. Absence of ossification signs in common carp larval samples at 5 DPH. Scale bar=1mm

At 17 DPH, in group A, the ossification process was visible on some head bones: parasphenoid, basisphenoid, ceartohyal, cleithrum, and the beginning of ossification is visible on the outer edges of the first 8 abdominal vertebrae (Fig. 18a). In group B, at the same stage, the ossification process was visible on most of the head bones (parasphenoid, basisphenoid, basioccipital, premaxillary, maxillary, dentary, hyomandibular, ceratohyal, opercle, cleithrum) (Figure 18b) and on abdominal and caudal vertebrae.

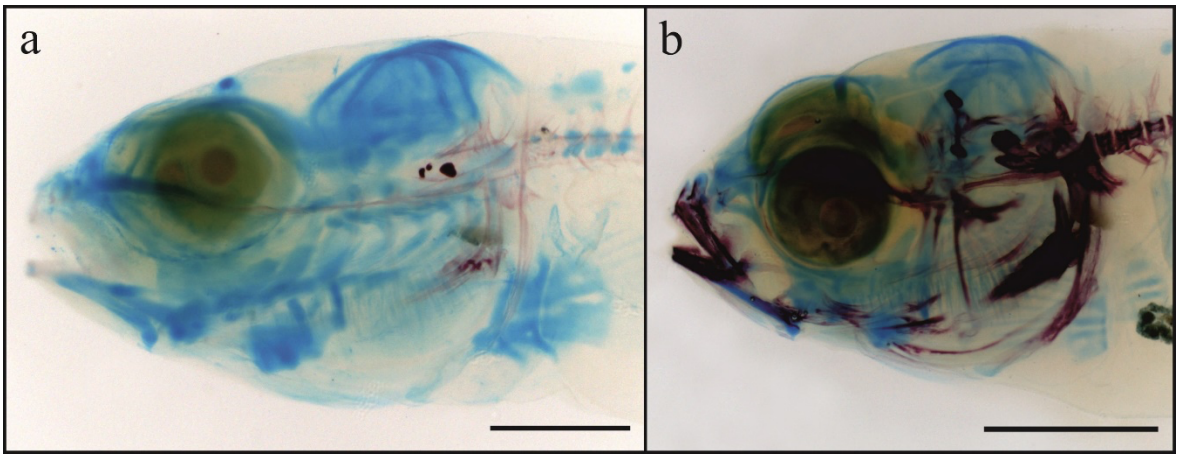

Figure 18: Ossification process visible on some head bones in group A (a) and on most of the head bones in group B (b), at 17 DPH. Scale bar=1mm

At 22 DPH, in group A, ossification started on basioccipital, premaxillary, maxillary, dentary, articular, quadrate, hyomandibular bone as well as on opercle, caudal vertebrae and on hypural bones of the caudal fin. In group B, ossification started on supraoccipital, articular and quadrate bone and the first sign of ossification was noticed on dorsal and caudal fins (Figure 19a and 19b) at this stage.

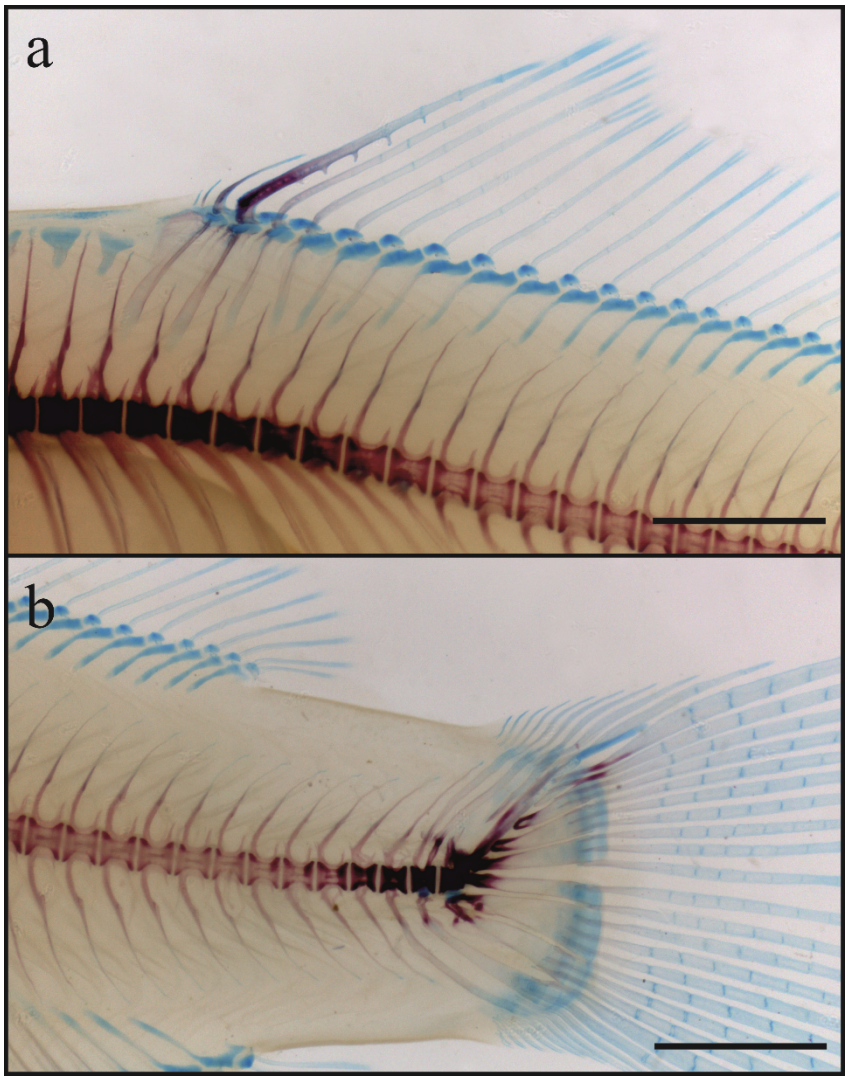

Figure 19. Ossification process on dorsal (a) and caudal fins (b) observed in group B at 22 DPH. Scale bar=2mm

At 24 DPH ossification was visible also on dorsal fin in group A, while new ossification signs on the head, frontal and parietal bone, as well as on anal, pelvic and pectoral fins was visible in group B.

At 26 DPH ossification started on anal, pelvic and pectoral fins in group A, while ossification was in progress or completed in group B.

Finally, at 29 DPH, frontal, parietal and supraoccipital bones started to ossify in group A. The process of ossification was either evolving or completed on the other investigated elements except fins where ossification was still not finished. At this stage ossification finished on anal fin while other fins still had cartilaginous parts in group B.

3.2.3 Pike perch health assessment: Case of mycobacteriosis

A high mortality among farmed pikeperch (0.2% d-1) cultured in an RAS was recorded. In total, five moribund pikeperch, ranging between 29 and 31 cm in length, were collected and examined from a single rearing tank. Stocking density in the tank was 48 kg m^{-3}. The source farm had a standing stock of approximately 6,000 fish, and farming was based on juvenile pikeperch imported from Hungary. Water temperature in the system was between 20.2 and 22.5°C, pH was between 7.4 and 7.8, and dissolved oxygen was between 7.0 and 8.7 mg/L.

Affected pikeperch were emaciated, lethargic, with visible skin lesions and discolouration. During necropsy, multiple greyish-white nodules were found in the spleen and kidney. These nodular lesions were larger in the spleen (2 mm in diameter) than in the kidney (0.06 to 0.95 mm in diameter), though severity of lesions was greater in the kidney than in the spleen. In the spleen, nodules were mostly found superficially (Fig. 20).

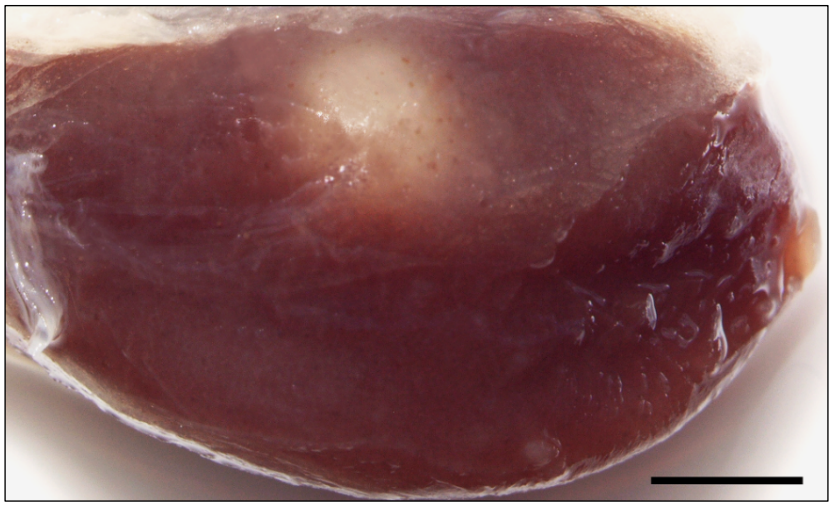

Figure 20. Superficial nodular lesion in the spleen of pikeperch (*Sander lucioperca*) caused by *Mycobacterium marinum*. Scale bar = 2 mm

Both organs demonstrated acid-fast bacteria within tissues and phagocytes (Figs. 21 & 22).

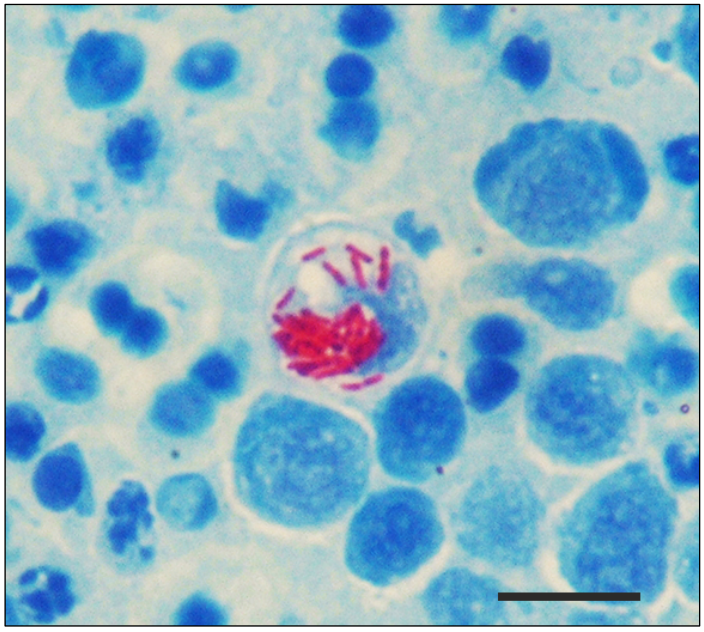

Figure 21. Ziehl-Neelsen stained kidney imprint with acid-fast mycobacteria within the macrophage. Scale bar = 10 µm.

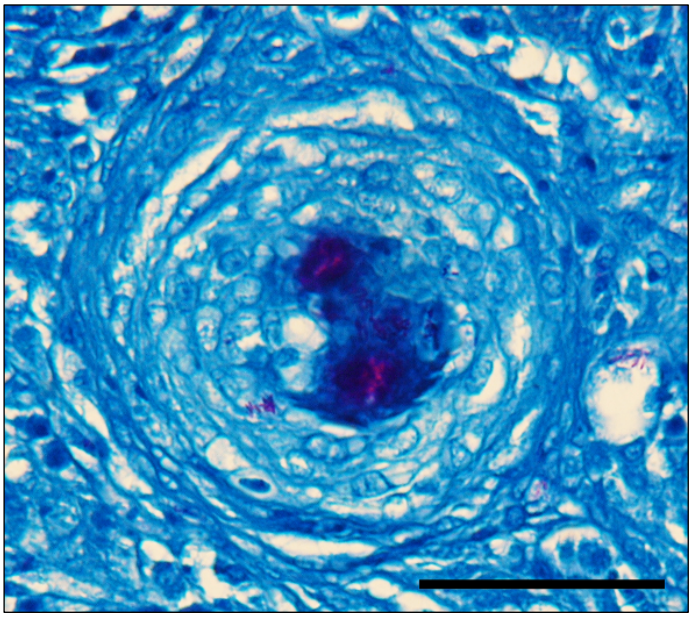

Figure 22. High magnification of early granuloma with a small number of acid-fast mycobacteria (Ziehl-Neelsen). Scale bar = 50 µm.

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Granulomas were composed mainly of epithelioid cells with or without central area of necrosis (Fig.23).

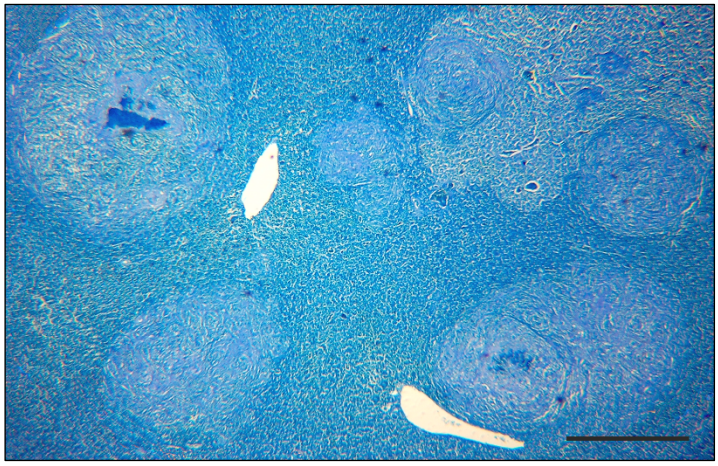

Figure 23. Histological section of a pikeperch kidney showing granulomatous response (Ziehl-Neelsen). Multiple poorly developed granulomas occupying a large portion of the anterior kidney

Connective tissue capsule was absent. Neither multinucleated giant cells nor dystrophic calcification in the granulomas were detected. Granuloma morphology was similar in both organs. Occasionally, adjacent granulomas appeared to fuse, resulting in large multinodular lesions (Fig.24).

