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Sustainable Strategies for Modern Fish Farming

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Abstract

With the increasing demand of aquatic products for consumption on a global level, aquaculture becomes indispensable as a sustainable way to provide them and lower the effects of overfishing. Fish farming over the last decades is tending towards intensive systems which usually have the potential to achieve the highest production rates. Recirculating aquaculture systems (RAS), in particular, are very popular for their high degree of water re-use and reliable manipulation of various factors affecting the production conditions. This makes them very adaptable and applicable to locations without adequate resources (e.g. difficult access to water) for more traditional methods. Besides commercial productions, RAS are used as the predominant type of system in animal experimentation facilities which require a strict control of the culture conditions and biosecurity for successful trials.

In this report I describe the RAS systems used in the facilities, the different tasks performed, and knowledge gained during my internship at CIIMAR, as well as provide theoretical context mainly for the components of recirculating systems.

Keywords: CIIMAR, RAS, biological filtration, water quality

Resumo

Com o aumento global da procura de produtos aquáticos para consumo, a aquacultura torna-se indispensável para o seu fornecimento sustentável e para ajudar a reduzir os efeitos da pesca intensiva. Durante as últimas décadas, as produções piscícolas têm adotado cada vez mais condições de cultura intensiva que, de uma maneira geral, têm o potencial para atingir as taxas de produção mais elevadas. Sistemas aquáticos de recirculação (RAS), em particular, são muito populares devido à sua grande capacidade de reutilização de água e manipulação de vários fatores que influenciam as condições de produção. Isto faz com que os sistemas de recirculação sejam altamente adaptáveis e possibilitam a sua aplicação em localizações sem recursos adequados para a utilização de sistemas de produção mais tradicionais (e.g. dificuldades no acesso a água). Para além de produções comerciais, RAS também são o tipo de sistema predominantemente usado em biotérios que exigem um controlo rigoroso das condições de cultura e biossegurança para a execução de ensaios experimentais com sucesso.

Neste relatório estão descritas as diversas tarefas que executei e conhecimentos que adquiri durante o meu período como estagiário no CIIMAR, assim como uma base teórica relacionada com as componentes dos sistemas de recirculação.

Palavras chave: CIIMAR, RAS, filtração biológica, qualidade da água

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1- Introduction

As the world population increases so does the consumption of protein rich products, specially the consumption of fish or aquatic food in general which has been rising, on a global scale, roughly two times faster than the population growth since 1961. In fact, global aquatic food consumption has surpassed the consumption of meat-based products originating from all terrestrial animals. Although the highest demand comes from developed countries with higher incomes, developing countries play an important role in exportation, due to developed countries often not being able to produce enough to support their demand. Not to mention that, in spite of usually having lower overall consumption, several developing countries have higher shares of fish protein in their diets, often relying on fish products for survival (FAO, 2018).

With such high demand for aquatic products there is inevitably a need for reliable food supplies and concern regarding sustainability. Capture rate of wild fish has been relatively unmodified for the last 40 years. Even with aquaculture practice gaining a lot of popularity and supplying roughly up to 50% of aquatic products consumed worldwide (including aquatic plants and algae), there are still a significant number of species which are fished at unsustainable rates. This applies to aquatic organisms for consumption but even more so to ornamental species, since a great number of species can only be obtained in the wild and have a very high demand making the market extremely profitable. This further highlights the need for expansion of the aquaculture sector and its potential as a steady, sustainable supply for the demand of aquatic products and in alleviating the stress put upon wild populations, eventually helping countries in achieving an economical and environmental stability (FAO, 2018).

1.1- Production systems

Aquaculture production systems, in general, fall roughly under three categories: extensive, semi-intensive, or intensive. The intensification of a system can be translated mainly into the increase in culture density and/or the level of external inputs and human interference (Tidwell, 2012). Systems may also be characterized as: static, with no water exchanges; open, if it is integrated in the environment which serves as the system and provides adequate ecological conditions (e.g. temperature, oxygen, waste removal); semi-closed, if the production units are mostly manmade but it still relies on the environment; and closed when it is not dependent on the environment and usually has a high degree of water

re-use. Many productions can be categorized using both concepts in conjunction (Tidwell, 2012; Lucas et al., 2019).

Extensive systems are the most traditional types, usually consisting of static ponds (natural or artificial) where aquaculturists rely on the food naturally present in the pond to sustain the animals with minimal external interference. Filtration components are not present since stocking densities are usually low (e.g. up to 250 kg/ha) and the established ecosystem of the pond can naturally deal with the relatively low amount of organic waste (Tidwell, 2012). This type of production is very common in Asia for freshwater fish and crustacean cultures (FAO, 2018). However, biomass production and control of water quality in extensive systems can be very limiting, thus more intensive systems may be more appropriate for when natural resources or space is not as available and if a higher degree of control of over the production conditions is desired.

Semi-intensive production generally uses systems that still rely on natural conditions but allow for higher culture densities and the manipulation of at least some factors. In open systems such as rafts, cages and net pens, human interference is usually limited to feeding and providing the structures to farm the organisms in a natural environment. Semi-closed systems allow for further manipulation generally by making water exchanges possible. Widespread systems used include raceways and ponds which, in contrast with extensive ponds or other open systems, allow for a higher control of inlet water and the incorporation of additional components such as pumping, water treatment units, aeration, etc (Tidwell, 2012; Lucas et al., 2019). This greater control of culture conditions also results in increased production, for instance, semi-closed ponds may surpass 5,000 kg/ha. In spite of these benefits, semi-closed systems have some disadvantages such as higher construction and equipment costs, management requirements and energy input (Tidwell, 2012).

Intensive systems usually allow almost complete control and manipulation over most factors regarding the production by using closed systems that minimize de dependence of environmental conditions. Closed systems are mostly known as recirculating aquaculture systems and strive to have as little water exchange as possible. Intensive conditions are known for considerably high culture densities, potentially up to 10 kg/m^3 (100,000 kg/ha) (Tidwell, 2012; Lucas et al., 2019).

There are several factors that may hinder the development of aquaculture such as: introduction and spread of aquatic diseases or parasites, environmental contamination, exposure and susceptibility to changes of climatic conditions, social conflicts, improper scaling of the facilities and equipment, limited production, and financing risks.

Between the different types of aquaculture, recirculating aquaculture systems (RAS) may offer solutions for some of these problems, namely water consumption (Verdegem et al., 2006), waste management and biological pollution control (Piedrahita, 2003; Zohar et al., 2005), and disease management (e.g. Summerfelt et al., 2009; Tal et al., 2009).

1.2- Recirculating aquaculture systems (RAS)

Recirculating aquaculture systems are set to re-use water to a certain degree by undergoing treatment. (Rosenthal et al., 1986). This recirculating technology was developed for intensive fish farming when resources like water and available space are limited, allowing for fish to be reared in higher densities while still being able to maintain water quality using the previously mentioned water treatment (Heinen et al., 1996). In addition to being very popular for inland aquaculture without easy access to water, RAS systems are the predominant type used in research laboratories and university bioteriums as well as small- and large-scale aquariums. Recirculating systems are also widely used as complements to traditional pond or net pen culture, giving support through the production of brood stock or fingerlings (Malone, 2013).

Designs may vary, but according to Malone (2013), RAS systems must be able to address 5 main points: clarification (solid removal), biological filtration, circulation, aeration, and degassing. This is assured using components such as: diverse water treatment equipment (mechanical filters, sand filters, biofilters, protein skimmers when working with saltwater, ozone and UV radiation); pumps to circulate the water between the sumps, culture tanks and different water treatment units; aeration is usually secured in the form of blown air, which also helps with degassing (Parker et al., 2012).

From these 5 points, solid removal and biofiltration are considered to be the most important in maintaining water quality (Badiola et al., 2012).

Biological filters are used to remove nitrogen waste from aquatic systems. Ammonia is a by-product excreted by fish that results from protein catabolism and is a main factor affecting water quality. It exists in water in two forms: un-ionized (NH_3) and ionized (NH_4^+) ammonia from which the former is highly toxic for most aquatic organisms. Concentrations as low as 0.6 mg/L can cause loss in appetite, reduced growth rate and death at high concentrations. In order to remove ammonia from the system, biofilters make use of three-dimensional structures made from non-corroding materials such as plastic, ceramic or fiberglass, that have large surface areas, to cultivate nitrifying bacteria. These bacteria exist naturally in water and under aerobic conditions can convert ammonia (NH_3) into nitrite (NO_2^-

), which is still toxic and nitrite into nitrate (NO_3^-). Nitrate is non-toxic at concentrations found in RAS (>200 mg/L) and is managed by periodic water exchanges (DeLong and Losordo, 2012; Masser et al., 1999).

Suspended solids can easily impact the performance of most RAS components by clogging them, directly affecting the biofilter by decreasing the specific surface area available for nitrifying bacteria to colonize and modifying water parameters (Jokumsen and Svensen, 2010). The reduced usable area for the bacteria will lead to less biofilter colonization and less ammonia and nitrite being converted, raising their concentration. Solids in the clogged components will be oxidized decreasing oxygen concentration in water and further contributing to the increase in ammonia and nitrite concentration, potentially leading to very stressful and harmful environments for cultured species as well as nitrifying bacteria. (Malone and Pfeiffer, 2006; Emparanza, 2009).

1.3- Prophylactic strategies

Other important issues affecting aquaculture success are health and nutrition, which are two complex fields by themselves but are also closely connected, especially regarding farmed animals. Farms under intensive culture conditions such as sea cages, usually have higher risks of disease outbreaks due to chronic stressful conditions (i.e. high fish densities), compromising the primary lines of defence such as mucosal surfaces through physical abrasion and, making it easier for pathogens to find their way into the host . The resulting reduced disease resistance can lead to strong negative effects on fish growth and survival, potentially causing severe economic losses, hence the great importance of disease prevention for successful aquaculture facilities (Klesius et al., 2004).

Vaccines have been used in aquaculture as early as the 1940s as a method to prevent disease outbreaks caused mainly by bacteria and virus which can commonly occur in productions (Ma et al., 2019). In general, vaccines possess substances in their composition which will function as antigens. These substances will stimulate the innate and/or adaptive immune response to a specific target pathogen in the treated organisms (usually fish). The most common methods of administration include immersion baths, oral delivery through the feed and intraperitoneal injection, the latter being generally more widespread (Sudheesh and Cain, 2017).

Substances used as antigens may be obtained and/or used in different ways, however most licensed vaccines in aquaculture nowadays are inactivated/killed or live vaccines (Ma et al., 2019). Inactivated/killed vaccines rely on the use of dead or inactivated

organisms which are processed in order to remove their ability to infect or replicate without eliminating their antigenic effect (Tlaxca et al., 2015). In live vaccines the pathogenic effects of the organisms are greatly attenuated through various possible methods (e.g. culture under unfavourable conditions, genetic manipulation, physical or chemical processes, etc) without killing them (Ma et al., 2019).

Vaccination has also showed success as a prophylactic strategy, for instance in the salmon industry (Sommerset et al., 2005), but despite their proven effectiveness, there is still a need to increase efficiency for cultured fish (Secombes, 2008). In general, such research has been conducted by feeding fish for a specific period and then evaluating immune parameters or survival after disease challenge (Waagbø, 2006). However, few studies have combined immunization with nutrient supplementation (Pohlenz et al., 2012). Under this scenario, certain additives may prove to be fundamental as they have been demonstrated not only to promote growth, but also to have an array of desirable immunological attributes in different fish species (Kiron, 2012). Therefore, more research is needed to support this new concept and to validate the potential role of novel and innovative diet formulations on improving vaccine efficiency.

Amongst other prophylactic strategies available, enhancing heath and disease resistance through the best possible nutrition is currently used in modern fish farming. Throughout the last 30 years, scientific evidence indicates that the inclusion of dietary nutrients or other additives in feeds can stimulate the immune system, which is important for farmed species as their access to nutrients is completely dependent on what they are fed with. This follows the concept of immunonutrition, providing animals with additional resources to support the protective mechanisms that counter stress factors or pathogen invasion such as regulatory cytokines, antioxidant defences, acute phase proteins and cellular responses (Kiron, 2012).

With this in mind, feeds are formulated in diverse ways, as the inclusion of dietary nutrients such as amino acids, lipids and vitamins or other antioxidant micronutrients can directly or indirectly influence immune functions. For example, as stated by Azeredo et al. (2019), dietary supplementation with tryptophan (above the levels required by the species for normal growth) might help counteract chronic stress induced by high stocking density, although the same supplementation to fish in lower densities might have an opposite effect. Therefore, it is important to verify the eventual negative effects of a certain additive or ingredient and take into account the level of supplementation and feeding time.

Other important additives widely used in animal farming are prebiotics, which are non-digestible and complex carbohydrates added to feeds to stimulate the growth of

desirable intestinal microorganisms. The most common are oligosaccharides, which are the only ones classified strictly as prebiotics. Although there are some positive results in health benefits, reviews suggest that further research is necessary as information available is still limited and not scalable to economic use in the feed industry (Merrifield et al., 2010; Ringø et al., 2010).

Probiotics are used more frequently than prebiotics and are one of the main focuses of modern aquaculture research to promote health and disease resistance, especially for their promising role as an alternative strategy to antibiotics. They consist in microorganisms that, administered in adequate quantities confer health benefits to the host (FAO/WHO, 2002). The benefits of enhancing intestinal microbiota include better digestion, absorption and storage of nutrients as well as protection against pathogen colonization by the production of antimicrobial factors and competition for nutrients (Neuman and Nanau, 2012). Indeed, probiotic dietary supplements are able to improve health and nutrition of livestock, but respective bacteria have mainly been isolated from terrestrial, warm blooded hosts, limiting an efficient application in fish. Native probiotics limited to the gastrointestinal tract of the respective fish species will establish within the original host more efficiently, and much work is being devoted to it (Van Doan et al., 2018).

1.4- Objectives

The main objective of this internship was the development of skills and accumulation of workplace related experience. With this in mind, the internship was roughly divided into two parts: collaboration with the staff of the bioterium of aquatic organisms (BOGA); and collaboration with the animal health and aquaculture laboratory (A_2S).

The collaboration with BOGA focused more on technical aspects of bioterium management. Activities consisted mainly in following staff routines as well as sporadic tasks, with special emphasis in managing recirculating aquaculture systems (RAS) and biofilter monitorization. Experience with biofilters included, independently starting new biofilter systems and accompanying the activation process of new and ongoing systems while learning to solve commonly associated problems.

The collaboration with A_2S lab also focused on RAS, albeit more related to the zootechnical aspect, serving as a complement to the overall internship experience, since at BOGA there is little contact with actual management of stocked RAS systems. This part of the internship consisted in managing recirculating systems in the context of two research trials: the first tested the immune response of gilthead seabream (*Sparus aurata*) following

a gut inflammatory stimulus and after being fed diets containing anti-inflammatory additives; the second trial tested whether an experimental diet Ygeia+ can improve vaccine efficiency on European seabass (*Dicentrarchus labrax*).

2- CIIMAR

The Interdisciplinary Centre of Marine and Environmental Research is located in Matosinhos, Porto, and shares a building with the cruise terminal of the port of Leixões. As a research centre it is at the forefront of ocean knowledge and innovation, harbouring a great number of interns, masters and PhD students from a wide range of different degrees and faculties. Associated institutions that make up the General Assembly include, Institute of Biomedical Sciences Abel Salazar, Faculty of Sciences, Farmacy and Engineering of the University of Porto.

A board of directors supervises scientific and technical activities as well as everyday management (fig. 1). A Scientific board, composed of all the PhD members of the Centre, also aids with advice and monitorization of scientific and technical activities. An external scientific advisory board provides further advice and evaluation of scientific activity.

