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STUDY OF THE EFFECTS OF AN AQUAPONICS SYSTEM WITH DIFFERENT FISH DENSITIES IN LETTUCE

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Abstract

Aquaponics is a sustainable food production system based on the interaction between fish, bacteria, and plants. Although it has existed for millennia, research begun a few decades ago and there is little practical knowledge about it. This experiment aimed to compare three different fish (*Oreochromis niloticus*) densities and study the effects on the growth of lettuce (*Lactuca sativa* var. *capitata*). The results showed that the lowest density (3.5 kg/1,000L) produced significantly smaller lettuces and with nitrogen deficiency symptoms. The middle (6.5 kg/1,000L) and highest density (13 kg/1,000L) formed bigger lettuces, with no significant differences between their weight. Nevertheless, considering the pH unstableness of the highest density and fish death due to competition, it was concluded that the fish density that best met the biological requirements of the system was 6.5 kg/1,000L.

Keywords

Aquaponics, Fish density, Lettuce, Growth, Tilapia

Resumen

La acuaponía es un sistema de producción de alimento sostenible que se basa en la interacción entre peces, bacterias y plantas. Aunque haya existido durante milenios, las investigaciones comenzaron hace unas décadas y el conocimiento práctico sobre el tema es reducido. El objetivo de este experimento fue comparar tres densidades de peces (*Oreochromis niloticus*) y estudiar los efectos en el crecimiento de la lechuga (*Lactuca sativa* var. *capitata).* Los resultados mostraron que la densidad menor (3,5 kg/1.000L) produjo lechugas significativamente más pequeñas y con síntomas de deficiencia de nitrógeno. La mediana (6,5 kg/1.000L) y la mayor (13 kg/1.000L) produjeron lechugas más grandes, sin diferencias significativas entre ellos dos en el peso. Sin embargo, teniendo en cuenta la inestabilidad en el pH del agua de la densidad mayor y la muerte de peces debido a la competición, se concluyó que la densidad de peces que mejor cumplió los requerimientos biológicos del sistema fue la de 6,5kg/1.000L).

Palabras clave

Acuaponía, Densidad de peces, Lechuga, Crecimiento, Tilapia

Laburpena

Arrain, bakteria eta landareen arteko elkarrekintzan oinarritzen den elikagai produkzio sistema jasangarria da akuaponia. Milaka urte izan arren, inbestigazioak duela hamarkada gutxi ekin ziren eta gaiaren inguruko jakinduria prkatikoa murriztua da. Experimentu honen helburua arrain (*Oreochromis niloticus*) dentsitate ezberdinak konparatu eta letxugaren (*Lactuca sativa* var. *capitata*) hazkuntzan zuten eragina aztertzea izan zen. Emaitzetan dentsitate txikienarekin (3,5 kg/1.000L) hazitako letxugak esanguratsuki txikiagoak zirela ikusi zen eta nitrogeno faltaren sintomak zituzten. Dentsitate ertainak (6,5 kg/1.000L) eta handiak (13 kg/1.000L) letxuga handiagoak produzitu zituzten, euren arteko pisuan diferentzia esanguratsurik aurkitu etzelarik. Hala ere, dentsitate handienaren uraren pH-aren ezegonkortasuna eta konpetizioagatik hildako arrainak kontuan izanik, eskakizun biologikoak hobekien betetzen zituen arrain dentsitatea 6,5 kg/1.000L zela ondorioztatu zen.

Hitz gakoak

Akuaponia, Arrain dentsitatea, Letxuga, Hazkuntza, Tilapia

INDEX

1. Introduction & Objectives

This study was framed inside Tknika's BioTkniFish project, linked to an internship done at the center. Tknika is the Basque Center of Applied Research and Innovation in Vocational Education and Training (VET), promoted by the Deputy Ministry of VET of the Education Department of the Basque Government. Through networking and direct involvement by the Basque Vocational Training teaching staff, the Centre develops innovative projects in the areas of technology, education, and management, with the objective of contributing to the improvement in the standards and quality of VET in Basque Country Autonomous Community.

BioTkniFish (Figure 1) is a project on sustainable aquiculture and vegetal production through aquaponics. It is structured as the focal point for aquaponics technology in the Basque Country Autonomous Community, with the aim of building the Basque technical aquaculture sector and supplying the markets with new quality products.

Figure 1. BioTkniFish Project. Source: Tknika

Although industrialization and technological development of the last century has led to benefits for humanity, it has also caused deterioration of environmental resources and future concerns, such as overpopulation. It is expected that by 2050 world's population will reach 9.8 billion, 30 percent higher than today (United Nations, Department of Economic and Social Affairs, 2017). In order to feed this larger number of people, food production may need to double in 30 years (Radford, 2016).

Therefore, producing more with less, while preserving and enhancing the livelihoods of small-scale and family farmers, is a key challenge for the future. Substantial improvements in resource-use efficiency and gains in resource conservation will need to be achieved globally to meet growing and changing food demand, and halt and reverse environmental degradation (FAO, 2017).

Aquaponics relies in a symbiotic interaction between fish, bacteria, and plants, which makes it an ecological and sustainable food production system. Although it has its origins centuries ago, developed aquaponic system components are not yet fully realized in view of either cost effectiveness or technical capabilities (Goddek et al., 2015). It is a promising subject to contribute to both global and urban sustainable food production and would help diminish pollution and need for resources, but needs to be scientifically studied and developed to get its place in the worlds' market.

From a commercial point of view of aquaponics, both the fish rearing and the hydroponic vegetable components must be operated continuously near maximum production capacity (Rakocy, Masser, & Losordo, 2006). This study consisted on evaluating the effect of three different fish densities on the growth of lettuce, as well as observing effects at any level on the system, so as to define the density that best meets the aquaponic ecosystem balance, not economically, but from a biological perspective.

1.1. Literature Review

1.1.1. Hydroponics

Hydroponics is the cultivation of plants in soilless media, which provide plant support and moisture retention. Irrigation systems are integrated within these media, thereby introducing a nutrient solution to the plants' root zones that provides all of the necessary nutrients for their growth (Somerville, Cohen, Pantanella, Stankus, & Lovatelli, 2014).

Without contact between plants and soil, hydroponics avoids the appearance of weeds and soil-borne pests. It leaves no toxic pesticide residue, the water- and fertilizer-use is highly efficient and there is a better control over nutrient and oxygen (FAO's Plant Production and Protection Division, 2018), making hydroponics the most suitable farming technique in arid regions or wherever nutrient dispersal is an issue for both environmental and economic reasons. Furthermore, as soilless media can be sterilized and reused between crops, hydroponics meets the particular demands of intensive production, allowing an increased crop quality and yields (Somerville et al., 2014).

Some substrates are even better than soil in terms of water-holding capacity and oxygen supply at the root zone. The manipulation, monitoring, and real-time control of nutrient availability is also better, which allows higher quantitative and qualitative productions (Somerville et al., 2014).

On the other hand, in hydroponics anything that ever comes into contact with plants or the nutrient solution needs to be sterilized, which makes it a very receptive system to disease outbreaks and they can be spread very quickly. Management is also more complicated and requires a different set of inputs, especially during installation, since electricity is generally required to circulate or oxygenate the water (Somerville et al., 2014). So the initial investment for this kind of production system is much higher than for conventional soil-based agriculture.

1.1.2. Aquaculture

Aquaculture is the farming of aquatic organisms in both coastal and inland areas involving interventions in the rearing process to enhance production (FAO, n.d.-a).

Since the beginning of the 21st century, and primarily in reaction to the problem of over-fishing throughout the world's oceans, aquaculture is an increasingly important source of global protein production. In fact, it now provides half of all fish for human consumption in the world, with a 73.8 million tonnes production in 2014 (FAO, 2016). It has the potential to decrease the pressure on the world's fisheries and to significantly reduce the footprint of less-sustainable terrestrial animal farming systems in supplying humans with animal protein (Somerville et al., 2014). At the same time, being one of the fastest growing-food production systems (WWF, 2017), aquaculture can be crucial for the prospective increasing food demand in the world for the next decades.

