Internship Report
Master in Marine Resources Biotechnology

Recirculating aquaculture systems and Ecotoxicology: Improving technologies to assess the impact of human activities in the marine environment

Inês Carvalho Pimparel

Peniche, *September 2017*

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RAS system and Ecotoxicology: Improving technologies used to assess the impact of the human activities in the marine environment

Inês Carvalho Pimparel

Internship report submitted to Superior School of Tourism and Maritime Technology, Polytechnic Institute of Leiria, as part of the requirements to obtain the Master Degree in Marine Resources Biotechnology. Internship held under the supervision of Doctor Simeon Deguara (AquaBioTech Group Company, Malta) and Professor Marco Lemos (School of Tourism and Maritime Technology, Polytechnic Institute of Leiria).

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Title: RAS system and Ecotoxicology: Improving technologies used to assess the impact of the human activities in the marine environment

Titulo: Sistemas RAS e Ecotoxicologia: Melhoramento de tecnologias usadas para avaliar o impacto das atividades humanas no ambiente marinho.

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ABSTRACT

An internship was held at AquaBioTech Group (Malta), a European company dealing with aquaculture and biotechnology, to complete the Master in Marine Resources Biotechnology of the School of Tourism and Maritime Technology of the Polytechnic Institute of Leiria.

Biotechnology and aquaculture have been contributing to the progress and improvement of fish production for years. The AquaBioTech Group is an example of a company that is able to integrate these areas to maximize their services. The company is located in Mosta (Malta) and operates in a sustainable way using Recirculation Aquaculture Systems (RAS) to maintain aquaculture species. In collaboration with several companies and institutions, the AquaBioTech Group is involved and supports the development of important international research projects. The present report focuses on two important parts of the internship that lasted a total of 6 months. During the first three months, the internship took place in the aquaculture facility where basic and advanced procedures were perfected and learned. The second part of the internship (last three months) was undertaken in the ecotoxicology laboratory to deepen and develop knowledge in assays conducted in the company. A trial was carried out in the laboratory with the intent of developing a model with gilthead sea bream embryos, Sparus aurata, as a model for Fish Embryo Acute Toxicity (FET) Tests.

The internship aimed to deepen the theoretical knowledge acquired during the academic training, develop professional skills, and provide an understanding of jobs available on the market.

Keywords: Aquaculture, AquaBioTech Group, Ecotoxicology, Marine Species, OECD 236, RAS.

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RESUMO

Este estágio foi realizado na AquaBioTech Group (Malta), uma empresa europeia dedicada à aquacultura e à biotecnologia, com o intuito de completar o Mestrado em Biotecnologia dos Recursos Marinhos da Escola de Turismo e Tecnologia do Mar, do Instituto Politécnico de Leiria.

A biotecnologia e a aquacultura têm contribuído para o progresso e para a melhoria da produção de peixes durante anos. A empresa AquaBioTech Group é capaz de integrar essas áreas de forma a maximizar os seus serviços. A empresa está localizada em Mosta (Malta) e opera de forma sustentável utilizando sistemas de recirculação (RAS) para manter as espécies de aquacultura. Em colaboração com várias empresas e instituições, a AquaBioTech Group está envolvida e apoia o desenvolvimento de projetos internacionais de investigação. O presente relatório de estágio divide-se em duas partes as quais duraram um total de seis meses. Durante os primeiros três meses, o estágio ocorreu nas instalações de aquacultura onde foram apreendidos e aperfeiçoados procedimentos básicos e avançados necessários ao trabalho em aquacultura. A segunda parte do estágio (últimos três meses) foi realizada no laboratório de ecotoxicologia para aprofundar e desenvolver conhecimento em ensaios realizados na empresa. Foi realizado um ensaio laboratorial com a intenção de desenvolver um modelo ecotoxicológico com embriões de dourada, Sparus aurata, para a realização de testes agudos de toxicidade com embriões de peixe (Fish Embryo Acute Toxicity Test, FET).

O estágio visou aprofundar conhecimentos teóricos adquiridos durante o percurso académico, desenvolver competências profissionais e compreender as oportunidades do mercado de trabalho.

Palavras-chave: Aquacultura, AquaBioTech Group, Ecotoxicologia, Espécies Marinhas OECD 236, RAS.

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EU	European Union
MAR	Malta Aquaculture Research Centre
EMS	Environmental Management System
RAS	Recirculating Aquaculture System
R&D	Research and Development
LSS	Life Support System
ABT	AquaBioTech
GMP	Good Manufacturing Practices
OECD	Organisation for Economic Co-operation and Development
ISO	International Organization for Standardization
ASTM	American Society for Testing and Materials
FET	Fish Embryo Tests
VMD	Veterinary Medicines Directorate
VRD	Veterinary Regulation Department
GCP	Good Clinical Practices
VICH	Veterinary International Conference on Harmonization
SOP	Standard Operating Procedures
GDP	Good Documentation Practices
GLP	Good Laboratory Practices
MS-222	Tricaine Methanesulfonate
FDA	Food and Drug Administration
VIE	Visible Implant Elastomer
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
MSDS	Material Safety Data Sheet
DI	Deionized water
FAO	Food and Agriculture Organization of the United Nations

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1 INTRODUCTION

1.1 Aquaculture

The oceans are clearly not an unlimited source of fishery products. Recently, scientists have concluded that the maximum sustainable yield has been reached, or it will be, for many species, while many species are already overfished (Ebeling and Timmons, 2012).

Aquaculture has been around for a long time. It started in China with the People's Republic of China (PR China), where *Cyprinus carpio* (common carp) was raised for food in ponds in 1100 B.C., and oyster farming was developed along Han Dynasty (206 B.C.–220 A.D.). Japanese cultured oysters for pearls, ancient Egyptians produced fish, the Greeks and Romans raised eels, and the Europeans cultured oysters (Bondad-Reantaso et al., 2005).

In 1988, the Food and Agriculture Organization (FAO) of the United Nations had defined Aquaculture as: farming of aquatic organisms, including fish, molluscs, crustaceans, and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated. For statistical purposes, aquatic organisms which are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture, while aquatic organisms which are exploitable by the public as a common property resources, with or without appropriate licenses, are the harvest of fisheries (Billard and Dabbadie, 1993; Edwards, 1997).

In recent years, aquaculture has been the fastest increasing animal food-producing sector in the world, and is progressively important to global food supply and economic growth. The world greatest goal for Aquaculture is to feed around 9 billion people by 2050 (FAO, 2016) in a scenario of climate change, economic and financial uncertainty, and growing competition for natural resources. The global supply of fish and shellfish increased 27% from 1992 to 2012 while the seafood supply increased from 155.8 million tonnes to 158.0 thousand tonnes from 2011 to 2012 (STECF, 2014). In 2014, the aquaculture sector's

contribution to the supply of fish for human consumption surpassed that of wild-caught fish for the first time (FAO, 2016).

The European Union (EU) is currently the largest market for fish in the world. In the past decades, the EU aquaculture production has been stagnant, and this has led to an increase of fish and seafood imports. The future goal is to increase production due to growing population and income and health benefits. This growing demand is a good opportunity for aquaculture production in the EU. Aquaculture in Europe Member States is mainly concentrated in Spain, United Kingdom, France, and Greece. The United Kingdom is the largest producer in EU with 22% total production in value, followed by France (21%), Greece (13%) and Spain (13%). Countries with a smaller value in total production are Ireland and Italy (4%), The Netherlands, Denmark, Poland, and Malta (Figure 1.1) (STECF, 2014).

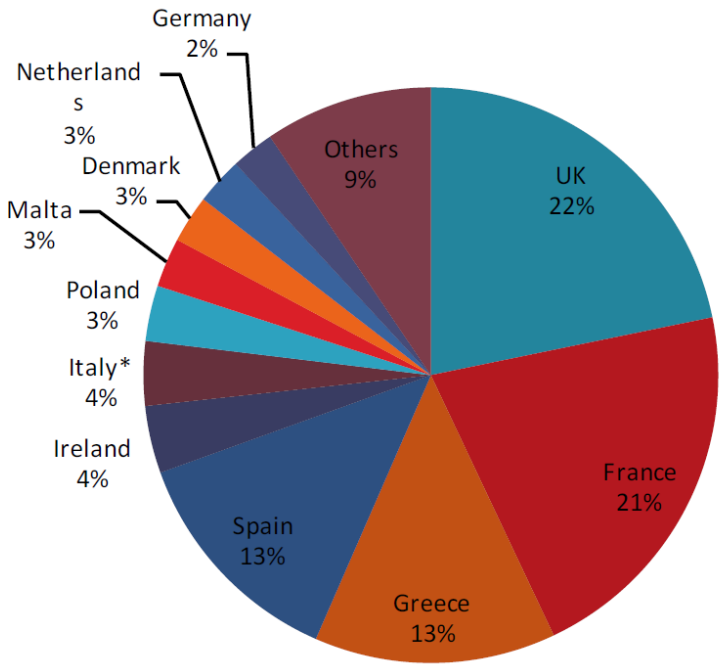

Figure 1.1: Aquaculture production in EU Member States in terms of value in 2012 (STECF, 2014).

European aquaculture is a diverse activity that can be characterized using different technologies for production. The most dominant production technologies in the EU are:

- Freshwater pond aquaculture or coastal pond aquaculture;
- Intensive freshwater/marine flow-through and partial recirculation systems;

- Freshwater or marine indoor recirculation aquaculture systems;
- Small cage systems (freshwater or sheltered marine cages);
- Marine large cage systems in exposed sites;
- Marine bottom culture (non-fed sedentary and attached animals and plants);
- Marine supported and suspended culture (non-fed sedentary and attached animals and plants).

Marine supported and suspended cultures are the most valuable production technologies in the EU with 600,000 to 700,000 tonnes of production, followed by large marine cages, intensive freshwater flow-through and partial recirculation systems, small cage systems, and finally marine bottom culture (Bostock et al., 2016).

Aquaculture in the Mediterranean Sea is mainly cage-based. It is entirely dependent on *Sparus aurata* (gilthead sea bream), *Dicentrarchus labrax* (european sea bass), *Argyrosomus regius* (meagre), and *Seriola dumerili* (amberjack) as "closed cycle species", and *Thunnus thynnus* (Atlantic bluefin tuna) farming, which is referred as capture based aquaculture. In Malta, the european sea bass and gilthead sea bream are currently cultured and mainly exported to Europe (especially Italy), and their production takes place in floating cages situated about one kilometre offshore. The Atlantic bluefin tuna is exported to Japan and situated in aquaculture zones between five and six kilometres offshore (Nature Trust Malta, 2015).

During the last years Malta has been struggling with some conflicts with the tourism industry due to lack of space around the island do to tourism and aquaculture operations. It is important that any future aquaculture developments continue to require appropriate environmental assessment including monitoring of water quality and sediments, benthic flora and fauna, and visual inspection of the seabed under the cages. Today the Malta Aquaculture Research Centre (MAR) is the only research facility on Malta having the infrastructure to hatch marine species for mariculture. They also accommodate trials with amberjack and Atlantic bluefin tuna larvae, with some success obtained with the Amberjack Project and on a EU level with the REPRODOTT, SELFDOTT, and TRANSDOTT projects. (FAO, 2005-2017; National statistical office in Malta, 2016).

1.2 The AquaBioTech Group

The AquaBioTech Group is an international consulting company located on the island of Malta. The focus of the company's work is related to the marine or aquatic environment, encompassing aquaculture developments, market research / intelligence, through to project feasibility assessments, finance acquisition, project management, technology sourcing, and technical support and training. The company is deeply committed to its responsibilities towards the environment. The Group has created an Environmental Management System (EMS) that plans to mitigate impacts that cannot be further reduced (AquaBioTech Group Company. Available in: https://www.aquabt.com). Within the AquaBioTech Group there are various divisions that focus on different business areas. These business areas are:

1.2.1 ABT Aquaculture (AquaCircTM)

This area has developed a number of highly efficient and cost-effective recirculating aquaculture systems (RAS). These can be applied to hatcheries, broodstock, aquatic research, aquaponic systems, and ongrowing operations. The company constantly strives to be at the forefront of the industry by testing and developing innovative technologies. ABT Aquaculture also offers consultancy services for all aquaculture related projects. This area has become well established as a provider of due-diligence and risk assessments for all forms of aquaculture operations, as well as consulting on operational issues and improvements in hatcheries, fish farms and processing facilities (AquaBioTech Group Company. Available in: https://www.aquabt.com).

