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Internship Report: Husbandry and Handling of Aquatic Organisms in the CIIMAR Bioterium (BOGA)

Henrique Ribeiro de Sousa

M

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Internship Report: Husbandry and Handling of Aquatic Organisms in the CIIMAR Bioterium (BOGA)

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar - Recursos Marinhos, especialização em Biologia e Ecologia Marinhas, submetida ao instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Orientador: Mestre Hugo Gabriel Maia da Silva Santos

Categoria: Técnico Superior

Afiliação: Centro Interdisciplinar de Investigação Marinha e Ambiental

Acknowledgements

I would first like to thank my family for providing me constant support and encouragement and always being there for me through this new journey in my life.

I would also like to thank my internship advisor Hugo Santos for providing me this experience and assisting me during my internship. Additionally, my gratitude goes towards the BOGA´s team: Olga Martínez, Ricardo Branco and specially Samuel Correia for the guidance, patience and knowledge provided whenever I needed the most.

I would also like to show my gratitude towards Professor Eduardo Rocha, for offering me this new opportunity.

Lastly, I would like to acknowledge all my friends that supported me through this internship and where always available to help and assist me.

Abstract

Investigation on aquatic organisms has been consistently growing for the last few years, as the artificial conditions created in laboratory are becoming very similar to the natural environment. This way, investigation can acquire results that are more reliable and consistent. RAS, just as they are applied in aquaculture, are used also in animal facilities due to the easy manipulation of parameters and creating conditions for the good welfare of aquatic organisms. Nevertheless, the husbandry in a RAS is also of extreme importance and therefore only qualified personnel can successfully manage it.

By performing an internship at BOGA-CIIMAR, knowledge and competences were acquired regarding the husbandry of aquatic organisms in Recirculating Aquaculture Systems. The knowledge gathered and tasks performed during the internship, as well as additional tasks, are described in this report.

Resumo

A investigação em organismos aquáticos tem crescido de forma consistente nos últimos anos, já que as condições artificiais criadas em laboratório têm-se aproximado cada vez mais do ambiente natural. Desta forma, a investigação pode adquirir resultados mais fiáveis e consistentes. Os RAS, muito usados em aquacultura, são também utilizados em biotérios de animais aquáticos devido a fácil manipulação dos parâmetros e a criação de condições de bom bem-estar para organismos aquáticos. No entanto, o cuidado de organismos aquáticos também é de extrema importância, uma vez que apenas pessoal qualificado pode cuidar de um RAS de forma bem-sucedida.

Ao realizar um estágio no BOGA-CIIMAR, foram adquiridos conhecimentos e competências no que diz respeito ao cuidar de organismos aquáticos em sistemas de recirculação. Todos os conhecimentos e tarefas realizadas, bem como tarefas adicionais, são descritos neste relatório.

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2- Introduction

For a long time, we have cultivated aquatic species mainly for consumption purposes. Several records were found that revealed ancient practices of aquaculture from primordial civilizations. Despite many sources believing that China would have cultivated fish in ponds since 2000 B.C., the oldest text is from 500 B.C (**Figure 1**) describing some methods and pond structures of cultivating common carps (*Cyprinus carpio*) (Rabanal 1988). Other cultures would also develop primordial aquaculture systems as a way of exploring the natural resources available: Egyptians would use saline soils with highly productive results and recreational fishing of tilapia (**Fig. 2**) ; Romans would cultivate mullets and trout in their plantations; Hawaiian populations would produce a wide variety of euryhaline and freshwater fish in small ponds created by deviation of streams (**Fig. 3**)(Spalding, Peyton et al. 2014).

Figure 1 - "The Chinese Fish Culture Classic".

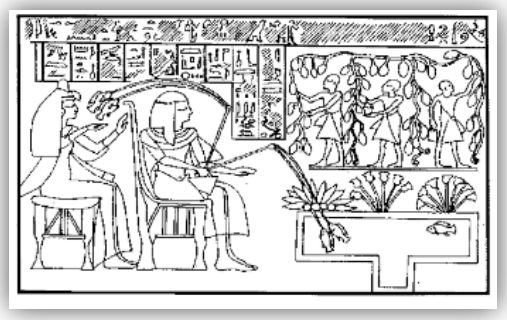

Figure 2 – Bas-relief from an Egyptian tomb

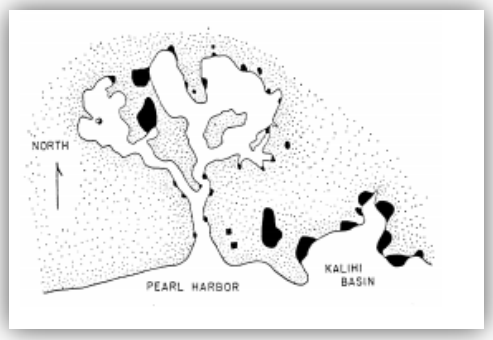

Image 3 – Structure of Hawaiian ponds.

As societies evolved, the increasing globalization and industrialization allowed communication and transfer of species. The crossing of information supported the development and improvement of aquaculture techniques and increasing interest from the World (Beveridge and Little 2002). These husbandry improvements would increase stock survivability and consequently lead to a selection of the most relevant species used in aquaculture(Rabanal 1988).

As a result, in the last 50 years, there has been an increasing concern for the welfare of animals in aquaculture, as many studies defended improvement would lead to less mortality and superior quality of the product (Rabanal 1988). Thus, major developments in the husbandry area would also bring more aquatic animals as a model for research into the discussion (Borski and Hodson 2003).

According to Hunn (1989), fish are being used in research on different fields for nearly 200 years. Most earlier fish experimentation studies were related to toxicology effects of some water contaminants in minnows (*Pimephales promelas*) for their sensibility and goldfishes (*Carassius auratus*) for their resilience, although other areas were also being explored: studies related to genetics, nutrition, renal physiology, embryology, parasitology, nerve physiology and endocrinology (Nigrelli 1953). Aquatic toxicology would be the first major developed area regarding fish research mainly due to the emergence of dangerous pesticides in the 1940s. Furthermore, from the 1970s forward, the idea of using fish as model for research gained support (Ostrander 2000). From the major categories of fish, jawless (Agnatha), cartilaginous (Chondrichthyes) and bony (Teleostei), the most studied fish were bony fish and the research was specially directed to cultivated species in aquaculture (Klontz and Smith 1968). Among the most used fish in experimentation, trout (*Oncorhynchus mykiss*), salmon and other salmonids species were at the top, mostly due to their mass use in aquaculture and quick adaptation in captivity. Various species of cyprinids were also being used, specially carps and goldfishes (Klontz and Smith 1968).

Several fish and other aquatic animals rose to the spotlight in research, although the most impactful one was the zebrafish (*Danio rerio*). Despite already being utilized several years before in toxicology and teratology, this species emergence came in the late 70s with its application in neuroscience (Vascotto, Beckham et al. 1997). As the time went on, *Danio rerio* has been applied in other areas, particularly in genetics, mostly due to relatively easy husbandry practices, short generation time and its

transparent embryos (Metscher and Ahlberg 1999). In the last few years, most projects regarding fish as a model for research is defined by the use of zebrafish (Kent, Feist et al. 2009).

Hence, in order to use fish in research, certain conditions need to be created to maintain animals in laboratory confinement and, if they are maintained in a stable and optimal way, good results can be obtained. In “Fish as Biomedical Research Models”, Lontz (1971) stated that “The first step (to achieve optimal conditions) is to recognize that the aqueous environment is capable of being manipulated on a micro-micro basis in closed systems and that constancy is essential.”, illustrating how the natural conditions can be simulated in closed aquatic systems. By simulating the original environment the animals live in, experimentation can be closer to the reality. The main problem regarding fish and other aquatic organisms in experimentation is their major diversity of species, requiring different conditions and husbandry procedures specifically dedicated to the animal in question (DeTolla, Srinivas et al. 1995).

In Bioterium of Aquatic Organisms BOGA-CIIMAR, where this internship took place, many aquatic organisms are maintained for research purposes, with daily husbandry practices to maintain the best conditions possible. As stated by DeTolla, Srinivas et al. (1995), the main systems used for maintaining animals in laboratory research can be “static , flow through, closed water recirculating, ponds or lakes and a net or cage placed in a body of water”. Even though there are many choices regarding the aquatic systems, the best decision must be made in accordance with the desired experiment. BOGA has some of these aquatic system designs in use, but the most common are the RAS (recirculating aquaculture system). Based on the application of RAS in aquaculture in a rather successful way (Zhang, Li et al. 2011), research facilities also apply these concepts and structures in order to enhance and control the water quality (Gutierrez-Wing and Malone 2006). The development of RAS, especially indoor, assisted the use of aquatic animals as research models (Gutierrez-Wing and Malone 2006), as several species could be maintained in an artificial environment for an extended period of time. Many aquatic animals can be maintained in these systems, including many species of fish and aquatic invertebrates (Helfrich and Libey 1991). Several reports refer the use of organisms in RAS for aquaculture and research purposes: quite a few species of salmonids (Good, Davidson et al. 2014, Mota, Martins et al. 2016); tilapia as a growing species in aquaculture and consequent use in experimental research (Mota, Martins et al. 2017); many species of catfish (Adamek,

Grecu et al. 2015); use of sea bass (Watts, Bright et al. 2016)and largemouth bass (Sammouth, d'Orbcastel et al. 2009); flatfish and puffer fish (Xue, Xu et al. 2017); some species of bivalves are maintained in RAS during the juvenile and adult stages (Kovitvadhi, Kovitvadhi et al. 2008); crustaceans, specifically crayfish (Seemann, Lorkowski et al. 2015) and shrimp (Brown 2013); and lastly some echinoderms are being adapted to RAS, namely the sea urchin (McBride 2005) and sea cucumber (Ying, Baoliang et al. 2015). Therefore, BOGA-CIIMAR has developed aquatic systems where they maintain some species cited above under research and maintenance, and will be explained in greater detail further down.

The purpose of this report is to describe the theoretical concepts learned and reveal knowledge regarding the husbandry procedures performed under the supervision of BOGA. Additionally, other projects were developed during the internship in the research facility –"Standard Operating Procedures" and "Biological Filter Tanks".

3- Internship at BOGA-CIIMAR

To obtain a Master degree in Aquatic Sciences-Marine Resources in ICBAS, in accordance with the Bologna agreement, a professional internship occurred in BOGA – CIIMAR. The choice was made to gain working experience regarding the functioning and organization of a scientific research aquatic facility, starting from the policies applied that need to be strictly followed, to the procedures in the husbandry of aquatic organisms.

The design of this work was made to contain the following objectives, taking into account all theoretical and practical concepts presented during the internship:

- Reveal knowledge of the different concepts that were learned during the internship;
- Describe the activities performed to gain experience;
- Present additional work performed to complement the internship: “Standard Operating Procedures” and “Biological filter tanks”.

3.1- Facility

BOGA, located next to the Law Faculty from Porto (**Fig. 5**), is an animal facility from the research center CIIMAR (**Fig. 4**) designed to provide the best conditions to perform animal experimentation with aquatic species. The main objective is to raise and maintain aquatic organisms in the best conditions, controlling the biotic and abiotic parameters regularly to ensure maximum quality of life and thus, obtaining the best results when conducting experiments.

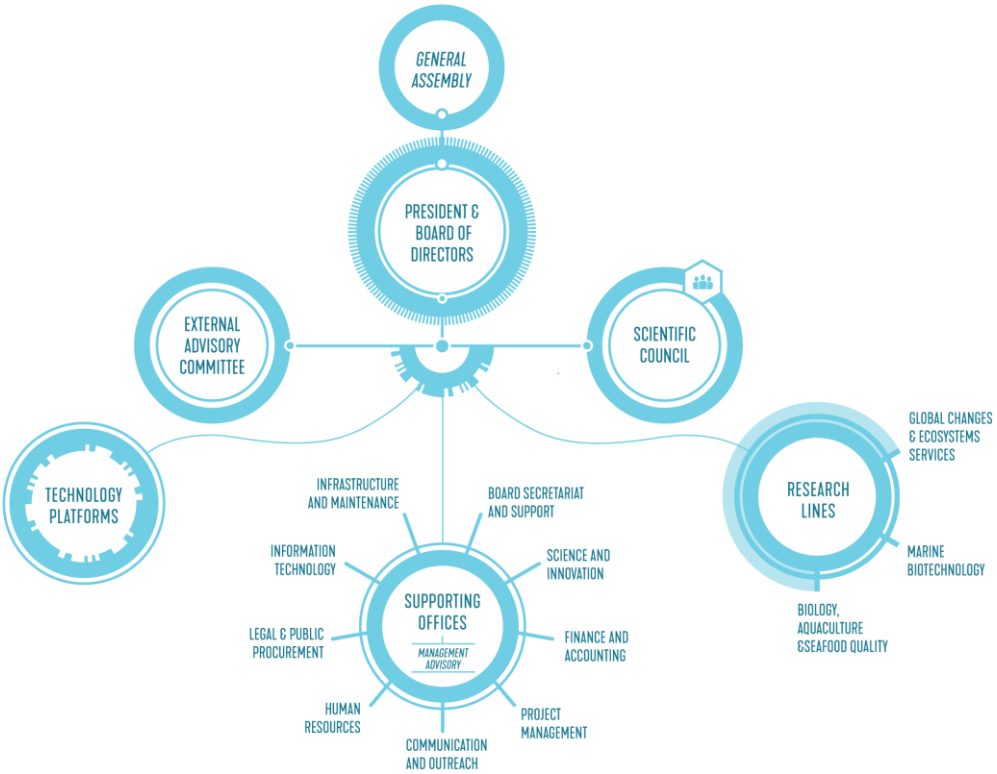

Figure 4 – CIIMAR organogram showing the different departments: The BOGA is under the "Technology Platforms" department.

Last year, CIIMAR changed its location to the new Marine Science and Technology Centre, located in Leixões harbor in Matosinhos (**Fig. 6**). However, due to some bureaucracies, the BOGA facility is still waiting for the legal confirmation to move to the new building. Nevertheless, the necessary arrangements for the relocation are happening gradually and it is expected for the aquatic facility to move to the new location within the next few months.

Figure 5 – Old BOGA-CIIMAR building.

Figure 6 – New CIIMAR building.

3.1.1- Access

Just like any other animal facility, the access to BOGA is restricted to the facility personnel and others that have the required authorization. Any other entrance is prohibited in order to prevent animal stress and the risk of pathogens spreading across the facility.

Upon getting the entry approval, some requirements need to be followed in order to maintain the organization and security of the facility. There are basic rules to follow in an animal facility, as well as the use of protective gear, necessary to protect from potential infections, namely rubber boots or lab shoes covers and lab coats. All these procedures need to be reviewed before entering the building and all the protective gear needs to be worn before breaking the physical barrier into the aquatic research facility. Nevertheless, these preventive rules and other procedures will be applied in the new BOGA location, under the form of Standard Operating Procedures. Some of these SOPs are described in this document as supplementary work for the internship in BOGA.

3.1.2- Plant and Rooms

All the access to the rooms is restricted and can only be permitted with the authorization of the BOGA staff and/or principal investigators. Therefore, whenever I entered a laboratory room, permission was always granted by the BOGA personnel. The BOGA building is divided into 3 floors: ground floor and second floor dedicated to the workplace; the top floor containing several machines essential to facility operation, water reservoirs and storage space.

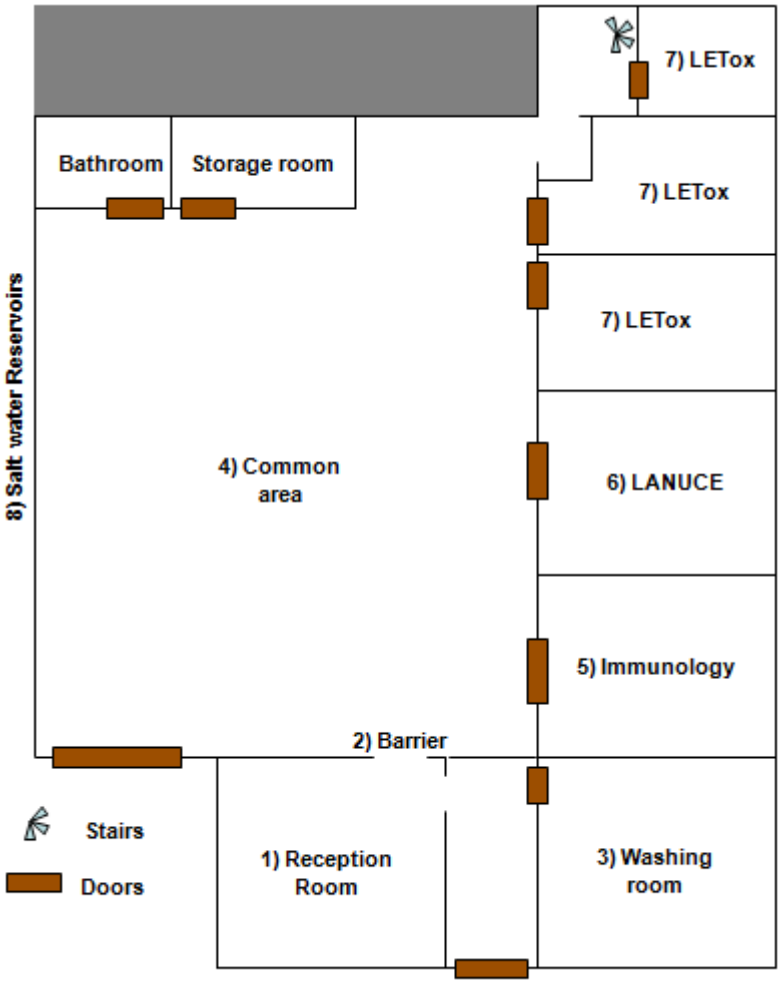

Figure 7 – Plant from the ground floor of the BOGA CIIMAR building.

3.1.2.1- Ground Floor (**Fig. 7**):

a) The first step upon entering the facility is to reach the reception room (1), where all the essential protective equipment is available; minimal protection is required to anyone, namely protective shoes; It is important to establish a barrier (2) in order to maintain a certain level of biosecurity before entering the aquatic facility.

b) Next to the reception room, the washing room (3) is used to prepare and clean the material before entering the facility and facilitate daily activities in the facility.

c) After crossing the barrier (2), the first zone to enter is the common area (4). In this spot, there are several RAS distributed across the room from different research groups. In this area, there are RAS dedicated to quarantine of newly-arrived animals, systems from the laboratories of CIIMAR, systems under the supervision of BOGA and biological filter tanks.

d) In the first floor there are several investigation rooms:

- Immunology laboratory (5);
- Growth, Nutrition and Fish Quality laboratory (LANUCE) (6);
- Environmental Toxicology laboratory (LETox) (7)

e) Water reservoirs are located in two places: 5 water tanks with the capacity of 3000L and one of 5000L placed outside the facility (8); 10 water reservoirs with the capacity of 1000L in the machine room located in the top floor. Overall, BOGA has the capacity to hold up to 30000L.

3.1.2.2- Second Floor (**Fig. 8**)

f) Other laboratories can be found in the second floor:

- Ecophisiology laboratory (LEcof) (9);
- Ecotoxicology laboratory (LEcotox) (10);
- Environmental Toxicology (LETox) (11);
- Microcosms (BOGA) (14);
- Molecular and cellular studies laboratory (LECEMA) (15);
- Ecophisiology laboratory (16);

- Ecotoxicology, genomic and evolution laboratory (LEGE) (17);
- Hiperbaric room from LEcof (18);
- Ecology and evolution of marine communities laboratory (LMCEE) (19).

f) The BOGA office is located in the second floor divided into two rooms (12).

g) Workshop room (13).