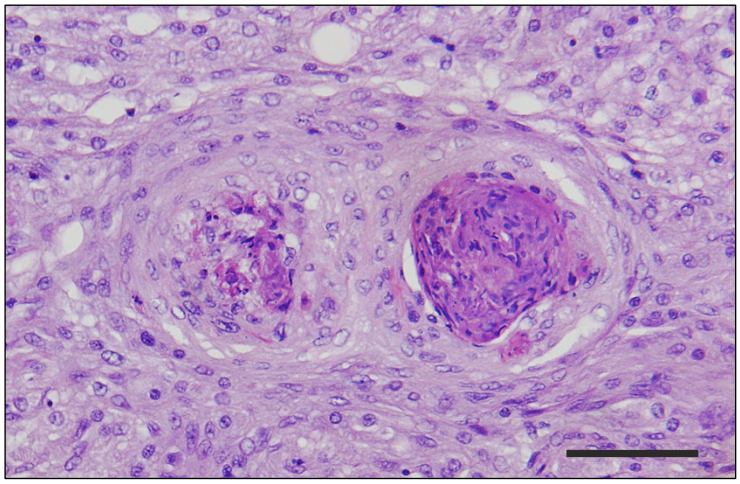

Figure 24. H&E stained section of a pikeperch spleen. Note a thick wall of epithelioid macrophages and necrotic debris in centre of granulomas. Scale bar = 50 µm.

Acid-fast bacteria were mostly limited to the inside of the granulomas (Fig. 22). The diagnosis was confirmed through isolation and identification of acid-fast bacteria. The culture of kidney samples for mycobacteria was positive, and growth was observed on liquid media after two weeks of incubation at 37°C. The isolate was identified by molecular methods as *Mycobacterium marinum*.

3.3 Microplastic occurrence in farmed gilthead sea bream and common carp

Seven hundred and 795 gilthead sea bream and common carp larvae respectively were examined for microplastic content. No plastic microparticles were observed in either species at this life stage. Number of specimens analysed and morphological characteristics, including the total body length (TL, cm) and the body weight (W, g) of fry and adults of both species are reported in Table 9.

Table 9. Data (length and weight) of the analysed samples of *Sparus aurata* and *Cyprinus carpio*, and corresponding number of microplastic particles (MPs)

Species		N° of samples	Length (cm) (Mean)	Weight (g) (Mean)	N° MPs	Item/specimen	Particle size (Mean÷SD)
Sparus aurata	**Fry**	60	6.84	5.41	13	0.21	1.84÷1.29
	Adult	20	25.6	253.8	26	1.3	1.96÷1.72
Cyprinus carpio	**Fry**	60	7.11	10.9	4	0.06	0.81÷0.64
	Adult	20	51.18	2740	5	0.25	0.80÷1
Total		160			48		

Representative images of MPs found in both species are shown in Figure 25.

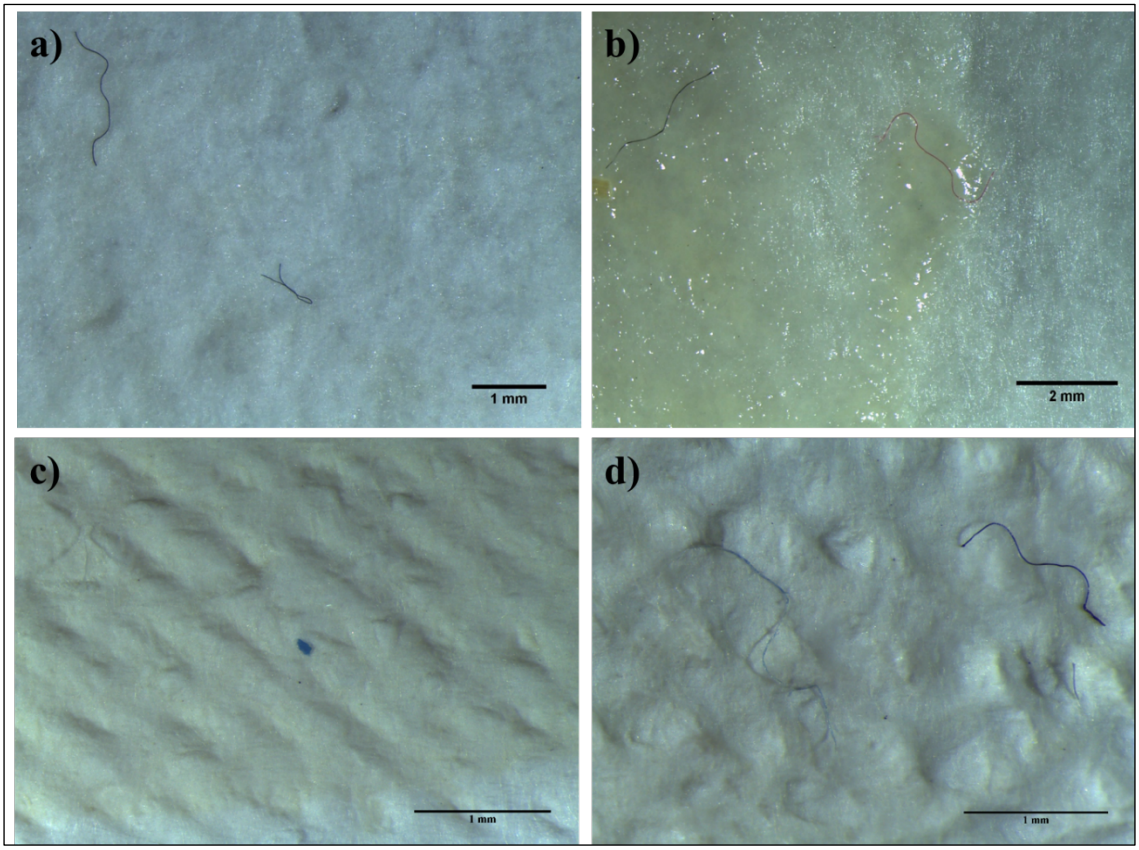

Figure 25. Representative images of microplastics found in fry (a) and adult specimens (b) of gilthead sea bream and in fry (c) and adult specimens (d) of common carp

In total, 39 plastic particles were isolated from the GITs of 80 gilthead sea bream specimen (0.48 items/specimen). 33.3% were isolated from fry, while 66.6% from adult individuals. MPs found were only in filamentous shape ranging in size between 0.24 and 8.86 mm. The dominant colour was black (46.15%), followed by azure (20.5%) (Fig. 26 a,b).

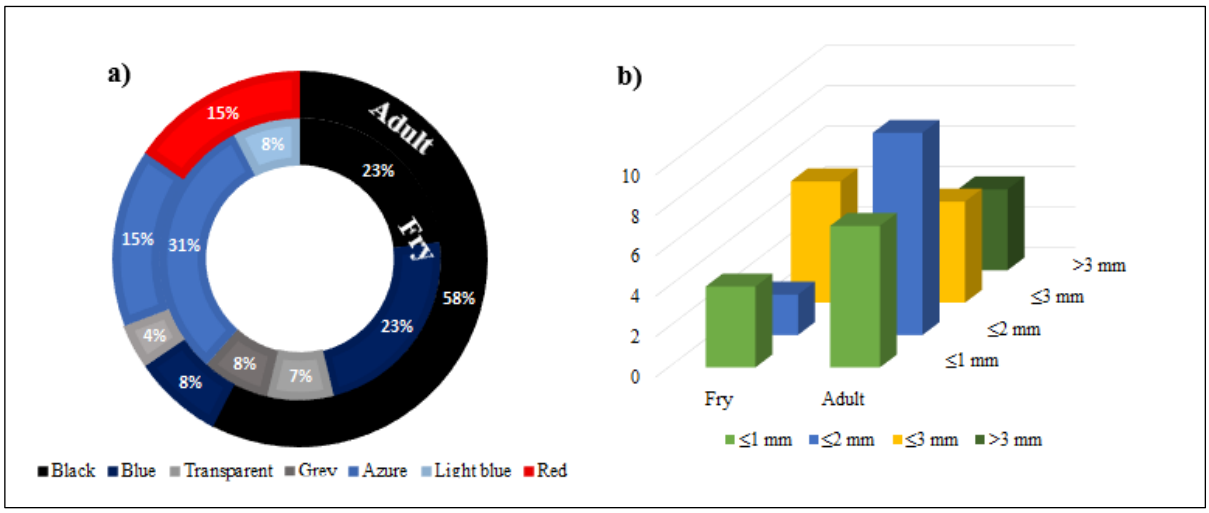

Figure 26. Percentage (%) of plastic particles classified by colour (a) and size (b) extracted from the gastrointestinal tract of reared fry and adult gilthead sea bream

From the GITs of 80 common carp specimens, in total 9 plastic microitems were isolated (0.11 items/specimen). 44.4% were isolated from fry, while 55.5% from adult individuals. The fibrous plastic debris represented 55.5%, while the fragments constituted 44.4%, ranging in size between 0.07 and 2.23 mm. The dominant colour was azure (55.5%), followed by black (22.2%), light blue (11.1%) and blue (11.1%) (Fig. 27 a,b,c). From the examination of the hepatopancreas no microplastics were found.

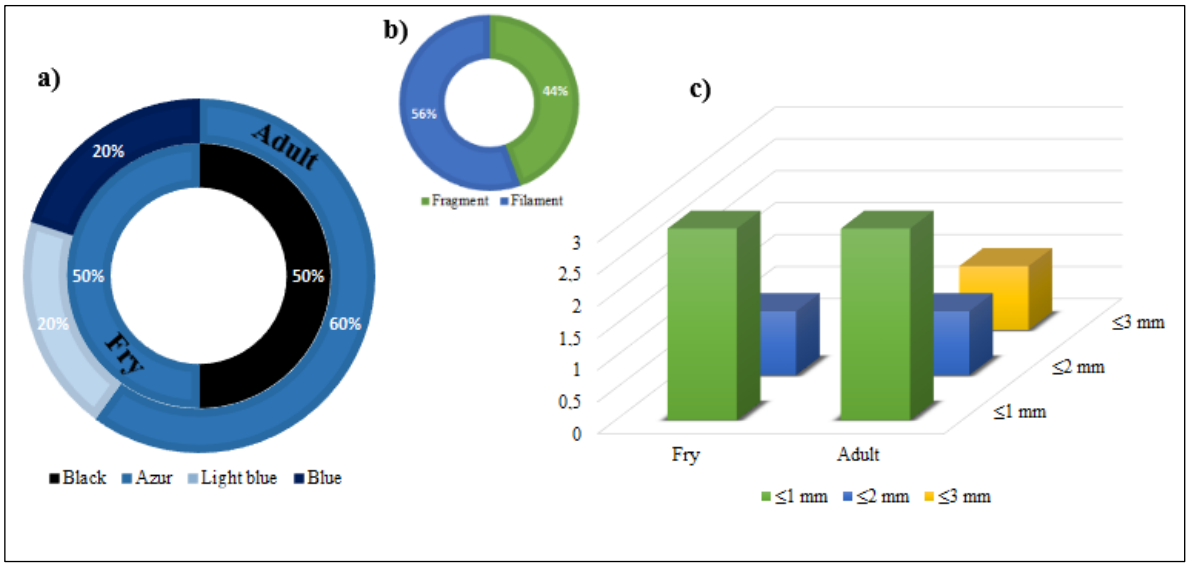

Figure 27. Percentage (%) of plastic particles classified by colour (a) shape (b) and size (c) extracted from the gastrointestinal tract of fry and adults of common carp

3.3.1 Microplastic identification

Among the 39 infrared spectra of samples isolated from specimens, 33 items were identified. Regarding composition, natural cellulose-based polymers (cotton, rayon, lyocell, linen), polyamide, nylon, polyester, polyacrylic and PTFE were identified. The number and the corresponding chemical types of the identified items found in the two species, are summarized in Tables 10 and 11.

Table 10. Microplastic concentration and polymer type of the identified items in the two investigated species

Sample		Counts(Items)	Chemical type
Common carp			
A	Fry	3	Polyester, PTFE, Linen
B	Fry	1	Rayon
C	Adult	1	Cotton: Polyester
D	Adult	1	Rayon
E	Adult	2	Lyocell, Cotton: Polyester
Gilthead sea bream			
F	Fry	5	Cotton: Polyamide, Rayon, Rayon, Polyester, Cotton
G	Fry	2	Cotton: Polyamide, Cotton
H	Fry	2	Rayon, Nylon
L	Adult	5	Rayon, Cotton: Polyamide, Polyacrylic, Cotton, PTFE
M	Adult	3	Nylon, Polyester, PTFE
N	Adult	4	Rayon, Cotton: Polyester, Wool: Polyester, Linen
O	Adult	2	Lyocell, PTFE
P	Adult	2	Rayon, Cotton

Table 11. Chemical type of the identified items and their percentages

Chemical types of the identified polymers			
Chemical type	Counts (items)	Percent (%)	Percent per class (%)
Natural/Artificial			
Linen	2	6	
Rayon	8	24	49
Lyocell	2	6	
Cotton	4	12	
Semi-synthetic			
Cotton: Polyester	3	9	
Cotton: Polyamide	3	9	21
Wool: Polyester	1	3	
Synthetic/Plastic			
Polyester	3	9	
Nylon	2	6	30
Polyacrylic	1	3	
PTFE	4	12	

The μ-FT-IR example spectra of the different microplastics found in the two species are shown in Figure 28 (common carp) and Figure 29 (gilthead sea bream), respectively.