1.2.2 ABT Innovia

ABT Innovia offers research services to support the development of vaccines, functional feeds, alternative protein sources, culture technologies, production techniques, and ecotoxicology with a wide range of commercially important species under any combination of culture conditions in its fully licensed and bio-secure R&D facilities (AquaBioTech Group Company. Available in: https://www.aquabt.com).

1.2.3 ABT Marine

This business area provides a range of services including marine surveying, construction support, and mapping/GIS. The techniques employed include bathymetric and side scan sonar surveys, bottom type assessments, sub-bottom assessments, data confirmation, and site inspections using both remote sensing and underwater video techniques (AquaBioTech Group Company. Available in: https://www.aquabt.com).

1.2.4 ABT Aquatics

This business area involves work ranging from initial feasibility studies, outline planning with concept development, architectural & structural design with engineering, filtration and life support system (LSS) development, through livestock supply, management support and turnkey operations for aquariums and ornamentals (AquaBioTech Group Company. Available in: https://www.aquabt.com).

Figure 1.2: AquaBioTech Group Logotype

1.3 Aims of the internship

This Internship was held in AquaBioTech Group at ABT Innovia and was divided into two main research areas: aquaculture and ecotoxicology. The aim of the first part of the internship was to learn at the facility, basic procedures important in a recirculating system, such as: biosecurity, cleaning, daily routines, water quality control and feeding. As well as how to proceed in a fish delivery and to manage diseases and mortality. The advanced procedures learned were: anaesthesia and euthanasia, sampling, tagging, growth performance, vaccination and their importance in a GMP facility.

The second part at the ecotoxicology laboratory aimed to develop knowledge and practical skills related with: laboratory routine, how to perform dilutions according to OECD guidelines, and assays, such as, algal growth inhibition tests (OECD, 2011; ISO 10253, 2006) and *Artemia salina* acute tests (ASTM E1440-91, 2012).

As the final experience, was developed an experiment aiming to propose *Spaurus aurata* eggs as model for the OECD Test No. 236: Fish Embryo Acute Toxicity (FET) Test (OECD, 2013).

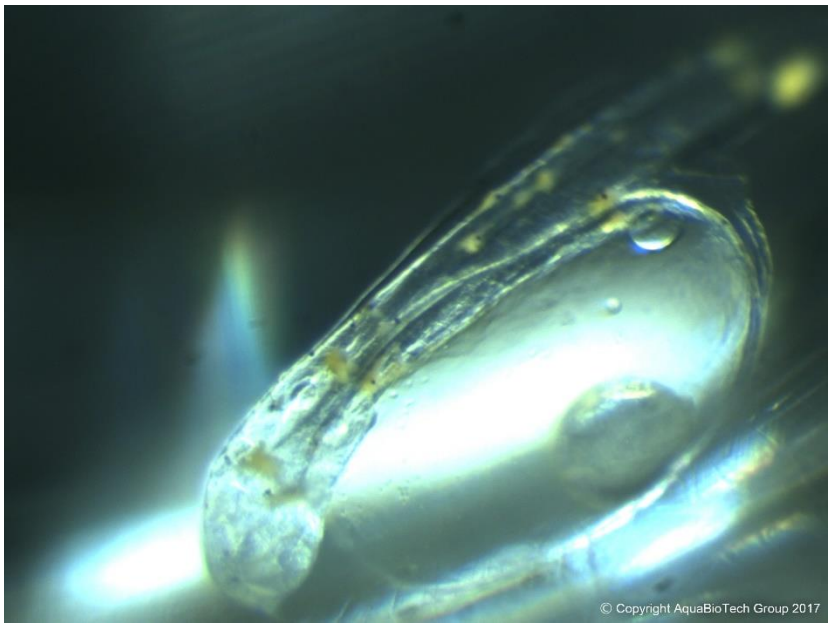

Figure 1.3: *Sparus aurata* larvae in AquaBioTech Group. Taken during the FET trial. (Source: AquaBioTech Group).

2 INTERNSHIP DESCRIPTION

2.1 Work experience: RAS Systems

ABT Innovia is an independent aquatic biotechnology and aquaculture research and training entity with its own dedicated Research & Development (R&D) facilities that forms part of the AquaBioTech Group. AquaBioTech Group is a GMP (Good Manufacturing Practice) company certified by the United Kingdom VMD (Veterinary Medicines Directorate) and Maltese VRD (Veterinary Regulation Department). It is also vital to note that all of the challenge trials performed are according to GCP (Good Clinical Practice) under the principles of the Veterinary International Cooperation on Harmonization (VICH) of Technical Requirements for Registration of Veterinary Medicinal Products. The facility is divided into numerous wet-labs that can be used for a variety of research purposes including: ornamental species, novel and carrier species, larviculture, veterinarian products and pathology, nutritional and ecotoxicology research (Figure 2.1).

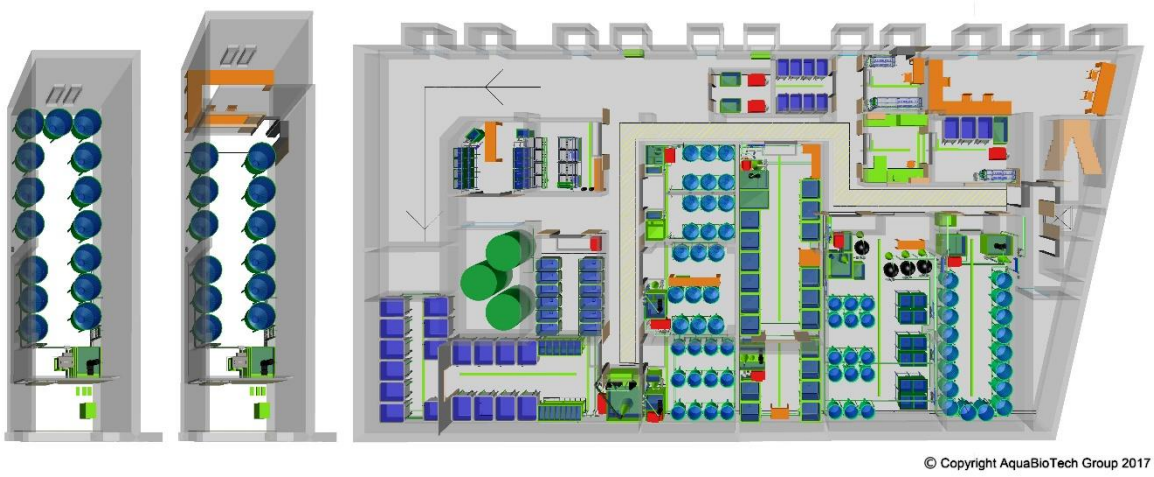

Figure 2.1: AquaBioTech Group Facilities (AquaBioTech Group Company. Available in: https://www.aquabt.com).

Each wet-lab has a RAS installed (Figure 2.2). A RAS is essentially a technology of farming aquatic organisms providing opportunities to reduce water usage, recycle nutrients, and to improve waste management. These qualities make RAS more sustainable (Martins et al., 2010). These systems use approximately 90 to 99% less water than conventional aquaculture

systems (Nazar, et al., 2013). Water is reused due to the removal of fish metabolites and feed waste and breakdown of products, such as solid and dissolved organic matter, which enhances water quality and improves fish welfare (Martins et al., 2010: Murray et al., 2014). The treatment of the wastewater by filters and pumps is designed to protect fish stocks from infection by disease agents and it helps to prevent disease outbreaks (Nazar, et al., 2013; Murray et al., 2014). In these systems, the water flows from the tanks to equipment where it is mechanical, biological, and chemically filtered. Suspended solids (debris, food scraps, and faeces) are removed by mechanical filtration using a drum filter. Toxic waste products such as ammonia are converted into nitrite which can then be converted to the less toxic nitrates by a solid medium (sand or plastic balls) that holds nitrifying bacteria. Chemical filtration reduces the abundance of bacteria and parasites in the water by using skimmers, ozonation, and UV tubes (Moe, 2009; AquaBioTech Group Company. Available in: https://www.aquabt.com; Nazar, et al., 2013).

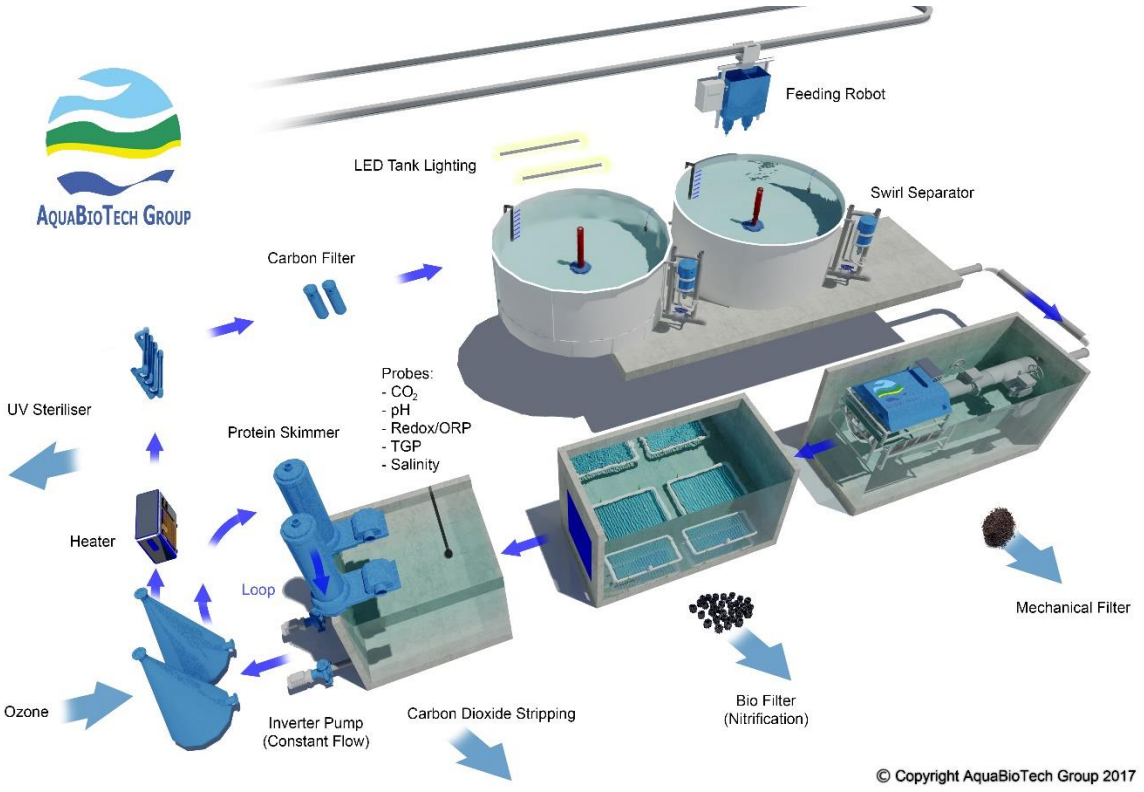

Figure 2.2: Basic water treatment of a recirculation system (AquaBioTech Group Company. Available in: https://www.aquabt.com).

2.1.1 ABT Innovia a certified facility

ABT Innovia has a GMP certified facility that carries out trials to ensure that all products are produced and controlled according to quality standards. The aim of GMP is to reduce the risk inherent in a production of a pharmaceutical, such as cross-contamination or mix-ups. Documentation is crucial to GMP compliance: “If it’s not written down, then it didn’t happen!”.

Documentation guarantees traceability of development, manufacturing and testing activities, providing at the same time, the capability of measuring the quality of operations. SOP’s (standard operating procedures) are an example of this, as they are considered a set of written instructions that document a routine or repetitive activity followed by an organization. SOP’s are known to increase the quality and consistency of the work, to record the workflow, and serve as a legal reference for clients or authorities.