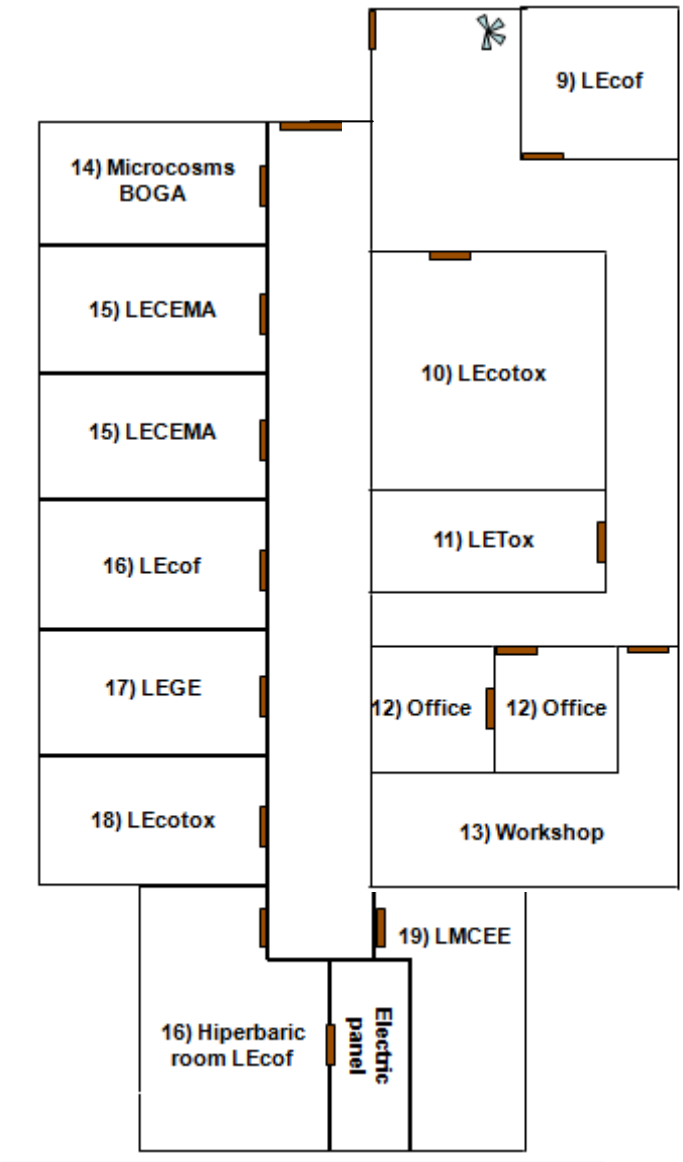

Figure 8 – Plant from the second floor of the BOGA CIIMAR building.

3.2- BOGA Staff

The team working in BOGA have numerous tasks that allow the facility to run efficiently: Maintenance of some aquatic systems; exclusivity in the maintenance of the quarantine organisms; assistance in the design of laboratory experiments; different levels of education of new people entering the facility; assistance during experiments and problem resolution that may emerge; among other tasks.

Throughout the internship, BOGA´s team taught and assisted me during the tasks I had to perform and corrected me whenever necessary to improve my skills and productivity. However, the main reason for my progress during the internship was questioning the decisions that I took, leading me to always question my thought process and decide if it was the best choice to take in a particular situation. Samuel Correia was my main supervisor inside the facility and guided me into performing the job correctly, as well as Olga Martínez and Ricardo Branco, whom, despite not being directly responsible for my tasks, were always available to help me whenever I needed.

4- Aquatic Systems in BOGA

The husbandry of animals in BOGA is manly made by RAS and static/semi-static systems, even though exceptionally another system can be used whenever it is required by a certain experiment. Being a land-based aquatic animal facility, some aquatic systems cannot be fully developed into dimensions closer to reality due to the structural and site limitations.

Static and semi-static conditions are normally used in toxicity tests (Solloch, Pechacek et al. 2015, Nishimura, Inoue et al. 2016), in order to maintain the initial concentration of the compound(s) in question and prevent concentration variations, although recirculation (Van Bussel, Schroeder et al. 2014) and flow through systems (Seitz, Bundschuh et al. 2013) can also be effectively used. The main advantage, which also becomes its main disadvantage, is not performing any or minimal water exchange, maintaining the concentration levels of any given compound but also leading to an aggravation of several parameters, namely low dissolved oxygen and high nitrogen compounds levels. Thus, the potential gain/loss must be well thought before executing the experiment, seeing that the results may be influenced by the extreme conditions created by poor water quality and not from the experience itself. My contact with still-water systems was in the husbandry of amphipods (*Gammarus locusta*) that I carried out weekly as part of my job in the aquatic facility and will be explained further below.

The main type of aquatic system used in BOGA is the RAS, which is also used in aquaculture. The use of RAS evolved from its specific use in hatcheries to a great variety of applications, namely the growth into adult life and maintenance of a great number of species (Blancheton 2000). This development enabled a more constant application of recirculating systems in experimentation. Contrary to still-water systems, there are several advantages in RAS that explain the growing popularity: simple manipulation of water conditions; capacity to work on larger densities of organisms; lower risk of contamination from diseases outside the facility; reuse/recycling of water; the application in different fields (aquaculture, investigation, recovery of endangered populations)(Bregnballe 2015). Beyond that, the use of recirculating systems enables the reduction of use of water and consequently discharges, as wells as less waste due to the good food conversion within a RAS (Gutierrez-Wing and Malone 2006).

Most of my experience handling organisms in BOGA was connected to RAS, which required a good knowledge on the functioning of these systems in order to perform a good job. Therefore, a more detailed information will be described below explaining most of the components that make a recirculating system work.

4.1- Recirculating Aquaculture Systems

According to Pillay and Kutty (2005) , all RAS must at least contain ways to "remove waste solids, oxidize ammonia and nitrite–nitrogen, remove carbon dioxide, and aerate or oxygenate the water before returning it to the fish tank", even though other upgrades and supplementary equipment can be introduced to improve the quality of the water in the aquatic system, in the event of specific requirements being considered necessary for a certain experiment or organism (**Fig. 9**).

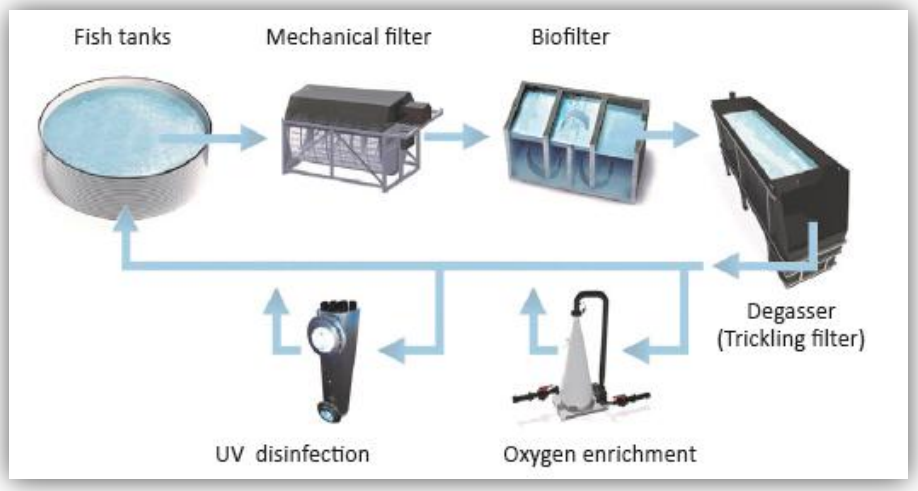

Figure 9 – Simple design of a RAS.

All these components included in a RAS are necessary to maintain water quality. Organisms living in a stagnant and untreated mass of water will cause gradual degradation of water parameters as the time passes by. Excretion products and

oxygen consumption are the main problem to tackle, as they naturally occur through the physiological needs of the organisms. The suspended residues will be retained in the mechanical filter while the smaller organic matter associated with phosphate and nitrogen compounds will be treated by naturally occurring bacteria living in the biological filter (Bregnballe 2015). Following this two filters, aeration becomes the next important step to improve the quality of the water. Subsequently, extra equipment may be added before the water returns to the organisms, namely UV disinfection, ozone and skimmer.

In BOGA, the design is as represented in **Figure 10**: water is captured by a pump inside the main tank and sent(1) into the initial rack where there is a mechanical filter (2); passes through and falls into the secondary compartment where the biological filtration occurs (3), sometimes even having additional racks just as its illustrated in this case; aeration also occurs when the water falls down into the system by a waterfall (4); Another pump (5) pushes the water to the skimmer (6), releasing the water into the cascade (7) (mechanical filter and biological filtration) ; and finally another source of aeration (8) is provided by air stones that are connected to a filtered air distribution pipe.

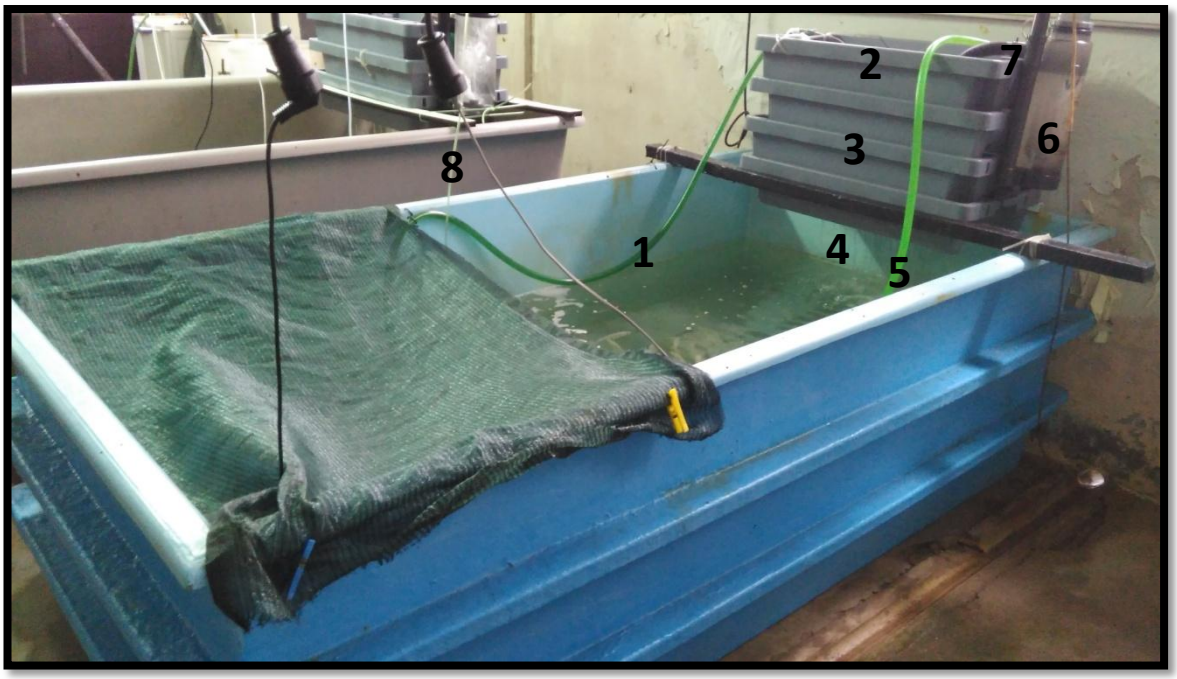

Figure 10 – Design of a RAS in BOGA

4.1.1- Mechanical Filtration

The main function of mechanical filters is to retain the suspended particles circulating in a RAS, namely feces, rotten fish, food that was not eaten and slough from biofilm present in the systems (Patterson and Watts 2003). Fish are a good example of the wastes produced by aquatic organisms, largely because they have restricted digestion, leading to high levels of excretion (36% of the food ingested is excreted). Furthermore, they require a high level of protein in their food and, being ammonia an end product of protein metabolism, nitrogen residues levels will increase in the water(Crab, Avnimelech et al. 2007). If these particles are not removed quickly from the systems, ammonia levels will increase rapidly and reduce dissolved oxygen levels , reduce the biological filter efficiency and even cause gill irritation in some fish, leading to reduced immune system and higher risk of pathology development (Khater, Ali et al. 2011) . Beyond that, system equipment can also be affected by clogging smaller orifices and pipes (Davidson and Summerfelt 2005).

A significant factor to consider when discussing filtration is the particle size and, depending on its dimensions, may be defined as suspended or dissolved. According to Castine, McKinnon et al. (2013), suspended solids are considered settleable if they are over 100µm and supracolloidal if they have dimensions between 1µm and 100µm (**Fig. 10**). Suspended particles, generally characterized in aquatic systems as solids of low density, high organic content and a large range of dimensions, can be mechanically filtrated to the size of 0,45µm.

On the other hand, dissolved solids are considered colloidal if they are within the range of 0.45µm to 1µm and dissolved if under 0.45µm. These particles are considered nearly impossible to remove mechanically and therefore need to be eliminated using others processes, namely foam fractionation (skimmers) and by biological filtration (**Fig. 11**).

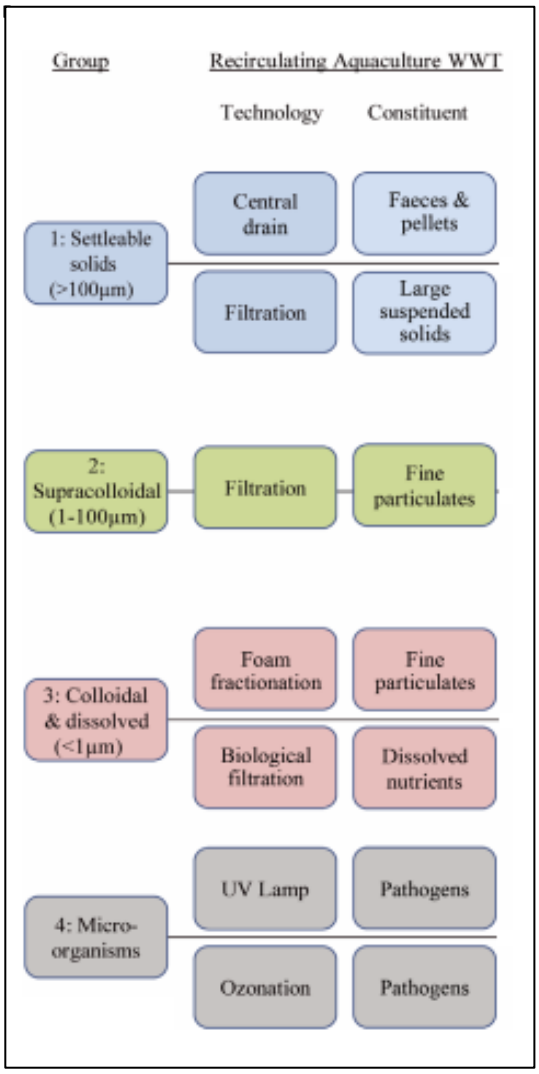

Figure 11 – Adapted from Castine, McKinnon et al. (2013). Particle sizes and principal treatments applied in a Recirculating Aquaculture system. Blue boxes represent pre treatment for 1º group; Green boxes represent primary treatment to 2º group; Pink boxes represent secondary treatment to 3º group; Grey boxes represent secondary treatment to the 4º group.

Mechanical filtration can be made using different methods, each of them with different advantages and disadvantages, which can be divided into sedimentation, micro screen or straining and sand filtration (Lekang 2007) . Sedimentation, as the name indicates, is to enable the solids to reach the bottom of the sediment tank by slowing down the water flow. Although it is one of the most inexpensive methods, it has a very low efficiency and high settlement time regarding solids smaller than 100µm, requiring sometimes others processes to complement the filtration (Chen, Stechey et al. 1994). Sand filtration, also called depth filtration, forces the water to pass through a certain granular material and, depending on the composition and size of the granular medium, suspended particles will be retained. Normally it is called sand filter due to the

extremely common use of sand as a medium in these equipments, but other materials, for instance gravel, can also be effectively used. The only downside from the sand filter is the potential clogging that will eventually happen as the time goes by, and generally needs a periodic cleaning using a backwash system (Lekang 2007).

Screen filter process revolves around creating physical restraints by passing the water through a certain material and the efficiency of that filtration will depend on the characteristics of that same material (Chen, Stechey et al. 1994). As the water flows, particles larger than the screen aperture dimension will be retained and accumulate. The problem resides on the fact that filtrating fabric is going to clog fast as the apertures become blocked and a constant cleaning is necessary (**Fig. 12**). Therefore, if a smaller aperture is needed for a determined system, the clogging will be even faster and regular cleaning is imperative (**Fig. 13**) (Lekang 2007). Despite being a method simple to implement, there are some disadvantages, specifically the removal of solids smaller than 50µm and reduced water flow (Barrut, Blancheton et al. 2013). Screen filters are the filtration process that is most used in BOGA and the one that I had the most contact with during my intern in the facility. The process was simple: a pump would push the water into a plastic board containing a filtrating material, forcing the water to pass through (**Fig. 12; Fig. 13**). The most used materials applied in BOGA were screen filters, glass wool and sponge, requiring a daily clean-up on most systems to prevent the clogging and consequently reducing filtration efficiency.

Figure 12 – Glass Wool as a mechanical filter in BOGA

Figure 13 – Screen as a mechanical filter in BOGA

4.1.1.1- Foam fractionation

A process commonly used for the removal of fine particles is the foam fractionation, normally performed by a device called skimmer. It consists of introducing air throughout the bottom of a column forming mall air bubbles and consequently foam (**Fig. 14**). Air bubbles will allow the smaller particles, mostly consisted of dissolved organic compounds (DOC), proteins and other small solids, to adsorb to their surface, "carrying" them to the top of the skimmer (Losordo, Masser et al. 1999). Alongside organic particles and proteins, it is also proven that skimmers can also remove not only toxic algae and parasites, but also viruses and bacteria, since they contain a surface-active components in their cell walls (Barrut, Blancheton et al. 2013). At the top of the skimmer a container exists that facilitates the removal of the foam containing surfactants. By applying a skimmer to a system, several advantages can be gain, namely a decrease in potential clogging of pipes and pumps, improve the color of the water by removing organic matter, remove some higher molecular height substances, pH stability and increasing the aeration levels (Lawson 1995). The size of the particles filtrated in a foam fractionation is extremely variable, ranging from 30µm to 10,6µm (Brambilla, Antonini et al. 2008), and its efficiency will depend on the design and capacity of the skimmer, but also the characteristics of the water being treated . The most important factors to have into account are the bubble dimensions and the time of contact between the bubbles and the surface-active compounds, as well as the concentration and surface chemistry of the particles in the water, which can facilitate the adsorption process (Barrut, Blancheton et al. 2013). Lastly, one popular skimmer design is the counter-current, also applied to a great extent in the BOGA facility. The process is rather easy, being illustrated in **image 15**: bubbles are produced at the bottom and rise across the column against the water flow, allowing the surface-active particles to attach to the bubbles as they burst when reaching the top, where the resulting foam can be collected (Brambilla, Antonini et al. 2008).

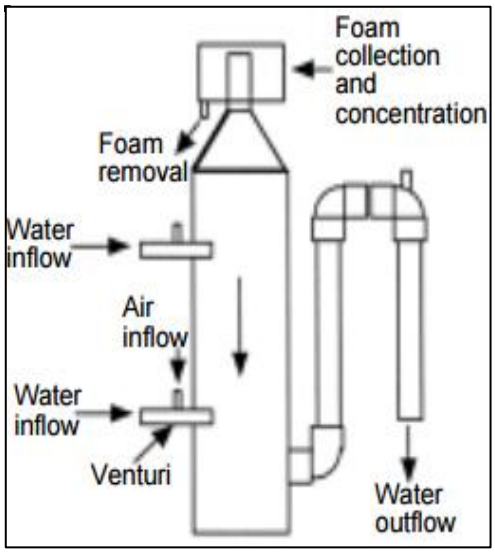

Figure 14 – Design and circulation of air and water in a Skimmer.

Figure 15 – Skimmer used in BOGA.

4.1.2- Biological Filtration

Aquatic organisms, as a result of physiological needs, will produce different wastes that need to be removed as quickly as possible. The most important excretion to be removed is ammonia, NH_4^+, mainly because it becomes toxic to organisms as the levels rise (Hochheimer and Wheaton 1998). Ammonia belongs to the nitrogen cycle, taking the inorganic forms ammonia-nitrite(NO_2^-)-nitrate(NO_3^-) in the aqueous environment. Normally, ammonia (NH_4^+) and ammonium (NH_3) are at equilibrium depending on temperature and pH levels, forming the Total Ammonium Nitrogen (TAN). Despite the two forms being toxic to organisms, the un-ionized form is the most toxic and, since the two forms are at equilibrium, removing one will automatically lower the levels of the other form (Crab, Avnimelech et al. 2007). Ammonia also transforms into nitrite through the process of nitrification by autotrophic bacteria (*Nitrosomonas*), which essentially means the oxidation of NH_4^+, and oxidation of nitrite to nitrate by *Nitrobacter bacteria*, a chemoautotrophic bacteria(Hall 1999). This process becomes essential since aquatic organisms have a higher tolerance to nitrate than ammonia or nitrite (Lekang 2007). At lower levels, NH_4^+ can cause reduced growth, create more susceptibility to diseases and less tolerance to handling and, at higher levels, ammonia can cause the death of all organisms within a system if not controlled (Francis-Floyd, Watson et al. 2009)

Figure 16 – Most used medium in BOGA for the development of nitrifying bacteria, bio balls.