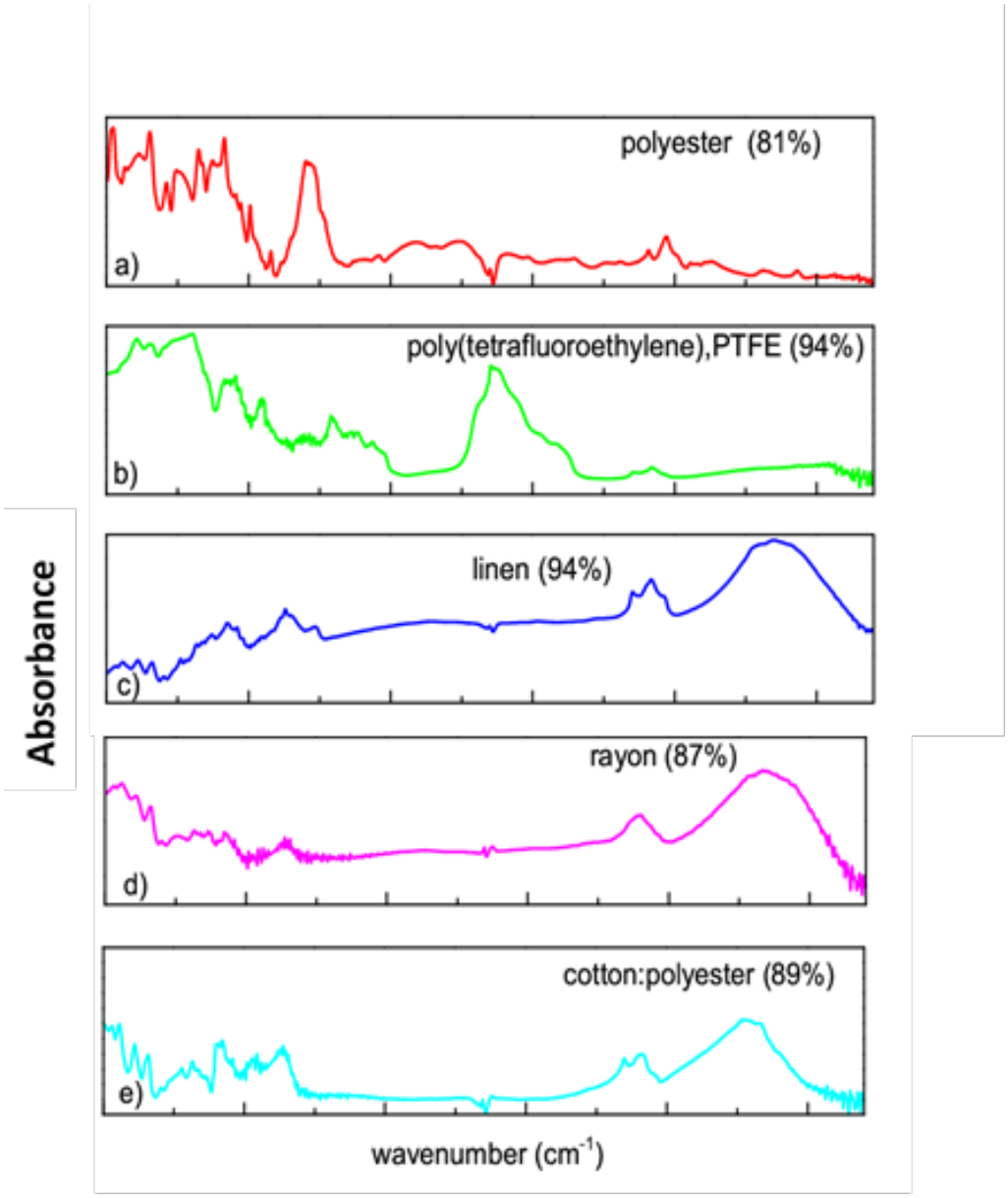

Figure 28. μ-FT-IR example spectra of the identified items in common carp specimens: a) b) c) spectra of items found in A sample; d) and e) spectra of items found in sample B and C, respectively.

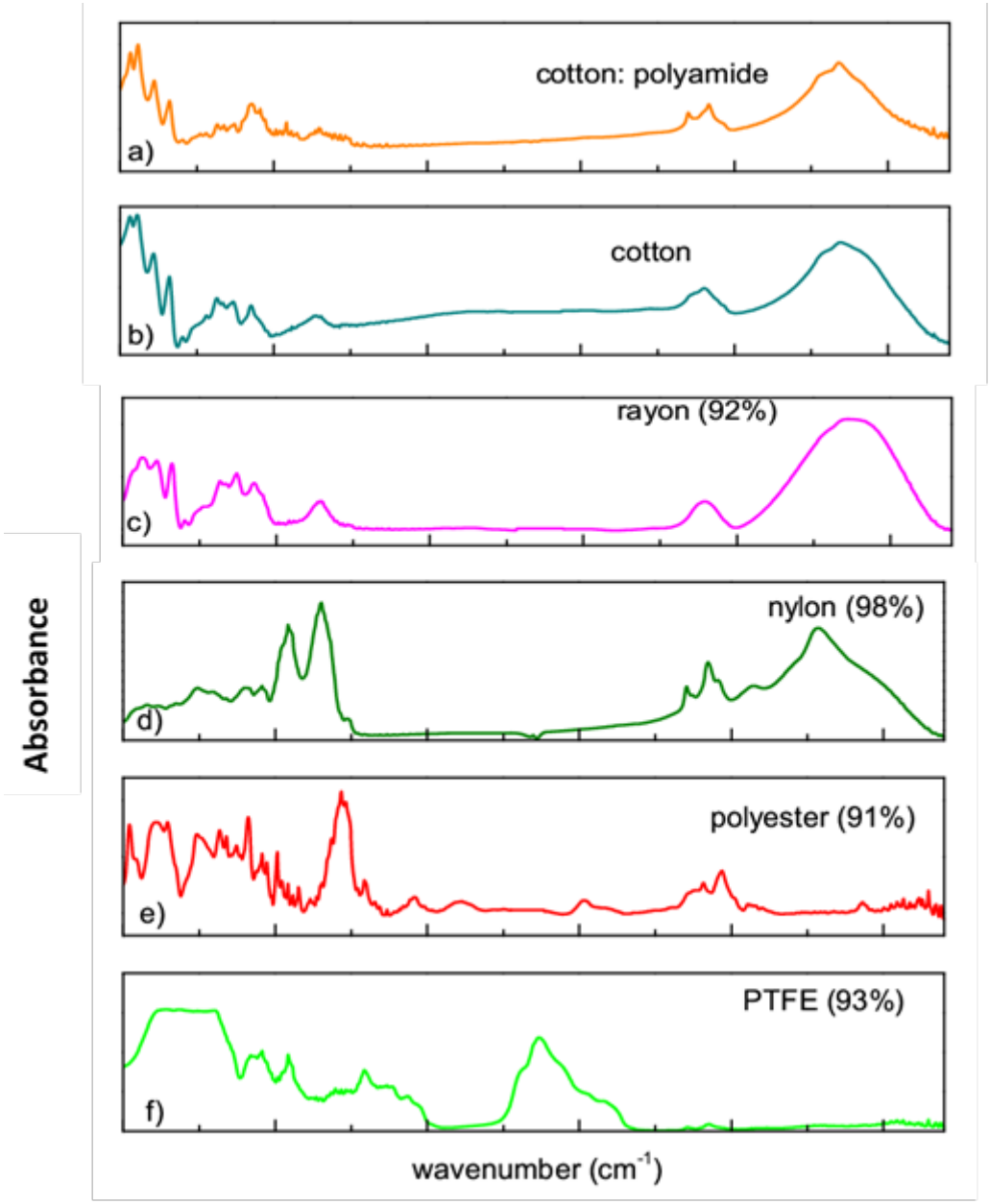

Figure 29. μ-FT-IR example spectra of the identified items in gilthead sea bream specimens: a) and b) spectra of items found in G sample; c) item found in H samples and d) e) and f) spectra of items found in M sample.

3.4 Evaluation of immunostimulant activity of Imoviral in *Sparus aurata*

3.4.1 Gene expression

To elucidate the immunostimulant effect of Imoviral as a dietary additive, the expression of immune-related genes involved in the acute phase response were investigated in the spleen of *Sparus aurata* fingerlings (Fig.30 a, b, c, d) (Tab. 12).

In general, the pro-inflammatory cytokine *tnf-α* was significantly up-regulated compared to the control group (CTRL), in both Imoviral-fed groups (infected and uninfected fish), although no significant variation was detected at time T168. *il-1β* was significantly up-regulated at 1 and 24 hpi in IVS compared to CTRL, while expression did not show any significant change between IPS and CTRL. Regarding the antimicrobial peptides, *hep* expression was markedly down-regulated compared to CTRL, in both IVS and IPS groups. *Def* expression was significantly down-regulated in IPS, while IVS showed no significant variation compared to CTRL.

All gene expression levels in noninjected and nonexperimentally fed animals (control) showed no statistically significant differences over time, with the exception of *hepc* (T1 vs. T72 and T168 vs. T72, $p<0.05$).

***Tnf-α* gene expression**. At 1 and 24 hpi, the IVS group showed the highest expression levels of *tnf-α* (0.222 ±0.014 and 0.210± 0.010 respectively), which were significantly different from the levels expressed in the PVS, as well as in CTRL, IPS, and PPS groups ($p <0.05$). Among uninfected group, IPS showed significant differences with CTRL ($p<0.05$), but no differences with PPS were observed ($p>0.05$)

Although a decrease in the expression of *tnf-α* was observed at 72 hpi in IVS _ (0.159±0.0012), this was still significantly higher than the levels expressed in the CTRL and in the IPS and PPS groups ($p <0.05$). At T72, *tnf-α* mRNA expression levels showed significant higher values in PVS group (0.406±0.014, $p<0.05$). Among uninfected group, IPS showed significant differences with PPS ($p<0.05$). At 168 hpi a decreased expression of *tnf-α* was recorded in IVS (0.095±0.001), showing a significant difference compared to the PVS group ($p <0.05$), which instead expressed the highest levels of *tnf-α* (0.087± 0.007). The lowest expression values of *tnf-α* at T168 were detected in the PPS group (0.087±0.007), varying significantly only from PVS groups ($p <0.05$). Note that the IPS and PPS groups did not show significant differences in *tnf-α* gene expression levels between time points within the same experimental group. The PVS group showed significant variations at all times investigated, with an increasing trend of expression

levels with increasing time. In contrast, the IVS group showed significantly decreasing levels of expression with increasing infection time ($p < 0.05$), except between T1 and T24 ($p > 0.05$) (Fig.30).

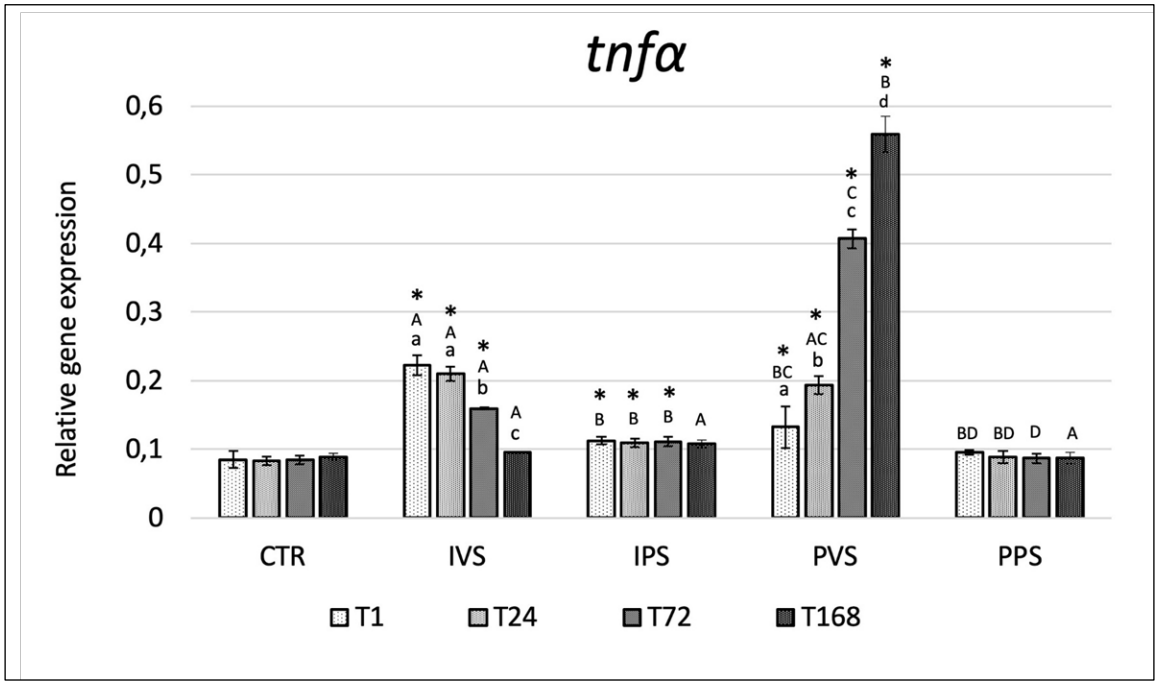

Figure 30. Relative gene expression of *tnf-α* mRNA in spleen tissue of *Sparus aurata* during experimental diet administration.

