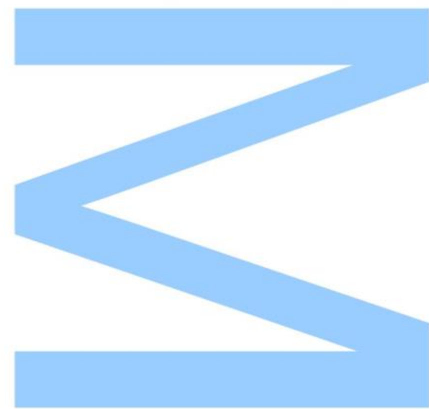
DTU Aqua and Nordsøen Oceanarium Internship:

The effect of turbidity and water exchange rate on pikeperch (*Sander luciperca*) larvae swim bladder inflation



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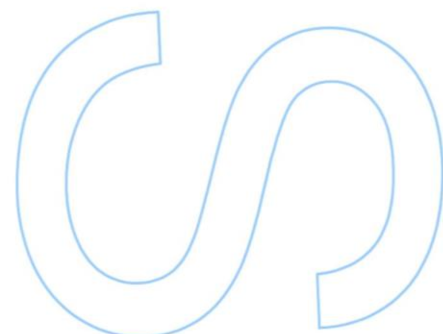

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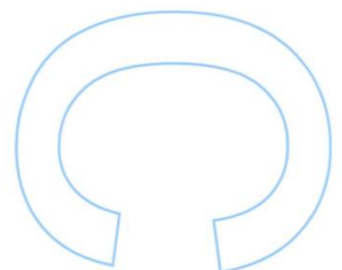

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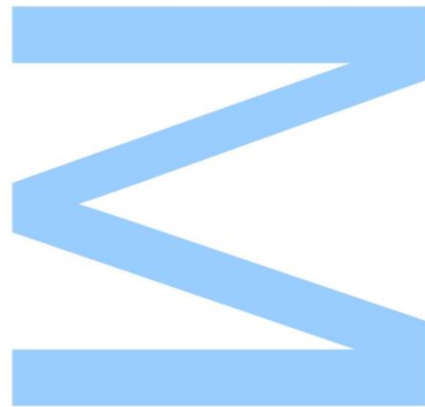

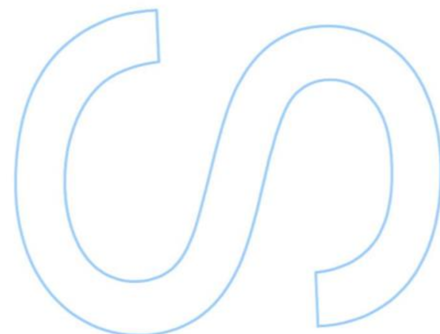

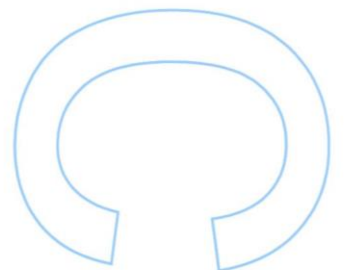

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Summary

This internship took place in partnership with DTU Aqua and Nordsøen Oceanarium, at their facilities in North Sea Science Park in Hirtshals, Denmark. This report aims to describe all the work I carried out throughout my stay in Denmark. My main trial was regarding the effects of water exchange rate (5%/h, 50%/h and 100%/h) and water turbidity (no turbidity, medium turbidity - 12.9 to 33.3 FNU - and high turbidity - 45 to 74 FNU) in the swim bladder inflation of pikeperch larvae (*Sander lucioperca*), as swim bladder non-inflation has proven to be a main bottleneck in previous trials carried in DTU's facilities and in most of the pikeperch larvae rearing facilities, causing high mortality. Other parameters were measured besides swim bladder inflation, such as growth (weight and body length), and different water parameters. Under high turbidities, swim bladder inflation was higher than in tanks with clear water, which might be explained by different factors, such as less cannibalism due to less encounters between the larvae, or less clinging behavior to the tank walls inducing the larvae to go to the surface. However, it is still uncertain if this are the real reasons behind the benefits of turbid water. Body weight proved to be significantly influenced by turbidity, as the tanks with higher degree of turbidity had the highest weight. Body length showed no significant differences when compared to the control. Regarding the water exchange rates, no significant changes were reported when compared to the control.

Throughout the internship I was assigned tasks in the daily routine of the institute technicians, such as feeding the fish in running trials, cleaning tanks, changing pumps and other tasks related to systems' maintenance. Afterwards, I helped in a temperature challenge pilot test in European lobster post larvae (*Homarus gammarus*), which aimed to evaluate the impact of low temperature on stress physiological response and growth of European lobster post larvae fed with experimental dry feeds specifically formulated for the species. As this was not my trial, I only helped with the daily procedures and maintenance of the system, and the stress related hormones analysis was carried out by a PhD student. Nevertheless, I was given the carapace length increment (iCL) and wet bodyweight increment (WBW) data and, as expected, it shows that higher temperature enables better growth in *H. gammarus* post larvae, both in iCL and WBW. Krill remains the best feed for this species, despite the current efforts to make pelletized feeds.

During my stay I helped in a European lobster rearing project at the Nordsøen Oceanarium, where I had to do the daily maintenance of the systems, and the daily cares of the broodstock and larvae (from newly hatched to post larvae). This project aimed to

re-stock the North coast of Denmark with European lobster, as the natural stock has been decreasing over the past years. Finally, I attended an “Advanced course in recirculating aquaculture systems: Design and application”, a two weeks course regarding RAS with lectures and different trials.

Keywords

Aquaculture; Broodstock; European Lobster; Larvae; Pikeperch; RAS; Stress; Swim bladder inflation.

Resumo

Este estágio foi realizado em parceria com a DTU Aqua e a Nordsøen Oceanarium, nas instalações localizadas no North Sea Science Park em Hirtshals, Dinamarca. Este relatório tem como objetivo descrever todo o trabalho que realizei durante a minha estadia na Dinamarca. O meu ensaio principal foi sobre os efeitos da taxa de renovação de água (5%/h, 50%/h and 100%/h) e da turbidez da água (sem turbidez, turbidez média – de 12.9 a 33.3 FNU - e turbidez elevada – de 45 a 74 FNU) na inflação da bexiga natatória de larvas de zander europeu (*Sander lucioperca*), visto que a não inflação da bexiga natatória provou ser o principal constrangimento em ensaios anteriores realizados nas instalações da DTU e na maioria das instalações de criação de larvas, causando uma elevada mortalidade. Outros parâmetros foram também medidos para além da inflação da bexiga natatória, como o crescimento em peso e comprimento corporal e diferentes parâmetros da água. Com elevada turbidez, a inflação da bexiga natatória foi muito maior do que em tanques com água limpa, o que pode ser explicado por diferentes fatores, como menos canibalismo devido à diminuição dos encontros entre as larvas, ou diminuição da natação agarrada às paredes do tanque fazendo com que as larvas vão à superfície. Mas ainda não é certo se esta é a verdadeira razão por trás dos benefícios da água turva. Apenas o peso corporal mostrou ser influenciado significativamente pela turbidez, pois os tanques com maior grau de turbidez apresentaram o maior peso. Em relação às taxas de renovação de água, nenhuma diferença significativa foi observada quando comparada com o controlo.

Ao longo do estágio foram-me atribuídas tarefas inseridas na rotina diária dos técnicos do instituto, tais como alimentar os peixes dos ensaios que decorriam no momento, limpar tanques, trocar bombas e outras tarefas relacionadas com a manutenção de sistemas. Posteriormente, ajudei num teste piloto relacionado com o efeito da temperatura em pós-larvas de lavagante (*Homarus gammarus*), cujo objetivo era avaliar o impacto de temperaturas baixas na resposta fisiológica ao stress e no crescimento de pós-larvas de lavagantes alimentados com rações secas experimentais, formuladas especificamente para a espécie. Como este não era o meu ensaio, apenas ajudei nos procedimentos diários e na manutenção do sistema. A análise das hormonas relacionadas com o stress foi realizada por um aluno de doutoramento responsável pelo ensaio. No entanto, recebi os dados do incremento do comprimento da carapaça (CL) e do incremento do peso corporal húmido (WBW) e, como esperado, estes resultados mostram que a temperatura mais elevada permite um melhor crescimento em pós-larvas

de *H. gammarus*, tanto no CL como no WBW. O Krill continua a ser o melhor alimento para esta espécie, apesar dos esforços atuais para fazer rações peletizadas.

Por último, durante a minha estadia ajudei num projeto de criação de lavagantes no Oceanário Nordsøen, onde fiz a manutenção diária dos sistemas e os cuidados diários dos reprodutores e larvas (desde recém eclodidos até pós-larvas). Este projeto teve como objetivo o repovoamento das populações de lavagantes da costa norte da Dinamarca, visto que o stock natural tem diminuído nos últimos anos. Por último frequentei um “Curso avançado em sistemas de recirculação de aquacultura: Desenho e aplicação”. Um curso de duas semanas sobre RAS, com aulas teóricas e diferentes ensaios práticos.

Palavras-chave

Aquacultura; Inflação da bexiga natatória; Larvas; Lavagante; Perca; RAS; Reprodutores; Stress

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1. Introduction

1.1 Global Aquaculture

Aquaculture is, and has been, one of the fastest growing industries and is expected to keep growing in the future, due to the high demand on commercial fish whose catches have stagnated, and to an increase in people's interest in a healthy diet (Lekang 2019b). But even though this growth rate is fast, it has been decreasing globally and heterogeneously among the different countries exploring this resource, which might be linked to the different constraining factors that aquaculture faces, such as broodstock maintenance, feed, farming areas, the market, social environment, among others (Nadarajah and Flaaten 2017). Nevertheless, in 2018 (Figure 1), a global record of 114.5 million tons of aquaculture produced live weight was achieved, from which 82.1 million tons consisted of aquatic animals, 47% of which was only from finfish, summing up to a total of 139.7 billion USD (FAO 2020).

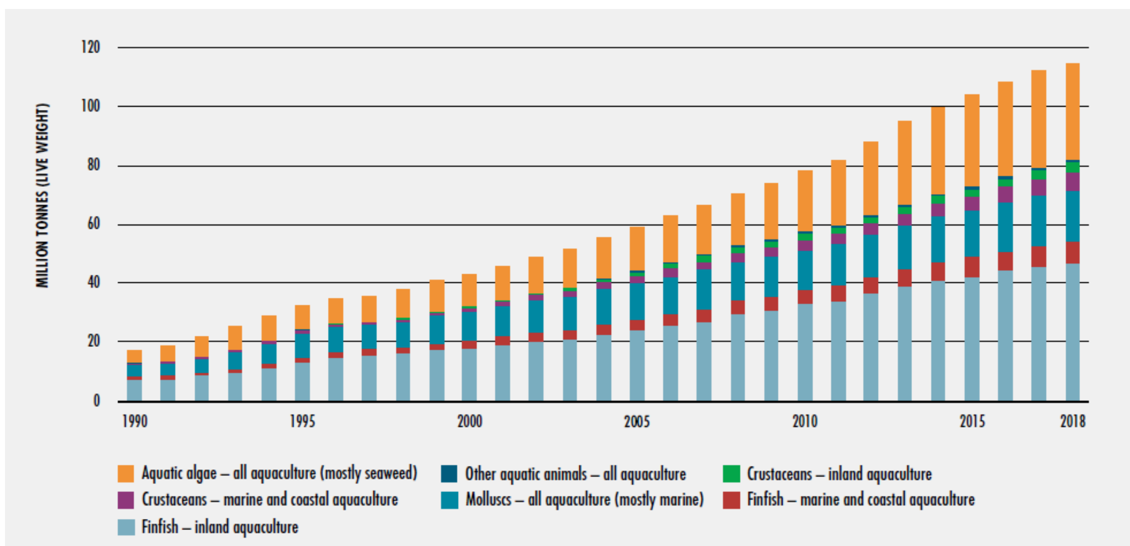


Figure 1 - World Aquaculture Production of Aquatic Animals and Algae, 1990-2018 (FAO 2020).