To create nitrification conditions, a medium needs to be provided to allow the bacteria to create biofilm and grow rapidly. This biolfim helps the bacteria to survive a variety of conditions and continue to perform the nitrification process (Malone and Pfeiffer 2006). In order to facilitate the biofilm formation, the medium needs to have a high surface area, have a design that prevents the biofilter from clogging and ensure proper circulation and contact with water. The most used mediums used today are plastic shapes (**Fig. 16**), but other materials can be used, namely gravel and sand (Lekang 2007).

After providing a substrate, several parameters need to be controlled to allow the biofilter to thrive: ammonia levels need to be above 3mg/L for a good bacteria expansion (Lekang 2007); temperature must be in accordance with the desired aquatic systems (Hochheimer and Wheaton 1998); dissolved oxygen is essential for nitrification and needs to be regularly checked, with levels always above 2m/L in the biofilter (Hochheimer and Wheaton 1998); pH is a limiting factor and needs to be between 7.5 and 9 (Hochheimer and Wheaton 1998); presence of organic matter may reduce biofilter efficiency by allowing other bacteria to grow and compete with nitrification bacteria (Lekang 2007); abrupt changes in salinity may hinge bacteria growth (Hochheimer and Wheaton 1998); presence of light must be minimal as it may be inhibitory to bacteria (Hochheimer and Wheaton 1998); understanding the toxicity of some substance to nitrifying bacteria, namely metal ions and medical substance (Lekang 2007). All these parameters need to be met to enable a productive biological filter.

4.1.2.1- Biological Filter Equipment

After defining the conditions, the biological filter setup is equally important to maximize the efficiency. Essentially, biological filters can have two configurations, specifically “attached growth” or “suspended growth” (Gutierrez-Wing and Malone 2006). Attached growth means that a media exists for the nitrification bacteria to attach and form a biofilm. Conversely, suspended growth signifies that the microorganisms are suspended in a certain mass of water, in general less used due to the association with poor water quality, instability and high management demands (Gutierrez-Wing and Malone 2006). Fixed biofilters are considered more popular relatively to the suspended ones, having also several configurations:

- Flow-through (submerged or trickling) biofilter;
- Rotating (biodrum or RBC) biofilter;
- Fluidized bed biofilter;
- Sand or granular filters.

Flow-through biological filters, as the name indicates, is allowing the water to flow and make contact with the nitrifying microorganisms. If the medium is submerged, there is a better circulation and contact of the water across the biofilm, despite the necessity to provide oxygenation. In contrast, trickling biofilters are not immersed and are located above the system, as the water is guided to pass through the medium containing the biofilm by cascading (Lekang 2007). The fact that this biological filter is not underwater gains the advantage of having a good oxygen circulation. On the contrary, submerged biological filters are dependent on oxygen levels carried in the circulating water (Malone and Pfeiffer 2006). Therefore, if an aquatic system is low oxygenated, all the organisms are being affected, including nitrifying bacteria. A rotating biofilter, or biodrum, gathers the best advantages of the two previous biological filters, having a partially submerged and emerged rotating drum. When revolving, the drum containing the medium will oxygenate out of the water, while removing CO_2 and reducing biofouling problems (Malone and Pfeiffer 2006, Lekang 2007).

Fluidized bed biofilters are also conventionally implemented in RAS due to their capacity to maintain a relatively good water quality (Crab, Avnimelech et al. 2007). In this case, the medium, where the biofilm is found, is in continuous motion in the water

column and the nitrification occur in the surface of the suspended solids (Lekang 2007). The system is normally aerated and the medium is maintained in suspension by the constant current, preventing the possibility of clogging. Therefore, the medium material used in these situations must have high surface areas and can have different characteristics, for instance granite, anthracite, sand, plastic, among others (Malone and Pfeiffer 2006). The growing popularity of this biofilter is attributable to the wide range of substrate concentration, the nitrification conversion levels (50-90% each pass), the constant efficiency in the face of different water flows and the straightforward management that it requires. The drawbacks are the constant aeration required, the minimal flow that is required or else the biofilter reduces drastically its nitrification efficiency and the pumping power necessary to lift and maintain the medium in suspension (Summerfelt 2006).

The design for a biological granular filter is the same required for the removal of solids. The container will enclose a material designed to uphold biofilm, generally spherical plastic called beads that are intended to have a positive buoyancy (Lekang 2007). This systems necessitates an aggressive backwash mechanism to prevent the inevitable clogging and control the biofilm growth (Malone and Pfeiffer 2006), becoming inevitably a downside when using this biological filter. Even though this system can also be used as a solid removal incorporated with ammonia removal capacity, the negative aspect regarding this system is the reduced nitrification rates in relation to the other biofilters.

Comparing all the biological filters (**table 1**), rotating biofilters have the better results when it comes to the ammonia removal rates, followed by trickling biofilters. Even though bead and fluidized filters reveal the lowest performance regarding nitrification, their expenses are reduced compared to the costly use of a rotating biofilter (Crab, Avnimelech et al. 2007).

Biofilter type	Average TAN areal removal rate (g TAN/m² day)	Cost (Euro/kg year)
Rotating biological filter	0.19-0.79	1.143
Trickling filter	0.24-0.64	1.036
Bead filter	0.30-0.60	0.503
Fluidized sand biofilter	0.24	0.198

Table 1 – Adapted from Crab, Avnimelech et al.(2007). A comparison between some commonly used biological filters regarding average removal of TAN and related costs.

4.1.2.2- Denitrification

Despite aquatic organisms being less susceptible to higher levels of nitrates compared to ammonia, a continuous accumulation of the final product of nitrification may lead to unsustainable levels of NO_3^- and eventually cause mortality. Although the levels of nitrates necessary to kill an organism are difficult to achieve, the variability among aquatic organisms may affect some more than the others and chronic exposure to high levels of nitrate may lead to other complications, for instance slow growth, low fertility and higher susceptibility to diseases (Gutierrez-Wing and Malone 2006). As said by Van Rijn, Tal et al. (2006), high NO_3^- affected the growth of several organisms such as shrimp, trout, eel and octopus. Beyond that, the removal of nitrate is equally important to comply with discharge regulations, constancy on the buffer capacity of the water and elimination of organic carbon and sulfides. The only way to lower the levels of nitrates in an aquatic system is by water exchange or denitrification (Hamlin, Michaels et al. 2008).

Denitrification is a process that removes nitrogen from the system while also recovering the alkalinity that was lost during the process of aerobic nitrification, transforming nitrite or nitrate into nitrogen gas that is release into the air (Klas, Mozes et al. 2006). More specifically, there are two anaerobic bacteria groups involved in the process of denitrification, one that reduces nitrate to nitrite or ammonia and the second one that converts nitrate (by nitrite) into nitrogen gas. Sometimes there is an accumulation of nitrite from nitrate mainly because there is one piece of the process missing, frequently referred as “incomplete denitrification” (Hamlin, Michaels et al. 2008). This fault is due to a lack of a carbon source, normally provided by organic

matter. In RAS systems, the carbon source can be provided by injection of methanol or acetate; however the amount inserted must be controlled not to intoxicate the bacterial culture (Qiu, Liu et al. 2016). Sometimes, in nitrification biofilters, denitrification can occur if there are anaerobic conditions and carbon availability in some levels of the biofilm (Van Rijn, Tal et al. 2006). If this happens, the biological filter may not be working efficiently.

Filters designed for denitrification must be anaerobic and have a constant font of carbon. Most designs require a large area for the bacteria to grow, just like nitrification filters, but need to be submerged to prevent the existence of oxygen, so trickling or drum filters are out of the question (Lekang 2007). In artificial systems, such as RAS, this final step becomes demanding and expensive to implement into large scale operations, despite the innumerous advantages that it will bring in the long term, namely reduction of water usage, improvement of the water pH and cleaner water discharges into the environment.(Van Rijn, Tal et al. 2006).

4.1.3- Aeration/Oxygenation

Next to the removal of particles, aeration is a crucial step that needs to be accomplished before the recirculating water reaches the tank. It is normal for water in RAS and other systems to accumulate gases as the time goes by and the removal of these gases, or degassing, is facilitated by the process of aeration (Bregnballe 2015). The most common gas that need to be maintained is the dissolved oxygen and additionally remove CO_2, mainly released from organism's respiration and from the biofilter, .N_2 may need to be removed if the process of denitrification is present in the system. At high concentrations, free nitrogen can cause bubble disease in fish, also called diving disease, and carbon dioxide can be toxic as well, having impacts growth and welfare (Lekang 2007). Moreover, under anaerobic conditions, Hydrogen sulphide (H_2S) can also be released and even the smallest concentration can induce death in a closed system (Bregnballe 2015). The purpose of an aeration system is to create conditions for the exchange of gases between water and air until they reach equilibrium. Since the concentration of N_2 and CO_2 are higher in the water, they will move into the air and oxygen, in higher concentration in the air will transfer into the water, taking place some oxygenation. An aeration system can have different

configurations if the desired process occurs, largely revolving around providing air into the water or providing water into the air (Lekang 2007). In BOGA, the most common aeration design is by creating a water cascade, forcing the air to contact with a larger surface area by gravity (**Fig. 17**) and by the use of air stones (**Fig 18**).

Figure 17 – Aeration method applied in most systems of the BOGA facility

To provide aeration through air stones, injection needs to occur to force the air to mix with the water (**Fig. 18**), by introducing into the water. The injection pressure will depend on the oversaturation needed or desired and the introduction can be done in several spots of the recirculating system (Lekang 2007). In BOGA, the aeration occurs inside the RAS, through several air diffusers normally located in the bottom of the tank. This way, localized air supply can be executed depending on the necessity and more sources of air can be provided if needed. Another benefit from providing oxygen rich air using this design is the gradient advantage, caused by providing oxygen in a below 100% oxygenation normally in the bottom of the tank. The use of a diffuser will also enhance the oxygenation process, since most of them are designed to create small bubbles to increase the surface area and assist gas transfer (**Fig. 19**).

Figure 18 – Aeration method applied in BOGA: Air stones place on the edge of an air providing system

Figure 19– Aeration in BOGA: different designs for the air stones.

Even though aeration helps oxygenation, introduction of oxygen to reach or even surpass the equilibrium (100%) is also an option to keep levels of O_2 elevated for the respiration of organisms and to reduce water waste, decreasing the necessity for water renewal (Bregnballe 2015). In most cases, pure oxygen is introduced in the system to ensure a good oxygenation and most of the times it is recommended to have an aeration system before the oxygenation, since the water will reach equilibrium levels before reaching the oxygenation system and consequently exceed normal diffusion levels (Lekang 2007). While pure oxygen injection is guaranteed to succeed and therefore must be considered as a way to reduce costs, there is a real risk of surpassing recommended oxygen levels in the water if not controlled properly. It was revealed that high concentrations of oxygen (150 to 200%) can cause some negative effects on organisms, for instance gill damage in some fish (Dong, Zhang et al. 2013).

Lastly, the transport of air through the BOGA facility is made by an air pump located in the top of the building, being distributed by series of pipes located in the ceilings of each floor (**Fig. 20**). When a new aeration source is needed, the process is simple: an air diffuser is connected to an air tube and consequently, the air tube is connected to an air pipe by a valve that simply opens or closes upon turning.

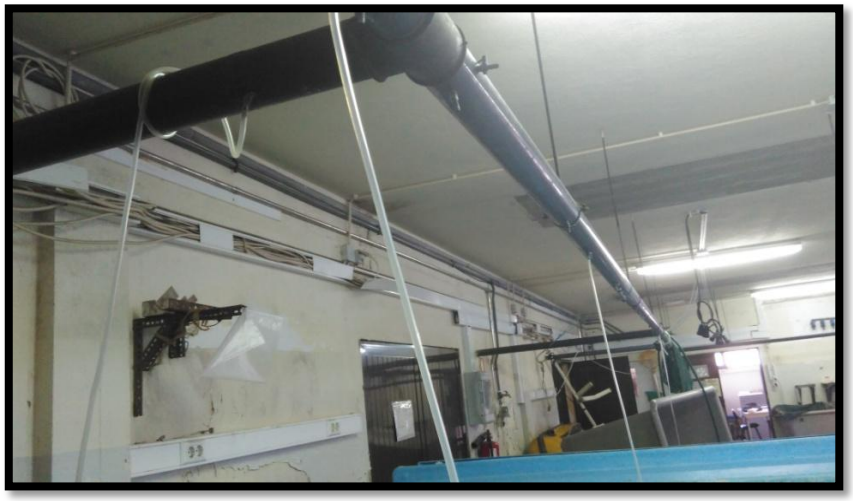

Figure 20 – Distribution of air pipes across the BOGA ceiling.

4.1.4- Physicochemical Treatments

In some situations, the reliance on biological treatments only for the removal of TAN and nitrate ions is not sufficient and can even bring some constraints. The susceptibility to temperature variations, the long activation time and slow recovery time in case of bacterial population decline can bring to the discussion other options (Gendel and Lahav 2013).

Ion exchange is a relatively simple process used, especially in aquaculture. The functionally is based on the fact that some molecules have the capability to attract others due to the charges of each one, and “exchange” them with molecules that are less attracted (Lekang 2007). Depending on the desired substance to retrieve from the system, a substance, usually a resin like material, is planted in contact with the water to perform the exchange process, existing the possibility to regenerate the substance later (Mook, Chakrabarti et al. 2012). For instance, if the desired molecule to be removed is ammonia, a cation exchanger is needed since NH_4^+ is positively charged and the substance capable of doing the exchange is, for example, mineral called zeolite (Lekang 2007).

Adsorption is another popular process for removing certain substances of the water. The definition of adsorption is described as the adhesion of substances, a gas or a liquid, to a solid surface and the effectiveness is largely dependent of the interfacial

area (Rouquerol, Rouquerol et al. 2013). The most commonly used adsorption material is the activated carbon, an adsorbent with a large and porous surface area, good at resisting temperature variations and low acid/base reactivity (Mook, Chakrabarti et al. 2012). Made from the activation of any carbonaceous matter, it has a high adsorption and regeneration capacity. It has several applications, being especially effective against metals (Goher, Hassan et al. 2015), but also micro pollutants (Mook, Chakrabarti et al. 2012) and non-degradable organic substances, namely pharmaceuticals (Ferreira, Calisto et al. 2016).

Another method, in most cases more expensive, is the reverse osmosis (RO). RO separates dissolved solutes by passing the water through a semi permeable membrane, also described as a “diffusion-controlled process” due to the control of the solution-solution mechanism (Wenten 2016). Reverse osmosis has the ability to not only remove ions, but also organic chemicals and proteins, while having high permeability to some ions and being environmentally friendly (Mook, Chakrabarti et al. 2012). Specifically, the use of RO for the removal of ammonia in RAS has been investigated with excellent results: the removal rate was between the ranges of 90% to 97% (Hurtado and Cancino-Madariaga 2014). The disadvantages are the energy costs, the occasional requirement for additional treatment and extreme caution with the membrane responsible for the whole operation. Additionally, the concentrate leftover from the RO filtration needs to be treated due to the high concentrations that, if released untreated, can lead to eutrophication and poor water quality (Wang, Wu et al. 2016).

4.1.5- Disinfection Methods

Beyond the removal of substances that could decline the quality of the water, sometimes the elimination of infectious organisms is also necessary. The origin of these pathogens can come from any direction: The water that is provided into an aquaculture or research facility may not have the best conditions; transfer between systems by any reason; introduction of pathogens by external people or even the facility workers. Occasionally, even the conditions in the water can allow the growth of pathogens, namely coliforms and heterotrophic bacteria (Gullian, Espinosa-Faller et al. 2012). After a infectious organism enters a RAS, the removal difficulty increases due to the constant water reuses and consequently the incorporation of pathogens in the biofilter can ensure the survival of the pathogen (Gonçalves and Gagnon 2011). Disinfection can be made by chemical or non chemical methods, although the use of chemical products, are being severely restricted due to the low number of approved chemicals, the high cost of production and the limited effectiveness due to the increasing bacterial resistance (Lakeh, Kloas et al. 2013). However, all these disinfection methods need to be located after the filtration processes, mostly because the presence of particles in the water reduces the effectiveness.

4.1.5.1- UV Radiation

UV light is an electromagnetic radiation that can be use effectively in the process of disinfection. It is especially successful between the wavelengths of 240 and 280nm, where the germicidal action takes place, damaging the genetic material and consequently leading to inactivation and death (Gullian, Espinosa-Faller et al. 2012) (Lakeh, Kloas et al. 2013). It is mostly used to prevent bacterial, fungal and viral diseases, despite viruses and other organisms, namely ciliates, nematodes and crustaceans, requiring a higher exposure dose to be damaged. The success of the UV disinfection will depend on different factors: water transparency, recirculation flow rate, microorganisms characteristics and wavelength applied (Gullian, Espinosa-Faller et al. 2012, Lakeh, Kloas et al. 2013). Beyond that, the UV lamp observation and attention is also a priority in these systems, since the cleanliness, age, use, distance between the

organisms, intensity and duration of the exposure will greatly affect the disinfection efficiency (Lekang 2007).

The design of a UV system normally revolves around the fact that the water circulates inside the chamber where the lamps are located. The lamps are protected with a quartz glass to prevent from cooling and biofilm formation and generally there are reflectors to improve the irradiation efficiency if there is turbidity (Lekang 2007).

As stated before, the localization of the UV system in a RAS or any other aquatic structure must be located after filtration systems because the UV works better with low concentration of solids and DOM (dissolved organic matter) (Lakeh, Kloas et al. 2013). If there are particles circulating in the water as the UV system are working, these solids will create shadows where the UV radiation does not reach and allow the microorganisms to survive. Additionally, according to Rizzo, Fiorentino et al. (2013), the incomplete effectiveness of UV radiation against antibiotic resistant bacteria can even promote the resistance among bacteria in a receiving water from an effluent, in this case *E.coli*. Moreover, the high operation and energy costs, as well as the "resource-intensive nature" limit the use of UV radiation as a financial and environmentally friendly system (Malara, Mielke et al. 2017).

4.1.5.2- Ozone

Ozone (O_3) is a highly reactive allotrope of oxygen, thus unstable in relation to atmospheric oxygen (O_2) and regarded as a very powerful oxidizing agent. Being unstable, it degrades into oxygen rapidly and thus ozone needs to be produced in the facility. Considered very toxic to any forms of life, it is a colorless gas that can be used as disinfection instrument (Powell and Scolding 2016). It is applied in various stages of water treatment to remove any microorganisms that can cause infections to animals by causing damage to the cell membranes and nucleic acids, causing its eventual inactivation (Lekang 2007, Powell, Chingombe et al. 2015).

Moreover, the disinfection properties are enhanced when ozone is in contact with seawater, largely because of the presence of bromide ion (Br^-). The reaction of this two compounds will created brominate compounds with disinfectant abilities (Gonçalves and Gagnon 2011). Other factors influencing the efficiency of ozone

include temperature, pH, flow rates, water quality, bacterial aggregates that can create protective shells, concentration and time of exposure (Powell and Scolding 2016).

Beyond their disinfection capacities, ozone can also assist in the improvement of the quality of the water by enhancing flocculation of any organic matter circulating in the treated water, which will lead to a superior filtration and skimming of colloids and suspended material (Schroeder, Croot et al. 2011). Additionally, ozone application can also oxidize yellow substances, normally non-biodegradable, to improve the visibility of the water and reacts with nitrite, transforming into nitrate.

The treatment with ozone can be done constantly, several times a day or by just introducing one time per day, normally associated with the feeding habits (Gonçalves and Gagnon 2011). This is mostly because of the ozone properties referred above, as they can also improve water quality. Therefore, the introduction into the system is done in a similar way as the aeration, demanding a pressure that can mix the water with the gas, usually with the use of a venturi. Also, the location where the ozone will be mixed and the retention time between the water and gas are equally important when designing an ozone system (Lekang 2007). Some systems introduced the gas in an inlet pipe while others use a retention tank to achieve a higher retention time. All these design choices will depend on the ozone dose needed and the characteristics of the aquatic system.

