escola superior de turismo
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Routines in a European aquaculture company and the development of a *Flavobacterium psychrophilum* dose response pre-model in adult Rainbow Trout

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An internship report submitted to the School of Tourism and Maritime Technology, Polytechnic Institute of Leiria as partial fulfilment for the requirements for the Master Degree of Science in Aquaculture, held under the scientific 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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Abstract

An internship in a European company dealing with aquaculture and biotechnology - AquaBioTech Group, Malta - was undertaken to complete the Master Degree of Science in Aquaculture of the School of Tourism and Maritime Technology of the Polytechnic Institute of Leiria.

Biotechnology and aquaculture are two areas that have been synergistically used to contribute for the progress and improvement of fish production. The AquaBioTech Group is an example of a company able to integrate these areas to maximizing their services. Located in Mosta (Malta) the company 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 performed during 6 months. Initially, it will cover the operation and constitution of the company, describing the routines and techniques acquired. Then, it will describe a pathology trial that forms the practical and scientific component of this report. Despite the limitation to describe some confidential assays, this trial consisted in the infection of Rainbow Trout (*Oncorhynchus mykiss*) with the bacterium *Flavobacterium psychrophilum* in order to evaluate the mortality rates over time.

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

Keywords: Aquaculture, AquaBioTech Group, Bacterial Coldwater Disease, Rainbow trout*, Flavobacterium psychrophilum*.

Resumo

Um estágio numa empresa europeia, que integra as áreas de aquacultura e biotecnologia - AquaBioTech Group, Malta - foi realizado para completar o Mestrado em Aquacultura na Escola Superior de Turismo e Tecnologia do Mar do Instituto Politécnico de Leiria.

A biotecnologia e a aquacultura são duas áreas que, quando empregadas sinergicamente, contribuem para o progresso e desenvolvimento da produção de peixes. AquaBioTech Group é um exemplo de uma empresa capaz de integrar estas vertentes de forma a maximizar os seus serviços. Localizada em Mosta (Malta), a empresa opera de forma sustentável utilizando sistemas de recirculação aquícola na manutenção das espécies de aquacultura. Em colaboração com outras empresas e instituições, AquaBioTech Group está envolvida no desenvolvimento e apoio de importantes projetos internacionais de investigação. O presente relatório centra-se em duas componentes essenciais do estágio realizado durante 6 meses. Inicialmente abordará o funcionamento e a constituição da empresa AquaBioTech Group, descrevendo as rotinas efetuadas e técnicas adquiridas. Em seguida, apresenta uma componente científico-prática no âmbito da patologia na aquacultura. Apesar das limitações para descrever alguns dos procedimentos confidenciais, este estudo consiste na infeção de Truta Arco-íris (*Oncorhynchus mykiss*) com a bactéria *Flavobacterium psychrophilum*, de forma a avaliar as taxas de mortalidade ao longo do tempo.

Este estágio serviu para solidificar conhecimentos teóricos adquiridos durante a formação acadêmica, desenvolver habilidades profissionais e elucidar sobre as oportunidades de emprego existentes no mercado.

Palavras-chave: Aquacultura, AquaBioTech Group, Doença bacteriana de água fria, Truta Arco-íris, *Flavobacterium psychrophilum*.

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1. Introduction

Aquatic organism production in aquaculture started to be developed in Asia as an activity being principally small scale and of non-commercial purpose. However, with continuous global population growth, the increasing demand for fish food has incremented at an average annual rate of 3,2 percent. This value outpaces the world population growth at 1,6 percent which means that fisheries alone can not supply all humanity food fish necessities (FAO, 2014). Creating and developing new techniques for aquaculture production appears to be the only solution to provide fish for food and satisfying the ever-increasing demand for their consumption. In this sector, China has been the leader in fish provision, owing to the fast expansion in its fish production from aquaculture. This contributes for its whole world spread, encompassing all aquatic environments and a diversity of culture species (Hishamunda and Subasinghe, 2009). Therefore, the aquaculture started producing several species in industrial levels, where they are often negotiated with high-value commercial and an international scale. The globalization of aquaculture opened many market opportunities and currently contributes to circa 45% of the world's fish supply for human consumption (FAO, 2014). With it is continued growth, it is expected that aquaculture will, in the near future, produce more fish for direct human consumption than fisheries capture (Subasinghe *et al*., 2009).

In Europe, the aquaculture industry development is one that has grown the fastest in the last three decades due to increasing of diversification, intensification and technological advances (Janssen *et al*., 2016). According to the different groups of aquatic species, the aquaculture production in the European Union is divided into three main sectors: marine, shellfish and freshwater production. Almost 50% of the total production resulted from shellfish aquaculture, namely from mussel followed by oysters and clams farming (Mente and Smaal, 2016). On the other hand, Europe also produces six main finfish species, namely Rainbow Trout (*Oncorhynchus mykiss*), Atlantic Salmon (*Salmo salar*), European Seabass (*Dicentrarchus labrax*), Gilthead Seabream (*Sparus aurata*), Turbot (*Scophthalmus maximus*) and Common Carp (*Cyprinus carpio*). In addition, the aquaculture industry is being viewed as an important financial and food contributor to the European economy as well as potentiating many employment offers (FAO, 2014; Janssen *et al*., 2016).

At the south of the European continent, in the middle of Mediterranean Sea, is the Republic of Malta. Due location and climate, the Republic of Malta has limited resources of water and increasing demands constituting a barrier to the attempt to support freshwater aquaculture facilities. Nevertheless, the government made some efforts to encourage aquaculture in abandoned salt pans, as suggested by Food and Agriculture Organization (FAO) in 1970, but concluding that such projects would be very expensive. The continuous increase in population density, the tourism industry expansion and climate conditions, such as limited rainfall, contributed for the annual water demand to surpass somewhat the amount of fresh ground water available. Thereby, all of the attempts to implement freshwater aquaculture were completely abandoned (Dill, 1993). Afterwards, in 1988, aquaculture started to be introduced into Malta by the National Aquaculture Centre (NAC). In own pilot marine hatchery, NAC begun with an annual production of 400,000 juveniles of European Seabass and Gilthead Seabream and quickly reached 1500,000 fingerlings on the following years owing to improvements in the technology and production. Thus it became aquaculture research pioneer in Malta offering expertise and technical support to young local industries. Currently it is known as the Malta Aquaculture Research Centre (MAR) and considered the only research facility for hatching marine species, encouraging other companies to invest in this area with the objective of increasing aquaculture in Malta (Fisheries and Aquaculture Department. Available in: http://www.fao.org/fishery/countrysector/naso_malta/en).

The aquaculture in Malta consists mainly in Atlantic Bluefin Tuna (*Thunnus thynnus thynnus*), European Seabass, Gilthead Seabream and a few productions of Meagre (*Argyrosomus regius*) and amberjack (*Seriola dumerili*). However, most of this production is maintained in floating offshore cages around the island. The Atlantic Bluefin tuna is cultured in five farms situated up to 6 km off the southeastern coast but European Seabass, Gilthead Seabream and Meagre are grown approximately one kilometer of the coast (Figure 1).

Figure 1 - Offshore aquaculture distribution in Malta (Google Earth®).

In 2013, European Seabass and Gilthead Seabream production was 2,677 tones whilst the Atlantic Bluefin tuna production was 6,123 tones, representing the total value produced of EUR 105,9 million. Statistical data from 2011 until 2014 proved a positive trend of development and aquaculture contribution to the economy of Malta, as shown in Figure 2 (National statistical office in Malta. Available in: https://nso.gov.mt/en/News_Releases/View_by_Unit/Unit_B3/Environment_Energy_Transport_and_Agriculture_Statistics/Pages/Aquaculture.aspx).

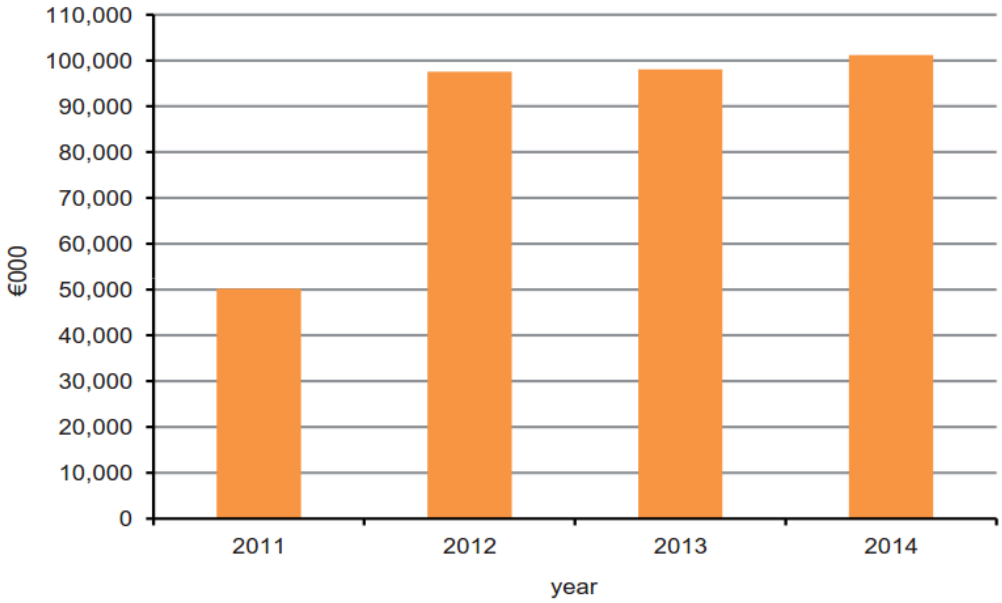

Figure 2 - Distribution of gross output of the aquaculture industry by year in Malta. (National statistical office in Malta, 2014).

The aquaculture sector expansion contributed to the country's economy through the unskilled and skilled labor, from the exports and increase in gross domestic product. More recent data demonstrated that the aquaculture sector created a total of 964 full-time jobs, with 197 of this job corresponding to the aquaculture sector itself directly and an additional 767 jobs generated by way of indirect activities, namely, transport and communication, financial intermediation and manufacturing sector (Sacchi, 2011). Currently, in Malta, formal opportunities to train technicians in aquaculture do not exist. However, this is been considered highly desirable to promote good and safe practices on the aquaculture sector. In a long term the government betting in strategies to promote investigations to increase sustainable aquaculture (Fisheries and Aquaculture Department. Available in: http://www.fao.org/fishery/countrysector/naso_malta/en). Thus, Recirculation Aquaculture Systems (RAS) started to be established to respond to aquaculture environmental concerns about land limitations and freshwater access in this country. This way, aquaculture in Malta has been maintaining stable production levels and diversifying to others marine species (Martins, *et al*., 2010; Fisheries and Aquaculture Department. Available in: http://www.fao.org/fishery/countrysector/naso_malta/en).

2. Aim of internship

The aims for the current internship in AquaBioTech Group were:

- Know the status of aquaculture in the Republic of Malta and understand the commercial aspects affecting this industry;
- Acquire competencies related to the management and organization of an aquaculture company;
- Understand the function and operation mode of a aquaculture company;
- Increase the practical experience and skills about the techniques of aquaculture recalculating systems;
- Maintain and take care of the main fish species of aquaculture and the life support systems (Figure 3);
- Control and manage water quality parameters;
- Acquire main techniques related with fish husbandry including experimental methodology and microbiology procedures;
- Learn and take part in the experiment carried out at the facility.

Figure 3 - Specie of tilapia (*Oreochromis niloticus*) produced in AquaBioTech Group.

3. AquaBioTech Group

a. Facilities description

i. The company

Biotechnology and aquaculture are two areas that have been developed in parallel to contribute for the innovation and progress of aquatic fish production. Founded in 1998, the AquaBioTech Group (Figure 4) is an international consulting company located in Malta (Mosta) which work focuses on the research and development aquaculture sector, fisheries and marine biotechnology. Over the years the company has established itself quickly, integrating these areas with environmental sustainability.

Currently the AquaBioTech Group became an independent consulting company in aquaculture area that operates on an international scale. To maintain the prominence in this sector, the philosophy of AquaBioTech Group focuses in two main perspectives: the environment and the humanity. First, the company ensures that all techniques and procedures implemented will aim to mitigate the impacts on the environment. Therefore, it strives to be be involved in projects to reduce impacts on the aquatic ecosystem, considering animal welfare as a priority. Second, the company believes that the welfare of the employees is reflected in a good working activity and consequently the customer satisfaction: *“Quality is the customer coming back – not just the service’’*.

Figure 4 - Logotype of AquaBioTech Group.

The AquaBioTech Group is divided into four different operating areas, each representing one specialized field of work:

- *ABT Innovia* centers on research into areas directly related with aquaculture productivity such as new products related to nutrition and fish health. For example, discovering new alternative protein sources or development of vaccines for use by the main commercially species. In this way, the research will improve fish growth and fish welfare, thereby improving productivity;
- *ABT Aquaculture* develops a wide range of aquaculture systems that indirectly will help creating the optimal conditions for fish growth. This involves work by designers and engineers to create aquaculture optimum production systems. Currently, the company focuses the work to develop sustainable systems, like Recirculation Aquaculture Systems (RAS) for hatcheries or aquatic research facilities and commercial production systems (Figure 5);
- *ABT Marine* provides different services related with the study of the seabed. For example, marine surveying, construction support and mapping. To carry out these studies, specialized techniques such as bathymetry and side scan sonar surveys, bottom type assessments, sub-bottom assessments using both remote sensing and underwater video techniques can be applied. The data obtained is analyzed specific programmers to provide information suitable to be able to create zones

Figure 5 - Production in Recirculation Aquaculture Systems.

