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1. Introduction

1.1 Definitions: prophylaxis, disease prevention and biosecurity

Prophylaxis can be defined as all the actions taken to prevent disease and therefore can be considered as a synonym of **disease prevention**. **Biosecurity** is also related to disease prevention but mainly consists of actions that minimize the risk of introduction and spreading of an infectious disease in a particular place and also spreading to other places. In aquaculture, therefore, **biosecurity** should be mainly related to those practices aiming at reducing the risk of specific infectious and parasitic diseases, whereas **disease prevention** and **prophylaxis** should be viewed with a wider scope, including measures and actions to reduce the susceptibility to infectious diseases and also non-infectious diseases and factors such as stress or environmental conditions, that are closely related to the emergence and expression of the diseases.

Prophylaxis, disease prevention and biosecurity should also be clearly differentiated from disease treatments and disease remediation, although in cases, certain measures and tools are used to prevent and also to treat. Treating European sea bass and gilthead seabream diseases has been already addressed in D3.3 of PerformFISH, i.e. Deliverable 3.3 "*Best Therapeutics Practices for Mediterranean Farmed Fish*".

1.2 Disease prevention in aquaculture

Disease prevention in aquaculture has been extensively addressed in specific literature. Amongst the general reviews and guidelines, the following publications make a comprehensive list:

- Fish Diseases: Prevention and Control Strategies. Edited by Jeney G., 2017, Academic Press.
- Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P., eds. 2012. FAO Fisheries and Aquaculture Technical Paper. No. 547. Rome, FAO. 207 pp.
- Aquaculture biosecurity: prevention, control, and eradication of aquatic animal diseases. Edited by A. David Scarfe, Cheng-Sheng Lee & Patricia J. O'Bryen. 2006. Blackwell Publishing
- Yanong, R.P.E g and Erlacher-Reid, C. (2012). Biosecurity in Aquaculture, Part 1: An Overview. University of Florida. SRAC Publication No. 4707.
- Yanong, R.P.E. (2012) . Biosecurity in Aquaculture, Part 2: Recirculating Aquaculture Systems. USDA- Southern Regional Aquaculture Center. SRAC Publication 4708.
- Yanong, R.P.E. (2013) . Biosecurity in Aquaculture, Part 3: Ponds. USDA-Southern Regional Aquaculture Center. SRAC publication 4712.
- Austin B. and Newaj-Fyzul A. (2017). Diagnosis and Control of Diseases of Fish and Shellfish. John Wiley & Sons Ltd. Chichester, West Sussex, UK, 329 pp.

In these reports, the main different aspects related with disease prevention are presented and briefly or extensively discussed. However, the different facets of aquaculture, including many

different orders of animals (mainly fish, mollusks and crustaceans), different species, different farming systems (extensive, semi-extensive, intensive, land based and water-based systems, flow-through, recirculation and integrated farming, amongst others) and different technological levels, draw a complex landscape that often renders these approaches too generalist and asks for more focused and detailed guidelines for each system.

1.3 Specific disease prevention strategies for Mediterranean aquaculture: the PerformFISH approach

1.3.1. Diseases and health management

WP3 of PerformFISH is devoted to health and welfare in gilthead seabream and European seabass farming. PerformFISH is exploring the most relevant factors related to fish health to provide efficient tools for improving health-related KPIs; prevention is one of the most relevant aspects included in this approach. This deliverable has been, therefore, designed and oriented to fulfill these needs and requirement. In this deliverable the different recommended strategies are mainly designed and adapted to the current production systems of gilthead seabream and European seabass farming in the EU Mediterranean countries. These recommended strategies have also considered the future trends in aquaculture development, with relevant changes in the design and management systems bearing also clear implications in health and welfare issues.

As complementary information to this document, it is strongly recommended to consult the other deliverables of WP3, as all of them have been designed in a complementary way. Concerning the aspects related to biosafety, characterization of the most relevant aspects concerning fish health management of the main European seabass and gilthead seabream production systems in the EU Mediterranean farming have been described in the Deliverable 3.2 "*Epidemiological status of Mediterranean farmed fish – Diseases in European sea bass and gilthead sea bream in European and Mediterranean farming: a comprehensive approach for a more efficient policy making*".

In addition to this deliverable, a specific exercise on identification and assessment of the relevance of the main risks associated to the most relevant diseases has also been developed in PerformFISH (Task 3.1). Part of the results of this exercise are included in this deliverable, as most of the prophylaxis recommendations are directly linked to the risks identified and how to minimise them.

Task 3.3 aiming at developing new vaccines and vaccination schemes is also closely related to disease prevention. Immunoprophylaxis (as vaccination) will be briefly covered in this document, mainly as one of the few preventive measures available in on-growing stages, yet a more extensive presentation of achievements in this topic is planned in Deliverable 3.5.

Last, but not least, it is very important to take into account that fish resistance and resilience to the diseases is clearly related to a series of additional factors, which, amongst others, are:

-Genetic and epigenetic background

-Nutrition and feeding strategies

-Water quality

-Fish management and welfare.

Most of these additional factors are also considered in WP1 (Genetic background), WP2 (Fry quality and epigenetics), and WP4 (nutrition). The present document highlights only general elements that can be relevant for prophylaxis, while detailed information is also delivered in the corresponding deliverables of these work packages.

1.3.2. Genetic background

It is well known that specific strains or lineages can be more resistant and more resilient to specific diseases. In European seabass and gilthead seabream, only limited information linked to resistance mainly to *Photobacterium damselae* subspecies *piscicida* is available Antonello et al, 2009, Massaut et al. 2011, Aslam et al, 2018). In PerformFISH, two specific subtasks in WP1 focus on disease resistance:

Task 1.3.1: The optimised, low-cost SNP set (Task 1.1) will be used first to reconstruct -family relationships and to estimate genetic parameters (heritabilities and genetic correlations) for resistance to VNN in larval and juvenile sea bream and response to nodavirus vaccination in juvenile.

Task 1.3.3: Genomic selection of European sea bass for one of the two parasitic disease challenges

The achievements of these substasks will be presented in:

D1.3 Report for EBVs for vibriosis and parasites infections

D1.6 Estimated genetic parameters and Low-density genome scan for SNPs associated with VNN resistance in sea bream

1.3.3. Larvae and juvenile management

The risk of disease during larval, postlarval and juvenile rearing is very high due to the particular physiological characteristics of those stages that make them particularly sensitive to changes of the environment or diseases. For this reason, some the most relevant prophylactic practices and mainly those related to biosecurity should be implemented in hatcheries and nurseries. Also, strengthening juvenile resilience is key to the improvement of the resilience of fish in more challenging environments. These and other aspects are mainly researched in WP2.

1.3.4. Health-Nutrition approach

Fish nutrition has relevant impacts on factors such as fish growth, farming economics, environmental and social sustainability and human nutrition but also on the fish performance against diseases and fish resilience in farming conditions. Indeed, the management of several dietary components has been found to substantially seal the defense system of European seabass and gilthead seabream by altering specific immune parameters (check Aquamax: https://cordis.europa.eu/project/id/16249/it; Arraina: http://www.arraina.eu/).

In WP4, novel feeds based in an improved knowledge of the gilthead sea bream and European sea bass requirements and also improved knowledge of the characteristics of the available raw materials / feeds are developed. This main objective is connected with a specific subtask

scheduled in 3.4.4, related to bioactive substances from Marine Natural Products) also with the objective to improve fish welfare and health.

1.3.5. Fish Welfare

European seabass and gilthead seabream welfare is also addressed in PerformFISH Task 3.5. This task mainly outlines practical methodologies for fish welfare assessment at farm level through the identification of Operational Welfare Indicators (OWIs). The results of this task will provide a valuable tool to allow the implementation of the most appropriate measures to improve the welfare level in the farm and this improvement will also have a direct effect on the capacity of fish to cope with the potential hazards related to diseases.

1.4. Mediterranean fish farming: production cycle and specific phases

As in many other aquaculture production systems, the Mediterranean marine fish production cycle is divided in distinct phases mainly related to the biological characteristics and requirements of the fish at different stages as follows:

-Broodstock

-Hatchery

-Nursery

-Pre-ongrowing

-Ongrowing.

In particular Mediterranean production areas (West Mediterranean, main production in offshore cages), pre-ongrowing tends to be clearly differentiated from the ongrowing phase. In this particular case, ongrowing farms require fry/juveniles of higher weight (10-30 g). Bigger fish are more adapted and resilient to the management in cages (feeding, basic husbandry), nets can have a higher mesh size and the total ongrowing cycle can be reduced. On the other side, large batches are required, which combined with the higher weight, usually puts extra pressure on the nurseries as exceeds their capacity.

Another specific characteristic of the European sea bass and gilthead sea bream production is the diversity in ongrowing systems used. Sea cages is the main system used for these two species. Cage farming can be operated in sheltered and calmed areas (mainly in Greece or Croatia) or also in more rough offshore conditions (Spain). Land-based intensive or semi-intensive ongrowing facilities based in tanks or ponds are also relevant in particular geographical areas (Italy, France), along with semi-intensive and extensive rearing pond and lagoons in Italy, Spain and Portugal. Each system has different characteristics and requirements.

Each one of these phases is developed in facilities designed for the purpose and practices are adapted to the characteristics and requirements of the life stage and age of the gilthead seabream and European seabass reared there. For this reason, the focus on prophylactic programs is different for each phase. In the following sections, the specific characteristics and requirements for prophylaxis are described in detail.

1.4.1 Production cycle stages: from broodstock to ongrowing

Different stages have different needs and for this reason are reared in separate facilities. This is also an opportunity for tailor-made prophylaxis and biosecurity protocols.

Each production stage (broodstock, hatchery, nursery, pre-ongrowing and ongrowing) has particular technical specifications, operational procedures and requirements and amongst these requirements, biosecurity and prophylaxis are some of the most relevant and will be described in detail hereunder. However, it is very important to highlight how relevant is for biosecurity, the fragmentation of the production cycle. This fragmentation can be regarded as a problem from the logistic or operational point of view but the fact that each stage operates independently and physically isolated from each other, with separate staff and supplies is considered as a positive strategy that reinforces biosecurity processes. However, gilthead seabream and European seabass production phases should not be considered as self-contained structures but as a linear process. If a pathogen or a disease emerge in the production system, these pathogens can be easily transmitted and dispersed throughout the entire production system if measures are not taken. For this reason, it is absolutely necessary to design and incorporate in the system, the adequate structures and measures to avoid or minimise the risk of pathogen expression in the system and the risk of spreading outside the system. One of the most efficient systems is the creation of “firewalls” that can allow the containment of the disease and pathogen and limit the propagation to other parts of the system. Physical isolation (separate buildings), distinct operational procedures (independent operations, supplies and staff) and a careful evaluation of the biological material (eggs, larvae, fry, juveniles) before to be transferred to the next stage are the basis of these “firewalls” concepts and will be detailed later on. It is important to stress the fact that in some cases, this concept of physical and operational separation of the different phases is not assumed or only partially in the general operational production structures in the Mediterranean. Broodstocks in some cases are maintained in the same building as larvae, with only some limited physical or operational isolation. In many of these cases, the isolation is limited simply to a wall, but with constant, non-restricted movements between the two areas of materials and staff through doors or entrances. In some of these facilities, footbaths are placed in these paths as a biosecurity measure, but in practice, the effectiveness of this measure is frequently questionable as the footbaths are not large, long and deep enough to ensure that staff stay in the disinfecting solution with both footwears immersed for the right time. In most of the cases and mainly if the path is frequently crossed, people tends to cross the footbaths very fast. Another frequent problem is the poor or absent maintenance of the footbaths: low disinfectant doses, lack of cleaning and substitution of the disinfection solution, lack of traceability of these operations or even dry footbaths are still frequently found bad practices in some farms. To avoid these problems, it is highly recommended to keep broodstock in a separate and isolated building, with specific entrance and paths, with designated staff or at least, specific and differentiated workwear, specific hygienic measures (cleaning and disinfection before and after the operations). If emergency exits should be placed in the building for security reasons, door/gate opening alarms should be placed to discourage any misuse of these paths. As eggs are the only biological material that can be transferred from broodstock area to hatchery, transfer can be done simply through a window (if buildings are side by side) or transferred in appropriate cleaned and disinfected containers. Egg disinfection should be performed in the broodstock building, after collection and cleaning and egg quality evaluation and before transfer to hatchery.

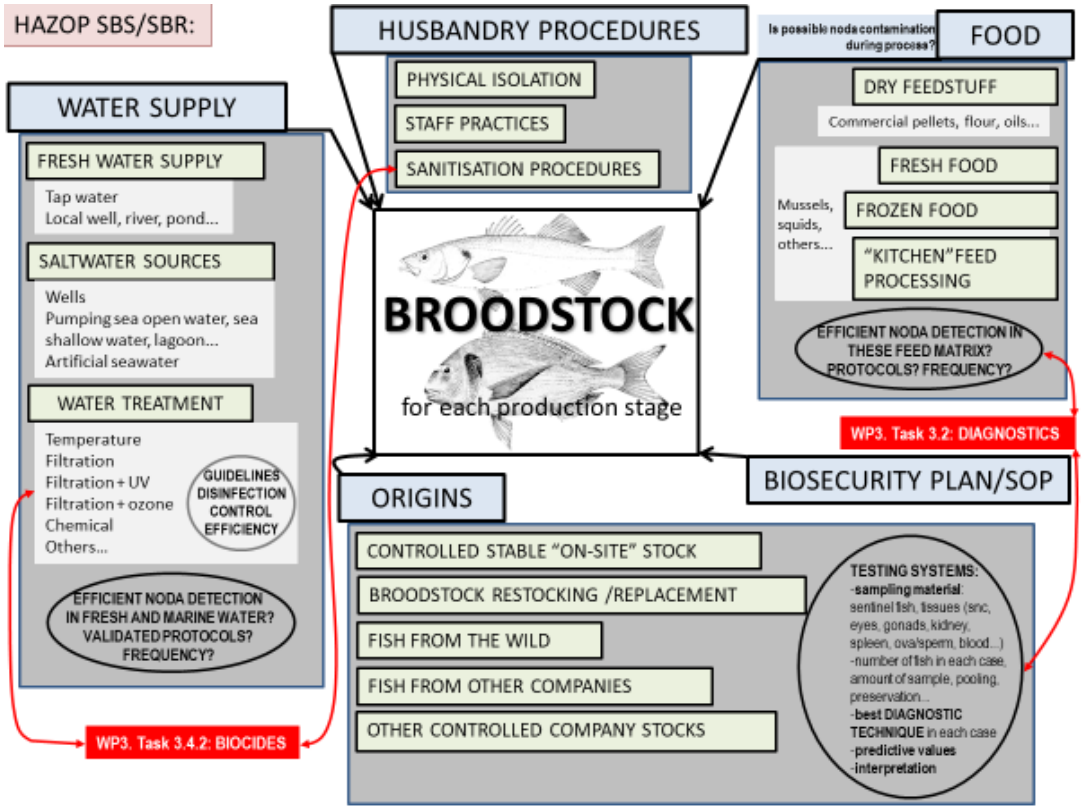
Similar approaches can be applied to hatchery/nursery or nursery/preongrowing transition although in these situations, biosecurity measures are not so strict as for broodstock and hatchery stages.

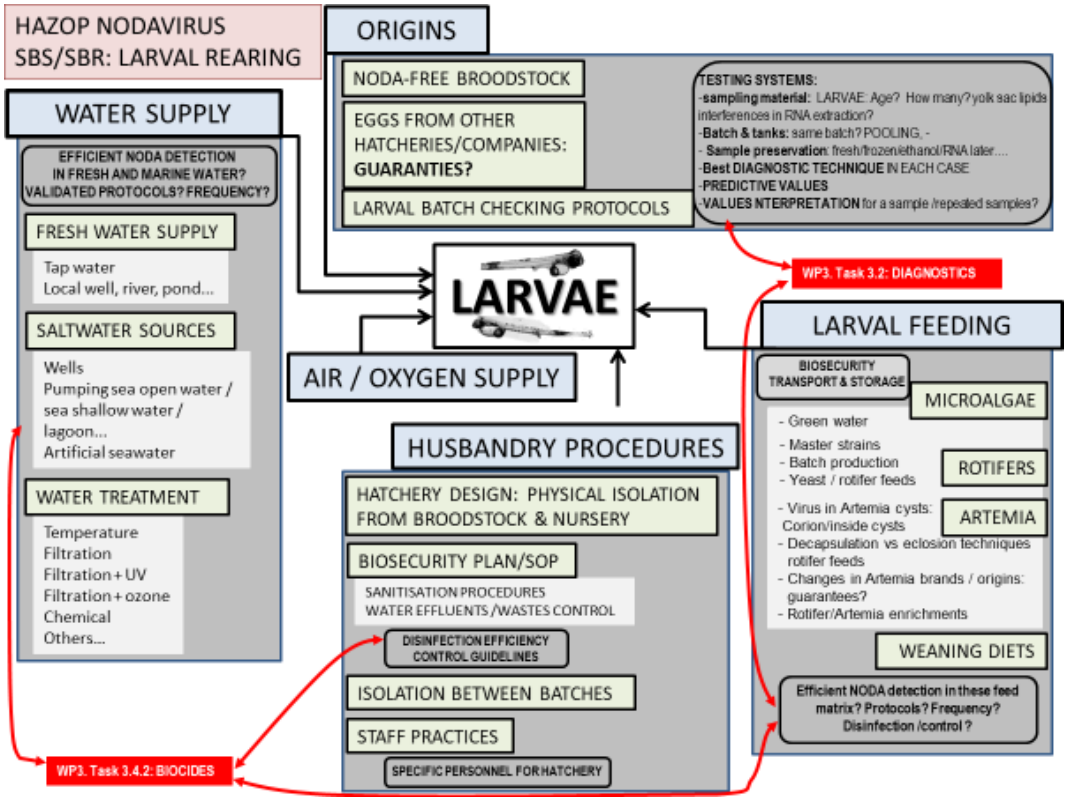
It is also important to notice that in some cases, in Mediterranean gilthead seabream and European seabass production, some fish farming companies operate under integrated rearing strategies, meaning that the same company has the different stages of the production cycle. In these cases, these companies tend to have harmonised prevention and biosecurity protocols, taking into account the specific characteristics and requirements of their own system. This usually allows a better control and traceability of these measures. However, in other cases, there are specific companies specialized in a part of the cycle and they act as egg, larvae, fry or juvenile suppliers for other companies. Also, in some cases and due to unexpected problems, companies operating under integrated strategies, occasionally also require supplies from external companies. In those cases, supplier and customer may have different prevention and biosecurity strategies and this can be also a potential source of problems. It is highly recommended to have a mutual evaluation of their strategies in order to harmonise the biosecurity levels.

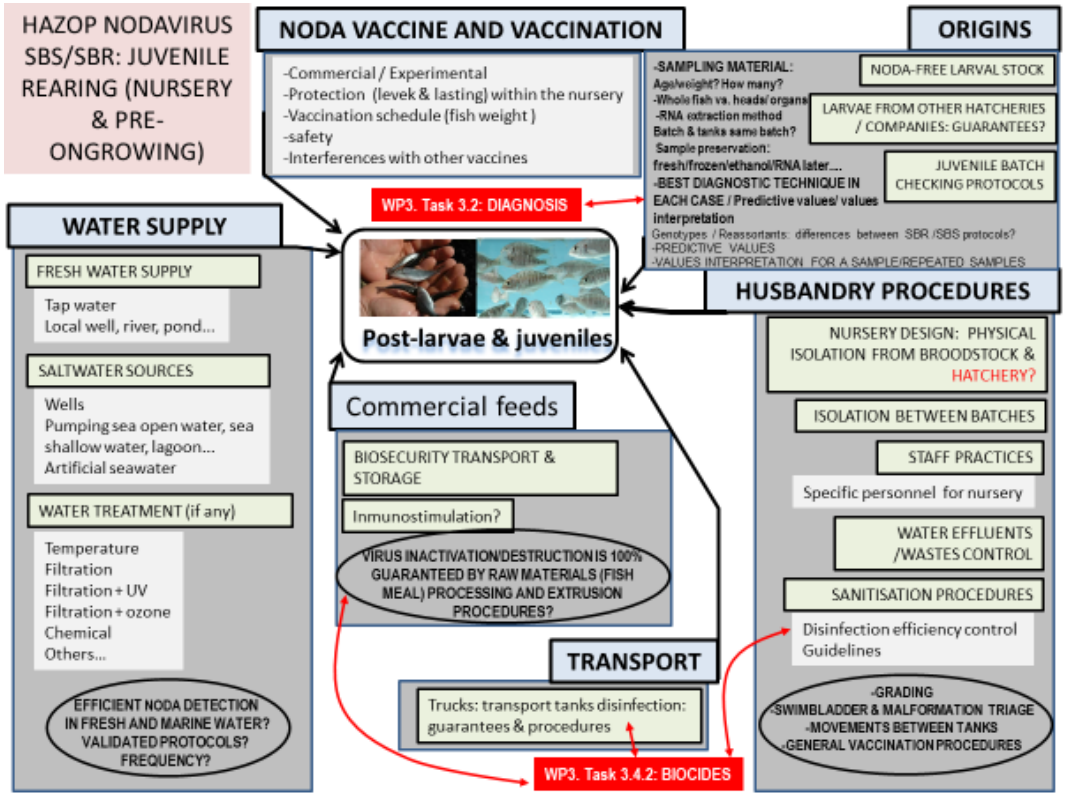
1.4.2 Disease risks

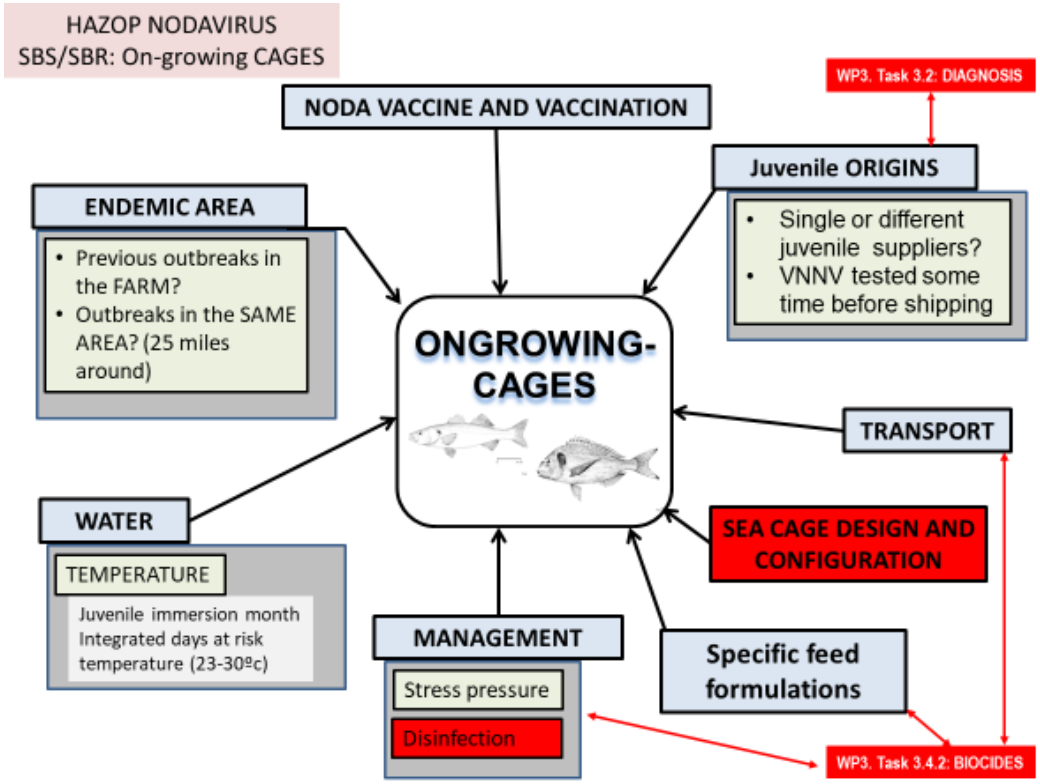
An exercise to identify the risks concerning the main diseases affecting gilthead seabream and European seabass, has been developed as part of task 3.1 (Epidemiology and Health Risk Assessment). A specialist working team formed by scientists identified the main risks associated to each production phase using a HAZOP (Hazard and Operability Study) evaluation system, combined with the information available from technical and scientific literature, as well from their personal experience. The results of this primary assessment for each production stage are indicated below. From this primary internal assessment, a more complete and detailed assessment on the relevance of each risk factor with experts on this field using DELPHI techniques is under development and results will be presented in the future. Results of the main risks for each relevant disease and each production stage are summarised in the graphs below.