Some crucial elements for GMP are: qualified and trained personnel, adequate premises and space, suitable equipment and services, correct materials, containers and labels, approved procedures and instructions, suitable storage, and transport. At AquaBioTech Group the personnel are required to follow Good Documentation Practices (GDP) which implies the following basic requirements:

- Record entries at the time of the activity, with date and initials;
- All records must be done using a ballpoint blue pen and using English Language;
- Pencil, white ink, or correction fluid cannot be used;
- Never sign for someone else;
- If a mistake has been done, a cross-out must be done over the mistake with initials, date and reason for the correction.

Self-inspections are performed inside the facility to monitor the GMP principles and to propose necessary corrective measures or improvements to the SOP in question. (Patel and Chotai, 2011: GMP guidelines: available in https://ec.europa.eu/health/documents/eudralex/vol-4_en).

2.1.2 Basic methods in RAS

2.1.2.1 Biosecurity procedures

Biosecurity in aquaculture is maintained by a combination of practices that minimize the risk of introducing infectious diseases and spreading it to the animals at the facility and the risk of spreading diseases from the facility to other study sites. AquaBioTech is fully committed to pathogen and people management. This management is controlled by a series of trainings given in the facility to working personnel and visitors to minimize the risks of contaminations. After a rigorous training, the permanent members of staff and interns can work inside the facility.

The facility is organized according to specific biosecurity rules. The aquaculture facility is divided into wet-labs called 'Bays' and each bay has an identification number - 1; 2; 3; and so on. The bays also have a Label on each door that informs the worker if it is a stock bay (green label) or a challenge bay (red label).

To follow everyday tasks, the staff has to follow basic biosecurity rules as soon as they enter the facility:

- The worker needs to dress specific clothing according with biosecurity requirements of the wet lab (Figure 2.3) on the day;
- Change footwear when entering the facility (from personal footwear change to clogs to boots);
- Disinfect footwear, gear, and hands at the entrance of every bay according to instructions specific to that Bay;
- Strict access control is maintained in challenge bays: individual sets of footwear need to be used (white boots) and changed at the entrance of the wet-lab. A person working in challenge bays is forbidden to enter stock bays.

Figure 2.3: Door biosecurity label; entrance of a bay (AquaBioTech Group Company. Available in: https://www.aquabt.com).

2.1.2.2 Cleaning

Cleaning practices are used to remove organic debris from the systems that harbour many pathogens. Manual cleaning is known to reduce the number of contaminating organisms from the system (Yanong and Erlacher-Reld, 2012). All stock bays in the facility are cleaned thoroughly twice a week to provide optimal sanitation conditions for fish maintenance. The technician must follow a checklist with tasks that are known to improve good sanitation conditions, such as:

- Internal and external cleaning of tank walls;
- Brush inlet and outlet pipes and air stones;
- Wipe probes;
- Syphon faeces and food wastes and purge the system;
- Clean equipment, tables, and floor;
- Check the presence of day-to-day consumables, such as gloves and other disinfectant products;
- Renew footbaths;
- Remove garbage.

In the allocated days for cleaning of the bays, also the corridor, cleaning area and facility office washing is required. At the end of the cleaning, the technician must make sure that everything is properly cleaned and sign the required paperwork. The cleaning procedure for challenge bays is different to that for Stock Bays.

The cleaning procedure for challenge bays is different from that of stock bays. It is carried out after the completion of the trial to avoid disease outbreaks. The cleaning SOP for challenge bays begins with a series of disinfecting agents and it finishes with an empty and completely disinfected system. Before using the system for a new trial, microbiological samples of critical surfaces are taken to prevent the use of a system that was not disinfected properly.

2.1.2.3 Daily Routines

A technician is responsible for the daily routines previously assigned by the technician supervisor. A checklist needs to be followed to organize the workflow of the facility. The checklist is slightly different according with the different bays, stock, or challenge bays. Stock bays need to be monitored five to six times a day and challenge bays at least twice a day. However, the tasks are mostly the same and include: fish delivery if applicable, feeding fish, removal and recording of mortalities, checking of health and fish welfare, monitor water quality parameters, removal of feed waste and faeces, and equipment maintenance. Data are collected during the day and is of the responsibility of the night shift person for stock bays, while for challenge bays person is responsible specifically for those bays.

2.1.2.4 Water quality control

In recirculating systems, good water quality parameters must be maintained for maximum fish growth. Changes in the levels of water quality parameters can be unfavourable or even toxic to fish. For this reason, it is important to avoid sudden changes in the physical and chemical parameters of the water (Bregnballe, 2015). Water quality may affect the well-being of farmed animals, such as fish or crustaceans, and for that very reason a wide range of parameters need to be controlled. It is important to understand the impacts on the environment and to relate them into the functioning of the system itself. If produced in an

adequate environment, the farmed fish will have a greater survival and growth rate. Typically, parameters such as oxygen, ammonia and others have a negative impact if not controlled, due to their stressful impact to the animals under culture (Simoes et al., 2007, Boyd and Tucker, 1998).

Water quality is measured on a daily basis in the facility for every system with some exceptions. If a system biofilter is already mature the water is evaluated twice a week while in the others, every day. The technician in charge of this task must take a sample from the systems and transport it to the water quality analysis table. At the table, the technician performs the analysis of the samples using colorimetric kits that measure ammonia, nitrites and nitrate, alkalinity, and salinity. Water quality colorimetric kits are suitable when a highest level of accuracy is not needed and when a quick analysis is desired (Boyd and Tucker, 1998).

Other parameters such as pH, oxygen, and redox are measured by probes. The probes are placed in different places of the recirculating systems: the pH and redox probes are placed in the sump of the system while the oxygen probes are placed in each tank of the system. The probes are connected to a computer system that retrieves the data and collects it in a database. If the parameters measured by these probes are out of range a visual alarm (low alarm) or a sound alarm (high alarm) will be triggered and the technicians alerted for a problem in a specific tank. To ensure accurate measures, all probes in the facility are calibrated on a monthly basis (Bregnballe, 2015).

All data from water quality is recorded on paper and computer files by the responsible technician to allow a temporal evolution of the systems and as a GMP requirement.

2.1.2.5 Fish deliveries

Before a fish delivery, the bay to be used needs to be disinfected and prepared to receive the fish according to the SOP. The SOP requires a disinfection procedure that takes a few days and different disinfecting agents to ensure maximum hygiene and safety. The technician in charge of the disinfection and bay preparation needs to guarantee that the bay is prepared to

receive fish to be delivered. The night shift technician is always in charge of the delivery to control the fish well-being overnight.

When fish arrive to the company, a veterinarian needs to be present to evaluate species-specific health parameters and diseases and paperwork to be filled out with all necessary information. Fish samples are taken for histology. Fish are acclimatized in the delivery bags and when parameters are stabilized they are transferred to the system and the quarantine period begins. Fish are only fed the next day to avoid feed waste and bad water quality parameters.

The tanks are labelled with batch and trial code, number and name of species, number of the tank, system and bay and fish markings. During the following days, the bay is kept under surveillance to prevent spreading of diseases. This bay is the last one to be checked to avoid contaminations.

2.1.2.6 Feeding

Feeding the fish in a recirculating system is a very important practice. If not made correctly it can affect the ammonia, nitrites, and suspended solids contents in the system and thus influencing fish (Ebeling and Timmons, 2012). Farmed fish require feeding several times a day. If fed properly, the feed is eaten and digested by the fish and used to supply energy and nutrition for growth and physiological processes (Bregnballe, 2015).

At AquaBioTech, feeding is performed three to four times a day. If fish needs to be handled, the feed will be given at non-scheduled times during a day (Figure 2.4), returning to normal the next day. Feed administration is based on fish size, number of fish in the tank, species, and temperature. Depending on characteristics, such as buoyancy, feed is administered in small amounts, allowing the animals to eat before the feed reaches the bottom of the tanks, avoiding waste. When administering food in nutritional trials some fish behaviour needs to be recorded and trial-specific requirements followed.

Figure 2.4: Label of a tank that is not to be fed (AquaBioTech Group Company. Available in: https://www.aquabt.com).

At the end of the day, the night shift technician collects all the feeding containers. The remaining feed is weighed and recorded in computer systems. Feed for the next day is weighed and the containers all placed again in the bays.

2.1.2.7 Disease and Mortality Management

Disease outbreaks can be controlled by good husbandry. Good husbandry implies the control of environmental conditions which if not controlled can weaken fish immune systems making them susceptible to disease (Yanong and Erlacher-Reld, 2012; Yanong, 2003). Occasionally, recirculating systems favour the outbreak of diseases. High densities, accumulation of biofilm and sediments in tank, sumps or filters and slower turnover of water can jeopardize fish health (Yanong, 2003).

The signs of disease in a fish population include changes in behaviour or appearance, reduced or absent feeding response, signs of morbidity (sick fish), and mortality (Figure 2.5) (Yanong, 2003). When signs of disease are observed, the veterinarian is contacted. In order

to give the best treatment, the veterinarian observes the presence of skin, fins and internal and external organ lesions, collects blood samples and tests the fish for presence of bacteria. The bay may be considered from now on a challenge bay, with high contamination risk. The treatment of the disease is normally made by antibiotic incorporation in the feed. During the treatment, mortalities need to be recorded and relevant samples taken as per the veterinarian's instructions.

Figure 2.5: Mortality collection (AquaBioTech Group Company. Available in: https://www.aquabt.com).

The presence of dead animals in a system jeopardizes the water quality due to organic matter decomposition by bacteria. For that matter, mortalities need to be removed as fast as possible from the system. After collection, the mortality bag needs to be labelled with batch/trial code, bay, system and tank code, date and technician initials, and stored in a freezer at -20 °C. The mortalities are kept in the facility's storage systems for mortality control at the end of the trial and then sent to an incineration company.

2.1.3 Advanced methods in RAS

Some specific practical techniques were acquired during this internship, such as: fish sampling, tagging, growth performance, and vaccination.

2.1.3.1 Anaesthesia and euthanasia of fish

Tricaine Methanesulfonate (MS-222) is used (and approved by the Food and Drug Administration (FDA)) as an ideal anaesthetic for fish. To minimize stress when handling fish, the facility uses MS-222 to carry out procedures such as vaccination, tagging, blood collection, and if necessary to weigh fish. The dose of MS-222 is calculated before use and it varies with the species. When anaesthetizing, fish behaviour needs to be monitored during all the procedures. After handling, the fish must begin to recover, gaining an upright swimming position after being inside the recovery tank (Popovic et al., 2012).

Euthanasia induces death with the least possible anxiety, pain and distress to an animal. MS-222 is also used as a euthanizing agent in the facility. Euthanasia is applied when diseased or moribund fish are found in tanks, to prevent disease outbreaks. It is also used to take samples during or at the end of a trial (Popovic et al., 2012).

2.1.3.2 Fish sampling/grading

Fish sampling is commonly used to determine fish growth in the systems. In fish, growth mainly depends on feed consumption, feed quality, and stocking density. So, from time to time fish growth needs to be evaluated. This parameter can be simply assessed by weighing or measuring the fish, followed of the necessary adjustment of feed size and quantity of feed given to optimize fish growth (Lugert et al., 2014).

2.1.3.3 Tagging

Tagging is a technique used in AquaBioTech with the purpose of distinguishing treated fish used in vaccination or nutrition trials. Visible implant elastomer (VIE) is a silicone-based subcutaneous tagging system used for individual identification and is widely used to mark fish and crustaceans. VIE is injected as a liquid that cures into a flexible and biocompatible solid. The fluorescent colours are visible under ambient light or fluoresced with a special VI light (purple light that causes fluorescence) (Fürtbauer et al., 2015). Before injecting the elastomer, the fish are anesthetized to ensure a visible mark can be inserted without hurting the fish. After preparing the elastomer, the syringe is introduced into a specific injector and

the needle bent at a certain angle to pierce just the surface of the skin. In the facility, the tag is placed near the eyes, however it can be placed in the caudal peduncle, near the anal fin, dorsal or ventral fin lobes, or anal fin (Figure 2.6).