The company considers that it is own philosophy of work needs to be based in practices that reflect the quality of performance. In addition, choice of eco-friendly operations is also a consideration applied during the work tasks (AquaBioTech Group Company. Available in: https://www.aquabt.com).

ii. Internal organization

To achieve a truly and independent solution to the clients, the company needs to be at the forefront of the latest technologies of aquaculture. In this way, the company has become multidisciplinary, working with qualified experienced team. The company operates with biologists, engineers and designers to provide technical support maintaining the systems and taking care of fish safety. Working also with financial expert and banking groups, financial houses and private investors, guarantees that all aspects of the project are covered. All of these characteristics enable the company to provide long-term support and management assistance to the clients.

The staff members of AquaBioTech Group must have experience according with the function that they will have. In this way, the company must ensure that every member hired has a professional or academic history related with the function that they play. On specific cases, like internships, the company does not require a great experience, but it is beneficial if the intern has had some training in the area. In the beginning, every new member of staff or intern has to read all of Standard Operating Procedures (SOP). This documentation consists in written procedures for repetitive techniques, in accordance with agreed upon specifications aimed at obtaining a desired outcome. Then, the company gives some adaptation and training days, accompanied by trained personnel and directs the staff to a specific area or responsibility. The salary is calculated according to parameters such as, professional experience and position to exercise and must be fair and adequate as well. All workers have a period of holidays and two days off per week, according to the activities that need to be carried out. Unlike the other workers, the aquaculture technicians are distributed by shift: Morning, afternoon and night shift.

The competent management of the team include consider some hierarchy functions inside of the company. This means that there is a responsible person to coordinate and manage all the main decisions, usually the Director. The Research Director is the responsible person to coordinate and manage all the research activities. The Research Director has the support of two Supervisors, who manage the research facility on a day-to-day basis and coordinates the staff activities. The client has direct contact with the Research Director and Trial Coordinators, so that the trial work is carried out according to the agreed protocols and results communication (Figure 6).

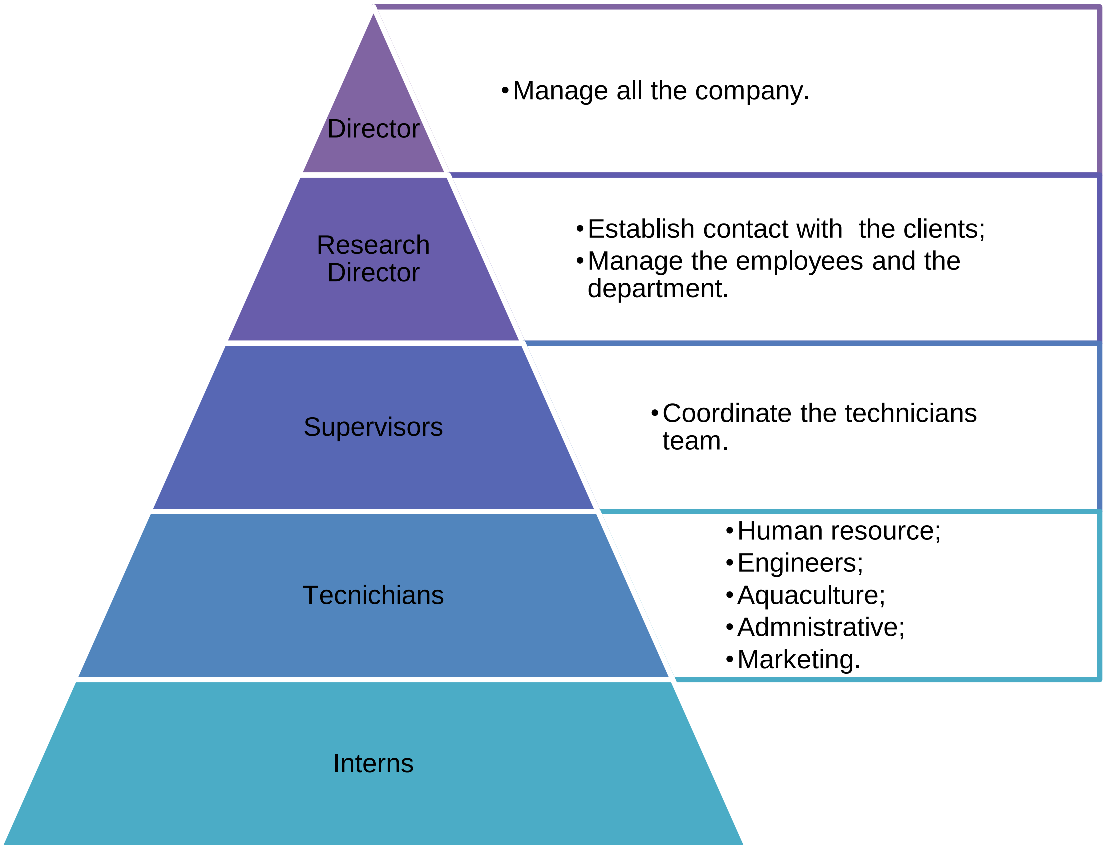

Figure 6 - AquaBioTech Group management hierarchy.

Despite this, everyone forming part of the team is considered as a unique individual, which means that the actions from a person should not produce a negative effect on the others. The employees deserve respect and dignity, have their merit recognized and must feel free to make suggestions and complaints. During the work, they must have a sense of security in their jobs and pride in the company they work for and also confidence in the people they work with. There must be equal opportunity for employment, development and advancement for those qualified as well as those who wish to improve themselves (AquaBioTech Group Company. Available in: https://www.aquabt.com).

iii. International recognition

The AquaBioTech Group is involved on several projects that are being developed in different countries, working with clients from around the world. For this reason the company needs to have staff able to respond to internationalization. This means the staff hired or the interns need to be comfortable to create relationships with people from different cultures and languages. In this way the company achieves an overview of the global aquaculture situation and also an international recognition. With a strong marketing strategy, the AquaBioTech Group have partnerships with other companies and involves in new projects, providing international visibility.

Figure 7 - European Business Awards contest.

The AquaBioTech Group is constantly looking to be at the forefront and in order to advertise these services and increase its reputation the company participates in congresses, events and contests (Figure 7). Until now the company has won already several awards in the contest European Business Awards. The AquaBioTech Group was selected as a National Champion of Malta at the

European Business Awards 2011, 2012/2013 and 2015/2016[1] (AquaBioTech Group Company. Available in: https://www.aquabt.com).

b. Research areas

The involvement and partnership with investigation projects may be considered as a way to maximize and generate more productivity in economic terms. In addition, with modernization and techniques optimization there has been an improvement in the practice of how things are done which has resulted in potential profitability of the industry.
In AquaBioTech Group all trials operating using the latest Recalculation Aquaculture System (RAS) technology allows undertake work on a number of species ranging from Rainbow Trout, Gilthead Seabream, European Seabass or tropical species such as Tilapia. Also the company is involved on different areas of aquaculture able to undertake projects ranging from ecotoxicology research, nutrition, fish health and disease prevention, development medicines or vaccinations.

Some examples of current areas of AquaBioTech Group research:

- *Pathology:* Trials are being developed to study several diseases caused by the main pathogens in aquaculture. The AquaBioTech group has a veterinary research unit, geared towards undertaking various aspects of applied research. This includes the development of test to detect easily some diseases and improving the efficacy of identification tests to pathogens. Such testing can be carried out for either new products or for products already established;
- *Pharmacology:* The AquaBioTech Group focuses its work in pharmacology area in partnership with the company PHARMAQ®. This company is the international leader in safe and efficient health products to the aquaculture sector. The AquaBioTech Group has equipment and all the conditions to test their products and the propose procedures. In this way, develops new techniques with the clients to improve the knowledge of the species health from aquaculture (Figure 8);

[1] Video prepared to the competition European Business Awards 2015/2016: https://www.aquabt.com/videos

Figure 8 - Pharmacology trial developed in AquaBioTech Group facilities.

- *Nutrition:* With partnership companies, the AquaBioTech Group has facilities specially designed to perform nutrition trials. In order to develop new feed formulations, the company testing new ingredients which could improve fish growth and health as well as reducing environmental impacts. In this type of study it is necessary measure a wide range of parameters allowing a detailed knowledge of the effects of the test substance. For example, the acceptance, the growth performance, water quality effects, feeding frequency and quantities, physical characteristics of a dry feed. Currently the AquaBioTech Group is carrying out research on how to use the feed as carriers of beneficial materials, such as probiotics or growth enhancers.

Currently, the AquaBioTech Group is involved on several investigations funded by different entities or European funds, ranging in the areas of aquaculture and biotechnology. Some examples of these projects are:

- *EnviGuard project*: Aims to develop a quite specific and precise environmental measurement device that can be used as an early warning system in aquaculture. This device will be composed by three different sensor modules to detect toxic microalgae, pathogens, toxins and chemicals *in situ* as quantitative and qualitative method. This way, it will be more economically profitable than the current devices once that will do several evaluation integrated into a unique portable device. Also will have the potential to save, display and send the collected data in real time to a server;

- *Biofouling:* The marine fouling provokes several problems on the hulls of vessels, aquaculture sea cages, ropes and others structures built under the sea. Therefore, many countries are fighting and spending their economic resources against biofouling, using toxic compounds that affect non-target organisms and bioaccumulate on the aquatic environment. The AquaBioTech Group facility provides laboratories to study and testing the antifouling activity of different concentration of toxic products. This way, the company developed ecotoxicological protocols with some vulnerable marine species (barnacle, bryozoans, mussels and algae) to study their toxic exposure (Figure 9).

Figure 9 - Biofouling project developed in AquaBioTech Group facilities.

- *TAPAS (Tools for Assessment and Planning of Aquaculture sustainability Aquaculture)*: This project aims to harness the potential of aquaculture production using sustainable practices to the environment. This way, it will contribute to support integrated multi-trophic aquaculture, develop innovative offshore technologies and evaluate tools for economic evaluation of aquaculture sustainability. Furthermore, promises to create a coherent and efficient legislation and strategies, with European member states support, to promote aquaculture sustainable growth.

(AquaBioTech Group Company. Available in: https://www.aquabt.com; PHARMAQ® Company. Available in: https://www.pharmaq.no)

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4. Recirculation Aquaculture Systems

a. Operation mode

In terms of sustainability, aquaculture has been one of the public's main concerns, leading many researchers to find solutions to minimize its impact on the environment. Over the years, Recirculation Aquaculture Systems (RAS) have been developed in this sector in order to reduce water consumption and promote the nutrients recycling (Piedrahita, 2003). Also, it establishes RAS has been seen as a great solution to respond the increasing environmental restrictions in countries with land limitations and water access (Martins, *et al*., 2010).

Considering the ecological friendly production by EU water management directive (Water Framework Directive 2000/60/EC 23rd Oct, 2000), RAS technology consists in closed-loop facilities where the tank systems are designed to retain and treats the water, allowing its partial reuse (Nazar, *et al*., 2013). This means that the water flows from the tank system to suffer different treatments in order to become healthy and safe to be returned to the fish tanks (Figure 10). Each treatment step consists on mechanical, biological and chemical procedures to remove solids (faeces, waste food), microorganisms (microalgae, bacteria) and chemicals (ammonia) wastes. This kind of system is designed to reuse circa 90-99 percent of water comparing with others aquaculture systems. Despite this, RAS can be applied to several fresh and saltwater species, providing good conditions to obtain fish densities satisfactory and control better the environmental parameters (Martins, *et al*., 2010; Nazar, *et al*., 2013). Currently the number of aquaculture companies applying RAS technology around the Europe is increasing, reflecting environmental, economic and social advantage for the country (Martins, *et al*., 2010). The AquaBioTech Group is an example of aquaculture company which operates with RAS showing that the high efficiency and the low costs helps to increase their own profitability (AquaBioTech Group Company. Available in: https://www.aquabt.com).

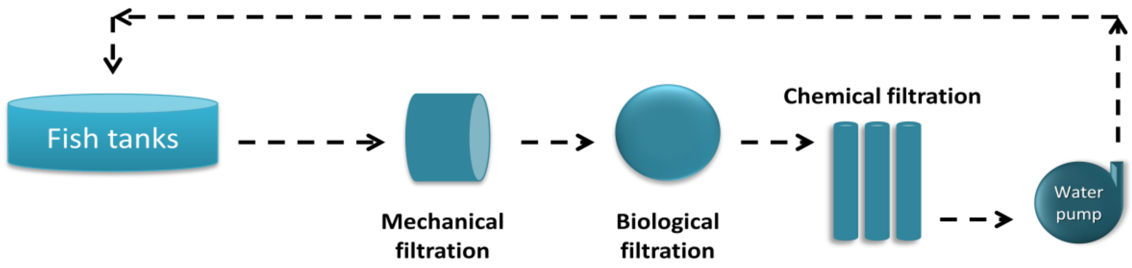


Figure 10 - Schematic of different process in Recirculation Aquaculture Systems (RAS).

b. Advantage and constrains to implement a RAS

The RAS is projected to make the aquaculture process more sustainable and obtain the best relation cost-effectiveness (Martins, *et al*., 2010; Ngoc *et al*., 2016). In this sense it presents a series of advantages when implemented on a large-scale production, for example:

- *Reduces water consumption*: The RAS is seen as the most cost effective way to reduce water consumption. Due to its main characteristic of treatment processes and water reuse it helps minimize many costs and save the water resources. RAS systems can reuse circa 90-99% of water and be almost independent of water resource to produce aquatic organisms, being also referred as indoor aquaculture systems. (Verdegem, *et al*., 2010);
- *Improves the waste management and nutrient recycling:* In aquaculture, the water quality is the most important aspect that should be ever monitored and controlled. The water quality parameters are related with a complex series of physical, biological and chemical reactions with direct effects on growth rates and fish health as well as the productivity. On other hand, the poor water quality produces pollutant effluents to the aquatic environmental (Piedrahita, 2003, Nazar, *et al*., 2013). The RAS works as integrated equipments, such as filters and pumps, to remove solid wastes and promote the nutrients converting. Some studies reported that removal RAS efficiencies is circa 85 -98 % to organic matter and suspended solids and 65 – 96 % for chemical wastes. Thus, RAS contributes to an efficient waste management, maintaining the

water quality stable and improving fish welfare (Pedersen *et al*., 2008; Martins, *et al*., 2010);

- *Improving biosecurity:* The RAS facility are often fully closed circuits and with entirely controlled parameters. Thus, the care on water quality control and good practices on waste management contributes to prevent disease outbreaks. Due of this high control, the entrance of any disease on the system become more difficult to when compared to most of other production systems (Martins, *et al*., 2010; Nazar, *et al*., 2013);
- *Production of a diverse range of species:* The application of RAS technology involves the production of a diverse range of species from the several aquatic environments. Also, it can produce high densities capable to cover investment costs and achieve new markets in order to become more profitable (Schneider *et al*., 2010).