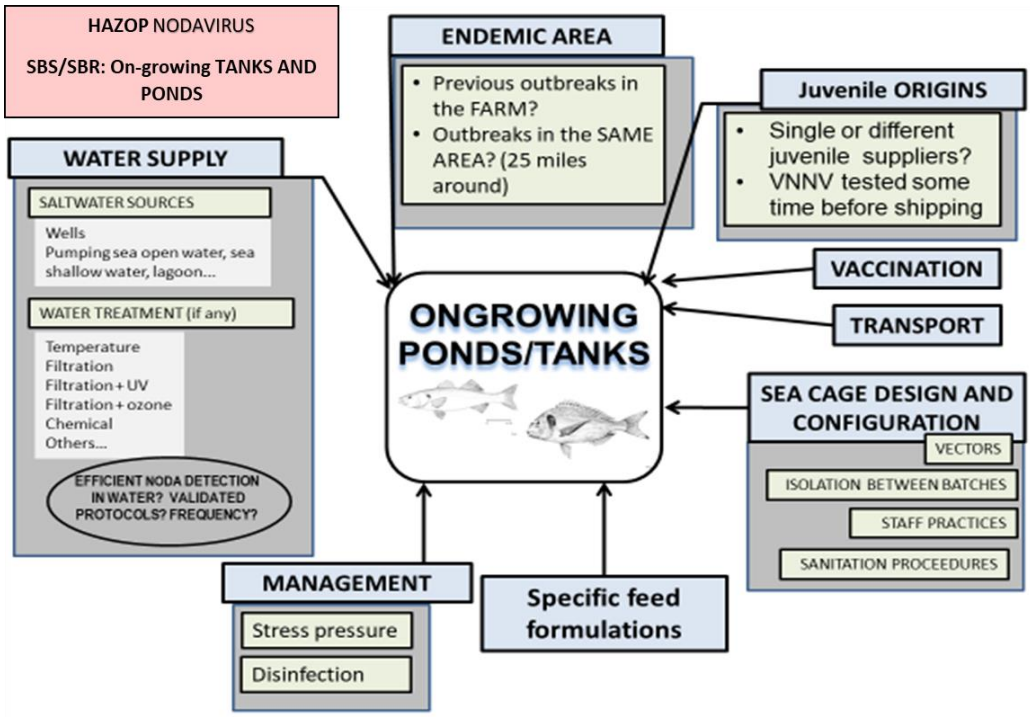
VIRAL NERVOUS NECROSIS / BETANODAVIRUS INFECTION



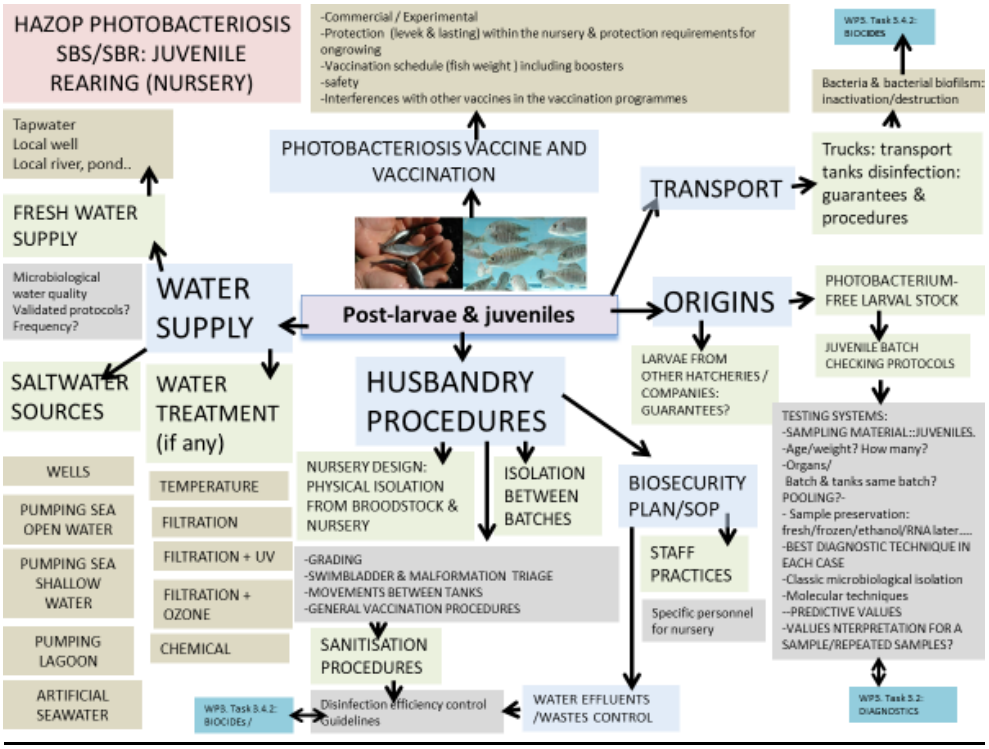


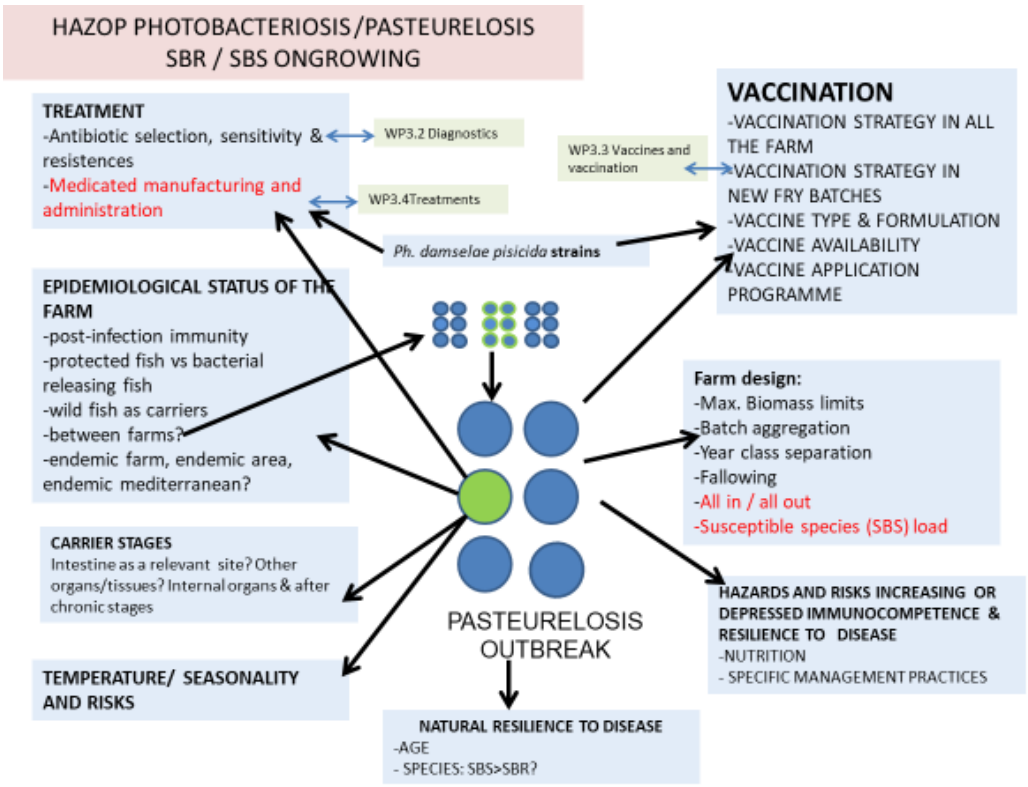

HAZOP NODAVIRUS SBS/SBR: JUVENILE REARING (NURSERY & PRE-ONGROWING)
NODA VACCINE AND VACCINATION
-Commercial / Experimental
-Protection (levek & lasting) within the nursery
-Vaccination schedule (fish weight)
-safety
-Interferences with other vaccines
ORIGINS
-SAMPLING MATERIAL:
Age/weight? How many?
-Whole fish vs. heads/ organs
-RNA extraction method
Batch & tanks same batch?
Sample preservation:
fresh/frozen/ethanol/RNA later....
-BEST DIAGNOSTIC TECHNIQUE IN EACH CASE / Predictive values/ values interpretation
Genotypes / Reassortants: differences between SBR /SBS protocols?
-PREDICTIVE VALUES
-VALUES INTERPRETATION FOR A SAMPLE/REPEATED SAMPLES
NODA-FREE LARVAL STOCK
LARVAE FROM OTHER HATCHERIES / COMPANIES: GUARANTEES?
JUVENILE BATCH CHECKING PROTOCOLS
WP3. Task 3.2: DIAGNOSIS
WATER SUPPLY
FRESH WATER SUPPLY
Tap water
Local well, river, pond...
SALTWATER SOURCES
Wells
Pumping sea open water, sea shallow water, lagoon...
Artificial seawater
WATER TREATMENT (if any)
Temperature
Filtration
Filtration + UV
Filtration + ozone
Chemical
Others...
EFFICIENT NODA DETECTION IN FRESH AND MARINE WATER? VALIDATED PROTOCOLS? FREQUENCY?
Post-larvae & juveniles
HUSBANDRY PROCEDURES
NURSERY DESIGN: PHYSICAL ISOLATION FROM BROODSTOCK & HATCHERY?
ISOLATION BETWEEN BATCHES
STAFF PRACTICES
Specific personnel for nursery
WATER EFFLUENTS /WASTES CONTROL
SANITISATION PROCEDURES
Disinfection efficiency control Guidelines
-GRADING
-SWIMBLADDER & MALFORMATION TRIAGE
-MOVEMENTS BETWEEN TANKS
-GENERAL VACCINATION PROCEDURES
Commercial feeds
BIOSECURITY TRANSPORT & STORAGE
Inmunostimulation?
VIRUS INACTIVATION/DESTRUCTION IS 100% GUARANTEED BY RAW MATERIALS (FISH MEAL) PROCESSING AND EXTRUSION PROCEDURES?
TRANSPORT
Trucks: transport tanks disinfection: guarantees & procedures
WP3. Task 3.4.2: BIOCIDES


HAZOP NODAVIRUS SBS/SBR: On-growing CAGES
WP3. Task 3.2: DIAGNOSIS
NODA VACCINE AND VACCINATION
Juvenile ORIGINS
• Single or different juvenile suppliers?
• VNNV tested some time before shipping
ENDEMIC AREA
• Previous outbreaks in the FARM?
• Outbreaks in the SAME AREA? (25 miles around)
ONGROWING-CAGES
TRANSPORT
WATER
TEMPERATURE
Juvenile immersion month
Integrated days at risk temperature (23-30ºc)
SEA CAGE DESIGN AND CONFIGURATION
MANAGEMENT
Stress pressure
Disinfection
Specific feed formulations
WP3. Task 3.4.2: BIOCIDES

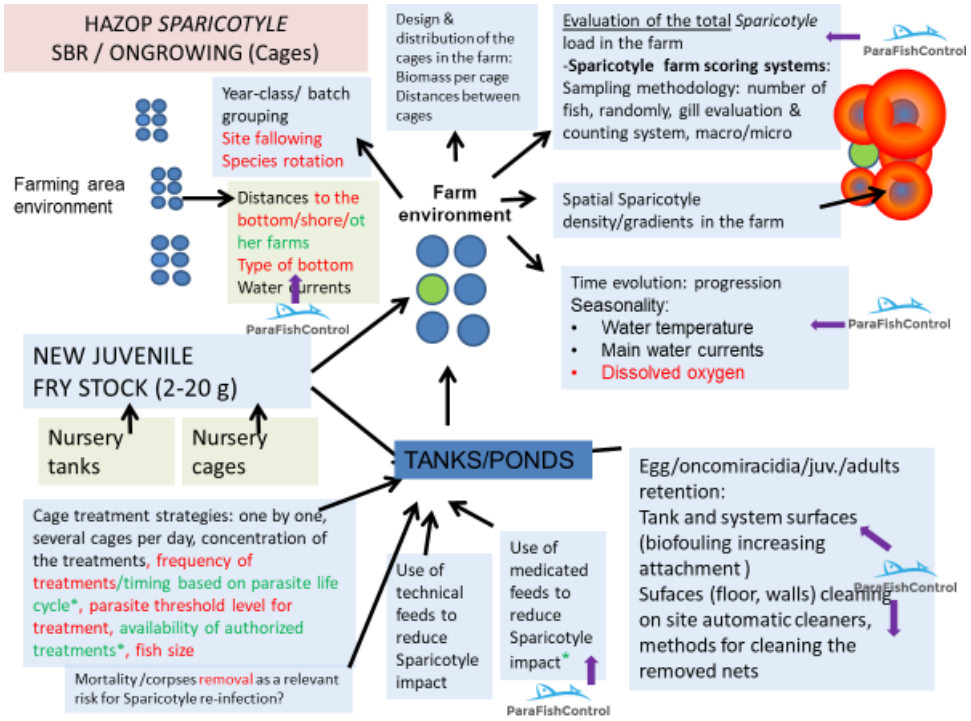

HAZOP NODAVIRUS
SBS/SBR: On-growing TANKS AND PONDS
ENDEMIC AREA
Previous outbreaks in the FARM?
Outbreaks in the SAME AREA? (25 miles around)
Juvenile ORIGINS
Single or different juvenile suppliers?
VNNV tested some time before shipping
WATER SUPPLY
SALTWATER SOURCES
Wells
Pumping sea open water, sea shallow water, lagoon...
WATER TREATMENT (if any)
Temperature
Filtration
Filtration + UV
Filtration + ozone
Chemical
Others...
EFFICIENT NODA DETECTION IN WATER? VALIDATED PROTOCOLS? FREQUENCY?
ONGROWING PONDS/TANKS
VACCINATION
TRANSPORT
SEA CAGE DESIGN AND CONFIGURATION
VECTORS
ISOLATION BETWEEN BATCHES
STAFF PRACTICES
SANITATION PROCEEDURES
MANAGEMENT
Stress pressure
Disinfection
Specific feed formulations

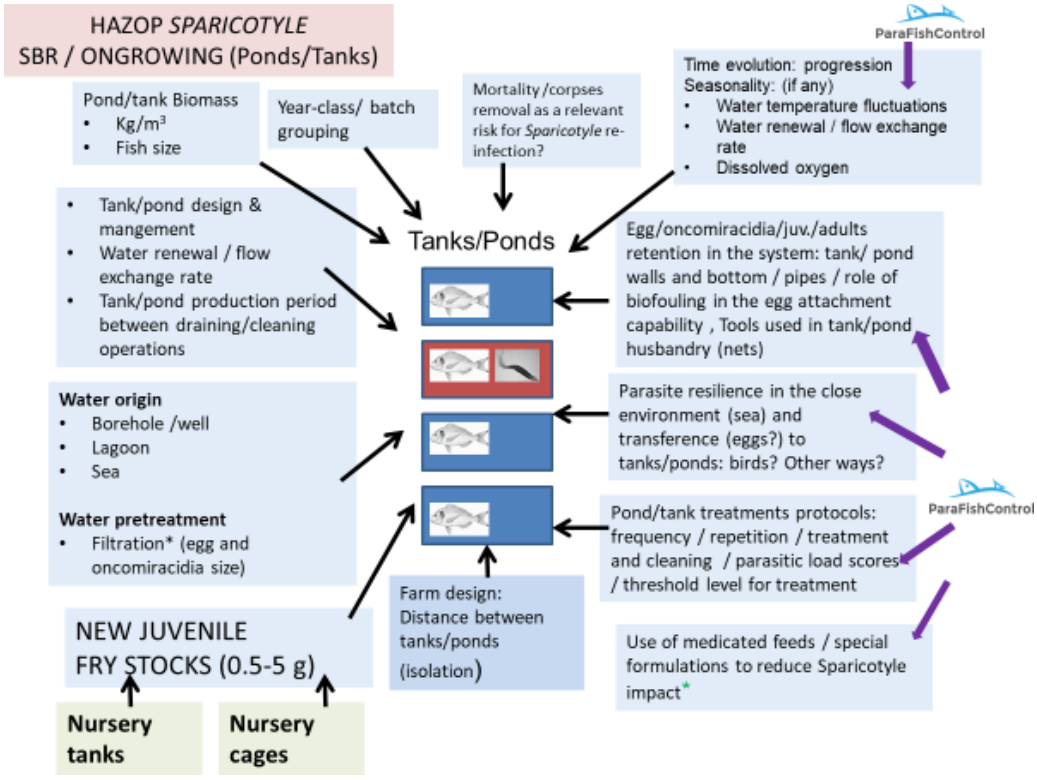
PHOTOBACTERIOSIS





SPARICOTYLE INFECTIONS





2. Specific prophylaxis and biosecurity aspects in each production stage

2.1 Broodstock

Appropriate genetic background is one of the most relevant issues for a high-quality production. The relevance of the selection for general fish resilience and resistance against specific diseases is addressed in WP1 of PerformFISH. In addition to that, another important factor to be taken into account is the health status of the broodstock and particularly the control of the pathogens that can be vertically transmitted to the offspring. Concerning this point, different aspects related to broodstock origin, organization and management should be considered:

2.1.1 Broodstock origin

Broodstocks can be set up with fish from different origins and with different health guarantee levels. In the best possible scenario, broodstock fish should be supplied from facilities specifically designed for the rearing and maintenance of high genetic value fish stocks, isolated from fish stocks from commercial circuits. These facilities normally offer the higher health standards and, in some cases, can achieve levels similar to specific-pathogen-free (SPF) conditions. This type of facilities are usually found to host fish for research and in specialized fish breeding companies mainly for salmon and trout. The same is also partially achieved in some of the current hatcheries of European seabass and gilthead seabream, although in most of them there are still some fluxes from commercial circuits. The same situation can also be found in some broodstock from research institutions. Therefore, it is very important to highlight the relevance of the potential risks for pathogen transfer from operating these fluxes without the appropriate risk assessment, surveillance and control measures.

Many hatcheries use fish selected from commercial batches as a main system of generational replacement and genetic improvement. Obviously, these strategies substantially increase the risk of entry of infectious diseases if appropriate measures are not implemented. To reduce this risk, it is highly recommended to maintain stocks of fish from genetic programs under controlled conditions in a similar way as salmon or other livestock genetic companies do. Another even riskier strategy is the incorporation of fish from the wild as part of the broodstock. This was done quite often in the past, in order to keep in the broodstock some “wild” morphological characters (shape, aspect) and without taking into account of the potential risk of introducing pathogens in the hatcheries. Nowadays, with the current knowledge on genetic selection, this practice is totally discouraged.

2.1.2. Broodstock pathogen free assessment and certification

In addition to the strategies related to the reduction of fluxes from commercial production circuits, all the broodstock populations and stocks should be reared under a health control plan, including an accurate plan for detection of asymptomatic carriers of potential pathogens. This plan should mainly focus on the diseases with potential vertical transmission, mainly VNN but also lymphocystis and Photobacteriosis. In this case, due to the high value of the broodstock specimens, diagnostic techniques based on non-lethal sampling methods should be the first

choice methodologies. If predictability of the results obtained are not robust enough, other epidemiological approaches using sentinel fish and lethal sampling should also be considered.

2.1.3. Broodstock groups configuration and isolation

The different groups of broodstock are organised according the necessities of the farm or the research center. Small familial groups (usually one female / two males) are usually kept in research units and in hatcheries where artificial fertilization is used (mainly in European seabass). Larger groups are more frequently found in commercial hatcheries, where large number of eggs per batch are required. In these cases, it should be borne in mind that these large groups should be considered as a single epidemiological unit. Groups & families in isolated tanks tend to have a higher protection than large groups in case of problems related to the pathogen entry in the facility.

Broodstock water supply and water treatment is also very relevant for biosecurity and prophylaxis. Broodstock facilities have different sources of water supply according to their particular geographical locations and water quality availability (pumping from open sea, coastal zones, lagoons, or wells). Most of them also incorporate specific systems of water treatment and disinfection, based on filtration & UV disinfection systems. The efficacy of disinfection may vary according to the technical characteristics of the equipment used and the maintenance applied, yet it should be adequate to guarantee the complete water disinfection against the main pathogens for Mediterranean aquaculture. In any case, it is highly recommended to evaluate routinely the efficacy of disinfection of these systems. For safety reasons, it is also recommended to have an oversized system to cope with potential peaks of water requirements and also have different systems running in parallel in order to minimize problems in case of technical failure of one of the systems and to allow maintenance operations.