Data are shown as mean± SD, n=3. Stars represent significant differences between the treatments and control group at the same time point. Letters are only present in the case of significant statistical differences. Different capital letters refer to significant differences between treatment groups at the same time points, and different small letters indicate significant differences between time points within the same treatment group. Differences were considered significant when $p<0.05$.

***Il-1β* gene expression**. At T1, significant variations of *il-1β* mRNA expression levels were observed between all experimental groups ($p < 0.05$), except between the IPS group and CTRL ($p > 0.05$). *Il-1β* was up-regulated in the IVS group (0.242±0.014), showing expression values significantly higher than in the CTRL, IPS and PPS groups (Tab. 12) ($p < 0.05$), but significantly lower than the PVS group (0.360±0.058), within which the gene was up-regulated compared to all the other experimental groups ($p < 0.05$). Uninfected groups IPS and PPS also showed significant differences, with a higher *Il-1β* expression observed in PPS (0.152±0.028). At T24, the greatest increase in *il-1β* was observed in the PVS group ($p < 0.05$). Among infected groups, at 24 hpi, in the IVS group there was a decrease in the levels of *il-1β* (0.158±0.030) compared to PVS, however it was higher expressed than in other groups ($p < 0.05$). No change in expression levels was

observed between uninfected groups IPS and PPS, nor with CTRL (p>0.05). At T72 IVS group showed no significant differences compared to IPS, PPS and CTRL groups, however the expression of *il-1β* was significantly lower than the levels expressed in PVS (p <0.05). PVS groups showed a decrease in the expression levels of *il-1β* compared to the previous time (p<0.05), while IPS and PPS showed a slight increase although not statistically significant. IVS, PVS and PPS groups showed variations between the time points analysed within the same group. In particular, IVS did not show significant variations in the expression levels of *il-1β* only between T24vsT72 (p> 0.05), showing a decreasing trend in expression levels from T1 to T168. In PVS between T72vsT168 no significant variation of *il-1β* (p> 0.05) was observed, while in PPS significant changes were detected only between T1vsT24 and T1vsT72 (p <0.05) (Fig.31)

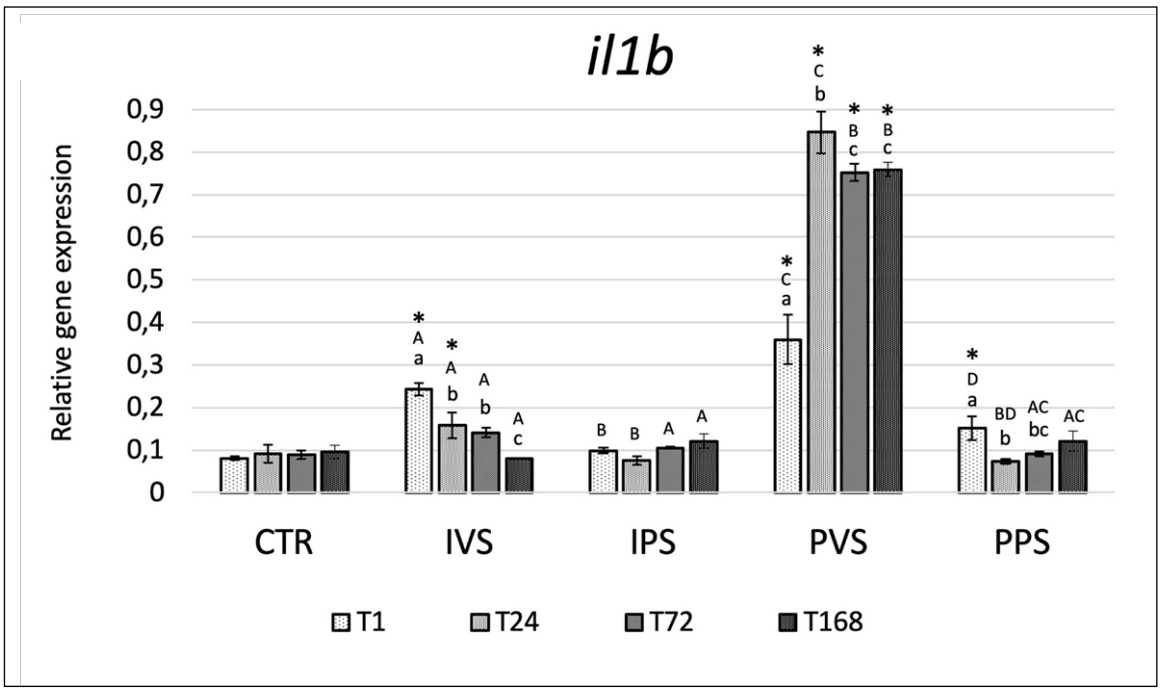

Figure 31. Relative gene expression of *il-1β* mRNA in spleen tissue of *Sparus aurata* during experimental diet administration.

Data are shown as mean± SD, n=3. Stars represent significant differences between the treatments and control group at the same time point. Letters are only present in the case of significant statistical differences. Different capital letters refer to significant differences between treatment groups at the same time points, and different small letters indicate significant differences between time points within the same treatment group. Differences were considered significant when p<0.05.

***Hep* gene expression**. At T1 *hep* was significantly down-regulated in both Imoviral-fed group IVS and IPS groups compared to CTRL (Fig.32) and significant variations where

also detected between them ($p > 0.05$). Regarding infected group, the expression of *hep* in IVS did not show significant differences with PVS. *Hep* expression levels varied significantly between uninfected IPS and PPS group. In fact, in IPS the gene was strongly down-regulated compared to PPS and also to the other groups ($p < 0.05$). At T24 only the IPS and PVS groups showed significant variations in *hep* levels compared to CTRL (Tab. 12) ($p < 0.05$). *Hep* levels showed significant variation only between infected IVS and PVS groups ($p < 0.05$), while no differences were detected between uninfected groups (IPS and PPS). *Hep* mRNA expression levels were markedly down-regulated in IPS, compared to PVS group, within which the gene was up-regulated compared to all the other experimental groups ($p < 0.05$).

At T72 only PVS showed a significant difference compared to CTRL ($p<0.05$), and *hep* was significantly higher expressed than in all the other groups ($p < 0.05$), while between Imoviral-fed group (IVS and IPS) no significant change was detected ($p > 0.05$). *Hep* levels showed significant higher expression in the uninfected PPS groups compared to IPS ($p<0.05$). At T168 the expression of *hep* varied significantly compared to CTRL in all groups ($p < 0.05$), except for PPS. *Hep* expression level was lower in IVS group (0.164 ± 0.0237) compared to all other groups ($p<0.05$), and strongly expressed in PVS (1.581 ± 0.017) ($p < 0.05$). An increase in *hep* expression levels was also observed in IPS compared to IVS ($p < 0.05$), although lower than the levels expressed in CTRL and PVS ($p < 0.05$). No significant differences were observed between the IPS and PPS groups ($p > 0.05$).

PVS group was the only one to show significant changes in *hep* expression over time ($p < 0.05$). Within the IVS group *hep* decreased significantly only at T168 compared to all other T ($p < 0.05$). IPS showed statistically valid differences only between T1 vs all other T ($p < 0.05$). No changes were recorded within PPS ($p > 0.05$).

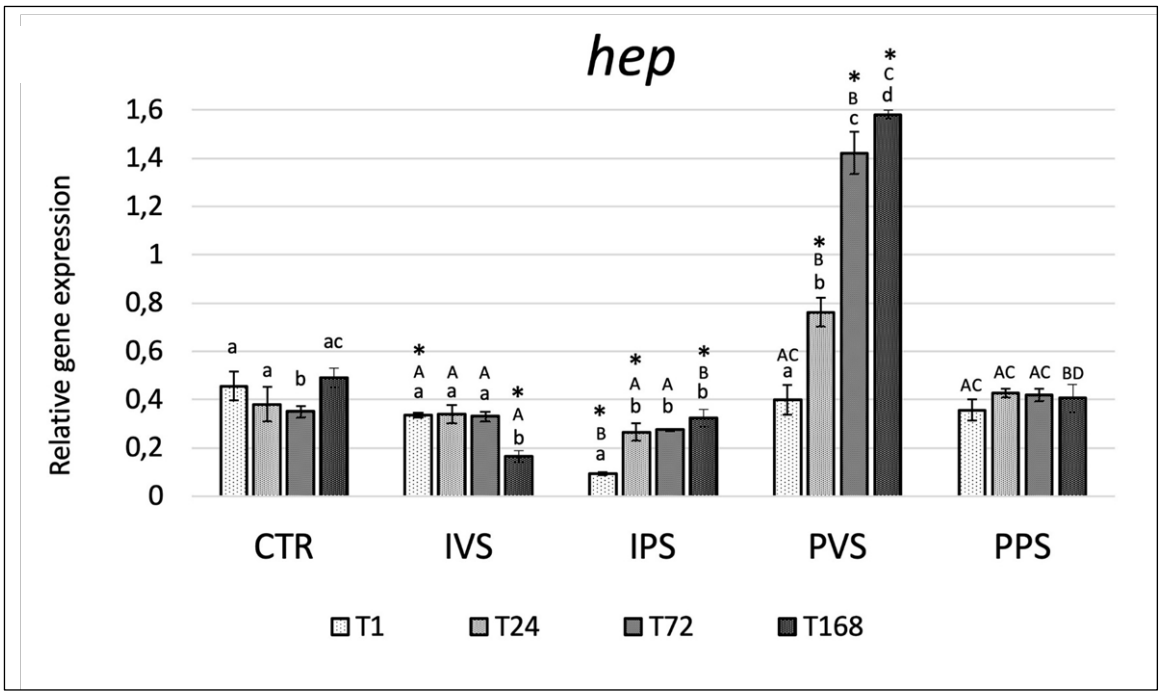

Figure 32. Relative gene expression of *hep* mRNA in spleen tissue of *Sparus aurata* during experimental diet administration.

Data are shown as mean± SD, n=3. Stars represent significant differences between the treatments and control group at the same time point. Letters are only present in the case of significant statistical differences. Different capital letters refer to significant differences between treatment groups at the same time points, and different small letters indicate significant differences between time points within the same treatment group. Differences were considered significant when $p<0.05$.

***Def* gene expression**. *Def* expression varied significantly between the CTRL and IPS and PVS experimental groups ($p <0.05$) over time. At T1 experimental fed IVS and IPS group did not show significant differences in the expression levels of *def*; in both groups it was strongly decreased compared to the PVS group ($p <0.05$). A significant increase in *hep* expression levels was also observed in PPS compared to IPS ($p <0.05$). At 24 hpi *def* expression in the IVS group showed marked differences compared to PVS, where the gene expression level was higher (Tab. 12, $p < 0.05$). and with IPS, where instead the gene was lower expressed. Significant changes in *def* expression were also highlighted between uninfected groups, where the gene expression level was higher in PPS than in IPS (Fig.33, $p <0.05$). At T72 and T168 the same differences described for T24 between the experimental groups were recorded. As with the expression of *hepc*, PVS group showed significant differences over time ($p <0.05$), IVS showed significant changes only between T168 vs all other time points ($p <0.05$). IPS showed significant changes only

between T1 and T24. Finally, PPS highlighted a significant variation between T72 and all other T ($p<0.05$).

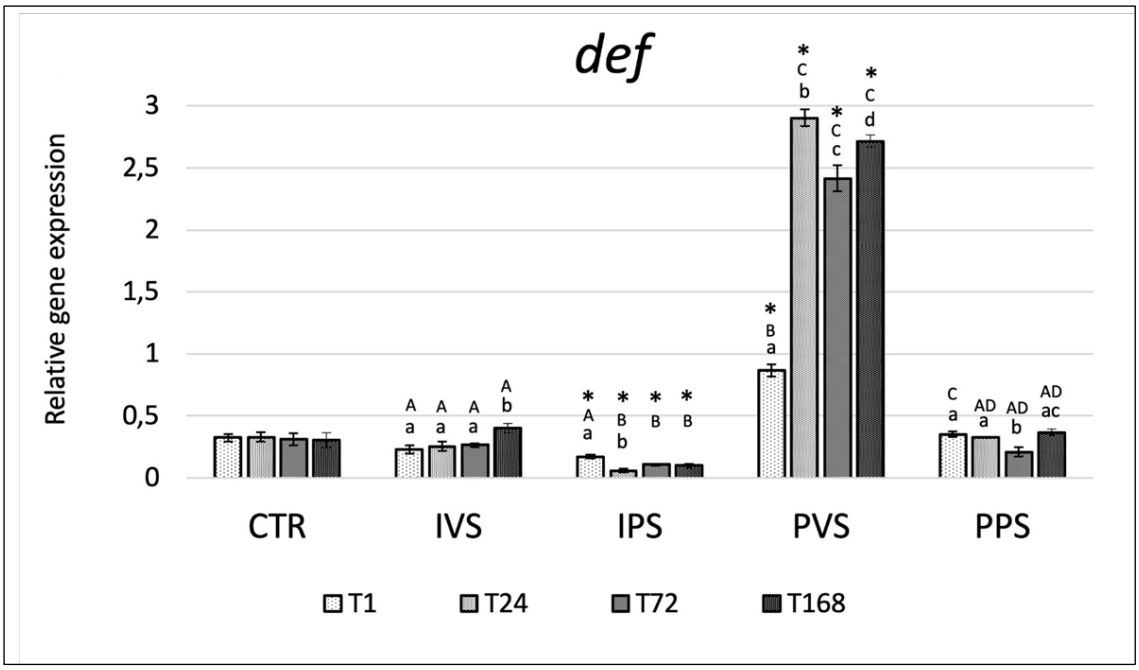

Figure 33. Relative gene expression of *def* mRNA in spleen tissue of *Sparus aurata* during experimental diet administration.