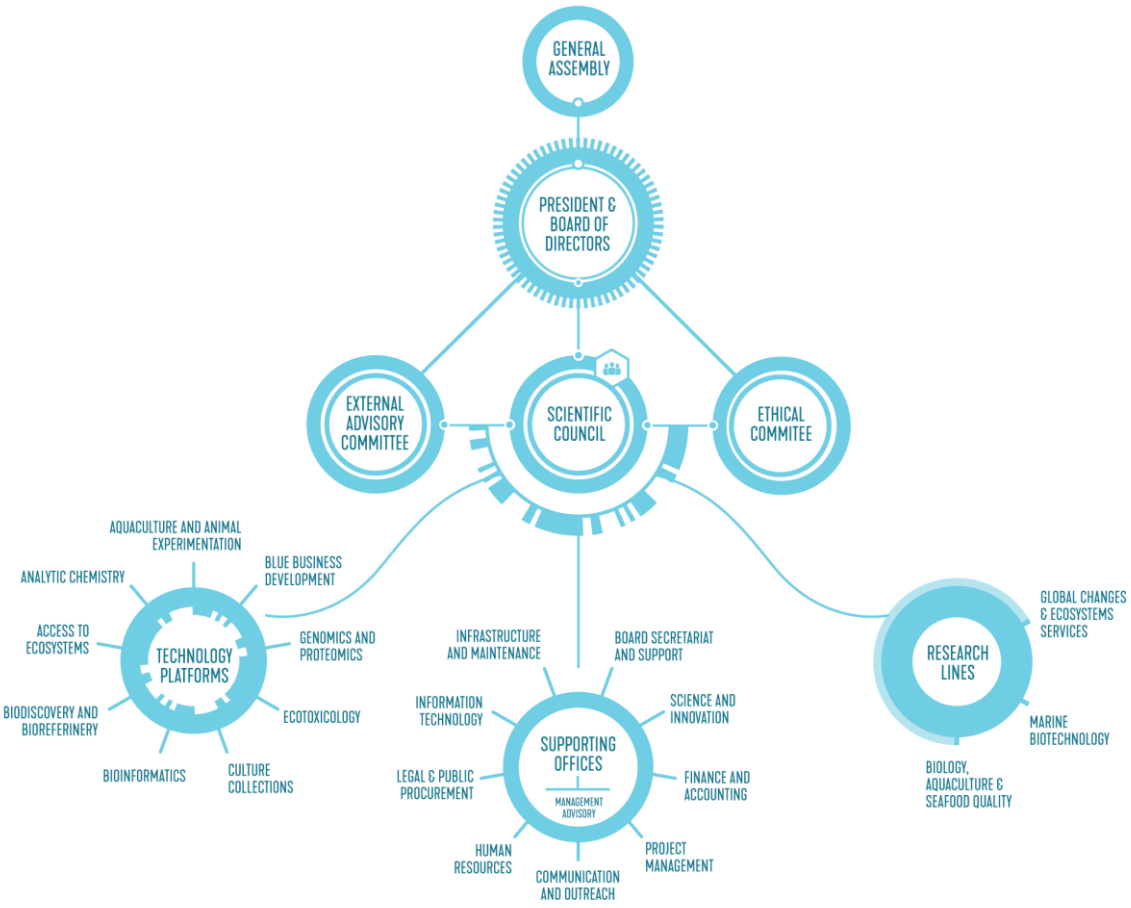

Figure 1 - CIIMAR organization. Source: ciimar.up.pt

There are three main research lines in the institute that branch into different research groups and teams, each with their specialized activities and principal investigators. The main research lines being: Biology, Aquaculture and Seafood Quality; Marine Biotechnology; and Global Changes and Ecosystems Services.

Additionally, the institute has several other professional supporting offices and technological platforms services that provide daily support, essential for the normal functioning of CIIMAR. Some amongst these are, administration, finance and accounting, human resources and even science communication and outreach, often organizing tours for scientific education to schools. Another supporting structure with great importance for the research institute is the bioterium of aquatic organisms (BOGA) where experimental trials are executed, and aquatic organisms kept by the various research groups are maintained in stock.

3- BOGA

The bioterium of aquatic organisms is situated at the basement level of the building and it is directed by the leader of the nutrition, growth and quality of fish research team, Luisa Valente, and managed on a day to day basis by the BOGA staff. The BOGA staff is composed of three members, Hugo Santos, Ricardo Branco and Olga Martinez which execute various daily routines and often provide aid and advice to researchers or other technicians that might be working on the various rooms that make up the bioterium.

Access is only available to authorized personnel, this mostly includes researchers and technicians who have ongoing activities (e.g. trials, maintenance, sampling) in active rooms of the facilities, and consequently must be regularly present. One must first acquire a CIIMAR card from the supporting offices and contact the BOGA staff to request entry to the bioterium. The card can then be used to enter the bioterium and only grants access to the rooms needed to conduct the specific work of a given researcher or technician.

In the basement floor, where the facilities are situated, one can find: an office where users can interact with the BOGA staff; a storage room; a workshop, used to store various tools and execute repairing works (i.e. tubing, pumps, electrical equipment, etc); a refrigerated room with various freezers where the different research teams can store feeds and frozen samples; and the Wet Lab that can be reserved by users, usually for sampling activities, equipped with freshwater and saltwater access, a sink, air tube openings and a digital precision scale.

All new BOGA users must attend an initial biosafety training conducted every two weeks, usually by Hugo of the BOGA staff. The user will be walked through a visit to the various rooms inside the bioterium and instructed on utilization norms and safety measures of the different common spaces.

3.1- Accessing the facilities

There are several requirements and mandatory procedures for accessing the facilities of BOGA, these are:

- Users must check in and out with the card.
- Personal protective equipment – consists of waterproof footwear and a lab coat of obligatory and exclusive use for the bioterium.
- Entering through the first door into the bioterium hall one can find changing rooms, a second entrance door to the actual facilities and an entrance door to the

quarantines. Handwashing and disinfection are mandatory upon entering and exiting the hall.

- Changing rooms – one changing room is reserved to the BOGA while the second is shared by the common users, both equipped with bathrooms and benches. The shared changing room can be used to store personal protective equipment as long as the user has to visit the facilities at least twice a week. During the year modifications have been made by adding several boot stands to the shared changing room, improving space utilization and organization.
- Footbath – one must place both feet inside the footbath for disinfection while entering or exiting the bioterium and the quarantines. Emphasis is given for following this rule rigorously since contamination via footwear is one of the most common for aquatic animal facilities.

3.2- Trial rooms

Most rooms inside the bioterium are dedicated to conducting research trials, as such, due to the diversity of research fields, BOGA has different types of experimental rooms. These include: aquaculture rooms, usually equipped with several tanks or aquariums and RAS filter systems with high volume used mainly by the Biology, Aquaculture and Seafood Quality research line's teams (e.g. Animal Health and Aquaculture team, A_2S; Nutrition, Growth and Quality of Fish team, LANUCE; Nutrition and Immunobiology, NUTRIMU); less specialized rooms that allow for more options of experimental designs of the culture room; microalgae culture rooms; and infection challenges rooms.

Access is limited to the users which have to perform tasks and cleaning equipment is usually provided by the BOGA staff. All culture rooms have a score sheet of the systems and a BOGA monitorization sheet. The score sheets are filled by researcher or technician in charge of the monitorization of the room in a given day and is essential for keeping track of the water quality of the systems and the general condition of the culture room. The BOGA monitorization sheet is used by the BOGA staff when inspecting rooms.

As a general rule, equipment used for a specific system of a given room in the bioterium should not be used for other rooms. If it is strictly necessary, the equipment must be properly cleaned and disinfected as to avoid cross contaminations. Water chillers used in their aquaculture systems are placed outside rooms to avoid that the heat released by them interferes with the room temperature. Every culture room has access to saltwater, dechlorinated water, and regular tap water. Tap water is used almost exclusively for

washing the room and system components since it can cause negative effects to the bacterial activity of the biofilter and organisms due to residual chlorine.

3.3- Rooms under BOGA management

There are specific rooms, under the BOGA jurisdiction that are, generally exclusive to them and used for the daily tasks related to the services they provide: biofilter systems room, p.15 (later moved to the quarantines); Zebrafish room, p.02; central storage room.

3.4- Washing room

The room is shared by all users and has two main components: a large sink, for cleaning lab equipment or larger system components which are not practical to wash in normal sized sinks; and a washing machine used to wash and disinfect mechanical filters.

Users have fill the utilization form in order to use the room that can be found on the washing machine with their name, respective research team and time of start and end of the washing process for the sake of organization, since it may have to be used multiple times a day by several people. Bleach must be added to the washing machine, as filters from different culture rooms are washed in the same machine, to avoid cross contamination. The room is under BOGAs management, but research teams have to provide their own bleach and users should always leave the rooms in clean conditions for the next user.

3.5- Sampling room

Similar to the wetlab outside the facilities, users can reserve the room for sampling. Equipment includes: a digital precision scale; sharps containers; salinity, pH, temperature, and oxygen probes; 2 photometers, distilled water, and a sink (fig. 2 and 3).

After using the probes in the sampling room, they must be washed with distilled water and one should not exchange the same sampling cup in between different systems without disinfecting it. The pH probe is immersed in a buffer solution and users have to take care not to leave the probe airdrying and always put it back in the buffer solution when it is not being used.

3.6- Carcass disposal

When working in an animal research facility, carcass disposal, is often necessary due to sampling events and to remove deceased fish from active systems. According to protocol set by the BOGA staff, when disposing of a carcass the following steps should be executed:

- Take a carcass bucket (can be found in the sampling room), collect the animal using a fishnet dedicated to the system where the mortality occurred and place it in the bucket.
- Register the mortality in the scoring sheet of the system.
- Take the bucket to the refrigerated room (S03) and place the carcass in the bag inside the “carcass freezer”, registering the information in the record sheet (name, research team, room, number of carcasses and species or common name).
- Wash and disinfect the carcass bucket in the washing room before returning it to the sampling room.

3.7- Organisms entry management

All new animals that enter the facilities must spend a minimum of 15 days in the quarantine before they can be moved to the experimental rooms. Researchers must fill a quarantine entry form in advance so that the BOGA staff can assess and prepare the quarantine room. Keeping the animals in quarantine has a cost for the research teams. Additionally, researchers must fill an animal utilization form and an animal transfer form. All forms are mandatory for any organism.

Figure 3 - Sampling room in BOGA.

Figure 2 - Salinity (left), pH (centre) and dissolved oxygen (right) probes in BOGA's sampling room.

4- Collaboration with BOGA

My entrance into the technician team of the bioterium as an intern was mediated by the coordinator of BOGA, Luísa Valente, and afterwards my supervision was entrusted to the BOGA staff. Hugo Santos, having the most experience as a technician, followed my work more closely, and alongside the rest of the staff, Ricardo Branco and Olga Martinez played an important role in tutoring me through technical procedures applied in the facilities, teaching me how to think critically to solve problems and providing help whenever it was needed. During the first days the staff instructed me on the routines until I was able to execute some of them myself.

4.1- BOGA – Routines

4.1.1- Saltwater reservoir check-up

The location of the research institute is advantageous in terms of saltwater access, being right by the sea in the port of Leixões, saltwater is collected directly into a collection tank (AVAC room, accessed through the parking lot) and subsequently passes through a sand filter and is stored in a saltwater reservoir (30 m^3) before it is distributed to the facilities systems. This is a significant improvement in terms of reliability compared to the method used in the previous facilities where saltwater was provided regularly by the firefighter department.

Every day a member of the staff checks the water level of the reservoir upon arrival to assess its availability to the facilities. This is done because, whenever it is possible, water from the AVAC tank is pumped into the reservoir near the time of the day when high tide is at its peak. The reason being to avoid pumping in water with bad physical or chemical properties as a result from descending tides from the port, additionally water quality from the AVAC tank must be checked before transfer to ensure it is safe to be used in the facilities. As such, considering that the hour of the peak of the high tide varies daily, checking the water level at the start of the day helps the staff to better manage the usable saltwater and inform the users, if needed, when there is little water available until the determined time of the day in which the reservoir will be refilled. For this, the staff keeps a tide calendar in the office and a board where the water level of the reservoir is displayed and changed accordingly by the members of the staff. If a member of the staff performs this task (checking the water level and refilling the reservoir) he should also write his identification and the hour of the next high tide on the board as to avoid confusion amongst members. A backwash must be performed for the sand filter after it is used to fill the reservoir.

After my collaboration with BOGA the saltwater collection system was further optimized. Currently it is equipped with automatized water collection and industrial scale filtration components (i.e. UV and ozone disinfection; activated carbon; ultra-fine filtration).

4.1.2- Daily inspections

The BOGA staff is responsible for daily inspections of the rooms in the bioterium. The three members of the staff rotate weekly to execute this task. The staff checks every experimental room individually, pointing out faults in the systems (e.g. leaks, faulty aeration, accumulation of solids) or regular room organization misconduct in the BOGA monitorization sheet that people have to check while using the room, if more urgent problems are encountered users may have to be contacted directly.

4.1.3- Zebra fish maintenance

The room p.02 is dedicated to the stock culture system of zebrafish under the management of the bioterium. These zebrafish are used almost exclusively for reproduction as a service to provide embryos for researchers. The room is composed of a single ZebTEC Active Blue Stand Alone rack (fig. 4) and is equipped with reverse osmosis system (fig. 5) to ensure quality freshwater is always available for water exchanges. This system almost fully automated and composed of a supporting structure with many components characteristic of RAS systems: several 3L tanks disposed in rows, mechanical and biological filtration, UV light disinfection, inbuilt heating, automated filter backwashing and a water distribution systems that enable the control of inlet water in individual tanks. Operators can monitor the system’s temperature, pH, and conductivity through a display monitor in the rack.

Figure 4 - ZebTEC Active Blue Stand Alone rack in BOGA.

Figure 5 - Reverse osmosis system in BOGA.

4.1.3.1- Zebrafish reproduction

During my collaboration with BOGA the zebrafish stock was used to provide eggs to CIIMAR's research teams throughout the year as a paid service. This, however, was changed and now the paid service only applies to external entities. For reproduction the staff makes use of a "cage" which consists of an aquarium structure without its glass base (for the brood stock), placed inside a larger, regular aquarium (for the eggs). The base of the smaller aquarium is switched for a mesh and marbles are placed on it to simulate the substratum.

Both aquariums are then filled with water and heated to the same temperature of the system for acclimation (usually between 27 and 29°C, 28°C being optimal) before transferring the brood stock. When zebrafish spawn their eggs will fall into the marbles and subsequently slide through them and the mesh, falling into the second aquarium until they are collected. This is useful to avoid predation of the eggs by their progenitors. The staff places the fish inside de aquariums in the evening and spawning will occur on the following day at the onset of light. The brood stock should also be left undisturbed from the moment they are placed in the reproduction aquariums for better reproductive rates. A member of the staff then uses a siphon to collect the eggs at the bottom of the aquarium. Afterwards, viable eggs have to be separated from non-viable ones and other types of solid matter that may be collected with the eggs. When researchers make the request to the BOGA staff,

they can specify if they will execute this work themselves or if they need the staff to isolate viable eggs.

4.1.4- Biofilter maintenance

The biological filter systems of BOGA are monitored daily by a different member of the staff every week. Water parameters are measured and, according to the needs of each system, the technician may have to adjust their levels (i.e. perform water exchanges; renew buffer solution; add ammonia to the system). The biofilter medium is usually used in the quarantine but may also be provided to other culture rooms when needed. This topic will be explored further in this report.

4.1.5- Quarantines

The quarantine room can only be accessed by the BOGA staff or by researchers and technicians that are responsible for animals that will be used on trials of their respective research teams. It is composed of two rooms (fig. 6); each one has two separate recirculating systems with 2000 L tanks and Tropical Marine Centre filtration systems with 600 L sumps. Before entering these rooms there is a small division where users sanitize their hands upon entry and where the new biofilter activation systems are located. BOGA also possesses systems with smaller dimensions with associated filtration up to scale (250 and 500 L).

Figure 6 - Quarantine rooms in BOGA.

New animals that enter the facilities must spend at least 15 days in the quarantine room. During this period, researchers and technicians attentively monitor the batches they are responsible for, assessing the health conditions of the animals and possibly single out individuals in poor conditions that may be sources of infection. Water quality and the status of the system components is also checked daily.

4.1.6- Minor or sporadic tasks

Footbath – as mentioned earlier both the entrance to the bioterium and to the quarantines possess footbaths for footwear disinfection. The footbaths have to be renewed according to their sanitary state (fig. 7 and 8), since several people enter and exit the facilities daily debris can accumulate and negatively affect the efficiency of the disinfectant. Virkon is the product of choice used by the BOGA staff, it has a strong bactericidal, fungicidal and virucidal activity. It comes in form of a powder and is added to the water in a 1:100 dilution. Gloves should be used when handling it as it can be harmful in contact with skin, additionally it is harmful when inhaled or in contact with the eyes.

Figure 7 - Renewed footbath.

Figure 8 - Worn out footbath with accumulated debris.

Equipment calibration and verification – as a rule, the probes in the sampling room are calibrated once a month by a member of the staff.

Entrance of new animals in the quarantine – when new animals are delivered to CIIMAR, the BOGA staff prepares quarantine systems in advance, adjusting the water parameters to the ones of the water they are transported in. The staff can also aid the researchers or technicians in the transport of the animals to the quarantine.