In flow-through systems, all the tanks are supplied with disinfected water and effluent water from the tanks is usually channeled in an appropriate and isolated way, so there are no major issues in these systems. However, some facilities can also operate on recirculation systems. In this case, similar systems of filtration and UV or similar water disinfection systems should be added after the general filtration system (mechanical filtration, skimmers, biofilters) as in flow-through systems. In addition, specific independent filtration and disinfection systems should be incorporated in to treat the make-up water used to compensate the water renewal.

2.1.4. Facility design

As previously discussed, it is highly recommended that broodstock facilities should be physically and operationally isolated from the other farming facilities. It is particularly relevant to limit or control the movement of staff between facilities and ban any entrance of unauthorised personnel. Visits should be limited as much as possible. The installation of large windows in some doors to inspect the activity in the facility from the outside, the use of cameras in the building and of specific videos for demonstration or educational purposes can be efficient alternatives to preserve biosecurity and reduce interferences with routine activities and potential stress sources for the broodstock. Isolation should also cover unexpected entrance of animals such as cats, rodents, birds, etc. (see also section 2.3.4 on nursery).

2.1.5. Operational procedures

Broodstock management is usually limited to specific routine operations or tank cleaning and feeding and to occasional manipulations related to biometrical samplings, biological material sampling, evaluation of gonadal development or stock reconfiguration. European seabass broodstock groups tend to require less frequent group reconfiguration than gilthead seabream which is a protandric species and stocks require constant replacement with new males. Eggs are normally obtained from natural spawning after temperature and photoperiod manipulations. Artificial spawning with or without hormonal management is occasionally performed in some European sea bass stocks. From the prophylactic and biosecurity point of view, all these operations should be performed with gentle and careful manipulation of the fish (use of anesthetics for sedation when necessary) to avoid harming the fish skin, using basic hygienic measures (use of gloves to handle fish, use of clean water, external disinfection on the fish after manipulation if required) and equipment (use of clean and disinfected landing nets, containers before and after use).

2.1.6. Staff, staff training, awareness and motivation

From the biosecurity point of view, broodstock premises should have a specific assigned staff. As routine management procedures are not very time demanding, the same staff members can work part time in other sections (hatchery, for example), but they should always completely separate the activities, applying personal hygienic measures (shower, disinfection) and change of work clothes and footwear after each activity. Clothes and footwear should be only used inside the facility. If possible, daily tasks in the broodstock facility should be scheduled and performed before any other task in the same day.

Maintenance and specific operations should only be performed by specialized staff with adequate training on specific prevention strategies and biosecurity procedures. Staff awareness and motivation for these aspects are highly recommended in order to minimize the risks associated to careless mistakes.

2.1.7. Broodstock feeding & biosafety

Biosecurity in broodstock feeds is considered a relevant risk in aquaculture and particularly in PerformFISH. Broodstock of gilthead seabream and European seabass can be fed with different types of diets according to the knowledge, experiences and preferences of each farm. Apart from the nutritional aspects and the effects on egg quality, feed safety is also a very relevant issue that should be stressed. Broodstock individuals can be fed on a natural based diet or on artificial feeds. Natural diets based on fish, mollusks or cephalopods have been used in the past and in some cases, natural diets are considered better than artificial ones in terms of reproductive performance. Natural diets were used as a basic diet or a complement during the spawning season and were used as fresh or frozen. In terms of biosafety, these natural diets should be considered as a major risk, as they are normally based on commercial fisheries material, with no specific health control. Taking into account that these organisms can harbor pathogenic viruses, bacteria or parasites that can be transferred to the fish stocks, its use as

food is not recommended. Specifically, for betanodaviruses, their presence in natural fish and shellfish populations has been widely confirmed (Gomez et al., 2008a; 2008b; Volpe et al, 2018) and they were detected even in trash fish/mollusk used for feeding cultured marine fish (Gomez et al., 2010). Freezing and deep freezing can inactivate some parasites in materials from animal origin, although the efficacy may vary (Franssen et al., 2019). Freezing does not inactivate bacteria and viruses. Other inactivation methods (gamma irradiation, high pressure/freezing) are used in food industry (Arvanitoyannis & Tserkezou, 2013; Arnaud et al., 2017) and could be also considered as alternative methods, but their application requires more complex logistics and no specific studies on inactivation of fish pathogens have been published.

Nowadays, there are several commercial fish feeds (extruded pellets) specifically formulated for broodstock, including European sea bass and gilthead seabream. Together with the changes induced in some components of the ingredients used in the formulation, extrusion has been described as a remarkable inactivation method due to the high temperatures reached during the extrusion process. This specific quality makes artificial food pellets a safe alternative for broodstock feeding.

2.1.8. Broodstock vaccination

Vaccine immunoprophylaxis can also be an extra measure for gilthead seabream and European seabass broodstocks, although high levels of biosecurity are usually considered as sufficient and vaccination is not strictly necessary. However, if the prophylactic measures applied are not the most suitable or if unavoidable risks are detected, then broodstock vaccination can be an option. Commercial vaccines used for ongrowing can also be applied to the broodstock fish using the characteristics on safety and protection described in ongrowing fish as a reference. However, very few studies have been published concerning vaccination in seabream or seabass broodstock and the protective effect on the vaccinated broodstock and this is maybe the reason why some commercial vaccines bear the indication that they are not suitable for broodstock vaccination. Hanif et al. (2004) described some effects of the vaccination on gilthead sea bream against Photobacteriosis. These authors described an increase in the total immunoglobulin level, specific antibody titer, anti-protease activity and lysozyme activity after vaccination and also increase of anti-protease activity, lysozyme activity and total immunoglobulin level were detected in the eggs. The same team (Hanif et al., 2005) also describes effects on the offspring, such as a slight delay onset in the mortality and also reduction of the mortality levels after challenging 67 days old larvae with *Photobaterium damselae* subsp. *piscicida*. Similar protective effects of broodstock vaccination has been suggested for nodavirus vaccination, yet in species other than European seabass and gilthead seabream. However, the number of studies on this topic is still low and results are controversial (Patel and Nerland, 2014).

Vaccination programs cannot substitute biosecurity measures in broodstock, but can be considered as a second-line measure mainly targeted to minimize the impact in terms of morbidity and mortality if the stock becomes infected.

2.1.9 Egg management and biosafety

Fertilized eggs are normally obtained by collection of eggs floating in the broodstock tanks using special egg collectors. Only in sporadic cases (mainly in seabass) eggs and sperm can be obtained

by manual stripping of female and male fish and then fertilized in a container. Floating eggs may have different levels of microbiological quality, as bacteria can be attached to the egg surface, mainly in dead or non-viable eggs. For this reason, the microbiological quality of the broodstock tanks should be monitored and controlled. As dead or non-viable eggs are also a relevant source of bacterial contamination, dead eggs should be removed from the egg batch as soon as possible. Viable, good-quality eggs should then be gently washed with clean water, taking special care to avoid any physical (temperature, movement) or chemical (salinity) shock during this process. The use of pre-cleaned eggs is also a recommended method to increase sanitization/disinfection efficacy.

As a routine process, it is highly recommended to sanitize or disinfect the eggs before moving these eggs to incubation. Iodine, glutaraldehyde and oxygen peroxide are the three most frequently used disinfectants. Different doses and exposure times have been tested with relevant efficacies in reduction of the bacterial contamination or even complete disinfection.

Iodine (50 ppm/5-10 minutes) is a frequently used disinfection protocol used in gilthead seabream and European seabass hatcheries and it was also described as an efficient and safe disinfection method for other cultured sparid species (Katharios et al., 2007). A particular study (Can et al., 2010) recommended higher dose and exposure times (300 ppm / 15-20 min /18ºC) to disinfect seabream eggs.

Glutaraldehyde disinfection (200 ppm / 4 minutes / 18ºC) has also been recommended as an efficient alternative method with no significant reduction of egg viability (Escaffre et al., 2001) in seabream eggs. Similar protocols using 200 ppm and different exposure times between 4 and 8 minutes allow significant decrease on the viable bacteria or even a complete egg disinfection (Can et al., 2010).

Similar results on disinfection efficacy on seabream eggs have been described using oxygen peroxide (300 pp /10 minutes /18ºC) (Can et al., 2010).

2.2. Hatchery

For this section, we strongly recommend to revisit the two volumes of the Manual on hatchery production of seabass and gilthead seabream, by Moretti and co-workers and edited by FAO (1999 and 2005). This is a very complete and practical review on European seabass and gilthead seabream hatchery procedures and with some specific references and recommendations on hygiene and prophylaxis.

Gilthead seabream and European sea bass larvae, like other similar larvae, are delicate organisms and very susceptible to physical, chemical or biological challenges. For this reason, strong prophylactic procedures should be applied in the hatcheries. These procedures can be summarized in the following aspects:

-Facility design

-Staff / Visits

-Larval health quality evaluation and management

-Live food microbiological quality

-General hygienic measures and operational procedures (including mortality removal)

-Technical stops and sanitary breaks

2.2.1. Facility design

Similar recommendations as for broodstock area can be given in this section. Hatchery should be physically and operationally isolated from the other activities and buildings. Hatchery can be physically built behind or in the vicinity of broodstock and nursery facilities but should be run as much independently from these other facilities as possible, minimizing the fluxes of materials and staff. Only eggs from broodstock should enter and only weaned larvae should be transferred to nursery. A single entrance to the hatchery is highly recommended in order to have a better biosecurity control on people and materials introduced in the hatchery. Auxiliary changing rooms with adequate equipment (toilets, showers, lockers) should be placed inside the hatchery, with a clear division between clean and not clean areas.

Emergency exits should be equipped with alarms in order to discourage any attempt to use these exits to bypass the main exit. Internal design of the hatchery should follow similar designs as for food industry, with an appropriate distribution of spaces, tanks and other elements that facilitate cleaning and disinfection operations. Tanks should have full visual and operational access, floors and walls should be designed in order to reduce as much as possible corners or dead spaces.

Tanks should be separated to avoid contamination by water splashes between neighbor tanks or alternatively, mobile plastic curtains/screens can be placed between them. Inlet and effluent water flows should circulate in different and independent systems. Tanks with larval batches should be grouped together and physically separated if possible.

Live food production should be produced in a separate, independent and controlled area, and only inspected live food can be introduced in the hatchery by specific delivery windows or pipes.

2.2.2. Staff / Visits

A member of the hatchery staff should have specific responsibilities on biosecurity design, supervision and implementation. A specific biosecurity plan, with corresponding guidelines should be prepared. All materials entering the hatchery should be approved and recorded.

Hatchery staff should be specifically assigned only to this specific site and to hatchery activities in order to minimize the entry and exit of personnel. Hatchery staff should follow strict personal hygiene measures, including shower and separate clothing (periodically cleaned and disinfected). Clothes should have a specific colour and should be clearly recognizable (mainly to prevent workers to exit the hatchery wearing hatchery clothes and footwear). If these measures can be applied, footbaths and other disinfection measures are not necessary within the hatchery.

Visits should not be allowed or at least should be strongly restricted. Any sporadic visit should follow the same strict hygienic and clothing rules as for hatchery staff. These mandatory rules are also helpful to discourage any unnecessary visits. Similar systems as described in broodstock

facilities (walls with wide display windows, cameras, video recordings) could be useful to show the hatchery facility without compromising biosecurity.

2.2.3. Larval health quality evaluation and management

Larval tanks and batches should be regularly checked in order to verify the larval distribution and behavior in the tank together with other technical measures related to water quality (physical & chemical parameters), available live food (counts) and larval quality (see also recommendations from PerformfISH WP2). Regular biological quality evaluation (microbiota, both quantitative and qualitative) could also be recommended in specific critical points and under certain conditions. Any deviations from the expected normal parameters should be recorded and reported accordingly. Larval mortality should also be accurately assessed after each tank siphoning and filtering. Dead larvae and any organic debris should be placed in appropriate containers and processed as organic biohazard. Any deviation or abnormal behaviour should be investigated. Periodical health and disease-free status controls should also be implemented.

2.2.4. Live food microbiological quality

Live food (rotifers, *Artemia*) and microalgae are normally supplied to the gilthead seabream and European seabass larvae. Microbiological controls should be implemented in the live food in order to evaluate the microbiological quality of the different live food batches.

Artemia nauplii microbiological quality should be assessed after a) hatching/decapsulation, b) filtering/washing/disinfecting, c) enrichment, and d) preservation/storage under hygienic conditions. Rotifers microbiological quality should also be assessed during the rotifer culture system and after the harvesting/washing and enrichment processes. Due to their high nutritional content, enriched rotifers and *Artemia* nauplii can easily become contaminated with bacteria, spoiling the nutritional value and increasing the risk of shifting microbiota in the rearing systems or of introducing harmful or pathogenic bacterial strains.

Different general disinfection methods are described in the literature using common disinfectants (Munro et al., 1999, Douillet & Pickering, 1999, Skjermo & Vadstein, 1999, Stefanakis et al., 2014) and some commercial products (Gimenez-Papiol et al., 2009). Commercial products specifically designed for bacterial control and disinfection of *Artemia* nauplii and rotifers are also available and can be used following technical protocols (Sanocare® products, INVE, Bio-Roticen®, Cenavisa).

If microalgae are used, periodical microbiological controls of the used microalgae (liquid culture, dehydrated, paste) before their release in the culture tanks should be performed.

2.2.5. General hygienic measures and operational procedures

Hatchery hygienic programs and protocols should be designed as in food industry. If the larval production is based on batches, it is highly recommended to work in an “all-in, all-out system”, including complete sanitary stops between production batches. These sanitary stops should include the complete emptying of the building of larvae and water. Once empty, careful cleaning

and disinfection of tanks, pipes and any kind of ducts in contact with the larvae or with the effluent water of the system should be applied. If internal surfaces are affected by mineral incrustation, appropriate mechanical and/or chemical descaling should be applied before cleaning and disinfection in pipes, heat exchangers, etc. As bacterial biofilms can also be a very relevant source for undesirable and/or pathogenic bacteria, accurate disinfection procedures should be applied. This is particularly relevant, as in most cases, larval tanks are supplied with disinfected (naïve) water. Therefore, in the first weeks after hatching, microbiota in the tanks are characterized by relatively low levels of bacteria but at the same time with a high level of instability concerning the bacterial species present in these tanks. If surfaces are not well disinfected, uncontrolled bacteria from biofilms can be re-inoculated in the system.

A specific water recirculation system with water and disinfectant agents can be used mainly in order to increase the time of exposure to all the surfaces. This method is mainly recommended for the pipes and pumps. Special care should be taken to avoid the use of disinfecting substances that can produce harmful or toxic residues in the system. The whole system (tanks, pipes) should be rinsed with clean water.

Disinfection of the air circuits is also necessary, mainly if humidity is detected to avoid problems with fungal contamination and growth.

Floors should be daily cleaned and disinfected after each workday and walls should be cleaned and disinfected regularly or between batches.

2.3. Nursery / Pre-ongrowing

Nursery phase in gilthead seabream and European seabass is usually considered a specific period of the production cycle comprising post-weaned larval/post-larval phases until they can be considered juveniles (1-2 g fish). After weaning, post-larvae feed on 100% artificial feeds but cannot be totally considered as juveniles as they still have not attained all the morphological and physiological characteristics of juveniles (scales, pigmentation, nutritional requirements, maturity of the immune system, sensitivity to management). During this phase, specific operations (e.g. passive grading) are progressively incorporated although all these procedures require major attention and delicateness in the management. In some cases, bigger fish (5-30 grams) are requested for some farms, mainly in West Mediterranean, as offshore cages operate with nets with bigger mesh sizes and fish must be adapted to feed delivery in open sea. For this reason, specific pre-ongrowing facilities are required. They operate with similar systems as nurseries (flow through systems or RAS, but with a bigger capacity (more tanks, bigger tanks) the keep the different batches of fish.

Mistakes in the management are attenuated by the relative physiological fragility of the post-larvae. In this scenario, problems related to opportunistic infections associated to inadequate management and chronic stress are not uncommon. This is the reason why hygienic conditions during management and husbandry are still necessary. At the same time, strict sanitary control in the facilities and practices is still necessary, as vaccination is not yet possible, due to the small size of the larvae and their immature immune system. Therefore, hygiene and prophylaxis can be considered as very relevant during this period.

2.3.1 Facility design

In this case, nursery design significantly differs from the broodstock or hatchery design. Gilthead seabream and European seabass nurseries require more tanks and tanks with different characteristics as post-larvae have different needs that implies different management. Again, it is highly recommended to have the nursery in a different building, but in this case, constant transfer of batches of post-larvae from hatchery to nursery and juveniles from nursery to pre-ongrowing or other facilities should be considered. This is the reason why, for the shake of logistics, nurseries can be placed adjacent to or in the vicinity of the supplying hatchery or the pre-ongrowing facilities. In any case, physical separation and some kind of isolation are highly recommended. Transport from the hatchery to the nursery can be done using specific pipes with passive flow or using transport containers (see specific section on live fish transportation). All this equipment should be cleaned and disinfected after each use and stored or maintained in a clean and separated area and should only be used for this purpose. For bigger juveniles, fish pumps can be used and in this case, fish pumps can be inspected and serviced regularly in order to reduce potential lesions of the fish due to malfunction. After each use, pumps, pipes and fish counters should be cleaned and disinfected according producer's recommendations. This is particularly relevant if this equipment is shared with other areas. Safety issues concerning potential toxicity of the residues of the products used (detergents, disinfectants) should also be assessed (see also Section 3).

Nursery does not require a strict control of internal entrances and exits as in hatcheries, so different doors or portals can be necessary to facilitate operations. Staff doors can be equipped with specific footbaths, but for bigger gateways with constant equipment movement it is much more difficult to control or apply biosecurity measures. The highest risks in terms of biosecurity are imposed when materials, equipment or machines have been in contact with fish or seawater outside the nursery and they have not been cleaned and disinfected appropriately. The risks are higher in machinery such as fish pumps, fish graders, nets (mainly if they are still wet) and transport tanks, when they are shared with other sectors in the farm (pre-ongrowing) or other farms and facilities and also if there are not appropriate cleaning and disinfection protocols or these protocols are not into effect. In any case, movements of materials & equipment should be authorized and supervised by the nursery coordinator.

2.3.2. Staff / Visits

Nursery normally requires much more frequent and larger-scale logistics (feeding, grading, transport, cleaning…) and , as a result, more staff than hatchery. Therefore, the labor and materials turnover is normally more intense. Under these circumstances it is much more difficult to keep strict hygienic measures and hygienic measures should be designed according to the particular risks and characteristics of the operations in each nursery. Standard hygienic measures (specific staff working only in the nursery, specific clothing and footwear, hands cleaning and disinfection before entering the facility) could be reasonable measures for staff.

Visits should also be discouraged for nursery, although in this case, visitors can be accepted if specific single-use lab coats and shoe covers are used and visitors should be warned about any contact with tanks, fish or water.

2.3.3. Juvenile health quality evaluation and management

Fish in nursery start to be reared in a more challenging system and put under more demanding management conditions (grading, sorting, competition during feeding, aggressive behaviour, cannibalism…). Covert chronic stress conditions may appear as well as physical damage due to rough handling. Water quality tend to be lower than in hatchery, mainly in terms of microbiological quality and control, so fry can be more exposed to pathogens than in hatchery. Although seabream and seabass juveniles are already considered as fully immunocompetent around 100-110 days after hatching, nursery is a particular susceptible period for fish stocks and disease episodes are normally expressed as acute and severe outbreaks. For this reason, a careful evaluation of the fish behaviour, feeding activity and daily mortality is highly recommended. A detailed pathological assessment of symptomatic or dead fish/carcasses collected during daily cleaning activities is also recommended when deviations from the normal pattern are detected. Together with the daily mortality assessment it is highly recommended to implement a health control plan, including periodical evaluation of the health Key Performance Indicators (KPIs, Deliverable 7.1) recorded and the evaluation of randomly-taken samples from all the batches reared in the nursery. This health control plan should include the recommended sampling protocols as well as the recommended diagnostic techniques for each disease/pathogen. As diseases in gilthead seabream and European seabass are not included as noticeable diseases by OIE or diseases under specific surveillance programs, Mediterranean industry should create and recommend their own standards for diagnostics. Diagnostics are also very well addressed in PerformFISH and a specific deliverable on diagnostics and diagnostic capacities is under development.

2.3.4. General hygiene measures and operational procedures

As post-larvae and juveniles reared in the nursery have already finished the weaning period in the hatchery and all of them feed on commercial feeds, the microbial quality assessment is not so demanding as in the life feed control. However, diets for young gilthead seabream and European seabass are characterized by high nutritional value and are more exposed to air oxidation and/or moistening. In these cases, measures to avoid feed spoilage are particularly required. Post-larval and juvenile feeds should be stored in dry and protected from the light places (specific warehouse) and only daily rations should be transferred to the nursery. The date of the opening of the container/bag should be written down on the container and the content of each opened container should be finished as soon as possible. Routine basic analysis of the general quality of the feeds (moisture, mold contamination) should also be implemented, aside from the nutritional quality analysis.

Nursery general hygiene programs and protocols should be designed according the juvenile production schedules. Production systems that avoid overlapping batches are the best ones to implement efficient hygiene measures. In these cases, when fish are not in the nursery, sanitary stops with accurate drying, cleaning and disinfection protocols can be applied in a more efficient and easy way. Comments on methodology are the same as for hatchery. If sanitary stops are not feasible, partial hygiene measures should be applied. In this case, tools and equipment should be cleaned and disinfected after each use and floors should be cleaned every day. As for hatchery, safety issues should also be assessed (see also Section 3).

In nursery/pre-ongrowing systems, specific operations such as vaccination, grading, sorting (swimbladder inflation, malformations) and transportation are frequently performed. Concerning hygiene and prevention measures, these are the main aspects and recommendations to consider, in addition to other considerations concerning efficacy, management, fish stress minimization and safety.

As for other sections, the presence of mammals (domesticated or feral cats, rodents) or birds (eg. Eurasian magpie, sparrows, pigeons) in the facilities and inside the pre-growing buildings should be avoided, not only for the health risks associated with them but for avoiding ruining structures (cables, devices) and causing stress to the fish. Fish feed bags should be placed in stored in safe places, feed pellets on the floor should be daily removed, discarded or dead fish should be disposed in suitable containers to reduce the attraction and availability of this material for the animals.