Data are shown as mean± SD, n=3. Stars represent significant differences between the treatments and control group at the same time point. Letters are only present in the case of significant statistical differences. Different capital letters refer to significant differences between treatment groups at the same time points, and different small letters indicate significant differences between time points within the same treatment group. Differences were considered significant when $p<0.05$.

Table 12. Quantitative expression (q-PCR) of immune genes in gilthead sea bream spleen of all experimental groups at all times analysed.

	Group	Control		IVS		IPS		PVS		PPS	
Gene	**T**	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
tnf-α	1	0.085	0.012	0.223	0.014	0.113	0.006	0.132	0.030	0.096	0.003
	24	0.083	0.007	0.210	0.010	0.110	0.006	0.193	0.013	0.089	0.009
	72	0.085	0.006	0.160	0.001	0.111	0.007	0.407	0.014	0.087	0.007
	168	0.090	0.004	0.095	0.002	0.108	0.006	0.559	0.026	0.087	0.008
il-1β	1	0.081	0.004	0.243	0.014	0.099	0.007	0.360	0.059	0.152	0.028
	24	0.091	0.021	0.158	0.030	0.076	0.010	0.847	0.049	0.073	0.006
	72	0.089	0.010	0.141	0.011	0.106	0.002	0.752	0.020	0.091	0.006
	168	0.096	0.015	0.080	0.002	0.121	0.016	0.759	0.016	0.121	0.024
hepc	1	0.457	0.058	0.336	0.011	0.094	0.007	0.400	0.063	0.357	0.043
	24	0.381	0.072	0.340	0.038	0.266	0.037	0.762	0.059	0.428	0.018
	72	0.351	0.023	0.332	0.020	0.275	0.005	1.422	0.087	0.419	0.027
	168	0.490	0.039	0.164	0.024	0.323	0.036	1.582	0.018	0.406	0.058
def	1	0.325	0.029	0.228	0.031	0.172	0.013	0.866	0.049	0.352	0.023
	24	0.330	0.038	0.255	0.036	0.060	0.018	2.902	0.067	0.324	0.003
	72	0.309	0.050	0.263	0.018	0.106	0.003	2.416	0.107	0.209	0.036
	168	0.302	0.062	0.402	0.040	0.098	0.020	2.715	0.050	0.367	0.029

3.5 Zebrafish embryo toxicity test to demonstrate the preferential removal of fungicides from water by molecular imprinting with TiO_2 photocatalysts.

The ZFET carried out to test toxicity of the different types of nanoparticles imprinted, did not show mortality nor sublethal effects in the embryos exposed. Also, the hatching rate did not show statistically significant differences ($p > 0.05$). However, we have highlighted statically significant differences in the hatching rate ($p < 0.05$) and in the viability of the larvae ($p < 0.05$) for the zebrafish exposed to imprinted nanoparticles with OPP fungicides.

Moreover, we evaluated the expression biomarkers MTs1 and HSP70. The immunohistochemical analysis performed in larvae exposed to different imprinted nanoparticles showed the presence of MTs1 in whole body (Fig. 31). In larvae treated with type 3 (3.8 x 10-5 mg/ml) response to the anti-MTs1 antibody was particularly marked

(Fig.31). In control samples (untreated) only the head region showed a positive response to anti-MT (Fig. 31).

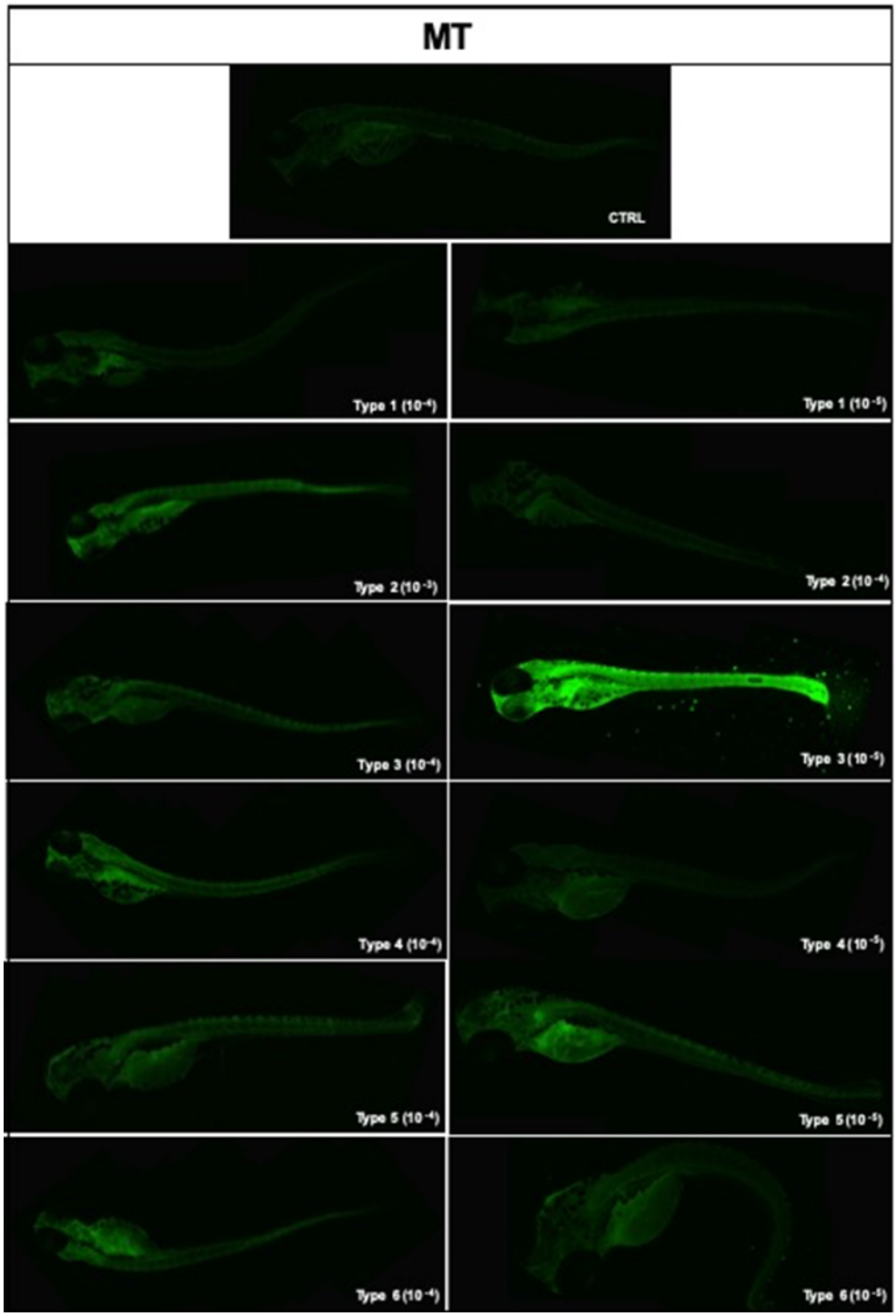

Figure 34. MTs1 antibody-staining of the 96-hpf zebrafish embryos after exposure to different types and concentrations of TiO_2 including untreated zebrafish embryos.

Significant differences were detected also for HSP70 expression in larvae exposed (Fig. 32). In particular, they are highly expressed in type 4 (3.6 x10-4 mg/ml) and in type 6 (6.9 x10-4 and 6.9 x 10-5 mg/ml) (Fig. 32).

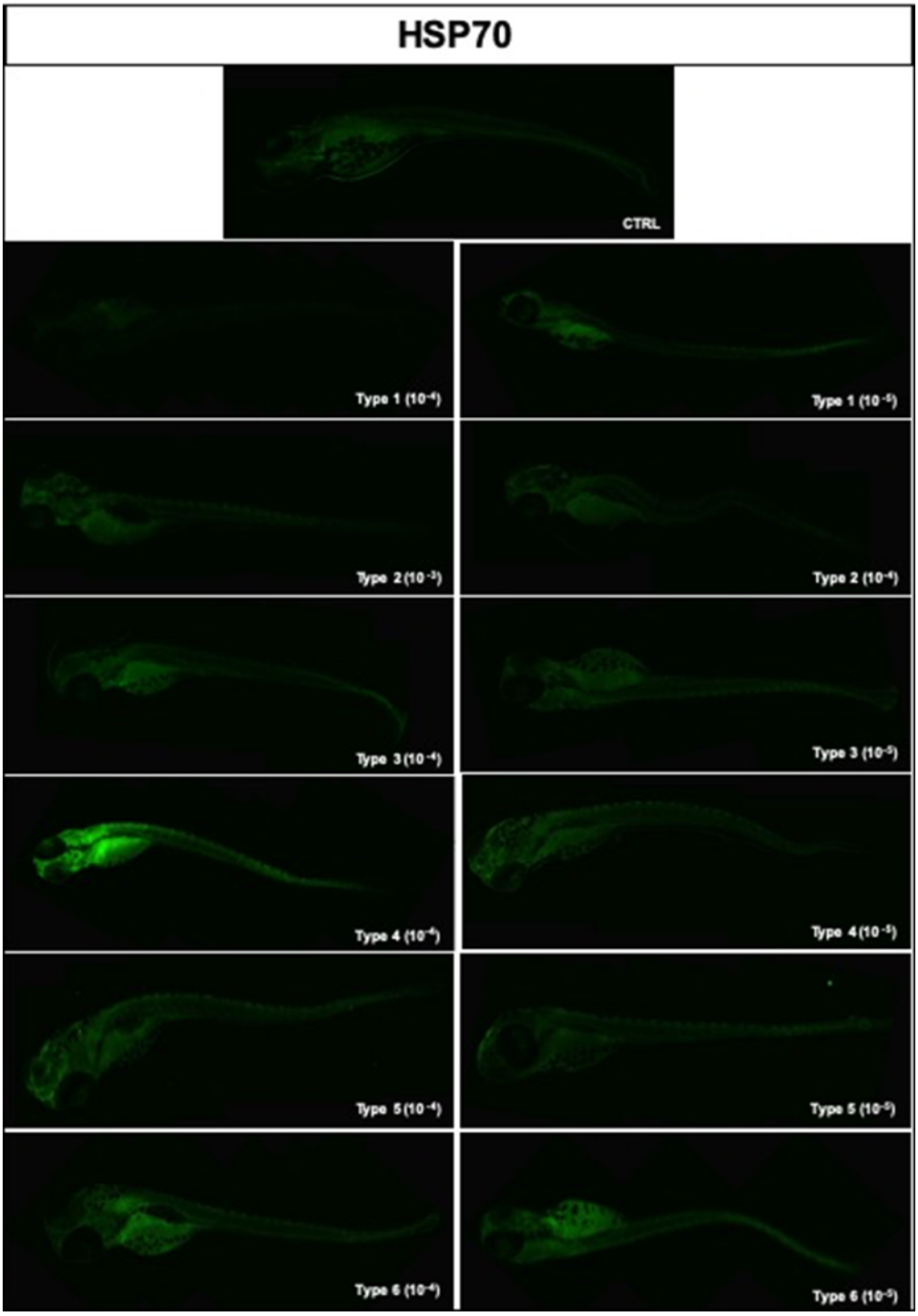

Figure 35. HSP7O antibody-staining of the 96-hpf zebrafish embryos after exposure to different types and concentrations of TiO_2 including untreated zebrafish embryo.

Zebrafish exposed to imprinted nanoparticles with OPP fungicide (1:1) died one hour after exposure and therefore immunohistochemical analysis showed no response to the biomarkers tested.