RAS are considered a closed circuit where all of the components are connected. Thus, if on one hand this is seen as an advantage, on the other hand it can be a problem. This means that once checked a fault on the one of components or parameter the entire system may be compromised causing catastrophic losses within a short period of time and affect all the production. Therefore, there are some constrains that until now has been seen as challenges to improve this sector (Nazar, *et al*., 2013). Some examples of RAS limitation are:

- *Excess suspended solids:* The most significant problem in RAS is related to the excess and accumulation of suspended solids. The presence of very fine particles such as faeces, uneaten feed and bacteria clusters potentiate the poor water quality. Nonetheless also compromise the performance of the water treatment units and whole the production (Nazar, *et al*., 2013). Without a fast repair action, the particulate matter will suffer a quickly biological degradation and support the growth of several bacteria. By consequence the oxygen availability for fish production will decrease and the toxic nitrogen compounds as ammonia will increase and be determinant to fish survival (Eding *et al*., 2006; Martins, *et al*., 2010);

- *Loss control on disease dissemination:* As already described, the high control of RAS parameters will potentiate a difficult entrance of disease. However the connection of all the units by water flows makes this system more susceptible to disease dissemination if any failure occurs. The water supply in association with poor management creates an entryway of pathogen and contamination way to the system. Once inside of the system, the pathogen will spread quickly and provoke disease on fish whereby the previous disinfection procedures should be ever performed (Martins, *et al*., 2010; Nazar, *et al*., 2013).

Despite its initial high investments, in the long term RAS implementation will be reflected in a cost-effective production (Ngoc *et al*., 2016). The control of various inputs and suitable designs which minimize height differences between compartments, like waterfall effect, reflects circa 50% more profitability in terms of energy comparing with others aquaculture systems. However to maintain the yield stable, RAS is important have the control system capable to measure all the parameters constantly. In this sense, the recent developments in this area are fighting to create innovative informatics mechanisms to revolutionize the RAS operation control (Martins *et al*., 2010).

c. RAS implemented in Europe

Although all environmentally and economical benefits, it is verified a slow adoption of RAS productions. Compared with others a method, RAS contribution to aquaculture is still almost insignificant due to the high initial investments required (Martins *et al*., 2010). However, many governments are working to support and encourage many aquaculture companies in order to implement RAS technology. In Europe the number of countries applying Ras is increasing (Schneider *et al*., 2006). It is also needed to consider that it is difficult to obtain data compiled available capable to evaluated the efficiency of RAS production. In this way, many projects (e.g. Consensus, SustainAqua and AquaeTreat projects) work hard to respond the most significant issues such as quantify the RAS sustainability and to create innovative technologies to improve it. To do this, some data are collected from interviews with relevant entities such as feed industry, farmers and associations from different European countries

(Martins *et al*., 2005; Martins *et al*., 2010). The most recent data show an increment in the interest to grow-out RAS production in Netherlands and Denmark, since 2005 and up to 2009 (Table I). Currently, the RAS is considered the most profitable aquaculture production method and has been increasingly adopted by aquaculture companies across the Europe (Schneider *et al*., 2006).

Table I - Grow-out production (metric tones/year) in RAS from 1986 until 2009 (Schneider *et al*., 2006).

	1986	1990	2002	2003	2004	2005	2006	2007	2008	2009
Belgium						10				
Bulgaria						5				20
Czech Republic										235
Denmark						2000				12000
Estonia									40	
Finland						130				
France						70				506
Germany			502	509	688	657		1257		
Hungary						650				24.5
Ireland										50
Lithuania								15		
Netherlands	300	950				9500		9635		9680
Poland						180				
Portugal								100	110	112
Spain						580				780
Sweden						490				
United Kingdom										100

The AquaBioTech Group is a company which only operates with RAS to increase the productivity and the cost effectively as well as reduce the impact on the environment. The systems versatility to apply to hatcheries, broodstock, aquatic research and on-growing operations put this company at the forefront of industry. RAS is clearly a technology that will improve the development of aquaculture to become the mainstream sector over the next years. (AquaBioTech Group Company. Available in: https://www.aquabt.com).

5. Internship description

a. Biosecurity procedures

Biosecurity is one of the most important aspects in fish production to prevent the introduction and disease dissemination within and between aquaculture facilities. The AquaBioTech Group operates with a high level of biosecurity in order to avoid the of pathogen transmission. This includes a rigorous training of the staff to follow the biosecurity procedures and the facilities are designed to keep the minimum risk for biosecurity. Developing studies with pathogens requires that these rules are fulfilled every day to maintain the quality of work and safety of the technicians. As example:

- According to the function required to execute in a particularly day, the worker should dress specific clothing and shoes (clogs) that are used only inside of particular areas of the facility. The only exceptions are the staff that work in offices and administrative part;
- All the divisions have disposable gloves, a bottle of alcohol to disinfect hands and footbaths with disinfectant;

Even the organization and infrastructure of the facility is planned to ensure the necessary biosecurity standards. The aquaculture section of the company is divided into different rooms, named 'Bays' where the trials with fish are carried out. Each Bay can be distinguished by: Challenge or Normal Bay. The Challenge Bays is where the trials that involve the infections with pathogens are carried out unlike the Normal Bays that there is no pathogen manipulation. This means that the biosecurity adopted inside of the different Bays follows different norms which will be more rigorous on the Challenge Bays (Figure 11).

Figure 11 - Biosecurity label on the Challenge Bay entrance.

The AquaBioTech Group is a GMP (Good Manufacturing Practice) certified company by the United Kingdom VMD (Veterinary Medicines Directorate) and Maltese VRD (Veterinary Regulation Department). It is also important to note that all of the experimental challenge trials are performed according to GCP (Good Clinical Practice) under the principles of the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) (AquaBioTech Group Company. Available in: https://www.aquabt.com).

b. Water quality control

In aquaculture, the guiding principle to obtain efficient production but also the major issue related with environmental impact is the water quality within the system (Munni *et al*., 2015). Feed wastes, faeces, metabolic products and any chemical components like antibiotics, that are added or formed are the main responsible to water quality deterioration. By consequence, it is had direct effects on fish growth, the disease susceptibility, mortality rates and low production. On the other hand, generates effluents with high concentration of organic matter, chemicals or others harmful contaminants which cause environmental pollution. Thus, the proper water quality control is essential to ensure the good fish health and mitigate the impact on aquatic environmental (Kurtz, 2002). This can only be achieved with a great waste management by technicians with knowledge of water quality and its effects on aquaculture species. Currently this management is facilitated by some portable instruments or kits test to evaluate the main water parameters (Boyd and Tucker, 2012).

In AquaBioTech Group, the water quality is measured daily in each system. The responsible aquaculture technician has the task to collect around 50 mL of water from each system and analyze it a specific place. Some parameters are analyzed using kits tests according with procedure already defined, such as aammonia, nitrites and nitrates as well as alkalinity. However, when colorimetric kits are used it should be kept in mind that the evaluation may be imprecise and dependent on the operator. First, the visual colour evaluation depends on the operator observation and other factors such as light that might also influence. Secondly, for each colour there is the correspondence of a specific and rounded value that is not the most correct. However, these tests are the commonly used because are a quick and easy procedures which provides an idea of the water quality general status (Boyd and Tucker, 2012). Other parameters are measured by instruments that provide a more accurate value, such as salinity, temperature, pH, Oxygen and RedOx. These parameters are controlled by sensors permanently present inside of the tank. The data are sent to a computer system that saves them and creates a database by systems and tank separately. Thus, if these values are out of optimal range conditions the alarm is triggered and through a general framework provides the indication of which Bay and tank the problem is occurring. After the measurement of all parameters of water quality, the data is recorded. Subsequently, this data is entered into the computer by the responsible technician who is identified with his initials. All data is saved over time, allowing a temporal evolution to be seen. Also, all aquaculture technicians receive an email with this information (see point 6.b).

c. Fishes deliveries

Fish deliveries are regularly made when a new trial is to be started. Every time that a fish delivery occurs, the Bays are properly cleaned and prepared previously with new material to ensure maximum hygiene and safety. The whole team needs to be ready and informed about all the details and recommendations from the clients. However, only two or three persons are necessary to receive them. Subsequently, the fish are introduced into the tanks and they are not fed during the day. As they are exposed to a high stress, the food would eventually be wasted due to less appetite. Finally, the tanks are labeled with the number and name of species, number of the Bay, system and tank and if the fish have marks and which ones. During the following days, the Bay is kept under hygienic conditions, security and surveillance. If high mortalities rates or signs of sickness occur are observed on the following days some procedures are strengthened come into effect.

d. Daily routines

Every day, someone from the team is responsible for the daily routines. To facilitate and organize tasks, there is a daily checklist which is filled six times per day in all the Bays composed by a list of tasks that include (e. g.):

- Monitoring of the whole system;
- Water quality parameters;
- Health and fish welfare;
- Removal of feed waste and faeces in tanks.
- Equipment maintenance;
- Feeding fish;
- Remove and record the mortalities.

The checklist has been specially formatted to simplify these types of observations, each of which had a specific field. Also, is performed different for the Challenge and Normal Bays and is filled inside them by a technician responsible to sign it. In the evening, after being carried out during the day, they are all collected and the data is recorded in the computer system. This procedure is a method to monitor all the systems and ensure the satisfactory operation and fish safety.

e. Cleaning

Two times per week all Normal Bays undergo a thorough cleaning in order to provide optimal hygiene conditions for good fish maintenance. The technician responsible must follow a series of tasks which includes:

- Cleaning the internal and exterior of the tanks;
- Cleaning whole system including the pipes and probes;
- Fill up the alcohol bottles and footbath;
- Remove faeces and food wastes by siphons or opening the purges;
- Check the gloves boxes;
- Remove the rubbish.

Also, this includes maintaining the corridors and aquaculture office tidy and organized. After this, the technician needs to make sure that everything is done and sign the cleaning list with their initial. However, in Challenge Bays challenge this procedure is only done after the trial be concluded, including rigorous disinfections steps of everything that is inside.

f. Feeding

Feeding is the most important task in the facility, because, if well executed, could ensure the good health of the fish. Usually the person responsible to do the daily checklist also feeds the fish. Feeding is performed in three or four daily cycles, varying from species to species (Figure 12). However, when the fish are included in nutrition trials, they have a specific feeding schedule. Exceptionally, feed is given at non-scheduled times when the fish have been exposed to stress conditions such as transportation, cleaning or handling.

As to how feed is given, it is important to understand how the particular species feeds in the water column. Some species prefer eating near the bottom, at the surface or in water column. Feed administration is given according to its buoyancy and the quantity needed to be fed to the fish. This means that if the feed has little buoyancy it is more often administered in small amounts. Thus the animals eat before the feed reach the bottom, avoiding waste. All of these characteristics need to be considered to ensure that all nutritional requirements are met. After the last feeding, the food containers are collected and taken to the aquaculture office. Feed remaining is weighed, recorded and the feed for the next day prepared as required.

Figure 12 - Feeding procedure.

Nutrition is central to the fish's health and its behavior is an indication of health. If the fish are not feeding it could be an indication that they have some disease. This way, the first prevention method should be immediately performed to avoid the disease spread and actions taken accordingly.

g. Disease management

The first signs of onset of disease are often related with the loss of appetite and according to the disease evolution, the physical signs visible will appear. When there is a suspicion or a disease occurs, the Bays are converted straightaway to Challenge Bay status, which means that biosecurity practices are strengthened to avoid contamination. Any disease needs to be diagnosed by a specialized veterinarian as fast as possible in order to be given the best treatment. An example of how sick fish is analyzed in the lab includes the following procedure:

- Observation and recording the presence of lesions on the skin, fins and other external organs;
- Dissection of the fish;
- Observation and recording of the internal organs;
- Collecting blood samples and others fluids;
- Plating of blood samples to test for the presence of bacteria.

The treatment is performed according to the disease but normally is through by antibiotic administration. However, the procedure depends on the type of antibiotic and the instructions of the manufacturer. During the following days, any mortality that occurs will be recorded, labeled, analyzed and relevant samples stored.

h. Mortality management

When animals die, the organic matter decomposition process starts, converting certain nutrients by bacteria and by the consequence the water quality will be deteriorated. Thus, a faster removal of dead animals is essential to prevent the reduction in the quality of the system's water and contribute to welfare of the others fish (Figure 13).

Figure 13 - Mortalities collection.