2.3.5 Vaccination strategies

Vaccines and immunoprophylaxis play a relevant role in prophylaxis and prevention in Mediterranean aquaculture. In PerformFISH, there is a specific and ambitious task on vaccine development and vaccination, the results of which will be presented in a specific deliverable. For this reason, only the most relevant aspects directly related to prophylaxis during vaccination processes will be presented in this document.

In Mediterranean Aquaculture, first vaccination is normally applied from 1-g-fish onwards, as it is widely accepted that immunological maturation in gilthead seabream and European seabass is not achieved until this stage. This is the reason why vaccination is one of the main activities in Mediterranean nurseries. For some vaccines, first vaccination is applied by bath vaccination and may require re-vaccination. Vaccines administration as an IP injection requires larger fish (10-30 g).

The following aspects of vaccination protocols related to hygiene and prophylaxis should be considered in all vaccination procedures:

-If possible, do not feed the fish 24 hours (summer) or 48-72 hours (winter) before vaccination. This strategy helps to reduce the risk of accidental injection in the digestive system and also reduces the amount of faeces in the water, contributing to hygienic conditions during the vaccination. Do not starve if aggressiveness in the stock is detected.

-In bath vaccination, water quality should be checked regularly and vaccination solution should be changed if too much mucus or foam is detected.

-After vaccination, fish should be placed in clean water for recovery. An additional external bath with disinfectants (oxygen peroxide, formalin) can also be used if risks of secondary bacterial external problems after vaccination are detected.

-Vaccines should be stored at 2-8ºC and protected from light and administered as IP injection at 15-20ºC.

-If possible, use the whole vaccine bottle/bag in a single vaccination application. Avoid the use of bottles previously used after mid or long term storage as contamination risk increases (vaccine bottles/bags opened more than 12-24 hours before should not be re-used).

-Containers/nets/net sticks, vaccination tables should be cleaned and disinfected after each vaccination procedure. If possible, use specific equipment only for vaccination.

- Vaccination guns and vaccination machines should be cleaned after each use and inspected before use. Vaccination needles should be checked (sharpness) regularly according to the needle/gun manufacturer recommendations. As a general recommendation, needles should be regularly checked to remove any scales or organic material and needles should be changed every 2000-3000 doses.

-Safety procedures for the staff should be carefully supervised (specific clothes, cleaning and disinfection hands and arms, use of new gloves after every batch or stop).

All these considerations on prevention/hygiene measures during vaccination processes should be considered together with the recommendations, vaccination protocols and quality controls in the vaccination manuals issued by the vaccine companies.

2.3.6. Grading

Grading operations should be performed under high hygiene standards. Before grading, all the devices (passive floating fish mesh/bar graders, automatic grading machines, fish pumps), pipes, buckets, containers, nets, rod nets, etc. should be cleaned and disinfected before use and should be cleaned and disinfected after grading a batch of fish. Grading devices should also be checked to detect any burring or alteration that can potentially harm the fish to be graded.

Water used during grading procedures should be the same inlet clean water used in the tanks.

2.3.7. Sorting (deformities, swimbladder inflation)

Similar recommendations for cleaning and disinfection devices and materials (pumps, buckets, containers, sorting tables, etc.) used in grading can be applied here.

As manual manipulation by sorting operators is relevant, similar hygiene considerations (cleaning and disinfection of arms and forearms, use of gloves, specific clothes…) are recommended.

2.4 Ongrowing in sea cages

Sea cage operations are based in a highly exposed environmental system where prophylaxis is really difficult. Most of the efforts should focus on:

- Fry health quality
- Appropriate configuration of the groups in cages according sanitary risks
- Design of the farm in order to concentrate single production batches in a same site
- Distances between cages, groups of cages and farms
- Sanitation period / fallowing should be applied if possible
- Regular control of the fouling in the nets and structures (rings, ropes…), net cleaning and/or net substitution

- Equipment / boats policies.

Under these exposed conditions, systematic and efficient vaccination programs (as immunoprophylaxis) should always be implemented.

2.4.1. Juvenile health quality evaluation and management

As for nursery and pre-ongrowing production systems, two of the main health measures to be implemented in cage production systems in the Mediterranean are:

a) to guarantee the sanitary level of the fry introduced in the cages

b) the protection level achieved, associated to the vaccination program.

All the juveniles/fry arrived to the cages should be previously certified for the absence of pathogens or at least, for the most relevant ones. Based on the diagnostic techniques and laboratory diagnostic capacities described and discussed both in PerformFISH and MedAID, it is very important that detection and diagnostic methodologies and protocols should be agreed by the industry in the future in order to harmonize and facilitate the mutual confidence on juvenile health quality between juvenile suppliers and cage ongrowing farmers. These harmonized protocols are very relevant when juveniles are produced in nurseries and pre-ongrowing facilities of the same companies but are even much more critical when juvenile suppliers are from a different company. As for nursery, details about the pathogens/diseases and diagnostic methods to be selected and implemented are widely discussed in the PerformFish Deliverable 3.2 "*Epidemiological status of Mediterranean farmed fish"* and in the documents on diagnostics issued in PerformFISH and MedAID.

Quarantine is a concept widely used in human and animal movement management and it is based in the restriction of movements, confinement or isolation of animal stocks or groups of humans to prevent the spread of diseases. Quarantine in aquatic animals has been widely considered and commented by Arthur et al. (2008) but for different reasons, quarantine procedures are usually difficult to apply in Mediterranean fish farming and also can be replaced by advanced sanitary control measures before the shipment of the fish. Currently, none of the diseases affecting gilthead sea bream or European sea bass are EU-regulated contagious diseases or are listed by the OIE. In this scenario, although evidence-based efficient systems for decision making for gilthead seabream and European sea bass diseases are required, these diseases are not under the same regulatory framework as some relevant diseases in other species such as VHS or IHN in salmonids. For these highly-regulated diseases, quarantines are much more indicated.

Quarantine implies the availability of specific and isolated facilities in the farms only for this purpose, but for the farms and the industry this cannot be feasible due to different reasons:

- High cost of these facilities, not only the cost of the construction, but also the inefficient economic return due to the limited use compared with the other.

-Size of the batches. There is a trend in Mediterranean aquaculture to increase the size of the batches. Therefore, the size of the quarantine facilities should be according to the size of these batches

-This only can be applied to inland facilities, not to cages, as only partial confinement is possible in cage farming.

-The availability of good, accurate and nearly real-time pathogen detection systems to evaluate the presence of pathogens in pre-shipment. All these techniques and systems are also widely addressed in Task 3.2 of PerformFISH. As the international fish farming industry (and also the Mediterranean fish farming) is moving forward to achieve high standards and strong certifications, strict fish health evaluation and health certifications are currently required to the fish (juvenile) suppliers, including the most relevant diseases.

Concerning vaccines and vaccination programs against the most relevant diseases, an introduction has also been included in PerformFish Deliverable 3.2 *Epidemiological status of Mediterranean farmed fish* and also a more detailed approach on new vaccines will be launched from PerformFish. Based on this knowledge, general and specific vaccination programs should be developed in the future by the industry. In this case, general and harmonized recommendations for the Mediterranean farmed fish can be established but as the epidemiology of the different diseases presents geographical differences, risks evolve over time and specific recommendations for each place and each moment should be developed. It is highly relevant, for health management in cages to take into account all the strategies and aspects that can reinforce protection aspects. These strategies should be based on the selection of highest protective levels by the vaccine and also the duration of the immunity. These two points are particularly relevant, as the most relevant diseases affecting European seabass and gilthead seabream can affect the stock during a period of 3-4 months following its introduction in cages and IP re-vaccination is technically difficult, as most of the ongrowing farms operate with big-size offshore cages in non-protected areas in frequently changing weather conditions. In the past, at least one commercial oral vaccine against Photobacteriosis was in the market, yet it is no longer available. Relatively low protection levels compared with IP vaccines and also problems related with regulatory gaps concerning their use in feed, together with other constraints probably limited its use at field level.

2.4.2. Sea cage design

With regard to disease protection, cages are the finfish production systems with higher exposure to diseases as they do not offer efficient isolation or containment for diseases and pathogens, whereas in some cases, cages may favor the establishment of pathogens and facilitate the development of diseases. Pathogens in the environment or harbored in wild aquatic organisms (invertebrates, other fish species) can freely enter the cages through the nets by the water flow and they can freely spread in the environment using marine organisms as vectors. This extremely reduced capacity of containment of marine cages is also responsible for a very limited capacity to apply the same prophylactic methods applied at hatchery or nursery levels. A certain containment, under these circumstances, can only be considered from an epidemiological point of view, in terms of site selection, cage distribution, batch management and production processes control and only to mitigate the effects of the diseases at farm level, not to prevent or reduce the risk of the disease. This reduced containment capability of the sea cages is one of the main reasons why salmon production is nowadays developing some new designs in cages to improve isolation mainly to fight against sea lice (partial or complete skirts, snorkels, fully closed

floating cages or "egg-concept") together with medical (treatments) or non-medical (thermal treatments, flushers, lasers, functional feeds and specific alternative feeding strategies, cleaner fish and others) solutions to mitigate the impact of the disease in salmon cages (https://globalsalmoninitiative.org/en/what-is-the-gsi-working-on/biosecurity/non-medicinal-approaches-to-sea-lice-management/).

The increasing impact of diseases (viral, bacterial, parasitic) in salmon industry and the also increasing economic costs of the measures to reduce this impact (vaccination, treatments, specific mitigation strategies) is revealing that diseases are putting salmon intensive production cages in future jeopardy and this is the reason why the salmon industry is now looking at recirculation systems (RAS) as potential alternative for the future, since the isolation and containment level is much higher.

Problems related to predatory birds (see also Section 2.5) should also be considered as a source of stress for the fish (Aguado-Giménez et al., 2018) but also a risk for pathogen transmission. Bird nets are frequently used to minimize predation and stress for fish, but it is important to bear in mind that these nets do not protect against potential transmission of pathogens through bird faeces.

2.4.3. Appropriate configuration of the groups of cages according sanitary risks

The design, distribution and production management of the cages in the farms is also paramount to mitigate the problems and impacts related to diseases. In current seabream and seabass farming in the Mediterranean, the general structure of the farms is based on groups of several floating cages (from 4 to 16 or more), with similar size and shape usually sharing common mooring systems (twin, orthogonal, radial…). Submersible cages are not so common as floating cages. In some cases, two or more groups of cages are placed in the same site, according to the characteristics of the marine administrative concession (size, maximum allowed produced…). In some places, different mooring sites can be found in the same area. Regulations for authorizing cage sites are different in the different EU and non-EU countries, they usually involve different administration levels and take into account health or epidemiological aspects barely or not at all. In wind/rough sea protected areas, smaller cages for fry (2 – 30 g) can be found. Sometimes these cages are located closer to the other cages or sometimes (few cases), cages for recently introduced fry are located in another and relatively distant place. In offshore sites, fish tend to be immersed at higher weights (15-30 g) as fish are more adapted to demanding conditions (stronger water currents), feeding is easier and the required mesh size is higher, reducing the problems of net clogging by fouling. As concession-operated aquaculture sites tend to be quite restrictive in terms of available space, cages tend to be concentrated with reduced distances between them, even if the fish sizes, fish species or the production batches are different. Under these circumstances, sizes, batches and sometimes species are not reared in different areas and frequently new batches are placed according to the availability of free cages following harvesting or grading. This situation leads to placement of new batches of young fry in the vicinity of cages with older fish, exposing the young ones (more sensitive) to a higher risk of disease transmission, as older fish (more resilient) can be pathogen carriers. This is a frequently observed scenario and is one of the main problems associated to the development of disease outbreaks in the Mediterranean gilthead seabream and European seabass production.

To reduce or minimize the disease risk and the intensity in the expression of the outbreaks, some simple but efficient principles should be considered to organize the structure of the cage sites. All these principles are partially described in the section 4 of the Aquatic Animal Code (OIE), particularly section 4.6, although the OIE is focused on the management under national and international regulations, not at farm level. Alternatively and in absence of specific regulations, Mediterranean finfish farming industry may play a relevant role in the promotion and coordination of these and other similar measures for certain areas.

All in-all out: This is a common strategy in animal farming based in the presence of a single batch of animals in the same place and at the same time and the complete emptying of the farming site after the end of the production cycle, allowing sanitization of the site before introducing a new batch. This strategy tend to guarantee the homogeneity of the stock in terms of health status (including age, origin, immunological level and disease background), avoiding cross-infections with animals from other batches, potentially with different immunological status and disease background. However, with the current size, design and characteristics of the Mediterranean fish farms (very large production capacity, many cages in the same area, requirements for continuous market-size supply and harvesting in the same farm) it is very difficult for nurseries and pre-ongrowing farms to produce at the same time very large batches of fish (several millions) to be immersed in a single farm. To sort out this problem, smaller production units, distant from each other could be a recommended strategy. However, the limited availability of places for finfish production in most of the Mediterranean countries and the increase of the production costs related to the dispersion of the different sites is a huge handicap in the current conditions in most of the Mediterranean countries. Generally, best methods are all-in all-out followed by fallowing period (2-3 months).

Year-class separation: It is a similar strategy as all-in all-out, but focusing on the different characteristics of the animals related to size /age. Particularly, in gilthead seabream and European seabass, young animals (2-50 g) have been reared in nurseries or pre-ongrowing sites under certain protective conditions and are moved to a most thought and demanding environment. For these reasons, during the first months, these fish are much more sensitive to environmental and farming conditions than older/bigger fish. Also, these fish face more risks associated to diseases present in the environment and are only partially protected against the specific diseases included in their vaccination programs applied during nursery/pre-ongrowing phase. Together with wild disease agent carriers (wild fish and other animals), older fish are a very relevant risk for these juveniles, as these fish usually are carriers of these pathogens and although they do not become sick, they can transfer these pathogens to the young fish. At this regard, one of the main sources for *Sparicotyle chrysophrii* infection in juveniles are the eggs and miracidia released by the infected older fish reared in neighbor cages. For this reason, if possible, cages should be grouped in the same site according to the size of the fish and with some distance between these groups of cages.

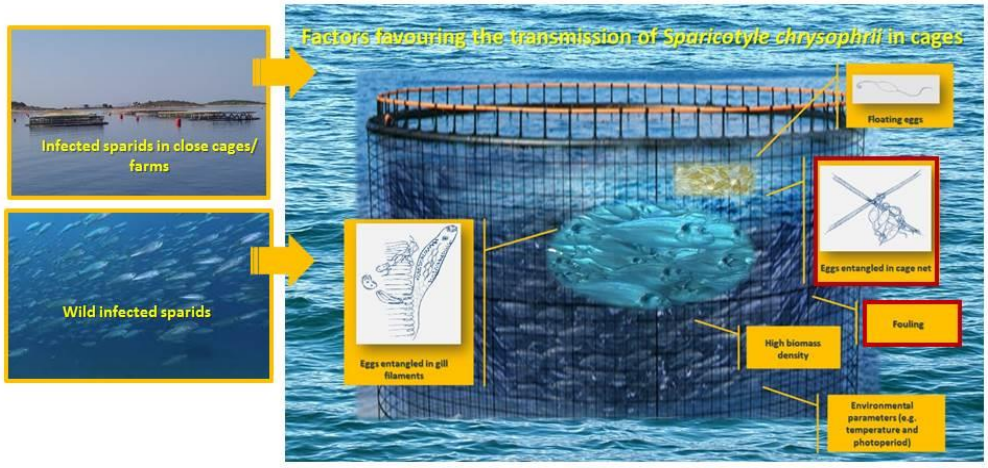
Other strategies that can be applied are **species rotation** (this mean, alternation of species as a method to reduce the impact of those diseases that are species-specific such as for example parasitic diseases due to monogeneans) **or site rotation** (this mean, several mooring sites, only one site in production). This concept is also strongly related to the fallowing periods, as in the temporal absence of the target animals for the pathogens (seabream and seabass), pathogen load tends to be progressively reduced. Fallowing also falls within the sanitary break concept

but it is not very realistically applicable due to the frequent space limitations in the Mediterranean.

2.4.4. Regular control of the fouling in the nets and structures (rings, ropes…), net cleaning and/or net substitution

Biofouling in the nets and cage structures can be a serious source of problems in Mediterranean aquaculture. Nets should periodically be cleaned or replaced because of the negative effects in the restriction of water exchange due to net occlusion, cage deformation and structural fatigue due to the increased weight of the structures but also for the disease risk associated to the presence of organisms in the fouling acting as potential reservoirs for pathogens (Fitridge et al., 2012; Pietrak et al., 2012). Therefore, fouling removal has relevant issues in the reduction of the risk of diseases. Fouling is an accumulation of different biological organisms including macrofouling (seaweed, cnidarians, bryozoans, mollusks, polychaetes, crustaceans, …) and also, microfouling (bacteria, protozoa). Bacteria (including pathogenic bacteria) can be found in the microfilms attached to structural surfaces and also can be found associated to macrofouling (externally attached, in the digestive system of filtering macrofouling) and some pathogenic protozoa can also survive as microfouling. Macrofouling can also act as reservoirs or intermediate host of fish pathogens and can also facilitate the attachment and permanence of reproductive stages of parasites. Particularly, in Mediterranean aquaculture, periodical removal of fouling can be relevant for the control and prevention of specific pathogens. In particular, mussels are capable of bio-accumulating bacteria such as *Vibrio anguillarum* and shedding virulent bacteria through their feces (Pietrak et al., 2012). Furthermore, clams were able to release infectious NNV via fecal matter and filtered water (Volpe et al., 2017). Also *Sparicotyle* and other monogenean eggs can be entangled in filiform biological structures. In some cases, net filaments (and mainly in damaged multifilament nets) can also increase the risk of egg attachment, so in addition to the increased risk of breakage, periodical net replacement is also recommended.

***Sparicotyle chrysophrii* in gilthead seabream: among factors favoring infection in cages, net fouling with presence of a high number of entangled eggs is of primary importance.**



Source: University of Bologna

2.4.5. Equipment disinfection

The absence of isolation conditions in the farms operating with cages makes internal disinfection procedures very inefficient compared with other facilities. Basic cleaning operations in order to prevent fouling development and to maintain hygienic conditions in the cages, anti-bird nets, boats, feed barges, feeders and diver's equipment can be enough in terms of prevention measures in the farm. However, a different approach should be taken if boats, machinery or diving equipment is also used in other facilities. In this case, the potential spread of pathogens to other facilities should be considered. A more detailed explanation about concepts on cleaning and disinfection of boats is detailed in the section related to transportation.

2.4.6. Interaction with wild fish and other aquatic animals

Particularly for ongrowing in cages, and in addition to piscivorous birds, interactions with other aquatic species (fish, marine mammals) can be a health-related problem. It is well known that relevant communities of wild fish and also fish escapees are usually found around cages, mainly due to the attraction by the feed pellets and particles lost during feeding operations. This specific problem is also well addressed in ParaFishControl H2020 project. In some cases, these fish communities are observed only around cages but some small-size species (bogues, sardines, round sardinellas and others) can also enter into the cages. Furthermore, some fish species such as blue fish (*Pomatomus saltatrix*) and greater amberjack (*Seriola dumerili*) can act as predators (Miralles et al., 2016) causing damage in the net structure and entering into the cages and feeding and stressing European seabass and gilthead seabream stocks. All these interactions can be a source of potential problems such as predation and stress but also disease transfer. Vicinity and/or contact with wild fish around the cages on in the water bodies from where water is pumped can act as a pathogen carrier. Prevention methods (specific external nets) and/or other instruments (ultrasonic deterrents) for keeping away seals, dolphins, tuna, amberjack attacking nets must be considered.

2.5 On growing in tanks/ponds

As for cage farming, these relevant factors should also be considered as paramount in inland ongrowing:

- Health quality of the fry/juveniles
- Facility design
- Design of the farm in order to concentrate batches in the same group of tanks/ponds/RAS systems
- Sanitisation / Cleaning / Disinfection in ponds / flowtrough tanks / RAS: needs and procedures
- Equipment (sorting equipments), nets
- Fry transport and harvesting transport.

Each of these factors will be detailed in specific sub-chapters or in the next section.

In inland on-growing operations, the exposure to environmental conditions is not so high as in sea cages. However, there are also differences related to the system used (intensive farming in concrete raceways, semi-intensive in ponds, extensive in lagoons). For outdoor ponds/raceways/tanks risks are mainly related to the source and the quality of the water used as possible pathogen entry in each farm. In addition, other risks associated to the entry of pathogens through biological vectors (piscivorous birds, animals) or by physical means (wind, materials), and the pathogen transmission between production units can be considered less relevant than in cages, as there is no continuity in the water bodies unless water reuse or recirculation. Concerning the source of water, the risk of pathogen entry is highly related to factors such as geographic and oceanographic characteristics of each site, presence of human or fish farming activities in the area and whether water is collected from the open sea, pumped near the shore or water supply comes from wells. For specific pathogens, it is obvious that the presence of other farms in the same area can increase the risk of presence of pathogens in the area. This is a similar epidemiological risk as already described for farms operating with floating cages. Saltwater wells (from marine saltwater intrusion) are considered much safer in terms of biosafety, as marine saltwater intrusion advances through coastal grounds. These grounds may have different geological origins and can be formed by different kind of materials (mud, sand, gravel, rocks…). These materials provide natural filtration to saltwater intrusion and this is the reason why saltwater from wells frequently presents biological characteristics comparable to sterility conditions.

Amongst coastal piscivorous birds, in Mediterranean environment, different species* of seagulls (*Chroicocephalus ridibundus, Chroicocephalus genei, Larus melanocephalus, Ichtyaethus auoduinii, Larus michahellis, Larus argentatus*), pelicans (*Pelecanus onocrolatus, Pelecanus crispus*), herons/Ardeidae (*Ardea cinerea, Egretta garzetta*) and cormorants (*Phalacrocorax carbo, Phalacrocorax aristotelis* and *Phalacrocorax pygmaeus*) are frequently found in coastal areas of the Mediterranean. In some cases, these species have relevant impact in the finfish farms (inland farms and cages) as predators, stressors (Aguado-Giménez et al., 2018) and obviously can also be vectors for diseases. The particular role of cormorants is presented in a report prepared by Ian G. Cowx for the EU entitled '[Between fisheries and bird conservation: the cormorant conflict]' where descriptions of the interactions between cormorants and aquaculture are provided, but there are no particular views on Mediterranean Aquaculture. In the same way, in the [2014 FEAP report], there was a reference on the impact of cormorant populations mainly for freshwater aquaculture.