Equipment or system components cleaning and disinfection – when systems are disassembled their components need to be thoroughly washed since most times, after their

use and prior to disassembly, they are disinfected by recirculating a solution of water and bleach. These instances are also important because some components can’t be cleaned while the system is functioning and can accumulate solids that may affect the quality of water.

5- Recirculating aquaculture systems

5.1- Mechanical filtration

As mentioned before, suspended solids are one of the main causes of decline in water quality and can easily impact the performance of most RAS components (e.g. clogging), thus it is important to be able to quickly and efficiently remove them from the systems (Han and Webb, 1996). This is the main purpose of using mechanical filters and other types of solid removal methods such as settling or granular filters.

Particulate matter in aquatic systems mainly originates from uneaten food and from the digestion waste (Masser et al., 1999). When these solids are oxidized by bacteria in the system, they consume oxygen and originate nitrogenous compounds which can impact the growth rate of organisms and cause mortality, hence the need for quick removal (Losordo et al., 1992). Particles can be classified according to their size: soluble or dissolved (<0.001µm); colloidal (0.001-1µm); supercolloidal (1-100µm); settleable (>100µm). As the size decreases particles became increasingly difficult to remove, thus mechanical filtration should be applied as the first line of water treatment and water that reaches the solid removal components should ideally be treated gently to avoid breaking the particles and reducing their size. Dissolved particles cannot be removed by traditional methods of mechanical filtration, therefore systems must include additional filtration components such as biofilters and protein skimmers (Lekang, 2007).

The use of granular filters, also known as depth filtration, relies on forcing water to flow through a layer of granular material (e.g. sand) where waste particles remain trapped. These types of filters can also partially function as biofilters due to medium they use (see 5.3.3- biofilters).

Settling, also called sedimentation, is an easy method that relies on the principle that suspended particles have a higher relative density than water to make solids sink into a settling basin where they can be siphoned out. Sinking speed will depend on the size of the particle (Lekang, 2007). In spite of being an effective way to remove larger particles with low effort, it is generally not capable of removing particles smaller than 100µm (Henderson and Bromage, 1988), thus it is normally used as a pre-treatment associated to the main filtration method (Cripps and Bergheim, 2000).

Mechanical filters, or micro screens, consist on applying a physical barrier through which inlet water passes but solids are retained. Different material can be used (e.g. perforated plates; screens; bar racks; filter socks; sponges; glass wool) and the size of their mesh or orifices will determine the size of particles it can trap (fig. 9 and 10). Generally, mesh sizes down to 30µm are used in recirculating systems. With mesh sizes as small as

20µm mechanical filters are also able to remove some parasites. These types of filters require frequently cleaning since they become easily blocked with solids, the clogging potential increases with smaller mesh sizes (Lekang, 2007).

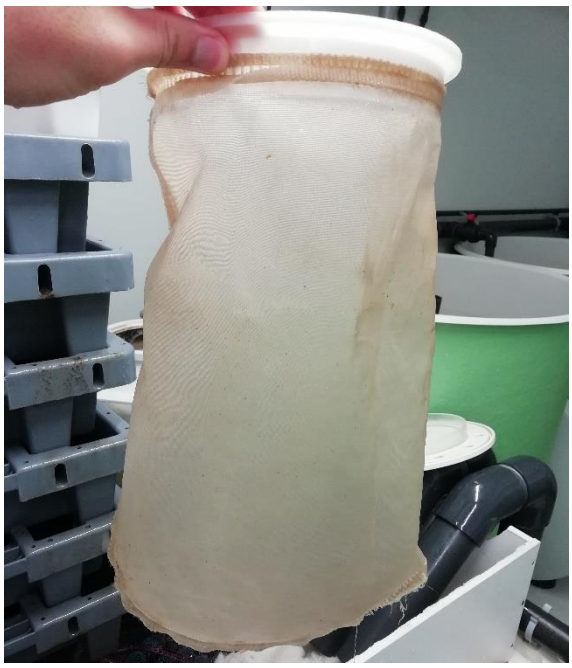

Figure 9 - Filter sock.

Figure 10 - Mechanical filters - glass wool (white); sponge (blue).

In recirculating systems with high degree of water re-use, suspended particles are predominantly smaller than 100µm and since approaches such as screens and settling are not very affective in removing particles bellow 50µm, in general, many systems may struggle in dealing with fine solids smaller than 30µm. As such, the application of granular filters which can remove solids sized down to 20µm is very useful for RAS and is highly suggest for most systems (Chen et al., 1994).

5.2- Foam fractionation

Foam fractionation, also called protein skimming, is a process widely used in aquaculture for a more efficient removal of dissolved and fine organic matter in water (<30µm) which are more prone to building up in recirculating systems. It consists in creating a stream of bubbles in a closed column of water, creating foam at the surface. As the bubbles ascend, particles adhere to their surface and are expelled through the foam. The foam being formed at the surface will start to build-up into a collection cup and is subsequently drained out of the skimmer (Losordo et al., 1992).

The efficiency of this process may be affected by several factors such as bubble size, suspended solids concentration and the chemical properties of the water as well as the particles (Brambilla et al., 2008). For instance, foam fractionation is more effective in saltwater or brackish water systems when compared to freshwater because bubble size decreases with increasing ionic concentration in the water. Smaller bubbles allow for a higher total area of contact with particles and bubbles produced in seawater are generally four times smaller than bubbles produced in freshwater (Slauenwhite and Johnson, 1999).

The benefits of using foam fractionation include: lower risk of clogging system components; removal of high molecular weight compounds such as proteins; better water clarification; removal of organic acids which helps stabilize pH; and an additional source of aeration (Lawson, 1995). Heterotrophic bacteria can also be effectively removed and, coupled with the rest of the organic particles removed improves the overall availability of oxygen. (Brambilla et al., 2008).

A typical design used in aquaculture consists in creating the bubble stream at the bottom of the water column either through introducing air directly with diffusers or by relying on a venturi associated to the skimmer. The bubbles are usually introduced in a counter current way, which means that the bubble stream flows in the opposite direction of the inlet water in the skimmer. This allows for higher turbulence and degree of contact between the water and the bubbles. Protein skimmers are also frequently chosen as a place to inject ozone due to high mixing potential created by the turbulence, also aiding in oxidizing organic matter (Lekang, 2007). Most protein skimmers used in BOGA follow this design (fig. 11).

Figure 11 - Protein skimmers in BOGA.

5.3- Biological filtration

5.3.1- Nitrogen waste

Ammonia (NH_3/NH_4^+), as mentioned before, is one of the most important parameters to take into consideration when monitoring any type of aquatic system, specially recirculating aquaculture systems due to their intensive nature (e.g. high culture density, higher risk of accumulation, larger feed administration) (Tomasso, 1994; Badiola et al., 2012). Most cultured aquatic organisms excrete ammonia directly into the water as a byproduct of protein metabolism (e.g. fish excrete ammonia through the gills). In addition, the oxidation of non-ingested feed or other solid accumulations (e.g. faeces) also raises the concentration of ammonia in the water, while also contributing to the depletion of the system's oxygen (Losordo et al., 1992; Masser et al., 1999).

Ammonia, in water, exists mainly in two forms: un-ionized ammonia (NH_3), and ionized ammonia (NH_4^+), together they are known as total ammonia nitrogen (TAN). The un-ionized form has a highly toxic effect on aquatic organisms compared to the ionized form. Lethal concentration can vary according to the species, there is a general agreement, however, that sub-lethal effects can be observed with concentrations as low as 0.6 mg/L**.** Negative effects of chronic exposure to sub-lethal concentrations may include, reduced growth, damage to gills and kidney, possible brain malfunctions and reduction of oxygen carrying capacity (Durborow et al., 1997a; Tilak et al., 2007).

NH_3 and NH_4^+ exist in balance and their respective proportions are largely dependent on the pH and temperature. At pH values below 8 most ammonia is in the less toxic ionized form (NH_4^+), this proportion, however, increases significantly as pH increases. The same is reported for temperature, for any given value of pH, more toxic un-ionized ammonia (NH_3) will be present in their proportion as water temperature increases. Generally speaking, for each increase in one unit of the pH value, the amount of un-ionized ammonia may increase up to ten times (Durborow et al., 1997a; Hargreaves and Tucker, 2004).

Nitrite (NO_2^-) occurs in water mainly as a result of ammonia oxidation and, in spite of not being as toxic as ammonia, is still very harmful to aquatic organisms and is usually monitored with the same degree of importance in aquaculture. High concentrations of nitrite can cause brown blood disease in fish due to nitrite binding with haemoglobin and making it incapable of transporting oxygen. Fish inflicted with this disease, or other types of respiratory deficiency, frequently exhibit a gasping behaviour on the surface of the water that can be a useful cue that the quality of the water is declining (Durborow et al., 1997b).

Nitrite can be further oxidized and converted into nitrate (NO_3^-) which is, generally, not as great of a threat as ammonia and nitrite in aquatic systems. Most aquatic organisms exhibit a great tolerance to high concentrations of nitrate, often being able to resist levels over 200 mg/L. Additionally, nitrate concentrations have a reduced probability of reaching these values in recirculating systems since regular water exchanges are performed (e.g. 5-10% of the total system volume daily) that avoid nitrate build-up (Losordo et al., 1992; Masser et al., 1999).

$$2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 4H^+ + 2\ H_2O$$

$$2\ NO_2^- + O_2 \rightarrow 2\ NO_3^-$$

5.3.2- Nitrifying bacteria

The process of oxidizing ammonia to nitrite, and nitrite to nitrate is mainly is executed by nitrifying bacteria that naturally exist in both saltwater and freshwater. These bacteria are the basis of the biological filtration used in various types of aquatic systems, from small- to large-scale production systems and aquariums. The bacteria convert these nitrogen compounds by oxidizing them, therefore the presence of oxygen is vital for this process to occur. There are mainly two types of nitrifying bacteria that play an important role in nitrification: bacteria which convert ammonia into nitrite (e.g. *Nitrosomonas* spp.); and bacteria which convert nitrite into nitrate (e.g. *Nitrobacter* spp., *Nitrospira* spp.) (Avnimelech, 2006; Foesel et al., 2008; DeLong and Losordo, 2012).

Although these are the most common groups, biofilter can house several other species of bacteria (e.g. Actinobacteria, Firmicutes, Proteobacteria) and, since aquatic organisms introduce their own microflora, biological filters are dynamic systems that can differ amongst them (Sugita et al., 2005; Schreier et al., 2010).

Additionally, there is a group of bacteria capable of converting ammonia and nitrite directly into N_2 (Planctomycetales) through a process called anaerobic ammonium oxidation and commonly known as anammox. As suggested by the name, these bacteria don not use oxygen for this process and, although they require completely anoxic environments to proliferate, some studies have suggested that they can be present in marine RAS biofilters. Their specific use is limited due to having slow doubling times, but they constitute an important part of denitrifying filters more used in systems with very high degrees of water re-use and organic loads. Denitrification filters convert nitrate into nitrite and subsequently into nitrogen gas, decreasing the need for daily water exchanges. Their microbial growth depends on a supply of organic carbon rather than oxygen (Kalyuzhnyi et al., 2006; Lekang, 2007; Lahav et al., 2009).

5.3.3- Biofilters

Biological filters exploit the nitrifying characteristics of the bacteria and use them mainly to remove nitrogen waste from aquatic systems. They are especially important and prominent in RAS systems, as they are more susceptible to high concentrations due to smaller culture volumes and having a higher level of water re-use (Lekang, 2007).

To make use of these bacteria, a suitable environment is provided for them to proliferate and create a biofilm. This consists in using a medium for the culture to grow, as well as adjusting water parameters to optimal levels. Materials with non-corroding properties (i.e. plastic, fibre glass, ceramic, sand) and high surface area, also known as specific surface area (SSA), are favoured for bacterial colonization (fig. 12). Although filter medium with high specific surface area usually achieves higher efficiency, it also tends to be more compact, which can be useful for reducing volume requirements but can also lead to clogging. Thus, it should be taken into consideration when choosing appropriate filter designs (Lekang, 2007; DeLong and Losordo, 2012). Regarding water quality, the main factors that influence the growth and efficiency of cultured nitrifying bacteria are for the most part ammonia and nitrite concentration, temperature, oxygen, and pH (Hochheimer and Wheaton, 1998; Lekang, 2007). More information will be presented further on the topic of biofilter starting and monitorization.

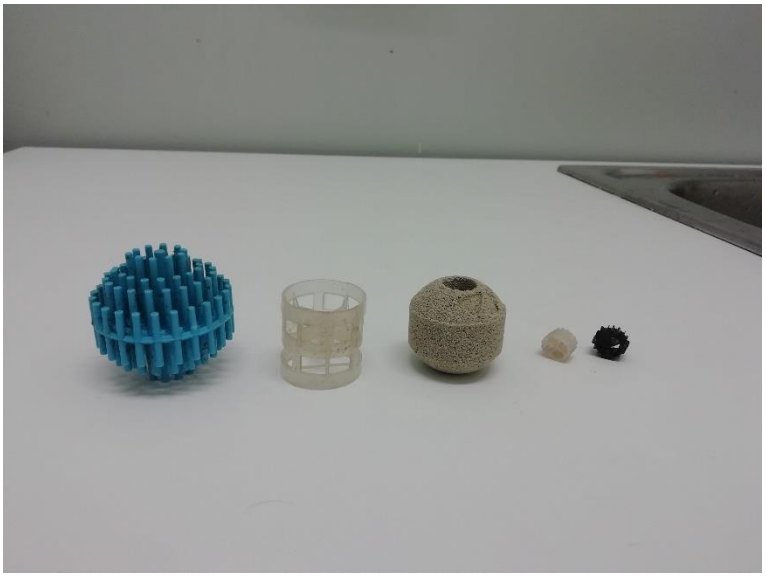

Figure 12 - Different types of filter media (“Bioballs”) used in BOGA.

Biofilters can be broadly characterized as: fixed film, also known as attached growth, that rely on a substratum for the bacteria to proliferate; and suspended growth, which consists in maintaining the bacterial culture in suspension (Gutierrez-Wing and Malone,

2006). The latter category is common in wastewater treatment facilities, yet they are not very used in aquaculture since they require a higher level of management and the general aquaculture community considers them to be unstable and more frequently related to poor water quality. Therefore, RAS biological filtration usually relies on fixed film filter systems (Gutierrez-Wing and Malone, 2006).

Biofilter designs can be very diverse, however, the most popular types used in recirculating systems are: flow-through systems; rotating biofilters; moving bed bioreactors; and granular filters (Lekang, 2007).

Flow-through systems consist in having a continuous flow of water passing through the filter medium and, in general, can be broadly divided into two categories depending on the method used to make the water flow through the medium: trickling filters; and submerged systems (Lekang, 2007).

In trickling filters water runs through the filter medium by gravity in a cascading motion (fig. 14). Filter medium is packed into a container placed above the water level that ideally allows for an even distribution of water (Lekang, 2007). This design helps to maximize oxygen transfer and aid in removal of carbon dioxide through the water trickling down the filters in addition to the base aeration of the system (Malone and Pfeiffer, 2006). Trickling filters are popular in aquaculture as well as wastewater treatment systems, offering advantages such as cheap installation, low maintenance requirements and high tolerance to hydraulic and organic load variation.

As in other designs, one of the most important aspects is choosing adequate filter medium. Important factors to take into consideration when choosing filter medium include: void ratio, defined as the volume occupied by air after filling a container with filter medium divided by the total filter volume. Lower void ratios can improve contact time between the water and established biofilm on one hand, but on the other hand can also result in easier clogging and reduced water flow and air transport; Specific surface area; Weight, lighter filter medium usually facilitates handling; Homogenous water flow, to minimize occurrence of dead zones that reduce nitrification rates; Economic availability (Lekang and Kleppe, 2000).

In submerged biofilters incoming water flows through the filter medium which is constantly submerged (fig. 13). Depending on the orientation that the water is circulated submerged filters may be characterized as up-flowing or down-flowing. These filters rely almost exclusively on the aeration of the system to keep oxygen available for the nitrifying process. These designs offer good conditions in terms of filter medium, allowing for a good distribution of water through the total volume of the filter, consequently enhancing the contact of water with the medium where the biofilm develops. In general, submerged biofilters are inexpensive and offer positive results, however, some common disadvantages can be singled out such as their dependence on a good system aeration and higher risk of clogging in systems with a heavy organic load (Malone and Pfeiffer, 2006; Lekang, 2007).