Every day, during the daily checklist performing, the deceased are collected, counted and recorded into sealed plastic bags. The bag is labeled with the date, number of individuals, Bay and tank number, trial code and technician initials. In the challenge Bays, the procedure is identical but the samples can be taken as required and analyzed as described below:

- Observation and recording of the external signs of disease;
- Dissection of the fish;
- Observation and recorded the state of internal organs;
- Collecting sample from the kidney;
- Plating the sampled on the bacteria medium;

- Labeling and incubation in incubators at the appropriate temperatures.

These mortality records can also be viewed as a stock control measure, especially to understand the cause of death, and developing new procedures in order to avoid future problems. Thus, the dead fish are stored in a freezer (at -20°C) until the end of the trial and then sent to an incineration company.

i. Salt and fresh water delivery

The water supply to AquaBioTech Group is performed by an external company providing fresh or salt water. The water is delivered by a purpose-built truck that connects a pipe to the water receiving system. After all the valves flow re opened and the water starts to fill up the reservoirs. When the reservoirs are completely full, the valves are closed and the pipe is disconnected. Finally, the responsible aquaculture technician needs to sign the water receiving record sheet.

j. Techniques acquired

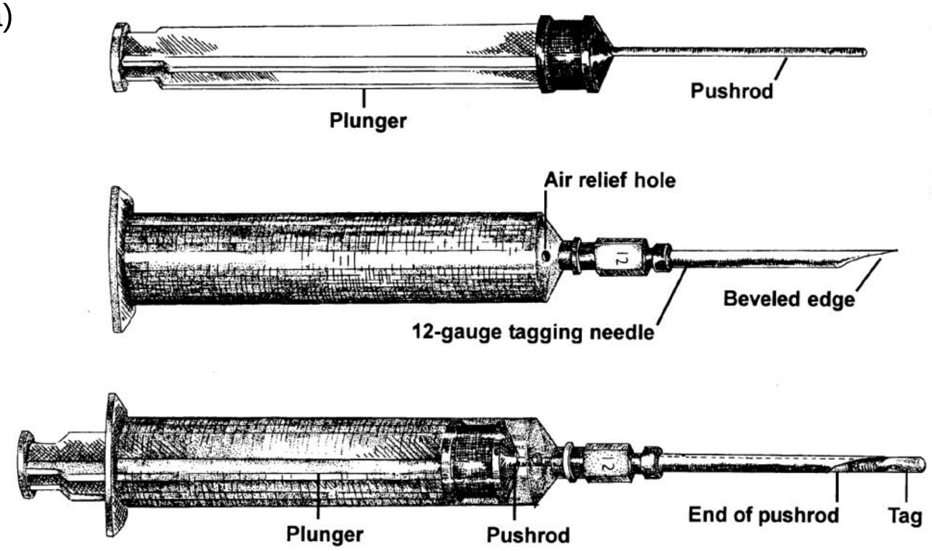
The AquaBioTech Group tries to contribute for the technicians learning new techniques that are important in aquaculture fish handling. Some examples of practical techniques that were acquired during the internship are:

- *Vaccination:* The vaccines are composed by antigens that when administrated induce a protective immune response without causing considerable side effects (Gudding *et al*., 2014). The vaccination procedure is a technique that requires fish manipulation and may cause more stress (Figure 14). However, to avoid excessive stress the fish are administered with an anesthetic. Then, using syringes the fish are injected immediately in the pelvic fins zone with minimum of risk of damaging the underlying organs. The time taken depends of the team skills which normally need around 2 or 3 people. In aquaculture, the vaccine production is an area under development and there are some companies, such as PHARMAQ®, that produces the vaccines and tests them. PHARMAQ® work in partnership with AquaBioTech Group that provides excellent conditions, equipment and experienced technicians to achieve good performance and get results. (PHARMAQ® Company. Available in: https://www.pharmaq.no; AquaBioTech Group Company. Available in: https://www.aquabt.com;)

Figure 14 - Fish vaccination procedure.

- *Tagging:* Tagging is a procedure with two main applications to study the fish in their natural habitat or to differentiate fish on the trials, for example different diets or vaccination. The tagging procedure comprises a viscous liquid with different colors which are introduced under transparent or translucent tissues (Willis and Babcock, 1998). This liquid is injected to form a permanent and biocompatible mark by a specific syringe inside the cylinder and a plunger (Fish Columbia Basin and Wildlife Authority, PIT Tag Steering Committee, 1999). When exposed to UV light, the compound fluoresces brightly and shows different colors. However, this technique requires some practice to manage the instrument and the fish (Willis and Babcock, 1998). First, the fish need to be anesthetized to make accurate markings, visible and without causing lesions. The injector is laid in the hand in certain position and the needle will penetrate the surface of the skin. Then, a slight pressure should be applied in order to make a visible mark. Commonly, the tag mark is performed near the eyes, on the left or on the right side, but can be done on the belly near the pectoral fins (Figure 15) (Fish Columbia Basin and Wildlife Authority, PIT Tag Steering Committee, 1999). However, the tag size depends of the fish size or technician requirements. Normally, the small fish are very difficult to handling and care must be taken to not damage internal organs. Also the large fish, over 200 mm are difficult to hold for people with small hands. Tagging method can be applied on the several fish species and Tagging method could be applied on the several fish species and for a considerable number of individuals (Fish Columbia Basin and Wildlife Authority, PIT Tag Steering Committee, 1999; Leblanc and Noakes, 2012).

a)



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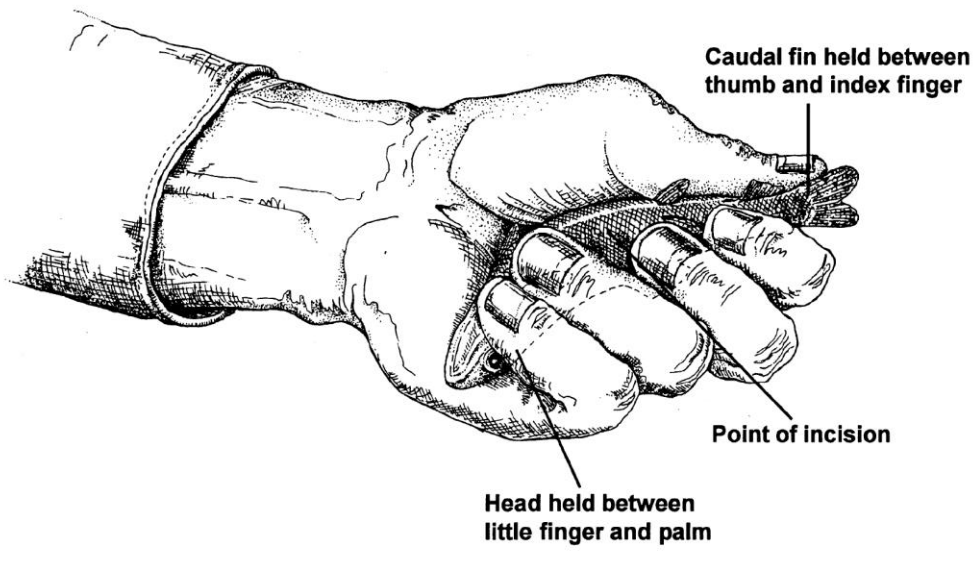

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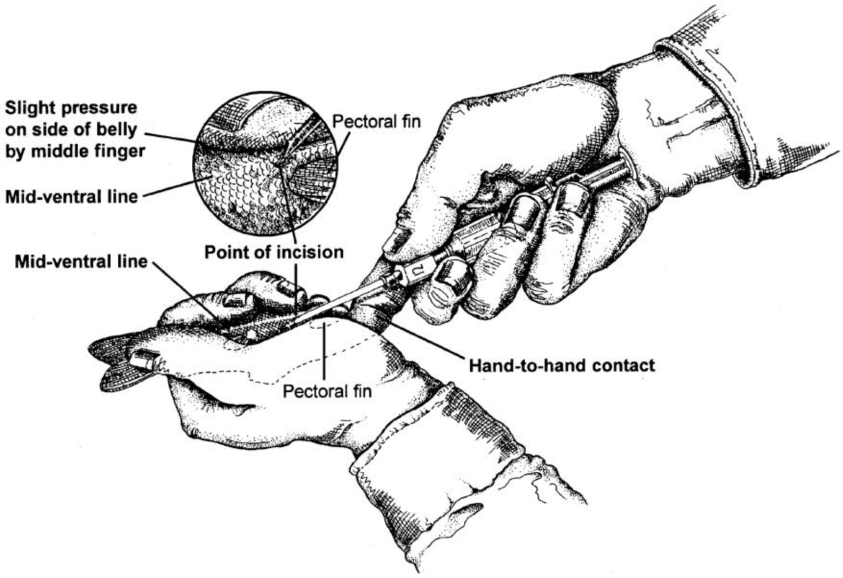

Figure 15 - Tagging technique: a) syringe, needles and instrument used to tagging fish; b) correct way to hold the fish during tagging; c) proper procedure to tagging fish (Fish Columbia Basin and Wildlife Authority: PIT Tag Steering Committee, 1999).

- *Stripping*: This technique aims to determinate the coefficient of digestibility through the periodic feces collection. Normally, is executed during nutrition trials to evaluate new feed diets from around twenty-five fish per tank previous anesthetizing. After being completely anesthetized the fish must be firmly secured with both hands. The fore and thumb finger will press and slide from the end of the pectoral fins to the beginning of the anus. The faeces should be immediately collected to Petri dishes and all scattered fragments must be collected to make the most amounts of faeces possible to analyze. Then the plates are dried in an oven at 60°C to remove the major part of water and labeled with the appropriate number of the tank and after stored until further analysis.
- *Sampling:* Sampling technique is usually performed after nutrition trials on AquaBioTech Group. In order to evaluate the effects of a diet, some fish are caught and killed with large doses of anesthetic in order to minimize pain. Then each individual is weighed and measured - total and fork length. After they are dissected and some parameters such as the weight of the viscera, and intestine length are measured. The upper, central and lower part of intestine is stored separately in 10% buffered formalin until future analysis.

- *Plating:* Plating is a technique used frequently in microbiology. In AquaBioTech Innovia, the plating is performed for the mortalities from the challenge Bays or when any disease outbreaks occur. The technique consists in dissecting the fish on the ventral side and collects a sample from the kidney. Then the sample is plated on a Petri dish which contains medium for bacteria growth. During these procedures some observations should be recorded.

For some techniques mentioned above, the use of anesthetics is indispensable to minimize the stress and allow handling of fish. Tricaine Methanesulfonate (MS-222) is commonly used and approved by Food and Drug Administration (FDA) as an ideal anesthetic for fish. However, its use requires some care (e.g. high concentrations and excess time exposure on anesthetic bath) can cause permanent damage or fish death. Thereby, the dose and the time of exposure depend of the species or fish size and should be verified before used. It is recommended a careful observation on fish behavior during all the procedure, especially the operculum velocity which can indicate limitation of oxygen and may cause fish asphyxia. Then, the fish should begin to regain the equilibrium and maintain an upright swimming position after be inside the recuperation tank (Fish Columbia Basin, Wildlife Authority, and PIT Tag Steering Committee, 1999; Leblanc and Noakes, 2012).

6. Facilities and life support systems

a. The facilities

The AquaBioTech group began as company with basic and small installations. Over the years the company expanded its facilities as well as the services it could offer. Currently occupies four floors of a building with numerous rooms for holding livestock, laboratories and administrative offices.

The third floor is a space designed exclusively for the aquaculture technicians and forbidden to persons outside the service. There the technicians can change their clothes and put on their work clothes and shoes. The employees can use their lockers to put their belongings. This floor has two accesses to the roof and remaining floors, by means of a lift and stairs. The second floor is intended only for administration and management of the company. In this place, staff involved in marketing, management, finance, human resources, informatics, system designers and other administrative. The office of the Technical Director of the company is also located on this floor. Therefore, this entire floor is equipped with informatics and office equipment. Is here that all bureaucratic issues are treated, some contacts are established and the documentation is stored, facilitating communication and work processes. The first floor is a mixed space with offices, socialization and meeting rooms. Here are located the offices of the Research Director and the veterinarian with equipment and office materials. It also has kitchen equipment that allows preparing light meals and storing some food. This whole floor is forbidden to persons outside the company, except the meeting room. The meeting room is used to discuss important internal and external issues. It is where staff members welcome new clients and representatives from companies, which are already clients to discuss relevant subjects. The main floor has an entrance hall that leads to a corridor and a bathroom. Also, there is access to the stairs and elevators to the other floors. On the right side there is a microbiology laboratory equipped with lab material and instruments such as laminar chamber flow, spectrophotometer, autoclave, two fridges and two incubators. In this lab work involving bacteria manipulation, including preparing culture media and others important analysis related to microbiology. Because of the constant manipulation of bacteria and the risk of contamination, this lab requires the maximum control of hygiene and safety. Opposite to the laboratory, there is Bay 0 which is separated by a biosecurity area. The system in Bay 0 is composed of 12 cylindrical tanks connected to a sump that allows water reuse (Figure 16). Usually, in this Bay growth and nutrition trials are performed because have big tanks which can support a high number of large fish. It also has a small room where bottles of oxygen are stored as well as all electronic equipment.

Figure 16 - Bay 0.