Nowadays, this is also a problem on marine aquaculture, including cages and inland facilities, although some changes in the behavior concerning aquaculture facilities have been detected in the West Mediterranean (Pablo Sanchez-Jerez, personal communication).

A particularly different approach should be used in those farms operating recirculation systems (RAS) and is based in the same principles indicated in the section of broodstock management. Recirculation systems allow a much more strict level of biocontainment and can substantially reduce the risk of pathogen entry if specific and efficient disinfection measures are incorporated in the design of the recirculation system. In this case, it is absolutely necessary to guarantee high levels of disinfection of the inlet water (make-up water). As the total daily water volume required in RAS is much lower than in flow-through systems, disinfection can be easily achieved

by water pre-treatment (heat, chemical) or with a combination of efficient filtration and UV disinfection. Some relatively large parasitic forms (monogenean juveniles and eggs) can be stopped by standard sand filtration (50 microns), but smaller parasites (protozoans, myxosporeans and microsporean spores), bacteria and viruses require much more selective filtration and the use of UV. Periodical tests should be performed to evaluate the proper operation of the system and also the efficacy of the disinfection.

**Species list was kindly summarized by Dr. Carola Sampera, from the Universitat de Barcelona.*

2.5.1. Health quality of the fry/juveniles

As for nursery and pre-ongrowing production systems, relevant health measures should be implemented in tanks and pond production systems in the Mediterranean to assess the sanitary level of the juveniles before their introduction in the tanks and the vaccination scheme is plammed. Both considerations are the same as for ongrowing in cages.

2.5.2 Facility design

For outdoor ponds/tanks, basic protection from strong winds and excessive sunlight is highly recommended under certain conditions. Use of external bird nets protecting the farm perimeter and covering the whole surface of the farm or bird nets over the tanks can reduce the risk of entry of potential pathogens harbored by these birds.

For indoor on-growing farms, facility design should follow the same recommendations as for nursery.

As on-site harvesting can also be performed in these facilities, tanks, nets and devices (fish pumps) used during harvesting should follow standard cleaning/disinfection procedures after each use. However, as fish in contact with these materials are in the end of the production cycle as they are harvested, stunned and slaughtered, these procedures should be performed to assure the hygienic condition of the harvested fish as food product.

Tanks / raceways should be arranged with enough space or physical distance to reduce the risk of casual transfer of fish (jumping) or water (splashes) between adjacent tanks/raceways. This is not so necessary for RAS, as tanks usually work as a single unit in terms of biosecurity.

Inland on-growing for gilthead seabream and European seabass in the Mediterranean normally operates with bigger tanks than in nursery or in large ponds and lagoons (*Valli* in Italy, *esteros* in Spain). As the on-growing period in inland farms is also relatively long and markets require continuous supply of market-size fish, different fish batches originated from nursery supply are present in the farm and also new batches after grading are created. All of them are reared simultaneously in the farm. In some cases, these batches are reared separately or in some cases, fish from different batches but similar sizes / weights (original nursery batches or new batches) are mixed. This problem has already been described in the section on cages. As mixing production batches is widely considered as a potential risk for health, it is highly recommended to keep production batches as much individualized as possible. In few cases, on-growing farms have their own nurseries in the close vicinity of the farm and fish are moved using pumps or other systems. In such cases, physical and operational separation is scarce and if sanitary

measures are not taken, linearity of the process can be altered and the probability of reverse transfer of pathogens from on-growing farm to the nursery can increase. In most cases, fish are mostly transported using fish transport lorries (see also Section 2.6.1). If cleaning and disinfection after transportation is performed under good standards, separation between nursery and land-based on-growing farms adds more health guarantees and reduces the risks mainly for the nurseries.

Land based on-growing farms usually require less strict policies of staff and material internal movements than in nurseries. For biosecurity reasons but also for other reasons (general control, security, theft prevention…), farms should be surrounded by walls or fences, and the entrance of vehicles, materials or people should be controlled and recorded.

Farm usually have big gates or portals to facilitate operations with heavy equipment and the traffic is usually quite busy as in many cases, fish are slaughtered and processed in separate buildings but in the same site. Staff doors can be equipped with specific footbaths, yet bigger gateways with constant equipment movement are much more difficult to control or apply biosecurity measures. The highest risks in terms of biosecurity are taken when materials, equipment or machines have been in contact with fish or seawater outside the nursery and have not been cleaned and disinfected appropriately. The risks are higher in machinery such as fish pumps, fish graders, nets (mainly if they are still wet) and transport tanks, mainly if they are shared with other areas in the farm (pre-ongrowing) or other farms and facilities and also if there are not appropriate cleaning and disinfection protocols or these protocols are not put into effect. Trucks, forklifts and pallet trucks have a lower risk compared with the other machinery that is in direct and frequent contact with water. In any case, movements of materials & equipment should be authorized and supervised by the nursery coordinator. Although the risks are less relevant that in hatchery or nurseries, visits should also be discouraged for land-based on-growing and particularly when operated in RAS. Visitors can be accepted if specific single-use lab coats and shoe covers are used (RAS) or appropriate disinfected shoes or boots for visitors are provided for outdoor facilities.

2.5.3. Sanitisation / Cleaning / Disinfection in ponds / flowtrough tanks / RAS: General hygiene measures and operational procedures- needs and procedures

RAS biosafety levels in on-growing are higher than in flow-through outdoor on-growing. For RAS, general hygiene measures should follow similar protocols as in nursery and broodstock (Section 2.1 and 2.3). Outdoor on-growing in tanks and ponds is much more exposed to environmental changes, so hygiene measures should be adapted to this characteristic. As the size of the tanks is usually bigger than tanks used in nursery and the fish stocks are reared in these tanks for longer time than in nursery, cleaning (tank bottom, walls) is not usually performed until the fish stock is harvested or moved to another tank. After tank is completely emptied, tank bottom and walls fouling, detritus, sediment and mud are removed using standard mechanical cleaning methods (brushes, high-pressure water) and abundant water flow and they are disinfected with standard disinfection methods. For this purpose, bleach or commercial disinfectants used for drinking water deposits are widely used. For larger earth ponds, mud layer tend to be much thicker than in ponds and sometimes should be removed using mechanical shovel excavators. Pond floor and walls should be allowed to dry-out completely under the sun when possible

(Mediterranean weather is favorable for that in most seasons). If it is not possible to dry, then it is necessary to use the classic methods applied in aquaculture for pond disinfection by raising soil pH: burnt lime (calcium oxide) or hydrated lime (calcium hydroxide), or using chlorine as disinfectant with chlorinated lime or high-test hypochlorite (calcium hypochlorite) (Boyd and Queiroz, 2014). Channels and pipes should also be cleaned and disinfected following the same procedures indicated for nursery and on-growing. These procedures should be mandatory after partial or total farm sanitary stops.

Prophylaxis-related measures in ongrowing farms should also be applied to commercial feed storage.

Nursery general hygiene programs and protocols should be designed according to the juvenile production schedules. Production systems without overlapping batches are the best ones to implement efficient hygiene measures. In these cases, when fish are not in the nursery, sanitary stops with accurate drying, cleaning and disinfection protocols can be applied in a more efficient and easy way. Comments on methodology are the same as for hatchery (Section 2.2). If sanitary stops are not feasible, partial hygiene measures should be applied; tools and equipment should be cleaned and disinfected after each use and floors should be cleaned every day. As for hatchery, safety issues should also be assessed (see also Section 3).

In tank/pond on-growing systems, specific operations such as classification and re-vaccination should be performed according the same recommendations as in the nursery (Section 2.3).

2.6 Live fish transportation: hygiene and prophylaxis concepts

Live fish transportation is a common activity in Mediterranean aquaculture. This activity is mainly focused on fry/juveniles transportation from nurseries/pre-ongrowing sites as only very few nurseries are placed in the vicinity of land based or floating cages ongrowing farms. Although fry can also be transported by well boats, only a small percentage of the seabream and seabass are transported using this system and most of the fry transport is conducted by trucks. This is the current scenario for the Mediterranean industry, but taking into account the relevance of well boats for fish transport in salmon aquaculture, both transport methods will be considered in this section.

2.6.1. Overland transport by trucks/lorries

Gilthead seabream and European seabass are transported in specific fish transport trailers/semitrailers specifically designed for this purpose. Each trailer has several tanks mounted on the trailer structure, each one with a hatch usually on top for fish loading, inspection and cleaning operations and a valve used as fish/water discharge point. In most of the trailers, individual temperature and oxygen sensors/probes are installed in each tank and measures can be monitored and tracked by the transporter. Tanks are made of fiberglass or plastic and usually have thermal insulation and hatch and valves are watertight. Trailers receive oxygen supply by several oxygen cylinders. In most of the trailers, tanks are externally mounted but in few of them, tanks are allocated inside a refrigerated van trailer. Some trailers may have small filtration/recirculation and water cooling/heating systems incorporated in the structure of the trailer.

Concerning prophylaxis, a relevant point should be stressed concerning the responsibility and control of cleaning and disinfection procedures. In many cases, fry are transported by independent fish transport companies. These companies offer their services to the fish farms and transport fry batches from the nursery or pre-ongrowing site to the ongrowing site. Trailers are normally cleaned and disinfected on site in the company according to their protocols before fish is loaded in the nursery/pre-ongrowing site. National legislation on biosafety in livestock transportation is developed in most EU countries following REGULATION (EU) 2016/429, and focuses mainly on general procedures for terrestrial animal transportation and not as much on specific aquatic animal transportation. For this reason, more specific and detailed cleaning and disinfection protocols and procedures are necessary. After delivering the fish, trucks can return to their basis or sometimes, to save time and money, have sequential transportations, with a new fish load in another fish farm and sometimes in a different company and even, a different fish species. In this case, they have to proceed with on-site post-download disinfection procedures, in intermediate disinfection stations. It is not recommended to proceed with cleaning and disinfection procedures in the destination farm. In other cases, farms own their trailer and truck fleets and usually they only operate internal trips. Therefore, cleaning and disinfection is a crucial point in the live fish transportation due to very frequent turnover of batch upload and download and in some cases, the frequent transport of live fish from different origins and even, fish species.

Due to the design of transport tanks (usually small hatches, difficult access to the tank internals, presence of valves and pipes) cleaning and disinfection is particularly difficult. In order to guarantee cleaning and disinfection efficacy, the following risks/recommendations should be taken into account:

-Presence of dead spaces or cavities in the tank: corners, surfaces in the upper part of the tank near the hatches or near the valves. For this reason, high pressure cleaning and disinfection systems using mobile heads are highly recommended. High-temperature and high-pressure cleaning and disinfection systems, similar to those used in food industry are also a very efficient system, as they can perform cleaning and disinfection all-in-one.

-Water discharge and biohazards: after fish download, faeces, scales, dead fish and detritus can be accumulated in the bottom of the tank. These materials impose a very high biosecurity risk. If possible, on-site tank cleaning should not be performed in the farm after download and should be done in appropriate truck cleaning stations, where water effluents are discharged to normal urban drainage systems.

-Rinsing should be performed only with tapwater freshwater, not with saltwater as salt can affect disinfection efficiency.

-It is important to highlight that in aquaculture, biohazard is concentrated in tanks and pipes system, not in the trailer or truck structures. Therefore, standard cleaning and disinfection measures (wheels or underneath the truck and trailer) are not relevant.

2.6.2 Well boats/ ferries

Well boat transportation is at this moment only scarcely used in the Mediterranean industry (European Commission, 2017; Papaharisis, personal communication). However, well boats are

widely used in the salmon industry, mainly in Norway, for slaughterhouse transportation and for smolts. A detailed explanation of the risks and strategies related to the use of barges and well boats are detailed in Lyngstad et al. (2015). Fouling in the boat keels and also water ballast can be considered as two main risks for pathogen biohazard.

In many cases, mainly in East Mediterranean and in island-based farms, trucks and trailers are transported by ferry and sometimes small ferries can moor in the vicinity of cages. In this case, both well boats but also ferries can be considered as an alternative. Boat keels can be covered with fouling and pathogens attached to this fouling and these pathogens can be transported to other facilities and sites if efficient cleaning measures are not adopted. When possible, ships that are moved to another place should be sent to a dry dock and keels should be cleaned and disinfected accordingly. Ballast water discharge is also widely recognized as a main risk for dissemination of biological material. For long-distance maritime transport, ballast water is a relevant source of transfer of non-native, exotic species. For aquaculture, ballast water can also be a source of transfer of pathogenic organisms from different sites. Well boats are also a risk in aquaculture for disease dissemination. Particular considerations will be developed in the following section on hygiene and prophylaxis in fish transportation.

3. General procedures for sanitization, cleaning, disinfection and sterilization

3.1. Main concepts

Sanitization, cleaning, disinfection and sterilization are different forms of the same concept related to prophylaxis: reduce or eliminate the presence or establishment of potential hazardous organisms or their habitats. Each one achieves different level of prophylaxis and also each one requires different levels of resources to achieve results. These differences can be easily understood at domestic level, where floors can be simply swept (sanitization), cleaned using a mop, water and commercial cleaner (cleaning), disinfected (hospitals) using a specific mop and specific floor disinfectants (disinfection) or undergo disinfection & sterilization of surfaces in operating room theatres (sterilization). The same concept can be applied to Mediterranean finfish aquaculture, where the more strict procedures should be applied mainly at broodstock and hatchery level.

For aquaculture, general concepts on disinfection can be found in Torgersen and Hastein (1995), Kasai et al., (2002), Danner and Merrill (2006), and Chapter 4.3 of the OIE Aquatic Animal Health Code (2019). We want also to highlight the excellent technical paper entitled “Farming and health management: Prevention and policy measures” by our colleague Alain Le Breton (Le Breton, 2009). This book covers most of the aspects included here in a particularly didactic and practical approach. It is also relevant to indicate that the book, compiled and edited by Chris Rodgers and Bernardo Basurco, was one of the first documents in prevention and disease management fully focused on Mediterranean Aquaculture.

These concepts are quite similar to the concepts used in the food industry. For a more detailed and comprehensive knowledge and practical use, it is highly recommended to complement this

document with reference works on this topic (hygiene in food industry) such as Sansebastiano et al. (2007).

Sanitization, in terms of aquaculture, should be considered as a first general step, with the main objective being the removal of organic and inorganic materials that can develop, or remains in the different surfaces in contact with water, or in the facilities. In a broad sense, sanitization can be used as a synonym of cleaning. In this section, we address general common dirtiness in the ground, channels, etc originated from the normal activities in the facilities. Food pellets dispersed on the ground is very common mainly in nursery and pre-ongrowing facilities due to little attention during routine feeding or to maladjustments of the automatic feeders. As this material is not attached to the ground surfaces, it can be easily swept away using scrubbing brushes or with a hose flows. A very relevant aspect to take into account is that wet floors are an important risk to be avoided. This is the reason that mainly after cleaning, it is necessary to let the floor dry. If humidity and temperature in the facility does not allow the floor to dry, floor drainers should be used to remove excess water. Another common problem in the different rearing systems is the accumulation of minerals deposits, organic matter and fouling, mainly in those facilities that have not been sanitized for a long time. These materials tend to be tightly attached to the surfaces in contact with water. Pipes and channels difficult to access are the places where these materials are most frequently found. Due to their nature, these materials are much more difficult to remove and require stronger physical or chemical methods to remove. For surfaces, energetic brushwork or high-pressure cleaning may be required. These methods can be applied in the surface of channels, tanks or mid-size ponds.

For pipes and devices with tubular structures, mechanical and chemical processes can be used. Mechanical systems include industrial scraper cleaning methods and high-pressure cleaning (when pipe segments are not too long). Chemical methods include the use of industrial products (most of them based on acids). Chemical methods should be applied in small size pipes and devices such as heat interchangers.

Cages and nets are a particular case for cleaning and disinfection. Net cleaning and replacement has been previously commented in Section 2.4.

Disinfection is the next level up to reduction of the pathogen load. Disinfection can be defined as the process which involves the elimination/deactivation of most pathogenic agents from surfaces or devices. In contrast of the term 'sterilization' (see below), disinfection does not guarantee 100% elimination or deactivation of the microorganisms, but at least, significantly reduces the presence of bacteria, viruses, fungi and other microorganism on the disinfected surfaces and consequently significantly reduces the probability of the presence of certain viable pathogenic agents and thus, reduces the risk of diseases.

Sterilization is the final and stricter step in these processes and is based on the elimination or deactivation of any form of life in a certain space. Sterilization is only necessary in some specific processes at hatchery and laboratory level, but not at normal husbandry level.

- Sanitization/cleaning: removal of organic and inorganic materials from surfaces that may allow pathogens to survive;
- Disinfection: deactivation/elimination of **most** of the potential pathogenic agents;
- Sterilization: deactivation/elimination of **all** the potential pathogenic agents.

3.2. Sanitization /Disinfection methods and processes

For an efficient sanitization and disinfection methods, standard protocols with different steps should be followed:

1st step: mechanical cleaning (solids/dirt removal)

2nd step: rinsing

3rd step: fat and oil removal

4th step: rinsing

5th step: disinfection

6th step: rinsing

Not all the steps should be followed in all the cases. Some steps can be avoided according to the characteristics and level of dirt and also according to the characteristics of the substances used.

3.2.1. Mechanical cleaning

Mechanical cleaning in aquaculture should be performed in facilities were solid deposits are found. These deposits can be found as loose material (organic matter, sediment) or solid materials tightly attached to surfaces (fouling and inorganic incrustations). Loose deposits can be simply removed by resuspension in water and draining, removed using discharge / water drainage valves or by syphoning. Inorganic encrustations and fouling may be removed by physical methods (brushes, high pressure jets, recirculation of cleaning balls or using metal descaling pipe cleaning devices) or using acid solutions. If encrustations or fouling are recurrently observed, specific measures should be taken (if possible) to reduce or minimise its impact through water treatment. These measures can include pre-filtration systems, periodical disinfections (fouling) or keeping high water velocity to avoid stagnant areas. Mechanical filters with small mesh can prevent to some degree ectoparasitic entrance in open flow systems.

3.2.2. Rinsing

Under normal conditions, rinsing should be done using an adequate volume or flow of clean water able to remove dirt, detergents or disinfectant substances previously used. Tap water or water from wells (if hygiene quality is acceptable) can be used. Saltwater can be used only after mechanical cleaning. This is due to the high amount of salt and the potential interference with detergents and disinfectants and also due to the fact that marine water may have different levels of microbiological quality.

3.2.3. Fat and oil removal

In some cases, fats and oils can be present in some parts and structures in the different facilities. Greasy surfaces can be frequently found mainly in nursery and ongrowing tanks, particularly when fish are fed with pellets with high amount of lipids and tanks have not been cleaned for a long time. In these cases, fat removal is recommended. The use of detergents or surfactants has

also been reported to reduce the microbial load, so in some cases, its use may facilitate the removal of bacterial biofilms. In cases that oil is not present, this step can be obviated. In any case, decisions can be taken according to the cleanliness level achieved after cleaning and rinsing.

3.2.4. Disinfection

Disinfection can be achieved using different methods. Two types of methods can be used: physical methods and chemical methods.

3.2.4.1 Physical methods

At practical level, these methods include: drying, sunlight, heat, and UV.

Drying is a particularly underrated disinfection system in aquaculture. Although it is widely accepted that sanitary breaks are a particularly efficient system to eliminate pathogens from aquaculture facilities, the way how sanitary breaks work is not well known. The efficiency of sanitary breaks is related to the combination of two relevant factors: depopulation and drying. Depopulation is particularly efficient for viruses and some parasites, as the survival of these pathogens is related to the survival of the hosts. Drying is also a particularly efficient system, as aquatic pathogens are strictly dependent on the aquatic environment, much more than terrestrial pathogens. Desiccation tolerance is a well-known phenomenon in protists (Potts, 1994) and also in other organisms, and adaptation to desiccation is a main evolutionary mechanism for terrestrial organisms (Alpert, 2006). Viruses may display different degrees of sensitivity to desiccation according to their structure but in general, most viruses are highly sensitive to desiccation. Some aquatic viruses such as Lymphocystis virus and Baculovirus penaei display a relative low resistance to desiccation (Wolf, 1962; Le Blanc & Overstreet, 1991). Desiccation can be an effective method to inactivate in few minutes the eggs of marine monogeneans which would be otherwise resistant to several treatments (Ernst et al., 2005).

Heat is also a common disinfection method used in animal farming and food industry and also another particularly underrated method for fish farming. Small pieces, tools or devices can be disinfected at sterilization grade using an autoclave. However, for tanks, equipment, floor and larger spaces, high pressure steam can be very useful. Moist heat (boiling/pressure steam) is widely accepted as an efficient method for disinfection but not for sterilization. In any case, it is important to highlight that the target in most of the hygiene operations is to achieve an acceptable level of disinfection, not sterilization. **Steam cleaning machines** are widely available and in most cases, steam flow higher than 140ºC have a good cleaning (mechanical cleaning effect by the strong steam flow) and also sanitation capacity (removing biofilms and exhibiting heat bactericidal and viricidal activity). Steam flow can also better access protected internal surfaces such as pipes. However, as it is not possible to standardize the exposure time it is not possible to guarantee a complete sterilization. It is also important to highlight that strong steam sprays can throw out some particles that still contain viable bacteria or virus, so it is important to use this method in all the area to be disinfected. It is also necessary to highlight that steam cleaning is a particularly good choice for environmental reasons as chemical disinfectants are not required and no post-disinfection rinsing is necessary (this is particularly relevant when disinfection is applied directly in contact with aquatic environment, like in cages). New steam cleaning machines are very efficient in terms of of tap water needed to operate. Steam flow use

is also safer, in contrast to dry heat (classic flaming), for many materials (plastic, PVC) that do not resist high temperatures. The use of high-pressure steam is, for example, a particularly good method for cleaning and disinfection of fish transport trailers and well boats.