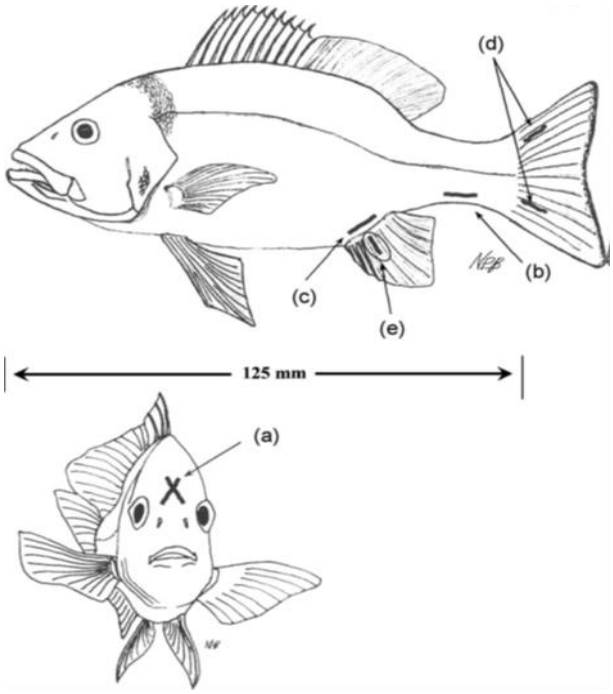

Figure 2.6: Body implant locations of visible tagging: (a) nose bridge, (b) caudal peduncle, (c) base of the anal fin, (d) dorsal and ventral caudal fin lobes, and (e) anal fin (Brennan et al., 2006).

2.1.3.4 Growth performance

Sampling techniques are usually applied at the end of a nutrition trial, and are used to evaluate the effects of a diet. The fish are taken from the tank and sacrificed with large doses of anaesthetic. Fish are weighed and measured (total and fork length) individually. After dissection, some parameters are taken, such as the weight of viscera, and intestine length is measured. The upper, central and lower parts of the intestines are stored in 10% buffered formalin for further analysis.

2.1.3.5 Vaccination

Vaccination is an easy, effective and preventive technique for protecting fish from diseases. The purpose of vaccines is to stimulate the immune system to induce a response against a pathogen. Vaccines can be delivered to fish by intraperitoneal injection, by immersion

(where animals are placed in a vaccine solution), or by oral administration (Rogers and Basurco, 2009). The vaccination technique used in AquaBioTech will depend on the experimental protocol. To avoid stress, fish are placed in anaesthetic solution and afterwards injected with the vaccine. During the injection period, fish are handled in a gentle manner to prevent mucus and scale loss, and checked for abnormalities. For injection, the needle needs to be chosen according to the fish size. Each fish is injected in the abdominal cavity without jeopardizing internal organs. Full injection of the vaccine is checked during the procedure. Vaccination is a teamwork, as two to four people are needed for the whole process.

2.2 Work experience: Ecotoxicology Laboratory

2.2.1 Advanced methods

The ecotoxicology research laboratory at AquaBioTech Group applies well-known techniques to test hazardous substances that are or may be released into the environment. Governmental regulations such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) allow industry to test chemicals under development to a variety of toxicity tests that ensure unwarranted toxicity is not a property of the material. Risk assessment is a tool that determines the environmental toxicology for certain chemicals and predicts their effects in the environment (Landis et al., 2003) The use of ecological models for risk assessment has become routine, since they can be used to extrapolate across levels of biological organization (individual to population) and across spatial and temporal scales (Galic and Forbes, 2014).

2.2.1.1 Laboratory routine

A laboratory used for research must follow general rules and procedures with no exceptions. Human mistakes can jeopardize the safety of laboratory staff. Training is carried out to ensure that the staff is informed about the laboratory procedures incorporated in the SOP's as based on GLP (good laboratory practices). After the training, basic rules need to be followed: do not work alone, do not eat or drink, do not wear jewellery, and keep the laboratory clean and organized. Staff needs to wear, as soon as they enter the laboratory, a protective lab coat, appropriate footwear, goggles, gloves, and if applicable a mask. The type

of goggles, gloves and masks will change according to the hazardousness of the work. The laboratory has a MSDS folder available in case of accidents, such as spills, or to be consulted with regards to the protective equipment used when handling a chemical.

Housekeeping is a crucial task in any working area. A clean and well-maintained work area assures safety by preventing accidents that can jeopardize the efficiency of the work performed. Work surfaces should be cleaned before and after use to prevent contaminations, if applicable, and possible health risks to other staff. The ecotoxicology laboratory at AquaBioTech has 4 cleaning plans: daily, weekly, monthly and every six-month cleaning.

In the laboratory, all glassware is physically (scrubbed) and chemically (1% nitric acid) cleaned and then rinsed with deionized water to avoid chemical residues. The equipment needs to be checked for cleanliness before and after use. Glassware cleaning is crucial, as dirty glassware will cause erroneous results. Dirty glassware can also affect the volume of liquid to be delivered altering the concentration of a dilution.

2.2.1.2 How to prepare dilutions within OECD guidelines

All test solutions of selected concentrations are prepared by dilution of a stock solution. The stock solutions are prepared by simply mixing the test chemical in the dilution medium by ultra-sonification. If the test chemical is difficult to dissolve in water, procedures described in the OECD (2022) Guidance Document No. 23 for handling difficult substances should be followed. The use of solvents should be avoided, but may be required in some cases to produce a suitable concentrated stock solution. Where a solvent is used to assist in stock solution preparation, the final concentration of the solvent should not exceed 100 µl/L and should be the same in all test vessels. When a solvent is used, an additional solvent control is required.

2.2.1.3 Algal growth inhibition tests OECD 201 and ISO 10253

Microalgae are responsible for a large percentage of all primary production in the marine ecosystems (Arai et al., 2009). They are the first level of the food chain to be affected by

pollution and therefore they offer valuable information on the environmental impact of pollution (Debelius et al., 2009). Microalgae toxicity tests have several advantages (Debelius et al., 2009; Zhang et al., 2016), such as:

- sensitivity to toxic substances;
- short growth period;
- easy operation;
- easy observation;
- not affected by animal-ethic constraints.

All the procedures involving the algae culture are performed under sterile conditions. Algae handling is performed under the flame and all the material autoclaved (120 ºC for 20 min). Three algae cultures are maintained at AquaBioTech: *Phaeodactylum tricornutum, Tetraselmis chuii*, and *Thalassiosira pseudonana*. *P. tricornutum* and *T. pseudonana* are diatoms, and T. chuii a green flagellated algae (Figure 2.7). The algae cultures are maintained in an incubator at 20 ± 1 °C, a 14L:10D photoperiod, and light intensity of 70 μE m^{-2} s^{-1}.

Figure 2.7 – Algae cultures at AquaBioTech Group (AquaBioTech Group Company. Available in: https://www.aquabt.com).

The algal growth inhibition tests performed in AquaBioTech were adapted from the OECD Guideline 201 (2011), ISO 1025 (2006), and Eisentraeger et al. (2003). The algae are cultured in Erlenmeyer flasks under sterile conditions and renovated each 4 to 7 day. To prevent possible evaporation, the Erlenmeyer flasks are covered with a cotton stopper. The

cultures are shaken twice a day, once in the morning and afternoon. The base medium used was F/2 Guillard's medium (Guillard and Ryther, 1962; Guillard, 1975). It is prepared by dilution of $NaNO^3$, $NaH_2PO^4.2H_20$, trace metals, and vitamins stock solutions in deionized water. For the culture of the algae 1 mL of each stock solution is added to one litter of seawater at 35 ± 2 °C as appropriate. For the culture of *T. chuii*, 1 mL of a silica solution also needs to be added.

To be used in a growth inhibition test, the algal culture needs to be four to seven days old. One day before the start of the test, all the 24-well plates need to be rinsed with deionized water (DI) and dried.

The stock solutions are prepared by diluting the compounds to be tested in culture medium 1h before each trial, to allow the solutions to be at room temperature. To avoid test medium evaporation, the borderline wells, need to be filled with deionized water, as shown in figure 8 in blue colour. The test solutions are added to 24-well plates from the lowest (C5) to the highest concentration (C1) with three replicates per concentration (Figure 2.8).

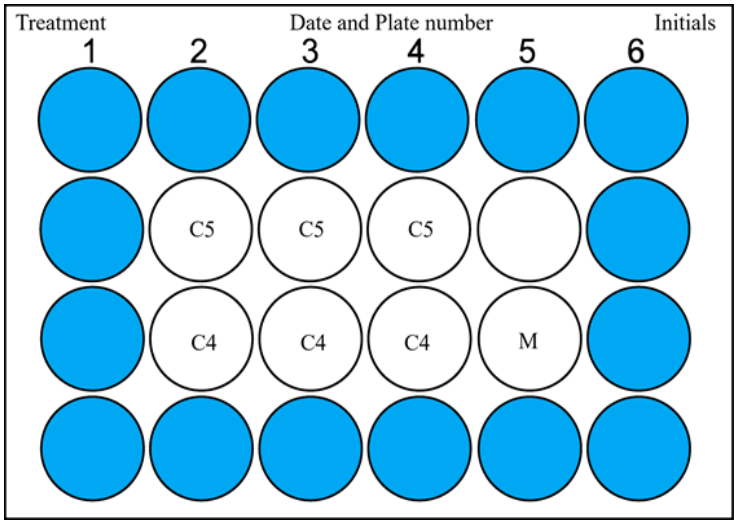

Figure 2.8: 24 well plate: blue circles indicate the wells to fill with DI. The wells in the middle are used for the test solutions: e.g. C5 and C4: concentrations, M: control 1 (only culture medium).

A sample of the exponentially growing culture is harvested and then the initial number of cells counted under the microscope. To start a trial with an initial cell concentration of 10^4 cells/mL for algae and 10^5 cells/mL for diatoms, a dilution needs to be made according to the following calculations:

For algae:

$$\text{average } 10\square * 25 = x * 10^4\ cells/mL$$

$$C1 \times V1 = C2 \times V2 \Leftrightarrow\ x * 10^4 cells/mL \times V2 = 50\ mL \times 1 * 10^4 cells/mL$$

$$V2 = x\ mL = x\ \mu L$$

For diatoms:

$$average\ 10\square * 25 = x * 10^4\ cells/mL$$

$$= x * 10^5\ cells/mL$$

$$C1 \times V1 = C2 \times V2 \Leftrightarrow\ x * 10^5\ cells/mL \times V2 = 50\ mL \times 1 * 10^5 cells/mL$$

$$V2 = x\ mL = x\ \mu L$$

As soon as the dilution is carried out, the algae/diatom are added to the test solution. To make sure that the cell concentration is reliable, the algae dilution is shaken again before adding to each well. The total volume in the wells is 1mL (900 µL test solution and 100 µL algae). Finally, the plates are placed in the incubator and a sample of the prepared dilution is counted under the microscope to correct the dilution value. The algae under test are shaken twice a day with a micropipette.

At the end of each trial (72 h), a sample is taken from each well and fixed in Lugol's solution to stop cellular growth. The algae are then counted using a haemocytometer under a light microscope. The data from the trials are recorded on paper and using a computer system.

2.2.1.4 Artemia salina acute tests (adapted from ASTM E1440-91 (2012))

The genus *Artemia* is characterized by the following features:

- adaptability to wide ranges of salinity, temperature and nutrients;
- short life cycle;
- high adaptability to adverse environmental conditions;
- parthenogenetic reproduction strategy (nauplii/cysts production);
- small body size.

These features make *A. salina* a reliable, feasible and cost-effective organism for ecotoxicological research. *Artemia* is by far one of the organisms most adapted to laboratory practice. Its use reduces the scale of test organisms by reducing test volumes needed, waste produced, and space needed to perform trials (Nunes et al., 2006).

The existence of *A. salina* cysts are an important factor for ecotoxicology. *A. salina* is hatched at AquaBioTech in a beaker with seawater and aeration at 25 ± 5 ºC, 35 ± 5 ‰ and pH of 8.1 ± 2. At AquaBioTech, *A. salina* is used for ecotoxicological studies using a methodology based in ASTM E1440-91 (2012).