Figure 13 - Submerged flow-through biofilter in BOGA (down-flowing).

Figure 14 - Trickling filters - biofilter activation systems in BOGA.

Rotating biofilters, generally known as biodrums or rotating biological contactors (RBC), consist of biofilter medium mounted on a shaft which rotates partially above water and partially bellow water. This format combines some of the advantages of the previous types, allowing a great contact of biofilm covered surfaces with the water when it is submerged, as well as consistent aeration when the medium is above water. In general, two possible designs are used regarding filter medium: a cylinder filled with bio balls; or parallel discs providing high surface are for biofilm to develop (Lekang, 2007).

Moving bed reactors, also called fluidized bed reactors utilize floating filter medium which are maintained in suspension by an up-flowing current of water or, more frequently, by strong aeration at the bottom of the reactor (Lekang, 2007). Filter medium used consist of relatively small elements with a slightly lower density than that of water which are easy to keep fluidized. Various materials can be used as substratum, ranging from polyethylene

or styrene bead types (1 to 4 mm) to larger plastic bio ball types (6 to 13 mm). Although sizes vary amongst types of medium, in general, most provide high surface area for biofilm growth and their constant movement in the fluidized state allows for abrasion between individual filter elements. This helps in removing old biofilm, keeping the more efficient, thin, fresh biofilm and makes the filter less prone to clogging, given there is decent mechanical filtration. These types of filters have relatively low assembly costs and operation requirements while also significantly contributing to the aeration and degassing of the systems they are used in (Lekang, 2007; Malone, 2013).

Lastly, granular filters are filters used for clarification which have also have suitable conditions to perform nitrification, frequently working as both a mechanical filter and a biological filter. Medium used is variable, but similar to fluidized systems it consists of small elements which provide a large surface area for biofilm development and filters can be designed with up-flowing or down-flowing water. Two of the most commonly used granular filters in aquaculture systems are bead filters and sand filters (fig. 15 and 16). Bead filters use floating plastic pellets much like those used for fluidized systems coupled with water flowing in an upward motion, trapping solids on a mostly static bed of beads. Sand filters are mostly designed to work with down-flowing water and possess a filtering layer composed of sand, with sizes ranging from 0.2 to 2 mm, deposited on a gravel support. Although Granular filters have the advantage of performing two types of filtration simultaneously, nitrification rates are not as effective as other biofilters and they must be frequently backwashed to remove waste particles (Lekang, 2007; de Oliveira, 2019).

Figure 15 - Sand filter in aquaculture room p.20.

Figure 16 - Sand filter in stocking room p.09.

5.4- Aeration/Oxygenation

As oxygen is vital for all aerobic reactions present in aquatic systems, its management is very important. In intensive conditions, a complete failure of the oxygenation system can result in high mortality of the cultured organisms in approximately half an hour (Losordo et al., 1992). Oxygen can be provided to the systems essentially in two ways, by aeration or by injection of pure oxygen (oxygenation), most established productions use at least aeration or a combination of both aeration and oxygenation. Having a stable method of maintaining optimal oxygen levels and degassing is especially helpful for recirculating systems as it increases the efficiency of water re-use (Lekang, 2007).

Gases can be dissolved in water and their concentration is usually referred to in terms of saturation. Water is fully saturated (100%) when the maximum amount of gas possible under normal atmospheric conditions is dissolved in it. This is also known as the state of equilibrium, when no more natural exchanges between the air and the water occur. Undersaturated water (<100%) contains less gas than what it can take, and oversaturated water (>100%) contains more gas than when it is fully saturated. Actual concentrations of a given saturation may vary according to water temperature (Lekang, 2007). Dissolved oxygen concentration in warm water recirculating systems tends to range between 5 to 6 parts per million, while in cool water systems ideally operate with levels above 8 parts per million (Malone, 2013).

The main purpose of aeration is increasing the concentration of dissolved oxygen by providing a large contact area between the water and the air. In doing so, the use of aerators aims to create conditions as close as possible to equilibrium between the gases in the air and in the water. In aquatic systems this means that generally oversaturated gases such as nitrogen and carbon dioxide will progressively leave the water, and undersaturated oxygen will be dissolved into the water. Thus, aeration also has an important role in degassing (Lekang, 2007). Although the build-up of these gases can be problematic, generally, they do not post immediate threats of toxicity in systems with sufficient dissolved oxygen (Losordo et al., 1992).

Aerators are designed to allow gas transfer either by supplying a flow of air into the water or by allowing water to pass through a layer of air. Gravity aerators follow the latter method and the two most common designs are packed column and cascade aerators. In both types water is transported by gravity and create conditions of high surface area contact between the water and the air, in packed columns by trickling down plastic media, much like trickling filters, and in cascade aerators by flowing through perforated plates or trays stacked upon each other (Lekang, 2007).

Supplying air to the water through blown air is a more basic and common method of aeration for most systems. Generally, air is forced through porous material (e.g. air stones) (fig.17) or diffuser hoses to create a fine stream of bubbles with high surface are for gas exchanges. These porous materials are prone to clogging and must be periodically maintained to work efficiently. Airlift pumps can also serve as aerators, but the use of air diffusers is more widespread due to the simplistic application and management, especially for smaller scaled systems (Malone, 2013).

Figure 17 - Example of air stone used in BOGA.

Injection of pure oxygen is generally the best way to increase the saturation of oxygen above 100%, if such is desired, since it is not possible to surpass the amount of oxygen at equilibrium levels with regular aeration (Lekang, 2007). Thus, pure oxygen systems are effective in keeping high oxygen concentrations and depending on the scale and costs of oxygen it can be effectively applied. Some productions also use them as backup in combination with aeration (Malone, 2013).

In BOGA most aeration is performed using air stones. All culture rooms possess air distribution tubes that can be connected to air diffusers. The predominant use of trickling filters also partly contributes to aeration. Users in BOGA generally measure oxygen levels with probes belonging to the individual research teams or with probes present in BOGA's sampling room.

5.5- Water disinfection

Disease spread is a common risk and concern for all aquatic systems as it can severely affect the health of cultured organisms and thus limit the development of aquaculture. The main source of diseases tends to be pathogenic microorganisms such as bacteria, fungi, viruses, or parasitic agents (Lekang, 2007).

Pathogens can enter the systems through various ways: most commonly with water introduced in the system; transmitted amongst cultured animals; with the food; and by vectors or fomites. Although aquaculture facilities apply biosecurity measures to reduce de risk of pathogen introduction (e.g. quarantines; footbaths; sanitation; etc), it usually is not enough to completely avoid their introduction to some degree (Yanong, 2003). Poor water conditions, especially the presence fine particulate organic matter can also create favourable environments for pathogens to proliferate (Gullian et al., 2012).

Applying water disinfection methods in culture systems serves the purpose of reducing the presence of these pathogens to tolerable levels thus lowering the risk of disease outbreaks (Lekang, 2007). This is particularly important for recirculating systems due to their high degrees of water re-use and which tends to keep pathogens in the system and can lead to problems with asymptomatic carriers and frequent re-exposure to infections (Gonçalves and Gagnon, 2011). The presence of dynamic populations of nitrifying bacteria associated to most systems in RAS also adds additional cares with disease and disinfection management. For instance, heterotrophic bacteria present due to poor water conditions proliferate at a much faster rate than autotrophic bacteria in biofilters, competing with them for oxygen and possibly reducing the efficiency of nitrification (Michaud et al., 2006).

Disinfection methods can be broadly categorized as physical or chemical, the two most commonly used being ultraviolet lights and ozone. Generally, inlet water is always disinfected as common procedure, but the outlet water can also be disinfected to avoid possible contamination of the body of water it will be discharged into. In RAS, generally, water is also disinfected before it is re-distributed to the culture tanks (Lekang, 2007).

5.5.1- Ultraviolet lights (UV)

The utilization of UV lights is a physical method useful for preventing the action of mainly bacteria, viruses, and fungi. UV disinfection can successfully reduce heterotrophic bacteria in the system which also increases oxygen available for nitrifying bacteria, lowering the risk of disease. Although ultraviolet light comprises radiation with a wavelength range between 1 and 400nm, UVC lights with wavelengths between 240 and 280nm are

considered to have the best germicidal action. The UV radiation acts by damaging the genetic material of microorganisms, causing their inactivation or death (Lekang, 2007; Gullian et al., 2012).

Lamps can be designed to be placed outside the water, irradiating light above the surface, or inside the water. The latter format is more common, usually lamps are enclosed in chambers through which water flows through in close contact (fig.18). The degree of effectiveness of this method can be limited by factors such as lamp intensity, age and surface cleanliness, therefore proper choice and management of lamps is important. For instance, lamps lose efficiency as time passes, thus they should ideally be replaced at least once a year. Pipes should be cleaned regularly as fouling may still occur (Lekang, 2007).

UV disinfection also depends on the distance between the lamp and the target organisms, and it is known that turbidity negatively affects their efficiency. Accumulated dissolved organic matter (DOM) or suspended solids can lower the UVC transmittance, providing bacteria with protection from the radiation. Therefore, it is important to have an efficient way of removing particles prior to UV disinfection, especially in systems with high culture densities and water re-use (Gullian et al., 2012; Lakeh et al., 2013).

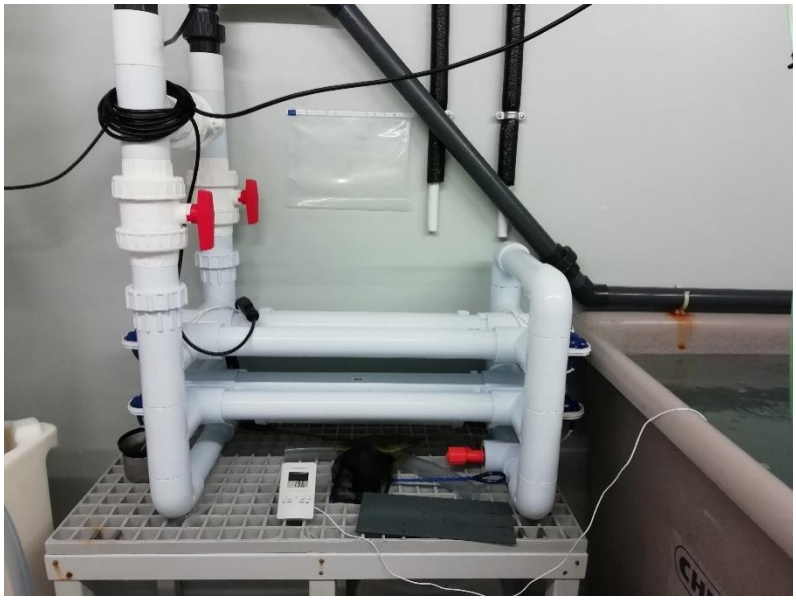

Figure 18 - UV disinfection lights used in BOGA.

5.5.2- Ozone

Ozone is applied in aquaculture as an efficient means to eliminate most pathogens commonly present in established aquacultures. Ozone is also capable of oxidizing organic and nitrogen waste, improving overall water quality while also creating favourable conditions for more oxygen availability to the nitrifying process (Sharrer and Summerfelt, 2007).

The ozone molecule is very unstable and decomposes quickly after its injection in the water, thus there is a need to produce ozone at the facilities with the aid of generators. It is extremely toxic to all lifeforms and acts by damaging cell membranes and nucleic acids. Taking this into consideration, ozone can also be harmful to both cultured organisms and operators due to overdosage, unwanted by-products or residual ozone. For instance, when seawater is used ozone reacts with bromide ions producing by-products such as bromate and bromoform regarded as cancerogenic substances with may affect the overall health condition of organisms (Lekang, 2007; Gonçalves and Gagnon, 2011).

Similar to how other gases are included in water, ozone has to be introduced with a method that ensures good mixing between the gas and water (e.g. venturi). Thus, ozone disinfection systems are composed of two main parts: a generator (fig.16) and an injection system (Lekang, 2007). Designs are variable, some of them also being used as aerators, however, most take into consideration common factors such as transfer efficiency, leak security and ozone resistant materials (Rakness, 2011).

Ozone may be administered as a single batch or a series of injection events per day, or it may be introduced continuously. Usually the latter method is preferred due to the additional benefits of water clarification by oxidizing various types of organic compounds and particles. Batch injection can also appropriate to deal with systems with moderate feeding events and reducing costs of ozone disinfection (Gonçalves and Gagnon, 2011). Treatment doses are variable amongst systems and are usually calculated according to daily feeding rates (King et al., 2001).

The most common method used for monitoring ozone activity in aquaculture systems is by measuring the redox potential which varies with the amount of ozone in the water. It is measured in mV and normal values found in aquatic systems are usually between 100 and 300 (Lekang, 2007). This type of probe is used in the aquaculture room p.20 of BOGA and redox potential values can be read in an associated display monitor (fig. 19).

Figure 19 - Ozone generator and the display monitor of the redox potential and temperature probe.

5.6- Water quality monitoring

The benefit of controlling most factors influencing the culture conditions that RAS provides comes with a higher degree of management requirements. Animals in closed systems depend completely on the quality of water maintained by the operators, and the failure of almost any component in RAS can quickly compromise the health conditions of cultured animals and potentially result in high mortality. Therefore, constant monitoring is vital for successive intensive systems. In general, the parameters that are given most importance in water quality monitorization include temperature, pH, salinity, dissolved oxygen, ammonia, and nitrite for their direct impact on animal health, feed intake, growth rate and carrying capacities (Fowler et al., 1994). These coincide with the parameters that can be registered on the system score sheet in experimental rooms in BOGA. Dissolved oxygen (see 5.4) and nitrogenous compounds (see 5.3 and 6.) were explored to some extent in previous chapters of this report.

5.6.1- Temperature

Depending on the cultured species, temperature in the system must be maintained within its respective optimum interval (Masser et al., 1999). Chronic exposure to temperatures outside tolerable range can lead to higher susceptibility to disease and reduced growth (Simbeye and Yang, 2014). Its manipulation can be very useful in aquaculture due to the influence it has on aspects such as feed intake and growth. Within the optimum temperature range of a species, in general, growth rates increase with increasing temperature. Coupled with the effect it has on gonadal maturation, temperature may be manipulated to speed up the development process of a fish and get it to mature or spawn within a desired period (Van Ham et al., 2003; Ranjan et a., 2019).

It also important to take into consideration that temperature affects other parameter such as dissolved oxygen as well as ammonia toxicity. With rising temperature less dissolved oxygen in present and water and ammonia toxicity is higher (Hargreaves and Tucker, 2004; Simbeye and Yang, 2014). Abrupt variations in the water temperature should be avoid as they can interfere with physiological processes of the organisms in the system (e.g. culture stock, nitrifying bacteria). Water may have to be heated or cooled depending on the ambience temperature of inlet water. Methods to raise the water temperature include submersible heaters, oil or gas burners and heat pumps. To decrease the temperature of water coolers are usually used (Lekang, 2007). In BOGA, submersible heaters and coolers are commonly used to manipulate water temperature (fig. 20 and 21).

Figure 20 - Submersible heater used in BOGA.

Figure 21 - Water coolers used in BOGA.

5.6.2- pH

The pH of a solution is used to measure to which degree it is acid or basic. Values range from 0 to 14. Lower values are associated with increasing acidity, whereas higher values indicate the solution is more alkaline. When pH is close to 7 it represents neutrality (Lekang, 2007). An adequate management of pH is vital for the proper functioning of recirculating systems due to its effects on other parameters and on the biological processes of the various organisms in the system (Losordo et al., 1992).

Negative effects on fish health can be caused either if values are too low or too high. Problems related to low pH include damage to tissues (i.e. gills, skin and eyes), reduced growth, higher solubility of metal ions and osmotic problems (Lekang, 2007). Higher pH can raise the toxicity of TAN as mentioned previously by increasing the proportion of non-ionized ammonia. It is also important to take into consideration that the activity of nitrifying bacteria can be greatly reduced at lower pH (Loyless and Malone,1997). Optimum pH can vary amongst species but in general it is maintained between 6.5 and 9 in aquaculture facilities (Lekang, 2007).