Sunlight: "Sunlight is the best disinfectant": this is a proverb referring how transparency prevents corruption in an organization, but also is addressing the main reality: sunlight is a well-known, efficient natural disinfectant; a cheap (is for free!), fast, sustainable and environmental friendly method; and something even much more relevant: remember, Mediterranean countries are well known by their sunny weather, so please use it! Sunlight acts through desiccation and also through the effects of sun radiation and there are even some scientific studies on this topic (Calkins et al., 1976) and even at domestic (!) level (Fahimipour et al., 2018). However, the use of sunlight cannot be standardized as it is difficult to control exposure dose/time as it depends on the weather conditions. Normally few hours are enough to dry the surfaces exposed to sun and give an acceptable level of disinfection. Some parts (tanks, tools) can be transported outdoors and exposed to sunlight, weather conditions permitting. Design of roofs in the buildings allowing a certain control of sunlight is maybe another possibility. To sum up, sunlight can be considered as an option, but with limited standardization concerning disinfection efficiency. Coupling this strategy with a wider "green" strategy as the use of sunlight as sustainable energy source for the facility, can also be considered.

Ultraviolet light (UV): the use of ultraviolet light for disinfection is widely known and extensively demonstrated at different levels (medical use, surface and air disinfection in food industry, laboratories using UV lamps and particularly in water treatments). UV is widely used as a method to sterilize drinking water and also in the biological control of wastewater. In aquaculture its use is also quite common (Summerfeld, 2003; Kasai et al., 2002), usually coupled with pre-filtration in land-based open and semi-open systems and in recirculation systems. Water disinfection by UV is the choice method mainly for broodstock and hatcheries as the volume of water to be treated is relatively low. As the water flow requirements increase, water UV disinfection requires higher dimension of the equipment and is much more expensive. Thus, as a strategy presents limited viability for ongrowing systems, unless they are recirculation systems (RAS). Particularly in RAS, UV disinfection devices can be highly efficient when laid in parallel with the make-up water entrance system as the water flow is normally reduced. For the general RAS system, UV can be laid after filtration and biofiltering. In this last case, the main purpose is water sanitization (in terms to keep under control the microbiological load of the water system) rather than disinfection. UV water treatment is a fast and reliable method for disinfection free from residues, chemical toxicity or other impact on the environment. It is also a safe system, as bulbs are placed in closed boxes and UV radiation cannot harm staff. However, the cost of the equipment is relatively high, as are the costs of servicing (UV lamp periodical evaluation and replacement) and electricity consumption. The use of UV is also related to factors such as absence of particles and water transmittance, as these two factors can significantly reduce the disinfection efficiency of the system. For this reason, UV treatments are usually coupled with prefiltration systems. Disinfection efficacy after UV treatment can be easily determined using viable bacteria count methodologies as an indirect evaluation method. Another advantage of UV is the possibility to adjust the required dose. As disinfection is mainly related to the intensity and wavelength of the radiation and exposure time, these two factors can be regulated in the UV device (number and type of bulbs used, water flow). The dosage is usually measured in

microjoules per square centimeter or microwatt seconds per square centimeter (µW.s/cm^2). Microorganisms have different sensitivities to UV radiation and in general, ranges between 2000-8000 µW.s/cm^2 are considered adequate to inactivate most of viruses and bacteria (Yoshimizu, 2009). For zoospore of oomycetes and actinosporean stages of myxozoans, lower doses seem to be sufficient, yet higher doses are required to inactivate pathogenic ciliates in Mediterranean aquaculture such as Trichodinids, Scuticilociliates and ciliate theronts. Particularly high doses (44000 µW.s/cm^2) are required to prevent infection by myxosporeans such as *Kudoa neurophila* (Cobcroft and Battaglene, 2012)

For aquaculture pathogens and particularly for some relevant pathogens in Mediterranean aquaculture, the doses in **Table 1** are recommended. Some of them are already summarized in Litvet et al. (1995).

3.2.4.2 Chemical disinfection: water treatments

Ozone

Ozone is a gas widely used in water treatment mainly for drinking water in Europe and in the U.S.A. Its use related to waste water treatment is expanding to other areas or to provide higher water disinfection in food processing industries, hospitals or other places where high water disinfection standards are required.

Table 1. Recommended UV doses for relevant fish pathogens

General Aquaculture pathogens	**Dose**	**References**
IPN	100-150 mW sec/cm2	Yoshimizu and Kimura, 1986
KHV	4.0 mW.s/cm^2	Kasai et al., 2005
IHNV	1.0-3.0 mW sec/cm2	Yoshimizu and Kimura, 1986
Mediterranean Aquaculture pathogens	**Dose**	**References**
Betanodavirus	290 mW.s/cm^2 200 mW.s/cm^2 100 mW.s/cm^2 150-250 mW.s/cm2 100 mW.s/cm^2 (SJNNV)	Frerichs et al. (2000) Commercial website https://edis.ifas.ufl.edu/fa180 https://edis.ifas.ufl.edu/fa180 Arimoto et al (1996)
Vibrio anguillarum	30 mW.s/cm^2	*references from commercial UV
Trichodinids	35-159 mW.s/cm^2	*references from commercial UV
Cryptocaryon irritans	280 mW.s/ cm^2	*references from commercial UV
Amyloodinium ocellatum	105 mW.s/ cm^2	*references from commercial UV

*https://www.rk2.com/uv-information.php

Ozone is also an emerging water disinfection system in aquaculture (Summerfelt et al., 1997; Kuhn et al., 2017; Powell and Scolding, 2018) and is particularly used in public aquaria since 90's as it can control biological quality of the water, but also provides higher water transparency (a very relevant aspect for visitors) due to the oxidation of organics, increases the oxidation of nitrites to nitrates and avoids the use of other substances. In aquaculture, ozone can be used for water disinfection in the water inlet (as water pre-treatment), for waste water disinfection or at lower doses to keep water microbiota under control (mainly in RAS). The regular use of ozone can also control and reduce the amount or organic matter due to its strong oxidizing properties. Ozone technology for aquaculture is nowadays widely available. Ozone generation units can be installed in the facility according to the design and the needs required. Specialized manufacturers can assist in the selection of the size and characteristics of each unit, where the unit should be placed and with other technological details related to the specific design of the water circuits in the facility. Ozone online measurement is extremely important in order to reduce the risk of excessive ozone levels in the system and adjust the right ozone delivery. Ozone level evaluation is usually done using oxidation-reduction potential meters. If high levels of ozone are used for initial water disinfection, ozone removal systems should be placed before the ozone-treated water is released to the system. Inadequate or excessive levels of ozone (chronic or acute exposures) can be very toxic for fish, producing chemical damage in fish skin and gills due the high amounts of oxidizing radicals present in the water and also can cause chronic stress. For this reason, it is highly recommended to frequently check the values and the proper operation of the oxidation-reduction meters. It is also important to highlight, mainly for Mediterranean aquaculture, that the use of ozone in saltwater induces the production of byproducts such as hypobromite and bromate, that are very toxic even at low concentrations and also these substances are much more stable in water than ozone and tend to accumulate in systems with low water renewal (RAS). For this reason, these two substances should be carefully monitored, mainly in hatcheries operating with RAS systems, as European seabass and gilthead seabream larvae/fry are particularly sensitive to these substances and they can lead to toxicity (Can et al., 2012) or generation of malformations (Ben-Atia et al., 2017) when ozone is used inadequately. Similar caution should be taken if ozone is used to disinfect live prey (rotifers, Artemia) in hatchery. In case ozone is used to disinfect inlet water, efficient ozone removal systems should be used. Removal can be easily achieved using ozone stripping methods such as aeration using columns, in the similar way as nitrogen degasifying columns.

Ozone levels for finfish pathogen inactivation are reviewed by Litvet et al. (1995). Values for Mediterranean relevant pathogens are as follows:

Betanodavirus	3(±0.3) mg/l (egg disinfection)	Grotmol & Totland (2000); Buchan et al (2006)
Vibrio anguillarum	0.12-0.20 mg/l [1] 0.081 and 0.123 mg.min/liter[2]	[1]Litved et al. (1995) [2]Sugita et al. (1993)
Photobacterium damselae piscicida	0.056 and 0.084 mg.min/liter	Sugita et al. (1993)

Oxygen peroxide

Oxygen peroxide (H_2O_2) is also a powerful oxidant, with similar properties to ozone. It has also been used in water treatment but in a lesser extent compared with chlorine and ozone. In

aquaculture is known for its use in external treatments (see PerformFish Deliverable 3.3: best therapeutic practices for Mediterranean farmed fish) but is not regularly used as water disinfectant. However, in particular cases such as disinfection water pre-treatment, oxygen peroxide can be used in high doses without problems, on the condition that residual peroxide after pre-treatment is removed by strong aeration before water is transferred to the aquaculture system. This method is particularly useful when large volumes of water can be stored and requires strong pre-treatments with complete oxidation of any kind of organism or organic compound. As no chemical residues are present (oxygen peroxide breaks into water and oxygen), no environmental concerns are associated to the use of this product. However, stabilizers (type and amount) used in H_20_2 formulations to minimize its decomposition during storage should also be considered.

Electrolysis

The use of electrolyzed water is an alternative to water disinfection. This technology is based on electrolysis of water containing low concentration of sodium chloride (0.1%) in specific devices with a diaphragm or so-called electrolytic cell between the two electrodes, producing high levels of chlorine and also alkaline and acidic water (Rasco and Ovissipour, 2015). As both high or low pH and chlorine have high disinfection properties, electrolyzed water can be obtained in-site in marine farm facilities (saltwater availability) and can be used for water pre-treatment or for surface disinfection. This technology has already been used in agriculture, food industries and also in disinfection in hospitals. The basis of the system is generation of chlorine and hypochlorous acid. Its potential use in aquaculture has been suggested (Jorquera et al., 2002; Katakose et al., 2007; Rasco and Ovissipour, 2015) although, due to the instability of chlorine in seawater and the potential generation of toxic products (bromine) associated to the use of chlorine, the risks of its direct use as disinfectant in the circulating water should be carefully evaluated.

3.2.4.3 Chemical cleaning substances and disinfectants for surfaces, tools and equipment

Chemical cleaning substances: detergents and soaps

As described above in some cases, when fat or oil is present on the surface of tanks or floors, use of specific surfactants / detergents is highly recommended in order to improve the efficiency of the following disinfection actions. A very complete explanation of the characteristics, types, effects and applications of surfactants/detergents is provided by Danner and Merrill (2006) and as no specifications for Mediterranean aquaculture in this section are necessary, we refer to this book chapter for more detailed information. On a practical level, commercial detergents and soaps or commercial products used for sanitization in terrestrial animals or in public health or food industry can be also used for the same objectives and with similar performances.

Chemical cleaning substances: disinfectants

As for soaps and detergents, a very complete description of the different chemical disinfecting agents (acids, alkalis, oxidizers, halogens, phenols, alcohols and aldehydes) used in aquaculture, including relevant information about efficacy and also safety aspects is provided by Danner and Merrill (2006). Therefore, in this document we will only describe the particular and relevant

characteristics of the different substances related to disinfection in Mediterranean aquaculture (see Section 4). Again, it is very important to highlight that some of these substances or agents can be used both for disinfection and also for treatments. Although the chemical substance/molecule is the same, the way of use of these substances may have completely different approaches mainly related to legislation. For this reason and for the frequent misunderstandings on that, it is very important to clarify the following points.

Substances used for cleaning / disinfection are usually commercial products labelled only for this specific purpose: sanitization of surfaces, tools, devices, etc. These products are in general designated in the same categories (chemical products and biocides) and their use is governed by specific legislations at European and also national/regional level. Concerning the EU legislation, these are the main regulations on the use of these products and substances:

- Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 "Concerning the Placing of Biocidal Products on the Market". Official Journal of the European Communities L 123/1 of 24.04.98. 1998
- Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC
- Directive Chemicals Agency 2006/121/EC of the European Parliament and of the Council of 18 December 2006 amending Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances in order to adapt it to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and establishing a European Chemicals Agency
- REGULATION (EU) No 528/2012 of the European Parliament and of the Council of of 22 May 2012 concerning the making available on the market and use of biocidal products.

It is particularly relevant to stress the fact that these products/substances can only be used for cleaning and disinfection of **FOMITES** (Fomites: objects or materials which are likely to carry infection, such as clothes, utensils, and furniture) and they can never be used for a different purpose. This means that according to the current legislation in the EU countries, these products cannot be applied to the fish for disease prevention or treatment. In this legislative context, a key thing to highlight is that everything used to treat fish, even if it is simple freshwater, theoretically should be used on veterinarian prescription.

If the same chemical substance is used for disease prevention or treatment then, completely different specific regulations for medicines apply (see EU regulations summarised in PerformFish Deliverable 3.3).

From this description, it seems crystal clear the different uses have different legislations or at least it is quite clear in terrestrial farming. However, in aquaculture, water and water management is a 'grey zone' as water is the environment were fish and the other farmed aquatic animals live, so the dichotomy between

fomite → disinfection and **fish → treatment**

is not always so clear. How should we describe the microbiological control of water? Water disinfection? Water treatment? and what if we call it water conditioning? All these words are usually used indistinctly but the consideration in one or the other sense has huge implications in many aspects. Maybe a comprehensive way to differentiate the two sides is to consider if the addition of the 'element' (physical or chemical) in the water is in the presence of fish or not. If we 'treat' water and the added substance (if any) is removed before 'treated' water is released to the fish, then it could be considered as disinfection. If the substance is added when the water body is already in contact with the fish then it should be considered as treatment. However, this apparently clear difference is not so clear if we consider for example the use of chlorine in drinking water or the use of chlorine, bromine, oxygen peroxide, ozone or benzalkonium chloride in swimming pools. In this case, these chemicals are in direct contact with people. Is therefore the concept 'to be in contact with the fish' so essential?

Maybe the most paradoxical and paradigmatic example is the use of oxygen peroxide in aquaculture, that is used as a disinfectant but also for treatments (baths). In these scenarios and although the same substance can be used, the legal status of each substance at EU level and also the availability of the licensed products as disinfectant or as medicines and their application should follow the different national regulations in each EU country. It is very important to highlight that licences and recommendations in one country do not necessarily apply in other EU or non-EU countries.

4. Biocides/ Disinfectants: current knowledge and new approaches in PerformFISH

4.1. Introduction

Biocides/disinfectants are considered paramount in the procedures associated to the routine husbandry and disease prevention.

Although there are some general references on disinfection capacity regarding the main groups of pathogens in Mediterranean marine fish farming, specific data on the efficiency of disinfection against the selected pathogens (Betanodavirus, *P.damselae* subsp. *piscicida* and *V. anguillarum*) in saltwater and surfaces are still required. For this reason, the main aim of this section is to describe the properties of the most reliable biocides and disinfectants for the most relevant pathogens as well as the recommended procedures to ensure efficient disinfection.

In this section, recommendations are based on the collection of the available information (**Table 2**, **Table 3**) on pathogens and disinfectant substances, evaluated by PerformFISH experts (see contributors to this Deliverable). The evaluation was reinforced with the results from laboratory experiments conducted with these specific pathogens and biocides mainly by experts in University of Bologna (Betanodavirus) and Universidad de Las Palmas de Gran Canaria (Bacteria).

Table 2. Disinfectants and doses for Betanodavirus

Substance	Quaternary ammonia	Iodine	Acids (pH 2)	Alkalis (pH 11-14)	Formalin (2%)	Chlorine
Dose/time	50 ppm / 10 min	25-50 ppm/ 30min[1] 100 ppm/ 5 min[1] 100 ppm/ 10 min[2]	15-42 days[1,2]	10 hours-15 days	6 h	25-50 ppm/ 5-30 min[1,2]
Reference	Arimoto et al. (1996)	[1]Frerichs et al. (2000) [2]Maltese & Bovo (2001)	[1]Frerichs et al. 2000 [2]Maltese & Bovo (2001)	[1]Frerichs et al. 2000 [2]Maltese & Bovo (2001) [3]Peducasse et al. (1999) [4]Arimoto et al, (1996)	Frerichs et al. (2000)	[1]Frerichs et al. (2000) [2]Arimoto et al. (1996)

In addition to the current available data on disinfection methods and on the use of disinfectants, PerformFISH focused on the development of two different sets of experiments in order to obtain more specific and detailed data on two relevant aspects:

a) Specific disinfection activity of Virkon against Betanodavirus (studies done by Dr Sara Ciulli and co-workers, Department of Veterinary Medical Sciences, University of Bologna).

b) Bacterial disinfection against Vibrios and *Photobacterium damselae* subs. *piscicida* using paracetic acid, Virkon® and oxygen peroxide in biofilms (studies done by Dr Felix Acosta and co-workers, Universidad de Las Palmas de Gran Canaria.

Table 3. Disinfectants and doses for Vibrio anguillarum

Substance	Chloramine-T	Iodine	Virkon ®	Chlorine
Dose/time	15 ppm (1 min)	50 ppm (1 min)	1% / 1 min	50-200 ppm / 1 min
Reference	Machen et al. (2008)	Machen et al. (2008)	Machen et al. (2008)	Machen et al. (2008)

4.2. Virucidal effect of Virkon S® towards nervous necrosis virus

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER) represents one of the most threatening diseases in the marine aquaculture (Doan et al., 2017). The causative agent of the disease, the nervous necrosis virus (NNV), is a small, non-enveloped, single stranded positive-sense RNA virus belonging to the genus *Betanodavirus*, family Nodavidae (Thiéry et al., 2012). Its genome consists of two molecules of RNA: the RNA1 that

encodes a non-structural protein with RNA-dependent RNA polymerase (RdRp) activity and the RNA2 that encodes the coat protein CP. Based on a partial nucleotide sequence of the coat protein gene, betanodaviruses are divided into four genotypes: Redspotted grouper nervous necrosis virus (RGNNV), Striped jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TPNNV) and Barfin flounder nervous necrosis virus (BFNNV) (Thiéry et al., 2012). Furthermore, reassortment events generated two reassortant betanodavirus strains, the SJNNV/RGNNV containing the RNA1 segment from the SJNNV genotype and the RNA2 segment from the RGNNV-type and the RGNNV/SJNNV whose genome is composed by RGNNV-type RNA1 and SJNNV-type RNA2. Actually, the RGNNV genotypes together with the RGNNV/SJNNV reassortant strain are the viral strains most frequently associated with European sea bass and gilthead sea bream mortality outbreaks in the Mediterranean Sea.

Betanodaviruses are highly resistant in the aquatic environment and can survive for a long time in seawater at low temperature (Frerichs et al., 2000). In intensive aquaculture, where single or multiple species are reared at high densities, infectious disease agents are easily transmitted between individuals (Shetty et al., 2012). Actually, no therapy is available to control VNN and prophylaxis is the best method to reduce its impact in European sea bass and gilthead sea bream farms. Recently, vaccines have been registered to be used in several European countries; however, several constraints limit the success of this tool in controlling the disease at different farm level. Vaccines can be used only in European sea bass, the vaccine protection is mainly towards to the RGNNV strain and they need to be administrated via intraperitoneal injection being usable only when fish weight is approximately 12-15g. For these reasons, direct prophylaxis plays still a major role in preventing NNV infection, particularly at hatchery level. Efforts must be concentrated on the means and tools to prevent entry, diffusion and persistence of the virus inside farms (Shetty et al., 2012; Doan et al., 2017). The use of direct prophylaxis for the control of infectious diseases requires a deeper knowledge of characteristics of pathogen resistance and the availability of products and disinfection protocols specific for the control of pathogens.

In order to evaluate the efficacy of disinfectant towards two different strains of betanodavirus (the RGNNV and the reassortant RGNNV/SJNNV strains representing the viral strains most frequently associated with European sea bass and gilthead sea bream mortality outbreaks in the Mediterranean Sea) *in vitro* trials have been performed for the commercial product Virkon S®, already used in veterinary medicine. Testing was based on the European protocol EN 14675 (**Table 4**), for which the effective standard is a 4 log(10) reduction in the viral titer. The product was tested under different conditions (concentrations, soiling, use of seawater as diluent). Time and temperature were set up at 5 minute at 20°C. Before conducting full-scale trials with a given disinfectant, pre-tests to determine cell cytotoxicity of the product were performed as described in the European protocol EN 14675. Two concentrations of the disinfectant were tested (0.5% and 1%). The presence of interfering substances was tested as low-level soiling (3 g/L bovine albumin solution) and high-level soiling (10 g/L bovine albumin solution and 10 g/L yeast extract).

All tested replicates produced a titer reduction; however, not all of them reached the effective standard requested by the European protocol EN 14675 and equivalent to a 4 log(10) reduction in the viral titer. Particularly, **Virkon S was effective at the manufacturer's recommended concentration 1% w/v at both low and high level soiling,** in fact, all replicates tested at 20°C for

5 minutes, have a titer reduction (TR) ≥ 4 log(10). The concentration of 0.5% w/v of the disinfectant was less effective with a TR≥ 4 log(10) only at low soiling conditions.

Concluding, the tested disinfectant was found to be suitable for NNV inactivation being effective under at least some of the conditions tested. However, the presence of the organic matter and the concentration of the product can significantly affect the result of the disinfection procedures. For these reasons, it is of paramount importance to set up a specific disinfection protocol considering the "cleaning" level that can be reached before disinfection.

Table 4. Efficacy of disinfection with Virkon S for Betanodavirus at two diferent soiling conditions

Compound	Time	Soiling conditions	Recommended Concentration	Dilution	Temperature
Virkon S	5 min	Low: 3 g/L bovine albumin solution	0.5-1.0 %	Sea Water	20°C
		High: 10 g/L bovine albumin solution and 10 g/L yeast extract)			

4.3. Effectiveness of three disinfectant substances on several *Vibrio* species and *Photobacterium damselae* subs *piscicida* and their biofilms in aquaculture

Bacteria are a common target of most of the disinfection protocols. To guarantee an efficient disinfection, these protocols are based on previous data on disinfection efficacy of different substances. Most of these data are obtained from laboratory tests, using classical microbiological techniques with exposure of bacterial suspensions to different concentrations of the disinfecting substances. However, specifically for bacteria and in the finfish rearing facilities, potentially harmful bacteria are usually found forming biofilms on the wet surfaces or on the surfaces in constant contact with water. Biofilms are syntrophic bacterial communities of aggregated bacteria and other microorganisms, attached to surfaces and protected by a self-produced matrix of extracellular polymeric substances. These matrices contribute with an added protection to these bacterial communities against a large number of changes of the external environment. Aquatic biofilms are particularly relevant in marine environments (de Carvalho, 2018). As biofilms play a major role in the permanence of potential bacterial pathogens in the facilities, the extra protection given by the biofilm matrix should also be considered in the assessment of the disinfection procedures. For this reason, specific studies on the effectiveness of disinfectant treatments against bacterial pathogens and its biofilms have been developed for PerformFISH in the Universidad de Las Palmas de Gran Canaria under the supervision of Dr Félix Acosta.