The aquaculture floor (Figure17), there is major research rooms, divided into different systems. In total, the company has 14 Bays on this floor, but has expansion plans to build more Bays. As already mentioned, the Bays are divided into Normal and Challenge Bays according to the biosecurity rules established. Depending of the trial that occurs, the company defines a Bay as Normal or Challenge but can switch between them. There is also an area designated for NaOH preparation, a cleaning area to wash any material and a feed store with cooled and dry conditions to prevent oxidation of the product. As well there is a place to store tools, material and carry out repairs and three water reservoirs in another room. The Bay 12 is a biotechnology lab where some ecotoxicology trials are performed. This lab is equipped with its own equipment, to carry out any analysis such as microscope, stereomicroscope, analytical balances, incubators, and other equipment to support the experimental trials being carried out at the facility. There is an adjacent aquaculture office where control of all systems is held, food prepared, water quality analysed and data introduced in the system. This area is equipped with computer equipment and office, feed boxes, some reagents and tests to determine the quality of water and some documentation such as older daily checklists. There is also a lab support composed with fridge and freezing chambers, where the mortalities are stored, and a workbench. This area requires high safety rules because are stored also mortalities from the challenges Bays in the exclusive freezer. All the Bays and the labs are divided into individual research units ensuring that biosecurity and client confidentiality is maintained at all times. The whole floor is restricted to the public visit except when there are previously programmed visits.

In accordance to the perspective of expansion, it is expected that a number of new wet labs and rooms that will be available by the end of 2016 in order to satisfy the growing sectorial demand. This will include quarantine and stock rooms as well as additional RAS facilities for Normal and Challenge Bays. (AquaBioTech Group Company. Available in: https://www.aquabt.com; ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf).

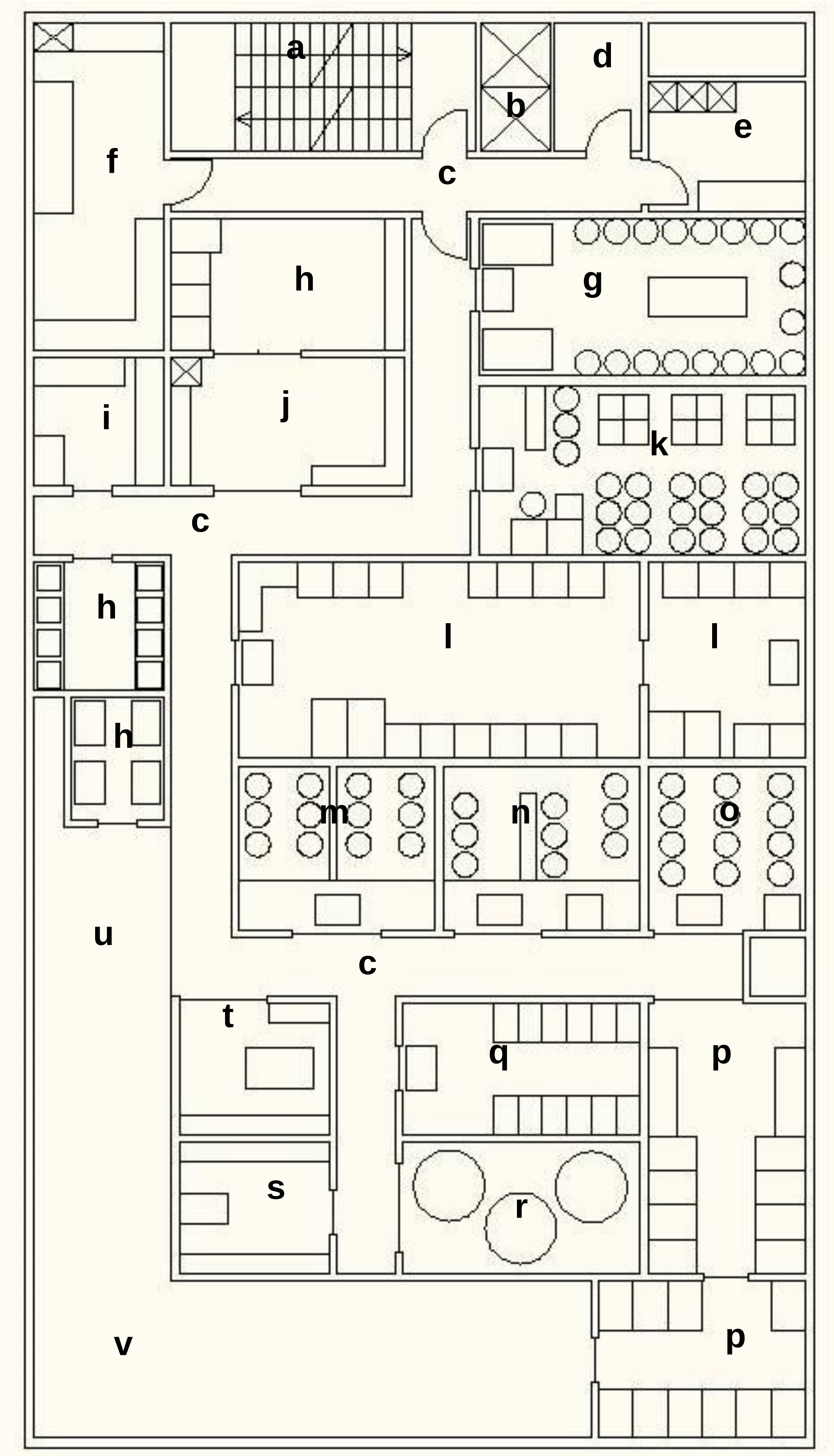

Figure 17- First floor plan: a) stairs; b) Lifter; c) Corridor; d) Bathroom; e) Mortalities storage room; f) Aquaculture office; g) Bay 1; h) New Bays in construction; i) Bay 11; j) Bay 12/ Biotechnology lab; k) Bay 2; l) Bay 3; m) Bay 4; n) Bay 5; o) Bay 6; p) Bay 7; q) Bay 8; r) Water reservoirs; s) Bay 10; t) Bay 9; u) Cleaning area, NaOH preparation room and feed stored room; v) Tools storage room (AutoCAD 2010®, Architecture).

b. Life support systems

The AquaBioTech Group building was renovated to be capable to have basic structures that provide an excellent fish life support system.

The water delivery circuit in a aquaculture company is essential to support all the systems. In AquaBioTech Group, the water delivery circuit connects all the systems between the reservoirs and water drain networks (Figure 18). As already mentioned, the company works only with RAS technology which means that the water flow movement follows a unique direction. The water effluent exits the system by a drain pipe present in each tank or by a purge. Then it flows into the sump where it is cleaned by mechanical, biological and chemical filtration. After, the water returns to the tanks in optimal conditions for fish (AquaBioTech Group Company. Available in: https://www.aquabt.com; ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf).

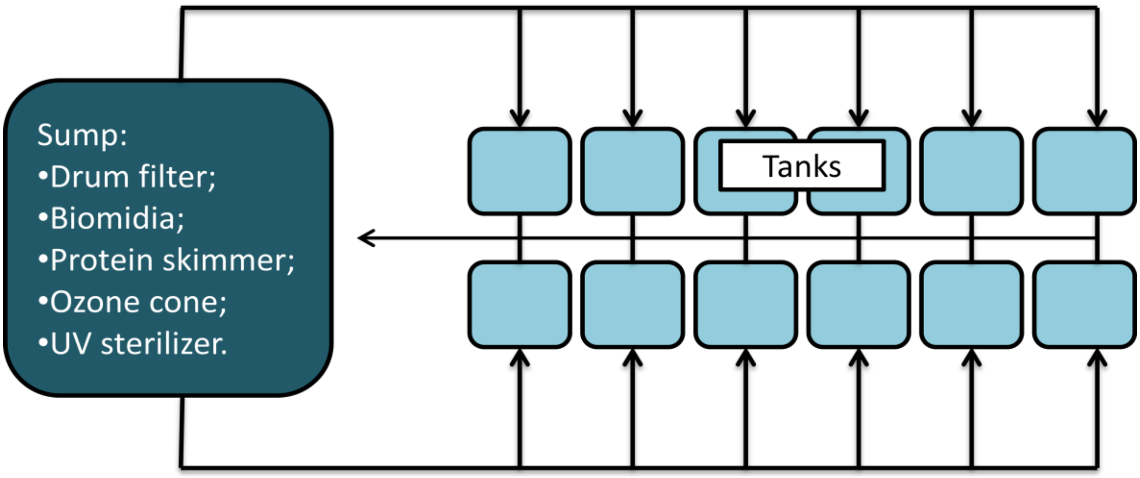

Figure 18 - Representative scheme of RAS system

Filtration is essential to maintain a good operation and sustain life support system. Each Bay consists of an independent RAS which contain the own sump to perform a mechanical, biological and chemical filtration (ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf).

There are three types of contaminants in the water: suspended solids, microorganisms and dissolved chemicals. The physical particles can have different sizes and the mechanical filtration is important to removes them from water of the system. This particle includes debris, food scraps, faeces and other waste. The biological filtration consists in conversion of waste toxic products such as ammonia and carbon dioxide into less toxic compounds. These compounds are formed from waste food and dead organisms in decomposition and are toxic for aquatic species. The main process during the biological filtration is nitrification performed by bacterial process that will reduce the amount of toxic compounds and increase the water quality. The chemical filtration methods involve the sterilization to removes all unwanted microorganisms. This means that will reduce the abundance of bacteria in the water and control parasitic infections (Moe, 2009).

In AquaBioTech Group, the effluents pass through a mechanical rotation cylinder filter (drum filter) where a centrifugal force collects the solid particles from the water. Subsequently, the water passes through a biofiltration system. A solid medium is used as a substrate for the attachment of the nitrifying bacteria where the sand or plastic balls are commonly used. The main aim is to provide a big active surface area for microflora colonization. Then, the water passes through a chemical filtration process that includes skimmers, ozonation and activated carbon. After, the water passes through UV light where the appropriate intensity depends on the fish species concerned (AquaBioTech Group Company. Available in: https://www.aquabt.com).

All the Bays have aeration systems which provide oxygen, from liquid oxygen bottles, that are introduced inside the tanks by air stones. This will produce air bubbles which rise quickly to the surface when the dissolved oxygen is low. The energy system supports all the pumps, chillers, lights, digital equipment and the blowers. Also, the Bays have an online monitoring system to control the water quality (Figure 19). Also, the Bays have an online monitoring system to control the water quality. This includes probes networks, submerged in each tank, that are connected with a board inside of each Bay. The probes give the information about the physical and chemical parameters of the water, such as temperature, CO_2, pH, salinity, dissolved oxygen and RedOx potential. The digital screen inside each Bay shows the values of these parameters per tank and the temporal information through a graph. However, it is also possible to control the input and output of oxygen. In the aquaculture office there is a main monitor showing water quality. This board is connected with beeping and light alarm systems that alert if some parameter is out of the optimal range. The alarm system is also connected to mobile phones and computers of technicians so they can act as quickly as possible (AquaBioTech Group Company. Available in: https://www.aquabt.com; ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf).

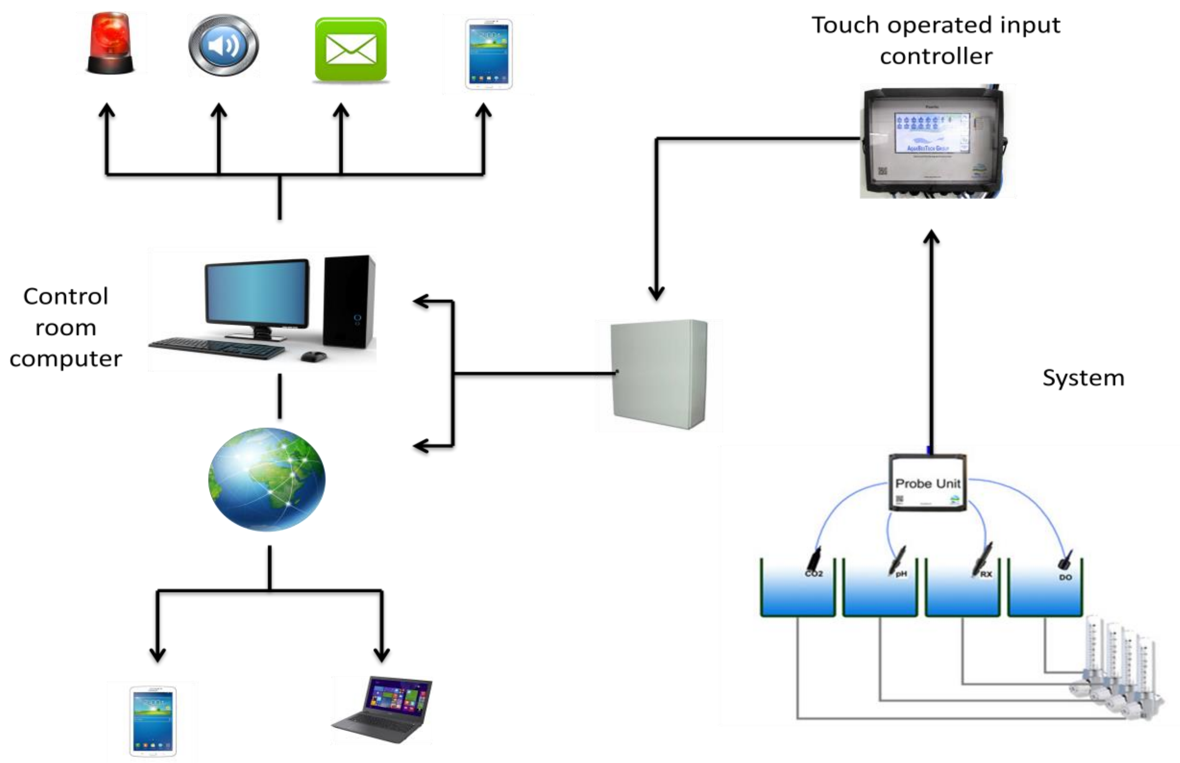

Figure 19 - AquaBioTech Group monitoring system.