1.2. Aquaculture systems

As seen in Figure 1, 86.5% (47 million tons) of all the fish produced in aquaculture come from inland aquaculture, and the 13.5% (7.3 million tons) left come from marine and coastal aquaculture. The production in aquaculture is done through various methods and systems that seek to fit the degree of its intensity, whether it is 'extensive', 'semi-intensive' or 'intensive', the latter being the most intense, and the first, the one that produces less biomass per volume/area unit (m^3/m^2) (Lekang 2019a). Aquaculture systems, in a very raw sense, need to properly provide to the fish three main environment parameters such as temperature, oxygen and waste removal, and the systems can be

categorized according to the degree of control that the system has on these parameters (Tidwell 2012b). The three most common categories in which the systems are divided are: open systems, semi-closed systems, and closed systems. “Open systems” category is where most of the marine and coastal aquaculture are inserted. This includes systems as floats, trays, and rafts (mainly used for bivalve culture), cages, net pens and some raceways. In these systems the functions are almost solely controlled by the environment (Tidwell 2012b). But, as these are systems assembled on already existing bodies of water or use water from natural sources without treating it, there are always environmental implications associated such as: organic matter released, in particulate or dissolved form, from fish waste and uneaten feed (Brummett and Beveridge 2015), the area occupied by the system’s structures that reduces the niche where species already lived, or even due to escapees, resulting in the introduction of new predators or genetic material (Troell et al. 2013). The “semi-closed systems” encompass ponds and raceways, where there is more control over what enters the systems when compared to the “open systems”, but the environment surrounding the system still has some effect on it. Finally, the “closed systems” provide the producer total control over every factors in the system and have less environmental impact than the previously mentioned systems (Tidwell et al. 2019).

1.3. Recirculating Aquaculture System (RAS)

The most prominent type of system, on the closed systems category, are the recirculating aquaculture systems (RAS). These systems are becoming more relevant due to their low environmental impact (Chen et al. 2019) and low usage of water, which does not overload the fresh water supplies, since it is a resource that is becoming limited (Brummett et al. 2015; Lekang 2019a). Huge investments are made to make RAS as sustainable as possible, whilst keeping the quality of the produced fish, and decreasing the environmental footprint (Papáček et al. 2019). These systems can have high degrees of recirculation, sometimes replacing only 10%, or less, of the used water per day (Midilli et al. 2012; Moreno-Andrés et al. 2020), and this can only be achieved through a highly technological water treatment. Along with the reduced water usage there is also a decrease in energy associated with heating and cooling, since there is not much new water coming to the system, and the re-heating or re-cooling of the water that is already circulating in the system, is not as energy consuming (Lekang 2019a). This control over the water temperature cannot be done with the same degree of preciseness in other in-land rearing systems, such as ponds or raceways (Summerfelt and Vinci 2008).

Since these systems have a very high dependency on technology, the maintenance and building costs are far greater than the other aquaculture systems (Lekang 2019a). However, RAS technology allows the species to be produced in an intensive regime and the culture environment to be highly controllable (Takeuchi 2017). This enables the producer to grow the species more efficiently in their optimal conditions, and when done in hatcheries allows the farmer to predict/define the harvesting season, while also allowing for various harvests throughout the year (Summerfelt and Vinci 2008; Tidwell 2012a; Timmons et al. 2018a; Lekang 2019a). But even though these systems might look as the perfect solution for every fish, for some species they are not the most cost-effective choice. For example, in the production of channel catfish (*Ictalurus punctatus*) ponds are the best choice cost-efficient wise and, therefore, the most used (Summerfelt and Vinci 2008).

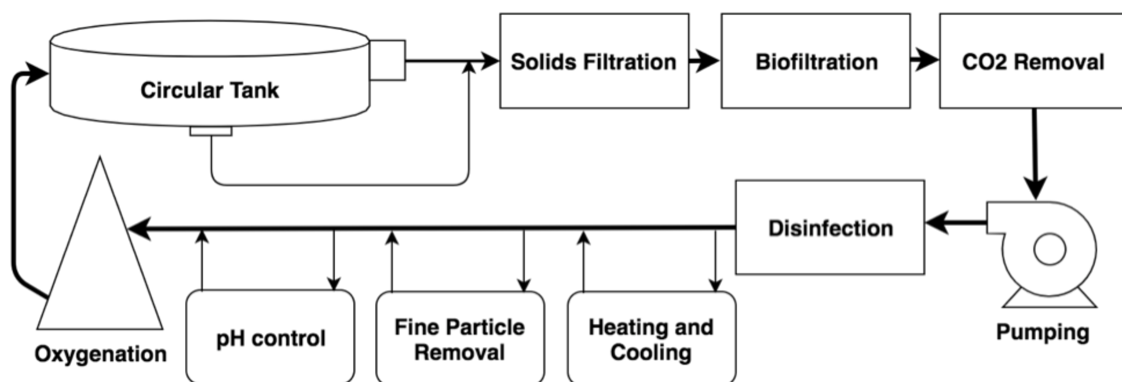


Figure 2 - Recirculating Aquaculture System processes

In Figure 2 it is visible the different processes that occur within a RAS unit. Each process needs a proper equipment and has a key role in keeping the system and the fish healthy. As fish are fed in the system, dissolved and particulate/suspended waste are produced (Dalsgaard and Pedersen 2011; Timmons et al. 2018c). Solid waste can have different sizes, and must be removed, usually beginning with the bigger ones, that are removed using gravity separation methods, such as settling basins or settling decks. After removing the bigger ones, smaller filtration is required, such as microscreen drum filters, disc filters or belt filter, and for particles smaller than 30µm, usually a foam fractionator is used if it is a saltwater system (Timmons et al. 2018b). After the solids removal, the nitrification processes take place in a biofilter, a unit where bacteria transform ammonia (NH_4^+) into nitrite (NO_2^-), and then into nitrate (NO_3^-). As seen before in mechanical filtration, there are also a vast number of different types of biofilters, such as trickling filters, moving bed bioreactors, fluidized sand filter, among others, each with its own purpose, advantages and disadvantages (Timmons et al. 2018d).

Additionally to all the aforementioned processes, other important factors are also monitored and controlled, such as temperature, dissolved oxygen and carbon dioxide, salinity, and pH, and further disinfection might be added, such as UV lights and Ozone (Timmons et al. 2018a).

1.4. Hatcheries

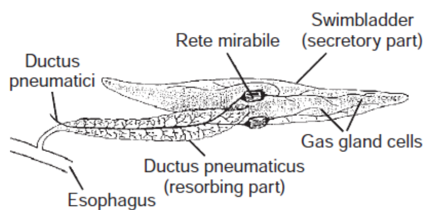
As previously mentioned, RAS allows the producer to have multiple harvests in one year and, for that to be possible, there needs to be an out-of-season spawning, made possible by hatcheries where it is possible to induce the spawning of the broodstock when necessary (Rónyai 2007; Duncan 2013). Some aquacultures still rely on broodstock captured in the wild (Mylonas et al. 2011), but a shift needs to be made into hatchery-cultured broodstock, as the quality and consistency of the wild caught finfish seeds is unstable and finite (Ranjan et al. 2019). Hatcheries are facilities where artificial breeding takes place, and where fish hatch and go through the first stages of life. These facilities are not only essential to provide juveniles to aquaculture facilities (Carter 2015), but also as a way to restock natural environments (Myers 2004; Rowland 2013) . To produce eggs and larvae, hatcheries need broodstock, and these fish are selected according to their characteristics in order to obtain fry/larvae with the best traits and improve the fish quality and genetics (Mylonas et al. 2011; Duncan 2013; Ranjan et al. 2019). Similarly to the different aquaculture production systems, hatcheries can also be categorized in extensive, semi-intensive and intensive, regarding the number of fry/larvae produced per volume of water (Lekang 2013). In these facilities all the environmental parameters are controllable (photoperiod and temperature being the most useful) and they are the quintessential for larvae rearing success and broodstock spawning (Mylonas et al. 2011; Duncan 2013; Lekang 2013; Ranjan et al. 2019). But, in many species, solely controlling the photoperiod and temperature is not enough to induce spawning, it needs additional hormone administration to complete the final gamete maturation, most commonly GnRHa (Gonadotrophin-releasing Hormone Agonists) (Mylonas et al. 2011; Fernández-Palacios et al. 2015; Zepeda et al. 2020).

1.5. Larvae rearing and its bottlenecks

After the spawning and hatching of the eggs, one of the most difficult and laborious stages of fishes' life cycle takes place, which is larval development (Mozes et al. 2011; Vadstein et al. 2018). This stage poses numerous constrains and bottlenecks in aquaculture, due to larvae's high sensitivity to the environment. Inadequately established

parameters such as light, temperature, salinity, feed, and others, can prove to be huge setbacks on larval rearing (Qin 2013). Also during the larval stages, many bone deformities that lead to eventual mortality can occur, being more common in larvae reared in manmade facilities, then when they are born in their natural habitat (Qin 2013; Guevara et al. 2014). But the major bottleneck is considered to be the non-inflation of the gas bladder (NGB), in pelagic species, which leads to increased mortality associated with lordosis, starvation, and cannibalism (Qin 2013), and to excessive energy consumption, due to swimming to maintain their position and not sink (Govoni and Forward 2008). Gas bladder plays a key role in fish neutral buoyancy, allowing fish to stay still in the water column without wasting energy, hence the importance of inflating it in larval stages (Pelster 2011). Without a functioning gas bladder, larvae would sink to the bottom of the water column, as the material composing their bodies are heavier than water (Govoni and Forward 2008; Pelster 2011).

(a) Physostome fishes (Eel)



(b) Physoclist fishes (Perch)

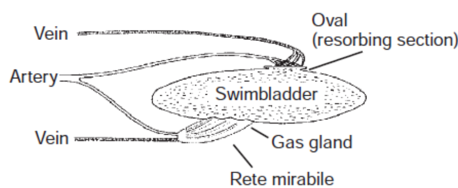


Figure 3 - Different swimbladder structures from physostomous fish (a) and physoclist fish (b) (Farrel et al. 2011).

There are two types of fish classified according to their swimbladder ontogenesis: the physostomes (Figure 3a) and the physoclists (Figure 3b) (Govoni and Forward 2008; Pelster 2011). All larvae are considered physostomes or transient physotomes (Sanabria et al. 2009; Price and Mager 2020) in their early stages, i.e., their swimbladder is formed through an invagination of the foregut and a pneumatic duct connects the swimbladder to the esophagus (Govoni and Forward 2008; Pelster 2011). The main difference between the two types of fish, is that in physostomes, this duct stays connected to esophagus even in the adult stages, whilst in physolcists fish, this duct only appears in a small window of time, after that, the swimbladder acts as a closed organ (Govoni and Forward 2008; Pelster 2011). But, since in all early larval stages the duct is still open, larvae inflate the swimbladder by going to the water surface and gulping air (Govoni and Forward Jr 2008; Pelster 2011; Villasante et al. 2017; Price and Mager 2020). Physostomes fish need to keep using this method to inflate their swimbladder in their adult stages, which sometimes can cause problems in aquaculture. For example, when Atlantic salmon (*Salmo salar*) is produced in grow-out submerged sea cages, it cannot inflate the swimbladder, as they do not have contact with the water surface. So, to counter this issue, air domes are added to the sea cage (Korsøen et al. 2012). On the

contrary, physoclist fish stop using this method once their pneumatic duct is closed and need to resort to other ways to control the inflation of their swimbladder. This is done via gas gland cells, that can be found in swimbladder's internal epithelium, which have a glucose driven metabolic pathway that reduces the gas-carrying capacity of blood cells, inducing the passage of O_2 and CO_2 to the swimbladder lumen through a partial pressure gradient, and also via the rete mirabile, a countercurrent system with numerous arterial and venous capillaries (Pelster 2011).

NGB might be due to many reasons: pathogenic action from bacteria, fungi or parasites (Pelster 2011; Villasante et al. 2017), water turbidity (Bristow and Summerfelt 1994; Rieger and Summerfelt 1997; Sanabria et al. 2009), gas supersaturation in water (Shrimpton et al. 1990; Elsadin et al. 2018), temperature, photoperiod, salinity, feeding and others (Sanabria et al. 2009; Partridge et al. 2011; Pelster 2011; Tielmann et al. 2017; Suchocki and Sepulveda-Villet 2019). But the main problem is related to an oil layer on the water surface formed due to lipids released from feeding, which larvae cannot penetrate in order to gulp the air, therefore preventing them to inflate the gas bladder. However, this problem has been almost overcome since many techniques have been created to remove the oil layer, such as skimmers, surface drains and spray nozzles (Pelster 2011; Qin 2013).

1.6. Species used during the internship

1.6.1. Pikeperch (*Sander lucioperca*)

Pikeperch (Figure 4) is a freshwater species widely distributed throughout the world, ranging from Germany, China, Russia, Sweden, and Finland (Steenfeldt 2015).

Figure 4 – Pikeperch (*Sander lucioperca*). Taken from Encyclopedia Britannica.

It is one of the main upcoming species in European aquaculture, which is mainly done in RAS (Robles et al. 2019), like in *AquaPri* (Denmark) where already 400 tons of pikeperch are produced per year using recirculating systems. The current main bottleneck for pikeperch production, tackled in a survey made by International Aquafeed, is the low knowledge on the currently used broodstock genetic pool, high larval mortalities, and high susceptibility to induced stress in husbandries and during handling (Robles et al.