Although there are several advantages to the application of ozone as a disinfection agent, there are also some constraints that need to be considered. The production of bromamine compounds by reacting with O_3 can create by derivates that are toxic and carcinogenic (Schroeder, Croot et al. 2011, Powell, Chingombe et al. 2015). Due to the high oxidation potential and risk of byproducts toxicity, most facilities isolate the use of ozone and also the removal of residual ozone before reaching any system containing organisms (Powell and Scolding 2016). Byproducts can be removed by activated carbon and UV radiation, which is one of the reasons why sometimes a combination of UV radiation and ozone exist. A combination of both disinfection systems produces highly reactive hydroxyl radicals, which elevates the power of oxidation (Bustos-Terrones, Rangel-Peraza et al. 2016). Despite the mutual benefits, these dual systems are applied when there is the need for highly restricted biosecurity, but sometimes the costs required for this implementation are too great for ever compensating the investment.

4.1.5.3- Chlorine

The use of chlorine-based substances as disinfection instrument has been applied for a very long time, mostly due to high efficiency and the low cost. Hypochlorite, chloramines and chlorine dioxide, but also chlorine gas, are some of the most commonly used chlorine-based disinfection agents applied in water (Simon, Berdalet et al. 2014). These compounds, when in contact with organisms, have the ability to break organic molecules, but the inactivation/death itself takes time and enough contact time between the organism and the disinfection agent is necessary. This is mostly because the chlorine-based agent must dissociate in the water, diffuse through the cell walls and reach certain enzymes to neutralize them (Lekang 2007).

When applying chlorine disinfectants, there is a risk of creating toxic byproducts, namely trihalomethanes and other substances (Rizzo, Fiorentino et al. 2013, Watson, Shaw et al. 2012). This formation of toxic substances is mostly due, just like ozone, by the existence of bromide compounds that are more prevalent in seawater than freshwater, reacting and forming substances that are carcinogenic, mutagenic and are difficult to remove (Simon, Berdalet et al. 2014). Therefore, areas where chlorine-based agents have been used must be thoroughly cleaned to wash away any residues.

In BOGA, the use of chlorine-based agents is relatively common for cleaning any contaminated material and even for the disinfection of entire aquatic systems. If a system is being deactivated, the main components are disassembled and the rest remains in a circulation of water mixed with bleach and hospital disinfectant (**Fig. 21**). After circulating for some days, the system is washed meticulously and is prepared for a sanitary break of several days. The main components are also well cleaned and disinfected to remove any debris and pathogens that may remain from the previous stock.

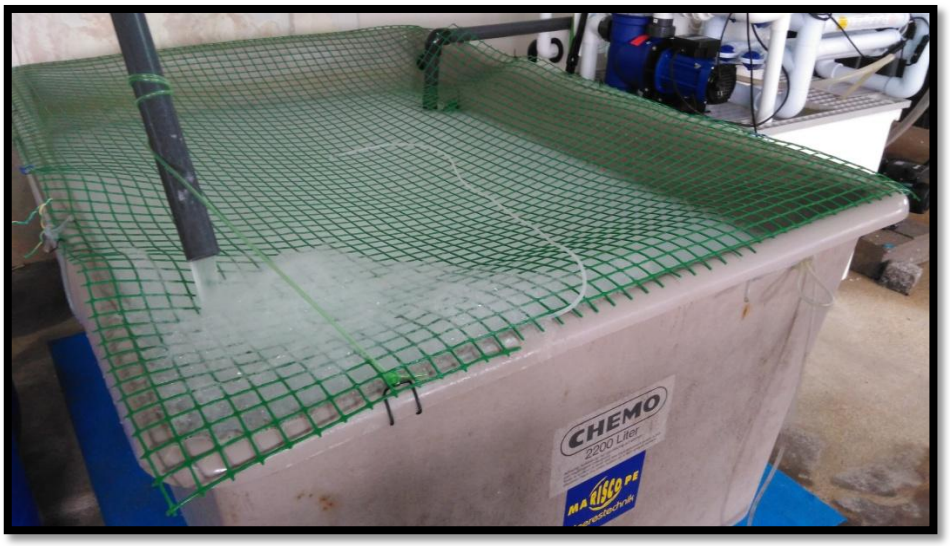

Figure 21 – Quarantine tank being disinfected by placing bleach and hospital disinfectant in circulation

4.1.6- Water Quality

The basis of water quality gathers the entirety of chemical, biological and physical conditions that have an effect on the growth and welfare and, as such, some water parameters need to be monitored constantly since it is the environment where the aquatic organisms live (Mallya 2007). Organisms will respond to poor water quality by stress which, if not controlled, can lead to other complications, namely a more elevated risk of a disease outbreak. Therefore, when environmental stress factors arise, the equilibrium tips in favor of pathogens, because the disturbances will “extend the adaptive responses of the animal beyond the normal range or affect the normal functioning to such an extent that chances of survival are significantly reduced” (Pillay and Kutty 2005).

Furthermore, the great diversity of aquatic organisms leads to a diverse range of parameters to control in a RAS. This means that the ideal parameters for a number of species may not be suitable for other species and the same goes for different stages of organisms. Normally, earlier stages require the best possible conditions that an artificial system can provide (Lekang 2007). In a RAS, the main parameters to control are: oxygen; carbon dioxide; temperature; pH; nitrogen compounds (ammonia, nitrite and nitrate); and salinity.

4.1.6.1- Dissolved Oxygen

Oxygen is an essential component of the aerobic respiration and therefore is a parameter to be controlled closely. Being a gas with low solubility in water, the amount of dissolved oxygen (DO) is primarily influenced by temperature and salinity, but also partial pressure in the atmosphere and other substances present in an aqueous environment (Mallya 2007). In warmer water, the amount of DO present is reduced in relation to colder water and the existence of substances in the water also reduces the concentration of dissolved oxygen. The solubility is normally expressed in mg/L (Lekang 2007).

Despite the oxygen content in the water determining its accessibility, the rate of diffusion across the gills is also central for the oxygen to reach the organisms blood and generate energy for body maintenance, movement and biosynthetic processes. This diffusion depends on the gill area, the partial pressure of oxygen in gills and the diffusion distance in the epithelia (Mallya 2007). According to McDaniel, Sugiura et al. (2005), maintaining high levels of DO in the system leads to increase feed consumption and efficiency, higher metabolism and consequently superior growth. If the dissolved oxygen levels are not sufficient, animals can behave abnormally. In fish, they start to become lethargic and start eating less due to the reduction of movement to save energy. In some extreme cases, fish can be seen reaching for the surface struggling to breathe and low DO levels can also compromise fertilization and larvae survival further down the road (Mallya 2007).

In RAS, the oxygen provided is not only for the organisms cultured, but also for the biological filter, which contains bacteria that need oxygen (Losordo, Masser et al. 1999). The lowest DO recommended for any system is 6mg/L and in the biological filter the nitrification efficiency reduces drastically if the dissolved oxygen concentration reaches below 2mg/L (Losordo, Masser et al. 1999). Therefore, DO saturation levels in water must always be above 80% (6mg/L) to provide a good environment and never drop below 30% (2mg/L), or the fish will eventually die (Mallya 2007). Finally, dissolved oxygen levels must be tightly controlled after feeding since the respiration will increase significantly and reduce DO concentration.

In case of pure oxygen use, there is a risk of the water becoming hyperoxic . Hyperoxia means that the water is supersaturated, reaching levels above 100% DO

saturation and bringing complications to the maintained organisms. Fish exposed to this water form bubbles in the blood that block capillaries and in case of a major artery block, the organism will die rapidly (Mallya 2007). To prevent oversaturation of the water, proper mixing and agitation of the water will allow the excess oxygen to be released into the atmosphere (Losordo, Masser et al. 1999).

4.1.6.2- Carbon Dioxide

As referred beforehand, carbon dioxide (CO_2) is a residual product of the metabolic processes of organisms, and therefore, in recirculating systems, accumulation of this molecule is going to be inevitable. Not only cultivated organisms produce it, but also microorganisms present produce acids, resulting in elevated levels of CO_2 (Losordo, Masser et al. 1999, Good, Davidson et al. 2010). One of the consequences of high carbon dioxide levels is the pH drop, which can result in complications related with respiration (Helfrich and Libey 1991). It is also demonstrated that high CO_2 concentrations can directly influence the feed intake, growth rate and development of nephrocalcinosis, resultant of high levels of carbon dioxide in the blood causing acidosis and impairment of oxygen-hemoglobin binding (Good, Davidson et al. 2010).

In RAS, for fish, it is advisable for the values to be between 10 and 20mg/L for good organism welfare, and never surpass 30 mg/L (Helfrich and Libey 1991, Good, Davidson et al. 2010). Nevertheless, the tolerance and optimal ranges need to be consulted for each species due to the enormous aquatic diversity. Additionally, the tolerance limit will vary due to the interaction of CO_2 with other factors, for instance oxygen saturation, alkalinity, higher solubility of metals and temperature (Good, Davidson et al. 2010).

CO_2 removal can be done by aeration, creating more contact area between the atmosphere and the water (consult **4.1.3-Aeration/Oxygenation** for more information).

4.1.6.3- Temperature

Biochemical and metabolic processes of cultivated organisms and living bacteria in the system are highly affected by temperature (ºC) and, in case of fish, no other parameters affects the maturity and growth as much as the temperature (Bregnballe 2015). It is also important to notice that temperature affects other water quality parameters. Most organisms have a range of temperatures where they tolerate the conditions and a narrow range where optimal growth is found. As the temperature rises, the growth will proportionally increase, although surpassing temperatures above the optimal range can have negative effects (Van Ham, Berntssen et al. 2003).

In general, cold water species cultivated in RAS have a temperature range of 10ºC to 18ºC, intermediate temperatures species between 18ºC and 25ºC and warmer species between the ranges of 25ºC to 32ºC. This is important to understand the factors when designing the recirculating system: the temperature tolerance levels of an aquatic organism and the optimal growth; the components required in the RAS that is going to contain the organisms, for instance the presence of heating or cooling equipment; and understanding the optimal nitrification levels to have an efficient biological filter (Helfrich and Libey 1991). The advantage of changing the water temperature is that water has a high specific rate, meaning that heats and cools slowly and can save energy costs after achieving the desired temperature.

Furthermore, temperature variations can bring complications to a stable aquatic system. Higher temperatures can affect enzyme and protein metabolic processes, becoming denaturated, as well as reaching optimal ranges for infectious microorganism to prosper and cause a disease outbreak. Warmer water reduces the dissolved gases content, reducing dissolved oxygen concentration (Lekang 2007). Beyond that, special attention needs to be given to sudden temperature changes, also called temperature shock. If this happens, organisms become stressed and are more susceptible to any pathogens.

4.1.6.4- pH

pH is defined by the determination of acidity and alkalinity in a certain aqueous solution, as a rule measured by a range of numbers between 1 and 14. pH 7 represents the middle ground and therefore neutrality; above the neutral number it is considered a alkaline solution and below it is considered a acidic solution (Lekang 2007). The measurement itself is the negative algorithm of the hydrogen ion concentration.

In aquatic systems, organisms normally tolerate pH in the range between 6.5 and 9, despite the optimal range being different for each species (Losordo, Masser et al. 1999, Lekang 2007). For seawater organisms, the range goes from 7.85 to 8.5 but, in a RAS, the pH tends to decrease as the time goes by due to the carbon dioxide excretions and the bacteria nitrification (Losordo, Masser et al. 1999, Abbink, Garcia et al. 2012, Bregnballe 2015). Nitrification also has an optimal pH range of activity, which goes from 7 to 8 and if the pH levels go down below 7, nitrification is inhibited (Losordo, Masser et al. 1999). Also, as referred above, pH and temperature have an influence on un-ionized ammonia levels, as higher alkalinity will lead to an increasing concentration of the more toxic ammonia form NH_3. Another problem related to the pH drop is the higher solubility of metal ions, for instance aluminum. The low pH transforms these heavy metals into more toxic forms, affecting the organisms in the system.

To increase the pH of an aquatic system, whose water is becoming acidic, a base needs to be added to remove the excess free hydrogen (H^+). The most common buffers used to raise the pH levels are sodium bicarbonate (Na_2HCO_3) and calcium carbonate ($CaCO_3$), but also calcium hydroxide ($Ca(OH)_2$) and sodium hydroxide (NaOH) (Losordo, Masser et al. 1999, Lekang 2007, Bregnballe 2015). For a more economic solution, partial water exchange of the system water can also assist in the raise of pH. In freshwater, problems with low pH can be solved by the introduction of small quantities of saltwater, which contains some carbonate and bicarbonate ions that can raise the pH, increase buffering capacity and enhance conductivity.

Nevertheless, sudden changes in pH are not advisable as it can induce lesions or cause stress in the cultivated organisms and damage the nitrifying bacteria culture. Exposure to low pH can also greatly damage the organisms. Reports of lesions in the

skin, eyes and gills, as well as creating osmotic problems by leakage of Na^+ and Cl^- have been described by (Lekang 2007).

4.1.6.5- Salinity

The concentration of salts in the water is defined by salinity and can be measured by PPT (parts per thousand) or mg/L. Salts are inorganic molecules that can easily dissolve into ions when In contact with the water. In saltwater, the main ions are Na^+ and Cl^-, forming NaCl in higher numbers and $MgCl_2$ in lower amounts, while freshwater is primarily composed of carbonates and HCO_3^-(Boeuf and Payan 2001).

It is important to understand the salinity and the related processes in order to perceive the osmoregulations occurring in the aquatic medium. Fish and other aquatic organisms spend between 20% and 50% of their total available energy on controlling the salt balance between their body and the exterior, revealing a large portion of energy devoted only to osmoregulation (Resley, Webb et al. 2006). In some cases, reducing the energy expenses on osmoregulation by controlling the exterior salinity can bring advantages to the cultivated organisms, as they can dedicate the remaining energy onto other processes, namely growth not only on marine species but also freshwater species(Boeuf and Payan 2001, Resley, Webb et al. 2006). According to Boeuf and Payan (2001) it appears that some marine species grow more on lower salinities and some freshwater species have higher growth rates on elevated salinities.

Salinity also plays an important role on feeding, as several reports have found that salinity interferes with consumption rates and the ability to digest feed and in some cases, like the greenback flounder (*Rhombosolea tapirina*), can directly affect egg fertilization and incubation (Boeuf and Payan 2001).

Given that salinity is a relatively easy parameter to manipulate, it can be used to control pathogens since some microorganisms cannot survive salinity changes and also cause the fish to produce more mucus, removing any external parasites that are stressing the organism. Despite this, some attention needs to be given to the biological filter, as nitrification in the presence of high levels of salinity is reduced due to the existence of chloride ions that affect bacteria growth, resulting in faster nitrification in freshwater than in saltwater (Lekang 2007).

Figure 22 – Salt used in the creation of artificial saltwater in BOGA.

4.1.6.6- Ammonia

The accumulation of ammonia in aquatic systems, specially RAS, is the result of organism metabolism byproducts and any uneaten food that remains in the water (Francis-Floyd, Watson et al. 2009). The excretion is made through the gills membranes and a small amount is excreted in the urine, under the form NH_3, which is the un-ionized form of ammonia and considered the most toxic (Losordo, Masser et al. 1999, Lekang 2007). In water, it is constantly in equilibrium between NH_3 and NH_4^+, meaning that the decreasing in one form will be balanced by transfer of the other (Helfrich and Libey 1991). This equilibrium between the two ammonia forms will depend on temperature, pH and also on salinity.

The presence of a biological filter in a RAS allows the removal of ammonia, transforming into other nitrogen compounds that are less toxic: aerobic bacteria will first transform ammonia into nitrite and later nitrite into nitrate (consult **4.1.2-Biological Filtration** for more information). If the ammonia levels are too high for the biofilter to handle, the first thing to do is to reduce feeding or even stop for small period of time. Additionally, water exchanges periodically will help stabilize the ammonia levels and reduce stress levels.(Francis-Floyd, Watson et al. 2009)

If the ammonia levels are not reduced quickly to acceptable levels, the water will eventually become toxic (Losordo, Masser et al. 1999, Lekang 2007). Different ammonia levels will have different reactions on organisms, despite being observed effects as low as 0,06mg/L (Losordo, Masser et al. 1999). High concentration of ammonia causes the organisms to become stressed, becoming more susceptible to bacterial infections and other pathogens. Furthermore, it was also reported to cause damage to the gills and other tissues (Francis-Floyd, Watson et al. 2009).

Temperature also has a close relation with un-ionized ammonia and pH. With increasing temperatures and low pH, the un-ionized form of ammonia nitrogen, also described as the more toxic form, increases too, leading to higher toxic levels of ammonia for the organisms, as described in **table 2**. Concluding, higher temperatures improve greatly the growth rate of organisms but also brings some disadvantages to the equation.

	Temperature (°C)								
pH	16	18	20	22	24	26	28	30	32
5.0	99.3	99.2	99.2	99.1	99.1	99.0	98.9	98.9	98.9
5.5	97.7	97.6	97.4	97.3	97.1	96.9	96.7	96.5	96.3
6.0	93.2	92.8	92.3	92.0	91.4	90.8	90.3	89.7	89.1
6.5	81.2	80.2	79.2	78.1	77.0	75.8	74.6	73.4	72.1
7.0	57.7	56.2	54.6	53.0	51.4	49.7	48.2	46.6	45.0
7.5	30.1	28.9	27.5	26.3	25.0	23.8	22.7	21.6	20.6
8.0	12.0	11.4	10.7	10.1	9.6	9.0	8.5	8.0	7.6
8.5	4.1	3.9	3.7	3.4	3.2	3.0	2.9	2.7	2.5
9.0	1.3	1.3	1.2	1.1	1.0	1.0	0.9	0.9	0.8

Table 2 – Adapted from Losordo, Masser et al. (1999). Table demonstrating the influence of temperature and pH on the concentration of un-ionized ammonia.

Since ammonia is colorless and odorless, detection can only be made by testing the water. Test for ammonia must always be performed on the system water and eventually on the source water, since ammonia can be present on the origin (Francis-Floyd, Watson et al. 2009).

4.1.6.7- Nitrite

Nitrite (NO_2^-) is a nitrogen compound that derives from the process of nitrification in the biological filter, where there is a transformation from ammonia to nitrite. The concentration of nitrite also needs to be controlled due to the toxicity, normally without surpassing the values of 0,5mg/L in a RAS (Helfrich and Libey 1991, Losordo, Masser et al. 1999, Lekang 2007), although it also varies depending on the species being used.

If nitrite levels surpass tolerance levels, it can cause a disease called “brown blood”(Losordo, Masser et al. 1999). It happens when hemoglobin is oxidized by nitrite, turning into methemoglobin, a respiratory pigment that cannot transport oxygen and turns the blood brown due to its coloration (Helfrich and Libey 1991). As a consequence, the fish behave as if there is a deprivation of oxygen. To reduce the nitrite concentration, the introduction of a salt can help, mainly due to the capacity of chlorine ions to block nitrite toxicity (Francis-Floyd, Watson et al. 2009). Normally, the use of salts is made at the proportion of 10:1, meaning 10mg/L of salt, usually sodium chloride, for every 1mg/L of nitrate. Another solution is by applying partial water changes in the system at risk.

The detection of high nitrite concentration can indicate that the biological filter may not be properly working or the biological biomass and respective excretions are too much for the biofilter to deal with (Losordo, Masser et al. 1999). In any case, it is imperative to check the parameters of the biological filter, most notably the dissolved oxygen.

4.1.6.8- Nitrate

The final product of nitrification is nitrate, where nitrite is oxidized in the biological filter by *Nitrobacter bacteria* (Helfrich and Libey 1991). Normally, since the toxicity of nitrate requires high concentration levels, the parameter can be overlooked and therefore has been considered non detrimental to aquatic organisms in the past, as some RAS could reach the concentration of 1000mg/l (Monsees, Klatt et al. 2016). However, it is been reported in some species that high nitrate concentrations can

indeed have some effects as low as 57 mg/l, and in juveniles as low as 5mg/l, despite taking into account the resistance of some organisms in relation to others.

If a system contains a denitrification system or performs regular water exchanges, it is very difficult to surpass nitrate tolerance levels (Losordo, Masser et al. 1999). If the nitrate levels continue to rise, it may start to impact the organisms in the system. As said by (Monsees, Klatt et al. 2016) the absorption of NO_3^- occurs by ingestion and the consequence reduction of nitrate to nitrite in the stomach. After nitrite is absorbed, oxidation of haemoglobin takes place in the blood, resulting in the same consequences as NO_2^- intoxication, which is the formation of the respiratory pigment methaemoglobin. As referred previously, this pigment turns the blood brown and cannot transport oxygen, increasing oxygen deprivation (Monsees et al., 2016).