One day before the start of the test, all the 24-well plates need to be rinsed with deionized water and dried. The *A. salina* used in the test should be 24 hours old. The stock solutions are prepared by diluting the compounds to be tested in autoclaved seawater 1h before each trial, allowing the solutions to cool to room temperature by the start of the test. The plates are identified as shown in Figure 2.9.

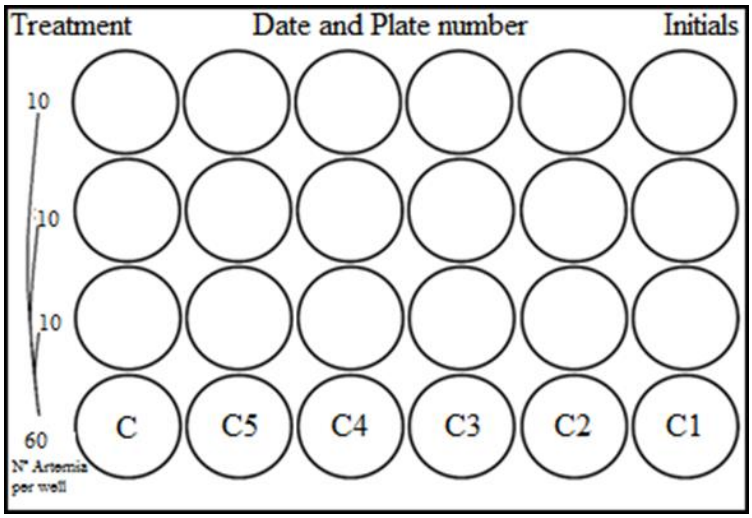

Figure 2.9: 24-well plate: identified from control to highest concentration. The three upper rows are used to perform the trial while the first row is used to minimize test solution dilution and is used to stock the upper wells.

Each column represents one test solution. The test solutions are added to 24-well plates, starting from the control and lowest concentration (C5) to the highest concentration (C1) with three replicates per concentration. The first row is filled with test solution and with an

average of sixty animals. To avoid dilution of the test solutions the animals are transferred from the lower horizontal well to the three replicates in the wells in the column above.

The plates are covered with parafilm to avoid solution evaporation and placed in the incubator for 24 h at 25 ± 5 ºC with no light. When counting, an *A. salina* is considered dead if there is lack of movement for at last 10 seconds the animal is considered dead. If there is more than 10% mortality in the control wells, the trial is considered not valid. After 24 h dead *A. salina* are counted and all data recorded on paper and on a computer system.

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3 EXPERIMENT: FISH EMBRYO ACUTE TOXICITY TEST

Sparus aurata as a model for the OECD Test No. 236: Fish Embryo Acute Toxicity (FET) Test

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Abstract

Currently, there are extensive regulatory requirements for fish acute toxicity data on individual chemicals, both for environmental risk assessment and hazard classification. Most of the methods used have been developed on freshwater species such as *Oncorynchus mykiss* (rainbow trout), *Danio rerio* (zebrafish), and *Oryzias latipes* (medaka). However, the use of a target marine species may be considered more appropriate to assess the impact of chemical anthropogenic substances on the marine environment. Recently, OECD proposed *Dicentrarchus labrax* (European sea bass) for the growth test (OECD No. 215).

In addition to scientific considerations, severe ethical concerns have arisen, since there is little doubt that fish subjected to acutely toxic concentrations of chemicals suffer severe distress and pain, which is clearly not compatible with current animal welfare legislation. The OECD Test No. 236 proposed a replacement alternative to the acute fish test. This method gives an excellent correlation with the acute fish toxicity test, and has the added advantage that nonfeeding developmental stages of fish are not categorized as protected stages according to the new European Directive 2010/63/EU on the protection of animals used for scientific purposes.

In this context, the present study is aimed at proposing the adaptation of the Fish Embryo Acute Toxicity (FET) test to gilthead sea bream embryos, Sparus aurata. For this purpose, the optimal conditions for cleavage and embryonic development of S. aurata at different temperature (10 °C to 30 °C) and salinities (30‰ to 40‰) were investigated, and when determined, a FET test was performed.

Temperature and salinity had significant effects on S. aurata embryos. 100% mortality was verified at 10 °C and 30 °C for all the salinities tested being impossible to calculate the total hatching (TH) and viable hatching (VH). At 20 °C the % of embryo mortality showed no statistical differences between the salinities tested. TH of ≥ 80 % was observed at both 35 ‰ and 40‰ showing statistical differences to the hatching observed at 30‰. VH was statistically different at all salinity treatments and highest at 40‰. At 25 °C at 30‰ the % of embryo mortality was statistical different to the % mortality at 20 °C at both 35‰ and 40‰ and for 25 °C at 35‰. At TH rate for 30‰ and 35‰ were statistical different and lower than 80%. No statistical differences

were found in the VH rate for all the salinities tested. 43 hours post fertilization (hpf) LC50 for saponin was 10 - 25 mg/L.

The optimal conditions for cleavage and embryonic development of S. aurata were 20 ºC at 40‰ of salinity. For the FET assays, all OECD requirements were fulfilled. A LC50 range was obtained due to S. aurata seasonality. Despite, Sparus aurata embryo showed to be a suitable a marine alternative specie for the OECD test No.236. due to its short duration and sensitivity.

Keywords: Embryonic development, OECD 236, *Sparus aurata*.

3.1 Introduction

In the European Union, fish acute toxicity data are an integral part and represent one of the most common tests for environmental hazard identification and risk assessment of chemicals, pharmaceuticals, biocides, feed additives, and effluents (Scholz et al. 2013). The fish acute toxicity is determined according to OECD test guideline 203 (OECD, 1992) or equivalent guidelines. However, the EU Directive (2010) on the protection of animals being used for scientific purposes aims to reduce the dependence on animal testing because of severe suffering and distress of the test animal (Braunbeck et al., 2005; Braunbeck and Lammer (2006); Embry et al., 2010 Busquet et al., 2014; Halder et al., 2014). Several suggestions to decrease or replace the number of animals for the acute fish toxicity test have been proposed, such as the use of fish embryos. The use of the Zebra Fish Embryo Tests has resulted in a reduction of tests on juvenile and adult fish required for aquatic toxicity testing (Kluver et al., 2015). Tests using early development stages are relatively fast, cheap, reduce volume of toxic wastes, and are sensitive to toxicants (Arufe et al., 2009). However, the use of freshwater species is not adequate to test substances in the marine environment because they do not test the potentially more sensitive species in this environment.

Fish produced commercially on a large scale in the Mediterranean Sea have been proposed as biological indicators to assess the impact of chemicals in the marine environment (Tornambe et al., 2015). *S. aurata* is typically reared in sea-based cages. It is an euryhaline and eurythermal (4–30 °C) species, with an optimal range of salinity [20–30 ‰] and temperature [14–28 °C], and is easy to maintain under laboratory conditions. In the past

years, embryos, larvae and juveniles of *S. aurata* have been used to evaluate the effect of chemicals (Oliva et al., 2008; Arufe et al., 2004; Arufe et al., 2009).

The aim of the present work was to verify if *S. aurata* embryos can be used as an alternative species in environmental risk assessment studies for the marine environment and propose this new species model for inclusion in animal testing following the OECD Test No. 236.

3.2 Materials and Methods

3.2.1 Test System and Test Reference

Fertilized eggs of *S. aurata*, were collected after spawning (19 ± 2 °C) from the broodstock facilities of the Malta Aquaculture Research Centre (MAR, Marsaxlokk, Malta), and then transported in 400 mL of oxygenated water placed in a 500 mL plastic container to the laboratory of the AquaBioTech Innovia (Mosta, Malta) facilities. Upon arrival, the embryos viability and size was checked under the stereomicroscope and fertilization rate calculated. The viable embryos were placed in an incubator at 20 ± 1 °C until they reached the morula stage before starting the tests described below.

Under the OECD Test No. 236, the model test compound Quillaja saponin, provided by ROTH (CAS-Nr. 8047-15-2) was tested for the present study. Stock solutions of 1000 mg/L were prepared in autoclaved filtered natural seawater (0.45µm) and then shaken vigorously in an ultrasonic bath for 10 min, to achieve maximum and homogeneous dissolution.

3.2.2 Effect of temperature and salinity on embryonic development of *S. aurata*

To determine the best temperature and salinity conditions to perform the fish embryo acute toxicity test, a preliminary assay was conducted using 4 batches of embryos. The diameter of 30 embryo from one batch was measured. Temperatures tested were 10 ± 1 °C, 20 ± 1 °C, 25 ± 1 °C and 30 ± 1 °C and the salinities tested were 30 ± 2‰, 35 ± 2‰ and 40 ± 2‰. The salinities tested were prepared by diluting filtered natural seawater (0.45µm) with deionized water, followed by autoclaving to avoid contamination during the testing period. Before the

experiment, the test solutions were aerated to ensure good oxygen conditions at the beginning of the experiment. After aeration, the solutions were placed in sterile polystyrene 96-well plates; each well was filled with 200 µL of the relevant test solution. The 96-well plates were previously acclimatized by incubating them to the temperatures tested and one embryo at morula stage was added to each well. For each temperature and salinity tested, three replicates of thirty embryos were used for four different batches. Every 24 hrs the embryos were transferred to plates containing new media.

Observations of the embryo development were made using an Optika Stereomicroscope (SZP-10) to help characterize the different stages of the embryo. A representative adapted figure of embryonic cleavage and development of sea bream eggs published by Kamaci et al., (2005) was used as guide for this step (Appendix I). Among others common morphological changes such as coagulated embryos, somite formation, rhythmic heartbeat, and tail detachment were observed and photographs were taken using an OptikamB5 Digital Camera (Appendix II).

Total hatch (TH) and viable hatch (VH) rates of the embryos were calculated. The total hatch rate was considered as the percentage of embryos that produced live larvae, both normal and abnormal. The viable hatch rate was considered as the percentage of embryo producing live larvae that were normal in appearance and behaviour (Mihelakakis and Yoshimatsu, 1998).

3.2.3 Fish embryo Acute Toxicity (FET) Assay

The experimental design of the assay was adapted from OECD Test No. 236 (OECD, 2013; Braunbeck et al., 2005). Embryos of *S. aurata* (at morula stage) were exposed to different concentrations of saponin, under 20 ± 1 °C, salinity of 40 ± 2‰ and a photoperiod of 16L:8D (LMS incubator). Three trials were performed with different saponin concentrations to determine the LC50. The concentrations used were: 39, 59, 88, 132, 198, 296, 444, 667, and 1000 mg/L; 5.8, 7, 8.4, 10, 12.1, 14.5, 17.4, 20.8, and 25 mg/L; and 9, 10, 11, 12, 14, 15, 17, 18, and 20 mg/L. The concentrations tested were a geometric series with a dilution factor 2, 1.2, 1.1 respectively, and prepared as described above (Section 2.1). Immediately after ultrasonication, aliquots were removed for the preparation of the final nominal test concentrations to start the experiments. Before the experiment, the test solutions were

aerated to ensure good oxygen conditions at the beginning of the experiment. The exposure was performed using sterile polystyrene 96-well plates with the embryos kept individually in 200 µL volumes of the test concentrations. Every 24 hrs the embryos were transferred to plates containing fresh suspensions, prepared as described above.

The embryos were examined during the following development stages: somite formation, rhythmic heartbeat, and tail detachment using an Optika stereomicroscope. Fourty-eight embryos per concentration were used. Apical observations performed on each embryo were: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. Coagulated embryos were characterized by a milky white appearance (Figure 3.1). Lack of somite formation is the non-appearance of spontaneous movement in the embryo. Non-detachment of the tail was observed when there was no posterior elongation of the embryonic body. A lack of heartbeat was characterized by no movement of the heart for at least one minute. At the end of the exposure period (43 hpf), surviving embryos were sacrificed (kept at <0 °C, until they were dead).

3.2.4 Data analysis

The OECD has developed and achieved consensus on practical guidance on principles and processes for the validation and acceptance of animal and non-animal test methods for regulatory hazard assessment purposes. To meet this goal each test has some validation criteria that need to be achieved. In the OECD Test No. 236 (OECD, 2013), the test results are considered valid when a) the overall fertilization rate of all eggs collected should be ≥ 70 % in the batch tested, (b) the water temperature should be maintained at 20 ± 1 °C in test chambers at all time during the test, (c) overall survival of embryos in the control should be ≥ 90 % until the end of the exposure, (d) hatching rate in the control should be ≥ 80 % at the end of the exposure, and (e) at the end of the of the exposure, the dissolved oxygen concentration in the negative control and highest test concentration should be ≥80 % of saturation.