2019). Focusing more on the larvae problem, as before mentioned, it is a very sensitive stage of fish life, and many bottlenecks may appear, the main being: high cannibalism which then lead to high mortalities, many deformities, great differences in size between larvae of the same batch, and NGB (which sometimes is the root problem of all the aforementioned constrains) (Steenfeldt 2015; Robles et al. 2019; Colchen et al. 2020). This species is a physoclist fish and its window of time in which the pneumatic duct is open is rather small, beginning at approximately 7 days post hatch (dph) and ending around 14 dph (Steenfeldt 2015), hence the importance to inflate it in the larval stages. With the increasing interest in pike perch as new species to aquaculture, the need to efficiently produce it requires studies to establish the perfect rearing parameters throughout the animal's life cycle. At DTU Aqua (Denmark), the lack of functional gas bladder in pike perch larvae has been a recurrent problem. Usually only 10-20% of the larvae will develop a functional gas bladder in their systems. Several modifications to tanks have been tried, such as: tank types, triangle skimmers and manual surface skimming, light/no light, spray nozzles (high /low intensity), feeding level, postponement of first-feeding until 5 dph (normally done at 3-4 dph), and feeding of unenriched live prey; all with no obvious effect. Further in this project, I will examine and evaluate the effect of two different potential external factors on gas bladder inflation: water turbidity and water exchange rate (Section 3).

1.6.2. European lobster (*Homarus gammarus*)

H. gammarus (Linnaeus, 1758), also known as the European lobster, is a benthonic crustacean that can be found throughout most of Eastern Atlantic and in the Mediterranean (Wahle et al. 2013). Its captures have increased in the last decade and a half (Figure 5) and their main purpose is for the food industry, as the European lobster is regarded as a high value food (Wahle et al. 2013; Goncalves et al. 2020), which sparked the interest in building lobster hatcheries (Jørstad et al. 2005).

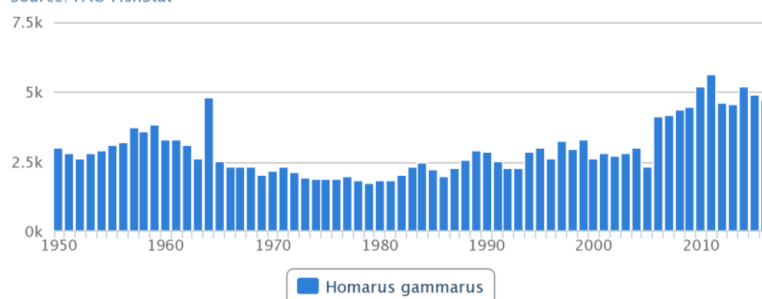


Figure 5 – Global capture production of European lobster (*Homarus gammarus*) since 1950 (FAO 2020).

To achieve great success in these rearing facilities, it is necessary to understand the complete life cycle of this species. Adult female European lobsters molt before mating, during which the male lobster deposits the spermatophore inside the female seminal receptacle, where the sperm might be stored throughout many years. During the extrusion of the eggs from the oviduct, the sperm is released and the external fertilization occurs, leaving the female lobster with an abdomen covered with fertilized eggs, which they carry for 9-11 months (Phillips 2013). These females, also called berried females, compose the broodstock of the hatching facilities and their hatching period can vary between three and fourteen days (females with more eggs tend to have bigger hatching periods) (Browne et al. 2009). The eggs do not hatch immediately into the first larval stage, firstly there is a short period of time where they are in prelarva stage (Phillips 2013). After that, they reach Stage I, then suffer two more molts: into Stage II and Stage III, which are still pelagic larval stages. The time it takes for larvae to reach Stage III varies greatly, reaching from two weeks to two months (Phillips 2013). This time frame can be shortened in the hatcheries by increasing the temperature (Browne et al. 2009). After Stage III, the larvae suffer metamorphosis into a postlarva stage (Stage IV), which is still pelagic for a few days, and then slowly shifts into a benthic state (Browne et al. 2009; Phillips 2013). Postlarvae then suffer consecutives molts throughout the years eventually reaching sexual maturity (Phillips 2013).

1.7. Internship objectives and task list

The main objective of this internship was to deepen the knowledge on RAS and on the daily procedures of an aquaculture research institute. The internship first started at DTU Aqua (Denmark), but an opportunity to work in a lobster rearing program (Section 5) at the *Nordsøen Oceanarium* (Denmark) appeared. The internship hours were then spent working at the oceanarium, whilst also helping at DTU's facilities.

The work done at DTU Aqua consisted of helping the institute technicians with daily tasks (Section 2) as well as preforming two trials, one carried out by me (Section 3), and another helping a PhD student (Section 4).

Task list:

- Daily work at DTU's facilities (Section 2);
- Trial regarding the effects of turbidity and water exchange rate in gas bladder inflation (Section 3), performed at DTU Aqua;

- Temperature challenge pilot test in European lobster post larvae (Section 4), performed at DTU Aqua;
- European lobster rearing project (Section 5), performed at the Nordsøen Oceanarium;
- Advanced course in recirculating aquaculture systems: Design and application, performed at DTU Aqua (Annex II).

2. Internship Description

DTU Aqua is a research center equipped with different facilities, each allocating a different research purpose. It comprises two quarantine rooms, one larval room, three different halls (one for digestibility trials, one for water quality trials, and one equipped with systems for fish trials with different purposes), four climate rooms, one extruder room, one separate digestibility hall, and one physiology hall. During my internship I had the opportunity to work in most of these facilities, and the tasks I carried out will be described according to each facility.

2.1. Digestibility hall

Starting with the digestibility hall, where different trials regarding fish nutrition were carried out, most of the work consisted of cleaning the tanks, collecting feces, feeding, and counting uneaten pellets (depending on the running trial). Exceptionally, some other labor was done in the facility, such as cleaning the automatic feeders between trials, weighing fish for new trials, dismantling a small system built for a single trial, and changing individual pumps that were not running properly. This facility had 12 large cylinder tanks, 18 medium cylinder tanks, 16 1x1 m^2 tanks, and 4 2x2 m^2 tanks (the exact volumes were not disclosed), and each set of tanks had a RAS system.

2.2. Physiology hall

The physiology hall was equipped with 12 medium tanks with individual RAS systems, 12 medium tanks that made up a single RAS system, and a respirometry laboratory. In this facility, I helped to load the fish inside the respirometry chambers, helped with a liver sampling in the end of a trial that was running in the 12 medium tanks system with rainbow trout (*Oncorhynchus mykiss*). During the “Advanced RAS Course” I also worked in this facility with the 12 tanks with individual RAS systems (trial described in Annex II).

2.3. Trial room

The pikeperch trial described in Section 3 was ran on a room located in one of the previously mention halls, equipped with 18 50L tanks, which compose one flowthrough

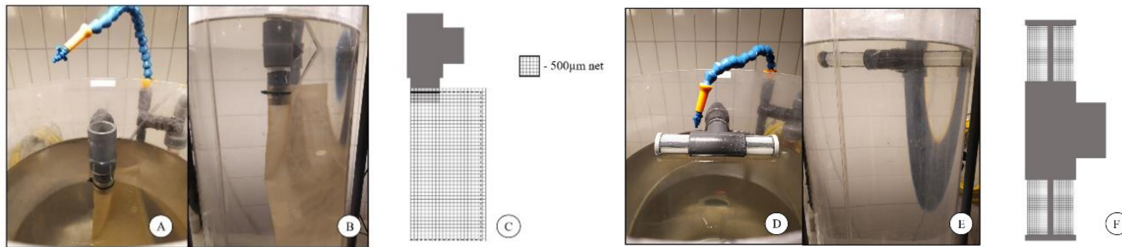


Figure 6 – A, B, C: Pictures and scheme of the initial tank outlet that did not promote the removal of the surface oil layer. D, E, F: Pictures and design of the newly built outlet, whose mesh is in contact with the water surface layer, hence removing the oil layer.

system, and 3 50L tanks to hatch Artemia. Before the beginning of the trial, the room and systems had to be prepared as follows: I firstly cleaned the freshwater reservoir, as it had not been used for a long time, and secondly I had to build new outlets for the 18 tanks, because the ones previously used (Figure 6 – A, B, C) did not break the surface tension. The new design (Figure 6 – D, E, F) consisted of a PVC T, two cut pipes in each end and two 3D-printed caps. These pipes had rectangle shaped holes cut in them which were then covered with a 500 μm mesh. This mesh would be on the water surface, enabling the water and the oil layer to come through, thus breaking the surface tension with the help of the spray nozzles already built in the system. Even though the new outlets were not 100% efficient on the removal of the surface oil layer, they were a big improvement over the old outlets. I was also in charge of setting up the pumps and buckets that would hold the clay solution, and, prior to the trial, I ran some tests with the pumps to be sure they were pumping the same volume out of all the tubes. Some turbidity tests should have been done as well, to make sure each treatment was achieving the same formazin nephelometric units (FNU) levels. Further during my stay, this room was also used to keep European lobster larvae, together with the oceanarium project.

2.4. Larval room

European lobster stage IV larvae were also kept at DTU Aqua, in a room with 4 raceways equipped with 3D-printed trays with 12 compartments each (3 x 4). These raceways partially recirculated the water, each having its own independent biofilter. In this room, I took care of the daily procedures of a trial regarding the effects of water temperature in European lobster post larvae, for 4 weeks, and when needed I was also in charge of the weekend feedings of the post larvae that were being kept in the room for further studies. The trial will be further explained in Section 4 of this report.

3. The effect of different turbidity levels and water exchange rates in swim bladder inflation of pike perch (*Sander lucioperca*) larvae

3.1. Introduction

Pike perch (*Sander lucioperca*) is a growing species in the aquaculture industry (Robles et al. 2019), and with this growing interest, comes the need to produce pike perch larvae in order to increase the productivity of this species. Being a physoclist fish, meaning its pneumatic duct closes during its larval stages, the gas bladder inflation (GBI) is key to a successful larval rearing. It can occur between 7 to 10 days post-hatching (dph) (Steenfeldt 2015; Blecha et al. 2019), and sometimes as early as 5 dph (Demska-Zakêœ and Kowalska 2003; Blecha et al. 2019). One of the biggest problems that *S. luciperca* hatcheries face, is the non-inflation of the gas bladder (NGB), which leads to bone deformities, lesser weight gain and high mortality rates (Demska-Zakêœ and Kowalska 2003; Szkudlarek and Zakęś 2007). This might be due to many different environmental factors, such as photoperiod, tank color, water temperature, bacteria, oil layer on the water surface, turbidity, stocking density, among others (Demska-Zakêœ and Kowalska 2003; Szkudlarek and Zakęś 2007; Clayton and Summerfelt 2010; Steenfeldt 2015; Blecha et al. 2019). Many of these factors have been tackled in different trials at DTU-Aqua, and none have had success in achieving high GBI ratios. So in this trial, we tested different turbidity levels, as pike perch thrives in turbid waters (Steenfeldt 2015), and many studies reveal that turbidity has a positive effect on SBI (Bristow and Summerfelt 1994; Clayton and Summerfelt 2010; Steenfeldt 2015). Additionally, different water exchange rates were also tested in order to achieve better water quality.

3.2. Materials and Methods

3.2.1. Experimental treatments

Each of the 5 treatments had 3 replicates (3 different tanks) and the conditions were the following: 3 tanks with 10 % water exchange per hour – Control (CTRL); 3 tanks with 100 % water exchange per hour (50 L/h) – High Water Exchange Rate (HWER); 3 tanks with 50 % water exchange per hour (25 L/h) – Medium Water Exchange Rate (MWER); 3 tanks with 10 % water exchange per hour (5 L/h) plus a solution of 4 g/L of clay at a rate

of 500 mL/h – High Clay (HCl); 3 tanks with 10 % water exchange per hour and plus a solution of 2 g/L of clay at a rate of 500 mL/h – Medium Clay (MCl).

3.2.2. Larvae rearing and daily procedures

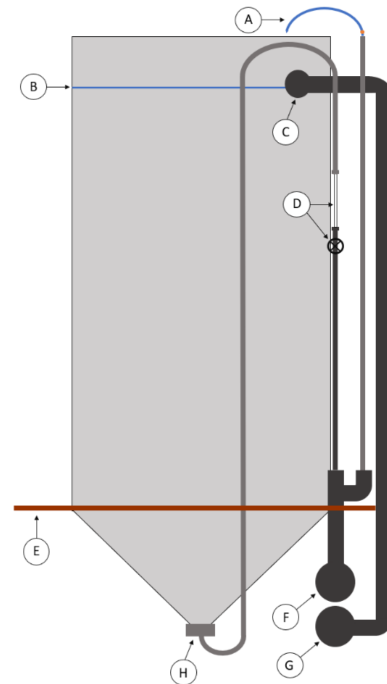


Figure 7 - 50L Tank scheme with each component: A- Spray nozzle and valve; B- Water surface; C- Water outlet (2µm mesh); D- Flowmeter and valve that controls the water flow; E- Table; F- Water inlet pipe; G- Outlet water pipe; H- 3-way water inlet valve.