5- Tasks performed at BOGA -CIIMAR

As part of my internship, I performed several tasks that were essential to my formation in the husbandry of aquatic species resident in BOGA. There were some tasks that needed to be done daily while others could be performed in a larger time spectrum. Nevertheless, if a task was required to be completed outside the normal range due to an emergence or for any other reason, it would be performed. During the internship, any decision I made or was asked to do in BOGA was questioned in order for me to understand the reasoning behind that decision, which facilitated my learning process. This way, whenever I performed an action, I would question and reflect on the consequences of that same action, as the tasks done in the facility would directly affect living organisms.

In this work, the tasks are divided between daily, weekly, monthly, irregular tasks and special tasks. Furthermore, every system in the BOGA facility had a spreadsheet where all the parameters and activities performed were registered in order to maintain a certain level of organization and observe the chronological evolution of the system.

5.1- Daily tasks

- **Observation of aquatic organisms and systems**
- **Replacement and washing of mechanical filters**
- **Measurement of parameters in aquatic systems**
- **Measurement of parameters in the biological filters**

5.1.1- Observation of aquatic organisms and systems

Upon entering the facility, the observation of aquatic systems was the most significant task to be performed before any other. The system verification was made in all aquatic systems within the common area, independently of the responsibility of the

BOGA`s team over a system. Specific rooms could also be verified at the request of researchers.

The purpose of this task was to detect any discrepancy in the aquatic systems and observe the behavior of the organisms. From one day to the other and even during the course of the day some components of a RAS system can fail and endanger the organisms: a pump that turned off or was clogged; aeration that may not be working; mechanical filter could be clogged and overflow. If these components fail, it could cause fatalities to some more susceptible organisms. It was important to check any components that were powered by electricity, for instance pumps (**Fig. 23**), skimmers, heating systems (coolers (**Fig. 24**) and heaters), and disinfection systems (ozone and UV radiation) in order to detect if they were disconnected from the electric font or were not functioning properly.

Figure 23 – Most commonly used RAS pump in BOGA

Figure 24 – Example of a cooler.

The next step was to observe the clogging levels of the mechanical filter and from every pipe or tube in the aquatic system in order to identify if any debris could prevent the normal water flow. Any reduction in the water flow could jeopardize the biological filter and decrease water quality. Water color is a visual evidence that can be detected early to identify poor quality, generally caused by the existence of suspended solids in the water.

Observation of organisms was also a significant process to perform, as many problems could not be revealed upon visualizing system components. Some abnormal

behaviors can indicate stress, meaning that water parameters may not be stable, whether it is related to the system equipment or water quality. Furthermore, organism's observation could sometimes reveal a fatality, which needed to be removed as quickly as possible so that the body would not decompose and consequently deteriorate water quality.

5.1.2- Replacement and Washing of Mechanical Filters

The use of screen filters and glass wool in a RAS is a common practice for suspended solids removal, especially in BOGA. As referred before, one of the disadvantages of this mechanical filtration is the labor involved, particularly if there is a use of a material with smaller mesh. Depending on the system and organisms involved, the type of material can be glass wool or screen filter for a more refined filtration.

Replacement of mechanical filters needed to be done every day, as they would be filtering all night and be clogged in the morning (**Fig. 25**). If there is a clogged material, the efficiency of the filtration reduces drastically and deteriorates water quality. Furthermore, substitution of the mechanical filters could occur more than one time a day depending on the system biomass and species, which could clog at a faster pace. Lastly, the unclean filter was placed in a bucket and transported into the cleaning room in order to prevent any microorganism's dispersal in the facility.

Figure 25 – Example of an unclean mechanical filter that needs to be replaced.

After retrieving the unclean filters carefully, the material needed to be cleaned before going into the washing machine. Normally, pressured water was enough to remove the bigger debris from the mechanical filter. After that, the material was placed in the washing machine where it was washed and disinfected by a mixture of bleach and hospital disinfectant. After putting the filter to dry, they were ready to be used again.

5.1.3- Measurement of parameters in aquatic systems

Most systems placed in the common area had a daily verification of some water parameters, while quarantines needed to have the full analysis of the water quality. While parameters were equal among most systems, others could have different conditions that would be dependent on the researchers requests. One of the first measurements was the pH and temperature, which normally was determined by the same device, a multi-parametric probe (**Fig. 26**). pH levels eventually drop in a system due to the respiration and degradation of organic matter. Low pH could reveal a high organic matter content, which would mean that a partial water exchange was needed. If the pH was constantly dropping, the implementation of a skimmer in the system would be considered. Nevertheless, a verification to detect the presence of dead organisms would also be necessary, as decomposition can bring the pH down. Additionally, temperature was always an important parameter to measure, especially because fish do not control their body temperature and therefore are extremely influenced by the water temperature. Moreover, thermal equipments could be used in some aquatic systems in days with very high or very low temperatures.

Figure 26 – Multi-parametric probe

Afterwards, the dissolved oxygen was also determined by a probe to discover if the system was well oxygenated. It was particularly important on high temperature days, where the levels of dissolved oxygen could drop drastically. In that case, an additional oxygen source could be implemented.

Subsequently, the salinity could be measured daily with the use of a refractometer (**Fig. 27**). If the salinity levels were outside the normal range, which was normally 35 ppm, the adding of water could be prepared by simply adding saltwater if under the normal levels or freshwater if superior to the normal levels.

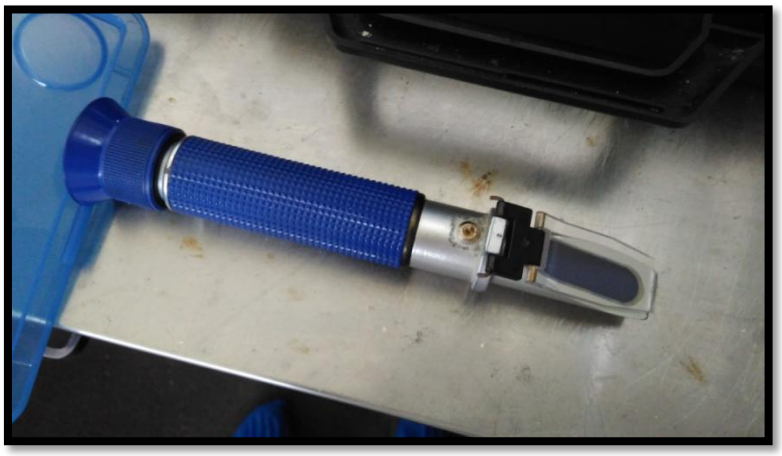

Figure 27 – Refractometer

The chemical tests to determine the levels of ammonia and nitrite were performed only one day a week in the common area systems, except in extreme cases. In those cases, corrective measures were performed and chemical tests were done daily until the aquatic system returned to acceptable levels. In contrast, chemical tests were done to quarantine systems every day, as newly arrived animals were still adapting to the new environment.

5.1.4- Measurement of Parameters in the Biological Filters

Alongside the measurement of parameters in the aquatic systems, biological filters parameters were also measured. Dissolved oxygen, temperature, pH and salinity were determined in the two existent biofilters in BOGA, one for saltwater and another for freshwater. From this four parameters, pH and dissolved oxygen were the most important to control, mainly because of their influence and the constant degradation of

ammonia. Dissolved oxygen needs to be maintained at high levels and pH needs to be above acidic levels or the nitrification reaction is impaired. The normal process of ammonia oxidation by bacteria will make the pH drop very rapidly if not controlled, and the adding of a buffer is sometimes necessary. The most common buffers used in BOGA are sodium bicarbonate ($NaHCO_3$) and calcium carbonate ($CaCO_3$).

The daily control of ammonia and nitrite was performed during some time in the internship to analyze the variations of the two parameters on the two biofilters and will be presented further below - Biological Filter Tanks. This task allowed me to understand how the biological filter maturates along the time.

5.2- Weekly Tasks

- **Measurement of Ammonia and Nitrite in BOGA Systems**
- **Analysis of Ammonia and Nitrite Values and Respective Responses**
- **Biological filters Maintenance**

5.2.1- Measurement of Ammonia and Nitrite in BOGA Systems

Weekly, the measurement of ammonia (NH_4^+) and nitrite (NO_2^-) was performed in the BOGA systems to understand the quality of the water, as these nitrogen compounds, especially ammonia, are highly toxic to most aquatic organisms. In BOGA, the measurement of nitrate is rarely done mainly because partial water exchanges are executed regularly, thus preventing the nitrate concentration from ever reaching toxic levels.

With the application of Palintest©, an equipment designed to test water quality, it was possible to measure the nitrogen compound levels. By testing ammonia in saltwater, a conditioner needed to be added previously to neutralize the existence of salts. It was a relatively simple colorimetric process, generally done by adding tablets into a sample of water and waiting for the color to develop (**Fig. 28**). Afterwards, the resulting colors were measured in a Palintest© multiparameter photometer (**Fig. 29**).

Figure 28 – Chemical Tests performed on several samples from different RAS.

Figure 29 – Palintest© multiparameter photometer.

Normally, the chemical tests were done on Wednesday, which is done on purpose to include any changes that could occur during the weekend, to observe the feeding influence on the ammonia and nitrite levels and to have sufficient time to do every corrective measure in the next days.

5.2.2- Corrective measures in RAS systems

Every time ammonia and nitrite values surpassed the normal spectrum, corrective measures needed to be taken to improve the water quality; otherwise the organisms would become stressed and could eventually die. In BOGA, the defined ranges for ammonia were 0,05mg/L and nitrite 0,5mg/L. One of the main responses to high levels of ammonia or nitrite was performing a partial water exchange. The exchange amount would depend on how high were the nitrogen compounds levels and therefore could dilute the water into safer concentrations. Another consideration when doing a water exchange was the temperature variations. The introduction of water needed to be done slowly due to the temperature differences between the water from the systems and the water source, to prevent thermal shock. Finally, if the levels stayed high even with some corrective measures, sometimes the best solution was to stop feeding the organisms during a small period of time for the system to recover and reach acceptable levels.

Some systems required a constant siphonage and therefore a partial water exchange, even without the measurement of parameters. Sometimes, if some tasks were not performed periodically and regularly, debris would accumulate in the aquatic systems and needed to be removed quickly to prevent deterioration of water quality (**Fig. 30**).

Figure 30 – RAS with excessive organic matter.

5.2.3- Biological filters Maintenance

Alongside measuring the nitrogen compound levels in the aquatic systems, the levels of ammonia and nitrite was also measured in the biofilters (**Fig. 31**). For a biological filter to work, ammonia needed to be provided to simulate the presence of organisms producing ammonia. NH_4^+ was provided in the form of ammonia chloride and the dose would depend on the efficiency of each biological filter. Together with ammonia chloride, a buffer (usually calcium carbonate) was also mixed to maintain pH levels above 7 in order to maintain the biofilter stable. Through the passing of days, the pH would continue to drop and thus more buffer would be provided until the ammonia reached low concentrations. Ammonia was controlled during the days to detect the degradation efficiency and when the time was right to introduce more ammonia.

Figure 31 – Saltwater Biological filter

5.3- Biweekly tasks

- **Maintenance and Feeding of Amphipods**

In a BOGA room, I had the responsibility of managing several aquatic systems containing amphipods (*Gammarus locusta*). There were four static systems, each one of them containing a significant number of organisms needing a total water exchange (**Fig. 32**). Since it was a static system, no filtration was used and only aeration was provided. Thus, total water exchange was performed on all systems twice a week, usually Tuesday and Thursday.

Figure 32 – Static systems containing amphipods.

Figure 33 – Different strainers used for filtration of water

Upon doing the water exchange, the water needed to be poured into two strainers, one to catch the feed (*Ulva spp*.) and larger amphipods and the other to catch smaller amphipods (**Fig. 33**). This way, a complete water exchange could be done without losing any organisms during the process. Before introducing the amphipods back into the system, pressure water was used to clean the substrate to remove any substances retained and prevent the formation of anoxia conditions, which could be very dangerous to a static system. After cleaning the four systems, feeding could be supplied. Usually, the feed was frozen *Ulva spp* provided in a generous amount whenever it was lacking.

5.4- Triweekly Tasks

- **Feeding of Animals in the BOGA Facility**

Another task for me to fulfill was feeding aquatic organisms in the common area and in some other rooms. The food was provided 3 days a week: Monday, Wednesday and Friday. Thus, the feeding time was spread as evenly as possible to reduce the feeding interval in the weekends, by feeding the last and first day of the week. The size, quantity and type of food would depend on the species, the life stage and the purpose of the system. In most RAS under the BOGA supervision, the purpose was to maintain the organisms, therefore only the essential quantity of feed was given. On the contrary, other systems existing in BOGA could have different purposes; hence a more appropriate feed could be given. During my internship, I feed a different number of species, detailed in the **table 3**.

Species	Life stage	Feed
European sea bass (*Dicentrarchus labrax*)	Juvenile; Adult	granulated
Gilt-head seabream (*Sparus aurata*)	Juvenile	granulated
European flounder (*Platichthys flesus*)	Adult	granulated
Small-Spotted catshark (*Scyliorhinus canicula*)	Juvenile; Adult	Frozen fish

Species	Life stage	Feed
Butterfly blenny (*Blennius ocellaris*)	Adult	Frozen fish
Zebrafish (*Danio rerio*)	Adult	flakes
Axolotl (*Ambystoma mexicanum*)	juvenile	Frozen fish

Table 3 – Different organisms fed in BOGA

5.5- Monthly Tasks

Every month some deeper cleaning needed to be made to the BOGA recirculating systems. As the time goes by, biofilm naturally starts to form in most fixed components, which could hinder the efficiency of a RAS system. Thus, pumps were removed from the system and opened, disassembled and cleaned to remove any debris. All the tubes and pipes within a system were also cleaned to improve the water flow. Additionally, skimmers were thoroughly cleaned, from the body where the bubbling formation occurred to the cup where the residues accumulated. Sometimes the skimmers cups would be cleaned more regularly due to the rapid accumulation of residues in some RAS.

5.6- Special Tasks

- **TMC© Filtration System Assembly**
- **Quarantines**
- **Other tasks**

5.6.1- TMC© filtration system assembly

As part of my formation, I helped in the assembly of a TMC© (Tropical Marine Centre) RAS filtration system with the purpose of being implemented in the quarantine zone (**Fig. 34**). The assembly of the system itself was straightforward, but the connections between the livestock tank and the TMC© tank revealed to be more intricate. The system was fully equipped: a main tank where the water was stored and the grid supporting the equipment; a mechanical filter composed of 4 bags; a fluidized sand filter containing oolitic coral sand; trickling biological filter tower; a skimmer with a venture system; and a UV equipment. During my internship, this system contained a stock of flounder (*Pleuronectes platessa),* which developed some problems along the way and are going to be discussed below.

Figure 34 – Partially assembled TMC filtration system with the main tank.

5.6.2- Quarantines

Quarantines exist in BOGA to isolate newly-arrived organisms during a period of time. By isolating, it allows the BOGA staff to understand the health state of the organisms and give time for adaptation to the new environment. During some days, I helped in the management of quarantine systems containing different aquatic species, namely the measurement of parameters and partial water exchanges. In order to work in quarantine systems, some strict rules needed to be followed. Some of the rules when dealing with quarantines were isolating not only the system components but also the water and material. Therefore, material used was specifically used only for quarantine and nothing else, reducing drastically the risk of spreading any pathogen that was carried by newly-arrived organisms. The same goes for the workers dealing with the quarantine: protection and hand disinfection was required before handling any other systems.

One particular quarantine case was important for me to understand the necessity of isolation before using the organisms, which was the quarantine of a flatfish, *Pleuronectes platessa,*

5.6.2.1- Case study

The flounders (*Pleuronectes platessa)* arrived at the BOGA facility and placed in quarantine in the 18th of January of 2017. These animals were captured from the wild and were transported in poor conditions into the facility, some of them even revealing some lesions. The quarantine had a TMC© filtration system fully equipped: separated tank with mechanical filtration, fluidized sand filter, biological trickling filter, skimmer and UV radiation, all connected to the livestock tank.

The parameters control was made daily, with the ammonia and nitrite values reaching high levels in the first week. This was expected, especially in newly-arrived quarantine systems, as the animals were more stressed and the fish biomass was relatively high. Furthermore, no food was given in the first days to control ammonia and nitrite levels because the biological filter was still maturing. Nevertheless, *Pleuronectes platessa* adaptation to artificial environments takes time, which means eating in the first weeks was difficult and uncommon.

After two weeks, the ammonia and nitrite levels continued elevated and almost daily water exchanges were needed. Due to the two powerful pumps and the UV radiation, the water temperature of the systems was always high and thus a refrigerator was added to decrease the temperature. In addition, mussel was given to see if the animals would eat, but they continued unresponsive.

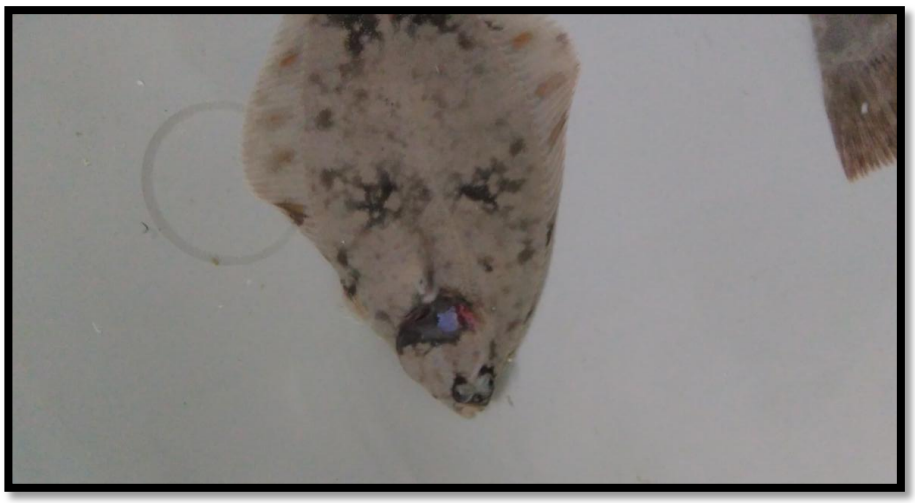

Figure 35 – individual infected and damaged by *vibrio spp*

Approximately one month after the introduction of the flatfishes, it was discovered that they had some sort of bacterial infection predominantly around the mouth. The ones that appeared to be infected and damaged were removed from the system and isolated (**Fig. 35**). At this time, ammonia and nitrite levels were always high and, for that reason, daily water exchanges became necessary.

After the first infection occurrence, more infected animals would continue to emerge and be separated accordingly throughout the following weeks. The ones that were first separated started to die after some days. After some tests, it was revealed that the flatfish stock developed a *Vibrio spp* infection, which means that treatment was very difficult to implement. Therefore, the stock of *Pleuronectes platessa* was euthanized some days later due to the visibly compromised health state. After the animal's removal, some water samples from different places in the systems were tested of *Vibrio spp*. So it was possible to confirm that the UV equipment was properly operating.

The development of *Vibrio spp* in the quarantine system may have occurred due to the high densities of fish together with the susceptibility of the animals to captivity. Additionally, the circumstances the animals came into the facility may have contributed to this disease outbreak. With these conditions, the animals would be permanently stressed and therefore became more susceptible to a pathogen infection. The animals stress would also cause the production of more substances and excretions, deteriorating the quality of the water and thus cause even more stress. Finally, it can be said that the bacterial overload was too much for the UV equipment to handle.

5.6.3- Other tasks

In BOGA, I performed other tasks that were somewhat infrequent to perform, although the importance was also noteworthy.

From time to time, saltwater filters from the water reservoirs would become clogged. For this reason, filter substitution needed to be performed immediately after the detection to ensure that the saltwater source that was provided across the facility

would not cease. Two filters needed to be replaced: one with larger mesh to catch the larger particles; the second with smaller mesh to catch smaller particles.

Cleaning of biological filter can also be significant to improve the efficiency. Through the passing of time the biofilm continues to grow until the biological filtration starts to be hindered, where the formation of anaerobic zones can also occur. To remove the excess biofilm, bio balls needed to be washed with water from the system being cleaned. By using this method, it ensured the survival of the nitrifying bacteria.

Another task that was demanding but needed to be done was the disinfection of bio balls. New and clean bio balls were needed, mainly to be introduced in the biological filters tanks in order to gain the nitrifying bacteria biofilm, which would take approximately one month. To disinfect, bio balls or other any high surface area material was dipped in water containing bleach and hospital disinfectant. It would remain one or more days in these conditions before being thoroughly washed and placed in the biological filters tanks for activation.