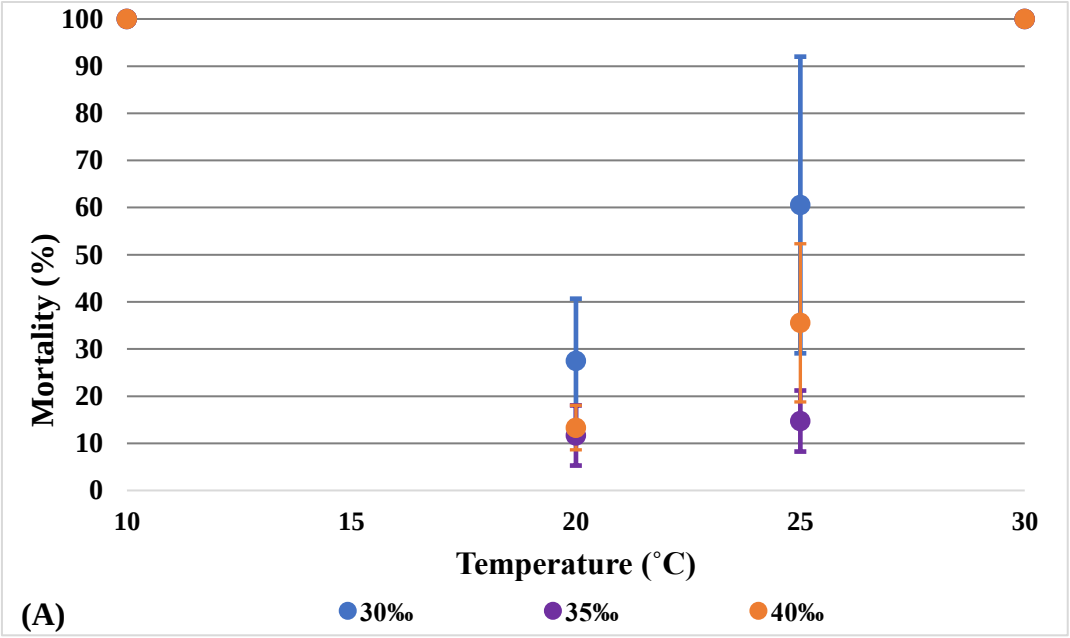
Statistical analysis of the results was performed using SPSS. Data on biological parameters were checked for assumptions of normality using the Kolmogorov-Smirnov homogeneity of variance using the Levene test. If both assumptions were met, data were analysed by one-

way ANOVA followed by Tukey's test. Data were analysed by the non-parametric Kruskal-Wallis test whenever either of the assumptions was not met. Differences were considered significant at $p < 0.05$ statistics.

3.3 Results

3.3.1 Effect of temperature and salinity on embryonic development of *S. aurata*

The average diameter of viable embryos used in this study was of 0.96 ± 0.05 mm (average ± S.D) (n=30). In this study, the embryonic development was affected by temperature and salinity (Figure 3.1A and 3.1B). 100% mortality was verified at 10 °C and 30 °C for all the salinities tested. Statistical differences were found when comparing mortalities at 20 °C and 25 °C at the different salinity treatments. At 20 °C the % of embryo mortality showed no statistical differences between the salinities tested. However, the % of embryo mortality for 25 °C at 30‰ was statistical different to the % mortality at 20 °C at both 35‰ and 40‰ and for 25 °C at 35‰ (Figure 3.1A and 3.1B).



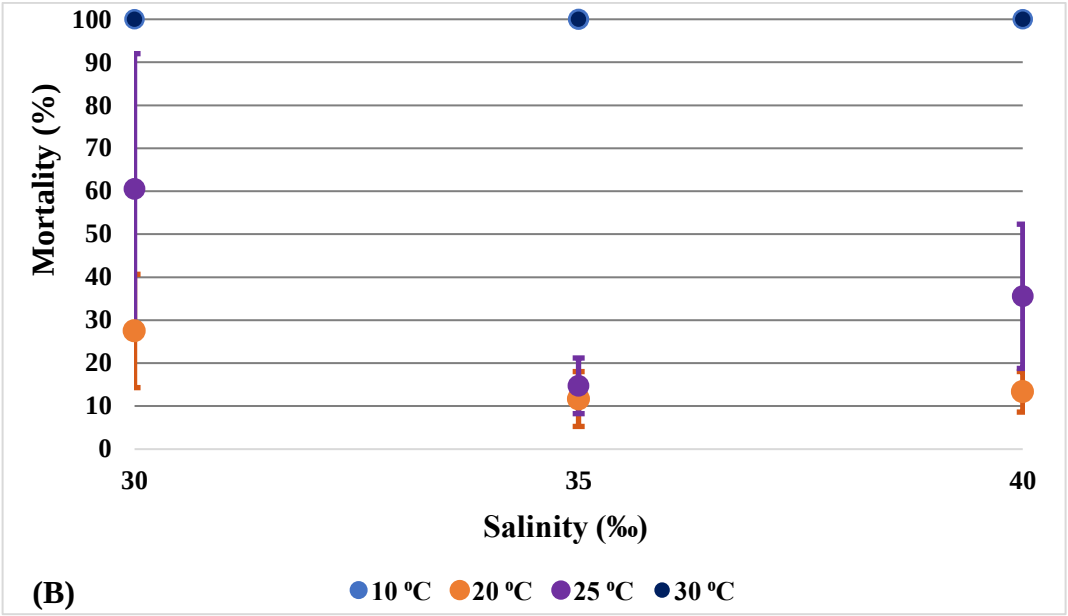


Figure 3.1: Percentage in mortality according to: (A) temperature (10 °C, 20 °C, 25 °C, 30 °C) at different salinities (30‰, 35‰, 40‰); (B) salinity (30‰, 35‰, 40‰) at different temperatures (10 °C, 20 °C, 25 °C, 30 °C).

Total hatching (TH) and viable hatching (VH) was also affected by temperature and salinity. TH and VH was not possible to determine at 10 °C and 30 °C as these temperatures were shown to be lethal to the embryos.

At 20 °C at 43 hpf TH calculated was 72.5%, 88.3% and 86.7% (Figure 3.2 A) and VH was 45.8%, 83.2%, 90.2% for 30‰, 35‰ and 40‰, respectively (Figure 3.2 B). At 20 °C at 43 hpf a TH of ≥ 80 % was observed at both 35 ‰ and 40‰ showing statistical differences to the hatching observed at 30‰. The VH results were statistically different at all salinity treatments and highest at 40‰.

At 25 °C TH at 27 hpf calculated was 60.6%, 14.7% and 35.6% (Fig. 3.2 A) and VH was 27.8%, 46.8%, 40.2% for 30‰, 35‰ and 40‰ respectively (Figure 3.2 B). TH for 30‰ and 35‰ were statistical different (Figure 3.2 A). However, in all the salinities tested (30‰, 35‰ and 40‰) the TH rate at 27 hours post fertilization (hpf) did not achieve 80 % according to OECD (2013) criteria, making these conditions unsuitable for future assays. There were no statistical differences in VH at all the salinities tested.

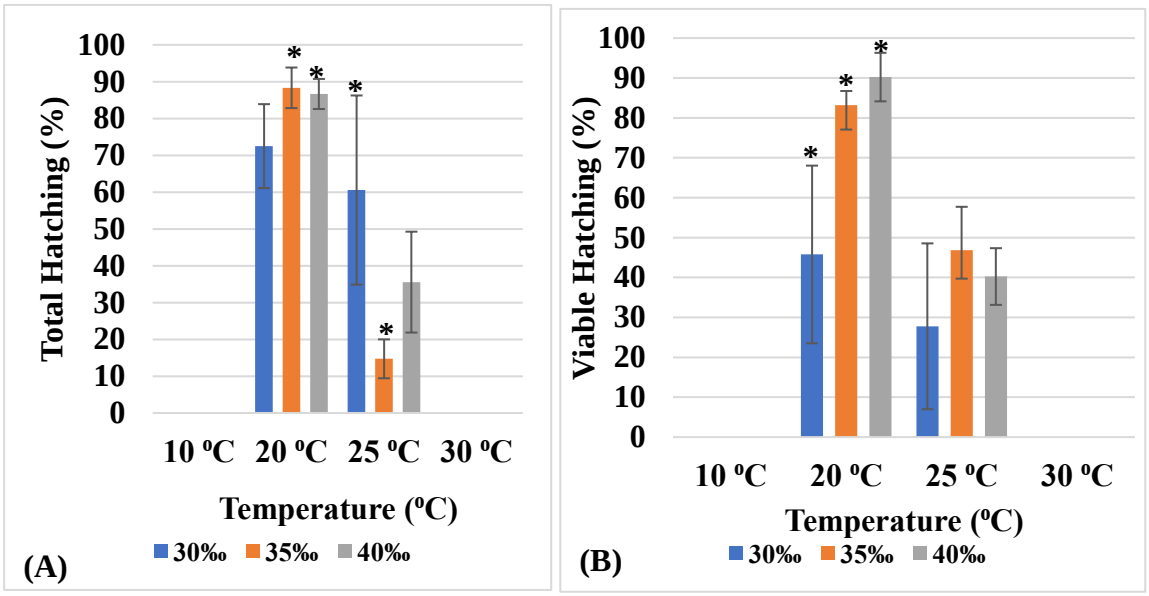


Figure 3.2: Percentage of total hatching (A) and viable hatching (B) at different temperatures (10 °C, 20 °C, 25 °C, 30 °C) and salinities (30‰, 35‰, 40).

At 10 °C the embryonic development of > 50% of embryos stopped at the morula stage, which was also the last stage observed as all the embryos died. At 20 °C, hatching was observed > 50% of embryos and the last observed at 43 hpf, for all the salinities tested. At 25 °C (27 hpf) this was also confirmed, except for 30‰ where the stage reached by >50% of the embryos was an increase of pigmentation by the embryo followed by hatching. At 30 °C the last stage observed by >50 % of embryos was a first pigmentation of the embryo at a salinity of 30‰ and the appearance of the heart at salinities of 35‰ and 40‰ and the first pigmentation for 30 ‰.

Table 3.1: Stages reached at temperatures (T) and salinities (S) tested by >50 % of embryos and the last stage observed the same, including the time reached by the last stage observed.

T (°C)	**S** (‰)	**Stage reached >50 % embryos**[#]	**Last stage observed**[#]	**Time to reach the last stage observed***
10	30	1F	1F	-
	35	1F	1F	-
	40	1F	1F	-
20	30	2H	2H	43
	35	2H	2H	43
	40	2H	2H	43
25	30	2F	2H	27
	35	2H	2H	27
	40	2H	2H	27

30	30	2B	2B	17
	35	2C	2C	17
	40	2C	2C	17

*The time when the majority (>50%) of embryos were judged to have reached that stage.

#Stages were indicated as per Appendix I

3.3.2 Fish embryo Acute Toxicity (FET) Assay

The FET assays performed were considered valid when the fertilization rate, survival of embryos in the control and hatching rate in the control were higher than ≥ 70%, ≥ 90%, and ≥ 80%, respectively. Temperature was maintained at 20 ± 1 °C during the trial and ≥80 % oxygen saturation at the end of the exposure. For the first FET trial (39, 59, 88, 132, 198, 296, 444, 667, and1000 mg of saponin/L), the fertilization rate was 99.2 % and survival of embryos and hatching rate in the control was 98%. 100% mortality was obtained at all the concentrations tested during the first assay.

In the second FET assay (5.8, 7, 8.4, 10, 12.1, 14.5, 17.4, 20.8, and 25 mg of saponin/L) the fertilization rate was 97.35% and survival of embryos and hatching rate in the control was 93.62%. During this assay, a 43 hpf LC50 value between 10 - 25 mg/L was determined (Fig. 3.4).