Nevertheless, aquaculture poses some environmental problems and concerns that need to be addressed to improve the sustainability of this agricultural technique. One major problem is the treatment of nutrient-rich wastewater. Some countries' environmental regulations do not oblige farmers to treat effluent, and without treatment, the release of nutrient-rich water can lead to eutrophication and hypoxia in the watershed and localized coastal areas, macroalgae overgrowth of coral reefs, and other ecological and economical disturbances (Somerville et al., 2014).

1.1.3. Aquaponics

Aquaponics is a sustainable production system of plants and fish that combine traditional aquaculture with hydroponics. The technique is based on a continuous recycling of the effluents, which maximizes the exploitation of the used resources and minimizes their waste.

In an aquaponic system, water from the fish tank cycles through filters, plant grow beds and then back to the fish tank (Figure 2). In the filters, water first passes through a mechanical filter that removes the solid waste and then through a biofilter where bacteria convert ammonium into nitrate. This process is called nitrification. As the water (containing nitrate and other nutrients) travels through the plant grow beds the plants uptake the nutrients, and finally the water returns to the fish tank purified. This process allows the fish, plants, and bacteria to thrive symbiotically and work together to create a healthy environment, provided that the system is properly balanced (Figure 3) (Somerville et al., 2014).

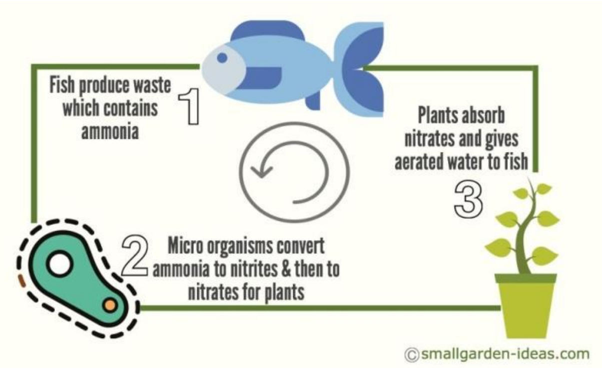

Figure 2. Aquaponic cycle.
Source: http://smallgarden-ideas.com/aquaponics-systems

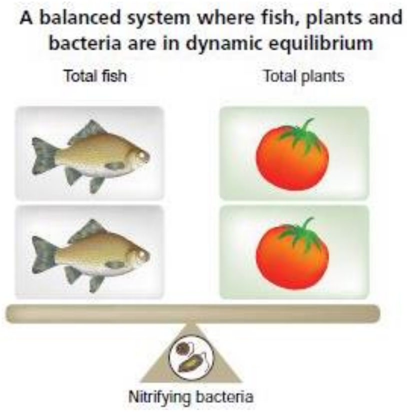

Figure 3. Balance of an aquaponics system. Source: (Somerville et al., 2014)

The concept of raising plants on rafts on the water surface has its origins back in 1000 AD, with early civilizations in both Asia and South America (ACS Distance Education, n.d.). Through the pioneering work of North American and European academic institutions in the late 1970s, and further research in the following decades, this basic form of aquaponics evolved into the modern food production system of today. Although in use since the 1980s, aquaponics is still a relatively new method of food production with only a small number of research and practitioner hubs worldwide with comprehensive aquaponic experience (Somerville et al., 2014).

Unlike in aquaculture, in aquaponics, the effluent is diverted through plant beds and not released to the environment, while at the same time the nutrients for the plants are supplied from a sustainable source. This minimization of water exchange reduces operating costs in arid climates and heated greenhouses where water or heated water is a significant expense (Rakocy et al., 2006). Beyond the benefits derived by integrating aquaculture and hydroponics, aquaponics has shown that its plant and fish productions can be equivalent to both systems (Somerville et al., 2014).

The principle drawbacks that this food production system faces are the extended superficial area required for its installation, the necessity of qualified staff for the maintenance of all the components, pest control that must be strictly biological, and the limited knowledge about the subject (Garcia-Ulloa, León, Hernández, & Chávez, 2005).

1.1.4. Nile tilapia (*Oreochromis niloticus*)

Figure 4. *Oreochromis niloticus*. Source: http://zomufish.com.pe/es/pj-categs/oreochromis-niloticus/

Oreochromis niloticus, nile tilapia (Figure 4) is an omnivorous grazer that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. It is a warm water fish, preferring temperature ranges from 31 to 36 °C, although it can tolerate temperatures from 11-12 ºC to 42 °C. Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg (FAO, n.d.-b).

Originated in Africa, worldwide distribution of the Nile tilapia occurred during the 1960s up to the 1980s. The development of hormonal sex-reversal techniques in the 1970s represented a major breakthrough that allowed male monosex populations to be raised to uniform, marketable sizes. In addition, research on nutrition and culture systems, along with market development and processing advances, led to rapid expansion of the industry since the mid-80s (FAO, n.d.-b).

Nile tilapia is one the most used fish in aquaponics due to the its commercial acceptance and its wide tolerance level to diverse environmental conditions (Rakocy et al., 2006).

1.1.5. Lettuce (*Lactuca sativa* var. *capitata*).

Lactuca sativa, lettuce or garden lettuce, is a leafy annual herb in the *Compositae* family. The species, which is not known in the wild but is thought to have been developed from the wild lettuce *L. serriola*, around 4,500 years ago in eastern Mediterranean basin, has been developed into diverse cultivars (Bradshaw, 2016). Today, it is the most widely used salad crop, cultivated commercially and in home gardens worldwide for its leafy greens.

The FAO estimates that total global commercial production of lettuce was 26.8 million metric tons (mmt) in 2016. China led production with 14.9 mmt, just over half the world total, while the second-ranked U.S. produced 4.1 mmt. India, Spain, and Italy were the next countries, with harvests of 1.1, 0.9, and 0.7 mmt, respectively (FAOSTAT, 2016).

The lettuce is a plant that facilitates its cultivation in aquaponic systems, due to the fact that it has a short productive cycle and, as its commercial interest is focused in leave production, it uses considerable nitrate quantities (Lee & Escobar, 2000).

The variety *capitata* has succulent leaves growing from basal rosette that forms heads that if not harvested turns into a flowering stalk (Ecocrop, 2007).

1.1.6. Bacteria & Nitrification

Two major groups of nitrifying bacteria are involved in the nitrification process: the ammonia-oxidizing bacteria (AOB), and the nitrite-oxidizing bacteria (NOB). The AOB oxidize the ammonium (NH_4^+) and create nitrite (NO_2^-) and the NOB further oxidize the NO_2^- into nitrate (NO_3^-). The genus *Nitrosomonas* is the most common AOB in aquaponics, and the genus *Nitrobacter* is the most common NOB, which are frequently used interchangeably in the literature (Somerville et al., 2014).

The biological cultures self-regulate according to the food available and the surface area they have to colonize. When the amount of ammonium increases, the bacterial culture also grows, as long as there is surface area for colonization (otherwise the system may unbalance, poisoning the water of the fish tank with high levels of ammonium). Whenever the level of ammonium falls, the number of bacteria reduces, leaving only those that are necessary to keep the system balanced (Sustaeta Zubillaga, 2015).

1.1.7. Water quality for each organism

As stated before, aquaponics is the management of a complete ecosystem that includes three major groups of organisms: fish, plants and bacteria. For the proper functioning of the system water parameters need to be adjusted to the needs of each one (Table 1), which is not always easy due to the fact that those needs can vary slightly depending on the specific species used for the aquaponics system.

Table 1. General water quality tolerances for fish (warm- or cold-water), hydroponic plants and nitrifying bacteria. Source: (Somerville et al., 2014)

Organism type	Temp (ºC)	pH	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	DO (mg/L)
Warm water fish	22-32	6-8.5	< 3	< 1	< 400	4-6
Plants	16-30	5.5-7.5	< 30	< 1	-	> 3
Bacteria	14-34	6-8.5	< 3	< 1	-	4-8

The water temperature at the fish tanks was set at 25 ºC.