I was also invited to help catch aquatic organisms on the coast for the “Mostra da Universidade do Porto”. The purpose was to simulate a coastal ecosystem by integrating representative organisms from that area: starfishes, anemones, crabs, shrimps, small fish, among others.

6- Supplementary Work developed in BOGA

6.1- Standard Operating Procedures (SOPs)

Standard Operating Procedures are necessary in any installation to assist workers to perform routine operations. The preparation of these protocols enables more efficiency between workers and interns since unnecessary communication can be avoided. Moreover, it creates uniformity of operations given that everyone follows the same rules when performing routine operations. The purpose of SOPs in BOGA is to have an instruction basis of different procedures that can be accessed by anyone that attends the facility. All the BOGA´s SOPs exist in a main document, divided into different topics depending on its purpose. During my internship, I was asked to review this document in order to understand the logic and importance of the SOPs, to detect any errors and also verify if the text was accessible when it comes to clarity and understanding of the writing. Additionally, I was asked to create some SOPs about some procedures that are performed in BOGA and therefore are listed in Annex I, as well as the index from the main document.

6.2- Biological Filter Tanks

As part of my functions as an intern in BOGA, I had the responsibility of managing the biological filters. Managing the biofilters involved replenishing bio balls when they were utilized in systems, feeding with ammonia and maintenance of the best conditions for the development of nitrifying bacteria. The management was described in some parts of this document and there is a resume in a SOP called ”Bio filters Tanks Activation, Feeding and Maintenance” which explains all the procedures to be made in a biofilter.

Additionally, I measured the ammonia and nitrite, as well as other important parameters (pH and temperature) during several weeks to observe the evolution of this two nitrogen compounds. All the values were registered and the results were illustrated in the form of graphs in Annex II.

7- Conclusion

The idea of using of fish as a research models as grown exponentially over the course of the last years. Therefore, the conditions needed to elaborate that same research has also developed greatly, leading to easier manipulation of conditions presented in the wild or any other artificial conditions. RAS was a major impulse in the development of fish research and is to a great extent applied in BOGA.

As an intern in BOGA, I gained experience in the husbandry of several RAS systems present in the facility. With the guidance of the BOGA´s team, I learned the best procedures to make in the different situations that could occur and to be prepared for the inevitable obstacles that I would face every day. Additionally, it was also important for me to create a work routine in order to be prepared for future jobs down the line.

Furthermore, the biological filter maintenance that I had under my responsibility was fundamental to realize the importance of biological filtration in the aquatic systems and also creating conditions for the activation of the biofilter.

Lastly, the development of Standard Operating Procedures was a different experience but ultimately an important one to create standardization for routine operations in BOGA.

In the end, I consider this internship a great experience, not just for the working skills and experience that I gathered during that time, but also all the people that I got to know, specially the BOGA´s team.

Annex I

Standard Operating Procedures (SOP)

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SECTION B – Animal Policies

B05 – Use of Organisms in a Classroom

1.0 Purpose:

To provide safe and healthy standards of practicing using live organisms in teaching.

2.0 Responsibility:

It is the responsibility of the principal investigator, the research technician, the student, and the animal technician entering the facility to follow the procedures established in this document.

3.0 Applicable Documents:

SECTION C – Fish Procedures

SOP B04 – Pain and Distress Policy

SOP D02 - Fish Husbandry

4.0 Guidelines:

4.1 All organisms used for teaching purposes need to follow Legal section F.

4.2 A justification for the use of organisms must be presented.

4.3 Proper analysis of the information available to choose the best candidate.

- 4.3.1 Can be essential to reduce the number of specimens used and improve welfare.

4.4 Guidelines must have been verified by the competent authority and ethics committee before being applied.

- 4.4.1 Constant update of the breakthroughs regarding animal welfare.

4.5 Personnel must be qualified when handling organisms.

- 4.5.1 Informed about the pain and distress behaviors in order to detect them.

4.6 Confirmation that the best possible conditions exist when handling the organisms.

- 4.6.1 Attendance to the biological characteristics of the animal under practice.
- 4.6.2 Provide the most feasible environment in accordance with the space and logistics.

4.7 Acquirement of organisms not under the facility must be certified.

- 4.7.1 For more information, check SOP A06 - Barrier Entry for Organisms.
- 4.7.2 In case transportation is necessary, verify the application of safe and healthy conditions.
  - 4.7.2.1 Check SOP B02 – Transport of Organisms into BOGA.

4.8 Procedures in laboratory or classroom must follow the rules imposed in the Section C – Fish procedures.

- 4.8.1 Always study the best possible scenario in which the organisms can't suffer or at least, suffer less.

4.8.1.1 Possibility of non-invasive procedures.

4.8.1.2 Multiple analysis on individual organisms should be considered to reduce the number of organisms used.

4.8.1.3 Usage of anaesthesia.

4.8.1.4 If the procedure reveals to be too painful for the animal(s), euthanasia can be a possibility.

4.8.2 For classroom purposes, consider the possibility of not using organisms for demonstration practices.

4.8.2.1 The necessities for laboratory research and classroom practices are very different.

4.9 Do not release laboratory organisms into the wild, unless that is the purpose of the study or class.

4.9.1 Organisms may have been affected and consequently alter their chances of survival.

4.10 When in doubt, always consult with the facility personnel and supervisor.

SECTION C – Fish Procedures

C01 – Daily Observation of Laboratory Organisms

1.0 Purpose:

To guarantee that each organism is properly observed and cared for in accordance with regulations and guidelines.

2.0 Responsibility:

It is the responsibility of the technician to perform the task to inform the facility manager and Principal Investigator responsible for the organisms and to follow the procedures established in this document.

3.0 Applicable Documents:

SOP A03 - Emergency Contact List
SOP B01 - Animal Identification
SOP B04 – Animal Pain and Distress Policy
SOP C02 - Water Quality Control
SOP D02 - Fish Husbandry
SOP F02 - Carcass Disposal
Registration sheet

4.0 Basic procedure for all animal rooms:

4.1 All organisms need to be observed daily.
- 4.1.1 Weekends and Holidays are included in this daily observation policy.
  - 4.1.1.1 Personnel shall rotate coverage on these days.

4.2 Before performing the room check, verify if there are any special conditions needed in the room.

4.3 Items to be checked and recorded on the daily census sheet:
- 4.3.1 Register the time and initials of technician performing the room check.
- 4.3.2 Temperature (room): normal range should be between 15 and 22ºC, but the ideal temperature depends on species.
- 4.3.3 Temperature (tank): normal range should be between 15 and 30ºC, but ideal temperature depends on species.
- 4.3.4 Humidity: normal range is 30-70%, but ideal humidity is dependent upon species.
- 4.3.5 Time checked & initials of technician performing the room check.
- 4.3.6 Check light/ dark cycle (12/12h).
- 4.3.7 Any scheduled feeding must be registered.

4.4 The water levels in the tanks should be checked.

4.4.1 If water levels are getting low, the principal investigator & animal facilities manager should be notified.

4.5 Additional items to be checked:

4.5.1 Water clarity.

4.5.2 Water conductivity.

4.5.3 Water pH.

5.0 Deviations:

6.0 If any deviation from the normal temperature and humidity ranges is noted, the animal facilities manager shall be notified immediately.

6.1 Verify if the deviations are there because that is the purpose of the experimental procedure and not from an anomaly.

7.0 Sick & Dead Organisms:

7.1 All Organisms should be checked daily – behavior and exterior appearance.

7.1.1 Animal wellbeing is especially important in investigation and observation can be the first step to detect organisms that are under stress.

7.2 The identification of sick organisms may differ between species, but some symptoms are common.

7.2.1 For fish: slow movement (lethargic), different color, lesions in the skin, loss of appetite (and consequent weight loss), bumping against the walls, swollen eyes, among others.

7.2.1.1 If more information is needed for evaluating if the fish are under stress, consult SOP B03 – Animal Pain and Distress.

7.2.2 For cephalopods: Some external and behavioral signs can be observed.

7.2.2.1 For more information, consult SOP B04 – Organisms Pain and Distress Policy.

7.3 If a sick fish/organism is found, then the Fish facility manager/veterinarian responsible should be notified immediately and a determination can be made whether to treat the animal or euthanize it.

7.4 If a dead fish is discovered, make a note on the tank, change the quantity of organisms on the tank, and record it on the daily census sheet.

7.4.1 The dead fish must be removed from the tank as soon as possible.

7.4.1.1 Check SOP F02 - Carcass disposal.

7.4.2 Once the Fish facility manager/ vet responsible is done collecting tissues, the carcass should be placed in the Animal Facility carcass freezer for disposal (SOP F02 – Carcass Disposal).

C03 – Fish Anesthesia

1.0 Purpose:

To induce the loss of sensation in order to reduce the pain inflicted during a certain procedure.

2.0 Responsibility:

It is the responsibility of the technician and investigator performing the task to inform the facility manager and to follow the procedures established in this document.

3.0 Applicable Documents:

SOP B04 - Animal Pain and Distress Policy

SOP C04 - Fish Blood Collection Procedures

SOP C07 - Necropsy Procedure

4.0 Anaesthesia Procedures:

4.1 Anaesthesia effects may vary depending on the administration route, pH, temperature, salinity, oxygenation, nitrogenous compounds and other water conditions.

4.2 Anaesthesia depth and recovery depends on its duration, anaesthetic concentration, organisms' body weight and metabolism, gill surface, fish health status, strain, age, and on the different particularities of fish species.

- 4.2.1 Bibliography needs to be revised in order to obtain the best results.
- 4.2.2 Anesthesia trials with small numbers of fish, i.e. pilot studies, must be performed to determine the optimal dosage and exposure time prior to the establishment of protocols.

4.3 Proper training and supervision of fish anaesthesia are essential to avoid complications that can lead to injury or death.

- 4.3.1 Not only anaesthesia should be carefully monitored but also complete fish recovery.
- 4.3.2 To learn more about the different stages of anaesthesia and the respective procedures that can be applied, consult Table 1 – Aneasthesia Stages in Fish.

4.4 Table 2 – Anaesthetic Agents provides a summary of the main anaesthetic agents used in fish.

4.5 For more detailed information, check the article below.

4.6 Aerated water should be used during anaesthesia and in the water recovery.

- 4.6.1 Dissolve oxygen levels must be monitored constantly.

4.7 Protocols for fish anaesthesia usually include only one anaesthetic agent instead of a combination. However, some studies have demonstrated that the mixture of two types of anaesthetic agents can result in a safer and more effective anesthesia.

4.8 Recovery container must exist.

- 4.8.1 The organism only leaves the recovery container when fully recovered.

4.8.1.1.1 Necessary to prevent attacks from other organisms in the stock.

Table 1 – Aneasthesia Stages in Fish

Anesthesia stages in fish.			
Stage of anaesthesia	Description	Physiological and behavioural signs	Clinical interest
0	Normal	Total equilibrium. Normal muscle tone. Normal reaction to visual and tactile stimuli. Normal respiratory rate.	
I	Light sedation	Slight loss of reaction to visual and tactile stimuli.	Can reduce stress and physical trauma during transport
II	Deep sedation	Slight decrease in muscle tone. No reaction to visual and light tactile stimuli. Small decrease in respiratory rate.	Suitable for close visual observation, weighing and measuring
III	Light narcosis / excitement phase	Partial loss of equilibrium/Weak responses to postural changes. Decrease in muscle tone. Increased reaction to visual and tactile stimuli. Respiratory rate increased and/or irregular.	Higher risk of physical injury or escape / jump from container or aquarium
IV	Deep narcosis	Total loss of equilibrium/Lack of responses to postural changes. No reaction to minor visual and tactile stimuli. Respiratory rate decreasing to almost normal.	Suitable for imaging techniques
V	Light anaesthesia	Complete loss of muscle tone. No reaction to painful stimuli. Decrease in respiratory rate. Decrease in heart rate.	Suitable for minor surgical procedures: external sampling, fin biopsies, gill biopsies, blood sampling
VI	Surgical anaesthesia	Absence of reaction to massive stimulation. Respiratory rate very low. Slow heart rate.	Suitable for major surgical procedures
VII	Medullary collapse/ Overdose	Flaccid muscle tone. Apnea – absence of respiratory rate, which can be followed in several minutes by cardiac arrest if anaesthesia depth is not decreased. Eventual death.	Suitable for euthanasia
Martins et al (2016)			

Table 2 – Anaesthetic Agents

Table 3 - Anaesthetic and analgesic agents tested in fish used in biomedical research			
Species	Anaesthetic or analgesic agent	Anaesthetic stage/Analgesia	Dose
Zebrafish	MS-222	Stage III – V	100 - 200 mg/L Immersion
Salmonid species	Metomidate	Stage III – VI	1-5 mg/L Immersion
	MS-222	Stage V – VI	40 - 200 mg/L Immersion
Sea Bass Finfish Rainbow trout	2-phenoxyethanol	Stage II- Stage VI	0,3-1 mg/L
	Buprenorphine	Analgesic	0.01 - 0.1 mg/kg (IM)
	Carprofen	Analgesic	1 - 5 mg/kg (IM)
	Lidocaine	Analgesic	0.5 - 2 mg/fish (SC)
Tilapias	MS-222	Stage IV	200 mg/L Immersion

C04 – Weight and Length Measurement

1.0 Purpose:

To obtain information regarding the size and weight of a certain number of fish.

2.0 Responsibility:

It is the responsibility of the technician, researchers and students performing the task to follow the procedures established in this document and to inform the facility manager.

3.0 Applicable Documents:

SOP B01 – Animal Identification

SOP B04 –Animal Pain and Distress Policy

4.0 Procedure:

4.1 Prepare all the necessary equipment before any procedure:
- 4.1.1 Measuring board.
- 4.1.2 Scale.
- 4.1.3 Anesthetic and container.
- 4.1.4 Recovery container.
- 4.1.5 Gloves.
- 4.1.6 Disinfectant.
- 4.1.7 Fish net and cloth.
- 4.1.8 Basic life support.

4.2 Humidify the equipment to minimize possible skin abrasions.

4.3 If live fish are used, evaluate if an anesthetic is necessary to reduce the movement and improve the quality of the measurements.
- 4.3.1 Consult the SOP for anesthesia for a more detailed information (SOP 04C – Anesthesia).

4.4 Fish must not eat in the 12-72 hours prior to the procedure.

4.5 Measurements need to be done quickly in order to diminish animal stress.
- 4.5.1 Maximum 30 seconds out of water.

4.6 Measurements:
- 4.6.1 Standard length.
- 4.6.2 Total length.
- 4.6.3 Fork length.
- 4.6.4 Weight.
  - 4.6.4.1 Always tare the scale before weighing.
  - 4.6.4.2 For small fish, remove the excess water before weighing.
  - 4.6.4.3 Small fish can also be measured submersed.

4.7 After the assessment, return the fish to a recovery bath (if anesthetic was used) and observe if they are recovering.
- 4.7.1 Monitoring can be extended to several days.

4.8 Dispose of the anesthesia baths (if an anesthetic was used) in the proper location and disinfect the place the place where the fish were handled.

C05 – Fish Blood Collection Procedures

1.0 Purpose:

To retrieve blood from the animal in order to run tests depending on the purpose of the study.

2.0 Responsibility:

It is the responsibility of the technician, researchers and students performing the task to follow the procedures established in this document and to inform the facility manager.

3.0 Applicable Documents:

SOP B01 - Animal Identification

SOP B04 - Animal Pain and Distress Policy

SOP C03 - Fish Anesthesia

4.0 Procedure:

4.1 Before any procedure, all the safety and disinfection measures need to be reviewed.

4.2 In case of doubt, always ask an experienced person to exemplify the task.

4.3 Prepare all the equipment before any procedure:

- 4.3.1 Disinfectant.
- 4.3.2 Anesthetic.
- 4.3.3 Gloves and other protection equipment.
- 4.3.4 Containers.
- 4.3.5 Air stones.
- 4.3.6 Scalpel or other sharp object and respective sharps container (SOP 05G – Sharps).
- 4.3.7 Sterile syringes and needles.
- 4.3.8 Tubes for blood collection.
- 4.3.9 Paper towel.

4.4 Always anesthetize or euthanize the animal before any procedure.

- 4.4.1 The dose of anesthetic will depend on the purpose of the procedure (consult SOP C03 – Anesthesia).

4.5 The different procedures are exemplified in the Table 3 – Blood Procedures.

4.6 If live fish were used during the procedures, monitoring is necessary to ensure that the fish are rapidly recovering.

- 4.6.1 Monitoring can be extended to several days.

4.7 In case there is a carcass to dispose of, consult the appropriate SOP (SOP F02 - Carcass Disposal).

4.8 Always disinfect the hands and equipment before and after each procedure.

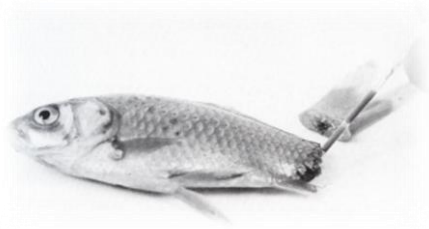
4.9 Dispose of the anesthesia baths in the proper location and disinfect the place the place where the fish were handled.

5.0 Equipment Cleaning After Exposure Studies:

5.1 Equipment used in exposure studies should be thoroughly cleaned according to data on the chemicals used, to removed toxins and harmful reagents.

5.2 Equipment that has been exposed to chemicals difficult to clean should be thrown away according to waste procedures (SOP F04 – Hazardous solid wastes).

Table 3 – Blood Procedures

Descrition of the different blood sampling procedures.		
Tail Ablation	-Performed in small fish due to the difficulty in the application of other procedures; -After euthanizing the fish, remove the caudal peduncle; -Retrieve the blood.	
Caudal Venous Puncture	- Insert the needle in the mid portion of tail below the lateral line; - Or Insert the needle in the ventral midline of the caudal peduncle; -Retrieve the blood when it reaches the column.	
Dorsal Aorta Puncture	-Introduce the needle in the dorsal midline of the mouth and above the secondary gill arch.	
Heart Puncture	-Difficult to execute (proper training is necessary); -Introduce the needle below the gill cover and the isthmus.	
In press		

C07 – Necropsy Procedure

1.0 Purpose:

To provide a sample of a group of organisms in order to reveal the health status and provide standardization in the procedures.

2.0 Responsibility:

It is the responsibility of the technician, researcher(s) and student(s) performing the task to follow the procedures established in this document and to inform the facility manager.

3.0 Applicable Documents:

SOP C04 - Weight and Length Measurement
SOP C06 - Fish Euthanasia
SOP F02 - Carcass Disposal

4.0 Early Considerations:

- 4.1 Procedures must be performed by a trained professional.
- 4.2 This procedure must be executed in the sample room.
- 4.3 Euthanasia must be performed before any procedure (check SOP C06 – Euthanasia).

5.0 Material:

- 5.1 Necessary to complement the information about the state of the animal.
- 5.2 Gather all the necessary equipment to execute the necropsy:
  - 5.2.1 Protection equipment - gloves, masks and lab coats.
  - 5.2.2 Procedure equipment - scalpels, scissors, knifes, syringes, swabs and brushes.
  - 5.2.3 Accessory equipment - microscopes, slides, trays, freezer bags and alcohol.
  - 5.2.4 Reagents – fixatives and anaesthetic.
  - 5.2.5 Labeled material for sample collection.

6.0 Exterior Procedures:

- 6.1 Before dissecting the animal, an exterior observation is necessary.
  - 6.1.1 Observation of the skin, gills, oral cavity and fins.
  - 6.1.2 Any exterior abnormality (skin lesions, fin lesions, among others) must be registered.
    - 6.1.2.1 Check for exterior lesions.
    - 6.1.2.2 Indication of any bacteria or parasite-induced lesion.
- 6.2 The next step is to gather information on the specimen being analyzed.
  - 6.2.1 Register data regarding sex, length and weight.
    - 6.2.1.1 Consult SOP C04 – Weight and Length measurement for more information.

7.0 Cytological Procedures:

7.1 At least one sample must be isolated for every lesion that is detected.

7.2 Imprint:

- 7.2.1 Collect samples from skin and eyes.
- 7.2.2 Gather samples from any lesion that is detected in the skin.
- 7.2.3 Any sample from any observable eye lesion must be collected.

7.3 Swab/Brush:

- 7.3.1 From any mucosa.
  - 7.3.1.1 Mouth.
  - 7.3.1.2 Gills.
- 7.3.2 From any lesion that was detected.

7.4 Scrapped:

- 7.4.1 From harder areas to remove a sample.
  - 7.4.1.1 From skin.
  - 7.4.1.2 From hardened lesions.