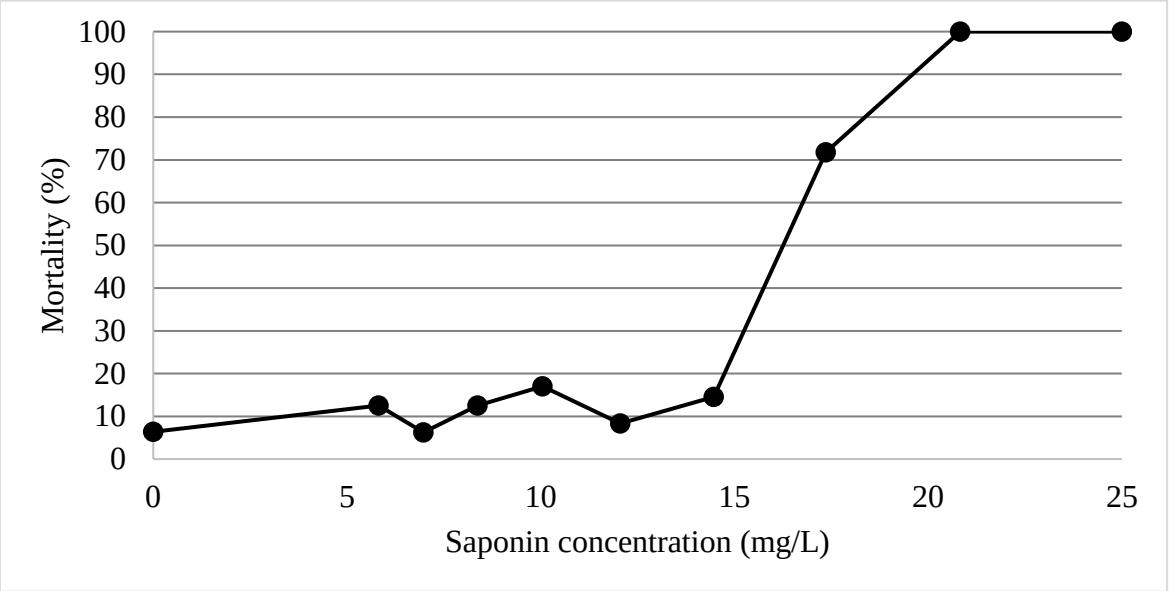


Figure 3.3: Percentage of mortality of S. aurata embryos when exposed to different concentrations of Quillaja saponin.

A third trial was performed with the aim of determining more accurately the LC50 value. However, data obtained was not considered, as the control showed a survival rate lower than 90% and repeating could not be made in the proposed report time-frame.

3.4 Discussion

Previous studies have reported that survival rate depends on egg size and that eggs obtained by hormonal treatment were relatively smaller in size (Boulineau, 1974; Nash and Kuo, 1975). During the tests described here, the average embryo diameter of viable eggs used was 0.96 ± 0.05 mm (average ± S.D) (n=30) which are similar with those found by Kamaci et al (2005).

Temperature and salinity are crucial parameters affecting the survival and development of fish embryos. Embryos are known to be sensitive to temperature variations due to their incomplete osmoregulatory system (Jin et al., 2017). Temperature is known to affect the rate and quality of fish embryonic development. It determines deterioration of the cellular symmetry and breaking-up of the oil globule. Temperature variations can also cause mass mortality decreasing in the rate of larvae production during gastrulation (Kamaci et al., 2005; Fuiman, 2003; Georgakopoulou et al., 2010; Mohammadi et al., 2012). Lower temperatures are known to retard the rate of embryonic development and higher temperature accelerate the process, affecting metabolic rates and cellular function (Mihelakakis and Yoshimatsu, 1998; Valeta et al., 2013). In this study, low temperatures (10 °C) were shown to be lethal for embryos, stopping their embryonic development at morula stage. A high temperature (30 °C) was found to accelerate the process, jeopardizing the proper development of the eggs as embryos developed until the appearance of heart, dying after this stage. In this study, it was found that embryonic development occurred best at 25 °C (27 hpf) and then at 20 °C (43 hpf).

A comparison of the embryonic development stages of D. rerio and S. aurata at different temperatures is presented in Table 3.2. The table confirms that the hatching occurs sooner for S. aurata at 18.5 °C and 20 °C than for D. rerio at 26 °C. However, the development times obtained by Uçal (1983) did not match with those obtained in this experiment. In this study, the embryo development was irregular as it proved to be slower until the morula stage

and faster from the gastrulation stage, achieving 100% hatching 3 hours earlier than observed by Ucal (1983). The results obtained in this study appeared to be more consistent in terms of normal development when compared with the results obtained by Braunbeck an Lammer (2006) and Kamaci et al. (2003).

Table 3.2: Comparison of embryonic developments in *Danio rerio* (A: Braunbeck and Lammer (2006)) and *Sparus aurata* (B: Kamaci et al. 2005; C: Uçal, 1983) and D: results of this study).

Reference of experiment	***A***	***B***	***C***	**D**
Species	*Danio rerio*		*Sparus aurata*	
Temperature	*26 °C*	*18.5 °C*	*20 °C*	20 °C
Stages of Embryonic development[#]	**Time**			
1B	*1:00*	*1:45*	*1:00*	1:30
1E	*4:40*	*3:00*	-	3:15
1F	*5:20*	*4:15*	*2:45*	4:30
1H	*6:00*	*14:00*	*9:15*	10:00
1M	*8:00*	*19:00*	*23:30*	17:00
2F	*25:00*	*36:00*	-	42:00
2H	*72:00*	*53:00*	*46:00*	43:00

**The time when the majority (>50%) of embryos were judged to have reached that stage.*

[#]Stages were indicated as per Appendix I

Kamaci et al. (2005) proved that during the embryonic period of S. aurata, developmental defects that jeopardize the incidence of viable larvae at hatching, are minimized at optimum temperature between 16–22 °C. Azab et al. (2015) showed that S. aurata larvae have an improved survival rate when at a salinity range of between 20 °C to 35‰. However, salinity can affect yolk utilization and larval growth and survival by influencing the amount of energy needed for osmoregulation (Azab et al., 2015), as demonstrated also in this study where a change in salinity significantly affected TH and VH.

According to the OECD (2013) guidelines, the TH rate is an essential parameter to evaluate toxicity in embryos. However, the VH rates are a better indicator of temperature and salinity effects than the TH rate. VH rates evaluate the presence of normal and abnormal larvae and provides information required for the prediction of the percentage of larvae that may achieve exogenous feeding and successive normal development (Mihelakakis and Yoshimatsu, 1998; Polo et al., 1991).

Taking this into consideration, the optimal conditions for embryonic development of S. aurata were 20 °C and 40‰, according to OECD test No. 236 criteria. Despite the fact that there were no differences between 35‰ and 40‰, 40‰, was chosen in this study because fertilization in the broodstock tanks was occurring at this salinity and this was the salinity of the water used to transport the embryos were being transported. Under these conditions hatching was obtained at 43 hpf. With the freshwater fish models used in the OECD test No. 236, hatching started after 48 hpf at 26 °C. This compares well with the work of Kamaci et al. (2005) obtained who obtained 100 % TH at 46 hpf.

The amount of test solution used in the test prohibited the measurement of dissolved oxygen concentration at the end of the test. However, this small volume in the wells created a high-tension surface which decreased oxygen dispersion (Braunbeck et al., 2005). That sufficient oxygen was available was demonstrated by the high TH and VH obtained in some of the treatments.

There is limited information about the effects of saponin in fish juveniles and fish embryos. MacPhee and Ruelle (1969) tested the relative effect of saponin (10 mg/L) on Ptychochellus oregonensis (squawfish), Oncorhynchus tshawlocha (chinook salmon), and Oncorhynchus kzsutch (coho salmon)). They concluded that the fish died between 3 to 7 hrs after exposure. Vinay et al (2013) studied the effect of a saponin vaccine. They showed that this saponin had effects in Paralichthys olivaceus (olive flounder) with a LD50 of approximately 105 μg/ fish (22.4 mg/kg), producing severe histological injuries in the liver, pancreas, kidney, spleen and intestine. A study conducted by Oliveira et al. (2012) showed a 96h LC50 of 22.546 mg/L (saponin), causing changes in heartbeat rate and in D. rerio length.

In embryos, saponin is known to have both beneficial and harmful effects. Lower concentrations can increase the absorption of ions that will increase cell proliferation and differentiation rates. High concentration can damage the membrane cholesterol, jeopardizing membrane fluidity, exhibiting shrinkage of the chorion followed by embryonic mortality (Hassan et al., 2008; Ansari and Ansari, 2012).

The LC50 value obtained for *Danio rerio* by Oliveira et al. (2012) was 22.546 mg/L. The LC50 range obtained in this study for *S. aurata* (10-25 mg/L), was close to the LC50 obtained by Oliveira et al. (2012) for *D. rerio*. In the present study, a LC50 value was not obtained due to *S. aurata* seasonality, more studies had to be performed to obtain a LC50 value.

The OECD Test No. 236 was designed to determine acute toxicity of chemicals on embryonic stage of fish (OECD, 2013). The aim of this study was to recommend a new model where eggs of *Spaurus aurata* can be presented as an alternative species to be used on risk assessment studies for the marine environment. OECD requirements were fulfilled for the assays considered as they presented: a) an overall fertilization rate of all eggs collected ≥ 70%; b) the temperature was maintained at 20 ± 1 °C in test chambers at all time during the test; c) the overall survival of embryos in the control was ≥ 90% until the end of the exposure; d) the hatching rate in the control was ≥ 80% at the end of the exposure; e) the dissolved oxygen was ≥80% of saturation. In summary, the short duration of *S. aurata* embryo-larval development and their sensitivity in the same range as *D. rerio* demonstrates that this model can be considered a suitable species for the OECD Test No. 236. Moreover, because the *S. aurata* test can run at a lower temperature it is an advantage when volatile or instable samples need to be tested.

3.5 Acknowledgments

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4 CONCLUSION

An internship performed in a company environment has a great number of advantages. It allows the intern to undertake a series of personal and professional challenges that are hardly achieved in a scholar environment. As AquaBioTech Group is a company located outside of Portugal that provides the opportunity to apply the knowledge acquired during the academic degree in different scenarios and learning new skills valuable as a professional. Also at a personal level, a sense of responsibility, organizational skills and development of good relationships are improved.

This internship was taken to complete the master in Marine Resource Biotechnology from the Polytechnic Institute of Leiria. This internship allowed the development of knowledge in the aquaculture facility and ecotoxicology laboratory. The AquaBioTech Group offers good facilities and equipment to perform high quality work, providing important tools during the internship period.

The final goal of the internship was to perform an experiment with *Sparus aurata*. Assays were performed to evaluate the optimal conditions for embryonic development and the effect of Quillaja Saponin in S. aurata embryos. The optimal conditions for cleavage and embryonic development of *S. aurata* were 20 ºC of temperature and at 40‰ of salinity. For FET assays, the LC50 range obtained was 10-25 mg/L.

Therefore, all work performed during the 6 months of internship proved to be a great opportunity to enter the work market. The company recognized the work performed as a job opportunity was given after the end of the internship.

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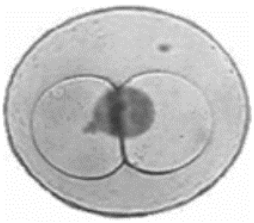
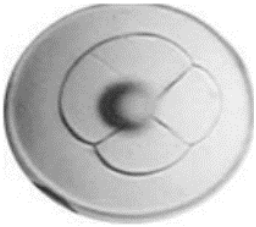
6 APPENDIX I

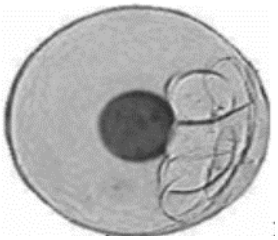
The cleavage (1A - 1M) and embryonic (2A – 2H) development of sea bream at 18.5 °C (adapted from Kamaci et al. (2005).