The design type of tanks used in AquaBioTech Group facility is versatile to adapt to the different life stages and fish species. The company operate with three different tanks designs ranging from small (Figure 20a) to large size, such as round (Figure 20b) and rectangular (Figure 20c) (AquaBioTech Group Company. Available in: https://www.aquabt.com; ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf). However, these differences are related with several aspects, such as the specie size or the costs associated with tanks cleaning. The small tanks are ideal to small rooms and suitable for the earliest stages of life which require be more controlled. The circular tanks have a appropriate design to minimizes the costs related with cleaning. In this type of tanks, the water is introduced from the surface and flows tangentially to the wall with a certain pressure. The incoming water move the mass of water below creating a circular flow pattern around the drain center, located at the bottom of the tank. In this way, the centrifugal forces transports and removed easily the solid waste, spending less energy. The main disadvantage to use circular tanks is the low profitability of the space around instead of rectangular tanks. This ones, with rounded corners, takes a better use of available space and also contribute to the smooth flow of water (Nazar *et al*., 2013).

Figure 20 - Different tanks design: a) small size; b) large with round form; c) large with rectangular form.

The AquaBioTech Group has been continually expanding its research and development facilities, which currently includes a total of twelve Bays with some specification but the company is able to expand (Table II) (AquaBioTech Group Company. Available in: https://www.aquabt.com; ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf).

Table II - The specifications of each Bay.

Bay	Number of systems	Number of tanks	Tanks capacity (L)	Specification
0	1	12	1500	Feed and faeces collectors.
1	1	18	500	Can be operated for nutritional or challenge trials.
2	1	6	300	System 3, 4 and 5 can be operated as one large system. Is a Bay where are performed only challenge trials.
	2	9		
	3	4	250	
	4			
	5			
3	1	6	1100	The Bay is dividing by a wall with two completely separate RAS units. Is currently used for quarantining and stock-holding fish.
	2	12		
4	1	6	500	The Bay is dividing by a wall with two completely separate RAS units. The system can be operated in two levels (350 or 500 L).
	2			
5	1	9	500	The system can be operated in two levels (350 or 500 L).
6	1	12	300	
7	1	18	1000	Used for on growing and immunization.
		8	60	
8	1	12	500	
9	1	8	100	All tanks have acrylic viewing windows for greater observation.
	2			
10	1	16	40	Composed with polycarbonate tanks with sliding lids
11	1	12	40	

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7. Challenge trial

The development of a *Flavobacterium psychrophilum* dose response pre-model in adult rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Flavobacterium psychrophilum is a bacterial agent causing “Bacterial Coldwater Disease’’ (BCWD) and it has been a challenge to discover solutions and effective treatments for over 100g Rainbow Trout (*Oncorhynchus mykiss*). The aim of this study was to follow the mortality rates of Rainbow Trout in the growing development stage, infected with *F. psychrophilum*. After the proper acclimatization, the fish were injected with different dilutions of bacteria: Non-diluted, 1:10, 1:100, 1:1000 and PBS solution. According to the results, the highest dose (non-diluted solution) gave the greatest mortalities, more than 70%, in comparison with the less concentrated dilutions. For the highest two doses, mortalities started to appear around day 3, while the first mortalities, registered in the 1:100 solution, appear on day 10. In this study, many fish showed signs of disease during the experiment, characteristic of *F. psychrophilum* effects. The symptoms verified were the abrasion of tissues, skin ulcerations and concurrent hemorrhage but any specifications about it were not recorded. The lack of normalized values for larger fish is a serious obstacle to further progress and it is probable that vaccination against *F. psychrophilum* was still not achieved successfully. This way it is important to understand the virulence factors and the identification of putative antigens to establish the standardized vaccination model. The present study was performed to provide some useful bases for future investigations. The necessity of further research is paramount to provide more answers about pathogenesis of *F. psychrophilum* and develop diagnosis preventive and treatments effective against BCWD in larger Rainbow Trout, which still remains poorly understood.

Keywords: *Flavobacterium psychrophilum;* Rainbow Trout; Bacterial Coldwater Disease; Mortality rates.

a. Introduction

The presence of pathogenic microorganisms and the consequent occurrence of disease outbreaks have emerged as a serious economic and ecological issue in the aquaculture sector. Although most pathogens are part of the normal water flora, they can cause disease when the cultured species are stressed due to poor environmental conditions, inadequate diet, or poor husbandry techniques (Barnes and Brown, 2011; Loch and Faisal, 2015).

Flavobacterium psychrophilum is a gram-negative bacterium which is present in the aquatic environment, particularly in freshwater (Barnes and Brown, 2011). This bacteria can survive for long time outside of fish host and is the agent causing what is known as “Bacterial Coldwater Disease’’ (BCWD). Normally, this disease occurs in association with certain pre-disposing factors, such as overcrowding, reduced dissolved oxygen, increased ammonia, and when water temperatures are below 10ºC (Madetoja and Wiklund, 2002; Starliper, 2011). However, it is transmitted through the water column but some evidence suggests that *F. psychrophilum* is also transmitted between fish interaction. Some studies have shown that this bacteria has been founded in Rainbow Trout (*Oncorhynchus mykiss*), Steelhead Trout and Atlantic salmon, in some fluids such as ovarian fluids, sperm and mucus samples (Madetoja and Wiklund, 2002; Barnes and Brown, 2011). After entering a fish farm, *F. psychrophilum* spreads quickly on the collagenous connective tissue where it produces enzymes that destroyed the collagen, fibrinogen and elastin. This results in skin abrasion and erosion of tissues (particularly on the peduncle and caudal fin), muscle degeneration, followed by progressive tissue necrosis (Nematollahi *et al*., 2003; Gómez *et al*., 2014). Also, this bacterium inhibits the nonspecific humoral defense and the necessity of specific antibody capable to induce an effective immune response against *F. psychrophilum* is of high importance (Madetoja and Wiklund, 2002; Barnes and Brown, 2011).

BCWD results in significant losses in aquaculture production facilities, causing high mortality rates on freshwater fish species, namely a wide range of salmonids (e.g.: *O. clarki*, *Salvelinus namaycush* or *O. tshawytsch*) such as *O. mykiss* (Barnes and Brown, 2011; Starliper, 2011). In *O. kisutch* the diseases mortalities as high as 50% have been reported to occur in fry stage, and in older fish losses are usually bellow 20%. *O. mykiss* is very susceptible to this disease leading to mortalities between

10% and 30% in the fry stage (in fish of size between 0,2-2 g) and quite often rising up to 70% (Nematollahi *et al*., 2003). Several studies reported that the mortalities and external signs are variable, depending on the fish species and their development stage. Until now, very little has been published on infections of *F. psychrophilum* in rainbow trout of over 100g (e.g., Nematollahi *et al*., 2003; Woynarovich *et al*., 2011; Gómez *et al*., 2014). Many researchers are looking for solutions to alleviate stressors in order to reduce the severity of outbreaks and then prevent further outbreaks. With BCWD, the mortality can rise quickly and be very high if the culture conditions are not improved quickly or a treatment is not promptly administered. Thus it is important that more studies about the mortality are done in more advanced development stage of their growth cycle to find preventive solutions or effective treatments. This disease on Rainbow Trout has been a recurring challenge and this has been suggested as a new niche for studying the virulent strains of the pathogen (Starliper *et al*, 2011).

The aim of this study was to follow the mortality rates in Rainbow Trout, *O. mykiss,* in the growing development stage which have been infected with *F. psychrophilum.*

b. Materials and methods

i. Fish acclimatization

In this experiment, fish species of *O. mykiss* were used, which had been hatched and grown up in the AquaBioTech Group facilities (Mosta, Malta), until a body weight average of 176g (±5.7g SD). During this period, the fish were fed with a commercial diet (Marico Supreme-22, 3.0 mm) three times a day, seven days a week. The fish had no previous health problems before the trial and all the experiments were carried according to European Union health and animal welfare regulations.

ii. Bacterial dilutions

To obtain the necessary volume of bacteria to be inoculated in fish, a stock of *F. psychrophilum* strain 259-93 (identified and isolated from fish with BCWD) was grown on the media recommended by Cepeda and colleagues (2004), which is the Tryptone Yeast Extract Salts Agar supplemented with Glucose (TYESGlucose). The composition of the media was the following Tryptone Yeast Extract Salts agar supplemented with Glucose (TYESGlucose), which composition consists in Tryptone: 1%; Yeast extract: 0,1%; NaCl: 0,8%; $CaCl_2$: 0,03%; Glucose: 0,05%; Agar: 1,5%.

The bacteria were plated in the previously described media supplemented with agar allowing them to grow for 10 days at 16ºC, as recommended by Long *et al*. (2014). After that, the colonies were resuspended in 10 mL of Dulbecco's Phosphate-Buffered Saline (PBS; Sigma®). From this solution, successive decimal serial dilutions were performed in 9mL of PBS in order to obtain four different solutions (Non-diluted, 1:10, 1:100 and 1:1000 solution).

To study the growth behavior of bacteria and determine the concentration of bacteria, from dilution, a volume of 100µL was inoculated in Petri dishes (90 mm diameter), in duplicate, containing TYESGlucose agar using the surface sampling technique. Then they were incubated for 10 days at a temperature of 16°C to count colonies. A volume of 1mL from each dilution was read by spectrophotometry at a wavelength of 525nm. This procedure allows the attaining a standard curve relating the optical density with the concentration of bacteria. All the procedures were performed under aseptic conditions and following biosecurity norms.

iii. Challenge trial

A total of 243 fish, previously starved for one day, were challenged in this experiment. To perform the injections, they were all anesthetized using Tricaine Methanesulfonate (Tricaine PHARMAQ®) at 80 mg L^{-1} and neutralized with the same amount of sodium bicarbonate, as suggested by the manufacturer. Groups of 27 fish were injected with each dilution of the bacteria, in duplicate, except for the 1:1000 dilution (Table III).

Table III - Injection procedure of *Flavobacterium psychrophilum* dilutions.

Treatment	Replica	Number of fish	Dilution
1	R1	27	Non-diluted solution
	R2	27	Non-diluted solution
2	R1	27	1:10
	R2	27	1:10
3	R1	27	1:100
	R2	27	1:100
4	R1	27	1:1000
	R2	-	-
5	R1	27	PBS solution
	R2	27	PBS solution

The pathogen was inoculated by an intramuscular injection with 0,05mL of the pathogen dilution, approximately 1 cm on the dorsal site of the lateral line, slightly posterior to the dorsal fin. The injection procedure started with the PBS group was followed by the lower to the higher concentrations of the pathogen. The injection was performed with an insulin syringe (Myjector U-100 insulin 0,3ml, TERUMO®). The fish were allocated in tanks with a capacity volume of 500L (Figure 21).

Figure 21 - System where the challenge trial was performed.

The challenge trial lasted 17 days and the biosecurity rules were maintained during the whole experiment. All fish were fed with a commercial diet (Marico Supreme-22, 3.0 mm) until satiation, three times per day. The study was performed using freshwater and their parameters were controlled and recorded every day until the end of the challenge (Table IV).

Table IV - Water quality parameters recorded during the

Parameters	Average (±SD)
Temperature	16.0 ºC (±0.3)
Oxygen	12.8 mgL^{-1} (±2.8)
pH	7.4 (±0.2)
Ammonia	0.3 mgL^{-1} (±0.17)
Nitrites	2.47 mgL^{-1} (±2.1)
Nitrates	305.9 mgL^{-1} (±117.1)

Behavior change or any fish health status indicators of disease (e.g. ulcers or lesions on skin disintegration) were reported. All mortalities were recorded and dead stored at -20°C. Every day, a streak sampling on one dead fish per replica was performed, plated on TYESGlu Agar and incubated at 16°C.

c. Results and discussion

F. psychrophilum reflects considerable economic losses whereby it is considered a challenge in aquaculture. Preliminary studies about the behavior and bacterial growth in culture medium are an important basis to understand their pathogenicity. However, studies about the disease consequences in later life stages of the fish are also essential to create effective prevention and treatment measures (Nematollahi *et al*., 2003; Gómez *et al*., 2014).

In microbiology it is important to choose the right medium and follow the protocol avoiding contamination routes. According to some studies, TYESGlu medium proved to be the most appropriate to isolate and inoculate *F. psychrophilum* (Cepeda *et al*., 2004). This bacterium shows a greater tendency to aggregate on TYESGlu, compared with other media. This is due to the presence of divalent cations ($MgSO_4$ and $CaCl_2$) in the composition of this medium that facilitates the aggregation by bridging on surface proteins present on these bacteria, making the most indicated medium to grow *F. psychrophilum* (Crump *et al*., 2001). Thus, in this study, TYESGlu medium was used to grow the colonies and no contamination was verified in the plates used to ressuspend the colonies in PBS. However, after performing the dilution and plating to count the colonies forming units, the presence of *F. psychrophilum* was not verified for any dilution and some contaminations were observed. In fact, the high absorbance observed for the less diluted solutions proves the presence of any bacteria and not specifically the presence of *F. psychrophilum.* Despite this, some evidences suggest that the bacteria used to the challenge were in fact *F. psychrophilum*:

- The presence of these bacteria is easy to verify because they have a characteristic yellow pigmentation phenotype (Figure 22), as observed in this experiment. However, many authors suggest more identifications analyses after the simple observation of their phenotype (Madetoja and Wiklund, 2002). This way, a Gram stain tests it was performed and presence of gram-negative rods was verified, which is suggesting a manipulation of the right bacteria.
- Although the TYESGlu medium used is ideal for the growth of *F. psychrophilum,* the slower growth is characteristic for this pathogen, as verified in this study. However some authors reported that the slow growth permits a easy inhibition by bacteria contaminants that grow faster (Madetoja and Wiklund, 2002);
- *F. psychrophilum* was identified and isolated from fish with BCWD, according with their phonotypical characteristics such as slow growth and yellowish pigmentation. The fish injected with *F. psychrophilum* solution, used in this challenge, showed typical lesions of BCWD which indicate the use of the appropriate bacteria during all the experiment.