Although according to the table above the pH could range between 6 and 7.5 to fulfill the requirements of the three organisms, the optimal pH was set from 6.5 to 6.8, since that is the margin where most of the nutrients are available for the plant. On the other hand, bacterial activity gets reduced below 7.

Regarding ammonia, nitrite, and nitrate, and dissolved oxygen (DO) concentrations, all of them were variables measured either for the control of the proper operation of the system or for its study.

2. Material & Methods

2.1.Facilities & Equipment

The study took place at the laboratory of Biotknifish (Figure 5) in Tknika, which is located in Errenteria (Basque Autonomous Country).

Figure 5. Laboratory of BioTkniFish.

The system infrastructure consisted on 9 fish tanks, 3 of each density of study, and each tank connected to two crop lines (Figure 6).

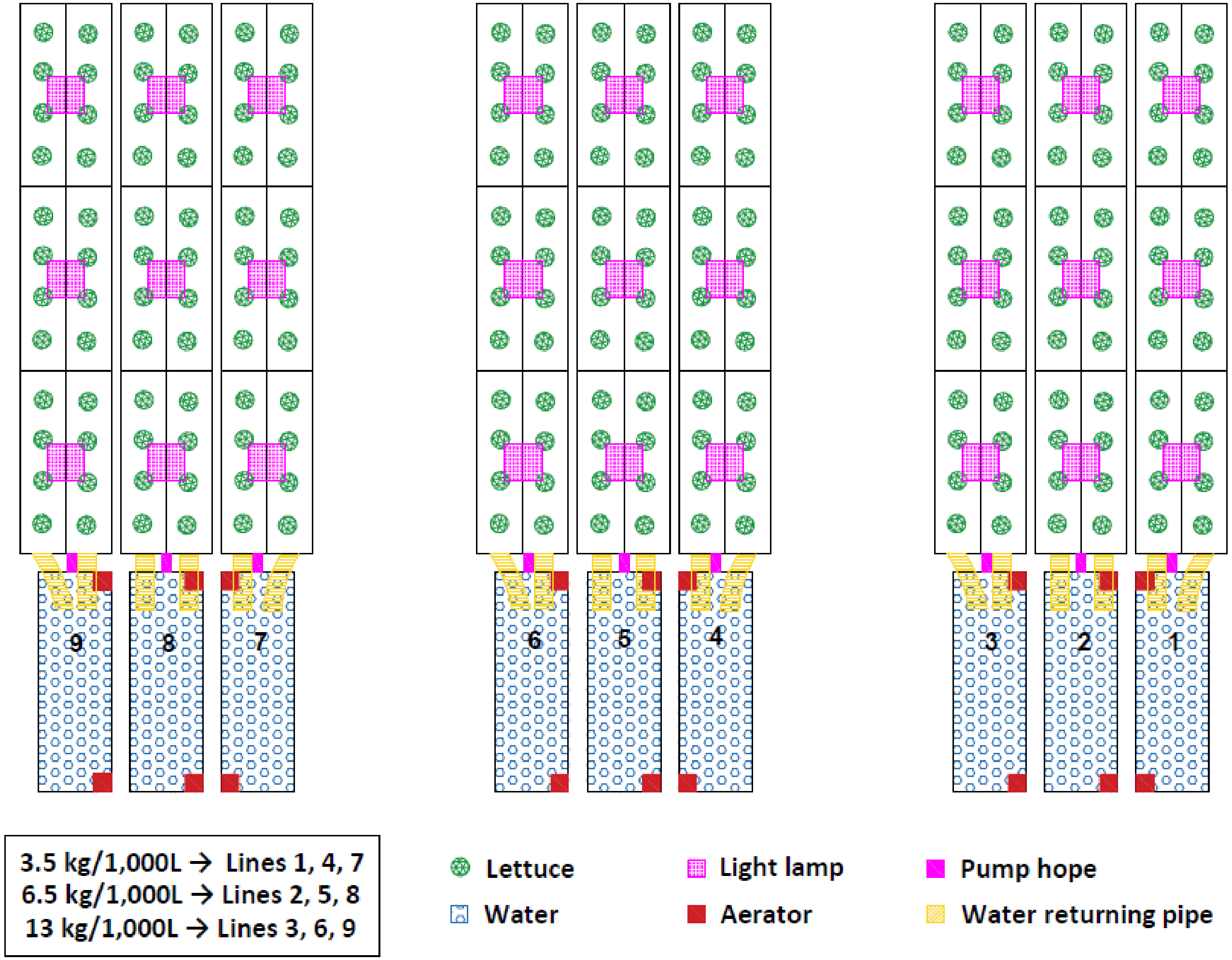

Figure 6. Blueprint of the aquponic system.

2.1.1. Tanks

The water tanks were made of glass, a material that does neither contaminate water nor harm fish and plants. Each tank had a capacity of 170 L.

2.1.2. Crop lines

Each tank was connected to two crop lines set on a cart at a height of 1 m. The crop lines consisted on triangular gutters made of plastic with circular holes on the above side that permitted the water enter. The dimensions were 2.8 x 0.25 m. Above each gutter there were 3 arlite sacs. Arlite is a chemically neutral expanded clay that guarantees air, water, and nutrients penetrate in plants' roots.

2.1.3. Pumps

To transport the water from the fish tanks to the crop lines water pumps were necessary. The pump used in this study was *Surface pump Natflow JPG 6005*, an external pump, which had the characteristics shown in Table 2.

Table 2. Characteristics of *Surface pump Natflow JPG 6005.*

Power	600 W
Maximum flow	3,000 L/h
Maximum pumping height	35 m
Maximum pressure	3.5 bar
Maximum suction height	8 m

2.1.4. Aeration system

Fish need oxygen to be able to breathe. Oxygen in the water can run out quickly depending on the biomass in the tank, so it is necessary to oxygenate the water. For that, membrane aerators were used, which generate small air bubbles, providing a greater surface area for contact between the bubbles and the water and thereby making the oxygen exchange more efficient.

Two aerators were placed in each tank, which supplied the same amount of oxygen to all of them.

2.1.5. Pipes and hoses

Pipes and hoses made the connections from the tanks to the crop lines and vice versa, as well as the connection between the fish tanks and the biological filters.

Water was absorbed from the fish tank and transported to the filters through a hose with 25 mm of interior diameter, and returned through a parallel hose to the tank.

To transport the water to the crop lines, it was pumped and run through a hose with 25 mm of interior diameter, which was connected to two thinner hoses that divided up the water to both crop lines of each tank. From there, water was distributed to each lettuce by drip irrigation.

For the returning of the water back to the fish tank, a pipe with 60 mm of diameter connected the crop line and the tank.

2.1.6. Lighting

Plants reflect the green light, due to the fact that chlorophyll absorbs blue (400-500 nm) and red light (600-700 nm) so as to perform the photosynthesis. Therefore, most plants can grow without green light.

For this study pink LEDs where used, *Urbi Line FF200-4P*, which provided the plants with a light spectrum shown in Figure 7. The dimensions of each lamp were 400 x 300 x 121 mm, and had an aperture of 120º.

By using this kind of lamps, electricity is saved (since LED lights are more efficient) and as plants might grow faster, water and time are saved as well.

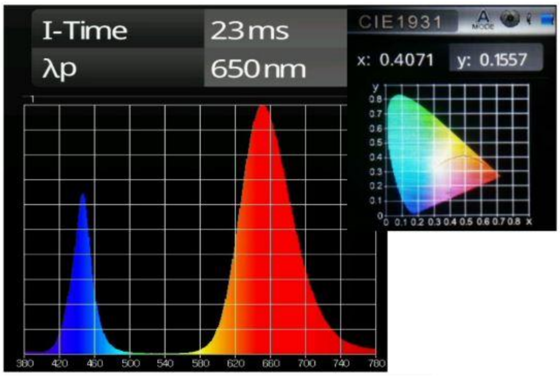

Figure 7. *Urbi Line FF200-4P* spectrum.
Source: Ingeniería Urbiline S.L.