Alkalinity, expressed in mg/L as $CaCO_3$, is used to measure the water's capacity to neutralize acids which, consequently, is important to maintain stables values of pH. Carbon dioxide present due to biological activity reacts with water to form carbonic acid and free hydrogen ions which will consume alkalinity and progressively reduce pH, especially in RAS. To counteract the rise in acids, water needs free bicarbonate and carbonate as bases to react with acids and serve as sources for alkalinity. Therefore, recirculating systems often have to rely on the addition of buffers to provide carbon to the water and help to stabilize pH within desirable ranges. Calcium carbonate and sodium bicarbonate are the most commonly used buffers, but other substances such as lime, calcium hydroxide, calcium oxide, and sodium hydroxide can also be used (Losordo et al., 1992; Masser et al., 1999; Lekang, 2007). In general, pH is measured with probes. As mentioned before, in BOGA a pH probe can be found in the sampling room.

5.6.3- Salinity

Salinity is usually measured in ppt (parts per thousand, ‰), although other units are also used (e.g. ppm). Salinity in water represents the presence of dissolved salts, the two most common being chloride (Cl^-) and sodium (Na^+). The natural salinity of seawater is 35 ppt and freshwater salinity should be close to 0 ppt. As with other parameters it is known to have an effect on feed intake and growth rates. Various marine species (e.g. Atlantic cod,

turbot) exhibit better conversion rates, feed intake and/ or growth rates in more brackish salinities (e.g. 12 to 19 ppt). Additionally, certain salinity levels may be optimum to help reduce energy spent in osmoregulation (Boeuf and Payan, 2001; Resley et al., 2006). Although the manipulation of salinity is relatively easy and can be useful, it should be taken into consideration that it can also influence other factors, as mentioned before. For instance, efficiency of foam fractionation is less effective in lower salinities. Conversely nitrification is less effective in saltwater due to the presence of chloride ions which affect biofilm growth (Lekang, 2007). The three most common ways to measure salinity are through probes, refractometers and by measuring conductivity, all of which are used in BOGA.

6- Biofilter starting and monitorization

While collaborating with the BOGA staff my main responsibility was the monitorization of their biological filter activation systems. Additionally, I had the opportunity to follow the setup and activation of a new saltwater biofilter system. Biofilters managed by BOGA fall under the category of fixed film trickling filters, and filter medium used are, generally, three-dimensional plastic structures commonly known as “bio balls”, with some instances where ceramic structures have been used.

Starting a biofilter consists in providing a system with nitrifying bacteria, a suitable medium for them to colonize, a source of nitrogen compounds and adequate water quality, while monitoring the growth and efficiency of the biofilm through water quality measurements. This process may also be referred to as biofilter activation.

One method that may be used for starting a biofilter is known as the cold start method in which cultured organisms are added to the system with non-activated biofilter. Taking advantage of bacteria introduced with the animals that are already acclimated to the culture conditions from where they originated and making use of their waste as a nitrogenous source. This method, however, is generally not optimal for the animals and operators since the bacterial colonies tend to take more time to settle and stabilize, resulting in a period where ammonia and nitrite concentrations can rise to high levels. Such conditions can create very stressful environments for the animals and operators will have to perform water exchanges more frequently to maintain good water quality (DeLong and Losordo, 2012).

Another method, usually preferred by systems managers, is developing the biofilm in the filter prior to the stocking of organisms. This way, stress to newly added animals is reduced since the nitrifying bacteria colonies are well developed and can rapidly oxidize the high levels of ammonia and nitrite. This method relies on naturally occurring bacteria in water, but bacterial preparations can also be used. These mixes of nitrifying bacteria are not essential, nevertheless, they can help reduce the total time needed to establish the biofilter (DeLong and Losordo, 2012).

When setting up a system for biofilter activation, the management of the various factors that influence the growth of nitrifying bacteria must be taken into consideration. Newly added water needs to be free of chlorine and parameters must be adjusted according to the characteristics of the system where the biofilters will be used, and must be monitored frequently (ideally on a day to day basis to better evaluate the state of the activation process and single flaws in the system). The most important parameters that should be measured are ammonia, nitrite, pH, and salinity. Considering there are no animals in the system,

substances such as ammonium hydroxide, ammonium chloride or regular household ammonia (unscented) can be used as nitrogenous compound sources for bacteria. Ammonia concentrations for biofilter activation should range between 3 and 5 mg/L, doses should be calculated according to the ammonia source used and/or the daily feed intake of the system they will be used in. (DeLong and Losordo, 2012). Excessively high levels of ammonia may have a negative effect on biofilm development (Lekang, 2007).

Since alkalinity is progressively reduced due to acids produced by the nitrification process, a buffer solution needs to be added as a source of carbon ($CaCO_3$) to adjust alkalinity and to help stabilize pH. Substances which can be used for buffer solutions in recirculating systems include calcium carbonate, sodium bicarbonate or sodium hydroxide. Some substances may create stronger buffers (sodium hydroxide) which cause more abrupt increases in pH and may stress nitrifying bacteria. Therefore, operators usually prefer substances with lighter effects on pH variation (e.g. sodium bicarbonate, calcium carbonate). Nitrifying bacteria are, generally, more efficient at pH levels between 7.5 and 9.0. Nonetheless, pH values closer to 7.0 can be sufficient to deal with ammonia concentrations usually found in aquatic systems. Additionally, a lower pH has the benefit of reducing the toxicity of ammonia that will be present in stocked systems (Hochheimer and Wheaton, 1998). It is important to take into consideration that nitrification rates can decrease drastically at pH values lower than 7.0 (Lekang, 2007).

Sudden variations in salinity can also affect nitrification, thus it should be maintained as constant as possible according to the salinity of the system they will be used in, and deliberate changes should be performed gradually (Hochheimer and Wheaton, 1998). Temperature must also be in accordance with the receiving system. Generally, bacterial activity can occur between 0 °C and 30 °C and increases with higher temperatures. Nitrification is usually more effective at temperatures above 20 °C, however bacteria can also acclimate to lower temperatures and achieve efficient nitrification rates. It is worth noting that at temperatures below 5 °C growth can be extremely slow and at temperature close to 30 °C the risk of mortality is higher (Hochheimer and Wheaton, 1998; Lekang, 2007). Dissolved oxygen is crucial for the nitrification process, bacterial activity can be negatively affected at levels below 4 mg O_2/L for *Nitrosomonas*, and below 2 mg O_2/L for *Nitrobacter*, thus concentrations should always be maintained above 4 mg O_2/L (Haug and McCarty, 1972). This usually is not a problem for RAS since abundant aeration is frequently associated to the systems (Lekang, 2007).

6.1- Biofilter systems in BOGA

There are two types of trickling filter systems under the BOGA staff management and similar activation method is applied to both: saltwater or freshwater, depending on the system, is circulated through plastic bio balls. Ammonia is provided through the administration of ammonium chloride (NH_4Cl) as “feed” to stimulate the growth of natural bacteria in the water.

The main difference between both types is the supporting structure of the filter medium and its layout. The first type uses stackable plastic boxes with perforated bases filled with bio balls and one or two pumps that transport the water from the sump, where the boxes are staked, to the topmost box, making the water flow through all the boxes and filter medium until it falls back into the sump.

The second type uses the same concept but a different design, the bio balls are inside individual cylindrical structures (TMC trickle bio-tower), instead of stacked boxes, and placed on top of a supporting grid above the sump. Several pumps then circulate the water between the sump and the bio-towers. The towers have a spraying mechanism that ensures an even distribution of water.

In the bioterium there are 3 systems dedicated to saltwater biofilters: saltwater biofilter 1 (340L), which uses the second type of design (fig. 23); and saltwater biofilters 2 and 3 (180L), which use the first type of design. Only one system is dedicated to freshwater biofilters (180L) since their demand tends to be lower (fig. 22). This freshwater system uses the first type of design as well.

Figure 22 - Freshwater biofilter activation system.

Figure 23 - Saltwater biofilter activation system 1.

6.1.1- Biofilter maintenance

All systems generally have the same maintenance requirements: adding ammonia to continue to stimulate the bacterial activity; adding a buffer solution to counterbalance the natural decrease of pH that results from the oxidation of ammonia by providing a source of carbon; and performing water exchanges to maintain desirable salinity levels.

Routinely, generally in the morning, water samples are taken from each biofilter system. Each system has their own sampling containers which are then taken to the sampling room where parameters are measured. For ammonia and nitrate Palintest testing kits are used, in which specific reagents are added to a water sample in a test tube and subsequently are measured in a photometer after a period of 10 minutes. Salinity, pH, and temperature are measured using the probes in the sampling room. Maintenance is then performed according to the needs of each system.

Ammonium chloride (NH_4Cl) must be added when ammonia or nitrate concentrations are low as to keep nitrogen compounds available for bacteria. Usually it is introduced when their levels drop below 1 mg/L. A set dose of 15g (in 180L for the smaller systems and in 340L for saltwater biofilter 1) of ammonium chloride is added by dissolving it in system water and introducing it in a recipient with a tube and a valve that is regulated to make the solution drip into the sump (fig. 24). This method avoids abrupt variations of ammonia concentration as the solution is introduced drop by drop progressively into the system.

The buffer solution used to regulate pH is obtained by dissolving sodium bicarbonate (Na_2CO_3) in water. The staff favours its use over calcium carbonate (Ca_2CO_3) for its higher solubility. Associated to the biofilter systems there are two reservoirs that distribute the buffer solution to all systems: one 20 litre bucket for the freshwater system and one 60 litre reservoir for the 3 saltwater systems (fig. 25). Both are placed above the systems relying on gravity to distribute the solution and using the same valve and dripping mechanism used for introducing ammonium chloride. In accordance with the pH of the receiving biofilter activation systems, the BOGA staff determined a quantity of 150g of sodium bicarbonate per system (7,5 g/L), using 150g for the freshwater reservoir and 450g for the saltwater

reservoir. When monitoring the pH of the systems it is important to not let the values drop below 7 as it will most likely inhibit the nitrification process. Therefore, if pH is dropping rapidly one has to control the system and make sure that the buffer solution is entering the system as valves can get clogged from the precipitation of sodium bicarbonate. Valves are usually regulated as to drop the solution periodically over roughly 2 to 3 days, thus, if the reservoirs are starting to become empty, they should be refilled. If the solution is properly dripping into the systems, but pH values are still dropping, more sodium bicarbonate can be added. When in doubt pH can be measured again in the afternoon to check its values are rising to desirable levels.

Figure 24 - Ammonium chloride solution container.

Figure 25 - Saltwater (top) and freshwater (bottom) buffer solution reservoir.

Sporadically, the buffer solution reservoirs have to be disassembled and cleaned to avoid excessive precipitation of sodium bicarbonate which can result in clogging and, consequently, harsh drops in pH. Specially the saltwater reservoir has to be monitored regularly since the solubility of sodium bicarbonates is lower and it is connected to the 3 saltwater systems, potentially affecting pH values in all of them.

Salinity levels should be approximately set at 35 ppm for saltwater systems and as close to 0 ppm as possible for freshwater systems. Since salinity tends to rise naturally in these systems, due to the continuous introduction of ions, partial water exchanges must be performed to correct salinity levels. When using freshwater for these water exchanges it is important to make sure that dechlorinated water is used to secure bacterial proliferation.

Bio balls that were previously being used in active systems can be re-used after proper disinfection. This consists in submerging them in a solution of dechlorinated freshwater and bleach for 24 to 48 hours to avoid cross contaminations (fig. 26). Afterwards bio balls must be thoroughly rinsed to remove any residual bleach that could disturb the systems they are re-introduced in.

Figure 26 - Container with filter medium undergoing disinfection.

Like in all RAS systems, if any kind of water loss is observed (e.g. leaks; water droplets spraying out of the system) it must corrected as soon as possible. This avoids significant losses of the total water volume of the system that can lead to pump malfunction due to dry running. It is also important to always check if aerators are functioning correctly to ensure the systems are being properly oxygenated.

6.1.2- Saltwater biofilter 1

Shortly after the start of my collaboration with BOGA, I followed the assembly of the biofilter system 1 for its activation process. Initially, two bio-towers were added to the system, each one filled with 30 L worth of filter medium. Bio balls used were previously disinfected and bio-towers were not filled totally with them, leaving some space between the spraying mechanism and the bio balls to ensure even distribution of water is not compromised. More towers were added during the time the system was active.

The BOGA staff also used this new system as an opportunity to test a probiotic and nitrifying bacteria culture mix they had in stock (Nitribiotic, Tropic Marin) to see if it could affect the activation time of the biofilter. The bacteria were added according to the

manufacturer's instructions (an initial dose of 1 mL per 100L of water volume and, subsequently, weekly doses of 1 mL per 200L of water volume).

After the system was assembled and set in motion, a dose of 15g of ammonium chloride was added. Water parameters were measured and registered on its respective system score sheet over the course of approximately two months (i.e. salinity, pH, ammonia, nitrite). The variation of ammonia and nitrite concentrations is represented in fig. 27 (to evaluate the stage of development and applicability of the biofilter).

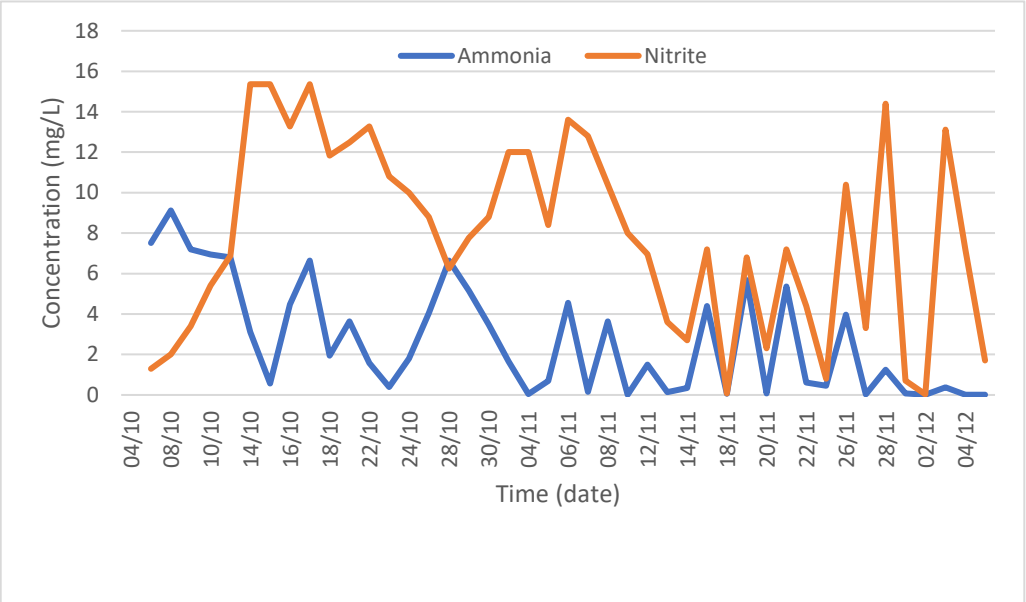

Figure 27 - Variation of ammonia and nitrite concentrations throughout the activation process of saltwater biofilter 1.

The initial spikes of ammonia and nitrite, characteristic of the activation process of a new system. Initial and subsequent spikes in the concentrations, generally, signal the moment in which bacterial colonies have developed enough biofilm to transform more nitrogenous compounds than are being created or added externally. The instances in which concentrations rapidly rise are mostly a result of the introduction of ammonium chloride doses.

In a first assessment one month after setting up the system, I could not affirm that the biofilter was ready to be used with full efficiency in active recirculating systems. Although ammonia converting bacteria showed signs of settling, the rates of ammonia and nitrite conversion were still not in accordance with desirable rates. A dose of NH_4Cl is supposed to simulate a general daily discharge of nitrogen waste in a working system. Biofilters, therefore, are expected to be able to convert these quantities over the course of one day. However, specific requirements may vary with a system's culture density and feeding rate.

During the second month, even though efficiency was still not optimal, conversion rates were significantly more stable as one can observe that both ammonia and nitrite were being transformed almost totally over the course of a few days in between NH_4Cl doses.

Additionally, I was not able make significant conclusions regarding the effect of the bacterial culture used. The starting time of the system did not follow the expected period of approximately one month as with previous systems activated in BOGA, therefore there was no apparent effect on shortening the activation process. However, I did not have a control system with the same activation conditions to make a proper comparison. Since the culture is composed of a mixture of probiotic and nitrifying bacteria it may be the case that it is not specifically design to optimize nitrifying activity. The mixture includes *Bacillus subtilis*, Nitrobacteria, Saccharomyces, Lactobacillus e purple bacteria. Amongst these, only nitrobacteria fall under the nitrifying category and the percentage they represent is not specified. It is possible that the use of this supplement is more directed to smaller systems or aquaria with more of an effect on improving overall water quality, nitrifying activity, and boosting the immune system of organisms.