The larvae used in the trial were purchased from *Fish2Be* (Belgium) and arrived at DTU's Aqua facility at 1 dhp and were acclimatized in three conical 50L tanks (Figure 7). At 2 dhp the larvae were separated into 15 50 L transparent tanks, approximately 1500 larvae in each tank, and were kept in the tanks without water exchange for one day. Throughout the trial the tanks were kept with a light cycle of 12h light-12h darkness (7 a.m. to 7 p.m.), with a light intensity of app. 20 lux and a gentle aeration provided by small air stones. The temperature fluctuated between 17.5 ºC and 19.4 ºC throughout the trial provided from a common 10 m^3 reservoir adjusted to approximately 18 ºC by a heat exchanger. At 4 dhp the water exchange rates were adjusted according to each treatment (5 L/h, 25 L/h or 5 L/h) and Clay (*Niigata Clay Mix, Denmark*) was added to the respective triplicate tanks for turbidity as follows: to 2 60L buckets were added two concentrations of clay (2 g/l and 4 g/l), and were re-filled with system water and clay when empty. To each bucket was connected a peristaltic pump (*LongerPump® BT300-2J*), each pump was loaded with three pump heads (*YZ1515x*) that carried the clay solution through a tube to the respective tanks at a rate of 500 mL/h. At 5 dph the larvae were fed manually with unenriched AF grade Artemia (Artemia Systems, Inve), 3 to 4 times a day until the lights were turned off. The cysts were incubated overnight (approx. 25 ppt, 28 ºC with high aeration), harvested the next day, and equally distributed to each tank to keep a density of app. 2-3 *Artemia*/mL. Every day at 8 a.m., each tank was individually "vacuum" cleaned by syphoning into a bucket the waste accumulated at the bottom of the tanks, composed of uneaten Artemia, and dead larvae. The tank bottom drain was also opened for 1-2 seconds into the same bucket, to collect the remaining waste, and the outlets' nets were cleaned with pressurized water from the spray nozzles. The dead larvae were counted, and mortality was registered. Alive larvae were put back into the respective tank. The tanks with the high clay concentration treatment caused some difficulties in the cleaning process due to high turbidity, which prevented observations of the tank bottom, making it difficult to syphon

the waste without removing a certain number of alive larvae. Counting the daily mortality in tanks with great turbidity was also more laborious due to the clusters created by the clay, dead Artemia and dead larvae, which might have led to an underestimation of the dead larvae. The trial ran for 17 days (until 18 dph) and at the end, the alive larvae were drained through the bottom of the tank to a strainer with a few cm of water. In order to keep larvae alive, these larvae were counted and used to calculate the total mortality. Afterwards, they were put in 2 different 50L tanks in case they were needed for another trial.

3.2.3. Sampling and measurements

Daily measurements of temperature (ºC) and dissolved oxygen (%) were carried throughout the trial in each tank with an Oxyguard® Handy Polaris. Every second day from 5 dhp, 500 mL samples were taken from each tank to measure ammonia (µg NH_4-N/L), hydrogen peroxide (H_2O_2) decomposition rate and turbidity (FNU - Formazin Nephelometric Units). Also starting from 5 dph ≥ 20 larvae were randomly taken from each tank and frozen for later bodyweight measurement. Every second day at 6 dph until the end of the trial, ≥ 20 larvae were taken from each tank for body length measurements and examination of swim bladder inflation. Total gas pressure was measured with an Oxyguard® Handy Polaris TGP inside each tank.

Swim bladder inflation development was examined under a Leica M125 stereo microscope connected to a computer with PROGRES GRYPHAX® Microscope Camera Software. It was calculated in % by dividing the number of larvae with inflated swim bladder with the total number of larvae in the sample. The software has a measuring tool which was also used to measure the total length of the larvae (from the tip of the mouth to the longer lobe of the caudal fin).

Water turbidity was measured in each sample using a Hach® 2100Qis Portable Turbidimeter. From the 500 mL of water sample, 42 mL were taken to measure H_2O_2 decomposition rate. This method was done according to a DTU Aqua in-house protocol that assesses microbial activity in water by enzymatic activity as described by Pedersen et al. (2019) (see Annex I). Ammonia was measured after filtrating 50 mL of the sample and analyzed following a DTU Aqua in-house protocol (see Annex I). In order to quantify bacterial activity, H_2O_2 decomposition rate constant (k-value) was calculated in each tank through an exponential regression analysis of the H_2O_2 decomposition curve, which was obtained via the protocol previously mentioned, and as described by Pedersen et al. (2019). With a low k, the decomposition rate is slower, therefore the bacterial activity is also lower and vice-versa. The larvae sampled for wet and dry body weight were stored

in labelled vials and stored at -80 ºC, until measurement. After unfreezing, excess water was removed from the larvae with a tissue, and each larva was weighed on a lab scale (± 0.1 mg). For determination of dry weight (DW), each larva was dried at 105 ºC for 2 h and weighed to obtain the pooled dry weight per tank.

3.2.4. Statistical analysis

To determine the significant differences between the control (CTRL) and the different levels of turbidity (HCL and MCl), and between the CTRL and the different water exchange rates (HWER and MWER), SPSS (*IBS-SPSS Statistics* v26, SPPS Inc., Chicago, IL, USA) was used to compared individual means. Initially the data was tested for homogeneity of variance by Levene's tests, and transformed whenever required, before submitting to a One-way ANOVA, followed by Tukey's multiple range test if the data showed significant differences. The differences were considered significant at *p*-value < 0.05. For graphic representation GraphPad (Graphpad Software Inc.) was used.

3.3. Results and Discussion

Daily measurements of temperature showed fluctuations between 17.5 and 19.4ºC throughout the whole trial, but all the tanks temperature fluctuated the same way (Figure 8A), as expected from a system running with water from one single reservoir.

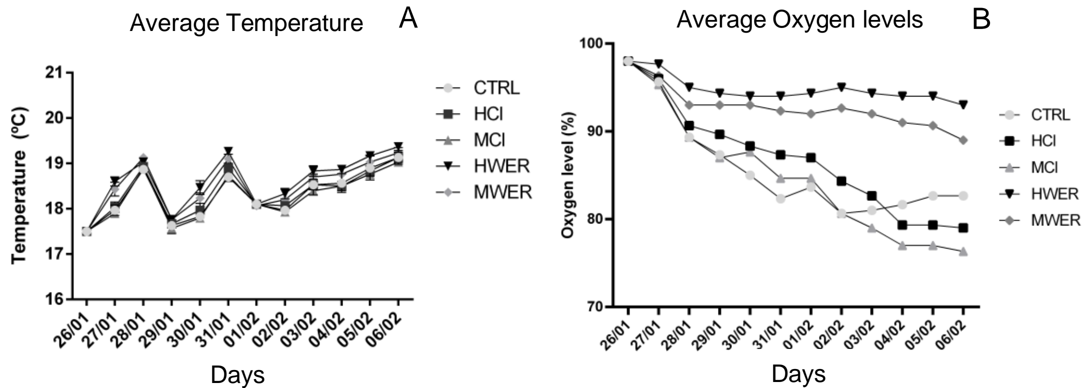


Figure 8 – A: average temperature (ºC) values throughout the whole trial with respective standard deviation lines, according to each treatment. B: average oxygen (%) values throughout the whole trial with respective standard deviation lines, according to each treatment.

At the beginning of the trial the dissolved oxygen (%) was the same in every tank but once the flows were adjusted according to the experimental design, the oxygen levels decreased (Figure 8B), and were in general lower in treatments with a water exchange rate of 10%/h (CTRL, HCl and MCl), than for the MWER and HWER treatments, which was expected taking into consideration that more water was being replaced per hour. Although the average dissolved oxygen content was highest in HWER and MWER and lower in tanks with lower water exchange rate (CTRL, HCl and MCl), levels never reached values below 73% (Figure 8A) considered to be within optimal rearing levels for all treatment groups.

Differences in oxygen, mortality, body length, wet body weight, dry body weight, ammonia, H_2O_2 k-value and gas bladder inflation between the control (CTRL) and turbidity levels (HCL and MCl) were represented in Table 1, and between the CTRL and the different water exchange rates (HWER and MWER) in Table 2.

Table 1 - Average values (± standard deviation) of oxygen, mortality, body length, wet body weight (WBW), dry body weight (DBW), ammonia, H_2O_2 k-value and gas bladder inflation at the end of the trial for the treatments CTRL, HCl and MCl.

	CTRL	HCl	MCl	p-value
Oxygen (%)*	85.8 ± 8.6	86.8 ± 6.4	84.7 ± 7.0	0.453
Mortality (%)	92.2 ± 4.5	84.9 ± 1.7	79.0 ± 8.4	0.072
Body length (cm)	1.39 ± 0.17	1.53 ± 0.22	1.14 ± 0.22	0.057
WBW (mg)	4.7 ± 0.5[a]	9.9 ± 1.0[b]	7.0 ± 1.4[a,b]	0.007
DBW (mg)	0.60 ± 0.05[a]	1.23 ± 0.18[b]	0.98 ± 0.22[a,b]	0.028
Ammonia (µg NH_4-N/L)	166.2 ± 72.3	172.3 ± 34.5	189.6 ± 52.5	0.789
H_2O_2 k-value	0.0261 ± 0.0054[a]	0.0353 ± 0.0103[a,b]	0.0601 ± 0.0142[b]	0.044
GBI (%)	38.8 ± 27.2[a]	97.9 ± 1.9[b]	94.5 ± 5.3[b]	0.000

* Oxygen values are the average from the whole trial.
Different letters in the same row, indicate statistical differences between treatments ($p<0.05$) (n=3).

Table 2 - Average values (± standard deviation) of oxygen, mortality, body length, wet body weight (WBW), dry body weight (DBW), ammonia, H_2O_2 k-value and gas bladder inflation at the end of the trial for the treatments CTRL, HWER and MWER.

	CTRL	HWER	MWER	p-value
Oxygen (%)*	85.8 ± 8.6[a]	94.8 ± 1.5[b]	92.7 ± 2.6[b]	0.001
Mortality (%)	92.2 ± 4.5	94.2 ± 2.2	93.5 ± 3.2	0.787
Body length (cm)	1.39 ± 0.17	1.34 ± 0.18	1.34 ± 0.17	0.458
WBW (mg)	4.7 ± 0.5	5.2 ± 1.5	6.1 ± 1.9	0.594
DBW (mg)	0.60 ± 0.05	0.61 ± 0.20	0.80 ± 0.19	0.444
Ammonia (µg NH_4-N/L)	166.2 ± 72.3[c]	23.7 ± 6.8[a]	56.8 ± 21.7[b]	0.000
H_2O_2 k-value	0.0261 ± 0.0054[b]	0.0089 ± 0.0024[a]	0.0079 ± 0.0029[a]	0.005
GBI (%)	38.8 ± 27.2	32.4 ± 44.1	25.2 ± 16.9	0.872

* Oxygen values are the average from the whole trial.
Different letters in the same row, indicate statistical differences between treatments ($p<0.05$) (n=3).

Mortality was not significantly affected by treatments (Table 1 and 2). As stated previously in the material and methods (3.2.2), mortality was affected by the daily cleaning of each tank. The ones with the highest turbidity were not as thoroughly cleaned as the others, due to the risk of syphoning out alive larvae and not being able to spot them inside the bucket. This might also be the cause of mortality's high standard deviation between the tanks within the MCI treatment, as seen on Table 1. On the other hand, the clusters created by the Artemia and dead larvae also caused a problem during the cleaning of tanks without clay, which might have led to the removal of alive larvae dragged with the cluster.

The higher water exchange rates were chosen under the assumption, that it would improve water quality parameters such as biochemical oxygen demand (BOD) and ammonia. This hypothesis was corroborated by the obtained data. O_2 (%) values were significantly higher in HWER and MWER than in the CTRL (P=0.001) (Table 2), as expected, since these tanks had a higher water exchange rate. Ammonia was significantly different in the CTRL, HWER and MWER treatments (P= 0.000). At 15 dhp, the CTRL tanks had an average of 166.2 µg NH_4-N/L, while the tanks with HWER and MWER had 23.7 and 56.8 µg NH_4-N/L, respectively (Table 2). When comparing the treatments with a one-way ANOVA, the data showed a significant difference between the CTRL, HWER and MWER (P= 0.000). HWER proved to be the most efficient in removing ammonia from the water, as the water exchange rate was the highest. The addition of clay did not seem to have an impact on the ammonia levels, as the HCl and MCl treatments were not significantly different (P = 0.789) from the CTRL tanks.