2.1.7. Substrate

As soil is not used in aquaponics, plants need some solid support to take root. Rockwool was the type of substrate used in this study, which is a type of wool used as thermal and acoustic insulation in the construction industry. In aquaponics, a special, more compact rockwool is used, which does not shed any fiber or residue that could be harmful to the fish.

2.1.8. Net pots

The net pots used in aquaponics are specially designed so that the substrate that keeps the plants in place gets very wet and ensures the nutrients come into contact with the roots. The pots are made of a mesh so that they hold the substrate but leave a large surface area free so that the water bearing the nutrients can flow without any problem through them.

2.1.9. Bacterial filters

The bacterial filter is another core part of an aquaponics ecosystem, since it is where nitrification takes place. In order to get a good bacterial filter, it was filled with expanded clay aggregate and biobarrels (small tubular pieces with a big specific surface area) that enable the bacteria to settle and colonize the filter.

The biofilter used in this study was *EHEIM professionel 4+ 350t*, indicated for aquariums from 120 L to 600 L. It had a prefilter that trapped large particles of dirt before entering into the biological filter.

2.1.10. Heaters

To regulate the water temperature heaters are needed, which consist on electrical resistors that are placed in the water to heat it directly. They had a thermostat that can be set at the desired temperature, so that when the water reaches that temperature, it automatically switches off until it falls below the setpoint again (Sustaeta Zubillaga, 2015).

Heaters were included in the biofilter *EHEIM professional 4+ 350t*, which allowed maintaining the water temperature between 24 and 26 ºC (the target value was set at 25 ºC).

2.2. Living being balance

2.2.1. Fish

Three different fish densities were studied in this project: 3.5 kg/1000L, 6.5 kg/1000L, and 13 kg/1000L. Therefore, three tanks were destined for each density. Table 3 shows the biomass quantity in grams introduced in each tank, as well as the number of fish and the average weight.

Table 3. Biomass, number of fish, and the average fish weight introduced in each tank.

Tank	Total biomass (g)	Quantity	Average weight (g)
1	655	12	54.58
2	1,022	17	60.12
3	2,280	46	49.57
4	634	11	57.64
5	1,177	21	56.05
6	2,261	40	56.53
7	581	11	52.82
8	1,276	19	67.16
9	2,269	47	48.28

2.2.2. Plants

Each crop line had three arlite sacs with 4 holes (around 200 mm from one to another). One lettuce was planted in each hole. Therefore, there were 24 lettuce connected to each fish tank, which makes 72 plants in total for each fish density (Figure 6).

2.3. Maintenance

2.3.1. NH_4^+ & NO_2^-

As explained in the introduction, ammonium is converted into nitrites and nitrites into nitrates. Nevertheless, both reagents can be accumulated in the water due to the fact that nitrification process can take longer than the input rate (for example, in the case of giving an excessive feed dose).

It is important to control these two parameters, since they can be very harmful to fish. In the case of ammonium, the risk appears when it is transformed into ammonia, which is very damaging to fish. It depends on the pH. As long as the pH is below 7, this H^+ interchange process does not take place and it remains as NH_4^+ (Figure 8). However, it is very advisable to keep the ammonium concentration below 1 mg/L.

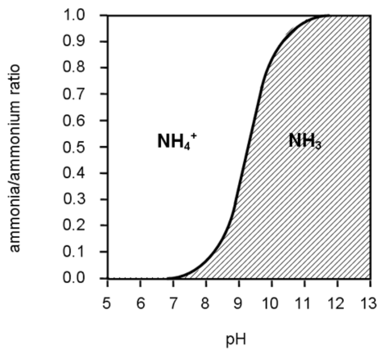

Figure 8. Ammonia/ammonium ratio dependent on pH. Source: http://www.nico2000.net/analytical/ammonium/NH4lib.html

Therefore, once a week on Wednesdays an ammonium analysis was done with API Test Kit (Figure 9).

Figure 9. Nitrite and ammonia/ammonium API Test Kits.

A high concentration of nitrites is always harmful to fish, so it is important to keep it below 0.5 mg/L. Nitrite analysis was also done with API Test Kit three times a week on Mondays, Wednesdays, and Fridays.

In cases where the concentrations exceed the red line, a change of water would be the safest procedure. Nevertheless, as this project aimed to study the different effects of different fish densities, it was important to avoid any water change. Therefore, nitrite and ammonium contents were conscientiously controlled and feed doses were adjusted to avoid the concentrations to rise.

2.3.2. Fish feed

The feed that was used in this study is *Dibaq Microbaq 165,* which is a complete feed for fingerlings.

- Composition: fish meal, pea protein, wheat gluten, fish oil, soya protein concentrate, corn gluten meal, pea starch, yeast extract, krill meal, canola oil, squid meal, soya lecithin, minerals.
- Components and analytical levels: 52% crude protein, 18% oils and crude fat, 1.2 % crude fiber, 8% total ashes, 1.4% Calcium, 1.2% Phosphorous, 0.31% Sodium.

During the first week of study 2.5% of the fish weight was supplied to them, divided in two doses, following the information supplied by Nerbreen Aquaponics Company from Hondarribia. However, it showed up to be excessive, since in the nitrite analysis performed on Wednesday the results were too high that could endanger the fish. Therefore, it was decided to reduce the dose to 1% of the fish weight. The feed supplied each day during each week is shown in Tables 4-7, as well as the total feed provided each week.

Table 4. Amount of feed (g) given each day and in total the first week in each fish tank.

Tank	Week 1											
	Mon		Tues		Wed		Thurs		Fri	Sat	Sun	Total (g)
1	10	5	10	5	10	-	-	-	6	6	-	52
2	20	10	20	10	20	-	-	-	-	-	-	80
3	40	15	40	15	40	-	-	-	22	22	-	194
4	10	5	10	5	10	-	-	-	6	6	-	52
5	20	10	20	10	20	-	-	-	11	11	-	102
6	40	15	40	15	40	-	-	-	-	-	-	150
7	10	5	10	5	10	-	-	-	6	6	-	52
8	20	10	20	10	20	-	-	-	-	11	-	91
9	40	15	40	15	40	-	-	-	22	No	-	172

Table 5. Amount of feed (g) given each day and in total the second week in each fish tank.

Tank	Week 2							
	Mon	Tues	Wed	Thurs	Fri	Sat	Sun	Total (g)
1	6	6	6	6	6	6	-	36
2	-	-	-	-	11	11	-	22
3	22	22	11	22	22	22	-	121
4	6	6	6	-	6	6	-	30
5	11	11	-	-	11	11	-	44
6	22	11	-	-	22	22	-	77
7	6	3	-	6	6	6	-	27
8	11	6	-	11	11	11	-	50
9	22	22	22	22	22	22	-	132

Table 6. Amount of feed (g) given each day and in total the third week in each fish tank.

Tank	Week 3							
	Mon	Tues	Wed	Thurs	Fri	Sat	Sun	Total (g)
1	6	-	-	-	6	6	-	18
2	11	-	-	-	11	11	-	33
3	22	-	-	-	22	22	-	66
4	-	-	-	-	6	6	-	12
5	11	-	-	-	11	11	-	33
6	22	-	-	-	22	22	-	66
7	6	-	-	-	6	6	-	18
8	-	-	-	-	11	11	-	22
9	22	-	-	-	22	22	-	66

Table 7. Amount of feed (g) given each day and in total the fourth week in each fish tank.

Tank	Week 4							
	Mon	Tues	Wed	Thurs	Fri	Sat	Sun	Total (g)
1	6	6	6	6	6	6	-	36
2	11	11	11	-	11	11	-	55
3	22	22	22	22	-	22	-	110
4	6	6	6	6	6	6	-	36
5	11	11	11	11	11	11	-	66
6	-	22	22	22	-	22	-	88
7	6	6	6	6	6	6	-	36
8	-	11	11	11	-	11	-	44
9	22	22	22	22	22	22	-	132

When nitrite concentration in the water was high, feed was not supplied until it descended to 0.25 mg/L. All the dashes (-) that appear in Tables 4-7 mean that no food was given to the fish in the tank due to the mentioned issue.