6.1.3- Main difficulties and possible solutions

One of the most common problems is managing pH values considering the direct effect it has on nitrifying efficiency. As mentioned before, although sodium bicarbonate is preferred for being more soluble than calcium carbonate, it can still easily precipitate and clog the reservoir system. This happens more frequently in the valves where the solution drips into the systems since the space where the solution passes through is very small, making them more susceptible to this problem.

In addition to cleaning the reservoir and tubing, one possible solution could be to slightly heat the buffer solution in the reservoir. This may, theoretically, reduce solute precipitation as solubility increases with higher temperatures. The heated buffer solution should also not have a significant impact on the overall water temperature of the system since it is introduced drop by drop, progressively over a period of days.

There is also some uncertainty regarding the amount of bicarbonate necessary to stabilize pH, since the quantity can vary according to factors such as the stage of development of the biofilm, quantity of biofilter medium or total water volume. For instance, the quantity of 150g of sodium bicarbonate per system set by the BOGA staff, effectively maintained good pH values, with some difference in the frequency they were renew. The full saltwater reservoir would usually last 2-3 days while the freshwater reservoir could last

up to 5 days. However, when saltwater biofilter system 1 still had a low bacterial activity and the other saltwater systems had a lighter load of bio ball boxes, 150 g of sodium bicarbonate could serve all 3 systems (total water volume of 700L) for the same period of 2-3 days.

To have a better grasp on the management of sodium bicarbonate, one could resort to measuring alkalinity. According to a pH management diagram, for desirable pH values between 7.5 and 8 at 25 °C, alkalinity levels should be maintained between 100 and 200 mg as $CaCO_3$/L or higher, however, levels above 400 mg/L are not advised (Masser et al., 1999). This may give more insight on the condition of the system when pH is still in an acceptable range, but technicians are uncertain whether pH values might drop and if additional buffer solution should be added. Having a better understanding on quantities needed could also lead to less sodium bicarbonate waste.

Considering that biofilter systems always require a starting time of approximately one month before they are fully functional, methods to reduce this initial waiting time could be beneficial. Some authors suggest that the use of bacterial preparations can speed up this process. Another possible method could be raising the temperature of the systems by a few degrees (2-3 °C) since bacterial activity in enhanced at higher temperatures (DeLong and Losordo, 2012). If this were to be applied, technicians must make sure that temperature is increased slowly as to not expose bacteria to a thermal shock. When desirable efficiency is starting to be observed due to the development of the biofilm, the water temperature should then be progressively adjusted to the standard conditions of the systems they are expected to be used on. The decrease in temperature will probably result in a decrease in nitrifying efficiency as well, still, if the objective of decreasing the initial latent period is achieved, it could possibly be a viable method.

During the process of biofilter activation, there is a certain difficulty in assessing the activity of the bacterial colonies that convert nitrite after the ones that convert ammonia are well developed. This happens because at this stage, ammonia introduced trough regular doses of ammonium chloride is almost totally converted into nitrite in the period of 24 hours in between daily measurements. Since new nitrite is constantly being created it becomes somewhat uncertain how much time is needed for the bacteria to convert all the nitrite resulting from a single dose of NH_4Cl (15g). A common solution is to perform daily measurements of the nitrate concentration for a better understanding of nitrite conversion.

On this note, throughout the activation process of the saltwater biofilter system 1, I attempted to measure nitrate concentrations. Since ammonia converting bacteria had already settled, I intended to check the activity of nitrite converting bacteria which were still not able to convert all of it during the span of 1 day. However, I was not able to make any

conclusions due to the results obtained for daily nitrate levels being too similar to each other, varying between 30 and 40 mg/L. Such variations do not coincide with the expected constant increase of nitrate, with occasional decreases due to water exchanges. One explanation for these incoherent results might be that the nitrate tests currently used in BOGA are destined for measuring concentrations up to 20 mg/L. Perhaps if alternative tests with a higher range were to be applied, results might be more consistent. Another reason could be that nitrate monitoring was not executed from the start of the activation process, as it was only tried after approximately one month from the assembly of the system.

7- Collaboration with A_2S

Animal Health and Aquaculture (A_2S) is a new team led by principal investigator Benjamin Costas and formed by pre-existing members of the Nutrition and Immunobiology research team. Both belong to the Animal Nutrition and Health group of CIIMAR's Biology, Aquaculture and Seafood Quality research line that focus on nutrition, immunology, and pathology of aquatic organisms, mostly of stablished species for aquaculture.

As aquaculture becomes increasingly necessary production trends tend to adopt more intensive approaches. Such intensive environments are more prone to unsuitable farming conditions and can lead to an intense use of traditional prophylactic and therapeutical treatments such as antibiotics, possibly resulting in problems related to microbial resistance. Thus, the A_2S team takes into consideration the important aspect of alternative and sustainable management strategies and aims to explore and innovate them to improve the health conditions of farmed animals.

The team's research focus includes: sustainable strategies to improve animal health, often relying on nutritional approaches to reduce the need of non-sustainable options (Machado et al., 2018; Ramos-Pinto et al., 2019); host-pathogen interactions, a crucial component to better understand and deal with infectious diseases; and neuro-endocrine/immune interactions as a means to evaluate the effects of stressful culture conditions on the physiology of aquatic organisms (Azeredo et al., 2017). On this note, the research team performs various trials to have a fundamental understanding of the processes involved in these topics and frequently collaborates and provides services to companies (e.g. hematologic analysis).

While collaborating with A_2S my main responsibility was to provide technical assistance on the work performed by the team in BOGA. This mostly included maintenance of recirculating aquaculture systems of stock and of systems used during experimental trials, as well occasionally providing assistance during sampling.

7.1- Stock room maintenance

Aquatic organisms under the responsibility of A_2S that are not being used in ongoing trials are usually kept in stock systems. For most of the time I collaborated with A_2S these stocks were maintained in individual systems in the room p.15, sharing it with the BOGA biofilter systems. Later during the year (2020), they were transferred to a newly constructed aquaculture room. Since the stock systems, in general, need daily maintenance, the A_2S

organizes itself to have members of the team capable of operating the systems take shifts to monitor the stock room.

7.1.1- Stock room p.15

In this room the animals were distributed between a maximum of five individual systems (i.e. S1, S2, S3, S4 and S5). Each system has approximately the same design concept, using open IBC containers as tanks (1000L), stackable boxes with mechanical and biological filters and air stones for aeration. Two of the systems also had additional protein skimmers incorporated in the systems most associated with higher organic loads (i.e. S2 and S5). Two of the systems also possessed purging valves that facilitated water exchanges (i.e. S2 and S3).

Although systems were equipped with functional filtration mechanisms, their efficiency in maintaining the water quality of the tanks was not optimal, therefore more care had to be put in their monitorization. Feed administered to the tanks was frequently limited to alternate days in an effort to reduce nitrogen waste and solid accumulation. Water exchanges generally had to be performed daily and in relatively high volumes (e.g. 50-60% of total water volume). Additionally, mechanical filter material (i.e. glass wool) had to be exchanged and protein skimmer cups cleaned on a daily basis. Water parameters and other information regarding the maintenance was registered in each system's specific score sheet.

7.1.2- Stock room p.09

This room was assembled during the period I collaborated with A_2S and was designed to have various tanks and 2 sumps that can be connected or isolated, allowing the room to function as an individual system or as two separate systems. Both are equipped with most essential components that characterize recirculating systems: mechanical filters, protein skimmers, sand filters, UV lights and trickling biological filters in stackable boxes (fig.29), similar to the concept used in the tanks of the room p.15 and BOGA's biofilters. Tanks are organized in rows (fig.28) and, generally, tanks in every row function as communicating vessels.

In terms of maintenance, the systems mostly have the same requirements as room p.15 with additional care regarding sand filter maintenance and handling of UV lights. Purging valves are also present that facilitate water exchanges for each row of tanks.

Figure 28 - Tanks in stocking room p.09.

Figure 29 - RAS components in room p.09 sump – biofilters; protein skimmer; mechanical filters.

7.2- Trial 1

The first trial I participated in was part of the FICA project in collaboration with SPAROS company. The trial's objective was to test 2 experimental diets, provided by SPAROS, on gilthead seabream (*Sparus aurata*). These diets contained additives with anti-inflammatory properties and immune-stimulatory (i.e. curcumin and beta-glucans) and their effect on the fish's immune response was evaluated after a gut inflammatory stimulus. The stimulus was induced through the administration of a diet containing a chemical compound with acute inflammatory properties, also provided by SPAROS.

I performed my activities under the supervision of two A_2S researchers responsible for the trial, Diogo Peixoto and Dr. Mariana Hinzmann. Since my focus was gaining practical experience in managing these systems, my responsibilities consisted mainly of the maintenance of the system where the trial was conducted. In general, researchers participating in trials work in shifts so that optimally, depending on the maintenance requirements, only person has to perform system maintenance in a given day. In this case I was responsible for monitoring the room from Monday through Friday while Diogo and Dr. Mariana took shifts for weekends. This allowed for me a more coherent experience in managing the system on a day to day basis and also slightly lightened the workload of the responsible researchers so they could focus on other tasks to be executed in the laboratory.

The gilthead seabream specimens used for the trial were previously maintained in the stock room p.15. Therefore, fish were first transported to the aquaculture room p.20 (where the trial took place), weighted in batches for their mean weight and acclimated for

approximately 3 weeks with a control diet prior to the start of the trial. During this period, specially, it was important to carefully monitor the fish, check on their health conditions and single out individuals that may serve as possible sources of infection.

Aquaculture room p.20 (fig. 32) is generally managed and setup by the BOGA staff and subsequently monitored by researchers which have requested its use. This aquaculture room is equipped with:

- 18 tanks (600L)
- TMC filtration system (fig.31) – 2 sumps, mechanical filters, 2-3 trickling biofilter towers, protein skimmer and UV lights.
- Ozone reactor
- Temperature and redox potential probe with a display monitor
- Sand filter
- 2 pumps – one circulates the water from the pumps, through the UV lights and the culture tanks (black pump); one circulates water between the sumps and the filter components (blue pump).

For this trial, additional biological filters were associated to the systems: 2 flow-through submerged biofilters and one fluidized biofilter (fig.30).

Figure 30 - Tank in aquaculture room p.20 used as a fluidized biofilter.

Figure 31 - TMC filtration system in aquaculture room p.20.

During the acclimation period fish were fed 1.5% of their mean weight daily. This feeding regime was subsequently changed to *ad libitum* administration for the duration of the trial.

Prior to its start, researchers randomly attributed the test and control diets to the 12 tanks (i.e. 6 control, 3 curcumin, and 3 beta-glucans). The respective diet for each tank was weighted at the beginning of the trial and after each maintenance to monitor their daily feed intake and posterior feed conversion ratio (FCR). When administrating any type of feeding regime, operators must make sure, whenever possible, that no un-eaten feed in left at the bottom of the tanks as it can contribute to poor water quality. This is especially important when feeding *ad libitum* in trials where feed conversion ratio has to be determined since non-ingested pellets cannot be accounted for, possibly leading to errors in FCR calculation.

The experimental diet feeding period lasted for approximately one month after which the first sampling event took place (T_0). Following the sampling T_0, the tanks were fed with the inflammatory inducing diet for one week until the second sampling event (T_1).

Maintenance requirements for the room p. 20 are listed in Annex I.

Figure 32 - Aquaculture room p.20.

7.2.1- Sampling

As mentioned before, this trial included 2 sampling events. In both instances 3 fish per tank were sampled for various laboratory analysis (i.e. haematology, gene expression, gut microbiome). Preparations such as labelling sampling containers, reagents and equipment checklists, and availability of members must be made in advance for the purpose of organization. Since these were the first sampling events I participated in, my contribution consisted mainly in collecting, euthanizing, and weighting the sampled fishes, although a I also had the opportunity to practice some blood sampling.

7.3- Trial 2

The following trial I participated in was part of the master's thesis of fellow colleague André Cunha, which tested whether an Ygeia+ diet had potential in improving the efficiency of vaccination in European seabass (*Dicentrarchus labrax*). Specimens were vaccinated in 2 different subsequent administrations, according to the duration of the period that each tank was fed with their respective dietary treatment (i.e. control diet 5 days; control diet 10 days; Ygeia+ 5 days; Ygeia+ 10 days).

This trial was also executed in the aquaculture room p.20, and mostly followed the same maintenance protocol applied to the previous trial. European seabass specimens were initially transported from the quarantine tank to the room p.20 and distributed through 8 tanks. Fish were individually weighted and fed 2% of their mean body weight with a control diet throughout the acclimation period. During this time the fish were expected to reach a mean body weight of approximately 15 g to begin the trial due to ideal body weight requirements for vaccine administration. After starting the trial, fish in each tank were fed ad libitum with their respective dietary treatment twice a day and their daily feed intake was registered.

After the administration of the vaccines, specimens were further separated into tanks according to their vaccination method and were fed with control diet for an additional period of 3 weeks. At the end of said period, specimens were sampled and submitted to a bacterial challenge to further compare and evaluate the effect on vaccine efficiency.

7.3.1- Vaccination

Vaccines were administered by intraperitoneal injection (fig. 33) according to their treatment, either being vaccinated after 5 or after 10 days of feeding with control or Ygeia+ diets. Half of the fish in every tank was vaccinated with a commercial vaccine (Hipra) (fig. 34) and the other half was administered a sham treatment with vaccine vehicle. Fish were then divided and distributed through the tanks making for a total of 16 tanks.

Figure 33 - Vaccination of European seabass (*Dicentrarchus labrax*) specimen.

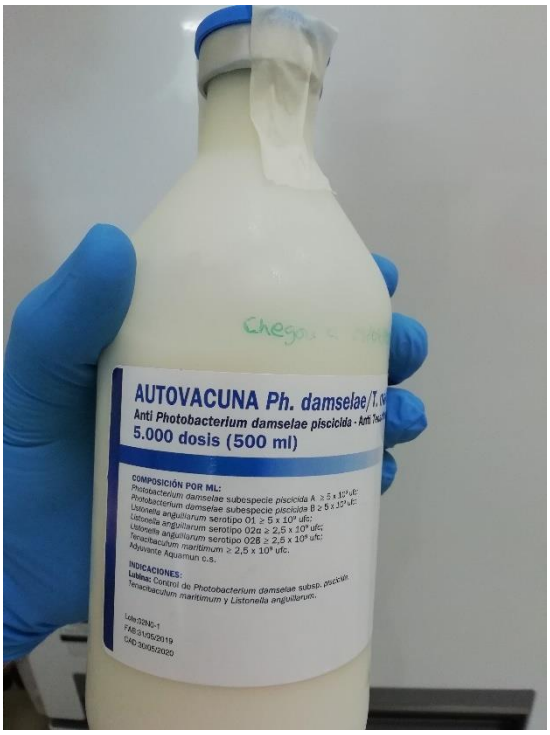

Figure 34 - Vaccine used for trial 2.

7.3.2- Sampling

Sampling events occurred twice, both were executed after 3 weeks of control diet feeding ended for each group of tanks treated with either 5 days of control or Ygeia+ or 10 days of control or Ygeia+ diets. Four or five specimens were sampled per tank for a total of 9 fish per treatment. Sampled tissues included gut, liver, head-kidney, and blood for RNA and haematological analysis. Fish which were not sampled were transferred to room p.14 for the infection challenge.

In the first sampling event I mainly aided in collecting, euthanizing, and weighting sampled specimens and in transporting the fish destined to the challenge room. In the second sampling event, however, I had the opportunity to practice some blood sampling and determination of haematocrit.

7.3.3- Challenge rooms

The bioterium possesses specific rooms reserved for trials that include exposing organisms to challenges, especially when infections are involved. These rooms are p.13 and p.14 and are isolated and accessible through a shared hall (fig. 35). To enter the hall,

as well as each room, users must disinfect their footwear in foot baths. Operators must use footwear and a lab coat exclusive to these rooms to when performing maintenance.

The hall contains a storage closet with various equipment and materials belonging to the A_2S team such as pumps, tubing, air stones, test kits, probes, etc. These are the ammonia and nitrogen test kits, oxyguard probe and refractometer used to measure water parameters in the stock room. Later during the year two additional oxyguard probes were acquired to be exclusively used for the challenge rooms.