A positive correlation between turbidity and larval wet weight has been previously reported (Bristow and Summerfelt 1994). However, other studies have registered no significant effects (Salonen and Engström-Öst 2013). When analyzing the obtained data at the last day of the trial (15 dph), the average wet weight of larvae from HCl treatment was significantly higher (9.9 mg) than the CTRL (4.7 mg) (P = 0.007; Table 1). Larvae from the MCl treatment had the second highest mean weight (7.0 mg), but this value was not significantly different when compared to the CTRL. Similar results were obtained for larval dry weight (HCl = 1.23 mg; MCl = 0.98 mg; CTRL = 0.60 mg) (P = 0.028; Table 1). Since it was the last day of the trial, it was not possible to say if these differences were due to the larvae reaching a point where the weight difference will start to be more noticeable due to the turbidity, or if turbidity really has no effect on bodyweight . HWER and MWER showed no differences in wet and dry weight when compared to the CTRL (Table 2).

Bristow and Summerfelt (1994) reported that turbidity could improve larvae total length, which was not confirmed here. Both HCl and MCl treatments average larvae body weight followed the same tendency as the wet weight data (Table 1). HCl had the highest body length average by the end of the trial (1.53 cm), MCl having the second greatest (1.41 cm), but none were significantly different from the CTRL (1.30 cm). HWER and MWER showed no differences in larvae body weight when compared to the CTRL (Table 2).

As previously written, turbidity was achieved by adding clay with two different pumps through the top of each tank with HCl and MCl treatments. Both pumps started with the same flow rate, but during the trial some malfunctions led to different turbidities among the tanks within the same treatment. Some of these malfunctions were due to broken pump heads, others due to the aeration inside the buckets which was used to keep the clay suspended. Thus, the equilibrium between not having too many bubbles that would compromise the continuous flow in the tubes, and having enough aeration to make the mix homogeneous, was hard to maintain. The latter problem made the concentration of clay fluctuate inside the buckets throughout the trial, even though the buckets were always refilled with solutions with the pretended concentrations (4 g-Clay/L and 2 g-Clay/L). For this reason, the turbidity in tanks (Table 3) with HCl treatment is categorized as high turbidity (45 to 74 FNU) and tanks with MCl treatment have medium turbidity (12.9 to 33.3 FNU). The water in tanks with no addition of clay was always clear and never reached 1 FNU, values were between 0.24 and 0.74 FNU.

Table 3 - Average turbidity values (± standard deviation) from the whole trial in each tank.

	Tank nº	Turbidity (FNU)
HCl	1	45.1 ± 20.7
	3	74.0 ± 15.2
	8	49.4 ± 19.4
MCl	7	12.9 ± 5.8
	14	22.8 ± 11.8
	15	33.3 ± 11.1

The experiment was carried out as a flow through set up and the added clay was never fully removed. Thus, it would settle on the bottom of the tanks due to the relatively low water exchange rate. This layer of settled clay was never fully removed because of the risk of removing alive larvae while doing so, which alongside with the particles in the water column increased the likelihood of bacteria build up in the tanks.

The tanks with higher water exchange rate behaved as expected and had very similar H_2O_2 k-values throughout the trial (Table 2). These values were significantly lower (HWER - 0.0089 and MWER - 0.0079) than the CTRL (0.0261) (P = 0.005). As these tanks renewed more water than CTRL, hence the less bacteria build-up and activity.

When adding clay, the number of particles in the water increased, as proven by the turbidity (Table 3), so a greater bacterial activity was expected, which was not observed in the treatment with high turbidity (Table 1). HCl had an average k-value of 0.0353 which was not significantly different from the CTRL average k-value (0.0261). In contrast, the MCl average k-value of 0.0601 was significantly higher than the CTRL. HCl low k-value might be explained by the adhesion of bacteria to clay particles (Lunsdorf et al. 2000; Kang et al. 2013; Unuabonah et al. 2018), which then settle on the bottom of the tank, removing most of the bacteria from the water column and "trapping" them on the layer of clay at the bottom of the tank. This layer was never remixed in the water column and part of it was vacuum during the morning routine. The conclusions drawn from these results might be dubious due to sampling size, i.e., only three water replicates (one sample from each tank). A better procedure might be to take multiple water samples from different points of the tank.

The GBI results showed a clear evidence on the beneficial effect of turbid water. By the end of the trial, HCl had a GBI average of 97.9 % and MCl of 94.5 %, whilst the CTRL only had 38.8 % (Table 1; P = 0.000). The different water exchange rates did not cause any significant differences in GBI (Table 2). However, one of the tanks of the HWER treatment had a GBI of 83.3 % while the other two only had 7.1 % and 6.7 %. This value did not correlate with any other data from this tank. Thus, turbidity was also low (0.32 FTU) and mortality was the fourth highest of all the 15 tanks, which might mean the 83.3 % GBI might be an outlier. Considering this hypothesis, a high water exchange rate might lead to a lower GBI, most likely due to the energy larvae waste to counter the current and due to fatigue caused by the constant swimming. Other problems might be associated with high current, such as lordosis which was observed in a few dead larvae that were syphoned from the tanks with HWER.

3.4. Conclusion

Turbidity had a positive effect on GBI of pike perch larvae. However, its implementation on commercial hatcheries might be difficult, mainly due to the disturbance that clay causes in a running system. Having high number of particles in a system is not ideal. Thus, if it is a RAS system, it will require a high degree of filtration, and if it is a flow-

through system it does not require filtration, but the effluent would need extra treatment to remove the clay, a problem that RAS would also face. So, a major challenge of implementing clay as a "GBI enhancer" would be the design of a system capable of removing the clay and treat it, or re-use it, to reduce the clay waste. Clay also has the aforementioned advantage of removing bacteria from the water column. Instead of clay, green water (i.e. microalgae water) can be used. Thus, it is turbid and causes no harm to the fish, if the cultured algae are controlled; and it is also removable using floating media and sand filtration (Kwon et al. 2014).

The most recent study to this date on pike perch larvae rearing, done by Colchen et al. (2020), suggested twelve abiotic, feeding, and biotic factors to improve larval rearing in recirculating systems. With the combination of all these twelve factors they were able to achieve a GBI of 92.6 %, while in our trial we achieved 97.9 % and 94.5 % in the HCl and MCl treatments, respectively just by adding clay, whilst also controlling temperature, light, and flow. This suggested that clay can be as beneficial as the combination of the twelve factors. It is necessary to keep in mind that Colchen et al. (2020) also had more improvements besides GBI, such as survival and specific growth rate (SGR), but it is nonetheless necessary to take water turbidity into consideration as an easy way to improve GBI. A cost-efficiency evaluation needs to be made to understand how to use turbidity in the most efficient way.

4. Temperature challenge pilot test in European lobster post larvae (*Homarus gammarus*)

4.1. Introduction

Climate change has affected water temperature and ocean's CO_2 concentrations, consequently acidifying them, in a global scale, which could be one of the reasons for the decline in European lobster populations around Denmark (Schmalenbach and Franke 2010; Rato et al. 2017). European lobster adults are tolerant to different temperatures (Albalat et al. 2019) as their environment temperature varies from 0 ºC to 25ºC (Rojo et al. 2013), but the acidification and warming of the sea waters affects *H. gammarus* larvae and decreases their fitness (Small et al. 2016; Rato et al. 2017). Another rising problem that can come from the rise of seawater temperature, is the anticipation of larval hatching, which might lead to low population's recruitment, due to the environmental conditions that are not optimal for larvae to survive (Schmalenbach et al. 2010).

Larval rearing of European lobster is very important, mainly for re-stocking or remediation of wild populations (Agnalt et al. 2007; Powell et al. 2017; Hinchcliffe et al. 2020), and is usually done in inland facilities. One of the most laborious tasks in these facilities is the feeding process, because it is hard to determine how much food is necessary to add to the system in order not to underfeed the larvae, as it can result in nutritional deficiencies and high cannibalism rates. Very few recent studies have been made with dry feed pellets, which seem to be one of the easiest feed forms, as they are easily stored, can have a consistent known nutritional value and reduce both labor costs and difficulty (Powell et al. 2017; Hinchcliffe et al. 2020). In *Homarus* hatcheries, the larvae are usually fed with Artemia, microalgae, krill, finely cut pieces of fish and shellfish (Browne et al. 2009; Powell et al. 2017), which sometimes are dependent from fisheries and cost more than dried pellets (Mente et al. 2001).

4.2. Material and Methods

The post larvae were fed with one of two formulated diets, one with high protein (High P) content and one with low protein (Low P) content (percentages were not disclosed), or with krill. Larvae were acclimated to two different temperatures, 18-19 ºC or 12-13 ºC,

the former being the optimal for European lobster larvae. With these different parameters, 6 treatment-groups were created, each with 9 larvae:

- 18-19 ºC fed with Krill (control group)
- 18-19 ºC fed with High P
- 18-19 ºC fed with Low P
- 12-13 ºC fed with Krill
- 12-13 ºC fed with High P
- 12-13 ºC fed with Low P

Two flow-through semi-closed raceways were used to achieve different water temperatures, as both systems ran with water from the same reservoir at a rate of 5 L/min. Two heaters were added in each end (inlet and outlet) of one of the raceways, so that the partially recirculated water would also stabilize. Three trays with twelve (3 x 4) 200 mL compartments were used in each raceway, one for each feed, where the post larvae were kept for 4 weeks and with a light cycle of 8 h light and 16 h darkness. In the beginning of the trial, all the larvae were carefully dried with paper towel and the wet body weight and carapace length were measured as stated in Gonçalves et al. 2020. Every day between 9:00 and 13:00 all the post larvae were fed according to its treatment, 1 pellet or 1 krill piece, and after 4 h the remains of the food were taken and stored in the freezer for later weighing and feed conversion ratio (FCR) calculation. Eventual deaths and molts were registered. In the end of the trial, the post larvae were weighed, the carapace length measured and the specific growth rate (SGR), and the carapace length increment (iCL) were calculated as follows:

$$Specific\ growth\ rate = [ln(BW_f) - ln(BW_i)] * days^{-1} * 100$$

where BW_f = final wet body weight; BW_i = initial wet body weight

$$Carapace\ length\ increment = (CL_f - CL_i) * CL_i^{-1} * 100$$

where CL_f = final carapace length; CL_i = initial carapace length

After being ethically killed, through an ice-cold seawater anesthesia, the post larvae whole bodies were frozen for further evaluation of stress related hormones (done by the PhD student in charge of the pilot trial).

Data were analyzed with the software SPSS (*IBS-SPSS Statistics* v26, SPPS Inc., Chicago, IL, USA). Initially, data were tested for homogeneity of variance by Levene's

tests and transformed whenever required before submitting to a two-way ANOVA, followed by Tukey's multiple range test, if the data showed significant differences. The differences were considered significant at *p*-value < 0.05. For graphic representation GraphPad (Graphpad Software Inc.) was used.

4.3. Results and Discussion

Temperature and dietary composition had no effect on iCL and SGR (p>0.05; Figure 9A and 9B). Such an effect was most likely related with the low number of replicates, as some larvae died during the process, and due to variability in weight and sizes of the larvae, which might have implications in their fitness as these were early postlarval stages.

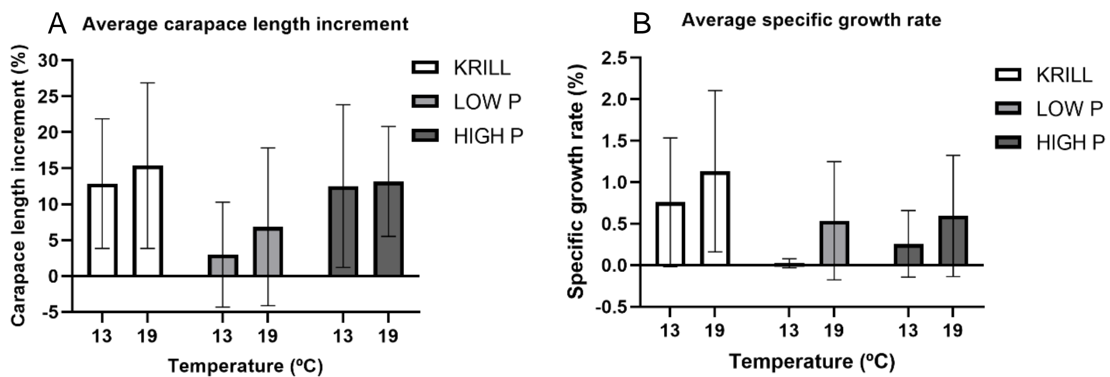


Figure 9 - A. Average carapace length increment according to temperature and diet. B. Average specific growth rate according to temperature and diet.