In this study, susceptibility of biofilms of several Vibrio species and *Photobacterium damselae* subsp. p*iscicida* to three disinfectants at different concentrations were tested. The aim of this study was to investigate the susceptibilities of fish bacterial pathogens and biofilms to different concentrations and exposition times of three disinfectant agents, two commonly used substances, peracetic acid (PAA) and Hydrogen peroxide (H_2O_2) and a commercial product, Virkon S.

4.3.1 Material and Methods

Bacterial strains, culture media and culture conditions

The microorganisms used in this study were *Vibrio anguillarum* (strain L-501), *Photobacterium damselae* subsp. *piscicida* (strain MED-1), *Vibrio harveyi* (strain SA-1) and *Vibrio algynoliticus* (strain SB-2). All strains have been previously isolated and identified in the ULPGC laboratory by classical microbiology and also PCR. The subcultures were prepared from stock cultures stored at -20°C, by inoculation in Brain Hearth infusion Broth BHIB (BHIB; Pronadisa, Spain) and incubated at 25°C for 24 h in aerobic and static conditions.

Chemical agents

Three chemical agents have been tested: Virkon S (Bayer Laboratories, Spain), Peracetic acid (PAA) (Sigma-Aldrich Chemical, USA) and Hydrogen peroxide (H_2O_2) (Sigma-Aldrich Chemical, USA). The disinfectants were used at different concentrations and times presented in **Table 5**.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines by two-fold serial broth microdilution (Clinical and Laboratory Standards Institute 2009).

Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) determination

MBIC and MBEC experiments were performed using a previously reported method (Reiter et al., 2013).

Table 5. List of disinfectant compounds tested at different doses and times

Compounds	Time	High-level soiling conditions	Recommended Concentration	Dilution	Temperature
Virkon S	1.5 and 10 minutes	10g/l yeast extract plus 10g/l bovine serum albumin solution)	0.5-1.0-1.5 %	Sea water	25ºC
Hydrogen peroxide			2.5-5-10 %		
Peracetic acid			0.0005-0.001-0.05%		

Test conditions for evaluating bactericidal activity in planktonic cells

For evaluation of bactericidal activity on each product against bacterial diseases of marine aquaculture, the minimum bactericidal concentration was used. Specifically, temperature was set at 22 ± 1°C and the contact time set as 1, 5 and 10 min ± 10s. Bacterial suspensions were prepared at a density of 1 x 10^8 CFU/ml and all products were tested in sterile seawater. Different concentrations of each product were prepared in sterile seawater.

Each test procedure involved adding 1.0 ml of interfering substance (10 g l^{-1} yeast extract plus 10 g l^{-1} bovine serum albumin solution) to 1.0 ml of a bacterial test suspension in a sterile glass container, after 8.0 ml of the product (at desired concentration) was added and the mixture was briefly vortexed. The mixture was incubated at 22 ± 1°C for 1, 5 and 10 min, following bacterial counts measured in agar plate. Colony forming units (CFU/ml) were converted into log10 CFU. Control tests were processed following a similar protocol, and excluding the use of the disinfectant product.

Test conditions for evaluating bactericidal activity in bacterial biofilms

20 µL of bacterial suspensions at a density of 1 x 10^8 CFU/ml were added to 180 µL BHIB, placed into a 96-well polystyrene flat-bottom microtiter plate (SPL), and incubated at 25°C for 24 hours without shaking to allow for bacterial attachment. The broth (containing non-adhered cells) was removed from each well and the plates were rinsed two times with 150 µL PBS. The dilutions of each chemical agent were prepared in a second microtiter plate with BHIB and were added to each well of the first plate in which the biofilm was formed. The mixture was incubated at 22 ± 1°C for 1, 5 and 10 min, following bacterial counts measured in agar plate. Colony forming units (CFU/ml) were converted into log10 CFU. Control tests were processed following a similar protocol, and excluding the use of the disinfectant product.

Statistical analysis

Surviving populations of bacteria were reported as the median CFU/ml from 3 replicate samples. For statistical analyses, CFU/ml values were log transformed to perform Tukey's Honestly Significant Difference (HSD) tests among different disinfectant types and time of exposure (minutes) on the reduction of bacterial growth. For all multiple comparisons and statistical tests, a *p* value of 0.05 was used to determine significance. All statistical analyses were performed using the Social Sciences (SPSS) software version 23.0 (SPSS, Chicago, IL, USA).

4.3.2 Results

Susceptibility of cultures to disinfectants

In the susceptibility test for Virkon S, the MICs of the marine pathogen were similar and in the range of 1% (**Figure 1A**), the MBCs were 1% for all except *Photobacterium damselae* subsp. *piscicida* that was 2% (**Figure 1B**).

In the susceptibility test for hydrogen peroxide, the MICs of *Vibrio anguillarum*, and *Vibrio algynoliticus* were similar and in the range of 5% (**Figure 1A**), whereas for *Photobacterium damselae subsp. piscicida* the MIC was 10% and for *Vibrio harveyi* the MIC was 2.5%. The MBCs were 5% for *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida,* and 10% *for Vibrio algynoliticus* and *Vibrio harveyi* (**Figure 1B**).

In the susceptibility test for peracetic acid, the MICs of *Vibrio anguillarum* and *Photobacterium damselae* subsp.*piscicida* were similar and in the range of 0.0005% (**Figure 1A**). *Vibrio harveyi* and *Vibrio algynoliticus* showed a MBC of 0.001% and *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida* showed a MBC of 0.0005% (**Figure 1B**).

Bactericidal effect of MBCs of disinfectants in cultures at different times of exposition

At 1 min, none disinfectant showed zero counts of CFU/ml but we have observed a statistically significant reduction of counts ($p < 0.05$) in the peracetic acid treatment with respect to the other two disinfectants **Figure 2**). Increasing time to 5 min resulted in zero counts for peracetic acids with statistically significant reduction of counts ($p < 0.05$) with respect the disinfectants (**Figure 2**). After 10 min of exposure, two disinfectants (Virkon S and Peracetic acid) were able to reduce the count to zero not showed statistically significant differences and Hydrogen peroxidase didn't reduce the count to cero and show statistically significant differences with the other treatments. Interactions between compounds, and times are shown in **Figure 2**.

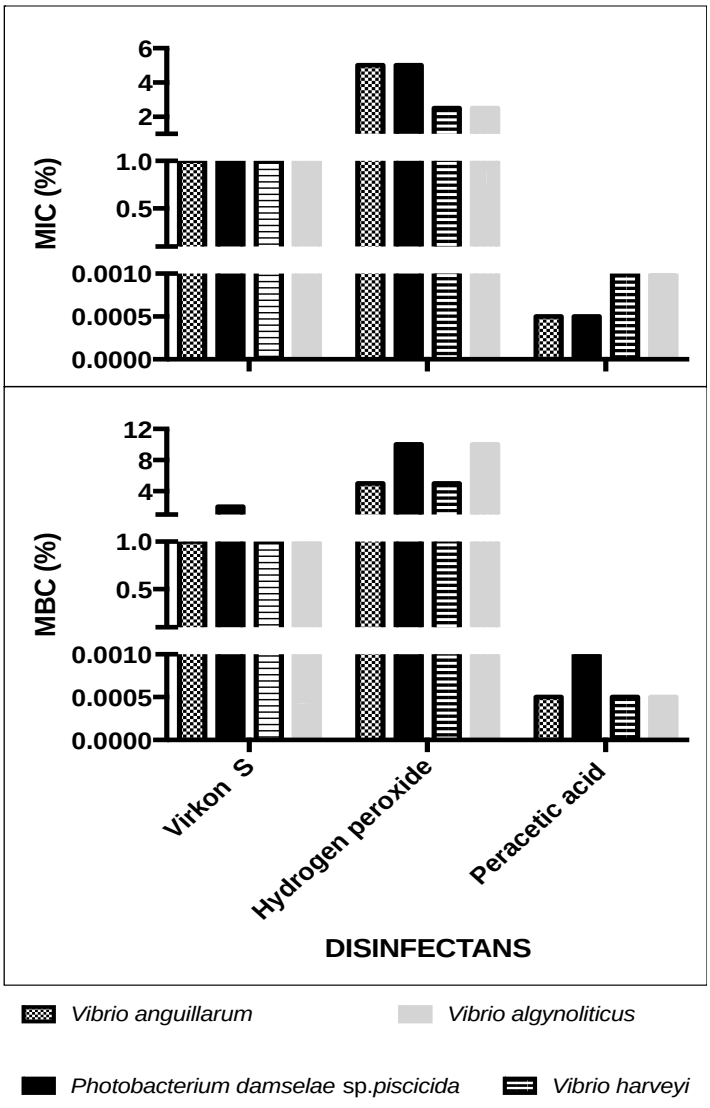


Figure 1. MIC and MBC values of the different disinfectants.

Susceptibility to disinfectants on biofilms

In the susceptibility test for Virkon S, the MBICs of *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida* were similar and in the range of 0.5% (**Figure 3A**), while for *Vibrio harveyi* and *Vibrio algynoliticus* the MBIC was 1%, for *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida* the MBECs was 0.5% and for Vibrio *algynoliticus* and *Vibrio harveyi* it was 1% (**Figure 3B**).

In the susceptibility test for Hydrogen peroxidase, the MBICs of *Vibrio anguillarum*, and *Photobacterium damselae subsp. piscicida* were similar in the range of 5% (**Figure 3A**), while for *Vibrio algynoliticus and Vibrio harveyi* the MBIC was 10%. The MBEC was 5% for *Vibrio anguillarum* and *Photobacterium damselae subsp. Piscicida,* while for Vibrio *algynoliticus* and *Vibrio harveyi* it was 10% (**Figure 3B**).

In the susceptibility test for peracetic acid, the MBICs of *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida* were similar in the range of 0.5% (**Figure 3A**) and for *Vibrio harveyi* and *Vibrio algynoliticus* the MBIC was 1%. The MBECs for *Vibrio anguillarum* and *Photobacterium damselae subsp. piscicida* were of 0.0005%, while for Vibrio *algynoliticus* and *Vibrio harveyi* they were 0.001% (**Figure 3B**).

At 1 min, only peracetic acid showed zero counts of CFU/ml against *Photobacterium damselae subsp.piscicida* (**Figure 4B**). It also showed statistically significant reduction of counts ($p < 0.05$) for the rest of bacteria with respect to the other two disinfectants (**Figure 4A**, 4C and 4D). Increasing time to 5 min resulted in zero counts for peracetic acid and statistically significant reduction ($p < 0.05$) against *Photobacterium damselae subsp.piscicida* for Virkon S with respect to Hydrogen peroxidase (**Figure 4B**). Virkon S also showed statistically significant reduction of counts ($p < 0.05$) with respect to Hydrogen peroxidase at 5 minutes of exposition against *Vibrio anguillarum and Vibrio harveyi* (**Figure 4A** and **Figure 4C**). After 10 min of exposure all disinfectants were able to eliminate *Photobacterium damselae subsp.piscicida* (**Figure 4B**). Also, Virkon S reduced the count to zero for *Vibrio anguillarum and Vibrio harveyi* (**Figure 4A** and **Figure 4C**) showed statistically significant differences and Hydrogen peroxidase.

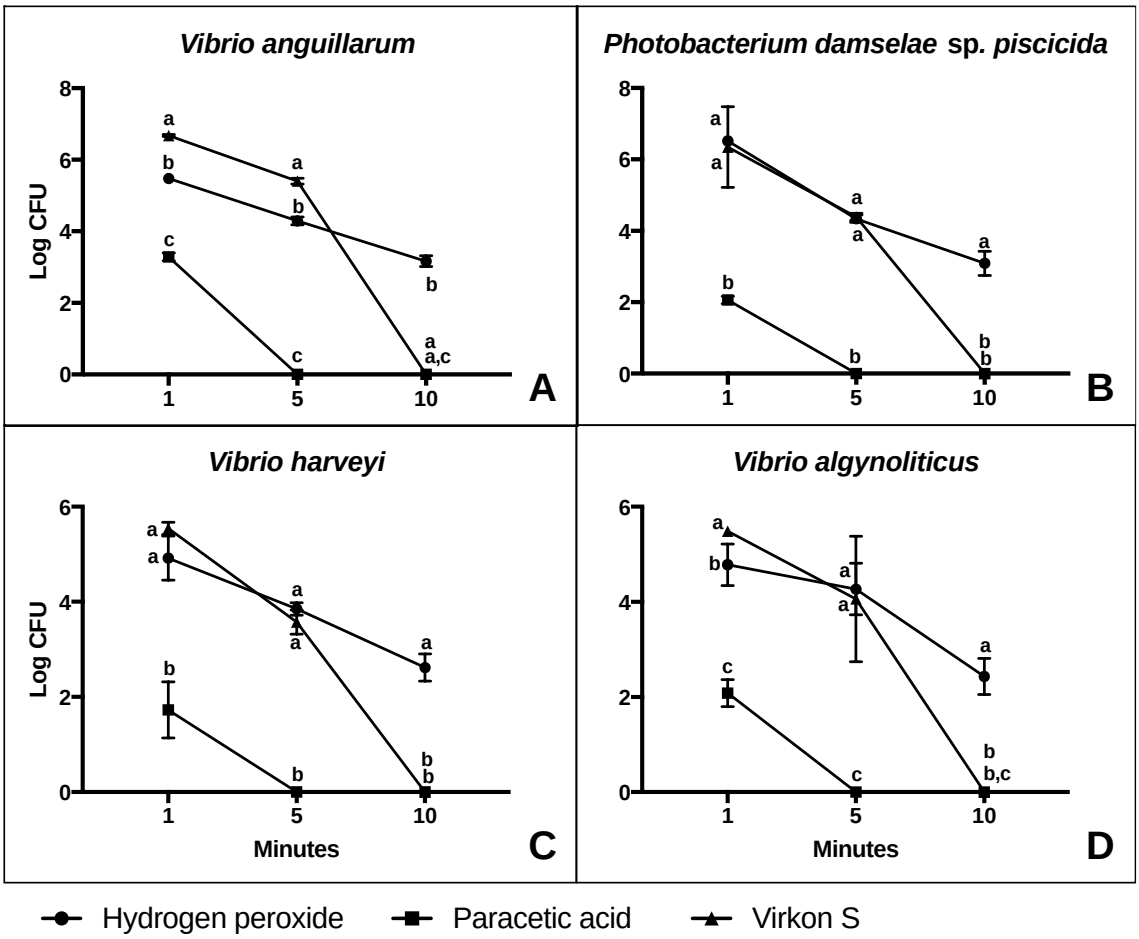


Figure 2. Bactericidal effect of the disinfecting substances/products at different exposure times.

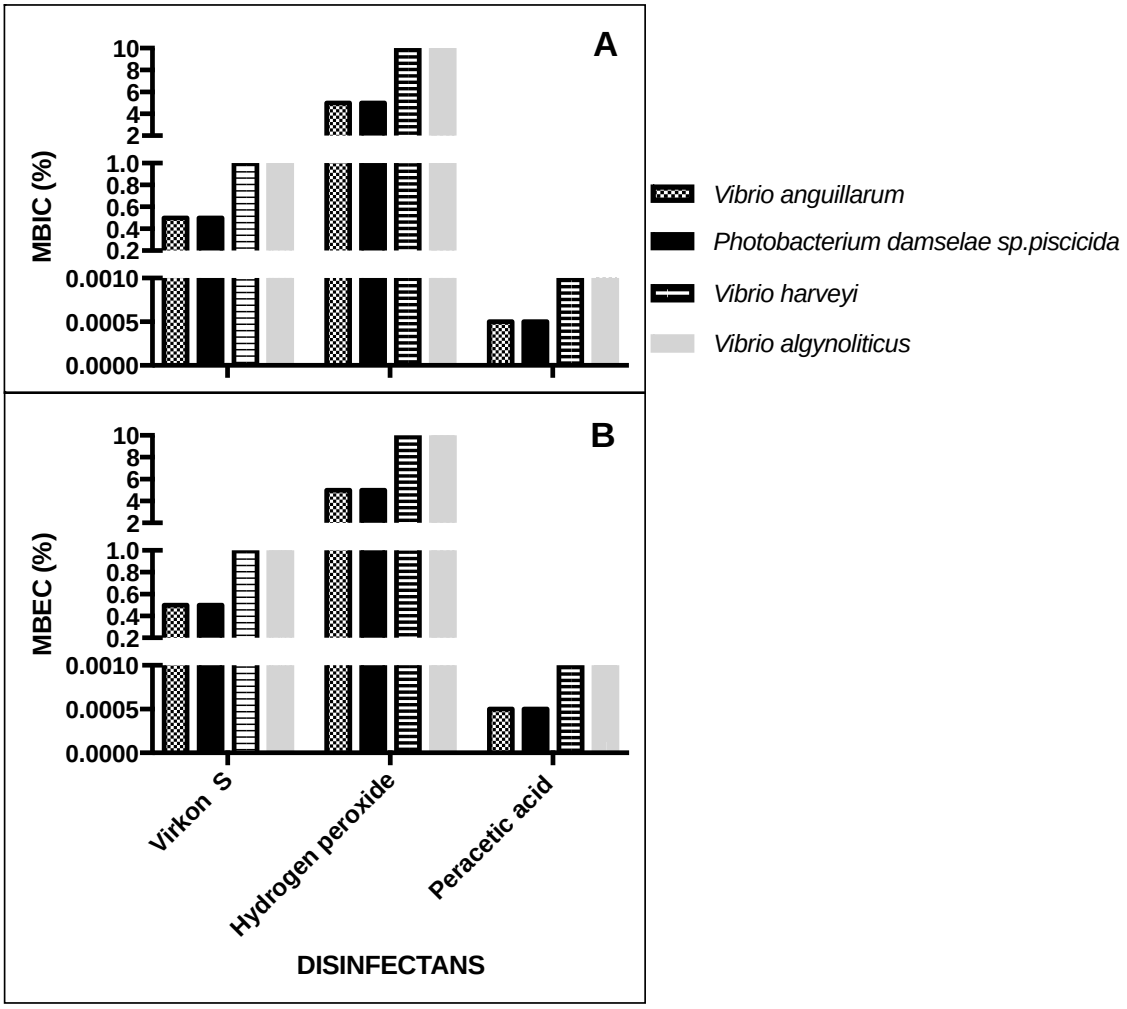


Figure 3. MBIC and MBEC values for the three disinfecting substances/products.

4.3.3 Conclusions

Peracetic acid is the substance that exhibits the greatest disinfectant activity against both suspension and biofilms, due to its strong oxidative activity (Block, 1991), which leads to the destruction of the bacterial cell wall (Straus et al., 2012) and the inhibition of the initial bacterial colonization (Kitis 2004). These results are in agreement on the high efficacy of peracetic acid in the control of other finfish aquaculture pathogens such us *Piscirickettsia salmonis* (Muniesa et al., 2019), *Aeromonas salmonicida* and *Yersinia ruckeri* (Meinelt et al., 2015; Yamasaki et al., 2017), *Vibrio parahaemolyticus* (Wong et al., 2018), and also in disinfection operations in food industry, displaying high efficacy in disinfection of biofilms on dry surfaces (Otterspoor & Farrell 2019) and particularly those of *Staphylococcus aureus* and *Salmonella* spp (Moreno et al., 2018). A reduction of 90% of the total aerobic bacterial load in culture water tanks treated with peracetic acid at a concession of 1 mg / L has also been described (Liu et al., 2018).

Hydrogen peroxide (H2O2) is also a powerful oxidant with activity against a wide range of microorganisms (Sattar et al., 1998). Regarding its use as a disinfectant, it has been tested with different fish pathogens such as Mycobacteria with survival ranges between 12.87% and 100% at a dose of 1.5% for 5 min (Chang et al., 2015); in *Aeromonas salmonicida* and *Yersinia ruckeri* products with higher molecular PAA:H2O2 ratios inhibited bacterial growth with minimum exposure time 5 min (Meinelt et al., 2015); H_2O_2 was the only chemical able to reduce the mortality caused by *A. hydrophila* infections (Pridgeon et al., 2011. In the current study, hydrogen peroxide displayed a lower disinfecting efficacy compared with the other 2 disinfectants tested. The bactericidal effect of H_2O_2 was not achieved in any of the incubation times used, unlike other studies where different degrees of reduction are achieved after 5 min incubation (Meinelt et al., 2015). Studies on the effect on hydrogen peroxide biofilms in other environments have been carried out with pathogens such as *P. aeruginosa*, where its action as a biocide is not clear because it seems that the ability of H_2O_2 to penetrate on the biofilm is very limited (Stewart et al., 2000), which may explain our results obtained for our pathogens with H_2O_2.

Within the disinfectants defined as broad spectrum, Virkon® is used in aquaculture as a disinfectant for footwear, nets, and equipment for protection from bacteria, viruses and one fungus. The efficacy of Virkon has been tested against different pathogens such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Pseudomonas anguilliseptica*, *Renibacterium salmoninarum*, *Vibrio anguillarum* and *Yersinia ruckeri* in different doses from 1: 100 to 1: 1000 (SYNDEL, Aquatic Life Sciences). Virkon® Aquatic has showed different results, with improved effect on vegetative forms (Hernández et al., 2000). Regarding the effect of the Virkon on biofilms, works with *Francisella noatunensis* subsp. *orientalis* have demonstrated the ability of this disinfectant with a MBEC of 1% (Soto et al., 2015).

In conclusion, for the most relevant bacterial pathogens in Mediterranean and Atlantic aquaculture (*Photobacterium damselae* subsp. *piscicida*, *V. anguillarum*, *V. harveyi* and *V. alginolyticus*) used in our work show that

1) Peracetic acid (PAA) is the most potent disinfectant against bacteria in **suspension and in biofilms**.

2) Virkon® has similar effects to the PAA but **at longer contact times**.

3) H_2O_2 display much less efficiency in the disinfection of biofilms.