At the end of the study, it is shown in Table 8 the total amount of feed that had been supplied for each density.

Table 8. Average of the total amount of feed given for each fish density during the four weeks of the experiment.

Density (kg/1,000L)	3.5	6.5	13
Feed (g)	135	214	458

2.3.3. KOH solution to rise pH

As stated in the section 1.1.7. of the Introduction, a target pH range was stablished for this study according to the optimal conditions for the three organisms that compose the system. That range went from 6.5 to 6.8. Nevertheless, pH values drop due to the oxygen consumption of fish, and therefore, it was necessary to intervene to maintain the pH as close as possible to those values so as to avoid any harm to the living beings.

A solution of 100 g/L KOH was used to raise the pH. An average of 6 mL was poured for each 0.1 that descended the pH from 6.6 down.

2.3.4. Formol bath to eliminate parasites

A monogenean trematode had been found in the hatchery of BioTkniFish. The trematode from the genus *Gyrodactylus* is an ectoparasite that parasites the body surface fish, as well as the gills of many species of *Oreochromis*. As it is viviparous, fish death is exponential.

Some fish died during the weeks the study lasted. Although necropsies did not show pathogenicity and the deaths seemed to be due to aggression, an intervention was required to make sure the trematode would not be present in the tank. Therefore formol baths were done to tanks 3 and 6 during the third week of the study. Fish were treated in buckets with a solution of 170 ppm of formol during an hour (Jiménez Guzmán et al., 1988).

2.4. Data collection

For the study of the effects of different fish densities two groups of parameters were analyzed: the growth of the plants and water parameters.

2.4.1. Growth of lettuces: diameter, number of leaves & weight

So as to take the measures of the lettuces, a sample of 18 plants was randomly taken for each density. For that, each plant was numbered from 1 to 72 (Figure 10) and then RANDOM() function was used in Microsoft Excel, obtaining random 18 numbers between 1 and 72.

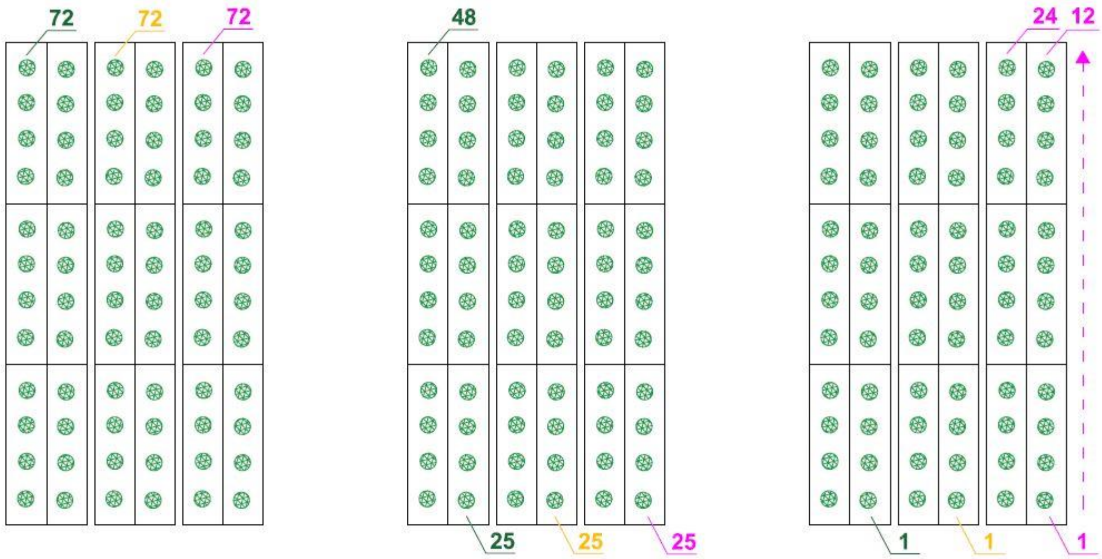

Figure 10. Numbering of the lettuces.

First of all, the diameter was noted down by measuring the radius of the longest leaf using a tape measure. Then, the quantity of leaves was counted for the 18 plants that were randomly chosen. Finally, lettuces were weighted with a weighing scale.

These three measures were taken once a week on Mondays, and these data were collected into a Microsoft Excel file.

2.4.2. Water parameters: pH, NO_3^-, and DO

Water pH was measured with a pH-meter that was calibrated every morning. A beaker was used to take water from each fish tank, a pill was put inside and it was placed on a shaker so as to homogenize the liquid. After a couple of minutes the pH value was stabilized and the value was noted down. This data was collected on Mondays, Wednesdays, and Fridays, and noted down in a Microsoft Excel file.

Nitrate concentration in water was measured as well, as it is the main component that affects plants' growth. A volume of 6 mL of each water tank were poured into a cell and this was introduced into the spectrophotometer to make the 'zero' value. Then an envelope of nitrate reactive was added to the cell and after mixing it gently for a minute, it was reintroduced into the machine. After 4' 30s the nitrate value was shown on the screen. The spectrophotometer had a range of 0 to 30 mg/L, and some waters exceeded this value. Therefore, in those cases, an API Test Kit was used, which even if less accurate, it covers a bigger range of values. Nitrate contents were measured twice a week, on Mondays and Fridays.

Regarding dissolved oxygen concentration, it was measured the last day of the study using an oximeter.

2.5. Statistical analysis

The analysis of the variance (ANOVA) establishes whether differences exist between different data series, and whether they are significant or not. That is, it proves if the means between two or more groups are significantly different or not. In those cases where the analysis of the variance has a positive result, a media comparative test (post-hoc) is performed to identify between which groups happen those differences (Pérez Roncal, 2015).

In this study an ANOVA of the single factor fish density was carried out, with a confidence interval of 95%, considering as variables the diameter, number of leaves, and weight of the lettuces. The media comparison was performed with the Scheffe Test.

Then, a multivariate analysis of the variance was performed (MANOVA) for each week, so as to test if the three studied variables together were affected by the factor, significantly differing each fish density.

For both analyses a null hypothesis was set: there is no difference between fish densities. If p-value is smaller than 0.05 ($p<0.05$), the null hypothesis is rejected, thereby concluding that the densities affect and create significant differences between the variables. Otherwise, it cannot be said that the density is a factor that affects them.

For the statistical analysis SPSS (Statistical Package for the Social Science) version 21 (SPSS Chicago, IL) was used.

2.6. Nutrient deficiencies

Apart from nitrogen, which is the main macroelement the plant nourishes with, other mineral elements are also essential for it to complete its vital cycle. Some of them are needed in big quantities (N, P, K, Ca, Mg, S), while some others in smaller portions (Fe, B, Mn, Cu, Zn, Mo, Cl), but all are necessary.

Therefore, during the study it was observed whether any deficiency symptom appeared in the lettuces.

3. Results & Discussion

3.1. Water parameters: pH, NO_3^-, and DO

As stated previously, target pH limits were set for this study and each fish tank's pH was analyzed three times a week. Figure 11 plots the average pH of the three tanks per density in time.