Although not statically different, it was observed that lower temperatures led to lower iCL and SGR (Figure 9A and 9B). Focusing on the carapace length increment, at 19ºC the post larvae fed with krill showed the highest iCL (15.4 ± 10.7%), and even under stress caused by low temperature, the ones fed with krill had a iCL similar to the high protein group at 19ºC (12.9 ± 8.4% and 13.2 ± 7.0%, respectively). This explains why krill is still the best feed for post larvae reared in systems, and is also corroborated by the SGR data, where Krill had the highest increment in both 13ºC and 19ºC (0.76 ± 0.73% and 1.13 ± 090%, respectively), when compared with the other diets.

Post larvae fed with low protein pellets (Low P) registered the lowest iCL and SGR within each temperature group, which might mean that protein levels are important for larval growth.

The number of molts and deaths are as shown in Table 4.

Table 4 - Number of molts and number of deaths according to each treatment

Temperature (ºC)	Diet	Number of molts	Number of deaths
13	KRILL	6	1
	LOW P	1	4
	HIGH P	7	0
19	KRILL	6	2
	LOW P	3	3
	HIGH P	6	2

As suggested by Hinchcliffe et al. (2019), it was expected that with the LOW P diet, the postlarvae mortality would be higher, which was observed at 13 ºC. However, mortality was similar between the three diets groups at 19 ºC. According to Powell et al. (2016) pelleted feed had no influence on the larvae mortality. One hypothesis might be that the postlarvae were able to endure the low nutritional value of the pellets if other parameters do not cause stress. But when reared at 13 ºC, the postlarvae were unable to withstand the protein deficit effect.

At 19 ºC, the number of total molts was higher in High P and Krill groups, 6 molts in each, and only 3 molts in the Low P group. Postlarvae reared at 13 ºC also showed more molts with Krill and High P treatments (6 and 7 molts, respectively), while Low P once again showing less molts (1 molt). This might be due to energetic trade-offs (Small et al. 2016), as the nutritional value is low in Low P diets and the stress caused by the low temperature leads the postlarvae to spend energy on survivability instead of growth and molts.

As this was a pilot trial, it helped to have a primary understanding on the effect of the tested treatments, and to have an idea on how to better carry out a future trial on the same subject. One of the main changes is to increase the number of larvae per treatment, as this trial had to be cut short due to many deaths on postlarvae reared at 13 ºC with LOW P diet. The available larvae were also limited at the beginning of the trial, hence the high variability of weights and sizes between the postlarvae. Overall, the next trial should be programmed according to the arrival of new berried females to the *Nordsøen Oceanarium*, as they are the supplier of larvae for the DTU Aqua trials. With these improvements, the effect of the different diets and temperatures might be more noticeable.

5. European lobster (*Homarus gammarus*) rearing project at the Nordsøen Oceanarium

5.1. Introduction

The increase in European lobster fishing might be one of the causes of the decrease in its stock (Agnalt et al. 2007), noticed by fishermen from Løkken to Hirtshals. The decrease in lobster catches took a toll on restaurants and markets throughout the northern Danish coast, which did not go unnoticed by the towns' populations and fisherman. Danish fishermen had to stop fishing this species, in order to letting it restore its population, and the lobsters found in the markets were mostly from by-catch. To re-establish the lobster stock on the Danish coast, the Danish Shellfish Center from Mors (Skaldyrcenter Mors), the North Sea Research Park (Nordsøen Forskerpark), and the Danish University of Technology (Dansk Teknologisk Universitet) joined efforts to develop a project to rear lobster larvae from stage I until releasable size, which I had the opportunity to help with. Since it was in everyone's interest to re-start lobster fishing, the local restaurants and tourism industry also invested in this project. After a few years, the Nordsøen Oceanarium had a room equipped with tanks for the fertilized broodstock, grow-out tanks, where the larvae would go from stage I to stage IV, and then transferred to a special system, Aquahive®, developed by Ocean On Land Technology.

5.2. System maintenance and daily procedures

The broodstock system consisted of a two-story rack, each story with twelve 40L boxes that acted as small scaled raceways, and the water flow would carry the hatched larvae through the outlet into a net (Figure 10).

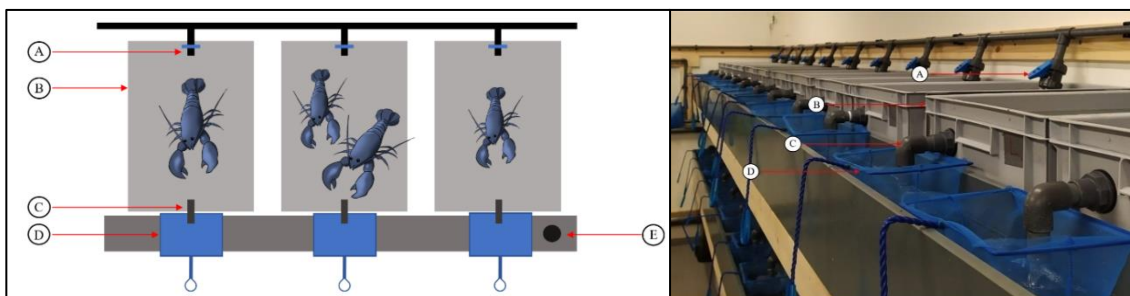


Figure 10 - Broodstock system top-view scheme (left) and picture (right). A- Water inlet with valve; B- 40L plastic box; C-Box outlet; D- Net to catch hatched larvae; E- Water collecting corridor with drain (hole).

As soon as Danish fishermen managed to fish berried females (females with fertilized eggs), they were brought to the Nordsøen Oceanarium facilities and, due to the limited capacity of the broodstock system (24 boxes), some boxes had more than 1 berried female with their claws taped shut. In order to not overcrowd all the boxes, some females

were put in a “stand-by tank” that ran at a temperature of 9ºC to delay the development and hatching of eggs. These females were then put in the broodstock system once the other female lobster no longer had eggs, making it possible to have a second batch of larvae while waiting for more berried females to be caught. The broodstock was fed with shrimp or mussels twice a day, once in the morning and once in the afternoon, and the remains were then syphoned out of the boxes.

The facility was equipped with six tanks (Figure 12) with different volumes destined to grow larvae from stage I through stage IV, all of them ran at 19ºC (with some fluctuation throughout the day) and with high aeration, to prevent larvae from clinging to each other, thus decreasing cannibalism inside the tank. Every morning, the newly hatched larvae were collected, counted, and put in one tank. In order to decrease cannibalism due to different size between larvae, each tank would only receive new larvae for three days. The larvae harvested on the fourth day would be put in a different tank, and the procedure would be repeated.

Figure 11 - Newly hatched larvae in a bucket with drawn lines to help the counting procedure

Before being put in the grow-out tanks (Figure 12), the larvae were transferred to a white bucket, with water syphoned from the broodstock tanks, where a picture was taken. With the aid of lines drawn using a smartphone software (Figure 11), the larvae were counted, and the number was registered on a white board.

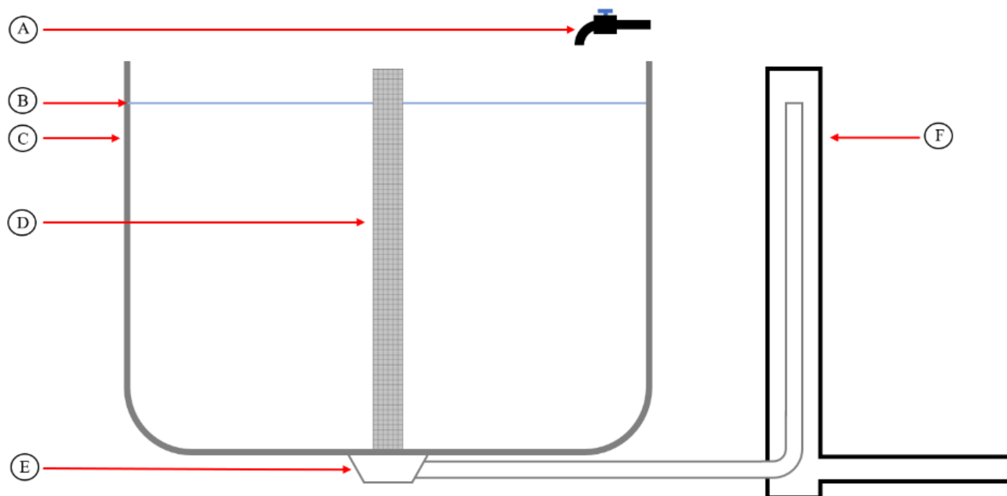


Figure 12 - Grow-out tank scheme. A- Saltwater inlet; B- Water surface; C- Tank; D- Outlet mesh connected to bottom drain (E); F- Effluent standpipe (to control tank water level).

After harvesting and counting the newly hatched larvae, a rough estimation of the number of larvae on the already running tanks was made in order to have a better understanding on the mortality of the larvae. The rough number was then registered on the same white board, thus enabled us to have a daily log of the number of larvae on the facility. When all the larvae were counted, krill was unfrozen and blended for a few seconds with salt water. This technique was then changed because it created too much foam, when the mix was added to the tanks, and made it harder to clean each tank. Instead, the krill was cut into very small pieces and spread uniformly in each tank. This was done twice a day, and before the second feeding the aeration was turned off to make the uneaten krill float to the top, making it easier to be removed. The latter technique, combined with the scrubbing of the center net, and the removal of slime on the tank walls, made up for the daily cleaning routine of the grow-out system.

When it was noticed that larvae started to molt into stage IV, every morning before the feeding, the stage IV larvae were harvested with a small hand net and transferred to a tray in the Aquahive (Figure 13). The Aquahive was design by Ocean On Land Technology to hold up to 28 trays (only 27 could be filled with larvae), each tray with 140 honeycombed compartments that could house one stage IV larvae. This unit contained six 90L cylinders and functions as a RAS system with mechanical filtration, biofiltration and two UV lamps, it is also equipped with six “feeding pumps” to recirculate the water inside each cylinder independently. The flow in each cylinder could be controlled by a valve located in the inlet. When putting the larvae in each honeycombed compartment, most of the compartments should be filled with larvae, otherwise it would build up bacteria after 3-4 days of feeding. After making sure that most of the 140 compartments had one larva, a new tray was put on top, so that the larvae did not swam away, and the locking nut was screwed.

Figure 13 - Aquahive 90L cylinder scheme, equipped with the maximum capacity of 28 trays (taken from Ocean on Land Technology Aquahive Flyer).

To feed the larvae in the Aquahive, both the cylinder’s outlet and inlet were closed, the feed pump valve was opened according to the desired flow and each pump was turned on. The krill quantity was roughly measured according to the number of larvae in the trays and then blended with saltwater. This mixture was then slowly poured in each cylinder, from the top into the central pipe, and would recirculate through the trays for 1h, twice a day. Due to the lack of larvae to fill every tray to the maximum, each cylinder needed to be clean every 3 days, and the larvae were moved to cleaned trays. This was

mainly due to the buildup of bacteria, either because of a compartment without a larva, or caused by the death of a larva. So, every third day, the larvae were changed to a new tray and distributed through most of the compartments. The dead larvae were removed, and the cylinder walls and central pipe were cleaned and scrubbed. Used trays were put inside a bucket with a Virkon™ S solution for a day or more, and then were thoroughly cleaned, dried, and stored for the next usage. Virkon™ S was also used to clean the tanks in between batches of hatched larvae, i.e., after all the larvae turned stage IV, and were put in the Aquahive, the tanks were emptied and cleaned with Virkon™ S.

The first phase of the pilot trail culminated with the release of 808 postlarvae lobsters on the coast from Løkken to Hirtshals. When getting ready for the second phase and waiting for the next batch of berried females, all the systems were cleaned and two new grow-out tanks were build, so that the room could handle more larvae hatching at the same time, and the tanks would not be over stocked. Once the new broodstock arrived, they were distributed throughout the tanks, but the amount of broodstock was over the capacity of the system, so some of them were put in tanks at 9ºC to delay the hatching of the larvae. When the first tank was set up, after one week, the decrease in the number of larvae was alarming, and after some analyzes a bacterium was discovered but unidentified. The cause of the high mortality rates was attributed to these bacteria due to its huge presence on the water and tank walls. These bacteria proliferated due to overfeeding and the system's incapability to remove all the uneaten food. At first, it only appeared on the grow-out tanks, as the larvae on the nets were analyzed and no bacteria were found, but after some time, even the broodstock had some hair on the eggs, when analyzed in a magnifying microscope. To advert this crisis, new measures needed to be taken and the work should be carried out more thoroughly.