7.5 Fine needle aspiration:

- 7.5.1 To recover fluid samples.
  - 7.5.1.1 From body cavities.
  - 7.5.1.2 From subcutaneous lesions.

7.6 After the collection of samples, do smears for posterior observation in the microscope.

8.0 Dissection:

8.1 Essential to observe and gather samples from organs.

8.2 Weigh and observe the organs for external abnormalities.

8.3 Gathering of samples for other analysis:

- 8.3.1 Imprint in the organs if necessary.
- 8.3.2 Histological samples.
  - 8.3.2.1 Define the major organs to be analyzed.
  - 8.3.2.2 Only a small piece of organ is needed for each procedure.
  - 8.3.2.3 Optical microscopy.
    - 8.3.2.3.1 Fixative compound: Bouin liquid.
    - 8.3.2.3.2 volume of fixative must be 20 times superior to the volume of the sample.
  - 8.3.2.4 Electron microscopy.
    - 8.3.2.4.1 Procedure needs to be fast.
    - 8.3.2.4.2 Cut the part of the organ into very small pieces.
    - 8.3.2.4.3 Fixative: glutaraldehyde.
    - 8.3.2.4.4 volume of fixative must be 20 times superior to the volume of the sample.
- 8.3.3 Molecular analysis.
  - 8.3.3.1 Procedure needs to be fast.

8.3.3.2 Cut a small part of the organ.

8.3.3.3 Adequate volume of fixative.

8.4 The final step is to organize all the samples that were collected and send them to the designated laboratories.

Note: Necropsy procedure can also be applicable to animal sentinel programs.

SECTION D – Husbandry Procedures

D01 – Observation/Maintenance of Aquatic Systems

1.0 Purpose:

To guarantee that each aquatic system is properly working by verifying each component.

2.0 Responsibility:

It is the responsibility of the technician to perform the task, inform the facility manager and to follow the procedures established in this document.

3.0 Applicable Documents:

SOP A03 - Emergency Contact List
SOP C02 - Water Quality Control
SOP D02 - Fish Husbandry
Experimental design information sheet

4.0 Basic Procedures:

4.1 Daily:

- 4.1.1 Check if the water is circulating across the system.
- 4.1.2 Verify if the pumps are working.
  - 4.1.2.1 If the pump is not working, check the electricity connection.
  - 4.1.2.2 If the pump still isn't working, warn the facility staff to retrieve and fix the pump if necessary.
- 4.1.3 Attention to the air flow.
  - 4.1.3.1 Examine if the air stone or other oxygenation system is properly placed and working.
  - 4.1.3.2 Check if the air system is pumping.
- 4.1.4 In some cases, controlling the temperature of a system is a necessity and needs to be controlled.
  - 4.1.4.1 Notice if the resistance or water chiller is properly working.
- 4.1.5 Check the skimmer.
  - 4.1.5.1 Notice if the cup is filled with organic matter.
  - 4.1.5.2 If necessary, remove the cup and dispose of the content in a safe and controlled way.
- 4.1.6 Observe if the mechanical filter is clean and not blocking the normal water flow.
  - 4.1.6.1 Remove and replace the mechanical filter if necessary.
- 4.1.7 Other equipment may be required to check depending on the system: UV lights, sand filters, among others.

4.2 Monthly:

- 4.2.1 Several components need to be cleaned after some time in order to maintain its efficiency.

4.2.2 Retrieve the pump and the respective tubes.

4.2.2.1 Dismantle the pump for a more competent cleaning.

4.2.2.2 The tubes need to be cleaned to remove any debris that are blocking the normal water flow and consequently reducing the efficiency of the pump.

4.2.3 Verify if the skimmer is clean.

4.2.3.1 If not, remove it from the system and clean all the components.

D07 – Bio filters Tanks Activation, Feeding and Maintenance

1.0 Purpose:

To maintain a supply of activated bio balls ready to be introduced in a new system.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Applicable Documents:

SOP C02 - Water Quality Control

4.0 Activation:

- 4.1 Bio ball activation occurs when the bio balls are covered in bacteria that will convert ammonia in nitrites, and others will convert nitrites in nitrates.
- 4.2 Verify if the conditions are gathered for the activation of the bio balls.
  - 4.2.1 pH - Between 7 and 9.
  - 4.2.2 Temperature.
    - 4.2.2.1 The temperature of the bio filter must be in sync with the destiny of the bio balls.
    - 4.2.2.2 Salt water – Close to 15ºC.
  - 4.2.3 Salinity.
    - 4.2.3.1 Fresh water bio filter – Close to 0 ‰.
    - 4.2.3.2 Salt water bio filter – Approximately 35‰.
  - 4.2.4 Oxygen.
    - 4.2.4.1 Bio filter tanks must be well aerated in order to perform.
    - 4.2.4.2 Close to 10mg/L is the desired oxygenation?
- 4.3 Disinfection of bio balls must be done before the introduction in the water.
  - 4.3.1 One day immersed in bleached water is enough.
  - 4.3.2 Wash the bio balls abundantly.
- 4.4 Activation of the bio balls will take 30 days.
  - 4.4.1 After this period, they are ready to be introduced in an aquatic system.

5.0 Feeding:

- 5.1 To stimulate the growth of bacteria in the bio balls, ammonia needs to be provided as the substrate.
  - 5.1.1 To simulate the presence of organisms producing ammonia.
- 5.2 Ammonia comes in the more stable form of ammonia chloride (NH_4Cl).
  - 5.2.1 The necessary calculations need to be made in order to determine the desired concentration of ammonia and the corresponding dose of ammonia chloride.
- 5.3 Introduction of ammonia should be done when the ammonia levels in the bio filter tanks is under 2mg/L.
- 5.4 Concentration of ammonia needs to be smaller in the beginning and can gradually be increased until the desired concentration is reached.

5.5 Upon introducing ammonia chloride, a complementary buffer needs to be added to stabilize the pH.
- 5.5.1 pH will drop during the process.
- 5.5.2 Sodium bicarbonate ($NaHCO_3$) or calcium carbonate ($CaCO_3$) are the most used buffers.
  - 5.5.2.1 Quantity of calcium carbonate is the same as the weight of the ammonia chloride that will be introduced (e.g.: 45g NH_4Cl = 45g $CaCO_3$).
  - 5.5.2.2 Sodium carbonate is 2 times stronger then calcium carbonate, therefore only half the weight of the ammonia chloride that will be introduced (e.g.: 45g NH_4Cl = 22,5g $NaHCO_3$).
  - 5.5.2.3 Other buffers can be used; however, its efficiency must be tested first.

5.6 Try to dissolve the ammonia chloride and the buffer in water before introducing in the system.

6.0 Maintenance:

6.1 When the ammonia chloride is introduced, is it crucial to check the parameters daily:
- 6.1.1 Ammonia.
  - 6.1.1.1 It is advised not to let the ammonia reach zero.
  - 6.1.1.2 Always maintain a certain level of ammonia.
- 6.1.2 Nitrites.
  - 6.1.2.1 High nitrites means that the reaction is happening.
  - 6.1.2.2 If nitrites are high and ammonia is not being transformed, bacteria may be dying.
    - 6.1.2.2.1 Solution: Check the different parameters referred above to observe any major deviations: pH, salinity, temperature and oxygenation.
- 6.1.3 pH.
  - 6.1.3.1 As the pH drops, the reaction will eventually stop.
  - 6.1.3.2 Consider introducing buffer when the pH is below 7.
  - 6.1.3.3 Be careful about the sudden pH drops.
    - 6.1.3.3.1 High pH drop may happen some days after the introduction of ammonia chloride.
    - 6.1.3.3.2 Consider water renewal.
  - 6.1.3.4 As the temperature lowers, so does the velocity of the reaction.
- 6.1.4 Salinity.
  - 6.1.4.1 Some buffers will increase the salinity of the system.
  - 6.1.4.2 When the ammonia is near zero, a partial water change may be needed to diminish the salinity.

6.2 It is necessary to renew the water every two weeks to renew the natural salts of the water.

6.2.1 Important to increase the hardness of the water.

6.2.2 Decrease the salinity.

6.2.3 50% partial water change is enough.

SECTION F – Waste Management and Treatment

F01 – Normal Waste

1.0 Purpose:

To ensure the facility is kept clean by disposing of materials in the designated container.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Applicable Documents:

SOP A04 - Basic Rules to Follow in the Animal Facility

4.0 Recycling:

4.1 The designated containers will be available throughout the facility, to all the people working on the animal facility.

4.1.1 Facility personnel, researchers and students.

4.2 The existing containers are:

4.2.1 Blue container.

4.2.1.1 Destined for paper.

4.2.1.2 Exceptions: metalized or plasticized paper; paper containing organic matter or toxic residuals.

4.2.2 Green container.

4.2.2.1 Intended for glass.

4.2.2.2 Exceptions: special glass; lamps; mirrors; cosmetic packages; glass from pharmaceutical or hospital origin.

4.2.3 Yellow container.

4.2.3.1 Metal/plastic container.

4.2.3.2 Exceptions: plastics with fat or toxic residuals; batteries; tools and household appliances.

4.3 For Hazardous wastes, check SOP F04 – Hazardous Solid Wastes and SOP F05 Hazardous Wastewater Treatment.

F02 – Carcass Disposal

1.0 Purpose:

To minimize the risks of public and environmental health and reduce the chances of pathogens spreading to the animal facilities and/or to personnel working within the animal facilities.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Applicable Documents:

SOP C07 - Necropsy Procedure

4.0 Carcass Disposal:

4.1 Animal carcasses should be placed in Zip bags or similar.

- 4.1.1 Large carcasses or large numbers of small and median carcasses should be placed in thick plastic bags.
- 4.1.2 All bags must be identified (content, dates, responsible...).
- 4.1.3 Those bags should be placed in plastic boxes in a dedicated freezer and held in frozen storage until disposal.
  - 4.1.3.1 The access to the freezer room only will be granted by the animal facilities technicians after the animal disposal recording sheet has been completed by the user.
- 4.1.4 All carcass bags must be sealed in order to prevent leakage of fluid or other biological materials.
- 4.1.5 Under no circumstances torn or leaking bags will be accepted for disposal by Animal Facilities.

F03 – Sharps

1.0 Purpose:

To minimize the risks of public and environmental health and reduce the chances of pathogens spreading to the animal facilities and/or to personnel working within the animal facilities.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Sharps:

3.1 All sharps are considered hazardous, even non contaminated ones.

3.2 Special attention when handling sharps.

- 3.2.1 Any distraction can induce injuries and if contaminated can bring aggravated health issues.

3.3 Sharps must be disposed in the right container specifically designed sustain sharpened objects.

- 3.3.1 Sharps container with a yellow box.

3.4 Materials that can be discarded in the sharps container:

- 3.4.1 Needles and syringes.
- 3.4.2 Blades such as scissors, x-Acto knife, scalpels, razors, etc.
- 3.4.3 Other objects designed to cut.
- 3.4.4 Some contaminated glass and plastics.

F04 – Hazardous Solid Wastes

1.0 Purpose:

To minimize the risks of public and environmental health and reduce the chances of pathogens spreading to the animal facilities and/or to personnel working within the animal facilities.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Applicable Documents:

SOP A07 - Quarantine Room Policy
SOP A08 -Infection Rooms
SOP A09 - Exposition Rooms

4.0 Hazardous Solid Wastes:

- 4.1 Any material that was in contact with dangerous chemical substances and/or infection trials.
  - 4.1.1 Must be discarded in the respective container before leaving the room.
    - 4.1.1.1 Container designed specifically for contaminated solid residuals.
- 4.2 Any waste from quarantine infection and exposition rooms are considered hazardous.
- 4.3 Some electric equipment may also be considered hazardous.
  - 4.3.1 Mercury switches, cathode ray tubes, batteries, activated glass, among others.
- 4.4 To verify if a certain material may be discarded as hazardous, consult the European List of Waste.

F06 – Quarantine, Infection and Exposition Room´s Wastewaters

1.0 Purpose:

To minimize the risks of public and environmental health and reduce the chances of pathogens spreading to the animal facilities and/or to personnel working within the animal facilities.

2.0 Responsibility:

It is the responsibility of the investigator, technician, or student performing the task to follow the procedures established in this document.

3.0 Applicable Documents:

SOP A07 - Quarantine Room Policy

SOP A08 -Infection Rooms

SOP A09 - Exposition Rooms

4.0 Wastewaters:

4.1 Extra care when handling hazardous wastewaters.

4.1.1 The disposal of the water should be done in a controlled and secured way in order to decrease the probability of contamination and/or contact with harmful reagents.

4.2 Wastewaters from Quarantine Infection and Exposition rooms will always be considered as hazardous and will have to be treated.

4.2.1 These rooms will have dedicated mobile treatment compact plants.

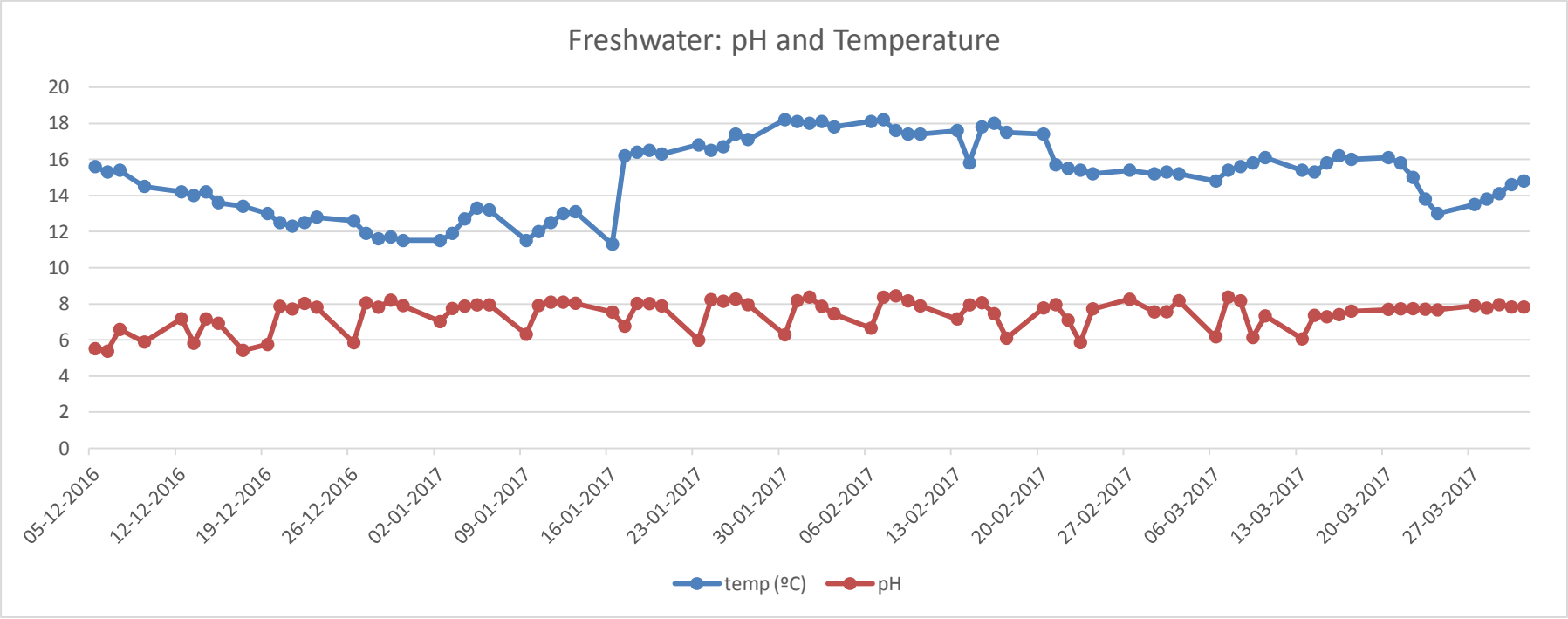
4.3 All the water discharged from the systems during water exchanges, tank bottoms siphoning and others, must be send to the treatment compact plant present in the room.

4.4 A pump and piping, also present in the room and identified as so, must be used to accomplish the transfer of water between the recirculation system and the treatment compact plant.

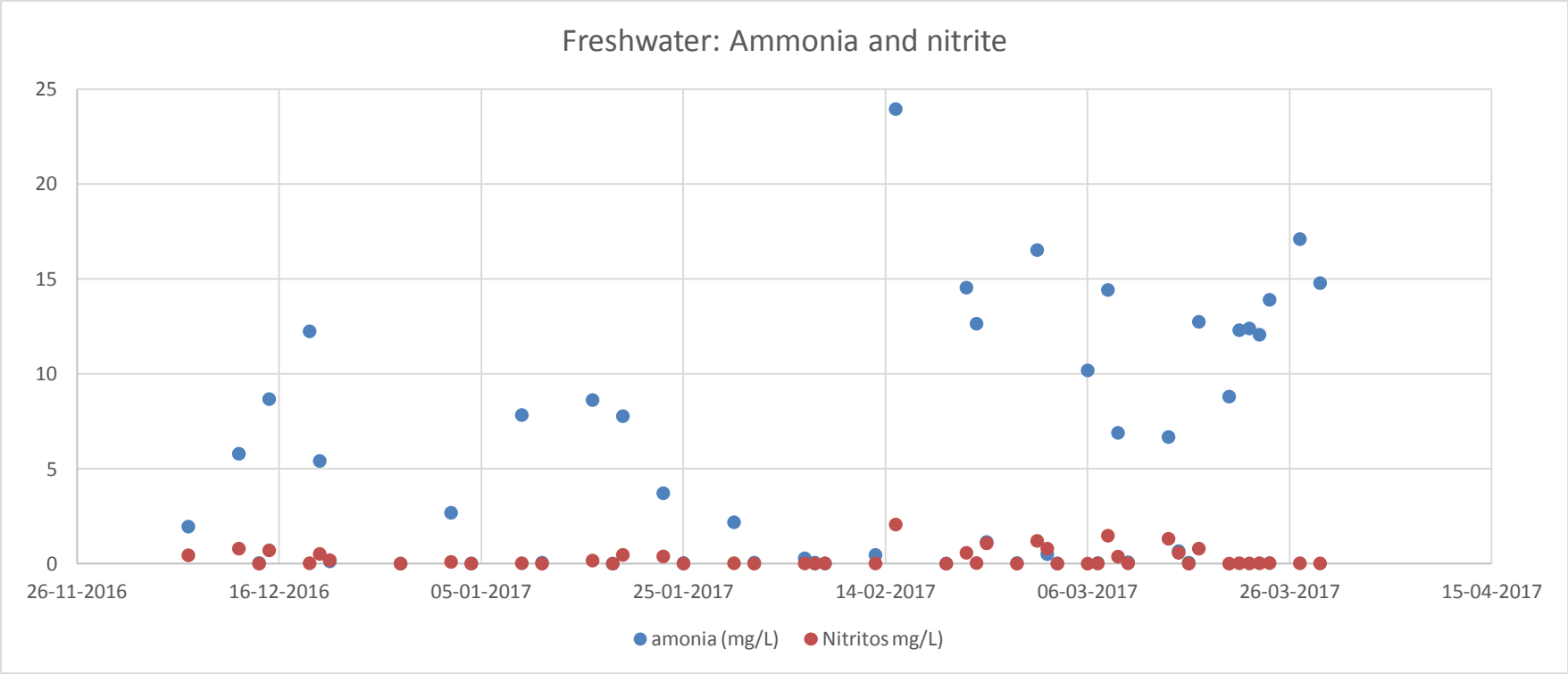
4.5 The water pump and UV light, from the treatment compact plant, must be switched on when receiving the water discarded from the aquatic systems.