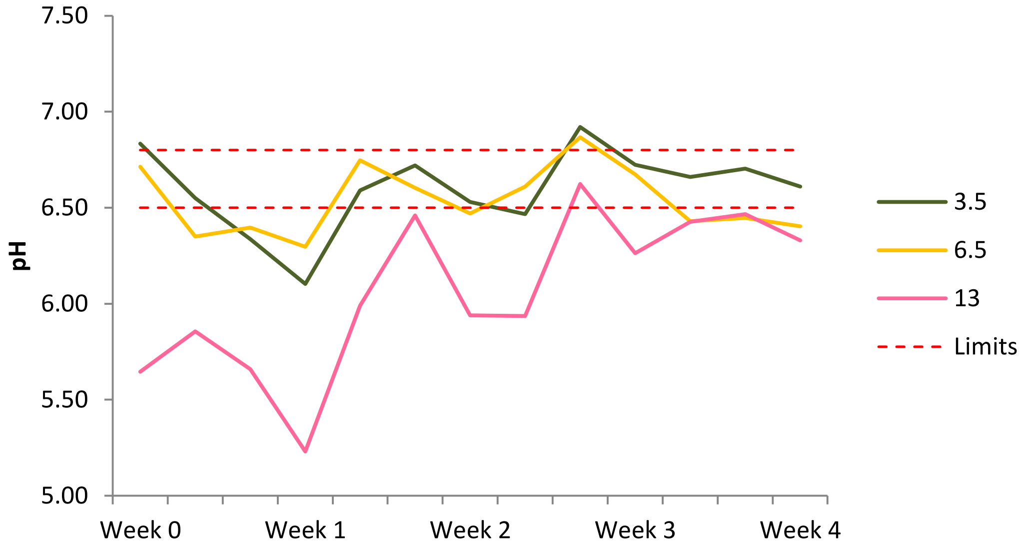

Figure 11. Average pH values registered during the study for each fish density (kg/1,000L).

It can be said that 3.5 and 6.5 kg/1,000L fish densities' pH could be maintained within the target range, while the pH of 13 kg/1,000L density was way below the minimum limit. This one reached a minimum pH value of 5.13, overcoming the required limits of the three organisms stated in Table 1 of the section 1.1.7. of the Introduction.

So as to avoid the dropping of the pH that could lead to harm to the living beings, as explained in Material & Methods, KOH solution was used to raise the pH. Table 9 shows the media of the total amount of KOH solution that was provided to each density.

Table 9. Average of the total amount of $KOH_{(aq)}$ poured for each fish density.

Density (kg/1,000L)	3.5	6.5	13
$KOH_{(aq)}$ (mL)	87	142	419

The pH needs to be analyzed looking to both, Figure 11 and Table 9, since the natural pH evolution would be different from what is shown in Figure 11. Therefore, it can be concluded that the density that remained closer to the target pH value was 3.5 kg/1,000L fish density. The middle density could be maintained within the limits as well, but a greater intervention was necessary for that (60%

more KOH solution). The pH of 13 kg/1,000L fish density had the tendency to drop drastically, exceeding the limits for the living beings and requiring a continuous control and intervention on the system (380% more KOH solution than 3.5 kg/1,000L density and 195% more than 6.5 kg/1,000L density).

Regarding nitrate concentration, it went increasing from the beginning of the study to the end of it (Figure 12), which might be the answer to the bacteria population growth over time.

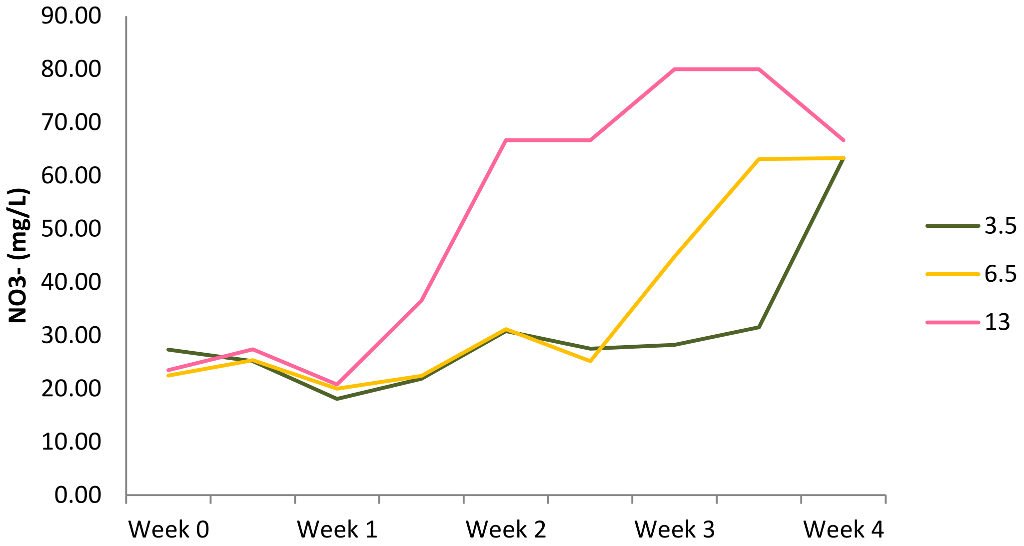

Figure 12. Average NO3- values registered during the study for each fish density (kg/1,000L).

In accordance with the results obtained in a study done in Italy that compared lettuce growth in aquaponics with two different fish densities and hydroponics, nitrate concentrations increased from the beginning to the end of the study (Pantanellaa, Cardarelli, Collab, Rea, & Marcucci, 2010).

The fish density of 13 kg/1,000 showed much higher nitrate concentration than the other two during the intermediate period. It reached the double nitrate content (almost 70 mg/L) than 3.5 and 6.5 kg/L densities (around 30 mg/L). However, the last day closer values to the densest ones were registered.

In the graph above it can be seen that the biggest increment in nitrate concentration came at different time for each density; between the first and the second week for the density of 13 kg/1,000L, around the third week for the middle one, and the last week for the 3.5 kg/1,000L fish density.

In the study of Pantanellaa et al. (2010), an ANOVA was done with nitrate concentration values obtained for low and high densities, and it resulted in significant differences between them.

Therefore, further study would have been necessary to see how the nitrate concentration evolved in time once the systems reached that highest value around 65 mg/L and get into a clear conclusion.

Finally, Table 10 shows the average DO for each fish density.

Table 10. Average DO for each fish density.

Density (kg/1,000L)	DO (mg/L)	DO (%)
3.5	6.33	93
6.5	6.20	91
13	5.90	86

The concentrations were suitable according to the optimal water quality parameters, but the difference on the oxygen content from the density of 6.5 to 13 kg/1,000L, was the double than what it was from 3.5 to 6.5 kg/1,000L.

This is connected to the water pH. The free CO_2 released during respiration (oxygen consumption) reacts with water, producing carbonic acid (H_2CO_3), and pH is lowered (Wurts & Durborow, 1992). Therefore, pH and DO results obtained and analyzed previously make total sense.

3.2. Fish death

Many fish appeared dead during the four weeks that lasted the study (from April 16th to May 14th) (Table 11).

Table 11. Fish death date, tank, and number of dead fish.

Death		
Date	Tank	Quantity
04/17/2018	3	1
04/23/2018	3	1
04/24/2018	9	1
05/01/2018	3	3
05/02/2018	6	2
05/11/2018	6	1
05/14/2018	6	1

All the deaths happened in the tanks that had the biggest density of fish. Necropsies were done and could not be diagnosed any pathogenicity while some aggression signs were detected, such as scale lack and broken lateral fins.

The presence of more than one male causes a great competition between them. Generally, male and female fish are differentiated and put a chip, but was not the case for this study, as they were not adults and the procedure was not done yet.

However, the conclusion from the registered deaths was that the higher the density, the higher the competition between fish.

3.3. Growth of lettuces

As explained in section 2.4.1. of Material & Methods, data of the four weeks that the study lasted was saved in a Microsoft Excel file. Figure 13, Figure 14, and Figure 15, show the evolution of the lettuces in diameter, number of leaves, and weight, respectively.