4. DISCUSSION

4.1 Fish health monitoring conducted at an Italian fish farm

From the survey conducted in the Italian fish farm it emerged that infectious diseases caused by bacterial or viral agents represent the main cause of mass mortality in reared gilthead sea bream larvae. Indeed, the rapid development and intensification of aquaculture production has increased fish species sensitivity to infectious diseases, due to stress factors such as overcrowding [300,301]. Although the pathologies to which gilthead sea bream is most susceptible are well known, the field of larval pathologies is still relatively little explored, and there is little information on the causes of mortality outbreaks that can occur in the hatcheries. With the present thesis, to our knowledge, we describe for the first time the co-occurrence of VER and LE, caused by reassortant betanodavirus RGNNV/SJNNV and *Vibrio alginolyticus* respectively, which in synergy or simultaneously, elicited mortality outbreak of *Sparus aurata* larvae raised in an Italian fish farm. Viral and bacterial co-infection episodes have been reported in both marine and freshwater fish species in *natura* [302]. Regarding cultivated Sparid fish, only one study described episodic co-infections by SJNN or VHSV and *Vibrio* spp. in farmed adult sea bream specimens collected from southern Spain [303]. Our results, in the field and in the laboratory, have demonstrated clinical signs caused by RGNNV/SJNNV reassortant strain in both 19 and 26-day old larvae groups analysed. Betanodaviruses are known to infect a large variety of farmed and wild marine fish species, as well as some freshwater species [33,304,305], though, some other species are generally considered asymptomatic or have low susceptibility to this disease [33]. These data can be misleading if we refer only to the RGNNV genotype, resulting, for example, highly infectious for sea bass but to which gilthead sea bream appears relatively resistant [34,306]. Betanodaviruses are classified into 4 major genotypes: RGNNV, BFNNV, TPNNV and SJNNV each of which exhibits a different ability to infect fish species [307]. More specifically, RGNNV is able to infect a wide variety of warmwater fish species. BFNNV seems restricted to marine cold-water fish species, while TPNNV affects only one species. Finally, regarding SJNNV type, in the past it had been reported only in some fish species present in Japanese waters [33], but it has recently been found in new host fish species such as Senegalese sole, gilthead sea bream and European sea bass cultured in the Iberian peninsula [308]. However, it has also been shown that a shift to new host fish species can occur by RNA reassortment, allowing the resultant reassortant strains, RGNNV/SJNNV and SJNNV/RGNNV, to create disease [309]. It would appear that reassortant RGNNV/SJNNV can cause severe disease and massive mortality in cultured gilthead sea

bream [37]. These results, also confirmed by a recent study conducted by Toffan and collaborators [310], could explain the increasing number of *betanodavirus* outbreaks in recent years as well as the few cases described in the past in the Mediterranean areas [311] finally demonstrating the susceptibility of this species to the disease

Histological sections revealed the presence of vacuolar lesions affecting retina and brain similar to those found by other authors in bream larvae affected by VER. Small and poorly distributed vacuolizations could be attributed to an acute phase of the infection [310,312].

Understanding the origin of an infection and therefore entry into the hatchery system remains of crucial importance. Fish nodavirus can be transmitted both vertically and horizontally [31,35]. In our case it is possible to exclude a vertical transmission because no recent introduction of new broodstock had been recorded in the farm prior the disease manifestation in larvae. Rather, horizontal transmission may have caused mass mortality of larvae due to two main reasons. First of all, a temporary malfunction of the UV disinfection system occurred in the farm, probably causing entry of a potential external contamination, and therefore from the environment to the system. Secondly, in the period before the outbreak, sea bass specimens imported from France were introduced into the farm, which may have been an asymptomatic vector of the disease. That could be a key factor in spreading the disease throughout all the hatchery system.

Additionally, our results showed that analysed gilthead sea bream larvae were affected by larval enteropathy infection caused by *Vibrio alginolyticus*, highlighting abdominal swelling and the presence of undigested food. This symptomatology generally refers to a larval enteropathy (LE), previously described as "distended gut syndrome" (DGS) [313]. Other authors have reported outbreaks of enteropatic syndrome, caused by *V. anguillarum*, *V harveyi*, in which the affected larvae showed almost the same symptomatology [313]. In general, it is believed that an overgrowth of *Vibrio* spp., associated with stressful conditions, such as the presence of other pathogens, or inadequate rearing and nutritional management, may be responsible for this condition [65,313]. The results of the histopathological examination were in agreement with what has been reported by previous studies [314]. The tissues of the affected larvae were histologically examined. Intense changes in the anterior intestine were observed. Marked hypertrophy of the intestinal epithelium was evident. The aforementioned injuries are typically attributable to *Vibrio alginolyticus* [65,314]. The infection was subsequently confirmed by microbiological analysis.

Co-posivity to a bacterial pathogen has never been documented in the few cases where RGNNV / SJNNV reassortant betanodavirus outbreak has been reported. Co-infections are

very common in nature and can have a devastating effect, altering the course and severity of different fish diseases. However, the data available on this topic are still scarce. The susceptibility of fish to various pathogens could change during mixed infections, causing sudden fish outbreaks [302]. Interactions between co-occurring pathogens can be synergistic or antagonistic; pathogens can compete with each other for resources within the host, resulting in the suppression of one or the other or both. Alternatively, a pathogen can sometimes weaken the host's immune response against subsequent infections by other pathogens [315,316]. *Vibrio alginolyticus* is commonly considered a stress-related pathogen, constituting a secondary invader, of already damaged tissue or a weak pathogen of stressed fish [55,317]. It is known that larval enteropathy does not have a unique, but multifactorial aetiology, and that the triggering factors are to be found among the biotic and abiotic parameters that regulate breeding. All these factors can alter the intestinal function of the larvae, compromising vitality, appetite, digestive and absorption capacity. The presence of any pathogens, due to poor hygienic-sanitary management, could also represent a further preferential way for the expansion of enteropathic syndrome [313]. Considering that we have not found high traces of pollutants that may have contributed to aggravate the mortality outbreak [55], in our case vibriosis, and consequent enteropathy, was diagnosed as a secondary infection, which may have spread due to a probable drop in the larvae immunity levels caused by nodavirosis. Unfortunately, this hypothesis is not yet supported by data in literature, although it has already been observed how SJNNV is able to alter interferon (IFN) response in gilthead sea bream [318].

Regarding parasitic diseases, our results showed that parasites occur infrequently in reared fish analysed and only *Cryptocaryon irritans* was identified and detected in few fry samples. This result is not surprising if we assume that conditions associated with intensive monoculture of aquatic animals mean that only a limited diversity of disease causing agents can successfully propagate, proliferate and harm aquaculture stock [8].

4.1.1 High deformity occurrence in reared gilthead sea bream

Gilthead sea bream (*Sparus aurata*) occupies the sixth position among aquaculture species produced in the European Union (13% of total production) [2,3]. In the EU market, sea bream is marketed as whole fish (~ 400g) and obviously deformed fish are excluded from the market. Therefore, deformed fish represent a serious economic problem, as they represent a non-marketable product, have a high mortality rate and cause an increase in production costs. In general, in marine hatchery an average of 5-20% of prevalence of skeletal deformities in juvenile products is observed. However, there are

cases in which deformities have also been observed in 45-100% of analysed fry [98]. In the present study, the survey on observed deformities showed that 4.5% of the fry examined were affected by skeletal anomalies. According to the literature, the main deformities observed related to cranial malformations such as pugheadness and deoperculation [319], although their occurrence frequency was lower than that reported by other authors (up to 80%: [103]; 5–20%: [320]). The causes of deformity are not yet clear. Nutritional, environmental or genetic factors have been identified as potential causes of the onset of skeletal anomalies. However, it has been observed that animals with a rapid growth rate are more likely to develop skeletal diseases [321]. In our case, anthropogenic pollution can be excluded as a possible cause of the onset of deformity considering that the analyses of the pollutants in the water samples examined showed values within the ranges provided by the environmental legislation. Thus, the causes may be related to diet, manipulation during the early life stages of fish or genetic causes.

4.2 Fish health monitoring conducted at a Croatian fish farm

From the survey conducted on common carp health status we identified multiple parasites, including protozoans, myxosporeans, monogeneans, cestodes and crustaceans. None of these parasites appeared to pose a significant health threat, since only minimal pathological changes were evident. However, heavy infections of ectoparasites under culture conditions might compromise osmoregulation [322]. Fish were more affected by parasites such as *Ichthyophtyrius multifilis* and *Dactylogyrus* spp. Indeed, parasitic diseases represent the most commonly encountered diseases in carp breeding and in particular diseases caused by parasitic worms such as *Dactylogyrus* spp. represent the most common parasites of fish. However, despite a quite frequent occurrence, it rarely causes intensive infections and massive mortality, according to our results [323].

In contrast, in presence of *Ichthyophtyrius multifilis* heavy mortality is common when the infection is intensive. *I. multifiliis* is the etiological agent that causes white spot disease which represents one of the most pathogenic parasitic diseases of warmwater fish. However, in our case, there were no mortality outbreaks, so it is possible that the transfer of fish stock to cleaner and larger ponds, to stop re-infection, has allowed the fish to develop resistance to this parasite [323].

As previously reported by other authors, CE was the most frequently observed bacterial disease during the survey [323]. CE, caused by atypical *Aeromonas salmonicida*, occurs mainly in cyprinids, evoking epidermal ulcers without septicaemia, causing a generally low

mortality. However, secondary infections may increase the mortality rate. For this reason, prevention and control of the spread of the disease plays a key role. In fishponds, especially in semi-intensive or intensive systems, the prevention of fish diseases is particularly important due to the low or limited exchange of pond water relative to the fish stock density. Given that in many countries, chemical treatments of disease are very expensive or, for both human and environmental reasons, unauthorized, prevention remains the best measure to reduce disease incidence.

In this perspective, rigorous control of the live fish trade can play a fundamental role in preventing the spread of diseases and high mortality, thus preventing the heavy economic losses reported in this study in a pike perch breeding farm, due to mycobacteriosis. In cultured fish, mycobacteriosis is generally associated with a high mortality rate [88,324]. In our case the mortality rate was 6% per month. As reported in previous studies, the host response to mycobacterial infection was granulomatous inflammation. Observed structure of granulomas mainly corresponds to previous descriptions [85,92]. However, granulomas were poorly formed and characterized by the absence of connective tissue capsule attributable to an early stage of granuloma development in accordance with data previously reported by other authors [325,326]. The structure of granulomas can depend on several factors such as maturity [85,326], causative agent, host species, diet and other factors [327,328]. The source of the pathogen is still unknown. However, one cannot exclude the possibility that *M. marinum* was introduced into the affected farm by infected juvenile fish coming from Hungary. In addition, poor diet or poor water quality could represent triggering factors as well as the presence of beluga sturgeon, already reported as a potential asymptomatic vector of mycobacteriosis [329].

4.2.1 Deformity occurrence and skeletal development in common carp

During the survey, the occurrence of deformity in common carp was evaluated only as presence/absence, for the sole purpose of quantifying the number of specimens affected by malformations. In our results, skeletal deformities were determined in 3.01% of 796 examined common carp. The reported percentage of deformed specimens was relatively lower than the results previously reported by Gjurcevic et al. [330] (22.8%) and Al-Harbi [331] (24.9%). These findings could depend on several factors, such as genetics, handling and degree of pollution of the area where the fish farm is located. Indeed, Eissa et al. [332] observed that in less contaminated areas the percentage of individuals with deformities was lower (2.85%). Despite the relatively low frequency, the presence of deformities remains a significant economic problem for fish farms.

Common carp is one of the oldest cultured and most domesticated fish in the world [333], however, ontogenetic processes are not fully elucidated and there is great variability and fragmentation in the data published. According to Osse and van den Boogaart [334] and Mabee et al. [335] development of morphological structures is strongly correlated with functional requirements. This conclusion was in accordance with the results of our study on common carp skeletal development.

At 5 DPH, there is no statistical difference in values of SL between groups A and B. In both groups the existing skeleton is made up of cartilage and there is no sign of ossification. The same was previously described by Itizawa [336] and Pashine and Marathe [337]. From 17 DPH to 26 DPH, values of SL between groups differ statistically. In both groups, ossification begins at 17 DPH on the parasphenoid, basisphenoid, ceratohyal and cleithrum, which could be explained by functional requirements in this life stage [338]. The parasphenoid and basisphenoid form the base of the cranium, give firmness to the skull and act as an attachment site for some masticatory muscles. The ceratohyal has a main role in articulation with branchiostegal rays. The cleithrum extends from the pectoral fin and forms the posterior edge of the gill chamber. At 17 DPH, in group B, ossification was present also on the basioccipital, premaxillary, maxillary and dentary structure as well as on the hyomandibular and opercle, Pashine and Marathe [339] described similar onset of ossification but in much smaller common carp larvae (total length 7.5 mm).

At 22 DPH in both groups, ossification is noticed on articular and quadrate bones. In group A, it was visible on the basioccipital, premaxillary, maxillary, dentary, hyomandibular and opercle. Additionally, in B group ossification started on the supraoccipital. Although the ossification sequence in the head is similar, a delay is visible in group A. At 24 DPH frontal and parietal bones started to ossify in B group while these two bones as well as the supraoccipital in S group showed signs of ossification only at 29 DPH.

On the vertebral column, in both groups the first sign of ossification is noticed on the abdominal vertebrae, at 17 DPH. Ossification started on the outer parts of the first three vertebral bodies and proceeded backwards. Caudal vertebrae started to ossify at 17 DPH in group B, while at 22 DPH in group A. The last three caudal vertebrae and urostyle ossify first and ossification proceeds forward. Concerning time and onset of ossification of vertebrae, the results of the present study do not coincide with those of Itizawa [336]. He reported that the ossification sequences of the vertebral column in the common carp larvae occur much earlier (5 DPH/SL 7.0 mm) and with a dissimilar pattern. The author placed special emphasis

on the first three vertebral bodies that ossify only after the backwards proceeding ossification has reached the 15th vertebra. Furthermore, Itizawa [336] pointed out that the ossification sequence in the caudal region of the vertebral column is complicated, and the whole process is completed at 15 DPH (SL 10.9 mm). On the other hand, Słomiñska and Jezierska [340] recorded end of ossifications on the vertebral column of common carp around 40 DPH, which is 11 days later than observed in this study.

Ossification of fins started at 22 DPH. In both groups, the dorsal fin ossified first. Simultaneously, in group B ossification was noticed also on the caudal fin. In group A, the ossification process on the caudal fin started at 24 DPH. Ossification of anal and pectoral fins in group B was noticed at 24 DPH while in group A it occured at 26 DPH. In both groups, pelvic fins started to ossify at 26 DPH. The same order is described by Itizawa [336], but the start point was much earlier (12 DPH/SL 9.3 mm).