The first step was reducing the feed, which proved to be harder than expect, because when giving less amounts of food the larvae were not eating enough (when the enzymes were analyzed by a PhD student, the levels were very low), but if we gave the right amount, it would still settle in the bottom of the tank and build up bacteria. Currently, trials with Artemia are being carried out, as it is a great feed for lobster larvae (Browne et al. 2009), does not settle on the bottom of the tanks, and can be easily washed out by the tank flow. Secondly, three different females were submitted to a "Betadine bath", by dipping their abdomen in a bucket with Betadine solution. The larvae hatched from these three lobsters were put on a separate tank, so that we could compare the effect of betadine bath on the larvae mortality. Before putting the larvae of these three females on the designated tank, they were put in a bucket with a low concentration of *Diversey™ Divosan Activ Disinfectant* (1 mL per every L of water). This was done as a preventive

measure, as it poses no threat to the larvae health, and kills the bacteria. Sometimes it was also used on the grow-out tanks, by previously stopping the flow, and then adding the Divosan solution.

The efforts done to cope with the bacterial crisis were not done fast enough, which resulted in the loss of a great number of larvae, but nevertheless gave us the opportunity to design a better protocol for daily maintenance and be prepare for similar crisis. Some aspects of this rearing project can still be improved, mainly regarding the stage IV system which takes most of the time and is not as practical as the system promised to be. DTU Aqua is equipped with raceways with 3D-printed trays design specifically for the European lobster larvae and postlarvae stages. But even though it is easier to maneuver the lobsters inside them, they take much more space than the Aquahive, as the raceways need long corridors, and it is much harder to feed the larvae in them, whereas the Aquahive has the trays built in height, and the feeding process take less than 5 min to prepare.

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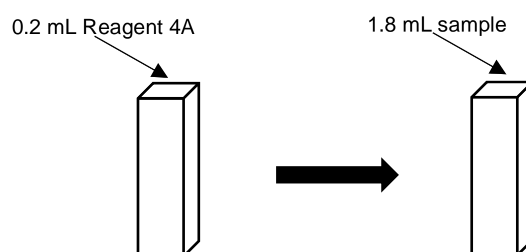
Annex I

Hydrogen Peroxide Determination

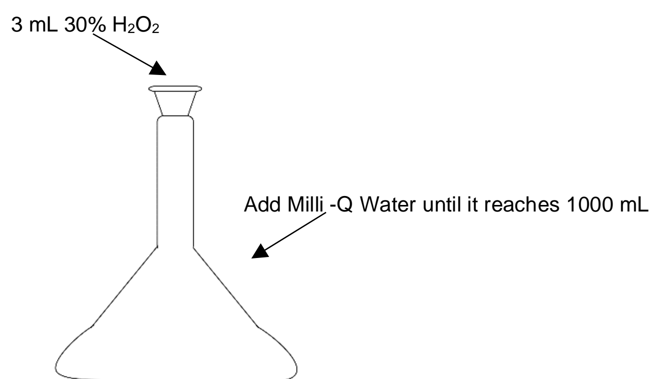
The determination is based on the Tanner and Wong (1998) method. H_2O_2 is fixated with an OPDV reagent (Reagent 4A) and forms a yellow/orange color. The process is stable after 15 min and is measured at 432 nm on a spectrophotometer.

Reagent 4A is prepared by mixing 1.2g NH_4VO_3 with 5.2g dipicolinic acid (recrystallized), 60mL Milli-Q water and 60mL concentrated H_2SO_4 followed by heating to dissolution.

Work order: 0.2mL of Reagent 4A is transferred to a cuvette and 1.8mL of sample can be added and fixated.



Spike solution (1000 mg/L H_2O_2)*:*



Degradation analysis:

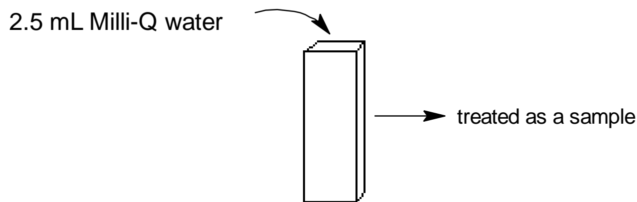
45mL of sample is transferred to a 50mL sterile vial and needs to be placed in water bath to reach 22ºC. Cuvettes with 0.2 mL of Reagent 4A are prepared for the sampling times: 0, 1, 15, 30, 45, 60 min, which means, 6 cuvettes per sample.

When the sample has reached 22ºC, 1.8mL of the sample is transferred to the "time 0" cuvette. To start the degradation, the 50mL sample vial is spiked with 0.43mL of the 1000 mg/L H_2O_2 solution and the time starts counting, after 1 min another 1.8 mL of the sample

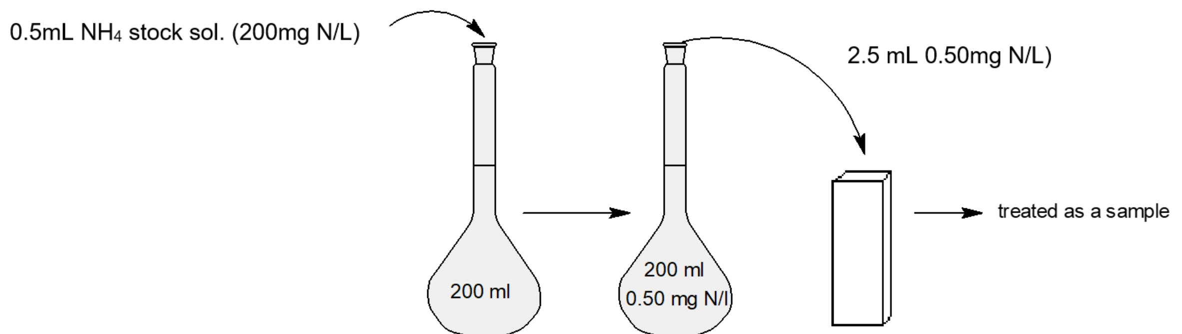
is taken and transferred to the “time 1” cuvette, the same procedure is done at 15, 30, 45 and 60 min.

Ammonia (µg NH_4-N/L) determination in freshwater

Blind sample:



Standard:



Reagent 2:

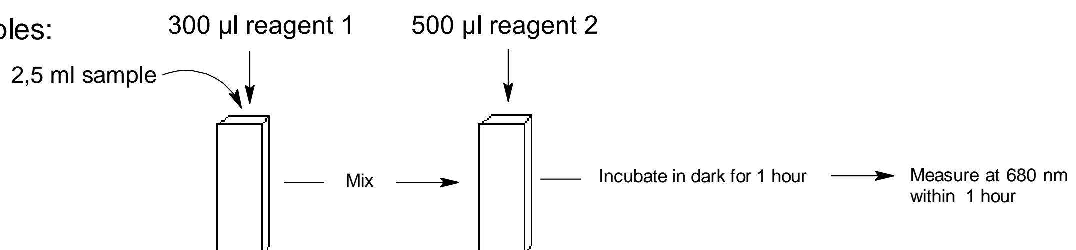
Reagent 2 is a mixture of: Milli-q water, Sodium hypochlorite and Alkali-citrate solution.

Reagent 2 is stable for 1 hour after mixing it.

Examples of different mixtures:

H_2O, mL	0.5	0.8	1	2	2.4	3	3.5	5.2
NaHClO, mL	0.25	0.4	0.5	1	1.2	1.5	1.75	2.6
Alkali-citrate, mL	6.75	10.8	13.5	27	32.4	40.5	47.25	70.2
Total volume	7.5	12	15	30	36	45	52.5	78

Samples:



Annex II

Advanced course in recirculating aquaculture systems: Design and application

In the end of the internship, I had the opportunity to join an advanced course in RAS, which is part of DTU's Aquaculture courses and had the duration of 10 days. During this 10 days, different lectures were taught regarding different RAS themes:

- Fish waste and its relation to feed composition.
- RAS components and focus areas.
- Waste and discharge – waste and end-of-pipe treatment.
- Biofiltration and chemical water quality; Components and operation; Important parameters.
- Particles in RAS.
- RAS input and components; Set-up and design calculations.
- Water chemistry and microbial water quality

Complementary to the theoretical lectures, two practical trials were carried out, one throughout the whole course, which was the main trial where different groups took care of their own recirculating systems, and one during an afternoon related to biofiltration kinetics.

1. First trial: Biofiltration Kinetics

Nitrite is only now becoming an emerging concern in RAS facilities, which seldom were equipped with denitrifying reactors, due to Nitrite's low toxicity in freshwater, and no monetary compensation for the removal of this compound from the water discharges (Suhr et al. 2013; Sander et al. 2018; Timmons et al. 2018d; Torno et al. 2018). This is a constrain that needs to be tackled especially in saltwater systems (Zhu and Liu 2017), due to the growing interest in marine species that leads to bigger productions and lower recirculation rates, to make the production more cost-efficient (Herreros, 2017). But there is still no definite truth regarding the effect of salinity on denitrification (Timmons et al. 2018e).

This trial was related to biofiltration kinetics and the total ammonia nitrogen (TAN) removal rate of a biofilter. The goal was to calculate the surface TAN removal (STR) by using a simple linear regression, and also to see the effect of different biomedia volumes

and of the salinity on the TAN removal rate. Thus, 3 different experimental setups were used and each setup had a dedicated group.

The first setup had two small-scaled biofilters, one with 1.25L of biomedia and the other with 2.5L, both having 4L of fresh water taken from a working RAS system from DTU'S facility, the biomedia was taken from this system's biofilter. The two biofilters were spiked with a solution of 5 ppm TAN.

The second experimental design was similar to the previous one, in biomedia volume and RAS water volume, but instead of being spiked with a TAN solution, the spiking was done using a 5 ppm Nitrite-N solution.

The third experiment had both biofilters with 2.5L of biomedia, but used 4L of 16‰ seawater, achieved by adding 2L of RAS water and 2L of seawater. Then one biofilter was spiked with the 5 ppm TAN solution, and the other with the 5 ppm Nitrite-N solution.

All three experiments were run at the same time by three different groups, and once the biofilters were spiked, samples were taken when 5, 15, 30, 45, 60, 90 min passed, which were stored in vials ate room temperature and properly labeled. Depending on the solution with which the biofilters were spiked, TAN concentration or NO2-N concentration was measured by the lab technicians. This step was not done by the students due to safety measures regarding the current pandemic

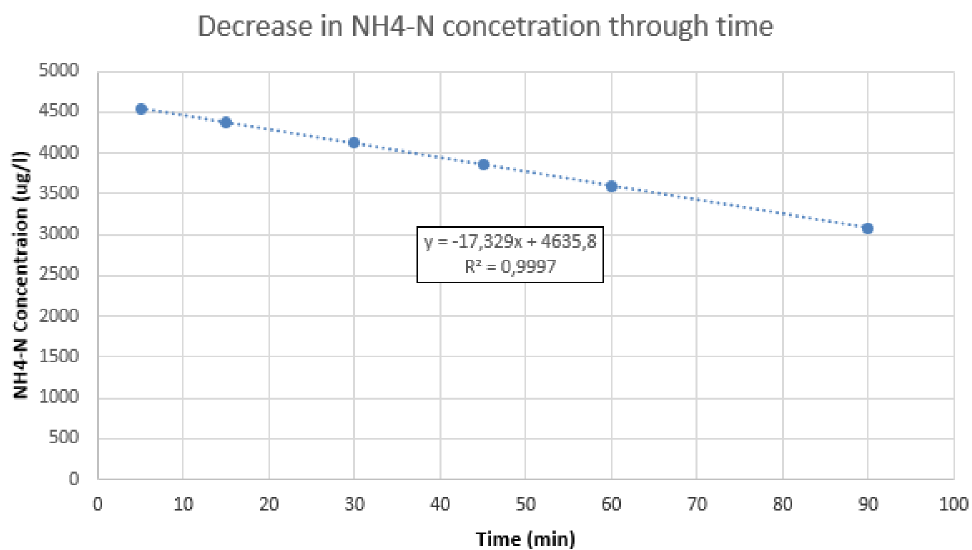


Figure 14 - Graph depicting the decrease in the concentration NH4-N after the biofilter is spiked with a TAN solution, values form the biofilters with 1.25L of biomedia, 4L of fresh water and spiked with a solution of 5 ppm TAN.

Once the concentrations were obtained, a linear regression (y = mx + b) was done with the concentration of TAN throughout the minutes and, using the slope of the linear regression, it was possible to obtain the TAN-µg/l of water/min value. From Figure 14 we take the slope: (-)17,33 TAN-g/l of water/min by doing the following equation whilst doing correct unit conversions, we were able to obtain the STR (g-N/m^2/day):

$$STR = \frac{|\text{Slope Value}| * \text{Volume of Water Used}}{\text{Surface Specific Area} * \text{Volume of Biomedia Used}}$$

Volume of water used – m^3

Surface specific Area – m^2/m^3

Volume of biomedia used – m^3

Once the results were obtained, troubleshooting and discussion took place, in order to compare the results and their accordance to the initial hypothesis. All the data cannot be shown here as the students did not receive the data from all the groups, only discussed it in class.