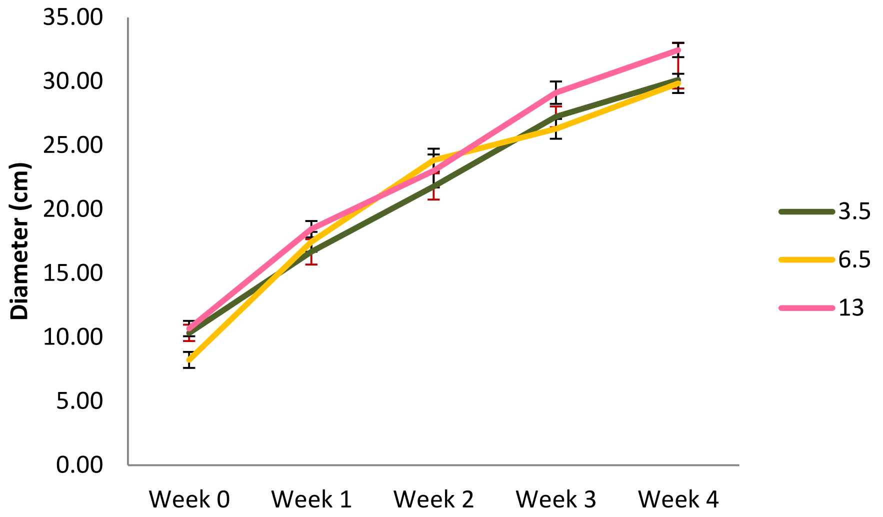

Figure 13. Evolution of the diameter (cm) of lettuces for each fish density (kg/1,000L).

The growth in diameter had a relatively lineal evolution. Although the highest density seems to promote the growth in diameter, the values crossed along the study and the tendency was similar in the three cases.

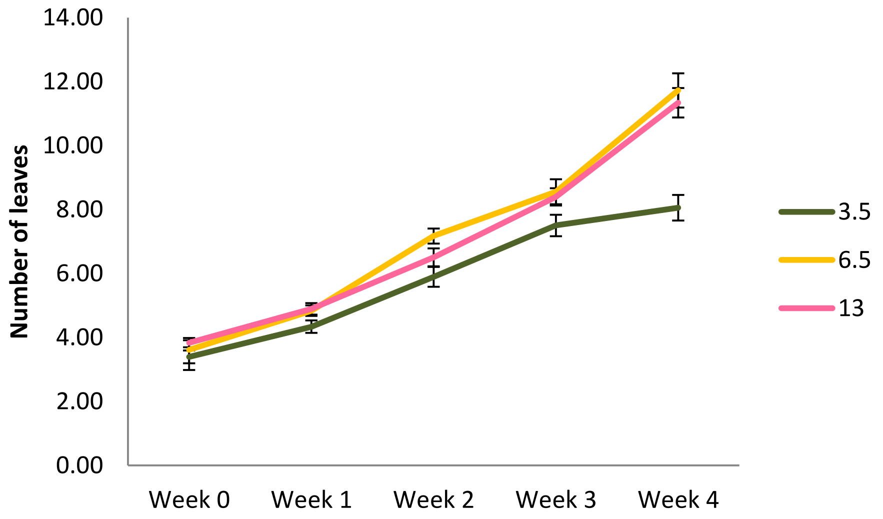

Figure 14. Evolution of the number of leaves of lettuces for each fish density (kg/1,000L).

The number of leaves also had a relatively lineal evolution until the third week. It is from the third week on when lettuces fed with 6.5 and 13 kg/1,000L fish densities' water highly increase their number of leaves, while the lowest density's lettuces rate experiences a decrease. As a consequence, the results obtained the last week show a clear difference between them.

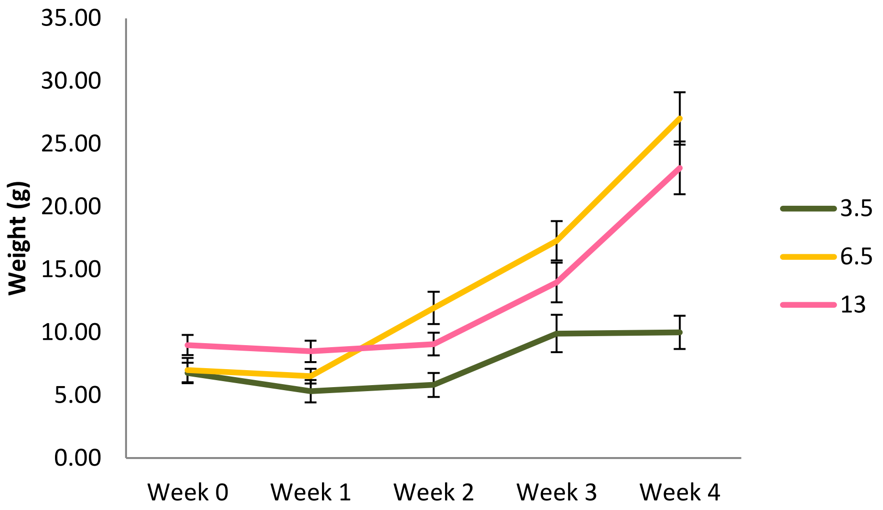

Figure 15. Evolution of the weight of lettuces (g) for each fish density (kg/1,000L).

Unlike what happened with the other two variables, growth in respect to weight seems to be more exponential. Similar to the number of leaves, the growth rate has a big increase from the third to the fourth week in lettuces fed with fish densities of 6.5 and 13 kg/1,000L, creating a leap from the other density.

So as to see if the difference perceived in the graphs between the three conditions was significant, every week data was analyzed statistically by an ANOVA (C.I. 95%) (Table 12).

Table 12. Results of ANOVA for the three variables.

	Diameter	Number of leaves	Weight	
Significance ($p<0.05$)	0.313	0.068	0.022	Week 1
	0.405	0.008	0.001	Week 2
	0.053	0.066	0.007	Week 3
	0.015	0.000	0.000	Week 4

The difference between densities in regards to diameter and number of leaves was significant according to the data collected the last week. However, density seemed to affect weight since the first week, creating significant differences between the three fish densities.

In order to see between which groups appeared those differences, Scheffe Test was done with the obtained data (Table 13).

Table 13. Averages, errors and results of Scheffe Test for the three variables.

	Diameter (cm)	Number of leaves	Weight (g)	Density (kg/1,000L)
Week 1	16.67±1.00 a	4.33±0.2 a	5.32±0.89 a	3.5
	17.44±0.78 a	4.83±0.17 a	6.52±0.6 ab	6.5
	18.44±0.64 a	4.89±0.18 a	8.49±0.85 b	13
Week 2	21.78±1.02 a	5.89±0.31 a	5.84±0.95 a	3.5
	23.83±0.89 a	7.17±0.23 b	11.95±1.29 b	6.5
	23.00±1.29 a	6.50±0.28 ab	9.07±0.90 ab	13
Week 3	27.22±0.81 a	7.50±0.34 a	9.91±1.48 a	3.5
	26.28±0.77 a	8.56±0.39 a	17.29±0.56 b	6.5
	29.11±0.87 a	8.39±0.27 a	13.97±1.58 ab	13
Week 4	21.78±1.02 ab	5.89±0.31 a	5.84±0.95 a	3.5
	29.83±0.76 a	11.72±0.54 b	27.03±2.09 b	6.5
	32.44±0.56 b	11.33±0.46 b	23.09±2.10 b	13

There was no significant difference in diameter between any group until the fourth week, when 6.5 kg/1,000L fish density's diameter was significantly smaller than 13 kg/1,000L, while the diameter of lettuces fed with 3.5 kg/1,000 fish density water was similar to both groups.

Regarding the number of leaves, significance differences appeared on the second week, although they disappeared on the third week. The last week the number of leaves of 6.5 and 13 kg/1,000L fish densities was significantly bigger.

The weight is the parameter used to measure lettuces for commercial aptitude. Therefore, it can be considered the most important between the three variables studied in this experiment. The second and the third weeks, two significantly different groups were differentiated: the lightest were those of 3.5 kg/1,000L, while those of 6.5 kg/1,000L gained more weight. Lettuces fed with 13 kg/1,000L water were similar to both groups until the fourth week, when differentiated from the lightest group.

Observing the results, it needs to be considered the fact that the crop line number 9 (with highest fish density) was next to the laboratory window. Although it cannot be demonstrated, there is the possibility that the light that entered from there affected those lettuces growth negatively, since the differences between line 5 and 6, or 2 and 3 were not visible but differences from 8 to 9 were obvious (Figure 16).