Annex II

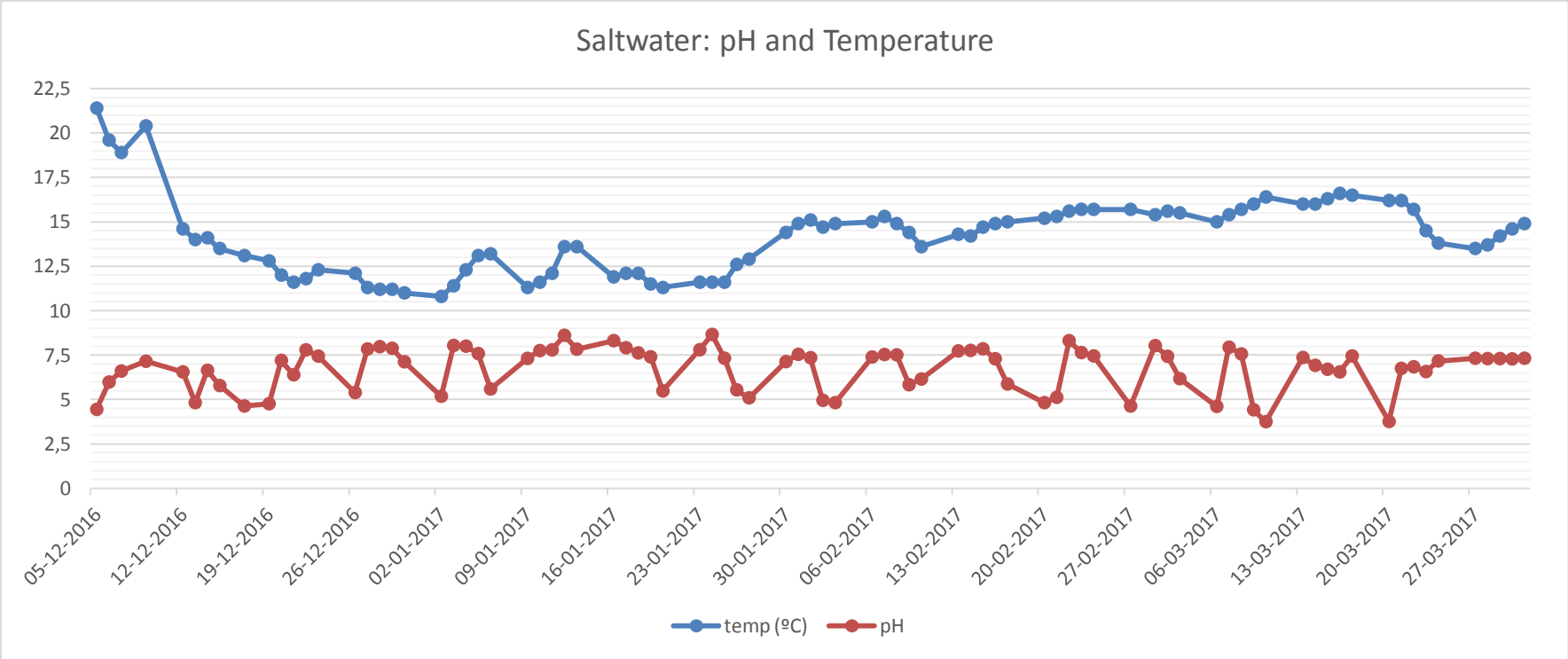
Biological Filter Tanks



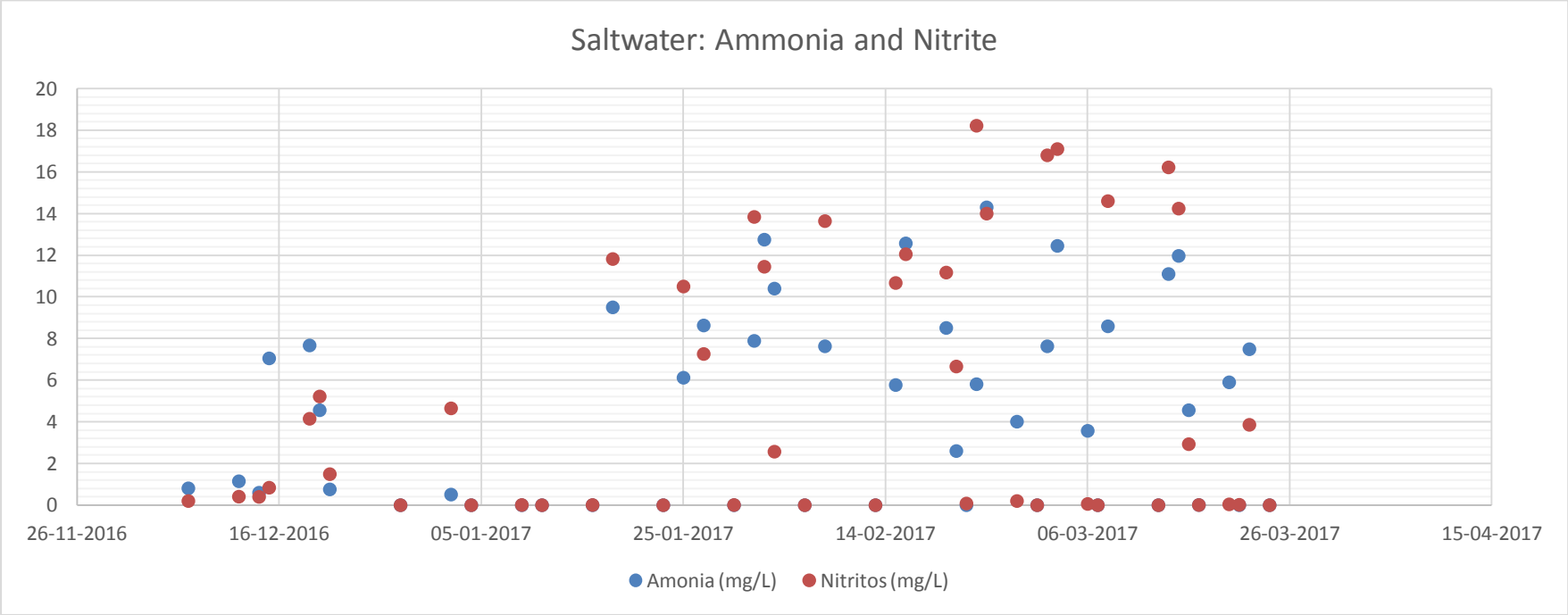
Freshwater Biological filter: pH and Temperature



Freshwater Biological filter: Ammonia and nitrite



Saltwater water Biological filter: pH and Temperature



Saltwater Biological filter: Ammonia and nitrite

7- References

Abbink, W., et al. (2012). "The effect of temperature and pH on the growth and physiological response of juvenile yellowtail kingfish *Seriola lalandi* in recirculating aquaculture systems." Aquaculture **330**: 130-135.

Adamek, Z., et al. (2015). "Processing traits of European catfish (*Silurus glanis* Linnaeus, 1758) from outdoor flow-through and indoor recycling aquaculture units." Journal of Applied Ichthyology **31**(s2): 38-44.

Barrut, B., et al. (2013). "Foam fractionation efficiency of a vacuum airlift—Application to particulate matter removal in recirculating systems." aquacultural Engineering **54**: 16-21.

Beveridge, M. C. and D. C. Little (2002). The history of aquaculture in traditional societies. Ecological aquaculture. The evolution of the Blue Revolution. B. A. Costa-Pierce. Oxford, Blackwell Science Limited**:** 3-29.

Blancheton, J. P. (2000). "Developments in recirculation systems for Mediterranean fish species." aquacultural Engineering **22**(1): 17-31.

Boeuf, G. and P. Payan (2001). "How should salinity influence fish growth?" Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **130**(4): 411-423.

Borski, R. J. and R. G. Hodson (2003). "Fish research and the institutional animal care and use committee." Ilar Journal **44**(4): 286-294.

Brambilla, F., et al. (2008). "Foam fractionation efficiency in particulate matter and heterotrophic bacteria removal from a recirculating seabass (*Dicentrarchus labrax*) system." aquacultural Engineering **39**(1): 37-42.

Bregnballe, J. (2015). A Guide to Recirculation Aquaculture: An introduction to the new environmentally friendly and highly productive closed fish farming systems. FAO, FAO**:** 100.

Brown, M. N. (2013). Microbial resource management in indoor recirculating shrimp aquaculture systems. Environmental Engineering, University of Idaho.

Bustos-Terrones, Y., et al. (2016). "Degradation of organic matter from wastewater using advanced primary treatment by O 3 and O 3/UV in a pilot plant." Physics and Chemistry of the Earth, Parts A/B/C **91**: 61-67.

Castine, S. A., et al. (2013). "Wastewater treatment for land-based aquaculture: improvements and value-adding alternatives in model systems from Australia." Aquaculture Environment Interactions **4**: 285-300.

Chen, S., et al. (1994). Suspended solids control in recirculating aquaculture systems. Aquaculture water reuse systems: engineering design and management. M. B. Timmons and T. M. Losordo. Amsterdam, Elsevier Science. **27:** 61-100.

Crab, R., et al. (2007). "Nitrogen removal techniques in aquaculture for a sustainable production." Aquaculture **270**(1): 1-14.

Davidson, J. and S. T. Summerfelt (2005). "Solids removal from a coldwater recirculating system—comparison of a swirl separator and a radial-flow settler." aquacultural Engineering **33**(1): 47-61.

DeTolla, L. J., et al. (1995). "Guidelines for the care and use of fish in research." Ilar Journal **37**(4): 159-173.

Dong, X., et al. (2013). "Acute ammonia toxicity and gill morphological changes of Japanese flounder *Paralichthys olivaceus* in normal versus supersaturated oxygen." Aquaculture Research **44**(11): 1752-1759.

Ferreira, C. I., et al. (2016). "Comparative adsorption evaluation of biochars from paper mill sludge with commercial activated carbon for the removal of fish anaesthetics from water in Recirculating Aquaculture Systems." aquacultural Engineering **74**: 76-83.

Francis-Floyd, R., et al. (2009). "Ammonia in aquatic systems." University of Florida IFAS Extension Publication **FA-16**.

Gendel, Y. and O. Lahav (2013). "A novel approach for ammonia removal from fresh-water recirculated aquaculture systems, comprising ion exchange and electrochemical regeneration." aquacultural Engineering **52**: 27-38.

Goher, M. E., et al. (2015). "Removal of aluminum, iron and manganese ions from industrial wastes using granular activated carbon and Amberlite IR-120H." The Egyptian Journal of Aquatic Research **41**(2): 155-164.

Gonçalves, A. A. and G. A. Gagnon (2011). "Ozone application in recirculating aquaculture system: an overview." Ozone: Science & Engineering **33**(5): 345-367.

Good, C., et al. (2014). "The impact of water exchange rate and treatment processes on water-borne hormones in recirculation aquaculture systems containing sexually

maturing atlantic salmon *Salmo salar*." Journal of Aquaculture Research & Development **5**(5): 260.

Good, C., et al. (2010). "The effects of carbon dioxide on performance and histopathology of rainbow trout *Oncorhynchus mykiss* in water recirculation aquaculture systems." aquacultural Engineering **42**(2): 51-56.

Gullian, M., et al. (2012). "Effect of turbidity on the ultraviolet disinfection performance in recirculating aquaculture systems with low water exchange." Aquaculture Research **43**(4): 595-606.

Gutierrez-Wing, M. T. and R. F. Malone (2006). "Biological filters in aquaculture: trends and research directions for freshwater and marine applications." aquacultural Engineering **34**(3): 163-171.

Hall, A. G. (1999). A comparative analysis of three biofilter types treating wastewater produced in recirculating aquaculture systems. Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University.

Hamlin, H., et al. (2008). "Comparing denitrification rates and carbon sources in commercial scale upflow denitrification biological filters in aquaculture." aquacultural Engineering **38**(2): 79-92.

Helfrich, L. and G. Libey (1991). Fish farming in recirculating aquaculture systems (RAS), Virginia Cooperative Extension.

Hochheimer, J. N. and F. Wheaton (1998). Biological filters: trickling and RBC design. Proceedings of the Second International Conference on Recirculating Aquaculture, Roanoke, VA.

Hunn, J. B. (1989). History of acute toxicity tests with fish, 1863-1987. Investigations in Fish Control, US Fish and Wildlife Service.

Hurtado, C. F. and B. Cancino-Madariaga (2014). "Ammonia retention capacity of nanofiltration and reverse osmosis membranes in a non steady state system, to be use in recirculation aquaculture systems (RAS)." aquacultural Engineering **58**: 29-34.

Kent, M. L., et al. (2009). "Recommendations for control of pathogens and infectious diseases in fish research facilities." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **149**(2): 240-248.

Khater, E. G., et al. (2011). "Solids removal in a recirculating aquaculture system." Agric. Eng. **28**(4): 1178-1196.

Klas, S., et al. (2006). "A conceptual, stoichiometry-based model for single-sludge denitrification in recirculating aquaculture systems." Aquaculture **259**(1): 328-341.

Klontz, G. W. and L. S. Smith (1968). Methods of using fish as biological research subjects. Methods of animal experimentation. W. I. Gay. Maryland, Academy Press. **3:** 383-385.

Kovitvadhi, S., et al. (2008). "A laboratory-scale recirculating aquaculture system for juveniles of freshwater pearl mussel *Hyriopsis (Limnoscapha) myersiana* (Lea, 1856)." Aquaculture **275**(1): 169-177.

Lakeh, A. A. B., et al. (2013). "Low frequency ultrasound and UV-C for elimination of pathogens in recirculating aquaculture systems." Ultrasonics sonochemistry **20**(5): 1211-1216.

Lawson, T. B. (1995). Recirculating Aquaculture Systems. Fundamentals of Aquacultural Engineering. Boston, MA, Springer**:** 192-247.

Lekang, O. (2007). Aquaculture engineering. Oxford, Blackwell Publishing.

Lontz, G. W. (1971). Fish as Biomedical Research Models. Animal Models for Biomedical Research, IV: Proceedings of a Symposium, Las Vegas, Nevada, National Academies.

Losordo, T. M., et al. (1999). "Recirculating aquaculture tank production systems. A review of component options." Southern Regional Aquaculture Center **453**.

Malara, D., et al. (2017). "Sustainable water treatment in aquaculture–photolysis and photodynamic therapy for the inactivation of Vibrio species." Aquaculture Research **48**(6): 2954-2962.

Mallya, Y. J. (2007). The effects of dissolved oxygen on fish growth in aquaculture. Fisheries Training Programme. Iceland, The United Nations University**:** 30.

Malone, R. F. and T. J. Pfeiffer (2006). "Rating fixed film nitrifying biofilters used in recirculating aquaculture systems." aquacultural Engineering **34**(3): 389-402.

McBride, S. C. (2005). Sea urchin aquaculture. American Fisheries Society Symposium, Phoenix, Arizona, American Fisheries Society.

McDaniel, N. K., et al. (2005). "Dissolved oxygen and dietary phosphorus modulate utilization and effluent partitioning of phosphorus in rainbow trout (*Oncorhynchus mykiss*) aquaculture." Environmental pollution **138**(2): 350-357.

Metscher, B. D. and P. E. Ahlberg (1999). "Zebrafish in context: uses of a laboratory model in comparative studies." Developmental biology **210**(1): 1-14.

Monsees, H., et al. (2016). "Chronic exposure to nitrate significantly reduces growth and affects the health status of juvenile Nile tilapia (*Oreochromis niloticus* L.) in recirculating aquaculture systems." Aquaculture Research **48**(7): 3482-3492.

Mook, W., et al. (2012). "Removal of total ammonia nitrogen (TAN), nitrate and total organic carbon (TOC) from aquaculture wastewater using electrochemical technology: A review." Desalination **285**: 1-13.

Mota, V. C., et al. (2016). "Cortisol and testosterone accumulation in a low pH recirculating aquaculture system for rainbow trout (*Oncorhynchus mykiss*)." Aquaculture Research **48**(7): 3579-3588.

Mota, V. C., et al. (2017). "Water cortisol and testosterone in Nile tilapia (*Oreochromis niloticus*) recirculating aquaculture systems." Aquaculture **468**: 255-261.

Nigrelli, R. F. (1953). "SECTION OF BIOLOGY: THE FISH IN BIOLOGICAL RESEARCH." Transactions of the New York Academy of Sciences **15**(6 Series II): 183-186.

Nishimura, Y., et al. (2016). "Using zebrafish in systems toxicology for developmental toxicity testing." Congenital anomalies **56**(1): 18-27.

Ostrander, G. K. (2000). The laboratory fish. Baltimore, Academic Press.

Patterson, R. and K. Watts (2003). "Micro-particles in recirculating aquaculture systems: microscopic examination of particles." aquacultural Engineering **28**(3): 115-130.

Pillay, T. V. R. and M. N. Kutty (2005). Aquaculture : principles and practices. Oxford, Blackwell Publishing.

Powell, A., et al. (2015). "The effect of ozone on water quality and survival of turbot (*Psetta maxima*) maintained in a recirculating aquaculture system." aquacultural Engineering **64**: 20-24.

Powell, A. and J. W. Scolding (2016). Direct application of ozone in aquaculture systems. Reviews in Aquaculture. [S.I.].

Qiu, T., et al. (2016). "Effects of solid-phase denitrification on the nitrate removal and bacterial community structure in recirculating aquaculture system." Biodegradation **27**(2-3): 165-178.

Rabanal, H. R. (1988). History of aquaculture. Coastal Fisheries Development Project FAO.

Resley, M. J., et al. (2006). "Growth and survival of juvenile cobia, *Rachycentron canadum*, at different salinities in a recirculating aquaculture system." Aquaculture **253**(1): 398-407.

Rizzo, L., et al. (2013). "Advanced treatment of urban wastewater by UV radiation: effect on antibiotics and antibiotic-resistant *E. coli* strains." Chemosphere **92**(2): 171-176.

Rouquerol, J., et al. (2013). Adsorption by powders and porous solids: principles, methodology and applications. London, Academic press.

Sammouth, S., et al. (2009). "The effect of density on sea bass (*Dicentrarchus labrax*) performance in a tank-based recirculating system." aquacultural Engineering **40**(2): 72-78.

Schroeder, J., et al. (2011). "Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems." aquacultural Engineering **45**(1): 35-41.

Seemann, U. B., et al. (2015). "Growth performance of Noble Crayfish *Astacus astacus* in recirculating aquaculture systems." Aquaculture international **23**(4): 997-1012.

Seitz, F., et al. (2013). "Nanoparticle toxicity in *Daphnia magna* reproduction studies: The importance of test design." Aquatic toxicology **126**: 163-168.

Simon, F. X., et al. (2014). "Seawater disinfection by chlorine dioxide and sodium hypochlorite. A comparison of biofilm formation." Water, air and soil pollution **225**(4): 1921-1932.

Solloch, S., et al. (2015). "Acute toxicity of peroxy sulfonated oleic acids (PSOA) to freshwater aquatic species and sludge microflora as observed in laboratory environments." Environmental Sciences Europe **27**(1): 21.

Spalding, M. J., et al. (2014). Sustainable Ancient Aquaculture. National Geographic: Newswatch, The Ocean Foundation.

Summerfelt, S. T. (2006). "Design and management of conventional fluidized-sand biofilters." aquacultural Engineering **34**(3): 275-302.

Van Bussel, C. G., et al. (2014). "Aquatic accumulation of dietary metals (Fe, Zn, Cu, Co, Mn) in recirculating aquaculture systems (RAS) changes body composition but not performance and health of juvenile turbot (*Psetta maxima*)." aquacultural Engineering **61**: 35-42.

Van Ham, E. H., et al. (2003). "The influence of temperature and ration on growth, feed conversion, body composition and nutrient retention of juvenile turbot (*Scophthalmus maximus*)." Aquaculture **217**(1): 547-558.

Van Rijn, J., et al. (2006). "Denitrification in recirculating systems: theory and applications." aquacultural Engineering **34**(3): 364-376.

Vascotto, S. G., et al. (1997). "The zebrafish's swim to fame as an experimental model in biology." Biochemistry and cell biology **75**(5): 479-485.

Wang, X.-X., et al. (2016). "Simultaneous nitrogen, phosphorous, and hardness removal from reverse osmosis concentrate by microalgae cultivation." Water research **94**: 215-224.

Watson, K., et al. (2012). "Chlorine disinfection by-products in wastewater effluent: Bioassay-based assessment of toxicological impact." Water research **46**(18): 6069-6083.

Watts, C., et al. (2016). "Evaluation of Stocking Density during Second-Year Growth of Largemouth Bass, *Micropterus salmoides*, Raised Indoors in a Recirculating Aquaculture System." Journal of the World Aquaculture Society **47**(4): 538-543.

Wenten, I. (2016). "Reverse osmosis applications: prospect and challenges." Desalination **391**: 112-125.

Xue, S., et al. (2017). "Impact of environmental bacterial communities on fish health in marine recirculating aquaculture systems." Veterinary Microbiology **203**: 34-39.

Ying, L., et al. (2015). "Recirculating Aquaculture Systems in China-Current Application and Prospects." Fisheries and Aquaculture Journal **6**(3): 1-3.

Zhang, S.-Y., et al. (2011). "An integrated recirculating aquaculture system (RAS) for land-based fish farming: The effects on water quality and fish production." aquacultural Engineering **45**(3): 93-102.