Differences in the ossification process in common carp could be explained with numerous factors, for example genetics, food sources, stocking density and water quality in the pond.

4.3 Microplastic occurrence in reared gilthead seabream and common carp

The widespread presence of microplastics in aquatic environments, both marine and freshwater, has attracted the attention of the scientific community. Microplastics may severely impact biotic and abiotic compartments of aquatic ecosystems. The ingestion of microplastics has been well documented in several fish species and at different life stages [212,213,215], and literature on the subject is constantly increasing. As such, the aquaculture industry may suffer from microplastic pollution, especially as plastic products are widely used for aquaculture. In this study, we evaluated the abundance and characteristics (shape, size and composition) of microplastics in the gastrointestinal tract of two reared fish species: larvae, fry and adults of gilthead sea bream (*Sparus aurata*) and common carp (*Cyprinus carpio*) specimens collected from the Italian and Croatian fish farms, respectively, examined in this thesis. In terms of number of items, both fish species showed lower abundance of microplastics than their wild counterparts [207,341] although gilthead sea bream showed a greater accumulation of plastic debris than carp (39 and 9 items respectively). In both species, no microplastics were found in the larval specimens. This is not surprising considering that larvae, in both farms, are raised inside a hatchery, equipped with filtration systems that probably mitigate the entry of microplastics through the water. However, this finding is in contrast to what has been reported in open water studies, where microplastics have recently been found in the digestive tract of wild fish larvae and juveniles belonging to

commercially important species of the English Channel and the Mediterranean Sea [216,217]. Analysing the results obtained for the gilthead sea bream, a significant difference was instead observed in the number of MPs present in fry (0.21 items/specimen) and in adult specimens (1.3 items/specimens). This difference can be linked to the type of production; while fingerlings are raised in raceways or tanks within the hatchery facility, adults are intensively reared in offshore sea cages. Therefore, adult specimens are more exposed to environment-derived microplastics. Existing data on the ingestion of microplastics by *S. aurata* are still very scarce, however, based on what Guven and collaborators reported [207], the number of MPs found in farmed sea bream is still lower than its wild counterpart (1.53 items/specimen). A low number of MPs was found in common carp specimens (fry: 0.06 items/specimen; adult: 0.25 items/specimens) showing no significant difference between fry and adult. On the basis of the existing literature, the common carp is a species which is not very susceptible to the ingestion of microplastics. Studies carried out in natural waters have shown in many cases low ingestion levels of plastic debris in this species compared to other species examined [341,342]. Unfortunately, there are no comparable data in literature on the load of microplastics in farmed carp. However, the low number of microplastics found could depend on two factors: 1) the location of the fish rearing plant and the level of contamination of the supplied water [342]; 2) the level of plastic contamination present in commercial feed [224]. Evidently, in our case both environment and feed contamination were very low. Regarding plastic size, much (30.7%) of the plastic debris found in gilthead sea bream ranged between 1-2 mm, while 66.6% of the plastics found in common carp specimens were smaller than 1mm. However, assuming that the species could not discern the size of particles for ingestion, such differences may be mediated by biological processes, such as mastication or digestion, which could modify the size of microplastics. According to previous observations [206,213,343] the MPs were found mostly in fibre forms in both species (100% in gilthead sea bream and 56 % in common carp).

Natural/artificial cellulose-based were the predominant polymers, contributing 49%. Semi-synthetic fibres are present in minor content (21%). The remaining polymers (30%) are made-up of synthetic and plastic fibres. The composition of most polymers is the typical one of textiles fibres. Thus, we suggest that the source of this microfiber pollution might be mainly from rivers, sewages, and maritime activities. The presence of a higher percentage of cellulose-based polymers fibres is in accordance with what has been recently observed in a study on the accumulation of microplastics in farmed aquatic species [343]. It is interesting

to note that in the present study polyethylene (PE), one of the most used polymers in aquaculture for ropes and floating rigs [178], was not found in the tested fishes. Polyethylene has a low density, and, rather than sink on the seabed, it tends to float on the water surface, thus being unavailable for the feeding of the studied fish species.

4.4 Evaluation of immunostimulant activity of imoviral in *Sparus aurata*

During the past decade, immunostimulants have received extensive attention revealing potential beneficial effects against various pathogens, enhancing the immune response of fish, minimising the risk associated with the use of chemical agents (including antibiotics) and preventing damage caused by toxic compounds both in fish and human. The use of natural extracts is highly recommended in aquaculture and considered as a safe, environment-friendly alternative approach to immunoprophylactic control [226,229].

In the present study, we fed gilthead sea bream fingerlings with commercial pellet supplemented with complex IMOVIRAL (25mg/10 gr pellet) in a four-week feeding trial and analysed the innate immune responses at the spleen level in uninfected and infected animals with *Vibrio anguillarum*. Imoviral is composed of exclusively natural extracts such as Uncaria (*Uncaria tomentosa*), Shiitake (*Lentinula edodes*), Beta-glucan and Blackcurrant (*Ribes nigrum*), whose immunostimulant properties of the individual extracts have already been demonstrated on teleosts, except for blackcurrant [240,344,345]. To our knowledge, this study represents the first investigation on the acute phase immune response in teleosts after Imoviral administration and on the potential synergistic effect of the natural extracts that compose it. Four genes considered important and representative were selected as target. These genes were chosen based on different criteria. Briefly, the proinflammatory cytokine genes *tnf-α* and *il-1β* were selected due to their importance as inflammation markers [346]. Hepcidin and defensin are antimicrobial peptides mediating the innate immune response showing strong antimicrobial activity against *Vibrio anguillarum* in *Sparus aurata* [347,348]. It has been shown that the administration of plant or fungal extract can alter the transcription of pro- and anti-inflammatory cytokine genes in different organs [349]. *Tnf-α* and *il-1β* are pro-inflammatory cytokines that show a generalized tendency to increase expression levels following the administration of natural immunostimulants such as *Salvia officinalis*, *Lippia citriodora*, fenugreek, microalgae in several fish species including gilthead sea bream [349,350].

Our results showed a modulation of gene expression dependent on different treatments and exposure times. In accordance with other authors, the levels of *tnf-α* were higher in the two

groups fed with Imoviral (IVS and IPS) at T1, T24 and T72, both compared to the control group and compared to the groups fed only on commercial feed and injected with PBS (PPS) [349,351]. It is worth noting that our results showed a differential response in the modulation of *tnf-α* and *il-1β* between the infected groups: IVS (fed with imoviral) and PVS (fed with commercial feed). As can be seen in Figure 30, IVS *tnf-α* levels are higher than in CTRL at T1 and T24; *tnf-α* expression then fell, demonstrating a strong initial response and then a regular expression decrease, probably due to the Imoviral effect. A different response was observed in PVS. In this latter group, *tnf-α* expression showed an increasing trend from T1 to T168. This result is in contrast to what reported by Espinosa et al. [268], where gilthead sea bream specimens, following the administration of *Jasonia glutinosa*, did not report significant changes in *tnf-α* expression.

Expression levels of *il-1β* (Fig.31) were higher in IVS (fed with Imoviral and infected with *V. anguillarum*) at T1 and T24 than in CTRL, while the IPS group (fed with Imoviral and injected with PBS) did not show significant changes compared to CTRL. Once again IVS and PVS showed a different trend in time-dependent expression levels (Tab. 12). IVS showed a moderate but significant increase in *il-1β* at 1 and 24 hpi while it was always up-regulated in PVS, showing a peak of expression at 24 hpi. The *Il-1β* gene down-regulation in the IVS experimental group compared to the PVS group indicates the potential immunostimulant of Imoviral against the vibrio infection. A previous study found that the levels of *il-1β* in specimens of gilthead sea bream fed with *Jasonia glutinosa* increased after 15 days from the start of the feeding trial but decreased after 30 days compared to CTRL [268]; in accordance with this study, we found a moderate expression of *il-1β,* evaluated after 30 days, in the IPS group compared to CTRL. Co-expression of *tnf-α* and *il-1β* was expected, however these cytokines have similar functions in the initiation of immune responses and therefore, maybe, expression was not necessary after treatment with Imoviral.

The fact that IPS showed moderate expression of *tnf-α* and did not show significant changes in *il-1β* expression compared to CTRL suggests that Imoviral does not induce a significant immune response under basal condition, but that it nonetheless provides protection in the case of infection and therefore for IVS. Proinflammatory cytokines are essential for mediating the inflammatory process produced by leukocytes activated in response to pathogenic signals. However, for *tnf* and other cytokines there seems to be a fine line between benefit and harm, a defence agent that is helpful in the local control of injury and infection may be toxic when it is released in large amounts or in the wrong place [352].

Furthermore, although not analysed in this study, it is necessary to take the role of genes associated to the anti-inflammatory response into account, which regulate and reduce the expression of pro-inflammatory cytokines when necessary, preventing collateral damage to host tissues and avoiding waste of bioenergetic resources [353]. In addition, it should also be noted that some of the extracts present in Imoviral have an anti-inflammatory activity, as in the case of *Uncaria tormentosa*, which could act by regulating *tnf-α* expression [353].

For both groups fed with Imoviral, *hep* expression decreased in comparison with CTRL, in particular at T1 and T168, while, in PVS, *hep* was always up-regulated both compared to CTRL and to IVS at 24,72 and 168 hpi (Fig. 32). *Def* levels significantly decreased in IPS compared to CTRL, while no significant difference was observed between IVS and CTRL. Again, the gene was strongly expressed in PVS (Fig. 33). Hepcidin and defensin genes are expressed in a wide range of tissues and exhibit upregulation after bacterial infection, therefore, the high levels of expression recorded in PVS (Tab. 12) are not surprising. The down-regulation of microbial peptide expression levels could depend on prolonged administration of the experimental diet (30 days) as previously observed. Furthermore, the low expression of *hep* could depend on the levels of *il-1β*. It is known that *hepcidin* is greatly stimulated by inflammation, and is principally induced by interleukin-1, interleukin-6, LPS [354], so it is possible that the low expression of *il-1*β influenced the expression of the microbial peptide.

4.5 Zebrafish embryo toxicity test to demonstrate the preferential removal of fungicides from water by molecular imprinting with TiO_2 photocatalysts.

In this study, a zebrafish embryo toxicity test (ZFET) was carried out to demonstrate the preferential removal of fungicides from water by molecular imprinting with TiO_2 photocatalysts. There are several studies in literature that analyse the performance of TiO_2 nanoparticles coated or supported with a layer of molecularly imprinted polymers that exhibited specific affinity toward various pollutants [355,356]. However, this approach still has some drawbacks which could be overcome with the development of materials with a molecularly imprinted inorganic framework as we propose in this research, a procedure which is simple and environmental-friendly. In particular, we synthesized, through the sol-gel technique, molecularly imprinted TiO_2 photocatalysts together with the OPP fungicide molecules. This is a new method that avoids the multistep, solvent-consuming procedures, typical of molecular imprinting [291]. Furthermore, the eventual toxic effects of the molecularly imprinted materials were evaluated by the ZETT, an alternative approach to

acute toxicity testing, to decrease the impact of experimental tests on live animals [280,299,357]. As biomarkers of exposure, we analysed MTs1 and HSP 70. The use of these biomarkers is supported by several studies. Some Authors showed that MTs1 proteins, a family of low molecular weight, could be a potential biomarker for metal nanoparticles contamination in aquatic environments [358–360] and some studies demonstrated a gradual decrease in the level of MT transcripts during the early embryonic stages [361]. In our study, MTs1 expression shows a high susceptibility to diverse imprinted TiO_2 and bare TiO_2, highlighting the importance of MTs1 with biomarkers for nanotoxicology. Also HSP70, molecular chaperones that play central roles in many cellular processes [362,363], are considered as an indicator of stress in cells after contact with nanoparticles [364]. However, tests carried out by exposing the larvae to the TiO_2 nanoparticles imprinted together with the OPP fungicide (1:1) did not give satisfactory results; all the larvae were already dead after one hour of exposure. Probably the concentrations of fungicide used were higher than the absorption capacity of the imprinted nanoparticles. In future, however, this technique could have great potential as eco-friendly materials to be used for water purification.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, infectious diseases represented the major problem detected in the two fish farms under study. In particular, results show that Viral Encephalopathy and Retinopathy, caused by Betanodavirus can be found in association with Enteropatic Larval Syndrome, primarily caused by pathogenic bacteria such as *Vibrio* spp., and that such infectious agents seem to synergistically exacerbate the severity of both diseases in larval gilthead sea bream, possibly causing severe distended gut syndrome. Future studies will be necessary to broaden knowledge on the factors that control the risk of co-infection, the circumstances in which pathogens can interact and the mechanisms behind these interactions. This is of fundamental importance for the development of suitable, integrated programs for the management of infectious diseases. Disease monitoring should be an essential part of any biosecurity program, accompanied by regularly scheduled health assessments of the entire stock in an aquaculture facility. Depending on the situation, this can include lethal or non-lethal sampling or both. Although none of these tests can guarantee that there are no potential pathogens in a fish population, they can help reduce the risk of maintaining a pathogen in a population. Periodic monitoring can also help determine the number of individuals within an infected population and the level or intensity of infection within that population. The main objective of an aquaculture biosafety program is to first prevent the introduction of any infectious organisms into an aquaculture facility. There are several potential sources of entry of an infectious agent into an aquaculture facility. These include adding new stocks; contaminated water or feed; asymptomatic carriers, humans, animals or equipment. Each of these potential sources must be evaluated and constantly monitored to prevent the entry of infectious organisms into the facility. Some of these potential sources may indeed explain the case of mycobacteriosis in pike perch. To our knowledge, this is the first case of mycobacteriosis reported in farmed pikeperch. Rigorous control strategies should include good management practices and avoid the introduction of pathogens into a recirculation system. Therefore, screening of broodstock and juvenile fish at supplier hatchery/farm is essential for any type of production system. Mycobacteriosis deserves special attention due to its zoonotic potential [365,366]. There is no effective treatment for fish mycobacteriosis [365,367] so, depopulation and disinfection of farms was the only solution available. In contrast, the carp sampled in the present study, despite harbouring a number of parasites, were in good condition. Consequently, there are no immediate concerns for this species, although continued monitoring is essential.