There are several reasons that might be responsible for the occurrence of this contamination but it will always be speculative. Thus it is convenient to repeat the process with enhanced care and additional steps in the protocol, avoiding contamination routes. As the presence of contamination was only observed after performing the dilutions with PBS solution, it could be relevant to perform also a PBS sterility control. This includes platting Petri dishes with only PBS solution, on the same medium used to growth *F. psychrophilum.* This way, it is possible to understand if the PBS solution was the contamination route of the whole process.

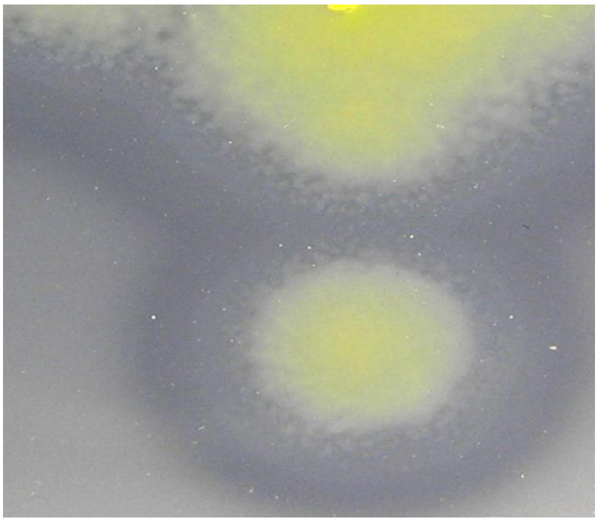

Figure 22 - Phenotype of *Flavobacterium psychrophilum* showing the yellow pigmentation characteristic (Madetoja and Wiklund, 2002).

Since it was not possible to obtain a calibration curve to determine which concentration of bacteria was injected into the trout, the results will be presented according to the different dilutions prepared to distinguish between treatments, such as non-diluted, 1:10, 1:100, 1:1000, and PBS solution.

Depending on the life stage of the fish, the infection method applied is different. In early life stages (<5 g), the infection by bath is the most appropriate method to infect all fish mimicking the natural route. However, for larger fish (>5g), that are normally less susceptible, the infection is typically performed by intramuscular or intraperitoneally injection (Fredriksen *et al*., 2013). In this study, the method used was by intramuscular injection, since it has been shown to be more effective to increase the mortality until 75 and 100% in other studies (Starliper 2011; Fredriksen *et al*., 2013). Although all ages of fish are affected by BCWD, the developed life stages are less vulnerable to infections comparing to the early life stages of the fish. Hadidi and colleagues (2008) developed some theories and evidence where this vulnerability could be explained by the immune system development. During the fish development, their immune system will specify and some organs and structures will develop. An example of this is the spleen that, in Rainbow Trout, is involved in several mechanisms of immune response, such as the Ab cells production and immunological memory. These authors reported, for the first time, a surprisingly high correlation between a physical trait (spleen weight) and bacterial disease resistance (provoked by *F. psychrophilum*), in a teleost fish. This suggests a functional relationship where less susceptible fish had a bigger spleen. Also Henryon and colleagues (2005) noted a correlation between trout body weight and *F. psychrophilum* resistance. It is known that Rainbow Trout spleen size increases in proportion to total body weight and this means an increase in the resistance against BCWD. However, this close correlation needs more evidence to be fully understood. Also a number of potential factors may influence the size of a certain organ, such as the spleen, that needs to be considered before adopt any strategy (Henryon *et al*., 2005; Hadidi *et al*., 2008). A depth investigation in this subject could be interesting to provide an easy measurement of disease resistance without requiring sophisticated equipment. Also, this relation could be considered as an indicator for selective breeding. In this study, no kind of relation between organ size and resistance of any disease was evaluated. However it was a principle to increase the concentration of bacterial solution to inject on the trout once there are no studies that mention the most appropriate *F. psychrophilum* doses to provoke mortalities in larger Rainbow Trout.

According to the results obtained, the dose response showed a positive relationship between the concentration of *F. psychrophilum* injected and the mortalities that occurred in Rainbow Trout. With this, it is possible to assume that bacteria affect larger fish but their effects depend on concentration. Therefore, the highest dose (non-diluted and 1:10 solution) had greater mortalities in comparison with the less concentrated dilutions, such as 1:100 and 1:1000 solution. As expect, and reported by Loch and Faisal (2015), the exposure to elevated concentration of bacteria induces early and devastating effects. In this study, fish injected with the higher concentration (non-diluted solution) showed more than 70% of mortalities whilst fish injected with 1:10 it did not reach 50% mortality. In other terms, the lethal dose of bacteria to kill 50% of fish could be between these two concentrations. However, to determinate this exactly dose it is necessary broaden the range of concentration between non-diluted and 1:10 solutions. For the highest two doses, mortalities started to appear around day 3 while the first mortalities registered in the 1:100 solution appear on day 10. This observation could be of particular importance when highly virulent strains of the pathogen spread on favorable condition in order to provide sufficient time to apply effective preventive measures. In this case, the increase of number of tank replicas could contribute to improve the resolution in the challenge results (Figure 23).

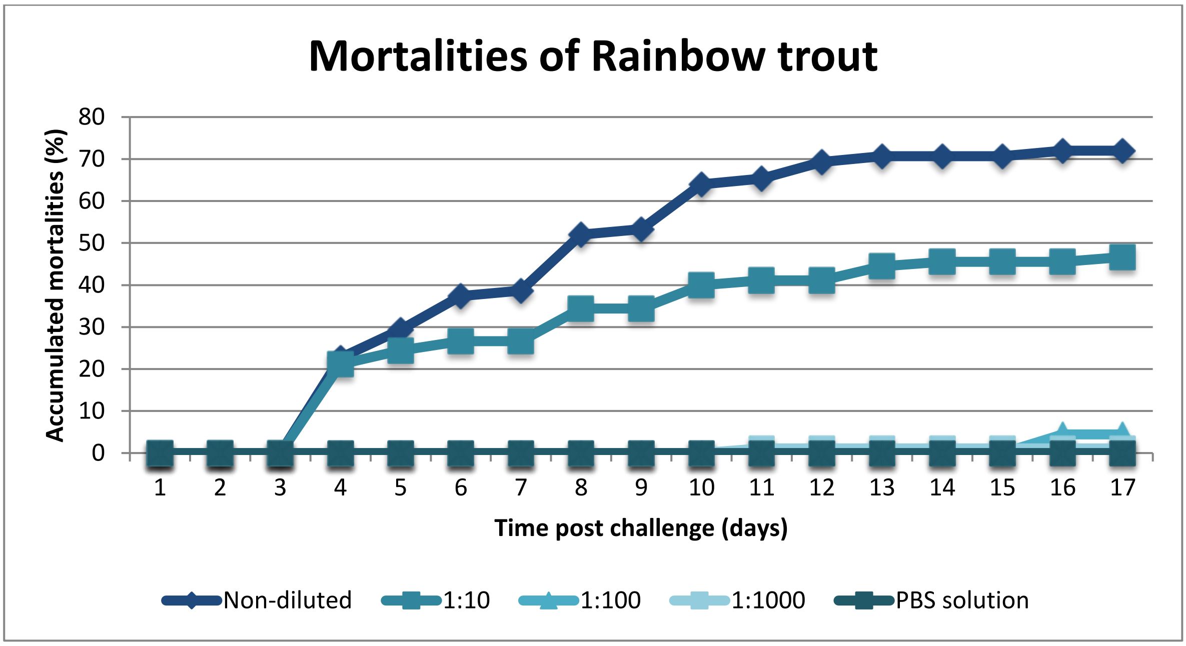

Figure 23 - Accumulated mortalities (%) of Rainbow Trout, during seventeen days post challenge, in different doses of *Flavobacterium psychrophilum*: Non-diluted, 1:10, 1:100, 1:1000 and PBS solution.

Coldwater disease manifests itself in many ways, producing characteristic open lesions on the external body surfaces of fish. In the early stage, these lesions are characterized by rough skin areas and fins tip fraying (Figure 24). As the infection develops, the necrosis evidences appear especially in adipose-caudal fin region. In advanced cases, the necrosis of the caudal region progress up to the caudal vertebrae and can lead to death (Barnes and Brown, 2011; Starliper, 2011). In these studies, many fish showed signs of disease during the experiment proving the presence of *F. psychrophilum* showing the expected symptoms. The verified signals were essentially the abrasion of tissues (particularly on the peduncle and caudal fin), skin ulcerations and concurrent hemorrhage. However, further analysis reporting the type of lesions or the number of fish that showed signs of disease were not registered.

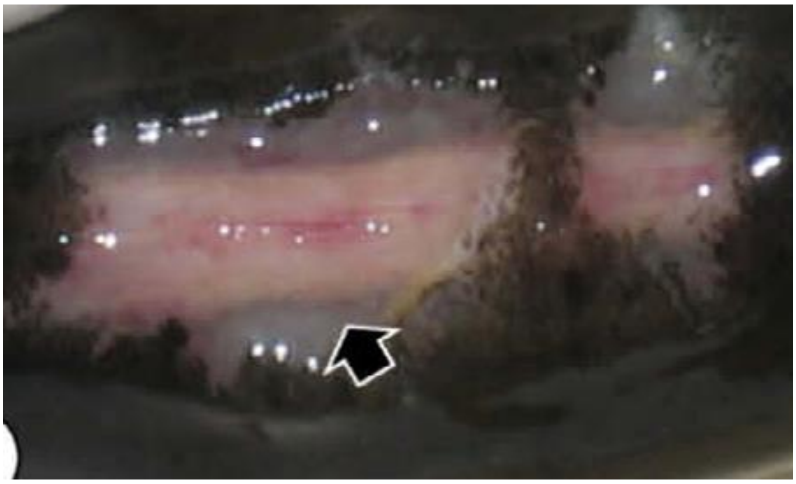

Figure 24 - Skin ulcerations provoked by *Flavobacterium psychrophilum* (Starliper, 2011).

Despite the symptoms reported being characteristic of *F. psychrophilum,* the absence of these bacteria during the growth in TYESGlu medium could raises doubts about if the injections were actually performed with *F. psychrophilum.* To outwit this hypothesis, some tests are suggested:

- Perform PCR and electrophoresis techniques to compare the DNA fragments from pure colonies with bacteria collected from infected fish (Madetoja and Wiklund, 2002);
- Histological examinations that prove the manifestations of disease caused by *F. psychrophilum* (Barnes and Brown, 2011; Starliper, 2011);

BCWD has been a recurring problem in aquaculture and further investigations for the analysis of the pathogenicity and its virulence is important. Any study which determines the dose of *F. psychrophilum,* which is causing acute and chronic signs of disease in large size Rainbow Trout, is essential to prevent or find effective treatments for this disease.

d. Futures prospective and conclusions

The present study was performed in order to provide some bases that are useful for future investigations. The necessity of further research is clear to provide more answers about pathogenesis of *F. psychrophilum* and to develop preventive diagnosis and effective treatments against BCWD in larger Rainbow Trout, which still remains poorly understood.

As with any fish disease, including BCWD, early advances should be directed towards preventing the disease. The prevention of diseases is the most prudent form of disease control and treatment, which provide the management strategy to minimize the risks of pathogen introduction or transmission, and to reduce the severity of overt disease outbreaks (Starliper, 2011). For this some points need to be worked out:

- Controlling the water quality of the system is the most important prevention measure. Several studies show that a regular analysis of the water supply's micro flora is important to prevent the spread of any disease (Nematollahi *et al*., 2003). Also, maintaining the parameters always controlled, for example above 10°C, can avoid the presence of *F. psychrophilum* - since this bacteria grows better at low temperatures and frequently causes disease when water temperatures are below 10ºC (Barnes and Brown, 2011);
- Good handling techniques will reduce fish stress minimizing the creation of *F. psychrophilum* entrance portals (Gómez *et al*., 2014). For example, physical handling provides the ideal point-of-entry for the pathogen and should be avoided whenever it is not strictly necessary (Madetoja and Wiklund, 2002). Reduce fish stress, such maintaining effective sanitization of the systems and equipment, are some preventive measure to avoid the entrance and spread of disease (Starliper, 2011);
- Good nutrition is another important point to prevent this disease. Some studies report that poor nutrition, like diets with high levels of oxidized lipids, show a greater mortality caused by *F. psychrophilum* after exposure to the pathogen (Starliper, 2011). The nutritional improvements with enhanced dietary formulations, that include research into the use of probiotics or immunostimulants, may lead to changes in BCWD susceptibility (Barnes and Brown, 2011; Gómez *et al*., 2014);
- Selective breeding showed to be a considerable promise. Some studies reported that the resistance to BCWD in Rainbow Trout is moderately heritable and found strong correlations between a physical characteristic and the resistance to the pathogen, allowing for genetic improvements of the broodstock. Also, avoiding the introduction of wild fish into existing fish stocks is another preventative measure (Henryon *et al*., 2005; Barnes and Brown, 2011);

- Develop more effective techniques of diagnosis that improves the speed, sensitivity, and precision for detecting and identifying *F. psychrophilum*. Currently some authors use the immunofluorescence method to rapidly diagnose of *F. psychrophilum* infections. Also with the refinement of molecular DNA techniques with considerable genetic variation precisions it is possible to differentiate the strains having virulence variation (Barnes and Brown, 2011).