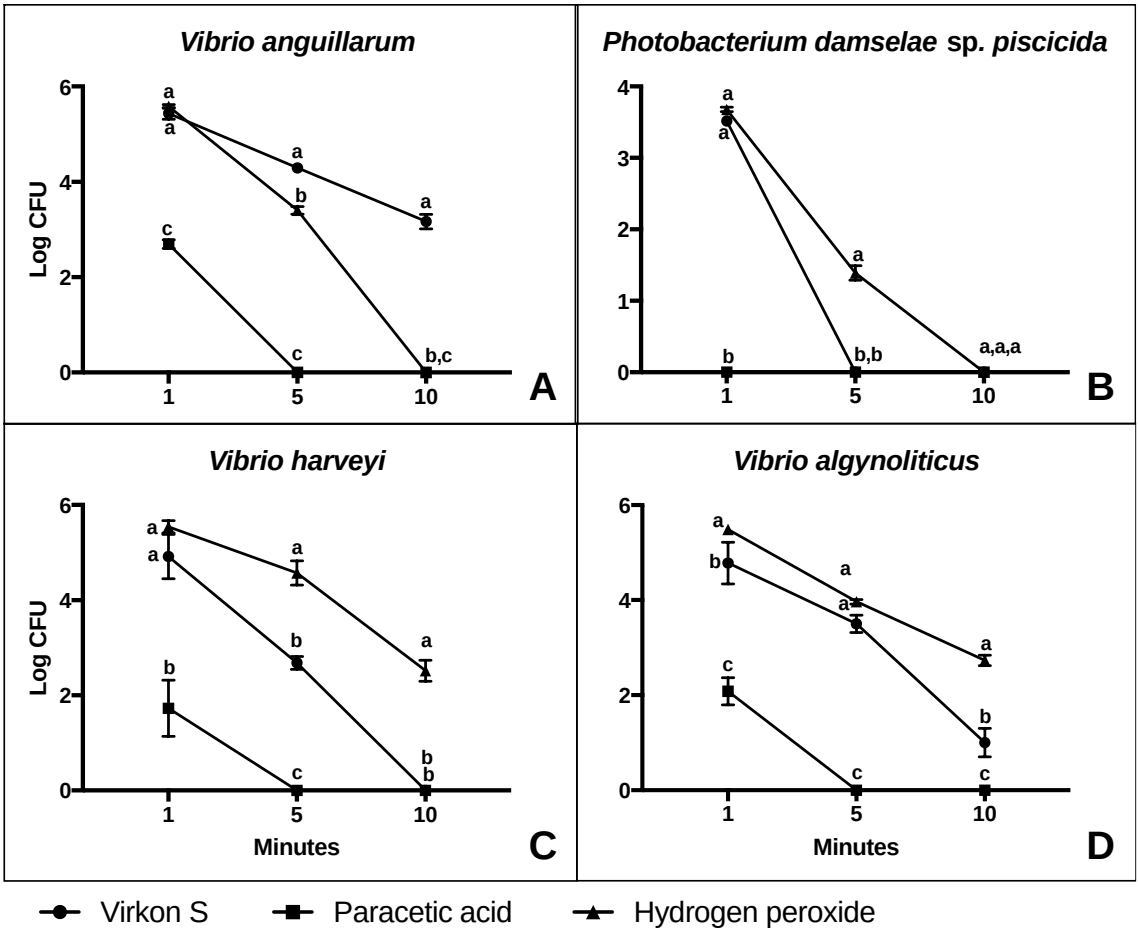


Figure 4. Bactericidal effect of the disinfecting substances/products in biofilms at different exposure times

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5. References

Aguado-Giménez, F., Eguía-Martínez, S., Cerezo-Valverde, J., García-García, B. (2018). Spatio-temporal variability of ichthyophagous bird assemblage around western Mediterranean open-sea cage fish ferms. Marine Environmental Research, Volume 140, 126-134.

Antonello, J., Massault, C., Franch, R., Haley, C., Pellizzari, C., Bovo, G., Patarnello, T., de Koning, D.J., Bargelloni, L. (2009). Estimates of heritability and genetic correlation for body length and resistance to fish pasteurellosis in the gilthead sea bream (*Sparus aurata* L.). Aquaculture, 298(1–2), pp 29-35.

Alpert, P. (2006). Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? Journal of Experimental Biology.209: 1575-1584.

Arnaud, C., de Lamballerie, M., Pottier, L., (2018). Effect of high pressure processing on the preservation of frozen and re-thawed sliced cod (*Gadus morhua*) and salmon (*Salmo salar*) fillets. High Pressure Research, 38:1, 62-79.

Arthur, J.R.; Bondad-Reantaso, M.G.; Subasinghe, R.P. Procedures for the quarantine of live aquatic animals: a manual. FAO Fisheries Technical Paper. No. 502. Rome, FAO. 2008. 74p.

Arvanitoyannis, I. S. and Tserkezou, P. (2013). Irradiation of Fish and Seafood. In Seafood Processing, I. S. Boziaris (Ed.). John Wiley & Sons, Ltd

Aslam, ML., Carraro, R., Bestin, A., Cariou, S., Sonesson, A.K., Bruant, J.S., Haffray. P., Bargelloni, L., Meuwissen T.H.E. (2018). Genetics of resistance to photobacteriosis in gilthead sea bream (*Sparus aurata*) using 2b-RAD sequencing. *BMC Genet*. Jul 11;19(1):43.

Ben-Atia, I., Lutzky, S., Barr, Y., Gamsiz, K., Shtupler, Y., Tandler, A. (2007) Improved performance of gilthead sea bream, *Sparus aurata*, larvae after ozone disinfection of the eggs. Aquaculture Research 38: 166– 173.

Boyd, C.E, Queiroz, J.F. (2014). The role and management of bottom soils in aquaculture ponds. INFOFISH International 2, 22-28.

Buchan, K.A.H., Martin-Robichaud, D.J., Benfey, T.J., , MacKinnon, A-M., Boston, L. (2006). The efficacy of ozonated seawater for surface disinfection of haddock (*Melanogrammus aeglefinus*) eggs against piscine nodavirus, Aquacultural Engineering,Volume 35, Issue 1, pp.102-107,

Calkins, J., Buckles, J.D. and Moeller, J.R. (1976). The role of solar ultraviolet radiation in 'natural' water purification. Photochemistry and Photobiology, 24: 49-57.

Can E, Karacalar U, Saka S, Firat K (2012). Ozone disinfection of eggs from gilthead seabream *Sparus aurata*, sea bass *Dicentrarchus labrax*, red porgy, and common dentex *Dentex dentex*. Journal of Aquatic Animal Health 24: 129– 133.

Can, E., Saka; S. and Firat, K. (2010). Disinfection of gilthead sea bream (*Sparus aurata*), red porgy (*Pagrus pagrus*) and common dentex (*Dentex dentex*) eggs from Sparidae with different disinfectants. Kafkas Univ Vet Fak Derg 16 (2): 299-306.

Chang, C.T., Colicino, E.G., DiPaola, E.J., Al-Hasnawi, H. J., C. M. Whipps. (2015). Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish. Comparative Biochemistry and Physiology. 178: 45–50.

Cobcroft, J., Battaglene, S. (2012). Ultraviolet irradiation is an effective alternative to ozonation as a sea water treatment to prevent *Kudoa neurophila* (Myxozoa: Myxosporea) infection of striped trumpeter, *Latris lineata* (Forster). Journal of Fish Diseases. 36(1): 57-65.

Danner, R. G. and Merrill, P. (2006). Disinfectants, Disinfection, and Biosecurity in Aquaculture. In Aquaculture Biosecurity (eds A.D. Scarfe, C. Lee and P.J. O'Bryen). Blackwell Publishing.

de Carvalho, C. C. C. R. (2018). Marine Biofilms: A Successful Microbial Strategy With Economic Implications. Frontiers in Marine Science (5) 126.

Doan, Q. K., Vandeputte, M., Chatain, B., Morin, T., & Allal, F. (2017). Viral encephalopathy and retinopathy in aquaculture: A review. Journal of Fish Diseases, 40, 717–742. doi.org/10.1111/jfd.12541.

Douillet P.A., Pickering P.L. (1999) Seawater treatment for larval culture of the fish *Sciaenops ocellatus* Linnaeus (red drum). *Aquaculture* 170, 113– 126.

Escaffre, A., Bazin, D. & Bergot, P. (2001). Disinfection of *Sparus aurata* eggs with glutaraldehyde. Aquaculture International, 9: 451.

Ernst, I., Whittington, I.D., Corneillie, S., Talbot, C. (2005) Effects of temperature, salinity, desiccation and chemical treatments on egg embryonation and hatching success of *Benedenia seriolae* (Monogenea: Capsalidae), a parasite of farmed *Seriola* spp. Journal of Fish Diseases 28(3):157-64.

European Comission. Welfare of farmed fish: Common practices during transport and at slaughter. Final Report. 2017. ISBN 978-92-79-75336-7 doi: 10.2875/172078 EW-07-17-005-EN-N

Fahimipour, A., Hartmann, E., Siemens, A., Kline, J., Levin, D., Wilson, H., Betancourt-Román, C., Brown, G.Z., Fretz, M., Northcutt, D., Siemens, K., Huttenhower, C., Green, J., Wymelenberg, K. (2018). Daylight exposure modulates bacterial communities associated with household dust. Microbiome **6,** 175.

Fitridge, I., Dempster, T., Guenther J.,de Nys, R. (2012) The impact and control of biofouling in marine aquaculture: a review, Biofouling, 28:7, 649-669,

Franssen, F., Gerard, C., Cozma-Petruț, A., Vieira-Pinto, M., Režek Jambrak, A., Rowan, N., Paulsen, P., Rozycki, M., Tysnes, K., Rodriguez-Lazaro, D., Robertson, L. (2019). Inactivation of parasite transmission stages: Efficacy of treatments on food of animal origin. Trends in Food Science & Technology, Volume 83, pp 114-128.

Frerichs, G.N., Tweedie, A., Starkey,W.G., & Richards, R.H. (2000). Temperature, pH and electrolyte sensitivity, and heat, UV and disinfecatant inactivation of sea bass (Dicentrarchus labrax) neuropathy nodavirus. Aquaculture, 185, 13-24.

Giménez-Papiol, G., Padrós, F., Roque, A., Estévez, A. and Furones, D. (2009), Effects of a peroxide-based commercial product on bacterial load of larval rearing water and on larval survival of two species of Sparidae under intensive culture: preliminary study. Aquaculture Research, 40: 504-508.

Gomez, D. K., Baeck, G.W., Kim, J.H., Choresca, C.H., & Park, S.C. (2008a). Molecular Detection of Betanodavirus in Wild Marine Fish Populations in Korea. Journal of Veterinary Diagnostic Investigation, *20*(1), 38–44.

Gomez, D. K., Baeck, G. W., Kim, J. H., Choresca, C. H. Jr, & Park, S. C. (2008b). Molecular detection of betanodaviruses from apparently healthy wild marine invertebrates. Journal of Invertebrate Pathology, 97, 197–202.

Gomez, D. K., Mori, K., Okinaka, Y., Nakai, T., & Park, S. C. (2010). Trash fish can be a source of betanodaviruses for cultured marine fish. Aquaculture, 302, 158–163.

Grotmol, S., Totland, G.K. (2000). Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea water inactivates nodavirus and increases survival of the larvae. Diseases of Aquatic Organisms 39, pp. 89-96.

Hanif, A., Bakopoulos, V., Dimitriadis, G.J. (2004). Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. Fish & shellfish immunology. 17. 411-35.

Hanif, A., Bakopoulos, V., Leonardos, I., Dimitriadis, G. (2005). The effect of sea bream *(Sparus aurata*) broodstock and larval vaccination on the susceptibility by *Photobacterium damsela* subsp. *piscicida* and on the humoral immune parameters. Fish & shellfish immunology. 19. 345-61.

Hernández, A., Martró, E., Matas, L., Martín M., Ausina, V. (2000). Assessment of in-vitro efficacy of 1% Virkon® against bacteria, fungi, viruses and spores by means of AFNOR guidelines. Journal of Hospital Infection 46: 203–209

Jorquera, M.A., Valencia, G., Eguchi, M., Katayose, M., Riquelme,C. (2002). Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment, Aquaculture, (207) 3–4, pp 213-224.

Kasai, H., Yoshimizu, M., Ezura, Y. (2002). Disinfection of Water for Aquaculture . Fisheries Science, 68(Supplement 1), 821-824. Proceedings of International Commemorative Symposium, 70th Anniversary of The Japanese Society of Fisheries Science.

Katayose, M., Yoshida, K., Achiwa, N., Eguchi, M. (2007). Safety of electrolyzed seawater for use in aquaculture. Aquaculture. 264: 119-129.

Katharios, P., Agathaggelou, A., Paraskevopoulos, S. and Mylonas, C.C. (2007), Comparison of iodine and glutaraldehyde as surface disinfectants for red porgy (*Pagrus pagrus*) and white sea bream (*Diplodus sargus sargus*) eggs. Aquaculture Research, 38: 527-536.

Kasai, H., Muto, Y., Yoshimizu, M. (2005). Virucidal Effects of Ultraviolet, Heat Treatment and Disinfectants against Koi Herpesvirus (KHV). Fish Pathology, 40(3):137-138.

Kitis, M. (2004) Disinfection of wastewater with peracetic acid: A review. Environ. Int., 30, 47–55.

Kuhn, D. D., Smith, S. A., Scott, D. T., Taylor, D. P. (2017). Ozone application in aquaculture. *Virginia Cooperative Extension Publications*, [FST-244P].

LeBlanc, B.D and Overstreet, R.M. (1991). Effect of desiccation, pH, heat, and ultraviolet irradiation on viability of Baculovirus penaei. Journal of Invertebrate Pathology, Volume 57, Issue 2,Pages 277-286.

Le Breton A.D. (2009). Farming and health management: prevention and policy measures. In : Rogers C. (ed.), Basurco B. (ed.). The use of veterinary drugs and vaccines in Mediterranean aquaculture. Zaragoza : CIHEAM, 2009. p. 207-220 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 86

Liltved, H., Hektoen, H., Efraimsen, H. (1995) Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. Aquacultural Engineering 14 (2), 107-122.

Liu, D., Straus, D.L., Pedersen, L.F. & Meinelt, T. (2018). Periodic bacterial control with peracetic acid in a recirculating aquaculture system and its long-term beneficial effect on fish health. Aquaculture, 485, 154–159.

Lyngstad, T.M., Høgåsen, H.R., Jansen, M.D., Nilsen, A. (2015). Risk of disease transfer with wellboats in Norway. Technical report. Veterinærinstituttets rapportserie 15-2015. Oslo: Veterinærinstituttet.

Massault, C. , Franch, R. , Haley, C. , de Koning, D. J., Bovenhuis, H. , Pellizzari, C. , Patarnello, T. and Bargelloni, L. (2011), Quantitative trait loci for resistance to fish pasteurellosis in gilthead sea bream (*Sparus aurata*). Animal Genetics, 42: 191-203.

Machen, J.W., Smith, S., Flick, G.. (2008*). Vibrio anguillarum* and *V. ordalii* disinfection for aquaculture facilities. International Journal of Recirculating Aquaculture. 9. 10.21061

Meinelt, T., Phan, T., Behrens, S., Wienke, A., Pedersen, L., Liu, D. & Straus D. L. (2015). Growth inhibition of *Aeromonas salmonicida* and *Yersinia ruckeri* by disinfectants containing peracetic acid. Dis Aquat Org 113: 207–213.

Miralles, L., Mrugala, A., Sanchez-Jerez, P., Juanes, F., Garcia-Vazquez, E. (2016) Potential Impact of Mediterranean Aquaculture on the Wild Predatory Bluefish, Marine and Coastal Fisheries, 8:1, 92-99.

Moreno, M. I. Gutierrez-Lomelí, M. Guerrero-Medina, P. J. & Avila-Novoa M. G. (2018). Biofilm formation by Staphylococcus aureus and Salmonella spp. under mono and dual-species conditions and their sensitivity to cetrimonium bromide, peracetic acid and sodium hypochlorite. Brazilian Journal of Microbiology. 49: 310–319

Moretti, A., Pedini Fernandez-Criado, M., Cittolin, G., Guidastri, R. Manual on hatchery production of seabass and gilthead seabream. Volume 1. Rome, FAO. 1999. 194 p.

Moretti, A.; Pedini Fernandez-Criado, M.; Vetillart, R.Manual on hatchery production of seabass and gilthead seabream. Volume 2. Rome, FAO. 2005. 152 p.

Muniesa, A., Escobar-Dodero, J., Silva, N., Henríquez, P., Bustos, P., Perez, A.M. & Mardones F.O. (2019). Effectiveness of disinfectant treatments for inactivating *Piscirickettsia salmonis.* Preventive Veterinary Medicine 167:196–201

Munro P.D., Henderson, R.J., Barbour, A., Birkbeck, T.H. (1999). Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture, Volume 170, Issues 3–4, Pages 229-244,

Otterspoor S., Farrell J. (2019). An evaluation of buffered peracetic acid as an alternative to chlorine and hydrogen peroxide-based disinfectants. Infection, Disease & Health, https. //doi.org/10.1016/j.idh..06.003

Patel, S and Nerland, A.H. Vaccination against diseases causes by Betanodavirus. In: Fish Vaccination (2014). R. Gudding, A. Lillehaug. and Evensen,Ø. Eds. Wiley Blackwell.

Pietrak, M.R., Molloy, S.D., Bouchard, D.A., Singer, J.T, Bricknell, I (2012) Potential role of *Mytilus edulis* in modulating the infectious pressure of *Vibrio anguillarum* 02β on an integrated multi-trophic aquaculture farm. Aquaculture 326−329: 36−39.

Potts M. (1994). Desiccation tolerance of prokaryotes. Microbiological reviews, *58*(4), 755–805.

Powell, A.; Scolding, J.W.S. (2018). Direct application of ozone in aquaculture systems. Reviews in Aquaculture, 10 (2), pp. 424-438.

Pridgeon, J.W., Klesius, P.H., Mu X., Song L. (2011). An in vitro screening method to evaluate chemicals as potential chemotherapeutants to control *Aeromonas hydrophila* infection in channel catfish. J Appl Microbiol. 2011 Jul;111(1):114-24.

Rasco, B., Ovissipour, M. (2015) Electrolyzed Water Applications in Aquaculture and the Seafood Industry. Journal of Aquaculture Research and Development 6: 293.

Reiter K. C., Villa B., Paim T. G., de Oliveira C. F., d'Azevedo P. A. (2013). Inhibition of biofilm maturation by linezolid in meticillin-resistant *Staphylococcus epidermidis* clinical isolates: Comparison with other drugs. J. Med. Microbiol., 62, 394-9.

Sansebastiano G., Zoni R., Bigliardi L. (2007). Cleaning and Disinfection Procedures in the Food Industry General Aspects and Practical Applications. In: McElhatton A., Marshall R.J. (eds) Food Safety. Springer, Boston, MA.

Sattar, S.A., Springthorpe, V.S., Rochon, M. (1998). A product based on accelerated and stabilized hydrogen peroxide: evidence for broad-spectrum germicidal activity. Can. J. Infect Control 123–130.

Shetty, M., Maiti, B., Santhosh, K.S., Venugopal, M.N., Karunasagar, I. (2012). Betanodavirus of Marine and Freshwater Fish: Distribution, Genomic Organization, Diagnosis and Control Measures. Indian J. Virol. 23(2): 114–123. DOI 10.1007/s13337-012-0088-x

Skjermo J. & Vadstein O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333– 343.

Soto E., Halliday-Simmonds, I., Francis, S., Kearney, M.T., Hansend J.D. (2015). Biofilm formation of *Francisella noatunensis* subsp. *orientalis*. Veterinary Microbiology 181:313–317.

Stefanakis, M.K., Anastasopoulos, E., Katerinopoulos, H.E. and Makridis, P. (2014), Use of essential oils extracted from three *Origanum* species for disinfection of cultured rotifers (*Brachionus plicatilis*). Aquac Res, 45: 1861-1866.

Stewart, P.S., Roe, F. Rayner, J., Elkins, J.G., Lewandowski, Z., Ochsner, A.,Hassett D.J. (2000). Effect of Catalase on Hydrogen Peroxide Penetration into *Pseudomonas aeruginosa* biofilms. Applied and Environmental Microbiology, 66,2: 836–838.

Sugita, H., Asai, T., Hayashi, K., Mitsuya, T., Amanuma, K., Maruyama, C., Deguchi, Y. (1993). Application of ozone disinfection to remove *Enterococcus seriolicida*, *Pasteurella piscicida*, and *Vibrio anguillarum* from seawater. Applied and environmental microbiology. 58. 4072-5.

Summerfelt, S.T. (2003). Ozonation and UV irradiation—an introduction and examples of current applications. Aquaculture Engineering 28, 21–36.

Thiéry, R., Johnson, K. L., Nakai, T., Schneemann, A., Bonami, J. R. & Lightner, D. V. (2012). Family Nodaviridae. In: A.M. Q. King, M. J. Adams, E. B. Carstens, E. J. Lefkowitz (Eds.), Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses (pp. 1061-1067). California: Elsevier Academic Press.

Torgersen Y, Håstein T. (1995). Disinfection in aquaculture. Revue Scientifique et Technique (International Office of Epizootics)14(2):419-434.

Volpe, E., Pagnini, N., Serratore, P., Ciulli, S. (2017). Fate of redspotted grouper nervous necrosis virus (RGNNV) in experimentally challenged Manila clam *Ruditapes philippinarum*. Diseases of Aquatic Organisms. 2017 Jun 19;125(1):53-61.

Volpe, E., Grodzki, M., Panzarin, V., Guercio, A., Purpari, G., Serratore, P., Ciulli, S., (2018). Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs. Journal of Fish Diseases, 41:603–611.

Wolf, K. (1962). Experimental propagation of lymphocystis disease of fishes. Virology. Volume 18, Issue 2, pp. 249-256.

Wong, H., Liao, R., Hsu, P. & Tang C. (2018). Molecular response of *Vibrio parahaemolyticus* to the sanitizer peracetic acid. International Journal of Food Microbiology 286., 139–147.

Yamasaki, M., Sakai, T., Ito, T. & Mori, K. (2017). Bactericidal Effects of Disinfectants on *Yersinia ruckeri*. Fish Pathology, 52 (4), pp: 198–201

Yoshimizu, M. (2009). Control Strategy for Viral Diseases of Salmonid Fish, Flounders and Shrimp at Hatchery and Seed Production Facility in Japan, Fish Pathology, 44(1) pp: 9-13.

Yoshimizu, M., Takizawa, H., Kimura, T. (1986). UV-susceptibility of some fish pathogenic viruses. Fish Pathology, 21, pp. 47-52