Since these rooms are frequently used for infections, water originating from them cannot be conventionally discarded as in regular systems in other rooms. Therefore, water leaving the systems drains into a shared reservoir associated to both rooms and passes through an additional UV disinfecting station. This is managed through the electric board and a monitor that displays the state of the reservoir (fig. 36), both found in the p.13/p.14 hall. Through the board the operator can turn the monitor on and control the circulation of the water in the reservoir through the UV lights and its discard. The monitor has an alarm system that rings when the reservoir is reaching its maximum capacity, notifying any operators near the room. As general practice when there are undergoing trials with infections, operators leave the water in reservoir recirculating through the UV lights overnight at the end of maintenance and discard it at the beginning of the next day. During my internship work was being undergone to add an activated carbon filtering component to the hall's reservoir.

Figure 35 - Challenge room p.13/p.14 hall.

Figure 36 - Challenge room hall electric board and reservoir capacity display monitor.

7.3.3.1- Challenge room p.14

This room is composed of two rows of aquaria and sumps and can be connected or isolated by a valve at will (fig. 38). Each row contains 16 individual aquariums that can be further isolated to function as two separate systems with 8 aquaria each. Every block of 8 aquariums has a specific temperature probe with a display monitor and is connected to a sump (fig. 37) which comprises mechanical filters, protein skimmers, UV lights (fig. 39) and enough space to include biofilters if necessary. Pumps distribute the water from the sump to the aquariums which subsequently overflows through a central tube, being transported through gravity back into the filtration components of the sump. The pumps are equipped with monitors which enable the regulation of the flow rates. Electric components are situated above the level of the tanks for safety, the same is true for all experimental rooms in BOGA. To avoid confusing the electric plugs, the A_2S team has identified them with tape with different colours for each component (fig. 40 and 41).

European seabass specimens used in this trial were transported to this room for the bacterial challenge. During this period the fish were initially exposed to *Photobacterium damselae piscicida* through intraperitoneal injection and the system was monitored daily by me or André to collect, weight and register any deceased specimens. Maintenance requirements during this period were very reduced since fish in most tanks would consume a very small amount of feed, if any at all.

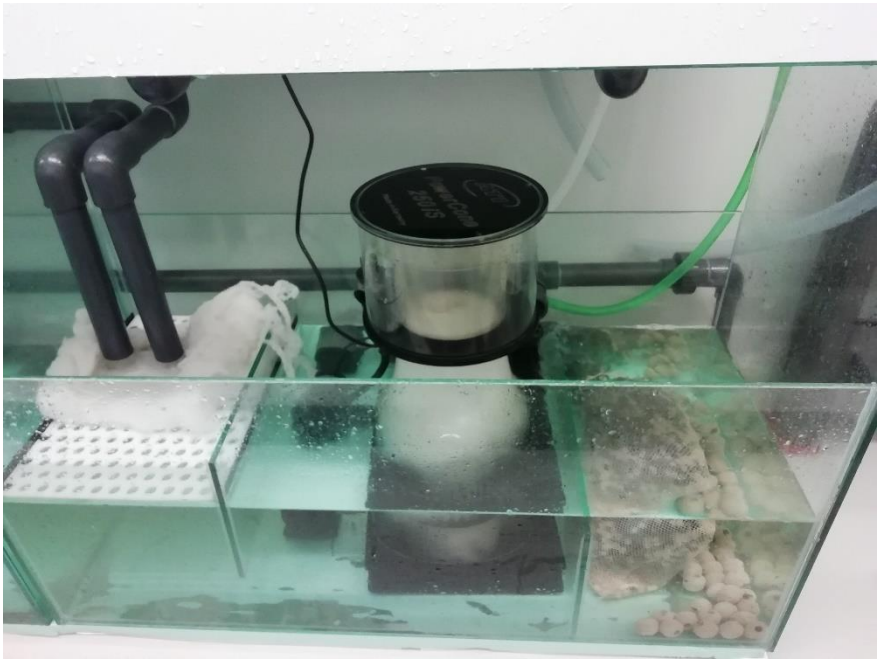

Figure 37 - Sump components in challenge room p.14: mechanical filter (left), protein skimmer (centre), biofilters (right).

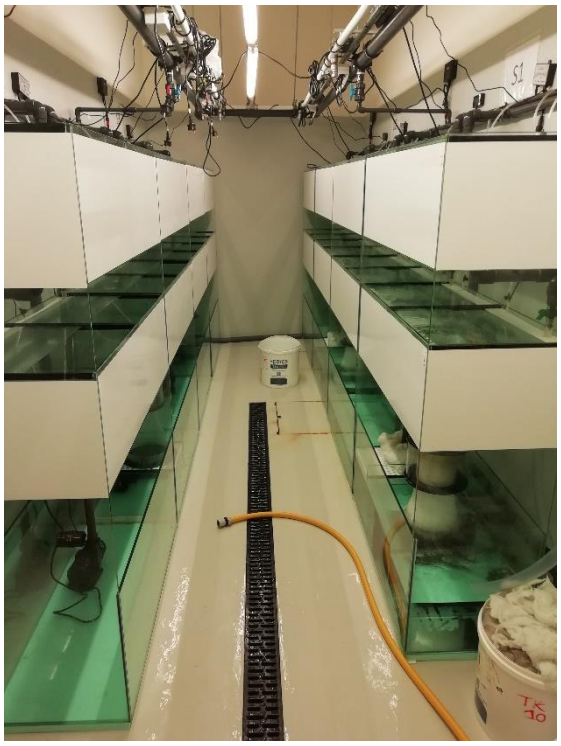

Figure 38 - Challenge room p.14.

Figure 39 - UV lights in challenge room p.14.

Figure 40 - Electric plugs in challenge room p.14.

Figure 41 - Identification code for the electric plugs in challenge room p.14.

8- Conclusion

The various components of recirculating systems enable the control and manipulation of most factors influencing their performance and, therefore, makes RAS application highly adaptable and increasingly popular. This is particularly useful for conducting research, as systems can be modelled according to the specific needs of the cultured species or the required experimental design.

Dividing the internship into a first contact with the technical management of an aquatic animal experimentation facility followed by the practical experience in activities related to a specific research team was very insightful for getting a wider understanding of the work performed in the bioterium through the point of view of both the BOGA staff and the regular users.

During my collaboration with BOGA I had the opportunity to accustom myself to the management of recirculating systems, especially through the monitorization of the biofilter activation systems. The knowledge and experience acquired during this period was very useful for a fundamental understanding of RAS components, water parameters and how they can influence each other, as well as the importance of proper monitorization to ensure their optimum performance.

The collaboration with A_2S served as a good complement to the activities I performed with BOGA, as I was able to apply some of the knowledge gained previously to manage active recirculating systems with live animals. Since most technical work was in the context of experimental trials, I also had the opportunity to experience some of the common procedures and difficulties encountered when dealing with recirculating systems used for research purposes.

Lastly, the component of haematology techniques gave me an insight on common laboratorial processing of samples which may be used in aquaculture facilities, or provided to them as a service, as is the case with the A_2S team.

Annex I

Protocols for standard procedures in BOGA

List of supplementary figures

Ammonia and nitrite measuring

1. Water needs to be sampled from the system to be measure.
2. Create three subsamples (10 mL test tubes), one will serve as a blank and the other two will be used to measure ammonia and nitrite levels (fig. S2).
3. For the ammonia testing two reagents are used (fig. S1). First the ammonia tablet nº1 is introduced, crushed, and dissolved inside the tube. Afterwards the same steps are repeated with the ammonia tablet nº2 in the same tube.
    3.1. If the sample consists of saltwater or brackish water, an ammonia conditioning reagent must be added before crushing the tablets to prevent the precipitation of salts. The amount of reagent necessary will vary with the salinity of the sample (fig. S4 and S5).
4. For the nitrite testing tube, only one reagent is necessary (fig. S3). The nitricol tablet is crushed and dissolved in a similar way as the ammonia tablets.
5. Both test tubes are then left to react for 10 minutes. If the temperature of the water sample is below 20°C they can be left for 15 minutes since the reaction time may be slower.
    5.1. During this time the colour of the sample will develop: samples for ammonia will start with a light yellow and progressively turn into darker shades of green the more ammonia is concentrated in the sample (fig. S6); samples for nitrite will start of as light pink and get progressively darker with higher concentrations.
6. After the 10 minutes pass the tubes can be placed in the photometer for measuring.
    6.1. First the target test must be selected (e.g. ammonia, nitrite). When in doubt one can consult the manual.
    6.2. After selecting the test, the blank must be defined (using the third tube with water from the system).
    6.3. Subsequently the dilution factor used must be specified. Highly concentrated samples may have to be diluted due to being above the test's range, this is indicated by the display of ">>" in the monitor. Distilled water must be used for the dilutions.
    6.4. When measuring the sample, the test tube must always be covered with the lid to ensure dark conditions.
7. When measuring is complete the samples must be discarded into the container destined for potentially toxic liquids present in the sampling room. Equipment should be thoroughly cleaning before storage.

Figure S1 - Ammonia test tablet nº1 (left) and nº2 (right).

Figure S2 - Sampling containers for biofilter activation systems and test tubes filled with their respective samples.

Figure S3 - Nitricol test tablet (Palintest).

Figure S4 - Sample for ammonia measuring with insufficient conditioning reagent.

Figure S5 - Sample for ammonia measuring with sufficient conditioning reagent.

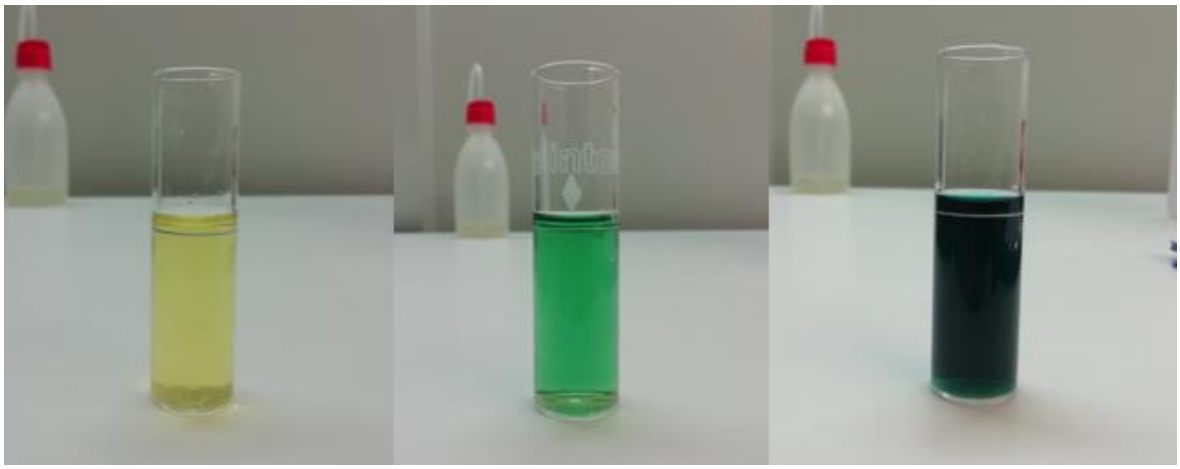

Figure S6 - Colour change in samples with progressively higher concentrations of ammonia (left to right).

Aquaculture room (p.20) maintenance

1. Feeding
    1.1- Feed is usually administrated in one of two ways, depending on the phase or objective of the trial: a set percentage of the animal's body weight; or *ad libitum*.
2. Mechanical filter exchange
    2.1- Remove filter socks from their support on the sump and take them to the washing room. Filters are then washed with bleach (half of a plastic cup) for 15 or 60 minutes in the washing machine depending on their quantity and how dirty they are.
    2.2- Filter should be transported in a bucket, ideally of exclusive use for the room.
    2.3- Depending on the culture density and feed administration filters should be exchange twice a day (usually when feeding *ad libitum*).
3. Sand filter backwash
    3.1- Whenever it is necessary to change the configuration of the sand filter, the pump (blue pump) connected to it must be turned off.
    3.2- Turn the valve from "filtration" to "backwash" and turn the pump back on. Water from the sump will be discarded through the sand filter.
    3.3- Wait until the sump is approximately at half of its volume.
    3.4- Turn off the pump and change the valve from "backwash" to "rinse". Turn the pump back on and rinse the filter for approximately 5 seconds.
    3.5- Turn off the pump, change the valve back to "filtration" and turn the pump on if there is no risk of dry running.
    3.6- Re-fill the sump – this step may be started while the sand filter is being rinsed.
        3.6.1- The sump can be filled with a tube placed directly into the sump and is regulated through a valve connected to the saltwater distribution of the room. Water usage can be monitored the water meter.
    3.7- If the sand filter is left on "backwash" or "rinse" the water from the sump will continue to be discarded even if the pump is turned off. Taking this into consideration the valve must not be left on these configurations unsupervised.
    3.8- If there is a need to turn off the distribution pump (black pump), the UV lights should also be turned off.
4. Water parameters measurement
    4.1- Ammonia and nitrite - measured through palintest test kits.
    4.2- Oxygen and temperature – measured with oxyguard probe.

4.2.1- After the probe is used it should be washed with distilled water and completely dried before storage.

4.3- Salinity – measured with a refractometer.

4.3.1- After it is used it should be washed with distilled water and dried before storage.

4.4- Alternatively, a water sample can be taken, and oxygen, temperature and salinity measured with the probes in the sampling room. Whichever measuring method is used, it is more important to use the same method for consistency.

5. Water exchange

5.1- Performing the sand filter backwash already exchanges approximately 250L of water.

5.2- If alarming water parameters are registered, especially ammonia and nitrite, additional water must be exchanged.

5.3- Water may be discarded from the system through the backwash function of the sand filter or through purging valves on the bottom of each tank. Using the purging valves is preferred when the sump has been recently refilled to avoid discarding newly introduced water.

5.4- Control the water level in the sump – if the tube connecting both sumps start to become visible, water discarding should be stopped to avoid possible pump dry running.

5.5- When refilling the sump take note of how much water was exchanged as it should be registered in the score sheet.

5.6- If possible, avoid changing large volumes of water in a single water exchange (>1000L). As the temperature of water entering the system is lower than the systems temperature it could possibly disturb the animals.

6. Protein skimmer maintenance

6.1- Connect the dechlorinated freshwater and the cleaning mechanism on the lid of the skimmer with a hose.

6.2- Fill and drain the skimmer cup until it is clean (usually 2 times).

6.2.1- Avoid filling the cup too much as water can enter the systems through it and potentially reduce the salinity in case saltwater is being used in the system.

6.3- The level of the skimmer can be regulated with the red circular valve if necessary.

7. Tank siphoning

7.1- Observe tanks with significant solid accumulation at the bottom and use a tube to siphon them.

7.2- Avoid siphoning tanks with animals if there is little solid accumulation to reduce stress applied to them.

7.3- If a considerable amount of water is removed as a result, the sump should be refilled to compensate the loss.

8. Cleaning the room

8.1- After maintenance is performed the rooms floor should be washed with freshwater to remove any detritus resulting from water exchanges and siphoning.

8.2- Periodically clean tubing components and pumps with dechlorinated freshwater to remove salt.

8.2.1- When cleaning the pumps avoid getting the electrical components wet.

8.3- If too much organic material is being accumulated on the sides of the tanks it should be wiped off.

Fish euthanasia

Fish which are sacrificed in sampling events at CIIMAR are usually euthanized through an anaesthetic immersion.

Equipment needed:

- Containers (e.g. 20L bucket).
- Air diffusers.
- 2-phenoxyethanol.
- Fishing net.

Procedure:

1. Prepare two containers (fig. S7):
    1.1- One filled with water from the system to place the fish.
    1.2- One filled with the anaesthetic solution – 2-phenoxyethanol at a concentration between 0.5 and 0.6 mL/L. Homogenize the solution and use gloves for safety.
    1.3- Ideally both containers should be aerated.
2. Collect the fish from the tank and place them in the container with system water.
3. Transfer fish to the anaesthetic bath and wait until no more vital signs can be observed (usually when the operculum stops moving).

Figure S7 - Container with anaesthetic solution (top) and container with live fish (bottom).

Blood sampling

Equipment needed:

- Gloves.
- Paper towels.
- Disinfectant.
- Sterile needles and syringes with heparin.
- Containers with heparin for the blood samples.
- Sharps container.

Procedure:

1. Disinfect the hands before and after blood collection and use gloves to prevent contamination.
2. Place the fish on a paper towel or another material that keeps it from slipping.
3. The puncture method used most in CIIMAR for euthanized or anesthetized fish is caudal venous puncture.
    - 3.1- Depending on the predisposition of the operator it may be performed in two ways:
        - 3.1-1. Ventral insertion – under the skin of the ventral midline of the caudal peduncle (fig. S8).
        - 3.1-2. Lateral insertion – under the scales in the central zone of the peduncle, slightly below the lateral line. The needle should be introduced at a 45° angle.
    - 3.2- Insert needle until you reach the column.
    - 3.3- Slightly withdraw the needle and carefully move it to the base of the column where the blood vessels are located until you start collecting the blood.
4. Discard the needle into a sharps container (fig. S9) and place the blood sample into a container for further processing.
5. If anesthetized fish were used, they should be returned to an aerated tank for recovery.