Further investigations about *F. psychrophilum* pathogenesis, which still remains poorly understood, will provide important information to develop effective treatments against BCWD. The identification and characterization of important antigens, both cellular and extracellular, seems to be the key for the development of recombinant vaccines (Crump *et al*., 2001). However there are still a number of fundamental points that have to be resolved before a vaccine against BCWD can be developed and commercialized. The lack of normalized values for larger fish is a serious obstacle to further progress and it is probable that vaccination against *F. psychrophilum* was not still achieved successfully. Also it is important to understand the virulence factors and identify putative antigens to establish the standardized vaccination model (Hastein *et al*., 2005; Gómez *et al*., 2014). In addition, reproducibility of *F. psychrophilum* vaccination is not yet well understood and experimental conditions needs to be improved in order to obtain reliable results (Decostere *et al*., 2000). Studies aiming to develop effective and easy to administer vaccines should continue to go hand in hand with a further elucidation of the pathogenesis of this disease. In this context, the necessity of an investment and the improvement of aquaculture and biotechnology techniques to understand certain aspects of prevention and treatment process are of paramount importance.

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8. Conclusion

To perform an internship in a company environment has many advantages but performing it abroad allows opening our mind and to broad our horizons. This internship allowed the application of knowledge acquired during the academic degree and to learn many other tasks that complement the training, offering a greater competence level and professional experience. Important personal skills are also acquired, where it is possible to learn, mandatory to follow the rules and establish good relationships. Moreover, responsibility and organization are highlighted qualities that are easily understood and refined under a company environment.

This internship performed to complete the masters in Aquaculture from the Polytechnic Institute of Leiria, allowed to master routine techniques in a number of aquaculture duties. Also allowed have an important research input in main aspects of fish pathology, such as the foundations for vaccine development of a serious global aquaculture problem. While presented with a highly learning environment, the aquaculture, marine biology, and biotechnology background received before this internship also allowed for important inputs in the routine works and more specialized research during the internship duration.

The AquaBioTech Group offers the intern good facilities and equipment to perform high quality work, providing important tools for the intern to evolve over this period. With prospects of expansion it is predictable that its facilities will keep improving and thus enhancing the quality of work of employees and trainees. Thus, all work performed during this 6 month of internship proved to be a great opportunity to enter the labor market and gave a good perception of aquaculture work in real company environment.

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9. References

ABT Innovia: *Capability Statement*. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 15th August 2016;

AquaBioTech Group Company. Available in: https://www.aquabt.com, viewed: 23th June 2016;

Barnes, M. E. and Brown, M. L., (2011). A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. *Open Fish Science Journal*, 4, pp.40-48;

Boyd, C. E. and Tucker, C. S., (2012). Pond aquaculture water quality management. *Springer Science and Business Media,* Mississippi, USA;

Bruun, M. S., Schmidt, A. S., Madsen, L. and Dalsgaard, I., (2000). Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. *Aquaculture*, *187*(3), 201-212;

Cepeda, C., García-Márquez, S. and Santos, Y., (2004). Improved growth of *Flavobacterium psychrophilum* using a new culture medium. *Aquaculture*, *238*(1), 75-82;

Cipriano, R. C. and Holt, R. A., (2005). *Flavobacterium psychrophilum*, cause of Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome. *National Fish Health Research Laboratory*, Kearneysville, USA;

Columbia Basin Fish and Wildlife Authority: PIT Tag Steering Committee, (1999). PIT tag marking procedures manual. *Basin Fish and Wildlife Authority*, Portland, USA;

Crump, E. M., Perry, M. B., Clouthier, S. C. and Kay, W. W., (2001). Antigenic characterization of the fish pathogen *Flavobacterium psychrophilum*. *Applied and environmental microbiology*, *67*(2), 750-759;

Dill, W. A., (1993). Inland fisheries of Europe. *Food and Agriculture organization of the United Nations*, California, USA;

Eding, E. H., Kamstra, A., Verreth, J. A. J., Huisman, E. A. and Klapwijk, A, (2006). Design and operation of nitrifying trickling filters in recirculation aquaculture: a review. *Aquacultural engineering*, *34*(3), 234-260;

FAO, (2014). The State of World Fisheries and Aquaculture 2014: Opportunities and challenges. *Food and Agriculture Organization of the United Nations*, Rome, ITA;

Fisheries and Aquaculture Department (Food and Agriculture Organization of the United), National Aquaculture Sector Overview: Malta. Available in: http://www.fao.org/fishery/countrysector/naso_malta/en, viewed: 30th April 2016;

Fisheries and Aquaculture Department (Food and Agriculture Organization of the United), Seed production: An overview. Available in: http://www.fao.org/docrep/field/003/ac182e/AC182E01.htm, viewed: 11th August 2016;

Fredriksen, B. N., Furevik, A., Gauthier, D., Egenberg, M., Paulsen, E. D. and Brudeseth, B., (2013). Intramuscular challenge of Rainbow Trout (*Oncorhynchus mykiss*) with two Norwegian field strains of *Flavobacterium psychrophilum*. *Fish and shellfish immunology*, *35*(2), 595-598;

Gómez, E., Méndez, J., Cascales, D. and Guijarro, J.A., (2014). *Flavobacterium psychrophilum* vaccine development: a difficult task. *Microbial biotechnology*, 7(5), pp.414-423;

Guidding, R., Lillehaug, A., Evensen, O., (2014). Fish vaccination. *Wiley Blackwell*, Oxford, UK;

Hadidi, S., Glenney, G. W., Welch, T. J., Silverstein, J. T. and Wiens, G. D., (2008). Spleen size predicts resistance of Rainbow Trout to *Flavobacterium psychrophilum* challenge. *The Journal of Immunology*, *180*(6), 4156-4165;

Henryon, M., P. Berg, N. J. Olesen, T. E. Kjaer, W. J. Slierendrecht, Jokumsen, and Lund, I., (2005). Selective breeding provides an approach to increase resistance of Rainbow Trout (*Onchorhynchus mykiss*) to the diseases enteric red mouth disease Rainbow Trout fry syndrome, and viral hemorrhagic septicemia. *Aquaculture 250,* 621–636;

Hishamunda, N., and Subasinghe, R. P., (2003). Aquaculture development in China: The role of public sector policies. *Food and Agriculture Organization of the United Nations*, Rome, ITA;

Janssen, K., Chavanne, H., Berentsen, P. and Komen, H., (2016). Impact of selective breeding on European aquaculture. *Aquaculture*, n.d.;

Kurtz, J., (2002). The simple guide to Marine Aquariums. *T.F.H. Publications, Inc.,* Neptune, USA;

Leblanc, C. A. and Noakes, D. L., (2012). Visible implant elastomer (VIE) tags for marking small Rainbow Trout. *North American Journal of Fisheries Management*, *32*(4), 716-719;

Loch, T. P. and Faisal, M., (2015). Emerging Flavobacterium infections in fish: A review. *Journal of Advanced Research*, 6(3), pp.283-300;

Long, A., Fehringer, T. R., LaFrentz, B. R., Call, D. R. and Cain, K. D., (2014). Development of a waterborne challenge model for *Flavobacterium psychrophilum*. *FEMS microbiology letters*, *359*(2), 154-160;

Madetoja, J. and Wiklund, T., (2002). Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farms. *Systematic and applied Microbiology*, 25(2), pp.259-266;

Martins, C. I. M., Eding, E. H., Schneider, O., Rasmussen, R., Olesen, B., Plesner, L., Verreth, J. A. J., (2005). Recirculation Aquaculture Systems in Europe. CONSENSUS. *Consensus working Group, European Aquaculture Society,* Oostende, Belgium;

Martins, C. I. M., Eding, E. H., Verdegem, M. C., Heinsbroek, L. T., Schneider, O., Blancheton, J. P., ... and Verreth, J. A. J., (2010). New developments in recirculation aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering*, *43*(3), 83-93;

Mente, E., and Smaal, A. C., (2016). Introduction to the special issue on “European aquaculture development since 1993: the benefits of aquaculture to

Europe and the perspectives of European aquaculture production". *Aquaculture International*, 24(3), 693-698;

Moe Jr. and M. A., (2009). Marine Aquarium Handbook – Beginner to Breeder; Revised and Expanded. *T.F.H. Publications, Inc.,* Neptune, USA;

Munni, M. A., Fardus, Z., Mia, M. Y. and Afrin, R., (2015). Assessment of pond water quality for fish culture: A case study of Santosh Region in Tangail, Bangladesh. *Journal of Environmental Science and Natural Resources*, *6*(2), 157-162;

National Statistical Office in Malta (European statistical system), Aquaculture: 2013. Available in: https://nso.gov.mt/en/News_Releases/View_by_Unit/Unit_B3/Environment_Energy_Transport_and_Agriculture_Statistics/Pages/Aquaculture.aspx, viewed: 30th April 2016;

Nazar, A. A., Jayakumar, R. and Tamilmani, G., (2013). Recirculation aquaculture systems. *Mandapam Regional Centre of CMFRI*, India;

Nematollahi, A., Decostere, A., Pasmans, F. and Haesebrouck, F., (2003). *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of fish diseases*, 26(10), pp.563-574;

Ngoc, P. T. A., Meuwissen, M. P., Cong Tru, L., Bosma, R. H., Verreth, J. and Lansink, A. O., (2016). Economic feasibility of recirculating aquaculture systems in pangasius farming. *Aquaculture Economics and Management*, *20*(2), 185-200;

Pedersen, P. B., Svendsen, L. M., Sortkjær, O., Ovesen, N. B., Skriver, J., Larsen, S. E., Rasmussen, R. S., Johanne A., Dalsgaard, T., (2008). Environmental benefits achieved by applying recirculation technology at Danish Trout farms (Model trout farm). *Proceedings of the aquaculture engineering society's fourth issues forum*, (n.d.);

PHARMAQ® Company. Available in: https://www.pharmaq.no/, viewed: 1st July 2016;

Piedrahita, R.H., (2003). Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. *Aquaculture*, 226, 35-44;

Roque d'Orbcastel, E., Blancheton, J.P., Aubin, J., (2009). Towards environmentally sustainable aquaculture: comparison between two trout farming systems using life cycle assessment. *Aquaculture Engineer*, 40, 113-119;

Sacchi, J., (2011). Analysis of economic activities in the Mediterranean: Fishery and aquaculture sectors. *Plan Bleu,* Valbonne, FR;

Schneider, O., Blancheton, J. P., Varadi, L., Eding, E. H., Verreth, J. A. J., (2006). Cost price and production strategies in European recirculation systems. *Linking tradition and technology highest quality for the consumer*, Firenze, ITA;

Schneider, O., Schram, E., Poelman, M., Rothuis, A., van Duijn, A., van der Mheen, H., (2010). Practices in managing finfish aquaculture using RAS technologies, the dutch example. *OECD workshop on Advancing the Aquaculture Agenda*, Paris, FR;

Starliper, C.E., (2011). Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research*, 2(2), pp.97-108;

Statistic explained (Eurostat), Fishery statistics. Available in: http://ec.europa.eu/eurostat/statistics-explained/index.php/Fishery_statistics . Viewed: 30th April 2016;

Subasinghe, R., Soto, D., and Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in Aquaculture*, 1(1), 2-9;

Verdegem, M. C. J., Bosma, R. H., Verreth, J. A. J., (2006). Reducing water use for animal production through aquaculture. *Int. J. Water* Resour. Dev., 22, 101-113;

Water Framework Directive, (2000). Directive 2000/60/EC 23rd Oct 2000 of the European Parliament: The Council establishing a framework for the community action in the field of water policy. *The European Parliament and the Council of the European Union,* Brussels, Belgium;

Willis, T. J. and Babcock, R. C., (1998). Retention and in situ detectability of visible implant fluorescent elastomer (VIFE) tags in *Pagrus auratus* (Sparidae). *New Zealand Journal of Marine and Freshwater Research*, *32*(2), 247-254;

Woynarovich, A., Hoitsy, G. and Moth-Poulsen, T., (2011). Small-scale Rainbow Trout farming. *Food and Agriculture Organization of the United Nations,* Rome, ITA.

10. Figure references

Figure 2 - National statistical office in Malta (European statistical system), Aquaculture: 2014. Available in: https://nso.gov.mt/en/News_Releases/View_by_Unit/Unit_B3/Environment_Energy_Transport_and_Agriculture_Statistics/Pages/Aquaculture.aspx, viewed: 30th April 2016;

Figure 3 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 23th June 2016;

Figure 4 - AquaBioTech Group Company. Available in: https://www.aquabt.com, viewed: 23th June 2016;

Figure 5 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 13th August 2016;

Figure 7 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 15th August 2016;

Figure 8 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 15th August 2016;

Figure 9 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 18th August 2016;

Figure 11 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 18th August 2016;

Figure 12 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 18th August 2016;

Figure 13 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 18th August 2016;

Figure 14 - PHARMAQ Company. Available in: https://www.pharmaq.no/, viewed: 20th August 2016;

Figure 15 - Columbia Basin Fish and Wildlife Authority: PIT Tag Steering Committee, (1999). PIT tag marking procedures manual. *Basin Fish and Wildlife Authority*, Portland, USA;

Figure 16 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 20th August 2016;

Figure 20 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 22th August 2016;

Figure 21 - ABT Innovia: Capability Statement. Available in: https://www.aquabt.com/assets/downloads/Capability-Statement.pdf, viewed: 22th August 2016;

Figure 22 - Madetoja, J. and Wiklund, T., (2002). Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farms. *Systematic and applied Microbiology*, 25(2), pp.259-266;

Figure 24 - Starliper, C.E., (2011). Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research*, 2(2), pp.97-108.

Declaration:

This report is signed by the legal representatives of the company AquaBioTech to prove that the contents of this is considered to be factual and true and performed with the collective knowledge.

Signed,

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