As expect in the first and second experimental design, the biofilters that had more biomedia volume had a bigger STR, as the amount of bacteria is bigger and therefore can nitrify more ammonia and nitrite at the same time. The results of the third experimental design, when compared to the ones obtain from the other experiments, also proved to be accoding to what was expected, i.e., when using biomedia from a freshwater system and adding saltwater, the bacteria cannot whithstand the difference in salinity and are not able to performer as they would in freshwater, resulting in very low STR.

2. RAS Trial

For the main trial, each group was composed of three people, each with one "specialty" (biofilter activity & chemical water quality, particles or microbial water quality). There were three groups in total and each group tested different component changes in a RAS system. As previously said, these trials were carried out in the physiology hall using 9 tanks with individual RAS systems, each system was composed by a 500L tank, a swirl separator, a 300L sump with a pump to return the water to the 800L submerged fixed bed biofilter, and a trickling filter (Figure 15). The species used was rainbow trout (*Onchorhynchus mykiss*). Every group had a different experimental setup, one group had different fish biomasses in each tank to study the effect of feed loading. Another had different levels of UV filtration (no lamp, one lamp and two lamps) to study the effects of UV radiation. The last, which was the group where I was inserted, had different degrees of microfiltration (none, 200µm cartridge filter, and 1µm cartridge filter) to test the effects of microparticle filtration. Every group's "specialist" had daily measurement practices for biofilter activity & chemical water quality (TAN; NO_2; NO_3; chemical oxygen demand (COD); alkalinity and biochemical oxygen demand (BOD5)), particles (distribution; numbers; area; volume; turbidity and UV transmission (UVT)), and microbial water quality (H_2O_2-degradation rate and Bactiquant). Each group member had the opportunity to try all the methods once, but the specialist did most of the measurements regarding their specialty.

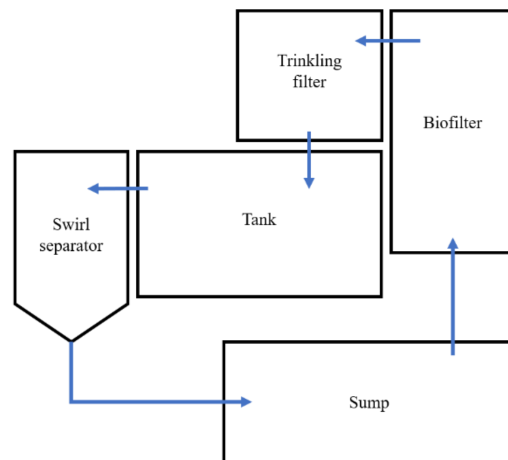


Figure 15 - Schematic design of the RAS units used during the trial (adapted from Gregersen et al. 2019 to enhance the image quality).

The daily routine was the same for every group and was proceeded as follows. At 8.30 a.m., 1L of water sample was collected from each swirl separator, which was then subdivided into different vials to make the different analyzes. After checking for dead fish, oxygen levels, pH and temperature were measured using a probe, and registered in the trial sheet. When pH was below 7, bicarbonate (HCO_3^-) was added. Then the feces collectors, located at the bottom of the swirl separator, were emptied, and checked for feed spill, which was also registered in the trial sheet. 40L of water were removed from the tank and then 80L of water were added to the water change reservoirs, which were equipped with valves to slowly release the new water into the sump. The fish biomass in

each tank and the amount of feed was predetermined by the teachers. The fish in each tank were fed 100g of feed, which was loaded into the automatic feeder's belt in the morning and then slowly poured into each tank. In our trial, the cartridge filters also needed to be changed daily, the 200µm cartridge was changed once every morning and the 1µm cartridge was changed twice per day, once in the morning and once during the afternoon, due to frequent clogging.

My role in the group was the particles "specialist", as my previous trial with pikeperch had to do with turbidity, and I felt it would be suitable for me to learn more about particles in RAS. As the particles "specialist", my assigned daily procedures consisted of: measuring the turbidity of the previously taken samples, using a *Hach® 2100Qis Portable Turbidimeter*, measuring UV transmission, using a proper equipment that measures absorbance in a chosen wave length, and measuring the number of particles/total volume/total surface area per particle diameter, using a *Multisizer 4e Coulter Counter*. The UV transmission (%) translates into the percentage of UV light that passes through 1cm of water. Thus, with this value it is possible to dimension the necessary intensity of the UV light in a system. The turbidity levels, obtained in FTU, correlate well with the number of particles in the water, as it is measured by a sensor that receives the light scatter from particles at 90º, and helps assess the water quality state, which is also a focal point in RAS systems. The Multisizer 4e Coulter Counter output, was a file with the number of particles, total volume and total surface area attributed to each particle size category, and with this data we can understand the degree of microfiltration needed, as it shows the most common size of the particles in the water column.

As the facility had a limited number of tanks, none of the trials had replicates and the trials only lasted one week, which then translated into data without scientific rigor. In spite of this, the main focus of these trials was to present the data obtained, troubleshoot and analyze the results, to see if it was according to what was expect and, if any irregularities were found, what could be their cause, which enabled a better understanding of different RAS parameters and what can affect them. On the last day of the course, each group presented the gathered data and discussed it with the class and present researchers/teachers.

3. Final assignment

During the course, we learned how to design a RAS system and how to dimension the key components of the systems. As the final assignment, each participant had to choose a species and tons of fish produced per year in the facility to be designed. Then we would be given the average density of this species population in aquaculture, the average specific growth rate (SGR) and the average food conversion ratio (FCR). With all this data we could then make the necessary calculations to design a RAS facility. This course was not graded numerically in the end. The classification system consisted of Pass or Not Pass, which I was attributed a "Pass" grade. My written assignment was projected for a pike perch aquaculture aiming to produce 700 tons per year, the calculations were the following:

Calculations for a pike perch RAS facility

The calculations in this report were done with the premise that the designed facility would produce and sell fish every day, and by using the following values for the species *Sander lucioperca*:

- Average density: 70 kg/m^3
- Average SGR: 0,8 %/day
- Average FCR: 1.1
- Water temperature: 27°C
- Salinity: 0 ppm
- Production size: 700 tons/year

A production of 700 tons/year means that the production per day is 1.92 tons. Considering the SGR, we can calculate the amount of fish in the tanks:

$$\frac{100\ \% * 1.92\ tons}{0.8\ \%} = 240\ tons$$

With the average density it is possible to calculate the total volume of the tanks:

$$\frac{240000\ kg}{70\ kg/m^3} = 3430\ m^3$$

I chose to use 10 tanks as follows:

- 4 big – 1600 m^3 in total – 400 m^3 each tank – radius: 7.14 m
- 3 medium – 1144 m^3 in total – 381 m^3 each tank – radius: 6.96 m
- 3 small – 686 m^3 in total – 229 m^3 each tank – radius: 5.39 m

FCR * Production per day = 1.1 * 1920 kg fish/day = 2112 kg feed/day

I chose biomar's pike perch 4.5mm grower feed (https://www.biomar.com/en/denmark/product-and-species/pike-perch/growers/#refid-2300) and by using the amount of feed per day and the waste model provided during the course (filled in with the composition of biomar's feed), I obtained the TAN and the urea produced per ton of feed:

42.11 kg-N/ton feed = 42.11 g-N/kg feed

Amount of N produced per day = 42.11 g-N/kg feed * 2112 kg feed/day = 88 936 g-N/day.

N-conversion at 12ºC = 0.2 g-N/m^2 of biomedia surface/day, since N-conversion increases 1.08 times per 1ºC (*rule of thumb*) and our systems runs at 27ºC, N-conversion in the biofilter will be 0.63 g-N/m^2/day.

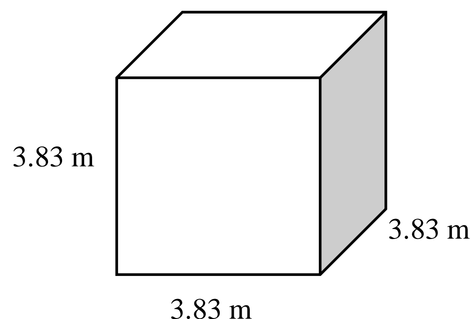
$$\frac{88936 \text{ g-N/day}}{0.63 \text{ g-N}/m^2/day} = 141\ 168\ m^2 \text{ biomedia surface area}$$

Using the biomedia from HEL-X model HXF13KLL+ which has a surface specific area of 955 m^2/m^3 (https://www.hel-x.eu/en/technical-details/?gclid=CjwKCAjwyo36BRAXEiwA24CwGZMkc_Kg51i6Zu4YsaVIKK019-19hzyItZBNmOG3NEv58b5p_MhVYxoCUikQAvD_BwE):

$$\frac{141168\ m^2}{955\ m^2/m^3} = 147{,}8\ m^3 \text{ of biomedia needed}$$

In a moving bed only 66% of the total volume is made up by the biomedia and the other 34% is "extra space" so that the biomedia can mix (*rule of thumb*), in this facility I chose to have 4 biofilters, each with 56.06 m^3:

- 66% = 37 m^3 of biomedia
- 33% = 19.06 m^3 of "extra space"



Flow = 3 * Total volume per hour = 3*3430 = 10 290 m^3/h

In a perfect scenario, the water that enters the tanks should have 100% Oxygen saturation or higher, but, in a real scenario, after passing the trickling filter the water has approximately 93% oxygen saturation. Using the calibration table for dissolved oxygen I obtained the value in mg-O_2/l of 100% oxygen saturation ate 27ºC and 0 ppt:

- 100% Oxygen saturation = 7.9 mg-O_2/l
- 93% Oxygen saturation = 7.35 mg-O_2/l
- 65% Oxygen saturation = 5.14 mg-O_2/l

In order to keep the fish healthy, we cannot let the oxygen saturation go below 65%, which means that in this case, the fish consumption would be .,21 mg-O_2/l (= 7.35 – 5.14).

Note: mg/l = g/m^3

Flow * Fish consumption = 10 290 m^3/h * 2.21 g/m^3 = 22 741 g-O_2/h = 545.76 kg-O_2/day, this is the amount of oxygen provided by the flow.

0.5 kg-O_2 is consumed per 1 kg of fish feed (*Rule of thumb*):

- 2112 kg feed/day * 0.5 kg-O_2/kg feed = 1056 kg-O_2 consumed per day
- 1056 kg-O_2/day – 545.76 kg-O_2/day = 510.24 kg-O_2/day that needs to be added by oxygen cones.

Oxygen cones have a maximum capacity of 0.30 g-O_2/h/cm^2 at 1bar, 34ppt, 25ºC and acceptable efficiency, and with these parameters the dissolved oxygen is 6.8 mg-O_2/l (with 100% saturation). Since the system is running with 1 bar, 0 ppt and 27 C, some corrections need to be made:

- Dissolved oxygen at 1 bar, 0 ppt and 27 ºC = 7,9 mg-O_2/l
- $\frac{7.9 \text{ mg-O}_2\text{/l}}{6.8 \text{ mg-O}_2\text{/l}} = 1.16$
- Cone's *corrected* maximum capacity = 1.16 * 0.30 = 0.348 g-O_2/h/cm^2

If the facility has 6 oxygen cones, then each cone needs to provide $\frac{510.24 \text{ kg-O}_2\text{/day}}{6}$ = 85.04 kg-O_2/day = 3540 g-O_2/h, and to calculate the area of the base of the cone:

- $\frac{3540 \text{ g}-\text{O2/h}}{0.348 \text{ g}-\text{O2/h/cm2}} = 10\ 172 \text{ cm}^2$

With an area of 10 172 cm^2, the diameter of each cone's base is 1.14 m. As a *rule of thumb,* the height of the cone should be 2.5 times bigger than the diameter of the base:

- 2.5 * 1.14 = 2.85 m
- Cone's total volume = 3.88 m^3

But, in order to be able to accommodate differences in the system, it is better to increase the cones capacity by 10%, which means the total volume should be 1.1 * 3.88= 4.27 m^3, in this case the cone would have the following measurements:

- 4.27 $= \frac{\pi * r^2 * (2{,}5 * 2r)}{3} \leftrightarrow r = 0{,}93$ m
- Diameter = 1.86 m
- Height = 2.5 * 1.86 = 4,.5 m

When considering which drum filter is more suitable, it is necessary to take the water flow into consideration, which in this case is 10 290 m^3/h. A suitable option would be the model MAT1401SS (https://mat-ras.com/files/2019/09/DRM-Group-Specification-Sheet.pdf) manufactured by *MAT FILTRATION TECHNOLOGIES*, but since it can only uptake a maximum flow of 300 m^3/h the facility would need 4 of these drum filters.

So, in conclusion, this facility would need 10 round tanks (4 x 400 m^3, 3 x 381 m^3 and 3 x 229 m^3), 4 moving bed biofilters (each with 56.06 m^3), one trickling filter, 6 oxygen cones (1.14 m base diameter and 4.65 m height) and 4 drum filters (capacity: 300 m^3/h).