Figure 16. Visual differences between middle and high density crop lines.

During the first crop cycle of the study of Pantanellaa et al. (2010) lettuces grown with high fish density (8 kg/1,000L) treatment showed to be significantly heavier (fresh weight) than those with low fish density (5 kg/1,000L). In the second crop trial, differences between 6 and 20 kg/1,000L were minimal. Analyzing those results and the results obtained in this study, it could be said that the optimum fish density (in these particular conditions) in regards to weight gain should be at some point between 6.5 and 13 kg/1,000L, since there are differences between 5 and 8 kg/1,000L, but 13 or 20 kg/1,000L seemed to be unnecessary.

Theoretically the effect of the three different densities would be accumulated the last week. Figure 17, shows the differences in diameter, number of leaves, and weight according to the data collected the last week of the study

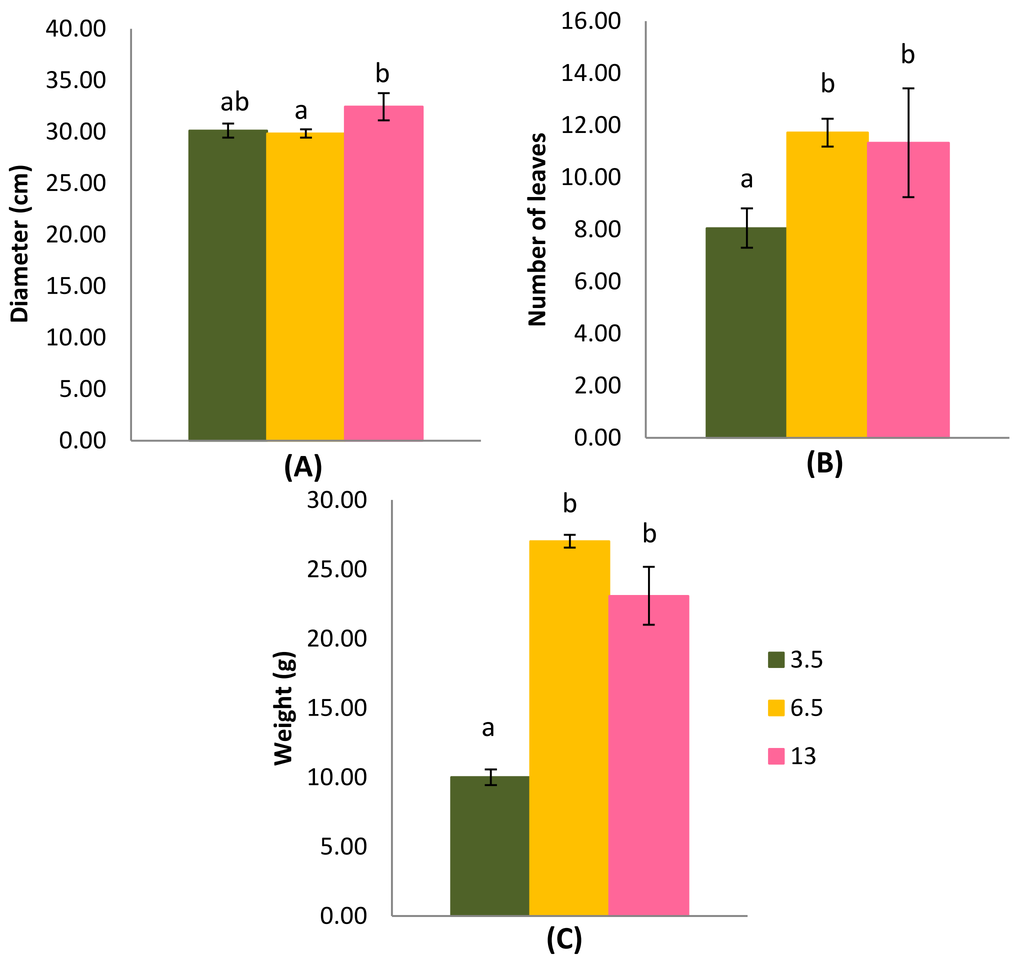

Figure 17. Average diameter (cm), number of leaves, and weight for each fish density (kg/1,000L) in the last week.

Significant differences were found during the last week of study for the three variables. The above graphs make it obvious the differences between the number of leaves (B) and weight (C) of 3.5 kg/1,000L and the other two fish densities.

Although there were significant differences in diameter (A) as well, it is not that apparent. In this case, 6.5 kg/1,000L density showed a smaller diameter. Repairing to the results of the other two variables and the simple appearance of the lettuces, it could be that nutrients were directed towards the invigoration of the leaves instead of their lengthening.

Following the premises that the last week is when the effect of fish density is most displayed and the weight the most important variable, it can be concluded that 6.5 and 13 kg/1,000L fish densities resulted in greater lettuces' growth. Between these two densities, although 6.5 kg/1,000L got better average results for number of leaves and weight, the differences were not significant, while the opposite happened in diameter and the differences in that case turned to be significant. However, in order to decide which density worked best in this study, water parameters and other issues should also be taken into account.

After analyzing the effect of the fish density on each of the three variables, Table 14 shows the MANOVA results that represent the effect on the general growth of the lettuces (diameter, number of leaves, and weight together).

Table 14. Results of MANOVA for each week.

	Sig. ($p<0.05$)
Week 1	0.095
Week 2	0.015
Week 3	0.001
Week 4	0.000

According to the MANOVA done with the registered data, the fish density factor affected lettuces' growth from the second week on when differences between the three fish densities are increasingly significant and the null hypothesis can be rejected. Therefore, it can be concluded that fish density does affect lettuce's growth.

3.4.Nutrient deficiencies

The second week yellowish spots were detected in leaves of those lettuce fed with water of 3.5 kg/1,000 fish density (Figure 18). This symptom appeared in the oldest leaves, which turned more and more yellow, dried, and finally died. This is called chlorosis, and it is a symptom of nitrogen deficiency.

Figure 18. Nitrogen deficiency symptoms observed on leaves.

This nutrient deficiency symptom is not only an evidence of nitrogen deficiency of the lowest fish density's water, but it also presents a problem for the commercialization of plants. There are regulations that regulate the quality and characteristics of each plant to be able to market them. This visible symptom is a reason to deny the commercialization of lettuces.

Therefore, it can be concluded that lettuces grown with 3.5 kg/1,000L fish density water cannot be considered as an optimum density in aquaponics.

4. Conclusions

After analyzing the results of this study, the first conclusion that can be extracted is that the lowest density (3.5 kg/1,000L) does not produce sufficient nitrate for the optimum growth of lettuces. The differences in nitrate concentration were big until the last week, when considerable values were obtained, but the growth during the four weeks that lasted the study was too poor and irreversible nitrogen deficiency symptoms appeared on the second week, which made these lettuces non-marketable.

The densities of 6.5 and 13 kg/1,000L showed to be more efficient and lettuces grew properly, without any deficiency symptom. Although not significant differences were found in weight and number of leaves between these two densities, the middle density obtained better averages. Nevertheless, the possibility that light had a negative effect on lettuce growth of the high density crop line next to the window was considered.

Regarding diameter, the results of 6.5 kg/1,000L fish density were significantly smaller. However, it was concluded that the plant might have allocated the absorbed nutrients towards the invigoration of the leaves instead of their lengthening.

Considering the analysis of the pH and its required control, it can be said that the highest density was much more demanding than the other two.

In addition, it needs to be into account the fact that several fish were found dead in the three tanks with the highest density. Aggression signs were detected at the time of the necropsies and parasite existence was dismissed, which lead to the conclusion that a high density of fish induces a greater competition between them.

All in all, it can be concluded that the preliminary fish density that best meets the biological requirements of an aquaponics system was 6.5 kg/1,000L fish density. Nevertheless, further studies would be necessary to adjust and define the optimum fish density, as well as to match it with commercial requirements and profitability.

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