1A: 2-cell stage

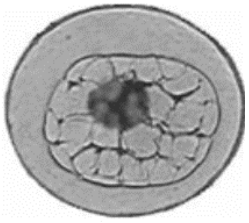


1B: 4-cell stage

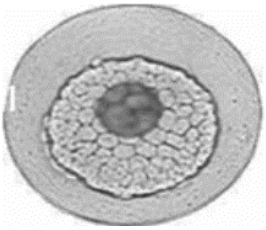


1C: 8-cell stage

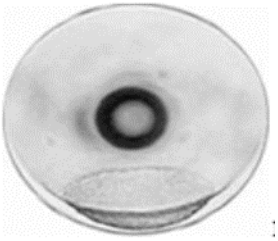
1D: 16-cell stage



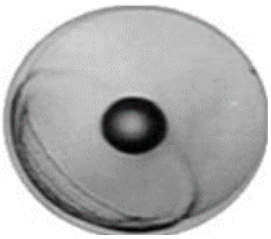
1E: 32-cell stage



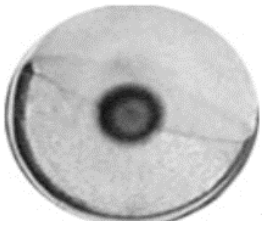
1F: Morula stage or Blastodisc formation



1G: Blastula stage

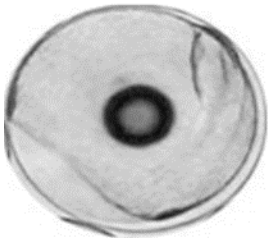


1H: Gastrulation stage

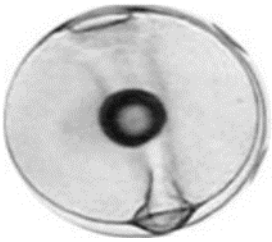


1J: Gastrulation stage

1K: Neurula stage

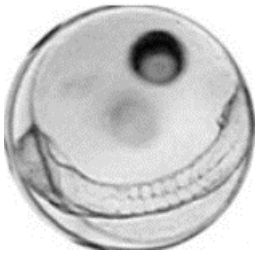


1L: Embryonic axis is visible

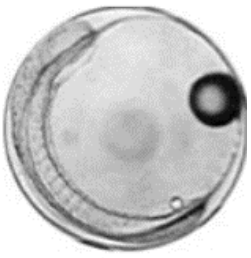


1M: Closing of blastopore with perfected embryonic axis

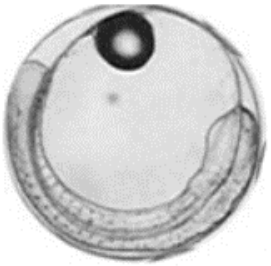
2A: Somits and Kupffer apparatus



2B: 1º pigmentation



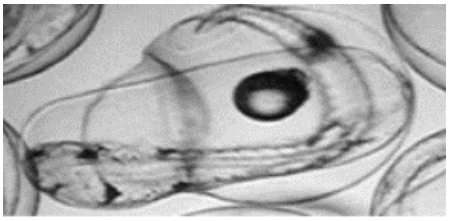
2C: Appearance of Heart



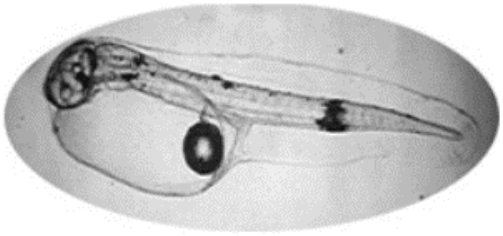
2D: Primordial fin

2E: Formation of optic lens

2F: Increasing of Pigmentation



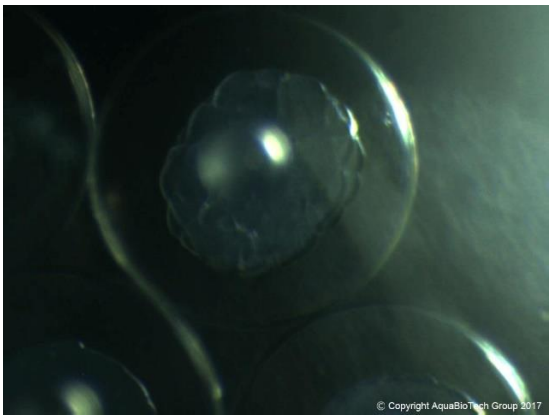
2G: Splitted the chorion



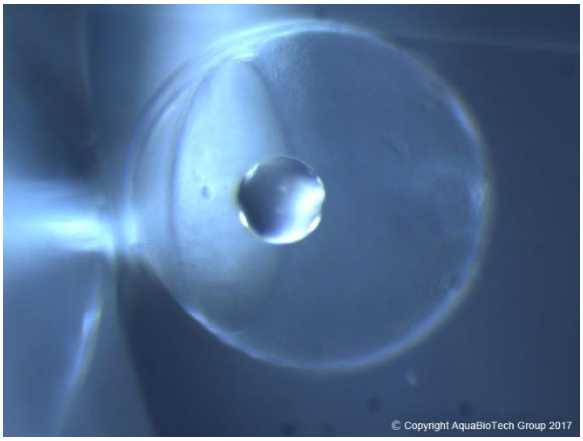
2H: Released from the egg

7 APPENDIX II

Pictures of the embryonic stages of *Sparus aurata.*

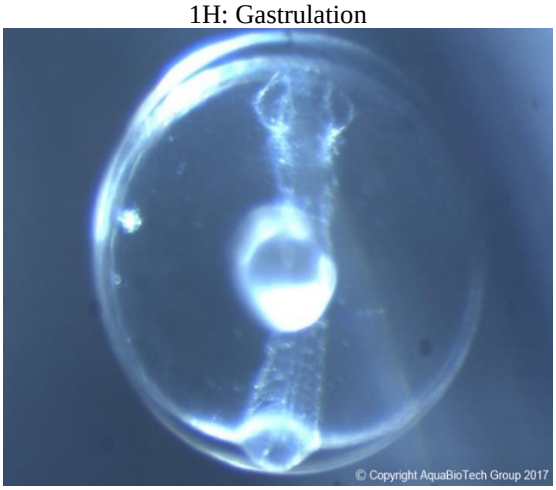


1F: Morula Stage

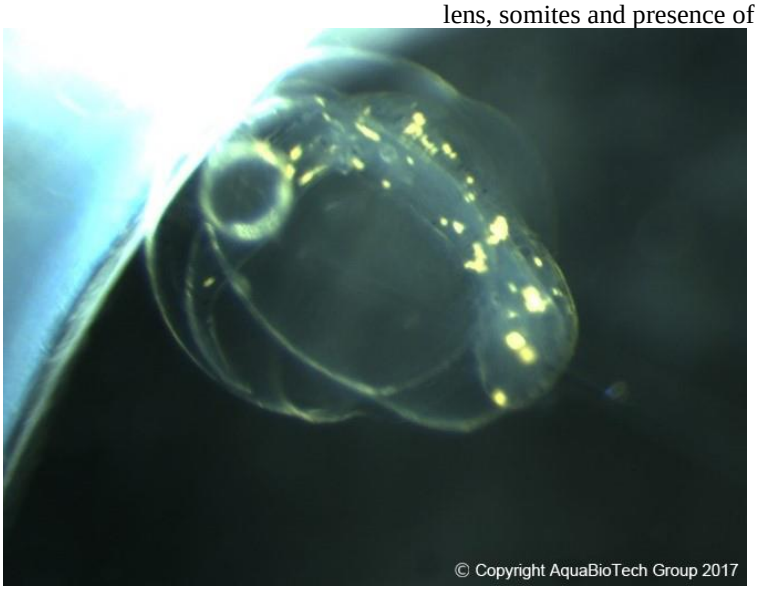


1H: Gastrulation

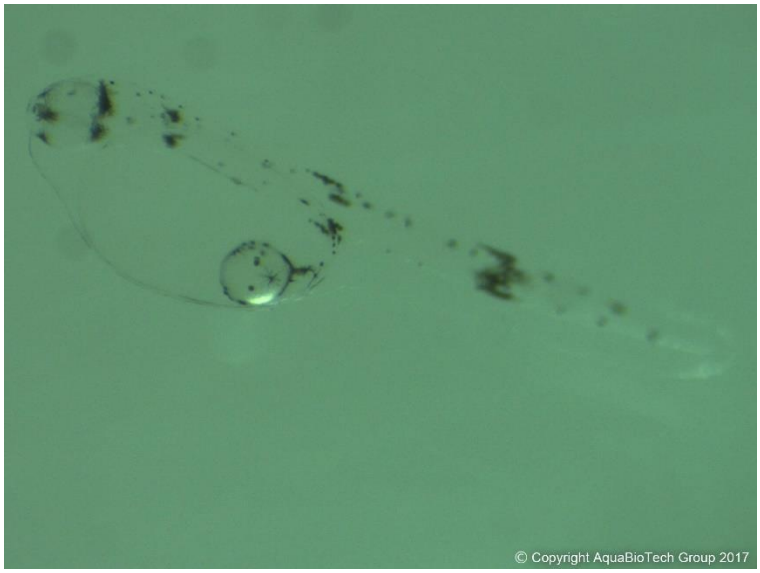
1M: Closing of Blastopore



1F: Increasing of pigmentation (formation of optic lens, somites and presence of heart beat)



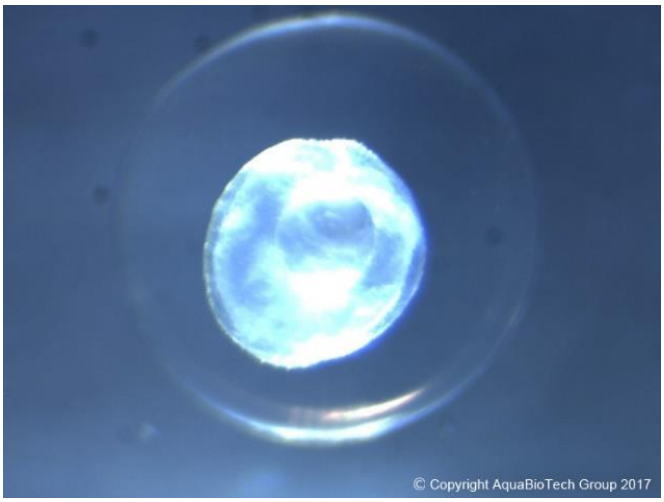
2G: Larvae emerging from the chorion



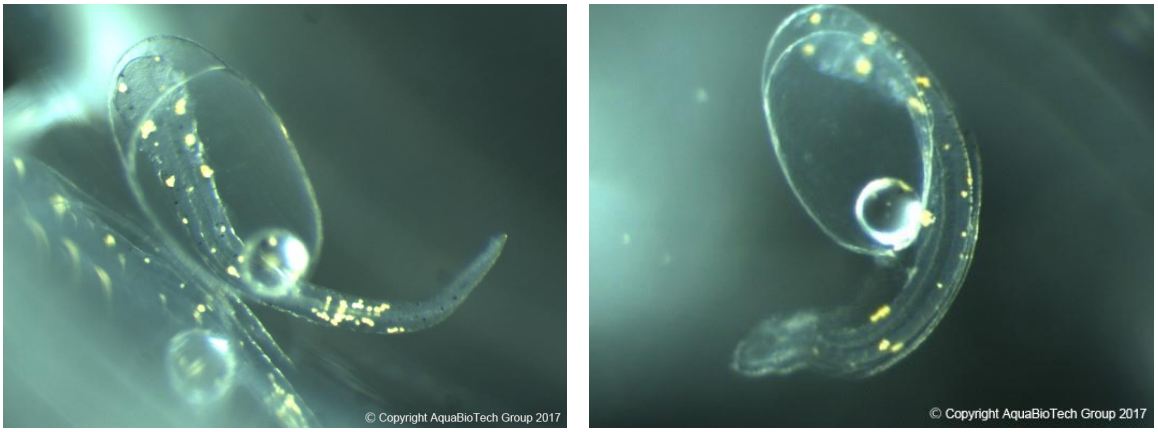
2H: Larvae released from the egg

8 APPENDIX III

Pictures of dead embryos (A) and abnormal larvae (B)



A: Dead embryo showing a milky white appearance



B: Larvae showing tail malformations