It is also clear that the problem of deformities is still unsolved and the economic losses they cause in aquaculture production are still significant for all fish species investigated. For this reason, the development of mitigation strategies to reduce the incidence and prevalence of these lesions is clearly necessary, as well as more in-depth studies on the potential causes that generate the onset of skeletal deformities.

Skeletal differentiation represents a fundamental event in larval ontogenesis. Bones and cartilage provide support for the body, protection for major organs, a storehouse for calcium and phosphorus, and provide a means of attachment for ligaments and muscles. As skeletal anomalies in reared juveniles are almost exclusively found in the trunk, fins and splanchnocranium, study of the development of these skeletal elements could represent a useful tool for early identification of possible anomalies during larval development. In our study, the onset of ossification was similar in both studied common carp groups but not completely identical. Inconsistency with data from literature was also found. Furthermore, we confirmed that in common carp the sequence of ossification was not determined by age (DPH). Significantly, larger, fast-growing specimens ossify earlier. Trade-off between fast growth and onset of ossification was not confirmed in our study.

It is therefore necessary to improve health surveillance methods, to guarantee animal welfare, good production and reduce economic losses. Expanding molecular and advanced genetic tools and new knowledge on diseases and disorders of aquatic organism used for both research and human consumption may help to improve health monitoring procedures. This may, in turn, contribute to a better understanding of the pathogenic mechanisms of aetiological agents. Better knowledge of disease transmission may allow for the development of efficient, synergistic preventive methods. Moreover, identification of new molecules of natural origin with antibacterial, anti-inflammatory and immunostimulant activity, to replace synthetic molecules, will hopefully encourage aquaculture progress and safety.

To improve fish diseases prevention and resistance, our finding suggests that a diet supplemented with Imoviral complex might provide enough immunostimulatory support in fish allowing them to have a faster response when challenged with a bacterial pathogen, highlighting the modulatory role of these functional feeds in the acute phase immune response. Its efficacy is further demonstrated by the high, increasing levels of expression of the target genes in the PVS group, lacking the adjuvant activity of Imoviral against the pathogen. Data provided supports the possible use of Imoviral as an interesting immunostimulant for farmed gilthead sea bream and as a valid alternative to prophylactic and

therapeutic plans based on vaccines and antibiotics. This study opens up the possibility for further studies: analysis of more genes related to immune response, different Imoviral doses, feeding times or administration to other species, as well as the possible protective effects against other fish diseases should be examined.

Poor water quality is a detrimental factor for any aquatic species and pollutants represent a possible indirect cause of economic loss in the aquaculture industry. Therefore, management of culture water represents one of the fundamental factors for the success of aquaculture production. A variety of different functional nanomaterials and nanocomposites can be used for water remediation, provided that they are not toxic for the environment and aquatic organisms. Zebrafish has routinely been used as a model to evaluate the phototoxic effects of TiO_2NPs and over the last 10 years it has played an important role in nanotoxicology research [368]. According to literature data, TiO_2 nanoparticles have proved their outstanding role in the treatment of pollutants in aqueous systems, in addiction the selective photodegradation of TiO_2 is enhanced by molecular imprinting process as reported by Fiorenza et al. [291]. The scarcity of water and its pollution is the most important problem of the modern society. One of the most critical industrial activities for the quality of aquatic ecosystems is the extensive use of agricultural products, such as herbicides, pesticides or fungicides [177]. In consideration of the exceptional result of imprinted NPs-TiO_2 applications, we have described a rapid approach to demonstrate that the imprinting technique could have great potential as an eco-friendly technique to be used for water purification thanks to our preliminary results. We did not observe high toxicity for the different types of imprinted NPs-TiO_2 and the expression of biomarkers highlighted the capacity of embryo zebrafish to respond to contaminants such as nanoparticles. Although further studies will be needed, this technique could have great potential as eco-friendly materials to be used for water purification in aquaculture.

Among pollutants, microplastics (MPs) represent a new emergent class of contaminant and seafood appears to be the most understood source of microplastics to humans. Our study represents the first evidence for microplastics detection in cultured fish samples. From the results obtained, it has been highlighted that the presence of microplastics in the gastrointestinal tract of cultured *S. aurata* and *C. carpio* was lower than in wild specimens. These results represent an important baseline in assessing cultured species food safety in term of microplastic ingestion demonstrating that fish farming could help in the reduction of human consumptions of MP contaminated fish. However, further study on nanoplastics are

essential, as there is concern that nanoplastics may have a high biological impact in both fish and humans [2].

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7. APPENDIX I. Ion concentration of water samples from the sea and borehole that supply water to Italian fish farm, expressed in mg / L.

	Chloride mg/L	**Bromide** mg/L	**Nitrate** mg/L	**Sulphate** mg/L	**Sodium** mg/L	**Potassium** mg/L	**Calcium** mg/L	**Magnesium** mg/L
Sea water	20321	42.6	17.3	3133	14538	455	487	1641
1	20654	41.8	<10	3003	14253	389	494	1508
1B	2176	46.7	< 10	2046	12331	450	480	1580
2	2033	50. 1	<10	2253	12883	460	433	1588
2B	22038	40.7	<10	2994	12994	467	503	1394
3	20004	50.7	<10	3051	13861	420	435	1598
3B	19813	47.8	11.1	2978	14322	442	399	1636
4	22600	45.7	19.1	3200	12896	543	500	1599
4B	22345	46.8	21.7	3155	13532	501	488	1477
5	18332	52.7	11.3	2834	14564	449	484	1495
5B	1931	43.8	15.5	3311	15432	502	493	1574
6	19453	44.1	12.4	3334	15621	530	500	1403
6B	20053	46.3	<10	3094	14861	540	520	1394
7	1893	51	12.4	2733	12964	490	480	1466
7B	18004	40.0	<10	3200	13053	480	<421	1602
8	18811	42.3	<10	3110	14221	466	465	1500
8B	18746	39.7	<10	3288	13633	476	398	1584
9	18220	50.3	<10	2993	15872	461	455	1600
10	18592	49.5	<10	2944	13777	492	488	1531
11	17562	47.5	<10	3244	12877	462	498	1481
12	18456	45.8	13.7	3003	13761	481	500	1487
Cianchino	18000	49.7	<10	3000	14000	471	485	1522

8. APPENDIX II. Metal concentration of water samples from the sea and borehole that supply water to Italian fish farm, expressed in mg / L, the concentration of Hg expressed in µg / L.

	B	P	Mn	Fe	Cu	Zn	Cr	Co	Ni	As	Cd	Pb	Hg
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	µg/L
Sea water	5.61	0.07	0.03	0.29	< 0.022	0.91	<0.010	< 0.006	0.05	0.02	<0.008	< 0.010	0.134
1	4.81	0.31	0.01	0.17	< 0.022	0.94	<0.010	< 0.006	0.04	0.01	< 0.008	<0.010	0.09
1B	5.22	0.45	< 0.018	0.22	< 0.022	0.78	<0.010	< 0.006	0.05	0.02	< 0.008	<0.010	0.07
2	4.94	0.8	< 0.018	0.26	< 0.022	0.94	<0.010	< 0.006	0.02	0.01	< 0.008	<0.010	0.02
2B	4.88	0.55	< 0.018	0.2	< 0.022	0.91	<0.010	< 0.006	0.03	0.01	< 0.008	<0.010	0.04
3	6.3	0.34	< 0.018	0.23	< 0.022	0.85	<0.010	< 0.006	0.04	0.01	< 0.008	<0.010	0.03
3B	5.2	0.31	< 0.018	0.18	< 0.022	0.81	<0.010	< 0.006	0.07	0.01	< 0.008	<0.010	0.04
4	4.78	0.72	< 0.018	0.25	< 0.022	0.86	<0.010	< 0.006	0.02	0.04	< 0.008	<0.010	0.03
4B	4.5	0.62	< 0.018	0.29	< 0.022	0.79	<0.010	< 0.006	0.03	0.01	< 0.008	<0.010	0.02
5	4.77	0.33	< 0.018	0.25	< 0.022	0.88	<0.010	< 0.006	0.01	0.01	< 0.008	<0.010	0.03
5B	5.01	0.42	< 0.018	0.19	< 0.022	0.93	<0.010	< 0.006	0.05	0.02	< 0.008	<0.010	0.03
6	4.94	0.2	< 0.018	0.26	< 0.022	0.71	<0.010	< 0.006	0.08	0.02	< 0.008	<0.010	0.06
6B	5	0.44	< 0.018	0.2	< 0.022	0.94	<0.010	< 0.006	0.04	0.03	< 0.008	<0.010	0.01
7	5.01	0.1	< 0.018	0.17	< 0.022	0.9	<0.010	< 0.006	0.04	0.03	< 0.008	<0.010	0.02
7B	4.76	0.41	< 0.018	0.21	< 0.022	0.77	<0.010	< 0.006	0.04	<0.04	< 0.008	<0.010	0.01
8	5.05	0.52	< 0.018	0.2	< 0.022	0.8	<0.010	< 0.006	0.04	0.05	< 0.008	<0.010	0.04
8B	5.32	0.12	< 0.018	0.22	< 0.022	0.82	<0.010	< 0.006	0.06	0.01	< 0.008	<0.010	0.05
9	5.3	0.43	< 0.018	0.25	< 0.022	0.7	<0.010	< 0.006	0.01	0.03	< 0.008	<0.010	0.02
10	4.88	0.3	< 0.018	0.28	< 0.022	0.8	<0.010	< 0.006	0.01	0.03	< 0.008	<0.010	0.06
11	5.2	0.65	< 0.018	0.21	< 0.022	0.86	<0.010	< 0.006	0.05	0.03	< 0.008	<0.010	0.01
12	5.66	0.5	< 0.018	0.16	< 0.022	0.89	<0.010	< 0.006	0.02	0.05	<0.008	<0.010	0.05
Cianchino	5.78	0.64	< 0.018	0.27	< 0.022	0.91	<0.010	< 0.006	0.03	0.01	< 0.008	<0.010	0.01

9. APPENDIX III. Polychlorobiphenyl (PCB) concentrations in water samples from the sea and borehole that supply water to Italian fish farm, expressed in µg / L.

	28	52	77	81	101	118	126	128	138	153	156	169	180	Σ PCB
	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Sea water	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 1B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 2B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 3 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 4 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 5 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 6 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 7 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 8 B	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 9	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 11	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Well 12	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
Cianchino well	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
														200

10. APPENDIX IV. Polycyclic aromatic hydrocarbon (PAH) concentrations in water samples from the sea and borehole that supply water to Italian fish farm, expressed in µg / L.

	Sea water	1	1B	2	2B	3	3B	4	4B	5	5B	6	6B	7	7B	8	8 B	9	10	11	12	Cianchino	Unit of measure	Reference values
Acenaphthyne	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	-
Anthracene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	0.1
Benz (a) anthracene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	-
Benzo (g, h, i) perylene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	0.004
Benzo (a) pyrene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	0.004
Benzo (b) fluoranthene	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	µg/L	0.004
Benzo (k) fluoranthene	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	µg/L	0.004
chrysene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	-
Dibenzo (a, h) anthracene	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	µg/L	-
fluoranthene	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	µg/L	0.1
Indeno (1,2,3-cd) pyrene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	0.004
Naphthalene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	-
phenanthrene	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	µg/L	-
Pyrene	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	-

11. APPENDIX V. Organochlorine pesticide concentrations in water samples from the sea and from borehole that supply water to Italian fish farm, expressed in µg / L.

	Sea water	1	1B	2	2B	3	3B	4	4B	5	5B	6	6B	7	7 B	8	8B	9	10	11	12	Cianchino	Unit of measure	Reference values
Aldrin	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	µg/L	0.1
Dieldrin	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	µg/L	0.1
DDD isomers	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
DDE isomers	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
DDT isomers	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
Eldrin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
HBC	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
α-HCH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
β-HCH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
γ-HCH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
heptachlor	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
Heptachloro epoxy	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
methoxychlor	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
mirex	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
oxychlordane	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
cis-chlordane	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
trans-chlordane	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
trans-nonachlor	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1
cis- nonachlor	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	µg/L	0.1

12. LIST OF ORIGINAL PAPERS

Gjurčević, E., Kužir, S., Žmak, L., Obrovac, M., Gudan Kurilj, A., Savoca, S., ... & Matanović, K. A case of mycobacteriosis in farmed pikeperch (*Sander lucioperca*) cultured in a recirculating aquaculture system. Aquaculture Research**. (**In Press).

Snježana Kužir, Krešimir Drašner, Krešimir Matanović, Lucija Bastiančić, Ivan Vlahek, Serena Savoca, Emil Gjurčević. Trade-off between fast growth and ossification process in common carp (*Cyprinus carpio*). Veterinarski Arhiv. (Accepted).