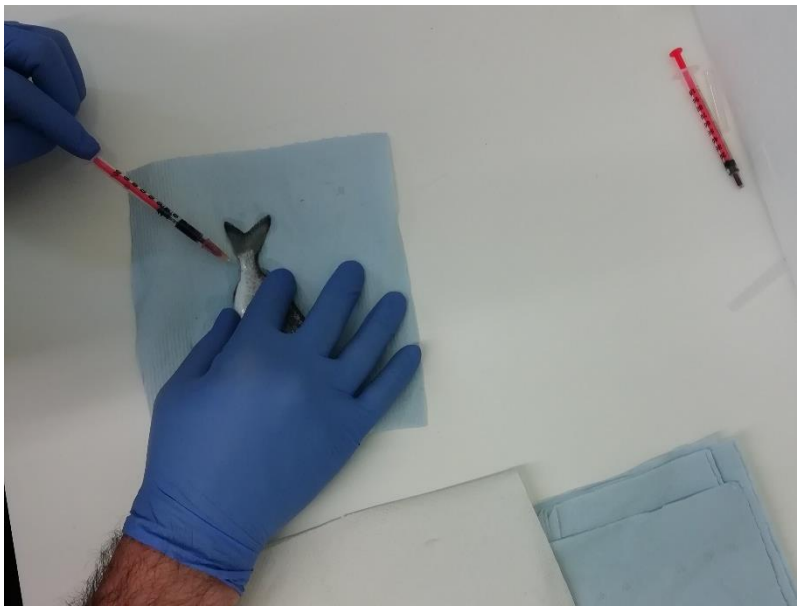

Figure S8 - Caudal venous puncture - ventral insertion.

Figure S9 - Sharps container used in BOGA's sampling room.

Annex II

Haematology techniques

Haematology techniques

Haematology techniques are used in trials to study the cellular components of the blood sampled from the fish. More specifically to assess the composition of red blood cells (erythrocytes), white blood cells (leucocytes) and platelets (thrombocytes).

Haematocrit

The haematocrit, also called packed cell volume (PCV) expresses the percentages of volume occupied by the blood cells and plasma and may be used as an alternative to red blood cell counting if a variation of erythrocytes in circulation between different samples is suspected.

Equipment needed:

- Blood samples.
- Heparinized capillary tubes.
- Capillary tube support.
- Plasticine.
- Haematocrit centrifuge.
- Haematocrit graphic reader.

Procedure:

1. Fill capillary tubes by immersing one of the extremities in the sample. Blood will enter through capillarity and should not exceed the mark on the tube.
2. Cover the other extremity with a finger to remove the tube from the sample and seal one of the sides with plasticine (fig. S10).
3. Place the tubes in the haematocrit centrifuge (fig. S11).
    3.1- Make sure that the tubes are balanced (same number of tubes on each side).
    3.2- The sealed extremity must be oriented towards the outer side of the rotor head.
    3.3- Secure the lid on top of the rotor head and close the centrifuge.
    3.4- Centrifuge for 10 minutes at 10,000 rpm.
4. Remove the tubes from the centrifuge and determine the percentage of the haematocrit through the graphic reader (fig. S12).

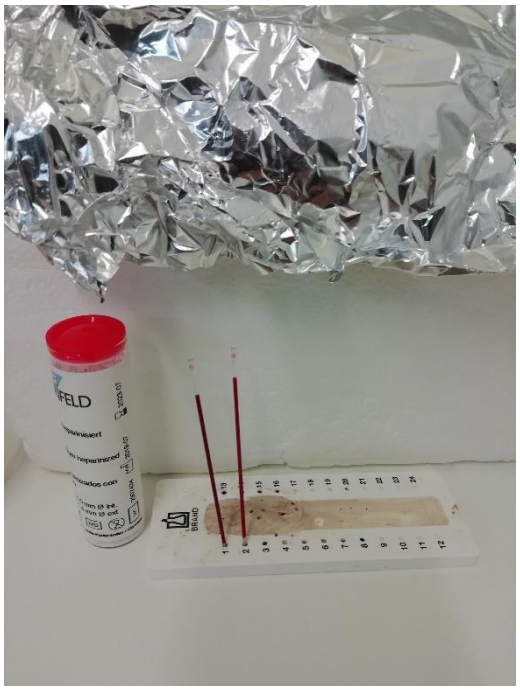

Figure S10 - Capillary tubes filled with blood samples, capillary tube support and plasticine.

Figure S11 - Haematocrit centrifuge with capillary tubes after centrifugation.

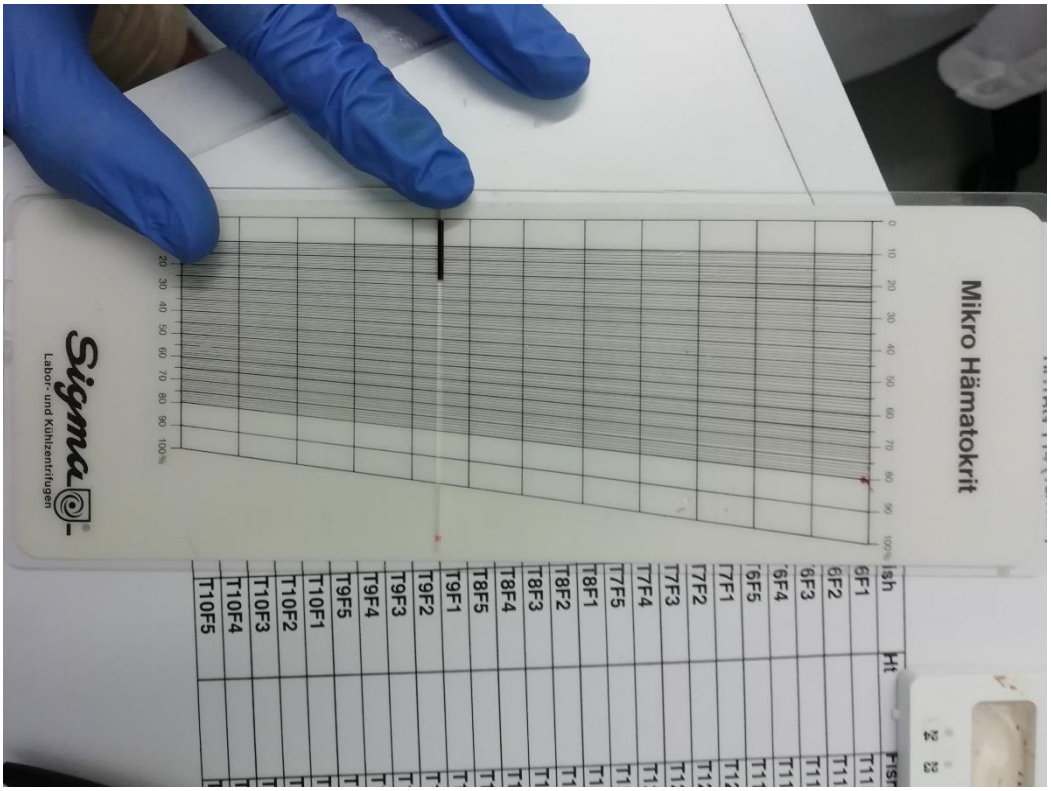

Figure S12 - Haematocrit graphic reader.

Cell counting

The total concentration of erythrocytes and leucocytes in a blood sample can be determined by counting cells in diluted blood samples using glass counting chambers called haemocytometers, commonly known as Neubauer chambers.

Stained blood smear can also be examined to estimate the composition of the different types of cells present in the blood sample.

Neubauer chamber

It is a thick glass slide with two central recessed areas containing finely ruled grids. These consist in 9 large 1 mm^2 squares subdivided into 16 smaller squares. The large central square is composed of 25 squares, each one subdivided into 16 smaller squares. This central square is used for counting red blood cells (RBCs) and the 4 corner squares are used for counting white blood cells (WBCs). When the covering slip is placed on the central grids, the depth of the chamber is 0.1 mm.

Procedure:

1. Add homogenized blood to sterile phosphate buffered saline (PBS) or Hank's balanced salt solution (HBSS) in a dilution of 1:20, for WBC (50µL of blood + 950µL of PBS/HBSS), or 1:200, for RBC (100µL of the solution for WBC + 900µL of sterile medium).
2. Mix properly.
3. Using a pipette introduce a portion of the dilution for WBCs and RBCs into the chamber with the cover slip ready, respectively, one in the upper grid and the other on the lower grid.
4. Let the cells settle for 2 minutes.
5. Use the x10 objective to focus on the grid. Switch to x40 objective for cell counting.
6. Counting blood cells:
    - 6.1- Red blood cells – count 5 groups of 16 small squares (centre square).
        - 6.1-1. Results are expressed in 10^6 cells/µL.
    - 6.2- White blood cells – count the 4 large corner squares.
        - 6.2-1. Results are expressed in WBC/µL.
    - 6.3- Cells that touch the upper and left lines of squares are counted to reduce the risk of counting the same cell twice.

Blood smear

1. Place a droplet of blood (<10µm) on one end of a slide, identified according to its respective sample (fig. S13).
2. Take an extra slide and slide it towards the droplet until it adheres to its edge, approximately at a 45° angle.
3. Quickly advance forwards with the extra slide as you slightly reduce the angle to obtain the blood smear (fig. S14).
4. Afterwards fix and stain the slides for counting.
5. Counting blood smears:
    - 5.1- Focus the blood smear with the x10 objective. Switch to x100 with immersion oil for counting.
    - 5.2- Cells counted include lymphocytes (fig. S15), monocytes (fig. S18), neutrophils (fig. S16), and thrombocytes (fig. S17). Ideally the whole blood smear is examined while counting.

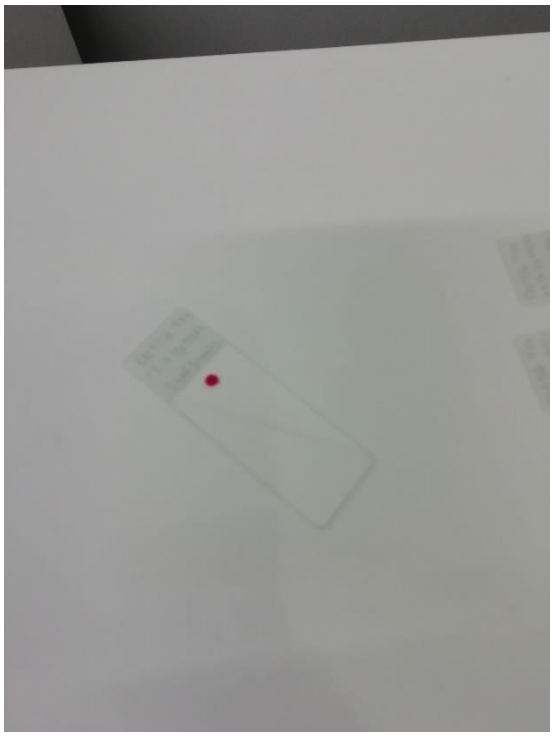

Figure S13 - Slide with droplet of blood for blood smear.

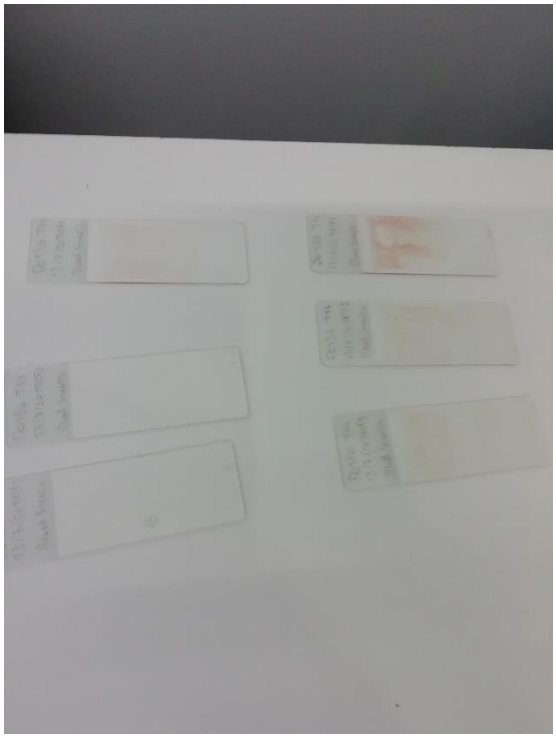

Figure S14 - Blood smears prior to fixing and staining.

Examples of blood smears observed under the microscope with x100 objective:

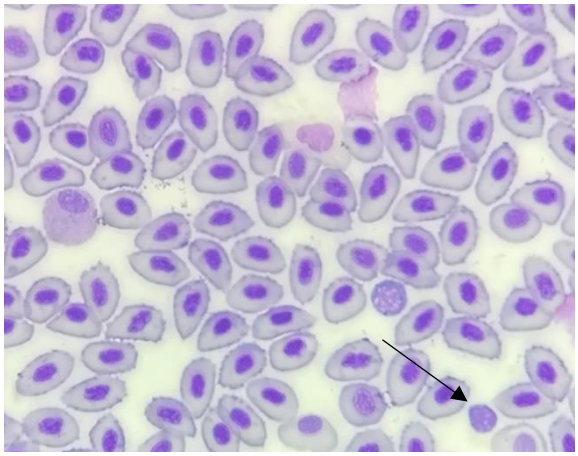

Figure S15 - Lymphocyte.

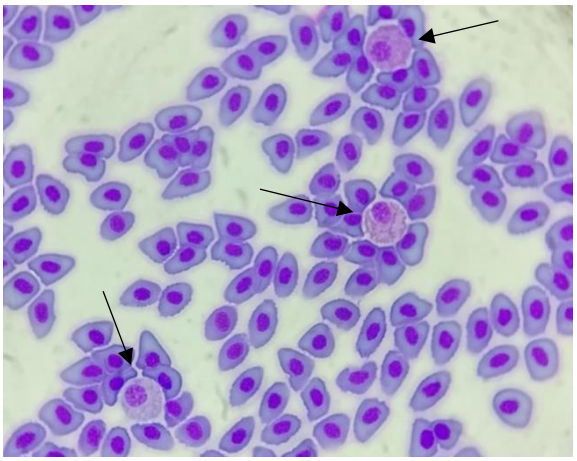

Figure S16 - Neutrophils.

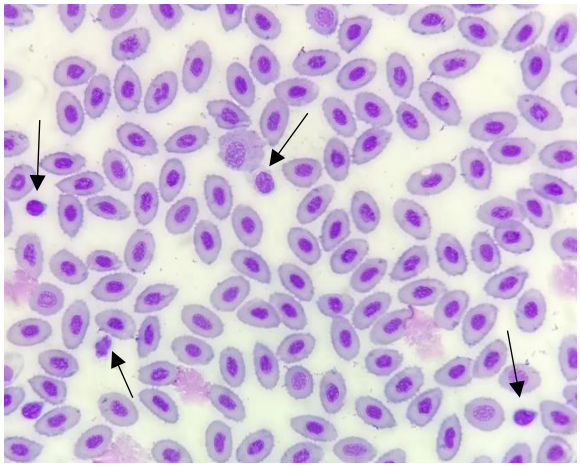

Figure S17 - Thrombocytes.

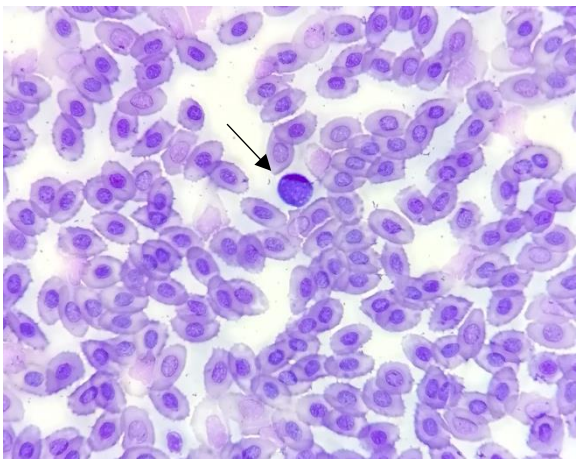

Figure S18 - Monocyte.

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